UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

NANOEMULSÕES CONTENDO ÓLEO DE *Pelargonium graveolens*: ESTUDOS DE ESTABILIDADE E ATIVIDADE ANTIBIOFILME EM MATERIAL MÉDICO HOSPITALAR

JANICE LUEHRING GIONGO

PORTO ALEGRE, 2015

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NANOEMULSÕES CONTENDO ÓLEO DE *Pelargonium graveolens*: ESTUDOS DE ESTABILIDADE E ATIVIDADE ANTIBIOFILME EM MATERIAL MÉDICO HOSPITALAR

Tese apresentada por **Janice Luehring Giongo** como requisito para obtenção do TÍTULO DE DOUTORA em Ciências Farmacêuticas.

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A presente tese foi redigida na forma de encarte de publicações para uma melhor organização e discussão dos resultados obtidos, e encontra-se dividida da seguinte maneira:

- Introdução incluindo a hipótese e justificativa do trabalho;
- Objetivos: Geral e específicos;
- Revisão da literatura;
- Capítulo 1: artigo publicado na revista International Journal of Pharmacy and Pharmaceutical Sciences;
- Capítulo 2: artigo submetido à revista International Journal of Pharmaceutics;
- Capítulo 3: artigo a ser submetido à revista Toxicology *in vitro*;
- Capítulos 4: artigo a ser submetido à revista Inflammation Research;
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- Discussão Geral;
- Considerações Finais;
- Referências bibliográficas.

Sem sonhos, a vida não tem brilho.

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Sem prioridades, os sonhos não se tornam reais.

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LISTA DE ABREVIATURAS E SIGLAS

AFM – Microscopia de Força Atômica

ANVISA - Agência Nacional de Vigilância Sanitária

ATCC - American Type Culture Collection

CAT - Catalase

CG - Cromatografia gasosa

CLSI - Clinical Laboratory Standart Institute

COX-1 – Ciclooxigenase 1

COX-2 – Ciclooxigenase 2

CV – Cristal violeta

DPPH - 2,2-difenil-1-picrilhidrazila

DSC - Calorimetria exploratória diferencial

ERO - Espécies reativas do oxigênio

FRAP – Poder antioxidante de redução do ferro

GO - Óleo de Gerânio

IL – Interleucina

NOx – Óxido nitríco

iNOS – Óxido nítrico sintetase induzida

INMETRO - Instituto Nacional de Metrologia, Normalização e Qualidade Industrial

LDH - Lactato desidrogenase

PDI - Índice de polidispersão

TEM - Microscopia eletrônica de varredura

mg - Miligrama

ml - Mililitros

MTT - 3-(4,5)-dimetiltialzolil-2,5- difeniltetrazólio

NC - Nanocápsula

NE - Nanoemulsão

NEG - Nanoemulsão contendo óleo de gerânio

NO - Óxido nítrico

nm - Nanômetro

PGE2 - Prostaglandina E2

RPM - Rotações por minuto

SOD – Superóxido dismutase

TBARS - Espécies reativas ao ácido tiobarbitúrico

TGA - Análise termogravimétrica

TNF - Fator de necrose tumoral

UFC – Unidade formadora de colônia

µg - Micrograma

RESUMO

As pesquisas por substâncias de origem natural, como óleos essenciais, têm aumentado progressivamente, devido principalmente à resistência dos microrganismos aos fármacos comumente comercializados. A finalidade de aumentar o arsenal terapêutico deve-se ao índice cada vez mais elevado de infecções microbianas, principalmente no ambiente hospitalar. Neste contexto, o presente trabalho utilizou o óleo de *Pelargonium graveolens*, conhecido popularmente como óleo de gerânio (GO) com o objetivo de verificar suas possíveis atividades antimicrobianas, antioxidantes, anti-inflamatórias e antibiofilme. Sabe-se que entre seus constituintes majoritários encontram-se o citronelol e o geraniol e os mesmos são responsáveis por algumas destas atividades citadas. A utilização do óleo puro não é muito vantajosa, pois a concentração dos constituintes pode ser afetada pela volatilização. A estratégia utilizada neste trabalho foi o desenvolvimento de sistemas nanoestruturados, como as nanocápsulas e nanoemulsões, a fim de aumentar e melhorar as atividades do óleo de gerânio. Após a realização dos diferentes testes, verificou-se que o óleo e as nanoestruras possuem atividade antifúngica e quando testados em material médico, demonstraram atividade antibiofilme. A atividade antioxidante demonstrou ser maior no óleo livre, entretanto quando as amostras foram utilizadas em macrófagos, as nanoemulsões demonstraram ter uma melhor atividade anti-inflamatória. O presente trabalho revela a importância do desenvolvimento de nanoestruturas contendo óleo de gerânio como uma alternativa no combate a formação de biofilmes em dispositivos médicos hospitalares.

ABSTRACT

Research by substances of natural origin, essential oils, have steadily increased, mainly due to the resistance of microorganisms to commonly marketed drugs. The purpose of increasing the therapeutic arsenal due to the increasingly high rate of microbial infections, particularly in the hospital environment. In this context, this study used the oil Pelargonium graveolens, popularly known as geranium oil (GO) in order to verify their possible antimicrobial activity, antioxidant, anti-inflammatory and antibiofilm. It is known that among its major constituents are citronellol and geraniol and are responsible for some of these cited activities. The use of pure oil is not very advantageous, because the concentrations of constituents may be affected by volatilization. The strategy used in this study was the development of nanostructured systems, such as nanocapsules and nanoemulsions in order to increase and improve the activities of geranium oil. After performing various tests, it was found that oil and nanoestruras have antifungal activity when assayed in medical and material shown antibiofilm activity. The antioxidant activity was shown to be higher in oil free, but when the samples were used in macrophages, nanoemulsions shown to have better anti-inflammatory activity. This study reveals the importance of developing nanostructures containing geranium oil as an alternative to combat the formation of biofilms in hospital medical devices.

INTRODUÇÃO

A resistência aos fármacos antimicrobianos tem sido um problema ao longo da história da humanidade e continua constituindo em uma ameaça para a população. Os microrganismos têm vindo a desenvolver mecanismos de resistência que têm contrariado os avanços alcançados no tratamento de infecções. Além disso, a prescrição inadequada do medicamento, duração prolongada do tratamento e aplicação de dose superior a limite também agravam a terapêutica do paciente (SHAB, ASLAM e AVERY, 2001; TENOVER, 2006).

Nos últimos anos, as infecções microbianas vêm ganhando destaque devido ao aumento de patologias que afetam o sistema imunológico, como AIDS, câncer, entre outras, bem como, ao aumento da utilização de algum material médico hospitalar nestes pacientes mais debilitados. Dados do Ministério da Saúde demonstram que mais de 70% dos microrganismos que causam infecções hospitalares no Brasil são resistentes a pelo menos um antimicrobiano e cerca de 80% de todas as infecções microbianas em humanos estão relacionadas à formação de biofilmes (RICHARDS *et al*, 2008).

Os biofilmes consistem em células microbianas viáveis, juntamente com as células mortas e uma vasta gama de substâncias poliméricas extracelulares (EPS) de sua própria produção, incluindo polissacarídeos, ácidos nucleicos (DNA extracelular a partir de bactérias), e proteínas (AKBARI & KYLLERUP, 2015). O uso crescente de dispositivos médicos e de implantes eleva a resistência de biofilmes aos tratamentos com antimicrobianos. Estes dispositivos podem servir como um nicho ideal para formação dos biofilmes, tornando-se um potencial para infecção sistêmica (LOCKHART *et al*, 2008; RICICOVÁ *et al*., 2010).

A problemática em relação à formação de biofilmes em dispositivos médicos refere-se, em primeiro lugar, ao reservatório de microrganismos formado sobre a superfície destes dispositivos, que podem conduzir a uma infecção crônica. Em segundo lugar, refere-se, à resistência da comunidade de microrganismo ao tratamento convencional, e por fim, às respostas do hospedeiro. Terapias antimicrobianas são frequentemente incapazes de eliminar

os microrganismos que crescem nos biofilmes e uma resposta inflamatória crônica no local do biofilme pode ser produzida (TURNER *et al*., 2009).

Observa-se em diversos países, um aumento crescente do número de casos de infecções microbianas, em especial as infecções fúngicas associadas a biofilmes, principalmente do gênero *Candida*. Este aumento é acompanhado por maiores taxas de mortalidade, aumento do tempo de internação e consequentemente, aumento dos custos associados ao manejo destes pacientes. Nos Estados Unidos os custos associados ao tratamento de infecções relacionadas a biofilmes excedem um bilhão de dólares por ano (PERCIVA, 2004; PITTET, 2005).

As infecções por *Candida* são consideradas como uma das principais causadoras de infecções nosocomiais. Estes microrganismos possuem a capacidade de formar biofilme em diferentes superfícies e vêm demonstrando bastante resistência aos antifúngicos disponíveis no mercado (WISPLINGHOFF *et al*., 2004; RAMAGE *et al*., 2006; YU *et al*., 2005; LAFLEUR *et al*., 2006; SENEVIRATNE *et al*., 2008).

Nas últimas décadas, a procura por novos medicamentos a base de plantas medicinais tem aumentado de forma significativa, com o intuito de descobrir novos compostos com efeitos *in vitro* e *in vivo*. Muitas dessas pesquisas já comprovaram que esses componentes de plantas apresentam atividade antimicrobiana, anti-inflamatória e antioxidante (VEIGA JUNIOR *et al*., 2007; BENMALEKE *et al*., 2013).

O óleo de *Pelargonium graveolens*, também conhecido como óleo de gerânio (GO) ou malva cheirosa, é extraído da árvore *Odorantissimum pelargonium* originária da África do Sul. Outros óleos podem ser produzidos por espécies dessa família como *P. odoranissimum, P. zonale e P. roseum* (BIGOS *et al*., 2012). Este óleo é usado popularmente para aromaterapia e no tratamento de inflamações, hemorroidas, como antibacteriano e antifúngico (MONDELLO *et al*., 2003; MARUYAMA *et al*., 2004).

Até o presente momento, há poucos estudos realizados com uso de nanoemulsões contendo óleo de *Pelargonium graveolens* em biofilmes formados por *Candida spp.* A nanotecnologia apresenta um grande número de vantagens, especialmente no desenvolvimento de carreadores de fármacos (com uma maior eficiência de encapsulação), além de uma liberação controlada. O fato de apresentar um tamanho bastante reduzido (o que pode facilitar a entrada do ativo na matriz polimérica do biofilme) também pode aumentar a potência antimicrobiana do óleo de gerânio.

O sistema nanoestruturado poderá aumentar a efetividade do óleo, aumentando, principalmente sua estabilidade e o tempo de contato sobre os biofilmes que poderão ser formados no material médico-hospitalar. Neste contexto, o desenvolvimento de nanoestruturas contendo óleo de gerânio aparece como uma estratégia interessante, visto que estes sistemas podem através de sua elevada superfície específica e reduzida tensão superficial, promover maior penetração de substâncias ativas (SALAMANCA-BUENTELLO, 2005).

A formação de biofilme é uma das características mais importantes para a virulência em espécies de *Candida*, e contribui para o aumento da resistência a fármacos antifúngicos. A relevância deste trabalho reside no fato de que nanoestruturas contendo óleo de gerânio podem constituir uma estratégia terapêutica a ser empregada em material médico-hospitalar (cateter) como agente antibiofilme, minimizando infecções hospitalares.

Cabe ressaltar que além de não haver estudos específicos sobre a incorporação do óleo de gerânio em nanoestruturas, a estabilidade, atividades antimicrobiana e antioxidante, citotoxicidade, genotoxicidade e atividade contra biofilmes ainda não foram estudadas, o que configura a originalidade desta proposta de desenvolvimento.

OBJETIVOS

Objetivo Geral

Desenvolver e caracterizar uma formulação nanoestruturada contendo óleo de gerânio e avaliar sua estabilidade físico-química, atividade antimicrobiana e antibiofilme em material médico hospitalar.

Objetivos Específicos

- Avaliar a atividade antifúngica e antibacteriana do óleo de gerânio puro e nanoestruturado;

- Desenvolver uma nanoemulsão contendo óleo de gerânio;

- Caracterizar a nanoemulsão em relação ao seu tamanho de partícula, índice de polidispersão, potencial zeta e pH;

- Desenvolver e validar um método indicativo de estabilidade por meio de CG, para a determinação quantitativa do óleo de gerânio na nanoemulsão;

- Conduzir estudo de estabilidade da formulação desenvolvida frente a principal condição de instabilidade do fármaco livre e também nanoestruturado;

- Caracterizar por análise termogravimétrica e calorimetria exploratória diferencial o óleo de gerânio puro e nanoestruturado;

- Realizar estudo de atividade antioxidante e citotoxicidade *in vitro* do óleo de gerânio puro e incorporado na nanoemulsão;

- Avaliar a capacidade protetora da nanoemulsão contendo o óleo de gerânio em macrófagos utilizando proteína solúvel de *Candida albicans* visando determinar o perfil inflamatório e influência na apoptose pela determinação de citocinas e das caspases 3 e 8;

- Avaliar a atividade antibiofilme do óleo de gerânio puro e incorporado na nanoemulsão em modelo de placas de polietileno e em cateter de poliuretano.

REVISÃO BIBLIOGRÁFICA

3. Revisão bibliográfica

3.1 Óleo de *Pelargonium graveolens*

Óleos voláteis derivados de plantas aromáticas possuem muitas propriedades biológicas e podem ser usados para prevenir e tratar doenças sistêmicas humanas, incluindo doenças infecciosas. Oferecem a oportunidade não apenas de combater a infecção, mas também inibem o crescimento da resistência microbiana (SIENKIEWICZ *et al*., 2014). Muitos deles têm alta atividade contra as bactérias Gram-positivas e Gram-negativas, assim como contra vírus e fungos (REICHLING *et al.*, 2009). Devido ao seu teor de agentes anti-infecciosos os óleos essenciais podem ser aplicados no combate contra as bactérias resistentes a fármacos e para a prevenção da formação de resistência de micróbios patogênicos (BIGOS *et al*., 2012).

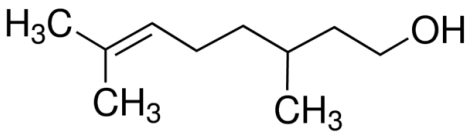
Pelargonium graveolens é uma das muitas espécies aromáticas do gênero *Pelargonium.* Seu óleo essencial também é conhecido como óleo de gerânio ou malva cheirosa. É obtido a partir de folhas, flores e talos e é originário da África do Sul. Pertencente à família Geraniaceae é composto por espécies herbáceas, arbustivas e subarbustivas (RAO, 2002). O gênero *Pelargonium* é bem adaptado ao clima das regiões sul e sudeste do Brasil, e por apresentar alta capacidade de hibridização, grande parte do material genético presente em nosso país é fruto de cruzamentos ocorridos naturalmente (MAY *et al*., 2007).

O óleo essencial de gerânio é um líquido pouco denso, brilhante, de coloração variando de amarelo pálido a amarelo esverdeado, odor por vezes de rosa, por vezes de menta (MAY *et al.*, 2007). Este óleo é comumente utilizado na medicina popular, entretanto, por ser perfumado, acaba sendo muito utilizado na indústria de cosméticos. É também usado como um agente aromatizante e como uma especiaria. As folhas desta espécie são recortadas e há a presença de pelos que lhes confere uma característica aveludada. Esses pelos possuem as glândulas que são responsáveis pelo armazenamento do óleo essencial, constituído de componentes altamente aromáticos (RANA, JUYAL, BLAZQUES, 2003).

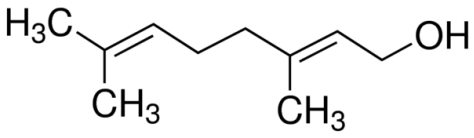
Figura 1: *Pelargonium graveolens.* Fonte: http://www.geraini.co.za

De acordo com a literatura, os dois componentes majoritários do óleo de *P. graveolens* são o citronelol e o geraniol. Estes podem ser determinados por Cromatografia Gasosa (CG) e alguns trabalhos têm empregado esta técnica analítica para a identificação e quantificação dos mesmos no óleo (LIS-BALCHIN *et al*., 2007; BIGOS *et al*.,2012).

O monoterpenoide citronelol é um composto de modelo linear naturalmente presente em plantas cítricas. O geraniol, por sua vez, é muito encontrado em espécie de flores, inclusive rosas, sendo estruturalmente parecido com o citronelol, diferem-se, porém pela adição de uma ligação dupla adicional conforme demonstrado na **figura 2** (HÖSCHLE & JENDROSSEK, 2005).



Citronelol



Geraniol

Figura 2: Estrutura química dos compostos majoritários presente no óleo de *Pelargonium graveolens* (Fonte: http://www.sigmaaldrich.com)

Estudos demonstram que o *Pelargonium graveolens* é bastante sensível às alterações climáticas. O rendimento e a concentração dos constituintes majoritários do óleo essencial variam conforme a sazonalidade, solo cultivado, a

idade da planta na colheita, a técnica utilizada na operação de destilação entre outros (MAY *et al*., 2007).

O geraniol apresenta valores inferiores nos meses de verão, período caracterizado por alta temperatura e baixa umidade, resultando na redução dos níveis fotossintéticos (RAO, 2002). Outros estudos conduzidos pelo mesmo autor demonstraram que no inverno o acúmulo de geraniol aumenta. O estresse sofrido devido à temperatura e umidade, associado ao longo fotoperíodo, provoca a conversão de parte do geraniol em citronelol e seus ésteres (RAO, 1996).

O óleo de gerânio demonstra ser atóxico, não irritante, geralmente não sensibilizante, e se desconhece quaisquer outros efeitos adversos (BOUKHATEM *et al*., 2013). Os efeitos terapêuticos incluem a aplicação no tratamento de disenteria, diarreia, condições das vias biliares, úlcera gástrica, diabetes, doenças de pele e para depressão. Os principais constituintes (citronelol, geraniol, linalol e isomentona) são os responsáveis pelas atividades biológicas (SHAWL *et al*., 2006; LIS-BALCHIN, 2004, DZAMIC, 2014).

Nos últimos anos a veiculação de óleos de origem vegetal em formulações nanoestruturadas vem sendo muito estudada com a finalidade de otimizar a liberação dos mesmos e protegê-los de uma possível volatilização e degradação. O desenvolvimento de drogas alternativas em sistemas de escala nanométrica possui a finalidade de melhorar a biodisponibilidade e eficácia de ativos fracamente solúveis em água ou compostos hidrofóbicos (KUO *et al*., 2008).

3.2 Atividade antioxidante

As plantas são conhecidas por produzirem algumas moléculas bioativas que podem reagir com outros organismos no meio ambiente e, por sua vez causarem a inibição do crescimento de bactérias ou fungos (atividade antimicrobiana) (SENGUL *et al*., 2011). Os óleos voláteis de plantas são constituídos por compostos terpênicos, que são especialmente conhecidos por sua atividade antioxidante (SACCHETTI *et al*., 2005).

O termo antioxidante tem natureza multiconceitual, entretanto no presente trabalho, pode ser definido como compostos de origem vegetal, que, presentes em baixas concentrações, comparativamente às biomoléculas que supostamente protegeriam, podem prevenir ou reduzir a extensão do dano oxidativo. Mais especificamente, antioxidantes agem nos organismos vivos por meio de diferentes mecanismos. Dentre estes, podem ser citados: a complexação de íons metálicos, a captura de radicais livres, a decomposição de peróxidos, a inibição de enzimas responsáveis pela geração de espécies reativas de oxigênio e nitrogênio e a modulação de vias sinalizadoras celulares (SENGUL *et al*., 2011).

Diversos ensaios foram desenvolvidos para screening de atividade antioxidante de compostos de origem vegetal. Os mais utilizados são o ABTS (ácido 2,2'-azinobis-(3- etil-benzotiazolino-6-sulfônico); DPPH (2,2-difenil- 1-picrilhidrazila), que é baseado na capacidade do DPPH reagir com doadores de hidrogênio; redução do íon férrico (FRAP), que está baseado na capacidade dos fenóis em reduzir Fe^{3+} em Fe^{2+} e capacidade de absorção de radical oxigênio (ORAC) (THAIPONG *et al*., 2006). Como alguns desses ensaios possuem mecanismos diferentes, sua resposta depende do tipo e da relação oxidante/ antioxidante utilizados (PAREJO *et al*., 2003).

3.3 Estresse Oxidativo

Os organismos aeróbios produzem constantemente espécies reativas como parte dos processos metabólicos. As espécies reativas do oxigênio (EROs) ou radicais livres são quaisquer espécies químicas que tem um ou mais elétrons desemparelhados (O_2^*, OH^*, H_2O_2). São produzidos endogenamente por processos metabólicos normais, porém, sua quantidade pode ser aumentada por fatores exógenos. Em consequência disso, há o aparecimento de danos oxidativos que podem provocar lesões celulares, muitas vezes irreparáveis (GOUVÊA, 2004; HALLIWELL, GUTTERIDGE 2007).

Entretanto, quando as EROs são produzidas sob condições normais nas células eucariotas, desempenham funções como: sinalização celular, proliferação e diferenciação; demonstrando grande importância para os processos celulares (DROGE, 2002; DENNERY, 2007).

O estresse oxidativo pode ser descrito como o desequilíbrio entre a formação de EROs e sua remoção do organismo, decorrente da geração excessiva de espécies reativas de oxigênio e/ou diminuição de antioxidantes endógenos. A ocorrência de um estresse oxidativo moderado, frequentemente, é acompanhada do aumento das defesas antioxidantes enzimáticas, mas a produção de uma grande quantidade de radicais livres pode causar danos e morte celular (ANDERSON, THOMAS, WILKINSON, 1996).

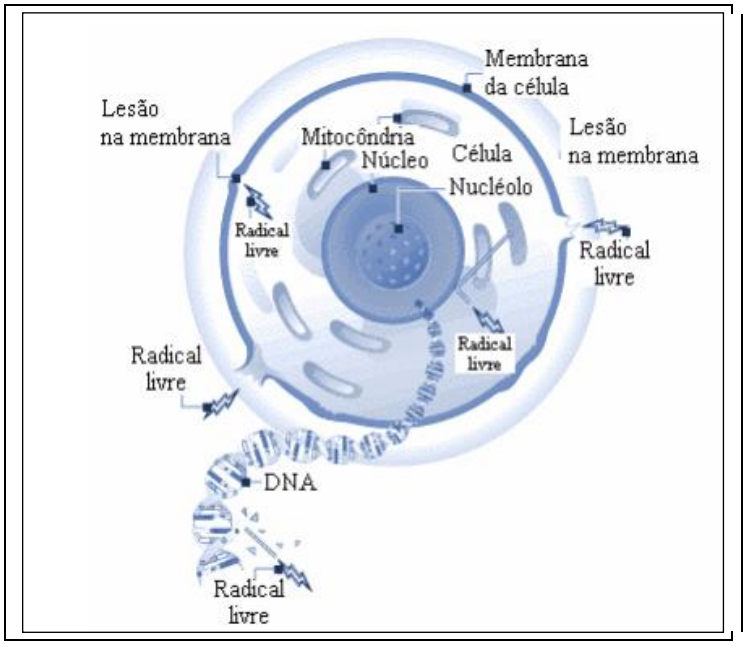


Figura 3: Ilustração de um ataque do radical livre na célula. (Fonte: http://pierre.senelart.com)

Devido a isso, o estresse oxidativo pode estar relacionado a alguns processos deletérios, como, por exemplo, a mutagênese, carcinogênese, envelhecimento, inflamação, isquemia cerebral, arterioesclerose, diabetes e demência (SIES, 1997; ALEXI *et al*., 2000; REN *et al*., 2001; JOHNSON, 2004).

Um complexo sistema de defesa antioxidante constituído de enzimas como a catalase, a superóxido dismutase, a glutationa peroxidase e a glutationa redutase, além de inúmeros sistemas de defesas não-enzimáticos incluindo as vitaminas A, E e C, flavonóides, ubiquinonas e o conteúdo de glutationa reduzida são responsáveis por neutralizar as EROs (ALEXI *et al.*, 1998; GIANNI *et al.*, 2004).

Neste contexto, surgem os biomarcadores, utilizados a fim de avaliar o balanço redox para análise dos danos causados pelo estresse oxidativo e com a finalidade de avaliar a deficiência das defesas antioxidantes. Os principais biomarcadores de estresse oxidativo são: espécies reativas ao ácido tiobarbitúrico (TBARS) (avaliam os danos em componentes celulares e estruturas lipídicas), compostos carbonílicos e sulfidrílicos (mensuram o dano nas proteínas), atividade das enzimas antioxidantes como a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx), medida de glutationa reduzida (GSH) e métodos fluorescentes como o diclorodihidrofluoresceínadiacetato (DCFH-DA).

Sabe-se que as plantas produzem uma grande variedade de substâncias antioxidantes contra os danos moleculares causados por EROs. Estudos recentes mostram que os antioxidantes naturais são indicados para atenuar os efeitos deletérios do estresse oxidativo celular. A partir desta perspectiva, as pesquisas buscam substâncias naturais com potente atividade antioxidante e baixa citotoxicidade (LIMA *et al*., 2006; ANDRADE *et al*., 2007; ROCHA *et al*., 2007; VICENTINO & MENEZES, 2007; BALESTRIN *et al*., 2008; IHA *et al*., 2008).

3.4 Citotoxicidade e Genotoxicidade

Os testes de toxicidade são realizados para determinar os riscos potenciais que novos produtos podem gerar na saúde e no meio ambiente como um todo (CASTAÑO e GÓMENEZ-LECHÓN, 2005). Assim, a citotoxicidade reflete os

efeitos sobre as estruturas celulares. A maioria das células devem mostrar uma resposta semelhante, e também responder de maneira similar quando a toxicidade é medida por vários critérios de viabilidade (BABICH, ROSENBERG e BORENFREUD, 1991).

A verificação da citotoxicidade nos óleos pode ser avaliada por uma série de bioensaios que são considerados mais ou menos sensíveis, expressando assim, resultados satisfatórios para utilização como triagem em relação à toxicidade dos mesmos (SOTELO-FÉLIX, 2002). Este ensaio *in vitro* é o primeiro teste para avaliar a biocompatibilidade de qualquer material para uso na biomedicina e consiste em colocar a amostra direta ou indiretamente em contato com uma cultura de células de mamíferos, verificando-se as alterações celulares por diferentes mecanismos (ROGERO *et al*., 2003).

Tendo em vista também o aumento da utilização de nanoestruturas contendo óleos de origem vegetal, faz-se necessário o emprego de um método rápido e eficiente para avaliar o seu impacto no ambiente e na saúde humana. Mesmo que modelos *in vivo* sejam eficazes para avaliação da toxicidade biológica, os modelos de cultura celular são altamente completos para o uso em estudos fisiológicos e para avaliar a toxicidade pré-clínica (CHUEHA *et al*., 2014).

A cultura de células fornece uma importante ferramenta para estudo da citotoxicidade de compostos com potencial atividade terapêutica, definindo o potencial de degeneração ou morte celular provocado pela amostra a ser testada. Assim, resultados positivos no ensaio de citotoxicidade descaracterizam a condição de inocuidade da amostra (CHORILLI *et al*., 2009; JONES; GRAINGER, 2009).

A viabilidade celular é um termo geral e pode ser investigada por ensaios que determinam um ou mais parâmetros celulares, tais como: 3-(4,5)-dimetiltialzolil-2,5- difeniltetrazólio (MTT) (atividade mitocondrial), enzima lactato desidrogenase (LDH), Vermelho neutro (VR), Azul Tripan entre outros.

Estes ensaios são geralmente adequados para medir efeitos tóxicos agudos de células cultivadas (SOENEN e DE CUYPER, 2009).

As nanoemulsões são preparações compostas de óleo, tensoativo e água e estão associadas com uma menor toxicidade (KAWAKAMI *et al*., 2002; LAWRENCE E REES, 2000). Estudos demonstram que algumas nanoestruturas podem interagir com as organelas celulares podendo muitas vezes chegar até o núcleo da célula atuando diretamente no material genético. Devido a isso, alguns parâmetros podem ser avaliados, como por exemplo: morfologia celular, a integridade do citoesqueleto, a funcionalidade das células e a genotoxicidade (dano diretamente ao DNA da célula) (MONTEIRO-RIVIERE, INMAN e ZHANG, 2009).

As agressões ao material genético podem resultar na indução ou promoção da carcinogênese, além de impactos na reprodução celular se o DNA de células germinativas for comprometido (DOAK *et al.*, 2011). O DNA pode sofrer mutações, afetar a estabilidade do genoma e levar ao desenvolvimento de doenças genéticas e câncer quando exposto a certos agentes (RILEY *et al*., 2008).

Atualmente existem técnicas capazes de marcar o DNA para que se possa correlacionar o dano causado ao núcleo de uma célula. O PicoGreen® é uma sonda fluorescente que tem a capacidade de se ligar ao DNA formando um complexo (DRAGAN *et al.*, 2010). As sondas fluorescentes podem interagir com os ácidos nucleicos desempenhando um papel importante nos estudos biofísicos das moléculas biológicas e seus complexos, proporcionando uma variedade de ensaios e técnicas (AHN, COSTA e EMANUEL, 1996).

3.5 Inflamação

A inflamação é um dos mecanismos de defesa contra a lesão dos tecidos causada por fatores biológicos, químicos ou físicos, ou seja, a inflamação é um mecanismo que provoca alterações no sistema vascular, nos componentes

líquidos e celulares, visando destruir, diluir ou isolar o agente lesivo. É a causa de uma ampla variedade de processos fisiológicos e patológicos (MEDZHITOV, 2008).

A inflamação é mediada por mediadores pró-inflamatórios (óxido nítrico (NO), prostaglandina E2 (PGE2) e citoquinas pró-inflamatórias, incluindo fator de necrose tumoral (TNF-α), interleucina (IL) -6 e IL-1β (JUNG et al., 2014). Os mecanismos dos processos inflamatórios no corpo humano são extremamente complicados e não podem ser atribuídos a um único mediador ou fator. O processo inflamatório possui basicamente duas fases, a fase aguda que é caracterizada por febre, dor e edema e a fase crônica que caracterizada pela proliferação celular (SÜLEYMAN *et al*., 2004).

A migração dos neutrófilos e eventos como exsudação plasmática caracterizam a fase aguda, pois esta refere-se a resposta inicial à lesão tecidual, é mediada pela liberação dos mediadores químicos, e em geral, precede o desenvolvimento da resposta imune. A inflamação crônica caracteriza-se por ter um período mais longo e por ter a presença de linfócitos e macrófagos, proliferação de vasos sanguíneos e necrose tecidual (ADER *et al*., 2006).

O resultado da resposta imune pode ser vantajoso para o hospedeiro quando permite que os agentes invasores sejam neutralizados ou fagocitados. Entretanto, pode ser deletério se resultar em inflamação crônica sem resolução do processo subjacente (GUYTON, 1996). O primeiro evento durante a resposta imune inata nos mamíferos é a ativação dos macrófagos, que fagocitam os patógenos invasores, secretam citocinas que medeiam outros eventos da resposta imune (ZANG & AN, 2007).

Enzimas como óxido nítrico sintetase induzida (iNOs) e ciclooxigenase-2 (COX-2) estão envolvidas na inflamação, tendo papel importante na resposta dos tecidos ao dano e a agentes infecciosos. Apesar da atividade fisiológica destas enzimas ser benéfica ao organismo, o aumento da expressão delas implica na patogenia de muitas doenças. Substâncias capazes de inibir a expressão ou a

atividade destas enzimas geralmente induzidas por citocinas inflamatórias são potenciais agentes anti-inflamatórios (RANG, 2007).

A ciclooxigenase-1 (COX-1) é expressa constitutivamente na maioria dos tecidos e os produtos desta enzima (prostaglandinas) são importantes para a manutenção de processos fisiológicos como a proteção gástrica e renal e a função plaquetária. A COX-2 normalmente não é detectável na maioria dos tecidos, mas é induzida rapidamente por citocinas, fatores de crescimento, hormônios, processos oncogênicos e durante processos patológicos como as inflamações (RUSSELL, 2001; FARROW & EVERS, 2002; KOKI & MASFERRER, 2002).

É importante em um processo inflamatório que ocorra a manutenção dos tecidos normais e a regulação da apoptose ou morte celular programada (LUZ *et al.*, 2009). A família das caspases caracteriza-se como a principal via de regulação da morte celular programada. Existem diferentes tipos de caspases que são expressas na maioria das células vivas, localizadas no citosol, na sua forma inativa (SCHWERDT *et al*., 2005).

A morte celular programada pode ocorrer através de duas vias principais, a via extrínseca e a intrínseca (FISHER, 2001). Pela via extrínseca, ocorrerá a ativação de receptores de membrana transmitindo sinais para o interior da célula e ativando, inicialmente a caspase-8 ou caspase-10. A via intrínseca, ativada por estresse celular, é mediada pela liberação do citocromo c pela mitocôndria, levando a ativação da caspase-9. Tanto a caspase-8 quanto a caspase-9 ativadas desencadeiam a ativação de outras caspases (caspases-3,-6,-7) capazes de clivar substratos celulares levando à apoptose (LORO *et al*., 2003).

A caspase-3, considerada a mais prevalente nas células, caracteriza-se como responsável pela maioria dos efeitos apoptóticos, como a quebra de muitas proteínas importantes na manutenção da homeostasia celular. A marcação da caspase-3, em imuno-histoquímica, tem sido utilizada para identificar células em apoptose, revelando fácil aplicabilidade e alta sensibilidade (LORO *et al*., 2003).

3.6 Gênero *Candida sp*

Nas duas últimas décadas a presença de micoses invasivas devido a fungos oportunistas demonstrou um aumento considerável, requerendo atenção, já que este expressivo aumento corresponde, também, em uma ascensão nas taxas de mortalidade. As infecções sistêmicas por *Candida* pertencem às enfermidades fúngicas mais frequentes que acometem os pacientes mais gravemente doentes (CALDERONE, 2002; WARNOCK, 2007; BIAŁON *et al.* 2014).

Candida é um fungo comensal que habita vários nichos do corpo humano, incluindo a cavidade bucal, trato gastrintestinal, vagina e pele de indivíduos sadios, sendo considerado também o mais frequente patógeno fúngico isolado de sítios clínicos (COLOMBO *et al.*, 2006). Dados demonstram que 25% das micoses superficiais são causadas por infecções por este gênero. Na população adulta e saudável, 80% possuem em seu tubo gastrointestinal colonizações deste gênero. Nas mulheres em torno de 20 a 30% possuem colonização de *Candida* na vagina. O trato intestinal é o reservatório mais significativo para fungos e leveduras, devido ao fato de que o suco gástrico quase não prejudica, por exemplo, a *Candida albicans* (JEHN *et al*, 2000).

O gênero *Candida* está representado por 200 espécies. A transição do inócuo comensal ao parasita depende de alguns fatores, tais como, o estado imune do hospedeiro e à versatilidade da *Candida* em se adaptar a uma grande variedade de habitats, bem como sua capacidade em formar biofilmes (SENEVIRATNE; SAMARANAYAKE, 2008).

As infecções causadas por leveduras do gênero *Candida* têm grande importância pela alta frequência com que infectam e colonizam o hospedeiro, representando uma ameaça crescente para a saúde humana. Os fatores que contribuem para que ocorram estas infecções, são o rompimento das barreiras cutâneas e mucosas, disfunção dos neutrófilos, defeito na imunidade mediada por células, desordem metabólica, exposição direta aos fungos, extremos de idade (recém-nascidos e idosos), desnutrição aguda, longo tratamento com antibióticos,

quimioterapia, transplantes, resistência a antifúngicos dentre outros (PFALLER & DIEKEMA, 2007).

Quando é abordado o termo resistência para espécies de *Candida*, é necessário diferenciar: resistência primária ou intrínseca da resistência secundária ou adquirida. A resistência intrínseca é uma resistência natural, ou seja, inata de uma espécie frente à determinada terapia antimicrobiana, ainda que o microrganismo jamais tenha estado em contato com tal agente antifúngico (LEWIS, 2009). No caso da resistência adquirida, os microrganismos tornam-se resistentes aos antifúngicos, devido a uma pré-exposição aos mesmos. Então, o uso prévio de antifúngicos pode selecionar algumas espécies menos sensíveis como a *C. glabrata*.

A incidência de infecções por estes microrganismos aumentou significativamente durante os últimos 20 anos. O uso de próteses, cateteres, tubos endotraqueais e marcapassos, também podem ser considerados fatores que pré-determinam a infecção por *Candida*. Esses dispositivos facilitam a colonização desses microrganismos permitindo a formação de biofilmes (WINGETER *et al*., 2007).

Muitos fatores de virulência têm sido propostos na patogenicidade das espécies de *Candida*, sendo que a adesão às células do hospedeiro, secreção de enzimas hidrolíticas, formação de hifas e desenvolvimento de biofilme aparentemente têm maior importância (WILLIAMS *et al*., 2011). O biofilme pode se desenvolver tanto em superfícies biológicas quanto superfícies inertes, como cateteres.

A formação de biofilme é importante na patogenicidade de *Candida* porque o torna uma barreira contra a penetração de fatores imunológicos do hospedeiro e de drogas antimicrobianas (VUONG *et al.*, 2004). Isto ocorre devido à formação de uma matriz extracelular polissacarídica, que inibe a difusão das moléculas do antimicrobiano para o interior do biofilme ou pela ação iônica do biofilme, que diminui sua ação esperada (AL-FATTANI, DOUGLAS, 2004; CLONTS *et al*., 2008).

Os biofilmes formados *in vitro* por *C. albicans* são descritos em três fases distintas: precoce (0-11 horas), intermediária (12-30 h) e de maturação (38-72 h). A fase prematura é caracterizada pela aderência e desenvolvimento de blastoconídios em microcolonias distintas. Em 24 h, a comunidade do biofilme pode ser vista como uma bicamada, compreendendo uma mistura de blastoconídios, pseudohifas e hifas jovens, além de produção da matriz caracterizando a fase intermediária. Na fase maturação uma espessa matriz abriga um denso emaranhado de células em todos os estágios de desenvolvimento de uma levedura (PERCIVAS, BOWLER, 2004).

A frequência de infecções em cateteres em decorrência de *C. albicans* está relacionada à colonização prévia do paciente e é justificada por seu potencial patogênico ou de virulência evidenciados pela capacidade de aderência à mucosas e epitélios, forte atividade secretória de enzimas (fosfolipases e proteinases), termotolerância e dimorfismo que auxilia a invasão de tecidos (LACAZ et al., 2002; BENDEL, 2003). A *C. tropicalis* é uma das espécies de *Candida* não-albicans mais prevalente. Muitos autores descrevem o isolamento de *C. tropicalis* em hemocultura de pacientes com câncer, leucemia e neutropenia (ALMIRANTE et al., 2005; NUCCI; COLOMBO, 2007). A *C. glabrata* também tem sido descrita como causadora de infecções hospitalares, entretanto sabe-se que é mais isolada de hemoculturas e ocorre mais frequentemente em idosos (PFALLER; DIEKEMA, 2002; PFALLER; DIEKEMA, 2007). Com menor frequência em fungemias, mas também importante, a *C. krusei* aparece em infecções, ocorrendo principalmente em pacientes com neutropenia, câncer, transplantados e naqueles infectados por HIV (HORN *et al*., 2009).

Apesar da *C. albicans* ser a espécie de maior prevalência em infecções, representando mais de 80% dos isolados, as outras espécies também são importantes e frequentemente relatadas. Além das *Candidas* não albicans já citadas acima, a *C. guilliermondii, C. parapsilosis, C. dubliniensis e C. kefyr* são relatadas em alguns estudos (MENEZES *et al*., 2004).

3.7 Biofilmes microbianos

Os biofilmes são agregados de microrganismos embebidos em uma matriz polimérica e aderidos a uma superfície sólida, formando uma estrutura porosa e altamente hidratada contendo exopolissacarídeos (EPS) e pequenos canais, abertos por entre microcolônias. Este tipo de organização é extremamente vantajosa a todas as espécies de microrganismos por fornecer proteção contra adversidades como desidratação, colonização por bacteriófagos e resistência a antimicrobianos (MELO, 2008).

O processo para formação do biofilme envolve fatores diversos, como ambientais, genéticos e físicos e pode trazer benefícios como malefícios para homens e animais (PLOUX *et al.*, 2007). Os biofilmes formam um ambiente dinâmico, no qual células microbianas se encontram em estado de homeostase organizadas de maneira a utilizar todos os nutrientes disponíveis. Podem se formar em próteses dentárias, cateteres e implantes médicos (CLONTS, 2008).

Os biofilmes são comunidades microbianas complexas, em que os microrganismos estão ligados em uma superfície natural ou artificial, envolvidos por uma matriz polimérica produzida por eles mesmos como uma forma de proteção as defesas do hospedeiro e aos agentes terapêuticos. Constituem um modo de crescimento protegido, que permitem a sua sobrevivência num ambiente hostil (DUNNE, 2002).

Um biofilme forma-se naturalmente em qualquer superfície sólida em contato com água não esterilizada (XAVIER *et al*., 2008). A formação e acumulação de biofilmes é resultado de vários processos de natureza física e biológica, conforme **figura 4.**

A vida dos microrganismos no interior do biofilme proporciona um grande número de benefícios quando comparados com os que se encontram de modo livre. Essas vantagens ocorrem devido ao fato dos agregados de microrganismos apresentarem maior disponibilidade de nutrientes, interferindo nas taxas de crescimento, cooperatividade metabólica e proteção aos fatores externos (BEHLAU e GILMORE, 2008).

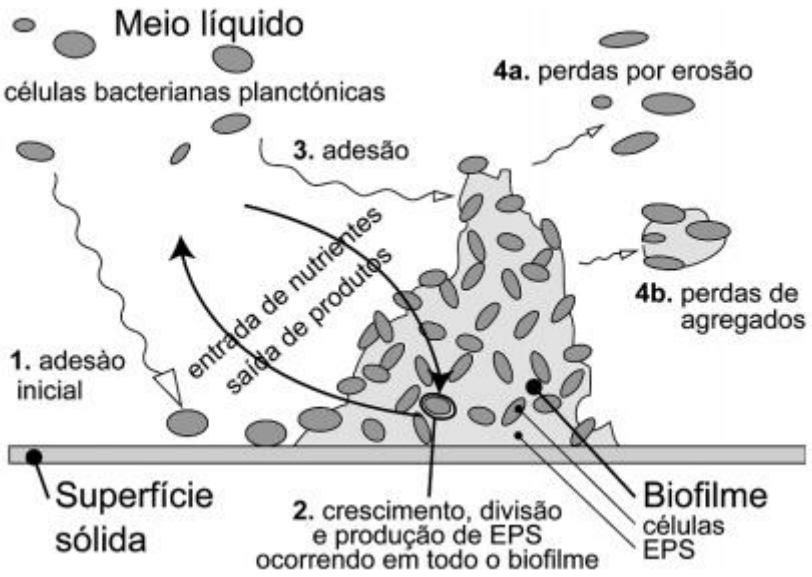


Figura 4: Processos envolvidos na formação e crescimento de biofilmes. Fonte: Xavier (2005)

A problemática da formação do biofilme ocorre devido ao fato de que estas estruturas podem ser 10 a 100 vezes mais resistentes aos agentes antimicrobianos do que as células planctônicas (não fixas). Essa resistência pode estar relacionada à dificuldade de penetração dos antimicrobianos na matriz composta por substâncias poliméricas extracelulares como EPS, com as alterações fenotípicas dos microrganismos no biofilme, com o desenvolvimento de mecanismos de resistência e, ainda, devido ao seu modo de crescimento estar associado à natureza crônica das infecções posteriores. Ou seja, apresentam um papel significativo no aparecimento de doenças infecciosas (T*URNER*, 2009; MORAN *et al*., 2009).

3.8 Biofilme em material médico hospitalar

Atualmente, a comunidade médica, já possui inúmeras evidências de que os biofilmes são os agentes causadores de várias infecções nosocomiais e crônicas, muitas das quais resistentes aos antimicrobianos disponíveis no mercado (ZHANG *et al.*, 2013; AKBARI & KJELLERUP, 2015). A maioria das infecções causadas por biofilmes está associada à utilização de implantes médicos invasivos (cateteres intravenosos e urinários, próteses ortopédicas, etc.),

além se estar relacionada à imunidade dos pacientes (KOJIC & DAROUICHE, 2004).

Um grande número de cateteres é inserido em pacientes todos os anos, e destes, mais de 60% acabam relacionados com a formação de biofilmes. Nestes casos, o período de hospitalização pode aumentar de 2 a 3 dias onerando em milhões de dólares, todos os anos, os custos associados ao manejo do paciente (SCOTT, 2009).

É conhecido que um número de espécies microbianas podem causar infecções na corrente sanguínea, entretanto os mais comuns são *Staphylococus* e espécies de *Candida* (AKBARI & KJELLERUP, 2015). Especificamente, espécies de *Candida* são referidas como sendo responsáveis por 5% a 71% de mortalidade e morbidade nas infecções sanguíneas de cateteres (DONLAN, COSTERTON, 2002; MORAN *et al*., 2009). Entre as espécies de *Candida*, a *C. albicans* mostrou ser comumente associado à formação de biofilmes na superfície de cateteres intravasculares (MARTINEZ *et al*., 2010).

A infecção se desenvolve, quando os microrganismos presentes na pele do paciente no local da inserção do cateter aderem à superfície do mesmo para formar um biofilme. Apesar da *C. albicans* estar entre as quatro principais causas de infecções sanguíneas oriundas de cateteres, outras espécies de *Candida*, tais como *C. parapsilosis*, *C. pseudotropicalis, e C. glabrata*, são capazes de formar biofilme. No entanto, são menos patogênicos que *C. albicans* (DOUGLAS, 2003).

Semelhante à maioria dos microrganismos, a *C. albicans* provoca a formação do biofilme e proporciona um ambiente protegido, além de aumentar a resistência aos antibióticos. Isto pode resultar na utilização de concentrações de antibiótico até 1000 vezes superiores ao necessário para o tratamento dos seus homólogos planctônicos (AKBARI & KJELLERUP, 2015). Assim, *C. albicans* tem uma resistência mais elevada aos antifúngicos tornando difícil evitar a formação dos biofilmes nestes dispositivos médicos (DOUGLAS, 2003).

Além das infecções sistêmicas causadas por espécies de *Candida*, os doentes que pertencem aos grupos etários extremos, como os

imunocomprometidos e em estado grave, geralmente precisam de terapias que utilizam métodos invasivos, o que facilita também a colonização por determinadas bactérias, como cepas de *Staphilococcus aureus* (CHENG *et al*., 2011; WARREN, 2012;) e *Pseudomonas aeruginosa* (GAYNES; EDWARDS, 2005).

Bonkat e colaboradores (2012) demonstram que os biofilmes que crescem em cateteres do trato urinário consistem basicamente de duas a seis espécies. A identificação de *Pseudomonas aeruginosa, Mirabilis Proteus, Providencia stuartii, Morganella morganii e Klebsiella pneumoniae* já foi feita em material médico hospitalar de uso prolongado.

A *P. aeruginosa* é um patógeno associado a diversas infecções nosocomiais, do trato respiratório e urinário, em queimaduras, feridas, olhos, dentre outras (BRANSKI *et al*., 2009; CHI *et al*., 2009; DRAGO, 2009). Tem como característica importante sua capacidade de adesão e propagação sobre dispositivos médicos como cateter, lentes de contato e próteses, resultando em muitos casos na formação de biofilmes (BRANSKI *et al.*, 2009; DIEC *et al.*, 2009).

Entres os anos de 1975 e 2003, observou-se um aumento de 9,6% para 18,1% das infecções hospitalares por este microrganismo, tornando-se a causa mais comum de infecções respiratórias agudas (GAYNES; EDWARDS, 2005). A formação de biofilme de bactérias sobre os dispositivos médicos, incluindo implantes, cateteres venosos centrais, cateteres do trato respiratório e urinário, tornou-se um problema grave e global (CHI, 2009; LASSEK *et al*., 2015).

Estima-se que 10 a 30% dos cateteres urinários estão associados a infecções e formação de biofilmes, o mesmo ocorre em 5 a 10% dos materiais de fixação de fraturas ósseas, além de 3 a 8% dos cateteres venosos centrais e 1 a 7% dos marca-passos, enxertos vasculares, próteses articulares e válvulas cardíacas mecânicas (DONELLI, 2006; VIALE; OPILLA, 2008). Os biofilmes associados às infecções urinárias e cateteres representam elevados custos hospitalares, além de altas taxas de morte (FINE, 2005).

3.9 Antifúngicos

As classes mais eficazes dos agentes antifúngicos utilizados no tratamento de infecções por *Candida sp.* são os azólicos, polienos e equinocandinas (COLOMBO, 2000), que representam o limitado arsenal de drogas antifúngicas no combate de infecções por estas leveduras (WHITE *et al.*, 1998; YU *et al*., 2005). Os antifúngicos azólicos são fungistáticos e seu uso é prejudicado pelo surgimento de espécies de *C. albicans* resistentes a azóis (YU *et al*., 2005).

Compostos azóis originais, como miconazol e econazol, e aqueles que se seguiram, tais como o cetoconazol, fluconazol e itraconazol, provaram ser importantes para combater as infecções fúngicas humanas. A eficácia clínica e a segurança do fluconazol fizeram com que seu uso se difundisse. A emergência resultante da resistência à azóis tem intensificado a busca por novos compostos que são ativos contra organismos resistentes (KHOT *et al*., 2006).

Os biofilmes são extremamente resistentes, por isso é necessário evitar a adesão de células livres a superfície. A correta higienização e sanitização tornam-se indispensáveis para evitar a formação de biofilme. Quanto mais denso o biofilme, mais difícil será para ele ser inativado ou removido. Por isso, é fundamental que exista um método para reduzir o número das células livres e prevenir a adesão. A baixa eficácia e o aumento da resistência dos biofilmes em relação aos agentes antimicrobianos limitam suas aplicações (DANESE, 2002; LAFLEUR et al., 2006).

Vavala e colaboradores (2013) investigaram a formação de biofilme em cepas de *Candida albicans* e o efeito de resistência do fármaco fluconazol sobre a formação de biofilme. A formação de biofilme em diferentes biomateriais (silicone, látex, policloreto de vinilo, polipropileno, nylon) foi analisada após 72 horas de incubação a 37°C. As cepas de *Candida* estudadas foram resistentes aos azólicos e anidulafungina e demonstraram forte capacidade de formar biofilmes nas superfícies de todos os biomateriais analisados.

Shinde et al, (2013) analisaram o potencial da cloroquina em combinação com quatro agentes antifúngicos, fluconazol, voriconazol, anfotericina B, caspofungina sobre o crescimento de biofilmes de *C. albicans.* Não houve

alteração significativa na sensibilidade dos biofilmes à caspofungina e anfotericina B quando combinadas com cloroquina, entretanto, este estudo, pela primeira vez demonstra que a cloroquina potencializa a atividade anti-biofilme do fluconazol e voriconazol.

As bases moleculares para o desenvolvimento de resistência intrínseca a antifúngicos têm sido estudadas. A determinação da resistência em micologia não está sujeita às mesmas condições prévias da bacteriologia. Por um lado, os fungos, como eucariontes, em contraste com as bactérias, nesta circunstância podem desenvolver secundariamente resistências de plasmídeos contra um antimicótico azólico primariamente eficaz ou antimicótico polieno. Por outro lado, estão sujeitos à corrente das mais diferentes influências de meio, pH, temperatura, tamanho do inóculo, entre outros (BENNETT *et al*, 2004; JEH *et al*., 2000).

A multirresistência dos microrganismos aos antimicrobianos comuns tornou-se uma das preocupações mais importantes na medicina moderna. O uso de plantas e nanoestruturas são uma nova alternativa para tratar com sucesso os microrganismos multirresistentes.

3.10 Nanotecnologia

Nos últimos anos, a nanotecnologia é um ramo da ciência que tem se destacado em diferentes setores da economia. A área da saúde é um dos setores mais beneficiados com os seus avanços, revolucionando o campo de liberação de fármacos e agentes de diagnóstico a sítios específicos (JAIN, 2005; SALAMANCA-BUENTELLO *et al*., 2005; HU *et al*., 2011). Sistemas nanoestruturados podem apresentar vantagens como direcionamento do fármaco a locais específicos, diminuição dos efeitos colaterais, liberação controlada e principalmente aumento da biodisponibilidade (KUMARI, YADAV e YADAV, 2010; SCHÄFER-KORTING, 2010). Dentre os diferentes nanomateriais que vêm sendo estudados, podemos citar as nanopartículas metálicas, os lipossomas, as nanopartículas lipídicas sólidas, as nanopartículas poliméricas e as nanoemulsões (TORCHILIN, 2007).

No entanto, as nanopartículas e as nanoemulsões normalmente apresentam-se como suspensões aquosas, com estabilidades físico-químicas limitadas. Neste sentido, diferentes técnicas têm sido propostas para desenvolver e produzir estas formulações, a fim de se obter um produto mais estável e versátil.

Nanoemulsões são sistemas isotrópicos dispersos com gotículas de tamanho que estão sendo definidos de forma diferente por diferentes autores, tais como 1-100 nm, (MASON *et al*., 2006), 20-200 nm (GUTIẺRREZ *et al*., 2008), 100-500 nm (LOVELYN, ATTAMA, 2011) e menores que 1000 nm (BOUCHEMAL *et al.,* 2004). Devido ao tamanho de suas gotas são cineticamente estáveis (SABERI *et al.,* 2013). Segundo CAPÉK (2004), as nanoemulsões apresentam característica translúcida quando o tamanho de glóbulos é inferior a 200 nm e aparência leitosa quando se encontram na faixa de 200-500 nm.

O desenvolvimento de uma nanoemulsão envolve a combinação de misturas isotrópicas com um componente oleoso e um agente tensoativo numa razão específica. Quando a relação correta é utilizada e for dispersa em meio aquoso sob agitação, o composto resultante terá a capacidade de formar uma nanoemulsão (SEO *et al*., 2013). O uso de tensoativos é o fator responsável pela estabilidade das nanoemulsões e esses compostos anfifílicos posicionam-se entre as duas fases da emulsão (aquosa e oleosa), originando uma película interfacial que estabiliza o sistema (FERNANDEZ *et al.*, 2004).

As nanoemulsões podem ser produzidas através de diferentes métodos. Os que utilizam alta energia ocorrem normalmente em duas etapas. Primeiramente, as fases aquosa e oleosa são aquecidas separadamente e emulsionadas através do uso de homogeneizadores de alta velocidade (como Ultraturrax®). Será obtida uma emulsão primária, com tamanho de gotícula micrométrico. Em uma segunda etapa, com o resfriamento, as gotículas são reduzidas a valores submicrométricos através da utilização de homogeneizadores de alta pressão ou outros equipamentos (YILMAZ & BORCHERT, 2005).

O diâmetro das gotículas produzidas por homogeneização à alta pressão pode ser afetado pela pressão, temperatura e número de ciclos de homogeneização. Estudos demonstram que o aumento do número de ciclos no processo reduz o tamanho de gotícula, entretanto, esta redução possui um limite a partir do qual não é mais relevante (TADROS *et al*., 2004). O uso de ultrassom para preparação de nanoemulsões também tem sido descrito por vários autores, entretanto algumas desvantagens são atribuídas a sua utilização, como aquecimento excessivo da amostra, contaminação do produto com resíduo de titânio provenientes da sonda, baixa reprodutibilidade em relação ao diâmetro de gotícula, além de problemas relacionados às dificuldades de transposição de escala (SOLÉ *et al*., 2006).

Os métodos de emulsificação de baixa-energia para produção de nanoemulsões utilizam a energia química armazenada nos componentes da formulação. A emulsificação espontânea é um exemplo. Este método utiliza uma fase orgânica, composta de óleo-solvente e uma fase aquosa, com subsequente evaporação do solvente e redução do volume sob pressão reduzida (SOLANS *et al*., 2005). O tamanho e a distribuição das gotículas são fortemente afetados pela natureza do solvente utilizado durante o processo (BOUCHEMAL *et al*., 2004).

A utilização de óleos essenciais em sistemas nanoestruturados vem demonstrando ser muito vantajoso, já que diminui a volatilização e reduz a instabilidade e perda do material. Este sistema pode proteger uma substância da ação do meio ambiente, além de melhorar a especificidade, a solubilidade e também a penetração intracelular nos tecidos biológicos (DANIELLI *et al*., 2013).

3.11 Estabilidade das formulações nanoemulsionadas

Sistemas emulsionados podem sofrer diferentes processos de instabilidade, tais como: cremeação, floculação e coalescência. Estes processos podem ser causados pela diferença nos valores de densidade entre os líquidos

dispersos, pela redução da estabilidade da interface entre outros fatores (SHUELLER, ROMANOWSKI, 2000). No caso das nanoemulsões, o tamanho reduzido dos glóbulos dificulta a deformação destes, diminuindo a instabilidade por coalescência, cremeação e sedimentação. A espessura do filme interfacial dessas partículas, em relação ao tamanho dos glóbulos, dificulta o rompimento destes glóbulos por redução natural deste filme (TADROS, 2004; MORAIS *et al.*,2006).

Entretanto, considerando-se que diversos fatores podem alterar a estrutura do sistema ao longo do tempo, modificando o potencial zeta ou a rigidez da camada de emulsificantes, devem ser realizados estudos de estabilidade em longo prazo (AKKAR & MULLER, 2003). As propriedades físico-químicas, influenciadas pela composição quali e quantitativa e pelas condições de preparação, devem ser estritamente controladas, visando à administração, principalmente quando esta é por via parenteral e a estabilidade do sistema (HUNG *et al.*, 2007). Um método muito utilizado para avaliar a estabilidade de uma emulsão é mensurar o tamanho dos glóbulos da fase interna e o potencial zeta (MORAIS *et al.*, 2006).

O tamanho dos glóbulos dispersos de uma emulsão irá determinar os processos de coalescência e floculação, além de determinar a polidispersividade. Quanto maior o índice de polidispersão maior será sua susceptibilidade ao fenômeno "Ostwald ripening" (CAPEK, 2004). Este fenômeno pode causar a movimentação de moléculas de óleo através de difusão passiva ou transporte assistido por micelas. Essas moléculas são transferidas dos menores glóbulos para os maiores, provocando a alteração da granulometria da fase dispersa (SOUNNVILLE-AUBRUN, SIMONNET, ALLORET, 2004).

Além das análises físico-químicas, as nanoemulsões contendo óleos voláteis devem ter sua estabilidade estudada realizando-se a quantificação dos compostos do óleo na formulação ao longo do tempo. A separação, identificação e quantificação de compostos em óleos voláteis devem ser realizadas e são úteis no controle de qualidade das formulações. Em sua grande maioria, utiliza-se a

cromatografia gasosa (CG), pois esta apresenta uma elevada capacidade de separação, capaz de determinar pequenas quantidades de substâncias. Outras técnicas de análise, como a técnica de *headspace*, também pode ser utilizada para a detecção de pequenas quantidades de componentes voláteis em plantas ou em matrizes complexas (LOCKWOOD, 2001).

A necessidade de assegurar a credibilidade de resultados obtidos após diferentes análises farmacêuticas está sendo cada vez mais reconhecida e exigida. Com esta finalidade, indústrias e laboratórios acadêmicos estão desenvolvendo e validando métodos que possam garantir dados analíticos confiáveis e reprodutíveis. A validação de métodos é uma das medidas universalmente reconhecida como parte fundamental do sistema de garantia de qualidade, principalmente na área analítica. Este processo engloba desde o planejamento até a execução experimental do protocolo, assim como o tratamento estatístico dos resultados (THOMPSON, ELLISON e WOOD, 2002; RIBANI *et al*., 2004).

Todo método analítico deve ser validado, para isso, existem recomendações a serem seguidas, as quais foram definidas e regulamentadas por diferentes organizações. No Brasil, a Agência Nacional de Vigilância Sanitária (ANVISA) e o Instituto Nacional de Metrologia, Normalização e Qualidade Industrial (INMETRO) disponibilizam guias e resoluções que descrevem e orientam como executar essa validação. Os parâmetros analíticos a serem validados devem ser baseados na intenção de uso do método (SHABIR, 2003; RIBANI *et al*., 2004).

Outras análises que podem ser realizadas e contribuem no estudo de estabilidade do óleo volátil puro e nanoestruturado são as chamadas análises térmicas. Estas abrangem um grupo de técnicas nas quais uma propriedade física ou química de uma substância, ou de seus produtos de reação, é monitorada em função do tempo ou temperatura, enquanto a temperatura da amostra, sob uma atmosfera específica, é submetida a uma programação controlada (MENCZEL e WILEY, 2009).

A caracterização térmica dos compostos químicos pode ser realizada mais comumente utilizando a análise termogravimétrica (TGA) e a análise de calorimetria exploratória diferencial (DSC). A determinação da estabilidade térmica dos compostos pode ser realizada a partir da análise de TGA, enquanto a análise de DSC permite a determinação das transições de fase onde fenômenos químicos (decomposição, combustão) ou físicos (mudanças de estado (fusão, sublimação e transições cristalinas) podem ser observados (FRIZZO *et al*., 2013).

A análise termogravimétrica (TGA) é a técnica na qual a mudança da massa de uma substância é medida em função da temperatura enquanto esta é submetida a uma programação controlada. Desta forma, é possível acompanhar as variações de massa envolvidas em um experimento, sendo seus resultados de ordem quantitativa (BROWN, 2001).

A calorimetria exploratória diferencial (DSC) é uma das técnicas mais utilizadas, pois fornece informações detalhadas sobre as propriedades físicas e energéticas das substâncias. Em uma análise térmica diferencial, a diferença de temperatura entre uma amostra e uma referência é medida em função de um ciclo de temperatura pré-programado. A amostra e a referência estão localizadas em panelas sob um termopar em um forno aquecido eletricamente. A diferença de temperatura entre amostra e referência é equilibrada variando o calor necessário para manter ambas as panelas com a mesma temperatura. Esta diferença de energia é plotada em função da temperatura da amostra (CLAS *et al*., 1999).

A análise de DSC pode ser aplicada para estudos de determinação de pureza, construção de diagramas de fase, determinação de entalpia de transições, determinação de temperaturas de transição vítrea, fusão, cristalização e grau de cristalinidade da amostra (FRIZZO *et al*., 2013).

CAPÍTULO 1: Atividade antifúngica e antibacteriana do óleo de gerânio puro e nanoestruturado

Resumo

O aumento de infecções decorrentes da resistência aos microrganismos é um fato bastante preocupante, requerendo atenção, devido principalmente, a uma ascensão nas taxas de mortalidade. As pesquisas por substâncias de origem natural estão sendo constantemente realizadas com a finalidade de aumentar o arsenal terapêutico no combate e controle destas infecções microbianas. Neste contexto, o presente trabalho utilizou o óleo de *Pelargonium graveolens*, conhecido popularmente como óleo de gerânio (GO) com o objetivo de verificar suas possíveis atividades contra algumas cepas de bactérias, micobactérias e fungos. O GO foi nanoencapsulado (NC1) e a formulação também foi testada. Sabe-se que as nanocápsulas fornecem alguns benefícios ao óleo, como por exemplo, os protegem da possível degradação e volatilização, além de auxiliar na liberação mais lenta da substância e vetorizar a mesma ao local de ação. Primeiramente, realizou-se a análise do GO por GC/ MS. As NC1 foram preparadas pelo método de deposição interfacial de polímero pré-formado, utilizando-se uma fase orgânica, composta pelo GO, monoleato de sorbitano, poly (ε- caprolactona) com a acetona e a fase aquosa foi composta de água purificada e polissorbato 80. Foram produzidas nanocápsulas controle (NC2) sem o GO, com a finalidade de verificar se algum componente poderia interferir nos resultados. As MICs foram determinadas para todos os microrganismos testados e realizou-se para *Candida albicans* o teste de inibição da formação do tubo germinativo, pois este é considerado o principal fator de virulência desta espécie. Todos os ensaios foram realizados em triplicata e o GO, NC1 e NC2 foram utilizados como amostras.Os resultados da análise do GO mostraram que os dois principais componentes são o citronelol e o geraniol, corroborando com a literatura. As nanocápsulas desenvolvidas (NC1 e NC2) apresentaram características manométricas. As NC1 apresentaram diâmetro médio de partícula de 188 nm, índice de polidispersão de 0,149, potencial zeta de -10,8 mV e pH igual a 5,5. Já as NC2 apresentaram diâmetro médio de partícula de 233,3 nm, índice de polidispersão de 0,185, potencial zeta de -10,7 mV e pH igual a 5,8. Os

resultados das MICs mostraram que o GO foi mais eficiente contra as espécies de *Mycobacterium*, apresentanto uma MIC de 17,9 µg/ml para *M. avium* e 35,9 µg/ml para *M. smegmatis*, *M. abscessos*, *M. massiliense.* Para as bactérias *E. faecalis, Streptococcus, Listeria monocytogenes*, *Pseudomonas aeroginosa* e *Salmonella enteritidis* a MIC encontrada foi de 149 µg/ml. Já a NC1 foi eficaz para *M. smegmatis* (149,7 µg/ml), *M. abscessos* (35,9 µg/ml), *M. massiliense* (35,9 µg/ml), *M. avium* (71,8 µg/ml), *Enterococcus faecalis*, *Streptococcus* (149,7 µg/ml) e *Listeria monocytogenes* (35,9 µg/ml). Quando o GO foi testado contra as *Candidas*, observou-se a MIC de 8.9 µg/ml para *C. albicans, C. kefyr*, *C. dublinienesis e C. glabrata.* Para NC1 foi realizado o teste de macrodiluição, a fim de aumentar o tempo de contato da formulação com as *Candidas*. A NC1 foi capaz de reduzir significativamente o número de células de *C. albicans e*m cerca de 5 log, 4 log para *C. dublinensis, C. glabrata, e C. krusei*, e 2 log para de *C. parapsilosis.* Para finalizar nosso estudo, os resultados obtidos no teste de inibição da formação do tubo germinativo para *C. albicans* demonstraram que o GO na concentração de 2x MIC possui atividade, entretanto a NC1 não inibiu este importante fator de virulência, sugerindo o desenvolvimento de uma formulação nanoestrutura que consiga liberar o óleo mais rapidamente.

ARTIGO 1: Antimycobacterial, Antimicrobial and Antifungal Activities of Geranium Oil-Loaded Nanocapsules

ANTIMYCOBACTERIAL, ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF GERANIUM OIL-LOADED NANOCAPSULES

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ABSTRACT

Purpose: The aim of this study was to perform the first ever investigation of the effect of activities in the nanocapsules containing Geranium oil (NC1) against different microbial of pathogens such as *Mycobacterium* spp. (both fast growing and slow growing), bacterial, and yeasts. Methods: The GO was analysed by GC and GC/MS. Nanocapsule suspensions (NC) were prepared by interfacial deposition of a preformed polymer method and the MICs were determined for the antimycobacterial, antimicrobial, and antifungal activities. Results: GO-loaded nanocapsules (NC1) presented nanometric mean diameters (188 nm), polydispersity indices below 0.149, pH (5.5), and zeta potentials (about -10.8 mV). The NC1 was effective against *Mycobacterium smegmatis* (MIC =149.7 µg ml^{-1}), *M. abscessos* (MIC = 5.9 µg ml^{-1}), *M. massiliense* (MIC= 35.9 µg ml^{-1}), *M. avium (MIC*= 71.8 µg ml^{-1}), *Enterococcus faecalis*, *Streptococcus sp.* (MIC= 149.7 µg ml^{-1}), and *Listeria monocytogenes* (MIC= 35.9 µg ml^{-1}). The NC1 was able to significantly reduce the number of cells of *C. albicans* (by approximately 5 log), 4 log the number of cells of *C. dublinensis*, *C. glabrata*, and *C. krusei*, and 2 log the number of cells of *C. parapsilosis* compared to the control group. Conclusions: Our study showed that the geranium oil- loaded nanocapsules have antimycobacterial activities similar to free oil. The GO was effective in inhibiting the formation of germ tubes of *Candida albicans*, and the nanocapsule containing GO failed to inhibit the formation of this important virulence factor.

Keywords: Nanocapsule**,** geranium oil, antimycobacterial, antimicrobial, antifungal, and nanotechnology.

INTRODUCTION

Recently, the clinical use of essential oils has expanded worldwide to include therapy against various kinds of diseases. The antimicrobial and antifungal properties of essential oils have been documented and have acquired greater importance. Essential oils can be effective in the treatment or prevention of fungal and parasitic diseases due to properties such as low density and rapid diffusion across cell membranes [1].

The oil *Pelargonium graveolens*, also known as geranium oil (GO) or mauve is extracted from the tree *Pelargonium odorantissimum* originating from South Africa. The *Pelargonium genus* (Geraniaceae) is represented by many essential oil producing species: *P. graveolens*, *P. odoranissimum, P. zonale*, *and P. roseum*. Geranium oil is obtained from the leaves, flowers, and stalks using steam or hydrodistillation. The GO has historically been used in the treatment of dysentery, hemorrhoids, inflammation, heavy menstrual flows, and even cancer [2]. The French medicinal community currently treats diabetes, diarrhea, gallbladder problems, gastric ulcers, liver problems, sterility, and urinary stones with GO. The main constituents responsible for biological activity are citronellol, geraniol, linalool, isomenthone, nerol, and citronellyl formate [3]. However, because of their chemical complexity, susceptibility to degradation, and volatility and insolubility in water, it is necessary to improve the oil's stability, contributing to the product's effectiveness.

Nanostructured systems appear as a potential system for asset management with lipophilic character. An important advantage of these systems is their small size (below 1 μm). In addition, further advantages are the possibility of increasing the effectiveness and stability of formulations or active substances, as well as their gradual release in adequate doses. Thus, one of the most promising areas for the use of nanocapsules is the vectorization of essential oils with antimicrobials activities [4–6].

Little is known about the biological activities of GO. The search for new antifungal and antibacterial agents is an important field. The prevalence of resistance among key microbial pathogens is increasing at an alarming rate worldwide [7]. The antibacterial activity of essential oils depends on their chemical composition, climate, season, geographical conditions, harvest period, and distillation technique. Bacteria have a genetic ability for transmitting and acquiring resistance to drugs. Recently, the antimycobacterial activity of *Melaleuca alternifolia* oil and nanoparticles across the different strains of mycobacteria were evidenced [8].

The fungi, like *Candida*, are opportunistic etiological agents. This means that the infection and the expansion occur only in the event of a predisposition of the host organism [9–12]. Studies indicate that geraniol, the major constituent of GO, shows activity against gram-negative bacteria and some *Candida* species [13]. Studies have revealed that nanostructures could be a delivery system to enhance the stability and water solubility of essential oils [14,15]. The advantages compared with conventional drug-delivery systems include improved efficacy, reduced toxicity, protection of active compounds, and enhanced biocompatibility [16]. Aiming to expand the utilization of GO and seeking to increase the use of this kind of medical form and take advantage of proven pharmacological actions of the essential oil, we evaluated the effect of GO-loaded nanocapsules (NC1) against different species of pathogens, such as *Mycobacterium spp.* (both fast growing as slow growing), bacterial, and yeasts for the first time.

MATERIAL AND METHODS

Acquisition of GO and reagents

The geranium essential oil (Lot STD1012) was purchased from Seiva Brázilis Ativos Naturais Ltd, São Paulo, Brazil; Dimethyl sulfoxide (DMSO) was used to dilute GO. Amphotericin B, for antifungal activity, and Amikacin, for

antibacterial activity, were used as controls in the experiments.

Geranium oil analysis

Oil composition and yield were analyzed by gas chromatography (GC), using an Agilent Technologies 6890N GC-FID system, equipped with a DB-5 capillary column (30 m x 0.25 mm x 2.5 mm film thickness) and connected to a flame ionization detector (FID). The injector and detector temperatures were set to 250°C. The carrier gas was helium with a flow rate of 1.3 ml/min. The thermal program was 100-280°C at a rate of 10°C/min. Two replicates of samples were processed in the same way. The injection volume of the GO was 1 µl [17]. GC-Mass Spectroscopy (GC-MS) analyses were performed on an Agilent Technologies AutoSystem XL GC-MS operating in the EI mode at 70 eV, equipped with a split/splitless injector (250°C). The transfer line temperature was 280°C. Helium was used as a carrier gas (1.5 ml/min) and the capillary columns used were an HP 5MS (30 m x 0.25 mm x 2.5 µm film thickness) and an HP Innowax (30 m x 0.32 mm i.d., film thickness 0.50 µm). The temperature programmed was the same as that used for the GC analyses. The injected volume was 1 µl of the essential oil.

Identification the constituents of GO was performed on the basis of retention index (RI), determined with reference to the homologous series of *n*-alkanes C_7-C_{30}, under identical experimental conditions, comparing with the mass spectra library search (NIST and Wiley), and with the mass spectra literature. The relative amounts of individual components were calculated based on the CG peak area (FID response).

Preparation of the formulation

Nanocapsule suspensions (NC) were prepared (n = 3) by interfacial deposition of the preformed polymer method. Briefly, an organic phase composed of GO (0.9 g), sorbitan monooleate (0.192 g), poly (Ɛ-caprolactone) (0.25 g), and acetone (67.0 ml) was added to an aqueous solution (133.0 ml) containing polysorbate 80 (0.192 g) and kept under moderate magnetic stirring

for 10 min. The nanocapsule containing GO was labeled NC1 and a control nanocapsule (NC2) containing the same constituents of NC1 was produced, but without GO. This nanocapsule was added to the (0.9 g) medium-chain triglycerides (MCT). The organic solvent was then eliminated from both NC1 and NC2 in a rotary evaporator (Fisatom, São Paulo, Brazil) at 60 rpm and 30-35°C temperature. The final volume of the formulations was fixed in 25 ml to obtain a concentration of 1% of oil (10 mg/ml).

The particle sizes and polydispersity index (n=3) were measured by photon correlation spectroscopy (Malvern Zetasizer/Nanosizer®) and zeta potential values were measured by electrophoretic mobility, after dilution of 20 µl samples in 20 ml of NaCl (1 mM). The pH value of the nanocapsules was analyzed by Digimed direct readings potentiometer (São Paulo, Brazil) at room temperature.

Dilution of GO and nanoparticles

The density of the GO (0.92 g ml^{-1}) was determined, and the same dilution (1: 1) was performed in DMSO to reach a concentration of 460 mg ml^{-1} (Solution I). Afterwards, dilution was made at 1:100 in a Middlebrook 7H9 base medium (antimycobacterial activity) or a Mueller-Hinton broth (antimicrobial activity) to yield a concentration of 4.600 µg ml^{-1} (Solution II). Then, 50 µl (antimycobacterial activity) or 200 µl (antimicrobial activity) of solution II were added to the first well of the microplates and, after homogenization, were moved to the same volume and so on, yielding final concentrations of: 2.300, 1.150, 575, 287.5, 149.7, 71.8, 35.9, 17.9, 8.9, 4.4 µg ml^{-1}. NC1 (575 µg ml^{-1}) and NC2 were added to the first well and after homogenization were transferred to the second, and so on to give final concentrations of 287.5, 149.7, 71.8, 35.9, 17.9, 8.9, 4.4 µg ml^{-1}, respectively.

Antimycobacterial activity

To evaluate the antimycobaterial activity of GO and NC1, the following microorganisms were used: *Mycobacterium smegmatis* ATCC 700084, *M. abscessus* ATCC 19977, *M. massiliense* ATCC 48898, and *M. avium* LR541CDC. The mycobacterial strains were thawed, picked to Lowenstein Jensen medium, and kept in an incubator until visible growth of the colonies. Subsequently, colonies were suspended in a Middlebrook 7H9 base medium supplemented with 10% OADC (oleic acid-albumin-dextrose-catalase) (Difco Laboratories, Detriod, Michigan) and 0.2% glycerol (MD7H9) then incubated for 3 to 7 days at 35 ± 2°C in a tube containing glass beads. This suspension was then homogenized in a vortex shaker and standardized to 0.5 on the Macfarland scale; the fast-growing mycobacteria suspension was further diluted in MD7H9 to a concentration of 10^5 UFC/ml. From this bacterial suspension, the assay was performed based on the protocol M7-A6 [18]. The assay was performed in microtiter plates of 96 wells in triplicate. Serial dilutions were performed GO, NC1, and NC2 as described above. A volume of 50 µl of each dilution was added to the well along with 50 µl of each bacterial suspension. Were also carried out controls of the medium, the microorganism, GO, NC1, and NC2. The plates were sealed with parafilm before being sterilely capped to prevent contamination occurred and volatilized oil. Subsequently, the plates were incubated at 35 ± 2°C for 5 to 7 days in a humid chamber. The results were observed by the formation of bacteria dotted at the bottom of the wells.

Antimicrobial activity

To evaluate the antimicrobial activity of GO and NC1, the following microorganisms were used: *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 7644, *Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* ATCC 35218, *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922 and *Pseudomonas aeroginosa* ATCC 340. Clinical isolates of *Streptococcus* sp, *Staphylococcus aureus*, *Klebsiella pneumoniae* KP+ (HCPA), *Klebsiella pneumoniae* KP+ (USP), *Klebsiella pneumoniae*, *Salmonella enteritidis*,

Enterococcus sp, and *Shigella flexneri.* The determination of the minimum inhibitory concentration was performed based on the protocol M7-A7 [19]. The bacterial suspension was prepared in saline with a turbidity equivalent to tube 0.5 of the MacFarland scale (1 x 10^8 UFC ml^{-1}). Then, this suspension was diluted at 1:100 in a Mueller-Hinton broth, yielding as inoculum 1 x 10^6 UFC ml^{-1}. This suspension was inoculated with 10 µl (1 × 10^4 UFC) into each well already containing 200 µl of different concentrations of the GO, NC1, and NC2 as described above. The microplates were incubated at 35 ± 2°C for 24 h, under aerobic conditions. The MIC (Minimal Inhibitory Concentrations) was defined as the lowest concentration of compounds that inhibits bacterial growth. This test was performed in triplicate on separate occasions. The 2,3,5-triphenyltetrazolium chloride was used as an indicator of bacterial growth.

Antifungal activity

For evaluating the antifungal activity of GO and NC1, the following microorganisms were used: *Candida albicans* ATCC 14053, *Candida tropicalis* ATCC 66029, *Candida glabrata* ATCC 66032, *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, *Candida geochares* ATCC 36852, *Candida magnoliae* ATCC 201379, *Candida kefyr* ATCC 66028, *Candida guilliermondii* ATCC 6260, *Candida catenulata* 10565, *Candida membranaefaciens* ATCC 201377, *Candida lusitaneae* ATCC 42720, *Candida dublinienesis* CBS 7987, and *Malassezia furfur* ATCC 14521. All strains were inoculated on sabouraud dextrose agar and incubated at 35 ± 2°C for 24 h before the tests. For *M. furfur* was added in sabouraud agar olive oil. Subsequently, five colonies were picked with a diameter of approximately 1 mm, which were suspended in 5 ml of sterile 0.85% saline. The resulting suspension was placed on a vortex mixer for 15 seconds, and the cell density was adjusted using a spectrophotometer, adding sufficient saline to obtain equivalent transmittance to that of a standard solution in 0.5 McFarland scale at 530 nm. This procedure provided a standard yeast suspension containing 1 x 10^6 to 5 x 10^6 cells per ml. The suspension was produced making a 1:50 dilution followed by a 1:20 dilution of the standard

suspension in RPMI 1640 medium supplemented with L-glutamine (Sigma Chemical Co., St Louis, Missouri, USA), buffered with MOPS [acid 3-(N-morpholino-propane sulfonic acid)] (0.165 mol l^{-1}) (Sigma), pH 7.0, to give the inoculum 2-fold concentrated used in the test (of 1 x 10^3 to 5 x 10^3 UFC ml^{-1}). After 100 µl of each concentration of GO, NC1, and NC2 (as described in item 2.5) was diluted in RPMI, each was then transferred to a well and added to 100 µl of inoculum. The final concentration after inoculation test was 0.5 x 10^3 the 2.5 x 10^3 UFC ml^{-1}, as recommended by the document M27-A3 [20]. The microplates were incubated at 37°C for 48 h in triplicate. The MIC was determined based on the lowest concentration of oil which completely inhibited the growth of yeasts. For a better understanding of the antifungal activity of the NC1 and NC2 front, the yeasts were held in the broth macrodilution method [21,22]. Briefly, the NC1 and NC2 were diluted to a final concentration of 50%, directly in RPMI 1640 supplemented with L-glutamine (Sigma Chemical Co., St Louis, Missouri, USA), buffered with 0.165 mol l^{-1} MOPS [acid 3-(N-morpholino-propane sulfonic acid)] (0.165 mol l^{-1}) (Sigma), pH 7.0. The method also follows the recommendations of the protocol M27-A (CLSI), but with modifications: sterile 11 x 70 mm tubes were used and the final volume in each tube was 1 ml; the incubation time with the NC1 and NC2 was increased to 72 hours and the quantification of the number of colonies present was held at the beginning of treatment and at the end of it. For this, the fungi were inoculated on Sabouraud dextrose agar and incubated at 35 ± 2°C for 24 h before the tests, the results were expressed as mean ± SD of log/UFC ml^{-1}. All experiments were performed in triplicate, being used as growth control only for the suspension of each fungus.

Inhibition of germ tube formation

The suspension of *C. albicans* ATCC 14053 was prepared from colonies growing on Sabouraud agar for 24 h dispersed in 0.85% saline. The suspension was standardized by spectrophotometer at 530 nm resulting in a concentration of yeast cells of 1 x 10^6 – 5 x 10^6 UFC $ml^{-1.}$ A volume of 100 µl of citrated human plasma was added to each well of a sterile microplate. Then, 100 µl of GO at

concentrations of 4.4, 8.9 and 17.9 µg ml^{-1} (MIC/2, MIC, MIC 2x), NC1 with 287.5 µg ml^{-1} and NC2 were added to the wells. Subsequently, 10 µl of the yeast suspension were added to each well. Control of germ tube formation was performed only with citrated human plasma and a yeast suspension. To verify the inhibition of germ tube formation, Amphotericin B (50 µg ml^{-1}) was used. The plate was incubated at 35°C ± 2 for 2 h. Inhibition of germ tube formation was estimated directly in a *Neubauer* chamber, and the results were expressed as percentage (%). All samples were tested in triplicate in two independent experiments.

Statistical Analysis

Results were subjected to analysis of variance (ANOVA) and Tukey´s test to verify the accuracy of the data. Values $P < 0.05$ were considered statistically different. In the comparisons between two variables, we used the nonparametric Wilcoxon Test; when comparisons involved three or more variables, we used the nonparametric Kruskal-Wallis.

RESULTS AND DISCUSSION

GC Analysis

The results of chemical analysis of the GO are presented in **Table 1**. The main components were citronellol (31.37%) and geraniol (10.34%). The chemical composition of the oil was similar to other previously studied species. Our study showed that the citronellol (31.37%), geraniol (10.34%), citronellyl formate (6.51%), and α-guaiene (5.13%) were the major compounds in the oil, with minor quantities of geranyl tiglate (2.07%) and geranial (2.18%). Other constituents were found in smaller amounts (< 2%). The rose geranium oil consisted mainly of oxygenated monoterpenes and oxygenated sesquiterpenes. The data presented here are consistent with previous reports of Boukhatem et al. [23], which demonstrated that geranium oils are characterized by citronellol (29.13%) and geraniol (12.6%). Some differences can be observed in the chemical composition of geranium oil; this is due to a number of factors

including differences in local climatic and geographical conditions, season at collection, and fertilization [24,25].

Table 1: Composition of the geranium essential oil.

Compounds	RI[a]	RI[b]	Geranium oil
			Area (%)
Linalool	1098	1098	3.46
Isomenthone	1164	1159	4.21
Citronellol	1228	1228	31.37
Geraniol	1255	1253	10.34
Citronellyl formate	1275	1275	6.15
α-Guaiene	1439	1443	5.13
6,9-Guaiadiene	1465	1465	5.09

Relative proportions of the essential oil constituents were expressed as percentages. [a]Retention indices from literature [17].[b]Retention indices experimental (based on homologous series of *n*-alkane C_7-C_{30}).

The essential oils are composed of specific compounds found in the leaves, flowers, seeds, stems and roots. The complex mixtures of volatile substances such as alcohols, esters, aldehydes, ketones, phenols, among others, are important properties and some of these hydrophobic components are responsible for antimicrobial and antifungal activities. The main constituents responsible for biological activity of GO are citronellol, geraniol, linalool, isomenthone, nerol, and citronellyl formate. Due to these components, the essential oil has a strong and antibacterial effect [26,27].

Physicochemical properties of nanocapsules

The NC containing essential GO (NC1) and NC containing MCT (NC2) appeared macroscopically homogeneous and opalescent. The physicochemical characteristics of the formulations are presented in **Table 2.** GO-loaded nanocapsules presented nanometric mean diameters (188 nm) as well as polydispersity indices below 0.149 indicating an adequate homogeneity of these systems. The formulation showed acid pH (5.5) and negative zeta potentials (about -10.8 mV). The negative zeta potential values presented by the samples are related to the presence of polysorbate 80, presenting a negative surface density of charge due to the presence of oxygen atoms in the molecules.

GO, as well as other essential oils, have a pronounced odor that sometimes should be masked in formulations. This way, we analyzed the odor of our formulation, comparing the intensity of their odor with the pure essential oil. It is important to point out that the incorporation of GO in nanocapsules allows for considerable reduction in the odor of the oil (Data not shown). This result is in agreement with the ability of polymeric nanocapsules to mask organoleptics properties of some substances [28].

Table 2: Physicochemical properties

Formulation	Particle size (nm)	PDI*	Zeta potential (mV)	pH
NC1	188 ± 0.025	0.149 ± 0.009	- 10.8 ± 0.08	5.5 ± 0.1
NC2	233.3 ± 0.030	0.185 ± 0.011	-10.7 ± 0.09	5.8 ± 0.09

* PDI: polydispersity index.

Antimycobacterial and antibacterial activity

The activity against *Mycobacterium* strains was analyzed. NC1 and GO showed to be active against *M. abscessos, M. massiliense, M. smegmatis, and M. avium.* The antimicrobial activity was also determined against different bacteria as described in item 2.7. The MIC demonstrated that NC1 and GO were able to

inhibit bacterial growth in small concentrations for these strains. These results are shown in **Table 3.**

After evaluating the antimycobacterial activity, it was observed that GO had activity against *M. abscessos, M. massiliense, M. smegmatis,* and *M. avium* with low MIC values (17.9 – 35.9 µg ml^{-1}).

The antimicrobial activity was also evaluated and observed for *S. aureus, Streptococcus, Sthaphylococcus, Listeria monocytogenes, Pseudomonas aeruginosa, and Salmonella enteritidis* (149.7 µg ml^{-1}). The obtained results for antimicrobial activity are also in accordance with the literature, showing that geranium oil has antimicrobial properties against all tested strains. The GO obtained from *Pelargonium graveolens* shows a very strong activity against the standard strain *S. aureus* (ATCC 433000) and also against the examined strains *S. aureus* obtained from the clinical materials. The values of MIC against clinical *S. aureus* strains ranged from 0.25 to 2.5 µg ml^{-1} [29]. Prabuseenivasan *et al*. [30] reported that oil obtained from *Pelargonium graveolens* used at concentrations higher than 12.8 mg ml^{-1} inhibited the growth of the *S. aureus* ATCC 25923. In another study, the aim was to determine the antimicrobial activity of GO against Gram-negative bacterial clinical strains. The microdilution broth method was used to check the inhibition of microbial growth at various concentrations of GO. The tested geranium oil was efficacious against Gram-negative pathogens [31].

In our investigation, it has been found that NC1 is effective against *M. abscessos, M. massiliense, M. smegmatis,* and *M. avium* with low MIC values (35.9 – 149.7 µg ml^{-1}) and *E. faecalis* ATCC29212, *Streptococcus* sp - IC and *L. monocytogenes* ATCC 7644. The NC1 showed no activity against other strains tested. Recently, Souza *et al*. [8] reported antimycobacterial activity of *Melaleuca alternifolia* nanoparticles with MICs ranged from 0.002 to 2.5%. To date, there are no reported studies using nanocapsules containing GO with antimycobacterial activity for comparison.

Table 3: Antimycobaterial and antimicrobial activity (MIC µg ml^{-1}) of geranium essential oil, nanostructures using microdilution method.

Microorganism	Geranium Oil	NC1	NC2
	MIC (µg ml^{-1})	**MIC (**µg ml^{-1})	**MIC (**µg ml^{-1})
M. abscessus ATCC19977	35.9	35.9	ND
M. smegmatis ATCC 700084	35.9	149.7	ND
M. massiliense ATCC 48898	35.9	35.9	ND
M. avium LR541CDC	17.9	71.8	ND
Enterococcus faecalis ATCC 29212	149.7	149.7	ND
Streptococcus sp - IC	149.7	149.7	ND
Sthaphylococcus aureus - IC	149.7	ND	ND
Listeria monocytogenes ATCC 7644	149.7	35.9	ND
Pseudomonas aeroginosa ATCC 340	149.7	ND	ND
Salmonella enteritidis - IC	149.7	ND	ND

ND: not detected

Antifungal activity

The determination of the Minimal Inhibitory Concentration (MIC) was measured after dilution of GO following the M27A3 protocol; the results can be found in **Table 4.** One can show that the GO showed a similar MIC (8.9 µg ml^{-1}) for the strains of *C. albicans, C. kefyr, C. dubliniensis, C. glabrata,* and *C. lusitaneae.* Interestingly, it also showed the same MIC to *Malassezia furfur*. *C. krusei* was observed to have an MIC of 17.9 mg l^{-1} and *C. guilliermondii* an MIC of 149.7 mg l^{-1}. This MIC value is observed for all the yeasts studied after contact with NC1. Because of this, the broth macrodilution method was used to evaluate the activity of NC1. The antifungal activity by the macrodilution method showed a reduction in the number of colony forming units (CFU ml^{-1}) between different species of *Candida* tested within 72 h. The NC1 was able to significantly reduce the number of cells of *C. albicans* (CA) by approximately 5 log, 4 log for *C. dublinensis* (CD), *C. glabrata* (CG) and *C. krusei* (CK) and 2 log for *C. parapsilosis* (CP) compared to control (**Figure 1**).

Fluconazole (FLC) susceptibility of isolates of *Candida* spp., (n = 42) the efficacy and the mechanism of anti-Candida activity of three constituents of geranium oil have been evaluated. No fluconazole resistance was observed among the clinical isolates tested. Geraniol and geranyl acetate were equally effective; fungicidal at 0.064% v/v concentrations, i.e., MICs (561 μg ml^{-1} and 584 μg ml^{-1}, respectively) and killed 99.9% inoculum within 15 and 30 min of exposures respectively [10]. Oliveira *et al*., [32] investigated the activity of the essential oil of *Cymbopogon winterianus* against fifteen strains of *C. albicans* by MIC. The MIC was determined by the microdilution method. The phytochemical analysis of the oil showed presence of citronellal (23.59%), geraniol (18.81%) and citronellol (11.74%). The GO showed antifungal activity, and the concentrations 625 μg ml^{-1} and 1.250 μg ml^{-1} inhibited the growth of all strains tested and it was fungicidal, respectively. These results corroborate with this study, where the GO showed high antifungal activity against *C. albicans* ATCC 14053, *C. kefyr* ATCC 66028, *C. dubliniensis* CBS 7987, *C. glabrata* ATCC 66032, *C. lusitaneae* ATCC 6258 *and Malassezia furfur* ATCC 14521.

Table 4: Antifungal activity (MIC μg ml^{-1}) of geranium essential oil, nanostructures using the microdilution method.

	Geranium Oil	**NC1**	**NC2**
Microorganism	**MIC (**μg ml^{-1})	**MIC (**μg ml^{-1})	**MIC (**μg ml^{-1})
C. tropicalis ATCC 66029	575	> 149.7	ND
C. geochares ATCC 36852	1150	> 149.7	ND
C. albicans ATCC 14053	8.9	> 149.7	ND
C. kefyr ATCC 66028	8.9	> 149.7	ND
C. parapsilosis ATCC 22019	1150	> 149.7	ND
C. guilliermondii ATCC 6260	149.7	> 149.7	ND
C. dublinienesis CBS 7987	8.9	> 149.7	ND
C. glabrata ATCC 66032	8.9	> 149.7	ND
C. krusei ATCC 6258	17.9	> 149.7	ND
C.lusitaneae ATCC 42720	8.9	> 149.7	ND
C. membranafaciens ATCC 201377	2300	> 149.7	ND

Malassezia furfur ATCC 14521	8.9	> 149.7	ND

ND: not detected

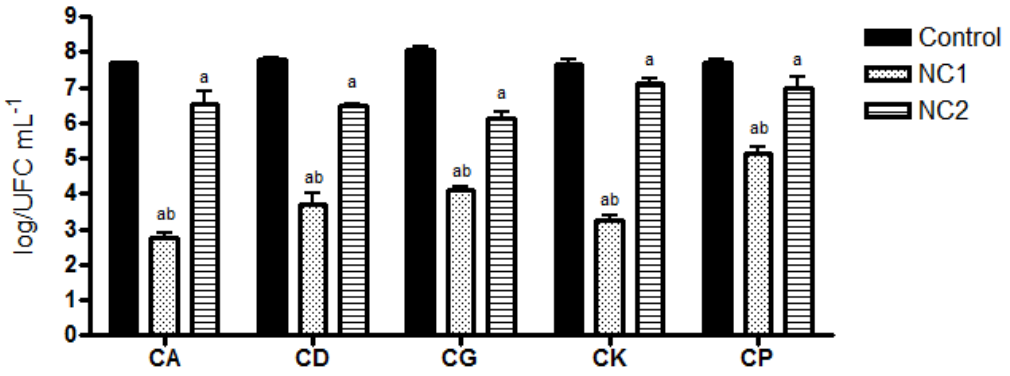

Figure 1: Antifungal activity of the nanocapsules containing Geranium oil after 72 hours incubation performed method by the macrodilution against different *Candida* species. *Candida albicans* (CA), *Candida dublinensis* (CD), *Candida glabrata* (CG), *Candida krusei* (CK) *and Candida parapsilosis* (CP). Values were statistically significant at $p < 0.05$ when compared to [a](growth control with NC1 and NC2) and [b](NC1 with NC2). Data are expressed as means ± SD of log/UFC ml^{-1} of at three independent experiments.

Inhibition of germ tube formation

The effect of GO, NC1, and NC2 on germ tube formation was evaluated using a suspension of yeast in human plasm. GO values relating to the determined MIC microdilution test, half the MIC, and two times the MIC were employed. As for the NC1 and NC2, the same values as for the macrodilution tests were used. As a positive control, Amphotericin B (50 µg ml^{-1}) was used. After the incubation period of 2 hours, the treated yeast were quantified microscopically in a *Neubauer* chamber. It was observed that the concentration of GO related to MIC and twice the MIC could significantly inhibit germ tube formation in *C. albicans*. In NC1, NC2, and the negative control, it was possible to observe the formation of characteristic structures of the germ tube indicating no inhibition of their formation (**Figure 2**).

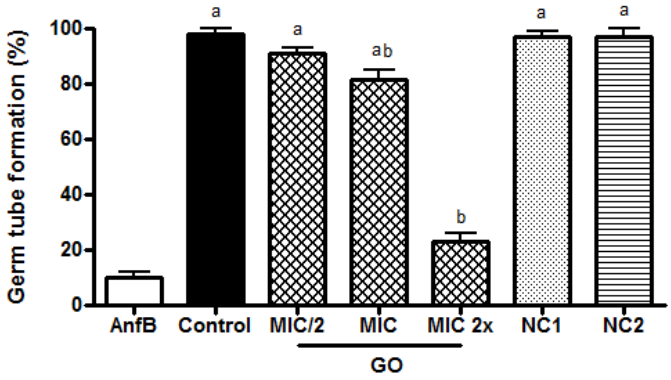

Figure 2: Inhibition of germ tube formation (%). Values were statistically significant at $p < 0.05$ when compared to [a](AnfB with growth control, GO, NC1 and NC2) and [b](growth control, GO, NC1 and NC2). Data are expressed as means ± SD of at three independent experiments.

C. albicans is a polymorphic fungus that can present various morphologies for better adaptation to the environment. The formation of hyphae, pseudohyphae, and chlamydospores are important in their persistence in the site of infection and resistance to antifungal drugs [33]. The germ tube is the passage from the yeast form to the filamentous form of the fungus, where this process helps the yeast to penetrate and adhere more easily in cells. In our study, it was possible to observe that the GO was able to inhibit germ tube formation when the concentration used was MIC; however, such inhibition became significant when the MIC value was doubled.

Budzyńska *et al*. [34] proved that GO showed an inhibitory effect on germ tube formation in 95–100% at a concentration of 0.097% (v/v) of the cells compared with the control using RPMI supplemented with fetal bovine serum. In another study, the oil of *Lavandula luisieri* showed an inhibition of 95% more than the tube formation [35]. However, NC1 has not inhibited germ tube formation of *C. albicans*. This may be due to the short exposure of the NC1, since this test was realized within two hours. To solve this problem, an alternative would be the use of nanoemulsions because they are systems in which the GO could be more easily released. Thus, the contact of GO against the fungus would be faster and would possibly exert its effect. Nanocapsules are used to

increase the solubilization and absorption of lipophilic drugs [36]. These systems function as carriers, releasing substances of low water solubility that could be associated with oil droplets nanometers in size and/or system interface [37].

CONCLUSION

The analysis of geranium oil presented citronellol and geraniol as major components. To prevent degradation of these compounds and increase their stability, nanocapsules containing GO were successfully produced. The obtained particles showed spherical conditions and mean diameters smaller than 200 nm. The physico-chemical characteristics showed homogenous formulation, with polydispersity index and zeta potential. Our study showed for the first time that the GO- loaded nanocapsules have antimycobacterial and antimicrobial activities similar to free oil. However, NC1 was not effective in inhibiting the formation of germ tubes of *Candida albicans*.

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CAPÍTULO 2: Desenvolvimento, caracterização e estudo de estabilidade de nanoemulsões contendo óleo de *Pelargonium graveolens*

Resumo

As nanoemulsões consistem em emulsões muito finas, em que gotículas de óleo são dispersas em uma fase aquosa externa, estabilizadas pela adição de tensoativos. O tamanho das partículas varia conforme diferentes autores, entretanto, sabe-se que estas formulações são mais estáveis, devido ao tamanho reduzido de suas partículas. A técnica de preparo e a quantidade de tensoativo utilizadas, também interferem na estabilidade deste tipo de formulação. A literatura relata, diferentes estudos sobre as vantagens das nanoemulsões para a incorporação de óleos essenciais. Além de melhorar as características organolépticas, elas protegem o óleo contra a degradação e volatilização, conseguindo liberá-lo de forma gradual. No presente estudo foi utilizado o óleo de gerânio (GO), já testado anteriormente na produção de nanocápsulas. Foram desenvolvidas, nanoemulsões contendo GO, denominadas de NEG, com a finalidade de aumentar a velocidade de liberação do óleo. Nanoemulsões sem a adição do GO (NEB) também foram avaliadas. A técnica de preparo com ultra-turrax levou em consideração a análise térmica do GO, que demonstrou que a temperatura inicial de decomposição é de 32 °C. As NEG e as NEB foram submetidas as temperaturas de 4 ± 2 ºC, 25± 2 ºC e 45 ± 2 ºC . A avaliação das características físico-químicas bem como a quantificação dos principais constituintes do GO foram realizadas no momento do preparo e após 7, 14, 30, 60 e 90 dias. Os resultados indicaram que as nanoemulsões apresentaram características nanométricas e que a estabilidade foi fortemente influenciada pela temperatura de armazenamento. O tamanho da gotícula aumentou rapidamente a temperaturas mais elevadas (45 ± 2ºC), porém, as NEG submetidas às baixas temperaturas (4 ± 2ºC) permaneceram com o mesmo valor de 164 nm. O citronelol e geraniol nas NEG tiveram suas concentrações reduzidas durante o período de teste e a temperatura influenciou neste processo. Houve uma redução significativa ao longo dos 90 dias nos dois constituintes, revelando que ambos possuem alta volatilidade, mesmo quando o GO está protegido por uma nanoestrutura.

Manuscrito 2: Development of nanoemulsion containing *Pelargonium graveolens* oil: characterization and stability study

Development of nanoemulsion containing *Pelargonium graveolens* oil: characterization and stability study

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Abstract

The oil *Pelargonium graveolens*, also known the geranium oil, has important therapeutic properties that are mostly associated to its two major components, citronellol and geraniol. Being a volatile oil, it can be degraded and losing the pharmacological properties. The objective of this study was to develop nanoemulsions containing geranium oil (NEG) and control nanoemulsions (NEB) and measure at different temperatures (4°C, 25°C, 45°C) for 90 days the particle size distribution, polydispersity indices and pH. The quantification of oil in the nanostructure was performed by GC-MS and thermal analyzes were used to contribute to stability studies. The results demonstrate that nanoemulsion stability was strongly influenced by storage temperature, with droplet size increasing rapidly at higher temperatures (45°C), which was attributed to coalescence near the phase inversion temperature. The NEG submitted the low temperatures (4 ± 2ºC) remained with the same particle size value (164 nm), however the citronellol and geraniol showed a significant reduction throughout the test even in these conditions of temperature.

Key-works: Geranium oil, citronelol, geraniol, nanoemulsion,

1. Introduction

Nanoemulsions are emulsified systems with droplets that are between 20 and 200 nm in size (Solans *et al*., 2005). Due to their characteristic size appear transparent or translucent to the naked eye (Gupta *et al*., 2010). Depending on the preparation method, different droplet size distributions might be achieved, explaining why the route of preparation can have an influence on the emulsion stability (Fernandes *et al*., 2014). The preparation of emulsions with droplet sizes in the submicrometer-range may be performed mechanically, which involves high-energy input that is generally achieved by high-shear stirring, high-pressure homogenizers, or ultrasound generators (Solans *et al*., 2005).

In contrast, nanoemulsions produced with low energy methods depend on the spontaneous formation of emulsions based on the phase behavior of certain surfactant, oil, and water systems (Komaiko and McClements, 2014). There is interest in using lower energy techniques in the emulsion formation process due to economic benefits and increasing amounts of research have been conducted to investigate the utility of different low-energy approaches (Anton, 2008; Wang, 2008).

Self-emulsifying systems offer a strategy for dealing with the low bioavailability of compounds (drugs and oils) that are not easily dissolved in water (Pal VK, 2011; Zhang *et al*., 2012).

The oil from *Pelargonium graveolens*, also known as geranium oil (GO) or mauve is extracted from the tree *Pelargonium odorantissimum* originating from South Africa. The *Pelargonium* (Geraniaceae) *genus* is represented by many essential oil producing species inter alia: *P. graveolens, P. odoranissimum, P. zonale and P. roseum*. Geranium oil is obtained from leaves, flowers and stalks by steam or hydrodistillation. The GO is composed of various chemical constituents such as linalool, citronellol, geraniol, and their esters (Lis-Balchin, 2002; Boukhris *et al*., 2012).

Further, the GO is non-toxic, non-irritant, generally non-sensitizing, and it is not known to cause any other side effects. Studies show that GO has therapeutic properties as: antidepressant, antiseptic and healing. It is used to

diverse dermatological problems such as oily or congested skin, eczema, and dermatitis (Zore *et al*., 2010).

However, few studies have explored the incorporation of GO in nanoemulsions. One of the main objectives of the current study was to investigate the formation of nanoemulsions by low energy isothermal methods using a well-defined model system: oil, non-ionic surfactant, and water. In addition, there is no study in the literature about the optimizing of nanoemulsion preparation in terms of droplet size, emulsion stability, and emulsification efficiency (EE). The produced formulations were analyzed and characterized in terms of physical properties such as particle size, zeta potential, morphology, entrapment efficiency and long-term stability.

2. Material and methods

2.1 Acquisition of Geranium oil and reagents

The GO, Geraniol, Citronellol and sorbitan monooleate were products of Sigma-Aldrich Co (São Paulo, Brazil), polysorbate 80 was supplied by Henrifarma (São Paulo, Brazil) and all other chemicals and solvents presented pharmaceutical or GC grade and were used as received.

2.2 Geranium oil analysis

Oil composition and yield was analyzed by gas chromatography (GC) using an Agilent 6890N GC-MSD system, equipped with DB-5 MS capillary column (30 m x 0.25 mm x 0.25 µm film thickness) connected to a mass spectrometer detector. The injector and detector temperatures were set at 250°C. Helium was used as the carrier gas, at a flow rate of 1.3 ml/min. The thermal programmer was 100-280°C at a rate of 10°C/min. Two replicates of samples were processed in the same way. Main components (citronellol and geraniol) were identified on the basis of retention times of the peaks of commercially available oils: Geraniol, Citronellol (from Aldrich) performed at under identical experimental conditions. Other components described in **Table 1** were identified by the comparison of mass spectra with the mass spectra library search (NIST),

and with the mass spectra literature. Component relative concentrations were calculated based on GC peak areas without using correction factors. 1 µl of the GO in a diluted solution CH_3CN was injected. GC-Mass Spectroscopy (GC-MS) analyses were performed on an Agilent 5975B EI/CI – MSD system operating in the EI mode at 70 eV, equipped with a split/splitless injector (250°C). The transfer line temperature was 280°C.

2.3 Preparation of nanoemulsions

Nanoemulsions (NEG) were obtained (n=3) after injection of oil phase (5% of geranium oil and 2% of sorbitan monooleate) in aqueous phase (2% polysorbate 80 and ultrapure water) under high agitation employing a T18 Ultra-Turrax (Ika) at 10.000 rpm. After the stirring was increased to 17.000 rpm and maintained for 1 h. The nanoemulsion containing GO was called NEG. For comparison, blank formulations (NEB) were prepared (n=3) using capric/caprylic triglyceride mixture (MCT) instead of geranium oil. All formulations were prepared in triplicate and stored under protection from light and at room temperature.

2.4 Physicochemical characterization of nanoemulsions

After preparation all formulations were characterized according following parameters: particle size distribution by laser diffraction (Microtrac S3500, Microtrac), particle size and polydispersity indices (PDI) by photon correlation spectroscopy (Zetasizer Nano ZS, Malvern Instruments) after dilution (500 times) of suspension in ultrapure water, zeta potential by electrophoretic mobility (Zetasizer Nano ZS, Malvern Instruments) after dilution (500 times) of formulations in 10 mM NaCl solution, and pH directly in formulations using a potentiometer previously calibrated (DM-22 Digimed).

2.5 Emulsion stability tests

After optimizing the production of the nanoemulsions, their storage stability at three different temperatures (4, 25 and 45ºC) was tested. These experiments were carried out using pre-established optimized parameters. All

formulations were analysed in triplicate and were tested for both particle size, zeta potential and pH after its production and after 24 hours, 7, 14, 30, 60 and 90 days.

2.6 Quantification of the constituents of NEG by GC/MS:

Quantification analysis was performed into the CG-MS system by injection of 1µl of the an aliquot of 100 ul of NEG diluted in 2 ml of acetonitrile after 0, 1,7,14,30,60 and 90 days. Quantification of the constituents of NEG was performed at same chromatografic conditions than those describe in GO analysis section. The amounts of geraniol and citronellol of NEGs were determined based on the linear calibration curves obtained by chromatographic peak area measurement of pure reference compounds at various concentrations.

2.7 Thermogravimetric Analysis (TGA)

The thermal stability of the compounds was determined by equipment TGA Q5000 (TA Instruments Inc. USA). The heating rate used was 10 °C/min and the inert atmosphere was N_2 (50 ml/min). The equipment was calibrated with $CaC_2O_4H_2O$ (99.9%). The mass was weighed sample of approximately 10 mg. The data were processed using the Software TA Universal Analysis 2000, version 4.5 (TA Instruments Inc., USA).

2.8 Differential Scanning Calorimetry (DSC)

The thermal events were studied by Modulated Temperature Differential Scanning Calorimetry (MTSDC) in equipment DSC Q2000 (TA Instruments, USA) with option MTDSC, equipment with cooling accessory RCS and as a purge gas N_2 (50 ml/min). The heating rate used was 5°C/min. The instrument was initially calibrated in the way DSC standard, with Indian (99.99%). The masses of pots and reference covers and samples weighed about of 50 ± 0.02 mg. The samples were sealed in aluminum pans with lids. The masses of the samples were weighed on a balance Sartorius (M500P) with a precision of the (± 0.001

mg). The data were processed using the Software TA Universal Analysis 2000, version 4.5 (TA Instruments Inc., USA).

2.9 Electron Microscopy

Morphological analyses were carried out at the *Centro de Microscopia Eletrônica* of the *Universidade Federal do Rio Grande do Sul* (Porto Alegre, Brazil) by transmission electron microscopy (TEM; Jeol, JEM 1200 Exll, Japan) operating at 200 kV. The combination of bright field imaging at increasing magnification and of diffraction modes was used to reveal the form and size of the nanoemulsion. In order to perform the TEM observation the nanoemulsion formulation was diluted with water (1/100).

2.8 Statistical analysis

A one-way analysis of variance (ANOVA) was used to statistically analyze, followed by Tukey's test. $p \leq 0.05$ indicated a statistically significant difference.

3. Results and discussion

3.1 GC Analysis

The volatile oil was analyzed by GC-MS. Qualitative and quantitative studies of the oil volatile profiles are listed in **Table 1** in order of their retention indices. In total, 20 compounds representing 83.5% of the GO were identified. The main components were citronellol (17.74%) and geraniol (14.73%). Mass spectra in full scan mode are shown in **Figure 1**.

Table 1. Chemical composition of geranium oil of *Pelargonium graveolens.*

Number	Compound	% (relative)	R_t
1	Linalool	0.727	6.773
2	cis-Rose oxide	0.678	6.913
3	cis Rose oxide	0.335	7.103
4	α-Citronellal	0.104	7.328
5	Isomenthone	2.118	7.459
6	Menthone	2.478	7.561
7	L-(-)-Menthol	0.119	7.801
8	**Citronellol**	**17.747**	**8.065**
9	cis-Citral	0.381	8.223
10	**Geraniol**	**14.73**	**8.309**
11	Isogeraniol	0.138	8.418
12	Citronellyl formate	5.966	8.512
13	Geraniol formate	3.825	8.757
14	cis Rose oxide	0.241	9.093
15	(6E)-2,6-Dimethyl-2,6-octadiene	0.563	9.208
16	α-Cubebene	0.155	9.332
17	Nerol acetate	0.677	9.468
18	Copaene	0.339	9.633
19	Geranyl ethyl ether 2	0.105	9.67
20	α-Bourbonene	0.939	9.729
21	Citronellyl propionate	0.434	10.019
22	α-Caryophyllene	0.592	10.068
23	Germacrene D	0.156	10.137
24	Aristolene	0.191	10.204
25	Geranyl propionate	0.835	10.269
26	α-Humulene	0.197	10.379
27	(+)-Aromadendrene	0.14	10.423
28	α-Muurolene	0.282	10.486
29	α-Cubebene	0.435	10.587
30	(+)-Ledene	0.634	10.669
31	Citronellyl butyrate	0.628	10.749
32	(-)-α-Cadinene	0.978	10.833
33	(-)-Calamenene	0.576	10.883
34	Geranyl isobutyrate	1.073	10.998
35	Cubenol	0.41	11.079
36	4a,7-Methano-4aH-naphth[1,8a-βoxirene, octahydro-4,4,8,8 tetramethyl-	0.865	11.191
37	Germacrene D-4-ol	0.158	11.233
38	Phenyl ethyl tiglate	1.549	11.315
39	(-)-Spathulenol	0.996	11.389
40	Caryophyllene oxide	0.667	11.461
41	Ledol	0.302	11.528
42	Cubenol	0.13	11.602
43	Viridifloro	0.258	11.639
44	Cubenol	0.55	11.675
45	10-*epi*-γ-Eudesmol	9.519	11.775
46	Cubenol	1.169	11.851
47	2-(3-Isopropyl-4-methyl-pent-3-en-1-ynyl)-2-methyl-cyclobutanone	0.561	11.901
48	γ-Eudesmol	0.389	11.935

% relative > 0.05%, R_t (min)

The data presented here are consistent with previous reports, which demonstrated that geranium oils are characterized by citronellol (22.0%- 32.9%) as the most important component (Rajeswara, 2002, Boukhris *et al*., 2012). However, our results diverge from those published by other studies in which the concentration of geraniol (23-38%) was higher than that of citronellol (21-28%) (Juliani *et al*., 2006; Verma *et al.*, 2010). The chemical composition of geranium oil will depend a number of factors, including differences in climatic conditions and geographical locations, season at the time of collection, and fertilization (Boukhatem *et al*., 2013).

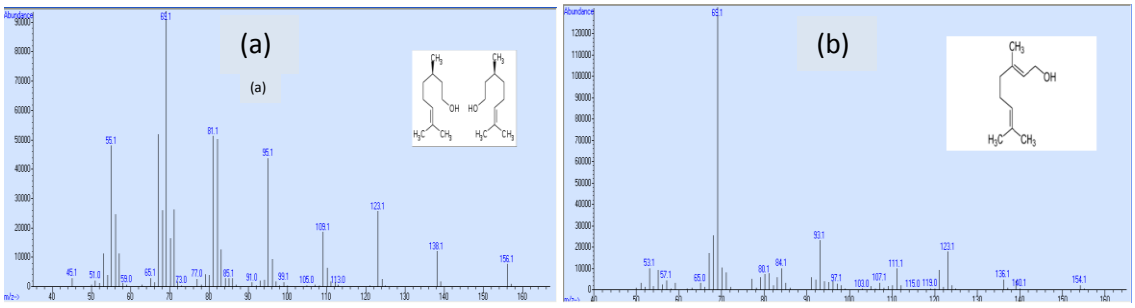

Figure 1. Mass spectra of citronellol (a) and geraniol (b).

3.2 Physicochemical properties of nanoemulsions

Nanoemulsion have been used as excellent vehicles to solubilize lipophilic drugs and significantly improve bioavailability (Ghosh *et al*., 2006; Khandavilli and Panchagnula, 2007; Shafiq *et al*., 2007; Shen and Zhong, 2006; Tiwari and Amiji, 2006).

The results presented in **Tables 2 and 3** showed that the technique with Ultra-Turrax has enabled the formation of nanometric particles in formulations, independent of the presence or absence of GO. The NEG showed reduced particle size (164 ± 3.5 nm) with a low polydispersity (0.25 ± 0.006) characterizing, therefore, colloidal systems with narrow particle size distribution. Mean droplet size analysis indicated that NEB presented the smallest mean diameter (130 ± 2.4 nm) and low polydispersity (0.12 ± 0.021). The formulations

where GO was added, on the macroscopic analysis, showed characteristics of nanoemulsions with tiny droplets size such as: bluish reflex, translucency and higher intrinsic stability. The GO not decreased the droplet size and also no provided a better stability of the system. It is well accepted that, for dispersed systems, the smaller the droplet size the higher the stability. The droplet size is an important data in the stability analysis, so faster the droplet size increases, faster will occur the instability process as creaming or phase separation (Jeong *et al.*, 2001).

Table 2: Nanoemulsions (NEG, NEB) droplet size values (nm) (Mean ± S.D.) subjected to different temperatures for 0, 1, 7, 14, 30, 60 and 90 days.

↓ Time (day)	(4 ± 2ºC)		(25 ± 2ºC)		(45 ± 2ºC)	
Formulations→	**NEG**	**NEB**	**NEG**	**NEB**	**NEG**	**NEB**
0	#	#	164± 3.5	130± 2.4	#	#
1	164± 4.12	131± 2.1	164± 1.5	140± 3.87	220± 3.8[a]	130± 4
7	161± 4.47	127± 2.3	183± 1.2	153± 4	712± 0.07[a]	126± 2.3
14	165± 4.43	146± 5.8[b]	185± 0.92	169± 2.6	*	182± 2.5[b]
30	167± 4.41	168± 1.7[b]	276± 14.9[a]	180± 2.2	*	384± 7.4[b]
60	160± 0.92	178± 0.97[b]	602± 16.40[a]	291± 3.6[b]	*	*
90	164± 3.9	181± 1.6[b]	*	1694± 10.1[b]	*	*

#: t=0 ; *: phase separation; [a]: statistical comparisons of day 0 to NEG with day 1, 7, 14, 30,60 and 90. Indicate significance when p≤0,05; [b]: statistical comparisons of day 0 to NEB with day 1, 7, 14, 30,60 and 90. Indicate significance when p≤0,05.

Table 3: Nanoemulsions (NEG, NEB) PDI values (Mean ± S.D.) subjected to different temperatures for 0, 1, 7,14,30,60 and 90 days

↓ Time (day)	(4 ± 2ºC)		(25 ± 2ºC)		(45 ± 2ºC)	
Formulations→	**NEG**	**NEB**	**NEG**	**NEB**	**NEG**	**NEB**
0	#	#	0.25± 0.006	0.12± 0.03	#	#
1	0.13+ 0.016[a]	0.22+ 0.015	0.25± 0.013	0.15± 0.009	0.22± 0.011	0.21± 0.02
7	0.13+ 0.011[a]	0.23+ 0.007[b]	0.38± 0.006[a]	0.16± 0.011	0.52± 0.074[a]	0.21± 0.011[b]
14	0.14+ 0.012[a]	0.26+ 0.05[b]	0.38± 0.008[a]	0.27± 0.008[b]	*	0.24± 0.04[b]
30	0.14+ 0.010[a]	0.22+ 0.004[b]	0.30± 0.039	0.29± 0.02[b]	*	0.25± 0.01[b]
60	0.14+ 0.01[a]	0.22+ 0.002[b]	0.51± 0.02[a]	0.44± 0.10[b]	*	*
90	0.15+ 0.01[a]	0.23+ 0.005[b]	*	0.93± 0.14[b]	*	*

#: t=0 ; *: phase separation; [a]: statistical comparisons of day 0 to NEG with day 1, 7, 14, 30,60 and 90. Indicate significance when p≤0,05; [b]: statistical comparisons of day 0 to NEB with day 1, 7, 14, 30,60 and 90. Indicate significance when p≤0,05.

The choice and amount of surfactant may influence the stability of the nanoemulsion. The proper balance between the surfactants and GO provides a smaller and uniform sized droplets because occurs uniform coverage of surfactant around the droplet which avoids aggregation. This clearly shows that, the proper balance between surfactant and GO will not only yield smaller and stable particles in terms of size, but also protect the emulsion from degradation. When there is excess surfactants system, there is increased formation of micelles, which facilitate can the mass transport of oil molecules from smaller to larger globules, which may result in an increase in particle size as a function of time (Rocha, 2014).

Some results suggest that every system should be investigated individually since factors such as the phase behavior of the surfactant–oil–water system and the physicochemical properties of the components greatly impact the effect of variables like stirring or mixing speed. It is frequently necessary to use blends, such as a pair of hydrophilic and lipophilic non-ionic surfactants, to achieve

droplets with small diameter (Fernandes et al.,2014; McClements, 2014; Sole et al., 2010).

Our study showed that NEG has not significant variation in the particle size of the system in the temperature remained of 4 ± 2ºC during the period of the study. At 25 ± 2ºC and 45 ± 2ºC, there were a change in the size of the droplets as well as in the polydispersity index, which is expected since the temperature increases the kinetic energy of the system, increasing the possibility to occur instability phenomena. Besides, the oily phase composed by volatile oil may have caused rupture of the interface by evaporation of its molecules.

When the formulations NEG were subjected to high temperature (45°C), it was found that after the seventh day there was phase separation (**Table 2**). This may be due to destabilization of the system by evaporation of its volatile constituents. The essential oils have a high vapor pressure, what means that it may volatilize in low temperatures. The phase separation can be due to the reorganization of the system, since the molecules of the essential oils, when submitted to heating might have caused the rupture of the interface followed by coalescence (Florence, 2003).

Zeta potential is a useful tool to predict the physical stability of colloidal systems. The zeta potential determines the electrostatic repulsion between the globules (Friberg, 1988). When the electrolyte concentration increases, the ionic double layer is compressed due to the ionic attraction forces, resulting in the reduction of its thickness and the extent of reducing electrostatic repulsion force.

The zeta potential values presented show that both the NEB as NEG (**Table 4**) have negative charges. The zeta-potential was from NEB and the NEG was around -10 mV. However, there was no significant difference when comparing the zeta potential of the NEG when subjected to different temperatures, only in the analysis 30 days at 4± 2°C. The NEB showed significant variation in zeta values after the 30 days when subjected to room temperature. Some authors explain negative values due to the structural

characteristics of the nanoemulsions interface components, especially polysorbate 80 used in a high concentration in the formulation. In this case, the hydrocarbon chain of the surfactant interacts with the hydrophobic region of the oily phase and could induce negative charges on the surface of the system (Mora-Huertas, Fessi e Elaissari, 2010).

Table 4: Nanoemulsions (NEG, NEB) zeta values (mV) (Mean ± S.D.) subjected to different temperatures for 0, 1, 7,14,30,60 and 90 days

↓ Time (day)	(4 ± 2ºC)		(25 ± 2ºC)		(45 ± 2ºC)	
Formulations→	NEG	NEB	**NEG**	**NEB**	**NEG**	**NEB**
0	#	#	-10± 1.7	-10± 1	#	#
1	-10.8+ 1	-8.3+ 3.8	-11± 1	-10± 0.8	-10.6± 1	-9.1± 2.4
7	-10.2+ 1.6	-9.5+ 1	-14± 1.2	-9.6± 0.8	-10± 1.2	-9.3± 1.1
14	-9.6+ 3.2	-8.3+ 0.8	-13± 1.6	-10± 1.1	*	-9.3± 0.2
30	-12.8+ 1.6a	-12+ 0.5	-12.5± 0.5	-18± 0.7[b]	*	-12.6± 0.8
60	-11.6+ 2.6	-13+ 1.5	-11± 0.1	-26± 1.1[b]	*	*
90	-11.7+ 2.1	-9.3+ 0.3	*	-22± 1.3[b]	*	*

#: t=0 ; *: phase separation; [a]: statistical comparisons of day 0 to NEG with day 1, 7, 14, 30,60 and 90. Indicate significance when p≤0,05; [b]: statistical comparisons of day 0 to NEB with day 1, 7, 14, 30,60 and 90. Indicate significance when p≤0,05.

A reduction in the electrical potential of the double layer causes a reduction in total electric potential, compromising the stability of the emulsion (Ruktanonchai *et al*., 2009). It is important to make a comparison between the zeta potential with the particle size results, because an increase in particle sizes of nanoemulsions was accompanied by an increase in negative surface charge values. The droplets size and zeta potential are the most representative parameters in the control of emulsion stability. To evaluate the emulsion

stability, these aspects were monitored for 3 months (**Table 2 and 4**). The zeta potential in NEG had a small variation during the stability test.

The pH value is a parameter for monitoring the stability since changes may indicate an occurrence of chemical reactions or microbial contamination. Formulations with vegetable oils may result in a decrease in pH from the hydrolysis of esters of fatty acid, which generate free fatty acids. The pH of the NEG had only a significant variation in temperature of 25± 2°C in an analysis of 60 days. However, it was observed that the NEB had their pH values decreased significantly after 30 days at 25 ± 2°C, 60 days at 4 ± 2°C and 7 days at 45 ± 2°C (**Table 5**).

Table 5: Nanoemulsions (NEG, NEB) pH values (Mean ± S.D.) subjected to different temperatures for 0, 1, 7,14,30,60 and 90 days.

↓ Time (day)	(4 ± 2ºC)		(25 ± 2ºC)		(45 ± 2ºC)	
Formulations→	**NEG**	**NEB**	**NEG**	**NEB**	**NEG**	**NEB**
0	#	#	3.7+ 0.12	6.4+ 0.21	#	#
1	3.7+ 0.14	6.4+ 0.21	3.7+ 0.17	6.4+ 0.21	3.7± 0.05	6.3± 0.24
7	3.8+ 0.15	6.3+ 0.27	3.7+ 0.08	6.4+ 0.27	3.4± 0.061	5.3± 0.37[b]
14	3.7+ 0.16	6.4+ 0.19	3.6+ 0.07	6.2+ 0.18	*	3.8± 0.18[b]
30	3.7+ 0.12	6.5 + 0.21	3.4+ 0.06	5.3+ 0.12[b]	*	3.4± 0.21[b]
60	3.7+ 0.04	6.8+ 0.16[b]	3.3+ 0.04[a]	3.8+ 0.12[b]	*	*
90	3.4+ 0.06	6.8+ 0.11[b]	*	3.5+ 0.04[b]	*	*

#: t=0 ; *: phase separation; [a]: statistical comparisons of day 0 to NEG with day 1, 7, 14, 30,60 and 90. Indicate significance when p≤0,05; [b]: statistical comparisons of day 0 to NEB with day 1, 7, 14, 30,60 and 90. Indicate significance when p≤0,05.

3.3 Evaluation of the constituents of NEG by GC / MS

The method for quantification of the constituents of NEG demonstrated to be linear in the range of 430 mg/l – 2000 mg/l and 420 mg/l – 2000 mg/l for geraniol and citronellol. The calibration equations y = 1643x - 5048 and y =

4179x-2933 showed excellent correlation coefficients, r = 0.9913 and 0.9911 for geraniol and citronellol. Therefore, the result confirms the absence of constant systematic error. The LOD and LOQ limits were 0.5 mg/l and 420 mg/l. The precision of the method was assessed considering repeatability. The precisions presented R.S.D. values lower or equal to 5.00% for geraniol and citronellol, which were considered satisfactory.

The GO was extracted from the nanoemulsions subjected to stability tests. The nanoemulsions containing GO were quantified by GC / MS. The **figure 2** show the components found in GO and after extracted from nanoemulsions submitted to temperatures of 4 ± 2ºC, 25 ± 2ºC and 45 ± 2ºC for 90 days. The citronellol has a significant reduction of its concentration in the NEG from the day 7 in the temperatures of 25± 2ºC and 45± 2ºC. The geraniol showed a significant reduction of its concentration in the NEG from day 1 in these same temperatures.

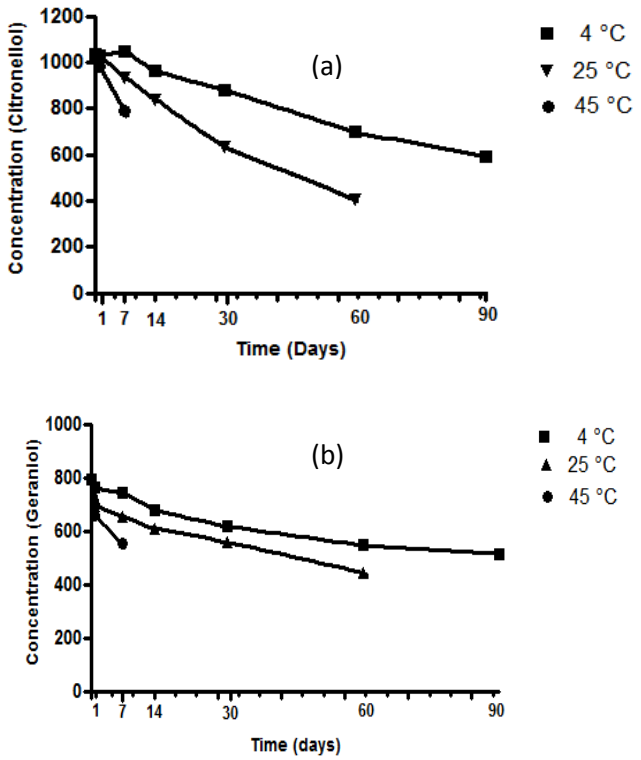

Figure 2: Concentration of components (ppm) found in GO after extracted from nanoemulsion Citronellol (a) and Geraniol (b). The results are represent the mean + S.D.

The **Figure 3** show a partial chromatogram performed after the production zero-day and after 90th days at 4 ± 2ºC. The possible hydrolysis of this compound can relate to the reduction of pH values observed in the formulations submitted to stability tests (**Table 5**).

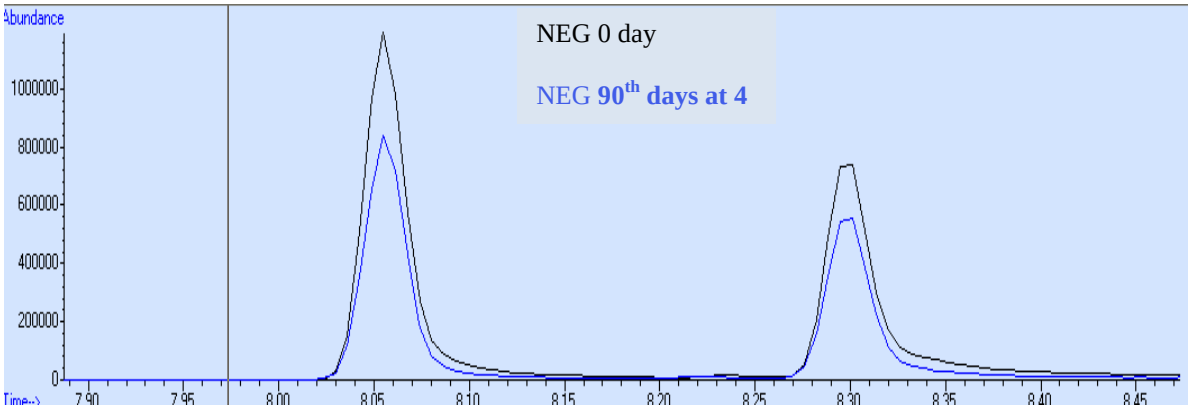

Figure 3: Partial chromatogram performed to NEG (day zero and after 90th days at 4 ± 2ºC)

3.4 Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC)

Thermograms of DSC were obtained for Citronellol, Geraniol, GO, NEG and NEB at same experimental conditions. The Citronellol, Geraniol thermogram display a glass transition temperature (Tg) at -59 and -57°C respectively. Similarly, GO thermogram shows a Tg at -58°C corresponding probably to main components citronellol and geraniol. The absence of endothermic and exotermic peaks in thermograms of citronellol and geraniol and GO indicated that they not crystalize and/or melting in the temperature range evaluated. Thus, it is possible affirm that they have an amorphous structure, but are able to organize themselves at low temperatures (approximately -60°C) (**Table 6**). On the other hand DSC thermogram (not demonstrate) of NEB and NEG show single thermal transition of water. Thermogram of NEB shows the cristalization peak at cooling cycle in -14.1°C and a melting was observed at 0.3°C. Thermogram of NEG shows the crystallization peak at the cooling cycle in -20.1°C and a melting was observed at

1.5°C. Results are indicating that the presence of GO in the nanoemulsion cause the greater shift of crystallization and melting of water. At the same time, the enthalpy of crystallization and melting was similar for NEB and NEG indicating that all water content that crystallized in cooling cycle had melting in heating cycle. However, it is important to note that the enthalpy of crystallization and melting of NEG was about 8 times greater than the enthalpy of crystallization and melting of NEB. The increase in the enthalpy of crystallization and melting in the presence of GO can be attributed to the rising of a complex lattice of intermolecular interactions between water, GO and other components of nanoemulsion that led to droplets formation.

Table 6: Thermic properties of NEGs determined by DSC and TGA

Sample	T_g (°C)[a]	T_d (°C)[b]	T_i (°C)[c]	T_f (°C)[d]
Geraniol	-57	126	33	180
Citronellol	-59	145	32	197
Geranium oil	-58	155	32	504
NEB	-[e]	54	29.3	177
NEG	-[e]	104	32	153

[a]Glass Transition (Tg). [b]Decomposition temperature (Td). [c] Inicial decomposition temperature (Ti). [d] Final decomposition temperature (Tf). [e]Thermogram show only thermal transitions (Tg and fusion) of water present in the formulation.

It was proved by thermogravimetric analysis that the initial decomposition temperature (T_d) is low for citronellol, geraniol, GO, NEB and NEG (around 30°C), indicating that at this temperature about 5% of mass was lost. However when we look at decomposition temperature, we can see that citronellol and GO were thermally more stable than geraniol. An an interesting observation was the thermal stability of NEG when compared with NEB. The T_d of NEG (104°C) was two times greater than T_d of NEB. In addition, GO and NEG show a perfil of decomposition in two steps. These two founds show that GO are in nanoemulsion. However when we compared T_d of GO with T_d of NEG, we observe a decrease in the T_d of GO when compared the NEG (**Table 6**). Finally,

it is worth note that GO was totally carbonized at 504°C while NEG was totally decomposed at 153 °C.

3.5 Electron Microscopy

In addition, the morphology of NEG and NEB were characterized by TEM (**Figure 4**) and the particles size were approximately the same as the diameters measured by the dynamic light scattering instrument. TEM images indicated that all the nanoemulsions particles were spherical and nanometric (**Figure 4**). These results corroborate those found for the particle size and polydispersity index obtained for suspensions of these nanoemulsions by dynamic light scattering technique**.**

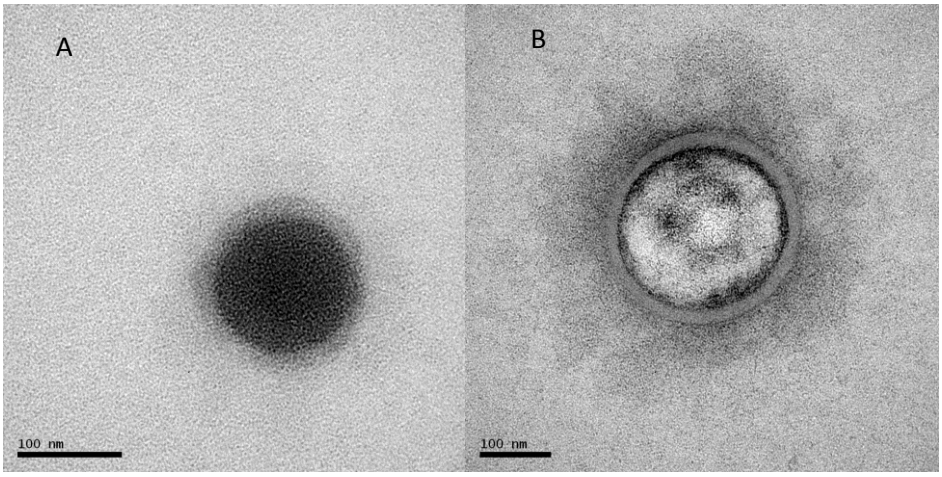

Figure 4: TEM images of droplets in the NEG (A) and NEB (B). Scale bars 100 nm.

4. Conclusion

In this work, we proposed a strategy to obtain nanoemulsions containing GO due to its important pharmacological properties already reported in literature. The formulations have been produced under high agitation employing a Ultra-Turrax, in order to incorporate the GO and prevent its volatilization. The

formulations presented good physicochemical characteristics, however the physicochemical stability for 90 days was only achieved in 4°C. These results allow us to suggest that the use the ultra-turrax is a strategy good to prepare nanoemulsions containing essential oil. Other studies can be realized with these formulations to check their potential biological activities.

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CAPÍTULO 3: Avaliação da atividade antioxidante e citotoxicidade do óleo de gerânio puro e nanoestruturado

Resumo

As nanoemulsões contendo óleos essenciais, quando objetivam a utilização em seres humanos devem ser avaliadas quanto a sua possível capacidade de causar dano celular. Com esta finalidade, as NEB e NEG juntamente com o GO puro foram avaliados quanto à citotoxicidade, *stress* oxidativo e quanto à atividade antioxidante. As análises físico-químicas das formulações confirmaram as características nanométricas. Para verificar as atividades antioxidantes do GO e NEG foram realizados dois ensaios diferentes, DPPH e FRAP. Em ambos os ensaios o GO apresentou nas diferentes concentrações testadas poder antioxidante, entretanto, este fato não foi observado com as NEG. Estas formulações apresentaram ação antioxidante, porém somente nas maiores concentrações testadas. O citronelol e o geraniol, constituintes majoritários do óleo, podem ter influenciado nestes resultados, pois a literatura relata esta atividade para estes dois compostos. Sabe-se que pela estrutura da NE o óleo de gerânio não está livre para exercer a atividade rapidamente. A realização do ensaio de citotoxicidade para as amostras através dos ensaios de MTT e LDH demonstrou que a NEB, NEG e o GO nas diferentes concentrações testadas não foram citotóxicos aos linfócitos e que as concentrações das amostras foram diretamente proporcionais ao aumento dos níveis de LDH. Entretanto, quando estas concentrações foram confrontadas ao controle com SDS, observou-se que os níveis de LDH foram significativamente inferiores. A análise da atividade hemolítica em eritrócitos humanos demonstrou que a NEB e a NEG, nas diferentes concentrações, não possuem a capacidade de lisar os eritrócitos. Este resultado é muito interessante e sugere a segurança da formulação para seu uso em humanos. Nos ensaios de *stress* oxidativo em que foram avaliados a peroxidação lipídica, com a formação de malondialdeído e a liberação da enzima catalase, novamente ocorreu proporcionalidade entre as concentrações das amostras e os resultados. Os níveis de TBARS e a liberação da CAT foram superiores no GO. A NEG mostrou um efeito de proteção quando os resultados

foram confrontados ao do GO. Conclui-se então, que a nanoestruturação do GO é eficiente e vantajosa na proteção contra a toxicidade e danos celulares.

MANUSCRITO 3: Evaluation *in vitro* of antioxidant activity, cytotoxicity, and oxidative stress caused by *Pelargonium graveolens* oil nanoemulsion

Evaluation *in vitro* of antioxidant activity, cytotoxicity, and oxidative stress caused by *Pelargonium graveolens* oil nanoemulsion

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Abstract

The following study aimed to evaluate the antioxidant activity, cytotoxicity and oxidative stress *in vitro* caused by *Pelargonium graveolens* oil ou Geranium oil (GO) and its nanoemulsion. Nanoemulsions (NEG, 5% Geranium oil and NEB, 5% medium-chain triglyceride as negative control) have been prepared under high agitation employing a T18 Ultra-Turrax. The particle sizes were determined as 164 and 130 nm for NEG and NEB, respectively and the zeta potential values as −10mV for both NE. The antioxidant activities of GO and NEG were examined applying two different biochemical assays namely diphenylpicrylhydrazyl (DPPH) and ferric reducing power (FRAP). In both trials it was possible to observe a higher antioxidant activity at the same concentrations of GO compared with NEG. After linear regression, it was obtained IC_{50} = 198 µg/µl for the NEG and IC_{50}= 4.72 µg/µl for GO in DPPH assay**.** The *in vitro* cytotoxicity of the GO, NEB, and NEG were evaluated in two different eukaryotic cells. Limphocytes were treated at different concentrations (0.1 – 1000 µg/µl) with GO, NEB, and NEG. Cell viability and plasma membrane integrity were checked by MTT and lactate dehydrogenase (LDH) assays. The hemolytic activity was measured in human erythrocytes. EC_{50} values to the GO and NEG in MTT were 1.252 and 10.893 µg/µl and hemolytic activities were above 221.4 and 724 µg/µl, respectively. Oxidative stress in the lymphocytes was determined by TBARS and CAT assays. The results showed that the NEB did not have a significant increase in TBARS compared to control cells, however, the TBARS of the GO was significantly increased in all tested concentrations. The results also indicated a significant increase in CAT activity in lymphocytes concentrations for all NEB, NEG and GO compared to the control cells, except for the concentration of 0.1 µg/µl NEB. All tested concentrations of GO showed a significant increase in CAT activity as compared to the same concentrations NEG. This study demonstrated for the first time the antioxidant activity NEG using the automatized FRAP assay, as well as cytotoxicity and oxidative stress *in vitro* of this nanoemulsion.

Keywords: Geranium oil, antioxidant activity, cytotoxicity, oxidative stress and nanoemulsion.

1. Introduction

The growth in the use of natural therapies has been increasing within the scientific and technological progress of modern medicine, arousing interest of researchers (Spadacio *et al*., 2010). Plants with therapeutic properties are important sources of new biologically active compounds, where the searches are even encouraged by the World Health Organization (Oliveira *et al*., 2006).

Among natural products, essential oils also known as volatile oils are described as products of great therapeutic and pharmacological potential (Edris, 2007). These oils are natural, volatile, and complex compounds consisting of about twenty to sixty compounds at various concentrations. Two or three components are often present at higher concentrations (20-70%) in comparison with other compounds. Different biological properties are characteristic of essential oils, such as antioxidant, analgesic, anti-inflammatory and antimicrobial activities (Caia *et al*., 2004; Fayed *et al*., 2009; Aidi Wannes *et al*., 2010; Mendes *et al*., 2010; Malik *et al*., 2011; Boligon *et al*., 2013; Santos *et al*., 2014; Vaucher *et al*., 2015).

Pelargonium graveolens L. oil, known as geranium oil (GO) or mauve smelling, has a very pronounced odor and has been historically important. The oil is almost a perfume and is originating in South Africa (Simon *et al*., 1984). Has a very pronounced odor and is widely used in the perfume, cosmetics and pharmaceuticals industry (Arrigoni-Blank, 2011). The therapeutic effects of the geranium oil find application in the treatment of dysentery, diarrhoea, biliary conditions, gastric ulcers, diabetes, cancer and skin diseases (Shawl *et al.*, 2006).

Recently was reported that geranium oil it has antimicrobial activity (Giongo *et al.*, 2015) and was able to significantly reduce the inflammatory symptoms associated with neutrophil accumulation and edema (Dzamic *et al*., 2014). This oil also has emotional and psychological actions, such as relaxing,

relieving anxiety, nervous tension and depression (Corazza, 2004). The main constituents responsible for biological activity are citronellol and geraniol (Lis-Balchin, 2002; Boukhris *et al*, 2012; Bigos *et al*., 2012).

Antioxidant properties of various medicinal plants are being investigated around the world; mainly due to toxicological problems associated to synthetic antioxidants and preservatives (Peschel *et al*., 2006). Compounds with antioxidant properties have scientific importance, since they can neutralize free radicals (Baba and Malik, 2014). It is known that many free radicals as superoxide, hydroxyl radical, nitric oxide and peroxynitrite can be observed in oxidative environments. There is a large number of concrete evidence about the role of free radicals in the development of many diseases, including inflammatory and neurodegenerative diseases and cancer (Halliwell, 1997; Halliwell, 2006; Ferguson, 2010).

Nanotechnology is showing strong growth, especially in incorporating essential oils. The objectives are: reduce volatilization and degradation of these oils and improving the biological activities. However it must be made some tests with these formulations, to increase security during use. The Nanoemulsion can protect the oil and reduce a possible toxic effect against the cells.

The present work aimed to investigate the antioxidant activity, cytotoxicity and oxidative stress *in vitro* of the GO incorporated into nanoemulsions. In this study was showed for the first time the use of the automatized FRAP assay to determine the antioxidant activity.

2. Materials and methods

2.1 Acquisition of Geranium oil and reagents

GO (PCode 1001742696), 2,2-diphenyl-1-picrylhydrazil (DPPH), butylated hydroxytoluene (BHT), potassium ferricyanide, trichloroacetic acid, 2,4,6-tris (2-pyridyl)-s-triazine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reagent, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sorbitan monooleate were products of Sigma-Aldrich Co (São Paulo, Brazil),

polysorbate 80 was supplied by Henrifarma (São Paulo, Brazil). All other chemicals and solvents were of analytical grade. All solutions were freshly prepapred in distilled water and all reagents were of analytical grade.

2.2 Geranium oil analysis

Oil analysis was was analyzed by gas chromatography (GC) using an Agilent 6890N GC-MSD system, equipped with DB-5 MS capillary column (30 m x 0.25 mm x 0.25 µm film thickness) connected to a mass spectrometer detector (Giongo *et al*., 2015- unpublished article).

2.3 Preparation of nanoemulsions

Nanoemulsions (NEG) were obtained (n=3) after injection of oil phase (5% of geranium oil and 2% of sorbitan monooleate) in aqueous phase (2% polysorbate 80 and ultrapure water) under high agitation, employing a T18 Ultra-Turrax (Ika) at 10.000 rpm. After the stirring, it was increased to 17.000 rpm and maintained for 1 hour. The nanoemulsion containing GO was called NEG. For comparison, blank formulations (NEB) were prepared (n=3) using capric/caprilic triglyceride mixture (MCT) instead of geranium oil. All formulations were prepared in triplicate and stored under protection from light and at room temperature.

2.4 Physicochemical characterization of nanoemulsions

After preparation, all formulations were characterized according to the following parameters: particle size distribution by laser diffraction (Microtrac S3500, Microtrac), particle size and polydispersity indices (PDI) by photon correlation spectroscopy (Zetasizer Nano ZS, Malvern Instruments) after dilution (500 times) of suspension in ultrapure water, zeta potential by electrophoretic mobility (Zetasizer Nano ZS, Malvern Instruments) after dilution (500 times) of formulations in 10 mM NaCl solution, and pH directly in formulations using a potentiometer previously calibrated (DM-22 Digimed).

2.5 DPPH radical scavenging activity

The antioxidant activity of the essential oil was evaluated by monitoring its ability in quenching the stable free radical DPPH, according to a slightly modified method previously described by Choi *et al*. (2002). Spectrophotometric analysis was used to measure the DPPH radical scavenging activity (%) and inhibitory concentration (IC_{50}). The GO, NEB, and NEG were diluted in absolute ethanol at a starting concentration of 100 µg/µl, and diluted 1:1 until the concentration of 0.04875 µg/µl, resulting into twelve different concentrations. The different concentrations were mixed with 1.0 ml of DPPH 0.3 mM in ethanol solution. After the samples were left for 30 min in the dark, at room temperature, the absorbance was measured in a spectrophotometer at 518 nm. As a negative control, it was used a solution of 2.5 ml of absolute ethanol with 1 ml of DPPH (0.3 mM) and BHT (1 µg/µl) as a positive control. The extract concentrations without DPPH correspond to white. Absolute ethanol was used to calibrate the spectrophotometer and the value of the NEB was deducted from the value of NEG. IC50 value denotes the concentration of sample, which is required to scavenge 50% of DPPH free radicals. The assay was performed in triplicate and the radical scavenging activity was calculated by the following formula:

$$\% \text{ Radical Scavenging Activity} = \frac{\text{Absorbance (control)} - \text{Absorbance (sample)}}{\text{Absorbance (control)}} \times 100$$

2.6 FRAP assay

For the determination of the antioxidant capacity of GO, NEB, and NEG was used the FRAP assay, employing the methodology described by Benzie and Strain (1996), adapted to the Cobas Mira® automated system (Roche Diagnostics, Switzerland). In the FRAP assay reagent was prepared from a combination of acetate buffer solution with TPTZ (2,4,6 - tris (2-pyridyl) -s-triazine), and aqueous ferric chloride solution. At acid pH, the complex Fe^{3+} is reduced to Fe^{2+} and forms an intense blue solution. As reference, FRAP reagent solution was used and the absorbance was measured at 600 nm, as Benzie and Strain (1996).

For the calibration curve, $FeCl_2$ was used as a standard in concentrations ranging from 50 µmol/l to 200 µmol/l. The value of the NEB was deducted from the value of NEG. The results were expressed in µmol/l.

2.7 Culture of lymphocytes

Peripheral blood samples were obtained from discarded samples of Clinical Analysis Laboratory School by venopuncture using a top Vacutainer® (BD Diagnostics, Plymouth, UK) and heparin tubes. The research was approved by the Human Research Ethics Committee of Centro Universitário Franciscano (CAAE: 31211214.4.0000.5306). The Histopaque-1077® (Sigma-Aldrich, St. Louis, MO) density gradient was used to separate lymphocyte cells using 20 ml blood samples. After further centrifugation for 30 min at 2500 x g, the cells were transferred to culture media containing 5 ml RPMI 1640 with 10% fetal bovine serum, 1% penicillin and 1% streptomycin. The cells were cultured in a 96-well microplate at an initial density of 2 x 10^5 cells at 37 ^oC in a 5% humidified CO_2 atmosphere to assess the viability cell (Costa et al., 2012). Then the cells were treated with various concentration of GO, NEB and NEG (0.1, 1, 10, 100 and 1000 µg/µl) were dissolved in medium and further incubated for 72 h. Subsequently, cytotoxicity in lymphocytes was determined using the MTT and LDH methods beyond the level of oxidative stress by determining the lipid peroxidation (TBARS) and catalase enzyme activity (CAT).

2.7.1 MTT assay

Cell viability was evaluated using the MTT assay, a colorimetric assay that measures the reduction of yellow 3- (4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase as described by Mosmann, (1983) with modifications. After treatment 20 µl of MTT (5 mg/ml) in phosphate buffered saline was added to each well and the plate was incubated at 37 °C for 4 h. The medium was removed and 100 µl of DMSO was added to each well. After 10 min of incubation at 37 °C, the plate was read at 570 nm using a microplate reader TP-Reader (Thermoplate, China). As a positive

control, 10% of SDS was used. In addition, a negative control; control wells without treatment, was employed and prepared under the same experimental conditions. All treatments were carried out in triplicates (in same 96-well plate) and repeated two times in independent experiments. The percentage of cell viability was calculated as: (absorbance of test/absorption of the control) ×100.

2.7.2 LDH assay

The LDH activity of the lymphocytes was performed using Labtest Kit (Labtest Diagnóstica SA) through the automatic analyzer CELM SBA 200® (CELM, Barueri/SP, Brazil). After treatment of lymphocytes as described above the culture supernatants were removed and LDH release was determined by two-point enzymatic kinetics. All tests were carried out in duplicate. The percentage of LDH release was calculated as A_T/A_C x 100; where A_T is the experimental absorbance of treated cells, A_C is the control absorbance of untreated cells.

2.8 Hemolytic activity assay

Hemolytic activity of GO, NEB, and NEG was measured spectrophotometrically using a hemoglobin release assay (Vaucher *et al*., 2010), with modifications. Briefly, the fresh defibrinated human erythrocytes were rinsed three times with PBS centrifuged for 15 min at 900 x g and resuspended at 4% (v/v) in PBS. Cells were treated with GO, NEB, and NEG (0.1, 1, 10, 100 and 1000 µg/µl) for 1h at 37 ºC and then centrifuged at 1000 x *g* for 5 min. Aliquots of the supernatant were then transferred to 96-well microplates, where hemoglobin release was monitored using a microplate reader TP-Reader (Thermoplate, China) by measuring the absorbance at 414 nm. Percentage of hemolysis was calculated as $(A_T–A_C)/(A_X–A_C)$ x 100; where A_T is the experimental absorbance of treated supernatants, A_C is the control absorbance of PBS-treated cell supernatant, and A_X is the absorbance of 10% (v/v) SDS lysed cells.

2.9 Lipid peroxidation

The determination of lipid peroxidation was assessed by determination of thiobarbituric acid reactive species (TBARS) according to the method described by Ohkawa, Ohishi, and Yagi (1979) and modified by Carrera-Rotlan and Garcia-Estrada (1998). After treatment, the lymphocytes were centrifuged for 5 min at 1000 x g to remove the culture medium. The supernatant was discarded and held two centrifugations with saline (0.9% NaCl) for 10 min at 1000 x g. After these steps, the supernatant was discarded and 100 µl was added butylated hydroxytoluene (BHT 10 mM), 500 µl of trichloroacetic acid (TCA 20%) and left a final centrifugation step for 5 min at 2000 x g. Immediately after centrifugation, 900 µl of the supernatant was mixed with a reaction medium containing thiobarbituric acid (TBA 0.8%) and incubated at 95°C for 1 h. The absorbance was measured at a wavelength of 532 nm in a spectrophotometer. The results were expressed as nmol MDA/10^6 cells.

2.10 Catalase enzyme activity (CAT)

The CAT was assayed spectrophotometrically by the Aebi method (1984) with some modifications involving monitoring the disappearance of H_2O_2 in the presence of the enzyme at a wavelength of 240 nm. After lymphocyte treatment, 100 µl of cell suspension was added to 1432 µl of potassium phosphate buffer (TFK) 50 mM, pH 7.0, and the enzyme reaction was started by adding 52.5 µl of H_2O_2. The results were expressed as U of CAT/10^6 cells.

2.11 Statistical analysis

Data were expressed as the mean ± standard deviation (SD) for three independent determinations for each experimental point. Data were analyzed with software package GraphPad Prism 4.00 for *Windows* (*GraphPad Software*, San Diego- CA, USA). All data of this study were submitted to analysis of variance (oneway ANOVA) followed by Tukey test ($p < 0.05$).

3. Results and Discussion

3.1 Characterization of GO and of NEG

Chemical analysis of the GO revealed the presence of main constituents: citronellol (17.74%) and geraniol (14.43 %). Nanoemulsions may represent an interesting alternative, increasing the stability, releasing the drug in the inflammatory site and decreasing the oil toxicity (Weiss *et al*., 2009). The optimization of nanoemulsion containing geranium oil (NEG) was performed using Ultra-Turrax. The results indicate an adequate homogeneity, with droplet size (diameter) less than 200nm and index polydispersity <0.25. One of method that can be used to determine the dispersion stability of emulsion is by measuring zeta potential value. The NEG showed zeta potential of -10mV. This result of the zeta potential is satisfactory, because when the nanostructure has a negative charge, system stability tends to be higher with a lower likelihood of aggregation of particles, and consequent precipitation of nanostructures (Friedrich *et al*, 2008). From the TEM, it was possible to observe the nanometric size of NEG and NEB, showing the successful development of the formulation.

The use of antioxidants has gained importance in the treatment of various metabolic disorders (diabetes mellitus, arthritis, cancer, aging, liver disorders, etc.) where free radicals are implicated. Worldwide, the scientific development programs aim to investigate the medicinal properties of plants for their potent antioxidant properties (Auddy *et al*., 2003; Shrestha *et al*., 2013). It has been reported that the antioxidant activity of GO showed positive correlation with anticancer activity, reported by some researchers. Such properties can be assigned to the GO composition, such as citronellol and trans-geraniol. It is known that the citronellol is a soluble oil component with anticancer and anti-inflammatory properties. Studies report that geraniol promoted significant inhibition (60-90%) of the growth of tumor cells of the pancreas (Fayed, 2009).

3.2 DPPH radical scavenging activity

The GO and NEG antioxidant activities were initially evaluated to determine DPPH free radical scavenging where different concentrations of GO and NEG (0.195 to 100 µg/µl) were compared with the antioxidant activity of

butylated hydroxytoluene (BHT) (1 µg/µl). As presented in **figure 1**, the main components of GO showed significant dose-dependent DPPH scavenging activities. At concentrations of 100 and 50 µg/µl of GO antioxidant activity was comparable to the activity observed for BHT. At a concentration of 25 µg/µl, there was no significant difference when compared to BHT, proving the antioxidant activity at this concentration. As the GO concentrations decrease, it can be evidenced a significant decrease in antioxidant activity to the concentration of 0.390 µg/µl oil. A significant decrease in antioxidant activity of NEG for the different concentrations tested compared to BHT was evidenced. After linear regression, it was obtained an IC_{50}= 198 µg/µl (Y=0.2506x + 0.1633, R^2=0.993) for the NEG and IC_{50}= 4.72 µg/µl (Y= 4.5278x + 28.595, R^2=0.9508) for GO.

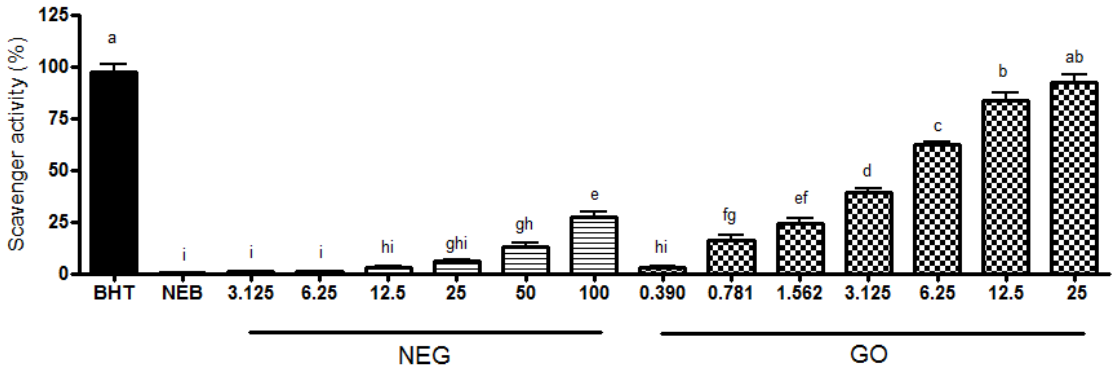

Figure 1. DPPH radical scavenging activity of GO and NEG. Scavenger activity (%) was measured by the scavenging of DPPH radicals and expressed as percent inhibition (%) and BHT was included as a positive control. Values are mean ± S.D. indicate statistical differences compared by one-way ANOVA followed by Tukey post hoc tests. (**small letters**) denotes $p < 0.05$ as compared to their control group respectively. The averages followed by the same letter are not statistically different from each other.

These results revealed that the nanostructuring of GO may have hindered the electrons transfer to the DPPH oxidizing compound. The GO has terpenoids as major compounds in its constitution. The geraniol and citronellol have groups such as hydroxyl (OH^-) or methoxyl (CH_3O^-) connected to insaturations to be

donated, resulting in increased free radical scavenging activity (Zhang *et al*., 2007).

In other studies, it has also been determined the antioxidant activity of GO. Recently, Džamić *et al.* (2014) evaluated the antioxidant activity by DPPH assay and reported an EC50 = 0.802 mg/ml. Cavar & Maksimović (2012), EC50 values obtained ranged 63.7 mg/ml and 64.88 mg/ml when the oil was extracted from the leaves and stems, respectively. Fayed (2009) reported that the antiradical activity of GO was significantly stronger than the activity of acetone extract of the plant (EC50 value of 14.49 mg/ml and EC50 = 66.45 mg/ml, respectively). Lis-Balchin (1996) also reported a pronounced antioxidant activity of GO.

3.3 FRAP assay

FRAP assay (Benzie *et al.*, 1996) is based on the ability of phenols in reducing Fe^{3+} to Fe^{2+}, the reduction is accompanied by the formation of a colored complex with Fe^{2+}. This complex has an intense blue staining, susceptible to be quantified spectrophotometrically at 593 nm, which is proportional to the amount of reducing species present in the sample (Sucupira *et al.*, 2012). From the observed results of antioxidant activity of GO and NEG in the FRAP experiments, the initial concentration of 25 µg/µl was chosen to investigate the ability of GO in reducing Fe^{3+} ferricyanide complex to the ferrous form, Fe^{2+}. It was shown that the GO increased FRAP values significantly at concentrations of 25µg/µl compared to the positive control (Vit E). When it was performed to compare the values of FRAP with GO and NEG was observed a significant reduction in FRAP values at concentrations of 6.25 and 25 µg/µl (**Figure 2**).

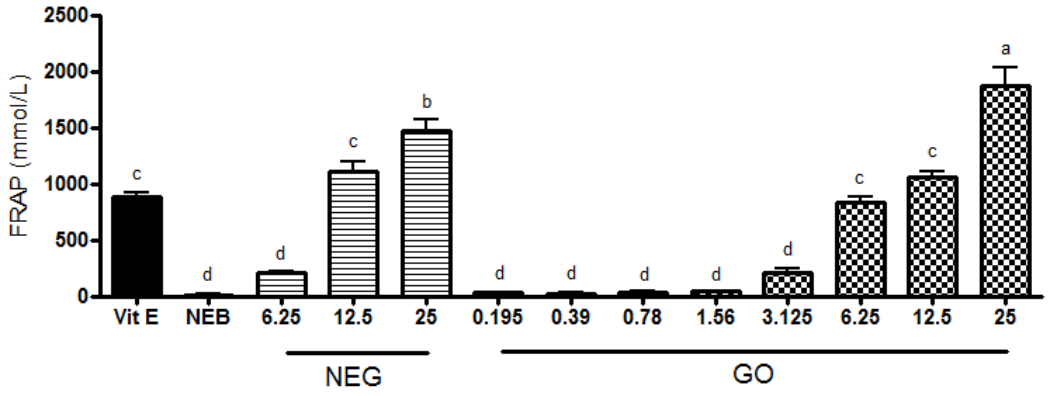

Figure 2. Antioxidant activity of NEG and GO were measured by ferric reducing/antioxidant power and expressed as FRAP (µmol/l) and Vit E was included as a positive control. Values are mean ± S.D. indicate statistical differences compared by one-way ANOVA followed by Tukey post hoc tests. (**small letters**) denotes $p < 0.05$ as compared to their control group respectively. The averages followed by the same letter are not statistically different from each other.

The results observed in the FRAP assay showed that although the NEG antioxidant capacity is less than the GO, this capacity remained and the incorporation of this oil in a nanostructure, did not significantly interfere with the antioxidant effect. However, there is no data in the literature to compare the results reported in this study having used the FRAP assay. It should be noted that the automated FRAP assay to determine the antioxidant activity of nanoemulsions has not yet been reported. This test may have advantages compared to manual methods, becoming an interesting alternative to the determination of the antioxidant activity of the nanoemulsions. Recently, Abla and Banga (2014) used the FRAP assay to determine the antioxidant activity of formulation of tocopherol used as nanocarriers.

3.4 MTT assay

The cytotoxicity of lymphocytes was evaluated by using MTT assay after 72 h of treatment. Five different concentrations of GO, NEB and NEG (0.1, 1, 10, 100 and 1000 µg/µl) were tested and the results are shown in **figure 3**. The GO was evident that concentrations higher than 1 µg/µl caused a significant decrease in cell viability compared to control cells, and this decrease was greater

as the concentration of oil increased. NEG not showed a significant decreased cell viability compared to control cells at all concentrations. However, when these concentrations were confronted with GO, lymphocyte viability was significantly increased at concentration of 1, 10, 100 and 1000 µg/µl, indicating a possible protective effect of the NEG. All concentrations of the NEB showed not to have a significant decrease in cells when compared to control cells. When compared to the positive control, all groups showed a significant increase in cell viability.

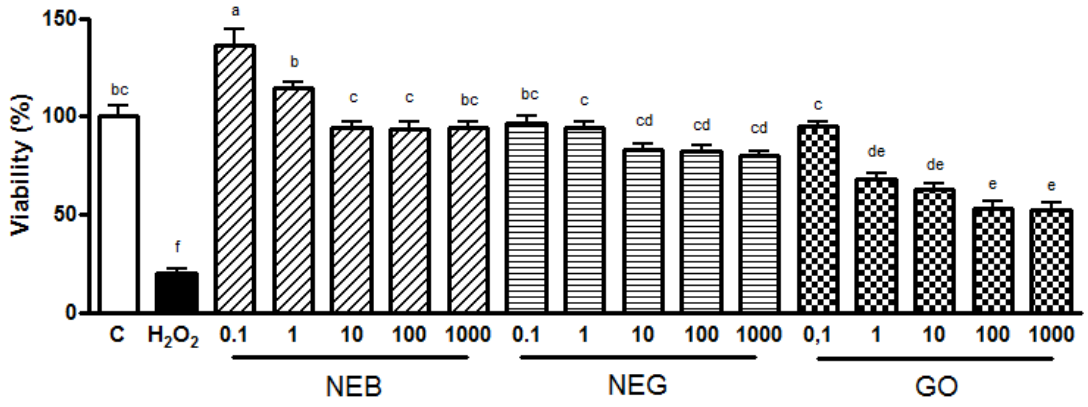

Figure 3. Lymphocyte viability evaluated by MTT assay after treatment with NEB, NEG and GO (µg/µl). Values are mean ± S.D. indicate statistical differences compared by one-way ANOVA followed by Tukey post hoc tests. (**small letters**) denotes $p < 0.05$ as compared to their control group respectively. The averages followed by the same letter are not statistically different from each other.

In these trials, it was evident that the therapeutic response of lymphocytes was dependent on the concentration tested, both for the NEG as to GO. The oil nanostructuring may explain the increase of the cell viability the formulation compared to GO. Nanoemulsions may represent an interesting alternative, increasing the stability, releasing the drug in the inflammatory site and decreasing the oil toxicity (Weiss *et al*., 2009).

3.5 LDH assay

The LDH assay measures the plasma membrane integrity in lymphocytes. The lymphocytes treated with five different concentrations of GO, NEB, and

NEG (0.1, 1, 10, 100, and 1000 µg/µl) resulted in different levels of LDH release (**Figure 4**). Concentration-dependent increase in extracellular LDH was observed for NEB, NEG, and GO when compared to the negative control. The treatment with SDS (positive control) resulted in high levels of LDH release, indicating extensive cell damage. Only the concentration of 1000 µg/µl evidenced a significant decrease in the levels of LDH when compared to NEG and GO, proving the possible protective effect of NEG as evidenced earlier in the MTT assay.

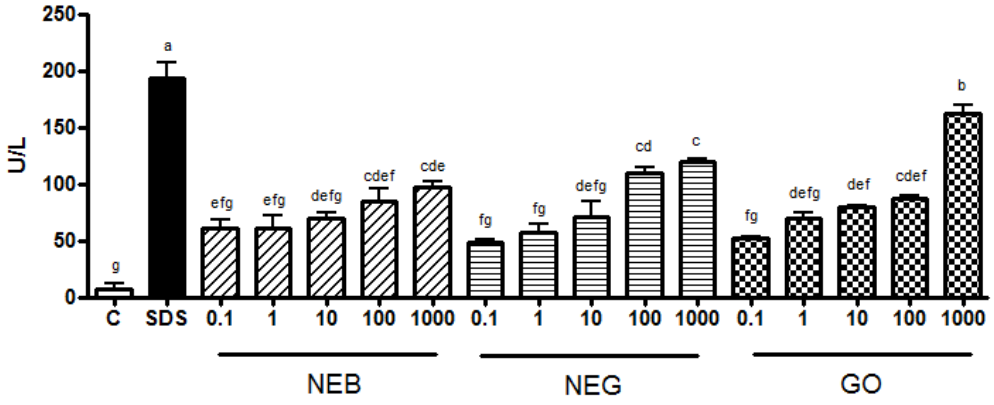

Figure 4. LDH release assay on lymphocyte after treatment with NEB, NEG, and GO (µg/µl). Values are mean ± S.D. indicate statistical differences compared by one-way ANOVA followed by Tukey post hoc tests. (**small letters**) denotes $p < 0.05$ as compared to their control group respectively. The averages followed by the same letter are not statistically different from each other.

It is known that in general the composition of target membranes is related to the different sensitivity to essential oils. Prashar *et al*. (2004) suggest that the cytotoxic mechanism of the oils may be associated with membrane damage. The essential oils can to cause a depolarization of mitochondrial membranes, affecting many ionic channels, reducing the pH gradient, and can change the fluidity of membranes, thus permitting the entry radicals, leading to cell death by apoptosis and necrosis (Hammer *et al*, 2000; Bakkali *et al*, 2008).

3.6 Hemolytic activity assay

The determination of cytotoxicity using the assay of hemolytic activity *in vitro* is important to predict damaged red blood cells when there is the need of administer the NEG or GO intravenously. Such care would prevent the hemolysis of red blood cells and the formation of a hemolytic anemia in tests "*in vivo*".

The hemolytic activities of the NEB, NEG, and GO were determined from of the lysis of human erythrocytes (**Figure 5**). According to the data in this study, GO showed significant increase in hemolytic activity in the concentrations of 100 and 1000 µg/µl compared to the control. NEB and NEG presented significant decrease in activity compared to the positive control (SDS). When the different concentrations between NEG and GO were compared, it was possible to notice a significant decrease in hemolytic activity of NEG at 100 and 1000 µg/µl, indicating that cytotoxicity is concentration dependent and that NEG decreases the toxic potential of GO.

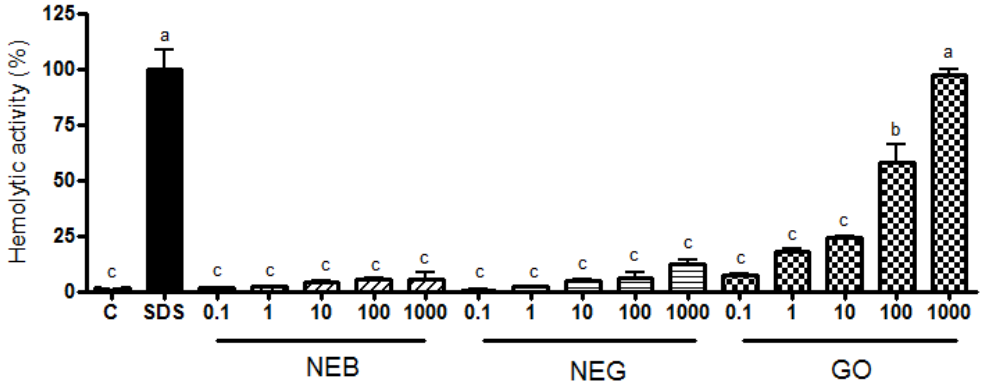

Figure 5. Hemolytic activity of the NEB, NEG and GO against human erythrocytes. Values are mean ± S.D. indicate statistical differences compared by one-way ANOVA followed by Tukey post hoc tests. (**small letters**) denotes $p < 0.05$ as compared to their control group respectively. The averages followed by the same letter are not statistically different from each other.

These results showed that NEG hampers the lysis in human erythrocytes caused by GO. From the MTT and hemolytic activity assays was possible to determine the EC50 in lymphocytes for NEG and GO using linear regression as shown in **Table 1**. A concentration of 10.893 µg/µl of MTT assay on NEG would

be necessary to cause death in 50% of cells and 724 µg /µl to cause hemolysis. There is also no comparative data available on EC50 in lymphocytes to compare the results obtained in this study.

Table 1. EC_{50} values for lymphocytes following exposure to different concentrations of NEG and GO for 72 h based on the dose-response curves derived from the MTT and hemolitc activity assays.

	EC_{50} (µg/µl)	
Assay	NEG	GO
MTT	10.893	1.252
Hemolytic activity	724	221.4

EC_{50} values were calculated by regression analysis of the dose-response curves for MTT [NEG ($Y=-0.003x+82.681$, $R^2=0.95$) and GO ($Y=-0.007x+58.769$, $R^2=0.91$) and hemolytic activity [NEG ($Y=0.0062x+5.10$, $R^2=0.99$) and GO ($Y=0.0595x+36.826$, $R^2=0.85$)].

3.7 Lipid peroxidation

There is increased interest in investigating pro-oxidants and antioxidants, since individuals with certain enzyme deficiencies are more likely to have oxidative damage (Colomé *et al*., 2003). In normal physiological conditions, there is a balance between the production of reactive species and antioxidant defenses. The unbalance of the redox homeostasis determines the degree of oxidative stress. Consequences of this stress include modification of cellular proteins, lipids and may cause DNA damage (Finkel & Holbrook, 2000; Moraes *et al*., 2010). TBARS assay reflect the amount of malondialdehyde formation, the final product of peroxidation of fatty acids and membrane lipid peroxidation that can be induced in vitro by the action of oxidants, probably due to the generation of free radicals (Sirtori *et al*., 2005).

TBARS levels in lymphocytes after 72 h of treatment with different concentrations of NEB, NEG, and GO are shown in **figure 6**. The results indicated that the NEB showed no significant increase in TBARS levels in any of the tested concentrations. To GO was observed a significant increase in TBARS levels at concentrations of 100 and 1000 µg/µl when compared to the control cell. For NEG this increase was significant only for the concentrations of 100 and 1000 µg/µl. The NEG and GO concentrations among themselves showed no statistical differences.

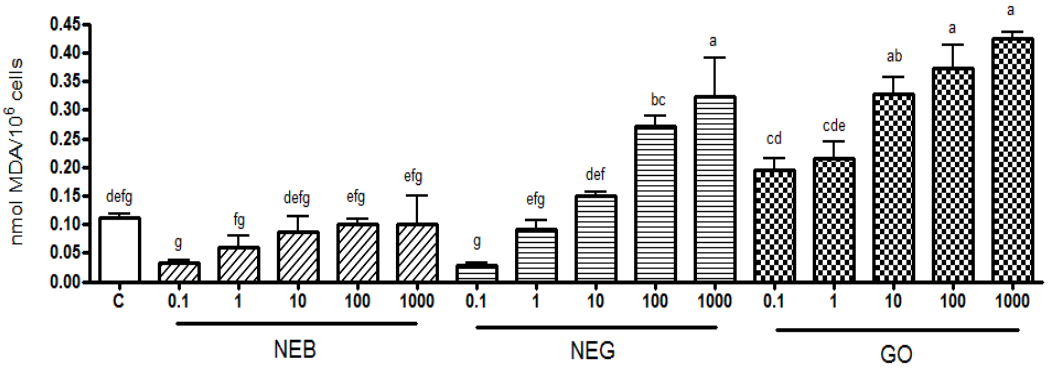

Figure 6. TBARS assays on lymphocyte after treatment with NEB, NEG and GO. Results were expressed as nmol MDA/10^6 cells. Cell control (C) cells in culture medium; Concentrations: (0.1, 1, 10, 100 and 1000 µg/µl). Values are mean ± S.D. indicate statistical differences compared by one-way ANOVA followed by Tukey post hoc tests. (**small letters**) denotes $p < 0.05$ as compared to their control group respectively. The averages followed by the same letter are not statistically different from each other.

An increase of TBARS at higher concentrations indicate strong lipid peroxidation caused by cytotoxicity of NEG or GO. Boukhris *et al.*, 2012, suggests that the administration of essential oil of *P. graveolens* may be helpful in the prevention of diabetic complications associated with oxidative stress. These authors used the TBARS assay to evaluate the hypoglycemic effect of the oil in rats and reported that after four weeks, the serum glucose significantly decreased and antioxidant perturbations were restored.

3.8 Catalase enzyme activity (CAT)

The enzyme catalase is part of the enzyme system of first line of cellular defense against oxidative damage, decomposing oxygen and hydrogen peroxide before their interaction to form more harmful reactive species such as hydroxyl radical (El-Missiry *et al.*, 2001).

CAT levels in lymphocytes after 72 h of treatment with different concentrations of NEB, NEG, and GO are shown in **figure 7**. Our study showed an increase in CAT activity when comparing all concentrations of GO and NEG. The results showed a significant increase in CAT activity in lymphocytes for all concentrations NEB, NEG and GO compared to the control cell, except for the concentration of NEB (0.1 µg/µl) who presented reduction.

This fact demonstrates the protective effect of NEG regarding potential oxidative damage caused by the GO treatment in lymphocytes. There is no study for comparative data in the literature that relates the cytotoxicity of GO with the analysis of CAT. However, Zhuang *et al*., 2009 reported the use of citronellol in 105 patients receiving cancer chemotherapy or radiotherapy. After six weeks of treatment cancer was observed decrease the depletion of leukocytes and neutrophils. In that study, it was proved that citronellol has anticancer and antiinflammatory properties, as well as wound healing promotion. Some studies have also demonstrated anti-inflammatory activity in vitro or in vivo of the GO (Maruyama *et al*., 2006; Su *et al*, 2010).

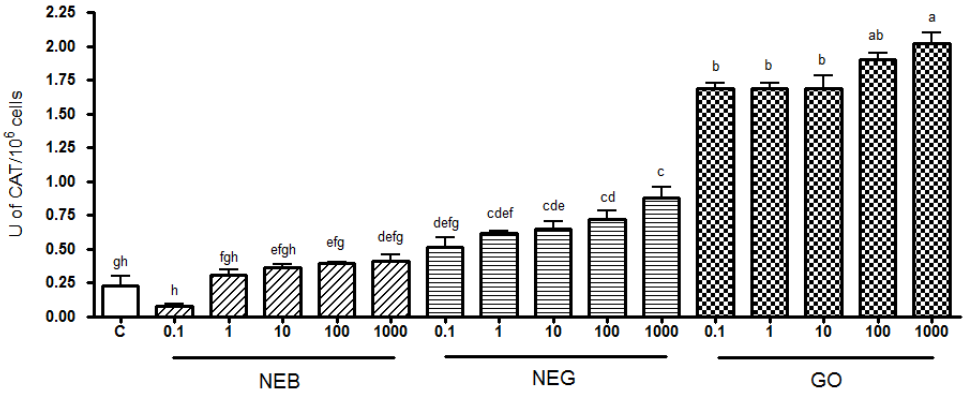

Figure 7. CAT assays on lymphocyte after treatment with NEB, NEG and GO. Results were expressed as nmol U of CAT/10^6 cells. Cell control (C) cells in culture medium; Concentrations: (0.1, 1, 10, 100 and 1000 µg/µl). Values are mean ± S.D. indicate statistical differences compared by one-way ANOVA followed by Tukey post hoc tests. (**small letters**) denotes $p < 0.05$ as compared to their control group respectively. The averages followed by the same letter are not statistically different from each other.

4. Conclusion

In conclusion, our results revealed that the NEG showed good homogeneity and average particle diameter in appropriate scale. The NEG presented a lower antioxidant activity when compared to GO at the same concentrations. Through the FRAP assay, we determined the antioxidant activity of NEG proving to be a trustworthy, fast and suitable method for the determination of nanoemulsions of antioxidant activity. The results also indicate that the NEG showed less cytotoxicity to lymphocytes and red blood cells at the concentrations tested when compared to GO. Although NEG increased CAT levels in TBARS assays, the results indicated that NEG can be used as an alternative to prevent or to safely treat oxidative stress.

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CAPÍTULO 4: Atividade antinflamatória do óleo de gerânio puro e incorporado em nanoestrutura

Resumo

A inflamação é um mecanismo de proteção do organismo contra agentes agressores (físicos e biológicos) que busca retomar a homeostasia. Porém em certas condições, esse mecanismo é exercido de forma inadequada, podendo ele mesmo lesar o organismo. Devido a esse fator, o desenvolvimento de novos medicamentos, principalmente oriundos de plantas é de suma importância. O óleo de gerânio (GO) como já descrito nos capítulos anteriores possui algumas atividades biológicas já comprovadas. Seus dois constituintes majoritários são descritos como responsáveis por algumas destas atividades. O objetivo deste estudo foi avaliar o potencial antinflamatório do GO livre e nanoemulsionado (NEG) em modelo de macrófagos contendo ou não proteína solúvel de *C. albicans*. Após o tratamento com o GO e a NEG nas concentrações de (½, 1, 2x MIC) foram determinados os níveis de óxido nítrico (NOx), interleucinas IL-1, IL-2, IL-6 e IL-10, fator de necrose tumoral (TNF-α), interferon (INFγ), caspase 3 e caspase 8, além da expressão gênica da IL-2, cicloxigenase-2 (COX-2) e óxido nítrico sintase induzida (iNOS) por PCR em Tempo- Real. O estudo deste painel foi importante, pois a NEG foi desenvolvida com a finalidade de ser utilizada em cateteres para diminuir a formação de biofilmes microbianos. Os resultados indicaram um aumento significativo dos níveis de NOx quando os macrófagos foram tratados com a NEG. Este mesmo tratamento não provocou aumento significativo para IL-6, mas o mesmo não ocorreu com a IL-10. A possível atividade antinflamatória da NEG, foi identificada através da redução dos níveis de TNF, IFNγ e caspase-3 quando os macrófagos foram induzidos com a proteína solúvel de *C. albicans*. Este estudo, apresenta resultados importantes e evidencia que a NEG em algumas concentrações, não provoca o aumento das citocinas inflamatórias, mas sim, diminui seus níveis, demonstrando uma interessante atividade antinflamatória.

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MANUSCRITO 4: Anti-inflammatory effect of Geranium nanoemulsion macrophages induced with soluble protein of *Candida albicans*

Anti-inflammatory effect of Geranium nanoemulsion macrophages induced with soluble protein of *Candida albicans*

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ABSTRACT

Pelargonium graveolens is a member of the Geraniaceae family and has been used in folk medicine in many countries because of its anti-inflammatory activity. No studies have yet been reported to evaluate the anti-inflammatory activity of a nanoemulsion containing geranium oil (GO) model in macrophages. In this study the anti-inflammatory effect of Geranium nanoemulsion (NEG) macrophages induced with soluble proteins of *Candida albicans* was investigated. GO presented citronellol (17.74%) and geraniol (14. 43%) as main constituents. The characterization in NEG was demonstrated, showing the particle size of 164 ± 3.5 nm, PDI of 0.12 ± 0.006 and zeta potential -10 mV ± 1.7. The MIC obtained for NEG and GO were 3.64 µg ml^{-1} and 1.82 µg ml^{-1}, respectively. The viability of the macrophages treated with NEG and GO concentrations (1/2 x, 1x and 2x MIC) was evaluated. There was a significant reduction of viability and the MTT assay was not confirmed after the LDH assay. Anti-inflammatory activity was evaluated by determining nitric oxide (NOx), cytokines (interleukin IL-1, IL-6 and IL-10), tumor necrosis factor-α (TNF) and the expression levels gene of interleukin (IL-2), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). The apoptosis inhibition capacity was assessed by determination of INFγ, caspase 3 and caspase 8. The results indicated that there was a significant increase of NOx in the levels after treatment with NEG and significantly reduced levels after treatment with GO. The cytokines (IL-1, IL-6, IL-10, and TNF) were evaluated and NEG (½ x, 1x MIC) decreased IL-1 levels by 1.25 to 1.37 times, respectively. The NEG did not decrease IL-6 levels and a significant increase was observed for IL-10. GO significantly decreased IL-6 and IL-10 levels. TNF levels were decreased after treatment with NEG and GO confirmg the possible anti-inflammatory activity in macrophages induced with the soluble protein of *C. albicans.* There was a significant decrease in IL-2 and COX-2 levels and increased levels of iNOs. The levels of IFNγ and caspase-3 after treatment with NEG decreased indicating an anti-inflammatory effect and can inhibit apoptosis. Finally, the levels of caspase-8 do not change.

Thus, pretreatment with NEG induced an anti-inflammatory effect against soluble proteins of *C. albicans* model macrophages.

Keywords: *Pelargonium graveolens*, nanoemulsion, anti-inflammatory, cytokines and RAW 264.7 macrophages.

INTRODUCTION

Inflammation is an essential part of innate immunity that protects the host from exogenous pathogens (Reuter *et al*., 2010). However, uncontrolled inflammation is closely associated with tissue damage, and consequently causes diseases with uncontrolled inflammation (Nathan & Ding., 2010). The first event during the innate immune response in mammals is the activation of macrophages that phagocytize invading pathogens (Gottipati *et al*., 2008). This occurs after the activation of pro-inflammatory mediators nitric oxide (NO), prostaglandin E2 (PGE2), and pro-inflammatory cytokines, including tumor necrosis factor (TNF-α), interleukin IL-6 and IL-1β and the production of reactive oxygen species (ROS) (Laskin *et al*, 2011; Jung *et al*, 2014).

Enzymes such as inducible nitric oxide synthase (iNOs) and cyclooxygenase-2 (COX-2) are involved in inflammation and have an important role in the response of tissues to injury and infectious agents. Although the physiological activity of these enzymes is beneficial to the organism, increased expression of these enzymes is part of the pathogenesis of many diseases. Substances able to inhibit the expression or the activity of iNOs and COX-2 induced by inflammatory cytokines are often potential anti-inflammatory agents (Rang, 2007). The inflammatory process can also occur maintaining normal tissues and regulating of programmed cell death or aptoptosis (Light *et al*., 2009). The caspase family is the main one characterizing the pathway for the regulation of programmed cell death. Different types of caspases are the expressed in most living cells, located in the cytosol, in its inactive form (Schwerdt *et al*., 2005).

Thus, the suppression of macrophage activation is considered an important strategy for the treatment of these inflammatory diseases (Davignon *et al*., 2013). Research is being conducted in order to demonstrate the anti-inflammatory activity of plants and their derivatives. Pelargonium graveolens (GO) oil known as geranium oil has proved to be non-toxic, non-irritating, and so far it is not known any other adverse effects (Boukhatem *et al.*, 2013). The therapeutic effects include the using the anti-inflammatory drug to treat arthritis and diarrhea,

biliary conditions, gastric ulcer, diabetes, skin diseases, and depression. It is believed that its main constituents (citronellol, geraniol, linalool, and iso menthone) are responsible for their biological activities (SHAWL *et al*., 2006; LIS-Balchin., 2004). Recently, Choi *et al*., (2015) investigated the mechanisms responsible for the anti-inflammatory activity of *Geranium thunbergii*. Su *et al*. (2010) also evaluated the effect of the main constituents of geranium oil, citronellol and geraniol, on lipopolysaccharide (LPS)-induced nitric oxide (NOx) and prostaglandin E(2) (PGE (2)) production in RAW 264.7 macrophages.

The use of nanostructures to protect this oil from a possible deterioration is very interesting, there are no reports on the effects of these nanostructures on the immune system cells. It is believed that nanomaterials can act on the immune system in many ways, inducing toxicity in immune cells, either directly or through oxidative stress and inflammation (Lozano-Fernández *et al*., 2014). Moreover, nanomaterials may interfere with the cellular system by interaction with proteins, DNA, lipids, membranes, organelles and biological fluids (Khan *et al.*, 2015). In this way, understanding the interactions between nanonaterials and the immune system is also a critical issue for their safe application in medicine (Lozano-Fernández *et al*., 2014).

However, no further studies were performed to evaluate the inflammatory activity of a nanoemulsion containing geranium oil in model macrophages. In this study, we investigated the anti-inflammatory effect of Geranium nanoemulsion macrophages induced with soluble proteins of *Candida albicans*.

MATERIALS AND METHODS

Acquisition of Geranium oil and analysis

The GO was purchased from Sigma–Aldrich (St. Louis, MO, USA). The GO was analyzed by gas chromatography (GC) using an Agilent 6890N GC-MSD system, equipped with DB-5 MS capillary column (30 m x 0.25 mm x 0.25

μm film thickness) connected to a mass spectrometer detector (Giongo *et al*. 2015- unpublished article).

Preparation of nanoemulsion and physicochemical characterization

Nanoemulsions (NEG) were obtained (n=3) after injection of oil phase (5% of geranium oil and 2% of sorbitan monooleate) in aqueous phase (2% polysorbate 80 and ultrapure water) under high agitation, employing a T18 Ultra-Turrax (Ika). For comparison, blank formulations (NEB) were prepared (n=3) using capric/caprylic triglyceride mixture (MCT) instead of geranium oil. After preparation, all formulations were characterized according to the following parameters: particle size and polydispersity indices (PDI) by photon correlation spectroscopy (Zetasizer Nano ZS, Malvern Instruments) after dilution (500 times) of suspension in ultrapure water, zeta potential by electrophoretic mobility (Zetasizer Nano ZS, Malvern Instruments) after dilution (500 times) of formulations in 10 mM NaCl solution, and pH directly in formulations using a previously calibrated potentiometer (DM-22 Digimed).

Minimum inhibitory concentration (MIC)

The determination of the MIC of GO against the strain of *Candida albicans* ATCC 14053 was performed as proposed by the M27-A3 protocol. The macrodilution method was used for NEG as the M44-A protocol and reported by Giongo *et al.* (2015).

Preparation of fungal inoculum

Candida albicans ATCC 14053 was inoculated on Sabouraud dextrose agar and incubated at 35 ± 2°C for 24 h. Five colonies with a diameter of approximately 1 mm were picked and suspended in 5 ml of sterile 0.85% saline. The resulting suspension was placed on a vortex mixer for 15 seconds and the cell density was adjusted using a spectrophotometer adding sufficient saline to obtain equivalent transmittance to that of a standard solution on a 0.5 McFarland

scale at a 530 nm wavelength. This procedure provided a standard yeast suspension containing 1 x 10^6 to 5 x 10^6 cells per ml. Subsequently, the yeast suspension was autoclaved and total protein determined by Bradford method (Bradford, 1976).

Cell culture

Murine RAW 264.7 macrophages cells (ATCC TIB-71) were cultured in 24-well microtiter plates with RPMI 1640 supplemented with 10% v/v fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA) and maintained at 37 °C with 5% CO_2 humidified air. Stimulated RAW 264.7 cells ($1x10^5$) were treated with or without soluble protein of *C. albicans* at a concentration of (1µg/well) for 24 h. Three different concentrations of GO (0.91, 1.82 and 3.64 µg ml^{-1}) and NEG (1.82, 3.64 and 7.28 µg ml^{-1}) equivalent to (½ x, 1x and 2x MIC), respectively were used to investigate the anti-inflammatory effect of treatment. After this time, the microplates were incubated for 24 h.

Viability assay

The GO and NEG effect on macrophage viability and proliferation was determined using the MTT assay, a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase (Choi *et al.,* 2002). Cell viability was presented as a percentage of the control value. For MTT assay, this reagent was dissolved in a 5 mg/ml phosphate buffer (PBS, 0.01 mol/l; pH 7.4), and then it was pipetted into a 96-well microplate containing the sample treatments, subsequently incubated for four h. Further, the supernatant was removed from the wells and the cells were resuspended in dimethyl sulfoxide. The absorbance was a read at wave length of 560 nm in a TP-Reader (Thermoplate, China). This assay was carried out in triplicate for each treatment.

LDH assay

The LDH activity of the macrophages was performed using Labtest Kit (Labtest Diagnóstica SA) with the automatic analyzer CELM SBA 200® (CELM, Barueri/SP, Brazil). After treatment of macrophages as described above the culture, supernatants were removed and LDH release was determined by two-point enzymatic kinetics. All tests were carried out in duplicate. The percentage of LDH release was calculated as A_T/A_C x 100; where A_T is the experimental absorbance of treated cells. A_C is the control absorbance of untreated cells.

Cytokine and caspase immunoassays

The analysis of caspase levels can indicate apoptosis pathway activation whereas analysis of some cytokine levels can indicate inflammatory route activation evaluated. The cytokines IL-1, IL-6, IL-10, TNFα, INFɣ, caspase 3 and caspase 8 were analyzed in the cells exposed to treatment using the Quantikine Mouse Immunoassay to measure culture supernatants in the cell according to the manufacturer's instruction. Briefly, all reagents and working standard were prepared and the excess microplate strips were removed. The assay diluent RD1W was added (50 µl) to each well. Further, 100 µL of standard control for our sample were added per well after which the well was covered with the adhesive strip and incubated for 1.5 h at room temperature. Each well was aspirated and washed twice for a total of three washes. The antiserum of each molecule analyzed here was added to each well and covered with a new adhesive strip and incubated for 30 min at room temperature. The aspiration/wash step was repeated and 100 µl of substrate solution were added to each well and incubated for 20 min at room temperature. Finally, the 50 µl stop solution was added to each well and the optical density was determined within 30 min using a microplate reader set to 450 nm. The values were reported as U/mg protein.

NO assay

NOx of the supernatant of macrophages was determined in Cobas Mira® automated clinical equipment using the modified Griess method as reported by Tatsch *et al*. 2011. The values were reported in µmol/l.

RNA extraction and expression of IL-2, Cox-2 and iNOs by real-time PCR

The total RNA of macrophages was directly isolated from microplate wells after the completion of treatment using TRIZOL® (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase (Invitrogen, Carlsbad, CA) and its quality was assessed by running samples on a 1% formaldehyde agarose gel. RNA was quantified spectrophotometrically. The primers used for the Real-Time PCRs were synthesized by Invitrogen (São Paulo, Brazil) and are described in **Table 1**. The real-time PCR amplification reaction was carried out using SYBR® Green One-Step qRT-PCR with Rox (Invitrogen, Carlsbad, CA), performed according to the manufacturer's protocol. cDNA was synthesized from 0.5 µg of total RNA, using the specific forward and reverse primers (20 µM) for each gene. PCR reactions were run on a Swift Spectrum Real-Time Thermal Cycler (ESCO, World Class, World Wide, Singapore) under the following conditions: 50 °C for 3 min, 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 30 s, after the dissociation curve was performed at 95 ºC for 10 s with a final step of 4 ºC. The assay was accomplished for each gene and included cDNA of the samples treated and control without template.

Results were obtained as C_T (threshold cycle) values. The software determines a threshold line at the basis of the baseline fluorescent signal, and the data point that meets the threshold is given as C_T, which is inversely proportional to the starting template copy number. The differences in C_T values between the control group treated group and endogenous control β-actin gene for each reaction (ΔC_T) were analyzed using the $2^{-\Delta\Delta CT}$ method. The relative expression genes after incubation were determined by dividing expression units of the treated group by the control group. All measurements were performed in duplicate in two independent experiments. The results were expressed as relative concentration calculated as described by Pfaffl (2001).

Table 1: Primers sequences used to evaluate the gene expression by Real-Time PCR.

Primers	Sequence 5′ → 3′
IL-2 f	AAATGGACAGAGCAGGACGG
IL-2 r	TAGCCCACACCCTCTTGGAG
COX-2 f	ACCCCCTGCTGCCCGACACT
COX-2 r	CCAGCAACCCGGCCAGCAATC
iNOs f	GCCTTGGCTCCAGCATGTACCCTCAG
iNOs r	CCTGCCCACTGAGTTCGTCCCCTTC
β-actin f	AGAGGGAAATCGTGCGTGAC
β-actin r	CAATAGTGATGACCTGGCCGT

Data analysis

Data are presented as mean ± standard deviation (SD). Statistical differences between groups were evaluated by one-way analysis of variance followed by the Tukey *post hoc* test. All statistical analyzes were performed where all p-values were two-tailed, and $p < 0.05$ was considered statistically significant.

RESULTS

Analysis of oil

Chemical analysis of the geranium essential oil revealed the presence of main constituents: citronellol (17.74%) and geraniol (14.43 %). These results are consistent with Giongo *et al.*, (2015) (unpublished results).

Physicochemical properties of nanoemulsions

The characterization of nanoemulsions demonstrated a particle size of 164 nm ± 3.5, PDI of 0.12 ± 0.006 and zeta potential -10 mV ± 1.7. The NEB presented size of 130 nm ± 2.4, PDI of 0.12 ± 0.03 and the same zeta potential as NEG.

Antifungal activity

The antifungal activity of GO and NEG was determined by conducting the MIC for *C. albicans*. The MIC obtained for the GO was 1.82 µg ml^{-1} and the

NEG was 3.64 µg ml^{-1}. The results demonstrated that both are powerful against this *Candida species*, because they showed a lower MIC.

Viability assay

The macrophage viability was assessed using the MTT assay after 24 hours of treatment. Were used for this assay macrophages without the addition induced with soluble protein (**figure 1A**) and challenged with induced with the soluble protein of *C. albicans* (**figure 1B**) treated with different concentrations of NEG and GO. Results showed a significant decrease in cell viability compared with control for concentrations of NEG (1x and 2x MIC) and GO (1x and 2x MIC), respectively.

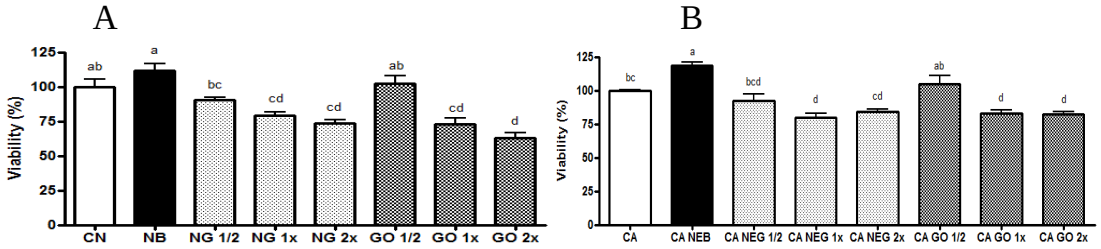


Figure 1. Viability evaluated by 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolic bromide (MTT) reduction among macrophage cells. A) without induced with soluble protein, B) induced with the soluble protein of *C. albicans*. Blank nanoemulsion (NEB), geranium nanoemulsion (NEG) (½ x, 1x, 2x MIC), geranium oil (GO) (½ x, 1x, 2x MIC), cell control (CN) and cell control with fungal inoculum (CA). Values are mean ± S.D. indicate statistical differences compared by one-way ANOVA followed by Tukey post hoc tests (**small letters**) denotes $p < 0.05$ as compared to their control group respectively. The averages followed by the same letter are not statistically different from each other.

LDH assay

The macrophages treated with different concentrations of NEG and GO (½ x, 1x, 2x MIC) resulted in different levels of LDH release. For these assays the culture supernatant of macrophages was used without the addition of fungal soluble protein (**figure 2A**) and challenged with soluble protein of *C. albicans* (**figure 2B**) treated with different concentrations of GO and NEG.

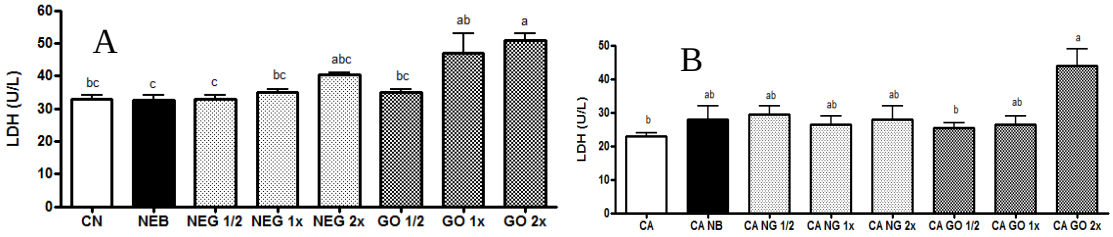


Figure 2. Determination of LDH in the supernatant of macrophages cell treated with NEG and GO. A) without induced with soluble protein, B) induced with the soluble protein of *C. albicans*. Blank nanoemulsion (NEB), geranium nanoemulsion (NEG) (½ x, 1x and 2x MIC), geranium oil (GO) (½ x, 1x and 2x MIC), cell control (CN) and cell control with fungal inoculum (CA). Values are mean ± S.D. indicate statistical differences compared by one-way ANOVA followed by Tukey post hoc tests. (**small letters**) denotes $p < 0.05$ as compared to their control group respectively. The averages followed by the same letter are not statistically different from each other.

As shown in **figure 2A** there is a clearly significant increase in LDH levels after treatment of macrophages with the GO concentrations (1x, 2x MIC). In figure 2B there is a clearly significant increase in the LDH levels after the treatment of macrophages induced with the soluble protein and with the GO concentration (2x MIC). These results demonstrate the possible protective effect of NEG and which was not found in the MTT assay.

NOx assay

In this assay supernatants of macrophages were used without the addition of soluble protein of *C. albicans* (**figure 3A**) and challenged induced with the soluble protein of *C. albicans* (**figure 3B**) treated with different concentrations of NEG and GO for 24 hours. As can be seen in the figures below there was a significant increase in the levels after treatment with NB, NEG (1x and 2x MIC) and significantly reduced levels after treatment with GO (1x and 2x MIC) compared to the control with no fungus macrophages. A similar effect was observed in macrophages induced with the soluble protein of *C. albicans*, where NEG (½ x and 2x MIC) caused a significant increase in NOx levels.

A B

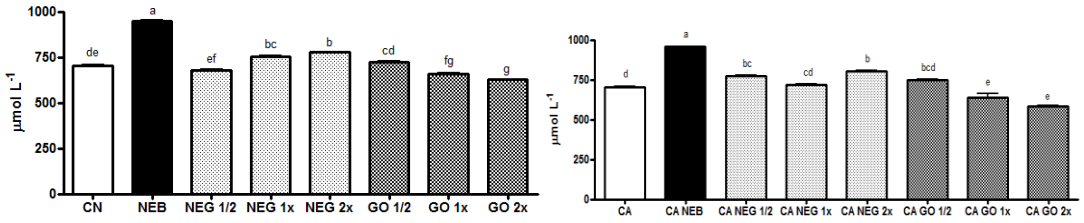


Figure 3. **Determination of NOx in supernatant of macrophages cell treated with NEG and GO.** A) without induced with soluble protein of *C. albicans*, B) induced with the soluble protein of *C. albicans*. Blank nanoemulsion (NEB), geranium nanoemulsion (NEG) (½ x, 1x and 2x MIC), geranium oil (GO) (½ x, 1x and 2x MIC), cell control (CN) and cell control with fungal inoculum (CA). Values are mean ± S.D. indicate statistical differences compared by one-way ANOVA followed by Tukey post hoc tests. (**small letters**) denotes $p < 0.05$ as compared to their control group respectively. The averages followed by the same letter are not statistically different from each other.

Evaluation of inflammatory cytokines and caspases

The production of cytokines (IL-1, IL-6, IL-10, TNF and INF) and caspases (3 and 8) in macrophages was assessed after 24 hours of treatment with different concentrations of NEG and GO (½ x, 1x and 2x MIC). After variance analysis by one-way ANOVA it was evident that IL-1 levels did not increase significantly in the group of fungi without macrophages (**table 2 without padding**). GO (2x MIC) significantly reduced IL-1 levels in the supernatant of macrophage culture. IL-6 levels were not significantly increased in the supernatant of macrophage culture. The levels of IL-10 decreased significantly after treatment with NB and NEG (1x and 2x MIC). TNF levels increased significantly after treatment with NEG (2x MIC) and GO (½ x MIC). INF levels increased significantly after treatment with NEG (2x MIC) and decreased significantly after treatment with the GO (1x and 2x MIC) when compared to control cells (CN). When caspase 3 was determined, a significant increase in treatment with NEG (1x and 2x MIC) and GO (1x and 2x MIC) was evidenced. Caspase 8 levels increased significantly after treatment with GO (2x MIC) when compared to control cells (NC).

After induced with soluble protein of *C. albicans* (**table 2 with faint gray fill**) it was possible to show a significant decrease in IL-1 levels for treatments with NEG (½ x and 2x MIC) and GO (1x MIC). The levels of IL-6 decreased significantly for the treatments with NB, NEG (½ x and 1x MIC) and GO (2x MIC). IL-10 levels increased significantly in treatments with NEG (½ x and 1x

MIC) and GO (½ x MIC), and we found a significant decrease after treatment with NEG (2x MIC). TNF levels increased significantly after treatment with NEG (2x MIC) and significantly decreased after treatment with NEG (½ x MIC) and GO (1x MIC). INF levels decreased significantly after treatment with NEG (½ x, 1x and 2x MIC) and GO (½ x, 1x and 2x MIC) when compared to control cells inoculated with the fungus (CA). The levels of caspase 3 were significantly increased after treatment with NEG (2x MIC) and GO (½ x MIC) and there was a significant decrease after treatment with GO (2x MIC). Finally for levels of caspase 8 a significant increase was found after treatment with NEG (2x MIC) and GO (½ x MIC) when compared to control cells inoculated with the fungus (CA).

Table 2: Determination of inflammatory cytokines and caspases in macrophages after treatment with different concentrations of NEG and GO.

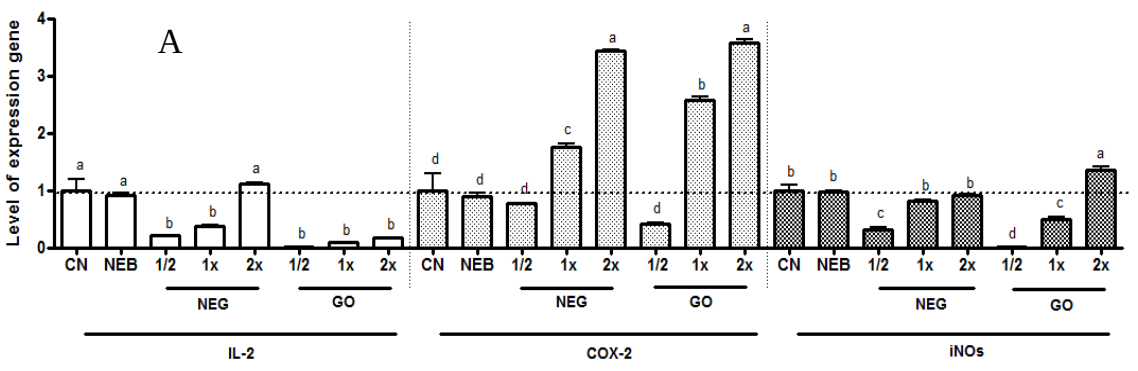
Treatment	IL-1 (pg ml^{-1})	IL-6 (pg ml^{-1})	IL-10 (pg ml^{-1})	TNF (pg ml^{-1})	INF (µg ml^{-1})	Caspase 3 U/mg protein	Caspase 8 U/mg protein
CN	25 ± 2^{ab}	33 ± 1^{a}	67 ± 3^{a}	43 ± 3^{c}	55 ± 5^{b}	4 ± 0.4^{d}	6 ± 0.3^{bc}
NEB	27 ± 1^{ab}	36 ± 3^{a}	58 ± 2^{bc}	45 ± 1^{bc}	52 ± 3^{b}	4.5 ± 0.2^{cd}	5.9 ± 0.5^{bc}
NEG ½	27 ± 6^{ab}	36 ± 2^{a}	60 ± 1^{ab}	44 ± 5^{bc}	58 ± 2^{ab}	4.5 ± 0.2^{cd}	5.7 ± 0.2^{c}
NEG 1x	28 ± 6^{ab}	37 ± 5^{a}	57 ± 3^{bc}	50 ± 4^{abc}	63 ± 4^{ab}	5.1 ± 0.2^{bc}	6.5 ± 0.4^{abc}
NEG 2x	35 ± 2^{a}	47 ± 6^{a}	50 ± 5^{c}	58 ± 2^{a}	74 ± 6^{a}	6.5 ± 0.5^{a}	7 ± 0.6^{ab}
GO ½	30 ± 5^{ab}	41 ± 4^{a}	64 ± 4^{ab}	52 ± 4^{ab}	65 ± 1^{ab}	5 ± 0.3^{bcd}	6.2 ± 0.3^{bc}
GO 1x	31 ± 2^{ab}	41 ± 3^{a}	60 ± 2^{ab}	48 ± 1^{bc}	54 ± 3^{b}	6 ± 0.1^{ab}	6.9 ± 0.5^{abc}
GO 2X	24 ± 3^{b}	34 ± 2^{a}	67 ± 2^{a}	45 ± 2^{bc}	56 ± 4^{b}	7 ± 0.7^{a}	7.6 ± 0.5^{a}
CA	31.5 ± 2.0^{ab}	45.5 ± 0.7^{abc}	53.5 ± 0.8^{bc}	54.5 ± 1.4^{bc}	66 ± 1.2^{a}	5.8 ± 0.2^{bc}	6.9 ± 0.3^{cd}
CA NEB	31.5 ± 3.5^{ab}	43 ± 1.4^{bc}	51.5 ± 2.0^{c}	55 ± 2.1^{bc}	63 ± 0.6^{a}	5.9 ± 0.14^{bc}	6.8 ± 0.11^{cd}
CA NEG ½	21.5 ± 2.1^{c}	36 ± 1^{de}	62 ± 1.4^{a}	46.5 ± 0.7^{de}	38 ± 1.3^{e}	6.0 ± 0.15^{b}	7.1 ± 0.13^{bc}
CA NEG 1x	28.5 ± 2.1^{abc}	39 ± 1^{cde}	60.5 ± 2.1^{ab}	51.5 ± 0.8^{cd}	49.5 ± 0.7^{de}	5.2 ± 0.28^{cd}	6.15 ± 0.2^{d}
CA NEG 2x	25.5 ± 1.6^{bc}	48.5 ± 0.7^{ab}	42 ± 2.8^{d}	68.5 ± 2.1^{a}	47.5 ± 2.0^{c}	7.1 ± 0.23^{a}	7.85 ± 0.18^{ab}
CA GO ½	34.5 ± 0.7^{a}	50 ± 1.4^{a}	66 ± 1.3^{a}	58.5 ± 2.0^{b}	58 ± 1.4^{b}	7.2 ± 0.25^{a}	8.1 ± 0.2^{a}

CA GO 1x	**25.5 ± 0.3^{bc}**	**33 ± 2.8^{a}**	**51 ± 2.6^{c}**	**42.5 ±0.7^{e}**	**45 ± 1.1^{cd}**	**4.5 ± 0.03^{a}**	**6.9 ± 0.14^{cd}**
CA GO 2X	**32 ± 2.8^{ab}**	**42 ± 2.0^{bcd}**	**52 ± 1.4^{c}**	**49.5 ± 1.8^{cd}**	**48.5 ± 2.1^{c}**	**6.05 ± 0.3^{b}**	**7.15 ± 0.21^{bc}**

Levels of cytokines and caspases in the supernatants of macrophages without induced with soluble protein (**without padding**) and induced with the soluble protein of *C. albicans* (**with faint gray fill**). Blank nanoemulsion (NEB), geranium nanoemulsion (NEG) (½ x, 1x and 2x MIC), geranium oil (GO) (½ x, 1x and 2x MIC), cell control (CN) and cell control with fungal inoculum (CA). Values are mean ± SD Indicate statistical differences compared by one-way ANOVA followed by Tukey post hoc tests (**small letters**) denotes p <0.05 as compared to their control group respectively.

Expression of IL-2, COX-2 and iNOs

The levels of gene expression of IL-2, COX-2 and iNOS were evaluated used the PCR technique in real time. Soluble protein of *C. albicans* without macrophages (**figure 4A**) and induced with soluble protein of *C. albicans* (**figure 4B**) were treated for 24 hours with different concentrations of NEG and GO (½ x, 1x and 2x MIC), and thereafter RNA was extraction from cells. The results showed that there was no significant increase in expression of IL-2 after the treatments performed with different concentrations of the NEB, NEG, and GO both without solube protein in macrophages and with the macrophages inoculated with soluble protein of *C. albicans*. A significant increase in relative levels of expression of COX-2 to concentrations of NEG (1x and 2x MIC), GO (1x and 2x MIC) and iNOS for GO (2x MIC) was evidenced after normalization of negative control without soluble protein in macrophages. After induction of macrophages with the soluble protein of *C. albicans* a significant decrease in the expression of COX-2 genes for all treatments tested and a significant increase in iNOS expression levels for GO for the treatment (2x MIC) could be observed.



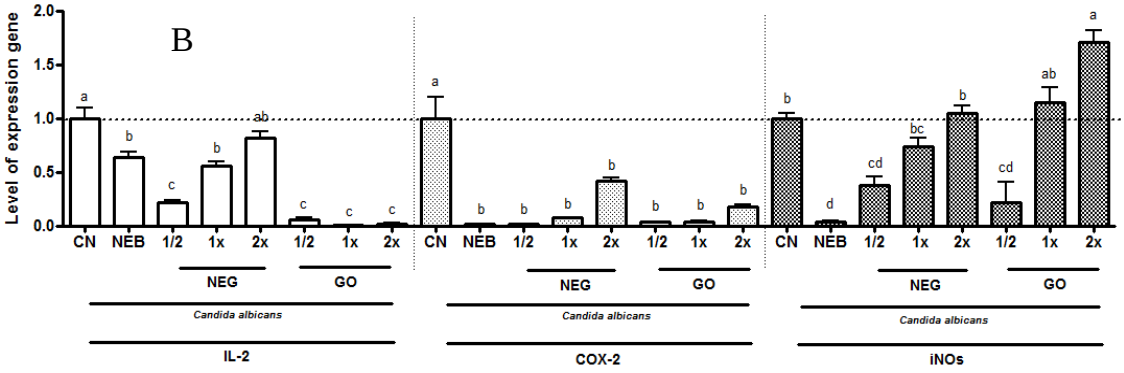


Figure 4: Levels of expression of IL-2 related genes, COX-2 and iNOS in no macrophages and induced with soluble protein of *C. albicans* treated with the NEB, and GO NEG (½ x, 1x and 2x MIC). The relative expression ratios are the average values from three replicate cultures. Bars indicate the 95% confidence interval of the mean (non-overlapping intervals denote significant differences at the level 0.05). This statistic is a conservative criterion of statistical significance. IL-2, COX-2 and iNOs expression levels were normalized using the geometric mean of β-actin gene.

DISCUSSION

Inflammatory response, a physiological reaction to infection or damage, plays a significant role in health and disease (Ran *et al*., 2012). Macrophages have a significant impact on immune response and inflammation. The cells inducing inflammation also initiate and maintain specific immune responses by secreting various types of cytokines (Heo *et al*., 2012).

In the present study, it was demonstrated that NEG exerted an inhibitory effect on inflammation induced with the soluble protein of *C. albicans* in non-toxic doses in murine macrophages. Some studies have demonstrated the anti-inflammatory activity of GO in models of inflammation in macrophages induced by LPS or IFN-γ (Su *et al.,* 2010, Boukhatem *et al*., 2013, Choi *et al*., 2015), however, this effect had not yet been reported for NEG. Classically activated macrophages are induced by combined stimulation with LPS and IFN-γ; these macrophages express a unique set of genes, including TNF-α, IL-1β, IL-6, cyclooxygenase-2 (*COX-2*) and iNOs (Drexler *et al*., 2008).

The characterization of GO revealed the presence of main constituents, citronellol and geraniol (Giongo *et al*., 2015- unpublished article). These constituents are reported as the main elements responsible for the biological activities. Nanoemulsions were prepared to contain geranium oil (NEG) and the physicochemical characteristics were determined. Results showed that the samples have a good homogeneity and particle diameter also appropriate to obtain the desired effect of vectoring (Grama *et al*., 2011). The minimum inhibitory concentration (MIC) against *C. albicans* was measured to verify the antifungal activity of NEG. The results obtained with na assay were utilized in order to investigate the influence of these concentrations on cell viability, NOx production, inflammatory cytokines, caspase expresssion and IL-2 genes COX-2 and iNOS in the presence of macrophages with soluble proteins of *C. albicans*.

The NEG showed a higher MIC compared to GO. Recently, Szweda *et al.* (2015) used essential oils, silver nanoparticles and propolis with alternative agents against fluconazole resistant *C. albicans, C. glabrata* and *C. krusei* clinical isolates. Sienkiewicz *et al*. (2014) reported the antimicrobial activity of GO against bacteria of genus *Acinetobacter* isolated from several clinical materials and from the hospital environment. These authors obtained MIC between 7.5 and 9.5 µl/ml. Giongo *et al*., (2015) showed that nanocapsules of GO have antimicrobial activity against different kinds of bacteria and fungi. Other essential oils such as *Thymus schimperi*, *Cinnamomum zelilanicum*, *Citrus limon* e *Eucalyptus* have also been reported in the literature as having a potent antifungal MIC of 0.016, 0.08, 2.5, 2.5 µg ml^{-1}, respectively (Nasir *et al*., 2015). Several studies have discussed the advantages of oil nanostructuring because the decrease of particle size justifies improvement in antifungal activity of these formulations, because passive transport across the cell membrane becomes easier (Donsiet *et al*., 2010, Chifiriuc *et al.*, 2012).

Cell viability of macrophages was evaluated using the MTT and LDH assays without the use of the soluble protein and after induced with soluble protein of *C. albicans*. It was possible to observe a significant decrease in

viability with NEG and GO concentrations (1x and 2x MIC). When performed to determine LDH release in supernatant of the cultures there was an increase in LDH levels after treatment of macrophages with the GO concentrations (1x and 2x MIC). A significant increase of LDH release in macrophages stimulated with protein *C. albicans* to the NEG concentration (2x MIC) was also observed. These results suggest a protective effect of NEG against of the cells not observed in MTT. Many methods have been described to assess cell viability in vitro, MTT and LDH are routinely used (Soenen and De Cuyper, 2009; Vaucher *et al*., 2010a, Vaucher *et al*., 2010b). Different cell lines have been employed for this purpose, model macrophages being widely used (Li *et al*., 2014). This model evaluates the capacity of anti-inflammatory compounds. In most studies involving these cells, LPS stimulation occurs and the treatments are performed at short time intervals, usually 6 h (Drexler *et al*., 2008). In the model of macrophages used in this study the treatment time was 24 h, treated with NEG and GO, so we believe that the time may have reduced macrophage viability, a fact verified in the MTT assay. NO levels of inflammatory cytokines and caspases were measured from the supernatant of macrophage cultures. It is known that NO serves as a pro-inflammatory agent by producing nitric oxide, the mediator of the inflammatory response (Korhonen *et al.*, 2005). It is also an important mediator of activated cytotoxic effector immune cells, capable of destroying pathogens and tumor cells, in addition to a role as a messenger / modulator in several key biological processes (Hibbs *et al*., 1987; Albrecht *et al.*, 2000).

For NO a similar effect was observed in macrophages with a significant increase in the levels after treatment with NEG and a significant reduction in the levels after treatment with GO. These results demonstrate that the NEG was able to increase the NO level, possibly due to a higher entry point into the cell. These results demonstrate a possible toxic effect of the NEG and GO particularly present in situations of oxidative stress, generation of oxygen intermediates and antioxidant system deficiency. Su *et al.* (2010) reported an inhibitory effect of citronellol and geraniol on nitric oxide and prostaglandin E_2 production in

macrophages. It notes that the laboratory measurement of NO is complex, and characterization of specific activators and inhibitors of NO synthesis constitutes a challenge to understanding their inhibition/induction mechanisms (Su *et al*., 2014).

The cytokines (IL-1, IL-6, IL-10, TNF and INF) and caspases (3 and 8) in macrophages were assessed after 24 h of treatment with different concentrations of NEG and GO (½ x, 1x and 2x MIC). It is known that the high production of cytokines, such as IL-1β and TNF is considered the main factor involved in the induction and maintenance of inflammation (Drexler *et al.,* 2008). With our results, we found that IL-1 did not increase significantly in macrophages induced with soluble proteins of *C. albicans*. However, compared to with macrophages without soluble protein, the NEG (½ x, 1x MIC) decreased IL-1 levels 1.25 and 1.37 fold respectively.

Choi *et al.* (2015) examined the mechanisms responsible for the anti-inflammatory activity of *Geranium thunbergii* in macrophages and they found a significative inhibition in the levels of interleukin-1β. Evaluating the levels of IL-6 under the same conditions, it was found that the NEG showed no anti-inflammatory activity because the IL-6 levels did not decrease significantly after treatment with NEG or GO. The levels of IL-10 apparently increased after treatment with NEG. However there was a reduction with the treatment with GO, indicating possible anti-inflammatory activity for that cytokine. TNF levels were decreased after treatment with NEG and GO confirming possible anti-inflammatory activity in macrophages induced with soluble proteins of *C. albicans.*

Santana *et al*. (2013) investigated the effect of citronellol on inflammatory nociception induced by different stimuli and they examined the involvement of the NO-cGMP-ATP-sensitive K^+ channel pathway and the results showed that citronellol at all doses tested inhibited the levels of TNF-α. Boukhatem *et al.* (2013) reported the anti-inflammatory activities of the essential oil of Rose Geranium (RGEO). The results indicated that RGEO may have a significant potential for the development of novel anti-inflammatory drugs with an improved

safety profile. Our results indicate that there was a decrease in INF levels after treatment with GO, however, this effect was more pronounced for the NEG model induced with soluble proteins of *C. albicans*. This result indicated the anti-inflammatory effect of NEG and a possible inhibition of apoptosis that can be induced in macrophages by increase this cytokine (Schroder *et al.,* 2006). Choi *et al. (*2015) recently demonstrated that Geranium exerted an inhibitory effect on IFN-γ-induced inflammation at non-toxic doses.

In this study, we report for the first time the determination of the levels of caspases following treatment with GO and NEG. It was observed that caspase-3 levels decreased significantly after treatment with the NEG and GO model induced with fungal protein and the levels of caspase 8 remained unchanged, indicating that there was no influence on the activation of apoptosis in these cells. In a recent study, Kumar *et al.,* 2015 investigated the *in vitro* cytotoxicity of essential oils from leaves and flowers of *Callistemon citrinus* from Western Himalayas and found that the levels of caspase 3 and 8 did not change significantly, and that apoptosis was not activated.

Levels of gene expression of IL-2, COX-2, and iNOS were determined in this study. It was possible to confirm the effect of anti- inflammatory activity of GO and NEG, due to the significant decrease of the gene expression of COX-2 in macrophages induced with soluble proteins of *C. albicans*. Our results corroborate those of Su *et al*. (2010) who reported a decrease in expression levels for COX-2. Pannee *et al.* (2014) used *Cinnamomum cassia* oil after treatment of macrophages and observed an inhibited mRNA expression of pro-inflammatory cytokines with significantly decreased expression of COX-2. In our study, we showed a significant increase in iNOS expression levels for GO in this treatment (2x MIC). Other studies using essential oils have been reported, with anti-inflammatory activity determined by the dosage or the gene expression of inflammatory cytokines (Hua *et al*., 2014; Karimian *et al*., 2014; Pannee *et al*., 2014; Rabelo *et al*., 2014; Sun *et al*., 2014; Xiao *et al*., 2014; Choi *et al*., 2015; Pinheiro *et al.,* 2015, Khodabakhsh *et al.,* 2015). It is important to conduct further studies to evaluate the anti-inflammatory activity of essential oils or plant

extracts, in order to better understand the mechanisms involved in such processes.

CONCLUSION

The inflammatory response stimulated by GO and NEG in macrophages induced with the soluble protein of *C. albicans* was evaluated. The results of the determination of NOx showed a significant increase in the levels after treatment with NEG. The cytokines (IL-1, IL-6, IL-10, and TNF) were evaluated and NEG (½ x, 1x MIC) decreased IL-1 and did not decrease IL-6 levels and a significant increase was observed for IL-10 and the TNF levels were decreased after treatment with NEG. These results indicated possible anti-inflammatory activity of GO when incorporated in nanoemulsions. Therefore, due to these anti-inflammatory activities, the NEG could be used in several applications in the future, including a possible utilization as a drug for treating inflammatory diseases.

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CAPÍTULO 5: Atividade antifúngica e antibiofilme do óleo de gerânio puro e incorporado em nanoemulsões

Resumo

As infecções hospitalares estão associadas a diferentes fatores de risco. Entretanto, sabe-se que pacientes imunocomprometidos, tempo prolongado de hospitalização e utilização de dispositivos médicos como cateteres centrais, intravenosos, enterais e urinários são os maiores fatores de risco (HHS, 2013; CORNEJO-JUÁREZ *et al*., 2015). O uso indiscriminado de antimicrobianos também facilita o surgimento de microrganismos cada vez mais resistentes aos fármacos disponíveis no mercado. A formação do biofilme é considerada uma das principais causas para a falha do tratamento com os agentes antimicrobianos. Esta forma de organização é muito vantajosa frente a todas as espécies de microrganismos, pois proporciona uma eficiente barreira contra a penetração de antimicrobianos (ASLMAN, 2008). Sabe-se que a formação de biofilmes nas superfícies de materiais médicos ocasionam graves infecções, aumentando os índices de morbidade e mortalidade em nosso país. A utilização de fontes alternativas para o combate deste tipo de infecção vem sendo o foco de estudo de inúmeros pesquisadores. O óleo de gerânio (GO) possui atividades antimicrobianas já comprovadas, entretanto ainda não há relatos na literatura sobre sua atividade antibiofilme na forma livre e incorporado a nanoemulsões. Foram desenvolvidas nanoemulsões contendo GO (NEG) e nanoemulsões controle (NEB) a fim de comparar estas nanoestruturas com o GO livre. Biofilmes de *C. albicans, C. tropicalis, C. glabrata e C. krusei* foram formados em placas de polietileno e em cateteres nasoenterais de poliuretano. Foi realizada a quantificação da formação dos biofilmes utilizando o cristal violeta, proteínas totais e o método de ATP-Bioluminescência. A microscopia de força atômica (AFM) foi realizada com o objetivo de verificar a rugosidade do biofilme na superfície dos materiais. Foi realizado um controle de formação de biofilme para cada espécie de *Candida* e realizou-se uma comparação com a Anfotericina B, GO, NEG e NEB. Os resultados demonstraram que o GO possui potencial atividade antibiofilme e que quando incorporado as nanoemulsões promove uma inibição significativa na formação dos biofilmes nas superfícies das placas de polietileno e nos cateteres de poliuretano. A AFM confirmou estes resultados,

mostrando a diminuição em nm da rugosidade dos biofilmes formados pelas diferentes espécies de *Candidas* nas placas de polietileno.

Manuscrito 5: Anti-*Candida* activity assessment of *Pelargonium graveolens* oil free and nanoemulsion in biofilm formation in hospital medical supplies

Anti-*Candida* activity assessment of *Pelargonium graveolens* oil free and nanoemulsion in biofilm formation in hospital medical supplies

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Abstract

Infections due to microbial biofilm formation on the surface of catheters and other medical devices are constantly reported as a major cause of morbidity and mortality in patients admitted to hospitals. Furthermore, sessile cells are more resistant to phagocytosis and most antimicrobial, which complicates the treatment of such infections. Researches aimed at new antimicrobial originating mainly from plants have increased in recent years and the development of new strategies for their release is critical in combating the formation of biofilms. Geranium oil (GO) has proven antimicrobial activity. Because of this, the aim of this study was to develop nanoemulsions containing this oil (NEG) and evaluate its activity after the biofilm formation of *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, and *Candida krusei* in hospital medical supplies. For quantification of the biofilm, crystal violet, total protein, and ATP-bioluminescence assays were used. The results revealed that GO and NEG showed lower MIC for *C. albicans* and *C. tropicalis*. The biofilms formed by different species of *Candida* on the surfaces of polyethylene and polyurethane were quantified. GO and NEG significantly inhibited the formation of biofilms in all species tested on the surfaces of polyethylene. However, NEG antibiofilm has had better activity than GO for *C. albicans*, *C. tropicalis* and *C. glabrata*, according to the surface potential analysis by AFM. The analysis of the biofilm formation on the polyethylene surface by ATP-bioluminescence and CFU showed similar results. In both methods the formation of biofilm in the catheter occurred in greater quantity for *C. albicans* and *C. tropicalis.* GO did not significantly inhibit the formation of biofilms only in *C. krusei*, although NEG significantly increased this activity GO in all species tested when compared to the control training biofilm. The following study shows that the development of NEG may become an effective alternative to reduce the adhesion of microorganisms and prevent infections resulting from the use of some hospital medical materials.

Keywords: nanoemulsion, biofilm, *Candida*, catheter, polyethylene, polyurethane

1. Introduction

The infections caused by species of *Candida* affect immunocompromised patients and are said to cause 5% to 71% of mortality and morbidity, respectively, in the hospital environment (Seneviratne *et al*., 2015). This has been associated with resistance to antifungal agents available on the market and biofilm formation on medical and hospital supplies; such as bladder probes, catheters, and prostheses (Akbari & Kjellerup, 2015). The biofilm development depends on the type and the number of cells that adhere to the device, the type of surface that constituted it and the medium or fluids wherein the microorganisms are exposed (Donlan, 2001).

Biofilms are defined as communities of microorganisms that are encapsulated in a self-produced extracellular polymeric substance (EPS) attached to a surface (Martinez *et al*., 2010). Biofilms constitute a growing protected mode, which enable their survival in a hostile environment. The life of the microorganisms within the biofilm provides a number of benefits when compared to the ones found on a free mode (Behlau & Gilmore, 2008).

Candida species are characterized as commensal organisms, but they can become pathogenic at times, where the host immune defense is not fully active (Kojic & Darouiche, 2004). Research shows that among the species of *Candida*, *C. albicans* is commonly associated with bloodstream infections related to the use of catheters (Seneviratne *et al*., 2008). *C. albicans* possesses an excellent ability to form biofilm communities on medical devices and tissue surfaces, which are extremely difficult to eradicate, giving dire consequences to the patients (Seneviratne *et al*., 2015). However, there are reports that *C. guilliermondii, C. tropicalis, C. krusei, C. parapsilosis, C. glabrata, C. dubliniensis* and *C. kefyr* also cause infections by the formation of biofilms. The increase in the prevalence of infections caused by species of non-albicans *Candida* is of concern, inasmuch as these species are more resistant to antifungal agents commonly used (Menezes *et al*, 2004, Li *et al*, 2007).

In recent years, there has been a great effort in the search for new agents to combat this type of fungal infection. In this context, the study of plants for this purpose has been realized and the use of essential oils incorporated in nanostructures appears as a new strategy. Our group has been studying the antifungal activities of *Pelargonium graveolens* nanostructured free oil and found that it possesses activity against *Candida* species (Giongo et al., 2015).

Pelargonium graveolens oil, known as geranium oil, belongs to the Geraniaceae family and consists of herbaceous, shrubs, and subshrubs species, (Rao, 2002). Geranium oil proves to be non-toxic, non-irritating, non-sensitizing generally, and it is not known any other adverse effects (Boukhatem *et al*., 2013). The therapeutic effects include the application in the treatment of dysentery, diarrhea, biliary conditions, gastric ulcer, diabetes, skin diseases, and depression. Its biological activities are related to its principal constituents, citronellol and geraniol (Shawl *et al*., 2006).

However, essential oils are characterized by instability and volatility. The nanostructures can be rightly used to prevent volatilization and increase the time of these compounds at the site of action (Donsi *et al*., 2011; Danielli *et al*., 2013). The nanoemulsions are ultrafine emulsion wherein the droplet size will depend on the structure and amount of surfactant. It has the capacity to dissolve hydrophobic active and good compatibility, which makes it an ideal vehicle for parenteral use formulations (Ravi & Padma, 2011). Thus, the aim of this study was to develop nanoemulsions containing this oil (NEG) and evaluate its activity after the biofilm formation of *Candida* species on polyethylene plate and hospital medical supplies.

2. Materials and methods

2.1 Acquisition of Geranium oil and analysis

The GO was purchased from Sigma–Aldrich (St. Louis, MO, USA). The GO was analyzed by gas chromatography (GC) using an Agilent 6890N GC-

MSD system, equipped with DB-5 MS capillary column (30 m x 0.25 mm x 0.25 μm film thickness) connected to a mass spectrometer detector (Giongo *et al*. 2015- unpublished article).

2.2 Preparation of nanoemulsion and physicochemical characterization

Nanoemulsions (NEG) were obtained (n=3) after injection of oil phase (5% of geranium oil and 2% of sorbitan monooleate) in aqueous phase (2% polysorbate 80 and ultrapure water) under high agitation, employing a T18 Ultra-Turrax (Ika). For comparison, white nanoemulsions (NEB) were prepared (n=3) using capric/caprilic triglyceride mixture (MCT) instead of geranium oil. The preparation technique was described by Giongo *et al*. 2015 (unpublished article).

After preparation, all formulations were characterized according to the following parameters: particle size and polydispersity indices (PDI) by photon correlation spectroscopy (Zetasizer Nano ZS, Malvern Instruments) after dilution (500 times) of suspension in ultrapure water, zeta potential by electrophoretic mobility (Zetasizer Nano ZS, Malvern Instruments) after dilution (500 times) of formulations in 10 mM NaCl solution, and pH directly in formulations using a potentiometer previously calibrated (DM-22 Digimed).

2.3 Antifungal activity

For evaluating the antifungal activity of GO and NEG, the following microorganisms were used: *Candida albicans* ATCC 14053, *Candida tropicalis* ATCC 66029, *Candida glabrata* ATCC 66032 and *Candida krusei* ATCC 6258. All strains were inoculated on sabouraud dextrose agar and incubated at 35 ± 2 °C for 24 h before the tests. The resulting suspension was placed on a vortex mixer for 15 seconds, and the cell density was adjusted using a spectrophotometer, adding sufficient saline to obtain equivalent transmittance to that of a standard solution in 0.5 McFarland scale at 530 nm. This procedure provided a standard yeast suspension containing 1 x 10^6 to 5 x 10^6 cells per ml. The suspension was produced making a 1:50 dilution followed by a 1:20 dilution

of the standard suspension in RPMI 1640 medium supplemented with L-glutamine (Sigma Chemical Co., St Louis, Missouri, USA), buffered with MOPS [acid 3-(N-morpholino-propane sulfonic acid)] (0.165 mol l^{-1}) (Sigma), pH 7.0, to give the inoculum 2-fold concentrated used in the test (of 1 x 10^3 to 5 x 10^3 UFC ml^{-1}). After 100 µl of each concentration of GO, NEG, and NEB (as described in item 2.2) was diluted in RPMI, each was then transferred to a well and added to 100 µl of inoculum. The final concentration after inoculation test was 0.5 x 10^3 the 2.5 x 10^3 UFC ml^{-1}, as recommended by the document M27-A3 (CLSI). The microplates were incubated at 37°C for 48 h in triplicate. The MIC was determined based on the lowest concentration of oil which completely inhibited the growth of yeasts (Giongo et al., 2015).

2.4 Substrates

As substrates for adhesion of the biofilm, polyethylene plates (Braskem®) were used, being subjected to a treatment with 10% peracetic acid solution (Peresal®) and sterile polyurethane catheters (Medicone®).

2.5 Formation and treatment of biofilms

Suspensions of *C. albicans* ATCC 14053, *C. tropicalis* ATCC 66029, *C. glabrata* ATCC 66032, and *C. krusei* ATCC 6258 were used for biofilm formation; all adjusted to the range of 1.0 McFarland turbidity. As a positive control, we used the antifungal Amphotericin B (50 µg/well). Also, for each plate, it was performed a control for biofilm formation, which was inoculated with each type of *Candida* added to the Brain Heart Infusion Broth, (Himedia®).

Briefly, 24-well microplate, polyethylene plates of 1 cm^2 were deposited in triplicate and added per well 1 ml of BHI broth. Thereafter, 100 µl of each fungus suspension was added to each microplate well and incubated at 35 ± 2 °C for 72 hs for the formation of biofilm. This formation was monitored daily, talking place the replacement of culture medium of microplate. The treatment of biofilms with NEG and GO were performed using the MIC previously determined for the four species of *Candida* studied. A treatment was also performed using NEB. Later, the microplates were again incubated at 35 ± 2 °C

for 72 hs. After this period, the treated polyethylene plates were washed three times with sterile distilled water to remove planktonic cells and transferred to 6-well microplate where the quantification assays were performed.

The experiments with the catheters were performed after their section in 3 cm fragments were packed in tubes (12 x 75 mm) previously sterilized containing 3 ml of BHI broth. Thereafter, 300 µl of each fungus suspension was added to each tube and incubated at 35 ± 2 °C for 72 hs for the formation of biofilm. This formation was monitored daily, taking place the replacement of the tubes culture medium whenever necessary. After the formation of *Candida* biofilms, they were treated as described above and incubated at 35 ± 2 °C for 72 hs. After this period, the catheters were washed three times with sterile water to remove the planktonic cells and transferred to new tubes in order to carry out the quantitation assays.

2.6 Crystal violet

It was initially used the method proposed by Gwendolyn *et al*. (2007) with some modifications for the quantification of biofilms treated. After transferring the polyethylene plates and catheters treated for 72 hs with Amphotericin B, NEB, NEG, and GO for 6-well microplate and new tubes, they were dried in an oven at 60 °C for 5 min. After this time, they were stained with 1% crystal violet for 15 min. The polyethylene plates and catheters in the tubes were washed with distilled water for 30 seconds and dried again in an oven at 60 °C for 5 min. Later, both were transferred to a new microplate and tube, added 3 ml of absolute ethanol to dissolve the crystal violet. To prevent evaporation, plates were sealed with *Parafilm*© and an outer layer of plastic film. The tubes were closed and incubated at room temperature for 48 h. After this time, the supernatants were transferred to 96-well microplates and the optical density (OD) measured spectrophotometrically at a wavelength of 570 nm.

2.7 Atomic Force Microscopy (AFM)

In order to visualize the biofilm by AFM, polyethylene plates after the completion of treatment as described above were fixed with absolute methanol for 1 minute. The images were obtained using an Agilent Technologies 5500 microscope. The images (5 μm × 5 μm and 1 μm × 1 μm) were collected in non-contact mode using PPP-NCL tips (Nanosensors, constant force = 48 N / m). The images were analyzed using software PicoView.

2.8 Biofilm quantification

Besides the quantification applying method crystal violet as described above, quantification was performed to estimate the number of fungi present in biofilms and the amount that remained after treatment with NEB, NEG, and GO. Two different methods were used: classic number of UFC ml^{-1} in agar (conducted for catheters and plates) and ATP-bioluminescence method (Rohof ATP Complet® system) used only for the quantification in catheters. Biofilms present on the plates and in the catheter after treatment were duly scraped with the aid of a sterile needle, washed with 1 ml of saline and collected in a microtube of 1.5 ml to carry out these measurements. With the aid of a calibrated loop (10 μl), it was performed by quantitative method BHI agar, the inoculation of each treatment, and the plates were incubated at 35 ± 2 °C for 24 to 48 hs. The count of the colonies and the results expressed in UFC ml^{-1} were conducted. Bioluminescence swabs were removed from a tube and dipped in microtubes containing the biofilm scraped catheter after treatment to quantify through the ATP-bioluminescence method. This swab is placed immediately after contact with the enzyme reagent luciferase/luciferin, which emits light that is directly proportional to the amount of ATP present in the sample. Such brightness is measured when the swab is placed on the ATP Complete® device, displaying the level of contamination and the results expressed in RLU (Relative Light Unit) (Costa *et al*., 2004; Oliveira & Canittieri, 2010).

2.9 Determination of protein concentration

The determination of protein concentration of the plates and the catheters biofilms was conduceted from wells that had the collected samples diluted in saline as described above. It was initially used the method described by Bradford (1976) with an adaptation technique (Zor & Selinger, 1996). For this assay was used Bradford reagent (Invitrogen, CA, USA). A standard curve with bovine serum albumin (BSA) (Sigma) was performed at concentrations of zero to 18 µg/ml. 200 µl of Bradford reagent were transferred into each well of a 24 well plate in triplicate and added to the standard curve points or samples of biofilms. The reaction was stirred, placed in an oven at 37 °C for 30 minutes and further subjected to reading absorbance at 595 nm in a microplate reader. The concentration of the samples was determined by comparison with BSA standard curve, plotted and analyzed by linear regression analysis curve in a program GraphPad Prism Version 6.0.

2.10 Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical differences between groups were evaluated by analysis of variance one-way followed by the Tukey *post hoc* test. All statistical analyses were performed where all p values were two-tailed, and $p < 0.05$ was considered statistically significant.

3. Results

GO was analyzed by GC/MS and most of the constituents found were citronellol and geraniol in concentrations of 17.74% and 14.73%, respectively. NEG were produced and characterized according to their physical and chemical characteristics. The results indicated a particle size of 164 nm, with a polydispersity index of 0.25, zeta potential of -10 mV and pH of 3.7. NEB analysis showed 130 nm particle size of 0.12 polidispersion index, zeta potential -10 mV and pH 6.4.

3.1 Antifungal activity of GO and NEG

The antifungal activity was determined according to the broth microdilution method as shown in **Table 1**. GO and NEG had lower MIC for *C. albicans* and *C. tropicalis*.

Table 1: Antifungal activity (MIC µg ml^{-1}) of GO and NEG using microdilution method.

Microorganism	MIC (µg ml^{-1})		
	GO	**NEG**	**NEB**
Candida albicans ATCC 14053	1.82	3.64	ND
Candida tropicalis ATCC 66029	1.82	7.29	ND
Candida glabrata ATCC 66032	14.6	29.2	ND
Candida krusei ATCC 6258	3.64	14.6	ND

ND: not detected

3.2 Biofilm formation and quantification plate

Figure 1 shows the formation of biofilms *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C.krusei* on polyethylene plates. The quantification was performed by counting in UFC and *C. albicans* proved to be the species that formed more biofilm, followed by *C. krusei*. There was no significant difference in the formation of biofilms between *C. tropicalis* and *C. glabrata.*

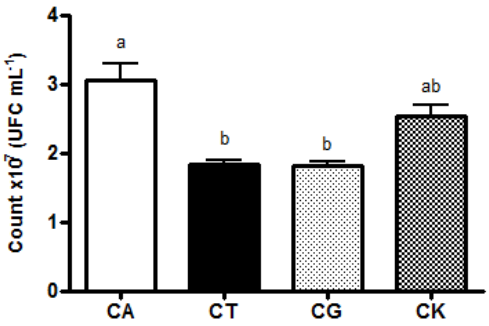


Figure 1: Biofilm formation in the polyethylene plates. *Candida albicans* (CA), *Candida tropicalis* (CT), *Candida glabrata* (CG), *Candida krusei* (CK). Values were statistically significant at $p < 0.05$ when same letters. Data are expressed as means ± SD of UFC ml^{-1} of at three independent experiments.

The influence of GO, NEG, and NEB in biofilm formation on the plates was evaluated by quantifying with CV. The results are shown in **Figure 2**. There was a change in color due to the attachment of the crystal violet, demonstrating the occurrence of biofilm formation by increasing absorbance of the samples. GO significantly inhibited the formation of biofilms *C. albicans, C. tropicalis, C. glabrata, and C. Krusei* when compared to biofilm formation control for each species.

NEG antibiofilm demonstrated activity in all species tested, since quantification by CV showed a decrease in the formation of biofilms compared to their respective controls. When GO was compared to NEG, it was observed that for *C. albicans* and *C. krusei* the nanoemulsion significantly decreased the amount of biofilm, however this difference was not observed for *C. tropicalis* and *C. glabrata* because both had similar antibiofilm activity.

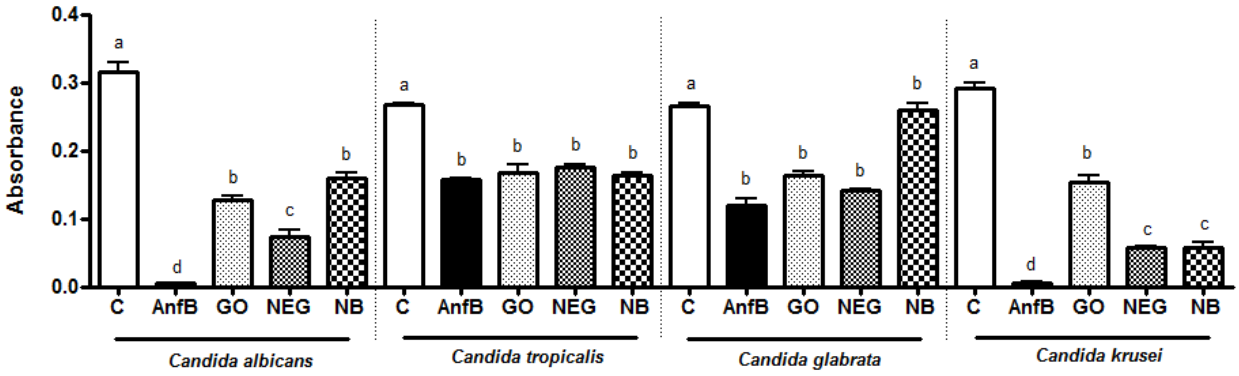


Figure 2: Quantification of biofilm by crystal violet after treatment of polyethylene plates. (C) Biofilm formation control, (AnfB) Amphotericin B, (GO) geranium oil, (NEG) geranium nanoemulsion, white nanoemulsion (NEB). The values were statistically significant for $p < 0.05$. When the treatments were compared between themselves and with the biofilm formation control (C) for each type of *Candida*. Data are expressed as means ± SD for three independent experiments. The averages followed by the same letter are not statistically different from each other

3.3 Total protein after treatment of the plates

The present study determined the amount of proteins present in biofilms after exposure to GO, NEG, and NEB. As seen in **Figure 3,** the amount of protein was significantly reduced in the presence of GO and NEG from all species of *Candida* tested. These results confirm what was obtained from CV measurement, since using this technique it was possible to see a reduction in the amplitude of biofilms.

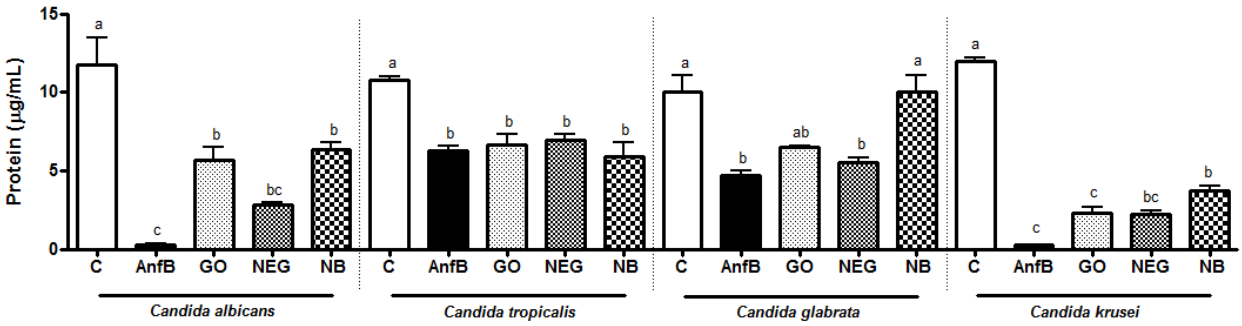


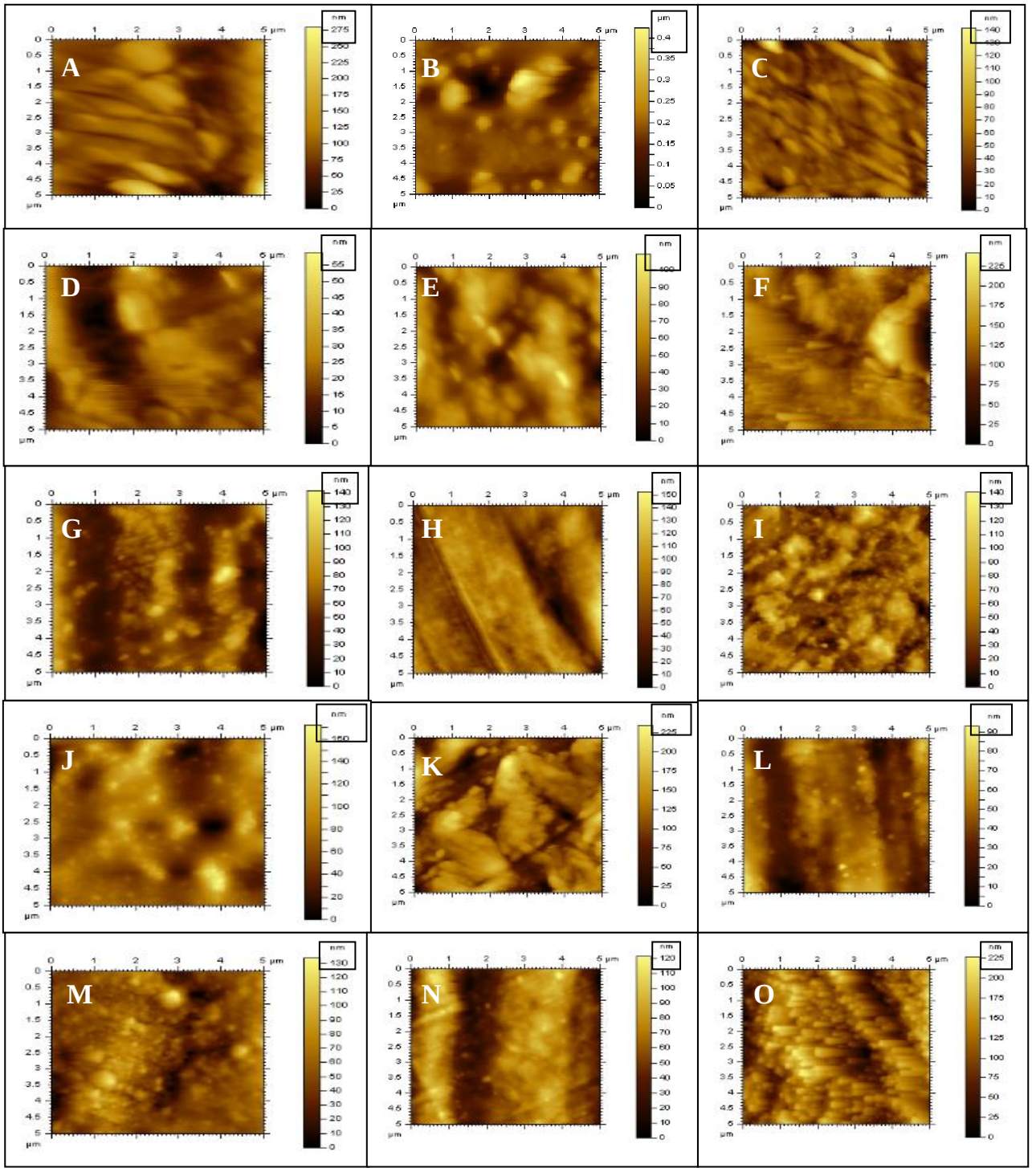
Figure 3: **Quantification of proteins of biofilms in polyethylene plates using Bradford.** (C) Biofilm formation control. (C) Biofilm formation control, (AnfB) Amphotericin B, (GO) geranium oil, (NEG) geranium nanoemulsion, white nanoemulsion (NEB). The values were statistically significant for $p < 0.05$ When the treatments were compared between themselves and with the biofilm formation control (C) for each type of *Candida*. Data are expressed as means ± SD for three independent experiments. The averages followed by the same letter are not statistically different from each other.

3.4 Atomic Force Microscopy (AFM)

The structural and morphological characterization of the biofilm formed on the polyethylene plate was conducted by examining the topographic images obtained by AFM. Atomic force microscopy (AFM) is an advanced technology that offers great advantages in mapping of biological samples such as biofilms and observation of biofilm growth in their natural complex environment (Qin *et al*., 2009; Andre *et al*., 2010).

Antibiofilm activities of the formulations GO, NEG, and NEB were evaluated for different species of *Candida*. The results are shown in **Figure 4** and corroborate the quantification of biofilms by CV and proteins listed above.

NEG antibiofilm had better activity than for GO for *C. albicans*, *C. tropicalis*, and *C. glabrata* according to the surface potential analysis to gauge resolution of the AFM. Frank et al (2015) reported that nanostructured systems tend to increase the interaction between the drug and the tissue, leading to increased penetration of the drug, or modified drug delivery. Samples with *C. krusei* both GO and NEG managed to reduce biofilm formation.



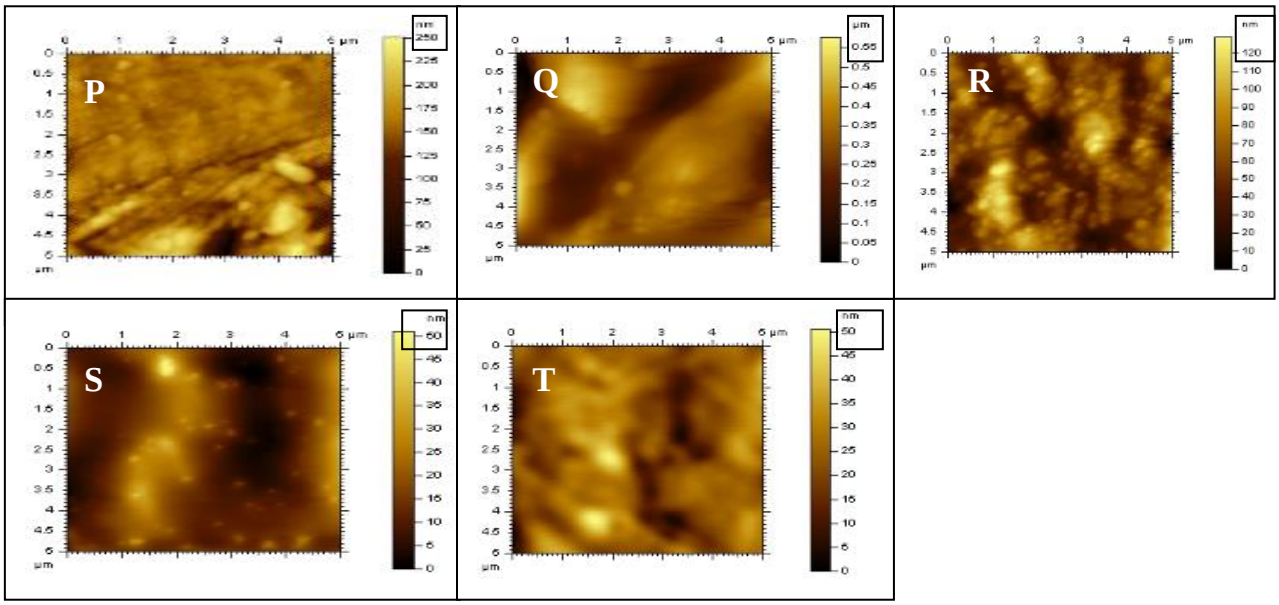


Figure 4: Atomic force microscopy (AFM) for the biofilms of species *Candida* after 72 hours of treatment. (CA) *C. albicans*, (CT) *C. tropicalis*, (CG) *C. glabrata*, (CK) *Candida krusei*. A) CA Negative control, B) CA Amphotericin B, C) CA NEB, D) CA NEG, E) CA GO, F) CT Negative control, G) CT Amphotericin B, H) CT NEB, I) CT NEG, J) CT GO, K) CG Negative control, L) CG Amphotericin B, M) CG NEB, N) CG NEG, O) CG GO, P) CK Negative control, Q) CK Amphotericin B, R) CK NEB, S) CK NEG, T) CK GO.

3.5 Biofilm formation in the catheter and quantification

Polyurethane catheters were used to verify the formation and adhesion of biofilms in this type of surface. **Figure 5** shows the formation of biofilms *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C.krusei* in catheters.

Besides the method for counting by UFC, ATP-bioluminescence method was also used. In both methods it was possible to observe the formation of biofilm in the catheter, having *C. albicans* and *C. tropicalis* a higher amount of formation. The results show that the kinetics of biofilm formation was the same for the applied methods. Thus, due to the fact that ATP-Bioluminescence method is faster and more widely used in hospitals, it was elected for the full quantification of biofilms after treatments.

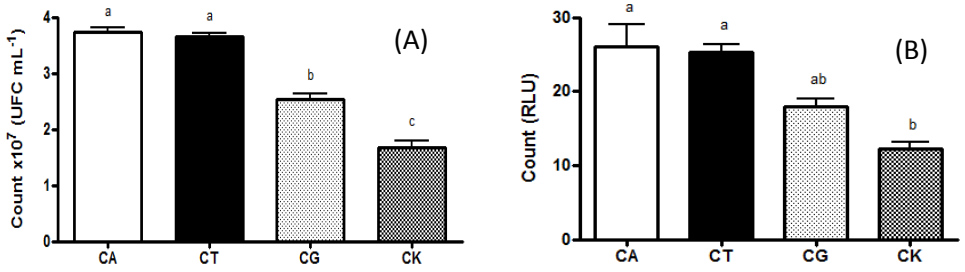


Figure 5: Biofilm formation on polyurethane catheters. (A) Count in UFC ml^{-1}. (B) Count in RLU. *Candida albicans* (CA). *Candida tropicalis* (CT). *Candida glabrata.* (CG) *Candida krusei* (CK). Values were statistically significant at p <0.05 when same letters. Data are expressed as means ± SD at three independent experiments.

The biofilms formed in the catheters were measured as RLU for each species of *Candida* and compared with the control biofilm formation (**Figure 6**). GO did not significantly inhibit the formation of biofilms only in *C. krusei;* for other species, it demonstrated a strong antibiofilm activity. NEG significantly increase GO activity in all species tested when compared to biofilm formation control. When GO was compared with NEG, it was observed a non-significant reduction for *C. albicans*, *C. glabrata*, and *C. krusei*. Only *C. tropicalis* presented significant reduction in the amount of biofilm after treatment with NEG.

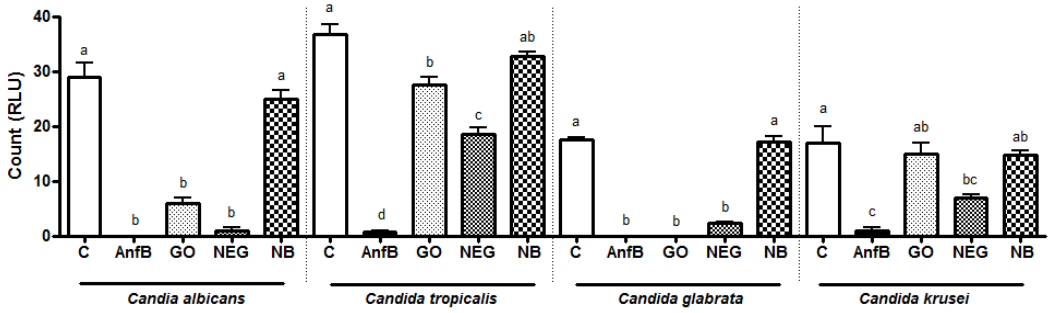


Figure 6: ATP Quantification (RLU) of biofilms on polyurethane catheters. (C) Biofilm formation control. (C) Biofilm formation control, (AnfB) Amphotericin B, (GO) geranium oil, (NEG) geranium nanoemulsion, white nanoemulsion (NEB). The values were statistically significant for p <0.05 when the treatments were compared among themselves and with the biofilm formation control (C) for each type of *Candida*.

Data are expressed as means ± SD for three independent experiments. The averages followed by the same letter are not statistically different from each other.

Besides ATP-bioluminescence method, CV was applied (**Figure 7**). The results demonstrated that GO and NEG significantly decreased the biofilm formation of all species of *Candida* tested for the control of biofilm formation.

NEG has increased antibiofilm activity related to control biofilm formation, confirming the results of quantification by ATP-bioluminescence. Biofilms *C. tropicalis* and *C. krusei* when treated with NEG had a significant decrease in its quantification compared to GO. However, this difference was not observed with *C. albicans* and *C. glabrata.*

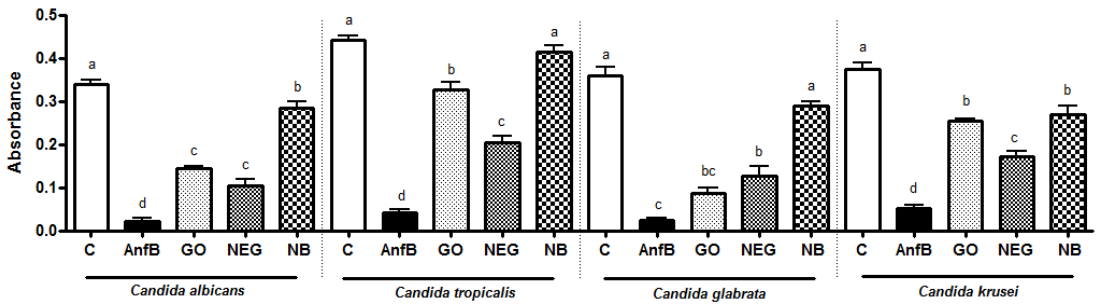


Figure 7: Quantification of biofilm by crystal violet after treatment of polyurethane catheters. (C) biofilm formation control, (AnfB) Amphotericin B, (GO) geranium oil, (NEG) geranium nanoemulsion, white nanoemulsion (NEB). The values were statistically significant for p <0.05 When the treatments were compared between themselves and with the biofilm formation control (C) for each type of *Candida*. Data are expressed as means ± SD for three independent experiments. The averages followed by the same letter are not statistically different from each other.

3.6 Total proteins after treatment catheter

The proteins were quantified after treatment of biofilms with GO, NEG, and NEB (**Figure 8**). The results corroborate the quantification kinetics by CV for *Candida* species. However, a significant reduction on antibiofilm effect of

GO was observed compared to control biofilm forming only *C. glabrata*. The quantification of the proteins to NEG was significantly reduced for *C. albicans, C. glabrata, and C. krusei.* The differences observed between the proteins and quantification CV may be due to the sensitivity of the methods.

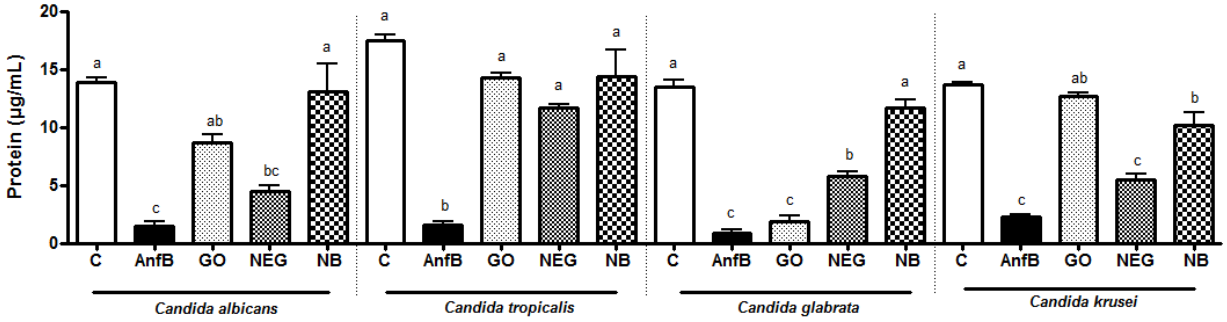


Figure 8: **Quantification of proteins of biofilms in polyurethane catheters applying Bradford.** (C) Biofilm formation control, (AnfB) Amphotericin B, (GO) geranium oil, (NEG) geranium nanoemulsion, white nanoemulsion (NEB). The values were statistically significant for $p < 0.05$ when the treatments were compared among themselves and with the biofilm formation control (C) for each type of *Candida*. Data are expressed as means ± SD for three independent experiments. The averages followed by the same letter are not statistically different from each other.

4. Discussion

GO presented in its constitution two major components: citronellol and geraniol. Studies indicate that they are responsible for the biological activities of the oil (Boukhate *et al.* 2013). NEG used presented nanoscale characteristics and good homogeneity. The use of oil incorporated into this type of formulation is advantageous, since the contact time of formula with the fungi and also the 200 nm particle size may activate the passive transport mechanism across the cell membrane (Donsì *et al*. 2010). The MIC obtained for NEG was similar to GO for *C. albicans*. In a previous study, our group ordered the MIC to nanocapsules containing GO and the results indicated a MIC higher than 149.7 µg ml^{-1} for

some fungi (Giongo *et al*., 2015). Thus, it is evident that NEG showed to be more effective at lower concentrations of the different *Candida* species tested.

Some studies also indicate that the surfactants can increase the microbial inhibition or even reduce it, by binding to phenolic and carboxylic groups (Orafidiya *et al*., 2006; Rodríguez-Rojo *et al*., 2012). However, this study used a nanoemulsion with the same concentrations of surfactants, but without GO in order to control such potential false-positive results.

The formation of biofilms by species of *Candida* has important clinical implications due to increased resistance to antifungal therapy and the ability of the cells within the biofilm resist defenses of the host immune system. Assuming that the ideal would be to prevent the formation of biofilms before treating infections caused by them and the isolated antimicrobial treatment only results in the elimination of planktonic cells, we are evident the importance of developing prevention and control strategies the formation of microbial biofilms (Stewart *et al.* 2004; Simões *et al.* 2009).

In order to quantify biofilm biomass formed by *C. albicans*,*C. tropicalis*,*C. glabrata*, and *C. krusei* on the polyethylene plates and polyurethane catheters, different methods were used. The assay using crystal violet (CV) is one of the most widely used methods to compare and quantify the development of biofilms of different pathogens. The CV dye binds proportionally to the biomass of the biofilm and has the advantages of low cost and a relatively fast technique and adaptable for use, and present with high performance microplates (Li *et al* 2003; Pitts *et al*, 2003).

ATP-Bioluminescence test is a quick technique being widely used in hospitals to determine the contamination on the surface of medical supplies. Oliveira & Canettieri (2010) evaluated the contamination in different types of materials and revealed that there was complete agreement between the counting methodologies plate and ATP-Bioluminescence, corroborating the results obtained in this study.

GO had antibiofilm activity on the plates and catheters, proving its antifungal activity. Alavarce *et al*., (2015) used acrylic plates to verify the adherence of biofilms formed by species of *Candida.* They demonstrated that the use of the extract *Equisetum giganteum* inhibits the formation of biofilm on the surface tested. Other studies show that the action of essential oils in biofilms formed by *C. albicans* may be due to reduced hydrophobicity of the *Candida* cell surface thus reducing its adhesion (Sudjana *et al*, 2012).

When GO was incorporated in nanoemulsions, it significantly decreased the biofilm biomass in some species of *Candida* which are considered extremely pathogenic in the hospital. This increased activity of antibiofilm nanoemulsions can be explained by the reduced diameter of the particle. The smaller the diameter, the greater the antifungal effect (the particle appears to be more able to penetrate the biofilm, causing an interference in the membrane and loss of cell viability) (Seil and Webster, 2012).

The investigation into the use of nanoemulsion as antimicrobial agent was prompted by the known problem of development of antimicrobial resistant strains experienced with the use of existing agents due to the widespread, and sometimes inappropriate, use of antibiotics, disinfectants and antiseptics. Studies on biofilms *C. albicans* demonstrated that the biofilm was able to withstand different concentrations of Terpinen-4-ol. However when lipid nanocarriers associated with Terpinen-4ol were used, the antifungal activity improved suggesting that nanocarriers could circumvent this problem (Sun *et al*, 2012).

Our results for inhibiting the biofilm formation by the action of NEG are in agreement with those previously reported by Myc *et al.*,(2001), where NE caused cell wall rupture of *C. albicans*, resulting in lack of viability. However, there are no reports in the literature on the use of NEG to prevent or reduce the formation of biofilms.

It is known that the main characteristics influencing the development of the biofilm include the adhesion and proliferation (including the development of

hyphae and forming germ tube) the production of extracellular matrix maturation and dispersal (Ramage *et al*, 2009). In addition, pH, amount of inoculum, hydrodynamic forces, characteristic of substrates, and availability of nutrients and oxygen are also involved in biofilm formation (Naves *et al*., 2008). Biofilms are not composed only by microorganisms. The matrix of these microbial communities is water, cells, proteins, products resulting from cell lysis, and EPS (Behlau & Gilmore, 2008). Proteins are components of all living cells and have been successfully used as a measure of cell biomass in biofilms. In this study it was observed that there was a reduction of protein on the plates and catheters, confirming the antibiofilm activity of GO and NEG before different species *Candida* tested.

AFM was used to determine the roughness of the biofilm formed on the plates after treatment with GO, NEB, and NEG. Surface topography of biofilm was characterized by its evaluating surface roughness. Surface roughness of biofilm is an important parameter that is characteristic of growth conditions and has been reported to have an effect on the rate of diffusion of nutrients, thickness of dissolved oxygen boundary layer and mass transfer resistance (Zhang *et al.* 1994). In our study, it was highlighted reduction in the roughness of biofilms treated with GO and NEG, especially for *C. albicans*. The literature reports that bacterial biofilm after treatment with metal nanoparticles synthesized from the extract *Plumbago zeylanica* also had a relative reduction in NM detected by AFM (Salunke *et al*., 2014).

Given the hydrophobic nature of the microbial surface, it is known that to prevent biofilm formation, one should be focused on two main aspects, reducing the presence of microorganisms in the environment and the development of new products with surfaces or special conditions disadvantage the primary membership (Tamura, 2007). In order to avoid the growth of the cells that adhered and colonized, research efforts have focused on two decades in the adsorption of materials or impregnation with antimicrobial agents (Renner & Weibel, 2011).

The physicochemical properties of the surface also exert a strong influence on the adhesion of microorganisms, which will adhere more easily on hydrophobic surfaces such as plastics and rubbers (Rodrigues *et al*., 2009). Considering that many microbial infections are related to the use of catheters, the anti-colonization and anti-adhesion strategies are quite interesting (Tamilvanan *et al*., 2008; Page *et al*., 2009). Developing formulations which can be adsorbed on the surfaces of catheters, such as NEG can become an effective alternative to reduce the adhesion of microorganisms and prevent infections arising from the use of this type of device.

Some micro- and nanoemulsions with antimicrobial properties have been developed and can be effective antibiofilm agents. It has been found, for example, that an o / w microemulsion of ethyl oleate, Tween 80 and n-pentanol is highly effective in the eradication of biofilms *Pseudomonas aeruginosa* which is a common nosocomial pathogen and very difficult to remove, especially when they form biofilms (Teixeira *et al*., 2007). Another pathogen responsible for infections, especially in catheters is *C. albicans.* This species has a greater ability to form biofilms on surfaces including polyurethane surfaces (Shinde *et al*., 2012). The results of this study *C. albicans* show that NEG was efficient and reduced the amount of biofilm in the catheter.

4. Conclusion

Catheters are widely used in medicine, allowing for administration blood, medications, and nutrition. However, their use is associated with a high risk of infections caused by colonization of microorganisms in biofilms. Among these, *Candida* species are responsible for most cases of contamination on these medical devices. These organisms can form highly resistant biofilms in catheters increasing its pathogenicity, which has been difficult to target by solely applying antimicrobials and antifungals. This low success rate in treatment of these infections using antimicrobials is pushing the need for alternative prevention and treatment strategies. The GO showed antifungal activity and NEG potentiated the

antibiofilm effect of oil on species tested. This result was evidenced in polyethylene plates and on the surface of polyurethane catheters. This formulation seems a novel strategy for effectively eliminate biofilm formation on indwelling devices and prevent growth of microorganisms on catheters and thus improve the patient's quality of life and life-span.

5. Acknowledgement

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DISCUSSÃO GERAL

O uso de substâncias originadas de plantas há muito tempo vem sendo realizado para o tratamento de doenças no ser humano. O mercado mundial desse grupo de medicamentos atinge vários bilhões de dólares. Estima-se que 40% dos medicamentos disponíveis na terapêutica foram desenvolvidos de fontes naturais e 25% são oriundos de plantas (NEWMAN, 2003). Os inúmeros trabalhos de pesquisa mostram que este dado é verídico e que essa fonte é muito importante nos estudos de desenvolvimento de novos medicamentos.

Sabe-se que o índice de resistência microbiana é crescente, principalmente no ambiente hospitalar. Os pacientes imunodeprimidos são os que mais sofrem com este problema, pois em sua grande maioria, precisam fazer uso de algum tipo de material médico-hospitalar durante o tratamento. O estudo de novas substâncias com potencial atividade antimicrobiana torna-se bastante relevante neste contexto e as plantas oferecem vantagens, como: grande quantidade de estruturas químicas, possibilidade de utilização como banco de moléculas para ensaios de alta velocidade, economia de tempo e recursos, fonte de pequenas moléculas para alvos moleculares complexos e mais importante, são capazes de serem absorvidas e metabolizadas pelo organismo (BALUNAS *et al*., 2010).

Dentro dessa concepção, os óleos essenciais acabam sendo escolhidos por apresentar propriedades farmacológicas fundamentais como: antifúngica, antibacteriana e anti-helmíntica, etc (BAKKALI *et al*., 2008; SOUZA *et al*., 2008). Em nosso estudo foi utilizado o óleo de *Pelargonium graveolens*, mais conhecido como óleo de gerânio (GO). Seus constituintes majoritários são o citronelol e o geraniol e a concentração dos constituintes no óleo depende principalmente das condições agronômicas.

O GO utilizado neste estudo foi analisado por CG-MS e encontrou-se uma concentração de citronelol na faixa de 17,74% a 31,37% e geraniol na faixa de 10,34% a 14,73%. Os resultados obtidos foram corroborados pela literatura revisada.

Sabe-se que o GO é utilizado popularmente como antisséptico, antidepressivo e anti-hemorrágico. No presente estudo, foram verificadas as

atividades antibacteriana e antifúngica do óleo puro e nanoestruturado. A maioria das plantas possui compostos que são antimicrobianos e que as protegem de microrganismos. A maior ou menor atividade dos óleos vegetais vai depender de sua constituição química (SILVEIRA et al., 2009). Os mecanismos de ação não estão totalmente elucidados, todavia é sugerido que esta atividade se dá pela modificação da estrutura da parede celular do microrganismo. Eles alteram a permeabilidade da membrana citoplasmática pela alteração do gradiente de íons de hidrogênio (H^+) e potássio (K^+). Isso leva a deterioração de alguns dos processos que são essenciais para a sobrevivência da célula. Assim ocorre a perda do controle quimiostático da célula e consequentemente sua morte (DORMAN & DEANS, 2000).

Os resultados demonstraram que o GO puro possui atividade contra *M. abscessos, M. massiliense, M. smegmatis and M. avium*, além das bactérias *S. aureus, Streptococcus, Sthaphylococcus, Listeria monocytogenes, Pseudomonas aeruginosa* e *Salmonella enteritidis.* Quando ele foi incorporado a uma nanocápsula (NC1) e esta foi colocada frente às mesmas bactérias, não se detectou atividade contra *Sthaphylococcus, Pseudomonas aeruginosa* e *Salmonella enteritidis*, entretanto, observou-se uma MIC de 35,9 µg/ml para *Listeria monocytogenes*.

Para avaliação da atividade antifúngica foram testadas diferentes cepas de *Candidas*. O GO teve uma MIC de 8,9 µg/ml para *C. albicans, C. Kefyr, C. dublinienesis* e *C. glabrata.* Como a NC1 apresentou a mesma MIC de 149,7 µg/ml para todas as cepas testadas, foi realizado o teste de macrodiluição por 72 horas. Com este método, foi possível verificar que a NC1 possui a capacidade de reduzir o número de células de *C. albicans, C. Kefyr, C. dublinienesis* e *C. glabrata.* Apesar do resultado ser extremamente importante, a realização do teste de inibição da formação do tubo germinativo mostrou que o GO na concentração de 2x MIC promoveu uma inibição deste fator de virulência da *C. albicans*, entretanto isso não foi observado com a NC1.

As nanocápsulas são formadas por polímeros biodegradáveis e podem aumentar a concentração da substância na região onde os microrganismos se localizam, além de induzir uma liberação lenta e controlada (WEI *et al*., 2009). Essa característica das nanocápsulas pode ser a razão pela qual foi observada a formação do tubo germinativo da *C. albicans*, pois o GO pode ter permanecido no interior da nanoestrutura não estando livre para exercer sua atividade antifúngica no período do teste. Esta hipótese corrobora com estudos sobre liberação *in vitro* relatados na literatura que reportam que o tipo de estrutura e a presença do polímero exercem importante papel no controle de liberação de substâncias (POLETTO *et al*., 2008; FONTANA *et al*., 2009).

Com as mesmas vantagens das nanocápsulas em relação à proteção do óleo, as nanoemulsões por não terem em sua constituição o polímero e serem basicamente formulações óleo em água podem promover a liberação do ativo mais rapidamente (LOVELYN & ATTAMA, 2011). Além disso, possuem a vantagem de diminuir a volatilização do óleo essencial, o que reduz a instabilidade e a perda de material (DANIELLI *et al*., 2015). Levando em consideração estes fatores, foi desenvolvida uma NEG a partir de uma técnica com ultra-turrax. A análise de estabilidade térmica do óleo demonstrou que a temperatura inicial de decomposição é de 32°C, esta informação foi útil para definição da técnica de preparo da formulação. O ultra-turrax torna-se vantajoso no preparo de nanoemulsões contendo óleo essencial, pois não utiliza elevação de temperatura e também possui um menor custo, já que não se faz necessário a utilização de solventes.

As NEG obtidas apresentaram características manométricas e com coloração translúcida. A TEM mostrou que as partículas da NEG são esféricas. O tamanho de partícula obtido foi de 164 nm $\pm$ 3,5, um PDI de 0,25 $\pm$ 0,006, potencial zeta de -10 mV $\pm$ 1,7 e pH de 3,7 + 0,12. A TEM determina a morfologia das nanoemulsões e a partir dela são obtidas imagens de alta resolução de fase dispersa. O índice de polidispersividade indica a qualidade ou a homogeneidade da formulação (LI *et al.,* 2011). Além disto, o resultado do

potencial zeta encontra-se satisfatório, pois, quando a nanoestrutura apresenta uma carga negativa, a manutenção de estabilidade do sistema tende a ser superior com uma menor probabilidade de agregação das partículas e, consequentemente, precipitação das nanoestruturas (SILVA et al, 2006; FRIEDRICH et al, 2008). O pH ácido pode ser devido ao óleo volátil, também ligeiramente ácido em solução. Os resultados obtidos em nosso estudo corroboram com resultados adquiridos por outros pesquisadores (BOUCHEMAL *et al*., 2004; CAPEK, 2004, DANIELLI *et al*., 2015).

Todas as características físico-químicas das formulações, bem como a quantificação de seus constituintes majoritários, foram verificadas durante o período de 90 dias, sob diferentes temperaturas (4 °C, 25 °C e 45 °C). O estudo de estabilidade de uma formulação refere-se à integridade química e física e a capacidade para manter a proteção contra a contaminação microbiológica (SHAKEEL *et al*., 2008). As nanoemulsões são conhecidas por fornecer uma maior estabilidade química às substâncias e devem sofrer alterações insignificantes quando submetidas às diferentes temperaturas (BABOOTA *et al*.,2007, SHAKEEL *et al*., 2008).

Em nosso estudo, as NEG que permaneceram estáveis durante todo o período de teste foram submetidas à temperatura de 4 °C. Estas não apresentaram diferença significativa quanto ao tamanho de partícula, entretanto o PDI, o potencial zeta e o pH sofreram variações significativas no decorrer dos 90 dias. Em contrapartida, as NEG submetidas à temperatura de 45 °C, já no sétimo dia tiveram variações significativas em todos os parâmetros analisados, o que resultou na separação das fases.

A quantificação do citronelol e do geraniol nas NEG demonstrou que o geraniol aparenta ser mais volátil que o citronelol. As formulações submetidas à temperatura de 45 °C apresentaram em pouco tempo uma redução significativa destes constituintes quando comparadas com as outras temperaturas. Este dado é de suma importância, pois são estes os compostos responsáveis pela maior parte das atividades biológicas do GO.

Em diferentes pesquisas são relatados as propriedades biológicas características dos óleos essenciais, tais como a atividade antioxidante (CHOI *et al*, 2002; CAIA *et al*, 2004; FAYED *et al*, 2009; AIDI WANNES *et al*, 2010; BOLIGON *et al*, 2013), analgésica, anti-inflamatória, antifúngica, antimicrobiana (SANTOS *et al*., 2012; SANTOS *et al*., 2014; VAUCHER *et al*., 2015) e atividade antitumoral (SILVA, 2006; FAYED *et al*., 2009). Considerando todas estas propriedades, os óleos têm vantagens importantes, como a volatilidade e composição de baixo peso molecular, o que lhes permite ser rapidamente eliminado pelo corpo (BANDONI & CZEPAK, 2008).

Segundo estudo conduzido por Su e colaboradores (2010), o citronelol e o geraniol são capazes de exercer atividade anti-inflamatória, pois ambos suprimem a produção de NO e PGE de uma forma dependente da dose. Outra atividade relatada para estes constituintes é a ação antioxidante. Sabe-se também que o citronelol possui propriedades anticancerígenas. Estudos relatam que o geraniol promove uma inibição significativa (60-90%) do crescimento de células de tumor do pâncreas (FAYED, 2009).

As atividades antioxidantes do GO e também das NEG foram determinadas pelos métodos de FRAP e DPPH. Em ambos os ensaios, foi demonstrado um efeito dependente da concentração utilizada do GO e da NEG. O GO apesar de ter apresentado uma maior atividade antioxidante quando comparado com a NEG (o que já era esperado) foi mais citotóxico para os linfócitos que a NEG, sinalizando um efeito protetor das mesmas. O ensaio de FRAP demonstrou também que a incorporação do óleo na nanoestrutura não interfere significativamente na atividade antioxidante Este resultado é interessante, pois o FRAP é um ensaio automatizado, que ainda não foi reportado na literatura para GO e NEG.

Avaliou-se também o LDH que tem a função de medir a integridade da membrana plasmática dos linfócitos. Normalmente valores elevados de LDH indicam destruição celular. Verificou-se, novamente, que a produção da enzima

LDH está diretamente ligada ao aumento das concentrações do GO e da NEG. Entretanto, houve uma redução significativa da U/L de LDH quando as amostras foram comparadas ao controle positivo (SDS) e na concentração de 1000 µg/µl a NEG apresentou efeito protetor quando comparada ao GO.

Segundo Prashar e colaboradores (2004) o mecanismo citotóxico dos óleos pode estar associado aos danos na membrana, o que explicaria este aumento na produção de LDH. Estes danos poderiam estar relacionados à capacidade que os óleos essenciais têm de provocar uma despolarização das membranas mitocondriais, afetando diversos canais iônicos, reduzindo o gradiente de pH, além de alterar a fluidez das membranas, permitindo assim a entrada de radicais, citocromo C, proteínas e íons, levando a morte da célula por apoptose e necrose (HAMMER et al, 2002; BAKKALI et al, 2008).

As NEG foram desenvolvidas com o objetivo de serem utilizadas em material médico hospitalar para auxiliar na inibição da formação de biofilmes e consequentemente, diminuir o risco de infecções em pacientes imunodeprimidos. Devido a isso, o teste de citotoxicidade torna-se primordial na avaliação das formulações desenvolvidas. Para completar os testes acima mencionados, realizou-se a atividade hemolítica em eritrócitos humanos. Os resultados indicaram que o GO e as NEG são doses dependentes para esta atividade, ou seja, quando maior a concentração utilizada, maior a lise. Todavia, apenas as concentrações de 100 e 1000 µg/µl do GO causaram atividade hemolítica significativa quando comparadas ao controle de células. Já as concentrações testadas para NEG e para NEB não tiveram a capacidade de lisar os eritrócitos.

Para avaliar o possível stress oxidativo do GO e da NEG em linfócitos, utilizou-se TBARS e os ensaios de CAT. O TBARS reflete a quantidade de formação de malondialdeído, o produto final da peroxidação de ácidos graxos e de peroxidação lipídica da membrana que podem ser induzidos *in vitro* através da ação de oxidantes, provavelmente devido à geração de radicais livres (SIRTORI *et al*., 2005).

Comparando diferentes concentrações do GO com a NEG, os tratamentos dos linfócitos após 72 horas não apresentaram aumento significativo na peroxidação lipídica de estruturas da membrana. Um aumento de TBARS poderia indicar forte peroxidação lipídica causada pela citotoxicidade de NEG ou GO, um fato que não foi verificado. Este resultado corrobora com o obtido no ensaio de LDH.

A enzima de catalase é parte do sistema de enzima da primeira linha de defesa celular contra os danos oxidativos, decompondo-se o peróxido de hidrogênio e de oxigênio antes da sua interação para formar espécies reativas mais prejudiciais tais como o radical hidroxila (EL-MISSIRY *et al*., 2001). Os resultados obtidos estão relacionados aos resultados do TBARS, em que o aumento das concentrações do OG e da NEG é proporcional ao aumento do dano e consequentemente induzem a liberação da catalase. Entretanto, a nanoestrutura demonstra efeito protetor em relação ao óleo puro, o que a torna muito vantajosa.

Dando continuidade aos testes e buscando ampliar a possibilidade de utilização da NEG, estudos *in vitro* a cerca da atividade anti-inflamatória foram realizados com macrófagos RAW 264.7 ATCC TIB-71. Tais ensaios foram realizados sem a presença de fungo e após estimulação com proteínas de *C. albicans.* Na viabilidade celular observou-se uma diminuição significativa das células para a NEG e GO nas concentrações de 1x MIC e 2x MIC. Após as dosagens dos níveis de LDH pode-se evidenciar um aumento significativo no sobrenadante da cultura dos macrófagos para o GO nas concentrações de 1x MIC e 2x MIC. Além disso, foi evidenciado um efeito protetor da NEG nos ensaios de MTT. Também foi realizada a dosagem do óxido nítrico (NOx) nos sobrenadantes dos macrófagos cultivados sem fungo e após a estimulação com as proteínas da *C. albicans*. Observou-se um aumento significativo nos níveis de NOx após tratamento com a NEG 1x MIC e 2x MIC e uma redução significativa com o GO nas mesmas concentrações nas células sem fungos. Quando os macrófagos foram cultivados com as proteínas da *C. albicans* foi observado um aumento significativo após tratamento com a NEG nas concentrações de ½ x

MIC e 2x MIC e novamente houve uma redução significativa do GO nas mesmas concentrações.

Sabe-se que o NO constitui um dos mais importantes marcadores de processos intra e extracelulares, apresentando um papel dúbio, às vezes benéfico e outras vezes prejudicial ao organismo. Constitui um importante mediador citotóxico de células ativadas, estando presentes em macrófagos e sendo capaz de destruir patógenos e células tumorais (LEE *et al*., 2009). Assim, neste estudo pode-se evidenciar que NEG aumentou os níveis de NOx após o tratamento dos macrófagos com proteínas solúveis do fungo, potencializando sua produção com o objetivo de ajudar na destruição de um patógeno agressor. Entretanto, a determinação laboratorial dos níveis de NOx é bastante complexa e a caracterização de ativadores e inibidores específicos da sua síntese constitui um desafio para o entendimento e tratamento das infecções.

As citocinas inflamatórias IL1, IL6, TNF e INF também foram determinadas nos sobrenadantes dos macrófagos após tratamento com a NEG e GO. Os resultados demonstraram que ocorreu um aumento significativo na produção da IL6 e o TNF nos macrófagos com proteínas fúngicas apenas para o tratamento com o GO na concentração de 2x MIC. Já os níveis de IL10 diminuíram significativamente nesta mesma concentração. Quando os dois grupos de macrófagos foram tratados com a NEG os níveis de IL10 aumentaram. Já os níveis de TNF foram diminuídos após tratamento com NEG e GO confirmando uma possível atividade antiflamatória em macrófagos induzidos com proteínas solúveis de *C. albicans.* Os resultados corroboram com os encontrados por Boukhatem *et al.* (2013), que relataram as atividades anti-inflamatórias do óleo essencial de gerânio. Entretanto não há na literatura relatos desta atividade para NEG.

As caspases também foram determinadas. Observou-se que os níveis de caspase-3 foram significativamente reduzidos após tratamento com NEG e GO nos macrófagos induzidos com proteína fúngica e os níveis de caspase 8

mantiveram-se inalterados, indicando não existir uma influência na ativação de apoptose nestas células. Os níveis de expressão dos genes IL-2, COX-2, e iNOS também foram determinados. Foi possível confirmar o efeito de atividade anti-inflamatória do GO e NEG, devido à diminuição significativa da expressão gênica de COX-2 em macrófagos induzido por proteínas solúveis de *C. albicans.*

A última etapa deste trabalho foi a verificação da atividade antibiofilme do GO e da NEG em superfícies de polietileno e poliuretano. As principais espécies de *Candida* (*C. albicans, C. tropicalis, C. glabrata e C. krusei)* responsáveis pela formação de biofilmes em ambiente hospitalar foram utilizadas nos ensaios. O biofilme, que está em muitos casos aderido a instrumentos da área médica, como cateteres, próteses e stents, possuem alguns mecanismos patogênicos que justificam sua gravidade.

Devido a isso, inúmeras pesquisas estão sendo realizadas com a finalidade de diminuir a adesão microbiana às superfícies e aumentar o poder de penetração dos ativos antimicrobianos nos biofilmes. Pensando nisso, este trabalho objetivou verificar se o GO e as NEG na concentração de 1 x MIC conseguiriam exercer esta atividade na superfície de um cateter.

Primeiramente, realizou-se a formação dos biofilmes das espécies de *Candida* em placas de polietileno. A *C. albicans* foi responsável pela maior formação de biofilmes, a quantificação foi realizada a partir de contagem total de colônias (UFC/ml). Este resultado vai de encontro com outros relatados na literatura, em que a *C. albicans* é citada como a mais preocupante e principal causadora de formação de biofilmes. As concentrações de 1x MIC do GO, NEG e NEB foram utilizadas a fim de verificar a influência das formulações na formação do biofilme nas placas. A quantificação foi avaliada através da utilização do ensaio de CV, proteínas totais e AFM. O GO inibiu significativamente a formação dos biofilmes das *C. albicans, C. tropicalis, C. glabrata e C. Krusei* quando comparado ao controle de formação de biofilme de cada espécie. A NEG demonstrou atividade antibiofilme em todas as espécies

testadas, pois a quantificação por CV demonstrou uma diminuição na formação dos biofilmes em relação aos seus respectivos controles de formação de biofilme. Quando o GO foi confrontado com a NEG, foi possível observar, que para *C. albicans* e *C. krusei* a nanoemulsão diminuiu significativamente a quantificação dos biofilmes. A quantidade de proteínas foi reduzida significativamente na presença do GO e das NEG em todas as espécies testadas. Este resultado vai de encontro com o encontrado no ensaio de CV.

Além destes dois ensaios, foi realizado a AFM para verificação da rugosidade dos biofilmes formados nas placas. Os resultados evidenciaram uma redução na rugosidade dos biofilmes tratados com o GO e a NEG, principalmente para *C. albicans.*

Para completar o estudo foram utilizados cateteres de poliuretano com a finalidade de verificar a formação e adesão dos biofilmes neste tipo de superfície. Estes cateteres são amplamente utilizados nos hospitais, principalmente em pacientes muito debilitados. Além do método de contagem por UFC, utilizou-se o método de ATP-Bioluminescência, que é um método considerado rápido e indicado para constatação de contaminação nas superfícies de dispositivos médicos. Em ambos os métodos foi possível observar a formação do biofilme no cateter, sendo que *C. albicans* e *C. tropicalis* apresentaram uma maior quantidade de formação. Sabe-se que o índice de infecção hospitalar em decorrência da formação de biofilmes por estas espécies de *Candida* em cateteres é extremamente alto (VIALE, P.; STEFANI, 2006).

Através das quantificações feitas por CV, por ATP-Bioluminescência e por proteínas totais para verificação da atividade antibiofilme do GO e da NEG na superfície dos cateteres, foi possível observar que ambos apresentaram atividade antibiofilme. A NEG demonstrou aumentar a atividade antibiofilme em relação ao controle de formação do biofilme e, em relação ao GO, em algumas espécies de *Candida*. Este aumento da atividade antibiofilme das nanoemulsões pode ser explicado pelo diâmetro reduzido da partícula. Quanto

menor o diâmetro, maior o efeito antifúngico, visto que a partícula possui maior capacidade de penetração no biofilme, causando uma interferência na membrana e perda da viabilidade celular (SEIL e WEBSTER, 2012). A natureza da NEG também pode ter influenciado na sua maior atividade, pois é conhecido que os microrganismos irão se aderir mais facilmente em superfícies hidrofóbicas.

Este trabalho evidenciou que o GO na forma livre e principalmente quando incorporado a nanoemulsões, pode ser uma alternativa se adsorvido na superfície dos cateteres para combater a formação de biofilmes. Esta estratégia se mostra segura, a partir dos resultados obtidos nos ensaios de citotoxicidade, atividade hemolítica e atividade antinflamatória previamente realizados. É fundamental que as pesquisas avancem nesta área, para que ocorra uma diminuição das taxas de morbidade e mortalidade causadas por processos infecciosos decorrentes da formação de biofilmes em dispositivos hospitalares.

CONCLUSÕES

- O óleo de *Pelargonium graveolens* apresentou como constituintes majoritários o citronelol e o geraniol.

- Neste trabalho, foi realizada uma proposta de obtenção de suspensões de nanocápsulas poliméricas (NC1) e nanoemulsões (NEG) contendo como ingrediente ativo o óleo essencial de *Pelargonium graveolens.* As formulações desenvolvidas apresentaram características físico-químicas satisfatórias.

- O GO e a NC1 apresentaram atividade antimicrobiana frente à algumas espécies de bactérias e fungos. Entretanto a NC1 não mostrou-se efetiva na inibição do tubo germinativo para *C. albicans.*

- As NEG desenvolvidas foram avaliadas durante 90 dias. Foi possível observar que a técnica empregada utilizando ultra-turrax protegeu o óleo contra a volatilização. Entretanto, no decorrer do teste a quantificação dos constituintes majoritários demonstrou uma diminuição significativa nas suas concentrações.

- A NEG mantida a 4°C apresentou durante os 90 dias estabilidade físico-química.

- A atividade antioxidante por DPPH e FRAP do GO e da NEG demonstraram que o GO possui poder antioxidante, entretanto, este fato não foi observado com as NEG, pois estas apresentaram ação antioxidante, somente nas maiores concentrações testadas.

- A citotoxicidade das amostras foi realizada através dos ensaios de MTT e LDH. Os resultados nos permitem afirmar que a NEB, NEG e o GO nas diferentes concentrações não foram citotóxicos aos linfócitos e que as concentrações das amostras foram diretamente proporcionais ao aumento dos níveis de LDH.

- A análise da atividade hemolítica demonstrou que nem a NEB, nem a NEG nas diferentes concentrações possuem a capacidade de lisar os eritrócitos.

- Os ensaios de *stress* oxidativo demonstraram que os níveis de TBARS e a liberação da CAT foram superiores no GO. A NEG mostrou um efeito de proteção quando os resultados foram confrontados ao do GO.

- Após a avaliação da atividade anti-inflamatória em macrófagos murinos induzidos por proteínas fúngicas de *C. albicans* foi possível observar que a NEG exerceu um efeito inibidor sobre a inflamação e doses não tóxicas para os macrófagos.

- A NEG foi capaz de reduzir os níveis de IL1, TNF-α e também INF nos macrófagos induzidos com as proteínas de *C. albicans*.

- O GO e a NEG nas concentrações testadas indicam possivelmente uma inibição da apoptose dos macrófagos, uma vez que os níveis de caspase-3 diminuíram e os de caspases-8 não se alteraram.

- Após análise da expressão gênica, observou-se que a NEG foi capaz de diminuir a expressão de COX e de iNOS em determinadas concentrações.

- A determinação da atividade antifúngica da NEG demonstrou uma MIC de 3.64 μg ml^{-1} e 7.24 μg ml^{-1}para *C. albicans* e *C. tropicalis*, respectivamente.

- Na superfície das placas de polietileno, o GO e a NEG inibiram significativamente a formação dos biofilmes de todas as espécies testadas. Contudo, a NEG demonstrou possuir uma melhor atividade antibiofilme do que o GO para *C. albicans, C. tropicalis* e *C. glabrata*, conforme a análise de potencial de superfície por AFM.

- A análise da formação de biofilme nas superfícies dos cateteres de poliuretano por UFC e ATP-Bioluminescência, demonstrou resultados similares. Em ambos os métodos, a formação do biofilme no cateter, ocorreu em maior quantidade para *C. albicans* e *C. tropicalis.*

- A NEG demonstrou aumentar significativamente a atividade antibiofilme do GO em todas as espécies testadas quando comparada ao controle de formação do biofilme nos cateteres.

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