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JANAÍNA ESMERALDO ROCHA

SÍNTESE, ESTUDOS ADMET, DOCAGEM MOLECULAR E ATIVIDADE MODIFICADORA DE ANTIBIÓTICOS DAS CHALCONAS DERIVADAS DA 2 – HIDROXIACETOFENONA

Crato – CE

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Tese de doutorado apresentado ao Programa de Pós-Graduação em Química-Biológica, da Universidade Regional do Cariri – URCA, como requisito parcial para a obtenção do Título de Doutora em Química-Biológica.

Orientador: Prof. Dr. Henrique Douglas Melo Coutinho

Coorientador: Prof. Dr. Francisco Nascimento Pereira Júnior

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Dedico este trabalho a minha família e a todas as pessoas que contribuíram na minha formação acadêmica e a todos aqueles que buscam o desenvolvimento da Ciência.

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"*Deus disse: eu irei com você e lhe darei a vitória*".

(EXÔDO 33:14)

ARTIGO 1: Antibacterial and antibiotic modifying activity, ADMET study and molecular docking of synthetic chalcone (E)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl) prop-2-en-1-one in strains of *Staphylococcus aureus* carrying NorA and MepA efflux pumps.

ARTIGO II: Synthesis, antibiotic modifying activity, ADMET study and molecular docking of chalcone (E)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one in strains of *Staphylococcus aureus* carrying MepA efflux pumps

1199B Cepa de *Staphylococcus aureus* portadora do Gene NorA

ABC Superfamília de "Cassetes de Ligação de ATP"

ADMET Método preditivo farmacocinético para "Absorção, Distribuição, Metabolismo, Excreção e Toxicidade"

ANOVA Análise de Variância

ATP Adenosina Trifosfato

ATR-FTIR Attenuated Total Reflection Fourier Transform Infrared

BHI Brain Heart Infusion

CCCP Carbonyl Cyanide 3-Chlorophenylhydrazone

CFU Colony Forming Unit

CIM (MIC) Concentração Inibitória Mínima

CLSI Clinical & Laboratory Standards Institute

CPMZ (CPZ) Clorpromazina

DMSO Dimethyl Sulfoxide

EB (EtBr) Ethidium Bromide

EPI Efflux Pump Inhibitors

EP Efflux Pumps

hERG human Ether-a-go-go-Related Gene

HIA Human Intestinal Absorption

IBE Inibidores de Bomba de Efluxo

K2068 Cepa de *Staphylococcus aureus* portadora do Gene MepA

LD50 Lethal Dosage to death of 50% of a group

LMBM Laboratório de Microbiologia e Biologia Molecular

MATE Família de "Extrusão de Multidrogas e Toxinas"

MDR Multidroga-resistente

MRSA *Staphylococcus aureus* resistentes à meticilina

MepA Gene que expressa a proteína de efluxo de mesmo nome

MepR Gene regulador de MepA

MFS "Superfamília de Facilitadores Principais"

MR Molar Refractivityxi

MW Molecular Weight

NBD Nucleotide Binding Domain

NMR Nuclear Magnetic Resonance

NorA Gene que codifica a proteína de efluxo de mesmo nome

PACE "Família de Efluxo de Composto Antimicrobiano Proteobacteriano"

PBP Penicillin Binding Protein

PSA Polaridade

RND Superfamília "Resistência-Nodulação-Divisão"

SA *Staphylococcus* aureus

SMR Família de "Pequena Multidroga Resistência"

SUMÁRIO

Síntese, estudos ADMET, docagem molecular e atividade modificadora de antibióticos das chalconas derivadas da 2 – hidroxiacetofenona.

RESUMO

A resistência bacteriana aos antibióticos de uso clínico vem crescendo de forma acelerada em todo o mundo, resultando no aumento do número de infecções com difíceis tratamentos e muitas vezes levando o paciente a óbito. Diante disto, faz-se necessário a busca por alternativas terapêuticas para inibição dos mecanismos de resistência bacteriana. Uma das alternativas promissoras é a busca por substâncias naturais, moléculas semissintéticas e sintéticas que possam vir a ter atividade antibacteriana intrínseca ou que sirva como adjuvante na antibioticoterapia. A partir de observações feitas através da literatura, pode-se observar que diversas chalconas apresentam atividade antibacteriana, desta forma sendo consideradas uteis para o estudo. Neste trabalho foram analisadas duas chalconas derivadas da 2-hidroxiacetofenona e avaliado sua interação com o mecanismo de Bomba de Efluxo presente nas cepas de *Staphylococcus aureus* 1199B e K2068 portadoras das proteínas de efluxo NorA e MepA, respectivamente. Para a síntese das chalconas foi utilizada a metodologia de *Claisen-Schimd* e para caracterização estrutural das mesmas foram utilizadas técnicas de Ressonância Magnética Nuclear e Espectroscopia de Infravermelho. Para avaliação da atividade antibacteriana foram realizados teste de microdiluição em caldo utilizando placas de 96 poços. Para verificação da inibição de bomba de efluxo, foi utilizada a concentração subinibitória das chalconas e dos inibidores padrões. As placas contendo essas substâncias foram microdiluídas com os antibióticos Norfloxacina, Ciprofloxacina e Brometo de Etídio, com concentrações variando de 512 µg/mL a 0,5 µg/mL. Técnicas computacionais foram utilizadas para observar características importantes fundamentais para possíveis candidatos a fármacos. Os dados obtidos foram analisados usando uma ANOVA de duas vias seguida pelo teste post hoc de Bonferroni, onde $p < 0,05$ foi considerado significativo. A média geométrica dos triplicados foi usada como dados centrais ± erro padrão da média. A estatística *GraphPad Prism* 5.0 programa foi usado para a análise. Foi observado que as chalconas conseguiram interagir com o mecanismo de bomba presente nas bactérias, essas interações foram confirmadas pela docagem molecular. As chalconas também potencializaram os efeitos dos antibióticos, onde a chalcona (E)-3-(2,4-diclorofenil)-1-(2-hidroxifenil)prop-2-en-1-ona apresentou resultados satisfatórios com ambos antibióticos utilizados no estudo. A ação das chalconas está relacionada com os ligantes químicos presentes nas mesmas. Os dados sumarizados no presente estudo nos permite concluir que as chalconas foram capazes de interagir no mecanismo de bomba de efluxo da bactéria, competindo pelos sítios ativos das proteínas com os substratos que seriam ligados, permitindo desta forma que o antibiótico consiga atingir o interior da célula bacteriana e produzir seus efeitos. Adicionalmente, novos estudos são requeridos para melhor identificar o perfil de não produzir efeito antibacteriano direto nas linhagens testadas.

Palavras-Chave: Resistência antibiótica. Chalconas. Bomba de Efluxo. NorA. MepA.

Agradecimentos: FUNCAP, CAPES e CNPq.

Synthesis, ADMET studies, molecular docking and antibiotic modifying activity of 2-hydroxyacetophenone-derived chalcones.

ABSTRACT

Bacterial resistance to clinically used antibiotics has been growing rapidly around the world, increasing the number of infections that are difficult to treat and often leading to death. Given this, it is necessary to search for therapeutic alternatives to inhibit bacterial resistance mechanisms. One of the promising alternatives is the search for natural substances, semi-synthetic and synthetic molecules that may have a intrinsic antibacterial activity or that can serve as an adjuvant in antibiotic therapy. From observations made through the literature, it can be observed that several chalcones have antibacterial activity, thus being considered useful for the study. In this work, two chalcones derived from 2-hydroxyacetophenone were analyzed and their interaction with the Efflux Pump mechanism present in Staphylococcus aureus 1199B and K2068 strains carrying the efflux proteins NorA and MepA, respectively, were analyzed. For the synthesis of the chalcones, the Claisen-Schimd methodology was used and for their structural characterization, Nuclear Magnetic Resonance and Infrared Spectroscopy techniques were used. To evaluate the antibacterial activity, a broth microdilution test was performed using 96-well plates. To verify the efflux pump inhibition, the subinhibitory concentration of chalcones and standard inhibitors was used. The plates containing these substances were microdiluted with the antibiotics Norfloxacin, Ciprofloxacin and Ethidium Bromide, with concentrations ranging from 512 µg/mL to 0.5 µg/mL. Computational techniques were used to observe important fundamental characteristics for possible drug candidates. The data obtained were analyzed using a two-way ANOVA followed by the post hoc Bonferroni test, where $p < 0.05$ was considered significant. The geometric mean of the triplicates was used as the central data ± standard error of the mean. The GraphPad Prism 5.0 statistical program was used for the analysis. It was observed that the chalcones were able to interact with the pump mechanism present in the bacteria, these interactions were confirmed by molecular docking. Chalcones also potentiated the effects of antibiotics, where chalcone (E)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one showed satisfactory results with both antibiotics used in the study. The action of chalcones is related to the chemical ligands present in them. The data summarized in the present study allows us to conclude that the chalcones were able to interact in the efflux pump mechanism of the bacteria, competing for the active sites of the proteins with the substrates that would be bound, thus allowing the antibiotic to reach the interior of the cell. bacteria and produce its effects. Additionally, further studies are required to better identify the profile of not producing a direct antibacterial effect in the tested strains.

Keywords: Antibiotic resistance. Chalcones. Efflux Pump. NorA. MepA.

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IMPORTÂNCIA/RELEVÂNCIA PARA A SOCIEDADE

O número de bactérias resistentes à antibióticos vêm crescendo de forma acelerada desde muito tempo e isso foi intensificado na Pandemia do COVID-19, onde o uso inadequado do medicamento utilizado por boa parte da população, contribuindo desta forma por um aumento no número de microrganismos multidrogarresistentes. Esse problema de saúde pública e econômica, vêm acarretando diversas consequências para a população, aumentando drasticamente o número de mortes ocasionadas por bactérias resistentes em todo o mundo. Por causa dessas resistências, é necessário que haja à procura por novos medicamentos que sejam eficazes contra esses grupos de bactérias. E essas novas drogas são na grande maioria, direta ou indiretamente, oriundas de produtos naturais. Com isso faz-se necessário a busca por substâncias naturais que apresentem atividade antibacteriana. Dentre essas substâncias temos as chalconas, onde no nosso trabalho foram sintetizadas duas delas derivadas da 2-hidroxiacetofenona. Foi visto no nosso estudo que essas chalconas podem agir modulando a atividade de antibióticos já utilizados na clínica, desta forma permitindo uma ação do antibiótico em menor concentração quando utilizado junto com as chalconas. Diversas chalconas já tiveram suas atividades biológicas comprovadas na literatura.

1.1 OBJETIVOS E QUESTIONAMENTOS

Houve um aumento global de infecções ocasionadas por bactérias multirresistente e a descoberta de novos fármacos que visam tratar tais infecções não conseguiu acompanhar ao aumento acelerado destas bactérias, desta forma, o aumento de doenças infecciosas sem tratamento tornou-se um problema de saúde pública alarmante. Há uma estimativa que essas infecções ocasionadas por bactérias resistentes a diversos antibióticos gerem uma quantidade aproximada de 700.000 mortes por ano, sendo que este número possa aumentar para 10 milhões de morte ao ano em 2050. Na Pandemia do COVID-19 o número de bactérias resistentes a antibióticos cresceu consideravelmente em relação a 2019, o ano pré-pandemia, tanto no exterior, quanto em várias regiões brasileiras, dentre elas o Nordeste. Este aumento tem várias consequências, dentre elas o aumento de mortes em todo o mundo, como também impactos financeiros. Na União Europeia, por exemplo, estima-se um gasto em torno de 1,5 bilhão de euros (cerca de 8,16 bilhões de Reais) anuais tanto na saúde pública quanto na perda de produtividade dos medicamentos (BLAIR *et al.*, 2015; NEILL, 2016; FIOCRUZ, 2022; ANVISA, 2021).

A resistência bacteriana ocorre quando as bactérias sofrem mutações genéticas ao serem exposta de forma exagerada a fármacos antimicrobianos. Desta forma esses microrganismos passam a apresentar uma proteção as ações bactericidas e/ou bacteriostáticas dos medicamentos, resultando em multiplicação bacteriana com consequente falta de tratamento e muitas vezes cura para determinadas doenças (FRACAROLLI; OLIVEIRA; MARZIALE, 2017; MIRANDA; SIMÕES; TEIXEIRA, 2017). A resistência pode ocorrer de forma natural ou adquirida, sendo que a primeira mostra que alguns microrganismos sempre apresentaram resistência a alguns fármacos ou os adquiriram por transmissão de material genético e a segunda, se refere a microrganismos que antes eram sensíveis aos antibióticos expostos (LOUREIRO *et al.*, 2016; COELHO, 2019). Os antimicrobianos pertencem ao grupo de medicamentos que possuem uma frequência maior de uso irracional em todo o mundo (LIMA *et al.*, 2019).

Muitas bactérias apresentam o mecanismo de resistência aos antibióticos, dentre elas, a bactéria *Staphylococcus aureus,* classificada como Gram-positiva e que mede aproximadamente 0,5 a 1,5 µm de diâmetro. É uma bactéria imóvel, que produz a enzima catalase e não é capaz de produzir esporos, sendo um patógeno humano, mas que também vive de forma comensal no organismo do homem. (TONG *et al.*, 2015). A *S. aureus* possui habilidade para adquirir resistência a quase todos os fármacos antibacterianos utilizados na clínica. Esta resistência ocorre comumente por mutações. E essa capacidade de se adaptar e de com isso adquirir resistência é uma grande preocupação para

saúde pública (FOSTER, 2017; FRIERI; KUMAR; BOUTIN, 2017). As cepas de *S. aureus* resistentes à meticilina (MRSA - *Methicillin-resistant Staphylococcus aureus*) são consideradas um problema mundial, visto que em grandes partes do mundo, pessoas sofrem com infecções causadas pelas mesmas, especialmente em países em desenvolvimento, onde possuem uma maior dificuldade visto que as alternativas para o tratamento das infecções causadas por esses microrganismos são escassas (CHESSA; GANAU; MAZZARELLO, 2015).

Alguns dos mecanismos que são responsáveis por promoverem resistência bacteriana aos antibióticos, são as enzimas que inativam antibióticos, diminuição da permeabilidade da membrana, modificação no alvo do antibiótico e efluxo ativo dos antimicrobianos (KUMAR *et al.*, 2013). Este último é capaz de realizar a extrusão do fármaco e desta forma consegue diminuir a concentração do medicamento para níveis que não são letais para a bactéria (VENTER, 2019).

Dentre as famílias portadoras de bomba de efluxo podemos citar a superfamília MFS (*Major facilitator superfamily*), que é a Superfamília de Facilitadores Principais, sendo esta a mais estudada e possui vários exemplos que são de interesse clínico, como por exemplo a bomba de efluxo NorA, que tem a capacidade de extrudar da célula bacteriana antibióticos fluoroquinolonas, compostos de amônia quaternária, dentre outros. A bomba NorA de *S. aureus* é o sistema de efluxo mais estudado e frequentemente utilizado para que se possa pesquisar a resistência bacteriana por bomba de efluxo presente nesta bactéria (ALAV; SUTTON; RAHMAN, 2018).

Outra família de bomba de efluxo, é a família MATE, que é a família de extrusão de compostos tóxicos e multifármacos, a qual a bomba de efluxo MepA presente em *S. aureus* faz parte. É um transportador que se utiliza de gradientes transmembrana de H^+ e/ou Na^+, para que assim consigam fazer a extrusão de substâncias tóxicas às células bacterianas (HANDZLIK *et al*., 2013; DU *et al.*, 2018).

Na tentativa de reverter a resistência bacteriana mediante o mecanismo de bomba de efluxo, tem-se feito a associação de substâncias capazes de inibir este mecanismo com os fármacos antibacterianos. Estas substâncias podem ser naturais ou sintéticas, normalmente as substâncias naturais advêm do metabolismo secundário de plantas. Os compostos que são capazes de inibir o mecanismo de resistência de bomba de efluxo possuem bastante interesse, pois os mesmos, apresentam potencial para aumentar a eficácia quando associados aos fármacos antibacterianos e podem atuar como adjuvantes aos tratamento (STAVRI; PIDDOCK; GIBBONS, 2007; PRASCH & BUCAR, 2015; BROWN *et al.*, 2015).

Diante deste contexto, visto que o aumento crescente das bactérias portadoras de mecanismos de resistência, onde muitas podem apresentar mais de um mecanismo na mesma cepa, como é o caso da bactéria *S. aureus* e visto também a presença das bombas de efluxo NorA e/ou MepA nesta espécie, viu-se a necessidade de pesquisar e avaliar substâncias alternativas que

pudessem reverter esse mecanismo de efluxo de forma direta ou em associação a fármacos utilizados na clínica.

Uma das substâncias encontradas amplamente na natureza, estando presente em várias plantas comestíveis, sendo de fácil acesso, são as chalconas. Estas possuem em sua estrutura grupos aromáticos (dois) que estão acoplados a uma cadeia lateral, constituída de três carbonos. Por causa da sua estrutura química relativamente simples, as chalconas podem sofrer diversas alterações em sua estrutura, resultando desta forma em diferentes atividades biológicas. Desta forma muitos pesquisadores estudam esse grupo de substâncias, na tentativa de encontrar alguma que possua, por exemplo, atividade antibacteriana. Muitas das atividades atribuídas as chalconas, tanto as naturais quanto as sintéticas, já foram relatadas na literatura, onde pode-se citar, a atividade antibacteriana que já foi estudada por nosso grupo de colaboradores (RAYEES AHMAD *et al.*, 2016; ROCHA *et al.*, 2021).

Diante da possibilidade de utilizar produtos de origem natural e/ou sintéticos para avaliar sua atividade antibacteriana direta e/ou avaliar sua capacidade de modular a ação de antibióticos frente a bactérias portadoras de bomba de efluxo, e vendo também as atividades biológicas presentes em diferentes chalconas, muitas delas advindo dos seus substituintes químicos, resolveu-se sintetizar chalconas derivadas da 2-hydroxyacetophenone e assim avaliar sua atividade antibacteriana frente as cepas de *S. aureus* 1199B portadoras da bomba de efluxo NorA e *S. aureus* K2068 portadora da bomba de efluxo MepA.

1.2 ESTRATÉGIAS DE PESQUISA

Os recursos que a natureza proporciona são considerados uma fonte para extração de substâncias com diferentes atividades biológicas, farmacológicas, que se apresentam potente e eficaz, desta forma, tanto os produtos naturais, quanto seus derivados semissintéticos e sintéticos tornam-se cada vez mais abundantes (DIMASI *et al.*, 2016; PATRIDGE *et al.*, 2016).

Para obtenção das chalconas sintéticas utilizadas no trabalho, foi utilizada a metodologia de *Claisen-Schimd*, através de uma condensação aldólica, resultando em duas chalconas derivadas da 2-hydroxyacetophenone. As chalconas resultantes foram (*E*)-3-(2,4-dichlorophenyl) -1-(2—hydroxyphenyl) prop-2-en-1-one e a (*E*)-1-2(2-hydroxyphenyl) -3-(2,4-dimethoxy-3-methylphenyl) -prop-2-1-one) (BANDEIRA *et al.*, 2019).

Para avaliação da atividade antibacteriana direta das chalconas, foi utilizada a metodologia de acordo com a CLSI (2015) com algumas modificações, para isto foi utilizada placas de 96 poços e ao final do teste pode se obter o valor da Concentração Inibitória Mínima (CIM) das substâncias

utilizadas no trabalho, ou seja, a menor concentração das chalconas capaz de inibir o crescimento bacteriano.

As bombas de efluxo presentes em bactérias são proteínas transmembrana que conseguem extrudar para o exterior da célula bacteriana substratos tóxicos as mesmas, onde se pode incluir quase que todas as classes de antibióticos. Exemplos dessas bombas de efluxo são as bombas NorA e MepA presente em cepas de *Staphylococcus aureus* 1199B e K2068, respectivamente. Uma forma de tentar reverter esse mecanismo de resistência é a pesquisa de Inibidores de Bomba de Efluxo (IBE) (BORGES-WALMSLEY; MCKEEGAN; WALMSLEY, 2003; SCHINDLER; JACINTO; KAATZ,2013).

Para avaliar se as chalconas agiam no mecanismo de bomba de efluxo de *S. aureus* foi utilizada a metodologia proposta por Oliveira-Tintino (2016), onde também se utiliza de uma microdiluição seriada, porém neste teste é utilizado inibidores padrões de bomba como a carbonil cianida m-clorofenilhidrazona (CCCP) e a Clorpromazina e também o corante tóxico brometo de etídio, que é utilizado para comprovação da bomba na bactéria feita através da redução da CIM deste composto (MAIS; BARBE, 2005; PATEL *et al.* 2010; PAGES; SHRIRAM *et al.*, 2018).

Para verificar uma possível interação entre as chalconas e as bombas de efluxo presentes nas bactérias, realiza-se a docagem molecular, e desta forma tenta-se evidenciar quais são os aminoácidos presentes nos sítios de ligação, e como a molécula candidata a possível IBE se acopla na proteína, não permitindo assim a extrusão de substâncias. E por fim, foi analisado as propriedades de Absorção, Distribuição, Metabolismo, Excreção e Toxicidade (ADMET) das chalconas (SCHWEIZER, 2012; TIAN *et al.*, 2018; MUNIZ *et al.*, 2021; ROCHA *et al.*, 2021).

1.3 ESTRUTURA DA TESE

A tese foi dividida em Introdução, Objetivos, Fundamentação Teórica Produção Científica, onde consta os dois artigos publicados vinculados a tese e Considerações Finais. Além disto, também se encontra ao final do trabalho os Anexos, com as comprovações cientificas realizadas nos quatro anos de doutorado. A organização segue o esquema abaixo (Esquema 1).

Esquema 1: Estrutura do relatório de tese.

2.1. OBJETIVO GERAL

Sintetizar e avaliar a atividade antibacteriana e de reversão de bombas de efluxo NorA e MepA em *Staphylococcus aureus* das chalconas sintéticas (E)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl) prop-2-en-1-one e a (E)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one.

2.2. OBJETIVOS ESPECÍFICOS

- Realizar a caracterização estrutural por espectroscopia de FT-Raman e FTR-Infravermelho;
- Determinar a Concentração Inibitória Mínima das chalconas sintéticas;
- Avaliar a atividade de reversão de bomba de efluo NorA e MepA;
- Realizar a docagem molecular das bombas de Efluxo NorA e MepA e sua interação com as chalconas;
- Analisar as propriedades de Absorção, Distribuição, Metabolismo, Excreção e Toxicidade (ADMET) das chalconas.

3.1 RESISTÊNCIA BACTERIANA AOS ANTIBIÓTICOS

As infecções ocasionadas por bactérias são responsáveis por diversas doenças que acometem os humanos e por significativo número de óbitos. Essas bactérias ao longo dos tempos desenvolveram vários mecanismos que possibilitaram a sobrevivência delas em ambientes desfavoráveis a sua existência, contribuindo desta forma, para resistência bacteriana aos medicamentos antibióticos (ROMPELOTTO, 2013 NIGAM *et al.*, 2014; JAMPILEK, 2017). Na resistência aos antibióticos ocorre uma adaptação as bactérias, permitindo que elas possam crescer e se reproduzirem mesmo em um ambiente com a presença do medicamento (BECEIRO *et al.*, 2013).

A resistência bacteriana pode ser resultado de uma variedade de mecanismos, onde estes podem ser desenvolvidos em nível comunitário ou celular, onde o primeiro é resultado de uma adaptação aos desafios encontrados no ambiente e o último por mutações cromossomiais ou transferência horizontal (LANGTON; HENDERSON; HERBET, 2005; PENESYAN; GILLINGS; PAULSEN, 2015; SCHILLAC *et al.*, 2017). Nos últimos anos houve um aumento significativo da prevalência das bactérias multirresistentes, ocasionada principalmente devido ao uso de forma insdiscrimada de antibióticos (LEAL *et al.*, 2020; REZENDE-JUNIOR *et al.*, 2020).

A resistência bacteriana aos medicamentos tradicionais diminui de forma considerável o repertório de fármacos que são eficazes no combate frente as infecções, resultando desta forma em um aumento no índice de mortalidade e custos nos tratamentos para infecções bacterianas multidrogas resistentes (MDR) (REDGRAVE *et al.*, 2014). Há uma estimativa que no ano de 2050 aproximadamente 10 milhões de pessoas morrerão por ano por doenças ocasionadas por bactérias multirresistentes, resultando desta forma em graves impactos sociais, ambientais, econômicos, dentre outros, em todo o mundo (NEILL, 2016; VENTER, 2019).

Dentre os principais mecanismos de resistência que as bactérias possuem frente aos antibióticos utilizados na clínica, podemos citar, (a) produção de enzimas que inativam os antibióticos, (b) modificação nos alvos do antibiótico, (c) mudança na permeabilidade de membrana e (d) bomba de efluxo. No primeiro caso, existe a produção de enzimas, como por exemplo, podemos citar a β-lactamase, que age quebrando o anel β-lactâmico, resultando na desativação da ação farmacológica dos medicamentos dessa classe. No segundo caso, ocorre uma ligação que confere proteção ou a diminuição do nível de expressão do alvo, desta forma resultando na diminuição da afinidade com o fármaco. Neste caso podemos citar modificações na proteína GyrA que conferem resistência a fluoroquinolonas (MUNITA; ARIAS, 2016; GARNEAU-TSOD; KOVA; LABBY, 2016; FOSTER, 2017; BELLO; DINGLE, 2018; EICHENBERGER; THANDE 2019).

A mudança na permeabilidade da membrana é utilizada como uma forma de impedir que o fármaco entre na bactéria, já que muitos destas têm seus alvos no interior da célula bacteriana eassim a ação farmacológica do medicamento é impedida. Pode ocorrer pela expressão de porinas mais seletivas, mutações nas porinas ou perda delas, desta forma os compostos hidrofílicos acabam sendo mais afetados quando ocorre a modificação na permeabilidade da membrana. Já as bombas de efluxo, são capazes de fazerem o efluxo de antibióticos, diminuindo a concentração do mesmo para níveis subtóxicos para bactéria. São considerados o mecanismo de resistência mais efetivo e mais rápido quando a bactéria está exposta a substâncias nocivas às mesmas, essa exposição é desencadeada por diversas reações complexas que podem resultar em uma expressão aumentada de vários genes transportadores (NICHOLS *et al.*, 2012; YELIN; KISHONY, 2018; VENTER, 2019).

Essa resistência bacteriana aos antibióticos é um fenômeno que acaba limitando as opções terapêuticas para o tratamento das infecções causadas por bactérias representando uma ameaça para saúde pública, onde o risco de falta desses medicamentos é listado por diversos estudiosos como o maior problema de saúde pública, seja ele em países desenvolvidos ou não (SILVEIRA *et al.*, 2006; KHAMENEH *et al.*, 2017; GUITOR; WRIGHT, 2018)

3.1.1 Bombas de Efluxo (BF) e Inibidores de Bomba de Efluxo (IBE) como alternativa para o mecanismo de resistência

As bombas de efluxo são proteínas encontradas na membrana que são dependentes de energia, sendo necessária para eliminar agentes antibacterianos para o ambiente extracelular de forma mais rápida que a difusão através da membrana plasmática, contribuindo para a resistência bacteriana. Essa ação faz com que a concentração intracelular da substância seja insuficiente para impedir as funções celulares na bactéria, tornando-se desta maneira ineficaz e impedindo que o antibacteriano consiga atingir seu sítio alvo. Bombas de efluxo são os fatores de resistência mais estudados (OPPERMAN E NGUYEN, 2015; KUMAR *et al.*, 2020).

Diante dos mecanismos existentes, as bombas de efluxo desempenham um papel importante, pois além do efluxo dos compostos tóxicos aos microrganismos, a superexpressão da bomba acarreta um grande risco de originar superbactérias que são ainda mais difíceis ao tratamento com terapias encontradas na clínica (FELICETTE et a., 2018). Desta forma, quando expressas essas bombas de efluxo, elas acarretam níveis altos de resistências aos antibióticos, que antes eram utilizados na clínica tradicional. As bombas elas podem ter uma especificidade a um substrato, porém muitas delas vem a transportar uma alta variedade de substratos diferentes, sendo desta forma chamada de bombas de efluxo de resistência a múltiplas drogas (*Multiple Drug Resistance* - MDR) (BLAIR *et al.*,2015).

As bactérias possuem vários genes que codificam bombas de efluxo de MDR em seus cromossomos, como também tais genes eles podem estar presentes exclusivamente em plasmídeos, que podem ser transferidos entre as bactérias da mesma espécie ou entre espécies de bactérias diferentes (DOLEJSKA *et al.*, 2013). As bombas de efluxo são reconhecidas como uma das principais causas de resistência presente nas bactérias a uma ampla variedade de antibacterianos, como por exemplo, as tetraciclinas, fluoroquinolonas, β-lactâmico, dentre outros, onde essas bombas vêm a proteger a célula bacteriana transportando estes antimicrobianos para o citoplasma das células, limitando a biodisponibilidade do fármaco (ZHANG; MA, 2010; SHARMA; GUPTA; PATHANIA, 2019).

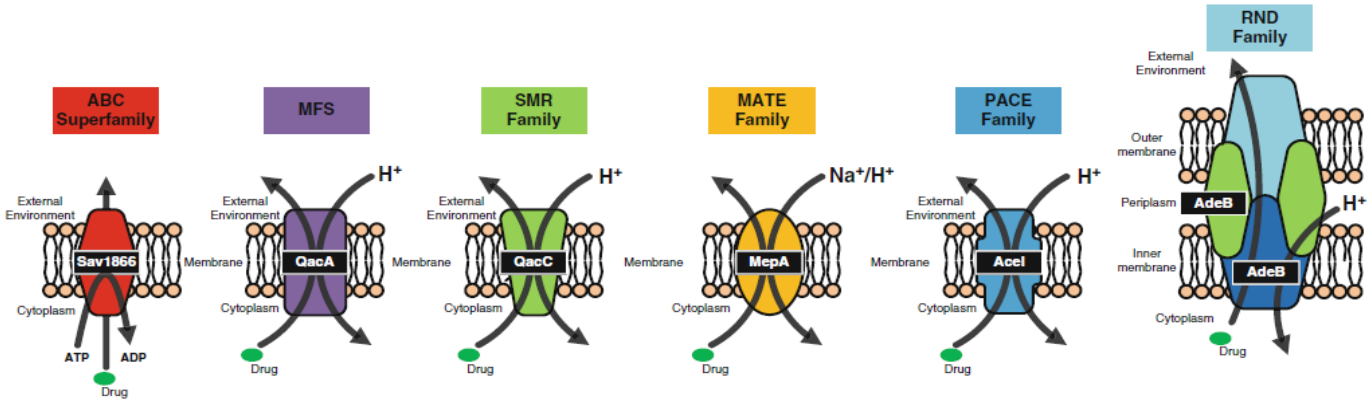
As bombas de efluxo são classificadas em seis famílias de acordo com a energia necessária para que promovam a expulsão das substâncias nocivas às mesmas. A classificação é dada em Superfamília Facilitadora – MFS (*major facilitator superfamily*), que utiliza o potencial eletroquímico de íons ou do soluto; Família de Resistência a Multidrogas – SMR (*family, the small multidrug resistance*); Família de Extrusão de Compostos Tóxicos e Multidrogas – MATE (multidrug and toxin extrusion), que utiliza os gradientes de Na^+ e H^+ para o transporte de substâncias; Superfamília de Cassete de Ligação de Adenosina Trifosfato – ABC (*ATP- binding cassette*), utiliza a energia da hidrólise do ATP; Superfamília de Divisão de Nodulação de Resistência – RND (*resistance-nodulation-cell division*); Superfamília de Efluxo de Compostos Antimicrobianos Proteobacterianos – PACE (*superfamily and the proteobacterial antimicrobial compound efflux*); e Superfamília do Metabólito Transportador de Drogas – DMT (*superfamily drug/metabolite transporter*). Com exceção da *ATP- binding cassette,* todas as outras vem a utilizar do gradiente de próton como energia necessária para fazer o transporte dos substratos (Figura 1) (HASSAN *et al.*, 2018; DU *et al.*, 2018; KUMAR *et al.*, 2020; HASSANZADEH *et al.*, 2020).

Diante da problemática, as bombas de efluxo se mostram como alvos necessários para inibição, e neste sentido muitas pesquisas foram realizadas para descobertas de substâncias que tenham a capacidade de inibir este mecanismo de resistência, a essas substâncias dar-se-á o nome de Inibidores de Bombas de Efluxo (IBE). A utilização dos IBE em combinação com os antibióticos é uma estratégia possível e possuem efeitos benéficos quando tem a capacidade de bloquear a saída do fármaco pela célula bacteriana, quando permite que antibiótico tenha sua ação desempenhada, e quando a dose do antibiótico é diminuída (KAATZ, 2016; SCHILLACI *et al.*, 2017; SEUKEP *et al.*, 2020; SCHINDLER).

Para se produzir um IBE alguns quesitos devem ser levados em consideração, como por exemplo, serem ser proteoliticamente estáveis, não devem possuir toxicidade para células humanas, não devem possuir atividade antibacteriana, dentre outros (BAMBEKE *et al.*,2006). Segundo Bhardwaj e Mohanty (2012), os IBE não devem possuir ação antibacteriana para que assim não

influenciem no desenvolvimento de resistência. A combinação de um IBE com um antibacteriano de uso clínico pode ser uma estratégia ótima e promissora (PIDDOCK, 2014; MELANDER; MELANDER, 2017).

FIGURA 01: Famílias de Bomba de Efluxo e seus respectivos mecanismo de ação.



Fonte: SAPULA BROWN, 2016.

3.1.2 Bactéria Sataphylococcus aureus

A bactéria *Staphylococcus aureus* é uma bactéria classificada como Gram-positiva, que possui formato de cocos, com tamanho que varia em torno de 0,5 a 1,5 µm de diâmetro, não móvel, que não forma esporos, é oxidase negativa, catalase e coagulase positiva. É encontrada na microbiota humana da nasofaringe e pele e está associada a diversas patologias nos humanos, como bacteremias, endocardites, dentre outras (HARRIS; FOSTER; RICHARDS, 2002; QUINN P, 2011; TONG *et al.*,2015; CROSBY *et al.*, 2016). A parede celular desta bactéria é principalmente composta de peptideoglicano, que tem como função primordial proteger a célula bacteriana de choques mecânicos e/ou osmótico (VERMASSEN *et al.*, 2019).

Entre as bactérias Gram-positivas, o gênero *Staphylococcus* é um dos que possuem mecanismos de patogenicidade mais notáveis, e a bactéria *S. auereus* é um dos patógenos que está mais presente nos hospitais e nas comunidades, o que pode resultar em infecções sérias, inclusive com risco de morte (TUCHSCHERR; LOFFLER, 2016; DOS SANTOS *et al.*, 2018). É uma bactéria comumente associada a infecções da pele e o tratamento para essas infecções é feito através de agentes antibacterianos tópicos, porém um número crescente destas bactérias desenvolveu resistência a estes antibacterianos, limitando assim a eficácia dos mesmos (KONING *et al.*, 2012; LARRU; GERBER, 2014; DOUDOULAKAKIS *et al.*, 2017).

As doenças que são causadas pela bactéria *S. aureus* em pacientes que possuem comorbidades são responsáveis por altas taxas de mortalidades nos humanos, onde as cepas que

possuem mecanismos de resistências foram surgindo com o uso de antibióticos, destacando-se entre elas a *Staphylococcus aureus* resistente à meticilina (MRSA), onde modificações na proteína de ligação a penicilina, tornou essa espécie resistente não somente a meticilina, mas também a outros β-lactâmicos, como a oxacilina, dentre outros (THOMER; SCHNEEWIND; MISSIAKAS, 2016).

Staphylococcus aureus resistente à meticilina (MRSA) é uma das principais preocupações em cuidados de saúde e ambientes comunitários. O que causa as infecções causadas por *S. aureus* um fator agravante, é que esta bactéria apresenta uma resistência a maioria dos medicamentos antibióticos de uso tradicional, como penicilina, vancomicina, fluoroquinolonas, β-lactâmicos, dentre outros, tornando desta forma dificultoso o tratamento de doenças e contribuindo de forma expressiva para o aumento das taxas de mortalidade e morbidade (HAVAEI *et al.*, 2010; PAL *et al.*, 2020).

A resistência a fármacos antibacterianos desenvolvida pela *S. aureus* acontece por meio de mecanismos moleculares que são muito eficientes, como por exemplo, através de enzimas que inativam o fármaco, modificação a nível molecular evitando que haja a ligação do fármaco ao local de ligação, barreira de permeabilidade da membrana externa, dentre outros, onde ainda, esses mecanismos podem ocorrer de forma concomitantemente. Esta bactéria é um patógeno presente nos seres vivos de grande importância por causa dos seus fatores de virulência e por conseguir adquirir resistência à uma grande quantidade de fármacos antibacterianos (STEFANI *et al.*, 2015; LAKHUNDI E ZHANG, 2018). A bactéria *S. aureus* também possui uma série de bombas de efluxo multidrogas resistentes, onde a mais estudada é a bomba de efluxo NorA (BUONERBA *et al.*, 2017).

3.1.3 Bomba de Efluxo NorA em cepas de Staphylococcus aureus

A bomba de efluxo NorA é classificada dentro da Superfamília de Facilitadores de Bomba de Efluxo (MFS), sendo esta uma proteína transportadora que se encontra presente principalmente nas bactérias de *Staphylococcus aureus*, sendo assim considerado um dos principais fatores que vêm a contribuir pelo crescente aumento de resistência de vários medicamentos a esta bactéria. Essa bomba é codificada pelo gene norA e tem sua superexpressão em cepas de *S. aureus* resistente a meticilina (MRSA) (PIDDOCK, 2006; ZGURSKAY *et al.*, 2015; SCHINDLER; KAATZ, 2016).

A proteína NorA, possui papéis importantes na resistência a diferentes fármacos com ação antibiótica, como podemos citar por exemplo, as fluoroquinolonas e compostos quartenários de amônio, brometo de etídio, dentre outros, essa resistência a diferentes substâncias é resultado do aumento da transcrição de norA ou do aumento na estabilidade de seu mRNA (KAATZ; SEO; RUBLE, 1993; RAO *et al.*, 2018). Essa resistência a múltiplos antibióticos que é causada pela bomba de efluxo NorA, faz com que o tratamento para doenças comuns causadas por essas bactérias, sejam

muito difíceis e complicadas. As MRSA possuem várias bombas de efluxo que são codificadas em cromossomos (SHARMA; GUPTA; PATHANIA, 2019; FIGUEREDO *et al.*, 2020).

A bomba de efluxo NorA é constituída por cerca de 388 aminoácidos e 12 segmentos transmembrana. Essa bomba tem sequências de aminoácidos com homologia com outras proteínas de efluxo encontradas em outras bactérias, como é o caso da proteína Bmr de *Bacillus subtilis*, e pode ser encontrada em bactérias Gram-negativas (KAATZ; SEO; RUBLE, 1993; BHASKAR *et al.*, 2016). A proteína NorA pode ser encontrada em cepas de *S. aureus*, como a SA-1199, SA-1199B, SA-1199-3, dentre outras, sendo que a cepa 1199B ela apresenta uma resistência a fluoroquinolonas de forma aumentada (superexpressando constitutivamente a proteína NorA) quando comparada a cepa 1199 (que expressa a proteína NorA de forma indutivelmente, sendo considerada a cepa selvagem) (KAATZ; SEO; RUBLE, 1993; KAATZ; SEO, 1995).

A bomba NorA usa a energia que é gerada pela força próton motriz (H^+/substância) para que assim ela consiga transportar substratos diversos e diferentes, e reduz a eficácia necessária nos sítios ativos (DEMARCO *et al.*, 2007, KUMAR *et al.*, 2020). Alguns mecanismos vêm sendo sugeridos para tentar explicar como um inibidor de bomba NorA tem sua ação, dentre eles, podemos citar, o vínculo competitivo na bomba, desta forma bloqueando a interação entre a bomba de efluxo e o antibiótico, evitando desta forma que o fármaco seja expulso da célula pela bomba (RATH *et al.*, 2019).

Outros mecanismos para os IBEs são a inibição para expressão do gene que é responsável pela bomba NorA, por competição entre substância e bomba, como falado anteriormente e por consumir a fonte de energia necessária para a bomba funcionar, como é o caso envolvendo a força de prótons. Neste último caso o CCCP, é um protonóforo capaz de inibir esta fonte de energia, dissipando o gradiente de prótons (KAATZ; SEO; RUBLE, 1993; NG; TRUCKSIS; HOOPER, 1994; COSTA *et al.*, 2016).

Alguns inibidores da bomba de efluxo NorA que não são antibióticos são a reserpina, verapamil, omeprazol, clorpromazina, dentre outros, onde apresentam em sua estrutura química grupos benzênicos e átomos em comum (GIBBONS, 2004; ROY *et al.*, 2013; JOSHI *et al.*, 2014; ASTOLFI *et al.*, 2017). Fontaine *et al.* (2014) demonstrou também que a bomba NorA pode ser inibida pela ação de produtos naturais. O transportador NorA é produzido normalmente em cerca da metade dos isolados clínicos, e é uma das bombas mais estudadas. O brometo de etídio é um substrato que é exclusivamente expulso pela célula bacteriana por bombas de efluxo (MARKHAM; NEYFAKH, 1996; BUONERBA *et al.*, 2017).

3.1.4 Bomba de Efluxo MepA em cepas de Staphylococcus aureus

A bomba de efluxo MepA faz parte da família de extrusão de compostos tóxicos e multifármacos (MATE). Dentre as famílias de bombas de efluxo esta foi a última a ser identificada e é a menos caracterizada (OMOTE *et al.*, 2006; HUET *et al.*, 2008). O primeiro membro desta família a ser identificado por sua capacidade de conferir o fenótipo de multidrogarresistente (MDR) foi o NorM de *Vibrio parahaemolyticus* (MORITA *et al.*, 1998). A proteína de efluxo MepA é superexpressada em cepas da bactéria Gram-positiva *S. aureus*, conferindo a esta cepa MDR (KAATZ; MCALEESE; SEO, 2005).

O gene MepA faz parte do operon mepRAB, onde também está compreendido o gene mepR, e este gene por sua vez, regula MepA. O gene mepR tem sua ação repressiva na bomba de efluxo MepA através da ligação específica no gene MepA, bloqueando desta forma sua transcrição (MCALEESE *et al.*, 2005; SCHINDLER *et al.*, 2013; AGAH *et al.*, 2014), mutações especificas no mepR diminui a afinidade ligação no DNA, levando a superexpressão da mepA (BIRUKOU *et al.*, 2013).

A bomba de efluxo MepA, codificada pelo gene cromossômico mepA, confere resistência a bactéria *S. aureus* K2068. A proteína MepA possui em sua constituição 451 aminoácidos e 12 segmentos de transmembrana, sua fonte de energia para que consiga transportar seu substrato vem do gradiente de prótons, sendo nesta bomba através de íons sódio antiporta, que é a mesma energia utilizada pela família MATE a qual essa bomba faz parte (HE *et al.*, 2004; KAATZ *et al.*, 2005; KURODA E TSUCHIYA, 2009).

Alguns dos substratos para proteína MepA são, fluoroquinolonas hidrofílicas, fluoroquinolonas hidrofóbicas, biocidas, brometo de etídio, biguanidas, tigeciclina, dentre outros (KAATZ *et al.*, 2005; MCALEESE *et al.*, 2005; KAATZ *et al.*, 2006; FERNANDEZ-FUENTES *et al.*, 2014; ARGUDÍN *et al.*, 2016). Em um estudo foi descrito a frequência de MepA, dentre outras proteínas de efluxo em isolados de *S. aureus*, sendo a proteína de efluxo MepA encontrada em 60% destes isolados (HASSANZADEH *et al.*, 2017).

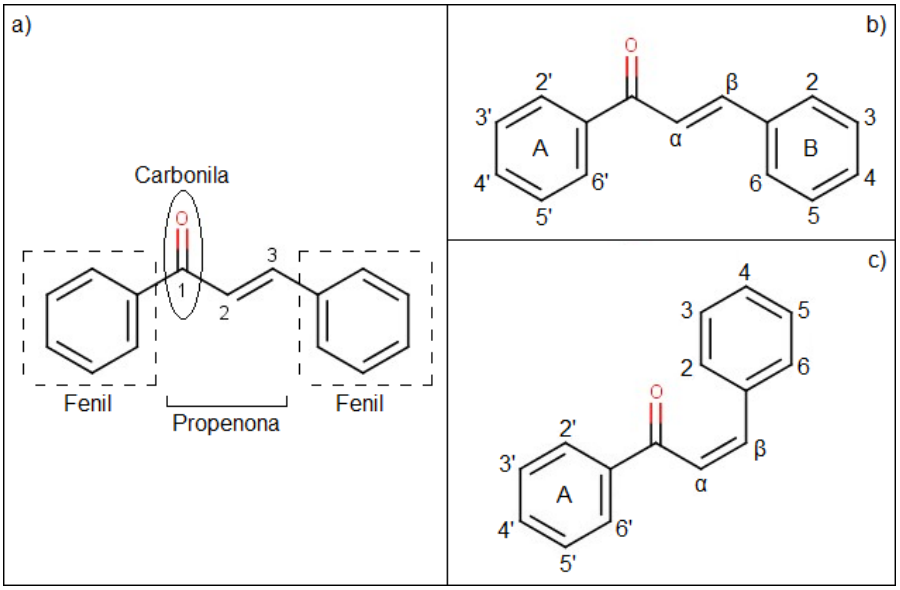
A bomba de efluxo MepA é codificada no cromossomo, assim como bomba NorA, onde a superexpressão da bomba pode acarretar fenótipos resistentes, quando as cepas bacterianas contendo estas proteínas são expostas a concentrações aumentadas de fármacos antibacterianos e de biocidas (COSTA *et al.*, 2013; COSTA *et al.*, 2018).

Visto o grande número de infecções causadas por *Staphylococcus aureus*, e por cepas portadoras de bomba MepA, NorA, dentre outras, várias pesquisas se fazem necessária para que se possa sintetizar substâncias novas com o objetivo de diminuir o mecanismo de resistência bacteriana e/ou tornar fármacos usados na clínica eficientes novamente (SCHINDLER; JACINTO; KAATZ, 2013).

3.2 CHALCONAS

As chalconas (1,3-diaril-2-propen-1-on), figura 02, são cetonas aromáticas, que existem como isômeros cis-trans, são derivadas dos flavonoides, que possuem dois anéis aromáticos, onde estes são interconectados por um sistema carbonil α, β-insaturado, que são altamente eletrofílico. São compostos naturais e que estão distribuídos de forma ampla nas plantas e possuem diversas atividades biológicas já comprovadas (FRIIS-MØLLER *et al.*, 2002; ACHANTA, 2006; ABE E MORITA, 2010; ORLIKOVA, 2011; ZHONG *et al.*,2015; MOLITOR *et al.*, 2016, TEKALE *et al.*, 2020).

FIGURA 2: Estrutura Geral da Chalcona.



Fonte: FREITAS, T.S. 2022. a) componentes formadores da estrutura das chalconas b) estrutura geral de uma chalcona na isomeria TRANS. c) Estrutura Geral de uma chalcona na isomeria CIS.

A estrutura química das chalconas permite que elas apresentem ações farmacológicas e assim apresentem diversas atividades biológicas, onde podemos citar chalconas com ação frente ao câncer, chalconas com atividade frente a microrganismos, com atividade anti-inflamatórias, antitumoral, atividade antioxidante, antibacteriana, inclusive resultando em ação sinérgica ao uso de antibióticos, dentre outras (ORLIKOVA, 2011; MANNA, 2016; JUNG *et al.*, 2017; COSKUN *et al.*, 2017; INAMULLAH *et al.*, 2017; CHU; GUO, 2017; DAS; TRATRAT *et al.*, 2019; UŠJAK *et al.*, 2019; MAHAPATRA; ASATI; BHARTI, 2019; BURMAOGLU *et al.*, 2019).

Dentro da química medicinal, as chalconas elas servem como uma base para que se possa descobrir novos candidatos a fármacos, isto devido elas poderem sofrerem modificações resultando em agentes medicinais menos tóxicos, com ações farmacológicas adequadas. Os anéis aromáticos das

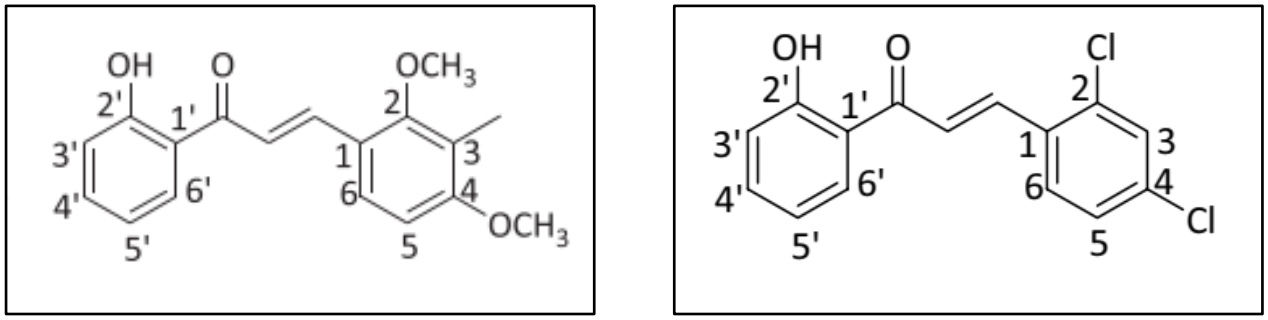
chalconas permitem uma grande variedade de substituintes, onde estes são responsáveis por suas ações farmacológicas (AWASTHI *et al.*, 2009; YERRAGUNTA *et al.*, 2013).

Através da reação de condensação (*Claisen-Schmidt*) é possível realizar a síntese de novas chalconas que podem ser usadas na terapêutica, essa reação envolve uma condensação catalisada por um ácido ou por base, entre um benzaldeído e acetofenona. Esta técnica é a mais utilizada, porém existem diversas outras (WINTER *et al.*, 2016; ZHUANG *et al.*, 2017).

Muitas chalconas sintéticas já tiveram sua ação antibacteriana demonstrada na literatura, muitas delas sendo analisada como possível agente capaz de inibir o mecanismo de bomba de efluxo presente em algumas cepas de bactérias, como nos trabalhos de (TRATRAT *et al.*, 2019; OLIVEIRA *et al.*, 2020; XAVIER *et al*., 2021; DA SILVA *et al.*, 2021; ROCHA *et al.*, 2021).

Como exemplo de chalconas sintetizadas, podemos citar as utilizadas no nosso estudo, que foram a (E)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl) prop-2-en-1-one (fig. 03a) e a (E)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one (fig. 03b), ambas apresentando atividade adjuvante frente bactérias portadoras de bomba de efluxo (ROCHA et al., 2021a; ROCHA et al., 2021b).

FIGURA 03: Estrutura das chalconas sintetizadas.



A **B**

FONTE: adaptado de ROCHA et al., 2021a; ROCHA et al., 2021b. Em (A) (E)-1-(2-hydroxyphenyl)-3-(2,4- dimethoxy-3-methylphenyl) prop-2-en-1-one e em (B) (E)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one.

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Antibacterial and antibiotic modifying activity, ADMET study and molecular docking of synthetic chalcone (*E*)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl) prop-2-en-1-one in strains of *Staphylococcus aureus* carrying NorA and MepA efflux pumps

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ABSTRACT

A large number of infections are caused by multi-resistant bacteria worldwide, adding up to a figure of around 700,000 deaths per year. Because of that many strategies are being developed in order to combat the resistance of microorganisms to drugs, in recent times, chalcones have been studied for this purpose. Chalcones are known as α, β-unsaturated ketones, characterized by having the presence of two aromatic rings that are joined by a three-carbon chain, they are a class of compounds considered an exceptional model due to chemical simplicity and a wide variety of biological activities, which include anticancer, anti-inflammatory, antioxidants, antimicrobials, anti-tuberculosis, anti-HIV, antimalarial, anti-allergic, antifungal, antibacterial, and antileishmanial. The objective of this work was evaluate the antibacterial and antibiotic modifying activity of chalcone (*E*)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl)prop-2-en-1-one against the bacteria *Staphylococcus aureus carrying* a NorA and MepA efflux pump. The results showed that chalcone was able to synergistically modulate the action of Norfloxacin and Ethidium Bromide against the bacteria *Staphylococcus aureus* 1199B and K2068, respectively. The theoretical physicochemical and pharmacokinetic properties of chalcone showed that the chalcone did not present a severe risk of toxicity such as genetic mutation or cardiotoxicity, constituting a good pharmacological active ingredient.

1. Introduction

A large number of infections are caused by multi-resistant bacteria worldwide, adding up to a figure of around 700,000 deaths per year, and this estimate may increase until the year 2050. The indiscriminate uses of antibiotics, as well as their misuse, resulted in the selection of bacteria

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resistant to known antibiotics, for which we have little or no treatment [1,2].

The Efflux Pump (EP) present in some bacteria, are considered to be one of the fundamental causes of drug resistance. These EPs carry out the active transport of small molecules to the extracellular medium of the bacterial cell. The NorA pump present in *Staphylococcus aureus* bacteria is an example of an efflux pump, this is a driving force-dependent proton pump. Another example is the MepA efflux pump belongs to the family of multidrug and toxin extrusion (MATE) [3–5].

Many strategies are being developed in order to combat the resistance of microorganisms to drugs, using new antibiotics, joining therapy with natural antibacterial substances, as well as the use of drug delivery systems. In recent times, many classes of natural products have been extensively studied for this purpose, especially against Gram-Positive and Gram-Negative multi-resistant bacteria [6–8].

Chalcones are present in several fruits, vegetables, plants, among others, being precursors of the synthesis of flavonoids and isoflavanoids, acting as intermediates that have their derivation in the amino acid phenylalanine. They are also known as α, β-unsaturated ketones, characterized by having the presence of two aromatic rings that are joined by a three-carbon chain, and have become very attractive due to their biological activities that are of great interest, such as for example, antimicrobial activity. This activity is related to the chalcone's structure, being especially attributed to the presence of phenolic groups in the molecule, where they have an affinity for proteins present in microorganisms and, in this way, managing to inhibit their development/growth [9–12].

Given the context, this study aimed to evaluate the direct antibacterial and antibiotic modifying activity of chalcone (*E*)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl)prop-2-en-1-one against the bacteria *Staphylococcus aureus carrying* a NorA and MepA efflux pump.

2. Materials and methods

2.1. Spectroscopic methods

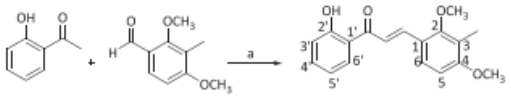
The chemical reagents were purchased from Sigma-Aldrich. ^{1}H and ^{13}C NMR spectra were obtained using a Bruker Spectrometer, model Avance DPX-500, operating at a frequency of 500 MHz for hydrogen and 125 MHz for carbon, respectively. The spectra were measured in $CDCl_3$, and chemical shifts are reported as δ values in parts per million (ppm) relative to $CDCl_3$.

Raman spectrum was collected Fourier Transform Raman spectroscopy (FT-Raman) with a Bruker RFS100/S FTR system and a D418-T detector, with a Nd:YAG laser emitting at 1064 nm as excitation source with 150 mW laser power, recording in the spectral range of 40–4000 cm^{-1} with a resolution of 4 cm^{-1}.

The infrared spectrum was measured by Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) using a Bruker vacuum spectrometer, model VERTEX 70 V with a HeNe laser source with 633 nm wavelength, recording in the spectral range of 130–4000 cm^{-1} with a resolution of 2 cm^{-1}.

2.2. Synthesis of the chalcone

The compounds 2-hydroxyacetophenone (2 mmol) and aldehyd2,4-dimethoxy-3-methylbenzaldehyde (2 mmol) were placed in a volumetric flask (25 mL). Then 5 mL of ethanolic NaOH (50%) solution was added and mixed with stirring for 48 h at room temperature. The progress of the reaction was checked by TLC (n-hexane: ethylacetate, 2:1). After 48 h the reaction mixture was neutralized with dilute HCl (10%) and ice water added. The product was obtained as a yellow solid filtered under reduced pressure, washed with cold water, and recrystallized from ethanol [13] (Scheme 1).



Scheme 1. Preparation of chalcone. a) NaOH 50% w v-1, ethanol, room temperature, 48 h.

2.3. Drugs

Chlorpromazine (CPZ), carbonyl-m-chlorophenyl hydrazone cyanide (CCCP) and ethidium bromide were obtained from Sigma Aldrich Co. Ltd. The antibiotics (Ciprofloxacin and Norfloxacin) and chalcone were dissolved in dimethylsulfoxide (DMSO) and then in sterile water. CPZ and ethidium bromide solutions were dissolved in distilled and sterile water, kept protected from light. The CCCP was dissolved in a 1:1 methanol/water solution. The final concentration of all compounds was 1024 μg/mL. The culture media used in the work were Brain Heart Infusion (BHI)-Agar (BHI, Acumedia Manufacturers Inc.), prepared according to the manufacturer and Brain Heart Infusion (BHI)-broth (BHI, Acumedia Manufacturers Inc.) prepared at a concentration of 10%.

2.4. Bacterial strains

The strains of *S. aureus* used were SA-1199B (overexpressed NorA) and SA-K2068 (which expresses MepA). The strains were provided by the Prof. S. Gibbons (University of London), being kept on blood agar (Laboratórios Difco Ltda., Brazil) and, before the experiments, they were grown for 24 h at 37 °C in solid Brain Heart Infusion (BHI)-Agar (BHI, Acumedia Manufacturers Inc.).

2.5. Determination of Minimum Inhibitory Concentration (MIC)

To determine the Minimum Inhibitory Concentration (MIC), the [14] with modifications was used.

2.6. Evaluation of NorA and MepA efflux pump inhibition

The inhibition of the Efflux Pump was tested using a Sub-inhibitory Concentration (CIM/8) of the chalcone and using the Effluent Pump Inhibitors (EPI) to verify the effect on the tested pumps, following the methodology proposed by [15].

2.7. ADMET study

The characterized molecule was designed in the MarvinSketch® academic license software [16] of the ChemAxon © software package Marvin JS (https://chemaxon.com/products/marvin) for theoretical calculation of the physicochemical properties of ionization (pKa), partition coefficient (logP), distribution coefficient (logD), water solubility (logS) and polarity (PSA), used as molecular descriptors for pharmacokinetic properties. Then, the SMILES of the molecule COc1c (/C = C / C (= O) c2ccccc2O) ccc (c1C) OC was uploaded to the online server admetSAR 2.0 (http://lmmd.ecust.edu.cn/admetsar2/) [17] and ADMETlab (http://admet.scbdd.com/home/index/) [18–20] for consensual prediction of pharmacokinetic properties of absorption, distribution, metabolism, excretion and toxicity (ADMET) models.

Then, the molecule was designed in the JSME molecular editor [21] implanted in the Pred-hERG 4.2 tool of the online server LabMol (http://predherg.labmol.com.br/), where the fragments were analyzed molecular molecules through a two-dimensional map of positive (green region) and negative (pink region) contributions to inhibition of hERG ion transport channels.

2.8. Molecular docking

The MepA model was generated by retrieving the protein sequence for the NCTC 8325 strain from the Uniprot [22] database. Then, the SWISS-MODEL [23] service was used to build the homology model. The template of the multidrug and toxic compound extrusion (MATE) transporter of the *Bacillus halodurans* (PDB-ID: 5C6N) was chosen for the homology model. The model was then uploaded to the Molprobity [24] service for protonation and then to the CASTp [25] server in order to identify possible binding sites. The largest site, which is accessible from the cytoplasmic side, was chosen. Thus, for the docking procedure, which was carried out using the Autodock Vina [26] software, a grid box of 80Åx80Åx80Å around the geometrical center of the model was used. This box envelopes the binding site completely. A representation of the binding site is provided as Fig. S5. Ligands were optimized using the Gaussian 09 Program [27]. Initial structures were created in the GaussView module [28] and were then optimized using the Density Functional Theory (DFT) method with B3LYP exchange-correlation functional [29] using the 6–31G* basis set. Partial Gasteiger charges were added to the protein and to the ligand atoms using the AutoDock Tools interface [30], non-polar hydrogen atoms were mixed while all other parameters were kept at their default values. Docking poses were chosen based on the best binding score.

2.9. Statistical analysis

The data were analyzed using a two-way ANOVA test, using the geometric mean of the triplicates as the central data and the Standard Deviation, using the statistical program GraphPad Prisma 5.0. Then, a post hoc Bonferroni test was performed (where $p < 0.05$ and $p < 0.0001$ are considered significant and $p > 0.05$ is not significant).

3. Results

3.1. Spectroscopic analyses

In the spectrum of ^{1}H NMR (Figs. S1 and S2, supplementary material) you can see the signals in δH 7.71 (J = 15.5) and 8.14 (J = 15.6) are attributed to two doublets referring to hydrogens α, unsaturated β, whose coupling constant (J) confirms the stereochemistry E of the double bond. The signals at δH 7.93 (d, J = 8.1 Hz, H6 ′), 7.03 (d, d, J = 8.3 Hz, H3′), 7.48 (t, J = 7.7 Hz, H4′), and 6.94 (t, J = 7.2 Hz, H5′) refer to aromatic hydrogens in ring A. While the signals at δH 6.72 (d, J = 8.6 Hz, H5) and 7.55 (d, J = 8.6 Hz, H6), and 2.19 (s, CH3) refer to aromatic hydrogens of ring B. In addition, hydrogens referring to methoxy groups were observed in δ_H 3.79 (s) and 3.89 (s). In the ^{13}C NMR spectrum (Fig. S3 and S4, supplementary material) there is the signal referring to α, unsaturated β carbonyl in δ_C 194,3 ppm. The ketone absorbs in 203.8 ppm, however, the presence of α, β unsaturation causes a displacement to high field and the probable cause is the displacement of charge by the benzene ring or by the double bond that makes carbonyl carbon less electron deficient. The olefinic carbons α and β are observed in δ_C 118.9, and 141.6 ppm and respectively. At δ_C 163.7 (C-2 ′), 136.2 (C-4 ′), 129.7 (C-6 ′), 120.4 (C-1 ′), 118.7 (C-3 ′) and 118.8 (C-5 ′) there are the signals referring to the carbons present in ring A. While the signals in δ_C 161.5 (C2), 159.8 (C-4), 127.5 (C-1), 121 (C6), 118.7 (C3), 106.8 (C5), and 9.1 (CH_3) refer to ring B carbons. In addition, the methoxy groups were observed in δ_C 61.7 and 55.9 ppm (Table 1).

The ATR-FTIR absorbance spectrum and Raman spectrum of polycrystalline sample of chalcone (*E*)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl) prop-2-en-1-one are shown in Fig. 1a and b, respectively. Table 2 lists a detailed description of the assignments of some vibrational modes associated with the Raman and infrared bands of this chalcone.

In the wavenumber region between 3700 cm^{-1} and 2750 cm^{-1} are localized the bands originating from the OH, CH and CH_3 stretching modes. The very weak infrared absorbance band observed at 3580 cm^{-1} in the ATR-FTIR spectrum is associated with the hydroxyl functional group, OH. In the infrared spectrum the bands seen at 2942 and 2835 cm^{-1} are associated respectively with the asymmetric and symmetric stretching modes of the methyl group, CH_3. The Raman bands in this region appear with low intensity but even in this manner is possible to note the presence of some bands after re-scaling the Raman intensity by a magnification of 50x.

The weak infrared band located at 1680 cm^{-1} is associated with the mixture of the C=O carbonyl stretching mode with the Cα-Cβ stretching mode, which are the unsaturated carbons connecting the aromatic rings. In chalcones, carbonyl stretching mode always appear conjugated with the Cα-Cβ stretching mode [31–34].

The more intense bands appearing in the infrared and Raman spectra at 1551 and 1557 cm^{-1}, respectively, correspond to the vibrational mode from B-ring deformation which is mainly associated with the displacements of CCH atoms.

The strong infrared band observed at 1487 cm^{-1} is associated with anti-symmetric bending of the of methyl group, and two strong infrared bands observed at 1273 and 1103 cm^{-1} are associated with from B-ring deformations due to the displacements of COC atoms of the ethoxy

Table 1
^{1}H NMR and ^{13}C data from chalcone (*E*)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl) prop-2-en-1-one on $CDCl_3$. The chemical shifts in δ_H and δ_C are in ppm.

C	δ_C	δ_H
1′	120.4	
2′	163.7	
3′	118.7	7.03 (d, J = 8.3 Hz)
4′	136.2	7.48 (t, J = 7.7 Hz)
5′	118.8	6.94 (t, J = 7.2 Hz)
6′	129.7	7.93 (d, J = 8.1 Hz)
C=O	194.3	
1	127.5	
2	161.5	
3	118.7	
4	159.8	
5	106.8	6.72 (d, J = 8.6 Hz)
6	121.0	7.55 (d, J = 8.6 Hz)
7	9.1	2.19 (s)
C_α	118.9	7.71 (d, J = 15.5 Hz)
C_β	141.6	8.14 (d, J = 15.6 Hz)
OCH3	61.7	3.89 (s)
OCH3	55.9	3.79 (s)

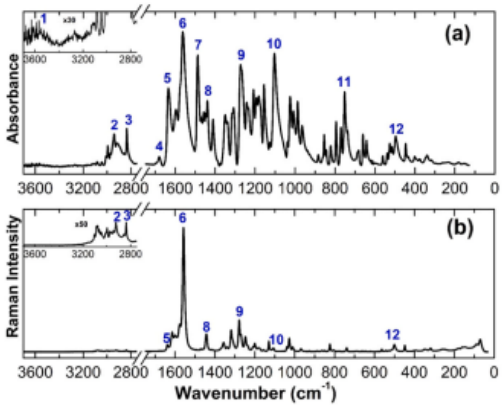


Fig. 1. (a) ATR-FTIR spectrum and (b) FT-Raman spectrum of chalcone (*E*)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl) prop-2-en-1-one.

Table 2
Infrared and Raman band positions in units of cm^{-1} with the respective intensities and assignment for some vibrational modes of the chalcone (*E*)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl) prop-2-en-1-one.

Modes	ν_{IR}	I_{IR} [a]	ν_{Raman}	I_{Raman} [b]	Assignment of the vibrational modes
1	3580	0.03	–	–	σ (OH)
2	2942	0.25	2944	0.01	σ_{as} (CH_3)
3	2835	0.28	2838	0.01	σ_s (CH_3)
4	1680	0.08	–	–	σ(C=O) + σ(CαCβ)
5	1635	0.58	1638	0.06	δ (CCH in both rings)
6	1561	1.00	1557	1.00	δ (CCH in ring B)
7	1487	0.83	–	–	δ_{as} (CH_3)
8	1439	0.49	1443	0.14	δ (C′C′H in ring A)
9	1273	0.76	1279	0.26	δ (COC in ring B)
10	1103	0.84	1107	0.03	δ (COC in ring B)
11	752	0.56	–	–	γ(C′C′C′H in ring A)
12	497	0.23	501	0.06	δ (all structure)

Nomenclature: ν_{IR} = wavenumbers of the infrared bands; ν_{Raman}= wavenumbers of the Raman bands. σ = stretching; σ_{as} = asymmetric stretching; σ_s = symmetric stretching; δ = deformation; δ_{as} = anti-symmetric bending; γ = deformation out of plane.

[a] Infrared absorbance intensities (I_{IR}) were normalized between 0 and 1 with highest absorbance band equal to 1.

[b] Raman intensities (I_{Raman}) were normalized between 0 and 1 with highest Raman band equal to 1.

group of the chalcone.

Three strong infrared bands observed at 1487, 1273 and 1103 cm^{-1} are associated with bending modes of the of the ethoxy group, the former corresponds to the anti-symmetric bending of the of methyl group, whereas the other two bands are associated with from B-ring deformations due to the displacements of COC atoms.

With the aforementioned spectroscopic data, we confirmed the molecular structure of the chalcone (*E*)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl) prop-2-en-1-one.

3.2. Antibacterial and antibiotic modifying activity

The association of chalcone (*E*)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl) prop-2-en-1-one with the antibiotic Norfloxacin, resulted in synergism, reducing the MIC of the drug from 128 μg/mL to 64 μg/mL, a reduction of 50% (Fig. 2). There was also a reduction in the antibiotic's MIC when it was used together with the standard efflux pump inhibitors, CCCP and Chlorpromazine, thus suggesting that the chalcone in question may be acting on the antibiotic's mechanism of action in the bacteria. When the chalcone was added to Bromide, there was no change in the result (Fig. 2).

The chalcone (*E*)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl did not have an antibacterial effect on the tested strain of *S. aureus* K2068 when associated with the antibiotic Ciprofloxacin. When the chalcone was used in association with ethidium bromide, it caused a bromide MIC reduction from 32 μg / mL to 12.6992 μg/mL (60.3% reduction), a similar result was observed when using the standard CCCP efflux pump inhibitor. Chlorpromazine, another efflux pump inhibitor, also promoted a reduction in the ethidium bromide MIC in these bacteria (Fig. 3).

3.3. ADMET study

In Fig. 4 (A), it is possible to observe the distribution of HS39 microspecies through the calculated pKa value. The value obtained in the order of 7.19, associated with the hydroxyl group of the phenolic fragment, suggests that there is a chemical balance between the neutral species (blue curve) and the ionized species (red curve), which tends to move towards the formation of the species ionized with increasing pH, dominant in physiological pH (approximately 7.4) [35].

The graph in Fig. 4 (B) expresses this behavior with the pH variation, where we can see that the initial value of logD in the order of 4.434 is equal to the index of logP, which suggests that the distribution remains constant until pH 6.0, where it begins to vary depending on the formation of the charged species. Thus, the achieved value of $logD_{7.4}$ 4.023 suggests that the compound may be slightly permeable to the biological barriers of the physiological system, but still presents lipophilicity considered ideal in the intestinal absorption phase [36].

Thus, the theoretical value of logP in the order of 4.434 indicates a lipophilic character within the established ideal, while the PSA evaluated at 55.76 suggests that methylchalcone is a good permeate of the various biological barriers, with a plasma protein affinity of approximately 90% (Table 3) [37–39]. The conduct of the ADMET calculations of the admetSAR 2.0 and ADMETlab servers indicated that the substance is a good permeate of colorectal adenocarcinoma cells, with subcellular mitochondrial location, since the logarithmic permeability coefficient of − 4722 cm / s (log Papp> −5.15 in 10^{-6} cm/s) calculated by the ADMETlab server justifies the good intestinal absorption evaluated in consensus [40]. Despite being a P-glycoprotein inhibitor, the servers indicated that the compound is very bioavailable after intestinal absorption, in an order of bioavailable molecular fraction greater than 30%. As a reflection of the permeability coefficient, the servers pointed out that the free molecular fraction is a potential permeate of the blood-brain barrier (BBB) (Table 3) [41].

Phase I metabolism is decisive in the excretion phase and in the determination of toxic effects caused by oral drugs. The cytochrome P450 (CYP450) isoenzymes are the main precursors of this metabolic activity, in particular the enzyme CYP1A2, responsible for the majority of drug biotransformations, and the CYP3A4 enzyme, responsible for the

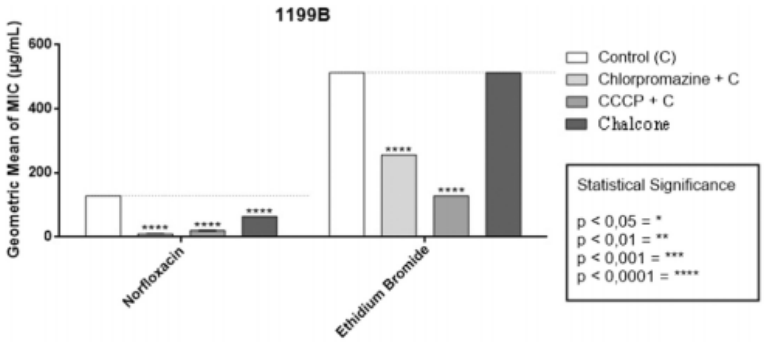


Fig. 2. Evaluation of antibiotic and ethidium bromide modification by chalcone against *Staphylococcus aureus* 1199B.

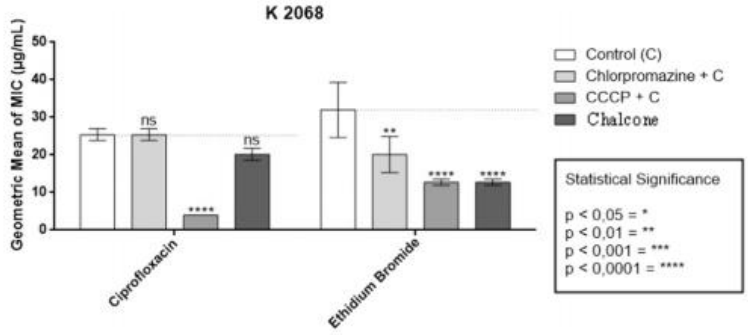


Fig. 3. Evaluation of antibiotic and ethidium bromide modification by chalcone against *Staphylococcus aureus* K2068.

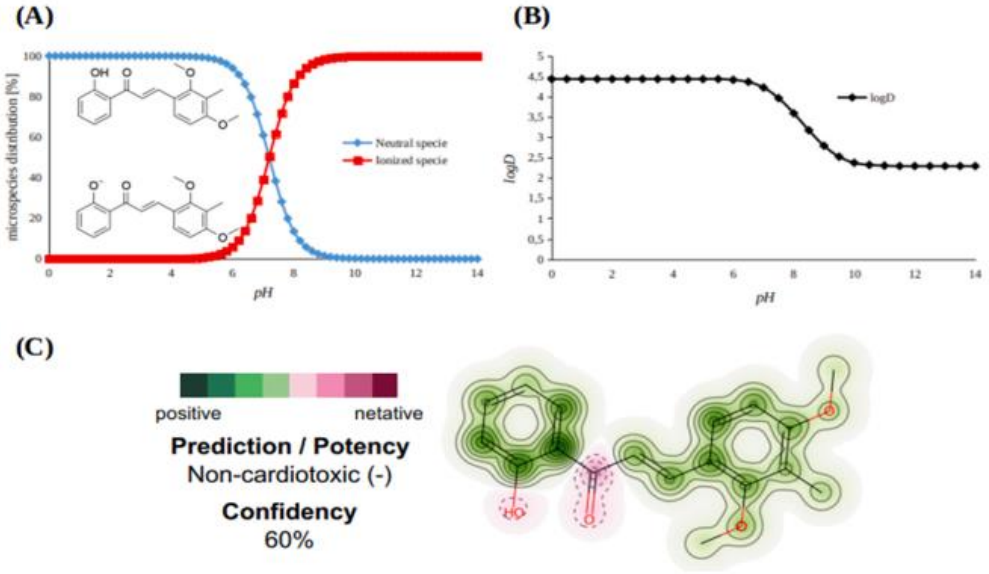


Fig. 4. (A) Graph of the distribution of chalcone microspecies as a function of pH; (B) Graph of the variation of the distribution coefficient (logD) of methylchalcone as a function of pH; (C) Two-dimensional map of molecular fragments of methylchalcone for hERG inhibition by the predictor LabMol.

Table 3
Physicochemical properties calculated.

Physicochemical descriptor	Value	Unit	Method
Ionization constant	7.194	PKa	ChemAxon
Lipophilicity	4.434	LogP	Consensus
Distribution at pH 7.4	4.023	$logD_{7.4}$	Consensus
Polar Surface Area	55.76	$Å^2$	ChemAxon
Plasma protein binding	0.905	100%	AdmetSAR
Acute Oral Toxicity	2.413	kg/mol	AdmetSAR

N–O-dealkylation oxidation reactions [42–46]. The servers pointed out, simultaneously, that methylchalcone is a potential inhibitor of CYP 3A4, 2C19 and 1A2, which suggests that the substance is slowly metabolized, with the risk of hepatotoxicity due to residual accumulation in the blood plasma and gastrointestinal tract fluids (Table 4).

The LabMol web server showed that the compound has no tendency to inhibit the hERG ion channels, with a 59% degree of reliability (Table 4). The two-dimensional map shown in Fig. 4 (C) shows that the negative contributions (pink region) associated with phenolic hydroxyl and carbonyl do not overlap the positive contributions (green region) of the rest of the molecule. The map has a 60% reliability index with a non-cardiotoxic effect [47]. Despite this, the ADMETlab server's database filter shows that a methylchalcone presents a cardiotoxic risk due to inhibition of hERG channels, unlike the admetSAR 2.0 server. Both servers use the AMES test database for mutagenicity, both of which are negative strategies for this toxicity model [48]. In addition, it is worth noting the approximation of the LD50 needs of the admetSAR 2.0 and ADMETlab servers, which, in the order of 2413 and 2.319 log mol/kg, belonging to toxicity class 3, which means that they are toxic only in high doses (Table 3) [49,50].

Table 4
Predicted pharmacokinetic properties by the ADMET predicted profile of admetSAR 2.0 server and ADMETlab web servers: HIA (Human Intestinal Absorption); Caco-2 (colorectal adenocarcinoma *cells); BBB (Blood Brain Barrier); + (positive result) and – (negative result).*

ADMET predicted profile	Result	Probability
HIA	+	0.9875
Caco-2	+	0.9406
BBB	+	0.9406
Human oral bioavailability	+	0.7143
Subcellular localzation	Mitochondria	0.8810
P-glycoprotein inhibitior	+	0.6976
P-glycoprotein substrate	-	0.9176
CYP3A4 substrate	-	0.5000
CYP2C9 substrate	-	0.7931
CYP2D6 substrate	-	0.8493
CYP3A4 inhibition	+	0.5188
CYP2C9 inhibition	-	0.9654
CYP2C19 inhibition	+	0.6722
CYP2D6 inhibition	-	0.9232
CYP1A2 inhibition	+	0.8002
CYP inhibitory promiscuity	+	0.7229
Ames mutagenesis	-	0.7400
hERG inhibition	-	0.5922
Hepatotoxicity	+	0.9250
Acute Oral Toxicity (c)	III	0.5556

3.4. Molecular docking

As mentioned before, there was no reduction on the MIC of EtBr (which is a known substrate of efflux pumps) when used in association with the chalcone against the *S. aureus* 1199B. As this strain overexpresses the NorA, the chalcone does not appear to be a NorA pump inhibitor. On the other hand, there was a reduction on the MIC of EtBr combined with chalcone against K2068, suggesting that the chalcone could be a MepA efflux pump inhibitor. Thus, in order to gain some insight on the efflux pump inhibition mechanism, we've created a MepA homology model for a docking procedure. The binding site of the MepA model consists of two regions, a proximal and a distal one. One possibility is that a substract entering the pump would initially bind at the proximal binding site and then be delivered to the distal site for subsequent extrusion, as was proposed for the AdeABC efflux pump [51]. On the boundary of these regions, one can name |Val283, Met363 and Phe335, for the proximal region, and Phe153, Tyr35 and Asn70 for the distal one. As it happens to other efflux pumps, such as NorA, there is a hydrophobic patch, in the distal site, consisting of several aminoacids, such as Phe153, Val149, Ala146, etc. A depiction of the binding site is provided as Fig. S5.

Both the neutral and ionized microspecies were docked to the MepA model, with binding affinities of – 7.4 and – 7.6 kcal/mol, respectively. Since one of the microspecies displays a net charge, it binds to a different region of the distal binding site, with very little overlap between the two poses, as can be seen in Fig. 5. The neutral species binds to the usual region of the binding site, displaying a hydrogen bond interaction of 2.25 Å with Asn205. van der Waals interactions with residues Thr201, Ser175, Val176, Asn179, Ser32 and many others are also present. Rezende-Júnior et al. [52] report that chalcones isolated from Arrabidaea brachypoda flowers bind to the same region of the binding site, with close contacts with residues Ser175, Val176, Asn179, Ser32, etc. Silva et al. [33] report that chalcones derived from acetophenone also bind to this region of the binding site, interacting with Tyr138, Phe62 and Tyr35. All of the best scoring poses bind to this region. A table with binding affinities and RMSD for all poses is provided as Table S1. A depiction of the best poses of both species clustered on the binding site of the model is provided as Fig. S6. A complete description of all interactions of the chalcone is shown as a 2D protein-ligand interaction diagram in Fig. 6. The binding region and interactions are typical of other chalcones with efflux pump inhibition capabilities, as described in Ref. [52].

Due to the loss of the hydroxyl proton, the best pose of the ionized microspecies interacts through a salt bridge of 2.91Å with Arg160, in a different region of the binding site. There's a Pi–Pi stacking interaction with Phe153 (5.5 Å), and close contacts with Glu156, Gln157, Phe152 and others. Its binding pose is such that it is much more exposed to solvent than the neutral species. Both species interact with residue in the range Ser175-Asn179 and Ser32-Tyr35. A 2D protein-interaction diagram of the ionized species docked to the model is provided as Fig. 7.

To validate our results, we docked EtBr (a known substrate) and chlorpromazine (a known inhibitor) to the MepA model. As can be seen on Fig. S6, both bind to the same region of the distal binding site, interacting with roughly the same residues as the neutral species and overlapping with the ionized one. As the chalcone bind the same region as a known substrate and inhibitor, one could thus argue that this chalcone act as a competitive inhibitor and that the ionized microspecies is probably less efficient in inhibiting the efflux bomb than the neutral one, but further investigation is needed in order to confirm this hypothesis.

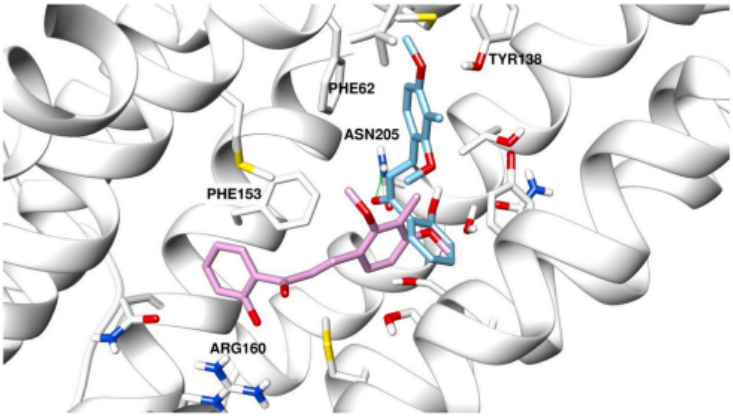


Fig. 5. Both microspecies (ionized in pink, neutral in blue) of the chalcone docked to the binding site of a MepA model. Hydrogen bonds are depicted in green.

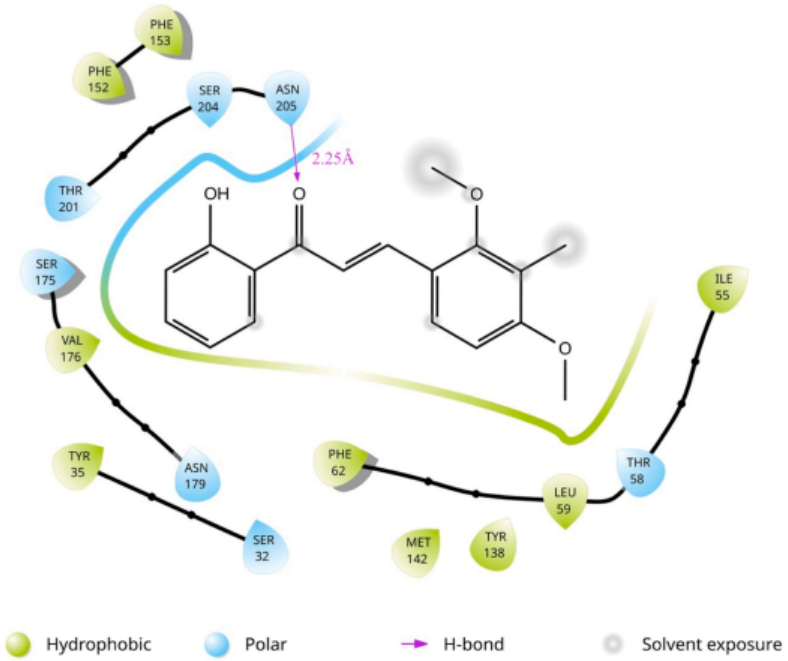


Fig. 6. A 2D protein-ligand interaction diagram of the neutral species docked on the binding site of the MepA model.

4. Discussion

The MIC of Ethidium Bromide was reduced when used with standard inhibitors, thus demonstrating that the bacterium in question has the effluent pump mechanism, since the mechanism by which bacteria dispense this DNA intercalant is through of the Eflux Pump [53].

In his work isolated several chalcones from the flowers of *Arrabidaea brachypoda* and evaluated their activity against different strains of *S. aureus* carrying efflux pumps. These chalcones had methoxy groups in their structure, such as those presented in chalcone (*E*)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl) prop-2-en-1-one. All chalcones had an effect against the bacterium SA1199B, where chalcone 4 had the best result in association with the antibiotic Norfloxacino, results that corroborate with the present study. However, its substances were also able to synergistically modulate the action of Ethidium Bromide, data that differ from our study [52]. Says in his study that methoxy groups present in the structure of the molecule benefits the interaction of the compound with the NorA efflux pump and our chalcone contains in its structure two methoxy groups, one linked to carbon two and the other to carbon four [54].

In this way, it can be concluded that chalcone acts as an inhibitor of the *S. aureus* MepA pump (Fig. 2), since Ethidium Bromide (EtBr) has been widely used as a model for checking the efflux pump in bacteria [55,56]. In a study with 3′, 4′-dihydroxy, 3, 4, 4′-trimethoxy-chalcone against the strain *S. aureus* K2068 in combination with the antibiotic Norfloxacin and Ethidium Bromide, the chalcone was able to reduce the MIC of both substances, however the present study this result was only observed for ethidium bromide [52].

As a way of justifying the pharmacokinetic behavior of chalcone, the theoretical physicochemical properties were calculated as descriptors of the absorption, distribution, metabolism, excretion and toxicity (ADMET) models, such as: ionization constant (pKa), partition coefficient (logP), distribution coefficient (logD), solubility coefficient (logS), polar surface area (PSA). As properties of the bioavailability domain of oral drug candidates, it is preferable to predominantly lipophilic compounds and not loaded in physiological medium [57–61].

The lipophilicity and permeability information provides information about the absorption and distribution of a bioactive compound. Most "drug-like" rules adopt the logP limit < 5 as the ideal lipophilicity range, stressing that a given oral drug candidate cannot be as lipophilic [59,62,63]. The distribution coefficient (logD) is directly related to lipophilicity and expresses the distribution of the bioavailable molecular fraction with the pH variation. Thus, values of distribution coefficient in physiological pH (logD7.4) less than 3 represent with stations with a good balance between absorption and distribution, as they present the ideal physicochemical conditions of lipophilicity and permeability [36,58]. The filter by Egan et al., 2000 refines compounds with intestinal absorption> 90% that are within the limits established between logP < 5.88 and PSA < 131.6 $Å^2$ [64].

Some chalcone derivatives have shown good antibacterial activity against Gram-positive and Gram-negative strains. However, these antibacterial activities are dependent on their pattern of structural replacement such as the introduction of a nitro group in ring A [65]. The biotransformation of the nitro group, releasing intermediates in the redox process and causing changes in the stability of membrane structures of several microorganisms [65]. Another important structural modification would be the introduction of methoxyl groups in ring A of the chalcone. The presence of methoxy groups has been considered as an important feature of many efflux pump inhibiting compounds [33,34,66].

5. Conclusions

We characterized by NMR, ATR-FTIR, and FT-Raman a promising

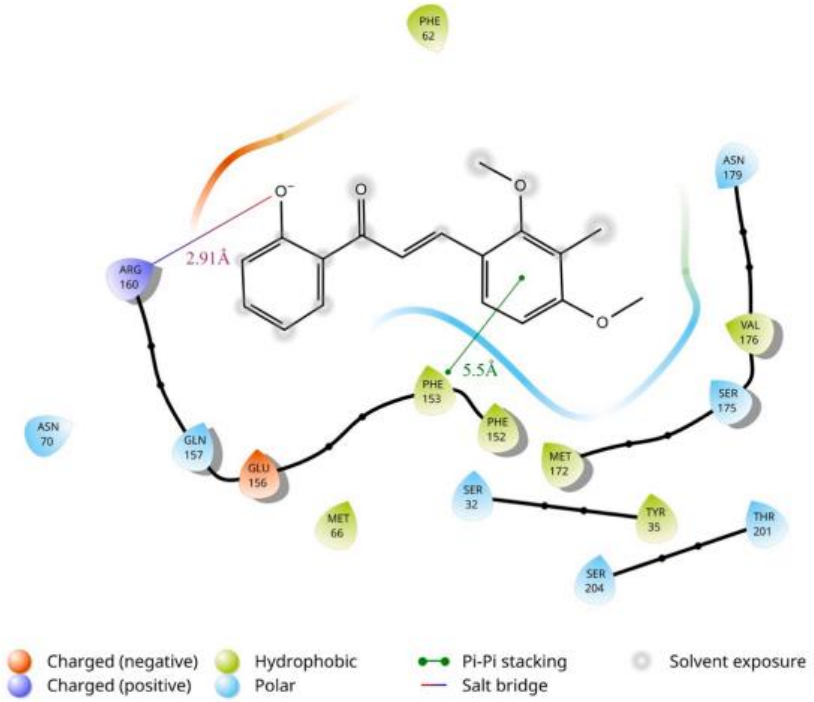


Fig. 7. A 2D protein-ligand interaction diagram of the ionized species docked on the binding site of the MepA model.

synthetic chalcone with potential application in antibacterial drug, and evaluated their pharmacokinetic properties. Many natural chalcones, semi-synthetic or synthetic, are being used to evaluate the antibiotic-modifying activity in bacteria carrying the efflux pump resistance mechanism. In this study, we can see that the synthetic chalcone (*E*)-1-(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl)prop-2-en-1-one was able to synergistically modulate the action of Norfloxacin and Ethidium Bromide against the bacteria *Staphylococcus aureus* 1199B and K2068, respectively. These data provide information for a potential use of these molecules as an Efflux Pump Inhibitor of the strains studied. The chalcone has high intestinal human absorption (HIA) and is active in the central nervous system (CNS), which characterizes its high molecular fraction available for the distribution phase. Despite the risk of hepatotoxicity by metabolic activation, the chalocne did not present a severe risk of toxicity such as genetic mutation or cardiotoxicity, constituting a good pharmacological active ingredient by oral administration.

CRediT authorship contribution statement

Janaína Esmeraldo Rocha: Investigation; Formal analysis; Writing - original draft. **Thiago Sampaio de Freitas:** Methodology. **Jayze da Cunha Xavier:** Methodology. **Raimundo Luiz Silva Pereira:** Methodology. **Francisco Nascimento Pereira Junior:** Data curation. **Carlos Emídio Sampaio Nogueira:** Software; Validation; Formal analysis; Writing – review and editing. **Márcia Machado Marinho:** Software; Validation. **Paulo Nogueira Bandeira:** Conceptualization; Resources. **Mateus Rodrigues de Oliveira:** Methodology. **Emmanuel Silva Marinho**: Formal analysis, Writing – review and editing. **Alexandre Magno Rodrigues Teixeira:** Formal analysis, Writing – review and editing. **Hélcio Silva dos Santos:** Formal analysis; Writing – review and editing. **Henrique Douglas Melo Coutinho**: Supervision; Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interestsor personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.111768.

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4.2 CAPÍTULO II: ARTIGO PUBLICADO NA REVISTA: ARCHIVES OF MICROBIOLY. FATOR DE IMPACTO: 2,55 – QUALIS – B1.

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ORIGINAL PAPER

Synthesis, antibiotic modifying activity, ADMET study and molecular docking of chalcone (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one in strains of *Staphylococcus aureus* carrying MepA efflux pumps

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Abstract
The *Staphylococcus aureus* bacteria is a Gram-positive, immobile, non-spore bacterium, with catalase and positive coagulase, among other characteristics. It is responsible for important infections caused in the population and for hospital infections. Because of that many strategies are being developed to combat the resistance of microorganisms to drugs, in recent times, chalcones have been studied for this purpose. Chalcones are found in parts of plants and can be found, for example, in the roots, leaves, bark, among others, but are mainly found as petal pigments, they are a class of compounds considered an exceptional model due to chemical simplicity and a wide variety of biological activities. This study aimed to evaluate the ability of chalcone (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one to reverse the efflux pump resistance, present in the bacteria *S. aureus* 1199B and *S. aureus* K2068. The synthetic chalcone (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one was able to synergistically modulate the antibiotic Ciprofloxacino and Ethidium Bromide against the bacterial strain *S. aureus* K2068, and with the antibiotic Norfloxacino against the strain 1199B. Thus, it is suggested that this chalcone may be acting by inhibiting the efflux pump mechanism of these bactéria. The theoretical physicochemical and pharmacokinetic properties of chalcone showed that the chalocne did not present a severe risk of toxicity, such as genetic mutation or cardiotoxicity. Molecular docking showed that the chalcone could act as a competitive inhibitor of the MepA efflux pump, as at hinders the binding of other substrates, such as EtBr.

Keywords Chalcone · Infections · *Staphylococcus aureus* · Efflux pump

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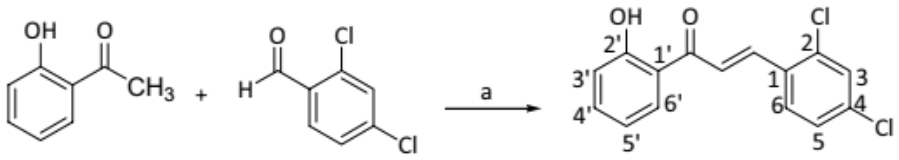

Scheme 1 Preparation of chalcone. **a** NaOH 50% w v-1, ethanol, room temperature, 48 h

Introduction

Chalcones (1,3-diaryl-2-propen-1-on) are aromatic ketones, which exist as cis–trans isomers, are derived from flavonoids, which have two aromatic rings, where they are interconnected by an α-carbonyl system, β-unsaturated, which are highly electrophilic. They are natural compounds that are widely distributed in plants and have several proven biological activities (Zhong et al. 2015; Molitor et al. 2016; Tekale 2020).

Chalcones are found in parts of plants and can be found, for example, in the roots, leaves, bark, among others, but are mainly found as petal pigments. These plants have been used in traditional medicine (Ni et al. 2004; Rozmer and Perjési 2016). A possibility to obtain compounds with activity against microorganisms is the guided synthesis of new compounds that have a certain similarity to the compounds of natural products, thus preventing the in vivo scarcity of the molecule (Umesha and Basavaraju 2014).

When talking about the synthesis of new chalcones, what has often been done is using an aromatic ketone in various condensation reactions with different aromatic aldehydes. This way, different groups are obtained that can be tested so that more effective candidates can be obtained for the various expected biological effects (Bertoldo et al. 2015).

Various techniques can be used to synthesize new chalcones, to modify the present structures. They have been extensively studied regarding their antibacterial activity (Matos et al. 2015; Mahapatra and Bharti 2016).

Among the Gram-positive bacteria, the *Staphylococcus* genus is one of those with the most notable pathogenicity mechanisms, and the S. auereus bacteria is one of the pathogens that is most present in hospitals and communities, which can result in serious infections, including life-threatening (Tuchscherr and Loffler 2016; dos Santos et al. 2018). It is a bacterium commonly associated with skin infections and treatment for these infections is done with topical antibacterial agents, but an increasing number of these bacteria have developed resistance to these antibacterials, thus limiting their effectiveness. (Koning et al. 2012).

New antimicrobials are needed to treat infections caused by these resistant pathogens, as well as new approaches to prevent these resistances. (Bell and Maclean 2018; Sommer et al. 2017; Baym et al. 2016). One of the resistance mechanisms of the *S. aureus* bacteria is the presence of efflux pumps, among them the Nora and MepA efflux pump, conferring resistance to fluoroquinolones, biocides, ethidium bromide, among others. Efflux pumps inhibitor (EPI) are substances that are used in therapies and have the ability to return the bacterial cell sensitivity to the drug and thus increasing its effect, since the EPI is able to maintain the antibiotic in the intracellular medium of the bacteria (Kapp et al. 2017). Thus, it is important to search for natural or synthetic substances that are capable of reversing the efflux pump mechanism present in *S. aureus*.

In view of this, this study aimed to evaluate the ability of chalcone (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one to reverse the efflux pump resistance, present in the bacteria *S. aureus* 1199B and *S. aureus* K2068.

Methodology

Spectroscopic methods

The chemical reagents were purchased from Sigma-Aldrich. ^{1}H and ^{13}C NMR spectra were obtained using a Bruker Spectrometer, model Avance DPX-300, operating at a frequency of 300 MHz for hydrogen and 75 MHz for carbon, respectively. The spectra were measured in $CDCl_3$, and chemical shifts are reported as δ values in parts per million (ppm) relative to $CDCl_3$.

Synthesis of the chalcone

The compounds 2-hydroxyacetophenone (2 mmol) and 2,4-dichlorobenzaldehyde (2 mmol) were placed in a volumetric flask (25 mL). Then 5 mL of ethanolic NaOH (50%) solution was added and mixed with stirring for 48 h at room temperature. The progress of the reaction was checked by TLC (n-hexane: ethylacetate, 2:1). After 48 h the reaction mixture was neutralized with dilute HCl (10%) and ice water added. The product was obtained as a yellow solid filtered under reduced pressure, washed with cold water, and recrystallized from ethanol (Scheme 1).

Drugs

Chlorpromazine (CPZ), carbonyl-m-chlorophenyl hydrazone cyanide (CCCP) and ethidium bromide were obtained from Sigma Aldrich Co. Ltd.

Bacterial strains

The strains of *S. aureus* used were SA-1199B (overexpressed NorA) and SA-K2068 (which expresses the MetA pump). The strains were provided by Prof. S. Gibbons (University of London).

Determination of Minimum Inhibitory Concentration (MIC):

To determine the Minimum Inhibitory Concentration (CIM), the CLSI methodology (2015) with modifications was used.

Evaluation of NorA and MepA efflux pump inhibition

The inhibition of the Efflux Pump was tested using a Sub-inhibitory Concentration (CIM/8) of the chalcone and using the Standard Efflux Pump Inhibitors (IPBE) to verify the effect on the tested pumps, following the methodology proposed by Oliveira-Tintino et al. (2018).

ADMET study

To perform the ADMET test the molecule was designed in the molecular editor Marvin JS implanted on the ChemAxon © web server (https://disco.chemaxon.com/calculators/demo/plugins/) for the calculation of the acid ionization constant (pKa) properties, partition coefficient (logP) and distribution coefficient (logD), used as molecular descriptors of the absorption and distribution models (Daina et al. 2017).

Molecular docking

To perform the molecular docking, the models and methodology proposed by (Waterhouse et al. 2018; Guex et al., 2009; Chen et al. 2010; Trott and Olson 2010).

Statistical analysis

The data were analyzed using a two-way ANOVA test, using the geometric mean of the triplicates as the central data and the Standard Deviation, using the statistical program GraphPad Prism 5.0. Then, a post hoc Bonferroni test was performed (where $p < 0.05$ and $p < 0.0001$ are considered significant and $p > 0.05$ is not significant).

Results and discussion

NMR data

About the spectrum of ^{1}H NMR and the ^{13}C NMR spectrum (Figures S1 and S2, supplementary material and Table 1), can be observed signals in 7.62 ($J = 15.5$) and 8.22 ($J = 15.5$) indicating the hydrogens α, unsaturated β, whose coupling constant (J) confirms the stereochemistry E of the double bond. Other signals as 7.04 (d, $J = 8.0$ Hz, H3'), 7.45–7.54 (m, H4'), 6.95 (t, $J = 7.6$ Hz, H5'), 7.88 (d, $J = 8.0$ Hz, H6 ') indicated the aromatic hydrogens in ring A, while the signs at 7.71 (s, H3), 7.45–7.51 (m, H4) and 7.54 (m, H5) refer to aromatic hydrogens of ring B. A signal referring to α, β unsaturated carbonyl in 193.4 and the ketone absorbs at 203.8. However, the presence of α, β unsaturation causes a shift to high field and the probable cause is the displacement of charge by the benzene ring or by the double bond that makes carbonyl carbon less electron deficient. The olefinic carbons α and β are observed in 140.0 and 118.9, respectively. At 163.8 (C-2´), 136.9 (C-4´), 130.5 (C-6´), 123.2 (C-1´), 119.2 (C-3´) and 118.9 (C-5´) there are the signals referring to the carbons present in ring A. While the signals in 130.4 (C-1), 136.9 (C-2), 129.9 (C3), 136.9 (C-4), 128.8 (C-5) and 127.8 (C-6), refer to ring B carbons (Table 1).

Table 1 ^{1}H NMR and ^{13}C data from chalcone (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one on $CDCl_3$. The chemical shifts in δ_H and δ_C are in ppm

	δ_C	δ_H
C		
1´	123.2	
2´	163.8	
3´	119.2	7.04 (d, $J = 8.0$ Hz)
4´	136.9	7.45–7.54 (m)
5´	118.9	6.95 (t, $J = 7.6$ Hz)
6´	130.5	7.88 (d, $J = 8.0$ Hz)
C=O	193.4	
1	130.4	
2	136.9	
3	129.9	7.71(s)
4	136.9	
5	128.8	7.45–7.51 (m)
6	127.8	7.54 (m)
C_α	118.9	7.62 (d, $J = 15.5$ Hz)
C_β	140.0	8.22 (d, $J = 15.5$ Hz)

Antibacterial and antibiotic modifying activity

In the bacteria carrying the NorA pump, the chalcone (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one when associated with Norfloxacin promoted synergism, thus decreasing the MIC of the antibiotic from 128 to 64 µg/mL. When CCCP and Chlorpromazine, efflux pump inhibitors, were used, there was also a reduction in the MIC of the antibiotic to 20.16 and 10.08, respectively. Thus, it may suggest that the chalcone studied may be acting on the antibiotic's pump resistance mechanism in the bactéria.

As for ethidium bromide, there was no difference when using chalcone, but there was a reduction in bromide MIC when using pump inhibitors, thus stating that the studied bacteria SA-1199B has an efflux pump (Table 2).

For the bacterium SA-K2068, according to Table 2, we can observe the reduction of the ethidium bromide MIC from 32 to 12.7 µg/mL (60.3%) when used in association with chalcone, this reduction also it was observed when the CCCP and chlorpromazine inhibitors were used, with chalcone having a more significant result than chlorpromazine. In this way, it can be said that chalcone acts as an efflux pump inhibitor in this bacteria.

Regarding the tested antibiotic, a reduction in Ciprofloxacin MIC from 25.39842 to 12.6992 µg/mL (50%) when associated with chalcone is also observed, with a reduction in MIC when associated with CCCP. In this way it can be suggested that (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl) prop-2-en-1-one may be acting on the efflux pump mechanism of this bacterium.

Table 2 MIC values from the efflux pump tests for strains 1199B and K2068

	MIC (µg/mL)	
	1199B	K2068
Antibiotic alone	128.00	25.40
+CPMZ	10.08 (12)	25.40 (0)
+CCCP	20.16 (6,3)	4 (6.4)
+(E)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one	64.00 (2)	12.70 (2)
Ethidium Bromide alone	512.00	32
+CPMZ	256.00 (2)	20.16 (1.6)
+CCCP	128.00 (4)	12.70 (2.5)
+(E)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one	512.00 (0)	12.70 (1.6)

Antibiotic of 1199B = Norfloxacin; Antibiotic of K2068 = ciprofloxacin, *CPMZ* Chlorpromazine, *CCCP* carbonyl-m-chlorophenyl hydrazone cyanide

The values in parentheses represent the reduction in the Minimum Inhibitory Concentration of the associations of substances + controls, when compared to the respective controls

The chalcones with modified chemical structures have already been studied to evaluate several pharmacological activities, such as anti-inflammatory, antioxidant, antibacterial properties, among others (Devia et al. 1998; Hassan 2011).

In the study Guptaa et al. (2019) the activity of several chalcones was analyzed for their inhibition in multiresistant *S.aureus* and potentiation of fluoroquinolone activity, among these chalcones studied IMRG4 [N- (5a-chlorine, 8a-triflouromethyl)-benzyl-N, 1a-dihydro-2H-O, N-isoliquiritigeninoxazine] exhibited a potent antibacterial activity against clinical isolates of *S. aureus* NorA overexpressing strain (SA-1199B) and a norA deletion mutant (SAK1758) of *S. aureus*. In our study, we also showed activity for strain 1199B, when it was associated with the antibiotic Norfloxacin.

Efflux pumps are transport proteins that are involved in the extrusion/expulsion of various antimicrobial agents, resulting in ineffective intracellular concentration (Webber and Piddock 2003).

In the study by Freitas et al. (2020) he evaluated the antibacterial activity of four chalcones, where chalcone 3 and 4 presented the presence of the chlorine group in its structure. Chalcone 4 showed the best synergistic effect with the antibiotics tested when chlorpromazine-sensitive efflux pumps were present. The author also states that in the presence of the chlorpromazine sensitive efflux pump, all chalcones tested were synergistic with the antibiotic. In another study, it is said that halogenated chalcones have moderate antimicrobial activity and that the activity of trifluoromethylated and chlorinated chalcones is slightly better compared to fluorinated chalcones (Basic et al. 2014).

ADMET study

As a rule, molecules that are not very lipophilic and neutrally charged have greater bioavailability after being absorbed in the intestine. Physico-chemical properties such as pKa, log P, logD and PSA can provide information on lipophilicity, solubility and permeability as key descriptors of the pharmacokinetic activity of an oral drug candidate (de Lange et al. 2005; Hay et al. 2009; Lipinski 2004; Perisic-Janjic et al. 2011; Veber 2002).

Based on the assumption, the microspecies of the molecular system of dichlorochalcone were evaluated. The theoretical value of the ionization constant (pKa) of 7.19, associated with the phenolic hydroxyl, suggests a moment of equilibrium between the neutral and ionized species at pH 7.19. The chemical balance moves towards the formation of ionized hydroxyl, considered a weak acid and, therefore, the main micro species in physiological pH (approximately 7.4).

Thus, the weak acid generated in physiological medium does not constitute a limiting action on the bioavailability of the compound (Fig. 1A).

Most of the criteria for discovering new oral drugs take an ideal range of logP < 5, which means that the tested compound cannot be so lipophilic as to interfere with its bioavailability (Ghose et al. 1999; Lipinski 2004; Muegge 2003). The distribution coefficient (logD) is directly related to the lipophilic character of the molecule. Thus, a statistical study by Fichert et al. (2003) shows that compounds with logD values at physiological pH below 3 have good intestinal absorption, as they present a balance between solubility and permeability (Fichert et al. 2003; Hay et al. 2009). The calculated value of logP in the order of 5.44 indicates that the compound exceeds the ideal lipophilicity range, but the graph in Fig. 1B shows that this value varies in physiological pH levels, depending on the formation of the ionized species. Thus, the given value of logD at pH 7.4 in the order of 5.03 indicates that the lipophilicity of dichlorochalcone tends to decrease with increasing pH and, therefore, being a substance with high lipophilicity and permeability. The calculated values can be found in Table 1.

The Brain Or IntestinaL EstimateD permeation method (BOILED-Egg) uses the molecular descriptors of the partition coefficient n-octanol/water of Wildman and Crippen (1999) (WlogPo/w) and polarity (PSA) of the method of Ertl et al. (2000) in the prediction of human intestinal absorption (HIA) and activity in the central nervous system (CNS) by penetrability in the blood–brain barrier (BBB), determinants in the bioavailability of an oral drug (Daina and Zoete 2016; Ertl et al. 2000; Wildman and Crippen 1999). The BOILED-Egg graph expressed in Fig. 1C shows that the dichlorochalcone is at the limit established by the values of 4 < WlogP < 6 and PSA < 79 Å^2, which indicates that the molecule has high intestinal absorption and has potential for permeability in BBB. However, the molecule is not a substrate for P-glycoprotein (P-gp), which limits its transport and efflux across different biological membranes, with a bioavailability score of 0.55 (from 0 to 1).

To determine the profile of metabolism, elimination and toxicity, a prediction of inhibition of cytochrome P450 isoenzymes (CYP450) was made, which can be seen in Table 1. The activity by inhibiting CYP450 indicates the biotransformations suffered by the drug in the phase metabolism I. The

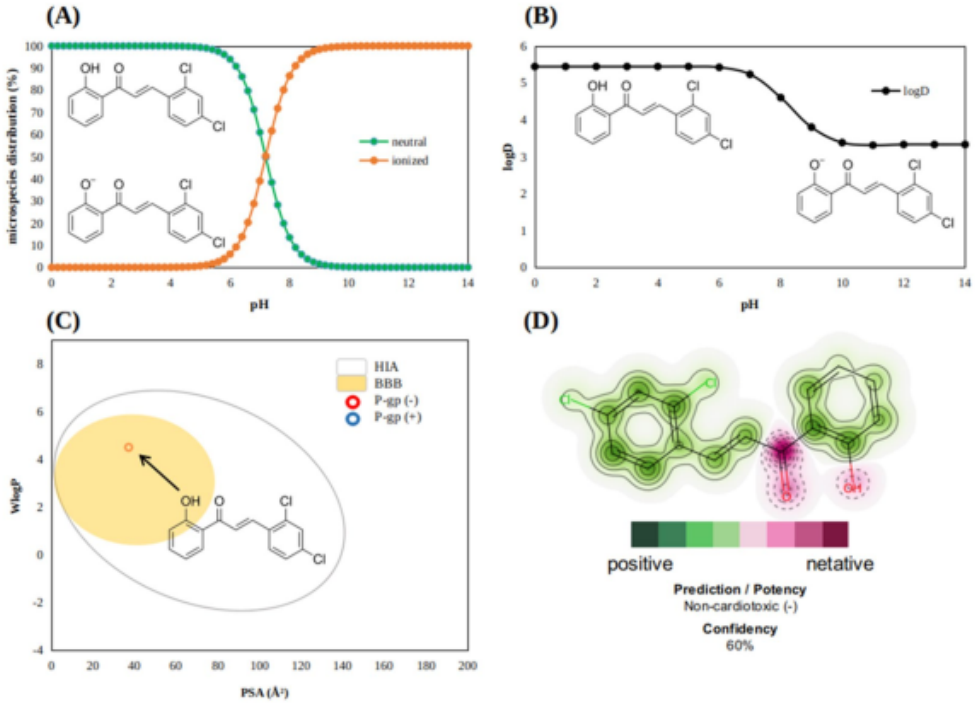

Fig. 1 **A** Microspecies distribution (%) of the dichlorochalcone as a function of the pH variation; **B** distribution coefficient (logD) of the dichlorochalcone as a function of the pH variation; **C** BOILED-Egg graph by the human intestinal absorption and blood brain barrier permeation models of the dichlorochalcone; and **D** two-dimensional cardiotoxicity map of the dichlorochalcone by the hERG inhibition model

enzymes evaluated were CYP1A2 and CYP2D6, responsible for most of the biotransformations of drugs by metabolic activation, CYP2C9 and CYP2C19, catalysts in the metabolism process, and CYP3A4, responsible for oxidation reactions, which determines the hepatotoxic activity of a drug orally (Azeredo et al. 2009; Eitrich et al. 2007; Matal et al. 2009; Terkeltaub et al. 2011; Zahno et al. 2011). The prediction showed that dichlorochalcone is a potential inhibitor of CYP1A2, CYP2C9 and CYP2C19, constituting a chemical entity with a slow renal elimination route, with a longer half-life, but is metabolized by CYP3A4, which suggests that oral administration of the compound does not present a risk of hepatotoxicity (Table 3).

In addition, it was possible to predict cardiotoxic activity through molecular fragments that present electronic contributions to inhibit the transport channels of hERG ions (human Ether-à-go-go-Related Gene) (Hakimelahi and Khodarahmi 2005; Khan et al. 2018). The two-dimensional map in Fig. 1D shows that the negative contribution associated with inhibition of the hERG channel by the carbonyl group of the dichlorochalcone (pink region) does not overlap over the positive contribution region of the remaining surface of the molecule (green region), constituting a non-cardiotoxic pharmacological active ingredient, with a 60% reliability level in the test.

Molecular docking

To gain insight about the efflux pump inhibition mechanism of the synthetic chalcone, we created a MepA homology model for a docking procedure. The chalcone and EtBr, a known substrate of the MepA efflux pump, were docked to the MepA model. As can be seen from Fig. 2, the best pose of the chalcone binds to the binding site of the model in roughly the same region as EtBr. There's a hydrogen bond between the carbonyl and residue Asn205 and another one between Asn205 and the hydroxyl. A Pi–Pi-stacked interaction between Phe62 and the dichlorophenyl ring is also present, as well as van der Waals interactions with residues Phe153, Met142, Leu59 and few others. A diagram depicting all interactions is provided in Fig. 3. In it, Hydrophobic and Polar interactions are colored-coded. On the other hand, Etbr appears to also interact with other residues, such as Gln284 and Arg281 (not shown in Fig. 2), while the chalcone does not. There is, however, a considerable overlap between the poses of these two molecules, and thus the chalcone could act as a competitive inhibitor of the MepA efflux pump, as at hinders the binding of other substrates, such as EtBr. It is worth mentioning that, as described at Rezende-Júnior et al. (2020), other chalcones with MepA inhibition capabilities also appear to bind to the same region of the binding site, displaying close contacts with residues, such as Ser32, Val176, Asn179, etc., just like the synthetic chalcone.

Conclusion

Bacterial resistance to antibiotics for clinical use has been growing at an accelerated rate, and therefore, it is necessary to search for new substances, natural or synthetic, that can reverse this situation. The synthetic chalcone (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one was

Table 3 Predicted physicochemical and pharmacokinetic properties of the chalcone

Property	Model name	Result	Unit	Source
Absorption	Lipophilicity	5.44	Numeric (ClogP)	Consensus
Absorption	Lipophilicity	4.49	Numeric (WlogP)	Wildman and Crippen (1999)
Absorption	HIA	High	Categorical (High/Low)	BOILED-Egg
Distribution	Ionization	7.19	Numeric (pKa)	ChemAxon
Distribution	Permeability	5.03	Numeric ($logD_{7.4}$)	Consensus
Distribution	PSA	37.30	Numeric ($Å^2$)	Ertl et al. (2000)
Distribution	BBB	Yes	Categorical (Yes/No)	BOILED-Egg
Distribution	P-gp substrate	No	Categorical (Yes/No)	SwissADME
Distribution	Bioavailability	0.55	Score (0–1)	SwissADME
Metabolism	CYP1A2 inhibitor	Yes	Categorical (Yes/No)	SwissADME
Metabolism	CYP2C19 inhibitor	Yes	Categorical (Yes/No)	SwissADME
Metabolism	CYP2C9 inhibitor	Yes	Categorical (Yes/No)	SwissADME
Metabolism	CYP2D6 inhibitor	No	Categorical (Yes/No)	SwissADME
Metabolism	CYP3A4 inhibitor	No	Categorical (Yes/No)	SwissADME
Toxicity	hERG inhibitor	No	Categorical (Yes/No)	LabMol

HIA Human Intestinal Absorption, *PSA* Polar Surface Area, *BBB* Blood Brain Barrier permeability, *P-gp* P-glycoprotein, *CYP* Cytochrome P isoenzymes, *hERG* human *Ether-à-go-go*-Related Gene

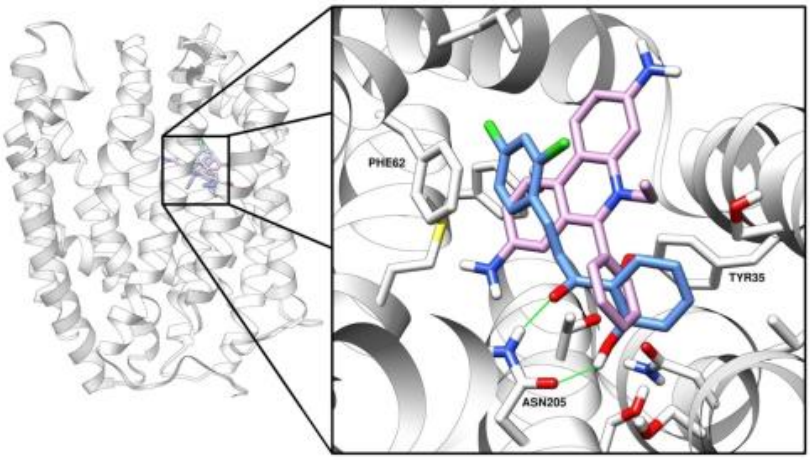

Fig. 2 Synthetic chalcone and EtBr docked to the binding site of a MepA model. Hydrogen bonds are depicted in green

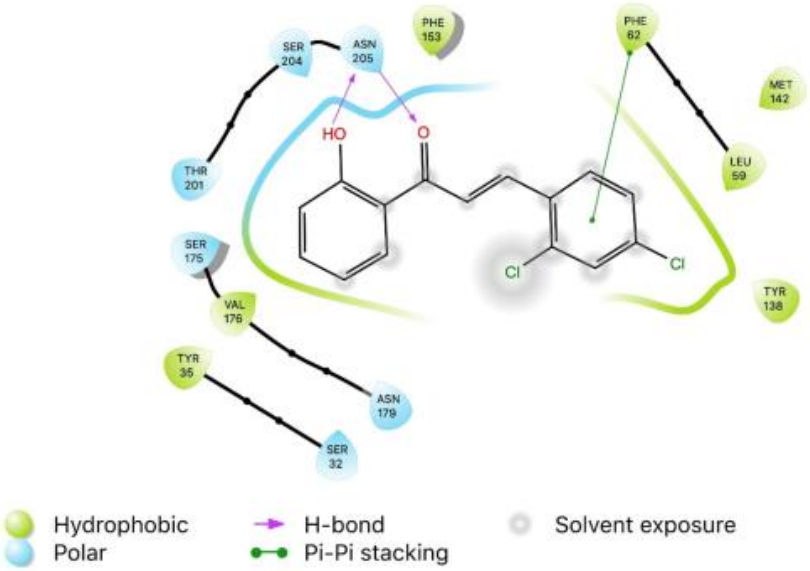

Fig. 3 2D protein–ligand interaction diagram of the synthetic chalcone docked on the binding site of the MepA model

able to synergistically modulate the antibiotic Ciprofloxacino and Ethidium Bromide against the bacterial strain *S. aureus* K2068, and with the antibiotic Norfloxacino against the strain 1199B. Thus, it is suggested that this chalcone may be acting by inhibiting the efflux pump mechanism of these bactéria. The calculated physical–chemical descriptors had a high degree of fidelity to the predicted pharmacokinetic properties. The chalcone has a chemical balance shifted to high lipophilicity and reduced water solubility, when subjected to theoretical calculation at pH 7.4. In this way, the filter that combines lipophilicity and polarity showed that the molecule is well absorbed in the intestine and has activity in the central nervous system (CNS). The chalcone could act as a competitive inhibitor of the MepA efflux pump, as at hinders the binding of other substrates, such as EtBr.

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Author contribution JER—methodology and writing. TSF—methodology. JCX—methodology. RLSP—methodology. FNPJ—validation and supervising. CESN—supervising. MMM—resources. PNB—supervising. MAAF—resources. ESM—resources. AMRT—supervising. HSS—supervising. HDMC—validation and supervising.

Data availability statement The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest There was no conflict of interest between authors.

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5.1 ASPECTOS GERAIS DA PRODUÇÃO CIENTÍFICA

As duas chalconas foram inicialmente sintetizadas por reação de *Claisen-Schimidt,* e ambas derivadas da 2-hydroxyacetophenone, essas estruturas foram confirmadas através da análise do espectro de RMN, com dados corroborados na literatura, como pode ser observado nos artigos um e dois publicados da tese.

Os efeitos antibacterianos das chalconas testadas apresentaram perfis similiares nos dois estudos publicados pelo grupo de pesquisa e que compõe a presente tese (*https://www.sciencedirect.com/science/article/pii/S0753332221005503?via%3Dihub* e *https://link.springer.com/article/10.1007/s00203-021-02666-z*), ou seja, nenhumas delas apresentaram efeitos antibacterianos diretos, desta forma apresentando um valor de CIM clinicamente sem significância estatística. Mas ao se analisar a atividade modificadora de antibiótico, registrou-se que estas chalconas apresentaram atividade biológica promissora.

Quando avaliada a atividade das chalconas como alternativa para reversão do mecanismo de resistência de bomba de efluxo, registrou-se que as duas chalconas apresentaram significativo perfil farmacológico na reversão dos mecanismos de resistência mediado por bomba de efluxo. Quanto a bactéria 1199B portadora da bomba de efluxo NorA, as duas chalconas conseguiram reduzir a CIM do antibiótico, mas não foram eficazes frente ao brometo de etídio, o que pode induzir que por mais que essas bactérias apresentem bombas de efluxo, provavelmente as chalconas estão agindo em outro mecanismo de ação, como por exemplo no mecanismo da topoisomerase, que também está presente na bactéria *S. aureus* 1199B. Esses dados corroboram com outros presentes na literatura para essa bactéria e que foram claramente explicitados em nosso referencial teórico.

Já em relação a bactéria *S. aureus* K2068, portadora da bomba MepA, a chalcona que possui como substituintes no anel B o cloro, tanto no carbono dois quanto no carbono quatro, apresentou atividades tanto frente ao brometo de etídio quanto frente ao antibiótico ciprofloxacino, conseguindo em ambos reduzir a CIM. Esses dados corroboram com outros da literatura, onde mostra que a presença de halogênios nessas posições, promovem uma ação frente a essas bactérias. Já a chalcona que possui no anel B, os grupamentos metóxi tanto no carbono dois quanto no carbono quatro, só apresentou atividade para o brometo, não demonstrando atividade modulatória para a ciprofloxacina.

Como pode-se observar, confrontando os dois artigos e também a literatura, a possível atividade de reversão do mecanismo de bomba de efluxo presente nas chalconas, pode ser atribuída

aos seus grupamentos químicos e as posições dos substituintes no anel dois, reforçando desta forma a importância dos mesmo quando feita a síntese das chalconas.

5.2 CONCLUSÕES GERAIS

- As chalconas testadas não apresentaram atividade antibacteriana direta;
- As chalconas (E)-1-2(2-hydroxyphenyl)-3-(2,4-dimethoxy-3-methylphenyl)-prop-2-1-one) e (E)-3-(2,4-dichlorophenyl)-1-(2—hydroxyphenyl)prop-2-em-1-one conseguem interagir com a bomba de efluxo MepA;
- A atividade da chalcona está diretamente correlacionada aos seus substituintes químicos;
- As chalconas possivelmente podem ser utilizadas como alternativa na reversão do mecanismo de resistência de bomba de efluxo, dependendo da bomba;
- As chalconas dependendo da dose não apresenta toxicidade;

5.3 PERSPECTIVAS DE INVESTIGAÇÕES FUTURAS

Com intuito de aprofundar melhor os resultados das chalconas estudadas, testes adicionais seriam interessantes para melhor verificar suas ações, como por exemplo, seria importante realizar testes com a metodologia de Checkerboard para verificação de sinergismo com os antibióticos Norfloxacina e Ciprofloxacina. Realizar teste de fluorimetria para observar o acúmulo do brometo de etídio dentro da célula bacteriana, quando este é utilizado isolado e concomitante com as chalconas. Avaliar a toxicidade, atividade ansiolítica, motora, comportamental, dessas substâncias em modelo de *Danio rerio* (zebra fish) e por fim avaliar a atividade frente a outras cepas de bactérias tanto Gram-positiva quanto negativas, portadoras de outros mecanismos de resistência e verificar a ação das chalconas com outras classes de antibiótico.

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