UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

PG-BGA

Produtos naturais: atividades antibacteriana e imunomoduladora *in vitro* e perfil bioquímico *in vivo*

Bruna Fernanda Murbach Teles Andrade

Tese apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração Biomoléculas: estrutura e função, linha de pesquisa: Atividades biológicas de produtos naturais.

Prof° Dr. Ary Fernandes Júnior

BOTUCATU – SP
2015

Instituto de Biociências - Seção Técnica de Pós-Graduação
Distrito de Rubião Júnior s/n CEP 18618-970 Cx Postal 510 Botucatu-SP Brasil
Tel (14) 3880-0780 posgraduacao@ibb.unesp.br

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Bruna Fernanda Murbach Teles Andrade

ORIENTADOR:

Prof° Dr. Ary Fernandes Júnior

CO-ORIENTADORES:

Prof° Dr. José Maurício Sforcin

Prof[a].Dr[a]. Ana Angélica Henrique Fernandes

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Orientador: Ary Fernandes Júnior
Coorientador: José Maurício Sforcin
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Palavras-chave: anti-inflamatório; antibacteriano; compostos majoritários; óleos essenciais; produtos naturais.

"Os problemas significativos que enfrentamos não podem ser resolvidos no mesmo nível de pensamento em que estávamos quando os criamos."
Albert Einstein

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*"A lei de ouro do comportamento é a tolerância mútua, já que nunca veremos senão uma parte da verdade sob ângulos diversos." **

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*"Nas grandes batalhas da vida, o primeiro passo para a vitória é o desejo de vencer" **

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*"De modo suave, você pode sacudir o mundo"**

À Deus e suas diversas manifestações que me fortaleceram e agiram de forma suave e transformadora em minha vida.

*Mahatma Gandhi

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Values are given as the mean±SD for each group of eight animals. [a]Significantly different from G1; $p \leq 0.05$; [b]significantly different from G2; $p \leq 0.05$; and [c]significantly different from G3; $p \leq 0.05$. G1: untreated control; G2: treated with geraniol; and G3: treated with essential oil.

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similar experiments. Different small letters indicate significant differences between the treatments ($P < 0.001$).

Figure 4 IL-10 production (pg/ml) by human monocytes incubated with (**A**) *Cymbopogon martinii* EO (0.1, 1, 5 and 10 μg/ml); (**B**) geraniol (0.057, 0.57, 2.87 and 5.74 μg/ml); 0.02% DMSO and LPS (10 μg/ml). Data represent median of ten similar experiments. Different small letters indicate significant differences between the treatments ($P < 0.001$).

Lista de abreviaturas

ALT: Alanine aminotransferase

AST: Aspartate aminotransferase

ATCC: American Type Culture Collection

BHI: Brain Heart Infusion

C. martinii: Cymbopogon martinii

CFU: Colony-forming unit

DMSO: Dimethyl sulfoxide

EO: Essential oil

EOs: Essential oils

GC-MS: Gas chromatography-mass spectrometer

GSH-Px: Glutathione peroxidase

IL: Interleukin

LH: Lipid hydroperoxide

LPS: Lipopolysaccharide

MHA: Mueller Hinton Agar

MIC: Minimal inhibitory concentration

MTT: 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide

NBT: Nitroblue-tetrazole

NCTC: National Collection of Type Cultures

ODs: Optical densities

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate buffered saline

PGE2: Prostaglandin E2

ROS: Reactive oxygen species

RPMI: Roswell Park Memorial Institute

SOD: Superoxide dismutase

TNF: Tumour necrosis factor

UFC: Unidade formadora de colônia

Resumo:

Produtos naturais, como os óleos essenciais, possuem diversas propriedades biológicas bem como são importantes fontes para a pesquisa. Além disto, os óleos essenciais estão presentes em nossa rotina, tanto em produtos como xampus, sabonetes e alimentos, assim como em alternativas terapêuticas como a aromaterapia. Objetivamos investigar atividades biológicas de produtos naturais, sendo os resultados apresentados na forma de três manuscritos. Investigamos a ação antibacteriana dos óleos essenciais de tea tree (*Melaleuca alternifolia*), gerânio (*Perlagonium graveolens*), palmarosa (*Cymbopogon martinii*) e seus respectivos compostos majoritários 1-terpinen-4-ol, citronelol e geraniol em sua fase líquida e gasosa (Capítulo I). Foi demonstrada a eficácia dos óleos essenciais e seus principais componentes como agentes antimicrobianos por contato direto e na forma de vapor utilizando três diferentes metodologias e foram destacados os danos causados por esses vapores utilizando informações de microscopia eletrônica de transmissão. Em outra parte do estudo, objetivamos analisar parâmetros bioquímicos séricos e o estresse oxidativo de ratos submetidos à inalação do óleo essencial de palmarosa e geraniol durante 30 dias (Capítulo II). Ratos tratados com os produtos naturais apresentaram níveis de colesterol total diminuídos bem como foi verificado efeitos benéficos do óleo essencial sobre o estresse oxidativo. Finalizando o estudo, avaliamos a ação imunomoduladora do óleo essencial de *C. martinii* e geraniol quanto à produção de citocinas pró- e anti-inflamatórias por monócitos humanos (Capítulo III). As diferentes concentrações testadas não mostraram efeitos citotóxicos em monócitos. A produção do fator de necrose tumoral (TNF)-α não foi afetada por *C. martinii* e geraniol, sendo que somente na concentração de 5 μg/mL de *C. martinii*, houve estímulo para a sua produção. Por outro lado, em todas as concentrações de *C. martinii* e geraniol houve aumento de interleucina (IL)-10 por monócitos humanos. Os resultados mostraram que as concentrações não citotóxicas do óleo e geraniol exerceram uma ação anti-inflamatória, verificada pelo aumento na produção de IL-10. Como importante conclusão, o geraniol parece ser o responsável pela atividade imunomoduladora do óleo essencial de *C. martinii* em nossa condição de ensaio. De forma geral, observamos uma ação efetiva dos compostos naturais nas atividades biológicas testadas, demonstrando atividade antimicrobiana *in vitro*, atividade anti-inflamatória em monócitos humanos e os efeitos da inalação *in vivo*.

Palavras-chave: produtos naturais; óleos essenciais; compostos majoritários; antibacteriano; anti-inflamatório; inalação; estresse oxidativo.

Abstract:

Natural products such as essential oils have diverse biological properties and are important sources for research. In addition, the essential oils are present in our daily lives, both in products like shampoos, soaps and foods, as well as alternative therapies such as aromatherapy. We aimed to investigate the biological activities of natural products, the results are presented in the form of three manuscripts. We investigated the antibacterial action of essential oils of tea tree (*Melaleuca alternifolia*), geranium (*Perlagonium graveolens*), palmarosa (*Cymbopogon martinii*) and their major compounds respective 1-terpinen-4-ol, citronellol and geraniol in liquid and vapor phase (Chapter I). The effectiveness of essential oils and their main components such as antimicrobial agents, was demonstrated by direct contact and vapor form using three different methodologies and the information of the damage caused by these vapors using transmission electron microscopy. In another part of the study, we aimed to assess serum biochemical parameters and oxidative stress in rats subjected to inhalation of palmarosa essential oil and geraniol during thirty days (Chapter II). Mices treated with natural products had total cholesterol levels decreased and it was found beneficial effects of the essential oil on oxidative stress. Concluding the study, we evaluated the immunomodulatory action of *C. martinii* essential oil and geraniol as the production of pro- and anti-inflammatory by human monocytes (Chapter III). Different concentrations tested showed no cytotoxic effects on monocytes. The production of tumor necrosis factor (TNF)-α was not affected by *C. martinii* and geraniol, and only the concentration of 5 mg/mL of *C. martinii*, there was a stimulus for their production. Furthermore, at all concentrations of geraniol and *C. martinii* increased by interleukin (IL) -10 by human monocytes. The results showed that the noncytotoxic concentrations of oil and geraniol exerted an anti-inflammatory action, as noted by increased production of IL-10. An important conclusion, geraniol appears to be responsible for the immunomodulatory activity of *C. martinii* essential oil in our test condition. Overall, we observed an effective action of natural compounds in the tested biological activities, demonstrating *in vitro* antimicrobial activity, anti-inflammatory activity in human monocytes and *in vivo* effects of inhalation.

Keywords: natural products; essential oils; major compounds; antibacterial; anti-inflammatory; inhalation; oxidative stress.

1. INTRODUÇÃO:

1.1 Óleos essenciais

Nas plantas, diferentes compostos orgânicos com estruturas de baixo peso molecular, perecem não ter função nos processos básicos de crescimento e desenvolvimento dos vegetais sendo historicamente referidos como produtos naturais ou metabólitos secundários (Gershenzon e Dudareva, 2007).

O metabolismo primário está ligado com as funções de crescimento e desenvolvimento da planta, enquanto que as funções do metabolismo secundário dizem respeito às funções adaptativas das plantas e todos os aspectos de interações químicas com o meio ambiente, são indispensáveis para a sobrevivência de uma população, porém dispensáveis para o crescimento do indivíduo. Os metabólitos secundários (Figura 1) provêm de precursores comuns do metabolismo primário (Hartmann, 1996).

A importância dos produtos naturais na medicina, agricultura e na indústria tem levado a inúmeros estudos sobre a síntese e as atividades biológicas destas substâncias. Os terpenos são o maior grupo de produtos naturais (Gershenzon e Dudareva, 2007).

Os óleos essenciais são metabólitos secundários normalmente líquidos, voláteis, límpidos e raramente coloridos, são compostos complexos que podem conter como por exemplo, aldeídos, compostos fenólicos e terpenos que são seus principais constituintes sendo estes divididos em hemiterpenos (C5), monoterpenos (C10), sesquiterpenos (C15), diterpenos (C20), triterpenos (C30) e tetraterpenos (C40). Um terpeno contendo oxigênio é chamado de terpenóide (Bakkali *et al.*, 2008)

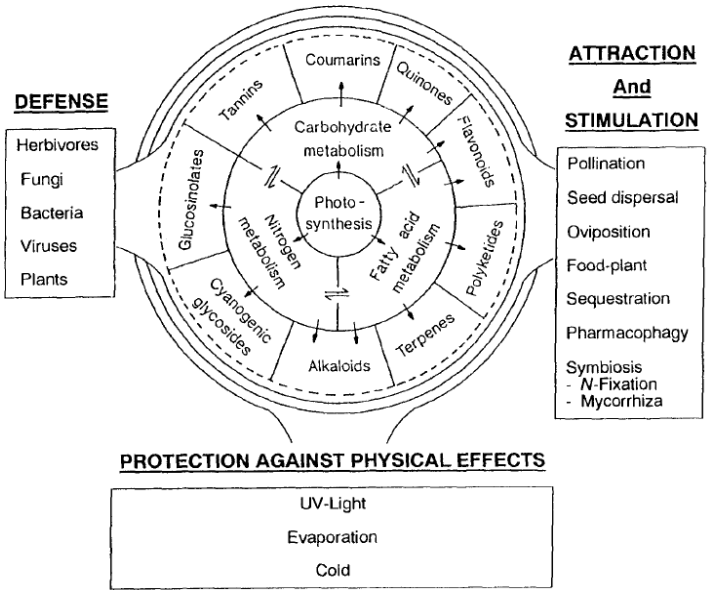

Figura 1. Produção de metabólitos secundários nas plantas a partir do metabolismo primário, estes compostos desempenham atividade na defesa, proteção contra efeitos físicos, atração e estímulo para outros indivíduos (Hartmann, 1996).

Os óleos essenciais são caracterizados por dois ou três principais componentes em concentrações elevadas quando comparados a outros componentes presentes em quantidades vestigiais, podem atuar na proteção das plantas como agentes antibacterianos, antivirais, antifúngicos e inseticidas (Bakkali *et al.*, 2008).

Podem ser extraídos de diferentes partes das plantas, por exemplo, folhas, cascas, flores, brotos, sementes, e assim por diante, existem vários métodos para a extração de óleos essenciais, alguns deles são a destilação á vapor (Figura 2) e a hidrodestilação (Bakkali *et al.*, 2008).

Os rendimentos na produção de óleos essenciais variam de acordo com a forma de obtenção e o material vegetal utilizado, como exemplo dos diferentes rendimentos por hidrodestilação (%v/v) entre diferentes plantas temos *Cymbopogon martinii* (2,05% v/v), *Mentha piperita* (2,22% v/v) e *Ocimum gratissimum* (3,38% v/v) (Duarte *et al.*, 2007).

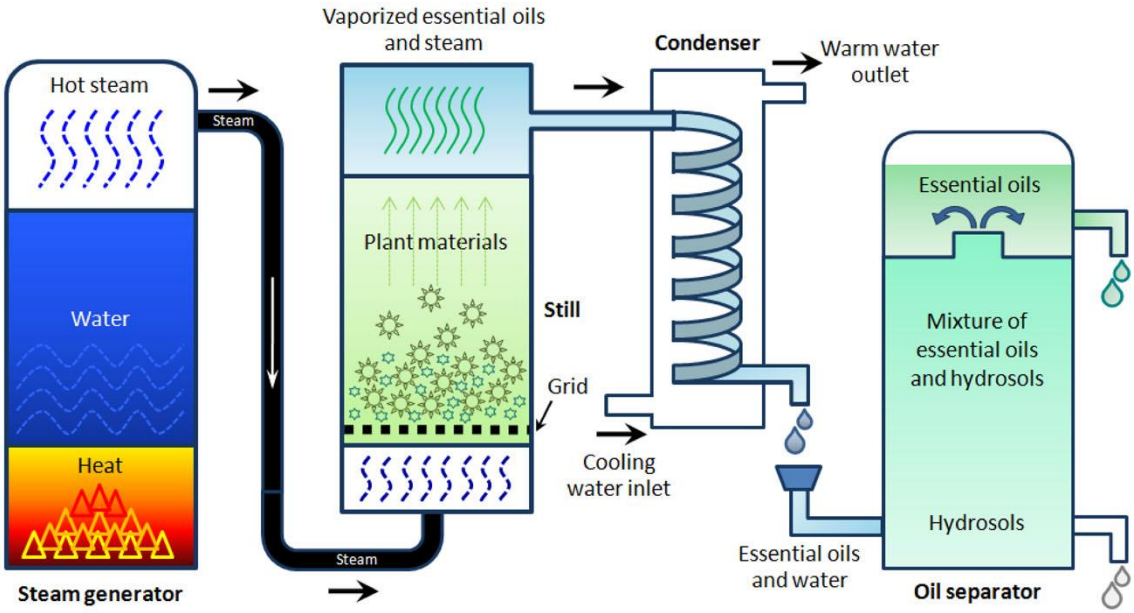

Figura 2: Processo de destilação á vapor de plantas para obtenção de óleos essenciais (Tongnuanchan and Benjakul, 2014), neste processo somente o vapor passa através do material vegetal carreando o óleo essencial, esta mistura do óleo volatilizado e vapor é condensada então óleo essencial e água são captados e separados pela diferença de densidade.

Produtos naturais como os óleos essenciais desempenham diversas atividades biológicas sendo grandes fontes para pesquisa, estão presentes em nossas rotinas, tanto nos produtos que consumimos como xampus, sabonetes e alimentos, assim como em alternativas terapêuticas como a aromaterapia (Bakkali *et al.*, 2008; Vigan, 2010; Tongnuanchan e Benjakul, 2014).

1.2 Propriedades biológicas dos óleos essenciais:

1.2.1 Propriedade antibacteriana

Dentre as inúmeras propriedades biológicas dos óleos essenciais são mencionadas a ação larvicida (Rajkumar e Jebanesan, 2010), atividade antioxidante (Aidi Wannes *et al.*, 2010), ação analgésica, anti-inflamatória (Mendes *et al.*, 2010; Murbach Teles Andrade *et al.*, 2014), fungicida (Carmo, De Oliveira Lima e De Souza, 2008) e antibacteriana (Andrade *et al.*, 2014).

Tão rápido quanto novos antibióticos são introduzidos, micro-organismos podem desenvolver resistência a eles, sendo que esta resistência pode ser codificada por plasmídeos transferíveis e trocada entre as espécies, gêneros e famílias de bactérias (Murray, 2009).

A atividade antimicrobiana dos óleos essenciais *in vitro* tem sido amplamente estudada sobre uma série de micro-organismos (López *et al.*, 2007; Andrade *et al.*, 2014) especialmente devido ao surgimento de bactérias multi-resistentes, representando um desafio no tratamento de infecções.

A propriedade antimicrobiana dos óleos essenciais é considerada de grande interesse para as indústrias alimentícias, farmacêuticas e cosméticas desde que o uso de aditivos naturais ganhou importância como tendência na substituição dos conservantes sintéticos artificiais (Okoh (Okoh, Sadimenko e Afolayan, 2010) *et al.*, 2010), assim como a atividade antioxidante (Olmedo, Nepote e Grosso, 2014).

Estudos sobre a atividade antibacteriana e os mecanismos de ação de óleos essenciais mostraram que o tea tree (*Melaleuca alternifolia*) causa lise e perda da integridade da membrana, devido à saída de íons e inibição da respiração celular bacteriana (Cox *et al.*, 2000; Burt, 2004; Carson, Hammer e Riley, 2006).

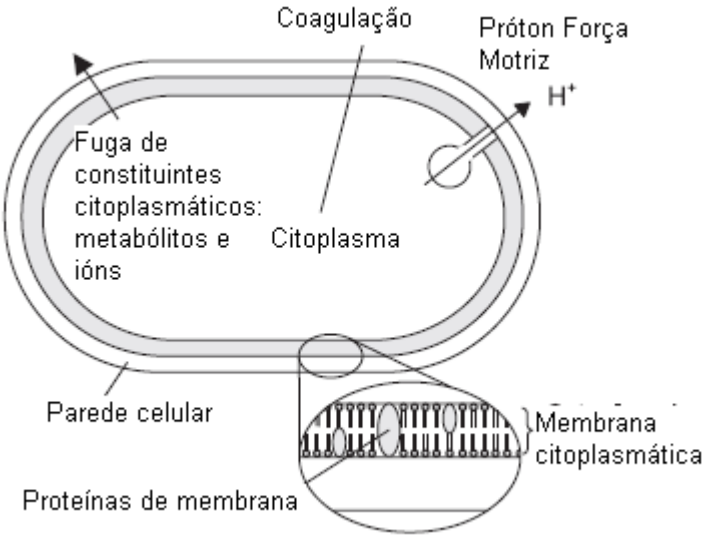

Figura 3. Locais e mecanismos de ação que podem ser sítios para ação de compostos naturais na célula bacteriana (Adaptado de Burt, 2004).

O óleo essencial de palmarosa (*Cymbopogon martinii*) apresenta atividade antibacteriana de amplo espectro, sendo o geraniol o seu composto antimicrobiano responsável por tal atividade (Duarte *et al.*, 2007), os valores da CIM de palmarosa contra treze sorotipos de *Escherichia coli* variou de 100 á 900 μg/mL.

Óleos essenciais de hortelã-pimenta, tea tree e tomilho podem agir como uma solução bucal anti-séptica intracanal eficaz contra patógenos orais, pois exibiram efeito inibitório significativo (Thosar *et al.*, 2013).

A redução de contagem bacteriana foi observada quando submetidas aos vapores de óleos essenciais, demonstrando o potencial antimicrobiano de óleos essenciais no seu estado gasoso e seu uso foi sugerido para desinfecção de ambientes fechados e sistemas de ventilação, *Staphylococcus aureus* exposto ao vapor do óleo essencial de *Satureja montana* durante 240 minutos a concentração de bactérias de 94 UFC/mL caiu para 0,5 UFC/mL (Pibiri *et al.*, 2006).

O vapor de carvacrol é eficaz na inibição do crescimento de *Salmonella* Enteritidis em ágar e na inibição e eliminação desta bactéria na superfície de frango, a concentração de 40% v/v de carvacrol em etanol foi suficiente para não se recuperar células viáveis (Burt *et al.*, 2007).

O vapor da combinação de óleos essenciais de laranja com bergamota (1:1v/v) reduziu *Enterococcus* sp. vancomicina resistentes e *Staphylococcus aureus* meticilina resistentes em superfícies de aço inoxidável de 1,5-3 $\log_{10}$ após 24 horas de exposição, houve redução nos biofilmes e a microscopia mostrou reduções na cobertura de biofilme do disco de aço inoxidável em até 99,5% (Laird, Armitage e Phillips, 2012).

A atividade antibacteriana em fase de vapor foi determinada pelo método do vapor em microambiente contra diferentes estirpes de *S. aureus*, incluindo *S. aureus* meticilina resistentes e isolados clínicos resistentes, os óleos essenciais testados foram *Allium sativum*, *Armoracia rusticana*, *Origanum syriacum*, *Satureja hortensis*, *Satureja montana*, *Thymus serpyllum* e *Thymus vulgaris*; o óleo essencial mais efetivo foi o de *Armoracia rusticana* com MICs variando entre 8,3 a 17 µL/L, os óleos essenciais testados podem ser considerados como produtos naturais anti-*Staphylococcus* eficazes com possibilidades de aplicação específica devido à atividade na fase de vapor, ao contrário da maioria dos agentes antimicrobianos atualmente utilizados para a desinfecção do ar, os óleos essenciais são de baixa toxicidade e podem ser utilizados em diferentes ambientes, eles possuem uma propriedade única de elevada volatilidade que não é observada em outros agentes antimicrobianos não-tóxicos (Nedorostova *et al.*, 2011).

1.2.2 Propriedade imunomoduladora e antioxidante:

O geraniol é abundante em inúmeras plantas e esta sendo amplamente utilizado como fragrância em produtos cosméticos e doméstico, bem como pode representar uma nova classe de agentes terapêuticos contra o câncer, além de apresentar propriedades biológicas incluindo a antimicrobiana, antioxidante e atividade anti-inflamatória (Chen e Viljoen, 2010).

Quanto à propriedade imunossupressora, investigaram *in vitro* o geraniol através da proliferação de linfócitos por meio do modelo de enxerto de transplante cardíaco em ratos, tendo verificado que este preveniu a rejeição aguda do enxerto (Ji *et al.*, 2002), enquanto que os óleos essenciais de capim limão, gerânio, menta e principais constituintes (citral, citronelol, geraniol e carvona) claramente suprimiram o fator de necrose tumoral-α (TNF-α) na concentração de 0,0125% (Abe *et al.*, 2003).

O óleo essencial de tea tree é tóxico para os monócitos na concentração de 0,016% v/v, embora os componentes solúveis em água do óleo em concentrações equivalente a 0,125% tenham suprimido significativamente a produção induzida por LPS de TNF-α, IL-1b e IL-10 (em cerca de 50%) e PGE2 (em aproximadamente 30%) após 40 h (Hart *et al.*, 2000).

Em um ambiente inflamatório, o aumento da vulnerabilidade das células e a liberação de radicais resulta em danos no DNA e mitocôndrias, com alterações apoptóticas em vários sistemas. Este esgotamento precisa ser superado repondo-se as defesas antioxidantes, o esgotamento das defesas antioxidantes devido à geração de espécies reativas de oxigênio, resultado da resposta imunológica celular, contribui para estes danos celulares (Soory, 2012).

O estresse oxidativo desempenha um papel central no desenvolvimento de doenças humanas, espécies reativas de oxigênio (ROS), que inclui o peróxido de hidrogênio, ânion superóxido, peróxidos lipídicos, hipoclorito e radical hidroxila estão envolvidos no crescimento, diferenciação, progressão e morte da célula, eles podem reagir com os lipídios de membrana,

ácidos nucleicos, proteínas, enzimas e outras moléculas pequenas. Baixas concentrações de ROS desempenham um papel indispensável na sinalização intracelular e defesa contra agentes patogênicos, enquanto que as quantidades mais elevadas de ROS desempenham um papel em doenças como a artrite, diabetes, aterosclerose e isquemia, por isso antioxidantes podem atuar contra o acúmulo de ROS e auxiliar em sua eliminação do sistema (Rajendran *et al.*, 2014).

A busca por antioxidantes naturais com a virtude de ser atóxico deu origem a um grande número de estudos sobre o potencial antioxidante de óleos essenciais (Amorati, Foti e Valgimigli, 2013)

O óleo essencial de *Artemisia argyi* demonstrou potente atividade antioxidante *in vitro* (Huang *et al.*, 2012).

Propriedades antioxidantes do óleo essencial de palmarosa e citronela foram testadas em células de linfócitos humanos, a atividade antioxidante foi avaliada por 2,2-difenil-1-picrilhidrazilo-DPPH + eliminação de radicais livres e ensaio de peroxidação lipídica, uma atividade antioxidante significativa dose dependente foi observada, podendo estes óleos essenciais constituírem uma fonte natural de antioxidante nova e segura. (Sinha, Biswas e Mukherjee, 2011)

1.3 Aromaterapia

A aromaterapia, termo este criado em 1927 pelo químico francês René-Maurice Gattefossé (Stevensen, 1998), é a terapia que faz uso dos óleos essenciais para a promoção e manutenção da saúde (Cavanagh e Wilkinson, 2002). Os óleos essenciais podem ser aplicados externamente como em massagem terapêutica misturado com um óleo vegetal, através de uma compressa, gel, spray, banho e inalação (Stevensen, 1998).

Segundo os premiados do Nobel de Fisiologia ou Medicina em 2004, mamíferos tem 1000 genes para receptores de odores dos quais 347 códigos para receptores de odor funcionais, os seres humanos podem cheirar entre quatro e dez mil diferente odores, e cada tipo de receptor responde a um conjunto de moléculas odoríferas, e cada odorante interage com uma variedade de receptores (Buck e Axel, 1991).

A aromaterapia é um tratamento tradicional que ao utilizar os óleos essenciais via inalação, a sua ação se inicia com uma molécula aromática combinada com um receptor para aroma, a molécula passa pela cavidade nasal e se adere ao epitélio olfatório que envia esta informação através de seu nervo diretamente para o hipocampo, sistema límbico e corpo amidaloide que consequentemente dispararão estímulos no controle do sistema nervoso autônomo e no controle secretório interno (Jimbo *et al.*, 2009).

Receptores olfativos estão sintonizados com propriedades físicas e químicas de moléculas odoríferas, compostos odorantes podem agir sobre o sistema neuroendócrino, neurotransmissores e neuromoduladores, influenciando o comportamento psicológico, bem como a inalação destes compostos modula vias fisiológicas (Angelucci *et al.*, 2014).

A pesquisa em aromaterapia tem sido cada vez maior para o uso como uma terapia adjuvante no gerenciamento de transtornos psiquiátricos e para a elucidação de seus mecanismos terapêuticos. A inalação de óleos essenciais podem enviar sinais para o sistema olfativo e

estimular o cérebro e neurotransmissores (serotonina e dopamina, por exemplo). A maioria dos estudos, bem como a experiência clínica aplicada, tem indicado que vários óleos essenciais, tais como a lavanda, limão e bergamota podem ajudar a aliviar o stress, ansiedade, depressão e outros transtornos do humor *(Almeida et al., 2004; Lv et al., 2013)*.

1.4 Objetivo geral:

O objetivo geral foi avaliar a atividade antibacteriana e anti-inflamatória *in vitro* e o perfil bioquímico e estresse oxidativo de ratos submetidos à inalação de óleos essenciais e compostos isolados. Esta tese está dividida em três capítulos conforme os objetivos específicos e escritos em forma de artigos que foram submetidos a periódicos indexados.

1.4.2 Objetivos específicos:

- Investigar a ação antibacteriana de três óleos essenciais que são frequentemente utilizados na aromaterapia, tea tree (*Melaleuca alternifolia*), gerânio (*Perlagonium graveolens*), palmarosa (*Cymbopogon martinii*) e seus respectivos compostos majoritários 1-terpinen-4-ol, citronelol e geraniol em sua fase líquida e gasosa (Capítulo I);
- Analisar o perfil bioquímico sérico e o estresse oxidativo de ratos submetidos à inalação subcrônica do óleo essencial de palmarosa (*Cymbopogon martinii*) e seu composto majoritário, o geraniol (Capítulo II);
- Avaliar a ação imunomoduladora do óleo essencial de palmarosa e geraniol quanto à produção de citocinas pró- e anti-inflamatórias por monócitos humanos (Capítulo III).

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Capítulo I*

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The antibacterial effects of *Melaleuca alternifolia*, *Pelargonium graveolens* and *Cymbopogon martinii* essential oils and major compounds on liquid and vapour phase

Bruna Fernanda Murbach Teles Andrade[a], Lidiane Nunes Barbosa[a], Fernanda Cristina Bérgamo Alves[a], Mariana Albano[a], Vera Lúcia Mores Rall[a], José Maurício Sforcin[a], Ana Angélica Henrique Fernandes[b], Ary Fernandes Júnior[a*]

[a]*Department of Microbiology and Immunology,* [b]*Department of Chemistry and Biochemistry, Biosciences Institute, UNESP, Campus of Botucatu, SP, Brazil, 18618-970.*

* Corresponding author. E-Mail:ary@ibb.unesp.br; Phone: (+55) 14 38800412; fax: (+55) 14 3815374 (Fernandes Júnior, A.)

Essential oils (EOs) are natural products from plant secondary metabolism. The antibacterial activity of EOs from *Melaleuca alternifolia*, *Pelargonium graveolens* and *Cymbopogon martinii* and terpineol, citronellol and geraniol, were investigated both in their liquid and vapour phases against Gram positive and negative strains. In microdilution tests geraniol showed a MIC value of 0.05 % v/v against almost all strains; with the inverted plate test the largest zone of inhibition (31 mm) was formed by terpineol against *Klebsiella pneumoniae,* and in determining the percent bacterial reduction in a vapour microenvironment *K. pneumoniae* was the strain most sensitive to the tested compounds. The effectiveness of compounds as antibacterial agents were demonstrated, highlighting the damage caused by *C. martinii* EO and geraniol vapours to strains by transmission electron microscopy, observing that geraniol was probably responsible for the antibacterial effect of *C. martinii* EO.

Keywords: essential oils; antibacterial; transmission electron microscopy.

Introduction

Essential oils (EOs) are secondary metabolites from plants and highly enriched in compounds with an isoprene structure, the terpenes. Such compounds may contain additional elements, usually oxygen, and are termed terpenoids, wich are synthesized from acetate units and originated from fatty acid metabolism (1). These products are typically liquid, volatile, clear and rarely coloured, characterized by a strong odour and display antibacterial, antiviral, antifungal, insecticidal (2), antioxidant (3), anti-inflammatory (4), antimicrobial (5), antidepressant and anxiolytic activities (6). Traditional treatments or therapy e.g. aromatherapy, using EOs volatilization have also been reported (7).

EOs have been used in the medical, food and cosmetics industries due to its antimicrobial action. EOs have been studied in liquid form (5, 8) as well as vapours (9), although studies have strongly focused on methods to clarify their properties based upon their liquid phase (10).

The use of EOs in the vapour phase could offer several advantages for antimicrobial activities such as efficacy without requiring direct contact, and the ease application (11) as well as the EO vapours could be used to air disinfection proceedings (12).

However, in developing fumigation processes for medical purposes, it is important to first evaluate the efficacy and safety of EOs in the gaseous state, although their antimicrobial activity and cytotoxicity have mostly been measured in their liquid phase. Thus, standard procedures for evaluating their activities have already been established, and consequently few studies have been carried out with EO vapours (13).

Infectious diseases constitute an important, global health burden, and bacterial control is usually achieved by disinfection (usually with liquid disinfectants). However,

liquid disinfectants exert an antibacterial effect at topically only (e.g. liquid phenol disinfectants), and aseptic environments such as surgical rooms, clinical and food microbiology laboratories and the pharmaceutical industry require special attention (14).

Community and hospital pathogens, including *Staphylococcus aureus*, *Salmonella*, *Enterococcus* sp., *Escherichia coli* and *Pseudomonas aeruginosa*, are some of the main multi-drug-resistant bacteria (15) and natural products and herbal derivatives exerting antimicrobial properties have been investigated due to its biological effects (16).

The plants, *Melaleuca alternifolia, Pelargonium graveolens and Cymbopogon martinii* used in this study have been used empirically for centuries.

Cymbopogon martinii is mainly used in perfume industry as well as employed traditionally in diabetes treatment and has been documented in Ayurveda medicine on urinary tract infections as anti-inflammatory and as diuretic properties (17).

Tea tree EO is derived from the australian native plant *Melaleuca alternifolia* is largely employed for its antimicrobial properties, incorporated as the active ingredient in many topical formulations used to treat cutaneous infections and marketed as a medication for various ailments (18).

Pelargonium graveolens belongs to the *Geraniaceae* family, and its leaves are popularly used as flavouring, insect repellent, in perfume and in aromatherapy for the treatment of gastrointestinal diseases and throat infections (19).

Antimicrobial properties of EOs from the leaves of *P. graveolens* have been associated with their high contents of oxygenated monoterpene, and were active against Gram positive than Gram negative bacteria revealing an *in vitro* antibacterial activity, confirmed by low minimal inhibitory concentrations (20).

Thus, gram positive and negative ATCC standard strains and a hospital environment strain were tested for their susceptibility to tea tree (*Melaleuca alternifolia*), geranium (*Perlagonium graveolens*) and palmarosa (*Cymbopogon martinii*) EOs and their major compounds terpineol, citronellol and geraniol, respectively, were evaluated in both their liquid and vapour phases. Transmission electron microscopy was also carried out to determine the effects of *C. martinii* EO and geraniol on bacterial structure.

Experimental

Essential oils and major compounds

Essential oils were commercially purchased from "By Samia", which markets essential oils in São Paulo, Brazil, and its identification is: Pure essential oil– By Samia 10 mL, with batch numbers, Geranium (*Perlagonium graveolens* L.) LOT 1221010BS, Palmarosa (*Cymbopogon martinii* L) LOT 2311NB5 and Tea Tree (*Melaleuca alternifolia*) (Maiden & Betche) Cheel LOT 341105BS, with chemical analysis by gas chromatography coupled to mass spectrometry (GCMS).

The percentage of compounds in each essential oil by GCMS, *Melaleuca alternifolia,* 1-terpinen-4-ol (45.48), gama-terpinene (18.77), alpha-terpinene (8.67), alpha-terpineol(4.18), para-cimene(3.66), 1.8 cineol (3.45), alpha-terpinolene (3.23), alpha-pinene (2.44), limonene (0.90), alpha-tujene(0.90), mircene(0.82), beta-pinene(0.71) alfa-phelandrene (0.35); *Cymbopogon martinii,* geraniol (57.49), geranyl acetate (13.56), linalool (1.71), beta-cariofilen (1.07), ocimene (0.27) and *Pelargonium graveolens,* citronellol (28.57), geraniol (20.99), menthone(5.76), alpha-muurolene (1.83), neryl acetate (1.50), isomenthone (1.32), rose oxide (1.26), alpha-bourbonene (1.15), geranyl acetate (0.92), citronellyl acetate (0.72).

The compounds were selected based on gas chromatography, these compounds are present in greater amounts in the essential oils of plants chosen, terpineol, citronellol and geraniol were purchased from Sigma Aldrich ® (purity > 98%).

Bacterial Strains

The standard *American Type Culture Collection* (ATCC) strains of *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 10100, *Salmonella* Enteritidis ATCC 13076, *Escherichia coli* ATCC 43895 and *Pseudomonas aeruginosa* ATCC 2785 as well as the strains isolated from human clinical specimens, *S. aureus, E. coli, Salmonella* Typhimurium*, P. aeruginosa* and *Klebsiella pneumoniae* and *S. aureus* strains were obtained from the hospital environments were assayed by susceptibility assays.

All strains were stored at -80°C in Brain Heart Infusion (BHI) broth plus glycerol in the culture collection of the Department of Microbiology and Immunology, Biosciences Institute, Unesp, campus of Botucatu. Prior to use, strains were seeded in blood agar to check their viability and purity.

Bacterial Sensitivity Tests

Microdilution assays

Bacterial strains were previously cultured (37 °C/24 h) in BHI broth and sensitivity assays were performed by broth microdilution to determine the minimal inhibitory concentration (MIC) values were recorded. Ninety six wells microplates with BHI plus Tween 80 0.5% were used and concentrations of 0.05; 0.1; 0.2; 0.5; 1.0; 2.0; 3.0; 4.0; 5.0; 6.0; 7.0; 8.0; 9.0 and 10% v/v of each compound were prepared. Each well received

a volume of bacteria from the standardized suspensions to attain approximately 10^5 CFU/mL (colony forming units).

The microplates were incubated (37°C/24 h) and the results were recorded after adding the indicator dye redox resazurin 0.01%. Bacterial growth is indicated by a colour change from violet to pink (or shade) and the lowest concentration without a colour change was taken as the MIC value (21).

Vapour diffusion assay by the inverted plate method

Sterile filter paper discs 9 mm in diameter containing 15µl of each substance with and without ethyl acetate (1:1) were fixed inside the covers of Petri dishes containing Mueller-Hinton ágar (MHA) and inoculated with ATCC standard and plus *Klebsiella pneumoniae* from hospital environment strains, then placed in an inverted position, so that only the vapour of each EO was in contact with the agar surface. After 37 °C/24 h, the zones of inhibition were recorded in millimeters (mm). Assays were performed in triplicate and ethyl acetate was used as a negative control (11). Ethyl acetate was used as a solvent because it is less toxic than other organic solvents containing halogens and benzenes, and volatilizes efficiently at room temperature (22).

Bacterial reduction count in vapour microenvironment

Open RODAC Petri dishes (120 cm³ of air) with MHA were inoculated with 5 µL of ATCC standard and *Klebsiella pneumoniae* from hospital environment strains using standardized bacterial suspension, 10^6 CFU/mL. Different concentrations of EOs and their major compounds (1000 µg/cm³ of air), with or without dilution in ethyl acetate (1:1), were placed on strips of filter paper 10 x 1.7 cm in dimensions, placed inside the Petri dishes and incubated at 37 °C/24 h.

Petri dishes were sealed with parafilm to preserve the vapour microenvironment generated by the compounds, adapted from Inouye (22). After this period colony forming units (CFU) were counted in each RODAC plates. Tests were performed in duplicate with control strains and ethyl acetate was used as negative control. The percentage of bacterial colonies was compared to the percentage of control without oil and compounds considered as 100%.

Transmission Electron Microscopy (TEM)

C.martinii EO and geraniol were chosen to exemplify a possible damage caused by the vapour of these compounds due to the results of geraniol in the sensitivity test performed in microdilution, as this is the major compound of C. *martinii* EO and geraniol were tested against Gram positive and Gram negative strains and conduced together with bacterial reduction count in vapour microenvironment test.

S. aureus, *P. aeruginosa*, *S.* Enteritidis and *E. coli* ATCC standard strains were incubated overnight in BHI at 37°C. RODAC plates containing MHA were then inoculated with 5 µL standardized bacterial suspension at 10^6 CFU/mL before placed in open Petri dishes (120 cm^3 of air). Then 15 µL of each compound was used to achieve 1000 µg/cm^3 air of *C. martinii* EO and geraniol were placed on 10 x 1.7 cm strips of filter paper inside the Petri plates and incubated. All treatments and controls were incubated at 37°C for 2 h and then centrifuged. Cells were washed twice with 0.1 M phosphate buffered saline -PBS (pH 7.4) and fixed with 2.5% (v/v) of glutaraldehyde in 0.1 M PBS overnight at 4 °C. Then, cells were post fixed with 1% (w/w) OsO_4 in 0.1 M PBS for 2 h at room temperature and washed three times with the same buffer before dehydration through a graded series of ethanol solutions (30%, 50%, 70%, 90%, and 100%). Stained bacteria were photographed using a transmission electron microscope.

Statistical analysis

Kruskal-Wallis One Way Analysis of Variance on Ranks ($p < 0.05$) was used in the microdilution assay, and the Mann-Whitney Rank Sum Test ($p < 0.05$) was used in reverse plating tests.

Results and Discussion

Our aim was to evaluate the antibacterial action of these EOs and their major compounds both in their liquid and vapour against Gram positive and negative bacterial strains.

According to the antibacterial microdilution susceptibility tests (Table 1), *P. aeruginosa* strain was highly resistance and geraniol showed an MIC value of 0.05 % v/v against the tested strains.

Table 1. Values of MIC (%v/v) in microdilution test with palmarosa EO, geraniol, geranium EO, citronellol, tea tree EO and terpineol against Gram positive and Gram negative strains.

Bacterial Strains	Palmarosa	Geraniol	Geranium	Citronellol	Tea Tree	Terpineol
S. aureus 25923	0.5	0.05	0.5	0.2	0.5	0.5
S. aureus(human clinical)	0.5	0.05	0.5	0.5	0.5	0.2
S. aureus(hospital environment)	0.5	0.05	1.0	0.2	0.5	0.2
S. epidermidis 12228	0.5	0.05	0.05	0.05	0.5	0.05
E. faecalis 10100	0.05	0.05	0.05	0.05	0.1	0.1
S. Enteritidis 13076	0.5	0.05	0.05	0.2	0.5	0.05
S. Typhimurium(human clinical)	0.5	0.05	0.2	4	0.5	0.1
E. coli 43895	0.5	0.05	0.5	0.1	0.2	0.1
E. coli(human clinical)	0.5	0.05	1.0	2.0	0.5	0.05
P. aeruginosa 27853	10.0	8.0	7.0	7.0	4.0	4.0
P. aeruginosa(human clinical)	9.0	10.0	8.0	8.0	4.0	0.5
K. pneumoniae(hospital environment)	0.5	0.05	0.5	0.1	0.5	0.1

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference when $p>0.05$.

Geraniol appeared to be a potent inhibitor of plasma membrane efflux mechanisms (23), what could explains at least in part its highest antimicrobial activities against almost all bacterial strains in this study.

Previous findings revealed that *P. aeruginosa* also showed greater resistance to the tested compounds and no MIC was obtained for these strains except with clove oil, with $MIC_{90\%}$ value of 8.29 mg/mL (24).

Vaara et al. (25) reported that the outer membrane of Gram-negative bacterial wall is impermeable to macromolecules and allows only limited diffusion of hydrophobic substances through its lipopolysaccharide-covered surface, and *P. aeruginosa* was once cited as a resistant bacteria among other Gram-negative species in susceptibility tests with EOs (26).

In addition to the presence of hydrophobicity outer membrane in the Gram negative wall, the *P. aeruginosa* synthesizes an exopolysaccharide called alginate in response to environmental conditions to protect from adversity in its surroundings and also enhances adhesion to solid surfaces (27). Thus, *P. aeruginosa* was less sensitive to natural products than the other tested Gram-negative bacteria and this may be due to the exopolysaccharide produced by this bacteria.

Another explanation regarding to its resistance may be due to the efflux pump.

The role of MexAB-OprM efflux pump in tolerance to tea tree EO and terpinenol interplay between the MexAB-OprM and MexCD-OprJ pumps may contribute to the tolerance to some components of tea tree EO, including 1,8-cineole and terpineol. This work further extends the broad range of substrates for MexAB-OprM to cyclic monoterpenes (28). In our study, the major compounds, citronellol and geraniol, are monoterpenes even as terpineol and this resistance was available for the other compounds by the efflux pump.

EO vapours have been the subject of other researches, showing an effective action through vapour contact (12, 29, 30). In the inverted plate test (Table 2) inhibition zones may be less than 9 mm although disk filter paper are 9 mm, because it is not in direct contact with the culture medium and the inoculated bacterium, halos are formed directly by vapour of these compounds, forming halos inhibition.

Table 2. Mean inhibitory zones (mm) from plate reverse test of each compound alone (C) and form by 30 μL of each compound added ethyl acetate (1:1) (EA) for Gram positive and Gram negative strains.

Bacterial Strains	Palmarosa		Geraniol		Geranium		Citronellol		Tea Tree		Terpineol	
	C	EA	C	EA	C	EA	C	EA	C	EA	C	EA
S. aureus 25923	7.0	9.0	12.5	12.0	10.0	11.5	12.0	15.5	12.0	16.0	17.5	21.0
S. epidermidis 12228	6.0	12.0	9.0	15.0	12.0	9.0	12.0	18.0	18.0	6.0	11.0	20.0
E. faecalis 10100	0.0	0.0	0.0	6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	13.0
S. Enteritidis 13076	0.0	0.0	0.0	0.0	6.0	0.0	6.0	0.0	17.5	14.0	20.0	23.0
E. coli 43895	0.0	0.0	0.0	0.0	14.5	0.0	0.0	0.0	15.5	19.0	18.0	19.5
P. aeruginosa 27853	0.0	0.0	0.0	6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
K. pneumoniae(hospital environment)	0.0	0.0	8.5	6.0	0.0	0.0	0.0	17.0	22.5	25.0	26.5	31.0

The difference in the median values between the groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference when $p>0.05$.

Tea tree oil produced inhibition zones, except with *P. aeruginosa,* but the largest inhibition zone was produced by terpineol against *K. pneumoniae* (31 mm) from hospital environment.

S. aureus and *S. epidermidis* were susceptible to the antibacterial effect of all compounds by plate reverse. Using orange essential oil in a disc-diffusion vapour assay mean halos for diverse *S. aureus* strains ranged from 17.8 to 78.8 mm (31).

Edwards-Jones et al. (29) also found inhibition zones by vapour contact, of which the largest was generated by tea tree EO on the *S. aureus* NCTC 6571 strain (25 mm).

Ethyl acetate used as a solvent control did not exert any antibacterial Inouye et al. (22), used a solution with essential oils and ethyl acetate for a rapid evaporation, and only essential oils lead to the most effective antibacterial action for *S. aureus* and *E. coli*.

According to the reduction percentage of bacterial numbers in the vapour microenvironment (Table 3), most of the strains showed a greatest sensitivity to tea tree EO, including 96,3% against *Salmonella* Enteritidis and 100% against *K. pneumoniae*.

K. pneumoniae strain was the most sensitive bacterial specie for all. This bacterium is a human nosocomial pathogen and an important agent in community-acquired infections (e.g pneumonia and urinary tract infections) (32).

Table 3. Percentage reduction of bacterial strains treated with 1.000 µg/cm³ of air for each compound alone (C) and with ethyl acetate (1:1) (EA)

Bacterial Strains	Palmarosa		Geraniol		Geranium		Citronellol		Tea tree		Terpineol	
	C	EA	C	EA	C	EA	C	EA	C	EA	C	EA
S. aureus 25923	60.5	45.8	56.0	45.8	94.0	36.6	76.4	55.0	85.2	96.6	93.0	86.6
S. epidermidis 12228	84.6	20.8	89.0	78.3	79.3	41.7	82.8	59.3	81.2	68.1	90.0	69.2
E. faecalis 10100	60.0	45.0	59.6	51.6	78.2	33.3	61.3	33.3	57.2	86.6	56.7	33.3
S. Enteritidis 13076	0.0	13.0	0.0	30.0	12.3	0.0	11.0	0.0	96.3	100.0	0.0	70.0
E. coli 43895	0.0	48.2	13.6	74.6	41.7	6.6	16.6	65.3	81.1	86.9	30.5	33.3
P. aeruginosa 27853	71.0	0.0	45.0	31.8	71.0	0.0	53.0	5.0	75.2	26.6	47.0	22.2
K. pneumoniae (hospital environment)	99.0	14.2	98.0	53.3	100.0	51.0	99.0	52.3	100.0	85.7	100.0	33.3

The percent inhibition found by López et al. (33) against *E. faecalis* and *L. monocytogenes* with clove and cinnamon EOs did not exceed 35%, while in our experiments tea tree EO was able to inhibit *E. faecalis* growth with 86.6% of count reduction.

A consistent mechanism of action has been described concerning tea tree EO, including loss of intracellular material, inability to maintain homeostasis, and inhibition

of respiration after treatment with tea tree EO and/or components, involving loss of membrane integrity (18).

Sealed box with a blend of oils reducted the growth of staphylococci species including 16 EMRSA (epidemic methicillin-resistant *S. aureus*), the compounds in this study, an EO blend containing lemongrass and geranium- BioScent^{TM} , therefore have potential use as volatiles to reduce numbers of this bacteria, the growth of *Staphylococcus* sp., including meticillin-resistant *S. aureus* (MRSA), were reduced to 38% after 20 hours of exposure to BioScent^{TM} vapour, the results showed likewise a greater susceptibility of the *S. epidermidis* strain while the *S. haemolyticus* strain was the least susceptible (12).

Experiment carried out using a sealed box demonstrated antibacterial activities by vapour contact against *S. aureus* strains, with EO concentrations ranging from 6.25 to 800 mg/L air (22).

The ATCC *S. epidermidis* and *S. aureus* strains were susceptible, even though resistance to antiseptic solutions has increased globally (15). An *in vitro* antimicrobial resistance assay showed the resistance of *S. epidermidis* ATCC 12228 to methicillin and tetracycline, whereas this strain was susceptible to fusidic acid, vancomycin, oxacillin, erythromycin, rifamycin, chloramphenicol and fluorquinolone (34).

In our research, was observed that with different methodologies and for all antimicrobial compounds, the *S. epidermidis* ATCC strain was highly inhibited by the vapour state as well as in the liquid phase of these products.

An important characteristic of EOs and major compounds is their hydrophobicity, which enables them to partition with the lipids of the bacterial cell membrane and mitochondria, disturbing cell structures and rendering them more permeable (15).

Goñi et al. (35) reported not statistically significant of inhibition zones between a combination of EOs and the isolated compounds, which agrees with our results that also there were no statistical difference between inhibition by EOs and their major compounds using gaseous phase assays.

Visual information is useful in providing insights into the microstructures of cells, characterizing the type and magnitude of changes that occur in cell composition in response to treatment. Transmission electron microscopy may help to understand how and why a treatment is effective against a particular organism, illustrating changes and explain possible mechanisms of action, including disruption of plasma membranes by localized hyper-acidification and disruption of membrane transport and/or electron transport (36).

Transmission electron microscopy (Figure 1) data revealed that there was a uniformity in cell walls. Control Gram-negative bacteria (B,C,D) were rod shaped, with double layers of the outer membrane closely apposed to the cytoplasmic membrane, and dispersed nuclear material.

In treatments with EO vapours (A1, A2, B2, D1), there was a evidence that the cytoplasmic membranes were bulging and/or ruptured, and cells appeared to be discharging intracellular materials. The cell wall became separated from the wall after treatment (C1, D2) and some vacuolization appeared in B1 and D2.

The features presented by bacteria treated with EO vapours were very similar to those subjected to direct contact with other products (11, 36).

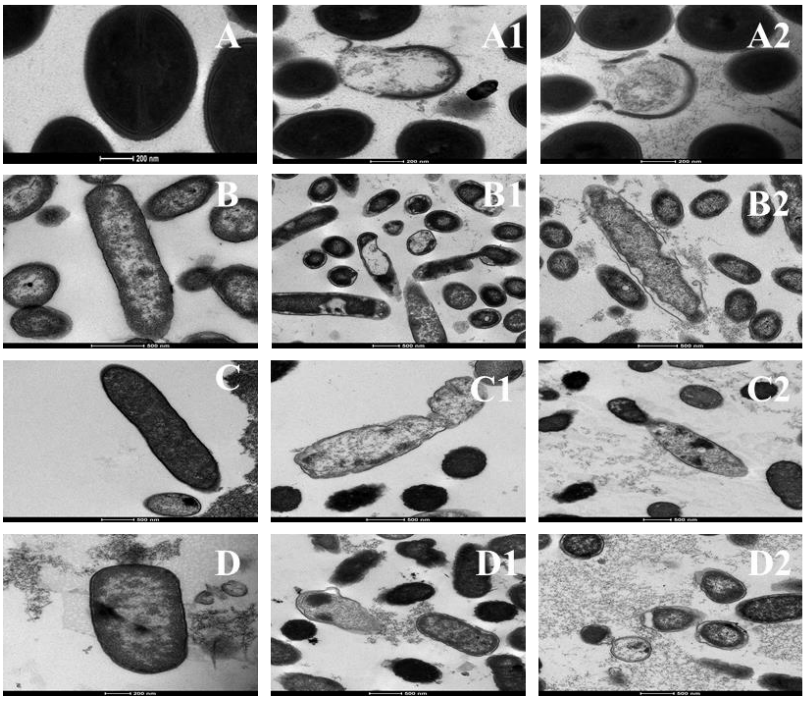

Figure 1. Transmission electron microscopy from ATCC standard bacteria, A- *S. aureus*, B- *P. aeruginosa*, C- *S.* Enteritidis, D- *E. coli* controls; A1, B1, C1, D1-treatments with 1000 μg/cm³ of *C. martinii* EO; A2, B2, C2, D2-treatments with 1000 μg/cm³of geraniol.

Geraniol is the major compound of *C. martinii* EO and its the concentration was 57.49%. According to the results of microdilution assays and through the damage found by transmission electron microscopy, one may suggest that geraniol was probably responsible for the antibacterial activity of *C. martinii* EO.

The use of EOs for topical administration or as penetration enhancers for antiseptics are promising. EOs could act as biopreservatives, reducing or eliminating pathogenic bacteria (15). According to Soković (37), EOs have a potential clinical use because of their very high specific activity; i.e., they may be used at low and non-toxic concentrations for the prevention and treatment of intestinal diseases in animals and humans caused by *E. coli*, *Salmonella* and other pathogenic bacterial species.

There are few published researches on the toxicity of EO vapours *per se*; however, in the future this needs to be explored before they can be utilized as commercial antimicrobial agents.

In conclusion, data revealed the effectiveness of these EOs and their respective major components as antibacterial agents, either by direct or vapour contact, and highlight the damage caused by these vapours to bacteria with clinical importance.
In microdilution test, the geraniol inhibited the bacteria growth at a lower concentration (e.g. 0.05% v/v), except for *P. aeruginosa* that was less sensitive than other bacteria.

These results may be due to the produce of exopolysaccharide of this bacteria, however, this protection by alginate should be the subject of future studies. The transmission electron microscopy illustrated the damage caused by EOs vapors and reinforced the idea that geraniol was probably responsible for the antibacterial effect of *C. martinii* EO.

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Research Article

Effect of Inhaling *Cymbopogon martinii* Essential Oil and Geraniol on Serum Biochemistry Parameters and Oxidative Stress in Rats

Bruna Fernanda Murbach Teles Andrade,[1] Camila Pereira Braga,[2] Klinsmann Carolo dos Santos,[2] Lidiane Nunes Barbosa,[1] Vera Lúcia Mores Rall,[1] José Maurício Sforcin,[1] Ana Angélica Henrique Fernandes,[2] and Ary Fernandes Júnior[1]

[1]*Department of Microbiology and Immunology, Institute of Biosciences, UNESP, 18618-970 Botucatu, SP, Brazil*
[2]*Department of Chemistry and Biochemistry, Institute of Biosciences, UNESP, 18618-970 Botucatu, SP, Brazil*

Correspondence should be addressed to Ary Fernandes Júnior; ary@ibb.unesp.br

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Th effects of the inhalation of *Cymbopogon martinii* essential oil (EO) and geraniol on Wistar rats were evaluated for biochemical parameters and hepatic oxidative stress. Wistar rats were divided into three groups ($n = 8$): G1 was control group, treated with saline solution; G2 received geraniol; and G3 received *C. martinii* EO by inhalation during 30 days. No signific nt differences were observed in glycemia and triacylglycerol levels; G2 and G3 decreased ($P < 0.05$) total cholesterol level. The e were no differences in serum protein, urea, aspartate aminotransferase activity, and total hepatic protein. Creatinine levels increased in G2 but decreased in G3. Alanine aminotransferase activity and lipid hydroperoxide were higher in G2 than in G3. Catalase and superoxide dismutase activities were higher in G3. *C. martinii* EO and geraniol increased glutathione peroxidase. Oxidative stress caused by geraniol may have triggered some degree of hepatic toxicity, as verifie by the increase in serum creatinine and alanine aminotransferase. Therefore, the beneficial eff cts of EO on oxidative stress can prevent the toxicity in the liver. This proves possible interactions between geraniol and numerous chemical compounds present in *C. martinii* EO.

1. Introduction

Plants synthesize around 200,000 secondary metabolites or specialized phytochemicals, of which essential oils (EOs) constitute an important group [1]. The e compounds can be extracted from plant tissues (e.g., stem, leaves, fl wers, and roots) by several procedures (e.g., hydrodistillation and steam distillation) [2]. The e compounds are mostly terpenes, which are commonly used in pharmaceutical industries and have therapeutic benefits and promote welfare, especially when used in aromatherapy procedures [3].

Cymbopogon martinii (Roxb.), Watson, popularly known as palmarosa, exhibits benefici l effects on several central nervous system pathologies, mainly neuralgia, epileptic, and anorexia [4]. There are a few reports on its eff cts; still *C. martinii* has attracted many researchers' attention due to its antimicrobial, antigenotoxic, and antioxidant activities [5–8]. Countries such as India, Brazil and Madagascar have the practice to produce EOs from this plant.

Geraniol, the major constituent of *C. martinii* EO, is an acyclic monoterpenoid that is abundant in many plants [9]. It may represent a new class of therapeutic agents against pancreatic [10] and colon cancers [11] and has several biological properties, including antimicrobial, antioxidant and anti-infl mmatory activities [12]. Geraniol is also an important constituent of ginger, lemon, lime, lavender, nutmeg, orange and rose EOs [13]. Also, it is used as a flavoring agent and was determined to be safe at the current levels of intake by the Joint Expert Committee on Food Additives

of Food and Agriculture Organization—FAO/World Health Organization—WHO [14].

Aromatherapy is a traditional treatment that uses EOs. Its effects begin when the aromatic molecule passes through the nasal cavity and adheres to the olfactory epithelium, causing nerve stimulation directly to the hippocampus and limbic amygdaloidal body. This consequently triggers stimuli that control the autonomic nervous system and internal secretory control by changing a number of vital reactions [15]. Th inhalation of aromatic compounds present in EOs is the reason for the name "aromatherapy" and this therapy may have sedating or stimulating effects on the individual [16].

Reports in the literature describe the benefits of using EOs in aromatherapy on the wellbeing of individuals, including improvements in mood, stress, anxiety, depression, and chronic pain, and promote so therapeutic, psychological, and physiological effects [17]. The inhalation of EOs elevated blood pressure and renal sympathetic activity, which enforces the idea that these components act in the central nervous system and pass through the blood-brain barrier [18].

Volatile organic compounds are highly lipophilic and may easily cross the blood-brain barrier and easily exert their neuropharmacological and toxicological effects. While studies on the toxic effects of these compounds are relatively easy to perform, the central effects induced by the perception of odor (e.g., in aromatherapy) are inherently complex. This is why the toxicological studies performed using volatile compounds are much more advanced [19].

Many studies have been conducted *in vitro* with the purpose of verifying the biological properties of EOs [2, 17, 20]; usually they are performed using *in vitro* assays. On the other hand, when the tests are performed *in vivo*, the products are usually administered in their liquid forms (e.g., by gavage or intraperitoneal), with few studies in the volatile state (i.e., by inhalation) [21]. Furthermore, EOs are used as fl voring agents in food products [14] and are also used in dermatology and in the fragrance and cosmetics industries. Specific lly, geraniol is extensively used in the manufacture of both household and cosmetics products [12].

Since people are often use EOs, it is important to evaluate the possible hepatotoxic effects of these oils. Liver is the main detoxific tion organ; the catabolism of both endogenous and exogenous compounds takes place in the liver. As a result it is exposure to toxic agents which can cause drug-induced hepatic dysfunction. Therefore, studies on serum activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are biomarkers of liver damage, are important. Th serum activity of ALT and AST is frequently used in clinical settings for diagnostic hepatic toxicity [22].

Numerous chronic degenerative diseases are associated with oxidative stress, which occurs when there is excess formation of reactive oxygen species (e.g., superoxide, hydroxyl, and hydrogen peroxide) and insuffici t defense by the antioxidant system (enzymatic and nonenzymatic). Thi imbalance between pro- and antioxidants may cause cell injury and death, which consequently lead to tissue dysfunction [23, 24]. It is well established that oxidative stress plays a fundamental role in the pathogenesis of hepatic disease, especially nonalcoholic steatohepatitis [25]. In addition, during hepatic catabolism of xenobiotics, excessive production of reactive oxygen species (ROS) occurs [26].

Our aim was to investigate the eff ct of inhalation of the *C. martinii* EO and geraniol on serum biochemical parameters, biomarkers of hepatotoxicity, and oxidative stress in hepatic tissue.

2. Materials and Methods

2.1. Cymbopogon martinii EO and Geraniol. *C. martinii* EO was supplied by the company *By Samia Aromaterapia* (São Paulo, SP) and showed the following chemical composition: geraniol (57.5%), geranyl acetate (13.6%), linalool (1.7%), β-caryophyllene (1.1%), and ocimene (0.3%) found by gas chromatography-mass spectrometer (GC-MS). The geraniol with 98% of purity was purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Animals and Experimental Procedure. Th experimental procedure was approved by the Ethical Committee from Institute of Biological Science, São Paulo State University, Botucatu, Brazil, and the animals experiments were carried out in accordance with the principles and guidelines of the Canadian Council on Animal Care as outlined in the Guide to the Care and Use of Experimental Animals.

Male Wistar rats (290–310 g) were reared in polypropylene cages maintained in a controlled environment (temperature 22 ± 3°C; 50–55% humidity; and a 12-hour light : dark cycle), with free access to water and food (Purina Ltd., Campinas, SP, Brazil).

Th rats were randomly distributed into three groups (n = 8). Th rats in the control group (G1) received saline solution by inhalation (saline = 0.9% g/v). The G2 group received geraniol by inhalation and the G3 group received *C. martinii* EO by inhalation.

Th rats from all groups were placed individually into chambers (180 mm × 300 mm × 290 mm) adapted from de Almeida et al. [27] and submitted to inhalation of geraniol (8.36 mg geraniol/L of air, which corresponds to 136.2 μL of geraniol/perspex box 14.5 L of air) and *C. martinii* EO (13.73 mg of *C. martinii* EO/L of air, which corresponds to 227 μL of *C. martinii* EO/perspex box 14.5 L of air) for 10 minutes every 48 hours for 30 days. Th geraniol concentration was calculated from the amount of geraniol found in the *C. martinii* EO.

Food and water consumption were measured daily at the same time and body weights were determined once a week.

2.3. Biochemical Measurements and Oxidative Stress. Afte 30 days, the animals were fasted overnight (12–14 h) and euthanized by cervical decapitation under anesthesia (solution containing 10% ketamine chloride and 2% xylazine chloride with a dose of 0.1 mL/100 g body weight). Blood was collected and the serum was obtained by centrifugation at 6000 rpm for 15 minutes. Serum glucose was determined using an enzymatic colorimetric method after incubation with glucose oxidase/peroxidase. Th total amount of protein

Table 1: General characteristics and serum protein levels after 30 days for all experimental groups.

Parameters	Groups		
	G1	G2	G3
Final body weight g	348.57 ± 43.65	328.40 ± 29.82	320.98 ± 39.90
Body weight gain g	37.47 ± 9.25	36.86 ± 8.22	38.53 ± 17.06
Final food consumption g/day	20.30 ± 4.40	19.25 ± 4.14	18.52 ± 3.78
Final water consumption mL/day	248.75 ± 21.84[c]	248.75 ± 16.20[c]	211.88± 10.67[a,b]
Serum protein g/dL	5,13± 0,83	5,39 ± 1,39	5,95 ± 1,13

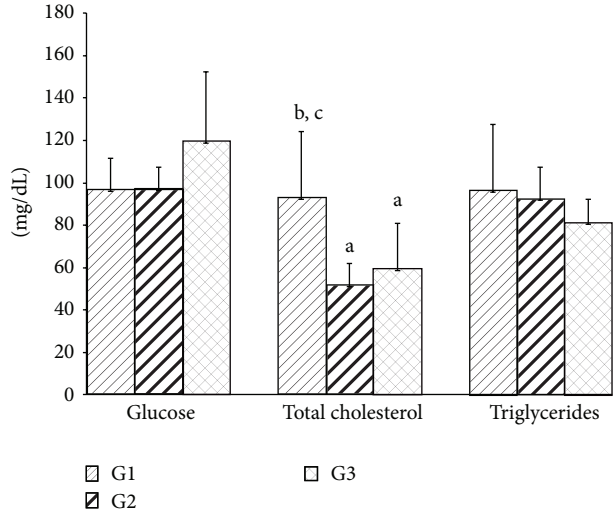
Values are given as the mean ± SD for each group of eight animals. [a]Significantly different from G1; $P \leq 0.05$; [b]significantly different from G2; $P \leq 0.05$; and [c]significantly different from G3; $P \leq 0.05$. G1: untreated control; G2: treated with geraniol; and G3: treated with essential oil.

was estimated using the biuret reagent and the total cholesterol concentration was determined using the cholesterol esterase/oxidase enzymatic procedure. Triacylglycerols levels were measured by enzymatic hydrolysis and the final formation of quinoneimine, which is proportional to the concentration of triacylglycerols present in the sample. Serum urea was determined by addition of urease and phenol-hypochloride, which leads to the formation of an indophenol-blue complex. Th serum creatinine levels were estimated using a reaction with picric acid in alkaline buffer to form a yellow-orange complex, whose color intensity is proportional to the creatinine concentration in the sample. ALT and AST activities were determined by using pyruvate and oxaloacetate as substrates, wherein NADH is converted into NAD+ proportional to the activities of these enzymes. Hepatic samples (200 mg) were removed and homogenized in 0.1M phosphate buffer, pH 7.4, using a Tefl n-glass Potter-Elvehjem homogenizer. Th homogenate was centrifuged (10,000 g for 15 minutes) and the supernatant was used to determine the concentration of hepatic lipid hydroperoxide (LH) and activities of antioxidant enzymes. Lipid hydroperoxide activity was determined by the oxidation of Fe^{+2} in the presence of a reactive mixture containing methanol, xylenol orange, sulfuric acid, and butylated hydroxytoluene. Catalase activity was assayed using phosphate buffer containing hydrogen peroxide. Th activity of glutathione peroxidase (GSH-Px) was determined in the presence of phosphate buffer, $NADPH_2$, reduced glutathione, and glutathione reductase. Superoxide dismutase (SOD) activity was assayed according to the method by measuring the rate of reduction of nitroblue-tetrazole (NBT) in the presence of free radicals generated by hydroxylamine.

2.4. Statistical Analysis. Results are expressed as the mean ± SD. The statistical significance between the groups was assessed using one-way analysis of variance (ANOVA) with Tukey's test to compare the means of the experimental group. The probability with $P \leq 0.05$ was considered signific nt.

3. Results

Inhalation of geraniol (G2) and of *C. martinii* EO (G3) had no effects on final body weight, body weight gain, and food intake of the rats (Table 1). No alteration in total hepatic protein was observed. While no signific nt differences were observed in the glycemia and triacylglycerol levels, geraniol



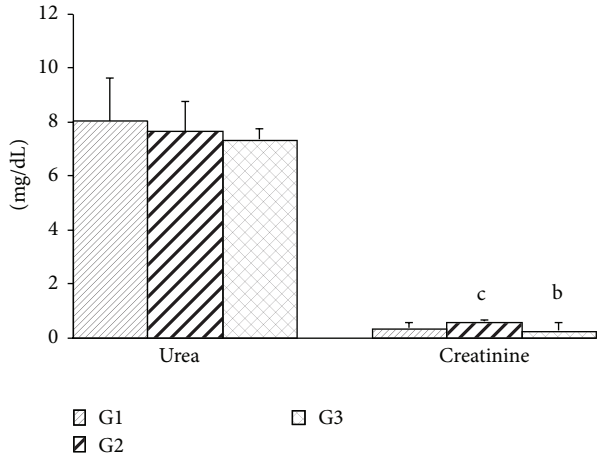
Figur e 1: Serum glucose, total cholesterol, and triglycerides levels after 30 days for all experimental groups. Values are given as the mean ± SD for each group of eight animals. [a]Signific ntly different from G1; $P \leq 0.05$; [b]signific ntly different from G2; $P \leq 0.05$; and [c]signific ntly different from G3; $P \leq 0.05$. G1: untreated control; G2: treated with geraniol; and G3: treated with essential oil.

(G2) and *C. martinii* EO (G3) decreased ($P \leq 0.05$) total cholesterol levels when compared with the control group G1 (Figure 1). There were no significant diff rences in serum urea levels between the groups. Creatinine levels increased in the presence of geraniol but decreased in the presence of *C. martinii* EO (G3; Figure 2).

ALT activity was higher in the group exposed to geraniol when compared to the other groups, which did not differ from each other. No change was found in the AST activity between the groups (Figure 3). LH was higher in the G2 group than in the G3 group (Figure 4). Catalase and SOD activities were higher in the G3 group when compared to the other groups. Both geraniol and *C. martinii* EO increased GSH-Px when compared to the control rats (Figure 5).

4. Discussion

EOs are widely used in aromatherapy procedures and there is interest in reports on the hepatic toxicity of these natural products. However, few studies have explored the effects of



Figur e 2: Serum urea and creatinine levels after 30 days for all experimental groups. Values are given as the mean ± SD for groups of eight animals each. [a]Signific ntly different from G1; $P \leq 0.05$; [b]signific ntly different from G2; $P \leq 0.05$; [c]signific ntly different from G3; $P \leq 0.05$. G1: untreated control; G2: treated with geraniol; G3: treated with essential oil.

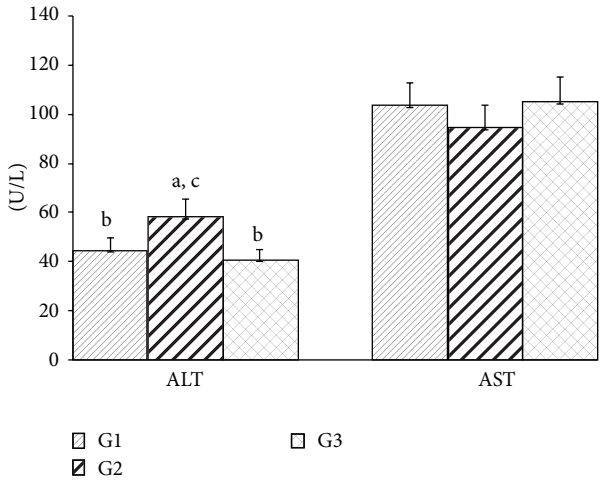


Figure 3: Serum activity of ALT and AST aft r 30 days for all experimental groups. Values are given as the mean ± SD for each group of eight animals. [a]Signific ntly different from G1; $P \leq 0.05$; [b]signific ntly different from G2; $P \leq 0.05$; and [c]signific ntly different from G3; $P \leq 0.05$. G1: untreated control; G2: treated with geraniol; and G3: treated with essential oil.

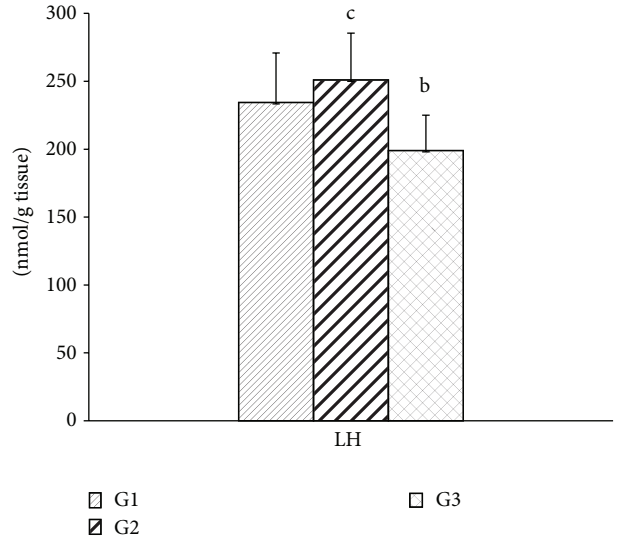


Figure 4: Hepatic lipid hydroperoxide levels after 30 days for all experimental groups. Values are given as the mean ± SD for each group of eight animals. [a]Signific ntly different from G1; $P \leq 0.05$; [b]signific ntly different from G2; $P \leq 0.05$; and [c]signific ntly different from G3; $P \leq 0.05$. G1: untreated control; G2: treated with geraniol; and G3: treated with essential oil.

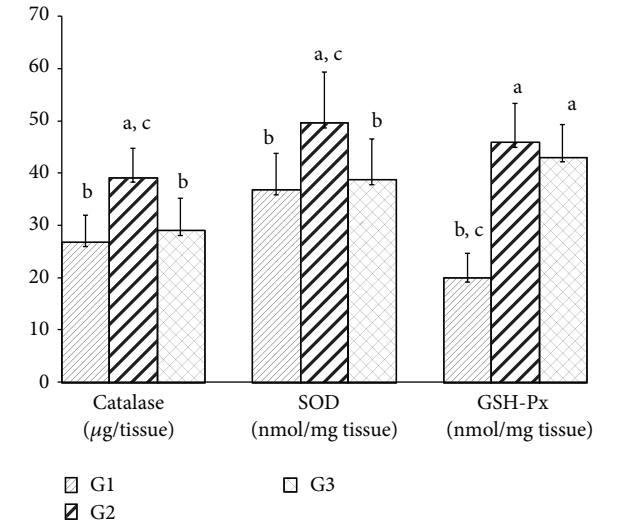


Figure 5: Hepatic activities of catalase, SOD, and GSH-Px after 30 days for all experimental groups. Values are given as the mean ± SD for each group of eight animals. [a]Signific ntly different from G1; $P \leq 0.05$; [b]signific ntly different from G2; $P \leq 0.05$; and [c]signific ntly different from G3; $P \leq 0.05$. G1: untreated control; G2: treated with geraniol; and G3: treated with essential oil.

EOs on an organism when administered by inhalation. Since EOs have been extensively used in aromatherapy due to their therapeutic properties and also used in food products, in dermatology, and in the fragrance and cosmetic industries [14], there is interest in investigating their hepatic toxicity, as well as dyslipidemia.

C. martinii EO reduced the fin l water intake of the rats, but without altering other parameters that we studied. No significi nt changes were observed in final body weight, body weight gain, final food intake, or total serum protein level, indicating no dehydration and no deficiencie of nutritionally important compounds in animals in the experimental groups. The e results disagree with others studies, which showed that the use of different EOs, for example, *Citrus aurantifolia* EO, decreases food intake and, consequently, weight gain [28, 29].

The e were no changes in serum glucose levels between the groups, indicating maintenance of glycemic homeostasis in these animals. However, other experimental studies have

demonstrated that *C. martinii* extracts exhibit antihyperglycemic activities through inhibition of α-glucosidase under diabetic conditions [30]. Assays with *C. citratus* aqueous extract (500 mg/kg/day; via oral) showed that the mechanism by which the extract induced hypoglycemia could be attributed to increased insulin synthesis and secretion or increased peripheral glucose utilization [31].

The inhalation of both geraniol (G2) and*C. martinii* EO (G3) reduced total cholesterol, but no changes in the serum triacylglycerol concentration were observed. Our results are in agreement with the result of Adeneye and Agbaje [31] and Burke et al. [10], who observed hypocholesterolemic effects using an aqueous extract of *Cymbopogon citratus* by oral administration. This reduction caused by EO can possibly be attributed to inhibition of 3-hydroxy-3-methylglutaryl CoA reductase, a key enzyme that regulates hepatic cholesterol synthesis [32, 33], or by reduction in the expression of these enzymes [34]. Yu et al. [35] demonstrated that geraniol inhibited the formation of mevalonate, a metabolic intermediate in the biosynthesis of cholesterol, in hepatomas. On the other hand, the administration of the highest EO dose (100 mg/kg) from *Cymbopogon* resulted in no change in total serum cholesterol [36].

Our results are also in agreement with Costa et al. [36] about total serum cholesterol; they reported that the biochemical parameters did not change aft r treatment with *Cymbopogon* but showed that there was a signific nt reduction in it ($F(4,27) = 3.061$; $P \leq 0.05$) after the administration of the highest EO dose (100 mg/kg) by gavage over a period of 21 days.

Since urea is formed in the liver and excreted by kidneys, estimation of this nitrogenous compound in the bloodstream is important to estimate both hepatic and renal functions. Animals treated with geraniol and EO did not show altered serum urea levels, suggesting a normal degree of protein catabolism, which was confi med by a normal concentration of hepatic protein. Although there was no alteration in the levels of serum urea in the G2 group, we cannot exclude the involvement of possible changes in the glomerular filtration rate in these animals. In clinical practice, serum creatinine, a biomarker for renal failure, is used as an indicator of renal function [37]. Curiously, serum creatinine levels were higher in the G2 group and lower in the G3 group, suggesting a decrease in renal excretion and some degree of renal insufficie y or early stages of kidney dysfunction when the major compound of *C. martinii* EO was administrated alone. The animals treated with both products had a tendency to have decreased levels of serum urea in our study. Serum creatinine was higher in G2, which may indicate lower renal excretion since the creatinine production was relatively constant [38].

Plasma membrane damage from some cells types, such as hepatic cells, is accompanied by release of cytosolic enzymes into bloodstream, a phenomenon that always occurs under several pathophysiological conditions [39]. Th aminotransferases ALT and AST are used for diagnosis of hepatic injury after toxic agents exposure [40]. The e was a signific nt increase in serum ALT activity in animals that inhaled geraniol (Figure 3). Since serum enzymatic activity of ALT is often used as a biomarker of hepatic toxicity, we can assume that there was some injury in hepatic tissue induced by geraniol aft r 30 days.

ROS, such as superoxide anion (O_2^-), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2), are formed through mitochondrial respiration during normal cellular metabolism. However, the cells have an enzymatic antioxidant defense system against ROS, but, under certain pathological conditions the excess formation of ROS results in suppression of antioxidant enzymes, the increase of ROS can occur in this way leading to oxidative stress [41].

Lipid peroxidation is an important toxic event because involves the removal of hydrogen from fatty acid chains mediated by ROS [42, 43] this way can lead to cell death and tissue damage.

Th endogenous antioxidant enzyme includes superoxide dismutase that catalyzes the dismutation of superoxide radicals [23]. Glutathione peroxidase catalyzes the reduction of hydrogen peroxidase to water through the oxidation of reduced glutathione. Catalase also participates in this conversion [44].

Signific ntly high LH was observed in rats exposed to geraniol (G2), while the benefici l effect of *C. martinii* EO was evidenced by the reduced LH in these animals. Th reductions observed in G3 can be attributed to a synergistic mechanism: a concomitant antioxidant action between other compounds, for example, linalool and β-caryophyllene, present in the *C. martinii* EO that showed antioxidant activity in other researches [45, 46]. Since free radical scavenger ability depends on the number of hydroxyl radicals in the molecule [43], the inhalation of the total *C. martinii* EO contributed to the reduction in the formation of ROS.

Rats exposed to geraniol (G2) had higher catalase, SOD, and GSH-Px activities, indicating that antioxidant enzyme activities were not sufficient to inhibit the ROS action and, consequently, the lipoperoxide generation in liver of these animals.

According to Koek et al. [47] the activity of antioxidants enzymes is increased early in nonalcoholic steatohepatitis but tends to decrease with progression of pathogenesis. Th activity of the SOD and catalase did not change in the G3 group, while GSH-Px increased in these animals, which showed lower values for LH. Buch et al. [4] observed increases in both SOD and catalase in the brains of rats treated with *C. martini* EO. Terpenoids, which are important components of EOs, lowered malondialdehyde levels and improved SOD activity in gastric mucosa [48]. Experimental data have shown that terpenoids, which are main components of EOs, are responsible for their antioxidant action [49, 50].

Since lipid hydroperoxide has been widely studied as marker of lipoperoxidation [51], a process that involves removal of hydrogen from fatty acids side chains by ROS, the result is referring to the mixture of compounds present in *C. martinii* EO (G3) that was effective in controlling oxidative stress and, therefore, lipoperoxidation by reducing the concentration of LH through a mechanism independent of the endogenous antioxidant enzymatic system.

In another study, geraniol reduced lipid peroxidation and inhibited the release of NO, indicating its possible

antioxidant potential in infl mmatory lung diseases, in which oxidative stress plays key role in these pathogenesis [14]. Moreover, the possible synergism between the compounds present in EOs can influence biological responses [52]. Thi can explain the results obtained for the G3 group. Thus, the effects we observed could be attributed to a constituent in a smaller proportion or synergism between compounds that are present in the oil [53]. For biological purposes, it is more informative to study the whole oil than some of its components because the concept of synergism appears to be more signific nt in the research on natural products [2].

5. Conclusion

In conclusion, *C. martinii* EO and geraniol maintained the glycemia, triacylglycerol protein, and urea levels but decreased cholesterol levels in Wistar rats. The oxidative stress caused by geraniol alone appears to trigger, to some degree, hepatic toxicity, as can be verifi d by the increase of serum creatinine and ALT. The e results suggest that benefici l actions of *C. martinii* EO on oxidative stress can prevent the toxicity in liver. Thi proves the possible interactions between geraniol and numerous chemical compounds present in *C. martinii* EO.

Abbreviations

C. martinii:	*Cymbopogon martinii*
EO:	Essential oil
EOs:	Essential oils
ALT:	Alanine aminotransferase
AST:	Aspartate aminotransferase
ROS:	Reactive oxygen species
LH:	Lipid hydroperoxide
GSH-Px:	Glutathione peroxidase
SOD:	Superoxide dismutase
NBT:	Nitroblue-tetrazole.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Paper

Cymbopogon martinii essential oil and geraniol at noncytotoxic concentrations exerted immunomodulatory/anti-inflammatory effects in human monocytes

Bruna Fernanda Murbach Teles Andrade, Bruno José Conti, Karina Basso Santiago, Ary Fernandes Júnior and José Maurício Sforcin

Department of Microbiology and Immunology, Biosciences Institute, UNESP, Botucatu, SP, Brazil

Keywords
Cymbopogon martinii; cytokines; essential oil; geraniol; monocytes

Correspondence
José M. Sforcin, Department of Microbiology and Immunology, Biosciences Institute, UNESP, Botucatu, SP 18618-970, Brazil.
E-mail: sforcin@ibb.unesp.br

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Abstract

Objectives In traditional medicine, plants have formed the basis of sophisticated systems that have been in existence for thousands of years and still provide mankind with new remedies. *Cymbopogon martinii*, known as palmarosa, has been used in aromatherapy as a skin tonic due to its antimicrobial properties. It has also used in Ayurvedic medicine for skin problems and to relieve nerve pain. The immunomodulatory action of *C. martinii* essential oil (EO) and geraniol was evaluated regarding the production of pro- and anti-inflammatory cytokines (tumour necrosis factor (TNF)-α and IL-10, respectively) by human monocytes *in vitro*.

Methods Monocyte cultures were incubated with EO or geraniol. After 18 h, cytotoxicity assays were performed using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide method, and cytokine production was determined by ELISA.

Key findings The variables showed no cytotoxic effects on monocytes. TNF-α production was not affected by *C. martinii* and geraniol, and only the concentration of 5 μg/ml of *C. martinii* stimulated its production. On the other hand, all concentrations of *C. martinii* and geraniol increased IL-10 production by human monocytes.

Conclusions Data showed that noncytotoxic concentrations of EO and geraniol exerted an anti-inflammatory action by increasing IL-10 production; moreover, geraniol seemed to be probably responsible for EO immunomodulatory activity in our assay condition.

Introduction

In traditional medicine, plants have formed the basis of sophisticated systems that have been in existence for thousands of years and still provide mankind with new remedies.[1]

Essential oils (EOs) are volatile complex compounds characterized by a strong scent from plant secondary metabolites and are widely used in pharmacy, medicine, food and beverages, aromatherapy, and cosmetics and perfumery, usually obtained by steam or hydrodistillation.[2,3]

EOs have been used in the skin (cosmetics and skin care products) and have a percutaneous absorption until bloodstream. After 5 min of an abdominal massage with lavender EO, it has been demonstrated that linalool and linalyl acetate – major components of lavender EO – were detected in blood, and after 90 min, the compounds were eliminated from the bloodstream.[4] Thus, it has become imperative to study the effects of these terpenes compounds in blood cells.

EO from palmarosa (*Cymbopogon martinii* Roxb.) Wats. var. *motia* Burk., Poaceae family syn. Graminaceae may be isolated by steam-distilling the leaves freshly harvested or partially dried. Palmarosa EO has been widely used in aromatherapy as a skin tonic because of its antibacterial and antiviral properties and mild anti-inflammatory quality. It has also been used in Ayurvedic medicine for skin problems and to relieve nerve pain.[5] Its major compound is geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol) – an acyclic monoterpene alcohol with the chemical formula $C_{10}H_{18}O$ (Figure 1) that may be separated through fractional distillation from the EO and used in flavour, fragrance and pharmaceutical industry.[5,6]

EOs from palmarosa foliage and inflorescence have shown an extensive importance in flavour, fragrance and perfumery industries because of its rose-note,[7] traditionally prescribed for central nervous system disorders such as neuralgia, epileptic fits and anorexia.[8]

Inflammation is a protective measure to eliminate the injurious stimuli and monocytes have a key role in providing an immediate defense against foreign agents. Monocytes are blood cells derived from bone marrow and precursors of macrophages in tissues. Upon activation, monocytes produce several mediators including cytokines,[2] playing a central role in innate and adaptive immunity.

The use of anti-inflammatory agents can be an effective tool in the therapeutic treatment of inflammatory diseases. In folk medicine, medicinal plants and their isolated compounds are employed to treat different inflammatory conditions, and in search for new bioactive natural products against inflammation, EOs have been increasingly referred as a rich source of such products.[9]

Because the modulatory effect of palmarosa EO and geraniol on macrophage-/monocyte-triggered inflammatory processes could contribute to the establishment of new therapeutic alternatives for the treatment of pathologies with a strong inflammatory component, the aim of this work was to analyse a possible immunomodulatory effect of palmarosa EO and geraniol on pro- and anti-inflammatory cytokine production by human monocytes *in vitro*, evaluating tumour necrosis factor (TNF)-α and IL-10 production, respectively.

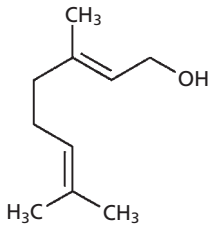

Figure 1 Chemical structure of geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol).

Materials and Methods

Cymbopogon martinii essential oil and geraniol

C. martinii EO was distilled from herb and purchased from *By Samia Aromaterapia* (São Paulo, SP, Brazil). Geraniol was purchased from Sigma Aldrich® (St Louis, MO, USA) with 98% of chemical purity. Palmarosa chemical analysis was performed in the Department of Chemistry and Biochemistry, UNESP, Campus of Botucatu, by gas chromatography-mass spectrometer (GC-MS) Shimadzu model QP5050A, according to operating conditions: CBP-5 capillary column (50 m × 0.25 mm × 0.25 μM), injector temperature of 250°C and helium (He) as a carrier gas. The energy of impact used in MS was 70 eV. Compounds identification in EO was carried out by mass spectra analysis according to the National Institute of Standards and Technology library.

Healthy blood donors and human monocytes isolation

Ten healthy blood donors (ageing 20–50 years) from the Biosciences Institute, UNESP, Campus of Botucatu, were included in the present work, which was approved by the Ethics Committee of Botucatu Medical School (CEP 3840-2011). An informed consent was signed by all blood donors.

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized (50 U/ml heparin) venous blood using Ficoll-Hypaque (density = 1.077, Sigma Aldrich) Briefly, 20 ml of heparinized blood were added to an equal volume of RPMI-1640 culture medium containing 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 40 mg/l gentamicin. Samples were added to 4 ml of Ficoll-Hypaque and centrifuged at 400*g* for 30 min at room temperature. The interface layer of the PBMC was aspirated and washed twice with phosphate buffer saline 0.1 M, pH = 7 containing 0.05 mM ethylenediaminetetraacetic acid and once with RPMI medium at 300*g* for 10 min. Cell viability, as determined by neutral red (0.02%) staining, was >95% in all experiments. Cells were resuspended at a final concentration of 1×10^6 monocytes/ml in RPMI medium supplemented with fetal calf serum. After 2 h, non-adherent cells were discarded, and monocytes were incubated with palmarosa EO diluted in 0.02% dimethylsulfoxide (DMSO, Sigma Aldrich) at the following concentrations: 0.1, 1, 5 and 10 μg/ml. Monocytes were also incubated with geraniol in proportional concentrations found in palmarosa EO (0.057, 0.57, 2.87 and 5.74 μg/ml) or with lipopolysaccharide (LPS, 10 μg/ml) and DMSO 0.02% as controls for 18 h.

Cytotoxicity assay

Cell viability was performed using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Aldrich) colorimetric assay.[10] Monocytes (1×10^6 cells/ml) were incubated with different concentrations of stimuli as previously described for 18 h at 37°C and 5% CO_2, in a final volume of 500 µl. Control cells were incubated only with culture medium. Culture medium was removed, and 300 µl of MTT (1 mg/ml) in complete RPMI were added to the culture cells for 3 h. Afterwards, MTT was aspirated and 200 µl of 0.02% DMSO was added to dissolve the formazan salt. Optical densities (ODs) were read at 540 nm in an ELISA reader, and the percentage of cell viability was calculated using the formula: (OD test/OD control) × 100. Assays were carried out in duplicate.

Cytokine determination by enzyme-linked immunosorbent assay

To evaluate cytokine production, monocytes (1×10^6 cells/ml) were distributed into 24-well flat-bottomed plates (Nunc, Life Tech., Inc., Frederick, MD, USA) and incubated with different concentrations of palmarosa EO, geraniol or LPS (10 µg/ml) for 18 h at 37°C and 5% CO_2. Afterwards, the supernatants were harvested for TNF-α and IL-10 measurement by ELISA, according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Briefly, a 96-well flat bottom Maxisorp (Nunc Maxisorp, San Diego, CA, USA) was coated with capture antibody specific to each cytokine. The plate was washed and blocked before 100 µl of the supernatants, and serially diluted specific standards were added to the respective wells. Following a series of washing, the cytokine was detected using the specific conjugated detection antibody. The substrate reagent was added into each well, and after color development, the plate was read at 450 nm, using an ELISA plate reader.[11]

Statistical analysis

For MTT and IL-10, analysis of variance was used to parametric data with normal distribution and homogeneity. For TNF-α, analysis of variance and Kruskal–Wallis test were used for nonparametric data. A P value of less than 0.05 was considered significant.

Results

Chemical analysis of palmarosa essential oil

The chemical analysis of palmarosa EO by GC-MS revealed that geraniol was its major compound (57.49%), followed by geranyl acetate (13.56%), linalool (1.71%), β-caryophyllene (1.07%) and ocimene (0.27%) (Table 1).

Table 1 Compounds of *Cymbopogon martinii* essential oil identified by gas chromatography-mass spectrometer

Compounds (%)
Geraniol (57.49%)
Geranyl acetate (13.56%)
Linalool (1.71%)
β-Caryophyllene (1.07%)
Ocimene (0.27%)

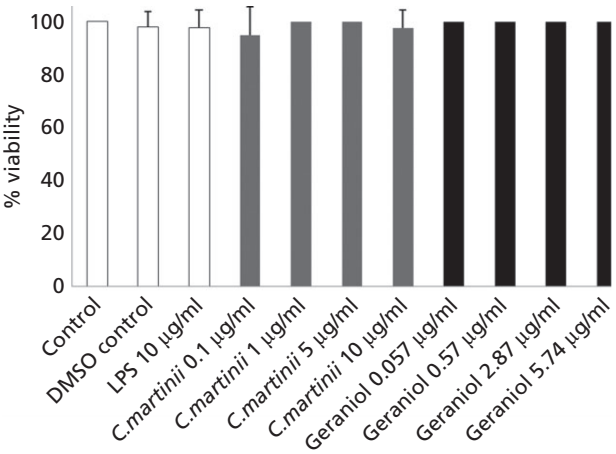

Figure 2 Monocytes viability (%) after incubation with *Cymbopogon martinii* essential oil (0.1, 1, 5 and 10 µg/ml); geraniol (0.057, 0.57, 2.87 and 5.74 µg/ml), 0.02% dimethylsulfoxide and lipopolysaccharide (10 µg/ml) for 18 h by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide method ($n = 10$; $P > 0.05$).

Palmarosa essential oil and geraniol effects on monocytes viability

No cytotoxic effects were seen after incubating monocytes with palmarosa EO or geraniol ($P > 0.05$), and cell viability was approximately 100% compared with control (Figure 2).

Tumour necrosis factor-α production

With respect to the effects of *C. martinii* EO on TNF-α levels produced by human monocytes, only the concentration of 5 µg/ml stimulated significantly ($P < 0.001$) its production compared with control (Figure 3a).

TNF-α levels were not altered after incubation with geraniol (Figure 3b), and its production was similar to control ($P > 0.05$).

Interleukin-10 production

Increased IL-10 levels were found after monocytes incubation with *C. martinii* but not with the lowest concentration ($P < 0.001$) (Figure 4a).

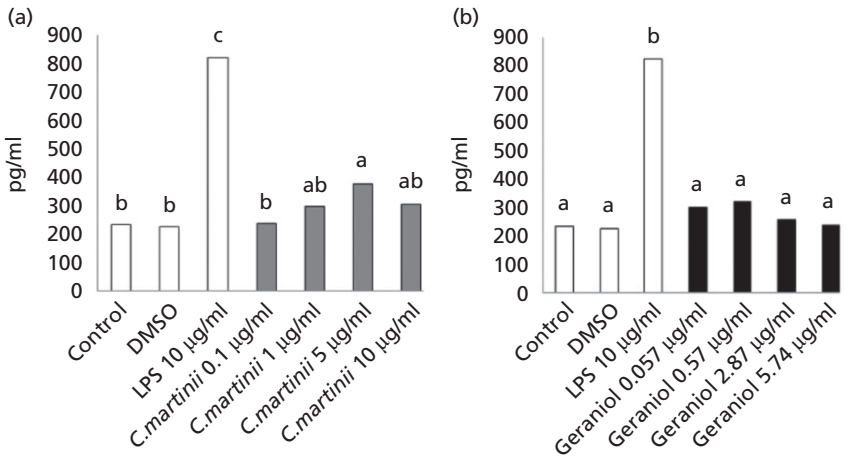

Figure 3 Tumour necrosis factor-α production (pg/ml) by human monocytes incubated with (a) *Cymbopogon martinii* essential oil (0.1, 1, 5 and 10 μg/ml); (b) geraniol (0.057, 0.57, 2.87 and 5.74 μg/ml); 0.02% dimethylsulfoxide and lipopolysaccharide (10 μg/ml). Data represent median of 10 similar experiments. Different small letters indicate significant differences between the treatments ($P < 0.001$).

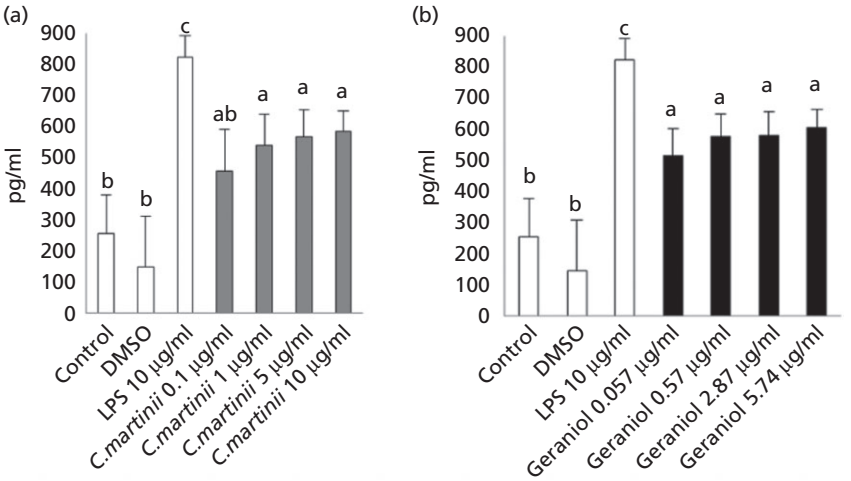

Figure 4 Interleukin-10 production (pg/ml) by human monocytes incubated with (a) *Cymbopogon martinii* essential oil (0.1, 1, 5 and 10 μg/ml); (b) geraniol (0.057, 0.57, 2.87 and 5.74 μg/ml); 0.02% dimethylsulfoxide and lipopolysaccharide (10 μg/ml). Data represent median of 10 similar experiments. Different small letters indicate significant differences between the treatments ($P < 0.001$).

Geraniol at all concentrations stimulated IL-10 production by human monocytes compared with control ($P < 0.001$) (Figure 4b).

LPS was used as a positive control and stimulated both IL-10 and TNF-α levels ($P < 0.001$), and DMSO 0.02% did not affect cytokine production.

Discussion

Pharmacognosy stems from the different systems of traditional herbal medicine and its 'reverse pharmacology' approach has led to the discovery of numerous pharmacologically active molecules and drug leads for mankind.[12] Medicinal plants and derived compounds have been a good source of new and specific inhibitors of the inflammatory process.[13] The treatment of exacerbated inflammation is important to alleviate the pain, fever or tissue damage associated with acute or chronic inflammation in clinical medicine.[14]

C. martinii has attracted researchers' attention lately because of its biological properties, such as antimicrobial, antigenotoxic and antioxidant ones.[15–18] However, little is known regarding its effects on the immune system, specifically on human monocytes.

One may verify that both palmarosa EO did not affect monocytes viability and their concentrations were used in

cytokine protocols. Geraniol, its major compound, did not affect cell viability as well. Phytochemical analysis of the EO of *C. martinii* (palmarosa) indicated the presence of geraniol (65–85%) and geranyl acetate (5–20%) as major components with noncytotoxic effects determined by MTT test, similar to our data.[19,20]

Data showed that *C. martinii* EO and geraniol increased IL-10 but not TNF-α production by human monocytes. This in an important finding as IL-10 is an important anti-inflammatory cytokine produced by activated immune cells, in particular monocytes/macrophages and T cell subsets. In monocytes/macrophages, IL-10 diminishes the production of inflammatory mediators, and the physiological relevance of this cytokine lies in the prevention and limitation of overwhelming specific and unspecific immune reactions, and contributes to induced tolerance.[21] Our data demonstrated the stimulatory action of *C. martinii* EO and geraniol in this cytokine production at noncytotoxic concentrations in human monocytes.

Previous works of our group with other natural products showed that clove (*Syzygium aromaticum*) extract and eugenol stimulated significantly IL-10 production by murine macrophages.[22] *Baccharis dracunculifolia* (Bd) and cafeic acid (Ca) stimulated IL-1β and inhibited IL-6 and IL-10 production in LPS-challenged protocols. Bd prevented LPS action either before or after LPS challenge, whereas Ca prevented LPS effects only after LPS addition in murine macrophages.[23] Lemongrass (*C. citratus*) extract modulated IL-1β and IL-6 production by macrophages depending on concentration. Its component citral was more efficient than *C. citratus* to inhibit IL-1β and IL-6 production.[24]

Although *C. martinii* and *C. citratus* belong to the same family, our findings are not in agreement with those from Bachiega and Sforcin because lemongrass extract and citral inhibited IL-10 production while *C. martinii* stimulated it. *C. citratus* extract exerted a therapeutic action by counteracting LPS stimulatory action, while citral showed preventive and therapeutic effects.[24]

The immunomodulatory effects of other natural products have been described as well. *Melaleuca alternifolia* EO, at a concentration of 0.01%, induced a twofold increase in IL-10 secretion by phytohaemagglutinin A-stimulated leucocytes but abolished it when used at 0.1%, suggesting a poor capacity of the EO to activate IL-10 secretion by human peripheral blood leucocytes.[25] A decreased IL-10 production because of higher concentrations of the compounds was not observed herein, as the concentrations of EO and geraniol increased IL-10 production similarly.

The effect of geraniol on bacterial-induced inflammation was evaluated in a monocytic cell line (Raw 264.7) and in BALB/c mice treated with pamidronate and geraniol. Geraniol diminished the levels of inflammatory markers induced by pamidronate stimuli both *in vitro* and *in vivo*.[24]

Palmarosa EO and geraniol did not affect TNF-α production by human monocytes in our results. EO from lemongrass, geranium, mint and their constituents (citral, citronellol, geraniol and carvone) clearly suppressed TNF-α at a concentration of 0.0125%.[26] Tea tree EO seemed to be toxic to monocytes at a concentration of 0.016% v/v, while the water-soluble components of the oil in concentrations equivalent to 0.125% significantly suppressed LPS-induced production of TNF-α, IL-1β and IL-10 (about 50%), and prostaglandin E2 (PGE2; approximately 30%) after 40 h. The individual components of the tea tree EO were analysed individually and only terpinen-4-ol suppressed the production of TNF-α, IL-1β, IL-8, IL-10 and PGE2 by LPS-activated human monocytes after 40 h.[27] Although we have used compounds rich in terpenes as mentioned earlier, our results showed no suppression of TNF-α production by human monocytes. Terpenoids have been extensively studied as pharmacological agents, and some have been found to possess specific anti-inflammatory properties.[13]

Assessing human lymphocytes, palmarosa oil induced significant DNA damage only at high concentrations (1000 μg/ml), while geraniol did not reveal any genotoxicity, what suggested their safety at low concentration for human consumptions.[20] Further investigation should evaluate palmarosa EO and geraniol in other targets, such as phospholipase A2 and free radicals, revealing the therapeutic relevance of basic research in the search of anti-inflammatory and antioxidant molecules. Several secondary metabolites from plants and marine sponges exhibited both anti-inflammatory and antioxidant properties, including terpenes, that are classified according to the number of isoprene units like hemiterpenes, monoterpenes, diterpenes, triterpenes, tetraterpenes and sesquiterpenes. Terpenes may act as powerful antioxidants protecting lipids, blood vessels and other body fluids against attack by free radicals such as hydroxyl, peroxides and superoxide radicals.[13,28]

Conclusion

In conclusion, EO and its major compound showed an anti-inflammatory action in our experimental model; moreover, the monoterpene geraniol seemed to be probably responsible for the immunomodulatory activity of palmarosa EO in human monocytes.

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Apêndices

Apêndice 1- Certificado favorável do Comitê de Ética em Experimentação Animal

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Campus de Botucatu

Instituto de Biociências de Botucatu - Unesp

Certificate

We certify that the protocol nº **290** about "Essential oils and major compounds: antibacterial and immunomodulatory activities in vitro and in vivo biochemical" agree with ETHICAL PRINCIPLES IN ANIMAL RESEARCH adopted by Brazilian College of Animal Experimentation (COBEA) and was approved by the BIOSCIENCE INSTITUTE/UNESP ETHICAL COMMITTEE FOR ANIMAL RESEARCH (CEEA), in **April 13, 2011.**

Botucatu, April 13, 2011.

Profª Drª Patrícia Fernanda Felipe Pinheiro
Presidente - CEEA

Instituto de Biociências – Diretoria Técnica Acadêmica
Distrito de Rubião Júnior s/n CEP 18618-970 Botucatu SP Brasil
Tel 14 3811 6013/6014 fax 14 3815 3744 e-mail secdta@ibb.unesp.br

Apêndice 2- Parecer favorável do Comitê de Ética em Pesquisa

unesp
Universidade Estadual Paulista
Faculdade de Medicina de Botucatu

Distrito Rubião Junior, s/nº - Botucatu – S.P.
CEP: 18.618-970
Fone/Fax: (0xx14) 3811-6143
e-mail secretaria: capellup@fmb.unesp.br
e-mail coordenadoria: tsarden@fmb.unesp.br

Ética
Comitê de Ética em Pesquisa

Registrado no Ministério da Saúde
em 30 de abril de 1997

Botucatu, 02 de maio de 2011. Of. 157/11-CEP

Ilustríssimo Senhor
Prof. Dr. Ary Fernandes Júnior
Departamento de Microbiologia e Imunologia do
Instituto de Biociências de Botucatu.

Caro Dr. Ary Fernandes,

De ordem do Senhor Coordenador deste CEP, informo que Projeto de Pesquisa **(Protocolo CEP 3840-2011) "Óleos essenciais e compostos majoritários: atividades antibacteriana e imunomoduladora in vitro e perfil bioquímico in vivo"**, a ser conduzido por Bruna Fernanda Murbach Teles Machado, orientada por Vossa Senhoria, Co-orientada pela Profª Drª Ana Angélica Henrique Fernandes, com a colaboração do Prof. Dr. José Maurício Sforcin recebeu do relator parecer favorável, aprovado em reunião de 02 de maio de 2.011.

Situação do Projeto: APROVADO. Ao final da execução deste Projeto, apresentar ao CEP **"Relatório Final de Atividades"**.

Atenciosamente,

Alberto Santos Capelluppi
Secretário do CEP.

Apêndice 3- Termo de consentimento livre e esclarecido

UNESP UNIVERSIDADE ESTADUAL PAULISTA
CAMPUS DE BOTUCATU
INSTITUTO DE BIOCIÊNCIAS

DEPARTAMENTO DE MICROBIOLOGIA E IMUNOLOGIA

BOTUCATU- SP-CEP-18.618-000 -FONE: (14) 3811-6058 -FAX: (14) 3811-6058236

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

PARTICIPAÇÃO EM PESQUISA DE INDIVÍDUOS SAUDÁVEIS

O Senhor (Sr.) está sendo convidado a participar da pesquisa "Óleos essenciais e compostos majoritários: atividades antibacteriana e imunomoduladora *in vitro* e perfil bioquímico *in vivo*"**, que avaliará células monomucleares do sangue periférico. Assim, quando ocorrer a doação do sangue, amostras de 20 mL serão colocadas em tubo estéril e heparinizado, e utilizadas nos laboratórios da disciplina de Imunologia, onde será desenvolvido o estudo.O sangue será coletado com material descartável (assim como é realizada a coleta para exames de sangue e colocados em tubos limpos, livres de contaminação), a quantidade de 20 mL é necessária pois utilizaremos somente os monócitos viáveis do sangue(somente uma parte do sangue será utilizada) estas células serão colocadas em contato com dois diferentes produtos naturais em cinco concentrações distintas, num total de dez tratamentos. Informamos que o Sr. possui total liberdade de recusar ou de retirar o seu consentimento de participação neste estudo, sendo que esta ação não causará qualquer transtorno à sua pessoa. Informamos também que os responsáveis por este trabalho estarão disponíveis para responder a quaisquer perguntas ou dúvidas, e que todas as informações obtidas neste estudo serão identificadas somente por número, sendo que seu nome não aparecerá quando os resultados do estudo forem apresentados e seu sangue(monócitos) será utilizado somente neste estudo. Qualquer dúvida adicional, você poderá entrar em contato com o Comitê de Ética em Pesquisa, através do fone: (14) 3811-6143**

Botucatu, de de

.

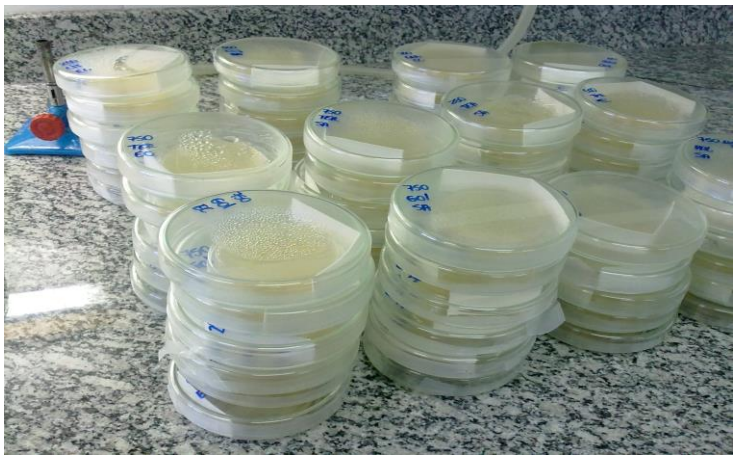
Nome do doador voluntário:_____

Assinatura do doador voluntário:_____

Prof. Dr. Ary Fernandes Junior
Responsável pelo Projeto
Depto. Microbiologia e Imunologia
IB – UNESP – Campus de Botucatu
Fone: 14-3811-6058 / ary@ibb.unesp.br

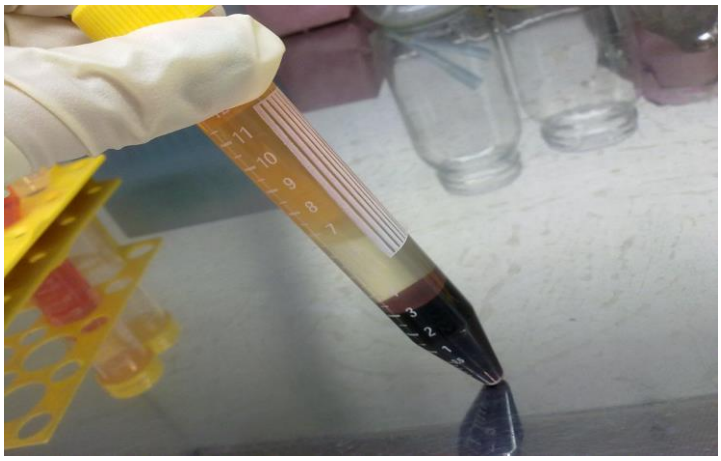
Bruna Fernanda Murbach Teles Machado
Doutoranda do Projeto
Depto. Microbiologia e Imunologia
IB – UNESP – Campus de Botucatu
Fone: 14-3811-6058
brunatura@hotmail.com.br

Apêndice 4- Fotos dos experimentos



Experimento microbiológico com vapor dos produtos naturais

Experimento com inalação em ratos



Apêndice 5- Fotos do grupo de pesquisa