GIOVANA CAROLINA BODNAR

ATIVIDADE ANTIBACTERIANA DE NANOPARTICULAS DE PRATA ASSOCIADAS AO EUGENOL

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Tese apresentada ao Programa de Pós-Graduação em Microbiologia como requisito parcial à obtenção do Título de Doutor em Microbiologia.

Orientador: Prof. Dr. Gerson Nakazato

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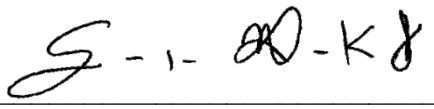

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Londrina, 04 de setembro de 2018.

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Bodnar, Giovana Carolina .
Atividade antibacteriana de nanopartículas de prata associadas ao eugenol / Giovana Carolina Bodnar. - Londrina, 2018.
103 f. : il.

Orientador: Gerson Nakazato.
Tese (Doutorado em Microbiologia) - Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Microbiologia, 2018.
Inclui bibliografia.

1. Nanopartículas - Tese. 2. Antimicrobianos - Tese. 3. Óleos essenciais - Tese. 4. Sinergismo - Tese. I. Nakazato, Gerson. II. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Microbiologia. III. Título.

Dedico este trabalho aos meus pais Alcides Bodnar e Rosilda Bodnar, por todo exemplo, amor, carinho, apoio e compreensão. E ao meu irmão Victor Bodnar por todo companheirismo e incentivo. Minha eterna gratidão a vocês.

AGRADECIMENTOS

À Deus por me guiar nos momentos mais difíceis, me dar forças para continuar a minha caminhada e por todas as oportunidades que foram colocadas no meu caminho.

À minha mãe Rosilda Aparecida Bodnar, por todo apoio e dedicação aos filhos, por sempre ter uma palavra amiga e de conforto nas horas difíceis permitindo que eu seguisse em frente com meus estudos. Ela com certeza foi minha maior incentivadora em não desistir pelo caminho.

Ao meu pai Alcides Bodnar, que sempre incentivou meus estudos, me apoia em tudo na vida e é um exemplo de dedicação e profissionalismo. Ele é meu espelho para me tornar uma excelente profissional.

Ao meu irmão Victor R. Bodnar, pela paciência, amizade e companheirismo; principalmente no período do Doutorado Sanduíche nos Estados Unidos.

À toda minha família que sempre me ensinou a lutar pelos meus objetivos.

Ao meu orientador Prof. Dr. Gerson Nakazato, pelas excelentes explicações, pelo profissionalismo e bom humor. E também por permitir que eu iniciasse outra graduação, mesmo em meio ao Mestrado e ao Doutorado. Pelo, apoio, dedicação e compreensão nos trabalhos realizados, e principalmente por entender minha ausência no laboratório em alguns momentos. Principalmente pelo incentivo na realização do meu Doutorado Sanduíche na *Oklahoma State University*, US.

À Prof. Dr. Renata Kobayashi, por todo apoio e paciência. Por tratar os alunos como “filhos”, sempre com preocupação e disposição a ensinar. Por permitir que os alunos participassem não só de pesquisas, mas de projetos de ensino. E por todas as conversas e conselhos.

À Prof. Dr. Marcia Perugini que incentivou minha jornada em Microbiologia antes mesmo do Mestrado e me apresentou ao Laboratório do Prof. Gerson. Também pela oportunidade de estágio no Hospital Universitário, onde aprendi muito sobre Microbiologia Clínica.

Aos colegas e amigos do Laboratório de Bacteriologia Básica e Aplicada da UEL (NIP3), por todo apoio, compreensão, união e momentos de descontração. Principalmente pelas dúvidas e explicações durante este último ano que eu estava nos Estados Unidos para realização do Doutorado Sanduíche. Em especial, Érica, Erick, Sara e Meiriele, agradeço por todo auxílio, explicação e paciência.

Aos alunos e professores do Laboratório de Biologia Molecular de Microrganismos (NIP 5) e Laboratório de Micologia Médica e Microbiologia Bucal (NIP 9), que direta ou indiretamente auxiliaram na pesquisa.

Aos professores e colegas de turma do Doutorado, em especial Paula e Anna Carolina.

Aos técnicos de laboratório Oswaldo e Ediel, que auxiliaram no preparo de material.

À Prof. Dr. Li Maria Ma, minha co-orientadora no exterior, pela oportunidade, por me receber tão bem, por todos os ensinamentos, por confiar em mim e na pesquisa desenvolvida.

Ao Prof. Dr. Peter Muriana, que concedeu espaço em seu laboratório no *Robert M. Kerr Food & Agriculture Products Center* da *Oklahoma State University* para o desenvolvimento de parte da pesquisa no exterior

À Claudia Diaz, minha colega de laboratório e principalmente amiga, durante o Doutorado Sanduíche na *Oklahoma State University*.

À todos os professores e alunos do *National Institute for Microbial Forensics and Food and Agricultural Biosecurity* (NIMFFAB) e do *Department of Entomology and Plant Pathology* da *Oklahoma State University,* que me receberam de portas abertas e de alguma forma me ajudaram ou fizeram parte do meu Doutorado Sanduíche. O aprendizado que obtive no exterior contribuiu muito para minha formação.

Aos meus amigos que por muitas vezes foram compreensivos e apoiaram sempre minhas decisões.

Ao Programa de Pós-Graduação em Microbiologia pela oportunidade de exercer a pesquisa e o aprendizado com excelentes profissionais.

À Universidade Estadual de Londrina, ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo apoio e suporte financeiro para realização desta pesquisa.

Sou eternamente grata a todos que contribuíram direta ou indiretamente para a realização deste trabalho.

BODNAR, Giovana Carolina. **Atividade antibacteriana de nanopartículas de prata associadas ao eugenol**. 2018. 98 f. Tese (Doutorado em Microbiologia). - Universidade Estadual de Londrina, Londrina, 2018.

RESUMO

Bactérias multirresistentes representam um grande problema que desafiam a saúde pública em encontrar estratégias utilizando os antimicrobianos atuais disponíveis no mercado para combater estas infecções. Por outro lado, o desenvolvimento de novos fármacos não ocorre no mesmo ritmo dos mecanismos de resistência desenvolvidos pelas bactérias. A resistência aos antimicrobianos não é apenas um problema da área clínica. No setor de alimentos diversos patógenos humanos vêm adquirindo resistência aos desinfetantes utilizados na indústria alimentícia. Sendo assim, pesquisadores buscam por novas substâncias antimicrobianas que podem auxiliar no tratamento das infecções multirresistentes pelo desenvolvimento de alternativas aos antibióticos e aos desinfetantes disponíveis. Os produtos naturais e a associação de compostos tornaram-se um importante campo de pesquisa para descoberta de novos antimicrobianos. O objetivo desta pesquisa foi avaliar a atividade antibacteriana de nanopartículas de prata biologicamente sintetizada pelo fungo *Fusarium oxysporum* em associação ao eugenol. Eugenol e bio-AgNP demonstraram efeito bactericida, com concentração inibitória mínima (MIC) entre 0.12 a 2% (v/v) e 39.4 a 630 µM, respectivamente; e todos os isolados clínicos de *Staphylococcus aureus* meticilina resistente (MRSA) analisados foram sensíveis aos compostos. Para a curva de crescimento e morte, eugenol agiu em menos de 2 h, enquanto as nanopartículas levaram de 0.5 a 1 h para eliminar totalmente as bactérias Gram-negativas e 24 h para as Gram-positivas. O efeito sinérgico ou aditivo foi observado quando os compostos foram combinados, com redução dos valores de MIC, para ½ MIC, 1/3 MIC ou até mesmo ¼ MIC, o que melhorou o tempo de ação da bio-AgNP comparado ao uso em isolado. A Microscopia Eletrônica de Varredura demonstrou alterações morfológicas celulares após os tratamentos com os compostos em isolado ou em combinação. No ensaio de biofilme, a combinação dos compostos reduziu em 5-log o número de células. As bio-AgNP associadas ao eugenol, composto derivado do óleo de cravo, são alternativas antimicrobianas que neste estudo demonstraram um importante papel contra bactérias Gram-positivas e Gram-negativas, incluindo também cepas multirresistentes, além de atuar contra o biofilme formado por estas bactérias. Sendo assim, podem ser uma estratégia alternativa para o controle de infecções, e apresentam um potencial não somente na área clínica/hospitalar, mas também aplicação na indústria.

Palavras-chave: Nanopartículas de prata. Óleo essencial. Eugenol. Antimicrobianos. Sinergismo.

BODNAR, Giovana Carolina. **Antibacterial activity of silver nanoparticles in association with eugenol**. 2018. 98 pp. Thesis (Doctorate Degree in Microbiology) - State University of Londrina, Londrina, 2018.

ABSTRACT

Multidrug resistant bacteria are a major public health challenge in finding strategies using the current antibiotics available in the market to fight these infections. On the other hand, the development of new drugs does not occur in the same rhythm as the resistance mechanisms developed by bacteria. Antimicrobial resistance is not only a clinical problem. In the food sector, several human pathogens have been acquiring resistance to disinfectants used in the food industry. Therefore, researchers are looking for new antimicrobial that can effort in the control of multidrug resistant infections, developing alternatives to the available antibiotics and disinfectants. Natural products and the association of compounds have become an important field of research for discovery new antimicrobials. The aim of this research was to evaluate the antibacterial activity of eugenol in association of silver nanoparticles biologically synthesized by a fungal *Fusarium oxysporum*. Eugenol and bio-AgNP showed bactericidal effect against all strains tested; with minimal inhibitory concentrations (MIC), ranging from 0.12 to 2% (v/v) and 39.4 to 630 µM respectively and all clinical isolates of methicillin resistant Staphylococcus aureus (MRSA) analyzed were sensitive to the compounds. Time-kill curves showed eugenol acted in less than 2 h, while the metallic nanoparticles took 0.5 h to 1 h to eliminate Gram-negative bacteria and 24 h to kill Gram-positive bacteria. A synergistic or additive effect was observed when the compounds were combined, which improved the time of action compared to bio-AgNP used alone and MIC reduction was to ½, 1/3 or ¼ MIC. Scanning Electron Microscopy (SEM) showed related morphological alterations in cells exposed to the compounds isolated or in combination. Biofilm lethality assay were able to reduce in 5-log cells the biofilm-formed. The bio-AgNP associated with eugenol, a compound derived from clove oil, are antimicrobial alternatives that in this study demonstrated an important role against Gram-positive and Gram-negative bacteria, also including multidrug resistant strains, besides acting against the biofilm formed by these bacteria. Thus, they may be an alternative for the control of infections, not only in the clinical or hospital area, but also in the industry.

Keywords: Silver nanoparticles. Essential oil. Eugenol. Antimicrobial. Synergism.

LISTA DE ILUSTRAÇÕES

REVISÃO DE LITERATURA

ARTIGO I

ARTIGO II

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

µL	Microlitros
µM	Micromolar
Ag^{+}	Íon prata
Ag^{0}	Prata metálica
Ag_2S	Sulfeto de Prata
$AgNO_3$	Nitrato de prata
AgNP	Nanopartículas de prata
Ag-O	Óxido de prata
ATP	Adenosina trifosfato
BHI	*Brain Heart Infusion* (Infusão de cérebro e coração)
bio-AgNP	Nanopartículas de prata biológicas
CC50	Concentração citotóxica a 50%
CDC	*Center for Disease Control and Prevention*
CFU	*Colony forming unit* (Unidade formadora de colônia)
CLSI	*Clinical and Laboratory Standards Institute*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO_2	Dióxido de carbono
DLS	Espalhamento dinâmico de luz
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
E	Eugenol
FDA	*Food and Drug Administration*
FIC	Fractionary inhibitory concentration (Concentração inibitória fracionada)
GRAS	*Generally Recognized as Safe* (Geralmente reconhecido como seguro)
HEp-2	*Human laryngeal epitelial carcinoma* (linhagem celular tumoral)
HU	Hospital Universitário
IC50	*Inhibitory Concentration at 50%* (Concentração inibitória máxima a 50%)
log	Logaritmo
LPS	Lipopolissacarídeos
m	metro
M	Molar

MEV	Microscopia eletrônica de varredura
mg	Miligrama
MH	Muller Hinton
MIC	*Minimal Inhibitory Concentration* (Concentração inibitória mínima)
min	Minuto
ml	Mililitro
mM	milimolar
MRSA	Methicillin resistant *Staphylococcus aureus (Staphylococcus aureus* resistente à meticilina)
MTT	Brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio
mV	Milivolts
nm	Nanômetro
NP	Nanopartícula
OE/EO	Óleo essencial
OMS	Organização Mundial da Saúde
OsO_4	Tetróxido de ósmio
OSU	*Oklahoma State University*
p<0.05%	Probabilidade menor que 5%
p>0.05%	Probabilidade maior que 5%
PBS	Solução Salina Tamponada de Fosfato
pH	Potencial de hidrogênio
RBC	*Red blood cells* (Hemácias)
RMN	Ressonância magnética nuclear
ROS	Espécies reativas de oxigênio
RPM	Rotação por minuto
s	Segundo
SD	Desvio padrão
SEM	*Scanning Electron Microscopy* (Microscopia eletrônica de varredura)
SI	Índice de seletividade
TEM	Microscopia eletrônica de transmissão
TiO_2	Dióxido de titânio
TSB	*Tryptic soy broth* (Caldo de soja tríptico)
UEL	Universidade Estadual de Londrina
US-FDA	*United States Food and Drug Administration*

UV/Vis	Espectroscopia UV/visível
V	Volts
v/v	Porcentagem em volume/volume
WHO	*World Health Organization*
XPS	Espectroscopia de fotoelétrons excitados por raios-X
XRD	Difração por Raios-X
ZnO	Óxido de zinco

SUMÁRIO

INTRODUÇÃO

Infecções causadas por bactérias multirresistentes são um grande problema para a saúde pública em todo o mundo. Estas aumentam o tempo de internação dos pacientes, além de elevar os custos com o tratamento. De acordo com um estudo realizado pelo governo britânico, 700 mil pessoas morrem por ano por infecções causadas por bactérias multirresistentes. E estima-se que após 2050 este número suba para 10 milhões de mortes por ano. Já em relação aos custos, nos EUA em torno de 20 bilhões de dólares são gastos por ano com pacientes com infecções hospitalares (O'NEILL, 2014; SMITH & COAST, 2013).

A pressão seletiva causada por antimicrobianos de amplo espectro induz à evolução e disseminação de microrganismos resistentes. Fazendo com que bactérias adquiram genes de resistência e assim reduza as opções de tratamento pelos antimicrobianos disponíveis no mercado. Outro mecanismo de resistência das bactérias é a formação de biofilmes, que dificultam a ação de antimicrobianos e sanitizantes, sendo um problema de grande importância em hospitais e também na indústria de alimentos.

Em 2017, a Organização Mundial da Saúde (OMS) divulgou uma lista com 12 famílias de bactérias que representam a maior ameaça para a saúde humana. Esta lista teve por objetivo orientar e promover a pesquisa e desenvolvimento de novos antimicrobianos. A lista é dividida de acordo com a urgência em que se necessita de novos antimicrobianos no mercado para tratamento de infecções (OMS, 2017). Das bactérias que são apresentadas, duas foram incluídas neste trabalho, uma *Enterobacteriaceae,* resistente à carbapenema e produtora de ESBL; e *Staphylococcus aureus*, resistente a meticilina, com sensibilidade intermediária à vancomicina.

Nos últimos anos diversas pesquisas tem sido realizadas para a busca de novas alternativas não apenas para o controle de infecções na área clínica, como também para o controle e prevenção de microrganismos na indústria de alimentos. Uma destas possíveis alternativas que tem chamado a atenção de pesquisadores é a combinação de compostos, que podem ser naturais ou sintéticos, com atividade antimicrobiana. Além disso, a busca por produtos naturais com ação preventiva e de tratamento e que ofereçam menor efeito adverso ao hospedeiro, melhoria dos efeitos antimicrobianos e diminuição de custos associados ao tratamento dos pacientes.

As nanopartículas tem sido amplamente investigadas para o seu uso na medicina, cosméticos, no ambiente e tecnologia, devido às propriedades quânticas que potencializam seus efeitos biológicos. Alguns estudos foram descritos como uso das nanopartículas de prata (AgNP) no combate a vários microrganismos causadores de importantes doenças humanas e de outros animais.

Outros tipos de compostos que também apresentam atividade antimicrobiana, são os óleos essenciais derivados de especiarias e que contém uma ampla variedade de metabólitos secundários que são capazes de inibir ou diminuir o crescimento de bactérias e fungos. O eugenol é um destes metabólitos que possui uma ampla gama de aplicações, como em perfumarias, aromatizantes, óleos essenciais e na medicina, anestésico local e antisséptico.

Estudos com a associação de compostos, como óleos essenciais e nanoparticulas tem despertado atenção de muitos pesquisadores. O que trouxe o interesse no desenvolvimento desta pesquisa como forma de compreender os possíveis alvo (s) e mecanismo (s) de ação antibacteriana do eugenol associado às nanopartículas de prata.

Esta Tese de doutorado é dividida entre revisão de literatura sobre o tema abordado, Artigo I “**Synergistic And Additive Effect of Eugenol Associate with Biological Synthesized Silver Nanoparticles against Gram-Positive and Gram-Negative Bacteria”** submetido ao “Journal of Nanoparticle Research”, e o Artigo II “**Synergistic Effect of Eugenol and Biologically synthesized Silver Nanoparticles against *Listeria monocytogenes***”, o qual será submetido posteriormente e referente aos resultados obtidos nos experimentos realizados durante o período de Doutorado Sanduíche na *Oklahoma State University*, Estados Unidos.

1. OBJETIVOS

1.1. OBJETIVO GERAL

Avaliar a atividade antibacteriana das nanopartículas de prata associadas ao eugenol contra bactérias Gram-positivas e Gram-negativas, incluindo isolados multirresistentes.

1.2. OBJETIVOS ESPECÍFICOS

- Verificar a sensibilidade bacteriana para nanopartículas de prata e eugenol.
- Analisar a cinética desta atividade antibacteriana.
- Avaliar o efeito dos compostos e da combinação contra biofilme.
- Determinar as alterações morfológicas causadas por estes compostos através da Microscopia Eletrônica de Varredura.

2. REVISÃO DE LITERATURA

2.1. NANOPARTÍCULAS

O termo “nano” é derivado da palavra grega que significa anão. O conceito de nanotecnologia foi dado por Richard Feynman em 1959, no entanto o termo “nanotecnologia” foi descrito em 1974 por Norio Taniguchi. Nanociência é definida como o estudo dos fenômenos e a manipulação de materiais na escala atômica, molecular e macromolecular, onde as propriedades diferem-se em larga escala (MAHENDRA et al., 2015).

Nanotecnologia foi previamente definida como sendo relativa a materiais, sistemas e processos, os quais se encontram a uma escala conhecida por nanômetros (10^{-9} m = 1 nm), com dimensões que não excedam 100 nm, em pelo menos uma dimensão (CASANOVA, 2010).

Já Iniciativa Nacional Americana de Nanotecnologia definiu nanotecnologia como “o entendimento e controle da matéria em dimensões de aproximadamente 1-100nm, onde fenômenos únicos possibilitam novas aplicações, abrangendo ciência, engenharia e tecnologia em nano escala, nanotecnologia envolve geração de imagens, medição, modelagem e manipulação de matéria em nano escala” (CHEN; WEISS; SHAHIDI, 2006). Em 2009 a Autoridade Europeia de Segurança em Alimentos definiu nanomaterial como “qualquer forma de material que tenha uma ou mais dimensões em nano escala” e nanopartícula (NP) como “uma entidade discreta que tem todas as três dimensões em nano escala” (MAHENDRA et al., 2015).

A partir do desenvolvimento e habilidade em produzir e manipular materiais em escalas nanométricas surgiu também a necessidade de aplicação dos novos materiais produzidos. Sendo assim a nanotecnologia passou a ser estudada por alguns ramos da ciência, como a biotecnologia, surgindo então a nanobiotecnologia. Esta entende-se como a interface entre a nanotecnologia e a biologia e apresentam como objetivos o estudo e desenvolvimento de produtos com aplicações biológicas (PINA et al., 2006).

As NPs apresentam características diferentes de partículas maiores e podem apresentar formas esféricas ou romboides, ocas ou massivas; sendo que as ocas podem ser utilizadas como carregadores de substâncias ativas, atuando então como nanocápsulas (FARIA-TISCHER; TISCHER, 2012).

Outra característica importante é o tamanho e o formato das NPs, que pode variar em relação ao material de origem. De acordo com estas variações, cada tipo de NP pode apresentar uma propriedade e coloração diferente, o que pode influenciar nas propriedades de absorção destas NPs (KHAN; SAEED; KHAN, 2017).

NPs são divididas em diferentes categorias de acordo com sua morfologia, tamanho e propriedades químicas. Com base em algumas características químicas e físicas algumas das classes mais conhecidas são: NPs baseadas em carbonos, NPs metálicas, NPs de cerâmica, NPs semicondutoras, NPs poliméricas e NPs baseadas em lipídeos (KHAN; SAEED; KHAN, 2017).

As NPs metálicas apresentam metais como seus precursores e pela ressonância plasmônica de superfície localizada (LSPR) estas NPs possuem propriedades óptico-elétricas únicas (KHAN; SAEED; KHAN, 2017).

2.1.1. Nanopartículas de Prata

A prata encontra-se no ambiente em quatro estados de oxidação: Ag^0, Ag^+, Ag^{2+} e Ag^{3+}. O estado mais abundante encontrado no ambiente é a prata livre Ag^+, estando associada a sulfito, nitrato, bicarbonato, sulfato ou com cloretos e sulfatos adsorvidos em material particulado na fase gasosa. Geralmente as AgNP são menores do que 100 nm em uma de suas dimensões e consistem em 20-15000 átomos de prata (KEAT et al., 2015); de fato, quanto menor a AgNP, maior a razão área de superfície para volume, e mais íons Ag^+ são liberados (CAMERON; HOSSEINIAN; WILLMORE, 2018). Comercialmente é conhecida como nanopartícula de prata ou prata coloidal (CAMERON; HOSSEINIAN; WILLMORE, 2018).

Em relação às AgNP, a capacidade antimicrobiana do composto é diretamente proporcional à capacidade de liberar íons prata e a proporção entre a área da superfície e o volume particular eleva a capacidade de liberação de Ag^+. Ou seja, quanto maior a proporção superfície/volume, maior a exposição da prata com o sítio alvo. Além disto, a atividade antimicrobiana é maior nas AgNP do que em relação aos íons prata ou sais de prata (NOWACK; KRUG; HEIGHT, 2011).

A prata como desinfetante foi usada empiricamente por vários milênios (CHERNOUSOVA; EPPLE, 2013; KLASEN, 2000a) e durante a década de 1960 foi implantada como sal ou nano-sistemas (coloides), principalmente para tratamento de feridas (KLASEN, 2000a).

O uso de prata como curativos e tratamentos de feridas é descrito desde o século 18. A atividade antimicrobiana dos íons prata foi primeiramente identificada no século 19 e a prata coloidal foi regulamentada para o tratamento de feridas na década de 1920 pela *Food and Drug Administration* dos Estados Unidos (US-FDA). No entanto com a introdução da penicilina na década de 1940 o uso da prata diminuiu (CHOPRA, 2007).

Devido à sua potente atividade antimicrobiana, as AgNP são um dos nanomateriais mais utilizados (DURÁN; NAKAZATO; SEABRA, 2016). As AgNP são utilizadas em diversos produtos devido ao seu tamanho e pelo seu potencial efeito antibacteriano (ASHKARRAN et al., 2012; DE FARIA et al., 2014; LEM et al., 2012).

Além disso, a prata pode ser absorvida e localizada na célula de diferentes maneiras, dependendo de sua NP ou forma iônica, o que resulta em efeitos característicos das NPs. A quantidade de íons Ag^{+} liberados pode depender de diversos fatores, como o tamanho da AgNP, o pH do ambiente em que se encontra, o revestimento da superfície ou a formação de uma proteína corona ao redor da AgNP (DURÁN et al., 2015; MANSHIAN et al., 2015; MAO et al., 2016; QIAN et al., 2015).

Nas células, a AgNP sofre transformação de prata elementar (Ag^{0}), em íons Ag^{+}, em espécies de óxido de prata (Ag-O–) e, finalmente, em espécies de sulfeto de prata (Ag-S–) até se ligar aos tióis (WANG et al., 2015). No ambiente, a AgNP sofre reações de sulfatação ao Ag_2S, reduzindo muito seu potencial de toxicidade (LI et al., 2017; LOWRY et al., 2012; SCHULTZ et al., 2016).

A produção de AgNP pode ocorrer por diferentes métodos, sendo estes químicos, físicos ou biológicos, que são apresentados no tópico a seguir.

2.1.2. Síntese de Nanopartículas de Prata

Diversos métodos podem ser empregados para a síntese de NPs, no entanto estes métodos são amplamente divididos em duas maiores classes: (1) síntese *"de baixo para cima"*; onde NPs são formadas a partir de substâncias mais simples, como a partir da manipulação direta do átomo e (2) síntese *"de cima para baixo"*; no qual moléculas maiores são decompostas em unidades menores e então convertidas em NPs (KHAN; SAEED; KHAN, 2017).

Entre essas classes os processos de obtenção podem ser físicos, químicos ou biológicos. Em métodos físicos, as NPs são preparadas por evaporação-condensação usando um forno tubular à pressão atmosférica (GURAV et al., 1994; KRUIS; FISSAN; RELLINGHAUS, 2000; MAGNUSSON et al., 1999; SCHMIDT-OTT, 1988). Os métodos físicos convencionais para a síntese de AgNP incluem a descarga de faíscas e pirólise (PLUYM et al., 1993; TIEN et al., 2008). As vantagens dos métodos físicos são a velocidade, a radiação usada como agente redutor e não há substâncias químicas perigosas envolvidas, mas as desvantagens são o baixo rendimento e o alto consumo de energia, a contaminação por solvente e a falta de distribuição uniforme (ABOU EL-NOUR et al., 2010; ELSUPIKHE et al., 2015; SHAMELI et al., 2010; TSUJI et al., 2005).

Os métodos químicos usam água ou solventes orgânicos para preparar as AgNP (TAO; SINSERMSUKSAKUL; YANG, 2006; WILEY et al., 2005). Esse processo geralmente emprega três componentes principais, como precursores de metais, agentes redutores e agentes de estabilização/encapsulamento. Além disso, os materiais utilizados para a síntese de AgNP, como citrato, boro hidreto, tio-glicerol e 2-mercaptoetanol, são tóxicos e perigosos (MALLICK; WITCOMB; SCURRELL, 2004). Além disso, as partículas fabricadas não são da pureza esperada, uma vez que as suas superfícies foram sedimentadas com produtos químicos. Também é muito difícil preparar AgNP com um tamanho bem definido, exigindo mais um passo para a prevenção da agregação de partículas (MALIK; O'BRIEN; REVAPRASADU, 2002). A vantagem da síntese química das NPs é a facilidade de produção, baixo custo e alto rendimento; no entanto, o uso de agentes redutores químicos é prejudicial aos organismos (GURUNATHAN et al., 2015a).

O processo tradicional de obtenção de NPs metálicas pode produzir grande quantidade de material tóxico e desnecessário assim como substâncias que podem ser perigosas ao meio ambiente (JAHANGIRIAN et al., 2017). No entanto, as NPs tem cada vez mais ganhado destaque pelo uso na medicina (como direcionamento de fármacos) (ADEYEMI; SULAIMAN, 2015), aplicação em materiais cirúrgicos (FURNO et al., 2004; RUPP et al., 2004), uso em tecidos (DURÁN et al., 2007; SILVER, 2003), tintas (KUMAR et al., 2008), cosméticos (KOKURA et al., 2010), embalagens para alimentos (CARBONE et al., 2016; SHARMA et al., 2017). E uma alternativa ao processo de produção química de NPs é a produção utilizando-se de sistemas biológicos.

Os métodos biológicos, também denominados “*ecofriendly*” ou “*green synthesis*” (síntese verde), utilizam extratos de plantas e microrganismos, os quais possuem a habilidade natural em reduzir íons metálicos na forma de NPs (HERMAN; HERMAN, 2014).

A síntese biológica das NPs depende de três fatores, incluindo (a) o solvente; (b) o agente redutor; e (c) o material não tóxico. A principal vantagem destes métodos é a disponibilidade de aminoácidos, proteínas ou metabólitos secundários presentes no processo de síntese, a eliminação do passo extra necessário para a prevenção da agregação de partículas, e o uso de moléculas biológicas para a síntese de AgNP é livre de poluição. As proteínas bacterianas ou extratos de plantas atuam como agentes redutores, sendo assim, pode-se controlar a forma, o tamanho e a monodispersão das NPs (GURUNATHAN et al., 2009a).

As outras vantagens dos métodos biológicos são a disponibilidade de uma gama de recursos biológicos, uma menor necessidade de tempo, alta densidade, alto rendimento, e alta estabilidade, além da pronta solubilidade de NPs preparadas em água (GURUNATHAN et al., 2015b; THAKKAR; MHATRE; PARIKH, 2010).

Entre vários métodos de síntese de AgNP, os métodos biológicos parecem ser simples, rápidos, não tóxicos, confiáveis e verdes, que podem produzir tamanho e morfologia bem definidos sob condições otimizadas (ZHANG et al., 2016).

De acordo com os parágrafos anteriores, a síntese biogênica apresenta diversas vantagens em relação ao tradicional processo de obtenção química ou física (SEABRA; DURÁN, 2015). Entretanto, a limitação da síntese biogênica ocorre devido ao processo de reprodutibilidade não ser tão eficiente quanto aos processos tradicionais. No entanto, a caracterização adequada é um passo crucial para a avaliação de sua atividade antimicrobiana.

A literatura descreve como analise básica para a estrutura de nanomateriais metálicos a difração por Raios-X (XRD). Porém, podem ser realizadas outras técnicas para analise, tais como, espectroscopia UV/visível (UV-Vis), Espectroscopia em infravermelho transformada de Fourier (Fourier transform infrared spectroscopy - FTIR), espectroscopia de fotoelétrons excitados por raios X (X-Ray photoelectron spectroscopy - XPS), ressonância magnética nuclear (nuclear magnetic resonance - RMN), microscopia eletrônica de varredura (scanning electron microscopy - SEM), microscopia eletrônica de transmissão (transmission electron microscopy - TEM), e espalhamento dinâmico de luz (dynamic light scattering - DLS), que além de

importantes também são complementares à caracterização (DURÁN; NAKAZATO; SEABRA, 2016).

A fim de abordar a questão da segurança para utilizar todo o potencial de qualquer material nano na finalidade do bem-estar humano, na nanomedicina, ou na indústria de cuidados de saúde, etc., é necessário caracterizar as NPs preparadas antes da aplicação (LIN et al., 2014). O aspecto característico dos nanomateriais, como tamanho, forma, distribuição de tamanho, área de superfície, solubilidade, agregação, etc., precisa ser avaliado antes de avaliar a toxicidade ou a biocompatibilidade (MURDOCK et al., 2008).

2.1.3. Atividade Antimicrobiana das Nanopartículas de Prata

As propriedades antimicrobianas das diferentes NPs tem sido comparadas dependendo do tipo de NP, método utilizado para produção, tamanho e forma das moléculas, e ação em diferentes cepas microbianas, inclusive em isolados multirresistentes (HERMAN; HERMAN, 2014).

A atividade biológica das AgNP depende de fatores que incluem química da superfície, forma, tamanho, distribuição do tamanho, morfologia e composição das partículas, revestimento/cobertura, aglomeração e taxa de dissolução, reatividade das partículas em solução, eficiência de liberação de íons, o tipo de célula, e o tipo de agentes redutores utilizados para a síntese de AgNP é um fator crucial para a determinação da citotoxicidade (CARLSON et al., 2008).

As propriedades físico-químicas das NPs aumentam a biodisponibilidade de agentes terapêuticos após administração sistêmica e local (JO et al., 2015; STAQUICINI et al., 2011) e por outro lado podem afetar a captação celular, a distribuição biológica, a penetração em barreiras biológicas e os efeitos terapêuticos resultantes (ALBANESE; TANG; CHAN, 2012; DUAN; LI, 2013). Portanto, o desenvolvimento de AgNP com estruturas controladas que são uniformes em tamanho, morfologia e funcionalidade são essenciais para várias aplicações biomédicas (GURUNATHAN et al., 2009b; PANÁČEK et al., 2009; SRIRAM et al., 2010; WONG et al., 2009; ZODROW et al., 2009).

A prata na forma de NP é conhecida por exibir um potente efeito biocida em diferentes espécies bacterianas (RAI; YADAV; GADE, 2009; SONDI; SALOPEK-SONDI, 2004), incluindo cepas multirresistentes (LARA et al., 2010b).

Em geral, o tamanho das células bacterianas está na faixa do micrômetro, enquanto suas membranas celulares externas possuem poros na faixa nanométrica. Como as NPs podem ser menores em tamanho do que os poros bacterianos, elas apresentam uma capacidade única de atravessar a membrana celular (AZAM et al., 2012).

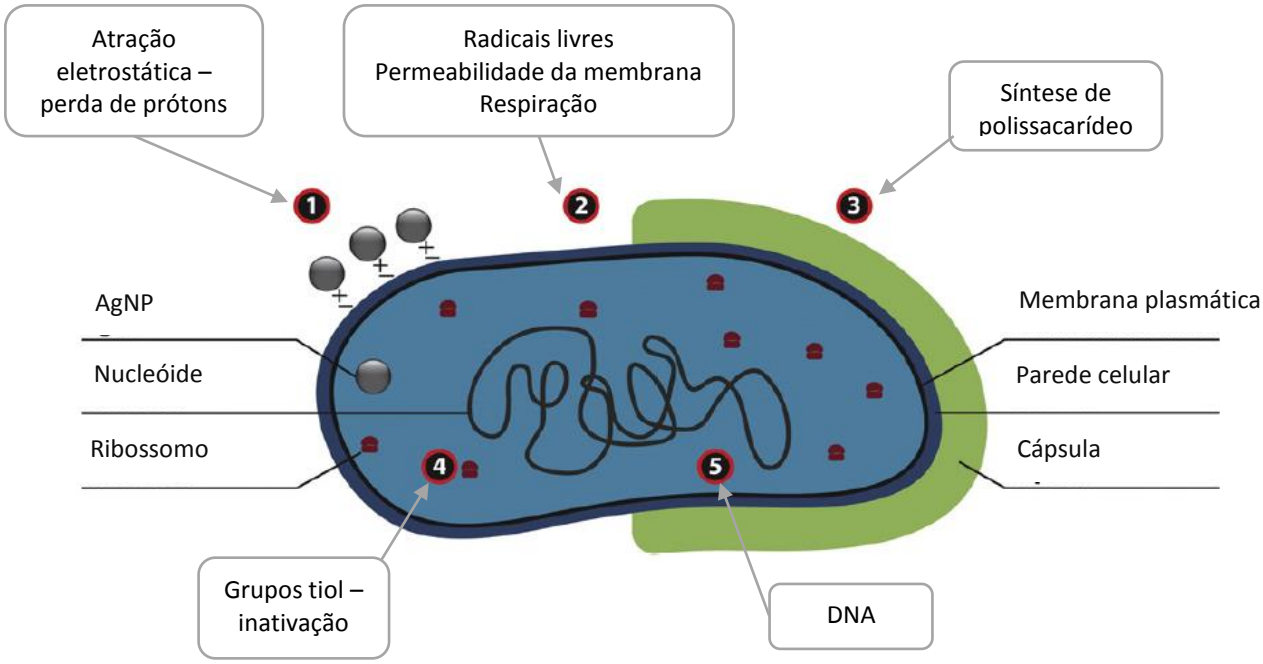
Uma importante afirmação de Lok et al. (2006) foi que a diferença de maior destaque entre AgNP e íons de prata está na sua capacidade antibacteriana: concentrações nanomolares no caso de NPs e faixas micromolares no caso de íons de prata. Um possível mecanismo de ação foi descrito por estes íons serem capazes de se ligar à estrutura da membrana celular, desestabilizando o potencial de membrana e causando a perda de prótons (GOGOI et al., 2006; MAILLARD; HARTEMANN, 2013).

Durán et al. (2010) enfatizaram as atividades antibacterianas centradas na ação dos íons de prata e as compararam com as de AgNP. Os dados sugerem principalmente que os íons de prata reagem com os grupos tiol das proteínas, produzindo inativação bacteriana. Esta foi uma boa suposição porque os níveis de μmol/L de prata diminuíram a replicação do DNA devido ao desacoplamento do transporte de elétrons respiratórios da fosforilação oxidativa, que inibe enzimas da cadeia respiratória e/ou interfere na permeabilidade da membrana.

O mecanismo das atividades antibacterianas das nanopartículas de prata (Figura 1) foi discutido por Marambio-Jones e Hoek (2010), que apresentaram três possíveis mecanismos de toxicidade: a) captação de íons livres de prata seguida de rompimento da produção de ATP e replicação do DNA, b) espécies reativas de oxigênio (ROS) geradas por nanopartículas de prata e íons de prata, e c) danos diretos à membrana celular por nanopartículas de prata. Os autores discutiram muitos estudos que correlacionaram a toxicidade das AgNP com a liberação de íons de prata. Alguns anos após a publicação desta revisão, Prabhu e Poulose (2012) chegaram às mesmas conclusões quanto aos mecanismos de ação da prata.

A atividade antibacteriana das NPs também depende da espécie dos microrganismos, o que foi destaque nos estudos de Kon e Rai (2013). Eles mencionaram o estudo de Kim et al. (2007), que compararam os efeitos das AgNP sobre *Escherichia coli* e *S. aureus*. Os dados mostraram uma maior sensibilidade de *E. coli* do que *S. aureus* devido a diferenças na concentração de peptideoglicano na parede celular de Gram-positivas.

Figura 1 – Mecanismo de ação das nanopartículas de prata.



Mecanismo de ação antibacteriana de nanopartículas de prata proposto por Durán e colaboradores (2015): 1 - atração eletrostática, 2 - produção de radicais livres, mudanças na permeabilidade, perturbação da respiração, extravasamento de conteúdo intracelular, 3 - modulação dos perfis de fosfotirosina das proteínas, envolvidos na progressão do ciclo celular e na síntese de polissacarídeos capsulares, 4 - interação com grupos tiol; inibição da síntese e função de proteínas, 5 - interação com moléculas contendo fósforo (DNA).
Fonte: Adaptado de Durán et al., 2015.

2.1.4. Aplicação

A prata como desinfetante foi usada empiricamente por vários milênios, e implantada como sal ou nano-sistemas (coloides) durante a década de 1960, principalmente para tratamento de feridas (CHERNOUSOVA; EPPLE, 2013; KLASEN, 2000a, 2000b). No entanto, pesquisas abrangentes sobre a ação antibacteriana de AgNP surgiram por volta de 2004 e aumentaram exponencialmente (SONDI; SALOPEK-SONDI, 2004). Os sistemas de nano-prata apresentam várias vantagens que os tornam muito interessantes para uso como agentes antimicrobianos. Eles possuem uma efetiva atividade contra uma gama de microrganismos e parasitas, mesmo quando doses baixas são usadas (a inibição completa do crescimento de bactérias pode ocorrer em apenas alguns mg/mL). Nessas doses, a prata apresenta muito pouca toxicidade sistêmica em relação aos seres humanos e é relativamente barata (LE OUAY; STELLACCI, 2015).

Como já foi descrito anteriormente, as NPs podem ser empregadas como direcionamento de fármacos (LEE et al., 2011), sensores químicos e biológicos (BARRAK et al., 2016), sensores de gás (MANSHA et al., 2016; RAWAL; KAUR, 2013; ULLAH et al., 2017), como captura de CO_2 (GANESH et al., 2017; RAMACHARYULU et al., 2015) e outras aplicações (SHAALAN et al., 2016).

As NPs são amplamente utilizadas na indústria cosmética e dermatológica, como a forma de dióxido de titânio (TiO_2) e óxido de zinco (ZnO), as quais são consideradas bloqueadores de amplo espectro UVA e UVB e protegem contra danos à pele causados pelos raios UV (HERMAN; HERMAN, 2014).

As AgNP são cada vez mais usadas em vários campos, incluindo os de uso médico, alimentos, saúde, consumo e industrial, devido às suas propriedades físicas e químicas únicas. Estes incluem propriedades óticas, elétricas e térmicas, alta condutividade elétrica e biológicas (GURUNATHAN et al., 2015b; LI et al., 2010; MUKHERJEE et al., 2001).

Devido às suas propriedades típicas, suas aplicações podem ser como agentes antibacterianos, em produtos industriais, domésticos e relacionados à saúde, em produtos de consumo, revestimentos de dispositivos médicos, sensores óticos e cosméticos, na indústria farmacêutica, indústria de alimentos, em diagnósticos, ortopedia, direcionamento de fármacos, como agentes anticancerígenos. Diversos estudos também demonstram que elas podem aumentar os efeitos contra o tumor em drogas anticâncer, além do efeito antifúngico e antiviral (CHERNOUSOVA; EPPLE, 2013; ELECHIGUERRA et al., 2005; KHATOON et al., 2015; KIM et al., 2007; LARA et al., 2010a; LONGHI et al., 2016; SUN et al., 2005).

Também foi descrito seu uso em sprays sanitizadores de ar, lenços umedecidos, embalagens para armazenamento de comida, xampu e pastas de dentes. Várias NPs ainda estão sob pesquisa e avaliação para serem utilizadas como aditivos em produtos cosméticos e de cuidados pessoais. Apesar do crescimento emergente do uso em produtos com diferentes tipos de nanomateriais, o efeito adverso em humanos ainda é desconhecido e necessita de maiores pesquisas (JEEVANANDAM et al., 2018).

As AgNP podem ser agentes antibacterianos alternativos aos antibióticos. No entanto, é necessário desenvolver AgNP como tais (ZHANG et al., 2016). NPs com menor tamanho de partícula tem sido relatadas como apresentando boa atividade antimicrobiana (JONES et al., 2008). A atividade antimicrobiana de NPs foi estudada

com bactérias patogênicas humanas, como *E. coli* (YOON et al., 2007), *S. aureus* (RUPARELIA et al., 2008), *Streptococcus agalactiae* (PERUGINI BIASI-GARBIN et al., 2015) e também bactérias multirresistentes (CARDOZO et al., 2013; SCANDORIEIRO et al., 2016).

2.2. Óleos Essenciais

Outro grupo de compostos com ação antimicrobiana são os óleos essenciais (OEs), sintetizados por espécies de plantas aromáticas, são misturas altamente complexas (HYLDGAARD; MYGIND; MEYER, 2012). Devido às suas importantes propriedades, eles foram propostos como alternativas aos agentes sintéticos (BASSOLÉ; JULIANI, 2012; TONGNUANCHAN; BENJAKUL, 2014). OEs tem sido amplamente utilizados em produtos farmacêuticos, cosméticos, alimentos, bem como em filmes comestíveis e materiais de embalagem (RAUT; KARUPPAYIL, 2014; SEOW et al., 2014). No entanto, sua baixa solubilidade aquosa e volatilidade limitam suas aplicações (KFOURY et al., 2016).

Especiarias como cravo, orégano, hortelã, tomilho e canela tem sido empregadas há séculos como conservantes de alimentos e também como plantas medicinais. Muitos relatos confirmam as propriedades antibacterianas, antifúngicas, antivirais e anticarcinogênicas destas especiarias (SHAN et al., 2005).

Estes OEs são líquidos hidrofóbicos de compostos aromáticos que são voláteis e oleosos na natureza e estão presentes em várias partes da planta, como galho, flor, folha, casca, semente e raiz. Muitos OEs de plantas são utilizados como aromatizantes em cosméticos, aditivos alimentares, sabões, resinas plásticas e perfumes. Além disso, a pesquisa sobre as aplicações de OEs que podem atuar como agentes antimicrobianos está crescendo devido à gama de atividades, origens naturais e geralmente estes compostos são reconhecidos como seguros (*Generally Recognized as Safe* - GRAS) pela *Food and Drug Administration* (FDA), dos Estados Unidos (PANDEY et al., 2017).

Os OEs são frequentemente estudados hoje em dia por seu efeito antifúngico (SINGH; TRIPATHI, 1999), antiúlcera (ĐORĐEVIĆ et al., 2007), anti-helmíntico (INOUYE; TAKIZAWA; YAMAGUCHI, 2001), antioxidante (MIMICA-DUKIĆ et al., 2003), anti-inflamatório (SINGH; MAJUMDAR; REHAN, 1996), repelente, inseticida (ISMAN et al., 1990; PANDEY; PALNI; TRIPATHI, 2014), citotóxico (SYLVESTRE et

al., 2007), antiviral (MAURYA et al., 2005), ovicida (PANDEY; SINGH; TRIPATHI, 2011), anestésico (GHELARDINI; GALEOTTI; MAZZANTI, 2001), moluscicida (FICO et al., 2004), imunomoduladora (MEDIRATTA; SHARMA; SINGH, 2002), larvicida (JANTAN et al., 2003), além do uso como conservante de alimentos (PANDEY et al., 2014).

Koul, Walia e Dhaliwal (2008) relataram que a maioria das plantas aromáticas e medicinais retém uma mistura odorífera de compostos voláteis que podem ser extraídos como um óleo essencial. Geralmente, diversos metabólitos secundários são produzidos por estes tipos de plantas, os quais podem ser: terpenóides, compostos alcoólicos (ex. geraniol, mentol, linalol), compostos ácidos (ex., benzóico, cinâmico, mirístico), aldeídos (ex., citral, benzaldeído, cinamaldeído, cânfora de carvona), corpos cetônicos (ex., timol, eugenol) e fenóis (por exemplo, ascaridol, anetol). Dentre estes metabólitos, os que apresentam papel importante na composição de OEs são os terpenos ou terpenóides (ex., pineno, mirceno, limoneno, terpineno, p-cimeno) e fenóis aromáticos (ex., carvacrol, timol, safrol, eugenol).

Alguns constituintes botânicos, tais como azadiractina, carvona, mentol, ascaridol, metil eugenol, toosendanina e volkensina, tem sido descritos pelo seu potencial em agir contra vários patógenos bacterianos e fúngicos, bem como contra pragas de insetos (ISMAN, 2006; PANDEY et al., 2012; PANDEY; SONKER; SINGH, 2016). Além disso, muitos deles possuem atividades bactericidas, fungicidas e inseticidas eficazes e podem ser responsáveis para melhorar o sabor ou conferir propriedades tóxicas para o ser humano.

Óleos essenciais de Acorus, Artemisia, Chenopodium, Clausena, Curcuma, Canela, Cymbopogon, Eupatorium, Foeniculum, Hyptis, Lippia, Ocimum, Putranjiva, Syzygium e Vitex são conhecidos por suas propriedades antimicrobianas que já foram descritas em estudos anteriores (PANDEY et al., 2012, 2013, 2014; SONKER; PANDEY; SINGH, 2015). As propriedades antibacterianas dos OEs e seus diversos compostos naturais ativos contra bactérias transmitidas por alimentos e suas aplicações em alimentos poderiam fornecer alternativas aos bactericidas e fungicidas convencionais (BURT, 2004; PERRICONE et al., 2015).

2.2.1. Eugenol

Syzygium aromaticum (S. aromaticum) (sinônimo: *Eugenia cariophylata*) comumente conhecido como cravo, é uma árvore de tamanho mediano (8-12 m) que pertence à família Mirtaceae nativa das ilhas Maluku no leste da Indonésia. Durante séculos, o comércio de cravo-da-índia e a busca dessa preciosa especiaria estimularam o desenvolvimento econômico dessa região asiática (KAMATOU; VERMAAK; VILJOEN, 2012).

Os maiores países produtores de cravo-da-índia são a Indonésia, a Índia, a Malásia, o Sri Lanka, Madagascar e a Tanzânia, especialmente a ilha de Zanzibar (KAMATOU; VERMAAK; VILJOEN, 2012). O cultivo de cravo é realizado no Brasil na região nordeste, no estado da Bahia, nas regiões de Valença, Ituberá, Taperoá, Camamu e Nilo Peçanha, onde são cultivados aproximadamente 8 mil hectares, produzindo cerca de 2.500 toneladas por ano (OLIVEIRA et al., 2009).

Devido aos inúmeros efeitos farmacológicos consolidados do uso tradicional há séculos e relatados na literatura, o cravo é uma importante planta medicinal (CORTÉS-ROJAS; DE SOUZA; OLIVEIRA, 2014).

Este representa uma das principais fontes vegetais de compostos fenólicos como os flavonoides, os ácidos hidroxibenzóicos, os ácidos hidroxicinâmicos e o propil hidroxifenílico. O principal composto bioativo do cravo-da-índia é o eugenol, encontrado em concentrações que variam de 9381,70 a 14650,00 mg por 100 g de material vegetal fresco (NEVEU et al., 2010).

Os demais constituintes principais, além do eugenol, são: acetil eugenol (SHAN et al., 2005), ácido gálico (PATHAK et al., 2004; SHAN et al., 2005; VARIYAR; BANDYOPADHYAY; THOMAS, 1998) e flavonóis, como quercetina e kaempferol (SHAN et al., 2005). Outros compostos (ácido p-cumárico, ácido protocatecuico e ácido siríngico) são encontrados em concentrações mais baixas (SHAN et al., 2005; VARIYAR; BANDYOPADHYAY; THOMAS, 1998).

Eugenol, quimicamente expresso como 1-alil-4-hidroxi-3-metoxibenzeno ou 4-alil-2-metoxifenol, apresenta fórmula molecular de $C_{10}H_{12}O_2$ e um peso molecular de 164,21 g/mol, é um dos principais componentes ativos do óleo de cravo, óleo canforado, óleo de folha de canela e óleo de noz moscada. Em temperaturas normais, o eugenol é um líquido oleoso viscoso amarelo pálido com um sabor forte

de cravo. Esta substância é ligeiramente solúvel em água e facilmente dissolvido em solventes orgânicos (KONG et al., 2014).

Este composto pertence à classe dos fenilpropanóides, que são assim nomeados como tal porque contém um grupo fenol aromático de seis carbonos e uma cauda de propeno de três carbonos a partir do ácido cinâmico, que é produzido durante o primeiro passo da biossíntese dos fenilpropanóides. Estes compostos representam uma porção relativamente pequena de OEs, sendo que eugenol, isoeugenol, vanilina, safrol e cinamaldeído são os fenilpropenos mais estudados. Os grupos hidroxila livres conferem a maior parte da atividade antimicrobiana dessas moléculas (LAEKMAN et al., 1990), sendo que a ação antimicrobiana do eugenol pode ser atribuída à presença de uma dupla ligação nas posições α, β da cadeia lateral e a um grupo metila localizado na posição γ (JUNG; FAHEY, 1983).

A estrutura particular e a disponibilidade imediata do eugenol transformaram o produto natural em um interessante material de partida e um componente útil para a síntese complexa de novas moléculas, bem como em um substrato valioso para várias biotransformações (KAUFMAN, 2015).

Como resultado de sua ampla gama de atividades farmacológicas e biológicas, os estudos sobre eugenol e produtos derivados do cravo ainda continuam sendo uma prioridade de pesquisa. É, portanto, de valor significativo unir racionalmente alguns dos achados de pesquisa mais importantes relacionados ao eugenol para destacar sua importância na saúde humana, bem como para elucidar seus mecanismos de ação quando possível (KAUFMAN, 2015), a fim de encontrar novas alternativas aos antimicrobianos atuais.

2.2.2. Atividade antimicrobiana dos óleos essenciais

A atividade antibacteriana de alguns óleos essenciais, tais como o de cravo-da-índia, orégano (*Origanum vulgare*), louro (*Pimenta racemosa*) e tomilho (*Thymus vulgaris*) foi testada contra *E. coli* O157: H7 mostrando os diferentes graus de inibição desses óleos essenciais (BURT; REINDERS, 2003). Da mesma forma, Pérez-Conesa, McLandsborough e Weiss (2006) testaram formulações contendo eugenol e carvacrol encapsuladas em um surfactante não iônico contra quatro cepas de *E. coli* O157: H7 e *Listeria monocytogenes,* dois importantes patógenos alimentares, e os resultados encontrados pelos autores reforçam o emprego do

eugenol para inibir o crescimento desses microrganismos em superfícies que estão em contato com alimentos.

Outro estudo *in-vitro* realizado por Biasi-Garbin e colaboradores (2015) demonstrou a ação antibacteriana deste composto contra *S. agalactiae*. Enquanto que em uma pesquisa realizada *in-vivo* com 40 ratos albinos, também foi confirmado a eficácia do extrato de cravo como antimicrobiano natural (KUANG et al., 2011).

Rana e colaboradores (2011) determinaram a atividade antifúngica do óleo de cravo em diferentes linhagens, e de acordo com as análises cromatográficas o eugenol foi o principal composto responsável pela atividade antifúngica devido à lise dos esporos e micelas. Devi e colaboradores (2010) também relataram um mecanismo de ação similar de ruptura de membrana e deformação de macromoléculas produzidas por eugenol.

Quando comparado a ação antimicrobiana de diferentes OEs, Badei e colaboradores (2002) relataram em sua pesquisa que o óleo essencial de cravo demonstrou maior atividade antimicrobiana e maior espectro antimicrobiano contra bactérias Gram-positivas e Gram-negativas, em relação ao óleo essencial de cardamomo e canela.

Geralmente, as bactérias Gram-negativas são mais resistentes aos OEs do que as bactérias Gram-positivas. Porém em relação ao eugenol e ao isoeugenol, interessantemente estes compostos exibem maior atividade contra Gram-negativas do que em bactérias Gram-positivas (HYLDGAARD; MYGIND; MEYER, 2012). Ou seja, nem todos os estudos de óleos essenciais concluíram que as bactérias Gram-positivas são mais suscetíveis (BUSATTA et al., 2008; PRABUSEENIVASAN; JAYAKUMAR; IGNACIMUTHU, 2006)

Esta diferença em relação à sensibilidade aos OEs pode ocorrer pois a membrana externa de bactérias Gram-negativas contém lipopolissacarídeos hidrofílicos (LPS) que atuam como uma barreira para macromoléculas e compostos hidrofóbicos, proporcionando maior tolerância a compostos antimicrobianos hidrofóbicos, como os encontrados em OEs (NIKAIDO, 1994, 2003; TROMBETTA et al., 2005). No entanto, é difícil prever a susceptibilidade de microrganismos aos OEs devido à amplitude das variações genéticas entre as espécies (PANDEY et al., 2017).

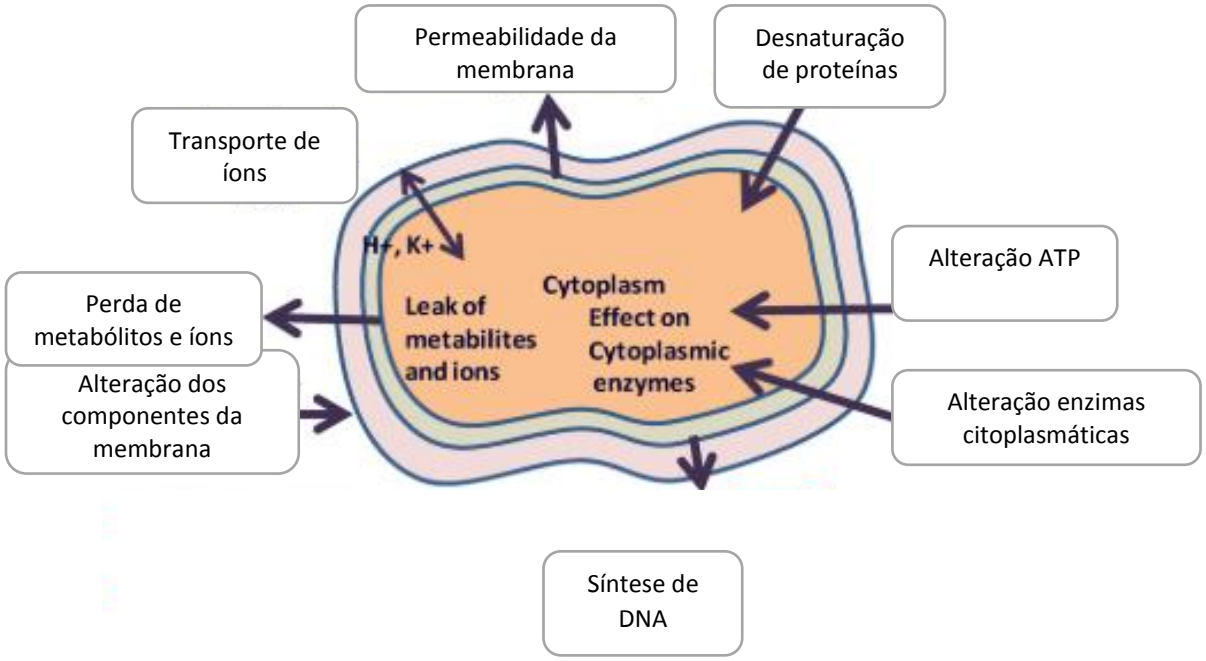
O efeito dos compostos fenólicos, que também estão presentes nos OEs, depende da quantidade do composto presente; em baixas concentrações, eles

podem interferir com enzimas envolvidas na produção de energia, e em concentrações mais altas, podem desnaturar proteínas (TIWARI et al., 2009). Nazzaro e colaboradores (2013) relatam que os mecanismos de ação dos OEs e/ou seus componentes (Figura 2) são dependentes de sua composição química e da quantidade de componentes individuais.

De acordo com alguns autores, o mecanismo de ação do eugenol pode ocorrer por alteração na membrana, afetar o transporte de íons e ATP e alteração do perfil de ácidos graxos de diferentes bactérias. O eugenol atua também contra diferentes enzimas bacterianas, incluindo ATPase, histidina carboxilase, amilase e protease (DI PASQUA et al., 2007; NAZZARO et al., 2013; THOROSKI; BLANK; BILIADERIS, 1989; WENDAKOON; SAKAGUCHI, 1995).

Xu e colaboradores (2016) afirmam que o cravo poderia destruir as paredes celulares e as membranas dos microrganismos, o que causa permeabilidade das membranas citoplasmáticas; ou então, penetrar nas células e, por conseguinte inibir a síntese normal de DNA e proteínas. Eugenol pode inibir a produção de amilase e proteases em *Bacillus cereus* e tem a capacidade de deterioração da parede celular e lise celular (BURT, 2004).

Figura 2 – Mecanismo de ação dos óleos essenciais e sítios-alvo nas células microbianas.



Fonte: Adaptado de Nazzaro et al., 2013.

2.2.3. Aplicação

Desde a antiguidade, as plantas medicinais tem sido a base da medicina fitoterápica tradicional entre os habitantes das áreas rurais de todo o mundo. Os produtos naturais integram os antigos sistemas tradicionais de medicina, incluindo Ayurveda, Chinesa e Egípcia. Atualmente, em torno de 40% da população mundial depende diretamente de medicamentos à base de plantas para seus cuidados de saúde. Portanto, o uso tradicional de plantas ou partes de plantas contendo eugenol para fins medicinais não é uma exceção (KAUFMAN, 2015).

O óleo de cravo é usado no tratamento de muitas doenças, incluindo acne, asma, artrite reumatoide, cicatrizes, verrugas e várias alergias; ele também é usado como analgésico, antiespasmódico e antisséptico geral na prática odontológica médica (MARCHESE et al., 2017).

Pesquisadores ressalvam que o declínio na pesquisa e desenvolvimento de novos agentes antibacterianos, que são capazes de inibir microrganismos causadores de doenças resistentes a antibióticos como o *S. aureus*, agrava a resistência antibiótica emergente (HÖGBERG; HEDDINI; CARS, 2010). Por conseguinte, os produtos naturais devem ser alvo de muita atenção, visto que estes poderiam ser usados como drogas eficazes no tratamento de doenças humanas, com alta eficácia contra patógenos e efeitos colaterais não tão significantes (LIU et al., 2017).

Nos últimos anos, pesquisas sobre o uso de OEs como conservantes alimentícios, a fim de aumentar o prazo de validade, foram realizadas com sucesso. Vários pesquisadores testaram OEs em forma pura ou em formulação, para aumentar a vida útil dos alimentos em diferentes tipos de armazenamento, como recipientes feitos de papelão, estanho, vidro, polietileno ou tecidos naturais e observaram um aumento significativo no prazo de validade (PANDEY; PALNI; TRIPATHI, 2014; TRIPATHI; KUMAR, 2007). Anos antes, um estudo relatou que alguns componentes dos OEs, como citral, citronela, citronelol, eugenol, farnesol e nerol, poderiam proteger de infecções fúngicas, sementes de pimentão e frutas por até 6 meses (TRIPATHI; ASTHANA; DIXIT, 1984).

O eugenol é amplamente aplicado em odontologia, como antisséptico contra doenças infecciosas como a doença periodontal devido às atividades antimicrobianas contra bactérias orais. É amplamente utilizado na medicina, como

anestésicos, analgésicos, agentes anti-inflamatórios, além do seu uso como agentes aromatizantes (CHAIEB et al., 2007).

Michiels e colaboradores (2008) relataram que, devido à solubilidade e volatilidade limitadas do eugenol, verificou-se ter melhor eficácia quando este foi administrado por via oral. O composto também foi rapidamente absorvido e metabolizado no fígado quando ingerido, enquanto 95% da dose foi excretada em 24 horas. Diversos OEs são absorvidos rapidamente pelo estômago e pelo intestino delgado proximal após a admissão oral, pulmonar e dérmica (MAJEED et al., 2016; MICHIELS et al., 2008). Portanto, convém realizar o encapsulamento do eugenol, para prevenir a absorção precoce e melhorar sua solubilidade e eficiência hídrica (ARANA-SÁNCHEZ et al., 2010; HILL; GOMES; TAYLOR, 2013; SHAH; DAVIDSON; ZHONG, 2013; WATKINS et al., 2015).

2.3. Associação entre compostos com atividade antibacteriana

Grandes avanços foram relatados no tratamento de doenças infecciosas no último século, mais precisamente após 1945. Estas descobertas promoveram uma diminuição na mortalidade e morbidade. No entanto, o desenvolvimento de resistência aos antibióticos vem crescendo drasticamente e há uma necessidade de procurar novos compostos com ação antimicrobiana (ALVES et al., 2018).

Um aspecto importante da atividade antimicrobiana das AgNP é o efeito sinérgico que ocorre quando essas partículas são combinadas com outros compostos naturais e sintéticos. Vários estudos relatam a ação sinérgica de AgNP com antimicrobianos convencionais, tais como amoxilina e polimixina B (HWANG et al., 2012; LI et al., 2005; RUDEN et al., 2009), dentre outros. Também foram descritas combinações sinérgicas de compostos naturais, como os óleos essenciais com vancomicina, β-lactâmicos, ciprofloxacin (HEMAISWARYA; DOBLE, 2009; VAN VUUREN; SULIMAN; VILJOEN, 2009).

Verificou-se que o uso de OEs em combinação com antibióticos promove uma ação sinérgica, o que reduz a dose mínima eficaz de antimicrobianos no tratamento de infecções e também reduz os efeitos adversos do antibiótico. A associação de antibióticos com óleos essenciais direcionados a bactérias resistentes pode ser uma alternativa importante, pois os OE podem exercer diferentes mecanismos de ação e o que leva a novas escolhas para superar a resistência microbiana (YAP et al.,

2014). Esta observação também pode ser direcionada em relação a AgNP, que apresenta diferentes mecanismos de ação antibacteriana, ou seja, possíveis alvos para antimicrobianos.

Li e colaboradores (2005) descrevem que as AgNP associadas à amoxicilina apresentou maior eficiência bactericida para *E. coli* do que quando foram aplicados separadamente. Ruden e colaboradores (2009) estudaram as interações entre AgNP e polimixina B e observaram efeitos sinérgicos para bactérias Gram-negativas.

No estudo realizado por Cardozo e colaboradores (2013) a associação entre AgNP e fenazina-1-carboxamida aumentam em 32 vezes o efeito antibacteriano contra cepas de *S. aureus* resistentes à meticilina (MRSA), resultando em alterações morfológicas da parede celular bacteriana. Biasi-Garbin e colaboradores (2015) mostraram um efeito sinérgico da atividade antibacteriana de AgNP com eugenol contra isolados de *S. agalactiae;* e Scandorieiro e colaboradores (2016) apresentaram o efeito sinérgico de OE de orégano e AgNP contra bactérias Gram-positivas, Gram-negativas e também contra isolados multirresistentes.

2.4. BIOFILME

O uso frequente de alguns antibióticos leva algumas bactérias a adquirir resistência, estas são capazes de sobreviver e até se multiplicar na presença do antimicrobiano. Isto leva a outro problema relacionado à resistência a antibióticos, que é a produção de biofilme (LYNCH et al., 2007). As bactérias frequentemente aderem às superfícies através de uma matriz de biofilme, um ambiente tridimensional, gelatinoso, altamente hidratado e localmente carregado. A adesão dessas bactérias a outros órgãos pode contribuir para a patogênese da infecção (ARCHER, 1998).

Os biofilmes são ecossistemas microbianos complexos formados por uma ou mais espécies imersas em uma matriz extracelular de diferentes composições, dependendo do tipo de ambiente e das espécies colonizadoras (GIAOURIS et al., 2015). A matriz extracelular é composta principalmente por polissacarídeos, como celulose, proteínas ou DNA exógeno. Esta matriz pode ser fixada a superfícies duras (equipamento da indústria alimentar, transporte, superfícies de distribuição e armazenamento, solo, etc.) ou a estruturas biológicas (vegetais, carne, ossos, frutos, etc.). A formação de biofilme confere muitas vantagens às células microbianas,

como resistência física, resistência mecânica e proteção química (FLEMMING et al., 2016).

Os biofilmes estão associados à placa dental (HOJO et al., 2009; PAJU; SCANNAPIECO, 2007), endocardite (PALMER, 2006), infecção pulmonar (LAU; HASSETT; BRITIGAN, 2005; WAGNER; IGLEWSKI, 2008) e infecção por dispositivos médicos (KHARDORI; YASSIEN, 1995; STOODLEY et al., 2013; VENKATESH et al., 2009). A formação de biofilme pode ocorrer também em diferentes superfícies, como em ambientes relacionados ao processamento de alimentos (GALIÉ et al., 2018).

Algumas associações entre compostos demonstraram eficácia contra o biofilme formado por bactérias em infecções ou também em diferentes superfícies.

Foi avaliado por Kalishwaralal e outros 2010 a eficácia das AgNP sintetizadas por *Bacillus licheniformis*, contra a formação de biofilme por cepas de *P. aeruginosa* e *S. epidermidis*. Os autores relataram que o tratamento com NP contendo nitrato de prata (AgNO3) reduziu em 98% o biofilme formado 24 h antes. Outro estudo realizado por Mohanty e outros 2012 demonstrou a eficácia dependente da dose de AgNPs contra o biofilme de *S. aureus* e *P. aeruginosa* após 24h de formação. O tratamento com 1 ou 2 μM (AgNPs) mostrou redução maior que 50% ou 85% na formação de biofilme, respectivamente.

Loo e colaboradores (2016) analisaram as possíveis interações sinérgicas e aditivas de AgNP e NP de curcumina, um extrato vegetal fenólico, contra *Pseudomonas aeruginosa* e *S. aureus*. A combinação dos compostos a 100 μg/mL foi eficaz no rompimento de 50% dos biofilmes bacterianos formados em placas de microtitulação.

Os dados sugerem que tanto a aplicação das nanopartículas de prata, quanto a de óleos essenciais, assim como a associação entre compostos são importantes alternativas ao uso dos antibióticos e também dos desinfetantes atuais.

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ARTIGOS

Os resultados e discussões foram apresentados na forma de artigo. O Artigo I foi submetido e segue as normas do “Journal of Nanoparticle Research” e tem como título: “**Synergistic And Additive Effect of Eugenol Associate with Biological Synthesized Silver Nanoparticles against Gram-Positive and Gram-Negative Bacteria”** e o Artigo II “**Synergistic Effect of Eugenol and Biologically synthesized Silver Nanoparticles against *Listeria monocytogenes***”, será submetido posteriormente e refere-se aos resultados obtidos nos experimentos realizados durante o período de Doutorado Sanduíche no exterior.

ARTIGO I

SYNERGISTIC AND ADDITIVE EFFECT OF EUGENOL ASSOCIATE WITH BIOLOGICAL SYNTHESIZED SILVER NANOPARTICLES AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

ABSTRACT

Studies have been demonstrated that eugenol essential oil and silver nanoparticles has strong antibacterial activity, also against multidrug resistant strains. The association between compounds are an interesting antimicrobial strategy. In this study, there were evaluated the antibacterial activity of the combination of silver nanoparticles produced by *Fusarium oxysporum* (bio-AgNP) with eugenol against Gram-positive and Gram-negative including multidrug resistant strains. Eugenol and bio-AgNP showed bactericidal effects against all strains tested; with minimal inhibitory concentrations (MIC) ranging from 0.12 to 2% (v/v) and 39.4 to 315 µM respectively and in none of the strains was observed resistance to these compounds. Time-kill curves showed that eugenol acted in less than 2 h, while the metallic nanoparticles took 0.5 h to 1 h to kill Gram-negative bacteria and 24 h to kill Gram-positive bacteria. A synergistic or additive effect was observed when the two compounds were combined, which caused a reduction of their MIC values and improved the time of action compared to bio-AgNP used alone. Scanning electron microscopy (SEM) showed related morphological alterations in *Staphylococcus aureus* (methicillin-resistant *S. aureus*, MRSA) cells exposed to three different treatments (eugenol, bio-AgNP and combination of the two), which appeared cell surface disturbing. For cytotoxicity assay with human red blood cells was not observed hemolysis higher than 50% for both compounds. The 50% cytotoxic concentration (CC50) for HEp-2 was 0.1859% for eugenol and 64.64 µM for bio-AgNP. This study described the antibacterial effect of eugenol in combination with bio-AgNPs, and our results indicated the combination as a possible alternative for controlling bacterial infection, mainly Gram-negative bacteria, with their safety uses.

Keywords: Biogenic nanosilver; synergism effect; antibacterial; clove oil; resistance.

INTRODUCTION

The proportion of associated health services caused by multidrug resistant pathogenic bacteria infections has increased significantly in recent decades, and the control of the infected patients is still a challenge in view of the available antimicrobials. New developments in present methods and novel strategies are urgently needed to cope with this problem (ALLAHVERDIYEV et al., 2011; WILLING et al., 2018). Some researchers are investigating alternative therapies to help the treatment of infections, resulting in improved antimicrobial effects, decreased costs associated with the treatment of patients, and fewer adverse effects to the host.

Active components of medicinal plants have been a source of therapeutics for the treatment of various illness since antiquity. The rapid spread of antibiotic resistance induced the research by active antimicrobial agents, and possibly even novel classes of antimicrobials, obtained from natural compounds. An alternative to the use of new antibacterial compounds is research of antibiotic synergists. Synergism in antimicrobial therapy is well known and it is used in order to describe supra-additive activity of antibiotics (HEMAISWARYA; DOBLE, 2009; XU et al., 2018).

Nanoparticles have a strong antimicrobial activity, being effective against various strains of bacteria, yeasts and molds, including antibiotic resistant (ZHANG et al., 2010). Metallic nanoparticles represent an effective solution for overcoming bacterial resistance, due to their antibacterial activities. These nanoscale agents allow more convenient routes of administration, lower therapeutic toxicity, extend the product life and reduce costs in healthcare (ALLAHVERDIYEV et al., 2011; HERMAN; HERMAN, 2014).

Nanotechnology is an important study to modify some materials, like silver metal in nanoparticle (NP) form. The development of biological method for the synthesis of nanoparticles has gained popularity, besides being more cost effective and ecofriendly than physical and chemical methods (DURÁN; NAKAZATO; SEABRA, 2016; SINGH et al., 2014; TALEBI; RAMEZANI; RAMEZANI, 2010). Green synthesis uses materials such as plant leaf extract, bacteria and fungi to reduce silver in AgNPs (SALAM et al., 2012).

Silver nanoparticles have unique physical, chemical and biological properties. They have been used in numerous cosmetic, medical and pharmaceutical area, including its use as antibacterial agent (HERMAN; HERMAN, 2014).

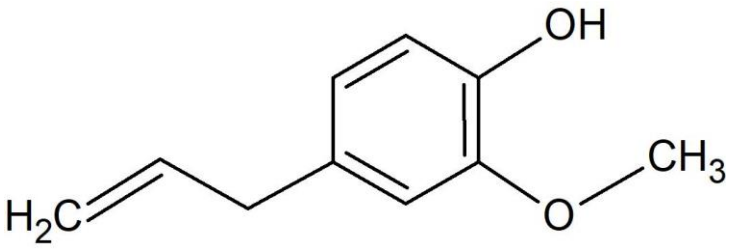
Action mechanisms of AgNP can describe these anchoring in the cell wall and then penetrate the bacteria, causing disturbance in cell membrane, such as membrane permeability dysfunction and cell death (DURÁN et al., 2016; SINGH et al., 2014). Also occurs formation of "holes" on the cell surface leading to the accumulation of nanoparticles (SONDI; SALOPEK-SONDI, 2004) and silver ions in the inner and outer membrane, which cause bacteria membrane destabilization (MORONES et al., 2005). Another AgNP action mechanism occurs by formation of free radicals leading to cell death (DANILCZUK et al., 2006; KIM et al., 2007). Researchers report one of major bactericidal activity mechanism is the ability of AgNP release Ag^+ ions (FENG et al., 2000; STOBIE et al., 2008), and these may interact with groups thiol present in many vital bacteria enzymes, and consequently inactivating them (FENG et al., 2000; MATSUMURA et al., 2003).

Bactericidal effect established by AgNP was described for *Escherichia coli, S. aureus, S. epidermidis, Streptococcus agalactiae, Bacillus subtilis, Klebsiella mobilis*, and *K. pneumoniae*, *Pseudomonas aeruginosa*, among others (GUZMAN; DILLE; GODET, 2012; MARAMBIO-JONES; HOEK, 2010; PATRA; BAEK, 2017; BIASI-GARBIN et al., 2015; SALOMONI et al., 2017; SCANDORIEIRO et al., 2016).

Another class of compounds which also have antimicrobial activity are the essential oils derived from spices that containing a wide variety of secondary metabolites which are capable of inhibiting or reducing the growth of bacteria and fungi. Phenolic compounds found in essential oils have been extensively researched by scientists for their health benefits potential (DEBIAGI et al., 2014; NAZZARO et al., 2013; SCANDORIEIRO et al., 2016).

Eugenol (4-allyl-2-methoxyphenol) (Figure 1), which is the active component *Syzigium aromaticum* (cloves), has a wide range of applications such as flavorings, essential oils and medicine as a local anesthetic and antiseptic. Some studies show that eugenol has several biological skills, including antimicrobial, antioxidant, anti-inflammatory, anti-carcinogenic, anti-mutagenic and anti-genotoxic (JAGANATHAN; SUPRIYANTO, 2012; QIU et al., 2010).

Figure 1 – Chemical structure of 4-allyl-2-methoxyphenol.



Phenolic compounds found in essential oils generally demonstrate activity against Gram-positive bacteria, however eugenol and isoeugenol presented higher activity against Gram-negative bacteria (HYLDGAARD; MYGIND; MEYER, 2012). The effects of this compound may depend; at low concentrations could interfere with enzymes involved in energy production and in high concentrations can denature proteins (TIWARI et al., 2009). Thus, essential oils can also demonstrate more than one action target in their activity, causing a cascade of reactions in the bacterial cell (NAZZARO et al., 2013).

Most of antimicrobial activity generated by eugenol and also other compounds called phenylpropenes like isoeugenol, vanillin, safrole and cinnamaldehyde is caused by their free hydroxyl group (LAEKMAN et al., 1990). The effects of eugenol may occur as a change in bacteria membrane, interference with the transport of ions and ATP in addition to modifying the profile of the chain fatty acids, thereby interfering with various enzymes such as ATPase, histidine, carboxylase, amylase and protease (DEVI et al., 2010; GILL; HOLLEY, 2004; HOLDER; BOYCE, 1994; THOROSKI; BLANK; BILIADERIS, 1989; WENDAKOON; SAKAGUCHI, 1995).

Studies have shown that eugenol also presented antibacterial activity to *E. coli, B. cereus, Helicobacter pylori, S. aureus, S. epidermidis, S. pneumoniae*, *S. pyogenes* and others (ALI et al., 2005; KAMATOU; VERMAAK; VILJOEN, 2012; MARCHESE et al., 2017; BIASI-GARBIN et al., 2015; ZHANG et al., 2017).

The development of new antimicrobial compounds or the modification of available; to improve antimicrobial activity for therapy, antisepsis, and disinfection is an area of high priority in the search.

Due to the emergence of bacterial resistance (multidrug resistance) and severity (high morbidity and mortality) for diseases related to these agents, there is a need for the use of new antimicrobials.

Recently, our group found that association of eugenol and AgNPs showed bactericidal synergistic action against *S. agalactiae* (BIASI-GARBIN et al., 2015) and other medical interest bacteria including multidrug resistant (patent deposited process Brazilian Patent PI BR 1020140215687).

The aim of this research is verify AgNps associated with eugenol against Gram-positive and Gram-negative bacteria, including multidrug resistant that cause important human infections and other animals.

MATERIALS AND METHODS

Bacteria strains

Gram-positive and Gram-negative standard strains, including multidrug resistant bacteria were used in this study. All antimicrobial assays were performed against reference strains from American Type Culture Collection (ATCC) provided by the Laboratory of Basic and Applied Bacteriology, of Department of Microbiology, of State University of Londrina (Londrina, Paraná, Brazil), and clinical strains provided from University Hospital (Londrina, Paraná, Brazil). The standard bacterial strains used were as follows: *Enterococcus faecalis* (ATCC 29212), *E. faecium* (ATCC 6569), *S. aureus* (ATCC 25923 and ATCC 29213), *S. epidermidis* (1E4248), *Acinetobacter baumannii* (ATCC 60114), *E. coli* (ATCC 8739 and ATCC 25922), *K. pneumoniae* (ATCC 10031 and ATCC 700603), *P. aeruginosa* (ATCC 9027 and ATCC 27853), *Salmonela enterica* serovar Enteritidis (ATCC 13076), *S. enterica* serovar Typhimurium (UK1), *E. coli* ESBL 176 (clinical isolates obtained from Londrina University Hospital were also tested ESBL-producing *E. coli*, from urinary tract infections (provided by Dr. Eliana Carolina Vespero, University Hospital, Londrina, Paraná, Brazil). *S. aureus* MRSA strain N315 (Kuroda *et al.*, 2001) and BEC9393 (Soares *et al.*, 2001) were provided by Dr. Elsa Masae Mamizuka (Universidade de São Paulo, São Paulo, São Paulo-SP, Brazil) and Dr. Agnes Marie Sá Figueiredo (Universidade Federal do Rio de Janeiro, Rio de Janeiro-RJ, Brazil), respectively. Were selected 27 *S. aureus* MRSA clinical isolates obtained from University Hospital, Londrina, Paraná, Brazil, previously described by Bodnar et al. (2016) and included in this study to evaluate if the strains showed some resistant profile against the tested compounds. The bacterial strains were stored in 25% glycerol (Sigma-Aldrich, St. Louis, US) at −80◦C.

Antimicrobial Agents

Eugenol

A stock solution of 50% eugenol (Sigma-Aldrich, St. Louis, US) was prepared containing 50% (v/v) dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, US). DMSO maximum concentration in assays was 4.0%.

Biological Silver nanoparticles from *Fusarium oxysporum*

Bio-AgNPs were obtained following the method of Durán et al. (2005). This method of production has been patented (Patent, 2006, PI 0605681-4A2; http://www.inpi.gov.br). The bio-AgNP were obtained after reduction of silver nitrate ($AgNO_3$ - Sigma-Aldrich, St. Louis, US) by *F. oxysporum*, strain 551, from the culture collection of the Molecular Genetics Laboratory of ESALQ-USP (Piracicaba, São Paulo, Brazil). After the growth of *F. oxysporum* culture, was added 100 mL of distilled water in 10 g of the biomass. Incubation was done by 72 h at 28°C, the solution components were separated by filtration, and $AgNO_3$ at concentration of 10^{-3}M was added. Sporadically, aliquots were removed and absorptions were measured from the solution system using an ultraviolet-visible spectrophotometry (Varian Cary 50 Probe); the peak at 440 nm corresponded to the surface plasmon resonance of silver nanoparticles. The diameter was determined by photon correlation spectroscopy, after bio-AgNP purification, using ZetaSizer NanoZS (Malvern), and zeta potential measurement was performed with the same instrument.

Antibacterial Activity of eugenol and Bio-AgNP Separately

Minimal inhibitory concentrations (MICs) were determined by micro-dilution assays according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2012), with necessary modifications. Single colonies of bacterial cultures grown in Muller-Hinton agar (MHA, Oxoid, Hampshire, UK) were diluted in saline solution and adjusted to 0.5 McFarland scale, which corresponds to 1.5×10^8 CFU/ml. Then, the bacterial suspensions were diluted in Mueller-Hinton broth (MHB, Oxoid, Hampshire, UK) and plated in 96-well plates at a density of 10^5 CFU/ml. Finally, tested concentrations of eugenol and bio-AgNP ranged from 0.125 to 2% and 39.4 to 315 µM, respectively. Mueller-Hinton broth alone, MHB plus eugenol and MHB plus bio-AgNP were tested as sterility controls, and untreated bacteria inoculated on MHB

alone and with 2% DMSO were tested as growth control. The plates were incubated at 37°C for 24 h, and MIC was defined as the lowest concentration of antimicrobial agent that inhibited visible growth. All assays were carried out in triplicate.

Drug interaction studies

To verify the antibacterial effects and interactions of eugenol combined with bio-AgNP produced by *F. oxysporum* against different strains, assays of microdilution in double-antimicrobial gradient were used. First, the MIC values for eugenol and bio-AgNP used alone were determined, and several concentrations of eugenol were combined with different concentrations of bio-AgNP. There were determinate a combination MIC, which was the lowest concentration of eugenol combined with the lowest concentration of bio-AgNP. In order to evaluate the interaction between both antimicrobials, the fractionated inhibitory concentration (FIC) index was used as described by (CHIN; WEITZMAN; DELLA-LATTA, 1997).

$$FIC = MIC(Ec) \div MIC(Ea) + MIC(Sc) \div MIC(Sa)$$

MIC(Ec) is the MIC of eugenol combined with the AgNP, MIC(Ea) is the MIC of eugenol alone, MIC(Sc) is the MIC of the AgNP combined with eugenol and MIC(Sa) is the MIC of the AgNP alone. FIC indexes were interpreted as follows: FIC ≤ 0.5 = synergic interaction; 0.5 < FIC ≤ 1.0 = additive interaction; 1.0 < FIC ≤ 4.0 = no interaction; FIC > 4.0 = antagonist interaction.

Curve of growth and viability

Regarding the measure of the eugenol and AgNPs effect on the bacterial growth, a time-response growth curve was obtained according to NCCLS (1999). Curve of growth and viability was done with *S. aureus* MRSA N315, *E. coli* ATCC 8739 and *K. pneumoniae* ATCC 10031. Firstly, a single colony forming unit of each strain was diluted in MH broth and grown for 24 h at 37°C, then, each culture was adjusted to 0.5 index in McFarland scale and inoculated at a cell density of ~10^6 CFU/ml in 2 ml of MH broth. For each strain culture was divided in four new cultures of 1 ml each. One culture received eugenol (1x MIC), the second received AgNP (1x MIC), other received eugenol combined with AgNP, both in different concentrations, and the last culture received no component (control). The bacterial cultures were

incubated at 37°C. In different times (0, 2, 4, 7, 10, 24 h for Gram-positive strains and 0, 0.25, 0.5, 1, 2, 4, 7, 10, 24 h for Gram-negative strains), an aliquot of the broth was collected, serial diluted in saline solution, plated on MHA and grown for 18-24 h at 37°C in order to determine the total CFU of each culture.

Scanning Electron Microscopy (SEM)

For scanning electron microscopy (SEM), there was used *S. aureus* N315 strain to evaluate the effects of the tests over the cell. To evaluate the action of these compounds front MRSA N315 was used MIC after 3 h of incubation. Suspensions of this bacteria (10^{10} CFU/ml) with and without the antibacterial compound (at about MIC) were spotted onto poly-lysine-coated (Sigma-Aldrich, St. Louis, US) glass slides. Each slide was then fixed by immersion in 1 ml of 2.5% glutaraldehyde (Sigma-Aldrich, St. Louis, US) and 2% paraformaldehyde (Sigma-Aldrich, St. Louis, US) in 0.1 M sodium cacodylate buffer (Sigma-Aldrich, St. Louis, US) (pH 7.2) for 12 h and then post-fixed in 1% OsO_4 (Sigma-Aldrich, St. Louis, US) for 2 h. The fixed samples were dehydrated in an ethanol (Merck, Darmstadt, DE) gradient (70, 80, 90 and 100°GL) and then were critical point dried in CO_2 (BALTEC CPD 030 Critical Point Dryer). Finally, the slides were taped onto stubs, coated with gold (BALTEC SDC 050 Sputter Coater) and observed under a FEI Quanta 200 SEM.

Cytotoxicity Assay with Human Red Blood Cells (RBC)

The cytotoxicity was determined by testing the hemolytic activity of eugenol and bio-AgNP using the method with some modification described by Izumi et al. (2012). Fresh human blood was collected in heparinized tubes (Vacutainer) to avoid coagulation from a healthy human donor with voluntary consent, which was approved by the human ethics committee (CAAE 47661115.0.0000.5231, No. 1.268.019 – UEL). Erythrocytes were separated by centrifugation (5000 rpm, 4°C, 5 min) and were prepared in sterile phosphate-buffered saline (0.1 M PBS, pH 7.2) at 6% (v/v). PBS was composed of 0.9% (w/v) sodium chloride (Merck, Darmstadt, DE), 0.2 M monobasic sodium phosphate (Sigma-Aldrich, St. Louis, US) and 0.2 M dibasic sodium phosphate (Sigma-Aldrich, St. Louis, US). A volume of 100 µl of RBC at 6% were added to 100 µl of PBS, in 96-well plates, with various concentrations of compounds individually and in combination. Supernatants were read at 550 nm to monitor release of hemoglobin after 3 h of incubation at 37°C. There was used as

control for 100% hemolytic activity Triton X-100 (Sigma-Aldrich, St. Louis, US) at 1% and hemolysis percentage was calculated for each compound concentration.

The concentration of eugenol and bio-AgNP ranged 0.0015 - 4% and 4.9 – 630 µM, respectively. The 50% cytotoxic concentration (CC50) was defined, as the antimicrobial concentration required reducing cell viability by 50% compared to untreated control. CC50 of each compound was determined by analysis for RBC cells and was calculated by GraphPad Prism Version 6.07 and the selectivity index (SI) were calculated using the equation: SI = CC50/IC50.

Cytotoxicity Assay with HEp2 cells

Cytotoxicity of eugenol and bio-AgNP to the human laryngeal epithelial carcinoma cell line HEp-2 was performed by the colorimetric dimethylthiazol diphenyl tetrazoliumbromide (MTT, Sigma-Aldrich, St. Louis, US) assay in 96-well plates. Cell line was grown as monolayers in RPMI medium 1640 (Gibco, Carlsbad, US) at 37°C in 5% CO_2. Non-adherent cells were removed using PBS and confluent cells were exposed to different concentrations of compounds individually and in combination during 24 h at 37°C in 5% CO_2. The medium was removed after incubation and each well was washed with sterile PBS. MTT solution (10 µl per well at 1.250 g/ml) was added to all wells, then plates were incubated at 37°C for 2 h. MTT solubilization solution (Sigma- Aldrich, St. Louis, US) containing 10% Triton X-100 in acidic isopropanol (0.1 N HCl) was added to each well to solubilize the formazan formed. After 15 min of homogenization, the plate was read at 570 nm. HEp-2 non-treated cells were used as control for 100% viability, and viability percentage was calculated for each compound concentration.

The concentrations of eugenol and bio-AgNP ranged 0.015 - 2% and 4.9 – 630 µM, respectively and the CC50 and SI were performed equal from previous cytotoxicity assay.

Statistical Method

Data were analyzed by ANOVA, and the difference among means was determined using Tukey's test ($p < 0.05$). The Student's t-test ($p < 0.05$) was also used for the analysis of means. All tests were performed with the statistical program RStudio version 1.0.136.

RESULTS

Bio-AgNP Characterization

Average bio-AgNP size and zeta potential were 81.25 nm and −36.4 mV, respectively (Supplementary Material – ANEXO 1).

Minimal Inhibitory concentration (MIC)

Eugenol and bio-AgNP inhibited the growth of all bacterial strains tested, including multidrug resistant strains. The eugenol MIC ranged from of 0.12 - 2% and the mean eugenol MIC for Gram-positive was 1.35 ± 0.63% and for Gram-negative was 0.54 ± 0.28%, with statistical difference ($p<0.05$) between Gram-positive and Gram-negative strains. MIC for bio-AgNP was ranged from 39.4 to 315 μM and the mean for bio-AgNP against Gram-positive was 106.9 ± 100.9μM and for Gram-negative strains was 74.9 ± 34.5 μM, with no statistical differences between Gram-negative and Gram-positive using bio-AgNP. These compounds do not have breakpoints because they are new antibiotics (Table 1). For 27 MRSA strains tested, eugenol MIC ranged from 0.25 – 1% and for bio-AgNP MIC ranged from 19.7 – 157.5 μM (Table 2).

According to MIC values *S. aureus* ATCC 29213, *S. aureus* MRSA N315, *E. coli* ATCC 8739 and *K. pneumoniae* ATCC 10031 were further selected for curve of growth and viability tests and MRSA N315 for scanning electron microscopy.

Drug interaction studies

Eugenol was combined with bio-AgNP for evaluation of synergic effect against Gram-positive and Gram-negative strains. MIC for these compounds combined were demonstrated in Table 1. FIC values ranged between 0.4 and 1, showed synergism or additive interaction according to different bacteria (Table 1) and only for *A. baumannii* (ATCC 60114) there was no interaction (FIC = 1.5). The major values showed synergism interaction both Gram-positive as Gram-negative strains, however with no statistic difference ($p>0.05$) between Gram-positive or Gram-negative strains.

Table 1 – MIC and synergistic effect generated by Eugenol and AgNPs against Gram-positive and Gram-negative bacteria.

Gram-positive Bacteria	MIC E% (v/v)	MIC AgNP (µM)	MIC E/AgNP	FIC	Interaction
***E. faecalis* (ATCC 29212)**	1	39.4	0.125/9.8	0.36	Synergic
***E. faecium* (ATCC 6569)**	2	157.5	0.25/78.8	0.62	Additive
***S. aureus* (ATCC 25923)**	0.5	39.4	0.125/19.7	0.74	Additive
***S. aureus* (ATCC 29213)**	1	78.8	0.25/19.7	0.5	Synergic
***S. aureus* N315**	2	315	0.5/78.8	0.5	Synergic
***S. aureus* BEC9393**	2	39.4	0.5/19.7	0.75	Additive
***S. epidermidis (*1E4248)**	1	78.8	0.25/39.4	0.75	Additive

Gram-negative Bacteria	MIC E% (v/v)	MIC AgNP (µM)	MIC E/AgNP	FIC	Interaction
***A. baumannii* (ATCC 60114)**	0.5	39.4	0.25/39.4	1.5	No interaction
***E. coli* (ATCC 8739)**	0.5	157.5	0.125/19.7	0.5	Synergic
***E. coli* (ATCC 25922)**	0.5	78.8	0.125/19.7	0.5	Synergic
***K. pneumoniae* (ATCC 10031)**	0.5	78.8	0.125/19.7	0.49	Synergic
***K. pneumoniae* (ATCC 700603)**	0.5	78.8	0.125/19.7	0.49	Synergic
***P. aeruginosa (*ATCC 9027)**	1	39.4	0.25/9.8	0.5	Synergic
***P. aeruginosa* (ATCC 27853)**	1	39.4	0.25/9.8	0.5	Synergic
***S. enterica serovar Enteritidis* (ATCC 13076)**	0.25	78.8	0.125/39.4	1	Additive
***S. enterica serovar Typhimurium* UK1**	0.125	78.8	0.06/39.4	1	Additive
***E. coli* ESBL 176**	0.5	78.8	0.25/39.4	1	Additive

MIC E – Minimal Inhibitory Concentration of eugenol used alone

MIC AgNP - Minimal Inhibitory Concentration of bio-AgNP used alone

MIC E/AgNP - Minimal Inhibitory Concentration of eugenol associated with bio-AgNP

FIC - Fractional Inhibitory Concentration

Table 2 – MIC by Eugenol and AgNPs against MRSA clinical isolates.

MRSA	MIC E% (v/v)	MIC AgNP (μM)
MRSA 102	1	39.4
MRSA 105	0.5	39.4
MRSA 106	0.5	39.4
MRSA 109	1	39.4
MRSA 110	1	39.4
MRSA 112	0.5	19.7
MRSA 114	0.5	39.4
MRSA 115	0.5	19.7
MRSA 116	1	39.4
MRSA 118	0.5	39.4
MRSA 119	0.5	19.7
MRSA 121	0.5	39.4
MRSA 123	1	39.4
MRSA 124	0.5	39.4
MRSA 301	0.5	39.4
MRSA 304	0.5	78.8
MRSA 306	0.5	39.4
MRSA 309	0.5	78.8
MRSA 402	0.5	78.8
MRSA 403	1	78.8
MRSA 404	1	39.4
MRSA 405	0.5	39.4
MRSA 406	0.5	39.4
MRSA 408	0.5	78.8
MRSA 410	0.25	78.8
MRSA 411	0.5	78.8
MRSA 413	1	39.4

MIC E – Minimal Inhibitory Concentration of eugenol used alone

MIC AgNP - Minimal Inhibitory Concentration of bio-AgNP used alone

Curve of growth and viability

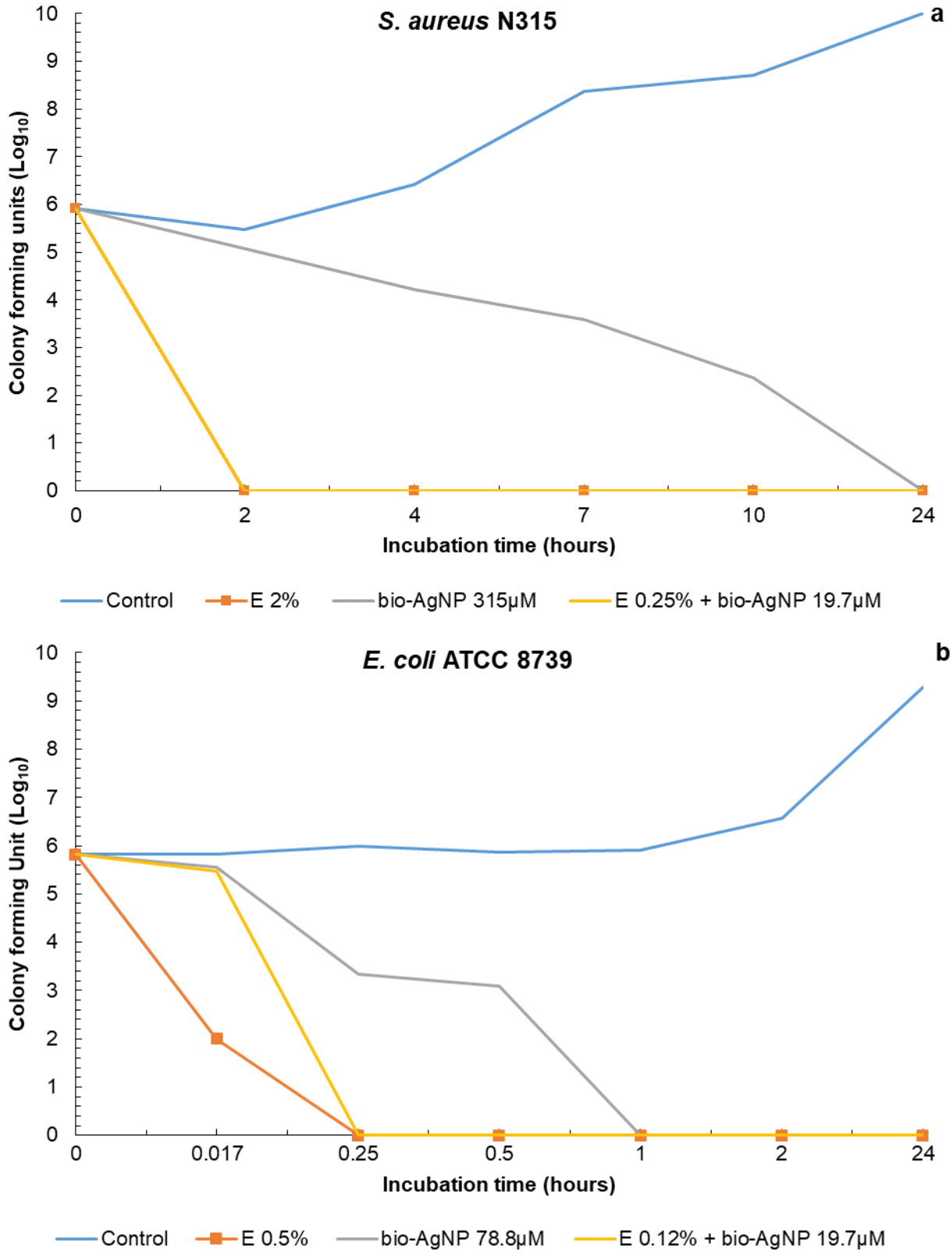
MRSA N315, *E. coli* ATCC 8739 and *K. pneumoniae* ATCC 10031 were selected to perform growth and viability tests. For each strain we used 1xMIC values

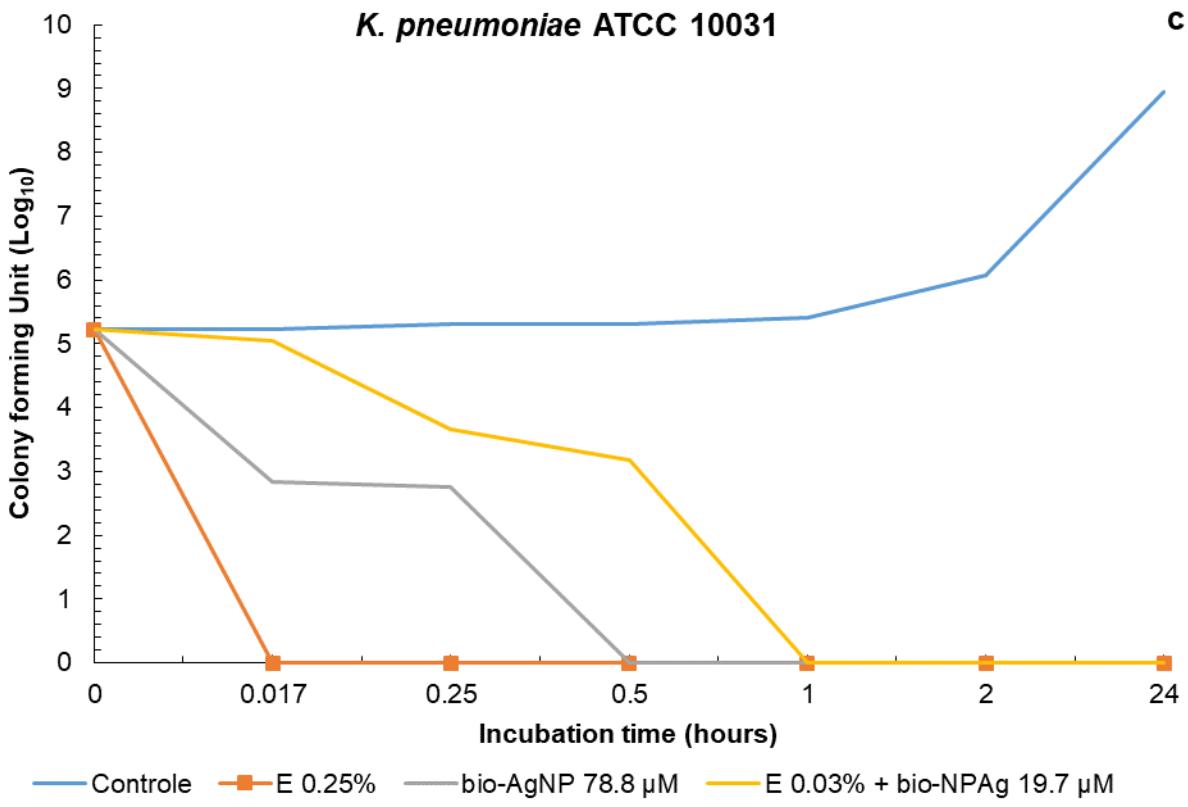
for eugenol and AgNP, and different values for eugenol combined with AgNP (Figure 2), according to each strain. In all strains, there were complete inhibitions of bacterial growth. The inhibition by eugenol at MIC (2%) acting against MRSA N315 was in 2h (Figure 2A), after the same time the combination of the compounds at MIC decrease (E 0.25% + bio-AgNP 19.7 μM) showed the reduction of 5.92 log. After 10h of incubation, bio-AgNP reduce 4.25 log and the completely inhibition was observed after 24h of treatment.

Gram-negative isolates showed different results than Gram-positive, the strains were more sensitive to compounds showed log reduction faster. Different times of incubation were applied for Gram-negative due to the bactericidal effect occurred in less than 2h after treatment.

Bio-AgNP at MIC (78.8 μM) showed 2.47 log reduction after 0.25 h from the initial treatment followed by total growth inhibition after 1h treatment, for *E. coli* ATCC 8739. The same strain when treated by compounds in combination at 1/4MIC (0.12% eugenol and 19.7 μM of bio-AgNP) showed bactericidal effect after only 0.25h (Figure 2B). For *K. pneumoniae* ATCC 10031, bio-AgNP and eugenol combined with nanoparticles after 2h incubation there were total growth inhibition (Figure 2C). Eugenol eliminate in 15 minutes all *K. pneumoniae* ATCC 10031 and *E. coli* ATCC 8739 strains at MIC (0.25% and 0.5%, respectively).

Figure 2 –Time-kill curves for *S. aureus* N315 (**2a**), *E. coli* ATCC 8739 (**2b**) and *K. pneumoniae* ATCC 10031 (**2c**) strains exposed to eugenol (E,) bio-AgNP and combination (E + bio-AgNP) of this two compounds.



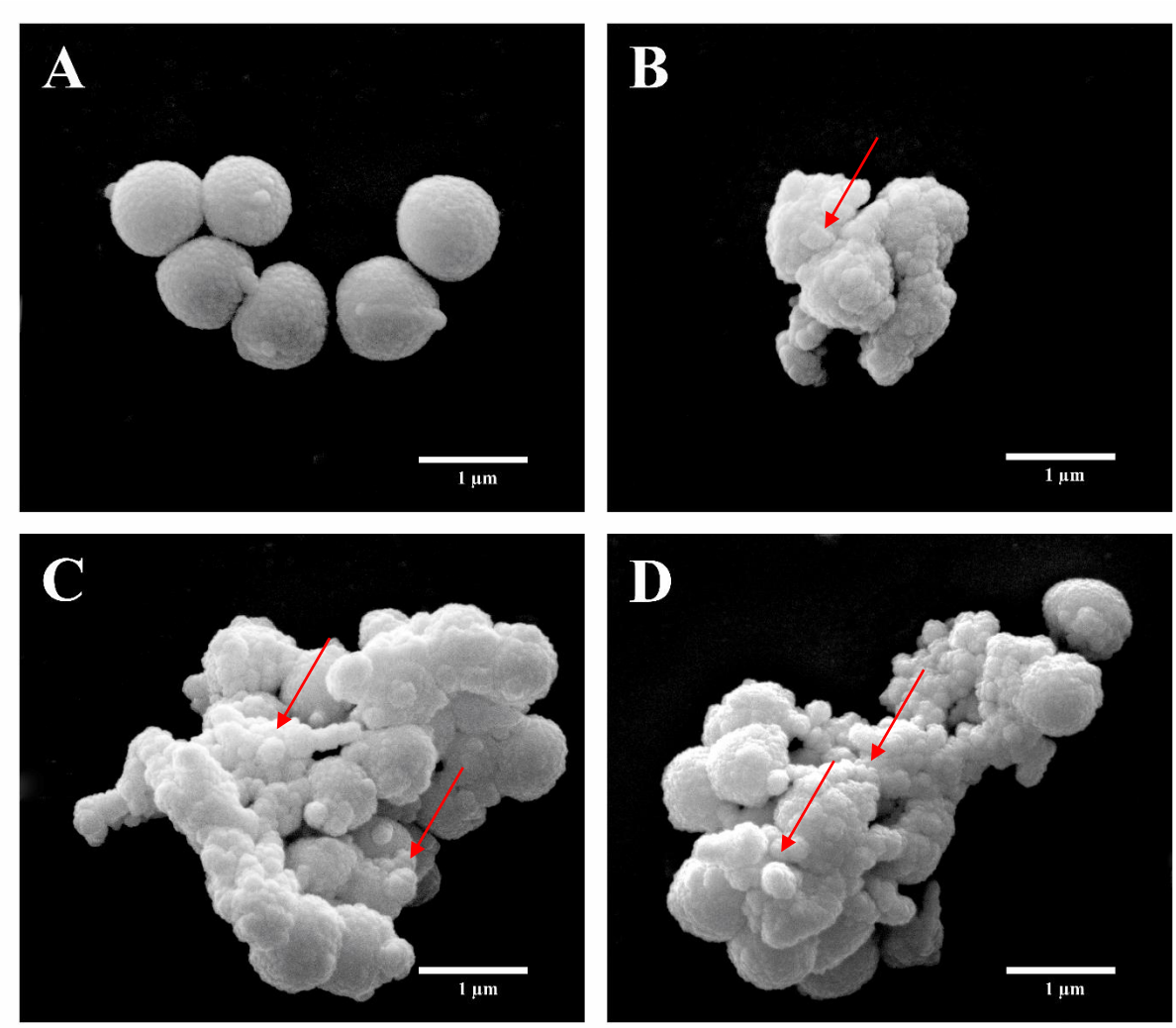


Scanning Electron Microscopy (SEM)

Through the images obtained from SEM, it was possible to observe changes in cell morphology of MRSA N315 in only 3 hours of incubation. The effects of treatments on bacterial cells are shown in Figure 3A to 3D. *S. aureus* N315 cells showed normal morphologies without any treatment (Figure 3A). Treatment with eugenol or bio-AgNP exhibit alteration cells morphology, with formation of prominence in the majority cells becoming deformed (Figure 3B and 3C). In treatment with bio-AgNP, some perturbations were visible on the surface of cells, with many fragments, indicating the damage of cell surfaces (Figure 3B).

In the images, we could observe a large amorphous mass, indicating the total destruction of the bacterial cell. Combination eugenol with bio-AgNP changes both showed the same images (Figure 3D). It is possible to identify cell prominences, characteristic of bio-AgNP, and formation of an amorphous mass, which could be indicated by eugenol characteristic.

Figure 3 - The effect of treatments on *S. aureus* N315 bacterial cells after 3 hours of treatment. (3a) this figure show control cells. (3b) figure demonstrate a treatment effect of 315µM bio-AgNP. (3c) this figure show cells treated with eugenol 2% in *S. aureus* N315 cells. (3d) demonstrate a treatment effect of eugenol (0.5%) associated with AgNP (19.7 µM).



Cells morphology with prominence in the majority cells becoming deformed; some perturbations are visible on the surface of cells, indicate by the arrows.

Cytotoxicity Assay with Human Red Blood Cells (RBC)

Results of human RBC assay showed that 2% of eugenol cause 42.27% hemolysis, according to Table 3 that show the toxicity of eugenol to RBC. Percentage of viability of RBC cells after eugenol (v/v) treatment in the range 0.0015% to 4% are indicate in Figure 4. The difference between the results from 4% and 2% eugenol concentration could be explain by the high concentration used of eugenol in 4% and

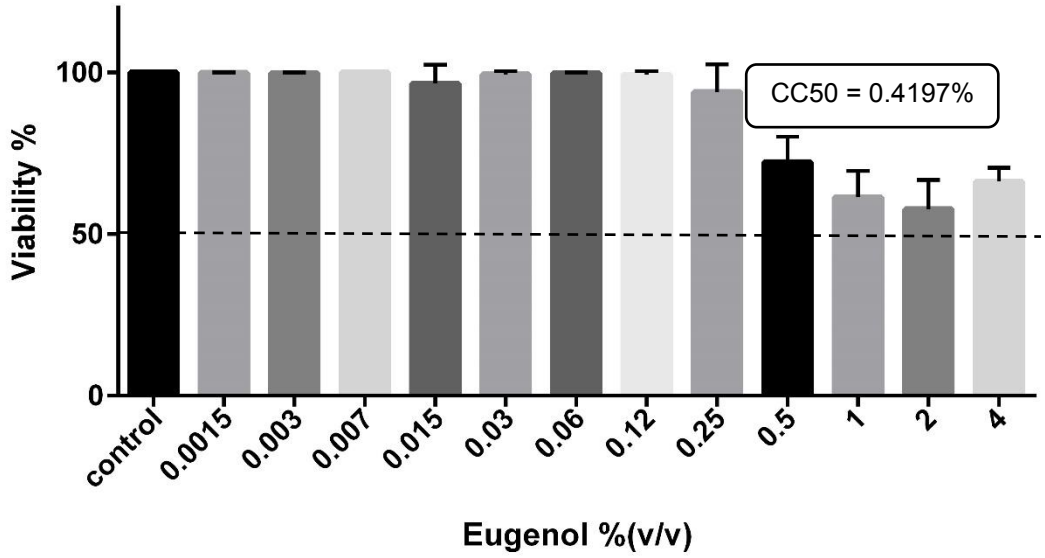
the oil couldn't mix with RBC and interfere in the bacterial nutrition depletion due to the highest concentration of the oil (Table 3, Figure 4). According to this experiment, there was not observed hemolysis higher than 50%. The CC50 for eugenol was 0.4197% with 95% confidence intervals between 0.3558 to 0.4951%. Eugenol SI values were higher than one only for *S. enterica serovar Enteretidis* (ATCC 13076) and *S. enterica serovar Typhimurium* UK1.

It was not possible to determine the 50% cytotoxic concentration of the bio-AgNP on RBC cells because even with the highest concentration tested (630 µM), 95% of the cells were viable.

Table 3 – Hemolytic activity of eugenol.

Eugenol % (v/v)	**% Hemolytic activity (Mean ± SD)**
0.0015%	0.09 ± 0.16
0.003%	0.20 ± 0.21
0.007%	0.00 ± 0.00
0.015%	3.38 ± 5.85
0.03%	0.57 ± 0.98
0.06%	0.18 ± 0.24
0.12%	0.63 ± 1.09
0.25%	5.98 ± 8.50
0.5%	27.68 ± 7.86
1%	38.59 ± 8.13
2%	42.27 ± 9.01
4%	33.70 ± 4.22

Figure 4 – Percentage of viability in RBC cells after eugenol (v/v) treatment.

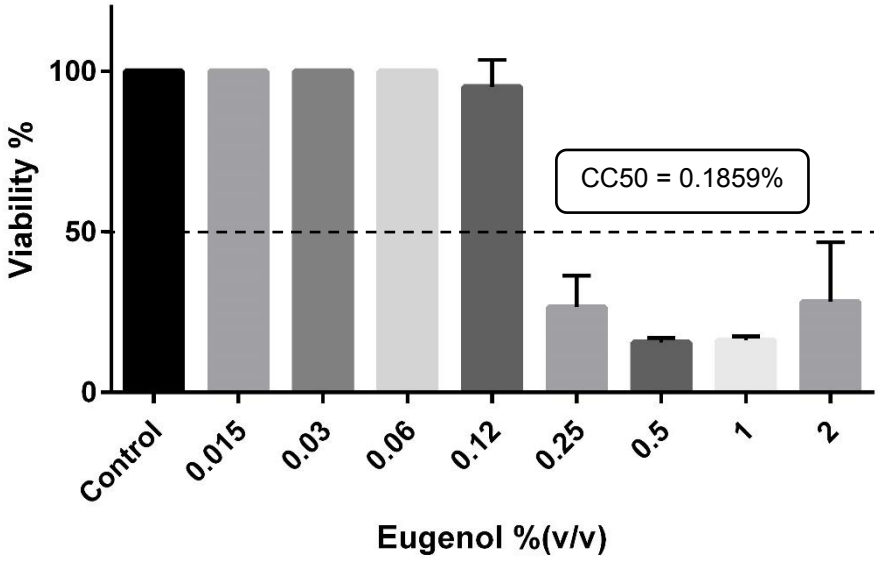


Percentage values of cell viability are the mean ± standard deviation.

Cytotoxicity Assay with HEp-2 cells

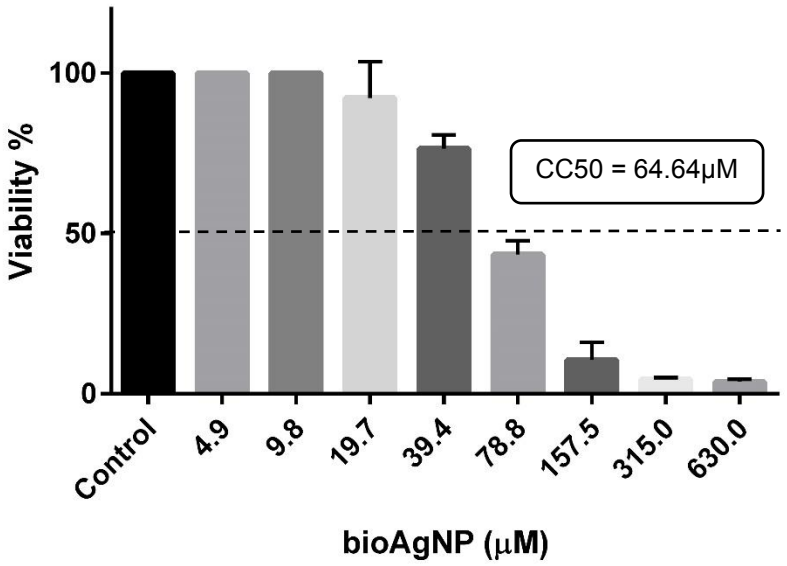
Results of MTT assay showed the eugenol cytotoxicity in tumor cells (HEp-2 cells), reducing cell metabolism by 50% or more at concentrations higher than 0.12% (v/v) (Figure 5) and CC50 was 0.1859%. However, this essential oil was less toxic to RBC (Figure 4). CC50 of bio-AgNP in HEp-2 cells was 64.64 µM (Figure 6).

Figure 5 – Percentage of viability in HEp-2 cells after eugenol (v/v) treatment.



Percentage values of cell viability are the mean ± standard deviation.

Figure 6 – Percentage of viability in HEp-2 cells after bio-AgNP (μM) treatment.



Percentage values of cell viability are the mean ± standard deviation.

DISCUSSION

This study showed that eugenol has potent bactericidal activity at low concentration with fast action, in agreement with previous studies (ALI et al., 2005; SINGH et al., 2016; THOSAR et al., 2013; YADAV et al., 2015a).

Fu et al. (2007) found for clove essential oil a MIC of 0.5% (v/v) against *P. aeruginosa* (ATCC 27853), 0.25% against *S. epidermidis* (ATCC 12228) and a MIC of 0.125% against *E. coli* (ATCC 8739). Sofia et al. (2007) observed the bactericidal effect of clove oil at 3% (v/v) concentration against *E. coli*, *S. aureus* and *Bacillus cereus*. Mith et al. (2014) reported MIC of 1 µl/mL against *S. enterica* serovar Typhimurium ATCC 14028 and MIC of 0.5 µl/mL against *E. coli* O157:H7 ATCC 35150. Xu et al. (2016) studied the essential oil obtained from clove buds, in which eugenol was the major component (76.23%), exhibited strong antibacterial activity against *S. aureus* ATCC 25923 with a MIC of 0.625 mg/ml. MIC values obtained in this study ranged from 0.125 to 2% (Table1), which were in line with literature results. The analysis showed that eugenol inhibited the growth in multidrug resistant bacteria tested at a low concentration, with MIC values of 2% against MRSA *S. aureus* N315 and *S. aureus* BEC9393 and 0.5% against *E. coli* ESBL176.

Time-kill assays showed that eugenol were bactericidal in *E. coli* ATCC 8739 (Figure 2B) and reduced cell populations in *K. pneumoniae* ATCC 10031 (Figure 2C) nearly 5-log (both after 1 min of treatment), so our results indicated that eugenol acted within a few minutes against these bacteria. Fu et al. (2007) showed that when *E. coli* ATCC 8739 at 10^5–10^6 CFU/mL was exposed to clove oil (0.125% v/v) there were no viable cells after the first 2 h of treatment.

Some studies have found that eugenol is active against Gram-positive and Gram-negative bacteria (DORMAN; DEANS, 2000; HEMAISWARYA; DOBLE, 2009; POATY et al., 2015). The results indicated that eugenol has broad-spectrum action in agreement with these previous studies, where it was equally effective according to MIC (Table 1), differing in time of action (Figure 2A to 2C), and with statistic differences between Gram-negative and Gram-positive bacteria ($p > 0.05$), in agreement of previous studies (HYLDGAARD; MYGIND; MEYER, 2012). The hydrophobicity of eugenol is an important factor affecting its antibacterial activity (KONG, 2014), most of this activity is conferred by benzene ring compound containing free hydroxyl group with antibacterial efficacy even when diluted to more than 2000 times (LAEKMAN et al., 1990).

Many studies suggest that essential oils have been known to possess antibacterial activity affect the cell membrane resulting in disruption and growth inhibition. Yadav et al. (2015b) related that the mode of bactericidal action of eugenol against *S. aureus* is through membrane disruption and further blocking the cell growth. Devi et al. (2010) showed the effect of eugenol on *S. enterica* serovar Typhimurium cell surface and observed that this essential oil made a deformation on cell surface. The authors related that bacterial membrane was disrupted and a complete loss of membrane integrity was also evident. Hemaiswarya & Doble (2009) showed in their study a loss of 50% of membrane integrity when Gram-negative bacteria where treated with eugenol for 10 min. In our study, SEM observations confirmed physical damage and considerable morphological alteration as cell surface blebbing in *S. aureus* N315 cells exposed to eugenol for 3 hours (Figure 3B), compared to control (untreated bacteria) showing no morphological changes after 3 hours (Figure 3A).

Other investigators have also found the lack of cytotoxicity of eugenol to erythrocytes. Hemaiswarya & Doble (2009) reported only 5.2% hemolysis at concentrations of 2.5 mM showing its low cytotoxic activity. In our studies, 0.25% (2.67 mg/mL) showed only 5.98% hemolysis. Since MIC values ranged from 0.25% to 2%, the highest value did not show 50% of hemolysis (Table 3).

Eugenol showed great bactericidal effect against all bacteria tested (Gram-positive and negative). Moreover, it showed low host cytotoxicity (hemolytic activity) increasing its potential use as antimicrobial. Another important aspect is the market availability and eugenol is a compound released for use as drug.

Concerning this study, bio-AgNP inhibiting growth of both Gram-positive and negative bacteria, presented broad-spectrum antibacterial action, in agreement with the literature. According with previous studies using silver nanoparticles produced by *F. oxysporum* that reported 125 µM MIC value (CARDOZO et al., 2013; BIASI-GARBIN et al., 2015; SCANDORIEIRO et al., 2016) the mean MIC value obtained in our study was 88 µM, considering similar to other studies. Some authors reported Gram-positive had been more tolerant to bio-AgNP than Gram-negative (AGNIHOTRI; MUKHERJI; MUKHERJI, 2014; DURÁN et al., 2010; JAIN et al., 2010; SCANDORIEIRO et al., 2016), was observed similar results, however with no statistical difference between the strains after the treatment with bio-AgNP (Table 1). However, time of action was faster in Gram-negative bacteria, reducing log

concentration in less than 1 hour, and the combination was fast and effective against *E. coli* ATCC 8739 in 15 minutes (Figure 2B and 2C). Fayaz et al. (2010) showed that the MIC of biogenic AgNPs against tested strains have a less significant effect on growth of Gram-positive bacteria than on Gram-negative bacteria. The structural difference in cell wall composition of Gram-positive and Gram-negative bacteria could explain this difference.

The size of nanoparticles is important on antibacterial activity. Some authors described that low size of nanoparticles showed higher antibacterial effect, but for biogenic and colloidal silver nanoparticles these phenomena did not occur (DURÁN et al., 2016). These bio-AgNP used in our study, with size range 70 to 100 nm, have higher antimicrobial activity against bacteria and yeast (LONGHI et al., 2016; OTAGUIRI et al., 2017; BIASI-GARBIN et al., 2015; SCANDORIEIRO et al., 2016).

SEM showed alterations in bacteria, in strains treated with AgNP, and the same perturbations were observed by Ghosh et al. (2013). Scandorieiro et al. (2016) showed surface protrusions on bio-AgNP treated *S. aureus* cells. Our researches observed extensive damage to the cell wall (Figure 2C) according to these authors.

Our MTT assay results showed that bio-AgNP was toxic to HEp-2 cells at CC50 equal 64.64 μM. Some authors have reported antitumor activity of silver nanoparticles (ANTONY et al., 2013; DEVI, 2012; SCANDORIEIRO et al., 2016), supporting our finding of bio-AgNP being toxic to HEp-2 cells and according to another study (SCANDORIEIRO et al., 2016).

The bacterial resistance to antibiotics has been comprehensively discussed in the literature, and some authors are studying the possible development of resistance to silver nanoparticles, but this mechanism has not been fully explored. Panáček et al. (2018) reported that some Gram-negative bacteria can develop resistance to silver nanoparticles after repeated exposure and Graves et al. (2015) utilized experiments to determine how quickly resistance to AgNPs can evolve in *E. coli* and indicates that bacteria can develop resistance to AgNPs. In our experiments, none of the bacteria tested showed resistant, including the multidrug resistant strains (Table 2), but care should be taken concerning the use of AgNPs as biocides or as unintentional environment exposure.

Some authors demonstrate synergic effect of AgNP in combination with antibiotics against Gram-positive and Gram-negative bacteria (CARDOZO et al., 2013; PERUGINI BIASI-GARBIN et al., 2015; SCANDORIEIRO et al., 2016). Fayaz

et al. (2010) observed the antibacterial activity of ampicillin, kanamycin, erythromycin, and chloramphenicol increase in bio-AgNP presence against *E. coli*, *S. aureus*, *S. typhi* and *Micrococcus luteus*. The results showed that the effective doses were different between Gram-negative and Gram-positive bacteria. Gurunathan et al. (2014) tested AgNP against *P. aeruginosa*, *Shigella flexneri*, *S. aureus*, and *Streptococcus pneumoniae* and observed more effect in Gram-negative strains. Was observed low concentrations of bio-AgNP being effect against in Gram-negative bacteria.

The study of eugenol associated with other compounds were described as effective by some authors (MILADI et al., 2017; BIASI-GARBIN et al., 2015; ZACCHINO et al., 2017). Miladi et al. (2017) was noticed a synergistic effect of essential oils (including eugenol) and tetracycline (TET) with a reduction rate ranged from 2 to 8-fold. In our studying, eugenol in association with AgNP showed the same reduction rate ranged according to different time of incubation and different concentration of the compounds (Figure 2a – 2c).

The association of the bio-AgNP with eugenol showed interesting results about antibacterial activity, leading a higher MIC decreasing of eugenol than bio-AgNP suggesting the great importance of nanoparticles against these bacteria. Moreover, the results of synergism showed higher antibacterial effect on Gram-negative bacteria than Gram-positive, probably due the high sensibility of Gram-negative for bio-AgNP, being the nanoparticles determinant for synergic effect.

The combined antibacterial effect of AgNP and eugenol on several bacteria was investigated in this study. Combination was highly effective against the bacterial cells and synergism effect was observed in almost all analyzed bacteria. The synergism importance is the reduction of both compounds concentration it may reduce the cytotoxicity too. A multidrug resistance strain *S. aureus* N315 e *S. aureus* BEC 9393 were also inhibited in the presence of both agents.

CONCLUSION

Bio-AgNP in combination with eugenol is interesting, mainly for Gram-negative bacteria. This association also suggests their safety uses due the high reducing on concentration of both compounds.

ACKNOWLEDMENTS

This study was supported by CNPq BIOTEC 402728/2013-0 and Graduate Program in Microbiology of Universidade Estadual de Londrina.

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ARTIGO II

Synergistic Effect of Eugenol and Biologically synthesized Silver Nanoparticles against *Listeria monocytogenes*

Abstract

Listeria monocytogenes is an important foodborne pathogen often associated with food processing environment. Natural antimicrobials, such as essential oil eugenol and biologically synthesized silver nanoparticles, may be used as effective sanitizer in food processing environment to minimizing such contamination. The aim of this study was to evaluate the antibacterial activity of essential oil eugenol and biologically-synthetized silver nanoparticles (bio-AgNP) against weak and strong biofilm producing *L. monocytogenes* strains isolated from food processing environment. Bio-AgNP were obtained after reduction of silver nitrate by *Fusarium oxysporum*. Microplate turbidometric growth inhibition assays was used to study the inhibitory action of eugenol and bio-AgNP at various concentrations, either alone or in combination, on *L. monocytogenes* isolates to evaluate the minimal inhibitory concentration (MIC). Biofilm lethality assay was performed to analyze the action of the compounds after biofilm formation. The results showed that when acting alone, the (MIC) of eugenol was 0.25% against weak and strong biofilm producing strain. MIC of bio-AgNP for the strong biofilm producing strain was higher (630µM) than that of the weak one (315µM). The compounds in combination were able to reduce in 5-log the biofilm. Synergistic and additive antimicrobial effect was observed when both compounds were used together. Both eugenol and bio-AgNP exhibited antilisteria activity at relatively low concentrations. Eugenol in combination with bio-AgNP offers a potential solution for new sanitation treatment of food processing equipment and environment.

Keywords: *Listeria monocytogenes*, essential oil, eugenol, biologically synthesized silver nanoparticle.

Introduction

Listeria monocytogenes strains are Gram-positive, rod-shaped ubiquitous bacteria that cause serious foodborne illness, listeriosis, which is typically linked to the consumption of contaminated ready-to-eat foods. This disease is associated with high hospitalization and mortality rates causing sepsis, meningitis, miscarriage, and stillbirth. Pregnant women, immunocompromised and the elderly are the most susceptible individuals to invasive forms of listeriosis (COLAGIORGI et al., 2017; OXARAN et al., 2017). In the United States, *Listeria* had the highest case fatality rates (CFR) (12.93 deaths per 100 infections) and the highest percentage of infections hospitalized (95.7%) in 2015 (CDC, 2017).

The transmission of this pathogen to humans occur by the ingestion of contaminated food (such as meat, poultry, dairy, vegetables, and ready to eat products) (EFSA 2008). This foodborne pathogen is capable to form biofilms, that are structured communities of bacterial cells fixed in a self-produced matrix of extracellular polymeric substances (EPSs), characterized by a well-defined architecture, and provides an optimal environment for the exchange of genetic material between cells (DONLAN, 2002). *L. monocytogenes* representing a serious concern for food safety because it could serve as source of contamination. This bacteria is able to form biofilm on several surfaces used in the food industry, attach to many food-contact surfaces, such as stainless steel, polystyrene and glass (DI BONAVENTURA et al., 2008).

The persistence in the processing environments is associated to the capability of the pathogen to survive at low temperatures, resist various food-related stresses and colonize surfaces in the form of biofilm-like structures (RIEU et al., 2008). Particularly, *L. monocytogenes* may adhere where food residues are accumulated to and grow on processing surfaces (GANDHI; CHIKINDAS, 2007; POIMENIDOU et al., 2009). This mechanism is a potential resistance to antimicrobial agents, biocides and heat (BOWER; DAESCHEL, 1999; CLOETE, 2003).

Food companies have the important assignment in eliminate the foodborne pathogenic and spoilage bacteria (GHABRAIE et al., 2016). In the food industry chlorine is widely used (SAGONG et al., 2011; VAN HAUTE et al., 2013); due to facility to apply, its relatively low price and wide spectrum of antimicrobial effectiveness (RAMOS et al., 2013). Though, this disinfectant can shows, limited efficiency in reducing microbial loads (YARON; RÖMLING, 2014), as it can be easily

inactivated by organic matter (RAMOS et al., 2013) (Parish et al., 2003), and its action is highly pH dependent (MEIRELES; GIAOURIS; SIMÕES, 2016).

The production of excessive amounts of harmful disinfection by-products (DBPs) in the water could be caused by the generation of chlorine gas due to the use of high chlorine concentrations in the production facilities (GOSLAN et al., 2009; HUA; RECKHOW, 2007; LEGAY et al., 2010; NIEUWENHUIJSEN; TOLEDANO; ELLIOTT, 2000; NOU; LUO, 2010). The use of chlorine in fresh-cut produce washing is prohibited altogether in some European Union countries such as Germany, Switzerland, the Netherlands, Denmark, and Belgium due to the possible generation of DBPs (ARTÉS et al., 2009; RICO et al., 2007; TIRPANALAN et al., 2011).

An alternative for the conventional disinfectants could be the natural compounds as essential oils and biological synthesized silver nanoparticles (RAI et al., 2017). Nanosilver has been used in animal breeding as a disinfecting agent used to sanitize transport chambers or the space used for the storage of animals (SCHIFFMAN, 1998). Bashir et al. (2011) demonstrated the action of AgNPs against *Escherichia coli* comparing to hypochlorite disinfection. AgNPs required 30 minutes for disinfection, however in contrast to hypochlorite, the minimum dose of the nanoparticles decrease to less than 1 part per million (ppm), as the incubation time increased.

Essential oils (EOs) are known as one of the best candidates to be used as preservatives in foods, due to their good antimicrobial properties and their safety; however, at high concentrations the application is limited because of their taste and odor impacts (GOÑI et al., 2009). Therefore, in order to use them in food without any changes in smell and taste it is necessary to determine their lowest concentration with acceptable sensorial level (TURGIS et al., 2012).

Valeriano et al. (2012) evaluated the anti-biofilm effect of disinfectant solutions formulated with EO against biofilm formation of foodborne. After 10 min of sanitizing solution, contact significantly reduced adhered bacterial populations for the EOs tested and cell counts were not detected after 20 and 40 min of treatment.

Regarding to find a good and alternative solution for food industry and processing environment, this study was to evaluate the antibacterial activity of essential oil eugenol and biologically-synthetized silver nanoparticles (bio-AgNP) against *L. monocytogenes* strains isolated from meat processing facilities.

Materials & Methods:

Antimicrobial Agents

Eugenol

A stock solution of 50% (v/v) eugenol (Sigma- Aldrich) was prepared containing 50% (v/v) dimethyl sulfoxide (DMSO- Sigma- Aldrich).

Biological Silver Nanoparticles from *F. oxysporum*

Briefly, AgNPs were obtained after reduction of silver nitrate by *F. oxysporum*, strain 551, from the culture collection of the Molecular Genetics Laboratory of ESALQ-USP (Piracicaba, São Paulo, Brazil). Subsequently the growth of *F. oxysporum* culture, was added 100 mL of distilled water in 10 g of the biomass follow by incubation during 72 h at 28°C, the solution components were separated by filtration, and $AgNO_3$ at concentration of 10^{-3}M was added. To purify and stabilize the silver nanoparticles, after all the reduction process, was done an ultracentrifugation (15000 rpm, 10ºC, 30 min) and sonication (80W, 100Hz, 30 min). The absorbance was determined at 440 nm that corresponds to the plasmon resonance value, after the system was kept for several hours. The diameter was determined by photon correlation spectroscopy, after bio-AgNP purification, using ZetaSizer NanoZS (Malvern), and zeta potential measurement was performed with the same instrument. The methodology used to prepare bio-AgNPs that were used in the assays were according Durán et al. (2005).

Bacterial Culture

Strains used in this experiment were *L. monocytogenes* weak and strong biofilm producing strains (CW35 and 9938, respectively), isolated from meat processing facilities from the culture collection of the Department of Animal Science of OSU (Oklahoma State University, Stillwater, Oklahoma, United States) (GAMBLE & MURIANA, 2007). *L. monocytogenes* from stock was grown in tryptic soy broth (TSB) at 37ºC for 16-18h.

Microplate broth dilution method

Minimal inhibitory concentrations (MICs) were determined by micro-dilution assay (CLSI, 2012). Overnight culture of *L. monocytogenes* in TSB were diluted and dispended in 96-well plates at a density of ~10^5 CFU/ml, than incubated at 37ºC during 24h. Predetermined concentrations of eugenol and bio-AgNPs were added and MIC was defined as the lowest concentration of antimicrobial agent that will inhibit visible growth. Minimal bactericidal concentration (MBC) was determined by sub culturing 10 µL from the broth dilution MIC, after 24 h of treatment, in TSA and MBC was defined as the lowest concentration with no growth in TSA plate after 24h of antimicrobial treatment.

Drug interaction studies

Assays of microdilution in double-antimicrobial gradient were used to verify the antibacterial effects and interactions of eugenol combined with bio-AgNP against *L. monocytogenes* strong and weak biofilm producer. First, the MIC values for the compounds used alone were determined, and several concentrations of eugenol were combined with different concentrations of bio-AgNP. There were determinate a combination MIC, which was the lowest concentration of eugenol combined with the lowest concentration of bio-AgNP. In order to estimate the interaction between both antimicrobials, the fractionated inhibitory concentration (FIC) index was used as described by (CHIN; WEITZMAN; DELLA-LATTA, 1997).

$$FIC = MIC(Ec) \div MIC(Ea) + MIC(Sc) \div MIC(Sa)$$

MIC(Ec) is the MIC of eugenol combined with the AgNP, MIC(Ea) is the MIC of eugenol alone, MIC(Sc) is the MIC of the AgNP combined with eugenol and MIC(Sa) is the MIC of the AgNP alone. FIC indexes were interpreted as follows: FIC ≤ 0.5 = synergic interaction; 0.5 < FIC ≤ 1.0 = additive interaction; 1.0 < FIC ≤ 4.0 = no interaction; FIC > 4.0 = antagonist interaction.

Microplate turbidimetric growth inhibition assays

The microplate inhibition assay used a mixture of *L. monocytogenes* and antimicrobials compounds and the methodology was according to described by Vijayakumar and Muriana (2015). *L. monocytogenes* was diluted and inoculated into TSB broth (~1 x 10^5 cfu/mL) from which 100 µL were distributed to various wells in a

clear 96-well flat bottom microtiter plate. Antimicrobials compounds (eugenol and bio-AgNPs) were added (final volume of 100µL each one, for each concentration) and mix by aspiration using a multi-channel pipette. Test concentrations of eugenol and bio-AgNPs ranged from 0.06% to 2% and 39.38µM to 630µM, respectively. Settings for the growth curve/turbidity analysis using a Beckman Coulter AD340 microplate reader were the follows: measure absorbance mode: absorbance; measurement wavelength: 570 nm; temperature 37ºC; shake duration (low): 10s; kinetic interval: 1800 s; and total measurement time: 24 h. The 96-well plate was sealed with UltraClear film to prevent evaporation of the liquid and well-to well contamination. The OD_{570} values obtained were plotted against time and were used to illustrate the antilisteria activity of eugenol and bio-AgNPs compounds against *L. monocytogenes*.

Biofilm lethality protocol

This assay were done according to Gamble and Muriana (2007), briefly overnight cultures (10^9 CFU/ml) of *Listeria monocytogenes* 99-38, were diluted 5-fold in BHI broth. A 200µl aliquot of each culture was allocated in triplicates into 96-well microplates flat bottom. Then, the microplates were incubated at 30°C for 24 hours. After the incubation, the wells were washed 3 times with Tris buffer (0.05 M; pH 7.4) with shaking for 30 sec at 150 rpm to wash off loosely adherent cells in addition to resuspending settled planktonic cells between each washing. This procedure was followed by the addition of fresh BHI (200µl) into the wells and an additional incubation for 24 hours at 30°C. The same process of washing with Tris buffer and adding fresh BHI into wells was repeated each day for three consecutive days. Then the final wash with Tris buffer was performed. The biofilms were washed three times with Tris buffer (0.05M, pH 7.4) and 200 µl of various concentrations of eugenol in combination of silver nanoparticles were added thereafter. After incubating compounds for the various assigned treatment periods, the treated microplates and control (with buffer treated) were again washed with Tris, aspirated and then 200µl Dey-Engley (DE) neutralizing buffer (Hardy Diagnostics, Santa Maria, CA) was added to the wells. The microplates were left for 5 minutes to neutralize the effects of the compounds.

After treatment with compounds and neutralization using DE buffer, the lethality of compounds was quantified by microplate biofilm detachment assay (enzymatic detachment and plating). The test wells (treated with compounds and

neutralized) were washed with Tris (0.05 M. pH 7.4) then 200µl of Trypsin-EDTA Solution (Sigma-Aldrich, 1X) was added into the wells and incubated for an hour at 37º C. The enzyme added wells were then harvested and the liquid was plated on Tryptic Soy Agar (TSA) plate. The liquid (200µL) were added in 1800µL of PBS (1X) followed by serial dilutions. The enumeration were done in triplicate dots in a TSA plate according to different dilutions of each concentration. The plates were then incubated for 24 hours at 37º C and enumerated for residual viable cells the next day. All different treatments in different times were done in triplicate.

Statistical analysis

Data were analyzed by ANOVA, and Tukey's test ($p < 0.05$) were applied to determine the difference among means. The Student's t-test ($p < 0.05$) was also used for the analysis of means. All tests were performed with the statistical program RStudio version 1.0.136.

Results

Bio-AgNP Characterization

Average bio-AgNP size was 81.25 nm and zeta potential was −36.4 mV, respectively (Supplementary material).

Antimicrobial activity of eugenol and bio-AgNP

The results showed that when acting alone, the minimal inhibitory concentration (MIC) of eugenol was 0.25% for weak biofilm producing strains and 0.12% for strong biofilm producing. MIC of bio-AgNP for the strong biofilm producing strain was higher (630µM) than for the weak one (315µM).

Drug interaction studies

Synergistic antimicrobial effect was observed when both compounds were used together. MIC for weak biofilm producer as 0.09%/78.8 µM (eugenol/bio-AgNP) and 0.06%/157.5µM for the strong biofilm producer. Data shown in Table 1. FIC values were 0.61 for *L. monocytogenes* weak biofilm producer and 0.49 for strong biofilm producer, with additive and synergistic interaction, respectively (Table 1).

Table 1. MIC and MBC of Eugenol and bio-AgNP against *L. monocytogenes* strains.

	***L. monocytogenes* weak biofilm former**	***L. monocytogenes* strong biofilm former**
Eugenol	MIC*/MBC** (0.25%)	MIC/MBC (0.25%)
Bio-AgNP	MIC/MBC (315µM)	MIC/MBC (630µM)
Eugenol + Bio-AgNP [a]	1/3 MIC + 1/4MIC	1/4 MIC+ 1/4 MIC
FIC [b]	0.61	0.49
Interaction	Additive	Synergism

*MIC: Minimal inhibitory concentration (no visible growth)

**MBC: Minimal bactericidal concentration (at least 3-log reduction of inoculum cells)

a: p-value ≤0.05

b: FIC: “synergistic” ≤0.5; “additive” >0.5 and ≤1; “no interaction” >1 and <4 and “antagonist” ≥4.

Growth inhibition assay

Growth inhibition assay showing the activity of Eugenol (E), bio-AgNP and combination (E+AgNP) (MIC, 1/3 and 1/4MIC) against *L. monocytogenes* weak biofilm producer after 24h of treatment. Was observed for control, ¼ MIC E and 1/3MIC bio-AgNP that still have some growth, for 1/4MIC bio-AgNP we do observed a late growth (Figure 1). For the treatment against *L. monocytogenes* strong biofilm producer, the growth inhibition assay showing inhibition for MIC E, MIC bio-AgNP and for the combination of 1/4MIC E and 1/4MIC bio-AgNP when the compounds where analyzed ¼ isolated MIC, we observed growth and late growth for ¼ Eugenol MIC (Figure 2). For all assays, the combination was statistical different ($p<0.05$).

Figure 1 – Growth inhibition assay showing the activity of Eugenol (E), AgNP and combination (E+AgNP) (MIC, 1/3 and 1/4MIC) against *L. monocytogenes* weak biofilm producer.

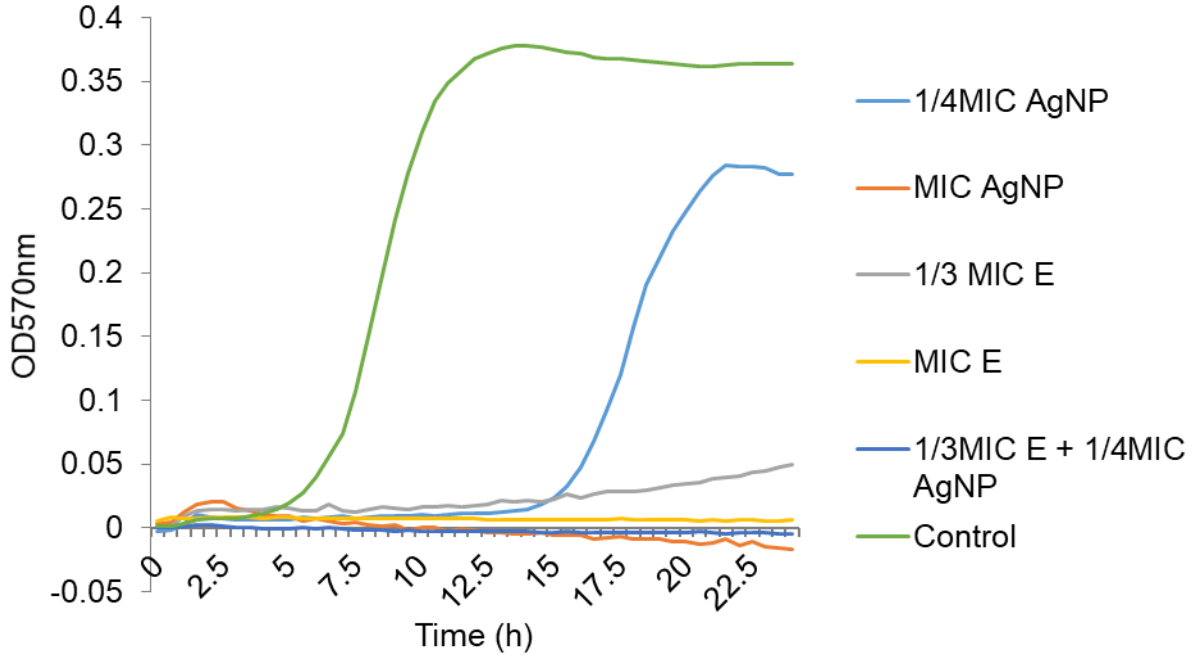
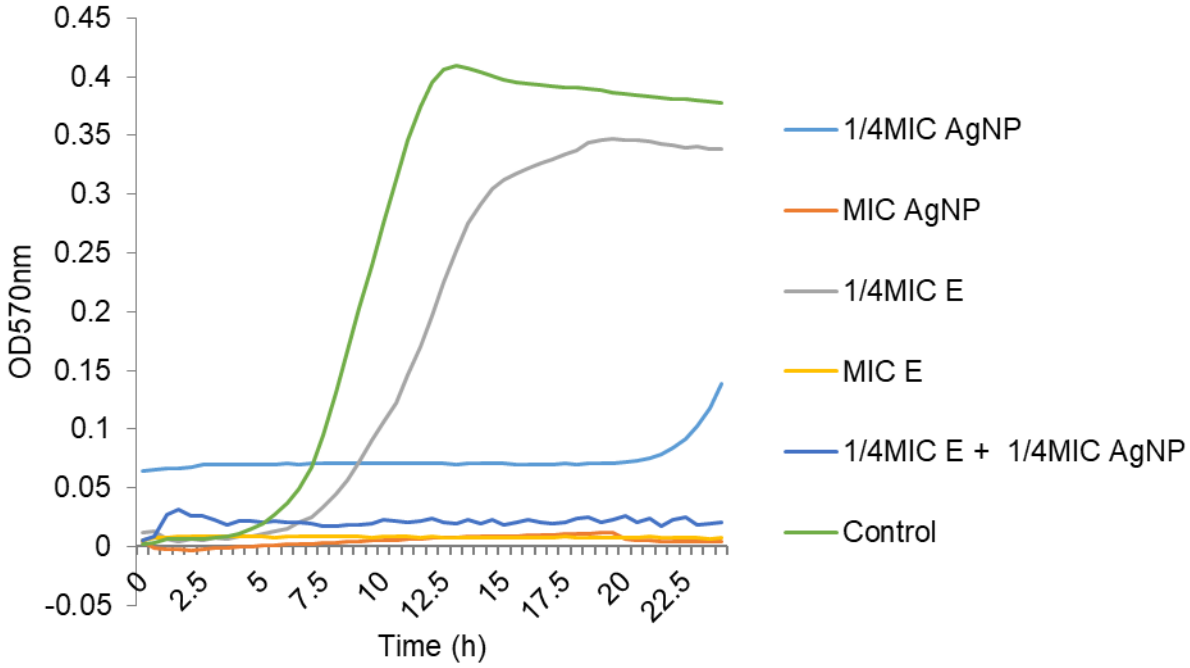

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
OD570nm
0
2.5
5
7.5
10
12.5
15
17.5
20
22.5
Time (h)
1/4MIC AgNP
MIC AgNP
1/3 MIC E
MIC E
1/3MIC E + 1/4MIC AgNP
Control

Figure 2 – Growth inhibition assay showing the activity of Eugenol (E), AgNP and combination (E+AgNP) (MIC, 1/4 and 1/4MIC) against *L. monocytogenes* strong biofilm producer.



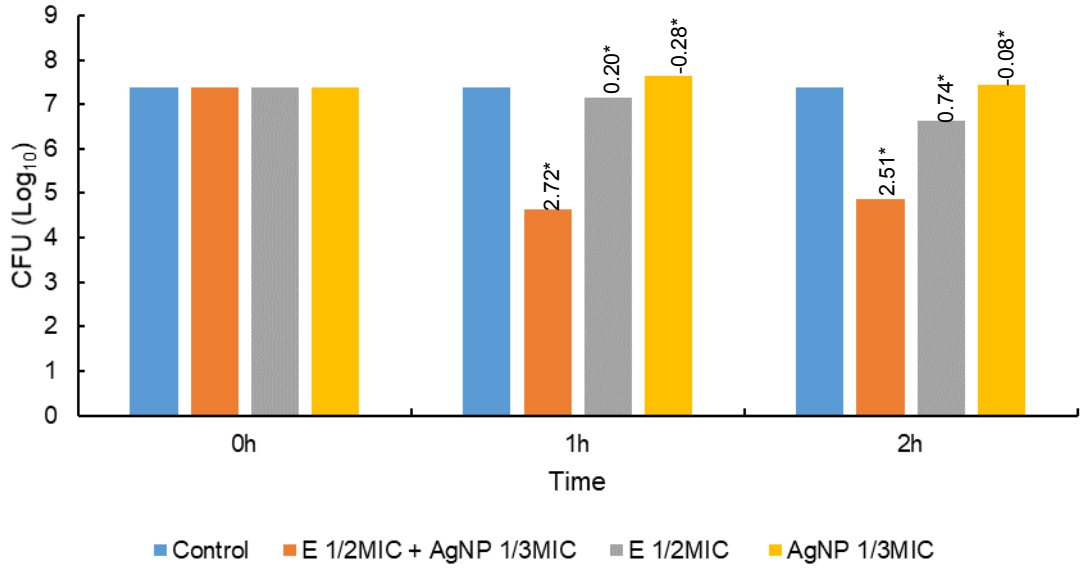
Biofilm lethality

According to each treatment was not observed statistic difference for E 0.12% and bio-AgNP 157.5 µM (1/3 MIC and ½ MIC) concentrations between 1h and 2h treatment with the compounds (isolated or in combination) (Figure 3). Bio-AgNP using alone (1/3 MIC concentration) demonstrate -0.08 log-reduction after 2h treatment, 0.74 log-reduction for eugenol 1/2MIC at the same time and the combination with the same concentration of the compounds (E 0.12% and bio-AgNP 157.5 µM) indicated 2.51 log-reduction for 2h. The results for combination were statically significance ($p<0.05$). The results was not effective in the compounds used isolated, but the combination was more effective after 1 h treatment with 2.72 log-reduction. Means of viable cells, standard deviation and log-reduction after treatments are demonstrated in Table 3.

Another treatment for biofilm lethality was performed with MIC of the compounds. According to each treatment was not observed statistic difference in isolated treatment with bio-AgNP 630 µM between the three times evaluated (0.25h, 1h, 2h), and the reduction comparing to control cells was less than 1 log-reduction (Figure 4). Bio-AgNP using alone reveal 0.82 log-reduction after 2h treatment, 5.02

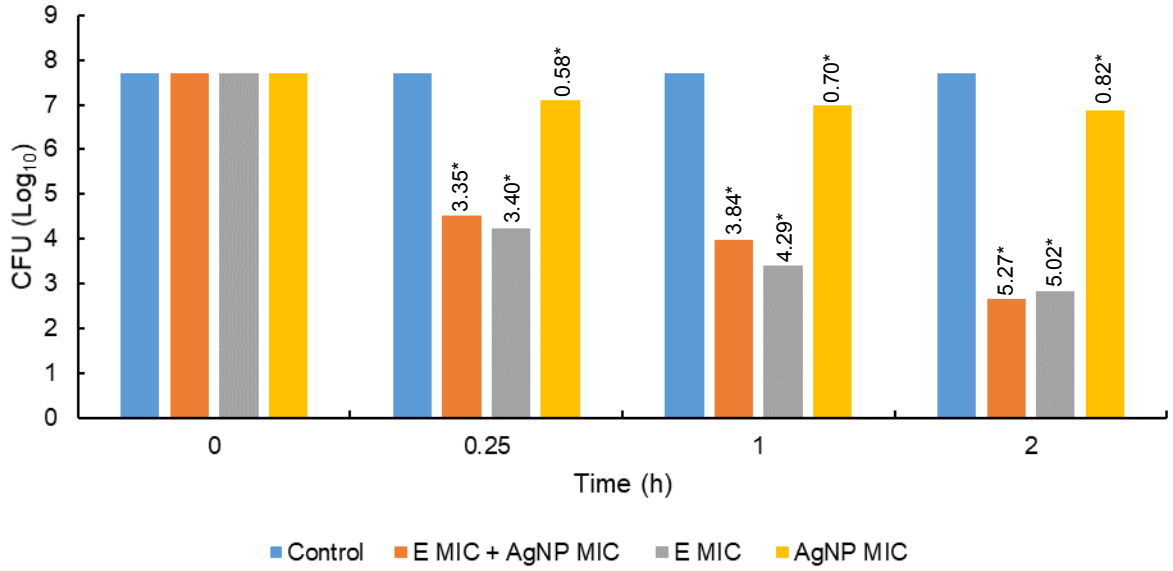
log-reduction ($p<0.05$) for eugenol at the same time and the combination with the compounds indicated 5.27 log-reduction ($p<0.05$). The results for combination were statically significance ($p<0.05$) from 2h comparing to another times. Table 3 indicates the means of viable cells, standard deviation and log-reduction after treatments.

Figure 3 – Viable cells after biofilm lethality assay in *L. monocytogenes* strong biofilm producing with different treatments and time after antibacterial action of eugenol (1/2 MIC) and bio-AgNP (1/3 MIC).



*Log-reduction comparing to positive control in time 0h.

Figure 4 – Viable cells after biofilm lethality assay in *L. monocytogenes* strong biofilm producing with different treatments and time after antibacterial action of eugenol (MIC) and bio-AgNP (MIC).



*Log-reduction comparing to positive control in time 0h.

Table 2 - Viable cells after biofilm lethality assay in *L. monocytogenes* strong biofilm producing with different treatments and time after antibacterial action of eugenol and bio-AgNP.

Treatments	Viable cells (CFU/mL) ± SD	Log reduction*
Positive Control	2.33E+07 ± 2.35E+07	
E 0.12% + AgNP 157.5 µM (2h)	7.23E+04 ± 2.74E+04	2.51 (p<0.05)
E 0.12% (2h)	4.02E+06 ± 4.33E+06	0.74 (p>0.05)
AgNP 157.5 µM (2h)	2.80E+07 ± 0.81E+06	-0.08 (p>0.05)
E 0.12% + AgNP 157.5 µM (1h)	4.34E+04 ± 6.12E+04	2.72 (p<0.05)
E 0.12% (1h)	1.46E+07 ± 0.96E+07	0.20 (p>0.05)
AgNP 157.5 µM (1h)	4.43E+07 ± 1.14E+07	-0.28 (p>0.05)

*Log reduction comparing to positive control in time 0h.

Table 3 - Viable cells after biofilm lethality assay in *L. monocytogenes* strong biofilm producing with different treatments and time after antibacterial action of eugenol and bio-AgNP.

Treatments	Viable cells (CFU/mL) ± SD	Log reduction*
Positive control	5.11E+07 ± 1.17E+07	
E 0.25% + AgNP 630 µM (2h)	4.44E+02 ± 5.09E+02	5.27 (p>0.05)
E 0.25% (2h)	6.67E+02 ± 1.15E+03	5.02 (p>0.05)
AgNP 630 µM (2h)	7.53E+06 ± 4.28E+06	0.82 (p<0.05)
E 0.25% + AgNP 630 µM (1h)	9.67E+03 ± 9.26E+03	3.84 (p<0.05)
E 0.25% (1h)	2.56E+03 ± 2.99E+03	4.29 (p<0.05)
AgNP 630uM (1h)	9.91E+06 ± 3.50E+06	0.70 (p>0.05)
E 0.25% + AgNP 630 µM (0.25h)	3.22E+04 ± 1.35E+04	3.35 (p<0.05)
E 0.25% (0.25h)	1.67E+04 ± 1.45E+04	3.40 (p<0.05)
AGNP 630 µM (0.25h)	1.31E+07 ± 9.79E+06	0.58 (p>0.05)

*Log reduction comparing to positive control in time 0h.

Discussion

Previous studies revealed that clove has anti-listeria effect and our results confirmed that eugenol, a major constituent of essential oil of clove, could respond to this effect (FRIEDMAN; HENIKA; MANDRELL, 2002; PÉREZ-CONESA; MCLANDSBOROUGH; WEISS, 2006; SMITH-PALMER; STEWART; FYFE, 2001). As showed in this study, eugenol had powerful antimicrobial activity against *L. monocytogenes* weak and strong biofilm producing.

Gill and Holley (2004) demonstrated that eugenol at 5mM was effective to reduce in more than 1-log the number of CFU within 1h. Our study showed eugenol acting in 0.25% for both strain tested with 5-log reduction after 24h of treatment. Filgueiras and Vanetti (2006) observed that eugenol promoted a delay on the growth of *L. monocytogenes* at concentrations of 100, 300 and 500 µg/mL and above 800 µg/mL the effect was bactericidal.

Several investigations has demonstrated the antibacterial activity of AgNPs, but the reported MIC values range through a wide extent of variation. Since there is no standard protocol for evaluation of antimicrobial activity of NPs and researchers have used different methods, it is hard to compare their results. Zarei, Jamnejad, and

Khajehali (2014) used a commercial AgNP against foodborne pathogens, including *L. monocytogenes* and the results showed a great effectiveness on pathogens and could be a good alternative for cleaning and disinfection of equipment and surfaces in food-related environments. Against tested bacteria, 3-log decrease, considered by the authors as the minimal bactericidal concentration (MBC), was observed after 7 hours of treatment at 6.25 µg/ml concentration. In this study was analyzed only 24h treatment and according to the previous authors, showed the completely inhibition of the pathogens with 5-log reduction.

The results of MIC and MBC tests revealed a higher value for *L. monocytogenes* comparing to the other researchers. A difference in these values could be related to the size of the nanoparticles, due to the fact that bactericidal property of nanoparticles is dependent on the concentration and size of nanoparticles and also the initial bacterial concentration (RUPARELIA et al., 2008). AgNPs with size of 1-10 nm have been reported to be most effective against bacteria through direct interaction with bacterial cells (MORONES et al., 2005). Bio-AgNP used in the assays has size of 81 nm.

In the microplate growth inhibition assay, was observed that some curves showed a late growth. According a study from Vijayakumar and Muriana (2015), that studied the effect of bacteriocins against *L. monocytogenes*; this effect could be by a slow lysis of the bacterial cells. As one of the mechanism of action of bio-AgNP and eugenol could be by form membrane pores in susceptible cells and/or the result of a change in cellular morphology upon nutrient depletion during prolonged stationary phase which may have an effect on optical density.

Remove bacteria by using disinfectant it is easy, compared with the elimination of biofilm. However, in certain environments, bacteria can develop into biofilm. This study performed the biofilm attachment in polystyrene surface and was observed that eugenol and bio-AgNP in combination had a good effect in the biofilm reduction. When the compounds were used alone, the effect was better for eugenol. Some authors described when the bacterial concentration was enhanced, the anti-biofilm activities of AgNPs decreased and the anti-biofilm ability of AgNPs depends on the form of application (PALANISAMY et al., 2014; WU et al., 2014; ZHAO et al., 2017). During the literature review was observed more studies with essential oils against antilisteria biofilm then with silver nanoparticles.

Desai et al. (2012) evaluated an 1-day-old biofilm cells in 24-well polystyrene plate assay and 4-day-old *L. monocytogenes* biofilms on stainless steel coupons. In the first assay, 0.1% essential oil concentrations were enough to inactivate biofilm cells completely, although concentrations of 0.25 to 0.5% were required to completely inactivate 4-day-old biofilm cells on stainless steel coupons. In previous studies, the efficacy of sanitization treatment was dependent on both age of the biofilm and the surface on which the biofilm was produced (ARIZCUN; VASSEUR; LABADIE, 1998; NORWOOD; GILMOUR, 2000; NOSTRO et al., 2007; YANG et al., 2009). In our experimental model, after 3-day-old biofilm was observed 3 and 5 log-reduction, according to the concentration and treatment time used with the combination of the compounds, thus the biofilm elimination is time and dose dependent.

Current anti-biofilm methods are not always sufficient for the removal of foodborne pathogens. In most cases, a single strategy of biofilm destruction is less used since the removal in such single methods is incomplete. Synergetic treatment would be a promising and efficient strategy for the prevention and removal of foodborne pathogens biofilms. Therefore, the union of these methods must be widely applied in the food industry.

Conclusion

When combined, eugenol and Bio-AgNP exhibited synergistic antilisteria activity both in planktonic form and in a biofilm, thus this combination could be a potential solution for new sanitation treatment in food industry and could substitute some compounds.

Acknowledgments

This study was supported by CNPq BIOTEC 402728/2013-0, PDSE/2016 CAPES, National Institute for Microbial Forensics and Food and Agricultural Biosecurity (NIMFFAB) and Oklahoma State University.

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CONCLUSÕES

- As perturbações causadas pelos compostos nas células bacterianas indicam um provável mecanismo de ação na membrana, causando alterações morfológicas como formação de vesículas.
- Também pode-se concluir que a combinação do eugenol com as bio-AgNP foi efetiva em patógenos de alimentos que causam infecções em humanos.
- A interação entre os compostos foi eficaz na eliminação do biofilme formado, porém quando analisado os compostos em isolado, o eugenol apresentou-se melhor como anti-biofilme.
- A combinação dos compostos gerou um depósito de patente denominada “Composição contendo nanopartículas de prata e um antibiótico obtido de cravo-da-índia e sua utilização” no Instituto Nacional da Propriedade Industrial (Número do registro: BR1020140215687. Depósito: 29/08/2014).
- O eugenol e as bio-AgNP são excelentes alternativas terapêuticas aos antibióticos presentes no mercado; e também aos sanitizantes utilizados na indústria.
- A aplicação destes compostos pode ser tanto na área clínica, como na indústria e processamento de alimentos.

ANEXOS

ANEXO 1

Size Distribution Report by Intensity

v2.2

Malvern

Sample Details

Sample Name: AgNP 2
SOP Name: mansettings.nano
General Notes:

File Name:	AgNP Durán.dts	**Dispersant Name:**	Water
Record Number:	2	**Dispersant RI:**	1,330
Material RI:	0,20	**Viscosity (cP):**	0,8872
Material Absorbtion:	0,400	**Measurement Date and Time:**	segunda-feira, 11 de janeiro ...

System

Temperature (°C):	25,0	**Duration Used (s):**	60
Count Rate (kcps):	450,5	**Measurement Position (mm):**	5,50
Cell Description:	Clear disposable zeta cell	**Attenuator:**	7

Results

			Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	81,25	**Peak 1:**	126,3	95,7	78,42
PdI:	0,296	**Peak 2:**	13,58	4,3	3,894
Intercept:	0,867	**Peak 3:**	0,000	0,0	0,000

Result quality : Good