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ATIVIDADES ANTIMICROBIANA, CITOTÓXICA E GENOTÓXICA DO ÓLEO ESSENCIAL DE *Ocimum basilicum* (LAMIACEAE) E DO LINALOL

João Pessoa

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Tese apresentada ao Programa de Pós-graduação em Produtos Naturais e Sintéticos Bioativos do Centro de Ciências da Saúde da Universidade Federal da Paraíba, como parte dos requisitos para a obtenção do título de DOUTOR EM PRODUTOS NATURAIS E SINTÉTICOS BIOATIVOS. Área de concentração: FARMACOLOGIA

Orientador: Profª. Draª. Edeltrudes de Oliveira Lima

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"Sonhe com aquilo que você quer ser,
porque você possui apenas uma vida
e nela só se tem uma chance
de fazer aquilo que quer.

Tenha felicidade bastante para fazê-la doce.
Dificuldades para fazê-la forte.
Tristeza para fazê-la humana.
E esperança suficiente para fazê-la feliz.

As pessoas mais felizes não têm as melhores coisas.
Elas sabem fazer o melhor das oportunidades
que aparecem em seus caminhos.

A felicidade aparece para aqueles que choram.
Para aqueles que se machucam
Para aqueles que buscam e tentam sempre.
E para aqueles que reconhecem a
importância das pessoas que passaram por
suas vidas."

Clarice Lispector

RESUMO

SILVA, V.A. Atividades antimicrobiana, citotóxica e genotóxica do óleo essencial de *Ocimum basilicum* (Lamiaceae) e do linalol.2015. Tese (Doutorado em Produtos Naturais e Sintéticos Bioativos, área de concentração: Farmacologia) CCS/UFPB, João Pessoa.

Staphylococcus aureus e *Pseudomonas aeruginosa* são micro-organismos de grande importância clínica, pois estão entre as espécies bacterianas com maior poder de causar infecções e que apresentam grande resistência aos antibióticos. Sabendo que bactérias resistentes a múltiplas drogas representam um desafio para o tratamento de infecções, é necessário encontrar novas substâncias que sejam eficazes no combate a estes micro-organismos. *Ocimum basilicum* L.(Lamiaceae) é conhecida popularmente como manjericão e faz parte de um grupo de plantas medicinais, aromáticas e condimentares de grande valor econômico. Este trabalho teve como objetivo avaliar a composição química de *O. basilicum* e determinar a atividade antibacteriana, citotóxica e genotóxica do óleo essencial e do seu composto majoritário. Sua composição química foi determinada por cromatografia gasosa acoplada a espectrometria de massa e a atividade antibacteriana dos compostos foram avaliadas pela determinação da concentração inibitória mínima e concentração bactericida mínima pela técnica de microdiluição. A cinética de morte microbiana e o estudo da associação dos compostos com antibióticos padrões também foram analisados. Para o estudo de citotoxicidade foi realizado o teste de hemólise em eritrócitos humanos e para a genotoxicidade o teste de micronúcleo em roedores. Entre os fitoconstituintes, o monoterpeno linalol (55,2%) apresenta-se como o majoritário. Os experimentos de atividade antibacteriana mostraram que o óleo essencial de *O. basilicum* e o linalol apresentaram atividade antibacteriana contra cepas de *S. aureus* variando entre 1024 a 512 μg/mL e 1024 a 32 μg/mL, respectivamente. Já para cepas de *P. aeruginosa* a concentração inibitória mínima do óleo foi de 1024 μg/mL, sendo algumas cepas resistente, e para o linalol variou de 1024 a 32 μg/mL. A atividade antibacteriana foi caracterizada como bactericida para as cepas de *S. aureus* na concentração dos compostos de CIMx4 e após 8h de contato. O estudo de associação dos compostos com antibióticos padrões mostrou que para as cepas de *S. aureus* a associação do óleo essencial ou do linalol com o imipenem apresentou efeito sinérgico. Já para a ciprofloxacina, a associação do óleo mostrou efeito antagonista e do linalol efeito aditivo. Em relação as cepas de *P. aeruginosa* a associação do óleo ou do linalol com o imipenem apresentou efeito sinérgico e com a ciprofloxacina a relação foi indiferente. O óleo essencial de *O. basilicum* e o linalol apresentaram baixa citotoxicidade. Estes dados foram confirmados através da análise da citotoxicidade frente a eritrócitos, que revelou valores de hemólise abaixo de 10 % para o tipo sanguíneo testado. A análise do potencial genotóxico dos compostos revelou que estes não foram capazes de causar danos no DNA das células do sangue periférico dos animais tratados. Em conclusão, estes resultados sugerem que o óleo essencial do *O. basilicum* e o linalol apresentam efeito antimicrobiano, sejam isolados ou em associação com antibióticos padrões, e que estes compostos possuem baixa citotoxicidade e genotoxicidade.

Palavras-chave: *Ocimum basilicum*, linalol, sinergismo, antimicrobiana, citotoxicidade, genotoxicidade.

ABSTRACT

SILVA, V.A. Atividades antimicrobiana, citotóxica e genotóxica do óleo essencial de *Ocimum basilicum* (Lamiaceae) e do linalol. 2015. Tese (Doutorado em Produtos Naturais e Sintéticos Bioativos, área de concentração: Farmacologia) CCS/UFPB, João Pessoa.

Staphylococcus aureus and *Pseudomonas aeruginosa* microorganisms are of great clinical importance because they are among the bacterial species with greater power to cause infections and which have great resistance to antibiotics. Knowing that multiple drug resistant bacteria pose a challenge for the treatment of infections, it is necessary to find new substances that are effective to combat these microorganisms. *Ocimum basilicum* L. (Lamiaceae) is popularly known as basil and is part of a group of medicinal plants, aromatic and culinary of great economic value. This study aimed to evaluate the chemical composition of *O. basilicum* and determine the antibacterial activity, cytotoxic and genotoxic of essential oil and its major compound. Its chemical composition was determined by gas chromatography and the antibacterial activity of the compounds was evaluated by determining the minimum inhibitory concentration and bactericidal inhibitory concentration by microdiluition technique, the kinetics of microbial death and those compounds association study with antibiotics standards were also analyzed. For the study of cytotoxicity was performed hemolysis test on human erythrocytes and genotoxicity the micronucleus test in rodents. Among phytochemicals, the monoterpene linalool (55.2 %) is presented as the main found. The antibacterial activity of experiments showed that the essential oil of *O. basilicum* and linalool showed antibacterial activity against *S. aureus* strains ranging from 1024 to512 µg/mL and 1024 to 32 µg/mL, respectively. As for *P. aeruginosa* strains the minimal inhibitory concentration of the oil was 1024 µg/mL, and some resistant strains, and linalool ranged from 32 to 1024 µg/mL. The antibacterial activity was characterized as a bactericide to the strains of *S. aureus* in the concentration of the compounds of MICx4 and after 8 hours of contact. The compounds of association study showed patterns with antibiotics for *S. aureus* strains the association of essential oil or linalool with imipenem showed a synergistic effect. As for ciprofloxacin, oil association showed antagonistic effect and linalool additive effect. Regarding the strains of *P. aeruginosa* oil pool or linalool with imipenem showed synergistic effect and with ciprofloxacin the relationship was indifferent. The essential oil *O. basilicum* and linalool showed low cytotoxicity. These data were confirmed by analyzing the cytotoxicity against erythrocytes, which showed hemolysis values below 10 % for blood type test. The analysis of the genotoxic potential of compounds revealed that they were not capable of causing DNA damage in the cells of peripheral blood from treated animals. In conclusion, these results suggest that the essential oil *O. basilicum* and linalool present antimicrobial effect, whether alone or in combination with antibiotics patterns, and that these compounds have low cytotoxicity and genotoxicity.

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Cytotoxic activity of *Ocimum basilicum* essential oil and the monoterpene linalool tested with human erythrocyte hemolysis

Assessment of genotoxic effect of *Ocimum basilicum* L. and Linalool

LISTA DE ABREVIATURAS

ATCC - American Type Culture Collection

CBM - Concentração Bactericida Mínima

CIM - Concentração Inibitória Mínima

CG-EM – Cromatografia gasosa acoplada a espectrometria de massa

CIF – Concentração Inibitória Fracionada

UFC - Unidades Formadoras de Colônias

OMS- Organização Mundial de Saúde

IRAS- Infecções Relacionadas à Assistência à Saúde

ESBL- Enzimas Betalactamases de Espectro Estendido

CIF- Concentração Inibitória Fracionada

ATCC- American Type Culture Collection

BHI- Brain Heart Infusion

CEP- Comitê de Ética e Pesquisa

SUMÁRIO

Introdução

1. INTRODUÇÃO

Apesar da grande diversidade de estruturas químicas e diferentes mecanismos de ação dos antibacterianos, o tratamento de infecções causadas por bactérias resistentes, principalmente *Staphylococcus aureus* e *Pseudomonas aeruginosa*, tem sido cada vez mais difícil (NETTEY et al., 2007; WARDAL et al., 2010). Não há, atualmente, um único antibacteriano em uso clínico, contra o qual não exista, pelo menos, uma cepa bacteriana a ele resistente. Desta forma, o sucesso no combate às infecções bacterianas e o controle sobre o aparecimento de bactérias resistentes é dependente do emprego criterioso dos antibacterianos e da descoberta de novas moléculas que possam ser disponibilizadas para uso em hospitais e na comunidade, o que tem levado de 10 a 15 anos para ocorrer (ERSON, 2005; ROLAIN e RAULT 2005; SEPUTIENE et al., 2010; CLANCY et al., 2010).

Paralelamente, a busca de antimicrobianos de origem natural que apresentem atividade sobre grande espectro de micro-organismo e que possam ser usados como alternativa aos antibióticos convencionais ou em combinação com os mesmos tem despertado o interesse da classe científica, sobretudo nas moléculas de origem vegetal, já que as plantas possuem grande potencial em sintetizar substâncias químicas com estruturas moleculares diversificadas muito superiores àquelas derivadas de produtos sintéticos, como sistema de defesa contra agentes patogênicos (RODRIGUES et al., 1997, PRADEEPA et al., 2014).

Entretanto, um dos principais problemas da utilização de produtos de origem vegetal é a crença de que eles são isentos de toxicidade. O uso milenar de plantas medicinais mostrou, ao longo dos anos, que determinadas plantas apresentam substâncias potencialmente perigosas, dentre elas, alcaloides pirrolizidínicos, antraquinonas e lactonas sesquiterpênicas. Por isso, o balanço entre a atividade biológica versus a toxicidade de um determinado produto natural é um parâmetro fundamental para verificar sua aplicação farmacológica (VEIGA JÚNIOR; PINTO; MACIEL, 2005).

Diante disso, a realização de estudos que investiguem além das atividades antibacterianas se faz necessária. As atividades citotóxicas e genotóxicas de compostos naturais mostram-se importante a fim de se garantir uma maior segurança do uso desses produtos pela população (SÁVIO et al., 2013).

Pensando nisso, o gênero *Ocimum,* da familia Lamiaceae, é uma importante fonte de óleos essenciais, tendo uso na medicina popular em vários países no tratamento de diversas doenças (VIEIRA; SIMON, 2000). *Ocimum basilicum* L., popularmente conhecido como manjericão, é tanto utilizado na indústria culinária quanto fitoterápica e na medicina tradicional, devido ao teor e composição do seu óleo essencial (SILVA et al., 2005). Diversos estudos mostram que o óleo essencial de *O. basilicum* possui como principais constituintes o linalol, eugenol e o geraniol, os quais, por sua vez, apresentam as mais variadas atividades farmacológicas, tais como: bactericida, fungicida, antiparasitária e antinociceptiva (OZCAN; CHALCHAT, 2002; KÉITA et al., 2001; GOVIN et al., 2000; VENANCIO, 2006) .

Assim, sabendo da importância clínica e da alta incidência de micro-organismos resistentes a antibióticos e do risco que o uso de produtos naturais sem estudos prévios de sua toxicidade representa para a população, estudos da atividade antibacteriana e da toxicidade de novos compostos são de fundamental importância para o desenvolvimento de novos fármacos. Baseado nesta afirmativa, esse trabalho se propôs a determinar a atividade antibacteriana, citotóxica e genotóxica do óleo essencial do *O. basilicum* e do seu composto majoritário, o monoterpeno linalol.

Referencial Teórico

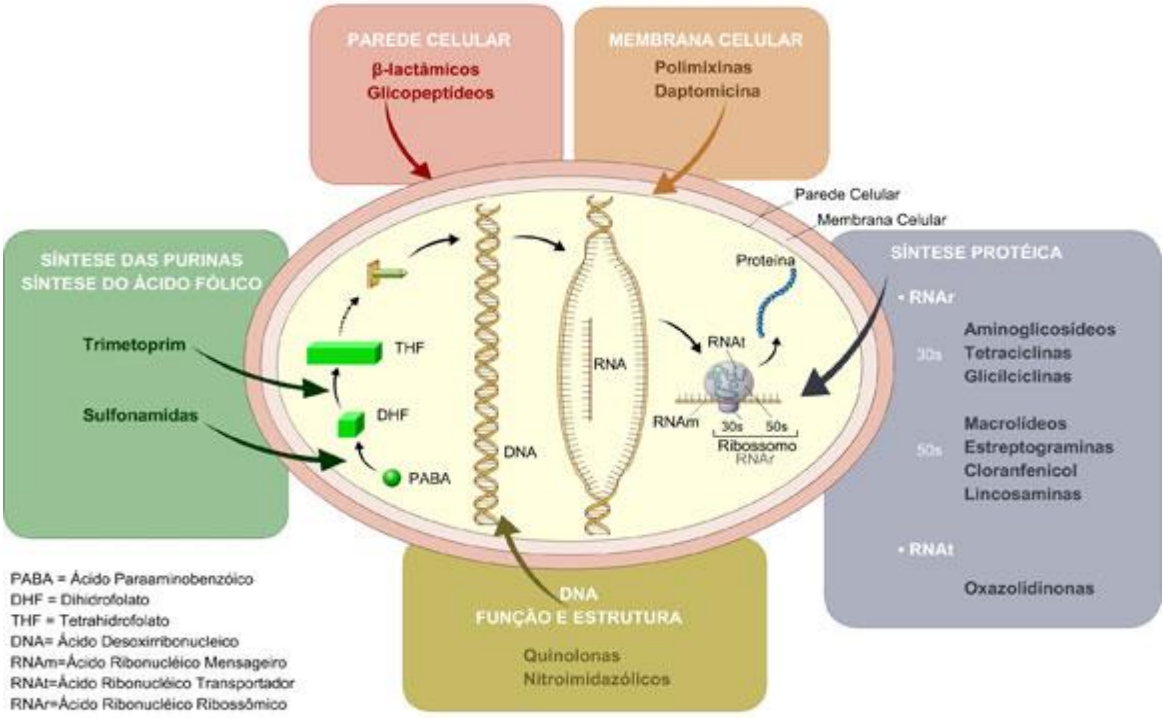
2. REFERENCIAL TEÓRICO

2.1 Antibióticos e Mecanismos de Resistência Bacteriana

A introdução dos agentes antimicrobianos na prática clínica representou um dos grandes avanços na medicina para o tratamento dos mais diversos tipos de doenças infecciosas. Os antibióticos podem apresentar duas funções distintas, a inibição do crescimento bacteriano através da ação bacteriostática, e a destruição de uma população bacteriana, por uma ação bactericida. A ação bacteriostática impede o crescimento das bactérias, mantendo o mesmo na fase estacionária (PANKEY; SABATH, 2013). Um antibiótico bactericida atua em processos vitais para a célula levando à morte celular (GOODMAN; GILMAN'S, 2008; KATZUNG, 2007; LAGO, 2011).

Estes efeitos ocorrem através da interferência sobre as vias metabólicas desses agentes infecciosos que podem alterar desde a permeabilidade (membrana externa) até os processos de síntese (parede celular, ácido fólico, DNA, RNA e proteínas) dessas bactérias (Figura 1) (CHOPRA et al., 2002; PAGES 2004; FLUHR et al., 2010).

Figura 1: Esquema representativo dos diversos mecanismos de ação dos antibióticos. (ANVISA, 2007)



Apesar dos excepcionais avanços médicos no desenvolvimento dos antibióticos, as infecções bacterianas continuam a ser uma importante preocupação na saúde pública devido ao aumento do surgimento de cepas resistentes e os aumentos correspondentes em relação aos custos na saúde e as taxas de mortalidade em decorrência da resistência bacteriana (GISKE et al., 2008).

No contexto da resistência aos antibióticos, os organismos procariotas podem apresentar um de três fenótipos fundamentais: resistência intrínseca, resistência adquirida ou susceptibilidade. A resistência intrínseca é a resistência natural exibida por todos os exemplares de determinada espécie. A título de exemplo, os organismos do gênero *Enterobacter* são naturalmente resistentes à cefoxitina, fenótipo que surge devido à produção de uma β-lactamase AmpC cromossômica (HONORE et al., 1986, CAVALLO et al., 2008).

A resistência adquirida aos antibióticos, por sua vez, pode resultar da mutação de genes reguladores ou estruturais, da aquisição de genes de resistência veiculados por elementos genéticos móveis ou da combinação de ambos os mecanismos. O fenótipo resultante da resistência adquirida não irá estar presente em todos os indivíduos da mesma espécie, existirá apenas nos indivíduos de uma linhagem bacteriana que derive de um organismo susceptível. A aquisição de genes de resistência faz-se, muitas vezes, através de elementos móveis, tais como plasmídeos ou transposons. Os genes que codificam β-lactamases surgem como exemplo de genes que são, muitos deles, disseminados por plasmídeos, os quais podem ser facilmente adquiridos por diversas bactérias patogênicas (transferência horizontal). Naturalmente, a susceptibilidade aos antibióticos resulta da ausência total de mecanismos de resistência que possibilitem a sobrevivência das bactérias na presença de determinados compostos (HARBOTTLE et al., 2006)

Apesar destes mecanismos variarem de patógeno para patógeno, a resistência é causada por alguns fatores básicos como: inativação do antibiótico por alterações químicas, geralmente promovidas por enzimas bacterianas (WRIGHT, 2005); modificação do alvo que leva à perda de sensibilidade ao antibiótico; mudanças na bomba de efluxo e permeabilidade externa da membrana que promovem a redução da concentração do antibiótico no interior da célula sem sua modificação química (ALLINGTON, 2001); transmissão do alvo – algumas bactérias se tornam insensíveis a alguns antibióticos porque são capazes de transmitir a inativação de uma determinada enzima, ou seja, os antibióticos com mecanismos de ação que envolve inibição enzimática tornam-se inativos por não terem o alvo para atuar.

Outro fator que contribui para tornar um antibiótico menos eficiente é a sua utilização indiscriminada e incorreta, o que vem a favorecer o surgimento de micro-organismos resistentes. Atualmente, algumas classes de micro-organismos representam extrema preocupação para a saúde pública por serem resistentes a múltiplas drogas. Dentre os que mais provocam mortes no mundo estão: *Staphylococcus aureus* meticilina-resistente (MRSA), *Staphylococcus aureus* vancomicina-resistente (VRSA), *Escherichia coli*, *Klebsiella pneumoniae* e *Pseudomonas aeruginosa*. Os processos infecciosos causados por estas classes de micro-organismos geralmente estão associados com alta letalidade e altos custos de tratamento (DEMAIN; SANCHEZ, 2009; NISHAMINY, 2006).

Em pleno século XXI, a resistência bacteriana é um desafio que se mostra ainda mais crítico, face ao crescente aparecimento de cepas bacterianas multirresistentes e troca de resistência entre as diferentes espécies de bactérias (Ex.: *E. faecalis, M. tuberculosis, N. gonorrhoeae, P. pneumoniae* e *S. aureus*) (MIN et al., 2007; ANDERSSON et al., 2010). Esse tema constitui um problema mundial de saúde pública, destacando-se como alvo para controle entre estratégias globais voltadas para garantir um cuidado seguro. Em 2011, a resistência antimicrobiana foi tema do Dia Mundial de Saúde, proposto pela Organização Mundial de saúde (OMS), chamando atenção para o desafio da implementação de ações imediatas de controle da disseminação destes micro-organismos, visando minimizar a progressiva limitação de opções terapêuticas para tratamento destes casos (OMS, 2011).

Com isso, fica evidente que o fenômeno da resistência é, de fato, algo muito preocupante uma vez que existem cepas resistentes a quase todos os antibióticos conhecidos atualmente e, neste sentido, há uma forte necessidade de se descobrir novas substâncias que não só tenham bom espectro de atividade, mas que possuam novos mecanismos de ação (NISHAMINY, 2006).

2.2 *Staphylococcus aureus e Pseudomonas aeruginosa*

Os *Staphylococcus aureus* são cocos Gram-positivos imóveis, não formadores de esporos, pertencentes à família *Micrococaceae*, que podem se apresentarem isolados, aos pares, tétrades, em cadeias curtas, porém, aparecem predominantemente agrupados em cachos irregulares, semelhantes a cachos de uva. São aeróbios e anaeróbios facultativos, com maior crescimento sob condições aeróbias, quando então produzem a catalase. Estes micro-organismos podem se

desenvolver entre 15 e 45ºC. Crescem em meio simples sem inibidores (JAWETZ et al., 2000; MURRAY et al., 2006; KONEMAN et al., 2008).

Cerca de 50% a 87% das infecções relacionadas à assistência à saúde (IRAS) têm como agente responsável *Staphylococcus aureus*, sendo que em 16% a 43% dos casos os pacientes evoluem para o óbito em função do amplo espectro de resistência deste micro-organismo (METAN, et al., 2005; SULLER; RUSSEL, 2000). Este micro-organismo é um dos patógenos mais isolados tanto em infecções no ambiente hospitalar quanto na comunidade e representa um grande problema para os sistemas de saúde pública devido à facilidade de adquirir resistência aos antimicrobianos utilizados. É responsável por uma grande variedade de infecções, atingindo desde tecidos superficiais até os mais profundos onde penetram através do rompimento das barreiras naturais, sendo assim associados a doenças de pele e tecidos moles, a infecções graves como síndrome do choque tóxico e sepse que podem ser fatais. Com isso, destaca-se por sua patogenicidade e alta frequência, causando doenças tanto em indivíduos imunocomprometidos quanto em sadios (LI et al., 2012; ADHIKARI et al., 2012; OTTO, 2010).

Pseudomonas aeruginosa é um organismo Gram-negativo aeróbio e ubíquo, que dificilmente causa doenças em pessoas saudáveis (HANCOCK; SPEERT, 2000), porém, assim como *S. aureus*, é uma das principais espécies bacterianas que ocasionam infecção em pacientes hospitalizados (GOLDBERG, 2010). Segundo Hauser e Ozer (2011), as principais doenças clínicas relacionadas à *P. aeruginosa* são: Infecções oculares, otológicas, respiratórias (acometendo também pacientes portadores de fibrose cística), do trato urinário, sanguíneas e de peles e tecidos moles (incluindo as feridas de pacientes com queimaduras).

Na América Latina *Pseudomonas* spp. é responsável por 7,5% das infecções de corrente sanguínea, por 31,2% dos casos de pneumonia e, por 13,8% das infecções da pele e dos tecidos moles (GALES et al., 2012).

Sua importância se deve pela expressão de múltiplos mecanismos de resistência, dificultando a ação de antibacterianos, ocasionando elevados índices de morbidade e mortalidade (GALES et al., 2004; LAMBERT et al., 2011; PELLEGRINO et al., 2002; POOLE, 2011). Dentre os múltiplos mecanismos de resistência ressaltam-se: enzimas modificadoras de aminoglicosídeos, super expressão de bombas de efluxo, perda de porina, alterações no sítio alvo (KANJ;KANAFANI,

2011; MULLER et al., 2011; POOLE, 2011; STRATEVA; YORDANOV, 2009; ZAVASCKI et al., 2010). Além disso, *P. aeruginosa* pode ser intrinsicamente resistente a diversos antimicrobianos devido à expressão constitutiva de genes codificadores de bombas de efluxo para os mesmos (NEVES et al., 2011; POOLE, 2011; STRATEVA, YORDANOV, 2009).

Tam et al (2014) mostraram que *S. aureus* e *P. aeruginosa* estão entre os micro-organismos mais associados a infecções de pacientes que se encontram em unidade de tratamento intensivo nos hospitais em Shangai.

Assim, as infecções causadas por estes patógenos são um desafio clínico, uma vez que *S. aureus* apresenta alta capacidade de desenvolver resistência devido maior adaptação sob pressão seletiva do uso intenso de antimicrobianos (WEIGEL et al., 2007) e *P. aeruginosa* é caracterizada pela suscetibilidade natural a um número limitado de agentes antimicrobianos (NOUÉR et al., 2005).

2.3 Produtos Naturais como fonte de medicamentos

Nos últimos anos, têm sido feitos esforços consideráveis para controlar a disseminação de agentes patogênicos com várias estratégias, incluindo o uso de substâncias alternativas como busca de novos medicamentos, dentre eles os antibacterianos (JONES et al., 1998; HAMILTON - MILLER, 2004).

Os produtos naturais tem sido a principal fonte de busca de novos antibacterianos. Nos últimos 80 anos, numerosas classes de antibacterianos de origem natural foram descobertos, logo estes produtos e seus análogos continuam a desempenhar um papel importante na medicina, sendo responsável por dois terços das novas terapias antibacterianas aprovadas entre 1980 e 2010 (NEWMAN; CRAGG, 2012).

Entre os produtos naturais largamente investigados nas últimas décadas, destacam-se os óleos essenciais. A ISO (International Standard Organization) define óleos essenciais como produtos obtidos de partes de plantas através de destilação por arraste com vapor d'água, bem como os produtos obtidos por compressão dos pericarpos de frutos cítricos (COSTA et al., 2008). Os óleos essenciais são compostos complexos, naturais e voláteis, límpidos, raramente coloridos, caracterizados por um forte odor e são produzidos por plantas aromáticas como metabólitos

secundários. Eles podem ser sintetizados por todos os órgãos da planta, ou seja, broto, flor, folha, caule, ramo, semente, fruto, raiz e casca, sendo armazenados em células secretoras, canais, células da epiderme ou tricomas glandulares (BAKKALI et al., 2008).

Os óleos essenciais podem ser obtidos pelos métodos de enfloração, arraste por vapor d'água, extração com solventes orgânicos, prensagem e extração por CO_2 supercrítico (SIMÕES et al., 2004) de diferentes partes da planta (ISMAN; MIRESMAILLI; MACHIAL, 2011). São compostos basicamente por dois grupos de origem biossintética distintas: o principal grupo é constituído por terpenos e o outro é constituído por componentes alifáticos e aromáticos. Ambos os grupos são caracterizados por moléculas com baixo peso molecular. Geralmente, o componente majoritário é o responsável pela atividade biológica apresentada pelo óleo essencial, porém o complexo não pode ser desprezado, uma vez que pode haver sinergismo entre as substâncias constituintes do óleo (BAKKALI et al., 2008).

Desde a antiguidade esse tipo de derivado vegetal tem sido utilizado no tratamento de diversas afecções e na preparação de perfumes, como aditivos alimentares e para o controle de pragas agrícolas (ISMAN; MIRESMAILLI; MACHIAL, 2011; ROMANO et al., 2013). Parte das propriedades farmacêuticas descritas para plantas medicinais são creditadas aos óleos. Estes produtos naturais têm mostrado algumas atividades biológicas como: antileishmania e imunomoduladora do óleo essencial de *Xylopia discreta* (LÓPEZ; CUCA; DELGADO, 2009), antioxidante na eliminação do radical ânion superóxido do óleo essencial de *Xylopia aethiopica* (Dun) A. Rich. (KARIOTI et al., 2004) e atividade moduladora da resistência bacteriana do *Staphylococcus aureus* ao antibiótico norfloxacino do óleo essencial de *Rollinia leptopetala* (COSTA et al., 2008).

Estudos mostram que muitos óleos essenciais têm atividade antibacteriana e que muitos compostos presentes nos óleos essenciais podem não ter forte atividade antibacteriana, mas podem acentuar a atividade de antibióticos clássicos por meio de interações sinérgicas (BHAVANANI; BALLOW, 1992; AHMAD; AQIL, 2007).

2.4 Associação entre produtos naturais e antimicrobianos

Uma estratégia empregada para responder aos mecanismos de resistência é o uso combinatório de drogas. Exemplo deste método é a utilização de inibidores de β-lactamases

(sulbactam, tazobactam ou ácido clavulânico) com drogas β-lactâmicas contra linhagens produtoras destas enzimas. No entanto, o frequente uso do ácido clavulânico, por exemplo, levou ao surgimento de cepas bacterianas resistentes. O aparecimento de β-lactamases de espectro estendido e resistentes contra as cefalosporinas e os carbapenens exigiu, ainda mais, a necessidade de desenvolver novos inibidores de β-lactamases (HEMAISWARYAA; KRUTHIVENTIB; DOBLE, 2008).

Além de suas propriedades antibacterianas intrínsecas, produtos naturais e seus derivados podem alterar o efeito de antibióticos, seja aumentando a atividade antimicrobiana ou revertendo à resistência aos antibióticos convencionais. A utilização destas substâncias pode representar um avanço contra os mecanismos de resistência desenvolvidos pelos micro-organismos que inativam antibióticos (CASTRO, 2010).

De acordo com Yim et al. (2011) a combinação de antimicrobianos com imipenem pode proporcionar uma opção de tratamento para infecções complicadas ocasionadas por *Enterobacteriaceae* produtoras de ESBL ou AmpC e que, a combinação de antimicrobianos pode reduzir o surgimento de resistência e elevar o espectro de atividade.

Existem dois métodos, amplamente aceitos, utilizados para avaliar a associação entre produtos. A cinética de morte microbiana que compara diferenças na contagem de colônias de um organismo ao longo de um determinado intervalo de tempo e o método de checkerboard que proporciona uma disposição bidimensional de concentrações diferentes das substâncias avaliadas, permitindo o cálculo do índice de Concentração Inibitória Fracionada (CIF) (HALL; MIDDLETON; WESTMACOTT, 1983; ODDS, 2003; ABREU et al., 2014).

Assim, o estudo e a descoberta de produtos naturais com princípios ativos que apresentem atividade antimicrobiana intrínseca ou combinada com antibióticos de uso comum podem representar uma nova forma de fazer frente aos micro-organismos multidroga resistentes. (COUTINHO, 2008).

2.5 Citotoxicidade e Genotoxicidade de Produtos Naturais

Infelizmente, a maior parte dos fitoterápicos que são utilizados atualmente por automedicação ou por prescrição médica não tem o seu perfil tóxico bem conhecido (CAPASSO, et al., 2000; VEIGA-JUNIOR, 2008). Por outro lado, a utilização inadequada de um produto,

mesmo de baixa toxicidade, pode induzir problemas graves desde que existam outros fatores de risco tais como contra-indicações ou uso concomitante de outros medicamentos (AMORIM et al., 2007; COELHO, 1998; CORDEIRO et al., 2005). Venancio (2006) estudou a toxicidade aguda do óleo essencial de *O. basilicum*. Seu resultado mostrou que o composto apresenta uma DL_{50} de 0,531 g/kg de peso do animal. Porém, até o presente momento não existem estudos mostrando a citotoxicidade e a genotoxicade do óleo essencial de *O. basilicum* nem do linalol.

A detecção de atividade citotóxica de um fitoterápico constitui uma medida prioritária, uma vez que vários compostos químicos podem ser capazes de causar efeitos tóxicos. A avaliação do potencial citotóxico em eritrócitos humanos constitui um modelo experimental *in vitro* eficaz para investigar os efeitos tóxicos e protetores de uma grande variedade de substâncias, visto que, a ocorrência de hemólise no eritrócito pode ser diretamente correlacionada com o efeito tóxico das substâncias testadas (BRANDÃO et al., 2005).

No Brasil, a Agência Nacional de Vigilância Sanitária – ANVISA – MS "Guia para a condução de estudos não clínicos de toxicologia e segurança farmacológica necessários ao desenvolvimento de medicamentos" que inclui os estudos de genotoxicidade *in vitro* e *in vivo* utilizando bactérias e células de roedores e de mamíferos (BRASIL, 2013).

A genotoxicidade é uma área da genética que estuda os processos que alteram a base da vida, em sua estrutura físico-química, o DNA, processo classificado como mutagênese. Os agentes que mudam a sequência do DNA são "tóxicos" para o gene e são, então, chamados de genotóxicos (SILVA; ERDTMAN; HENRIQUES, 2003).

É bem documentado que mutações gênicas atuam em etapas do processo de carcinogênese e que ensaios que detectam componentes genotóxicos permitem identificar substâncias com risco potencial aos seres humanos. Substâncias genotóxicas têm em comuns propriedades químicas e físicas que permitem suas interações com os ácidos nucléicos. Devido à sua alta reatividade, podem levar a defeitos hereditários através de mutações em células germinativas, e quando a mutação ocorre em células somáticas, a consequência mais comum é a formação de tumores benignos ou malignos. Além disso, recentemente foi proposto que as mutações em células somáticas podem também estar envolvidas na patogênese de algumas doenças crônicas degenerativas tais como as

cardiovasculares e neurodegenerativas, em adição ao processo de carcinogênese (ANDREASSI, et al., 2000; ARUOMA, 2003; DE FLORA, 1996, ROSS E MARGOLIS, 2005).

Um teste utilizado para identificação da atividade genotóxica é o teste de micronúcleo, um teste rápido e de baixo custo. Este é realizado em mamíferos *in vivo* e detecta substâncias mutagênicas que quebram os cromossomos (substâncias clastrogênicas) ou que interferem na formação do fuso mitótico, alterando a distribuição equitativa dos cromossomos durante a divisão celular (substâncias aneugênicas) (FLORES; YAMAGUCHI 2008). Este ensaio é internacionalmente aceito como parte da bateria de testes recomendada na avaliação do potencial mutagênico para o registro de novos produtos químicos que entram no mercado mundial e como um método de triagem no desenvolvimento de novos fármacos (HAYASHI et al., 2000; RIBEIRO, 2003). A alta confiabilidade e o baixo custo da técnica contribuem para o sucesso mundial e adoção desse biomarcador para estudos de danos genéticos *in vivo* (BONASSI et al., 2007).

2.6 *Ocimum basilicum* L. e Linalol

A planta *Ocimum basilicum* (Figura 2) pertence à família Lamiaceae, é conhecida popularmente como manjericão e pode ser encontrada na Ásia Tropical, África, América Central e América do Sul. Faz parte de um grupo de plantas medicinais, aromáticas e condimentares de grande valor econômico, muito utilizada para diversos fins como ornamental, condimentar, medicinal, aromático, na indústria de perfumaria e de cosméticos (CAROVIC-STANKO, 2010). Na gastronomia, as folhas verdes são utilizadas em massas, saladas e condimentos “in natura”, folhas secas inteiras ou moídas integram molhos de tomate (DeBAGGIO; BELSINGER, 1996). As cultivares com folhas arroxeadas ou púrpuras, também são utilizadas como plantas ornamentais (LORENZI; MATOS, 2002; SANTOS, 2007).

Figura 2: *Ocimum basilicum* L. (manjericão).

Fonte: http://plants.usda.gov/core/profile?symbol=OCBA, acessado em: 12 de junho de 2015.

Dentre as diversas espécies pertencentes ao gênero *Ocimum*, *O. basilicum* L. é a mais cultivada comercialmente devido às suas folhas verdes e aromáticas que são utilizadas secas ou frescas como condimento ou na obtenção de óleo essencial. A composição dos óleos essenciais extraídos das folhas e dos ápices com inflorescência do manjericão varia de acordo com a espécie e a localização geográfica, sendo classificados em quatro quimiotipos, de acordo com os componentes majoritários do óleo: quimiotipo linalol-metil chavicol (Europeu), metil chavicol (Reunião), metil cinamato (Tropical) e quimiotipo eugenol (Java). O óleo essencial desta espécie contém pelo menos cinco ácidos graxos: palmítico, esteárico, oléico, linólico e linoleico (LOUGHRIN, 2001).

Em função de suas propriedades terapêuticas, o manjericão é amplamente utilizado na medicina popular como antitérmico, auxiliando também na digestão e no combate de infecções bacterianas e parasitárias intestinais (TELCI et al., 2006). O seu chá é estimulante digestivo, antiespasmódico gástrico, antireumático, (LORENZI; MATOS, 2002). Na aromaterapia é utilizado para aliviar ansiedade, stress, depressão e frieza emocional, fadiga e reanimador e fortalecendo o sistema nervoso central (GROSSMAN, 2005).

Umar et al. (2010) relatou que o extrato de *O. basilicum* têm atividade anti-hipertensiva. Shirazi et al (2013) mostraram que o óleo essencial de *O. basilicum* apresentou atividade antibacteriana contra cepas de *S. aureus, Salmonella enteritidis* e *E. coli*; e antifúngica contra cepas de *Aspergillus niger* e *Candida albicans.*

Venancio (2006) mostrou que o principal constituinte químico do óleo essencial do *O. basilicum* é o linalol (constituinte majoritário), geraniol, 1-8 cineol, acetato de nerila e α-trans-bergamopteno e que o óleo apresentou atividade antinociceptiva periférica e central.

O linalol, 3,7- dimetilocta-1,6-dien-3-ol, (Figura 3) é um monoterpeno alcoólico terciário de cadeia aberta encontrado em várias espécies de plantas aromáticas como espécie de perila (*Perilla frutescens)*, alecrim (*Rosmarinus officinalis* L.) e aroeira (*Pistacia lentiscus*) (SUGAWARA et al., 2000; LETIZIA et al., 2003; BURDOCK; CARABIN, 2009; MASUMOTO; KORIN; ITO, 2010; PARASCHOS et al., 2011; OJEDA-SANA et al., 2013). É o constituinte majoritário do óleo de *Ocimum basilicum* (ZHELJAZKOV et al., 2008).

Figura 3: Estrutura química do Linalol

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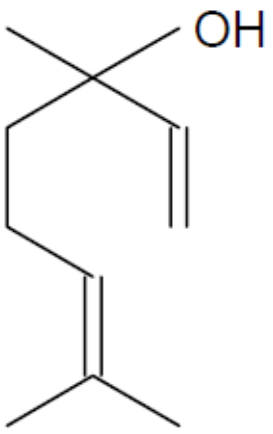

O linalol é um líquido incolor ao amarelo pálido que é comercializado como ingrediente de muitos produtos cosméticos como shampoos, perfumes, sabonetes dentre outros. É também utilizado em produtos de limpeza como detergentes (LETIZIA et al., 2003). Estudos demonstraram algumas atividades biológicas do linalol como antitumoral (CHUNG et al., 2006; MITI´C-

CULAFI´C et al., 2009), anti-inflamatório (HUO et al., 2013; PEANA et al., 2002), analgésico (PEANA et al., 2004; PEANA et al., 2006; BATISTA et al., 2008) e anticolesterolêmico (KLADNIEW et al., 2014).

Bassolé et al. (2010) mostraram que o linalol apresentou atividade antibacteriana contra cepas de *L. monocytogenes, E. aerogenes, E. coli* e *P. aeruginosa.*

Objetivos

3. OBJETIVOS

3.1 Objetivo Geral

Determinar a atividade antimicrobiana, a citotoxicidade e a genotoxicidade do óleo essencial de *Ocimum basilicum* e do linalol.

3.2 Objetivos Específicos

- Verificar o perfil de sensibilidade dos isolados clínicos de *Staphylococcus aureus* e *Pseudomonas aeruginosa* frente aos antibióticos convencionais;
- Analisar os componentes químicos do óleo essencial de *O. basilicum;*
- Determinar a Concentração Inibitória Mínima (CIM) do óleo essencial de *O. basilicum* e do linalol sobre cepas ATCC (American Type Culture Collection) e isolados clínicos de *S. aureus* e *P. aeruginosa*;
- Avaliar e caracterizar a cinética de morte microbiana dos produtos que apresentarem atividade inibitória sobre a espécie bacteriana.
- Avaliar o efeito da associação do óleo essencial e do linalol com antibióticos padrões;
- Avaliar a atividade citotóxica *in vitro* do óleo essencial de *O. basilicum* e do linalol por meio da atividade hemolítica.
- Avaliar o potencial clastogênico e aneugênico do óleo essencial de *O. basilicum* e do linalol através do teste de micronúcleo em medula óssea de roedores *in vivo*.

Materiais e Métodos

4. MATERIAIS E MÉTODOS

4.1 Local de trabalho

Os estudos de atividade antibacteriana foram realizados no Laboratório de Micologia do Departamento de Ciências Farmacêuticas, do Centro de Ciências da Saúde (CCS), da Universidade Federal da Paraíba (UFPB). Os testes de citoxicidade e genotoxicidade foram feitos no Laboratório de Bioquímica, Genética e Radiobiologia (BioGeR) do Departamento de Biologia Molecular (DBM), do Centro de Ciências Exatas e Naturais (CCEN) – UFPB em pareceria com o Biotério do Centro de Biotecnologia da UFPB.

4.2 Posicionamento ético

Para realização deste trabalho foi levado em consideração os aspectos éticos e legais da pesquisa envolvendo seres humanos (eritrócitos humanos-Teste de Hemólise) e da pesquisa envolvendo animais (Teste de Micronúcleo). O projeto foi aprovado pelo Comitê de Ética em Pesquisa do Centro de Ciências da Saúde, da Universidade Federal da Paraíba, com o Protocolo CEP/CCS n° protocolo 0285/11; e pelo Comitê de Ética no Uso de Animais do Centro de Biotecnologia, da Universidade Federal da Paraíba, com o Protocolo CEUA/CBiotec nº 0101/11.

4.3 Obtenção das substâncias teste

O óleo essencial de *Ocimum basilicum* L. e o (±)-linalol (62140) foram obtidos comercialmente, respectivamente, da Quinari (Paraná-Brasil) e da Sigma Aldrich (St. Louis, MO, USA).

4.4 Eritrócitos humanos

Os eritrócitos humanos foram oriundos de bolsas contendo concentrado de hemácias que não poderiam mais ser utilizados para transfusão. As bolsas foram obtidas na Unidade Transfusional do Hospital Universitário Lauro Wanderley/UFPB. A manipulação e o descarte dos eritrócitos foram realizados de acordo com as Normas de Segurança seguidas pela referida unidade.

4.5 Animais

Nos modelos experimentais, foram utilizados camundongos *Mus musculus* albinos machos e fêmeas, linhagem Swiss pesando entre 25-35 g, todos procedentes do Biotério Prof. Thomas George /UFPB. Os animais foram aclimatados às condições do biotério local, por cerca de sete dias antes dos ensaios experimentais, sob temperatura (21 ± 2ºC) e ciclos claro-escuro controlado de 12 horas. Os animais foram alimentados com ração e água *ad libitum*, sendo distribuídos nos diferentes grupos experimentais, ao acaso.

4.6 Análise do óleo essencial de *O. basilicum*

A extração dos componentes foi feita por meio de destilação a vapor e o método de análise foi a cromatografia gasosa de alta resolução na Universidade Federal de Minas Gerais (UFMG).

A separação cromatográfica foi realizada utilizando uma coluna capilar HP5 - 5 (30 m x 0,25 mm x 0,25 mm). A temperatura do forno da coluna foi programada para passar de 50 ° C (3 min), 3°C / min, até 170°C. A temperatura do injetor e do detector foi de 200 ° C. O volume de injeção foi de 1,0 µL (concentrado 0,5% em clorofórmio).

A identificação dos constituintes do óleo essencial foi efetuada junto ao sistema de computação e processamento de dados (workstation) interligado ao CG-EM. O sistema é equipado com uma biblioteca Wiley, 6 ª Edição da classe-5000, 1999, com 229,119 espectros.

4.7 Ensaios Microbiológicos

4.7.1 Meios de cultura

Os meios de cultura utilizados nos ensaios de avaliação da atividade antimicrobiana foi o meio sólido ágar Mueller-Hinton e o caldo BHI (Brain Heart Infusion) adquiridos da Difco Laboratories, USA. Os meios foram solubilizados em água destilada e esterilizados em autoclave, a 121 °C por 15 minutos.

4.7.2 Linhagens Bacterianas

As linhagens bacterianas de origem clínica utilizadas foram cedidas pelo laboratório de análises clínicas Hemato localizado na cidade de João Pessoa-PB. Foram utilizados no total 16 cepas bacterianas, sendo 2 cepas de *Staphylococcus aureus* e 2 de *Pseudomonas aeruginosa* ATCC (Americam Type Culture Collection), e 12 isolados clínicos, sendo 6 de cada espécie.

4.7.3 Preparação do inóculo bacteriano

As cepas selecionadas foram inoculadas em caldo BHI e foram mantidas a 35-37°C durante 24-48 horas. O inóculo foi preparado e padronizado em solução fisiológica estéril a 0,9% com o auxílio do tubo 0,5 da escala de McFarland obtendo concentração final de aproximadamente 10^6 UFC/mL (BAUER et al.,1966; CLEELAND; SQUIRES, 1991; HADACEK; GREGER, 2000).

4.7.4 Determinação da Concentração Inibitória Mínima (CIM)

Para determinação da concentração inibitória mínima (CIM) do óleo essencial de *O. basilicum* e do linalol foi utilizado a técnica de microdiluição descrita por Eloff (1998). Nos 96 orifícios foram adicionados 100 µL de caldo BHI. Em seguida, foram distribuídos 100 µL da substância teste no primeiro orifício da linha A até H. A partir da concentração inicial, foram feitas as diluições seriadas à razão de 2 nos orifícios de 1 a 10, obtendo concentrações de 1024 até 2 µg/mL. Após, foi adicionado 10 µL do inóculo bacteriano. Como controle positivo do experimento, calculou a CIM dos antibióticos imipenem e da ciprofloxacina, baseado no perfil de sensibilidade das cepas, do crescimento bacteriano e do caldo BHI.

As análises foram realizadas em triplicata e incubadas a 35-37°C durante 24-48 horas. Posteriormente foi realizada a primeira leitura dos resultados e em seguida adicionado 20µL de uma solução 0,01% (p/v) de resazurina sódica (SIGMA), preparada com a água destilada estéril. Nova incubação foi feita 35-37°C por uma hora aproximadamente. A CIM foi revelada pela menor concentração do óleo ou do linalol que promoveu a inibição do crescimento bacteriano, verificado por uma não mudança na coloração. A atividade antibacteriana foi classificada segundo os métodos de classificação de Sartoratto et al. (2004) onde o óleo é considerado com forte atividade antibacteriana quando apresentar CIM até 500 µg/ml, moderada com CIM entre 600 e 1500 µg/ml e fraca atividade antibacteriana com CIM >1500 µg/ml.

4.7.5 Determinação da Concentração Bactericida Mínima (CBM)

Para determinar a CBM, alíquotas de 1 µL da CIM, CIM X 2 e CIM X 4 do produto foram inoculadas em placas contendo ágar Muller Hinton e incubadas em estufas a 37ºC por 24 horas. A leitura para avaliar a CBM foi realizada com base no crescimento ou não dos micro-organismos. A CBM foi definida como a menor concentração do produto que inibiu o crescimento ou produziu crescimento inferior a quatro UFC, resultando em 99,9% de atividade bactericida. Os ensaios foram

realizados em duplicata e o resultado expresso pela média aritmética dos valores de CBM obtidos nos dois ensaios (ESPINEL-INGROFF et al., 2007; ERNST et al., 1999; KLEPSER et al., 1998; CLEELAND; SQUIRES, 1991).

4.7.6 Determinação da Cinética de Morte Microbiana

O óleo essencial do manjericão e o linalol foram testados quanto à viabilidade das cepas bacterianas através do método de contagem das colônias. A partir dos resultados obtidos na CIM, através da técnica de microdiluição, foram preparados os testes nas seguintes concentrações: CIM/2, CIM, CIM x 2, CIM x 4 e o controle com o antibiótico padrão. Foi preparada uma suspensão bacteriana em solução salina 0,9%, equivalente ao tubo 0,5 da escala de McFarland, contendo aproximadamente 10^6 UFC/mL. Em tubos de ensaios de 150 x 15 mm foi adicionado 9 ml de caldo BHI estéril, o produto teste na concentração definida e 1 ml da suspensão bacteriana. As soluções testes foram incubadas a 37ºC e durante os tempos determinados (0, 1, 2, 4, 6, 8, 12 e 24 horas), uma alíquota de 10µL foi inoculada em uma placa de Agar Mueller-Hinton a qual foi incubada a 37°C por 24 horas. Em seguida foi feita a contagem de colônias, onde a média do número de colônia contadas ($\log_{10}$ UFC/mL) foram marcadas versus o tempo para cada cepa e usadas para comparar a média e a dimensão da atividade antibacteriana em várias concentrações. A análise dos resultados para o produto teste foi considerada como atividade bactericida quando reduzir a morte microbiana $\geq$ 99,9% ($\geq$ 3 $\log_{10}$) e bacteriostática $\leq$ 99,9% ($\leq$ 3 $\log_{10}$) em consideração ao inóculo inicial. O ensaio foi realizado em triplicata (RASOOLI; MIRMOSTAFA, 2006; ERNST et al., 1996; KEELE et al., 2001; KLEPSER et al.,1998).

4.7.7 Ensaio da associação dos compostos com antibióticos padrões (Método Checkerboard)

Inicialmente, 100 µL do meio de cultura com 10% da suspensão bacteriana foram adicionados nas cavidades da microplaca estéril contendo 96 poços com fundo em "U" (Alamar®). Em seguida cada microplaca foi preenchida no sentido vertical com 100 µL do óleo essencial ou do linalol na concentração inicial de 2048 µg/ml que foi diluída seriadamente até concentração final de 4 µg/mL, e no sentido horizontal com 100 µL dos antibióticos (baseado no perfil de sensibilidade das amostras) imipenem, na concentração inicial de 32 µg/ml, e ciprofloxacina, na concentração inicial de 16 µg/ml sendo diluídas seriadamente na proporção 1:1 no caldo. Nas duas últimas colunas foram adicionados caldo BHI com a suspensão bacteriana. Em uma coluna foi

adicionado 100 µL do antibiótico e na coluna seguinte 100 µL do óleo essencial que foram diluídos seriadamente. O ensaio foi incubado à 35-37 ºC por 24 horas e o crescimento bacteriano foi evidenciado pelo uso da resazurina.

O efeito combinado dos antibióticos com óleo essencial ou linalol foi calculado e expresso por meio do índice CIF (Concentração Inibitória Fracionada) que é calculado através da soma do $CIF^A + CIF^B$, onde A representa o antibiótico e B o produto em teste. O CIF^A, por sua vez, será calculado pela relação CIM^Acombinado/CIM^A sozinho, enquanto que o CIF^B será CIM^B combinado/CIM^B sozinho. Este índice foi interpretado da seguinte maneira: sinergismo (≤0,5), indiferença (>0,5 - 4,0) ou antagonismo (> 4,0) (ODDS, 2003).

4.8 Ensaios toxicológicos

4.8.1 Avaliação da Atividade Citotóxica sobre eritrócitos humanos.

A avaliação da atividade hemolítica do produto teste foi realizada com uma suspensão de eritrócitos humano tipo O a 0,5%. Para isto, uma amostra de sangue (2 mL) tipo O foi misturado com NaCl 0,96 %, na proporção de 1:30, e centrifugada a 2000 rpm durante 5 minutos para obtenção dos eritrócitos. Este procedimento foi repetido mais duas vezes e o sedimento da última centrifugação foi ressuspenso em uma concentração final de 0,5 %. As amostras foram adicionadas até obter uma concentração final de 1000, 100, 10 e 1 $\mu g.mL^{-1}$ as quais foram adicionadas a 2 mL da suspensão de eritrócitos. O controle negativo foi montado com suspensão de eritrócitos mais NaCl 0,96 % (0 % de hemólise) e o controle positivo com suspensão de eritrócitos mais 100 µL de Triton X-100 1% (100 % de hemólise). As amostras foram incubadas sob agitação lenta e constante (100 rpm) por 1h a 22 ± 2 °C. Decorrido este tempo foram centrifugadas a 2000 rpm durante 5 minutos e a hemólise foi quantificada por espectrofotometria a 540 nm (RANGEL et al., 1997). Todos os experimentos foram realizados em triplicata e os resultados expressos em porcentagem do grau de hemólise.

4.8.2 Investigação do potencial genotóxico em eritrócitos de roedores *in vivo*.

Os procedimentos experimentais foram realizados de acordo com a Resolução Nº 90/2004 da Agência Nacional de Vigilância Sanitária –ANVISA (BRASIL, 2004). Grupos de três machos e três fêmeas receberam, por via oral, doses de 100 mg/kg e 200 mg/kg da solução do produto teste. Um grupo controle (negativo) recebeu apenas o dispersante da amostra e o outro grupo controle

(positivo) recebeu o agente mutagênico ciclofosfamida numa dose de 50 mg/kg de peso do animal. Vinte e quatro horas após os animais foram sacrificados com xilasina (5 mg/Kg) de acordo com as normas vigentes para evitar ansiedade ou medo (stress) (ANDRADE et al., 2006) e em seguida foram retiradas amostras de sangue da veia caudal para o preparo das lâminas as quais foram analisadas para observação da presença ou não de micronúcleos em eritrócitos de cada animal. Foram contados cerca de 2000 eritrócitos por animal. As lâminas foram coradas com corante panótico e observadas ao microscópio óptico no aumento de 1000x para a contagem dos micronúcleos (HAYASHI et al., 1994). Os resultados foram expressos como a média mais ou menos erro padrão da média.

4.9 Análise Estatística

Os resultados obtidos nos experimentos tiveram seus valores expressos em média ± erro padrão da média (e.p.m.) e analisados empregando-se o teste *t* de Student não pareado, para análise de duas colunas. Os resultados foram considerados significativos quando $p < 0,05$. Para a análise dos dados utilizou-se o programa estatístico GraphPad Prisma versão 5.0®.

Resultados e discussões

5.1 **Antibacterial Activity of *Ocimum basilicum* Essential Oil and Linalool on Bacterial Isolates of Clinical Importance**

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Antibacterial Activity of *Ocimum basilicum* Essential Oil and Linalool on Bacterial Isolates of Clinical Importance

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ABSTRACT

Ocimum basilicum, popularly known as Basil, is a Lamiaceae family species widely known to treat different diseases. This species has as its main compound the monoterpene linalool. This study aimed to determine the antibacterial activity of *O. basilicum* essential oil and linaloolagainst *S. aureus* and *P. aeruginosa* strains, as well as times to bacterial death facing each substance. The extraction of the *O. basilicum* components was made by steam distillation. The Minimum Inhibitory Concentration (MIC) was calculated using microdilution technique, and assessment of bacterial kinetics was performed with time-to-kill methodology. The results showed that *O. basilicum* essential oil and linalool display antibacterial activity against both *S. aureus* and *P. aeruginosa*, with certain strains of *P. aeruginosa* being resistant to the oil. Bacterial kinetics testing showed bacteriostatic activity against the strains in almost all concentrations, while only the MIC x 4 concentration of either essential oil or linalool against *S. aureus* displayed bactericidal activity. We conclude that the *O. basilicum* essential oil has antibacterial activity characterized as bacteriostatic or bactericidal against clinical isolates, and this activity is likely associated with linalool, its major compound.

Key words: Basil, bacterial resistance, medicinal plants, linalool.

INTRODUCTION

The appearance of antibiotics was a milestone in the history of health because it brought forward the possibility of effective combat and treatment of the numerous diseases caused by microorganisms. Unfortunately, what looked like a problem solved became a worldwide public health problem due to the emergence of resistant bacteria, the beginning of the era of bacterial resistance to existing antibiotics had begun. The current situation of drug resistance has its origin in many factors, including selection of resistant mutants thru exposure to antimicrobial agents; genetic transfer of resistance determinants among bacterial strains; and clonal spread of resistant strains between both hospitalized patients and hospitals. The consequences are increased patient morbidity and mortality, a reduced number of usable drugs for future generations, and the economic impacts brought by the cost of infections[1].

The species *Pseudomonas aeruginosa* is responsible for a variety of infections; affecting the skin, urinary tract, the eyes, and the ears. A wide distribution of *Pseudomonas* in the environment is ensured by its non-fastidious growth requirements, and *Pseudomonas* possesses many structural factors, enzymes, and toxins that enhance virulence. This also makes them resistant to most common antibiotics[2].

Staphylococcus aureus is often found colonizing the natural microbiota, especially the skin. With the breakdown of skin barriers or immunity *S. aureus* can become pathogenic. It causes a variety of skin and subcutaneous infections, post-surgical infections, osteomyelitis, pneumonia, abscesses, endocarditis and bacteremia[3].

Plants used in traditional health care with therapeutic properties are an important source of new biologically active compounds. They have been part of traditional health care in many parts of the world for decades, and have aroused the interest of many researchers[4].

Ocimum basilicum (Lamiaceae) is widely distributed in tropical and warm temperate regions. It is a multi-purpose medicinal herb commonly used in folk medicines to treat different diseases like upper respiratory tract infections, diarrhea, headaches, eye problems, skin disease, pneumonia, coughs, fevers, and conjunctivitis[5].

Linalool, 3,7 dimethylocta-1,6-dien-3-ol, is a monoterpene found in most aromatic plant essential oils. It is the major constituent of *Ocimum basilicum* oil. It has been widely used as starting compound for several important syntheses, such as ethyl linalyl acetate, and is a certified acaricide,

bactericide and fungicide. In medicine it has been applied successfully as a sedative, and is currently being analyzed for its anticonvulsant properties. Thus, linalool enjoys wide application in various areas of human knowledge, necessitating its production in ever greater quantities[6].

Based on the above, this study aimed to evaluate the antibacterial activity of *O. basilicum* essential oil against isolated *S. aureus* and *P. aeruginosa,* and to determine the time of bacterial death for sensitive bacteria.

MATERIALS AND METHODS

Compounds

The essential oil of *O. basilicum* was acquired commercially from Quinarí® (Ponta Grossa-PR), and linalool from Sigma-Aldrich .

Antibiotics

The antibiotics used in this work were Imipenem and Ciprofloxacin acquired commercially from Sigma-Aldrich, based on the sensitivity profile of the strains

Bacterial Strains

16 bacterial strains were used as follows: two strains of *Staphylococcus aureus* ATCC (25926 e 6538*),* 2 strains of *Pseudomonas aeruginosa* ATCC (25853 e 9027) and 12 clinical isolates, 6 of each species. The strains of clinical origin used were provided by the clinical analysis Laboratory of Hematology in João Pessoa- PB-Brazil. All other microorganism strains were obtained from the Laboratory of Mycology collection. Bacteria were kept on Nutrient Agar (NA) slants at 4 °C. Inoculum was obtained from overnight cultures grown on NA slants at 37 °C, and diluted in sterile saline solution (NaCl 0.85% w/v) to provide a final concentration of approximately 10^6 colony forming units per mL (CFU mL^{-1}), and adjusted according to turbidity at 0.5 McFarland tube scale.

Determination of Minimum bactericidal concentration (MBC)

After reading the MIC results, the determination of minimum bactericidal concentration (MBC) was performed; three 10 µL dilutions from the MIC were inoculated in Mueller-Hinton broth (100 µl/well) medium in sterile microdilution plates, and then were incubated at 35-37 ° C

for 24-48 hours. Then, 20 uL of resazurin was added. The plates were incubated for 24 hours at 35-37 °C and then to confirm the concentration capable of inhibiting the overall growth of bacterial species, checked by no-change in the indicator dye staining (MANN; Markham, 1998; Palomino, et al, 2002).

Determination of bacterial kill time

The essential oil of basil, and linalool were tested against bacterial strain viability by the colony counting method. From the MIC results obtained using the microdilution technique, the tests were prepared at the following concentrations: MIC/2, MIC, MIC x 2, and MIC x 4, control, and standard antibiotic. A bacterial suspension in saline 0.9%, equivalent to 0.5 McFarland tube scale, containing approximately 10^6 CFU/mL was prepared. To the 150 x 15 mm test tubes was added 9 ml of sterile BHI broth, the test product in the defined concentration, and 1 mL of the bacterial suspension. The test solutions were incubated at 37 °C, and during scheduled times (0, 1, 2, 4, 6, 8, 12 and 24 hours) an aliquot of 10μL was inoculated into a Mueller Hinton agar plate and incubated at 37 °C for 24 hours. Then, colony counting was done, where the average numbers of colonies (log10 CFU/mL) were labeled versus time for each strain, and used to compare the mean, and the extent of antibacterial activity at various concentrations. The analyses results for the test product were considered bactericidal if causing microbial death ≥ 99.9% (≥ 3 log10), and bacteriostatic if ≤ 99.9% (≤ 3 $\log_{10}$) taking into account the initial inoculum. The assay was performed in triplicate[10,11,12,13].

RESULTS AND DISCUSSION

The indiscriminate use of antibiotics has fomented the emergence of bacterial resistance to commonly used drugs and, consequently, the need (and search) for new products that can replace those which are no longer effective [14].

For more than 50 years, natural products have served us well in combating infectious bacteria and fungi. Microbial and secondary plant metabolites have helped to: double our life span during the 20th century, reduce pain and suffering, and revolutionized medicine. Essential oils are involved in many important processes related to plant survival, playing a prominent role in defense against microorganisms [15].

Among the various species of the genus Ocimum, *O. basilicum* L. is the most widely commercialized, due to its green and aromatic leaves which are used dried or fresh, as a condiment, or for obtaining essential oil. The composition of the essential oils extracted from the leaves and apices with basil inflorescence varies according to the species and the geographical location, being classified into four chemotypes, (according to the major components of the oil): linalool-methyl chavicol (European), methyl chavicol (Reunion), methyl cinnamate (Tropical), and eugenol (Java). The essential oil of the species contains at least five fatty acids: palmitic, stearic, oleic, linolic, and linoleic [16].

The results obtained for *O. basilicum* essential oil chromatography are shown in Table 1. It is observed that the oil has as its major compound, the monoterpene linalool.

Table 1- Chromatography of essential oil of *Ocimun basillicum*

RI	**Compounds**	**%**
928	α-pinene	0.4
972	β-pinene	1.1
987	Myrcene	0.7
1034	1,8-Cineole	8.8
1041	*trans*-β-Ocimene	0.6
1099	Linalool	55.2
1182	Terpinen-4-ol	0.9
1356	Eugenol	3.2
1421	B-Caryophyllene	0.4
1439	α-*trans*-Bergamotene	7.0
1489	Germacrene D	2.2
1515	γ-Cadineno	2.9
1638	Muurolol	2.9

RI: Retention index

This result corroborates studies by Veloso et al[17] which identified two major constituents present in the essential oils of the evaluated *O. basilicum* samples (from different regions): one monoterpene (linalool), the majority in both cultivars, and phenylpropanoid (E) cinnamate methyl), the majority in wild accessions.

The results for antibacterial activity of *O. basilicum* essential oil on *S. aureus* and *P. aeruginosa* strains can be seen in Table 4. The activity, in both cases, was measured in terms of

presence of microorganism growth**.** The sensitivity profile of the strains (table 2 and 3) is also revealed.

Table 2: Phenotypic sensitivity profile of the species of *S. aureus*

S. aureus	**Amoxicillin**	**Amoxicillin / Acid clavulonic**	**Ampicillin**	**Azithromycin**	**Cefalexina**	**Cefalotina**	**Ciprofloxacin**	**Clarithromycin**	**Clyndamicin**	**Erythromycin**	**Oxacillin**	**Penicillin**	**Teicoplanin**
72-1	R	S	R	S	S	S	S	S	S	S	S	R	S
M-289	R	S	R	R	S	S	S	R	R	R	S	R	S
A-197	R	S	R	R	S	S	S	R	R	R	S	R	S
M-177	R	S	R	R	S	S	S	R	R	R	S	R	S
M-137	R	S	R	S	S	S	S	S	S	S	S	R	S
M-117	R	S	R	S	S	S	S	S	S	S	S	R	S

R=resistance; S= sensible;

Table 3: Phenotypic sensitivity profile of the species of *P. aeruginosa*

P. aeruginosa strains	**Ciprofloxacin**	**Levofloxacin**	**Polymyxin B**	**Gentamicin**	**Amikacin**	**Ceftazidime**	**Cefepime**	**Piperacillin-tazobactam**	**Imipenem**	**Meropenem**	**Ceftriaxone**
M 116-1	R	R	S	S	S	S	S	S	S	S	NT
166.22.260	R	NT	S	NT	S	R	R	NT	S	S	R
166.23.39	S	NT	S	NT	NT	R	S	NT	S	S	R
M-163	S	S	S	S	S	S	S	S	S	S	NT
LAC-21-1	S	S	S	S	S	S	S	S	S	S	NT
LM-07	S	S	S	S	S	S	S	S	S	S	NT

R=resistance; S= sensible; NT= not tested

Table 4 - Minimum Inhibitory Concentration of *O. basilicum* essential oil on *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains.

	Microrganism	Control (Mo)	OB (µg/mL)		LIN (µg/mL)		IMP (µg/mL)	CPF (µg/mL)
			MIC	MBC	MIC	MBC		
S. aureus	**ATCC 25923**	+	1024	>1024	32	>1024	8	2
	ATCC 6538	+	1024	>1024	1024	>1024	4	2
	72-1	+	512	>1024	64	>1024	2	2
	M-289	+	1024	>1024	1024	>1024	4	2
	A-197	+	512	>1024	128	>1024	4	4
	M-177	+	1024	>1024	1024	>1024	4	2
	M-137-2	+	512	>1024	512	>1024	2	2
	M-117	+	512	>1024	512	>1024	2	2
P. aeruginosa	**ATCC 25853**	+	1024	>1024	1024	>1024	4	2
	ATCC 9027	+	1024	>1024	1024	>1024	2	2
	M 116-1	+	R	>1024	1024	>1024	16	4
	166.22.260	+	1024	>1024	1024	>1024	4	R
	166.23.39	+	1024	>1024	1024	>1024	4	2
	M-163	+	R	>1024	1024	>1024	2	2
	LAC-21-1	+	R	>1024	32	>1024	2	2
	LM-07	+	R	>1024	1024	>1024	2	2

Mo= microrganism; MIC= Minimum Inhibitory Concentration; MBC= Minimum bactericidal concentration; (+) Bacterial Growth; OB= O*cimum basilicum* essential oil; LIN =linalool; IMP= imipenem; CPF= ciprofloxacin; R= resistance.

In Table 4, we observe that all *S. aureus* strains were sensitive to the *O. basilicum* essential oil and linalool with MICs in the ranges of 1024-512 µg/mL, and 1024-32 µg/mL, respectively. According to the Sartoratto et al.[18] classification methods, antibacterial activity can be classified as moderate for the oil, and between moderate and strong for linalool. As for the *P. aeuruginosa* strains tested, it was observed that 50% of the strains were resistant to *O. basilicum* essential oil, and those which were sensitive had an MIC of 1024µg/mL, which also characterizes moderate antibacterial activity.

The antimicrobial activity of basil essential oil has been linked in part to the presence of high amounts of the monoterpene linalool. Researchers have found that basil oil and linalool compounds display antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Aspergillus niger*[19]. According to the literature, the antimicrobial activity of the essential oil could be a result of the high percentage of oxygenated monoterpenes (94.47%), which are particularly active against microbial cells[20]. In this study, we observed that linalool showed antibacterial activity for all of the tested *P. aeruginosa* strains, including those that were resistant to the essential oil, which supports the idea that linalool, is the substance primarily responsible for the antibacterial activity of *O. basilicum* oil.

Microbial death kinetic studies are commonly used in investigations of new antimicrobial agents because they are relatively easy to perform and economically viable[21]. They relate microorganism growth inhibition with exposure to various test drug concentrations over time, showing whether the same has bactericidal or bacteriostatic action.

It is observed that the bacterial kinetics (Figure 1 to 4) of the *S. aureus* and *P. aeruginosa* samples against the *O. basilicum* essential oil and linalool showed bacteriostatic activity at nearly all times and concentrations since there was a reduction lower than 3 $\log_{10}$CFU/mL (<99.9%) of the initial inoculum. Only at the concentration of MIC x 4 did *O. basilicum* essential oil and linalool show bactericidal activity against *S. aureus,* being a reduction in bacterial growth greater than 3 $\log_{10}$ CFU/mL (> 99.9%) of the initial inoculum after 8 hours of contact. The bacteriostatic action of a compound means that it prevents the growth of the bacteria, maintaining the same in the stationary phase, while bactericidal action kills the bacteria[22].

Figure 1 - Curve of bacterial kill time, *Staphylococcus aureus* strain 72-1 by *O. basilicum* essential oil.

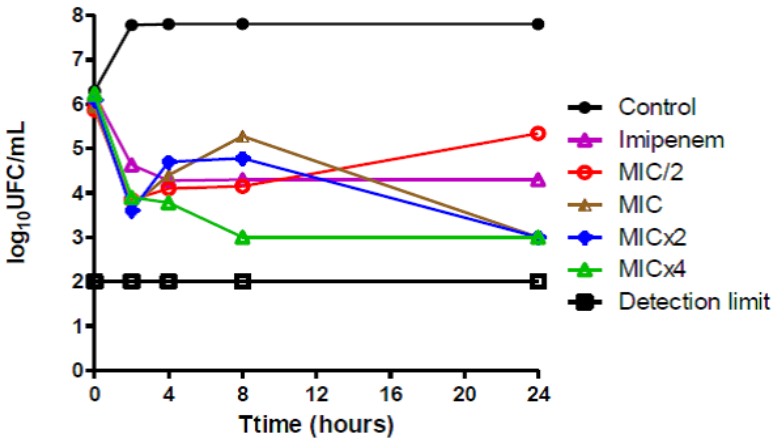

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Figure 2- Curve of bacterial kill time, *Staphylococcus aureus* strain 72-1 by linalool

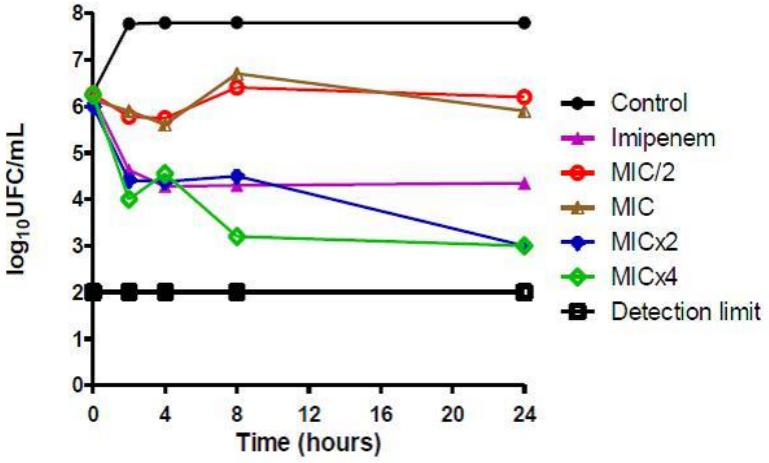

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Figure 3- Curve of bacterial kill time, *P. aeruginosa* 166.23.39 strain by *O. basilicum* essential oil.

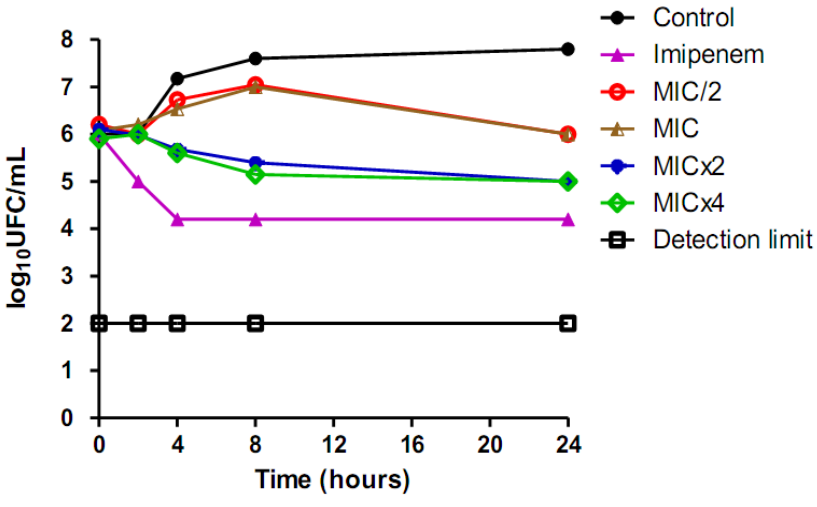
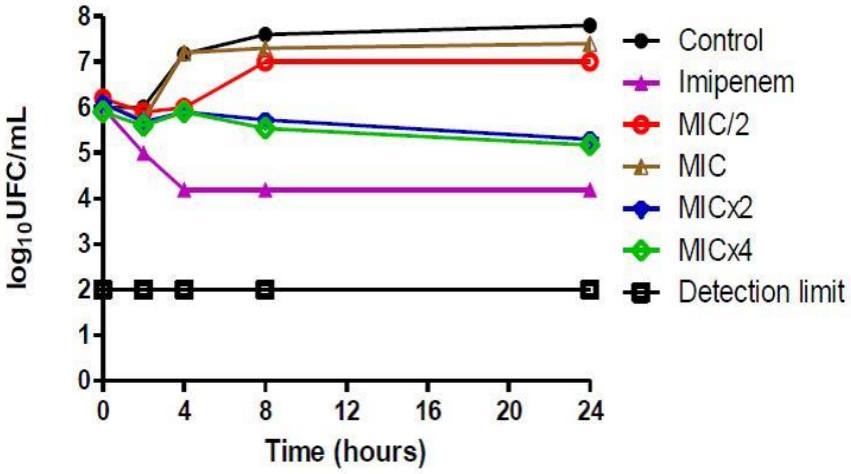


Figure 4- Curve of bacterial kill time, *P. aeruginosa* 166.23.39 strain by linalool.



According to Greay & Hammer[23], monoterpenes such as linalool interfere with the integrity and function of the cell membrane; changing the membrane potential, causing loss of cytoplasmic material, and inhibiting the respiratory chain. Exposure to terpenes can interfere with the expression of virulence factor encoding genes, considered when producing strains of *S. aureus* enterotoxins[24], and the expression of cytoplasmic and membrane proteins in *Salmonella enterica*[25].

The results obtained in this study suggest that the compounds present considerable antibacterial effect against both Gram positive and negative bacterial species. Thus, further studies are necessary to explore this effect, investigate toxicities, and delineate mechanisms of action.

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5.2 *Ocimum basilicum* L.: Antibacterial activity and association study with antibiotics against bacteria of clinical importance

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Ocimum basilicum: Antibacterial activity and association study with antibiotics against bacteria of clinical importance

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Abstract

Context

Ocimum basilicum L. (Lamiaceae) (Peckolt, 1852), popularly known as basil, is part of a group of medicinal plants widely used in cooking and known for its beneficial health properties, possessing significant antioxidant effects, antinociceptive and others.

Objective

To determine the pharmacological effects produced on the bacterial strains *Staphylococcus aureus* and *Pseudomonas aeruginosa* when standard antibiotics and *O. basilicum* essential oil are combined.

Materials and methods

The extraction of *O. basilicum* (leaves) components was done by steam distillation. The Minimum Inhibitory Concentration (MIC) was calculated using microdilution technique, where the oil concentrations varied from 2 to 1024 µg /mL. The combinations of *O. basilicum* oil with ciprofloxacin or imipenem were analyzed by the checkerboard method where fractional inhibitory concentration (FIC) indices were calculated.

Results

O. basilicum essential oil, imipenem, and ciprofloxacin showed respective MIC antibacterial activities of 1024, 4, and 2 µg/mL, against *S. aureus*. In *S. aureus*, the oil with imipenem association showed synergistic effect (FIC = 0.0625), while the oil with ciprofloxacin showed antagonism (FIC = 4.25). In *P. aeruginosa*, the Imipenem/oil association showed additive effect for ATCC strains, and synergism for the clinical strain (FIC = 0.75 and 0.0625). The association of *O. basilicum* essential oil with ciprofloxacin showed synergism for clinical strains (FIC = 0.09).

Conclusion

O. basilicum essential oil associated with existing standard antibiotics may increase their antibacterial activity, resulting in a synergistic activity against bacterial strains of clinical importance. The antibacterial activity of *O. basilicum* essential oil may be associated with linalool.

Keywords: *Ocimum basilicum*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Imipenem, Ciprofloxacin.

Introduction

The phenomenon of bacterial resistance is known to be directly related to the formation of antibiotic (non-sensitive) strains. These are able to grow even in the presence of higher than normal concentrations of antibiotics. This is due to both, the natural development of microorganisms and irrational use of these agents in medical, agricultural, and veterinary practices (Wannmacher, 2004; Hoefel *et al*., 2006).

Staphylococcus aureus and *Pseudomonas aeruginosa* are the most common multidrug-resistant agents of nosocomial infections. They cause great clinical and economic impact due to their continued presence, particularly in hospitals (Bernardes *et al*., 2004; Sader *et al*., 2010). Infections caused by these pathogens are of particular clinical challenge due to their frequent adaptations; they develop resistance under the selective pressure of intense antibiotic use and are characterized by susceptibility to only a limited number of antimicrobials agents.

Many plants have been assessed that not only demonstrate their antimicrobial activities directly, but also serve as sources of compounds with the potential to modulate antibiotic action (Gibbons, 2004; Gurib-Fakim, 2006). Several chemical compounds of synthetic origin, such as the phenothiazines, or from natural sources such as flavonoids, terpenes and others provide direct antibacterial activity. They can also increase the activity of specific antibiotics, while reversing some types of bacterial resistance to certain antibiotics. They may also promote the elimination of plasmids (that carry resistance determinants) or inhibit transport functions (of some classes of antibiotics), in the plasma membrane. Increases in antibiotic activity or the reversal of resistance caused by non-conventional natural or synthetic compounds identifies them as antibiotic activity modifiers (Molnar *et al*., 2004; Wolfart *et al*., 2006).

Natural products discovery of ingredients that have intrinsic antibacterial activity, and/or which may be used in combination with standard antibiotics, may prove a viable alternative for production of effective new drugs against multi-resistant microorganisms.

Ocimum basilicum L. (Lamiaceae), also known as basil, is an aromatic herb used extensively for its distinctive aroma and for food flavoring. The leaves can be used fresh or dried as a spice. Essential oils extracted from the fresh leaves and flowers can be used as food aroma additives, and

in pharmaceuticals and cosmetics (Javanmardi, 2002). Traditionally, basil has been used as a medicinal plant in the treatment of headaches, coughs, diarrhea, constipation, warts, worms, and kidney malfunctions (Simon *et al*., 1999)

Based on the above, this study observed the effects of combinations of *O. basilicum* essential oil with standard antibiotics used in clinical practice against *S. aureus* and *P. aeruginosa* strains.

Materials and methods

Essential oil

The essential oil of *O. basilicum* was acquired commercially from Quinarí® (Ponta Grossa, Paraná, Brazil) essential oil.

Antibiotics

The antibiotics used in this work were imipenem and ciprofloxacin acquired commercially from Sigma-Aldrich (St. Louis, MO, USA), as based on the sensitivity profile of the strains.

Bacterial Strains

As follows four bacterial strains were used: 2 strains of *Staphylococcus aureus* (ATCC 6538 and M-177*),* and 2 strains of *Pseudomonas aeruginosa* (ATCC 25853 and 1662339). The strains of clinical origin used were provided by the clinical analysis Laboratory of Hematology in João Pessoa- PB-Brazil. All other microorganism strains were obtained from the Laboratory of Mycology collection. Bacteria were kept on Nutrient Agar (NA) slants at 4°C. Inoculum was obtained from overnight cultures grown on NA slants at 37°C, and diluted in sterile saline solution (NaCl 0.85% w/v) to provide a final concentration of approximately 10^6 colony forming units per mL (CFU mL^{-1}), and adjusted according to turbidity at 0.5 McFarland tube scale.

Chromatography of O. basilicum essential oil

Components extraction was made by steam distillation, and the analysis method used high resolution gas chromatography. Chromatographic separation was performed using a DB-5 capillary column (30 m x 0.25 mm (HP). The temperature of the chromatography oven was programmed

from 50°C (3 min), 3°C/min, to 170°C. The temperature of the injector and detector were 200°C. The split was at 1/200, and the detector FID 200°C. The injection volume was 1.0 µL (0.5% concentration in chloroform). Identification of individual components was based on their mass spectral fragmentation; two computer library MS searches (Wiley 229), and by retention index (RI).

Determination of Minimum Inhibitory Concentration (MIC)

The microplate bioassay was used to determine the minimum inhibitory concentrations (MIC) for (Imipenem, Ciprofloxacin, and the Essential oil). For this purpose, 96-well plates were prepared by dispensing 100 µL of double strength Nutrient Broth (NB) inoculated with the bacteria into each well prior to the assay. Aliquots (100 µL) of each compound (at its respective concentrations) were transferred into six consecutive wells. The highest substance concentration (1024 µg/mL) solution was added to the first well with the smallest concentration (2 µg/mL) in the antepenultimate well. The penultimate and the last wells containing 200 µL of the NB were respectively inoculated with the microorganism suspension, and Imipenem (100 µg/mL), being the negative control and positive controls. The microplate was aseptically sealed, and incubated at 37 °C for 24 h (Viljoen *et al*., 2003; Sahin *et al*., 2004).The antibacterial activity was detected using colorimetric method adding 20 µL of resazurin (0.1 g/100 mL) aqueous staining solution to each well at the end of the incubation period. The MIC was defined as the lowest sample concentration able to inhibit the bacterial growth as indicated by resazurin staining (dead cells not being able affect the color staining - visual observation – blue to red) (Burt & Reinder, 2003). All experiments were carried out at least twice with consistent results.

Association studies using the checkerboard method

The strains were tested using the microdilution checkerboard technique (Eliopoulos & Moellering, 1991). Suspensions of 10^6 CFU/mL were prepared and distributed into micro-titer plates containing various concentrations of the different drugs. The inoculated plates were incubated at 37°C for 24h, and then evaluated for bacterial growth. In order to determine the activity of the drug combinations, fractional inhibitory concentration (FIC) indices were calculated as $FIC^A + FIC^B$, where FIC^A and FIC^B represent the minimum concentrations that inhibited bacterial growth for drugs A, and B, respectively: $FIC^A = MIC^A$ combination/MIC^Aalone, and FIC^B

= MIC^B combination/MIC^B alone. A mean FIC index was calculated based on the following equation: FIC index = $FIC^A + FIC^B$, interpretation as follows: synergistic (≤0.5), additive (>0.5 but <1), indifferent (≥1 but <4), or antagonistic (≥4.0).

Results

Chromatography of the *O. basilicum* oil revealed linalool as the principal compound (Table 1).

Table 1- Chromatography of essential oil of *Ocimun basillicum*

RI	**Compounds**	**%**
928	α-pinene	0.4
972	β-pinene	1.1
987	Myrcene	0.7
1034	1,8-Cineole	8.8
1041	*trans*-β-Ocimene	0.6
1099	Linalool	55.2
1182	Terpinen-4-ol	0.9
1356	Eugenol	3.2
1421	B-Caryophyllene	0.4
1439	α-*trans*-Bergamotene	7.0
1489	Germacrene D	2.2
1515	γ-Cadineno	2.9
1638	Muurolol	2.9

RI: Retention index

The results of the *O. basilicum* oil / standard antibiotics association study are shown in Tables 2 and 3.

Table 2: Antibacterial activity of the isolated compounds and in combination against *S. aureus* strains.

Compounds		*S. aureus* ATCC 6538			*S. aureus*M-177		
		MIC (µg/mL)	**FIC (Index)**	**Type of interaction**	**MIC (µg/mL)**	**FIC (Index)**	**Type of interaction**
Alone	**OB**	1024			1024		
	IM	4			4		
	CP	2			2		
Association	**OB+IM**	32 / 0.125	0.0625	Synergism	32 / 0.125	0.0625	Synergism
	OB+CP	4096 / 0.5	4.25	Antagonism	4096 / 0.5	4.25	Antagonism

MIC: Minimum Inhibitory Concentration; FIC: Fractional Inhibitory Concentration Index; OB= Essential oil of *O. basilicum*; IM=Imipenem; CP= Ciprofloxacin.

Table 3: Antibacterial activity of the isolated compounds and in combination against *P. aeruginosa* strains

Compounds		*P. aeruginosa* ATCC 25853			*P. aeruginosa* 1662339		
		MIC (µg/mL)	**FIC (Index)**	**Type of interaction**	**MIC (µg/mL)**	**FIC (Index)**	**Type of interaction**
Alone	**OB**	1024			1024		
	IM	4			4		
	CP	2			2		
Association	**OB+IM**	256 / 2	0,75	Additivity	32 / 0,125	0,0625	Synergism
	OB+CP	32 / 2	1,03	Indifferent	32 / 0,125	0,09	Synergism

MIC: Minimum Inhibitory Concentration ; FIC: Fractional Inhibitory Concentration Index; OB= Essential oil of *O. basilicum*; IM=Imipenem; CP= Ciprofloxacin.

Observing Table 2, the respective MIC results for *O. basilicum* essential oil and Imipenem used alone against the *S. aureus* strains tested were 1024 µg.mL-1, and 4 µg.mL^{-1}. The associations

revealed that *O. basilicum* oil in combination with Imipenem reduced the MIC to 32 and 0.125 μg/mL, respectively. Thus, the FIC was 0.0625, and the compound association was characterized as synergistic for the tested *S. aureus* strains. Regarding ciprofloxacin, the MIC of the antibiotic alone was 2μg/mL. For the association of ciprofloxacin and *O. basilicum* oil, we observed an MIC increase to 4096 $\mu g.mL^{-1}$, and a reduction in the MIC of ciprofloxacin to 0.5 $\mu g.mL^{-1}$, resulting in an FIC of 4.5 indicating antagonism.

In Table 3, we observe that for the strains of *P. aeruginosa;* standard and clinical isolate, the *O. basilicum* oil MIC was 1024 $\mu g.mL^{-1}$, for Imipenem it was 4$\mu g.mL^{-1}$, and for Ciprofloxacin it was 2 $\mu g.mL^{-1}$, (compounds used alone). *O. basilicum* oil associations with Imipenem were classified as additive or synergistic. For Ciprofloxacin, the combination with *O. basilicum* oil, for the strain ATCC was classified as indifferent.

DISCUSSION

Resistance to antibiotics is increasing globally, and at a very alarming rate (Stuart & Bonnie, 2004). In general, bacteria have the genetic capacity to both gain and transmit resistance to drugs used as therapeutics.

Associations of antimicrobials are evaluated for their ability to suppress the emergence of resistant mutants, and to produce *in vivo* synergistic effects. Extending the useful life of current antimicrobials might be possible if they were used in combination with natural products. These combinations could represent therapeutic alternatives for the treatment of infections (Musumeci & Berberis, 2003).

In this study, *O. basilicum* essential oil, either alone, or in combination with Imipenem (resulted in synergism) displayed antibacterial activity against *S. aureus* strains. For the *P. aeruginosa* strains tested, the oil combinations with Imipenem and Ciprofloxacin were synergistic, but for the ATCC strain, the association with Ciprofloxacin was indifferent.

Depending on variations in chemotype, leaf, and flower color, aroma, and origin of the plant *O. basilicum* essential oils exhibit a wide and varying array of chemical compounds (Da-Silva *et al*., 2003). The chief constituents include chavicol methyl ether (or estragole), linalool, and eugenol (Hussain *et al*., 2008; Omidbaigi e*t al*., 2003). Studies in the literature suggest that linalool, a monoterpene, is the main ingredient responsible for antibacterial activity (Ravid *et al*., 1997).

In studies by Bassolé *et al*. (2010), *O. basilicum* had as its main compounds; linalool (57%) and eugenol (19.2%). The essential oil showed antibacterial activity against strains of *S. aureus*, *E. faecalis*, *L. monocytogenesm*, *E. aerogenes*, *E. coli*, *S. enteric*, and *S. typhimurium*. Using the checkerboard method, associations of *O. basilicum* and eugenol showed synergetic effects, confirming the roles of certain components in the interaction.

Based on the hypothesis of Pei *et al*. (2009), we suggest that synergetic effects might be due to increases in one of three factors which determine monoterpene antimicrobial character: their lipophilic properties, the potency of their functional groups, and the resulting aqueous solubility when in a paired combination (Knobloch *et al*., 1998).

Conclusion

This work demonstrated that *O. basilicum* essential oil in association with existing antibiotics can result in increased antibacterial activity through molecular synergism against bacterial strains of clinical importance. Considering the high incidence of *S. aureus* and *P. aeruginosa* in infectious diseases, and their increasing resistance to existing antibiotics, the search for alternative compounds is of great importance. Natural products may well serve as a source for these compounds by producing new medicines, or by enhancing existing ones.

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Declaration of interest

The authors declare no conflicts of interest.

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5.3 Antibacterial activity of the monoterpene linalool: alone and in association with antibiotics against bacteria of clinical importance

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Antibacterial activity of the monoterpene linalool: alone and in association with antibiotics against bacteria of clinical importance

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ABSTRACT

Antibacterial activity studies of new molecules, either alone or in combination with existing antibiotics, are of great importance considering the resistance acquired by microorganisms in recent times. Linalool is a phytoconstituent found in the essential oils of various plant species. It is a monoterpene widely used in perfumery, cosmetics, and the food industries. Our objective was to determine the pharmacological effects produced on the bacterial strains *Staphylococcus aureus,* and *Pseudomonas aeruginosa* when combining standard antibiotics with linalool. The Minimum Inhibitory Concentration (MIC) was calculated using microdilution technique, where the linalool concentrations varied from 2 to 1024 µg /mL. Combinations with standard antibiotics were analyzed by the checkerboard method where the fractional inhibitory concentration (FIC) indices were calculated. Linalool, Imipenem, and Ciprofloxacin showed respective MIC antibacterial activities against *S. aureus* of 1024, 4, and 2 µg/mL. In *S. aureus*, the linalool with Imipenem association showed a synergistic effect (FIC = 0.0625); while with ciprofloxacin, the linalool showed additivity (FIC = 0.75). In *P. aeruginosa*, the Imipenem/linalool association was

synergistic for both the ATCC and clinical strains (FIC = 0.0625). The association of *linalool* with ciprofloxacin was indifferent. We conclude that Linalool associated with existing standard antibiotics may increase antibacterial effectiveness, resulting in synergistic activity against bacterial strains of clinical importance. This makes the molecule potentially important for production of new, therapeutically effective drugs against resistant microorganisms.

Key words: natural products, antibacterial activity, synergism, linalool.

INTRODUCTION

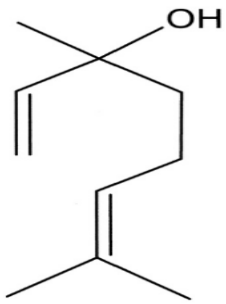
In the 21st century, given the growing number of multiresistant bacterial strains, and resistance exchanges between different species, bacterial resistance has become a critical challenge, (Ex.: *Neisseria gonorrhoeae, Pneumocystis pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus*)[1,2]. As a global public health problem, the theme was proposed by the World Health Organization (WHO) in 2011, and emphasized on World Health Day as a controlling target among global strategies to ensure safe healthcare. Attention was also drawn to the challenges of implementing immediate actions to control the spread of resistant microorganisms in order to minimize the progressive deterioration of therapies handling such cases.

Among the pathogens considered important in relation to bacterial resistance, one might highlight *S. aureus* and *P. aeruginosa*. Infections caused by these pathogens are a clinical challenge, due to adaptations under the selective pressures of intense antimicrobial use; *S. aureus* has achieved a great ability to develop resistance[3], and *P. aeruginosa* is already characterized by limited susceptibility to any number of antimicrobial agents[4].

Because of the great resistance that microorganisms, such as *S. aureus* and *P. aeruginosa*, have acquired to a wide range of antibiotics in recent years, the search for new compounds has been the subject of intensive research. The fight against this emerging problem of pathogenic organism resistance has in the present day employed two divergent approaches: the development of completely new antibiotics, and/or combinations of substances already in use[5]. The adoption of combination therapy often occurs in cases where the etiological character is poly-microbial, making it difficult to achieve mono-therapeutic healing[6].

Linalool (Figure 1), 3,7-Dimethyl-1,6-octadien-3-ol, is a widely used monoterpene in perfumery, cosmetics, and the food industries. It has been used as the starting compound for several important syntheses, such as linalyl acetate. It has been used successfully as a sedative, and has anticonvulsant, hypnotic, and hypothermic properties, affecting the central nervous system as a depressant. It is also being analyzed for its bactericidal and fungicidal properties. More studies on its properties are necessary[7,8].

Figure 1- Quemical structure of Linalool



.

The compound is a constituent of the essential oils of various plants of the Brazilian flora, such as rosewood (*Aniba roseadora*), several species of the *Piper* and *Croton* genres, Coriander (*Coriandrum sativum*), Tangerine (*Citrus reticulata*), and the Bergamot variation (*Citrus bergamia*), as well as other citrus fruits, and even the basils; (*Ocimum basilicum*) and (*Ocimum gratissimum*)[9,10,11,12].

Based on the above, this study aimed to observe the effects of combinations of linalool with standard antibiotics used in clinical practice against strains of *S. aureus* and *P. aeruginosa*.

MATERIALS AND METHODS

Linalool

Linalool was acquired commercially from Sigma-Aldrich.

Antibiotics

The antibiotics used in this work were Imipenem and Ciprofloxacin acquired commercially from Sigma-Aldrich, as based on the sensitivity profile of the strains.

Bacterial Strains

As follows four bacterial strains were used: 2 strains of *Staphylococcus aureus* (ATCC 6538 and M-177*),* and 2 strains of *Pseudomonas aeruginosa* (ATCC 25853 and 1662339). The strains of clinical origin used were provided by the clinical analysis Laboratory of Hematology in João Pessoa- PB-Brazil. All other microorganism strains were obtained from the Laboratory of Mycology collection. Bacteria were kept on Nutrient Agar (NA) slants at 4°C. Inoculum was obtained from overnight cultures grown on NA slants at 37°C, and diluted in sterile saline solution (NaCl 0.85% w/v) to provide a final concentration of approximately 10^6 colony forming units per mL (CFU mL^{-1}), and adjusted according to turbidity at 0.5 McFarland tube scale.

Determination of Minimum Inhibitory Concentration (MIC)

The microplate bioassay was used to determine the minimum inhibitory concentrations (MIC) for (Imipenem, Ciprofloxacin, and the linalool). For this purpose, 96-well plates were prepared by dispensing 100 µL of double strength Nutrient Broth (NB) inoculated with the bacteria into each well prior to the assay. Aliquots (100 µL) of each compound (at its respective concentrations) were transferred into six consecutive wells. The highest substance concentration (1024 µg/mL) solution was added to the first well with the smallest concentration (2 µg/mL) in the antepenultimate well. The penultimate and the last wells containing 200 µL of the NB were respectively inoculated with the microorganism suspension, and Imipenem (100 µg/mL), being the negative control and positive controls. The microplate was aseptically sealed, and incubated at 37 °C for 24 h[13,14]. The antibacterial activity was detected using colorimetric method adding 20 µL of resazurin (0.1 g/100 mL) aqueous staining solution to each well at the end of the incubation period. The MIC was defined as the lowest sample concentration able to inhibit the bacterial growth as indicated by resazurin staining[15]. All experiments were carried out at least twice with consistent results.

Association studies using the checkerboard method

The strains were tested using the microdilution checkerboard technique[16]. Suspensions of 10^6 CFU/mL were prepared and distributed into micro-titer plates containing various concentrations of the different drugs. The inoculated plates were incubated at 37°C for 24h, and then evaluated for bacterial growth. In order to determine the activity of the drug combinations, fractional inhibitory concentration (FIC) indices were calculated as $FIC^A + FIC^B$, where FIC^A and FIC^B represent the minimum concentrations that inhibited bacterial growth for drugs A, and B, respectively: $FIC^A = MIC^A$ combination/MIC^A alone, and $FIC^B = MIC^B$ combination/MIC^B alone. A mean FIC index was calculated based on the following equation: FIC index = $FIC^A + FIC^B$, interpretation as follows: synergistic (≤0.5), additive (>0.5 but <1), indifferent (≥1 but <4), or antagonistic (≥4.0).

RESULTS

The results of the linalool/standard antibiotics association study are shown in Tables 1 and 2.

Table 1: Antibacterial activity of the isolated compounds and in combination against *S. aureus* strains

Compounds		***S. aureus* ATCC 6538**			***S. aureus*M-177**		
		MIC (µg/mL)	**FIC (Index)**	**Type of interaction**	**MIC (µg/mL)**	**FIC (Index)**	**Type of interaction**
Alone	**LNL**	1024			1024		
	IM	4			4		
	CP	2			2		
Association	**LNL+IM**	32 / 0.125	0.0625	Synergism	32 / 0.125	0.0625	Synergism
	LNL+CP	512 / 0.5	0.75	Additivity	512 / 0.5	0.75	Additivity

MIC: Minimum Inhibitory Concentration; FIC: Fractional Inhibitory Concentration Index; LNL= Linalool; IM=Imipenem; CP= Ciprofloxacin

Table 2: Antibacterial activity of the isolated compounds and in combination against *P. aeruginosa* strains

Compounds		*P. aeruginosa* ATCC 25853			*P. aeruginosa* 1662339		
		MIC (µg/mL)	**FIC (Index)**	**Type of interaction**	**MIC (µg/mL)**	**FIC (Index)**	**Type of interaction**
Alone	**LNL**	1024			1024		
	IM	4			4		
	CP	2			2		
Association	**LNL+IM**	32/ 0.125	0,0625	Synergism	32 / 0,125	0,0625	Synergism
	LNL+CP	256 / 2	1,25	Indifferent	1024/ 2	2	Indifferent

MIC: Minimum Inhibitory Concentration; FIC: Fractional Inhibitory Concentration Index; LNL= Linalool; IM=Imipenem; CP= Ciprofloxacin

Observing Table 1, the respective MIC results for linalool and Imipenem each used alone against the *S. aureus* strains tested were 1024 and 4 µg.mL^{-1}. Linalool in combination with Imipenem reduced the MIC of these compounds to 32 and 0.125 µg/mL, respectively. The FIC was 0.0625, and the compound associations were characterized as synergistic for the tested *S. aureus* strains. Regarding ciprofloxacin, the MIC of the antibiotic alone was 2 µg/mL. For the linalool/ciprofloxacin association, we observed that the MIC decreased to 512 µg.mL^{-1} for linalool, and the MIC of ciprofloxacin decreased to 0.5 µg.mL^{-1}, resulting in an FIC of 0.75 (indicating additivity).

In Table 2, we observe that for the standard and clinical isolate strains of *P. aeruginosa*; the linalool MIC was 1024 µg.mL^{-1}, for Imipenem it was 4 µg.mL^{-1}, and for Ciprofloxacin it was 2 µg.mL^{-1} (compounds used alone). Linalool, in association with Imipenem was classified as synergistic for the clinical isolate and standard strains. Ciprofloxacin, in combination with linalool for the strain ATCC was classified as indifferent (FIC=2).

DISCUSSION

The combined use of antimicrobial agents is a routine clinical practice; always seeking an increase in the drug's therapeutic role[17]. Studies on aspects of plant derivatives and the possibility of synergism with conventional antimicrobial drugs are common[18]. Antibiotics interacting synergistically in combinations with herbal extracts against resistant microbial strains are a new strategy for treating infections which allows the use of antimicrobial drugs that when used alone are not effective on certain bacterial strains[19]. Studies with combinations of natural products from plants (or phytochemicals) together with synthetic drugs are still limited, but the results are often positive.

In this study, we evaluated antibacterial activity of linalool (a monoterpene found in many essential oils) /antibiotic associations in combination with antibiotics used in clinical practice against strains of hospital importance. The results showed that linalool, either alone or in combination with Imipenem (resulting in synergism), displayed antibacterial activity against *S. aureus* and*P. aeruginosa* strains. For the *P. aeruginosa* strains tested, the linalool combination with ciprofloxacin was indifferent.

Mossa et al[20] documented the synergism of linalool and α-terpineol from *Melaleuca leucodendron* when combined with ampicillin and kanamycin. Bassolé et al[21] found FIC indices ranging from 0.11 to 2.47 for paired combinations of *L. multiflora*, *Mentha* x *piperita*, and *O. basilicum* essential oils. All of the paired combinations had synergetic effects; inhibiting *E. faecalis*, *L. monocytogenes*, and *E. coli*. Combinations of *L. multiflora* with *Mentha x piperita*, or *O. basilicum* had synergetic effects inhibiting *S. typhimurium*, and *S. dysenteria*.

There are a few generally accepted mechanisms of antimicrobial interaction that produce synergism. They include sequential inhibition of a common biochemical pathway, inhibition of protective enzymes, and the use of cell wall agent activity to enhance the uptake of other antimicrobials[22].

Based on the hypothesis of Pei et al[23], we suggest that the synergetic effects observed might be amplified due to increases in one of three factors which determine a monoterpene's antimicrobial character: its lipophilic properties, its functional groups' potencies, and the paired combination's resulting aqueous solubility[24].

CONCLUSION

This work showed that linalool is able to potentiate the antibacterial activity of existing clinical antibiotics thru synergistic interactions; the molecule could be an alternative for the production of new drugs which are effective against multiresistant microorganisms.

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DECLARATION OF INTEREST

The authors declare no conflicts of interest.

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5.4 Cytotoxic activity of *Ocimum basilicum* essential oil and the monoterpene linalool tested with human erythrocyte hemolysis

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Cytotoxic activity of *Ocimum basilicum* essential oil and the monoterpene linalool tested with human erythrocyte hemolysis

[La actividad citotóxica de aceite esencial de *Ocimum basilicum* y linalol monoterpeno través de la prueba de hemólisis de eritrocitos humanos]

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ABSTRACT*:* Knowledge of the toxicity of natural products is of fundamental importance for safe use by the population. This study aimed to assess the cytotoxic activity of *Ocimum basilicum* essential oil and its major constituent linalool (a monoterpene), through hemolysis testing on human erythrocytes. Red blood cells were obtained from the Transfusion Unit of the University Hospital Lauro Wanderley/UFPB. Hemolytic activity was evaluated using a 0.5% erythrocyte suspension which was treated with differing concentrations of the test product, and Tritox-X as the positive control (100% hemolysis). The hemolytic activity (using hemoglobin (Hb)) was quantified by spectrophotometer. The results showed that the essential oil of *O. basilicum* and linalool present no significant hemolytic activity.

Keywords: *Ocimum basilicum*, linalool, hemolysis, cytotoxicity.

Resumen: La toxicidad de un producto natural es de importancia fundamental para esto se puede utilizar de forma segura por la población. Este estudio tuvo como objetivo evaluar la actividad citotóxica de aceite esencial de *Ocimum basilicum* y su componente principal, el linalool monoterpeno, a través de la prueba de hemólisis de eritrocitos humanos. Las bolsas de las células rojas de la sangre se obtuvieron en la Unidad de Transfusión del Hospital Universitario Lauro

Wanderley / UFPB. La actividad hemolítica se evaluó utilizando un 0,5% de suspensión de eritrocitos que fueron tratados con diferentes concentraciones del producto de ensayo y Tritox -X como control positivo (100% de hemólisis). El efecto hemolítico se cuantificó por espectrofotometría , mediante la cuantificación de la hemoglobina (Hb) . Los resultados mostraron que el aceite esencial de *O. basilicum* y linalool no mostró actividad hemolítica significativa.

Palabras clave: *Ocimum basilicum*, linalool, la hemólisis, la citotoxicidad.

INTRODUÇÃO

Ocimum basilicum (Lamiaceae) is popularly known as basil; it can be found in tropical Asia, Africa, Central America, and South America. It is part of a group of medicinal, aromatic, and culinary plants of great economic value, which are widely used for various ornamental, medicinal, and aromatic reasons; as a spice, and in both the perfume and cosmetics industries (Carovic-Stanko, 2010).

In folk medicine, it is used as an antipyretic, in aiding digestion, and also to combat bacterial and parasitic intestinal infections (Telci *et al*., 2006). Its tea is a digestive stimulant, gastric antispasmodic, and anti-rheumatic, (Lorenzi; Matos, 2002). In aromatherapy it is used to relieve anxiety, stress, depression, emotional coldness, and fatigue, and also for refreshing and strengthening the central nervous system (Grossman, 2005). Umar *et al*. (2010) reported that *O. basilicum* extract has anti-hypertensive activity.

Linalool, 3,7 dimetilocta-1,6-dien-3-ol is a monoterpene found in most of the essential oils of aromatic plants. It is the major constituent of *Ocimum basilicum* oil. It has been widely used as starting compound for several important syntheses, such as ethyl linalyl and is certified as an acaricide, bactericide, and fungicide. In medicine it has been applied successfully as a sedative and is currently being analyzed for its anticonvulsant properties (Radünz, 2004).

Cytotoxic activity detection of an herb is a priority since so many natural chemical compounds are toxic. The evaluation of cytotoxic potential against human erythrocytes is an effective experimental *in vitro* model to investigate both protective and toxic effects for a large

variety of substances. The toxic effects of the tested substances can be directly correlated to erythrocyte hemolysis (Brandão et al., 2005).

The erythrocyte membrane is a delicate structure which can be significantly altered by drug interactions (Aki; Yamamoto, 1991). Several studies indicate that certain compounds isolated from plants, such as polyphenols, glycosides, saponins, and triterpenoids can cause changes in the membranes of red blood cells that subsequently produce hemolysis (Ng Li; Yeung., 1986; Bader et al, 1996; Grinberg et al., 1997; Zhang et al, 1997).

This work aimed to evaluate the cytotoxic effects produced by *O. basilicum* essential oil and linalool thru hemolytic activity in human erythrocytes.

MATERIAL E MÉTODO

Obtaining test substances

The *Ocimum basilicum* L. essential oil and the monoterpene linalool were obtained commercially from Quinari, and Sigma Aldrich, respectively.

Human erythrocytes

Human red blood cells (type O) were from red blood cell bags that could no longer be used for transfusion. They were obtained at the Lauro Wanderley/UFPB University Hospital Transfusion Unit. The handling and disposal of erythrocytes were performed according to the safety standards followed by the unit.

Chromatography of O. basilicum essential oil

Components extraction was made by steam distillation, and the analysis method used high resolution gas chromatography. Chromatographic separation was performed using a DB-5 capillary column (30 m x 0.25 mm (HP). The temperature of the chromatography oven was programmed from 50°C (3 min), 3°C/min, to 170°C. The temperature of the injector and detector were 200 °C. The split was at 1/200, and the detector FID 200ºC. The injection volume was 1.0 µL (0.5% concentration in chloroform). Identification of individual components was based on their mass spectral fragmentation; two computer library MS searches (Wiley 229), and by retention index (RI).

Hemolysis assay

The red blood cells (RBC) bags were obtained in Transfusion Unit of the University Hospital Lauro Wanderley / UFPB. A sample of human blood was mixed with 0.9% NaCl at a ratio of 1:30 and centrifuged at 2500 rpm for 5 minutes to obtain the erythrocytes. This procedure was repeated two more times and the last centrifugation the pellet was resuspended in 0.9% NaCl to obtain a suspension at 0.5%. The test product samples at different concentrations were added to 2 mL of the cell suspension to a final volume of 2.5 mL. An erythrocyte suspension was used as a negative control (0% hemolysis) and a cell suspension plus Triton X-100, 1% as a positive control (100% hemolysis). After that, the samples were incubated for 1 hour at 22 ± 2 ° C under constant slow (100 rpm) stirring. After this time the samples were centrifuged at 2500 rpm for 5 minutes and hemolysis will be quantified by spectrophotometry at a wavelength of 540 nm (Rangel, 1997). All experiments were performed in triplicate and expressed as a plus or minus the standard error of the mean average.

Statistical analysis

The degree of hemolysis in each concentration of extract for hemolytic and anti-hemolytic test were presented as means ± standard deviation. Paired t-test was used to compare the treated and control groups. A significance level at $p< 0.05$ was adopted. In Stat Graph pad software was used to perform statistical analysis.

RESULTS

In this work, chromatography showed that the majority phytochemicals for *O. basilicum* essential oil were linalool (55.2%), and 1,8-Cineole (8.8%) (Table 1).

Table 1- Chromatography of essential oil of *Ocimun basillicum.*

RI	**Compounds**	**%**
928	α-pinene	0.4
972	β-pinene	1.1
987	Myrcene	0.7
1034	1,8-Cineole	8.8
1041	*trans*-β-Ocimene	0.6
1099	Linalool	55.2
1182	Terpinen-4-ol	0.9
1356	Eugenol	3.2
1421	B-Caryophyllene	0.4
1439	α-*trans*-Bergamotene	7.0
1489	Germacrene D	2.2
1515	γ-Cadineno	2.9
1638	Muurolol	2.9

RI: Retention Index

In the evaluation of *O. basilicum* essential oil and linalool cytotoxicity against human erythrocytes a low hemolytic activity (hemolysis rate <15%) was observed (Figure 1) for the products as compared with the groups treated with Triton- X (Control +); indicating no damage to cell membranes of human erythrocytes.

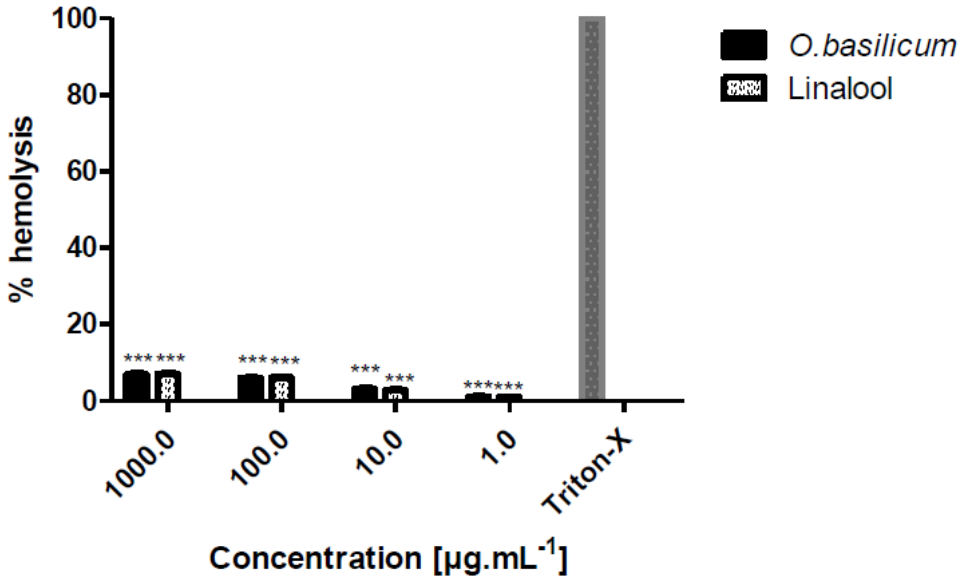

Figure 1: Percent hemolysis of human erythrocytes following treatment with the *O. basilicum* essential oil and linalool. The columns and the bars represent the mean ± standard error of triplicate experiments with a 95% confidence interval. The comparison of the groups was made by t test, *** p <0.001 compared to the control group (Triton X = 100% hemolysis) using the Graph Pad Prism version 4 program.

DISCUSSION

Among more than 65 species of the genus *Ocimum*, basil is the major essential oil crop which is cultivated commercially in many countries (Sajjadi, 2006). Basil has a characteristic odor and sharp taste. The plant probably originated in India, Afghanistan, Pakistan, Northern India and Iran, and now is cultivated worldwide. Traditionally, basil has been extensively utilized in food as a flavoring agent, and in perfumery and medical industries (Telci et al., 2006). However, recently the potential uses of *O. basilicum* essential oil, particularly as antimicrobial and antioxidant agents have also been investigated (Lee et al., 2005; Wannissorn et al., 2005). The *O. basilicum* essential oils exhibited a wide and varying array of chemical compounds, depending on variations in chemotypes, leaf and flower colors, aroma and origin of the plants (Da-Silva et al., 2003). One of the main constituents of the essential oil *O. basilicum* is linalool.

Linalool has known bioactivity (Cheng et al 2012). Lang et al (2012) points out that essential oils containing linalool are active against microorganisms including fungi. However, Chang et al

(2000) showed that the activity of the essential oil of *Cinnamomum osmophloeum*, (rich in linalool) is less intense against wood-destroying fungi than either eugenol or cinnam aldehyde oils (of other chemo-types).

Although natural products are widely considered less risky if compared with synthetic drugs, they are not completely free of toxicity or other adverse effects (De Smet, 2004).

The hemolytic assay is one of the most widely used experimental *in vitro* models to verify toxicity, being able to evaluate the cytotoxicity of various xenobiotics. The erythrocyte membrane is very sensitive and its structure can be significantly altered by molecular interactions, making it possible to estimate potential *in vivo* erythrocyte damage.

Thus, with the results obtained in this study, one can infer that *O. basilicum* essential oil and linalool have low toxicity against eukaryotic cells at the concentrations tested. The values of % hemolysis are only considered high when above 15 %, since they indicate promotion of extensive erythrocyte membrane damage (Prete et al., 2011).

Venancio (2006) showed that the major compound found in *O. basilicum* essential oil was linalool, which in the acute toxicity studies presented an LD_{50} of 0.5321 g/kg of body weight.

There are studies involving the hemolytic activity of substances extracted from different parts of plants. Silva et al. (2008), evaluated the activity of essential oil obtained from "guacatonga" (*Casearia sylvestris*), and observed hemolysis induction in seven different kinds of erythrocytes, indicating the need for caution and restraint when using elaborate preparations of this plant. Subsequently, Tariku et al. (2010) found that essential oils of *Artemisia abyssinica* and *Satureja punctata* ssp. caused damage, 50% erythrocyte lysis (LC_{50}) at concentrations of 0.35, and 1.52 $\mu L.mL^{-1}$ respectively. Tariku et al. (2011) studied the hemolytic effects of *Artemisia absinthium*, and *Echinops kebericho* essential oils obtaining LC_{50} values of 1.52, and 2.62 $\mu L.mL^{-1}$ respectively, indicating again the need for restraint in the use of essential oils.

In this work, it was concluded that *O. basilicum* essential oil and linalool do not damage the human erythrocyte membrane at the concentrations tested, indicating that these compounds possess low toxicity against eukaryotic cells.

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5.5 Assessment of genotoxic effect of *Ocimum basilicum* L. and Linalool

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Assessment of genotoxic effect of *Ocimum basilicum* L. and Linalool

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Ocimum basilicum L. is a plant that belongs to the Lamiaceae family, popularly known as basil and has the Linalool monoterpene as its major constituent. In this study, the essential oil of *O. basilicum* and Linalool were evaluated for its genotoxic effects. The micronucleus test on peripheral blood cells was used in the study for activities. Groups of three mices males and three females received, by gavage, the compounds in dose of 100 and 200 mg/kg of animal weight. The negative control group received only the dispersant of the sample (distilled water) and positive control received Cyclophosphamide 50 mg/kg of animal weight. Twenty-four hours after treatment, the animals were sacrificed and blood was collected from the caudal vein and made a smear on the slide. The obtained results showed the absence of genotoxic effect of tested compounds.

Uniterms: *Ocimum basilicum*, micronucleus, linalool, genotoxic.

Ocimum basilicum é uma planta da família Lamiaceae, conhecida popularmente como manjericão e possui como seu constituinte majoritário o monoterpeno Linalol. Neste estudo, o óleo essencial de *O. basilicum* e o Linalol foram avaliados para os efeitos genotóxicos. O teste de micronúcleos em células de sangue periférico foi usado neste trabalho. Grupos de três camundongos machos e três camundongos fêmeas receberam, via gavagem, os compostos na dose de 100 e 200 mg/kg do peso do animal. O grupo controle negativo recebeu apenas o dispersante da amostra (água destilada) e o controle positivo recebeu ciclofosfamida 50 mg/kg do peso do animal. 24 horas após o tratamento, os animais foram sacrificados, o sangue da veia caudal foi coletado e feito lâminas de esfregaço. Os resultados obtidos mostraram a ausência de efeito genotóxico dos compostos testados.

Unitermos: *Ocimum basilicum*, micronúcleo, linalol, genotoxico.

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INTRODUCTION

It is known since antiquity population makes use of medicinal plants as an alternative source for the treatment of various diseases. Because they are natural, many adopted mistakenly the concept that they are not toxic to the body. However, this consumption should be monitored in order to warn of possible effects on living organisms , since they are often exposed to mutagenic substances that can cause cell damage (Costa, Menk; 2000).

Worldwide, has found strong relationship between exposure to genotoxic agents and the development of many adverse health effects. There is a growing concern about the mutagenic and carcinogenic effect of genotoxic agents in exposed occupational populations or accidentally, or by lifestyle (Flores, Yamaguchi, 2008). Genetic toxicology tests are assays designed to detect direct or indirect genetic damage induced by chemical compounds. Fixation of DNA damage can result in gene mutations, loss of heterozygosity, chromosome loss or gain, and chromosome aberrations. These events may play an important role in many malignancies. Thus, identifying genotoxic/mutagenic effects is important for the risk/benefit assessment of substances, in particular those which are part of the dietary habits of any populations (Doppalapudi *et al*., 2007).

The micronucleus test "in vivo" is widely accepted by international agencies and government institutions as part of the recommended battery of tests to establish the evaluation and registration of new chemicals and pharmaceutical annually entering the world market and that may have mutagenic activity (Ribeiro, Salvadori and Marques, 2003).

Basil (*Ocimum basilicum* L.), a member of the Lamiaceae family is an annual herb which grows in several regions around the world. Traditionally, basil has been extensively utilized in food as a flavoring agent, and in perfumery and medical industries (Telci *et al.,* 2006). The leaves and flowering tops of the plant are perceived as carminative, galactogogue, stomachic and antispasmodic in folk medicine (Sajjadi, 2006). However, recently the potential uses of *O. basilicum* essential oil, particularly as antimicrobial and antioxidant agents have also been

investigated (Lee *et al*., 2005; Wannissorn *et al*., 2005).The studies in the literature suggest linalool, the monoterpen, as the main active agent responsible for antibacterial activity (Ravid *et al*., 1997).

Considering the absence of studies on the toxic effects of this plant, the aim of the present study was to evaluate the genotoxic activities of *O. basilicum* and linalool using the micronucleus test on peripheral blood cells.

MATERIAL AND METHODS

Compounds

The *Ocimum basilicum* L. essential oil and the monoterpene linalool were obtained commercially from Quinari, and Sigma Aldrich, respectively.

Chromatography essential oil *O. basilicum*

The oil was obtained from the leaves of plants, the extraction of components was made by steam distillation and the analysis method was gas chromatography high resolution. Chromatographic separation was performed using a DB-5 capillary column (30 m x 0.25 mm (HP). The temperature of the chromatographer oven was programmed from 50°C (3 min), 3°C/min, to 170°C. The temperature of injector and detector were 200 °C. The split 1/200 and detector FID 200ºC. The injection volume was 1.0 µL (0.5% concentrated in chloroform). Identification of individual components was based on their mass spectral fragmentation based on two computer library MS searches (Wiley 229) and retention index.

Animals treatment

The use of animals was approved by the Ethics Committee for Animal Research Laboratory of Pharmaceutical Technology/UFPB under registration number 0101/11. For the realization of experimental models were used five to six-week old albino Swiss mice (*Mus musculus*), weighing approximately 30 g from the Biotery Prof. Thomas George -UFPB. The animals were acclimated to the bioterium local conditions for about seven days before the experimental tests under temperature (21±2 ° C) and controlled light-dark cycle of 12 hours. The animals were fed chow and water ad libitum and were distributed in the different experimental group sat random.

Micronucleus test

To perform the micronucleus test, the animals were sacrificed with xylazine (5mg/kg) in accordance with existing regulations to prevent anxiety or fear (stress) (Andrade; Pinto; Oliveira, 2006) and then blood samples were collected from the caudal vein of mices.

The micronucleus test on peripheral blood cells was carried out as described by Hayashi *et al*. (1994), who concluded that bone marrow cells can be replaced by peripheral blood as material for the micronucleus assay. This is allowed because, alternatively in mice, the micronuclei can be analyzed in circulating normochromatic erythrocytes (NCE, erythrocytes), whereas the spleen of mice did not hijack the blood micronucleated erythrocytes.

Groups of three mices males and three females received, by gavage, the essential oil of *O. basilicum* or linalool in dose of 100mg/kg to 200 mg/kg. The negative control group received only the dispersant of the sample (distilled water) and positive control received Cyclophosphamide 50 mg/kg of animal weight. Twenty-four hours after treatment, the animals were sacrificed, blood was collected from the caudal vein and made a smear on the slide.

Analysis of the slides

The slides were stained with Panotic and observed under an optical microscope (Zeiss) increasing 1000x (objective = 100 x with eyepiece = 10 x) for counting the micronucleus. Were assessed at least 2,000 NCE per slides (Hayashi *et al.,* 1994).

In this study, the presence of micronucleus in erythrocytes of mice in the positive control was not influenced by gender ($p>0,05$), so data were pooled to determine the average number of micronucleus to calculate the standard error of the mean and to assess differences between groups.

The data from the micronucleus assay were statistically analyzed using Student's t-test, comparing the treated groups with controls (Pereira, 1991). The significance level considered was $p<0.05$. Results were expressed as mean±standard error of mean.

RESULTS AND DISCUSSION

The detection of cytotoxic activity, genotoxic and / or mutagenic is a priority measure in the production of a herbal medicine , since various chemical compounds may be capable of causing toxic effects and even modify the genetic information contained in DNA. Obtaining data on the

toxicity of these agents should be anticipated by experiments that can provide, with reasonable safety margin, an indication of the risks involved in their use (Benigni, 2005).

Through phytochemical prospecting of the essential oil of *O. basilicum,* it was possible to determine the presence of diverse compounds. The chromatography results are shown in table I. It was observed that the essential oil of *O. basilicum* presented as major compound linalool.

The arial parts of *O. basilicum* are reported to have strong medicinal use like antimicrobial and antiviral property and with high vitamin and mineral content (Chiang *et al*, 2005).It contains a chemical, eugenol, which is antimicrobial. Studies show that the chief constituents include chavicol methyl ether or estragole, linalool and eugenol (Hussain *et al*., 2008; Omidbaigi *et al*., 2003). The studies in the literature suggest the monoterpene linalool as the main active agent responsible for antibacterial activity.

Table I- Chromatography of essential oil of *Ocimun basillicum*

Compounds	**%**
α-pinene	0.4
β-pinene	1.1
Myrcene	0.7
1,8 cineol	8.8
trans-β -ocimene	0.6
Linalool	55.2
Terpinen-4-ol	0.9
Eugenol	3.2
β -Caryophyllene	0.4
Bergamotene	7.0
Germacrene D	2.2
γ- Cadineno	2.9
Muurolol	2.9

The evaluation of micronucleus induction is the main test in vivo in a battery of genotoxicity tests and is recommended by enforcement agencies around the world as part of the safety assessment of chemicals and natural products. The test, when performed correctly, detects both effects: clastogenic and aneugenic (Krishna; Hayashi, 2000).

Micronuclei are indicative of numerical and/or structural chromosome aberrations during cell mitosis. Other authors have used the micronucleus test as a biomarker for chromosome

instability and malignancy, observing higher frequencies of micronucleated cells among cancer patients than among healthy individuals (Kamboj, Mahajan, 2007; Lou *et al.*, 2007).

The results showed that the essential oil *O. basilicum* and its major compound linalool at doses of 100 and 200 mg/kg showed no genotoxic activity (Table II), since the amount of micronuclei formed was significantly smaller than those formed in the groups treated with cyclophosphamide positive control (p<0,05) (Figure 1, 2 and 3).

Table II- Micronucleus frequency in 2000 found peripheral blood erythrocytes of mice of different experimental groups.

Experimental group	**Number of micronucleated erythrocytes (mean ± s.e.m)**
Negative control	1.5 ± 0.42***
Cyclophosphamide (50 mg/Kg)	43.5 ± 5,89
E.O *O. basilicum* (100 mg/Kg)	1.167 ± 0.3***
E.O *O. basilicum* (200 mg/Kg)	1.5 ± 0.34***
Linalool (100 mg/Kg)	3.33 ± 0.49***
Linalool (200mg/Kg)	5.3 ± 0.61***

E.O: Essential oil. Tests were performed in triplicate (n=6) with a confidence interval of 95%. The comparison between groups were performed using the *t* test for the program GraphPadPrism 5. ***p<0.001 compared with positive control.

FIGURE 1: Micronucleus in mice red blood cells treated with *O. basilicum* at doses of 100 mg/Kg (A) and 200 mg/Kg (B).

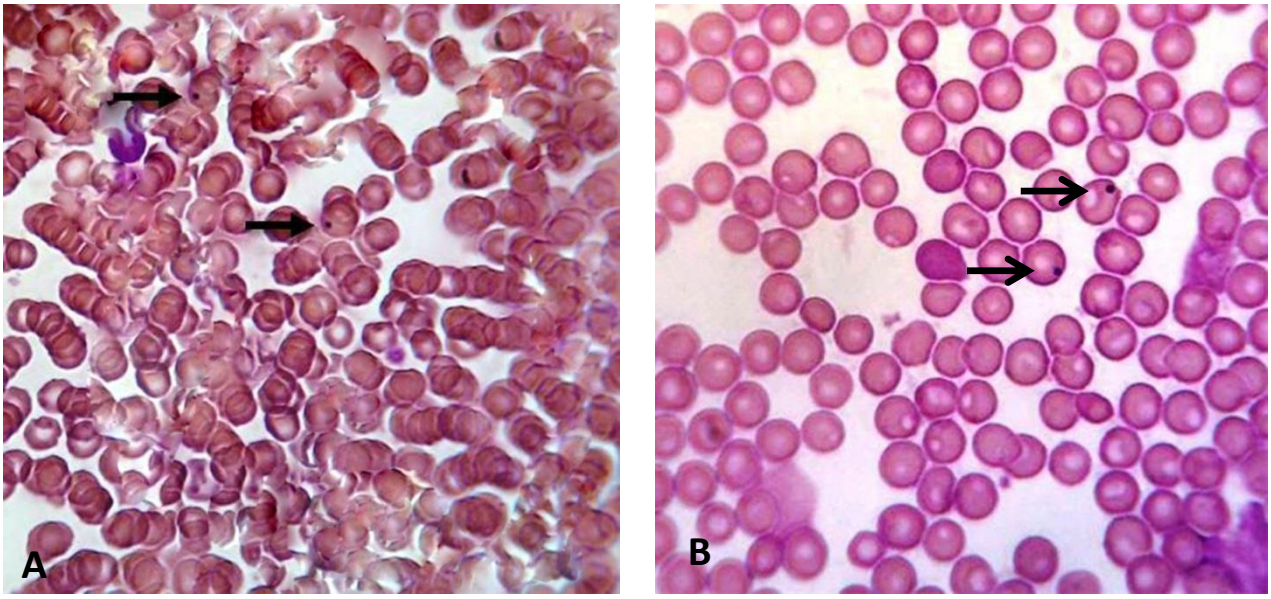


FIGURE 2: Micronucleus in mice red blood cells treated with Linalool at doses of 100 mg/Kg (A) and 200 mg/Kg (B).

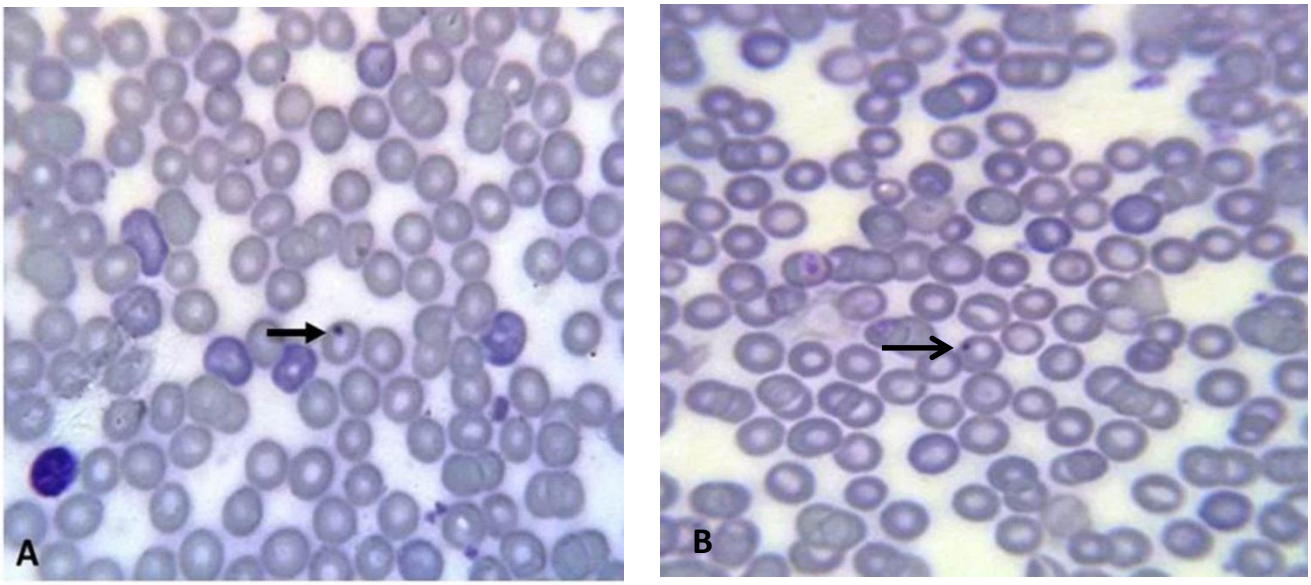
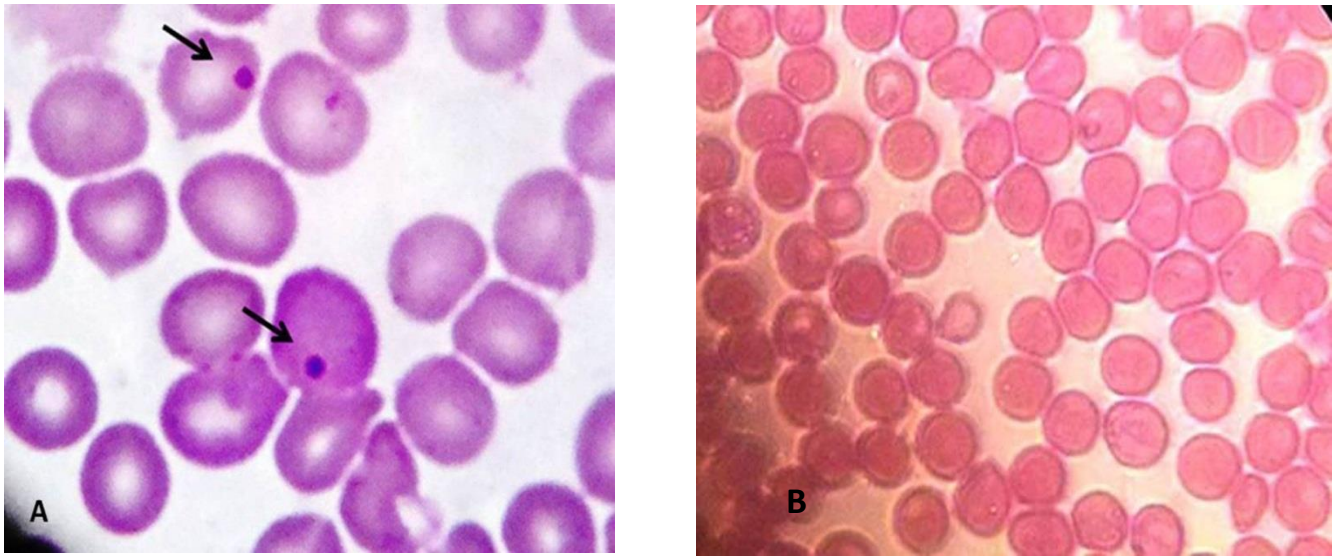


FIGURE 3: Micronucleus in mice red blood cells treated with cyclophosphamide (50 mg/Kg) (A) and water (B).



Fletcher *et al*. (2005) evaluating the genotoxicity of *Malaleuca angustifolia* oil as well as the main oil component, 4- terpineol , and the results showed 4- terpineol showed toxicity only at the highest dose used.

Santos (2011) showed that the essential oil of oregano did not induce micronucleus formation in Wistar rats revealed no genotoxicity in the essential oil.

CONCLUSION

Through the results obtained, it can be concluded that the essential oil of *O. basilicum* e and your major compound linanool does not induce an increase in the frequency of the micronucleus characterized as an agent not mutagenic in these conditions. Further studies of toxicity need to be made to the use of this essential oil in the treatment of diseases to be stimulated.

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Conclusão

6. CONCLUSÕES

Com base nos estudos de atividade antimicrobiana, citotoxicidade e genotoxicidade do óleo essencial de *O. basilicum* e do monoterpeno linalol realizados neste trabalho pode-se concluir que:

- O linalol foi o constituinte majoritário do óleo essencial do *O. basilicum* utilizado nesse estudo;
- O óleo essencial do *O. basilicum* apresentou atividade antibacteriana contra cepas de *S. aureus* com CIM variando entre 1024 a 512 μg/mL e *P. aeruginosa* com CIM de 1024 μg/mL, sendo algumas destas cepas resistentes. A CBM para ambas as cepas foi maior que 1024 μg/mL.
- O linalol apresentou atividade antibacteriana contra cepas de *S. aureus* e *P. aeruginosa* com CIM variando de 1024 a 32 μg/mL. A CBM para ambas as cepas foi maior que 1024 μg/mL.
- A cinética de morte microbiana do óleo e do linalol demonstrou que na concentração de CIMx4 e após 8h de contato ambos compostos possuem atividade antibacteriana caracterizada como bactericida frente a cepas de *S. aureus*.
- A associação do óleo de *O. basilicum* ou do linalol com o imipenem apresentou efeito sinérgico frente a cepas de *S. aureus.* Já para a ciprofloxacina, a associação do óleo mostrou efeito antagonista e do linalol efeito aditivo para as mesmas cepas.
- A associação do óleo de *O. basilicum* ou do linalol com o imipenem apresentou efeito sinérgico ou aditivo frente a cepas de *P. aeruginosa*. Já para a ciprofloxacina, a associação do óleo mostrou efeito indiferente ou sinérgico (cepa de origem clínica) e do linalol efeito indiferente.
- O óleo essencial de *O. basilicum* e o linalol apresentaram um baixo poder citotóxico.
- O óleo essencial de *O. basilicum* e o linalol apresentaram um baixo efeito genotóxico em camundongos tratados por via oral.

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Apêndice

Apêndice A- Artigo publicado na revista International Journal of Pharmacognosy and Phytochemical Research (IJPPR)

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Research Article

Antibacterial Activity of the Monoterpene Linalool: Alone and in Association with Antibiotics Against Bacteria of Clinical Importance

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ABSTRACT

Antibacterial activity studies of new molecules, either alone or in combination with existing antibiotics, are of great importance considering the resistance acquired by microorganisms in recent times. Linalool is a phyto-constituent found in the essential oils of various plant species. It is a monoterpene widely used in perfumery, cosmetics, and the food industries. Our objective was to determine the pharmacological effects produced on the bacterial strains *Staphylococcus aureus,* and *Pseudomonas aeruginosa* when combining standard antibiotics with linalool. The Minimum Inhibitory Concentration (MIC) was calculated using microdilution technique, where the linalool concentrations varied from 2 to 1024 µg /mL. Combinations with standard antibiotics were analyzed by the checkerboard method where the fractional inhibitory concentration (FIC) indices were calculated. Linalool, Imipenem, and Ciprofloxacin showed respective MIC antibacterial activities against *S. aureus* of 1024, 4, and 2 µg/mL. In *S. aureus*, the linalool with Imipenem association showed a synergistic effect (FIC = 0.0625); while with ciprofloxacin, the linalool showed additivity (FIC = 0.75). In *P. aeruginosa*, the Imipenem/linalool association was synergistic for both the ATCC and clinical strains (FIC = 0.0625). The association of *linalool* with ciprofloxacin was indifferent. We conclude that Linalool associated with existing standard antibiotics may increase antibacterial effectiveness, resulting in synergistic activity against bacterial strains of clinical importance. This makes the molecule potentially important for production of new, therapeutically effective drugs against resistant microorganisms.

Key words: natural products, antibacterial activity, synergism, linalool.

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ORIGINAL ARTICLE

Ocimum basilicum: Antibacterial activity and association study with antibiotics against bacteria of clinical importance

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ABSTRACT

Context: *Ocimum basilicum* L. (Lamiaceae), popularly known as basil, is part of a group of medicinal plants widely used in cooking and known for its beneficial health properties, possessing significant antioxidant effects, antinociceptive, and others.

Objective: The objective of this study is to determine the pharmacological effects produced on the bacterial strains *Staphylococcus aureus* and *Pseudomonas aeruginosa* when standard antibiotics and *O. basilicum* essential oil are combined.

Materials and methods: The extraction of *O. basilicum* (leaves) components was done by steam distillation. The Minimum inhibitory concentration (MIC) was calculated using microdilution technique, where the oil concentrations varied from 2 to 1024 µg/mL. The combinations of *O. basilicum* oil with ciprofloxacin or imipenem were analyzed by the checkerboard method where fractional inhibitory concentration (FIC) indices were calculated.

Results: *Ocimum basilicum* essential oil, imipenem, and ciprofloxacin showed respective MIC antibacterial activities of 1024, 4, and 2 µg/mL, against *S. aureus*. In *S. aureus*, the oil with imipenem association showed synergistic effect (FIC = 0.0625), while the oil with ciprofloxacin showed antagonism (FIC value = 4.25). In *P. aeruginosa*, the imipenem/oil association showed additive effect for ATCC strains, and synergism for the clinical strain (FIC values = 0.75 and 0.0625). The association of *O. basilicum* essential oil with ciprofloxacin showed synergism for clinical strains (FIC value = 0.09).

Conclusion: *Ocimum basilicum* essential oil associated with existing standard antibiotics may increase their antibacterial activity, resulting in a synergistic activity against bacterial strains of clinical importance. The antibacterial activity of *O. basilicum* essential oil may be associated with linalool.

KEYWORDS

Ciprofloxacin, *Pseudomonas aeruginosa*, imipenem, *Staphylococcus aureus*

HISTORY

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Research Article

Antibacterial Activity of *Ocimum basilicum* Essential Oil and Linalool on Bacterial Isolates of Clinical Importance

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ABSTRACT

Ocimum basilicum, popularly known as Basil, is a Lamiaceae family species widely known to treat different diseases. This species has as its main compound the monoterpene linalool. This study aimed to determine the antibacterial activity of *O.basilicum* essential oil and linalool against *S. aureus* and *P. aeruginosa* strains, as well as times to bacterial death facing each substance. The extraction of the *O. basilicum* leaves components was made by steam distillation. The Minimum Inhibitory Concentration (MIC) was calculated using microdilution technique and assessment of bacterial kinetics was performed with time-to-kill methodology. The results showed that *O. basilicum* essential oil and linalool display antibacterial activity against both *S. aureus* and *P. aeruginosa*, with certain strains of *P. aeruginosa* being resistant to the oil. Bacterial kinetics testing showed bacteriostatic activity against the strains in almost all concentrations, while only the MIC x 4 concentration of either essential oil or linalool against *S. aureus* displayed bactericidal activity. We conclude that the *O. basilicum* essential oil has antibacterial activity characterized as bacteriostatic or bactericidal against clinical isolates, and this activity is likely associated with linalool, its major compound.

Key words: Basil, bacterial resistance, medicinal plants, linalool.