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THIAGO SAMPAIO DE FREITAS

SÍNTESE, ESPECTROSCOPIA, ESTUDOS *In Vitro* E *In Silico* DE ATIVIDADE ANTIBIÓTICA E INIBIÇÃO DE BOMBA DE EFLUXO POR CHALCONAS DERIVADAS DE 2-HIDROXI-3,4,6-TRIMETOXIACETOFENONA, ISOLADA DE *Croton anisodontus* Müll.Arg.

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

Orientador: Prof. Dr. Alexandre Magno Rodrigues Teixeira
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Dedico este trabalho a todas as pessoas que direta ou indiretamente contribuíram na minha formação acadêmica.

“Precisamos vencer a fome, a miséria e a exclusão social. Nossa guerra não é para matar ninguém - é para salvar vidas”.

Luiz Inácio Lula da Silva

RESUMO

A resistência bacteriana aos antibióticos de uso clínico é um grave problema de saúde pública em todo o mundo. A inibição dos diversos mecanismos de resistência bacteriana por meio da combinação de compostos ativos a fármacos, mostrou-se uma estratégia efetiva para a recuperação da eficácia dos antibióticos. Entre os possíveis alvos desses adjuvantes, destacam-se as bombas de efluxo. Neste trabalho, 4 chalconas sintetizadas a partir do composto 2-hidroxi-3,4,6-trimetoxiacetofenona, isolado da casca do caule de *Croton anisodontus*, foram testadas quanto a suas propriedades antibióticas, bem como seu uso como adjuvante na antibioticoterapia contra cepas resistentes. Técnicas de Ressonância Magnética Nuclear e Espectroscopia de Infravermelho foram utilizadas para confirmar as chalconas. Estudo *in silico* baseado nas propriedades físico-químicas dos compostos predisseram as propriedades de Absorção, Distribuição, Metabolismo, Excreção e Toxicidade (ADMET) das chalconas. A metodologia de diluição em caldo, utilizando placas de microdiluição, foi empregada nos testes microbiológicos. No teste de Concentração Inibitória Mínima, placas contendo meio nutritivo com inóculo foram microdiluídas com as chalconas em concentrações que variaram de 512 µg/mL a 8 µg/mL. No modelo de adjuvante, as chalconas foram adicionadas em sua concentração subinibitória ao meio com inóculo e as placas foram microdiluídas com antibióticos. Para o teste de inibição de bombas de efluxo, as chalconas e inibidores padrões de bombas de efluxo foram adicionados em concentrações subinibitórias ao meio nutritivo e as placas foram microdiluídas com Brometo de Etídio, substrato para as bombas de efluxo. As concentrações dos antibióticos e do Brometo de Etídio variaram de 512 µg/mL a 0,5 µg/mL. Dados de Infravermelho confirmaram a presença do grupamento C=O e da dupla ligação nas bandas de 1631 e 1559 cm^{-1}, respectivamente. As espectroscopias de ressonância magnética nuclear do Carbono e do Hidrogênio confirmaram a presença da α-β insaturação, com uma constante de acoplamento (J = 15.5 Hz), confirmando a estereoquímica trans. Notadamente, as chalconas não apresentaram atividade antibiótica relevante contra nenhuma cepa testada. No modelo de adjuvante, as chalconas conseguiram reverter a resistência bacteriana para os antibióticos ciprofloxacina e gentamicina na cepa de *Staphylococcus aureus* e para o antibiótico gentamicina nas cepas de *Escherichia coli* e *Pseudomonas aeruginosa*. As chalconas também foram capazes de inibir as bombas de efluxo NorA e MepA para o substrato Brometo de Etídio e fluoroquinolonas. Diferenças entre o efeito adjuvante das chalconas foram relacionadas aos substituintes, bem como a posição desses substituintes na molécula, sendo o melhor efeito obtido pela Chalcona 3 (átomo de Cloro na posição 4 do anel B). As Chalconas 1 e 2 apresentaram melhor

capacidade de absorção e distribuição no organismo. Todas as chalconas apresentam permeação moderada da barreira sangue-cérebro, significando uma absorção moderada das chalconas pelo Sistema Nervoso Central. Quanto ao metabolismo e excreção, elas interagem com as enzimas do citocromo P450 e apresentam uma eliminação lenta via renal. Nenhuma chalcona apresentou risco cardiogênico, porém, os testes predisseram um efeito mutagênico sobre células humanas para as chalconas 1 e 2. A Chalcona 4 foi a única dentre as chalconas testadas a demonstrar-se como substrato e não-inibidora da enzima 3A4 do complexo citocromo P450, não apresentando desta forma, risco de hepatotoxicidade por ativação metabólica. Em Conclusão, as chalconas funcionaram como modificadoras dos mecanismos de resistência bacteriana em modelos Gram-positivos e Gram-negativos, incluindo inibição de bombas de efluxo. Os efeitos antibacterianos, bem como as propriedades farmacocinéticas dependem dos substituintes presentes na molécula, bem como de sua posição na mesma.

Palavras-chave: Flavonoide; Resistência bacteriana; Bomba de efluxo; Espectroscopia; ADMET

ABSTRACT

Bacterial resistance to clinically used antibiotics is a serious public health problem worldwide. Inhibition of the different mechanisms of bacterial resistance through the combination of active compounds with drugs, proved to be an effective strategy for recovering the effectiveness of antibiotics. Among the possible targets of these adjuvants, efflux pumps stand out. In this work, 4 synthesized chalcones from the 2-hydroxy-3,4,6-trimethoxyacetophenone compound, isolated from the stem bark of *Croton anisodontus* were tested for their antibiotic properties as well as their use as an adjuvant in antibiotic therapy against resistant strains. Nuclear Magnetic Resonance and Infrared Spectroscopy techniques were used to confirm the chalcones. *In silico* study based on the physicochemical properties of the compounds predicted the Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties of the chalcones. The broth dilution methodology, using microdilution plates, was used in the microbiological tests. In the Minimum Inhibitory Concentration test, plates containing nutrient medium with inoculum were microdiluted with the chalcones in concentrations ranging from 512 µg/mL to 8 µg/mL. In the adjuvant model, chalcones were added in their subinhibitory concentration to the medium with inoculum and the plates were microdiluted with antibiotics. For the efflux pump inhibition test, the chalcones and standard efflux pump inhibitors were added in sub-inhibitory concentrations to the nutrient medium and the plates were microdiluted with Ethidium Bromide, substrate for the efflux pumps. The concentrations of antibiotics and Ethidium Bromide ranged from 512 µg/mL to 0.5 µg/mL. Infrared data confirmed the presence of the C=O group and the double bond in the 1631 and 1559 cm-1 bands, respectively. Carbon and Hydrogen nuclear magnetic resonance spectroscopy confirmed the presence of α-β unsaturation, with a coupling constant (J = 15.5 Hz), confirming the trans stereochemistry. Notably, chalcones did not show relevant antibiotic activity against any strain tested. In the adjuvant model, chalcones were able to reverse bacterial resistance to the antibiotics ciprofloxacin and gentamicin in the *Staphylococcus aureus* strain and to the antibiotic gentamicin in the *Escherichia coli* and *Pseudomonas aeruginosa* strains. The chalcones were also able to inhibit the NorA and MepA efflux pumps for the substrate Ethidium Bromide and fluoroquinolones. Differences between the adjuvant effect of chalcones were related to the substituents, as well as the position of these substituents in the molecule, with the best effect being obtained by Chalcone 3 (Chlorine substituent at position 4 of the B ring). Chalcones 1 and 2 showed better absorption and distribution capacity in the body. All chalcones show moderate permeation of the blood-brain barrier, meaning a moderate absorption of the chalcones by the Central Nervous System. As

for metabolism and excretion, they interact with cytochrome P450 enzymes and have a slow elimination via the kidneys. No chalcones presented a cardiogenic risk, however, tests predicted a mutagenic effect on human cells for chalcones 1 and 2. Chalcone 4 was the only one among the tested chalcones to demonstrate itself as a substrate and non-inhibitor of the 3A4 enzyme of the cytochrome P450 complex, thus not presenting a risk of hepatotoxicity by metabolic activation. In conclusion, chalcones functioned as modifiers of bacterial resistance mechanisms in gram-positive and gram-negative models, including inhibition of efflux pumps. The antibacterial effects as well as the pharmacokinetic properties depend on the substituents present in the molecule, as well as on its position in it.

Keywords: Flavonoid; bacterial resistance; efflux pump; spectroscopy; ADMET

IMPORTÂNCIA/RELEVÂNCIA PARA A SOCIEDADE

Chalconas apresentarem um largo espectro de atividade farmacológica e uma variedade de possibilidades para construção de novas moléculas, seja por troca de átomos, e/ou grupos funcionais, e/ou por prolongamento da cadeia enona, e/ou por alteração nos seus anéis aromáticos. Chalconas naturais são encontradas no caule, na folha, na casca e na raiz de uma variedade de árvores e plantas e também ocorrem em maior concentração nos pigmentos de pétalas, levando-os a ter um papel importante na polinização das plantas. Há séculos que as populações humanas utilizam produtos naturais para combater infecções. Chás, raizadas e partes das plantas *in natura* são utilizadas para isto. Mas será que substâncias sintéticas derivadas de plantas teriam um efeito combativo a infecções, sem causar danos aos usuários? A resposta está neste trabalho. Veremos ao final da leitura desta tese, que chalconas derivadas de 2-hidroxi3,4,6-trimetoxiacetofenona isolada das folhas de *Croton anisodontus* Müll.Arg. são capazes de aumentar o efeito de antibióticos para combater bactérias resistentes, o que torna esta pesquisa bastante relevante, visto que alguns antibióticos já não são eficazes contra infecções causadas por bactérias multirresistentes, e que a Organização Mundial de Saúde (OMS) estima que todos os antibióticos atuais perderão eficácia em 2050, as chalconas derivadas de 2-hidroxi3,4,6-trimetoxiacetofenona poderão ser uma alternativa no desenvolvimento de novos antibacterianos.

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ARTIGO 1 - Direct antibacterial and antibiotic resistance modulatory activity of chalcones synthesized from the natural product 2-hydroxy-3,4,6-trimethoxyacetophenone

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LISTA DE ABREVIATURAS E SIGLAS

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"
AMES	Teste de Ames (Bruce Ames e colaboradores) para detecção de mutagenicidade
ANOVA	Análise de Variância
ATCC	American Type Culture Collection
ATP	Adenosina Trifosfato
ATR-FTIR	Attenuated Total Reflection Fourier Transform Infrared
BBB	Blood-Brain Barrier
BHI	Brain Heart Infusion
CCCP	Carbonyl Cyanide 3-Chlorophenylhydrazone
CFU	Colony Forming Unit
CIM (MIC)	Concentração Inibitória Mínima
CMC	Comprehensive Medicinal Chemistry
CNS	Central Nervous System
CPMZ (CPZ)	Clorpromazina
DMSO	Dimethyl Sulfoxide
EB (EtBr)	Ethidium Bromide
EC	*Escherichia coli*
EPI	Efflux Pump Inhibitors
EP	Efflux Pumps
hERG	human Ether-à-go-go-Related Gene
HIA	Human Intestinal Absorption
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
MepA	Gene que expressa a proteína de efluxo de mesmo nome
MepR	Gene regulador de MepA
MFS	"Superfamília de Facilitadores Principais"
MR	Molar Refractivity

MW	Molecular Weight
NBD	Nucleotide Binding Domain
NCTC	National Collection Type Culture
NMR	Nuclear Magnetic Resonance
NorA	Gene que codifica a proteína de efluxo de mesmo nome
PA	*Pseudomonas aeruginosa*
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"
SUB I	Concentração Sub-inibitória

SUMÁRIO

INTRODUÇÃO GERAL

1.1 OBJETIVOS E QUESTIONAMENTOS

A motivação para abordar essa temática no projeto de tese surgiu ainda no mestrado, no tocante a minha área de trabalho. Sou enfermeiro de formação e quando fui prestar seleção para o mestrado de Bioprospecção Molecular procurei a área mais próxima ao meu trabalho cotidiano que era a microbiologia. Comecei a pesquisar com produtos naturais, (extratos, óleos essenciais e compostos isolados) no combate à resistência bacteriana e, no decorrer do curso, pude perceber a real importância destes compostos como fonte de novos medicamentos, diante da problemática da resistência bacteriana.

A descoberta dos antibióticos foi um marco crucial para o tratamento de doenças infectocontagiosas que assolavam a saúde humana, porém, juntamente com essas drogas milagrosas, houve o aparecimento de cepas bacterianas que apresentavam resistência intrínseca ou adquirida aos mesmos, mesmo com o desenvolvimento de diversas classes de antibióticos ao longo dos anos (LI; WEBSTER, 2018). Nos últimos anos, cepas bacterianas resistentes trouxeram uma realidade perturbadora em escala global (CHRISTAKI; MARCOU; TOFARIDES, 2020).

O mundo natural ainda nos fornece material abundante para a prospecção de moléculas bioativas com capacidade antibacteriana e os avanços em diversas áreas da biologia molecular nos permite criar um banco de dados com tais substâncias, estruturas e efeitos nas células bacterianas (JACKSON; CZAPLEWSKI; PIDDOCK, 2018).

Ainda durante o mestrado, aprendi que as substâncias presentes nos produtos naturais podem não ser utilizadas como estão nos extratos e óleos essenciais, mas servindo como modelo para a síntese de novas estruturas capazes de atuar na quebra da resistência antibacteriana.

Os diversos constituintes isolados de produtos naturais fornecem uma variedade de estruturas químicas, com atividade biológica, capazes de serem sintetizadas, servindo como molde para novos antibióticos (ATANASOV *et al*., 2021).

Durante a seleção do doutorado entrei para a área de síntese e caracterização dos compostos, fazendo com que eu tivesse um novo olhar quanto a relação entre a estrutura dos compostos e seus efeitos biológicos. O trabalho com Chalconas surgiu a partir do meu

orientador e coorientadores, que tiveram a ideia de sintetizar as chalconas a partir do composto natural 2-hidroxi-3,4,6-trimetoxiacetofenona, extraída da casca do caule de Croton anisodontus, e modificar sua estrutura.

Dentre numerosos compostos derivados de fontes naturais, as chalconas apresentam-se como fortes candidatas a modelos estruturais de fármacos. Possuem, genericamente, uma estrutura formada por dois anéis aromáticos ligados por uma cadeia enona (Cβ=Cα-C=O) que contém um grupo funcional cetona e a ligação dupla Cβ=Cα. A estrutura molecular representativa de uma chalcona é comumente denominada por 1,3-difenil-2-propen-1-ona (ZHUANG *et al*., 2017). Esta estrutura fornece um arcabouço propício para derivações, através das quais surgem compostos com bioatividades seletivas (CHOWRASIA *et al*., 2016). Graças às possibilidades de mudança em sua estrutura, as chalconas podem adquirir diversas propriedades biológicas, que incluem atividade anticâncer (GAO; HUANG; XIAO, 2020; NGAMENI *et al*., 2021), antimalária (QIN *et al*., 2020; CHENG *et al*., 2020), anti-inflamatória (WANG *et al*., 2019; MAHAPATRA; BHARTI; ASATI, 2017), antioxidante (GUAZELLI *et al*., 2021), antibacteriana (DAN; DAI, 2020; XU *et al*., 2019) e antifúngica (LAGU *et al*., 2020; AHMAD *et al*., 2017).

A partir desta narrativa, explicitamos o porquê da utilização das chalconas neste trabalho: As chalconas são compostos com estruturas simples e moldáveis e que apresentam numerosas atividades biológicas. Desta forma, foram sintetizadas quatro chalconas derivadas de 2-hidroxi3,4,6-trimetoxiacetofenona isolada de *Croton anisodontus* Müll.Arg., que divergem estruturalmente na posição 2 e 4 do anel B, que nos propiciou fazermos observações variadas sobre os átomos substituintes, bem como à própria estrutura básica.

A literatura já reporta que chalconas com os substituintes cloro e flúor, possuem atividades antibacterianas (BEHESHTI *et al*., 2021; DU *et al*., 2021; NASKER *et al*., 2018; ZHENG *et al*., 2021) e seus efeitos são intrinsecamente dependentes de sua estrutura molecular (VO, *et al*., 2019). Desta forma, poderíamos observar se a estrutura das chalconas derivadas de 2-hidroxi3,4,6-trimetoxiacetofenona nos propiciaria um modelo que pudesse ser usado futuramente para o desenvolvimento de um composto antibacteriano e as possíveis interferências dos substituintes.

As chalconas utilizadas nesta tese foram: 1) (*E*) -3- (2-fluorofenil) -1- (2-hidroxi-3,4,6- trimetoxifenil) prop-2-em-1-ona; 2) (*E*) -3- (4-clorofenil) -1- (2-hidroxi-3,4 , 6-trimetoxifenil) prop-2-em-1-ona; 3) (*E*) -3- (4-fluorofenil) -1- (2- hidroxi-3,4,6-trimetoxifenil)

prop-2-em-1-ona e 4) (*E*) -3- (2,4-diclorofenil) -1- (2-hidroxi-3,4,6-trimetoxifenil) prop-2-em-1-ona.

As perguntas-chave para nossa tese foram:

i) As chalconas derivadas de 2-hidroxi3,4,6-trimetoxiacetofenona isolada de *Croton anisodontus* apresentam atividades antibacterianas? A partir deste questionamento buscamos verificar se as chalconas apresentariam atividade antibacteriana intrínseca, ou seja, se elas conseguiriam inibir o crescimento de cepas bacterianas.

ii) Se as chalconas derivadas de 2-hidroxi3,4,6-trimetoxiacetofenona isolada de *Croton anisodontus* não tiverem atividade antibacteriana, poderiam elas ser utilizadas como adjuvantes no tratamento de infecções? Sabendo que diversas substância não apresentam atividade antibacteriana intrínseca, mas conseguem reverter a resistência bacteriana aos antibióticos, nos propomos a verificar uma possível utilização das chalconas como adjuvantes de antibióticos.

iii) Qual o provável mecanismo pelo qual estas chalconas atuam sobre as bactérias? Uma vez que as chalconas apresentassem a capacidade de atuarem como adjuvantes, nos propomos a investigar o mecanismo de resistência bacteriano de extrusão antibiótica, conhecido como bomba de efluxo.

iv) Qual a relação entre os diversos substituintes presentes nas chalconas e sua atividade antibacteriana, ou a ausência desta? Este questionamento tratou-se de entender a relação entre os átomos substituintes e capacidade de reversão da resistência bacteriana, para a qual poderia obter melhor resultado ou se alguma(s) dela(s) anulariam ou teriam um efeito adjuvante.

1.2 ESTRATÉGIAS DE PESQUISA

Para tentar responder a primeira pergunta-chave (hipótese) a respeito da atividade antibacteriana intrínseca do composto, utilizamos a metodologia proposta por Javadpour e colaboradores (1996) adaptada. As adaptações necessárias foram feitas baseadas na metodologia proposta na CLSI (2013). Volumes de inóculos e proporções entre meio e a substância a ser diluída foram obtidas da CLSI, enquanto a ordem de preenchimento da placa, bem como a forma de microdiluição foi obtida de Javadpour. Desta forma, poderíamos aqui falar de CLSI adaptada.

Essas adaptações buscaram otimizar o método, de modo a trabalhar com a menor quantidade de substância possível, obtendo resultados confiáveis e que não extrapolassem o preconizado pelas agências que regulam tais testes. Com isso, diminuímos quantidade de substância necessária ao teste, diminuímos o risco de contaminação das amostras e otimizamos o tempo necessário à realização do teste, tudo de acordo com o que dispomos em termos de métodos e estruturas laboratoriais.

A metodologia de Javadpour (1996) é uma metodologia já consagrada na área de microbiologia. Nosso grupo de pesquisas já utiliza esta metodologia há mais de 13 anos. O professor Dr. Henrique Douglas Melo Coutinho, coordenador do Laboratório de Microbiologia e Biologia Molecular da URCA estabeleceu as bases fundamentais do uso de metodologias para ensaios de atividade antibacteriana (COUTINHO *et al*., 2008), de acordo com os seguintes pressupostos: i) As metodologias não devem ser trocadas de forma contínua (a menos que apresentem erros identificados ou substituídas por uma claramente superior no uso), a fim de não gerar dúvidas quanto a obtenção dos resultados. ii) Além da fácil aplicação, ela também é de simples reprodução, um dos requisitos necessários para a comprovação dos resultados dos testes em qualquer trabalho científico publicável.

Para testarmos os efeitos das chalconas como adjuvante, utilizamos a técnica desenvolvida por Coutinho e colaboradores (COUTINHO *et al*., 2008). Nesta metodologia, a substância é colocada em todos os poços da placa de microdiluição em uma concentração sub-inibitória correspondente a CIM/8 do valor obtido no teste de efeito intrínseco. Neste teste, a intenção é que as chalconas não exerçam nenhuma atividade antibacteriana direta, apenas haja em alvos de resistência, inibindo-os e permitindo que o antibiótico exerça seu efeito.

Um dos questionamentos mais levantados a respeito desta metodologia é o porquê da utilização do valor CIM/8. A resposta dada por Coutinho e colaboradores (COUTINHO *et al*., 2008) é que este valor foi obtido de forma experimental. Segundo ele, o valor CIM/4 não era garantidor de que as substâncias testadas não estivessem exercendo um efeito direto e a CIM/16 resultava em valores iguais ao controle, o que poderia ser indícios de um efeito placebo.

As metodologias acima mencionadas estão bem consolidadas dentro do nosso grupo de estudo, bem como por pesquisadores de outros laboratórios parceiros localizados em Fortaleza (MORAIS *et al*., 2017), Pernambuco (QUEIROZ DIAS *et al*., 2019) e Piauí (LEAL *et al*. 2019).

Para testar os mecanismos de resistência nos quais as chalconas agem, elas foram testadas com bactérias que expressam bombas de efluxo. A técnica usada é basicamente a mesma metodologia da reversão de resistência descrita acima, com a diferença que os controles são produzidos com a adição de inibidores padrões de bomba de efluxo como a clorpromazina e o Carbonyl Cyanide 3-Chlorophenylhydrazone (CCCP), a fim de possibilitar comparações. Essa metodologia é complementada com estudos de docagem molecular para avaliar em quais resíduos da proteína há a interação com as cepas portadoras de bombas de efluxo, e também por estudos das propriedades ADMET (absorção, distribuição, metabolismo, excreção e toxidade) para avaliar o comportamente farmacocinético das chalconas testadas. Esses estudos *in sílico* (docagem molecular e ADMET) são bem conhecidos na literatura e já vem sendo usados pelo nosso grupo de pesquisa (MUNIZ *et al*., 2021; SANTOS *et al*., 2018; XAVIER, *et al*., 2021).

1.3 ESTRUTURA DA TESE

Esta tese está organizada em Introdução Geral (Dividida em: Objetivos e questionamentos, Estratégias de pesquisa e Estrutura da tese) e mais quatro capítulos (Fundamentação teórica, 1º. Artigo publicado, 2º. Artigo publicado e Considerações finais), seguido pelo apêndice.

- **Capítulo 1:** Fundamentação Teórica. Este capítulo foi dividido em 6 tópicos:
    1. Chalconas: Neste capítulo é descrito as características gerais das chalconas, sua estrutura e a reação mais utilizada para sua síntese;
    2. Resistência bacteriana: Neste capítulo é descrito os mecanismos gerais de resistência bacteriana, intrínsecos e adquiridos, e uma breve descrição da resistência em duas cepas bacterianas de modelos distintos (Gram-positivo e Gram-negativo);
    3. Resistência mediada por bombas de efluxo: Descrição das principais famílias de bombas de efluxo, suas estruturas e fontes de energia e a importância dos inibidores de bombas para a reversão da resistência bacteriana;
    4. Proteínas de efluxo NorA e MepA: Compilação dos trabalhos que descreveram estas duas proteínas de efluxo, sua estrutura, fonte de energia, nível de resistência induzida na bactéria mutante e diferenças para a cepa selvagem parental.

5. Inibidores de Bombas: Retrata, de forma geral, as interações dos inibidores com as bombas de efluxo, bem como os mecanismos de ação dos Inibidores-Padrão utilizados nesta tese: carbonil cianida m-clorofenilhidrazona (CCCP) e Clorpromazina.
6. Processo ADMET em Pesquisas: Breve explicação dos processos de absorção, distribuição, metabolismo, excreção e toxicidade no organismo humano e de modelos de predição ADMET em estudos pré-clínicos.

- **Capítulo 2:** Artigo publicado na revista: *FEMS Microbiology Letters*, 2020, Vol. 367, No. 15; FATOR DE IMPACTO 1,987 – QUALIS B2: Artigo mostrando a caracterização estrutural das 4 chalconas sintéticas e o efeito delas sobre cepas multirresistentes de *Staphylococcus aureus*, *Escherichia coli* e *Pseudomonas aeruginosa*, relacionando o efeito com o substituinte e posição do mesmo.
- **Capítulo 3:** Artigo publicado na revista: Microbial Pathogenesis, 2021, Vol. 161, Parte B; FATOR DE IMPACTO 2,914 – ISSN 0882-4010 – QUALIS B2: Segundo artigo desta tese, nele foi dado um passo a mais sobre os mecanismos de ação das chalconas, testando-as em cepas portadoras de bombas de efluxo. Neste artigo também foi realizado docagem molecular e estudos ADMET das chalconas;
- **Capítulo 4**: Considerações finais: Este capítulo foi dividido em três subtópicos (Discussão geral, Conclusões gerais e Perspectivas de investigações futuras).
- **Apêndice 1**: Material Suplementar do 1º. Artigo, publicado na *FEMS Microbiology Letters*.
- **Apêndice 2**: Material Suplementar do 2º. Artigo, publicado na Microbial Pathogenesis.
- **Apêndice 3**: Produções científicas. Nesta sessão da tese estão listadas todas as produções científicas realizadas durante o Curso de Pós-Graduação *Strictu sensu* em Química Biológica, nível doutorado. Foram 63 publicações ao longo do doutorado, sendo 02 artigos relacionados com a tese, 60 artigos não relacionados e 01 capítulo de livro publicado não relacionado com a tese.

CAPÍTULO 1: FUNDAMENTAÇÃO TEÓRICA

1.1 CHALCONAS

1.1.1 ASPECTOS GERAIS

Chalconas são compostos fenólicos, caracterizados quimicamente como cetonas aromáticas, presentes em uma ampla gama de vegetais, que participam da biossíntese de flavonoides e isoflavonoides, sendo consideradas seus precursores (RAMMOHAN *et al*.,2020). Sua cor é decorrente do grupo cetoetilênico, bem como de outros possíveis cromóforos presentes em sua estrutura (GAONKAR; VIGNESH, 2017). Possuem uma coloração amarelada, tendendo ao bronze, característica esta, que deu origem ao seu nome, derivado da palavra grega "chalcos" (NURKENOV *et al*., 2018; SIMÕES *et al*., 2017). São encontradas em diversas partes comestíveis de plantas, como frutas, sementes, folhas e raízes (MAZZONE *et al*., 2015).

Sua estrutura química simples permite a síntese de compostos derivados, capazes de interagir em numerosos alvos, gerando grandes interesses farmacológicos (MAHAPATRA; BHARTI; ASATI, 2017). Seja na sua forma natural ou sintética, exibem importantes bioatividades já registradas na literatura (KANT *et al*., 2016). Dentre as atividades citadas, são referidos efeitos antimaláricos (SINHA *et al*., 2019; SHARMA, 2015; SYAHRI *et al*., 2017), anticâncer (KOCYIGIT, 2018), anti-inflamatório (REDDY *et al*., 2017; LI *et al*., 2017), antioxidante (ZAINURI *et al*., 2017), antiprotozoário (ZHEOAT *et al*., 2021; BETECK *et al*., 2019), anti-HIV (TURKOVIC *et al*., 2020; ELKHALIFA *et al*., 2020), antibacteriano (KOUDOKPON *et al*., 2018) e antifúngico (MELLADO *et al*., 2019; GUPTA; JAIN, 2015).

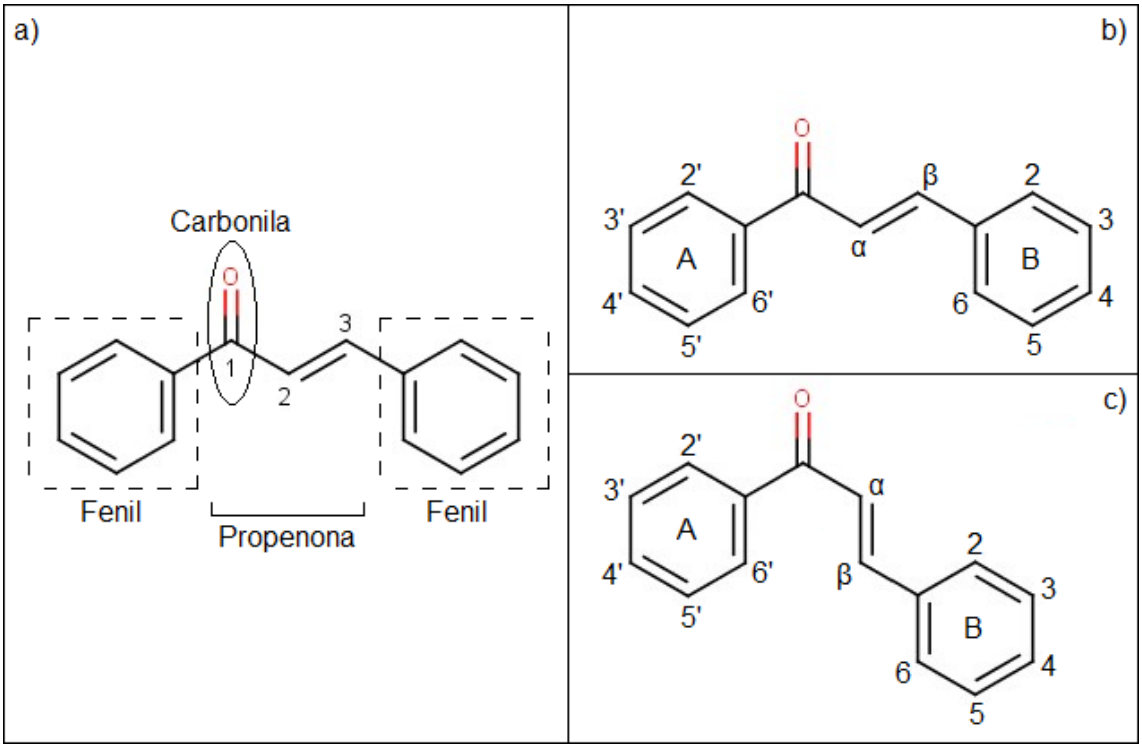
1.1.2 ESTRUTURA

O grande espectro de atividades farmacológicas das chalconas deve-se as diferentes possíveis estruturas moleculares que as chalconas podem possuir, seja por alterações no tipo de átomo presente, grupos funcionais, ou mesmo de seus anéis aromáticos que conectam com a cadeia enona (CHOI *et al*., 2018). Essas modificações também podem ser reponsáveis pelo

aumento de uma atividade biológica prévia de estruturas-modelo (MAZZONE *et al*., 2015; ZAIDI *et al*., 2015).

Como pode ser observado na Figura 1, a estrutura de uma chalcona é composta por dois radicais fenis ligados a uma propenona, que contém uma porção carbonila e uma ligação de carbonos α, β insaturados, apresentando fórmula química geral 1,3-difenil-2-propen-1-ona (EKBOTE *et al*., 2017).

Figura 1: Estrutura Geral das Chalconas



Fonte: Autor. Produzido com o programa MarvinSketch 17.13. 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.

As estruturas básicas das chalconas proporciona estereoisomeria, apresentando-se nas formas *Cis* e *Trans*, sendo o isômero *Trans*, o mais estável termodinamicamente (ZHUANG *et al*., 2017).

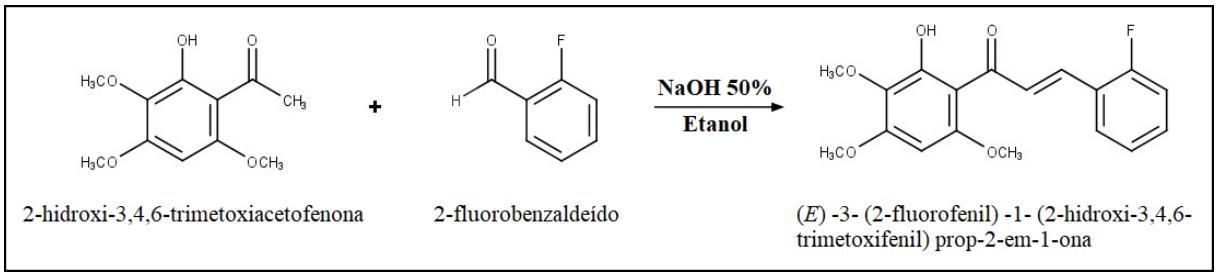
As estruturas simples das chalconas permite modificações nos substituintes de ambos anéis aromáticos, conduzindo a alterações nos padrões físico-químicos das moléculas (NAIR *et al*., 2018) e por consequência, em suas bioatividades, podendo ocorrer redução ou ampliação de uma propriedade bioativa (DE MELLO *et al*., 2018). Outra parte da molécula essencial para que ela desempenhe suas funções, está na sua porção substiutinte dos anéis fenis, na qual, a presença da insaturação, juntamente com o grupamento cetona, torna-se

essencial para a versatilidade de suas atividades biológicas (SAHU *et al*., 2012; SINGH; ANAND; KUMAR, 2014; GUPTA; JAIN, 2015).

1.1.3 SÍNTESE DE CHALCONAS

As chalconas são sintetizadas através de numerosas técnicas de condensação básica ou ácida, dentre as quais, a reação de Claisen-Schmidt é a mais usada (Figura 2). Esta reação pode ser feita por catálise ácida ou básica, através, respectivamente, de um mecanismo enol ou aldol, entre cetonas e aldeídos, tais como derivados da acetofenona e o benzaldeído (JIOUI *et al*., 2016; WINTER *et al*., 2016; ZHUANG *et al*., 2017).

Figura 2: Reação de Claisen-Schmidt



Fonte: Autor. Produzido com o programa MarvinSketch 17.13

Classicamente, esta reação ocorre pelo uso de catalisadores básicos homogêneos, como o hidróxido de sódio, hidróxido de potássio e o hidróxido de bário, na presença de solventes como metanol ou etanol (JIOUI *et al*., 2016; ZHUANG *et al*., 2017).

1.2 RESISTÊNCIA BACTERIANA

O surgimento da resistência bacteriana é um problema que afeta a saúde pública no mundo inteiro. Os mecanismos de resistência podem surgir de forma intrínseca através de mutações ou serem adicionados a uma cepa através da transferência horizontal de genes a partir de reservatórios genéticos, que podem ou não ser bactérias patogênicas (PETERSON; KAUR, 2018).

Mecanismos intrínsecos diferenciam-se dos adquiridos por não serem específicos para um agente agressor, enquanto os adquiridos são geralmente específicos (VAN DUIJKEREN *et al*., 2018). Também se diferem pela sua localização, geralmente no cromossomo central

procarioto para os intrínsecos, enquanto os extrínsecos estão situados em nível de plasmídeos, o que facilita sua dispersão (PETERSON; KAUR, 2018).

Além de fatores biológicos, diversos são os condicionantes responsáveis pela ampliação da resistência bacteriana, tais como o uso indevido de antibióticos, seja na saúde humana ou nos sistemas de produção agropecuários, o encurtamento de distâncias em um mundo globalizado e lacunas no conhecimento do tema (DADGOSTAR, 2019).

A carga dessa resistência possui efeitos multicêntricos, se estabelecendo sobre o paciente acometido, com resultados que variam de maior tempo de internação à morte (TILLOTSON; ZINNER, 2017); impactos sobre o sistema de saúde no que se refere ao aumento do número de internações com sepse (GOLDSTEIN *et al*., 2019) e a diminuição do arsenal de drogas de uso clínico pelo estabelecimento de resistência a várias classes de antibióticos (PAUL; ABDULATEEF, 2019); além de impactos econômicos que contribuem para a manutenção da pobreza em algumas partes do mundo (AHMAD; KHAN, 2019).

Em 2017 a Organização Mundial da Saúde publicou uma lista com 12 espécies de patógenos resistentes, prioritárias para a busca de novos antibióticos, devido sua associação com os impactos à saúde humana. Dentre os patógenos presentes nesta lista, destacam-se a *Pseudomonas aeruginosa* resistente a carbapenem e *Staphylococcus aureus* resistente à meticilina (WHO, 2017).

Staphylococcus aureus resistente à meticilina é uma cepa mundialmente preocupante, isolada em 72% das infecções por *Staphylococcus aureus* em ambiente hospitalar e que apresenta resistência cruzada a outros antibióticos (GAROY *et al*., 2019). Sua resistência à meticilina está relacionada a modificações nas proteínas de ancoragem do antibiótico (PBP – penicillin binding protein) reduzindo a afinidade ao sítio de ligação (CRAFT *et al*., 2019). É uma bactéria altamente adaptável, provocando epidemias sustentadas (LAKHUNDI; ZHANG, 2018) e altas taxas de morbi-mortalidade tanto nos ambientes hospitalares quanto em comunitários (KOURTIS *et al*., 2019).

Pseudomonas aeruginosa resistente a carbapenem é uma bactéria oportunista geralmente associada a infecções pulmonares (MUNITA *et al*., 2017). Sua resistência ao carbapenem advém de variados mecanismos, tais como a diminuição à permeabilidade das drogas através de alterações em porinas e mecanismos de efluxo, bem como da ativação de enzimas β-lactamases, codificados no cromossomo central ou em elementos móveis, aumentando com isso a preocupação quanto à disseminação destes mecanismos (WALTERS

et al., 2019). Resistência cruzada à aminoglicosídeos e fluoroquinolonas é uma realidade entre as cepas resistentes de *Pseudomonas aeruginosa* devido aos mecanismos variados (ROSTAMI *et al*., 2018).

A depender da estrutura da molécula do antibiótico e seu mecanismo de ação, as bactérias possuem um arsenal de mecanismos de resistência, tais como modificações na membrana, tornando-se menos permeável através de porinas de difusão geral ou por via mediada por lipídeos (GHAI; GHAI, 2018), modificação da molécula alvo, como a modificação da Serina no sítio de ligação da topoisomerase (PHAM; ZIORA; BLASKOVICH, 2019), inativação dos antibióticos através de enzimas que os degradam ou que os modificam, como no caso das várias enzimas β-Lactâmicas (LIMA *et al*., 2020) e enzimas acetiladoras (BURCKHARDT; ESCALANTE-SEMERENA, 2020) e mecanismos de efluxo, através de proteínas transmembranares que capturam o antibiótico intracelular e os extraem para o meio externo (KAPOOR; SAIGAL; ELONGAVAN, 2017).

1.3 RESISTÊNCIA MEDIADA POR BOMBAS DE EFLUXO

Bombas de efluxo são estruturas presentes em quase todos os organismos vivos, sejam eles eucariotos ou procariotos (KANJI; HASAN; HASAN, 2019). Tratam-se de proteínas transmembranares cuja principal função é retirar agentes agressores do interior celular e eliminá-los ao meio externo. Porém, elas possuem outras funções celulares como: comunicação célula a célula (ALCALDE-RICO *et al*. 2020), interação com outros organismos, incluindo organismos eucariotos pluricelulares (PASQUA *et al*., 2019) e associação com genes reguladores da virulência bacteriana (DU TOIT, 2017). Elas podem ser expressas em níveis basais ou ser superexpressas, em decorrência da presença de um agente agressor ou da seleção de um mutante (GRIMSEY *et al*., 2020a).

Como descrito anteriormente, bactérias multirresistentes apresentam bombas de efluxo capazes de diminuir a concentração antibiótica no interior celular, diminuindo sua suscetibilidade ao mesmo. Estas bombas de efluxo podem envolver mecanismos intrínsecos de mutantes selecionados, ou de mudanças fenotípicas causadas por um agente agressor de forma temporária. As bombas de efluxo que fazem parte do arsenal de resistência intrínseca de numerosas bactérias costumam ser expressas em níveis basais, enquanto as cepas mutantes podem superexpressar esse mecanismo de defesa (ALCALDE-RICO *et al*., 2016).

As bombas de efluxo podem ser divididas de acordo com sua constituição, força-motriz e atividades desempenhadas em seis classes distintas, a saber: ABC (Superfamília de Cassetes de Ligação de ATP), RND (Superfamília Resistência-Nodulação-Divisão); SMR (Família de Pequena Multidroga Resistência); MATE (Família de Extrusão de Multidrogas e Toxinas); MFS (Superfamília de Facilitadores Principais); PACE (Família de Efluxo de Composto Antimicrobiano Proteobacteriano) (DU *et al*., 2018). Em uma outra classificação mais abrangente, as bombas de efluxo podem ser divididas em primária ou secundária, de acordo com a energia utilizada para seu funcionamento (HASSANZADEH *et al*., 2020). As primárias utilizam-se diretamente da energia fornecida pela hidrólise de ATP (superfamília ABC), enquanto as secundárias utilizam-se dos gradientes de concentração de H^+ e Na^+, sendo chamadas de antiportadores ou trocadores (VARELA, 2019). Todas as outras famílias mencionadas anteriormente, com exceção da ABC, são antiportadores.

As bombas de efluxo podem ser geneticamente codificadas no cromossomo central bacteriano, como no caso das bombas de efluxo NorA (família MFS) e MepA (família MATE) ou codificadas no plasmídeo, como nas bombas MsrA (superfamília ABC), QacA e QacB (superfamília MFS) e QacC (família SMR) (HASSANZADEH *et al*., 2020).

Os transportadores ABC são proteínas transmembranares presentes em muitas formas de vida, desde organismos simples à multicelulares. Utilizam a energia do ATP para deslocar vários tipos de compostos, incluindo antibióticos, lipídeos, vitaminas e íons para o meio extracelular (THOMAS; TAMPÉ, 2018). A sua estrutura é marcada por duas regiões transmembranares responsáveis pelos deslocamentos das diversas substâncias e duas regiões responsáveis pela ligação do substrato, chamadas NBDs, cuja sequência de nucleotídeos é altamente preservada entre os domínios da árvore da vida (THOMAS; TAMPÉ, 2020).

As bombas de efluxo ABC estão relacionadas à resistência de células cancerígenas aos quimioterápicos (BASU *et al*., 2017); à doenças cardiovasculares devido o envolvimento em funções corporais relacionadas ao sistema; bem como, na farmacocinética de medicamentos (SCHUMACHER; BENNDORF, 2017). Elas estão implicadas na etiopatogenia da doença de Alzheimer (PEREIRA *et al*., 2018), e também são responsáveis por promover resistência em fungos (CIESLIK *et al*., 2020) e bactérias (SHIADEH *et al*., 2019; PÉREZ-VARELA *et al*., 2019).

As bombas de efluxo RND são proteínas transmembranares que atravessam as duas membranas presentes em bactérias Gram-negativas, expelindo uma ampla gama de compostos devido sua múltipla especificidade (VARGIU *et al*., 2018), sendo encontrada apenas neste

tipo de bactéria (ALMATAR *et al*., 2020). A sua capacidade de atravessar as duas membranas decorre de sua estrutura tripartida, formada por uma bomba na membrana interna e um canal na membra externa, unidos por uma proteína adaptadora no periplasma (COLCLOUGH *et al*., 2020). A presença dessas bombas está associada à resistência em cepas de *Escherichia coli* e *Pseudomonas aeruginosa* (PUZARI; CHETIA, 2017), *Acinetobacter baumannii* (LUCAßEN *et al*., 2021) e em cepas do gênero *Burkholderia* (SAROVICH *et al*., 2018; KRISHNAMOORTHY *et al*., 2019; PERRIN *et al*., 2018).

Bombas de efluxo da família MATE, assim como as ABC, estão presentes em bactérias e diversos eucariotos, transportando inúmeros compostos catiônicos e xenobióticos (CASTELLANO *et al*., 2021). Sua estrutura tem a forma de um "V", cujos braços são formados por várias hélices que atravessam a membrana, ficando aberta para o meio externo, enquanto fica fechada para o meio interno ou fechando para o meio externo, e abrindo para o meio interno. Sua atividade de transporte se dá pela mudança conformacional destas hélices, através de uma rede de pontes de hidrogênio (MIYAUCHI *et al*., 2017).

A estrutura das bombas de efluxo MFS, de uma forma geral, é composta por 12 hélices transmembranas, das quais oito formam uma cavidade e quatro formam uma estrutura responsável pela comunicação com o meio externo (SUN; DENG; YAN, 2014). As MFS são largamente distribuídas entre as bactérias e são responsáveis por mecanismos de virulência e interação com hospedeiros (PASQUA *et al*., 2019). Algumas bombas de efluxo pertencente a esta família são a NorA, NorB, NorC, MdeA, SdrM, LmrS, QacA e QacB, associadas a diversos organismos patogênicos (HASSANZADEH *et al*., 2020).

Como o próprio nome sugere, as SMR são um grupo de proteínas extrusoras estruturalmente pequenas, formadas por apenas quatro hélices transmembranas que formam uma estrutura com duas disposições equivalentes, mudando entre essas conformações para realizar a atividade de efluxo (DU *et al*., 2018). A presença destes transportadores está associada a *Pseudomonas aeruginosa* resistentes a quaternários de amônia (MITCHELL; STONE; DEBER, 2019).

As proteínas de efluxo PACE são uma família recém-descoberta, associadas a cepas resistentes de *Acinetobacter baumannii*, codificadas por uma sequência preservada de genes e que utilizam a energia de um gradiente eletroquímico de prótons (HASSAN *et al*., 2019). Possuem estrutura e tamanhos parecidos com as proteínas de efluxo SMR, com dois domínios transmembranares formados por quatro α-hélices, presentes em um grupo variado de Gram-

negativas (HASSAN *et al*., 2018), mas não são codificadas em *Escherichia coli* (DU *et al*., 2018).

1.4 PROTEÍNAS DE EFLUXO NorA E MepA

Diversas bombas de efluxo possuem importância clínica quanto à resistência em cepas de *Staphylococcus aureus*, podendo até mesmo coexistir genes para diferentes bombas importantes em uma mesma cepa bacteriana (HASSANZADEH *et al*., 2020). Dentre estas cepas, discutiremos a 1199B, portadora do gene NorA, e a K2068, portadora do gene MepA.

O gene NorA, presente no cromossomo de bactérias, codifica uma bomba de efluxo em cepas de *Staphylococcus aureus*, através da produção uma proteína transmembrana com domínios hidrofóbicos e uma região central hidrofílica, formada por 388 aminoácidos, responsável pelo efluxo de fluoroquinolonas, e que provavelmente teve uma origem natural no curso da evolução, apresentando homologia com proteínas de efluxo de diversas cepas, incluindo cepas Gram-negativas (KAATZ; SEO; RUBLE, 1993).

A variante 1199B apresenta uma maior resistência à fluoroquinolonas quando comparada a cepa 1199, sendo o aumento transcricional do gene NorA, o mecanismo responsável, constatado pelo aumento de RNA mensageiro e do produto proteico do gene, na cepa 1199B (KAATZ; SEO; RUBLE, 1993; KAATZ; SEO, 1995; KAATZ; SEO, 1997). Apesar de ocorrer um fenômeno de indução do aumento do transcrito, o mecanismo NorA na cepa 1199B é constitutivo (KAATZ; SEO, 1995).

Uma outra diferença entre as cepas 1199 e 1199B é a presença de uma mutação na sequência codificadora da Topoisomerase IV (substituição do aminoácido alanina na posição 116 pelo aminoácido ácido glutâmico), que é o alvo de ação principal das fluoroquinolonas e desta forma, uma mutação nesta enzima pode estar relacionada ao aumento da resistência nesta cepa (KAATZ; SEO, 1997).

A inibição da bomba de efluxo NorA mostra-se uma estratégia promissora para a reversão da resistência bacteriana à antibióticos de uso clínico, com estudos *in vitro* mostrando diversas substâncias capazes de promover este bloqueio, seja tais compostos, sintéticos ou naturais (MUNIZ *et al*. 2021), extratos ou substâncias isoladas (ESPINOZA *et al*., 2019; RIBEIRO *et al*., 2019; CRUZ *et al*., 2020). Pesquisas recentes mostram que as chalconas podem inibir o mecanismo de efluxo regulado pelo gene NorA (SILVA *et al*., 2021; REZENDE-JÚNIOR *et al*., 2020; SIQUEIRA *et al*., 2021).

Apesar da força-motriz das bombas MFS ser advinda dos gradientes de prótons e sua inibição ocorrer pelo bloqueio da força motriz por inibidores como a CCCP (KAATZ; SEO; RUBLE, 1993; KAATZ; SEO, 1995), um bloqueio competitivo pelas regiões de afinidade aos substratos, também pode ocorrer (KAATZ; SEO, 1997).

A cepa SA-K2068 é uma cepa de *Staphylococcus aureus* resistente à fluoroquinolonas que apresenta fenótipo diferenciado da 1199-B, comprovado por um perfil de substrato que, apesar de sobreposto, é diferente do perfil de substrato apresentado por outras cepas de *S. aureus*, incluindo portadoras de NorA (KAATZ; MOUDGAL; SEO, 2002).

Estudos revelaram que esta cepa não apresenta modificações em regiões codificadoras de resistência à quinolonas (*gyrA, grlA*, *gyrB* e *grlB*) e que a expressão de NorA é comparável a cepa selvagem parental, ou seja, não há superexpressão desse gene, o que significa que a bomba de efluxo MepA é responsável pelo fenótipo apresentado (KAATZ; MOUDGAL; SEO, 2002).

A proteína MepA é uma proteína transmembrana, composta por 12 segmentos, pertencentes a família MATE, codificada pelo Operon MepRAB. O MepR é um gene regulador MepA e possui sequências alteradas na cepa K-2068, quando comparada com a cepa parental, o que provavelmente explica a superexpressão de MepA na cepa K-2068, um vez que MepR trata-se de um gene repressor, sendo esta proteína de efluxo (MepA) responsável pelo fenótipo de resistência nesta linhagem (KAATZ; MCALEESE; SEO, 2005).

Estudo com mutantes que superexpressam MepA demonstra que as mutações favoráveis à melhoria da eficiência do efluxo ocorrem em resíduos localizados na porção terminal da proteína, em uma região próxima a face interna da membrana plasmática ou nas alças citoplasmáticas, sugerindo que esta região é a responsável pela captação dos substratos carregados pela bomba de efluxo e que estas mutações aumentam a afinidade pelo substrato ou favorecem sua translocação para o meio externo e desta forma, a captação dos substratos ocorrem ou na interface da membrana com o citoplasma, ou no interior da bicamada lipídica (SCHINDLER *et al*., 2013).

Assim como para as bactérias portadoras da bomba de efluxo NorA, uma estratégia promissora para a revitalização de antibióticos que perderam sua eficiência diante de cepas portadoras da bomba MepA, é o garimpo de substâncias que possam atuar na inibição do efluxo produzido por esta proteína, seja através da síntese de novas substâncias, seja através

de produtos extraídos de fontes naturais, que funcionem ativamente ou que sirvam como moldes estruturais para novos compostos (SCHINDLER; JACINTO; KAATZ, 2013).

Diversos estudos microbiológicos *in vitro* e *in silico* apontam as chalconas como inibidores eficazes de bombas MepA em cepas de *Staphylococcus aureus* K-2068 (REZENDE-JÚNIOR *et al*., 2020; SILVA *et al*., 2021; XAVIER, *et al*., 2021; ROCHA, J.E. *et al*., 2021).

1.5 INIBIDORES DE BOMBAS

Devido a seriedade destas proteínas na resistência de bactérias de importância clínica, a prospecção de inibidores de bombas de efluxo é uma estratégia importante no tratamento antibiótico.

Os inibidores de bombas de efluxo são uma importante estratégia no controle de fatores de resistência, agindo principalmente em cepas cuja expressão seja significante, atingindo numerosos mecanismos em diferentes tipos de bombas de efluxo (ALMATAR *et al*., 2020). Os inibidores podem exercer seus efeitos ao se ligarem com os antibióticos, reduzindo sua afinidade pelas bombas, bloqueando a força motriz das bombas, afetando a expressão gênica das mesmas, ligando-se às bombas de maneira competitiva ou não-competitiva e através de atividade tampão (AHMAD *et al*., 2019).

Um dos mecanismos de inibição mais afetados é o bloqueio da fonte energética da bomba de efluxo, como no caso da carbonil cianida m-clorofenilhidrazona (CCCP) que age inibindo bombas dependentes de gradientes de concentração de prótons. Uma outra forma de bloqueio decorre da ligação do inibidor ao sítio de ligação do antibiótico, agindo desta forma como inibidor competitivo (ALMATAR *et al*., 2020).

A clorpromazina é uma droga antipsicótica derivada da fenotiazina que penetra no sistema nervoso central, agindo nos receptores de dopamina (BOYD-KIMBALL *et al*., 2018). A clorpromazina é muito usada em testes microbiológicos de inibição de bombas de efluxo (GEORGE *et al*., 2019; DA SILVA *et al*., 2020; OLIVEIRA *et al*., 2021), agindo através dos dois mecanismos citados, inibindo a força motriz do gradiente de prótons (CHAN; ONG; CHUA, 2007), bem como através do mecanismo de inibição competitiva pelo sítio ativo do substrato (GRIMSEY *et al*., 2020b).

Alguns dos compostos sabidamente inibidores de bombas de efluxo acabam sendo tóxicos ao organismo humano e, por isso a síntese de novos compostos, ou a prospecção de produtos naturais com características não tóxicas aos nossos sistemas são imprescindíveis (RAO *et al*., 2018), sejam os produtos naturais na forma de extratos (LU *et al*., 2019; ANDRADE *et al*., 2020), estejam eles na forma de compostos isolados (REZENDE-JÚNIOR *et al*., 2020; SILVA *et al*., 2021).

1.6 PROCESSO ABSORÇÃO, DISTRIBUIÇÃO, METABOLISMO, EXCREÇÃO E TOXICIDADE (ADMET) EM PESQUISAS

Estudos ADMET (acrônimo para Absorção, Distribuição, Metabolismo, Excreção e Toxicidade) surgiram da necessidade de entender os processos farmacocinéticos das diversas drogas que utilizamos em nossos arsenais terapêuticos, compreender a interação entre suas propriedades físico-químicas e os componentes biológicos dos organismos e relacionar as doses e vias de administração dessas drogas com seus efeitos farmacológicos (TALEVI; QUIROGA, 2018). A incorporação de estudos ADMET tornou-se importante parte na descoberta de novas drogas, incluindo o item segurança aos testes (LUCAS *et al*., 2019).

As drogas podem ser administradas por várias vias, dentre elas, a oral, a intramuscular e a subcutânea e atingem a circulação sanguínea em um processo conhecido por absorção (BOLLEDDULA *et al*., 2022). A depender da via de administração, os fármacos passam por diversas barreiras até atingir o interior dos vasos sanguíneos ou a superfície-alvo e dependem de suas características físico-químicas para atravessarem essas barreiras e evitarem ser degradados (GHADIRI; YOUNG; TRAINI, 2019). Além de suas propriedades físico-químicas como solubilidade e constante de ionização, os fármacos também dependem da anatomia e da fisiologia do órgão-alvo para terem uma absorção devida (TALEVI; QUIROGA, 2018). Diversos modelos *In vivo* em ratos e outras espécies de mamíferos avaliam em etapas pré-clínicas a absorção de novas drogas no organismo, prevendo o comportamento no organismo humano (BOLLEDDULA *et al*., 2022).

Distribuição se refere ao movimento dos fármacos não metabolizados do sistema circulatório para os diversos tecidos do organismo, nos quais farão seu efeito (ONETTO; SHARIF, 2021). Os fármacos serão distribuídos diversificadamente nos diferentes tecidos que compõe o organismo e, desta forma, a distribuição dependerá de fatores relacionados ao fármaco, bem como ao tecido-alvo. Dentre os diversos fatores estão a lipofilicidade do

composto, sua ligação à proteínas específicas, o PH tecidual, bem como a presença ou ausência de barreiras naturais (VAN DEN ANKER *et al*., 2018).

Metabolizar uma droga significa transformar essa droga em produtos divergentes do absorvido, através de um complexo enzimático presente no nosso corpo, facilitando sua eliminação (ZHANG; TANG, 2018).

O metabolismo dos fármacos envolvem reações de biotransformações que podem ser divididas em reações de fase I e de reações de fase II. As reações de fase I acrescetam substituintes à droga e criam um ponto de ancoragem para uma futura conjugação (peça chave para a eliminação do xenobiótico), enquanto as reações de fase II fazem a conjugação das substâncias decorrentes da fase I ou podem fazer uma conjugação direta do fármaco absorvido à cofatores como ácido glucurônico, glutationa ou sulfato, preparando-o para excreção (ULENBERG *et al*., 2020; SHARMA; DURAIRAJ; BUREIK, 2020).

As reações de fase I ocorrem em grande parte no retículo endoplasmático liso das células do fígado, são realizadas por enzimas redutases, hidrolases e oxidases, das quais, as mais importantes são as enzimas do CYP450 (MANNAN; UNNISA, 2019). A metabolização dos fármacos têm por princípio a inativação dos mesmos, porém pode-se formar metabólitos tóxicos ao organismo, provocando necrose hepática, necrose renal e câncer de fígado (TALEVI; QUIROGA, 2018).

Ao contrário das reações de fase I, na fase II, raramente os produtos são compostos ativos ou tóxicos. Nestas reações, são adicionados substituintes polares, tornando as substâncias mais hidrofílicas (CHEN, 2020). Os produtos destas reações são transportados por difusão simples ou bombas de efluxo. A reação de fase II mais presente é a glucuronidação e o local mais preponderante das diversas reações é o citosol, embora também ocorra nas mitocôndrias e no retículo endoplasmático (BANERJEE, 2020).

A excreção é a retirada do xenobiótico, metabolizado ou não, do organismo, sendo a via urinária e a via biliar as duas principais vias de excreção do organismo. A via urinária é decorrente da excreção renal, enquanto a biliar ocorre a excreção intestinal (MAXWELL, 2020).

Na excreção renal, o sangue atinge o glomérulo na cápsula de bowman com pressão suficiente para fazer a filtração através das células endoteliais, que bloqueiam moléculas maiores e deixam passar para a cápsula, pequenas moléculas. Os fármacos também são

transportados dos vasos sanguíneos para o lúmen do túbulo proximal, em um processo conhecido como secreção tubular (HADDAD; NONG, 2020).

A excreção biliar é a via principal para moléculas grandes que são atraídas por transportadores presentes nas células hepáticas e lançadas na bile, que por sua vez, direciona tais metabólitos para o instestino, por onde são excretados para fora do corpo através das fezes (MAXWELL, 2020; TALEVI; QUIROGA, 2018).

Instrumentos que façam a previsão *in silico* das propriedades ADME das substâncias em testes pré-clínicos ajudam na seleção de fármacos, bem como evitam a perda de tempo em etapas posteriores, não excluindo a validação experimental (DURÁN-ITURBIDE; DÍAZ-EUFRACIO; MEDINA-FRANCO, 2020).

A absorção do fármaco pode ser prevista em testes *in silico* através da análise de suas propriedades de hidrofobicidade, lipofilicidade, tamanho e área da molécula. Para a distribuição, os modelos se baseiam na estrutura cristalina do complexo droga-proteína. Quanto ao metabolismo, a interação dos substituintes presentes na molécula, bem como de sua estrutura total, com o complexo enzimático CYP, é levada em consideração. Para a excreção, dados físico-químicos e estruturais são utilizados para fazer a previsão (MAXWELL, 2020; TALEVI; QUIROGA, 2018).

Os compostos primários podem conter estruturas tóxicas ao organismo ou gerar metabólitos tóxicos ao reagir com o complexo CYP (TALEVI; QUIROGA, 2018). Através de técnicas utilizando hepatócitos humanos, hepatócitos de ratos e modelos de estrutura-atividade e estrutura-propriedade, foi possível a criação de técnicas *in silico* capazes de prever toxicidade de compostos para células hepáticas humanas, toxicidade mitocondrial, mutagenicidade, cardiotoxicidade e dose terapêutica recomendada (FERREIRA; ANDRICOPULO, 2019).

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CAPÍTULO 2 – ARTIGO PUBLICADO NA REVISTA: *FEMS Microbiology Letters*, 2020, Vol. 367, No. 15; FATOR DE IMPACTO 1,987 – QUALIS B2

Direct antibacterial and antibiotic resistance modulatory activity of chalcones synthesized from thenatural product 2-hydroxy-3,4,6-trimethoxyacetophenone

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One sentence summary: Chalcones are strong candidates for structural models for the development of antibiotics.

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ABSTRACT

Antibiotic for clinical use lose its effectiveness over time due to bacterial resistance. In this work, four chalcones with modifications in their ligands were synthesized from the natural product 2-hydroxy-3,4,6-trimethoxyacetophenone, characterized by nuclear magnetic resonance (NMR) and infrared spectroscopy, and tested in bacterial models to investigate the direct and modifiers effects of the antibiotic activity of these four novel chalcones. The tests followed the broth microdilution methodology to obtain the Minimum Inhibitory Concentration (MIC). The MIC/8 of the products were used in the resistance reversion test. The

chalcone 2 showed the best result in terms of direct activity, with MIC 645 μg/mL for *Staphylococcus aureus* and 812 μg/mL for *Escherichia coli*. While, for the bacterial resistance reversal test, the chalcones presented several synergistic interactions, being that chalcone 4 had the best interaction with the tested antibiotics. It was found that the type of ligand, as well as its position in the ring, interferes in the modulation of the antibiotic activity. Our results show that chalcones are strong candidates to be used as antibacterial drug or in combination with antibiotics for the treatment of infections caused by multidrug-resistant (MDR) strains.

Keywords: chalcones; antibacterial activity; modulation of antibiotic activity

INTRODUCTION

Antibiotics traditionally used in medicine throughout the world have been losing their effectiveness over the years due to the emergence of resistant pathogenic species (Yousefi *et al*. 2017). Multiple resistance mechanisms, propagated by horizontal transmission through mobile genetic elements (Sharma *et al*. 2016), may coexist in the same strain making these multidrug-resistant strains a serious public health problem (Baquero *et al*. 2015).

Multidrug-resistant bacteria cause numerous infections across the globe, generating roughly 700.000 deaths per year, with even darker estimates being reported for the next 30 years (Willyard 2017). Developing new technologies capable of reverting this bacterial resistance is necessary for the continued clinical treatment of infections. Thus, synthetic products exhibiting direct antibiotic activity or activity in conjunction with standard antibiotics can be very useful.

Chalcones are naturally occurring substances in vascular plants, from pteridophytes to angiosperms (Lokesh, Prasad and Shaik 2017), which participate in the biosynthesis of flavonoids and isoflavonoids in these beings (Qin *et al.* 2015; Das and Manna 2016). Chalcones can also be synthesized by numerous techniques (Matos *et al.* 2015), as well as have their structures modified in the laboratory, making them potential therapeutic agents (Das and Manna 2016; Mahapatra and Bharti 2016). Moreover, compounds synthesized based on the structure of chalcones have several biological effects (Matos *et al.* 2015), some of which have approved clinical uses (Sahu *et al.* 2012), which demonstrates the importance of these compounds.

Earlier, our research group isolated for the first the acetophenone 2-hydroxy-3,4,6-trimethoxyacetophenone from the stem bark of *Croton anisodontus* and performed a study on their structural and spectroscopic proprieties (Santiago *et al.* 2018). This natural compound presented potential antibacterial activity (Oliveira *et al.* 2014). More recently, we have synthesized the chalcone 2*E*-1-(2-hydroxy-3,4,6-

trimethoxyphenyl)-3-(phenyl)-prop-2-en-1-one, and observed which it also presented the antibacterial action in combination with antibiotics (Teixeira *et al*. 2019). These results encouraged us to synthesize new derivatives from natural acetophenone and investigate the antibacterial potential of them.

In this study, a series of four synthetic chalcones were tested for their direct antibacterial effects or antibiotic modulatory activity, focusing on structural ligand changes and their relationship with the tested bioactivity.

MATERIALS AND METHODS

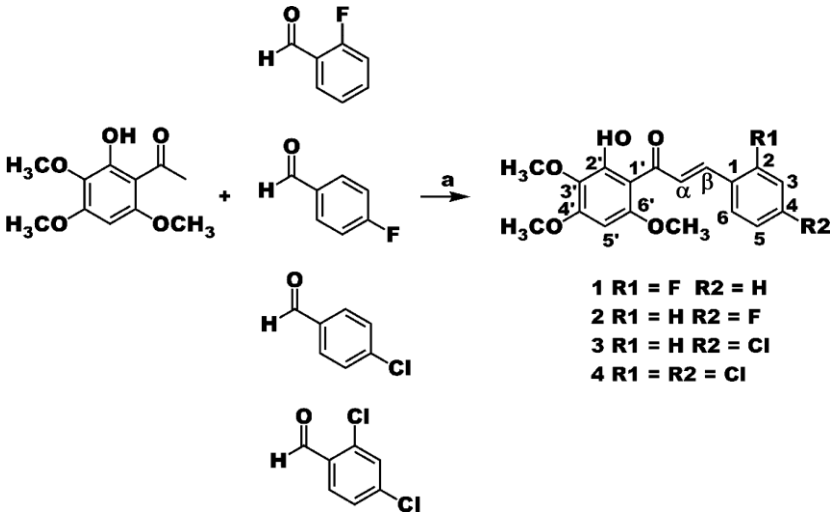
General procedures

The chemical reagents were purchased from Sigma-Aldrich Chemical Corp., (St.Louis, Mo., United States). ^{1}H and ^{13}C NMR spectra were obtained using a Bruker Spectrometer, model Avance DRX-500, operating at a frequency of 500 MHz (11.7 T) for hydrogen, and 125 MHz (2.9 T) for carbon, respectively. The spectra were measured in $CDCl_3$ solvents, and chemical shifts are reported as δ values in parts per million (ppm) relative to $CDCl_3$.

The Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectra of polycrystalline samples were recorded at room temperature using a CARY 600 FT-IR spectrometer in the spectral regions from 680/cm to 3600/cm with a resolution of 4/cm and accumulating 60 scans per spectrum.

Synthesis of the chalcones

The description of the procedure of the synthesis of the chalcones is shown in Scheme 1. The chalcones (1–4) were synthesized by a Claisen–Schmidt condensation reaction in basic medium (Bhat *et al*. 2005). The compounds 2-hydroxy-3,4,6 trimethoxyacetophenone (2 mmol) and benzaldehydes (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 48h at room temperature. The progress of the reaction was checked by TLC (n-hexane: ethylacetate, 2:1). After 48h the reaction mixture was neutralized with dilute HCl (10%) and ice water added. The product was filtered under reduced pressure, washed with cold water, and recrystallized from ethanol.



Scheme 1. Preparation of chalcones (1–4). a) NaOH 50% w/v, ethanol (5 mL), room temperature, 48 h.

Bacterial strains

Multi-resistant and standard *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* strains were used in both the direct and modulatory antibacterial activity assays, with the Gram-positive and Gram-negative bacterial models in mind. The standard strains are from the American Type Culture Collection (ATCC). In this work were used the *S. aureus* ATCC 25 923, *E. coli* ATCC 25 922 and *P. aeruginosa* ATCC 9027. Multi-resistant strains are bacterial strains isolated from hospitalized patients and numerically designated according to their specific serotype in *S. aureus* 10 (SA 10), *E. coli* 06 (EC 06) and *P. aeruginosa* 03 (PA 03). All the strains were obtained from cultures grown at the Laboratory of Microbiology and Molecular Biology (LMBM) of the Regional University of Cariri (URCA). The source for obtaining multidrug-resistant serotypes and the classes of antibiotics associated with this resistance are described in Table 1 below.

Table 1. Antibiotic resistance profile of the tests.

Bacteria	Source	Resistance profile
Staphylococcus aureus 10	Rectal swab	*β*-Lactamics, Macrolides and Quinolones
Pseudomonas aeruginosa 03	Catheter tip	*β*-Lactamics and Quinolones
Escherichia coli 06	Urine culture	*β*-Lactamics

Source: Autor.

Direct antibacterial activity test

The broth microdilution procedure recommended by Javadpour and collaborators (1996), with adaptations, was adopted to determine the Minimum Inhibitory Concentration (MIC). The inoculums were prepared in 0.9% physiological saline (PS) using 24 h bacterial cultures, with a turbidity of 0.5 as measured according to the McFarland scale ($1x10^8$ CFU/mL). A BHI (Brain Heart Infusion Broth) broth was prepared at a concentration of 10% (10g BHI/100mL of sterile water). Subsequently, 900μL of this BHI broth was poured into Eppendorf® tubes together with 100μL of the bacterial inoculum, completing a final volume of 1000μL per Eppendorf® tube. Then 100μL of the tube was then used to fill each as well of the microdilution plates.

Microdilutions were performed at a 1:1 ratio using 100μL from each compound at an initial concentration of 1024 μg/mL and microdiluting it towards the column. The MIC was read with a resazurin reagent following 24 h of incubation at 37°C in a bacteriological incubator, where concentrations ranged from 512 μg/mL in the first well to 8 μg/mL in the penultimate well. The last well served as bacterial growth control.

Antibiotic modulatory activity test

The methodology proposed by Coutinho and collaborators (2008) was employed for this assay. This technique consists of the same procedure as the previous assay, however, with the following modifications: eppendorfs® are filled with the BHI solution, the inoculum and a Chalcone, at a volume corresponding to a sub-inhibitory concentration (MIC/8) of the previous step, with the plate microdilution being performed with clinically used antibiotics.

Control plates were prepared from eppendorfs® tubes containing only the 10% BHI medium and bacterial inoculum. Ciprofloxacin, gentamicin and cephalexin, all at a concentration of 1024 μg/mL, were the antibiotics used in the modulation assays. At this point, the plates were used in their numerical sense, with concentrations ranging from 512 μg/mL in the first well to 0.5 μg/mL in the penultimate well.

In an attempt to infer a possible mechanism of action for chalcones, a standard Efflux Pump Inhibitor (EPI), Chlorpromazine (CPZ), was added at sub-inhibitory concentrations to the antibiotic modulatory activity test. The antibiotic MICs in the presence and absence of the standard efflux pump inhibitor were compared to the antibiotic MICs in the presence and absence of chalcones.

Lower MICs, with statistical significance, in the presence of the standard inhibitor compared to antibiotic MICs alone suggest the presence of an efflux pump for a given antibiotic for a given bacterium.

Statistical analysis

All bacteriological tests were performed in triplicates. Data were analyzed using a two-way ANOVA followed by Bonferroni's post hoc 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.

RESULTS AND DISCUSSION

Structural data of the chalones

The infrared data corroborated the confirmation of the structure, affirming the presence of stretch bands characteristic of C=O with values of system conjugated to C-sp^2 and stretch bands of the trans type demonstrating the formation of the double bond C=C. These and other values are found in Table 2.

Table 2. Infrared data for chalcones (1–4).

	Infrared bands positions for the chalcones (1–4) in units of cm^{-1}			
Main vibrations	1	2	3	4
ν [C = O]	1631	1625	1635	1631
ν [C = C]	1559	1554-1413	1559-1406	1559-1426
ν [C-O]	1121	1125	1127	1121
δ [C(C = O)C]	1333	1211	1210	1333
δ [C-F]	1013	1018		
δ [C-Cl]			1025	1013

Nomenclature: ν = Stretching mode; δ = Bending mode.

The structures were determined by NMR, IR and mass spectra. The ^{1}H NMR spectra of the chalcones synthesized showed three signals between 3.84 and 3.96 relative to the hydrogens of methoxy groups MeO-3, MeO-4 and MeO-6, respectively. Between 7.79 and 8.06 ppm (J = 15.5 Hz) were attributed to doublets referring to α, β unsaturated hydrogens, whose coupling constant (J) confirms the stereochemistry *E*. The singlets observed between 6.01 and 6.07 refer to hydrogen attached to the carbon 5^r-carbon of ring A. In the ^{13}C-NMR spectrum of the chalcones synthesized it was possible to observe a sig- nal concerning α, β unsaturated from 192.6 to 193.5 ppm. The ketone absorbs 203.8 ppm, however, the presence of α, β unsaturation causes displacement to the high field, and the probable cause is the delocalization of charge by the benzene ring or by the double bond that makes carbonyl car- bon less electron deficient. The olefinic carbons α and β are observed between 127.4 and 141.5 ppm, respectively. The signals between 56.1and 60.9 refer to the carbons of the

methoxy groups. The IR and NMR spectra are shown in Figures S1–S21 (Supporting Information).

(*E*)-3-(2-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (1)

Yellow solid (Yield: 84,07%), m.p. 144.3–144.8°C. ^{1}H NMR ($CDCl_3$, ppm): 3.96 (s, MeO-3^{r}); 3.96 (s, MeO-4^{r}); 3.84 (s, MeO-6^{r}); 6.07 (s, H-5^{r}); 7.67 (m, H-3); 7.44 (m, H-4); 7.25 (m, H-5); 7.18 (m, H-6); 7.82 (d, Hα, J = 15,5 Hz); 8.06 (d, Hβ, J = 15.5 Hz). ^{13}C NMR ($CDCl_3$, ppm): 193.5 (C = O); 60.0 (MeO-3^{r}); 56.2 (MeO-4^{r}); 56.1 (MeO-6^{r}); 107.1 (C-1^{r}); 158.9 (C-2^{r}); 130.4 (C-3^{r}); 159.6 (C-4^{r}); 87.3 (C-5^{r}); 158.8 (C-6^{r}); 131.7 (C-1); 135.4 (C-2); 131.6 (C-3); 131.1 (C-4); 116.3 (C-5); 116.6 (C-6); 124.7 (Cα); 135.5 (Cβ). MS (EI) m/z ($M^{+\cdot}$ 322), calcd for $C_{18}H_{17}FO_5$/332.

(*E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (2)

Yellow solid (Yield: 69,53%), m.p. 144.3–144.8°C. ^{1}H NMR ($CDCl_3$, ppm): 3.96 (s, MeO-3^{r}); 3.96 (s, MeO-4^{r}); 3.84 (s, MeO-6^{r}); 6.07 (s, H-5^{r}); 7.63 (d, J = 8.58, H-2/6); 7.65 (d, J = 8.58, H-3/5); 7.82 (d, Hα, J = 15,6 Hz); 7.83 (d, Hβ, J = 15.6 Hz). ^{13}C NMR ($CDCl_3$, ppm): 193.2 (C = O); 60.9 (MeO-3^{r}); 56.3 (MeO-4^{r}); 56.2 (MeO-6^{r}); 107.1 (C-1^{r}); 158.7 (C-2^{r}); 130.4 (C-3^{r}); 159.6 (C-4^{r}); 87.4 (C-5^{r}); 158.7 (C-6^{r}); 131.9 (C-1); 131.2 (C-2/6); 131.9 (C-3/5); 141.5 (C-4); 127.4 (Cα); 141.5 (Cβ). MS (EI) m/z ($M^{+\cdot}$ 322), calcd for $C_{18}H_{17}FO_5$/332.

(*E*)-3-(4-chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (3)

Yellow solid (Yield: 74,68 %), m.p. 94.3–96.8°C. ^{1}H NMR ($CDCl_3$, ppm): 3.96 (s, MeO-3^{r}); 3.96 (s, MeO-4^{r}); 3.94 (s, MeO-6^{r}); 6.02 (s, H-5^{r}); 7.38 (d, J = 8.35, H-2/6); 7.53 (d, J = 8.35, H-3/5); 7.79 (d, Hα, J = 15.6 Hz); 7.83 (d, Hβ, J = 15.6 Hz). ^{13}C NMR ($CDCl_3$, ppm): 193.5 (C = O); 60.9 (MeO-3^{r}); 60.9 (MeO-4^{r}); 60.9 (MeO-6^{r}); 106.5 (C-1^{r}); 159.0 (C-2^{r}); 129.7 (C-3^{r}); 159.6 (C-4^{r}); 87.4 (C-5^{r}); 159.2 (C-6^{r}); 134.2 (C-1); 131.2 (C-2/6); 131.7 (C-3/5); 136.2 (C-4); 128.2 (Cα); 141.3 (Cβ). MS (EI) m/z ($M^{+\cdot}$ 348), calcd for $C_{18}H_{17}ClO_5$/348.

(*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (4)

Yellow solid (Yield: 73,93%), m.p. 164.3–164.8°C. ^{1}H NMR ($CDCl_3$, ppm): 3.98 (s, MeO-3^{r}); 3.94 (s, MeO-4^{r}); 3.85 (s, MeO-6^{r}); 6.01 (s, H-5^{r}); 7.62 (d, J = 8.5, H-3); 7.46 (d, J = 1.5, H-5); 7.30 (d, J = 1.5, H-6); 7.80 (d, Hα, J = 15,5 Hz); 8.06 (d, Hβ, J = 15.5 Hz). ^{13}C NMR ($CDCl_3$, ppm): 192.6 (C = O); 60.7 (MeO-3^{r}); 56.1 (MeO-4^{r}); 56.1 (MeO-6^{r}); 106.8 (C-1^{r}); 158.7 (C-2^{r}); 130.4 (C-3^{r}); 159.4 (C-4^{r}); 87.1 (C-5^{r}); 158.6 (C-6^{r}); 134.2 (C-1); 136.8 (C-2); 135.5 (C-3); 136.0 (C-4); 131.7 (C-5); 128.5 (C-6); 127.5 (Cα); 130.1 (Cβ). MS (EI) m/z ($M^{+\cdot}$ 383), calcd for $C_{18}H_{16}Cl_2O_5$/383.

Direct antimicrobial activity

The chalcones antimicrobial activity was calculated as described in the methodology and expressed as Minimum Inhibitory Concentration (MIC) values in Table 3.

Table 3. MIC values (μg/mL) in the presence of the chalcones (1–4) in standard and multi-resistant bacteria.

Chalcones	Standard and multi-resistant bacteria					
	S.A. ATCC 25 923	**E.C. ATCC 25 922**	**P.A. ATCC 9027**	**S.A. 10**	**E.C. 06**	**P.A. 03**
1	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024
2	645	812	≥1024	≥1024	≥1024	≥1024
3	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024
4	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024

S.A., *Staphylococcus aureus;* **P.A.**, *Pseudomonas aeruginosa;* **E.C**., *Escherichia coli;* **ATCC**, American Type Culture Collection.

All substances obtained MIC values ≥ 1024 μg/mL, with the exception of the chalcone 2 against standard *S. aureus* and *E. coli* strains, where MIC values of 645 μg/mL and 812 μg/mL, respectively, were obtained.

In an attempt to establish a comparison standard for antimicrobial activity suggested that an isolated substance would have a satisfactory inhibitory effect when its MIC was less than 100 μg/mL, with possibly promising results for those obtaining MIC values below 10 μg/mL (Ríos and Recio 2005). Subsequently, Santos and collaborators (2014) used previous methodologies to categorize the antibacterial effect of synthetic nanoparticles into three classes: when the MIC was less than 100 μg/mL the substance was considered having a good antibacterial effect; when the MIC ranged from 100 to 500 μg/mL, its effect was considered moderate; between 500 and 1000 μg/mL the effect was considered as low, and substances obtaining values above these were considered as inactive. The antibacterial effects of each substance related to its structures were summarized in Table S1 (Supporting Information), included in the supplementary material.

Our data show that the chalcones analyzed did not present an antibacterial effect against *P. aeruginosa,* being therefore considered inert for this strain, with respect to clinically relevant doses. The chalcone 2 obtained a weak effect against standard *S. aureus* and *E. coli* strains. However, this was not considered satisfactory. The remaining chalcones analyzed were considered inert against the remaining strains.

This greater resistance of Gram-negative bacteria to the action of substances has been demonstrated in studies with chalcones, where a lower activity has been observed for this group (Kucerova-Chlupacova *et al.* 2016; Marliyana and Syah 2017; Koudokpon *et al.* 2018). Moreover, within the Gram-

negative group, *P. aeruginosa* has been demonstrated to be resistant to various compounds (Kucerova-Chlupacova *et al.* 2016), not suffering any antibacterial action as verified by minimum bactericidal concentration (MBC) analysis (Amole, Bello and Oyewale 2019).

In a study by Ávila and collaborators (2008), 31 chalcones were tested against four bacterial strains, 2 Gram-positive and 2 Gram-negative, in an attempt to establish an association between structural ligands and antibacterial activity. The results presented a lack of activity against *P. aeruginosa*, and *E. coli* strains at clinically relevant doses for all tested chalcones, with eight chalcones obtaining a satisfactory effect only against the Gram-positive *S. aureus* and *Bacillus cereus* strains. The data from the aforementioned study corroborates with the present results, showing that morphological differences between the bacterial groups are a barrier to the action of chalcones against Gram-negative strains, especially *P. aeruginosa*. In the same study, the composition, as well as the positions of the ligands in both rings, were found to interfere with the antibacterial activity of the molecule. In our study, given the composition of ring A was kept constant and the presence of $–OCH_3$ ligands were not associated with an increased antimicrobial effect (Liu, Xu and Go 2008), antibacterial activity depended on the ligands and their positions in ring B. This effect can be observed when comparing the antibacterial effect of the chalcones 2 and 3 (ligand change) and chalcones 2 and 1 (position change) pairs against the *S. aureus* and *E. coli* strains.

Antibacterial effects associated with the position of halogenated ligands in ring B have been reported against several fun- gal, bacterial and mycobacterial strains, with prominent activities against *S. aureus* and without effects against Gram-negative *E. coli* and *P. aeruginosa* strains (Kucerova-Chlupacova *et al.* 2016).

It can be inferred that the fluorine ligand at position 4 of ring B confers an antibacterial effect to the original chalcone structure, which presented no direct bacterial effect against the aforementioned strains (Teixeira *et al.* 2019). While observing the structure of chalcones, the closer proximity of the fluorine ligand to the ring A radicals, when this is located at position 2, may cause interactions between these and, consequently, result in the loss of the antibacterial activity of the compound.

This antibacterial action of the fluorine atom has been evidenced in other studies testing chalcone series against *S. aureus* and *E. coli* strains, showing more significant effects against the Gram-positive strain, where this effect is associated with the presence, position and quantity of the fluorine atom in the molecule (Chu *et al.* 2018; Amole, Bello and Oyewale 2019), acting by depolarization and increased membrane permeability (Chu *et al.* 2018).

A more pronounced effect against Gram-positive strains was also found in two studies with chlorinated chalcones. However, chlorine atoms at positions 2 and 4 of ring B were observed to increase the antibacterial activity of chalcones (Al-Jorani *et al.* 2019), and this antibacterial activity extended to *P. aeruginosa*

strains (Venkatesh *et al.* 2016). These findings are discordant with the present results. However, the composition of ring A in the aforementioned study was different, which suggests the composition of ring A ligands may interfere with the action of ring B ligands, which may explain the low antibacterial activity of the chalcone 4 in this test.

Antibiotic modulatory activity

Bacterial resistance reversal was tested using two intracellular antibiotics, ciprofloxacin and gentamicin and a *β*-Lactamic (Cephalexin), whose action is on the cell wall. The strains used in this test for potentiation of antibiotic activity were the multidrug-resistant (*S. aureus* 10, *E. coli* 06 and *P. aeruginosa* 03). An efflux pump inhibitor (EPI) was added as a modulation control to try to establish a probable mechanism of action of chalcones in bacterial resistance modulation.

Efflux pumps (EP) are a mechanism present in the cells of almost all living beings, wherein bacteria this acts in the extrusion of antibiotics, as well as in intercellular communication and pathogenicity (Blanco *et al.* 2018), which makes these a desirable target for inhibition. Modulation data are expressed in Figs 1–3, which show the effect of chalcones against Gram-positive and Gram-negative strains.

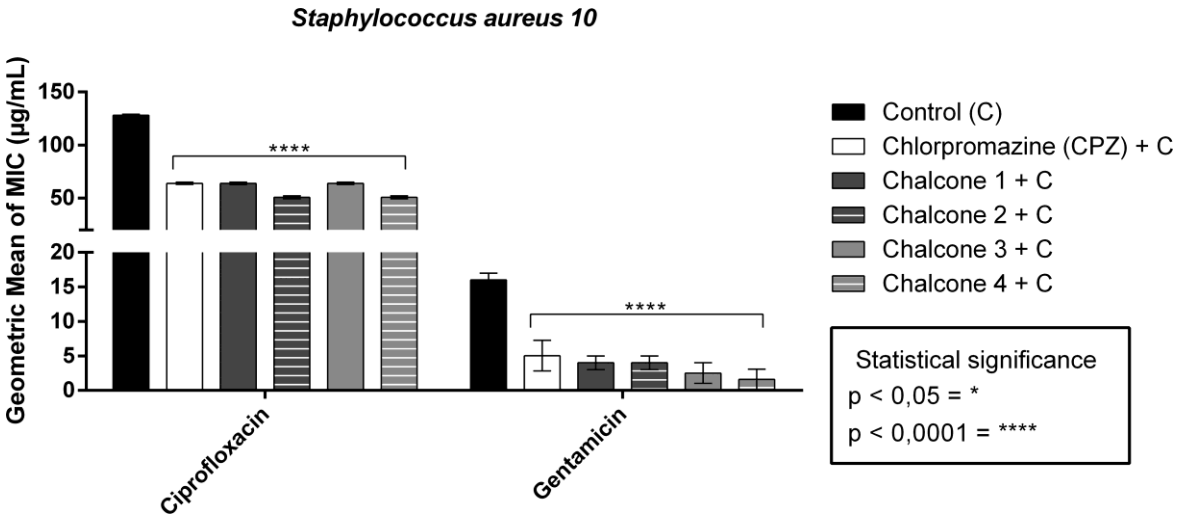


Figure 1. Antibiotic modifying activity effect of synthetic chalcones against *Staphylococcus aureus* 10.

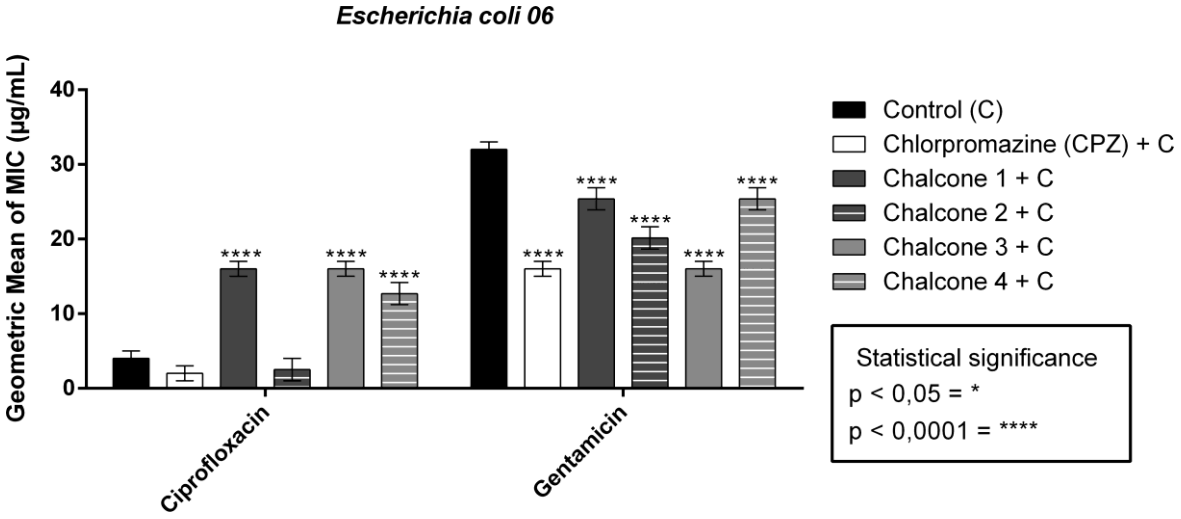


Figure 2. Antibiotic modifying activity effect of synthetic chalcones against *Escherichia coli* 06.

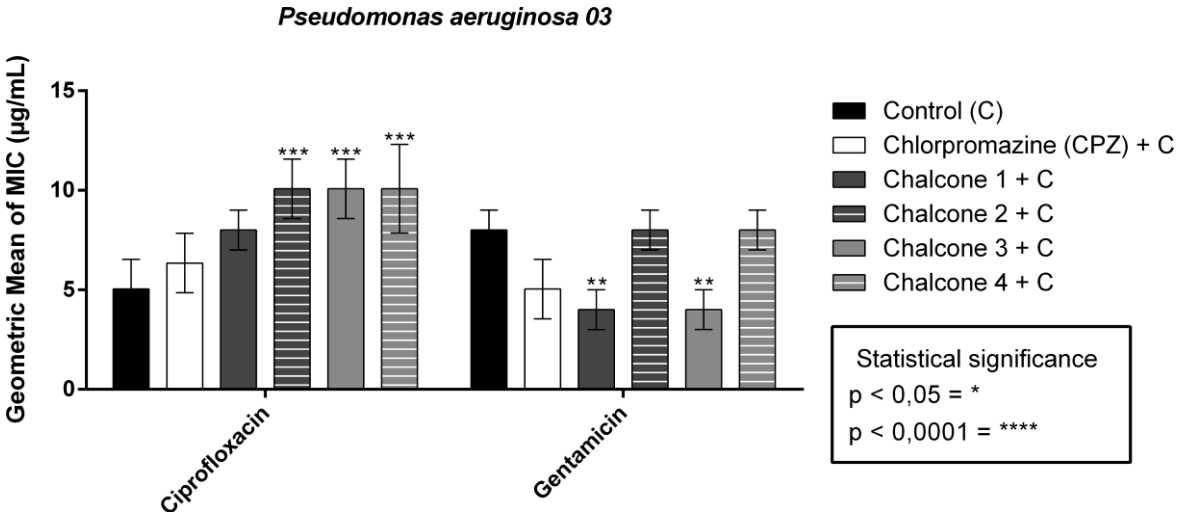


Figure 3. Antibiotic modifying activity effect of synthetic chalcones against *Pseudomonas aeruginosa* 03.

All except *P. aeruginosa*, the used strains were sensitive to chlorpromazine (CPMZ) with one or both tested antibiotics, which indicates the presence of efflux pumps acting in the extrusion of ciprofloxacin and gentamicin in the *S. aureus* strain, and in the extrusion of gentamicin in the *E. coli* strain. As for *P. aeruginosa*, the present results indicate it possesses other mechanisms of action responsible for its resistance to these antibiotics, or it presents CPMZ-insensitive efflux pumps. It was precisely in the presence of EP mechanisms that chalcones presented the greatest effect.

Chlorpromazine is a well-known EPI that acts by inhibiting calcium uptake, which is essential for the energy supply required for pump activation (Blanco *et al.* 2018). In the presence of chlorpromazine-sensitive EPs, all

chalcones, whether fluorinated or chlorinated, presented synergism with the antibiotic where the inhibition of this mechanism is the suggested action of chalcones.

Individually, the most relevant Minimum Inhibitory Concentrations (MICs) against the Gram-positive strain were those presented by the chalcones 2 and 4 for ciprofloxacin, which presented a better effect than the CPMZ control. This indicates a greater sensitivity of the efflux pumps to the products, inducing a lower need for antibiotics to exert their effects *in vitro*. For gentamicin, no statistical differences between chalcones and CPMZ control were observed.

A synergistic modulatory effect for ciprofloxacin and gentamicin against resistant *S. aureus* strains have been previously reported in the literature and associated with the presence of ligands in ring B (Bozic *et al.* 2014).

When compared to its position isomer, the chalcone 2 presents a better modulatory effect over ciprofloxacin's antibacterial activity, as well as its direct antibacterial effect. In the chlorinated series, the number of chlorine atom ligands directly influenced the modulatory effect. The chalcone 3, although synergistic for the antibiotics in question, presented an effect comparable to the standard EPI used, while the chalcone 4 presented more pronounced synergistic effects compared to other com- pounds in the series and compared to CPMZ. The chalcone 4 also obtained the lowest Minimal Inhibitory Concentration (MIC) when associated with gentamicin, despite no statistical difference when compared to the CPMZ control.

This again underscores the importance of the number of ligands and their localization in the antibacterial effect of a given chalcone and, in this instance, their ability to reverse bacterial resistance indirectly.

Synergistic results using quinolones against *S. aureus* were also found in a chalcone series, revealing the inhibition of efflux pumps as a mechanism of synergism, as proven by the increase in intracellular ethidium bromide accumulation inside bacterial cells treated with chalcones (Gaur *et al.* 2015).

Results for the Gram-negative models were not as promising as the results obtained with *S. aureus* (Figs 2 and 3). Synergistic effects were shown by all chalcones against *E. coli* when associated with the antibiotic gentamicin. These data corroborate with the suggested mechanism of action since this bacterium presented EPs for this antibiotic. On the other hand, antagonistic effects for ciprofloxacin were obtained with the chalcones 1, 3 and 4, while non-significant interactions were exhibited by the chalcone 2. These data reinforce the proposed mechanism of action since chalcones lost their synergistic effects in the absence of EPs, in addition to demonstrating the relevance of the data of the chalcone 4 for its specificity with CPMZ-sensitive efflux pumps since this substance obtained the best modulatory effect against *S. aureus* in the presence of EPs against ciprofloxacin.

A direct relationship between the presence of ligands at position 4 of ring

B and the modulatory effect in the presence of EPs in Gram-negative strains was observed. The chalcone 2 obtained a lower MIC than the chalcone 1, and statistical significance demonstrates a loss of effect in the chlorinated series modulating gentamicin: chalcones 3 and 4. The presence of a second ligand in position 2 also interfered with the interaction with the antibiotic. The presence of chlorine was also more efficient than the fluorine ligand.

The worst chalcone effects were found with *P. aeruginosa*, where the absence of efflux pumps for the tested antibiotics is a factor that supports the results that have been explained so far and allows us to make some inferences. In this strain, three chalcones presented synergism for the antibiotic gentamicin with the chalcones 1 and 3, suggesting a second mechanism of action for these substances or an inhibition of CPMZ-insensitive efflux pumps. The second mechanism of action may explain the synergism of the chalcone 1 in this model since the ligand in position 4, which is associated with EP inhibition, was not statistically significant.

Again, the position of the ligands was instrumental in the bacterial resistance reversal effect, with position 4 of ring B standing out as a possibly privileged position for ligands associated with antibacterial effects.

A chalcone fluorinated at position 4 presented a direct antibacterial effect against Gram-positive and Gram-negative strains, synergistically modulating the quinolone norfloxacin against all tested Gram-negative strains, including *P. aeruginosa*. This effect was associated with an interaction of the chalcone with DNA gyrase (Liu *et al.* 2017).

Corresponding interactions were found in a study with fluorinated chalcones associated with ciprofloxacin and amikacin against Gram-negative strains, in which a greater synergis- tic potential for the aminoglycoside tested against *Acinetobacter baumannii* was observed. No synergistic interactions were observed against the resistant *P. aeruginosa* strain, only additive antibiotic effects associated with the inhibition of pyocyanin production. In this study, synergism occurred with chalcones fluorinated at position 4 of ring B (Uš jak *et al.* 2019), strengthening the theory of the importance of a ligand at position 4 and the antibacterial effect of a chalcone.

The lowest effect being observed against the resistant *P. aeruginosa* strain is compatible with the literature, which highlights several resistance mechanisms for this Gram-negative bacteria such as efflux pumps, enzymatic mechanisms and reduced membrane permeability, as well as a high mutagenic capacity, which permits its growth even in the presence of antibiotics (Cabot *et al.* 2016; Chatterjee *et al.* 2016).

The results of the association of the chalcones with cephalexin did not indicate any interaction, neither synergism nor antagonism, with all the values of the Minimum Inhibitory Concentration presenting $\geq$ 1024 μg/mL. For this reason, they were not plotted in the graphics. This absence of interaction should be a

result of the mechanism of bacterial resistance on this antibiotic, which is commonly related to the production of enzymes β-lactamases (Dowling, O'Dwyer and Adley 2017).

CONCLUSION

The position and ligand type present in ring B of the chalcone influence its direct antibacterial effect. The position and number of ligands influence the reversibility of bacterial resistance, with respect to the mechanisms presented by the efflux pumps. Chalcones modified with a fluorine ligand at position 4 of ring B obtained the best direct antibacterial effect. The chalcone 4 presented the best synergistic effect with the tested antibiotics when chlorpromazine-sensitive efflux pumps were present. The position 4 of ring B, together with a chlorine ligand presented the best association for the bacterial resistance reversal effect.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflicts of interest. None declared.

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In vitro and *in silico* studies of chalcones derived from natural acetophenone inhibitors of NorA and MepA multidrug efflux pumps in *Staphylococcus aureus*

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Abstract

Bacterial resistance induced by efflux pumps is a frequent concern in clinical treatments involving multi-resistant bacteria. *Staphylococcus aureus* is a microorganism responsible for several types of infections and has several strains carrying efflux pumps, among them are the strain 1199B (NorA overexpresser), and the strain K2068 (MepA overexpresser). In this work, four chalcones derived from *Croton anisodontus* with modifications in the B ring in their structures were tested regarding their ability to inhibit NorA and MepA efflux pumps. The efflux pump inhibition mechanism was tested with the ethidium bromide substrate in the presence and absence of standard efflux pump inhibitors. The minimum inhibitory concentration values were also compared to those of strains that do not overexpress these efflux pumps. In order to gain some insights about the efflux pump mechanisms of these chalcones, two homology models were created (NorA and MepA) for a docking procedure. In addition, the ADMET properties (absorption, distribution, metabolism and excretion) were also evaluated. The tested chalcones promoted synergism of the norfloxacin antibiotic by inhibiting associated efflux pumps. All four tested chalcones appear to bind to the binding sites of the efflux pump models in the same fashion as other chalcones with efflux pump inhibition capabilities. It was also verified that the chalcones 1-4 are well absorbed in the intestine, but with a decrease in their bioavailability, resulting in a low volume of distribution in the blood plasma, in addition to having a mild CNS activity. However, the chalcone 3 and 4 were not toxic due to metabolic activation. Whereas the chalcones 1 and 2 present a mutagenic risk, depending on the oral dose administered. The tested chalcones have not antibacterial activity; however, they are capable of inhibiting efflux pumps for the 1199B and K2068 strains. They promoted synergism of the norfloxacin antibiotic by inhibiting associated efflux pumps, as well as other associated mechanisms.

Keywords: Chalcone; NorA; MepA; Efflux pumps; ADMET; Molecular docking.

1. Introduction

Infections by resistant bacteria remain a matter of concern as cause of death in developing countries [1]. Bacterial resistance to antibiotics is found in numerous bacterial communities worldwide, from bacteria with no clinical relevance to the most well-known strains involved in public health problems. Through horizontal transfer mechanisms, genes associated with resistance migrate to strains that cause disease in humans, and among them, pathogenic strains, making them multidrug-resistant, which makes clinical treatment difficult [2].

Overall , the bacterial resistance mechanisms can be grouped into 4 main groups: changes in the binding sites, whether external or internal; enzymatic mechanisms that degrade antibiotics; decrease in membrane permeability; and efflux pumps, which promote the extrusion of compounds, reducing their concentration inside the bacteria to non-lethal levels [1].

Efflux pumps are present in several organisms, being expressed as basal or overexpressed form, depending on the presence of aggressive agents or selection of mutants capable of higher gene expression and are involved in both bacterial resistance and virulence [3]. According to the energy source used and the structure of these pumps, they are classified as: ABC (ATP-Binding Cassettes), the only family of efflux pumps that use ATP energy directly; MFS (main facilitator superfamily), consisting of two units that, when opening into the cell, close outward and when opening outward, close inward, capturing the antibiotic inside and throwing it out; and MATE (multidrug and toxin extrusion family); the proteins that constitute this efflux pump family, acquire a “V” shape, opening outward of the cell; RND (resistance-nodulation cell division superfamily), usually have a structure formed by three equal parts [4, 5]; SMR (small multidrug resistance family), can be constituted of homodimers or hetero-oligomers, which protect bacteria against several classes of aggressive agents; and PACE (proteobacterial antimicrobial compound efflux family), consisting of two transmembrane domains [5].

Belonging to the MFS family, the NorA efflux pump is encoded by three different alleles located in the circular chromosome DNA of the *Staphylococcus aureus* strains [6], promoting resistance to antibiotics belonging to the fluoroquinolone class [7]. The ineffective treatment of bacterial infections are related to the presence of the NorA gene in multidrug-resistant strains. The MepA efflux pump belongs to the MATE family, encoded by genes also located in the central bacterial DNA of *Staphylococcus aureus* strains, granting these bacteria resistance to fluoroquinolones [8]. Strains from clinical isolates that contain genes capable of

encoding and overexpressing these two pumps showed high values of minimal inhibitory concentrations [9], demonstrating the need to inhibit these efflux pumps when fighting bacterial resistance to antibiotics [10].

Chalcones are products of secondary metabolism of plants that have a privileged and simple structure, They have a diversity of natural sources, in addition to being easily synthesized by previously established methodologies, generating a multitude of synthetic compounds that, depending on the ligand associated with the main structure, bind to several biological targets, providing an increase in the clinical applications of this group of substances [11].

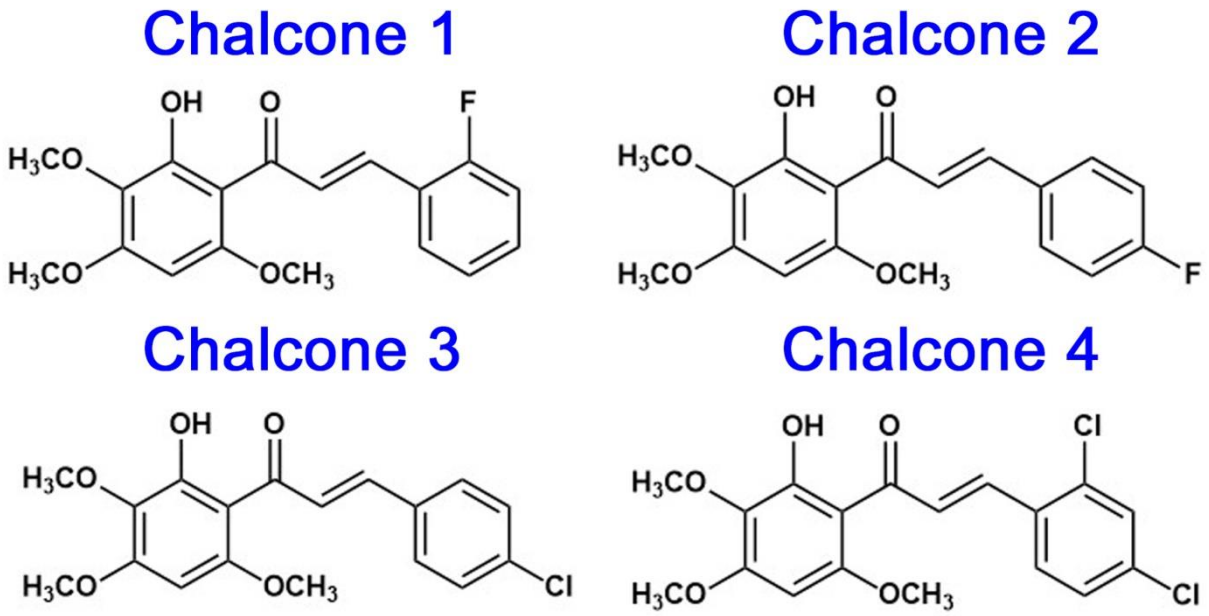
The substitutions involving the hydrogens present in the aromatic rings in their main structure by several ligands are the main causes of their wide bioactivity [12]. Among the effects that have been previously demonstrated in laboratory tests we find: anticancer [13], antioxidant [14], anti-inflammatory [15], antimalarial [16], antifungal [17] and antibacterial activity [18, 19].

In the present study, four chalcones derived from the natural product 2-hydroxy-3,4,6-trimethoxyacetophenone, with substitution of the ligands in the B ring of its main structure, were tested for their antibacterial activities in two strains of *Staphylococcus aureus* that carry efflux pumps, that is the 1199B strain (carrying the NorA pump) and the K2068 strain (carrying the MepA pump). *In silico* study by docking molecular were done, in order to understanding the efflux pump inhibition mechanisms. In addition, the ADME properties (absorption, distribution, metabolism and excretion) were also evaluated.

2. Material and Methods

2.1 Compounds

Figure 1 shows the structural representation of the chalcones derived of acetophenone isolated from *Croton anisodontus* Müll.Arg. with the following nomenclature: Chalcone 1: (*E*)-3-(2-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one; Chalcone 2: (*E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one; Chalcone 3: *(E)*-3 (4-chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one; and Chalcone 4: (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl) prop-2-en-1-one. These chalcones were synthesized by Claisen-Schimdt aldol condensation, and their structural data were reported by Freitas and collaborators [20]. The nuclear magnetic resonance (NMR) spectra of ^{1}H and ^{13}C, as well as the mass spectra of the chalcones 1-4 are given in the supplementary material (Figures S1-S20).



Standard inhibitors chlorpromazine (CPMZ) and carbonyl-m-chlorophenyl hydrazone cyanide (CCCP), as well as ethidium bromide (EtBr) were obtained from Sigma Aldrich Co. Ltd. A total of 10 mg of each of the compounds used in the tests were weighed. The chalcones, chlorpromazine ((SANOFI, São Paulo SP, Brazil) and the norfloxacin (SANDOZ, Cambé PR, Brazil) and ciprofloxacin ((GEOLAB, Anapolis GO, Brazil) antibiotics were initially diluted in 0.5 mL of dimethyl sulfoxide (DMSO) (LABSYNTH, Diadema SP, Brazil) and then 9.265 mL of sterile water were added, comprising a total of 9.765 mL and a final concentration of 1024 µg / mL. The carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (SIGMA-ALDRICH, ST. LOUIS, EUA) was first diluted in methanol and, subsequently, in distilled water, at a 1:1 v/v ratio, with a final concentration of 1024 µg / mL. the ethidium bromide was diluted directly in 9.765 mL of sterile water, reaching a concentration of 1024 µg / mL.

2.2 Bacterial strains

The strains used in this test were strain 1199B, which carries the NorA gene and overexpresses efflux pumps against fluoroquinolones [21], provided by Professor S. Gibbons (University of London) and kept on blood agar (Laboratorios Difco Ltda., Brazil) and the K2068 strain, which overexpresses the MepA efflux pump [22]. Both strains were grown on BHI agar (microMED – ISOFAR, Duque de Caxias RJ, Brazil) 24 hours before testing and kept in a bacteriological incubator (SANYO, model MOC-17AC, Japan) at 37ºC in the

Laboratory of Microbiology and Molecular Biology (LMBM) of Universidade Regional do Cariri (URCA).

2.3 Antibacterial activity test

The protocol developed by JAVADPOUR et al. (1996) was used in the experiments. Based on the cultivated strains, inoculants were prepared in 0.9% saline solution (SS), with turbidity being measured according to the 0.5 McFarland scale (1 x 10^8 CFU/Ml, where CFU means Colony Forming Units). Eppendorf® microtubes (CRALPLAST, Cotia SP, Brazil) were filled with 900 µL of 10% BHI (Brain Heart Infusion) broth and 100 µL of the bacterial inoculum. The solution in each Eppendorf® microtubes (CRALPLAST, Cotia SP, Brazil) was responsible for the filling up of a vertical row, in alphabetical order, of the microdilution plate (CRALPLAST, Cotia SP, Brazil) with 100 µL in each well.

The rows were microdiluted with the chalcones and with the chlorpromazine and CCCP (SIGMA-ALDRICH, ST. LOUIS, EUA) standard efflux pump Inhibitors (controls) at a 1:1 ratio, with 100 µL of each compound. The solutions of these two inhibitors containing the bacterial strains were microdiluted in a microdilution plate (CRALPLAST, Cotia SP, Brazil). The concentration values of the compounds in each well ranged from 512 µg/mL to 8 µg/mL in the second to last well. After 24 hours of incubation inside of the microdilution plate (CRALPLAST, Cotia SP, Brazil) at 37 ºC in a bacteriological incubator (SANYO, model MOC-17AC, Japan) the Minimum Inhibitory Concentration (MIC) values were measured using the Resazurin reagent (VETEC, Rio de Janeiro RJ, Brazil). The test was performed in triplicate.

The resazurin method is widely used and accepted in microbiology for evaluation of antimicrobial activity [23]. The resazurin reacts with products of the bacterial metabolism indicating the presence or absence of bacterial activity [24]. This method is simple, fast, efficient, accurate and reproducible, and it can be used to perform assays of extracts, fractions of extracts and pure compounds. It is a low cost and sensitive technique even in small volumes, just like in the microdilution plate [25]. The resazurin is only added at the end of the antimicrobial assay, after the compound and antibiotic had already exerted their effects, so there is no change in the result by associations between resazurin and the products.

2.4 Efflux Pump Inhibition Test

The methodology proposed by Coutinho and collaborators [26] was used, after adaptations. The seeding of the bacteria and the preparation of the inocula followed the same

technique as the previous topic. After the MIC was verified in the antibacterial activity test, the subinhibitory ([SubI]) concentrations of all compounds were calculated using the following formula [SubI] = MIC / 8. Subsequently, this subinhibitory concentration was calculated in terms of volume to be placed in Eppendorf® microtubes (CRALPLAST, Cotia SP, Brazil), using the formula $C_1xV_1 = C_2xV_2$, in which C_1 is the concentration of the prepared stock solutions (1024 μg / mL),V_1 is the volume to be removed from the stock solution and placed in the microtube, C_2 is the subinhibitory concentration of each product and V_2 is the total volume of the microtube. The total volume of each microtube was 1500 μL, comprising inoculum (150 μL), the volume corresponding to the subinhibitory concentration for each substance and 10% BHI volume, enough to complete the 1500 μL of the Eppendorf® microtube (CRALPLAST, Cotia SP, Brazil).

Antibiotic control was also prepared in Eppendorf® microtubes (CRALPLAST, Cotia SP, Brazil) containing only 1350 μL of 10% BHI medium (microMED – ISOFAR, Duque de Caxias RJ, Brazil) and 150 μL of bacterial inoculum. The ethidium bromide and specific antibiotics for each strain were microdiluted. In the microdilution process, it is added 100 μL of the ethidium bromide solution at an initial concentration of 1024 μg/mL, into the first well of the plate at a determined column, which contains 100 μL of the solution that was prepared in Eppendorf® microtubes, achieving at a final volume of 200 μL.The analysis of efflux pump presence was performed by comparing the Minimum Inhibitory Concentrations of ethidium bromide (SIGMA-ALDRICH, ST. LOUIS, EUA) in the presence of the standard inhibitors (CCCP and chlorpromazine) and in their absence (Control). Lower MICs in the presence of standard inhibitors means that the ethidium bromide efflux was stopped and, therefore, the presence of this resistance mechanism has been verified.

We performed a test with the ethidium bromide (SIGMA-ALDRICH, ST. LOUIS, EUA) in the presence and absence of efflux pump inhibitors to evaluate the phenotypic expression of the NorA and MepA bacteria. The ethidium bromide test is a method that is commonly used for microbiological assays with bacteria that overexpress efflux pumps, as in the case of 1199B and K2068. This test was validated in an experiment carried out by Amaral and collaborates [27] in which they demonstrated the accumulation of ethidium bromide inside efflux pump carrier strains after the addition of inhibitors such as CCCP and Chlorpromazine. This is an inexpensive and easy-to-implement method commonly used in microbiology laboratory for identifying multidrug-resistant strains that overexpress efflux pumps [28]. The use of the CCCP inhibitor as a methodology for evidencing efflux pumps

were demonstrated in several literatures by the intracellular accumulation of ethidium bromide or antibiotics after exposure to the inhibitor [29, 30].

2.5 Statistical analysis

The microbiological tests were performed in triplicate. The central data used in the statistical tests consist of geometric means of these central triplicates ± Standard Deviation. A one-way ANOVA test was performed for each strain and its due substrate (Ethidium Bromide and Antibiotic), followed by Tukey's post-hoc test (where $p < 0.05$ was considered significant). The statistical program GraphPad Prisma 5.0 was used for the statistical analysis.

2.5. Pharmacokinetic by the "drug-like" criteria and ADMET properties prediction

Using the plug-ins available in the MarvinSketch® code [31], the 2D structures of the chalcones were plotted and the properties of the ionization coefficient (pKa), lipophilicity coefficient (log P), distribution coefficient (log D) were calculated, polarity (PSA) and molecular weight (MW) to be used as pharmacokinetic descriptors.

The physicochemical properties of log P, PSA and MW were plotted on radar charts, according to representative models of Egan et al., [32, 33] and Ghose et al., [34] for human intestinal absorption (HIA) and activity in the central nervous system (CNS), to support the prediction of absorption and distribution models.

The molecules' SMILES were loaded on the pkCSM web server (http://biosig.unimelb.edu.au/pkcsm) to predict the absorption, distribution, metabolism, excretion and toxicity (ADMET) properties through an internal systematic graphic analysis of high efficiency [35].

The molecules were designed in the JSME molecular editor [36] implanted in the Pred-hERG 4.2 tool available on the LabMol © web server (http://predherg.labmol.com.br/predict), where they were toxic fragments with cardiotoxic potential through the inhibition of hERG channels were analyzed (human Ether-à-go-go-Related Gene).

2.6 Docking Procedure

The NorA protein model was created using the same procedure as described by Oliveira and collaborators [37].

The MepA model was generated by retrieving the protein sequence for the NCTC 8325 strain from the Uniprot database [38]. Then, the SWISS-MODEL service [39] 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 docking procedure was carried out using the Autodock Vina [40] software. The grid box defined as a 70 Å x 70 Å x70 Å box around the geometrical center of the model. Partial Gasteiger charges were added to the protein and to the ligand atoms, non-polar hydrogen atoms were mixed while all other parameters were kept at their default values. Docking pose was chosen based on the best binding score.

3. Results

3.1 Antibacterial activity

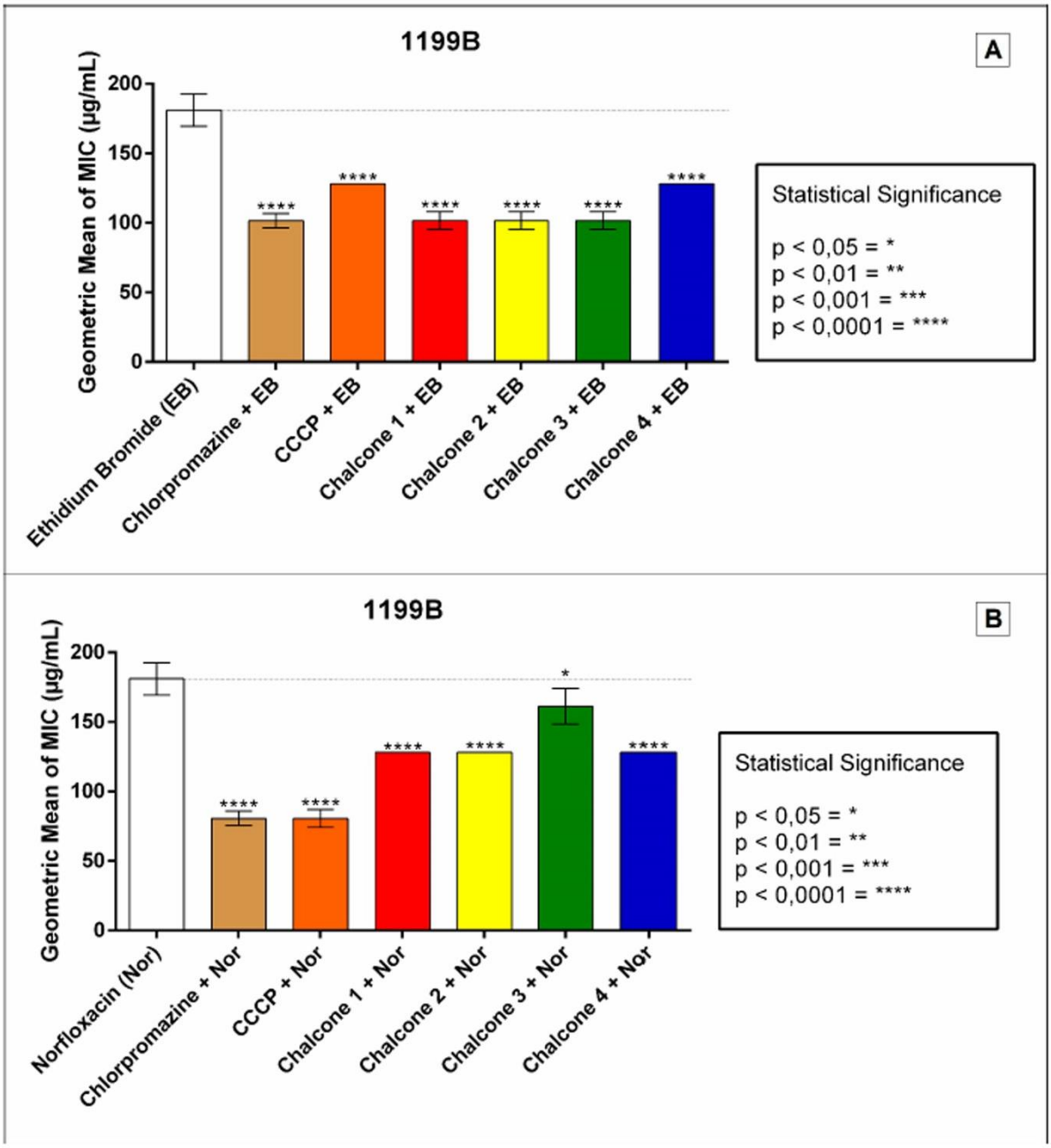
The antibacterial activity of chalcones is expressed in Table 1 as µg/mL. *In vitro* assays have shown that concentrations ≥1024 µg/mL have no clinical relevance on *Staphylococcus aureus* strains that are carriers [41, 42] or non-carriers [43, 44] of efflux pumps.

Table 1. Minimum Inhibitory Concentration (MIC) for chalcones 1-4.

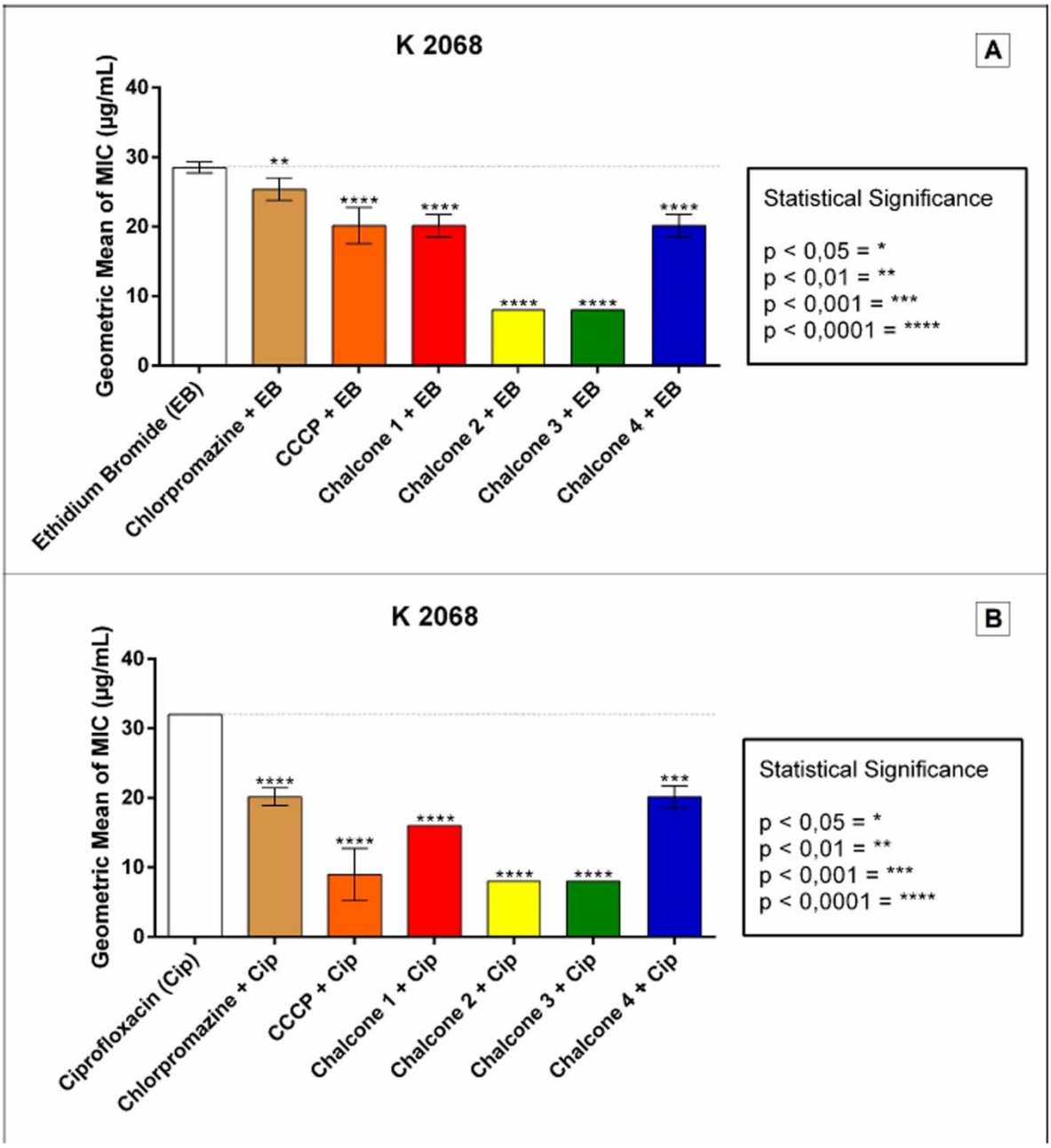
Substance	1199B	K2068
Chalcone 1	≥ 1024 µg/mL	≥ 1024 µg/mL
Chalcone 2	≥ 1024 µg/mL	≥ 1024 µg/mL
Chalcone 3	≥ 1024 µg/mL	≥ 1024 µg/mL
Chalcone 4	≥ 1024 µg/mL	≥ 1024 µg/mL

3.2 Resistance Reversal and Efflux Pump Inhibition

Chalcone modulation on the bacterial resistance mechanism concerning ethidium bromide and antibiotics is given in Figure 2 (strain 1199B carrying the NorA pump) and Figure 3 (strain k2068 carrying the MepA gene).



Legend: Strain 1199B carrying the NorA gene. A = Effect of chalcones on the efflux pump mechanism associated with Ethidium Bromide; B = Effect of chalcones on the mechanisms of resistance to the antibiotic Norfloxacin; * = express the significance of associations of chalcones in relation to controls, when $p < 0.05$.



Legend: K2068 strain carrying the MepA gene. A = Effect of chalcones on the efflux pump mechanism associated with Ethidium Bromide; B = Effect of chalcones on the mechanism of resistance to the antibiotic Ciprofloxacin; * = express the significance of associations of chalcones in relation to controls, when $p < 0.05$.

3.3 Phenotypic expression of the NorA and MepA bacteria

The phenotypic expression of the NorA and MepA bacteria, which overexpress efflux pumps was evaluated by assays with the ethidium bromide together with the chalcones against the Sa ATCC® 25923 strain that does not possess efflux pumps. The results of this test are presented in Table 2. The differences in the MICs of ethidium bromide in the different strains confirm the phenotypic difference from the strains that overexpress efflux pumps.

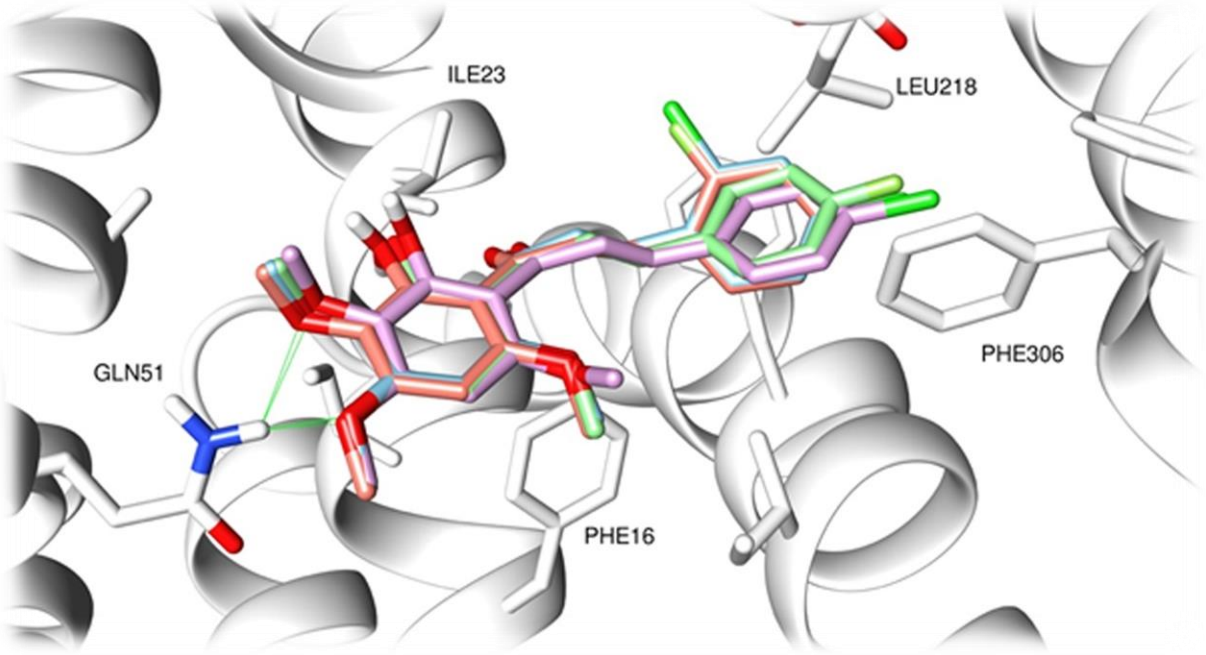
Table 2. MICs of the Ethidium Bromide in several *Staphylococcus aureus strains*.

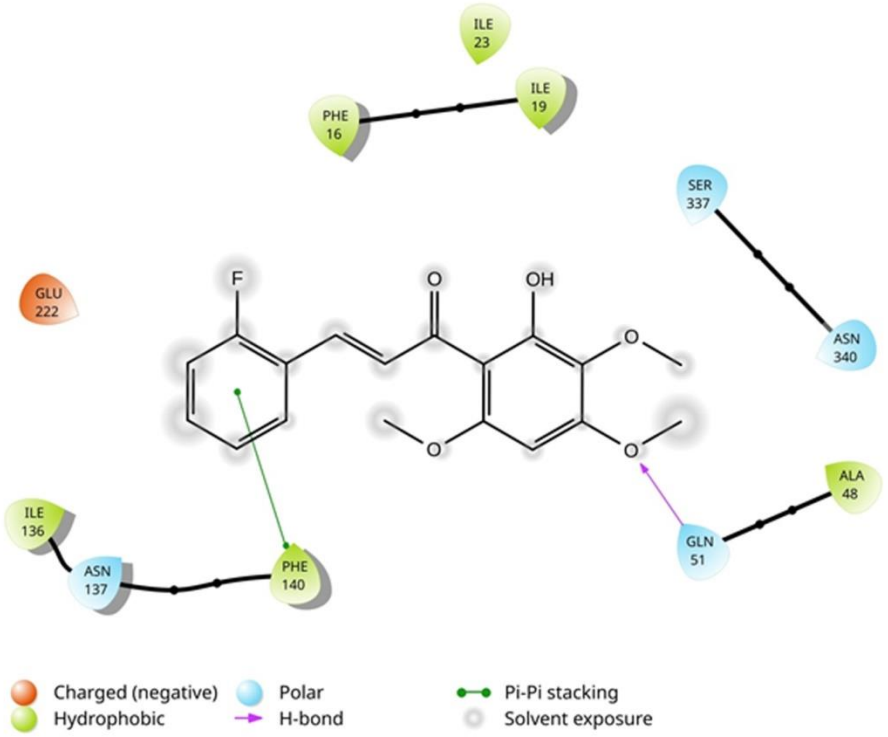
Substances	MICs for bacteria in values in µg/mL.		
	1199B (NorA)	**K2068 (MepA)**	**Sa ATCC® 25923**
Ethidium Bromide (EB)	181.5 ± 11.6	28.5 ± 0.8	5.04 ± 1.4
CPMZ + EB	101.6 ± 5.1 p < 0.0001	25.4 ± 1.6 p < 0.01	8.0 ± 0 p < 0.05
CCCP + EB	128.0 ± 0 p < 0.0001	20.2 ± 2.6 p < 0.0001	6.4 ± 0.4 p > 0.05

Legend: 1199B (NorA) = *Staphylococcus aureus* strain that expresses the NorA efflux pump; K2068 (MepA) = *Staphylococcus aureus* strain that expresses the MepA efflux pump; EB = Ethidium Bromide; CPMZ = Chlorpromazine; CCCP = carbonyl-m-chlorophenyl hydrazone cyanide. Sa ATCC® 25923 = *Staphylococcus aureus* American Type Culture Collection non efflux pump carrier; Values are described as mean of the triplicates ± standard deviation of the mean. Statistical significance is given according to the "p" values just below each association, in relation to the control.

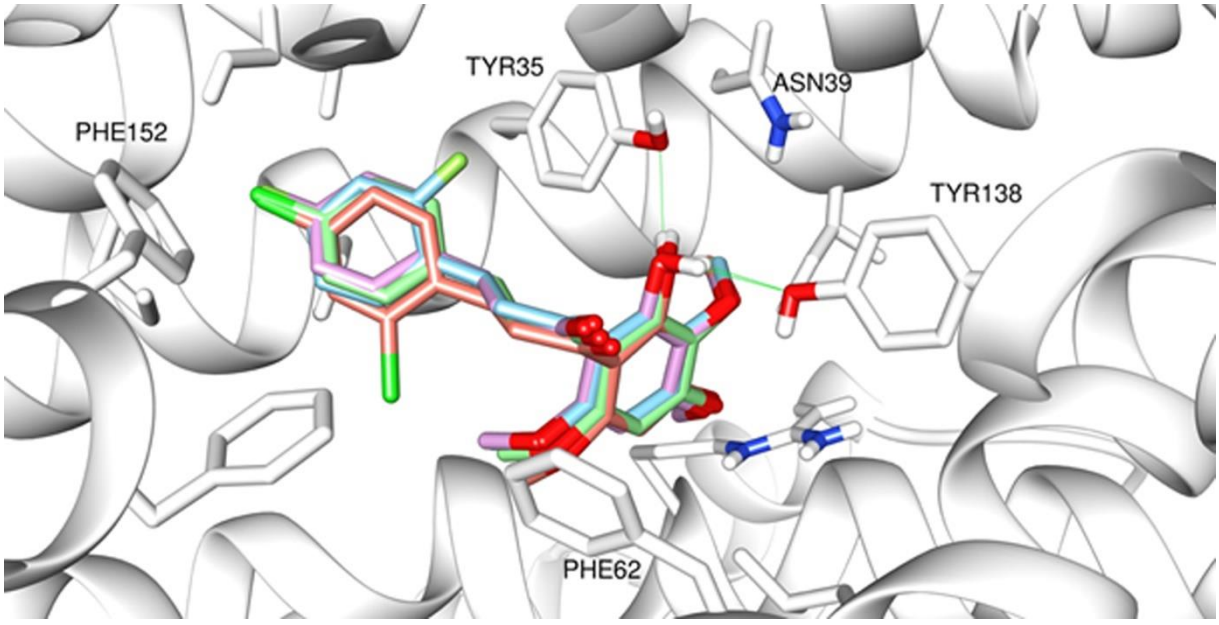
3.4 Molecular Docking

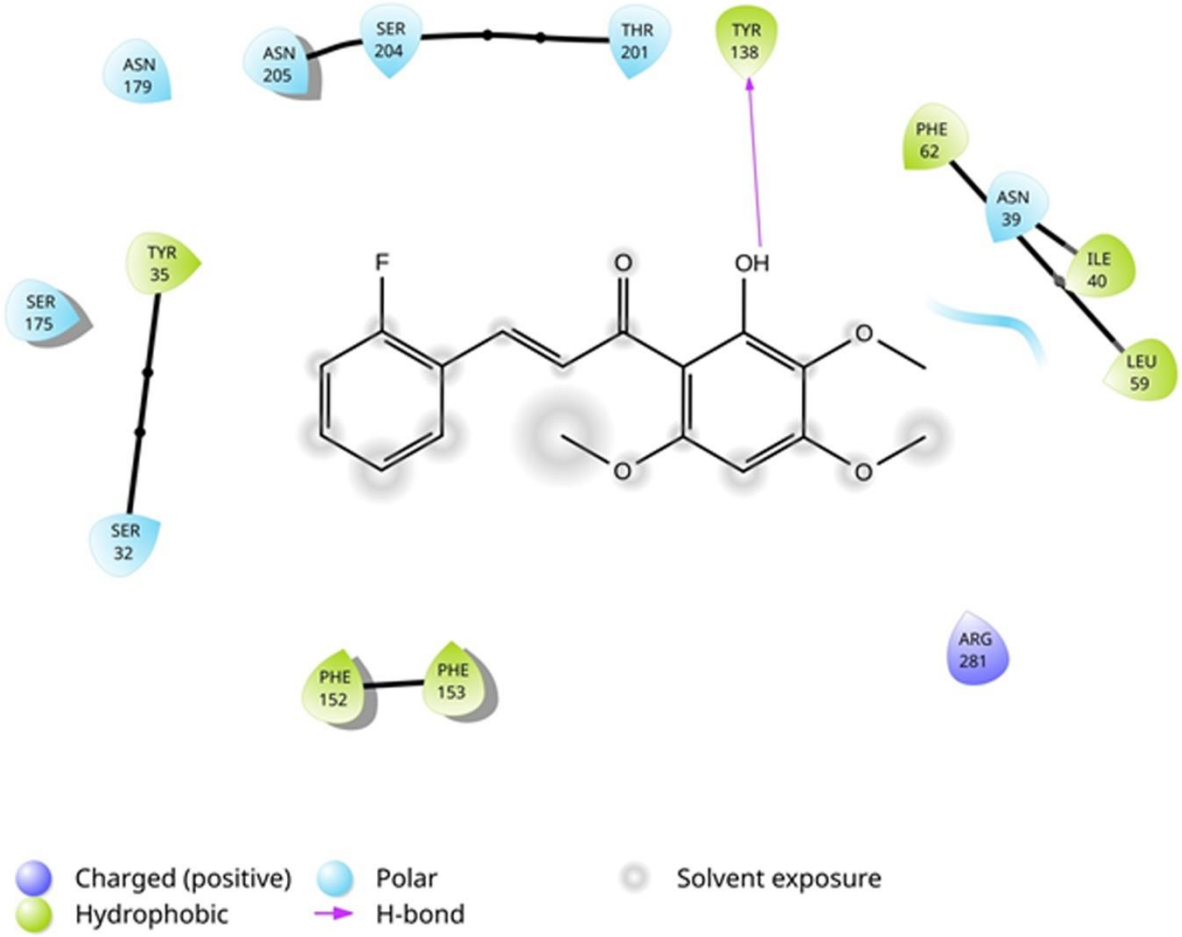
In order to gain some insights about the efflux pump inhibition mechanisms of the chalcones 1-4, two homology models were created (NorA and MepA) for a docking procedure. In Figure 4 shows the chalcones 1 to 4 (colored orange, green, pink and blue, respectively) docked to the binding site of a NorA model. hydrogen bonds are depicted in green. The 2D schematic diagram of protein-ligand interactions on the binding site of the NorA model for chalcone 1 is shown in Figure 5.





The results of molecular docking for chalcones 1 to 4 (colored Blue, pink, green and orange, respectively) in the MepA model are given in Figure 6. Whereas, the 2D schematic diagram of protein-ligand interactions on the binding site of the MepA model for chalcone 1 is shown in Figure 7.





3.5 Pharmacokinetic by the "drug-like" criteria and ADMET properties prediction

The calculation of theoretical physicochemical properties is fundamental in the process of discovering new oral drugs. Most drug-like criteria that refine bioactive compounds with pharmacological potential include properties such as molecular weight (MW), lipophilicity (log P), polarity (PSA), molar refractivity (MR) and molecular size by the number of atoms (NA) as determinants of pharmacokinetic behavior, providing information on solubility, permeability, absorption and distribution. Figure 8 shows the behavior pharmacokinetic for chalcones 1-4, which are evaluated on drug-like criteria. In Table 3 is given the physicochemical and pharmacokinetics parameter values for chalcones 1-4. Whereas the prediction of the metabolism, excretion and toxicity properties of the chalcones 1-4 is presented in Table 4.

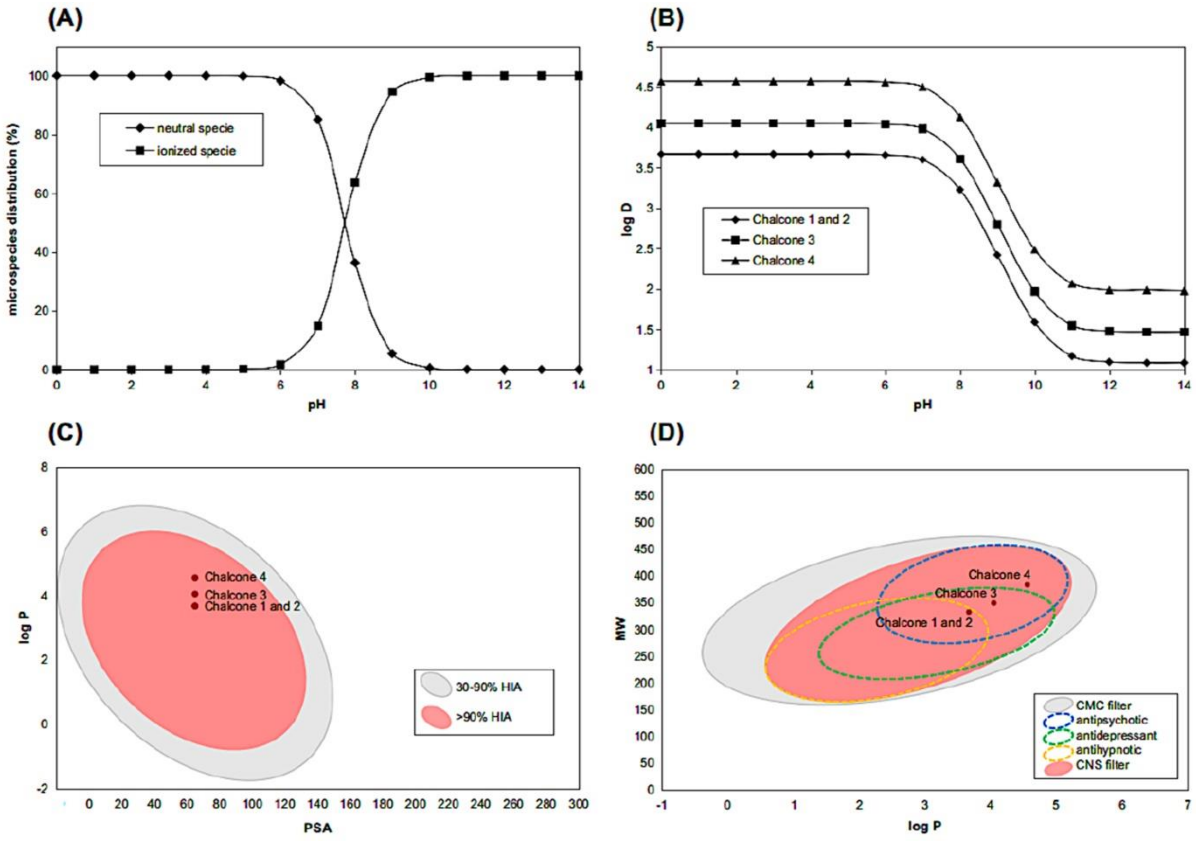


Table 3. Predicted physicochemical and pharmacokinetics properties of the chalcones 1-4.

Compound	Physicochemical properties					Absorption				Distribution		CNS activity	
name	Ka	log P	log $D_{7.4}$	PSA	MW	log P_{app}	%HIA	Pgp (s)	Pgp (i)	log VD_{ss}	Fu	log BB	log OS
Chalcone 1	7.76	3.90	3.75	64.99	332.32	1.15	96.08	No	Yes	-0.38	0.08	-0.05	-2.95
Chalcone 2	7.76	3.90	3.75	64.99	332.32	1.07	95.51	No	Yes	-0.42	0.08	0.20	-2.93
Chalcone 3	7.76	4.36	4.21	64.99	348.78	1.29	94.41	No	Yes	-0.22	0.03	0.32	-2.23
Chalcone 4	7.76	4.97	4.81	64.99	383.22	0.74	93.62	No	Yes	-0.13	0.03	0.26	-2.12

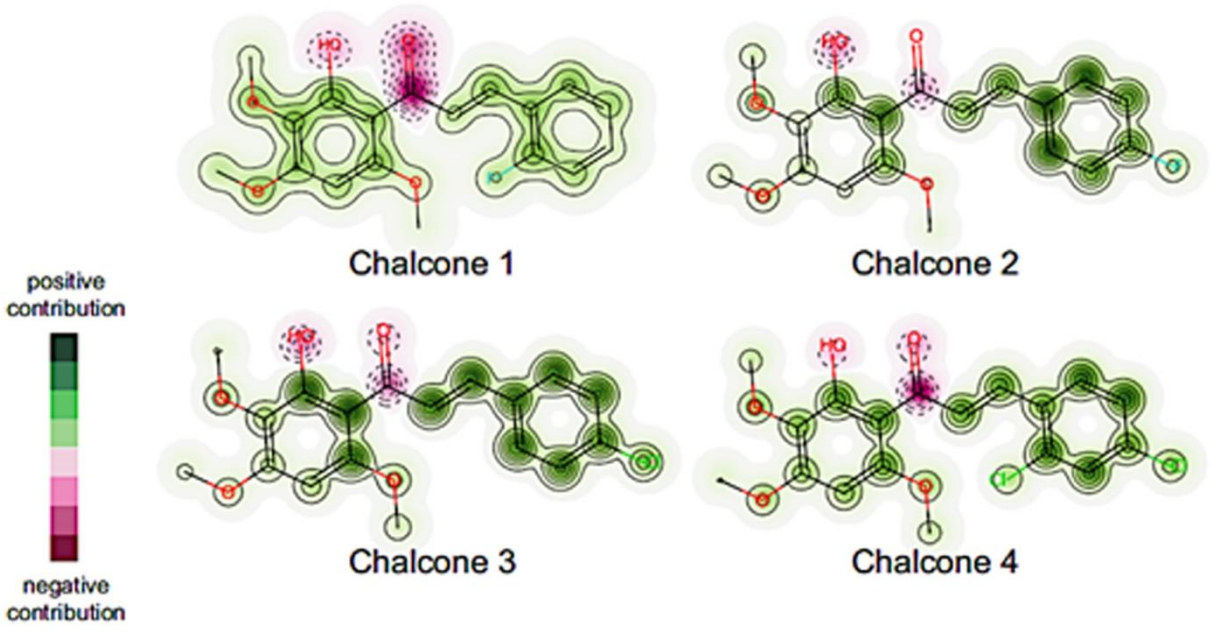
Note: pKa (ionization constant); log P (partitioning coefficient); log $D_{7.4}$ (distribution coefficient at pH 7.4); PSA (Polar Surface Area (Å²)); MW (Molecular Weight (g/mol)); log P_{app} (apparent permeability coefficient (cm/s)); %HIA (Human Intestinal Absorption percentage); Pgp (P-glycoprotein (s)(substrate) and (i)(inhibitor)); log VDss (steady state of volume distribution coefficient (L/kg)); Fu (Fraction unbound (score)); log BB (distribution in Blood-Brain coefficient); log PS (Permeability-Surface Area product coefficient)

Table 4. Predicted metabolism, excretion and toxicity properties of chalcones 1-4.

Compound Name	Metabolism							Excretion		Toxicity			
	CYP450 substrate		CYP450 inhibitor					log CLtot	OCT2(s)	LD_{50}	Hepatic	AMES	hERG(i)
	2D6	3A4	1A2	2C19	2C9	2D6	3A4						
Chalcone 1	No	Yes	Yes	Yes	Yes	No	Yes	0.24	No	2.46	No	Yes	No
Chalcone 2	No	Yes	Yes	Yes	Yes	No	Yes	0.14	No	2.40	No	Yes	No
Chalcone 3	No	Yes	Yes	Yes	Yes	No	Yes	0.52	No	2.49	No	No	No
Chalcone 4	No	Yes	Yes	Yes	Yes	No	No	0.45	No	2.67	No	No	No

Note: CYP450 (Cytochrome P450 isoenzymes); log CLtot (Total Clearance coefficient (mL/min/kg)); OCT2(s) (Organic Cation Transporter 2 substrate); LD50 (Lethal Dosage to death of 50% of a group of test animals (mol/kg)); AMES (AMES mutagenicity); hERG(i) (human Ether-à-go-go-Related Gene inhibition).

The analysis of the molecular fragments can help to identify toxic activity, depending on the toxicity model evaluated. Molecular fragments with inhibitory activity of hERG ion transport channels (human Ether-à-go-go-go-Related Gene) may indicate a cardiotoxic potential of a compound [63, 53]. In Figure 9, it is possible to observe the molecular fragments of the cardiotoxicity model of the chalcone 1-4.



4. Discussion

4.1 Antibacterial activity

In a study by Freitas et al. these chalcones were tested in standard and multi-resistant strains of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* [20]. Chalcone 2 showed antibacterial activity against the standard *S. aureus* 25923 strain and its effect was associated with the fluorine atom at position 4 of the B ring.

Regarding the multidrug-resistant *S. aureus 10* strain, which carries an efflux pump for ciprofloxacin, the results corroborate those of this article, considering that none of the 4 chalcones showed antibacterial activity.

Chalcones derived from the flavonoid isoliquiritigenin were tested against several strains of resistant *S. aureus*. One of the 6 tested chalcones showed important results against all strains, including SA 1199B. This chalcone had chlorine and fluorine atoms as ligands and caused damage to the bacterial cell membrane, increasing its permeability [45]. The divergent result may be due to the structural difference in the chalcone skeleton in relation to those used in this article.

The compound 2-hydroxy-3,4,6-trimethoxyacetophenone was used in the synthesis of two chalcones, HYTFURFURAL and HYTTHIOPHENE, which obtained the same MICs found in this test for both strains [46]. It was also used in the synthesis of two nitrogenated chalcones, tested for the NorA strain, with MICs $\geq$ 1024 μg/mL [47].

In another study, chalcones isolated from the flowers of *Arrabidaea brachypoda* did not show antibacterial activity against the strains of *S. aureus* 1199B and K2068, either [48]. The chalcones of the aforementioned study had a skeleton that was highly similar to the chalcones of this article, with differences regarding only the ring where the modifications occurred (A ring) and the fact that the modifications involved the addition of the hydroxyl ligand in some positions.

4.2 Resistance Reversal and Efflux Pump Inhibition

Ethidium bromide is a DNA intercalant that works as a substrate for several classes of efflux pumps [49]. The decrease in the Minimum Inhibitory Concentration (MIC) of ethidium bromide, when associated with sub-inhibitory concentrations of standard efflux pump inhibitors, is a widely used methodology when seeking to demonstrate the presence of pumps [50].

Based on the basic premise of the bromide test, one can observe that the bromide MICs in the presence of the standard inhibitors are reduced in relation to the control, and this reduction was statistically significant, thus demonstrating the presence of efflux pumps sensitive to chlorpromazine and CCCP. Therefore, we have established that the mechanism involved in the resistance of this strain to Bromide is the efflux pump mechanism. Notably, the 4 investigated chalcones can also significantly reduce the ethidium bromide MIC, demonstrating their effect on the efflux pumps.

There is no statistically significant difference related to the action of chalcones 1, 2 and 3 when compared to chlorpromazine. Chalcone 4, despite having an effect on the efflux pump, showed a poor performance when compared to the other chalcones. This difference in activity brings us to its structural difference. This chalcone has two chlorine atoms at positions 2 and 4 of the B ring. This represents a single difference in structure when compared to chalcone 3, which has 1 chlorine atom at position 4 of the B ring. Thus, we propose that the position and number of ligands are essential for their activity in the bacterial resistance mechanism.

The 1199B strain is due to a mutation in a parental strain (1199), showing an overexpression of the NorA gene, in addition to possible combinations with other resistance mechanisms arising from the parental strain [21].

Significant differences ($p < 0.0001$) between the associations of standard inhibitors with norfloxacin when compared to antibiotic controls confirmed that the overexpression of the NorA gene in this strain is an important resistance mechanism; however, one cannot rule out other involved mechanisms. The results demonstrated that all chalcones had synergism with the antibiotic agent, reducing the amount of antibiotic required to obtain an inhibitory effect on bacterial growth.

As the action of chalcones on NorA efflux pumps has been previously demonstrated, this mechanism is certainly affected by them in the antibiotic test. Chalcones 1, 2 and 4 showed the best synergistic effects when compared to each other. Chalcone 3 showed loss of effectiveness when compared to itself in the bromide test and Chalcone 4 showed improvement, when using this same analysis. This allows us to infer that, when changing the substrate for the NorA pump, the structures once again play an important role in reversing the resistance regarding the affected mechanisms.

The presence of other mechanisms may justify the improvement in chalcone 4 activity, as it may be showing its performance in two mechanisms at the same time. The reduction in the effect of chalcone 3 can demonstrate that the other mechanisms are important

resistance factors against fluoroquinolones and, possibly, that its action on efflux pumps is not sufficient to reduce the minimum inhibitory concentration comparable to other chalcones.

Silva and collaborators [46] carried out an experiment involving chalcones also derived from 2-hydroxy-3,4,6-trimethoxyacetophenone, and they observed an antagonistic effect in the ethidium bromide test for strain 1199B and a synergistic effect for the association with Norfloxacin. This demonstrated the presence of other mechanisms associated with antibiotic resistance in this strain and, therefore, possible differences in the modulating activity of chalcones, once again supporting the variation of results in this study.

Two nitrogenated chalcones, with the ligand NO_2 in positions 3 and 4 respectively, acted synergistically to ethidium bromide against the NorA strain; however, they antagonized Norfloxacin [47]. This fact reinforces the presence of other resistance mechanisms associated with the antibiotic agent, as well as the importance of ligand action in these mechanisms. Ligands that inhibit the NorA efflux pump might not inhibit other resistance mechanisms, just as ligands that inhibit the other associated mechanisms might not inhibit the efflux pump.

A study with the *S. aureus* 1199B strain showed that the IMRG4 chalcone was able to inhibit the NorA efflux pump in the ethidium bromide test, with an effect similar to that of the inhibitor reserpine. Moreover, it was also able to reduce the MIC of the tested antibiotic by 16 fold when associated with Norfloxacin and by 4 fold when associated with Ciprofloxacin, by inhibiting efflux pumps associated with these antibiotics [45].

A series of 4 chalcones, tested at two subinhibitory concentrations (MIC/4 and MIC/8), against the 1199B strain, was able to synergistically modulate both ethidium bromide and the antibiotic Norfloxacin, with one of them reaching rates similar to the standard inhibitor chlorpromazine on both occasions [48], showing the role of chalcones in both the efflux mechanism and in secondary mechanisms related to the resistance of this strain.

The K2068 strain is a *Staphylococcus aureus* strain that has a mutation in the Operon where the MepA gene is located; however, it does not have a topoisomerase mutation related to its resistance profile. Thus, the resistance demonstrated by this lineage to biocidal agents of different classes and to fluoroquinolones, is due to the overexpression of efflux pumps [22].

The presence of these efflux pumps is demonstrated by the decrease in MICs of ethidium bromide when associated with chlorpromazine and CCCP. All chalcones show synergism for ethidium bromide, so all of them also act as inhibitors of MepA efflux pumps.

Chalcones 1 and 4 showed a higher level of activity on the MepA pump than the standard inhibitor Chlorpromazine and comparable to the standard inhibitor CCCP. However, statistically, the activities of chalcones 2 and 3 were even more remarkable, reducing the MIC

of ethidium bromide to less than half of the CCCP + bromide association. Chalcone 2 has the same altered ligand as Chalcone 1, varying only its position in the B ring (position 4). Chalcone 3 has the same ligand as Chalcone 4, varying its quantity and location in the B ring. Chalcone 3 has Chlorine as a ligand in position 4, whereas Chalcone 4 has one additional chlorine atom in position 2. When analyzing the chalcones that have the same ligand, one can infer that position 4 of the B ring, regardless of the halogen atom, is associated with the blockade of the efflux mechanism in MepA.

When the substrate was changed to the fluoroquinolone Ciprofloxacin (Figure 3-B), we found basically the same results for the ethidium bromide substrate. All chalcones were able to reverse bacterial resistance to this fluoroquinolone and once again chalcones 2 and 3 were the ones that showed the best results in relation to the standard inhibitors and between themselves.

As the K2068 strain has no additional fluoroquinolone resistance mechanisms, this result supports the findings in the ethidium bromide test and reinforces position 4 of the B ring as the most active on efflux pumps in this strain.

4.3 Phenotypic expression of the NorA and MepA bacteria

According to the results of MICs of the Ethidium Bromide in several *Staphylococcus aureus strains* (Table 2), there was an antagonism when the ethidium bromide was associated with the chlorpromazine and in the case of the CCCP there was no statistical significance when compared to the control. In both cases, it was demonstrated the absence of efflux pumps in the referred strain. The 1199B and K2068 strains showed MICs 36x and 5x higher than the strain that does not possess efflux pump, demonstrating a phenotypic change in relation to standard strain (an Sa ATCC® 25923) due to a differential mechanism of resistance between them, demonstrating the expression of the NorA and MepA efflux pumps. When chalcones were added to ethidium bromide at their subinhibitory concentrations against Sa ATCC® 25923 strain, they were not capable of modulating the activity of the ethidium bromide (Table 2), showing MICs statistically non-significant in relation to the control, reinforcing the data of interference in the efflux pumps in the 1199B and K2068 strains.

A study by Rezende-Júnior et al. [48] tested a chalcone that had been previously selected for its synergistic action to norfloxacin and ethidium bromide in a *S. aureus* 1199B strain, against a strain carrying the MepA gene. In that study, the chalcone was able to reduce the MIC of bromide by 2 fold and the MIC of the antibiotic agent by 4 fold, demonstrating the effect of chalcones on efflux mechanisms.

A synthetic chalcone, derived from the same natural product (2-hydroxy-3,4,6-trimethoxyacetophenone) of the chalcones analyzed in this article, also showed synergistic activity when associated with the antibiotic Ciprofloxacin and ethidium bromide in *in vitro* tests against the K2068 strain, showing effects that were comparable to the standard CCCP inhibitor [51]. This same result was found for 2 of the 4 chalcones in this article, both with modified ligands at position 4.

The chalcones HYTFURFURAL and HYTTHIOPHENE, mentioned before, were also tested for their capacity to reverse resistance in the K2068 strain. These two chalcones, which were not able to inhibit NorA efflux pumps, inhibited the MepA efflux pumps in the ethidium bromide test, in addition to showing synergistic activity for the antibiotic Ciprofloxacin (Silva et al., 2021). These findings point to the structural difference between the NorA and MepA efflux pumps, further demonstrating the fact that all chalcones in this article were able to inhibit the two tested efflux pumps. The A ring of HYTFURFURAL and HYTTHIOPHENE were exactly the same as the A ring of the 4 chalcones tested here, the difference being in the B ring. One can infer that the B-ring structure is essential for the activity of reversing bacterial resistance in multiple classes of efflux pumps and differences in the quality of ligands, as well as in their position associated with the B ring, are also crucial factors for the obtained results.

4.4 Molecular Docking

Regarding the NorA model, the best poses of all chalcones interact with essentially the same residues of the binding site of the model. All chalcones interact through hydrogen bonds with residue Gln51. Two of the methoxy groups serve as hydrogen bond acceptors, as seen in Figure 4. Hydrogen bonding with this residue appears to be associated with efflux pump inhibition of the NorA efflux pump [46, 52]. Also notable are Pi-Pi stacking interaction with residue Phe140. Additionally, there are close contacts with residues Ile136, Asn127, Phe16, Asn340 and many others, as seen in Figure 5.

The best poses of the chalcones on the MepA model also cluster together, as seen in Figure 6. The hydroxyl groups are positioned such that they may act as hydrogen bond donors with either Tyr35 or Tyr138. The position of the chalcones on the binding site match that of other chalcones with MepA inhibiting properties, as described in Rezende-Junior et al. [48]. In particular, there are close contacts with residues Ser32, Phe15, Asn179 and others, as seen in Figure 7.

As the best poses of all chalcones bind to the binding sites of the efflux pump models in the same fashion as other chalcones with efflux pump inhibition capabilities, one could argue that these chalcones could indeed act as efflux pump inhibitors.

4.5 Pharmacokinetic by the “drug-like” criteria and ADMET properties prediction

As a filter that combines the "drug-like" methods used in recent years, molecules with a neutral physiological charge, not so lipophilic and with polarity in a band that favors transport and effective penetrability in biological membranes, as characteristics of a good oral bioavailability [32, 34, 53-55].

The calculated pKa value in the order of 7.76 for Chalcone analogs 1-4 indicates a balance between the neutral molecular volume (neutral specie) and the weakly formed acid species (ionized specie), which moves towards the acid species as the most dominant only at pH levels above 7.7. Thus, it is possible to observe in the graph of Figure 8(A) the dominance of the neutral species in physiological pH levels (approximately 7.4), following a concentration in the order of 69.47%.

In order to define the chemical balance between solubility and permeability, the distribution coefficients (log D) of the micro species of the Chalcone analogs 1-4 were calculated. Directly related to lipophilicity, log D represents the degree of distribution of a compound at different pH levels, as a function of the dominant microspecies. Thus, log D values at pH 7.4 below 3 represent the best absorption and distribution conditions [56, 57]. In the graph of Figure 8B it is possible to observe this behavior as a function of pH, where log values D7.4 follow the order Chalcone 4> Chalcone 3> Chalcone 1 and 2 suggests that analogs 1 and 2 are closer to the ideal range of absorption and distribution, at the same time as the log Papp values of 1.15 and 1.07 (respectively) reflect the difference in permeability between the benzene substituents Ph-*o*-F and Ph-*p*-F of these substances, in addition to indicating the permeability in colon adenocarcinoma cells (Caco2) are organized as follows: Chalcone 3> Chalcone 1> Chalcone 2> Chalcone 4 (Table 3).

The use of statistical analyzes based on molecular descriptors is a fundamental tool in the discovery of new drugs. The graphic models developed by Egan et al., [33] use the lipophilicity (log P) and polarity (PSA) descriptors of compounds tested by Pharmacopeia, Inc. to refine compounds with high human intestinal absorption (HIA). Thus, the ellipse formed by positive log P values greater than 5.88 and PSA less than 131.6 Å² correspond to compounds in the database with intestinal absorption> 90%. This representation can be seen

in Figure 8C, with the indicators generated by the log P vs. PSA values of the Chalcone 1-4 analogs. The molecules obtained the same PSA value of 64.99 Å², where the absorption was defined by the log P values that vary between 3.90-4.97, within the absorption ellipse> 90% (Fig. 8C). The log P values of the compounds organized in the order Chalcone 4 (4.97)> Chalcone 3 (4.36)> Chalcone 1 and 2 (3.90) suggest the lowest values with the highest HIA of this prediction, organized in the order Chalcone 1 (96.08%)> Chalcone 2 (95.51%)> Chalcone 3 (94.41%)> Chalcone 4 (93.62%) (Table 3). However, the compounds have reduced bioavailability because they are P-glycoprotein (Pgp) inhibitors, which reduces their transport and efflux potentials through biological membranes and their respective distribution volumes (VD), with VDss log values that vary between -0.42 and -0.13 L / kg, where the molecular fraction not bound to plasma proteins (Fu) follows an order of 0.08 for chalcones 1 and 2 and 0.03 for chalcones 3 and 4.

Quantitative “drug-like” characterization by Ghose et al., [34] uses compounds from the Comprehensive Medicinal Chemistry (CMC) database and constitutes a “drug-like” library Efficient in the discovery of new drugs. The graph in Figure 8D is a radar with ellipses defined by the qualification range that governs 80% of CMC compounds with activity in the central nervous system (CNS) through the molecular log P and molecular weight descriptors. The ellipse formed between the log P intervals between 0.5 and 5.2 and molecular weight between 162 and 464 encompasses compounds with antihypnotic, antidepressant and antipsychotic activity. Thus, the combination of the log P values and the molecular weight of the chalcones 1-4 is defined in the CNS activity area (Fig. 8D), where the log BB values between -0.05 and 0.32 suggest that the compounds permeate moderately the blood-brain barrier (BBB). Thus, the predicted values of the product coefficients permeability-surface area (log PS) between -2.95 and -2.12 indicate a moderate absorption of these molecules in the CNS (Table 3).

With the prediction of the interaction with cytochrome P450 isoenzymes, it was possible to define the metabolic profile by oral administration of Chalcone 1-4 analogs. As responsible for the phase I metabolism of drugs, they perform the redox reactions, such as the reactions of N- and O- dealkylations by the enzyme CYP3A4 and the biotransformations made by CYP1A2, constituting chemical entities more easily eliminated [58-62]. The prediction showed that all compounds tested are potential inhibitors of the enzymes CYP1A2, CYP2C19 and CYP2C9, in addition to not being substrates of the renal organic transport protein (OCT2) constituting compounds with a slow renal elimination route, where the total release coefficient (log CLtot) between 0.14 and 0.45 mL / min / kg. However, it is curious to

note that Chalcone 4 is the only substrate and non-inhibitor of CYP3A4 in this test, which suggests that the compound does not present the risk of metabolic activation hepatotoxicity among the other compounds, with the highest LD_{50} obtained in the test. 2.67 mol/ kg, at the same time that the chalcones 1 and 2 obtained the lowest LD_{50} values and, therefore, present a toxic risk by administration, testing positive for mutagenic toxicity (Table 4).

In the analysis of the molecular fragments of hERG blockage of the chalcones 1-4 (Figure 9), it is interesting to highlight the positive contributions (green region) of the halogen substituents Ph-oF from Chalcone 1, Ph-*p*-F from Chalcone 2, Ph-*p*-Cl from Chalcone 3 and Ph-*o,p*-Cl2 from Chalcone 4 as non-inhibitors hERG channels. Despite the negative contributions (pink region) associated with the phenolic hydroxyl and carbonyl group, it is not superimposed on the positive contributions and, therefore, are chemical entities without cardiotoxic risk.

5. Conclusion

The tested chalcones do not have antibacterial activity; however, they are capable of inhibiting efflux pumps for the 1199B and K2068 strains. They promote synergism of the norfloxacin antibiotic by inhibiting associated efflux pumps, as well as other associated mechanisms. Different resistance mechanisms require different ligands in their reversion; the position and quantity of the ligands are essential for their activity in the bacterial resistance mechanism. The best effects on the MepA efflux pump were observed when the ligands were at position 4 of the B ring. Therefore, the B-ring substituents may be related to the inhibition activity of multiple efflux pumps. Through the molecular descriptors of lipophilicity, polarity and molecular weight of the “drug-like” statistical models, it was possible to predict the pharmacokinetic behavior of intestinal absorption and CNS activity. Chalcone 1-4 are well absorbed in the intestine, but with a decrease in their bioavailability, resulting in a low volume of distribution in the blood plasma, in addition to having a mild CNS activity. However, Chalcone analogs 3 and 4 were not toxic due to metabolic activation. Whereas the Chalcone 1 and 2 present a mutagenic risk, depending on the oral dose administered. In conclusion, the Chalcone 1-4 substituting halide groups are non-inhibitory molecular fragments of hERG ion transport channels, generating no toxic risk due to the accumulation of the drug in the blood. Our results indicated that all chalcones bind to the binding sites of the efflux pump models in the same fashion as other chalcones with efflux pump inhibition capabilities, one could argue that these chalcones could indeed act as efflux pump inhibitors.

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CAPTION FOR THE FIGURES:

Figure 1. Structural representation of the chalcones 1-4 derived of acetophenone isolated from *Croton anisodontus* Müll.Arg.

Figure 2. Effect of the chalcones 1-4 on the NorA efflux pump.

Figure 3. Effect of the chalcones 1-4 on the MepA efflux pump.

Figure 4: Chalcones 1 to 4 (colored orange, green, pink and blue, respectively) docked to the binding site of a NorA model. hydrogen bonds are depicted in green.

Figure 5. 2D protein-ligand interaction diagram of chalcone 1 docked on the binding site of the NorA model.

Figure 6. Chalcones 1 to 4 (colored Blue, pink, green and orange, respectively) docked to the binding site of a MepA model. Hydrogen bonds are depicted in green.

Figure 7. 2D protein-ligand interaction diagram of chalcone 1 docked on the binding site of the MepA model.

Figure 8. (A) graph of microspecies distribution and **(B)** distribution coefficient (log D) of chalcones 1-4 as a function of the pH variation; **(C)** human intestinal absorption radar by the Egan-egg models and **(D)** CMC filter by the Ghose criteria to CNS activity.

Figure 9. Molecular fragments of hERG blockage of the chalcones 1-4.

CAPTION FOR THE TABLES:

Table 1. Minimum Inhibitory Concentration (MIC) for chalcones 1-4.

Table 2. MICs of the Ethidium Bromide in several *Staphylococcus aureus strains*.

Table 3. Predicted physicochemical and pharmacokinetics properties of the chalcones 1-4.

Table 4. Predicted metabolism, excretion and toxicity properties of chalcones 1-4.

CAPÍTULO 4: CONSIDERAÇÕES FINAIS

4.1 DISCUSSÃO GERAL

Os efeitos antibacterianos das chalconas foram similares quando comparou-se os resultados em ambos os artigos. As chalconas não apresentaram efeito antibacteriano sobre as cepas resistentes de *Staphylococcus aureus* em ambos os testes, porém conseguiram modificar a ação antibiótica nos dois cenários retratados.

No primeiro artigo desta dissertação todas as chalconas potencializaram o efeito do antibiótico ciprofloxacina contra a cepa de *Staphylococcus aureus* multirresistente, repetindo o padrão no segundo artigo, com a cepa MepA, que apresenta um mecanismo de bomba de efluxo associado. A cepa *S. aureus* 10 utilizada no primeiro artigo também apresentava bombas de efluxo sensíveis à clorpromazina. A associação destas informações reforça os achados de ambos os artigos sobre o mecanismo de atuação das chalconas, bem como o fato de que o antibiótico usado influencia no efeito de reversão, pois, apesar de todos potencializarem a ação antibiótica, os efeitos individuais se diferenciam.

A hipótese levantada no primeiro artigo quanto a melhor atuação da chalcona 4 (2,4-dicloro) sobre bombas de efluxo, não se confirmou nos testes mais específicos realizados no segundo artigo. Isolando os resultados com o Brometo de etídio (substrato específico para as bombas de efluxo em análise) para as duas cepas com mecanismo de efluxo e comparando com os resultados nas cepas multirresistentes do primeiro artigo, foi possível inferir que a posição 4 do anel B é de relevância para a inibição de bombas de efluxo, suscitando a hipótese levantada no primeiro artigo sobre a presença de bombas de efluxo insensíveis à clorpromazina na *Pseudomonas aeruginosa* utilizada no teste. Uma vez que houve um melhor efeito do antibiótico gentamicina quando associado à chalcona 3, em relação ao controle antibiótico, no modelo de *Pseudmonas aeruginosa*, foi entendido que o átomo de cloro na posição 4 do anel B seria o substituinte relacionado com a maior presença de potenciações antibióticas.

Reforça-se deste modo, a relevância do tipo de átomo e da posição do substituinte para a obtenção de um efeito de reversão da resistência. Também influenciam a reversão da resistência, o antibiótico utilizado, bem como a cepa sobre a qual o antibiótico exerce o seu efeito.

4.2 CONCLUSÕES GERAIS

- As chalconas testadas não apresentam atividade antibacteriana intrínseca;
- As chalconas apresentam a capacidade de reversão da resistência bacteriana à classe das fluoroquinolonas (ciprofloxacina) e aminoglicosídeos (gentamicina) no modelo Gram-positivo (*Staphylococcus aureus*) e à classe dos aminoglicosídeos (gentamicina) nos modelos Gram-negativos (*Escherichia coli* e *Pseudomonas aeruginosa*);
- O mecanismo de bomba de efluxo é afetado pelas chalconas, mas a eficiência do bloqueio deste mecanismo depende da cepa, bem como do substrato utilizado;
- A capacidade das chalconas de reverterem os mecanismos de resistência bacteriano se altera com o tipo de substituinte, bem como da posição do substituinte;
- A posição 4 do anel B mostrou-se importante para a reversão do mecanismo de resistência de efluxo.

4.3 PERSPECTIVAS DE INVESTIGAÇÕES FUTURAS

Este trabalho poderá ser complementado futuramente com técnicas que consigam mensurar os níveis de mRNA das bombas de efluxo NorA e MepA, além de Western blots para demonstrar os níveis de proteína. Desta forma, uma comprovação mais sensível demonstrará de forma inequívoca a inibição destas bombas de efluxo, ao comparar os níveis de mRNA e de proteínas entre as bactérias que sofreram a ação das chalconas e as bactérias do controle. Métodos de fluorimetria para determinar o acúmulo do Brometo de etídio no interior da célula bacteriana também será efetivo para investigações futuras. Outro teste de importância é a microscopia eletrônica, com o objetivo de verificar danos na estrutura bacteriana principalmente na membrana. Quanto as possibilidades mutagênicas apontadas nos estudos de ADMET, testes com performance *in vivo* das chalconas também poderão ser executados. Métodos utilizando camundongos ou *Drosofila melanogaster* podem oferecer apoio à discussões futuras. Outro modelo bastante utilizado em testes de toxicidade é o peixe zebra-fish (*Danio rerio*), cujos embriões apresentam genes homólogos aos de humanos. Outro ponto de importante avanço em uma perspectiva futura são experimentos *in vivo* do potencial

antibacteriano das chalconas como adjuvantes (reversão dos mecanismos de resistência), em tratamentos clínicos. Para isto, o modelo *Danio rerio* também pode ser bastante útil.

APÊNDICE 1 – MATERIAL SUPLEMENTAR DO ARTIGO PUBLICADO NA REVISTA: FEMS Microbiology Letters, 2020, Vol. 367, No. 15; FATOR DE IMPACTO 1,987 – QUALIS B2

Direct antibacterial and antibiotic resistance modulatory activity of chalcones synthesized from the natural product 2-hydroxy-3,4,6-trimethoxyacetophenone

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SUPPLEMENTARY MATERIAL

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Figure S1: *IR spectrum of compound* the chalcone (*E*)-3-(2-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

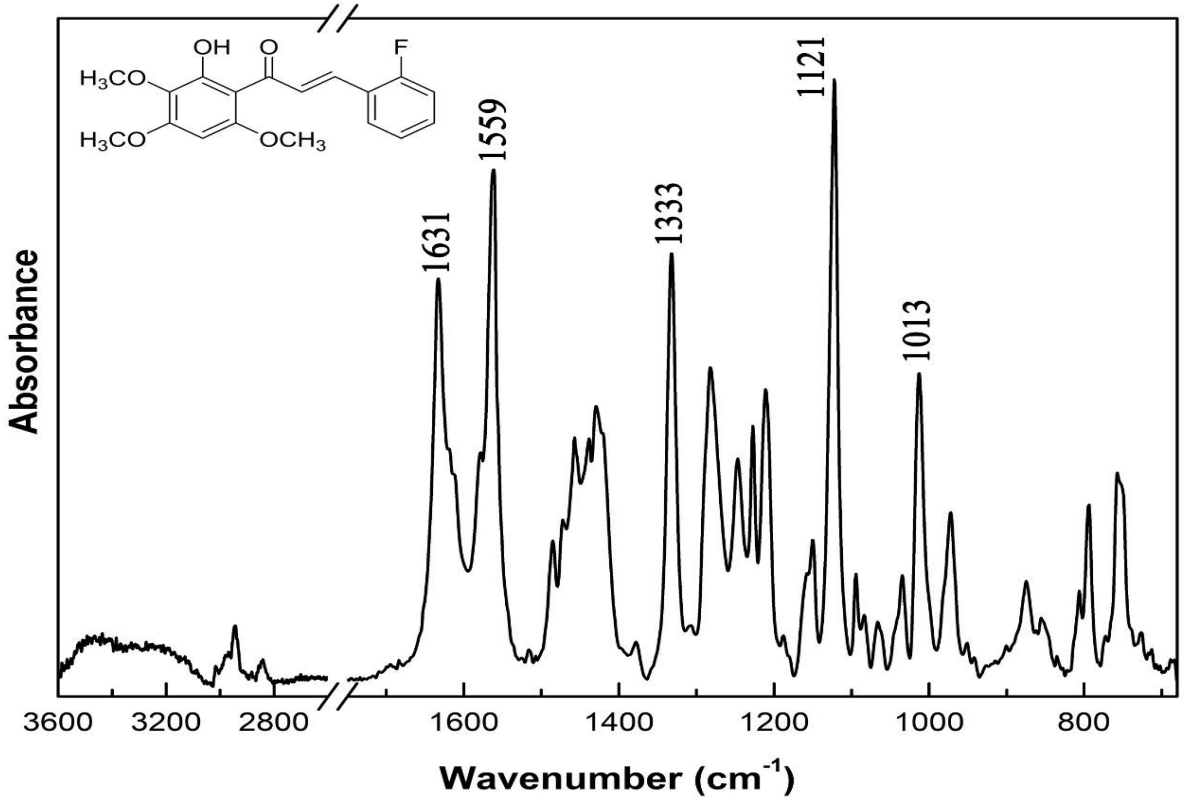

Figure S2: ^{1}H NMR spectrum of the chalcone (*E*)-3-(2-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

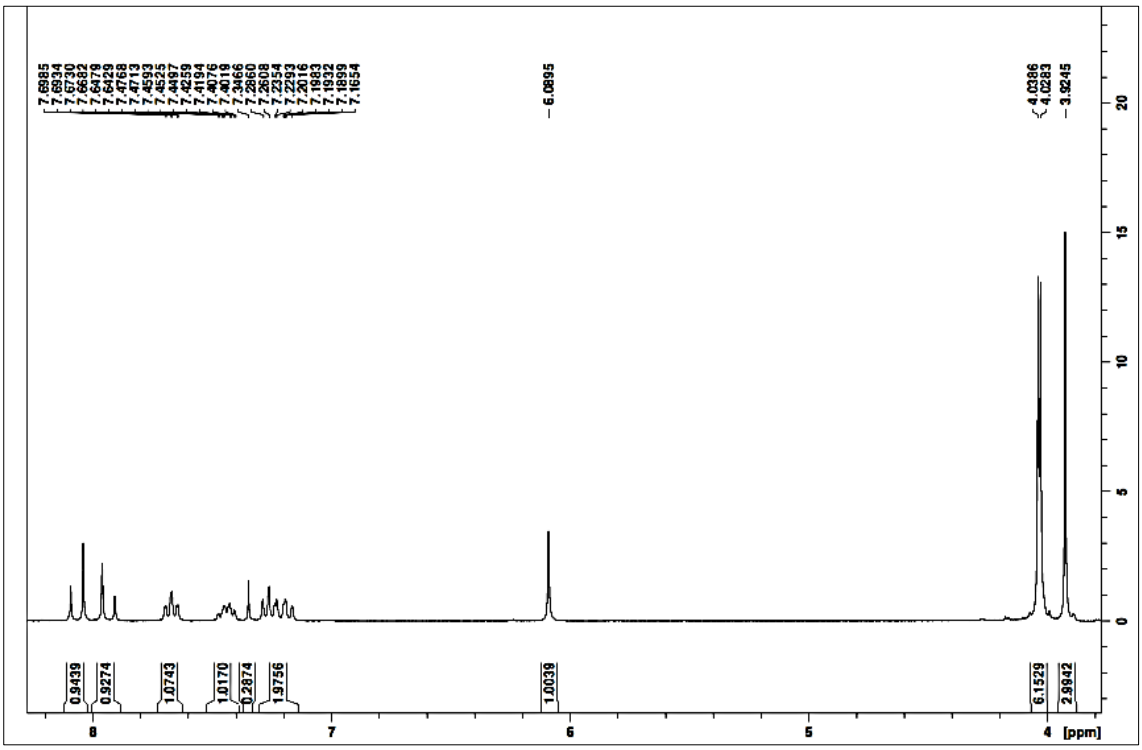

Figure S3: Expansion of the ^{1}H NMR spectrum of chalcone (*E*)-3-(2-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

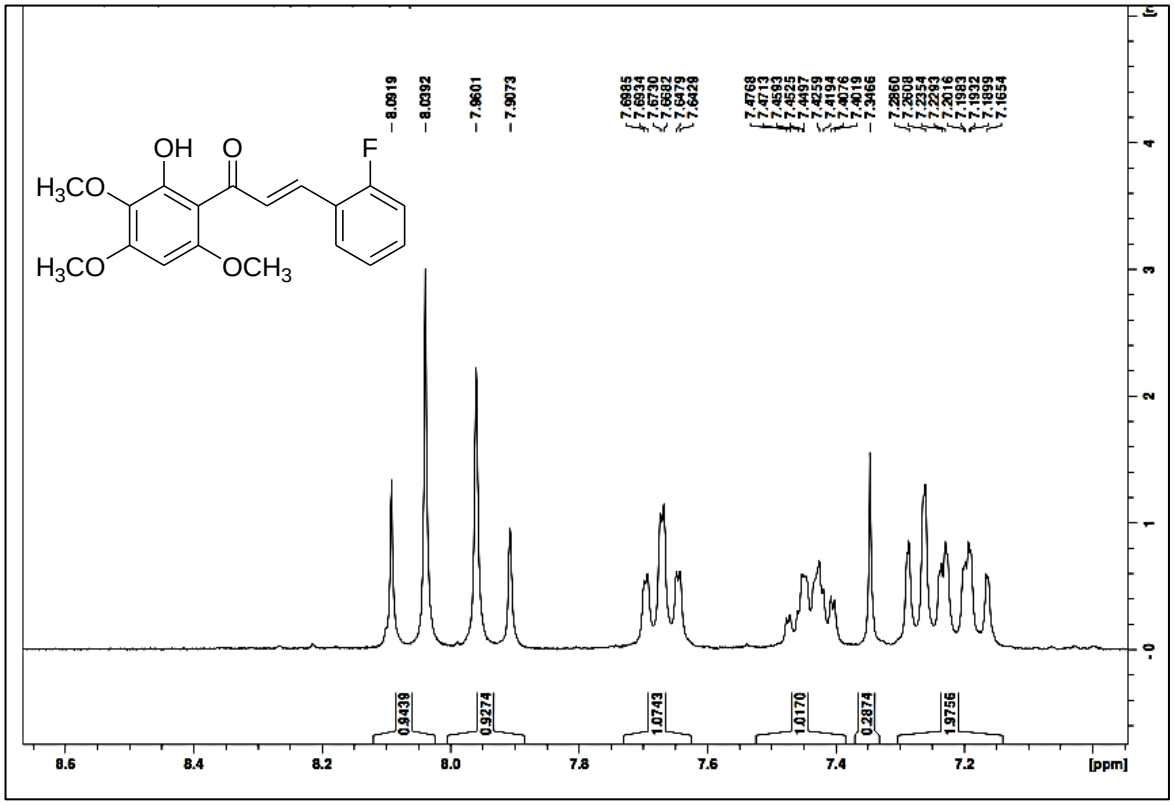

Figure S4: ^{13}C NMR spectrum of the chalcone (*E*)-3-(2-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

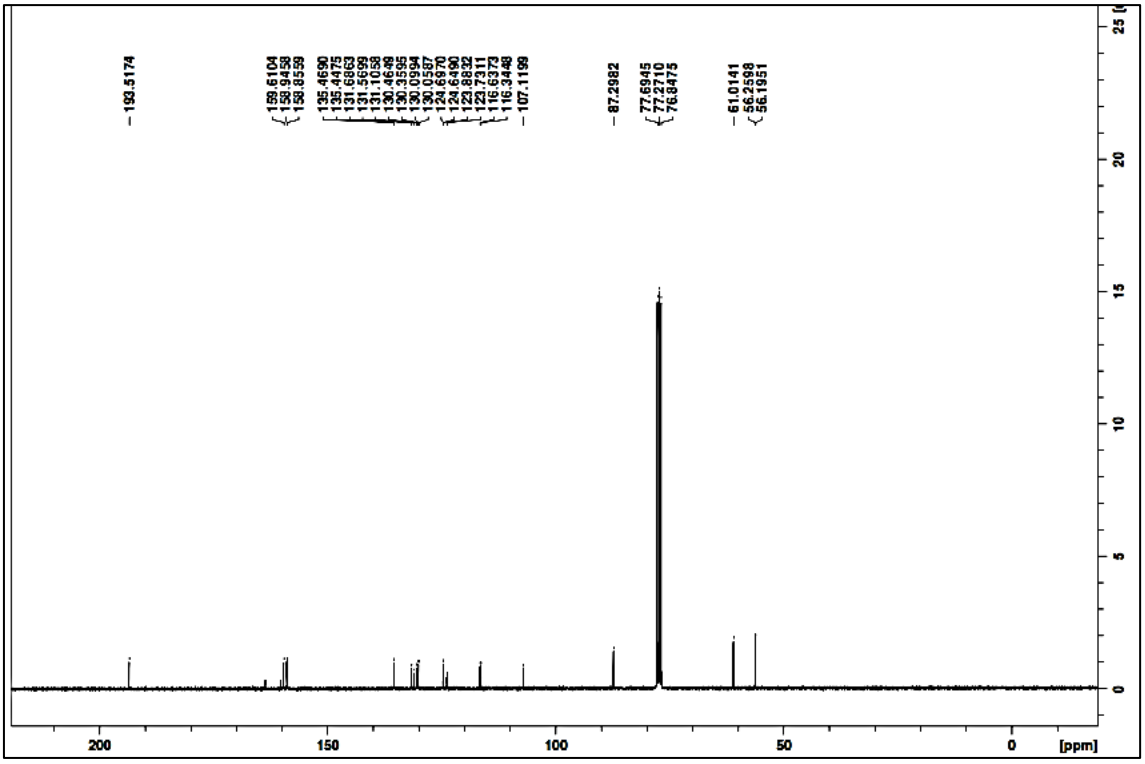

Figure S5: Expansion of the ^{13}C NMR spectrum of chalcone (*E*)-3-(2-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

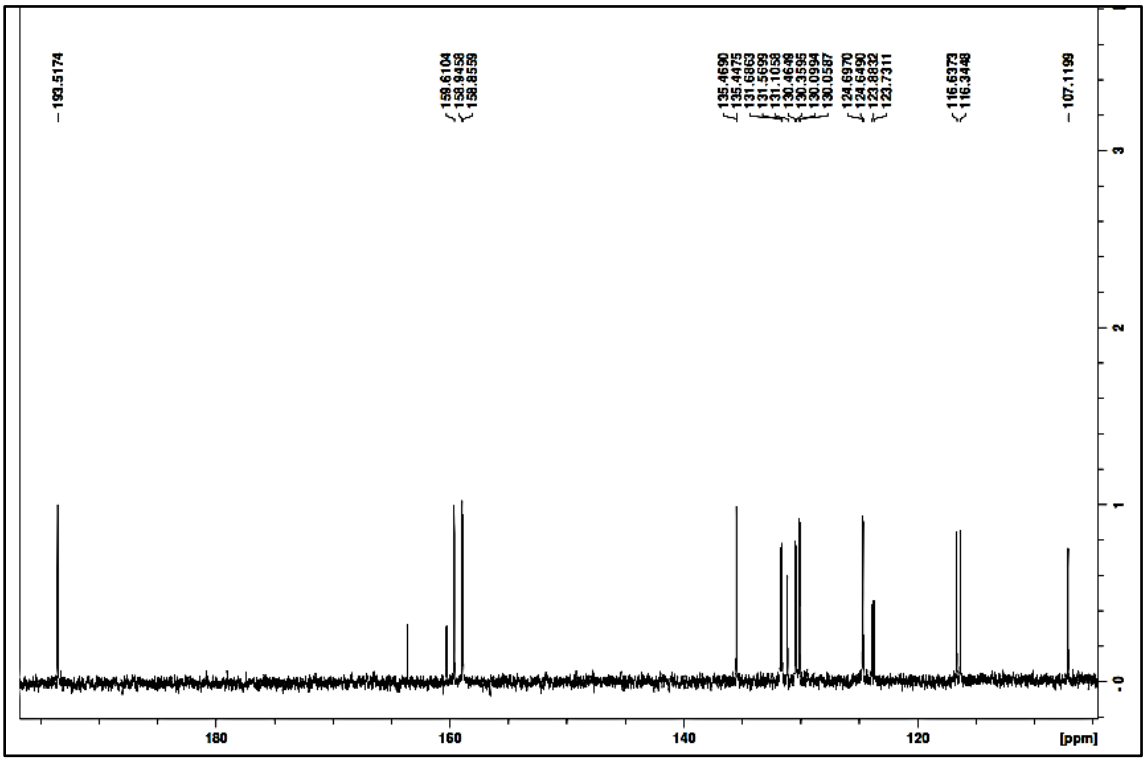

Figure S6: *IR spectrum of compound* the chalcone **(***E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

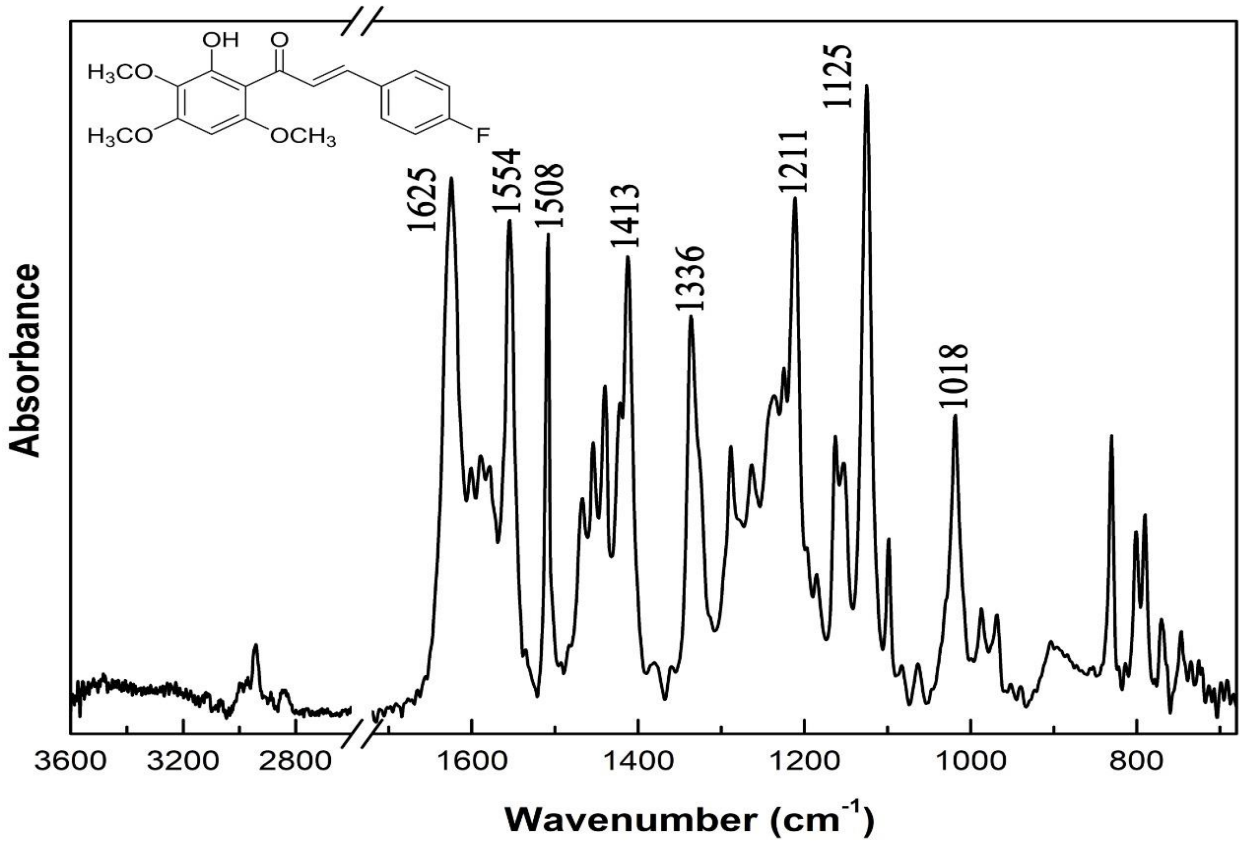

Figure S7: ^{1}H NMR spectrum of the chalcone **(***E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

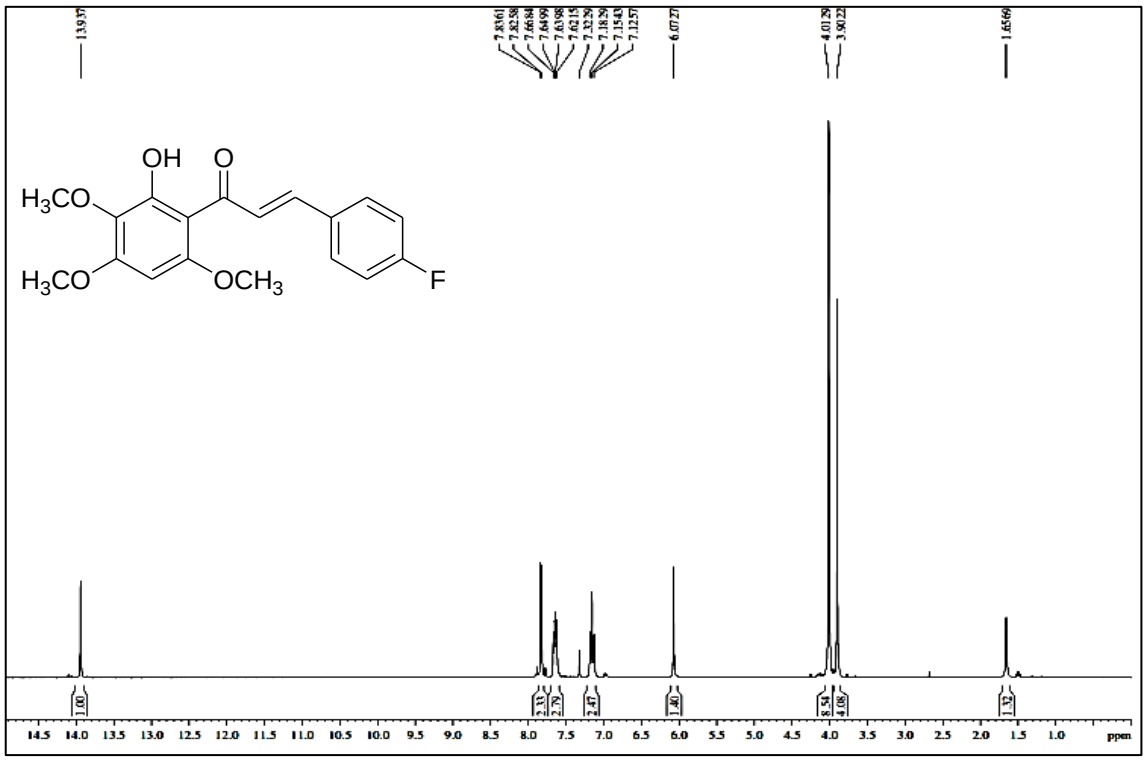

Figure S8: Expansion of the ^{1}H NMR spectrum of chalcone **(***E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

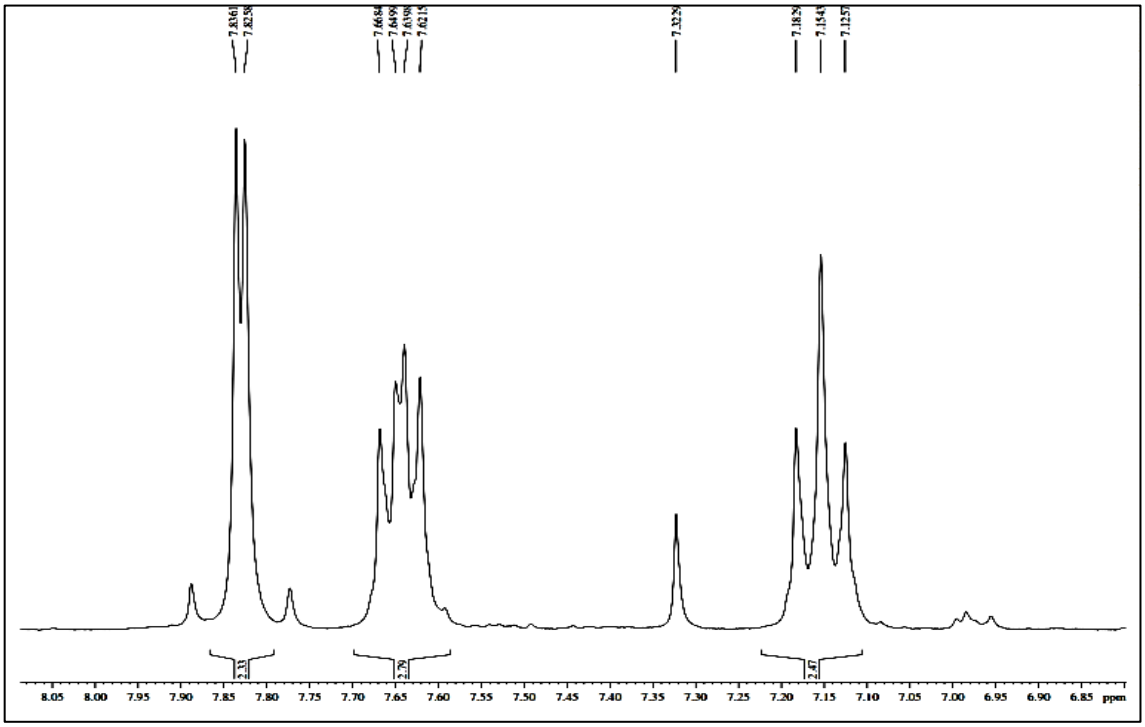

Figure S9: ^{13}C NMR spectrum of the chalcone **(***E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

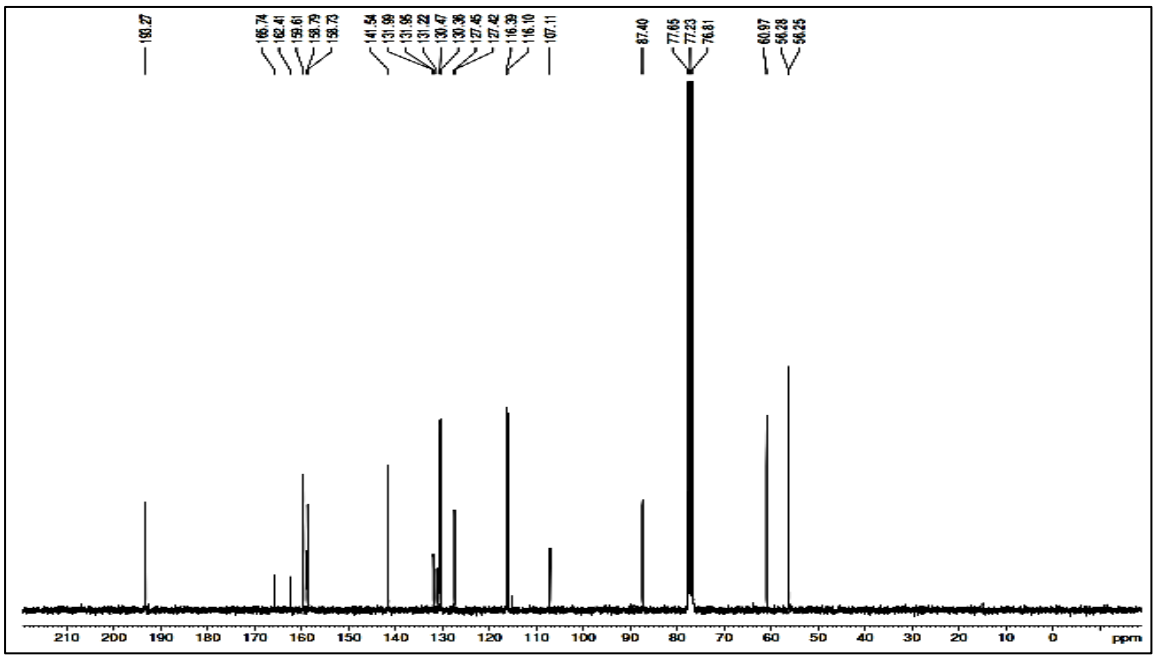

Figure S10: : Expansion of the ^{13}C NMR spectrum of chalcone **(***E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

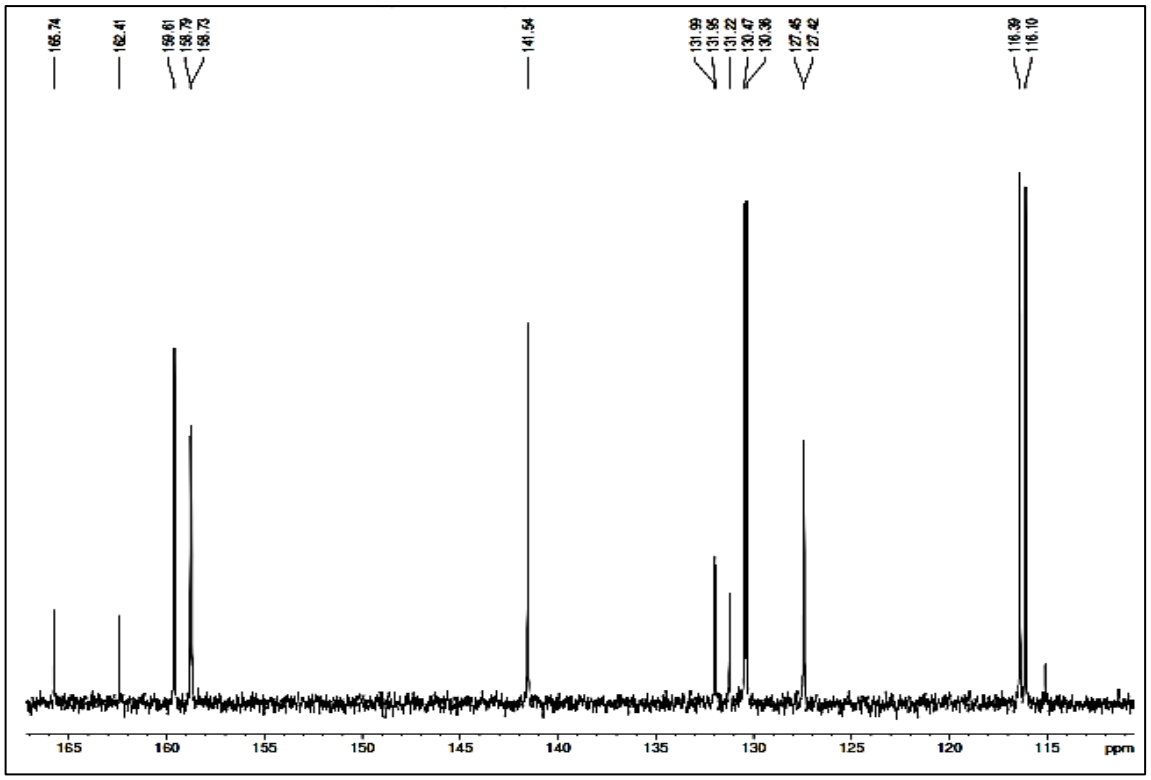

Figure S12: *IR spectrum of compound* the chalcone (*E*)-3-(4-chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

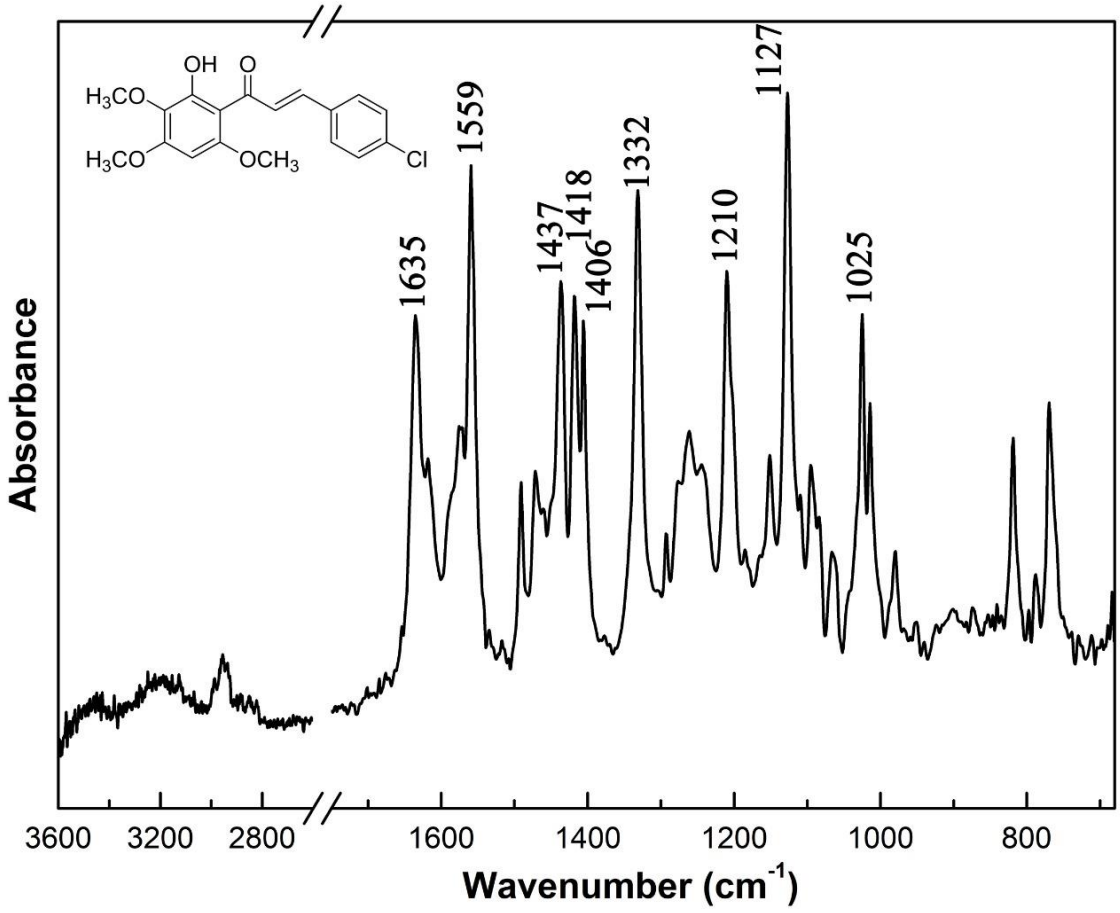

Figure S13: ^{1}H NMR spectrum of the chalcone (*E*)-3-(4-chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

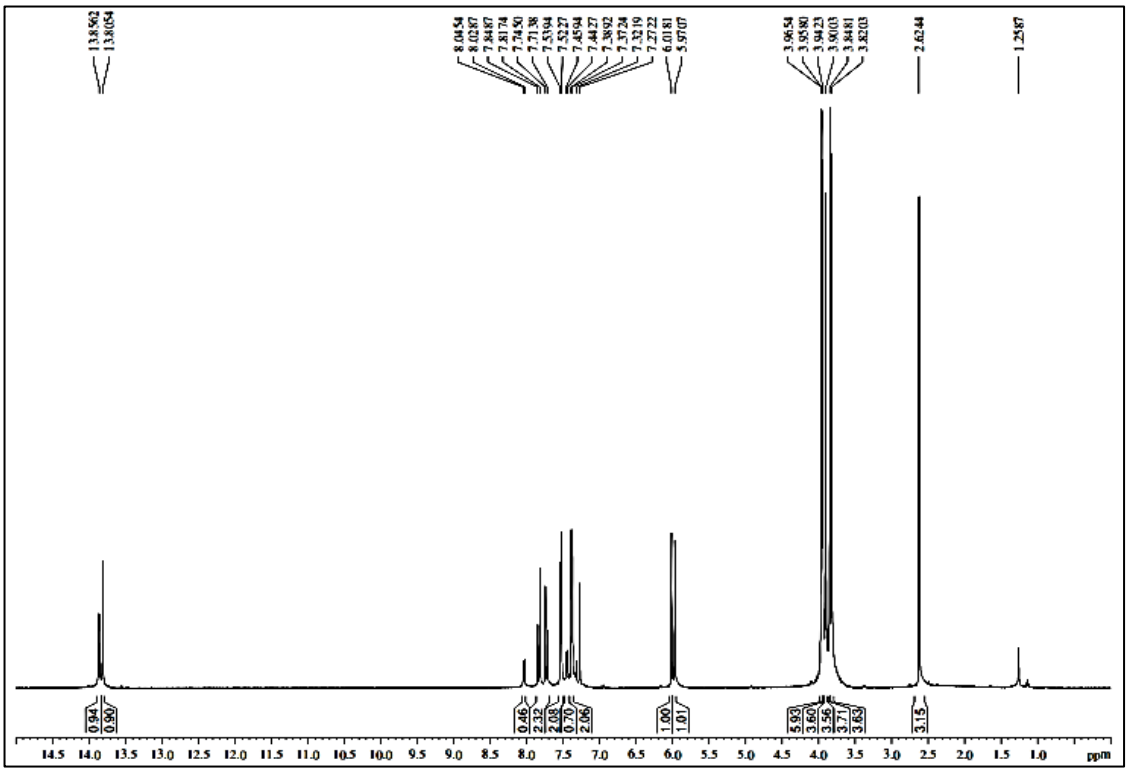

Figure S14: Expansion of the ^{1}H NMR spectrum of chalcone (*E*)-3-(4-chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

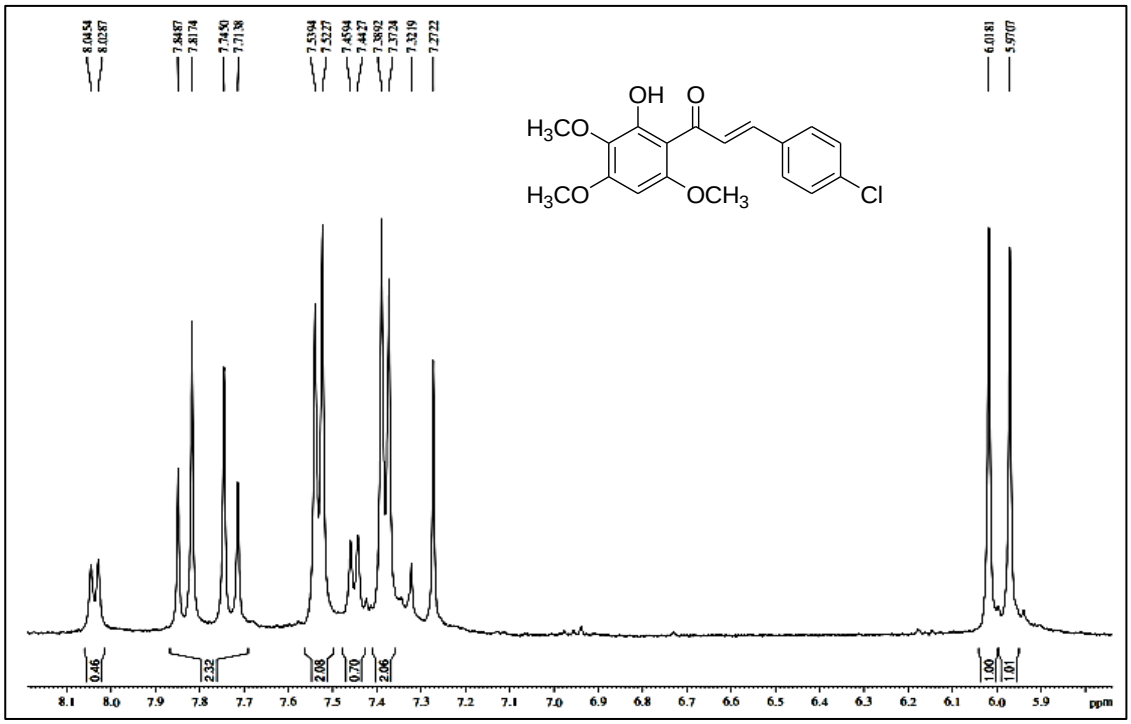

Figure S15: ^{13}C NMR spectrum of the chalcone (*E*)-3-(4-chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

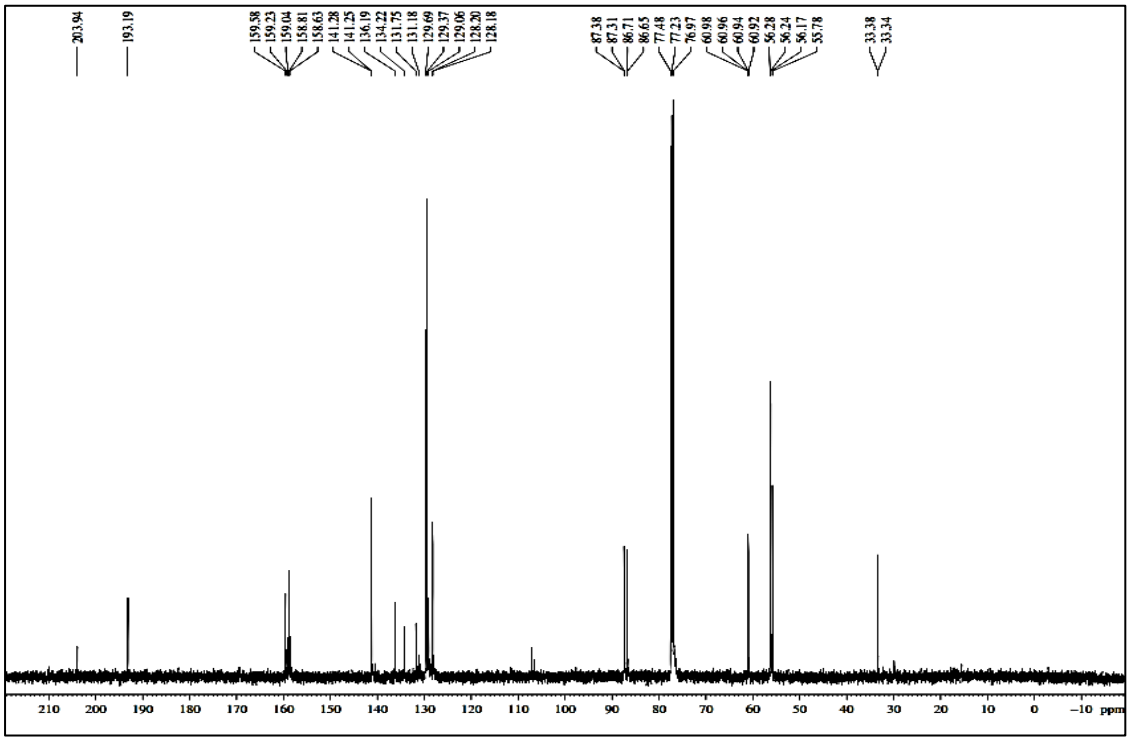

Figure S16: Expansion of the ^{13}C NMR spectrum of chalcone (*E*)-3-(4-chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

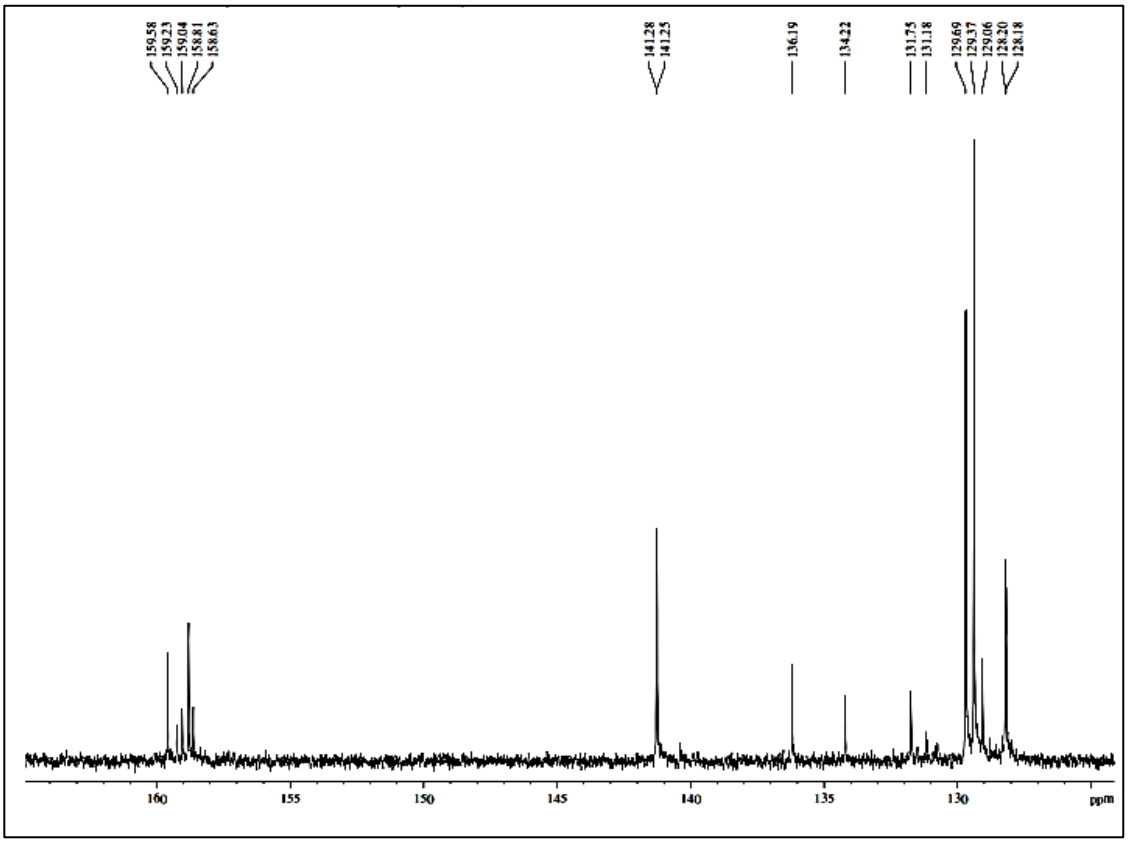

Figure S17: *IR spectrum of compound* the chalcone (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

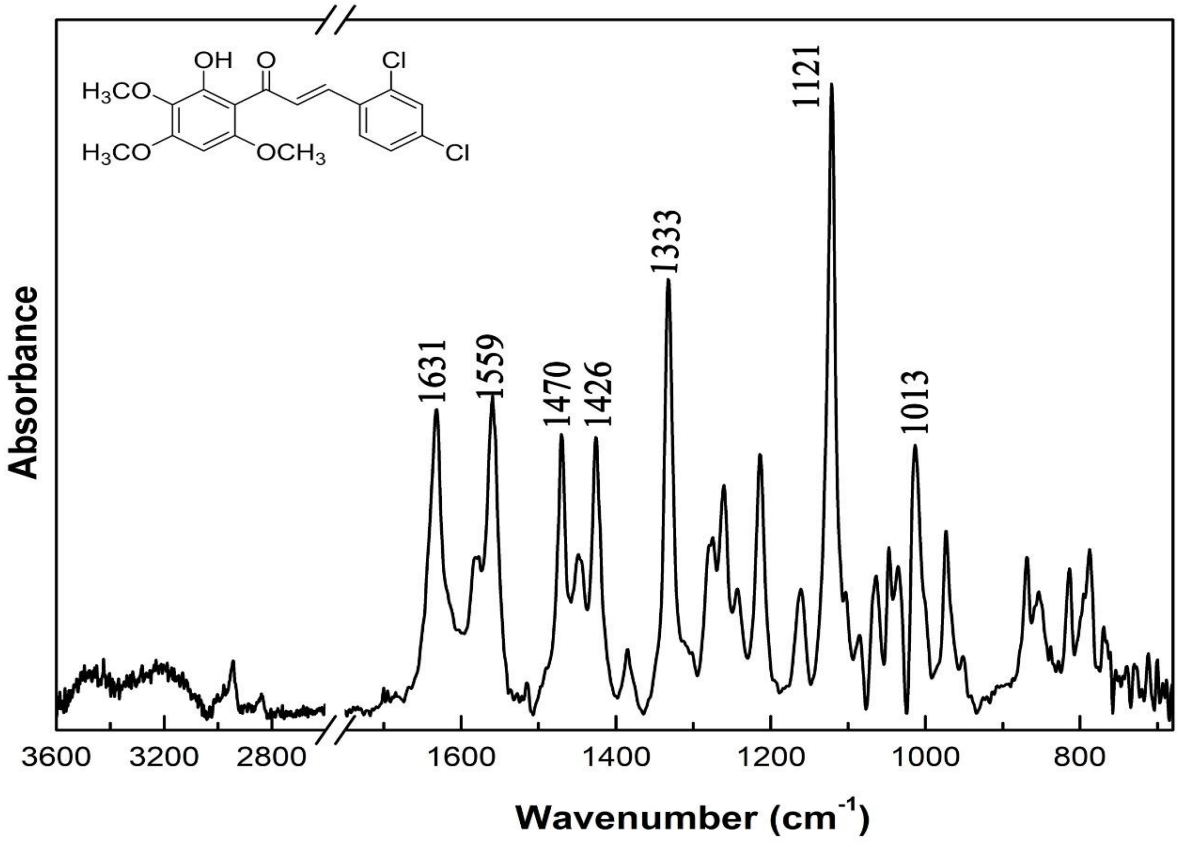

Figure S18: ^{1}H NMR spectrum of the chalcone (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

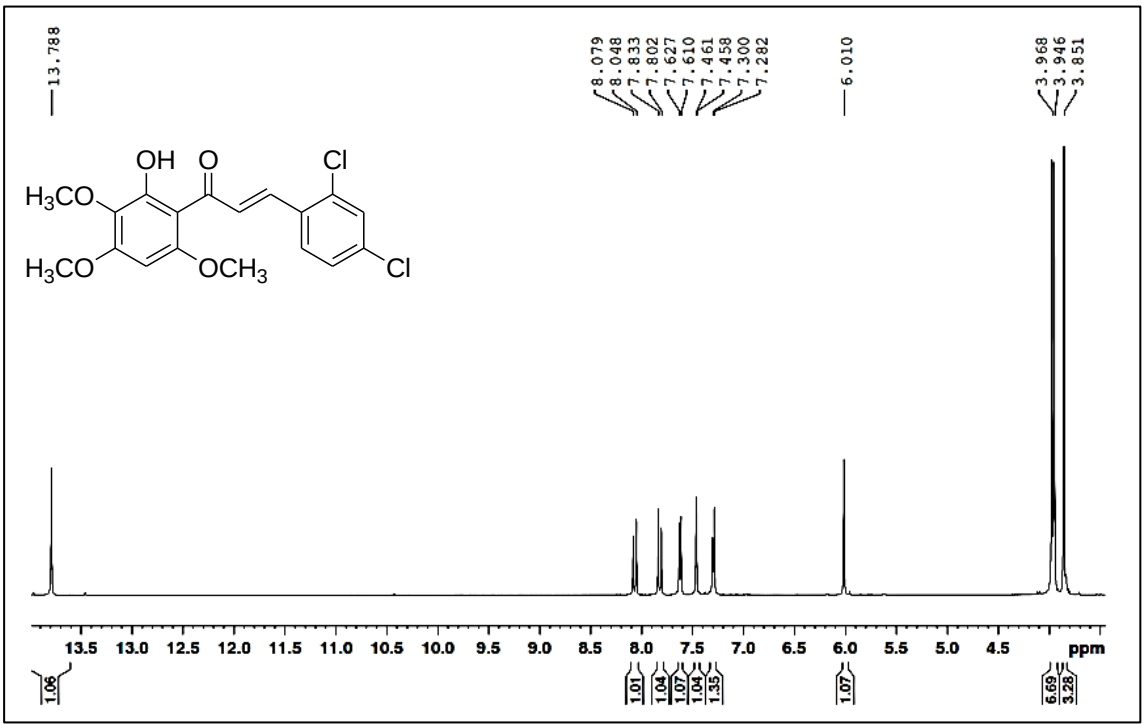

Figure S19: Expansion of the ^{1}H NMR spectrum of chalcone (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

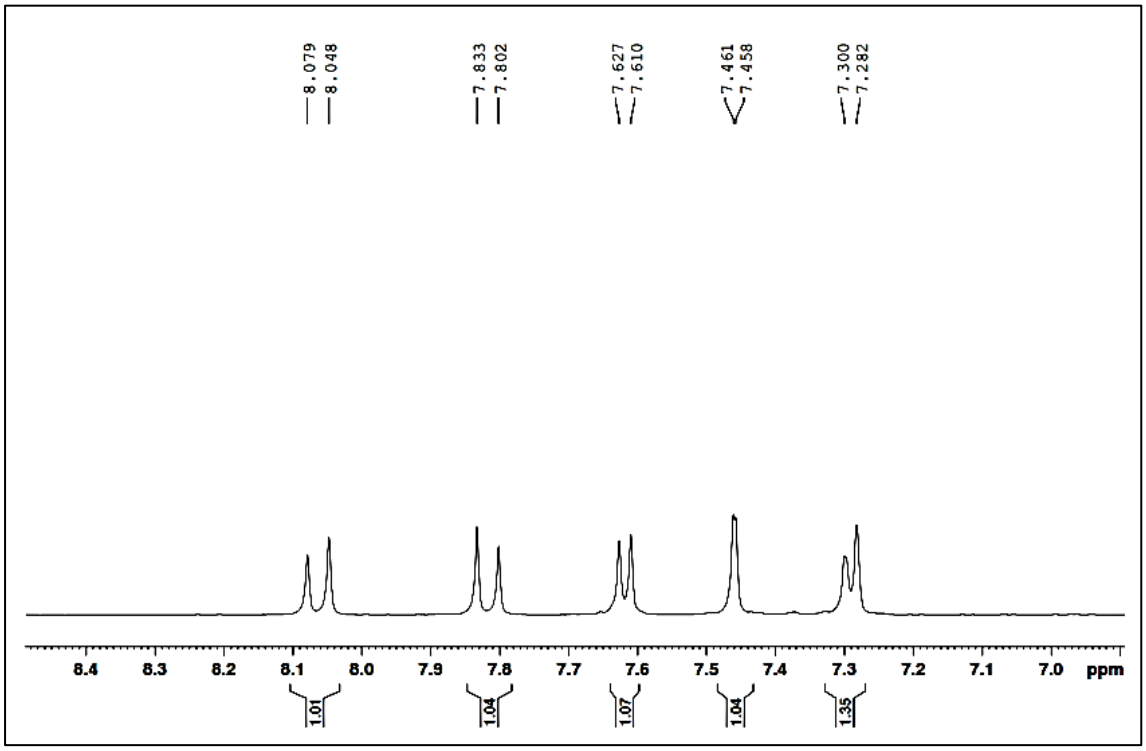

Figure S20: ^{13}C NMR spectrum of the chalcone (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one

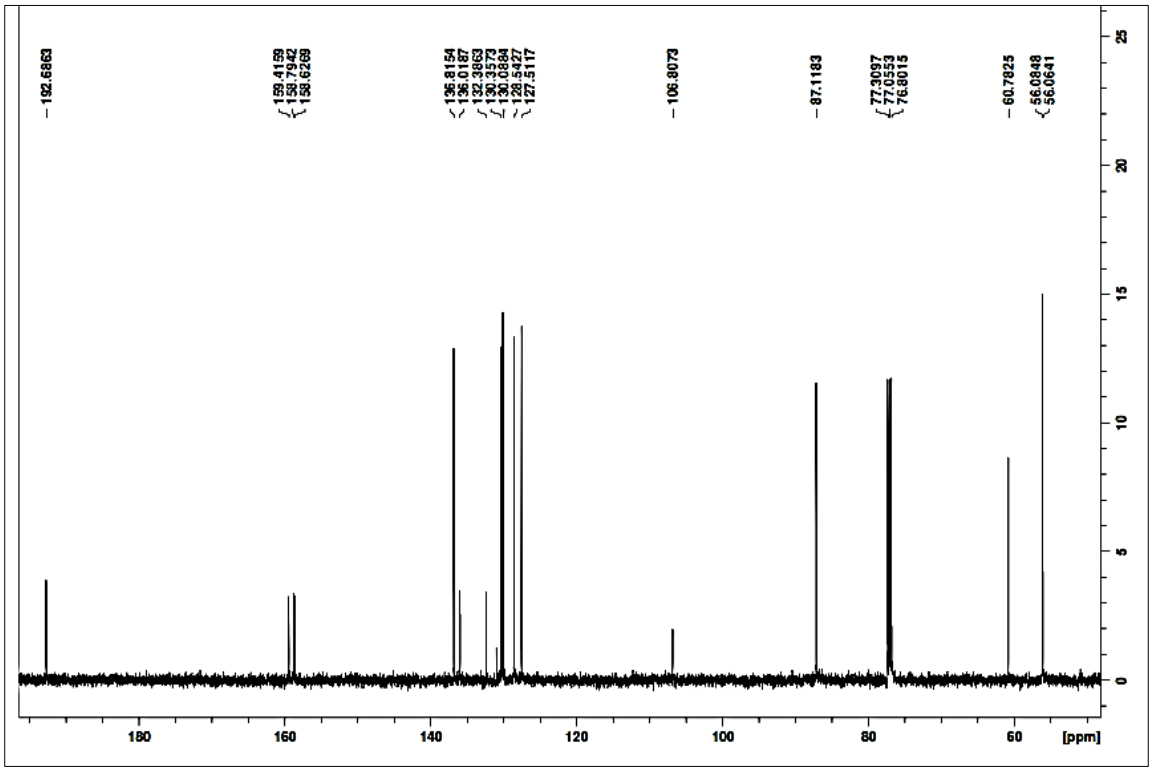

Figure S21: Expansion of the ^{13}C NMR spectrum of chalcone (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one.

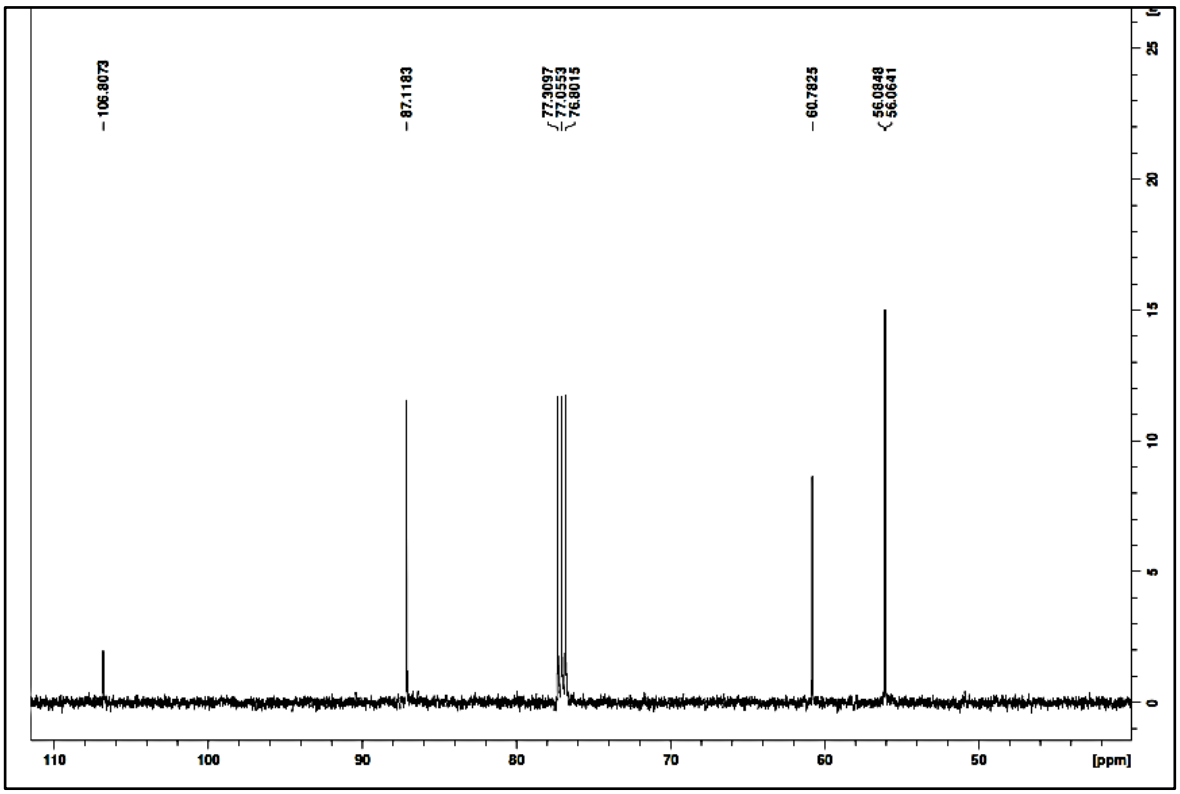

Table S1. Antibacterial effect in Gram (+) and Gram (-) bacteria against the tested chalcones.

Chalcones	Gram (+)	Gram (-)
1	Inert	Inert
2	Weak effect	Weak effect
3	Inert	Inert
4	Inert	Inert

-

APÊNDICE 2 – MATERIAL SUPLEMENTAR DO ARTIGO PUBLICADO NA REVISTA: Microbial Pathogenesis, 2021, Vol. 161, Parte B; FATOR DE IMPACTO 2,914 – ISSN 0882-4010 – QUALIS B2

In vitro and *in silico* studies of chalcones derived from natural acetophenone inhibitors of NorA and MepA multidrug efflux pumps in *Staphylococcus aureus*

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Figure S1. ^{1}H NMR spectrum of the (*E*)-3-(2-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 1).

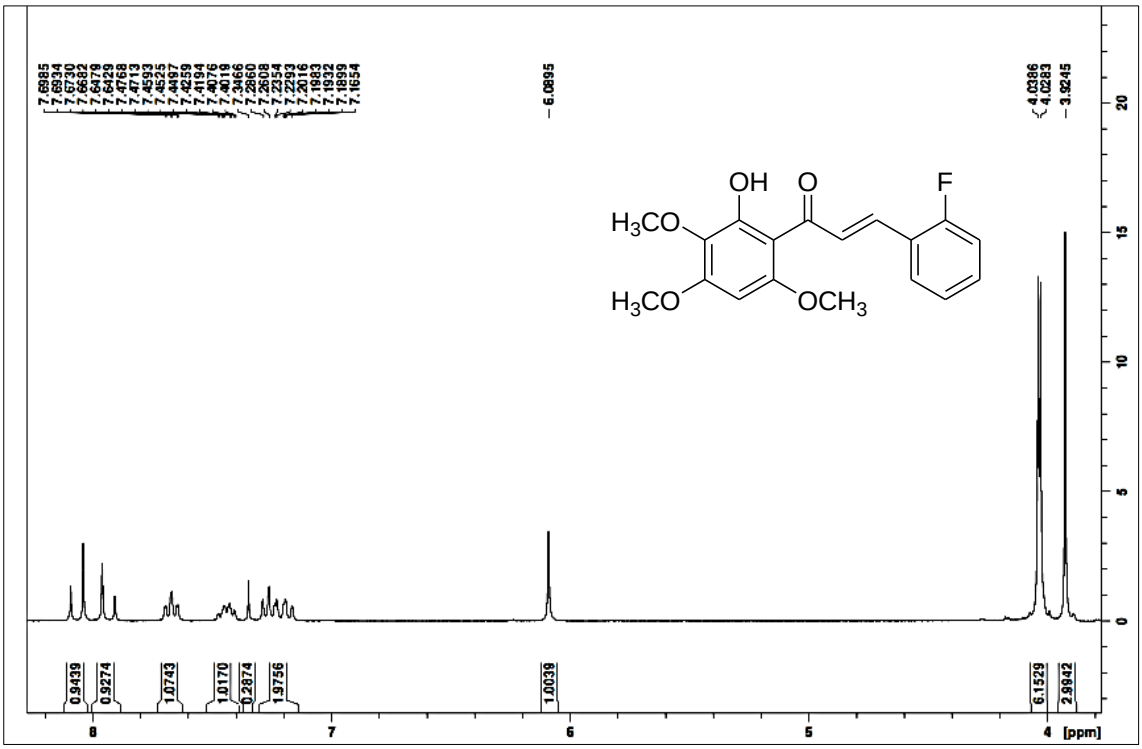


Figure S2. Expansion of the ^{1}H NMR spectrum of (*E*)-3-(2-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 1).

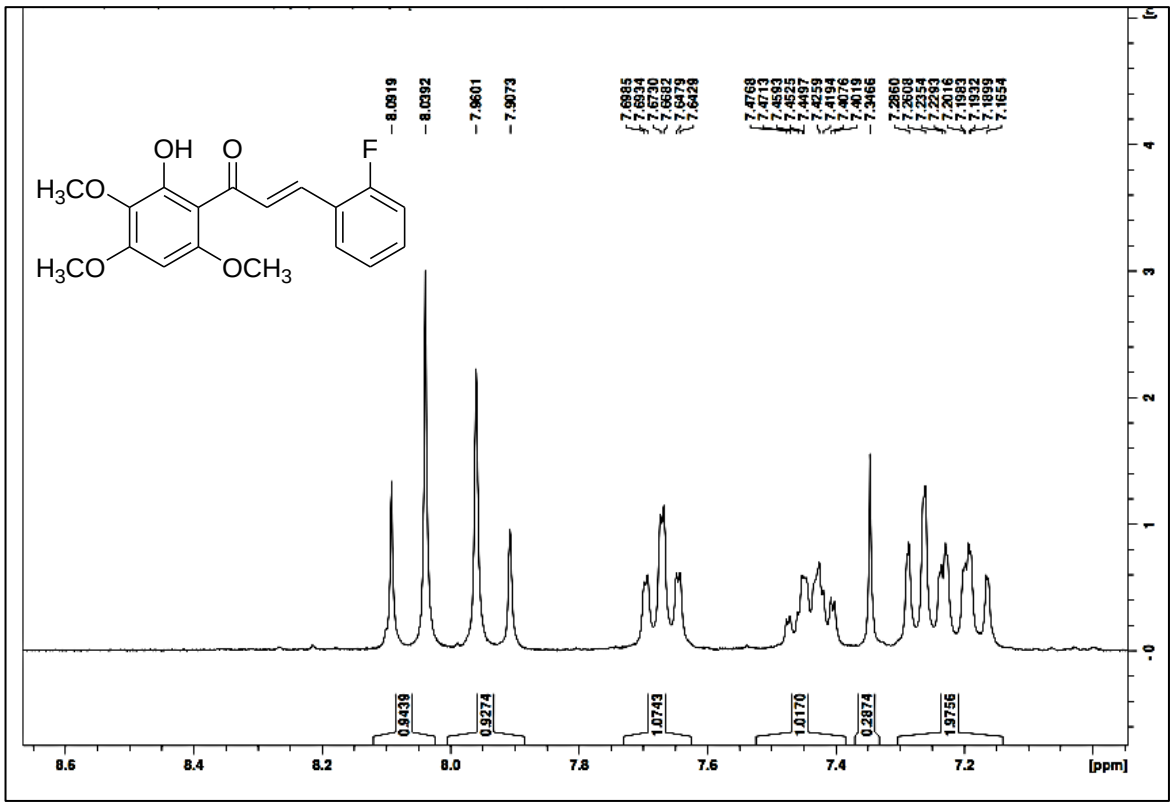


Figure S3. ^{13}C NMR spectrum of (*E*)-3-(2-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 1).

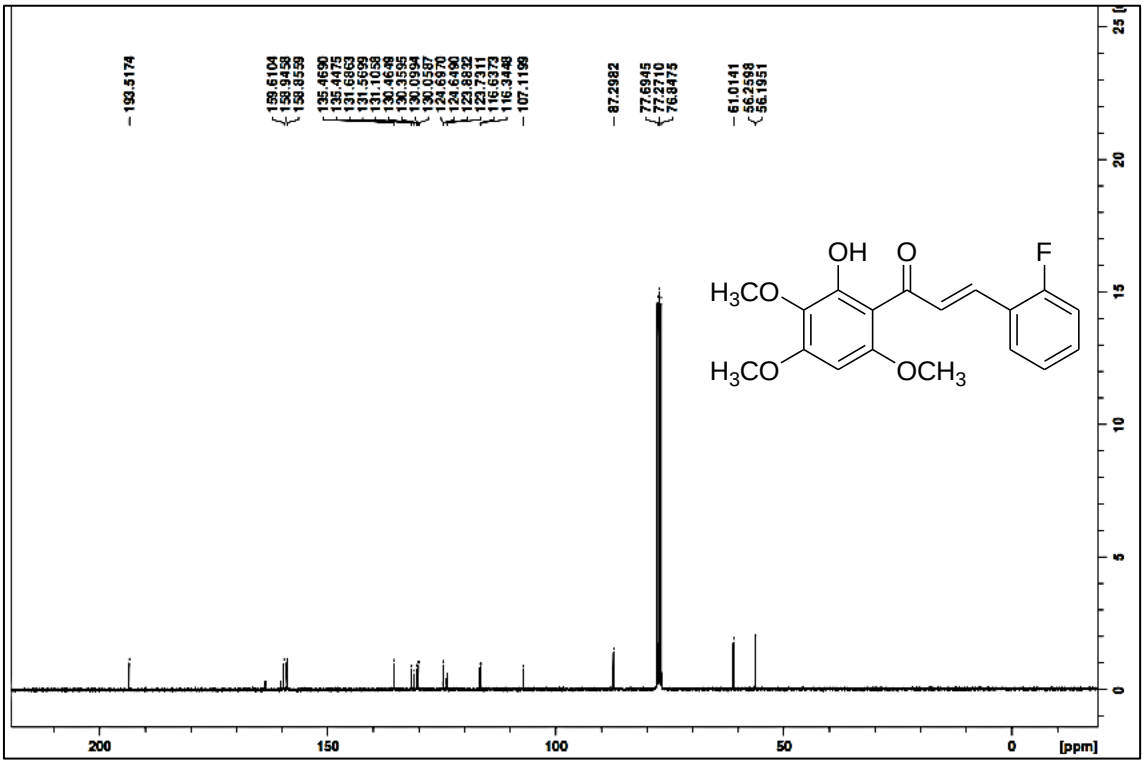


Figure S4. Expansion of the ^{13}C NMR spectrum of (*E*)-3-(2-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 1).

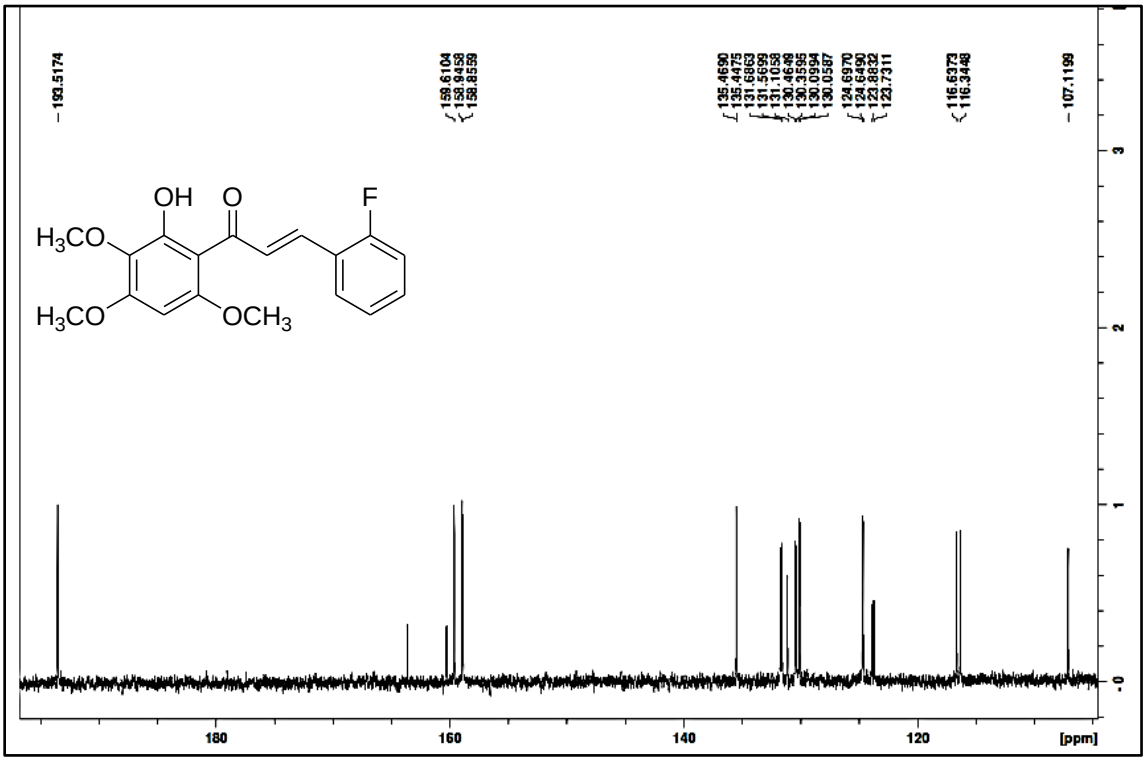


Figure S5. *Mass spectrum of* (*E*)-3-(2-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 1).

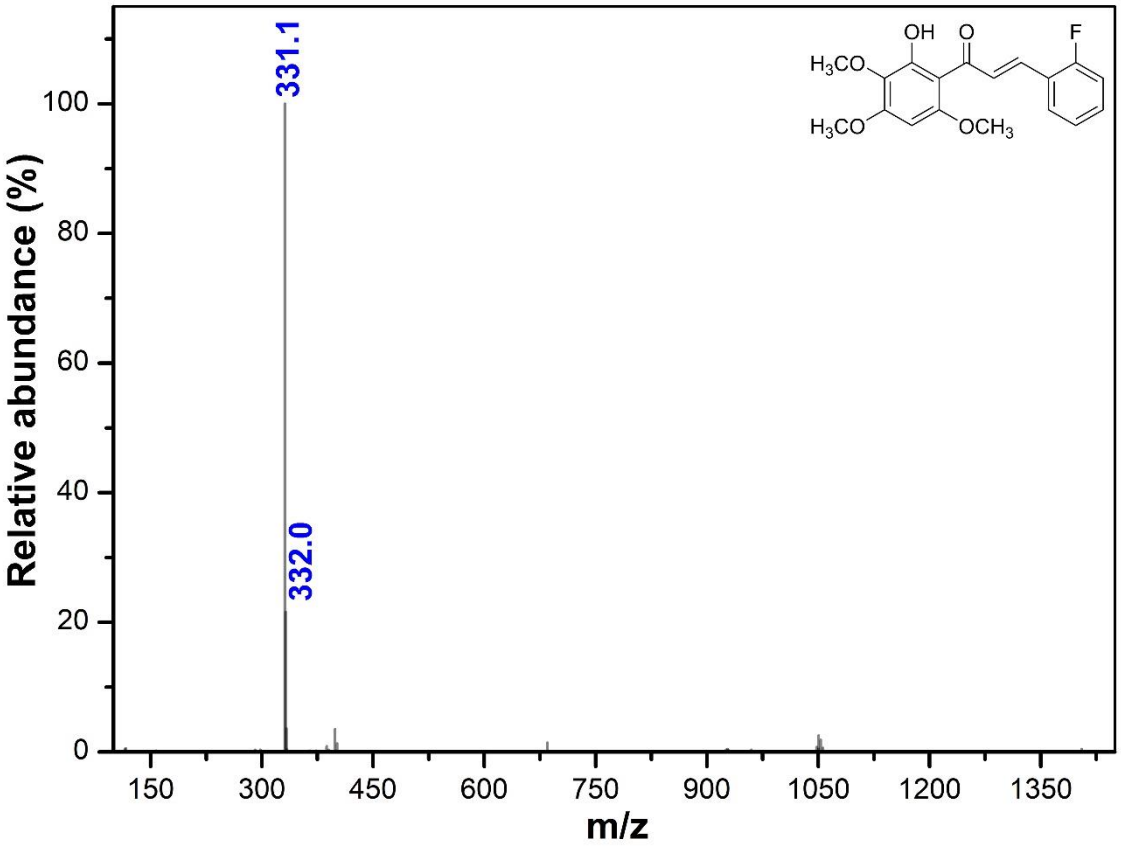


Figure S6. ^{1}H NMR spectrum of **(***E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 2).

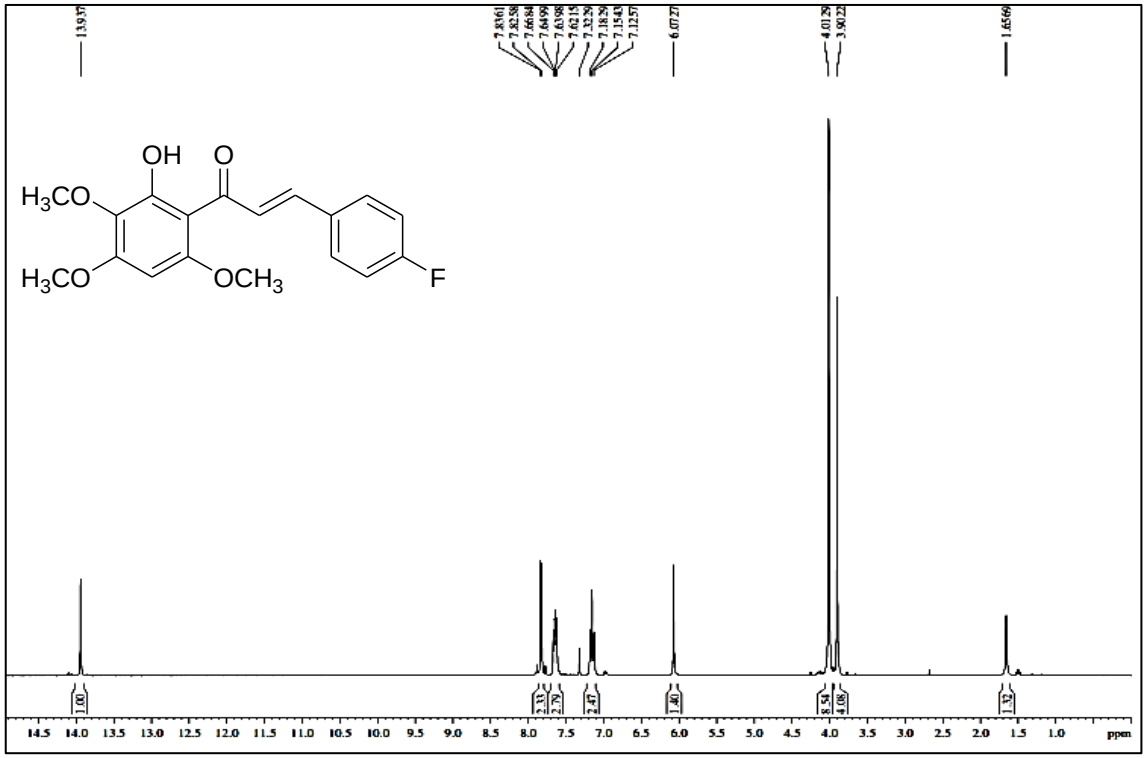


Figure S7. Expansion of the ^{1}H NMR spectrum of **(***E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 2).

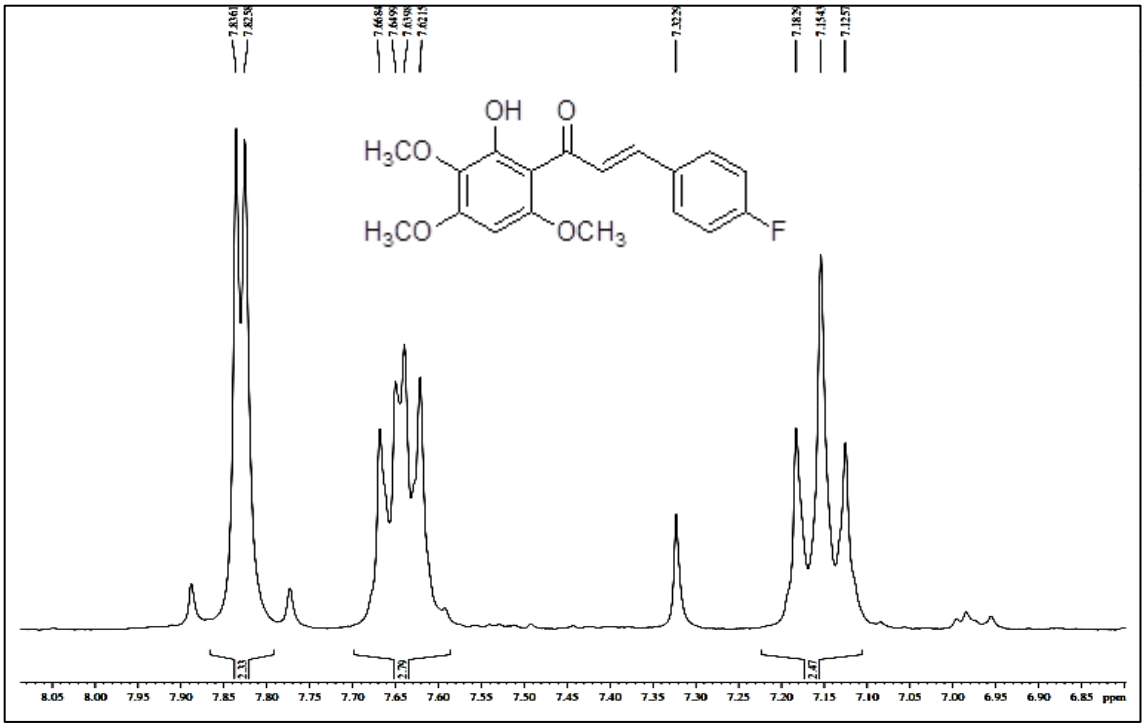


Figure S8. ^{13}C NMR spectrum of **(***E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 2).

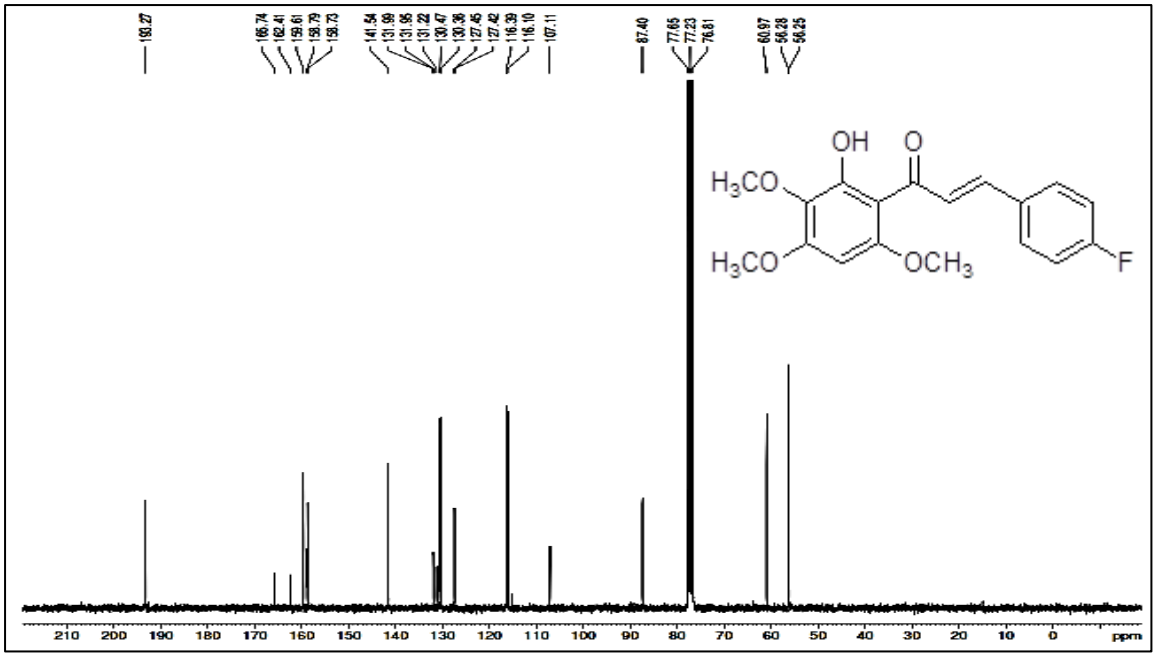


Figure S9. . Expansion of the ^{13}C NMR spectrum of **(***E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 2).

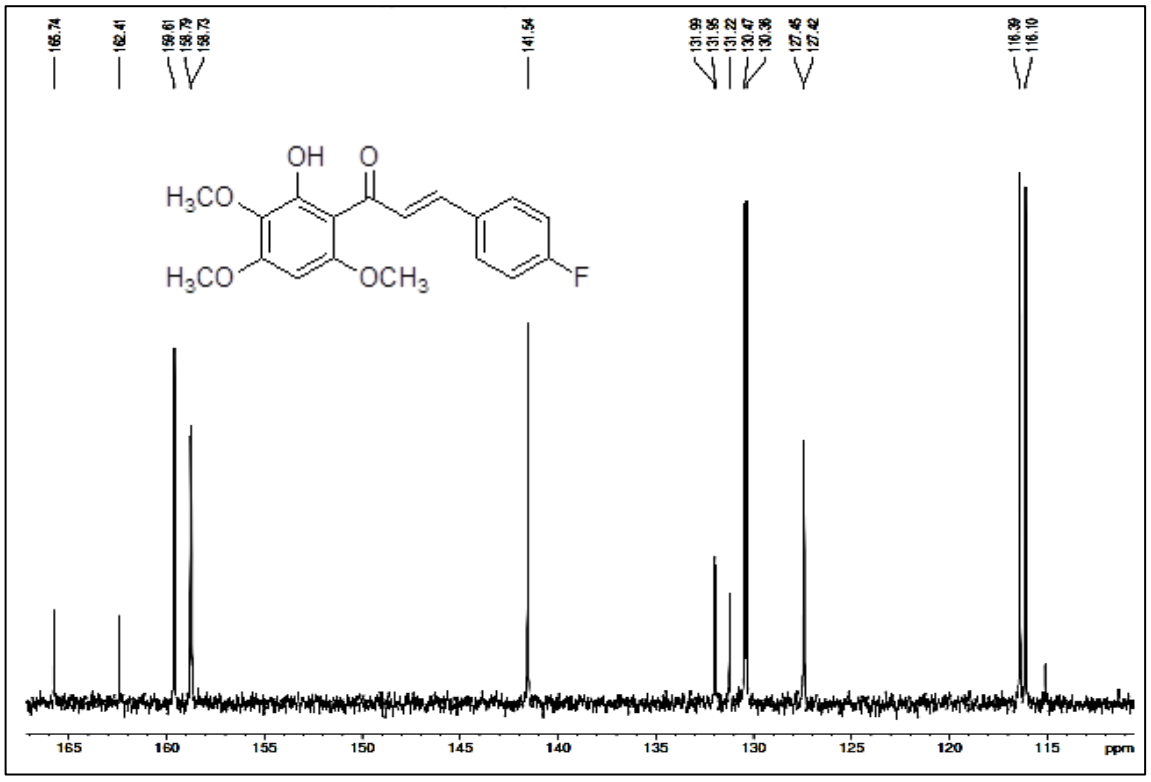


Figure S10. *Mass spectrum of* **(***E*)-3-(4-fluorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 2).

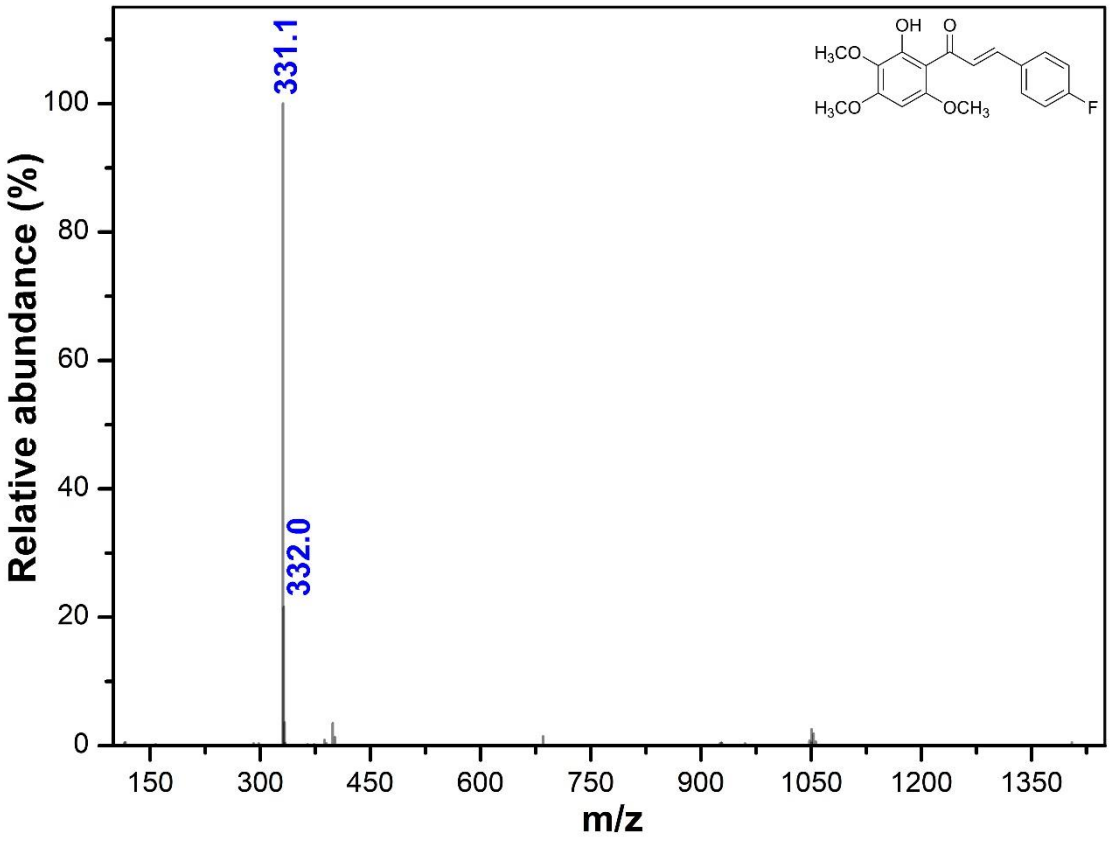


Figure S11. ^{1}H NMR spectrum of (*E*)-3-(4-chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 3).

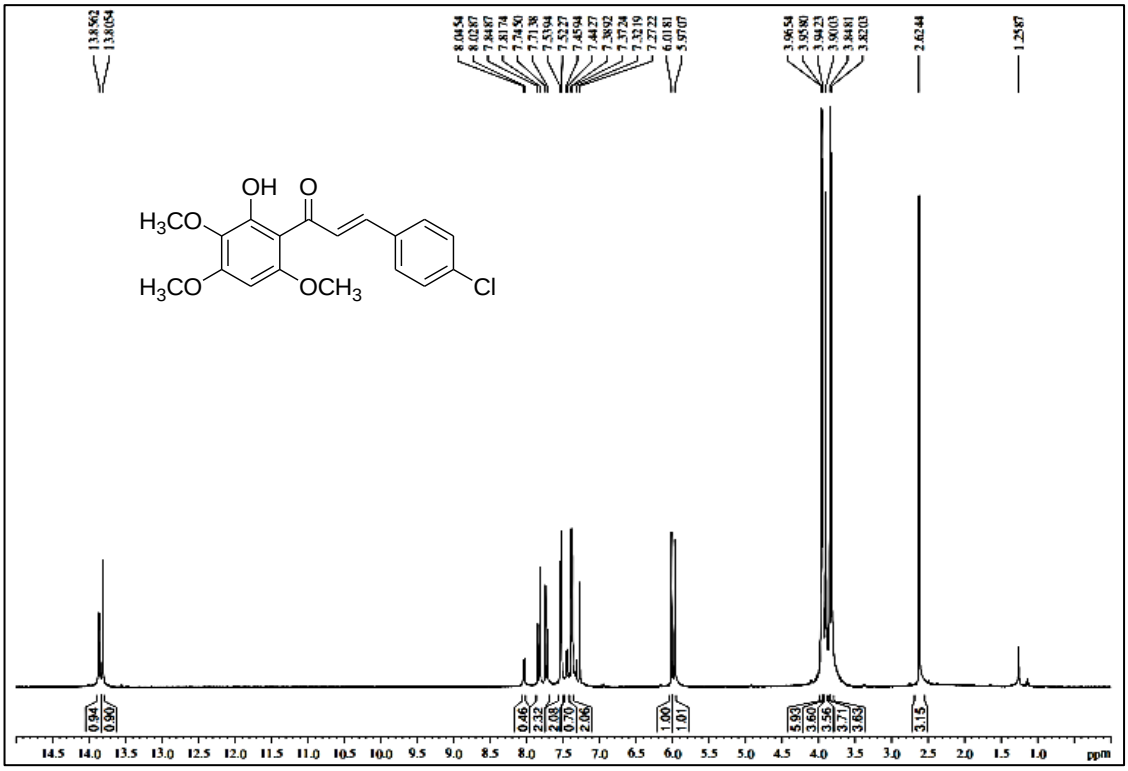


Figure S12. Expansion of the ^{1}H NMR spectrum of (*E*)-3-(4-chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 3).

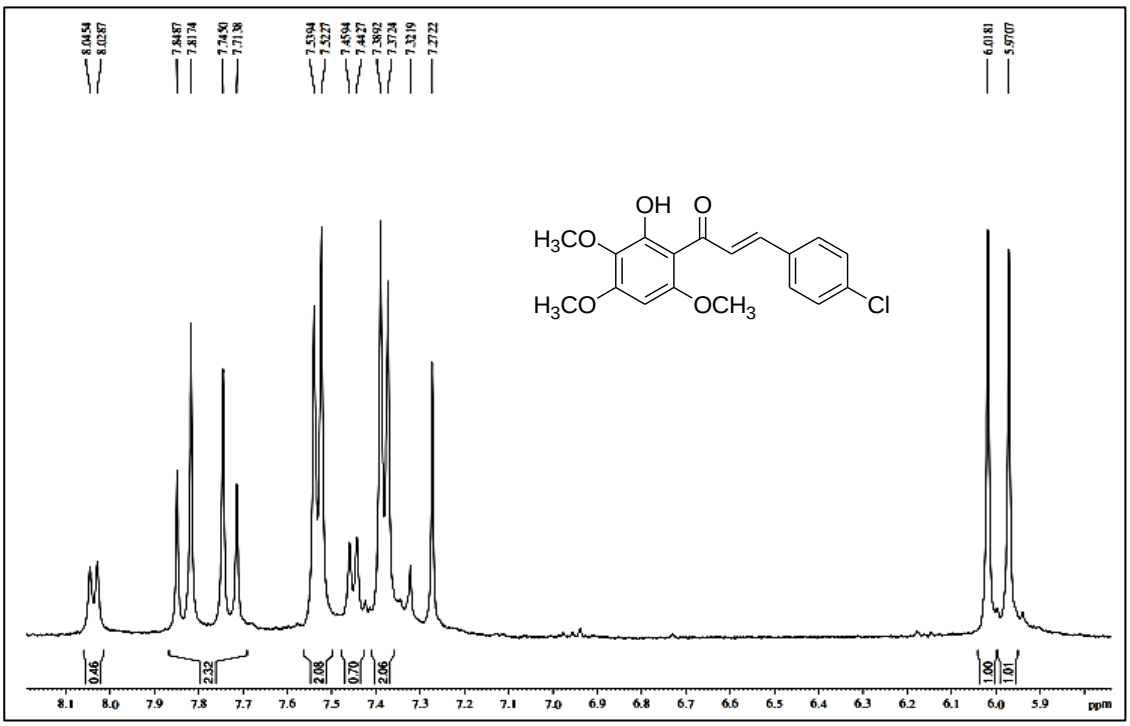


Figure S13. ^{13}C NMR spectrum of (*E*)-3-(4-chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 3).

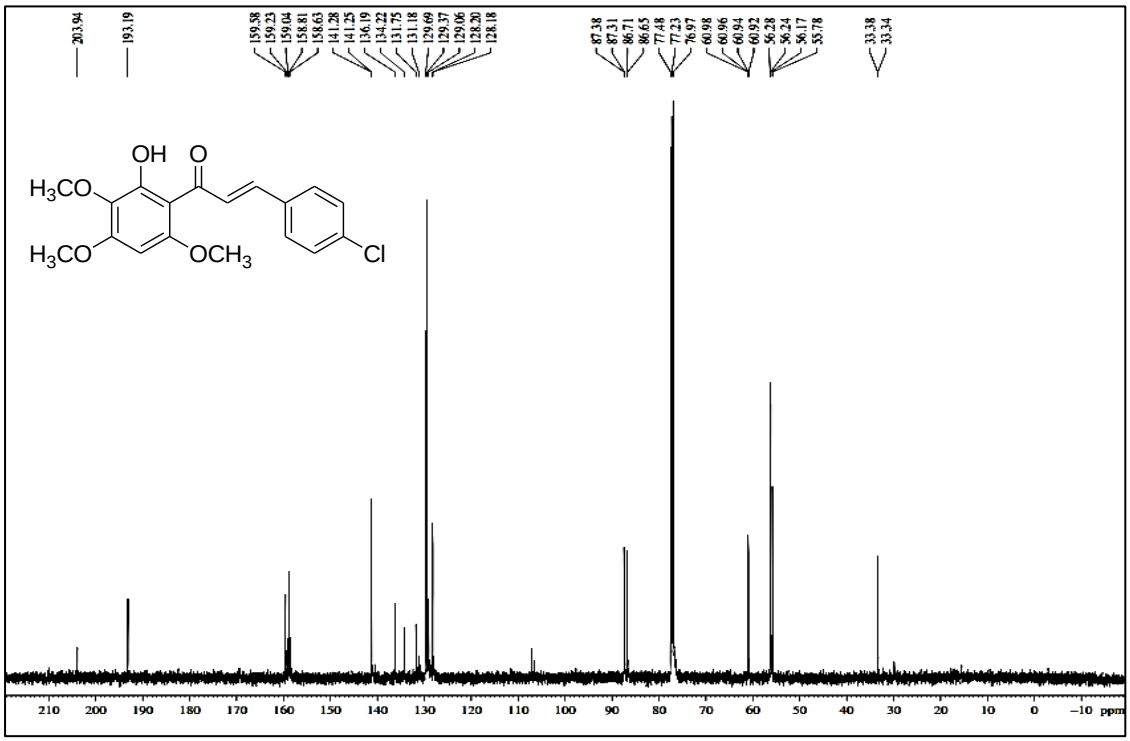


Figure S14. Expansion of the ^{13}C NMR spectrum of (*E*)-3-(4-chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 3).

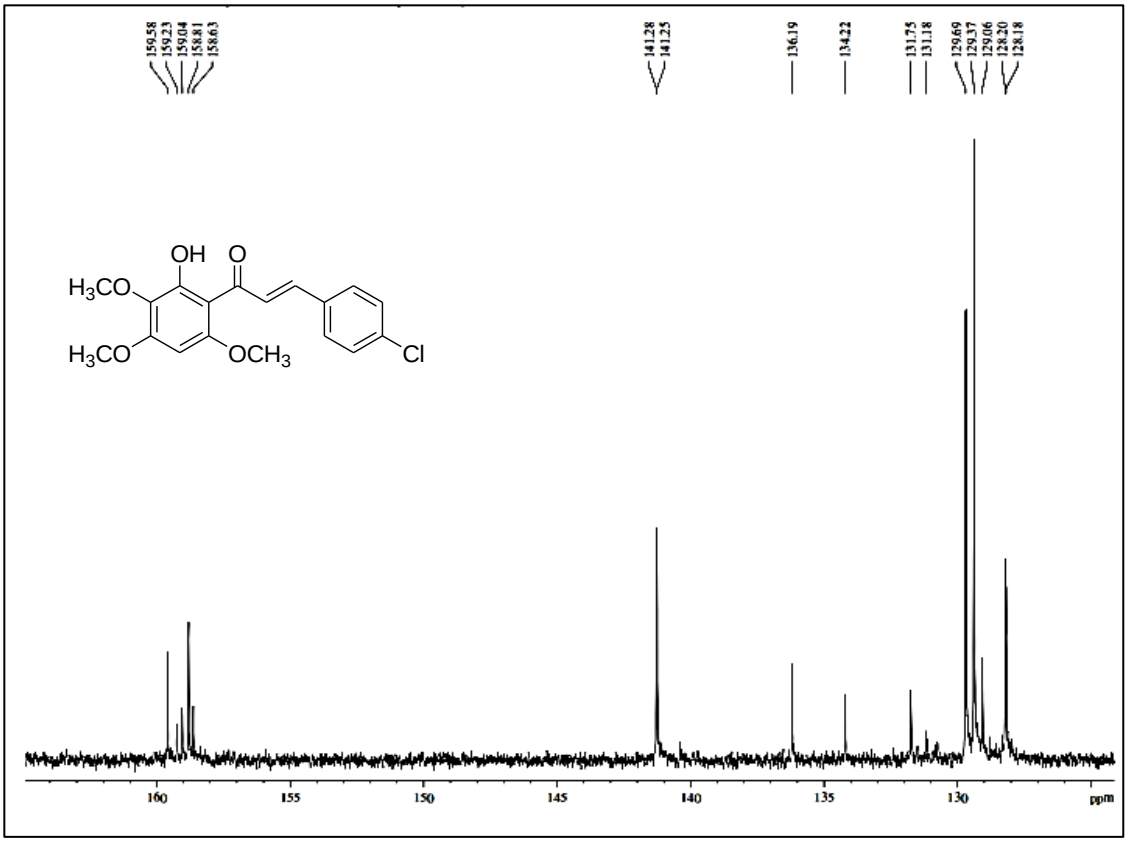


Figure S15. *Mass spectrum of* (*E*)-3-(4-chlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 3).

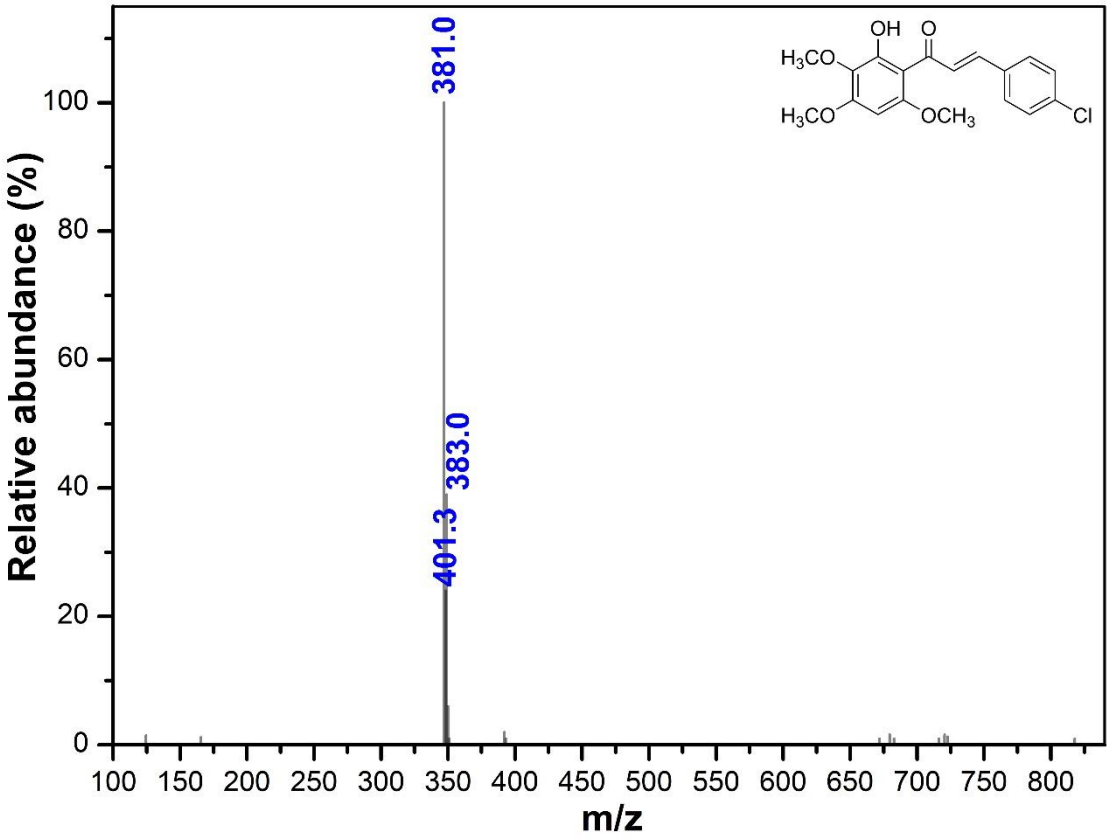


Figure S16. ^{1}H NMR spectrum of (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 4).

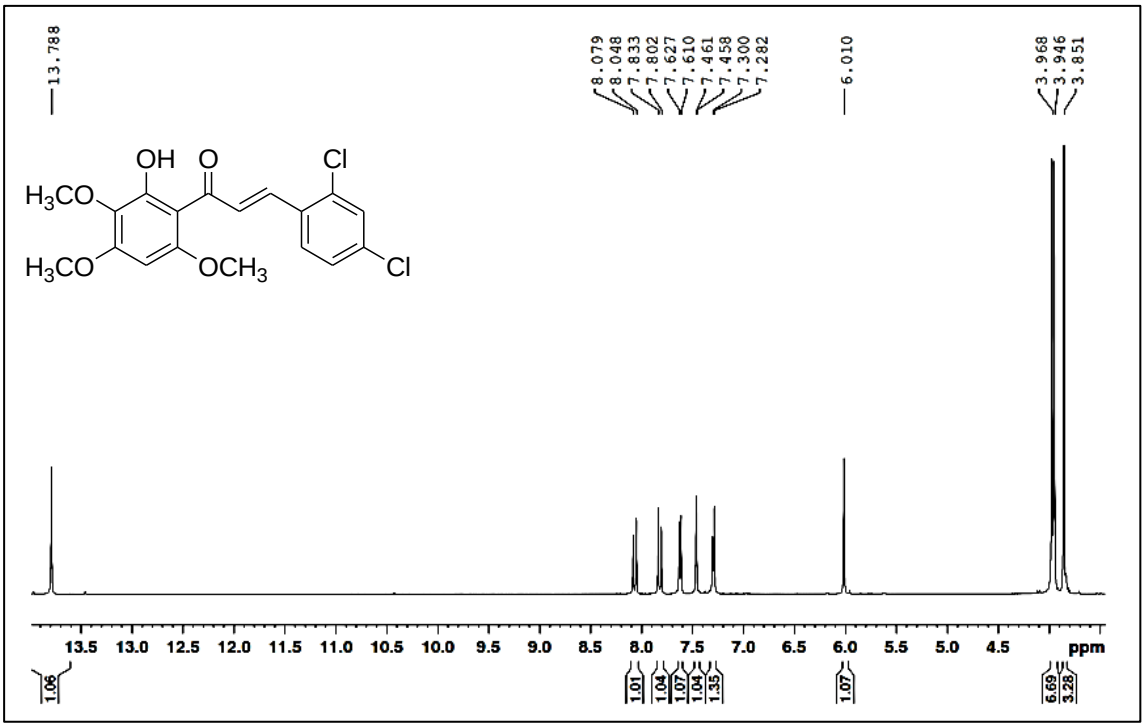


Figure S17. Expansion of the ^{1}H NMR spectrum of (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 4).

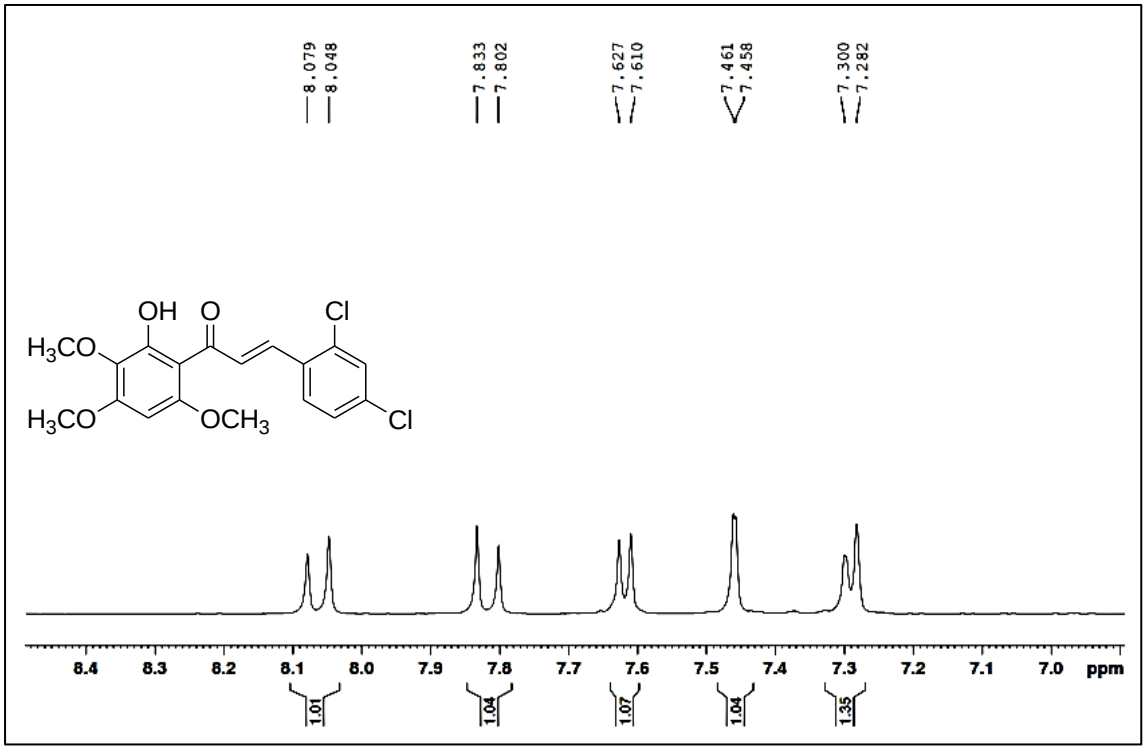


Figure S18. ^{13}C NMR spectrum of (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 4).

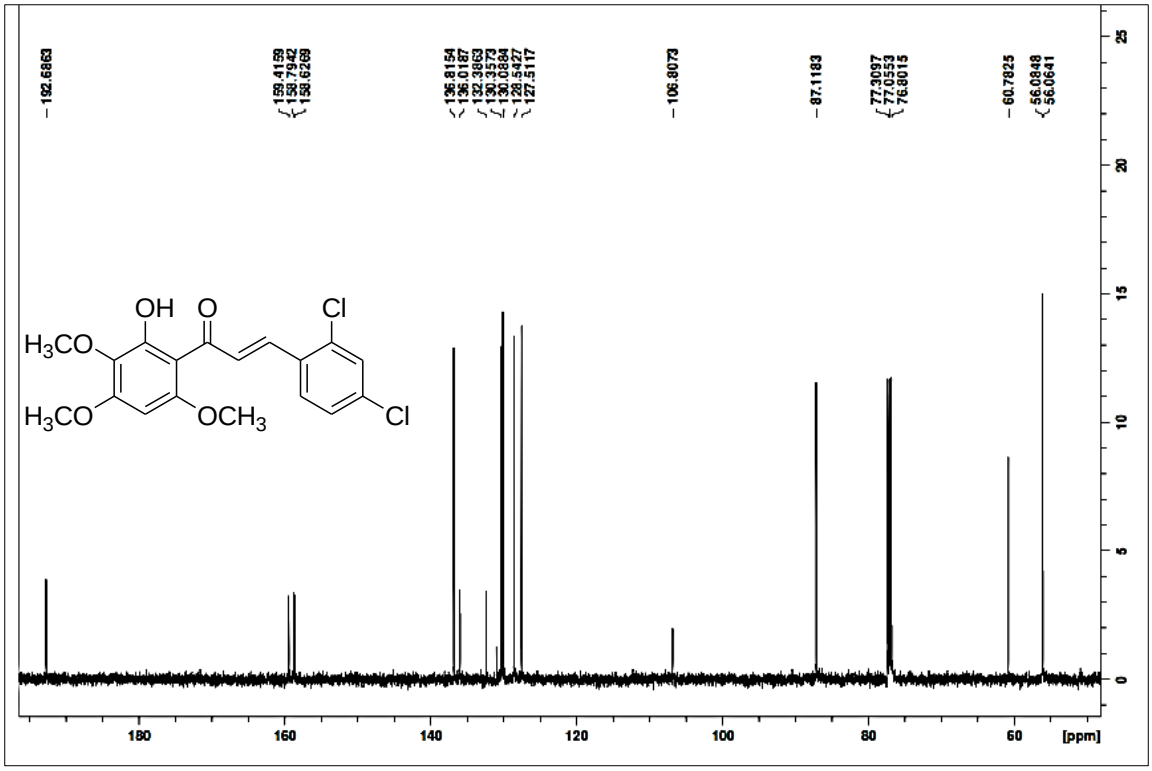


Figure S19. Expansion of the ^{13}C NMR spectrum of (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 4).

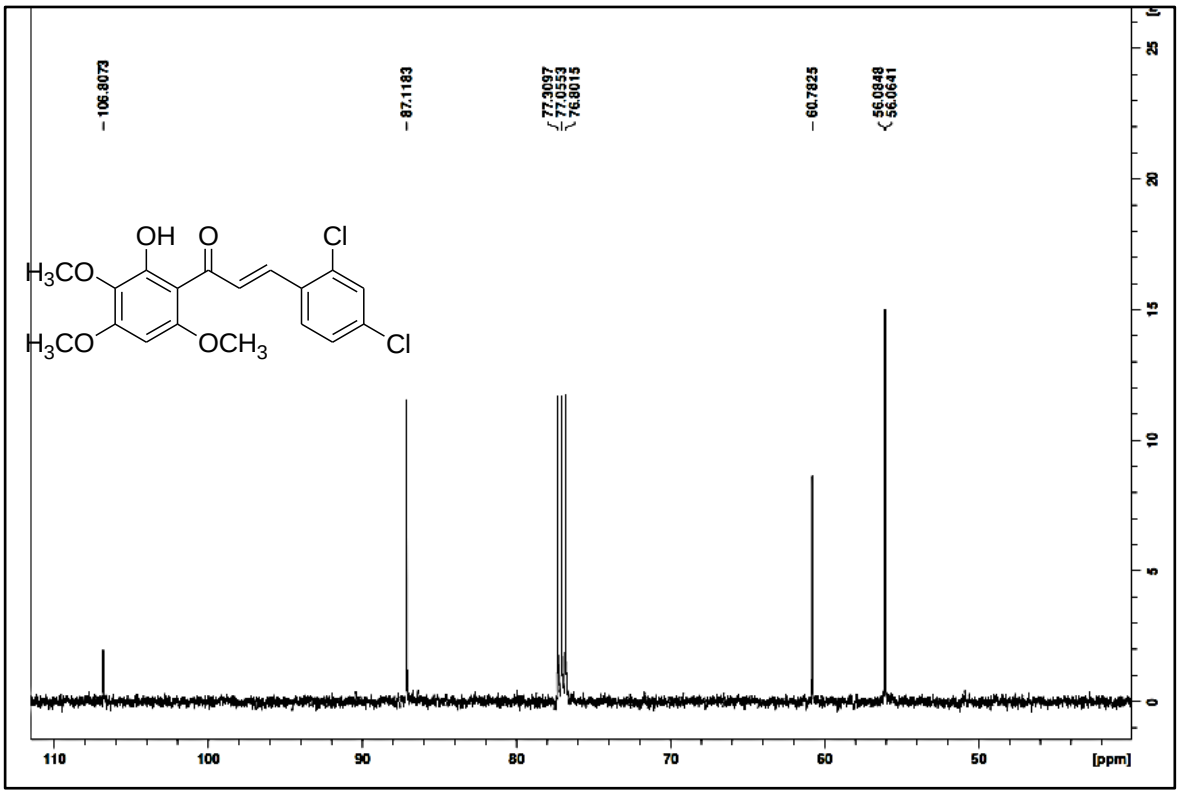
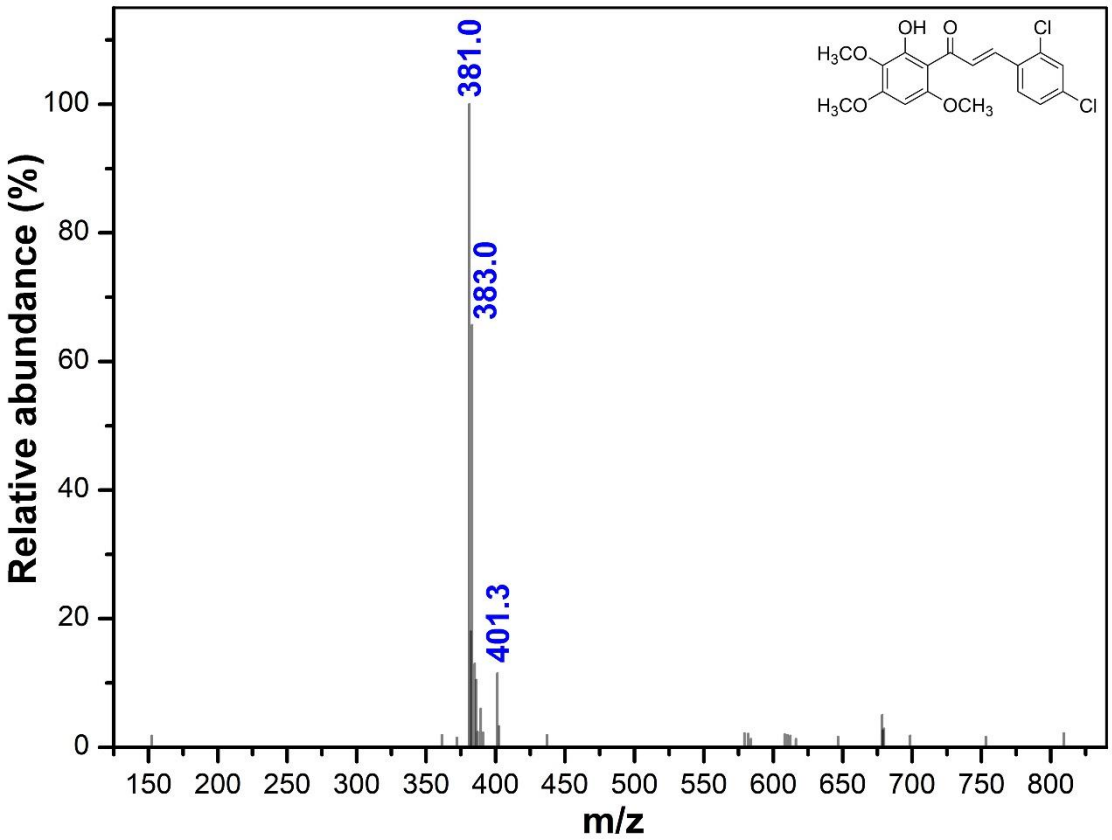


Figure S20. *Mass spectrum of* (*E*)-3-(2,4-dichlorophenyl)-1-(2-hydroxy-3,4,6-trimethoxyphenyl)prop-2-en-1-one (Chalcone 4).



APÊNDICE 3: PRODUÇÕES CIENTÍFICAS

Artigo publicado relacionado com a tese:

1. FREITAS, T.S. *et al*. Direct antibacterial and antibiotic resistance modulatory activity of chalcones synthesized from the natural product 2-hydroxy-3, 4, 6-trimethoxyacetophenone. **FEMS microbiology letters**, v. 367, n. 15, p. fnaa124, 2020.

2. FREITAS, T.S. *et al*. In vitro and in silico studies of chalcones derived from natural acetophenone inhibitors of NorA and MepA multidrug efflux pumps in Staphylococcus aureus. **Microbial pathogenesis**, v. 161, p. 105286, 2021.

Artigos publicados não relacionados com a tese:

1. DE FREITAS, M.A. *et al*. HPLC–DAD analysis and antimicrobial activities of *Spondias mombin* L.(Anacardiaceae). **3 Biotech**, v. 12, n. 3, p. 1-15, 2022.

2. AGRESSOTT, E.V.H. *et al*. Na-TiNT Nanocrystals: Synthesis, Characterization, and Antibacterial Properties. **Bioinorganic Chemistry and Applications**, v. 2022, 2022.

3. LOPES, T.P. *et al*. Pharmacological activities of allylbenzene and allylanisole phenylpropanoids: Inhibition of antibiotic resistance targets and toxicity profile in a *Drosophila melanogaster* model. **Journal of King Saud University-Science**, p. 101995, 2022.

4. MACEDO, N.S. *et al*. Phytochemical prospection, evaluation of antibacterial activity and toxicity of extracts of *Libidibia ferrea* (Mart. ex Tul.) LP Queiroz. **Arabian Journal of Chemistry**, v. 15, n. 2, p. 103632, 2022.

5. ROCHA, J.E. *et al*. 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. **Biomedicine & Pharmacotherapy**, v. 140, p. 111768, 2021.

6. PEREIRA, R.L.Silva *et al*. Antibacterial and modulatory activities of β-cyclodextrin complexed with (+)-β-citronellol against multidrug-resistant strains. **Microbial Pathogenesis**, v. 156, p. 104928, 2021.

7. DOS SANTOS BARBOSA, C.R. *et al*. Effect of Carvacrol and Thymol on NorA efflux pump inhibition in multidrug-resistant (MDR) *Staphylococcus aureus* strains. **Journal of bioenergetics and biomembranes**, v. 53, n. 4, p. 489-498, 2021.

8. BEZERRA, A.H. *et al*. Effect of estragole over the RN4220 *Staphylococcus aureus* strain and its toxicity in *Drosophila melanogaster*. **Life Sciences**, v. 264, p. 118675, 2021.

9. DOS SANTOS, J.F.S. *et al*. Enhancement of the antibiotic activity by quercetin against *Staphylococcus aureus* efflux pumps. **Journal of Bioenergetics and Biomembranes**, v. 53, n. 2, p. 157-167, 2021.

10. DA COSTA, R.H.S. *et al*. Evaluation of antibacterial activity and reversal of the NorA

and MepA efflux pump of estragole against *Staphylococcus aureus* bacteria. **Archives of Microbiology**, v. 203, n. 6, p. 3551-3555, 2021.

11. NETO, L.J.L. *et al*. Evaluation of Benzaldehyde as an Antibiotic Modulator and Its Toxic Effect against *Drosophila melanogaster*. **Molecules**, v. 26, n. 18, p. 5570, 2021.

12. DE SOUSA JÚNIOR, D.L. *et al*. Evaluation of isoeugenol in inhibition of *Staphylococcus aureus* efflux pumps and their toxicity using *Drosophila melanogaster* model. **Life Sciences**, v. 285, p. 119940, 2021.

13. LUNA, E.M. *et al*. Evaluation of phytochemical composition, toxicity in *Drosophila melanogaster* and effects on antibiotics modulation of *Plathymenia reticulata* Benth extract. **Toxicology Reports**, v. 8, p. 732-739, 2021.

14. DE MATOS, Y.M.L.S. *et al*. FTIR analysis and reduction of the phytotoxic effect of mercury dichloride by rutin. **Rhizosphere**, v. 19, p. 100393, 2021.

15. Xavier, M.R. *et al*. Modulating antibacterial activity against multidrug-resistant *Escherichia coli* and *Staphylococcus aureus* of the flavonoid pectolinarin isolated from *Lantana camara* leaves. **J Anal Pharm Res**, v. 10(6), p. 217-220, 2021.

16. BRAGA, A.L. *et al*. *Piper regnellii* (Miq.) C. DC.: Chemical composition, antimicrobial effects, and modulation of antimicrobial resistance. **South African Journal of Botany**, v. 142, p. 495-501, 2021.

17. ROCHA, J.E. *et al*. 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. **Archives of Microbiology**, v. 204, n. 1, p. 1-9, 2022.

18. FREITAS, T.S. *et al*. Synthesis of Cu-TiNT, characterization, and antibacterial properties evaluation. **Materials Today Chemistry**, v. 21, p. 100539, 2021.

19. DA SILVA, P.T. *et al*. Synthesis, spectroscopic characterization and antibacterial evaluation by chalcones derived of acetophenone isolated from *Croton anisodontus* Müll. Arg. **Journal of Molecular Structure**, v. 1226, p. 129403, 2021.

20. FREITAS, T.S. *et al*. UPLC-QTOF-MS/MS analysis and antibacterial activity of the *Manilkara zapota* (L.) P. Royen against *Escherichia coli* and other MDR bacteria. **Cellular and Molecular Biology**, v. 67, n. 1, p. 116-124, 2021.

21. SIQUEIRA, M.M.R. *et al*. Aminophenyl chalcones potentiating antibiotic activity and inhibiting bacterial efflux pump. **European Journal of Pharmaceutical Sciences**, v. 158, p. 105695, 2021.

22. BEZERRA, C.F. *et al*. Antifungal activity of farnesol incorporated in liposomes and associated with fluconazole. **Chemistry and Physics of Lipids**, v. 233, p. 104987, 2020.

23. BEZERRA, C.F. *et al*. Antifungal Properties of Nerolidol-Containing Liposomes in Association with Fluconazole. **Membranes**, v. 10, n. 9, p. 194, 2020.

24. Júnior, J.P.S. Avaliação da Atividade Antifúngica do Extrato Aquoso das Folhas de

Libidibia ferrea (Mart. Ex Tul.) L. P. Queiroz (Pau Ferro). **Cadernos de Cultura e Ciência (URCA)**, v.19, p.1 - 16, 2020.

25. Neto, R.N.O. Avaliação do Potencial Antifúngico e Ação Moduladora do Extrato Aquoso das Folhas de *Ricinus communis* L. (MAMONA) Frente a *Candida spp*. **Cadernos de Cultura e Ciência (URCA)**, v.19, p.10 - 22, 2020.

26. SCHERF, J.R. *et al*. Effect of terpinolene against the resistant *Staphylococcus aureus* strain, carrier of the efflux pump QacC and β-lactamase gene, and its toxicity in the *Drosophila melanogaster* model. **Microbial Pathogenesis**, v. 149, p. 104528, 2020.

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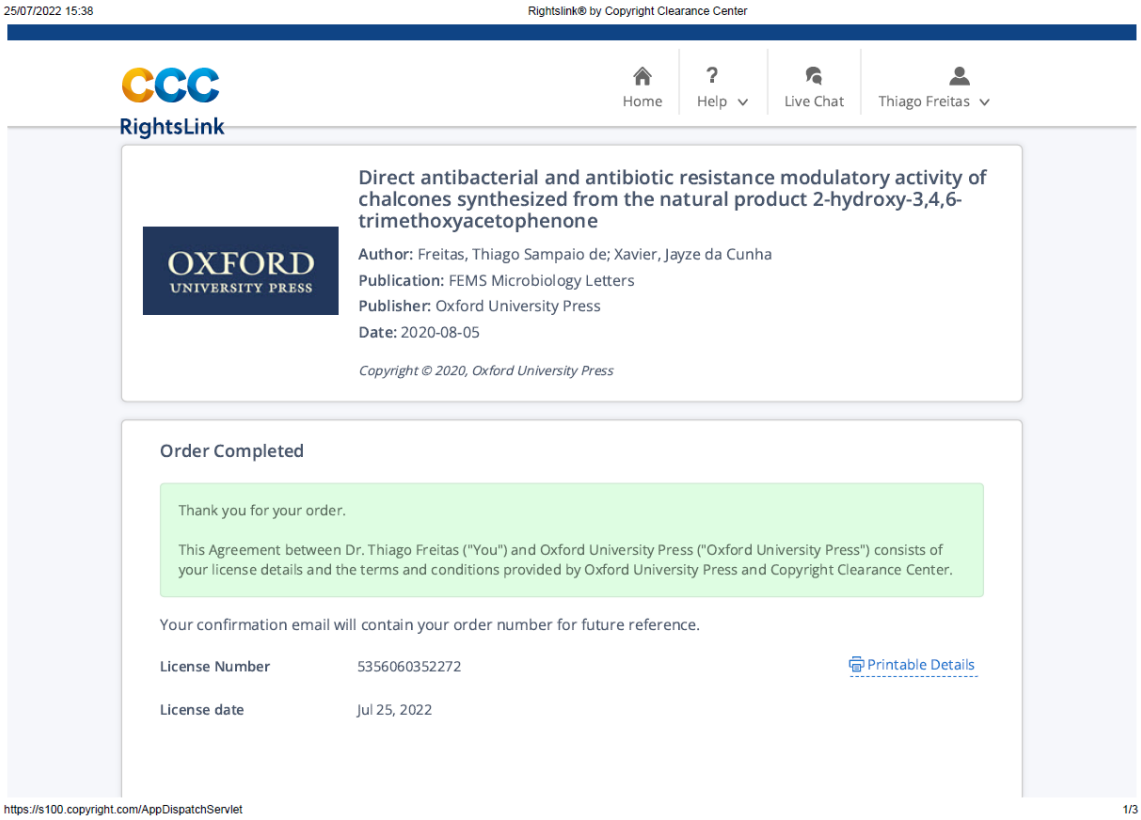

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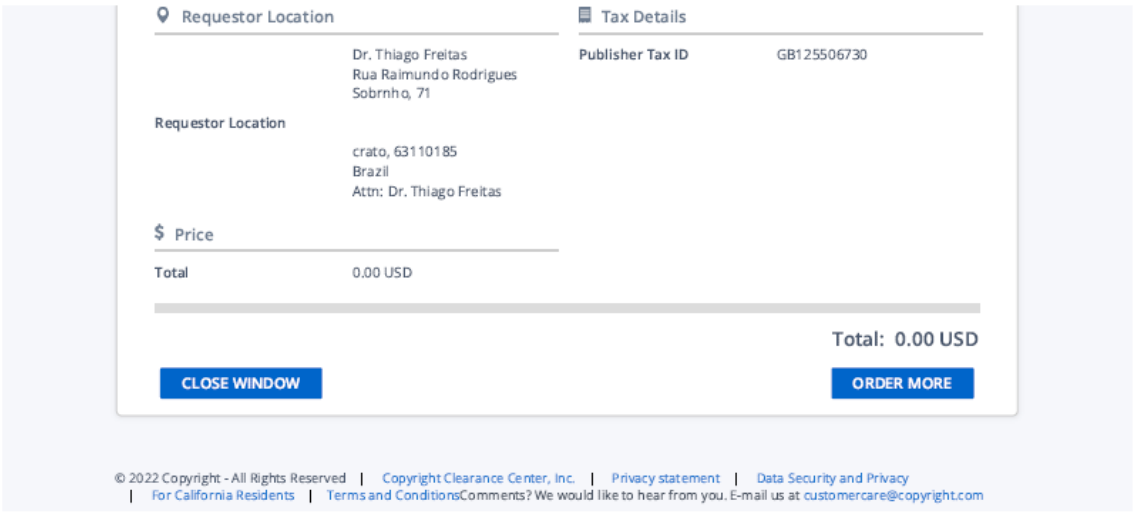

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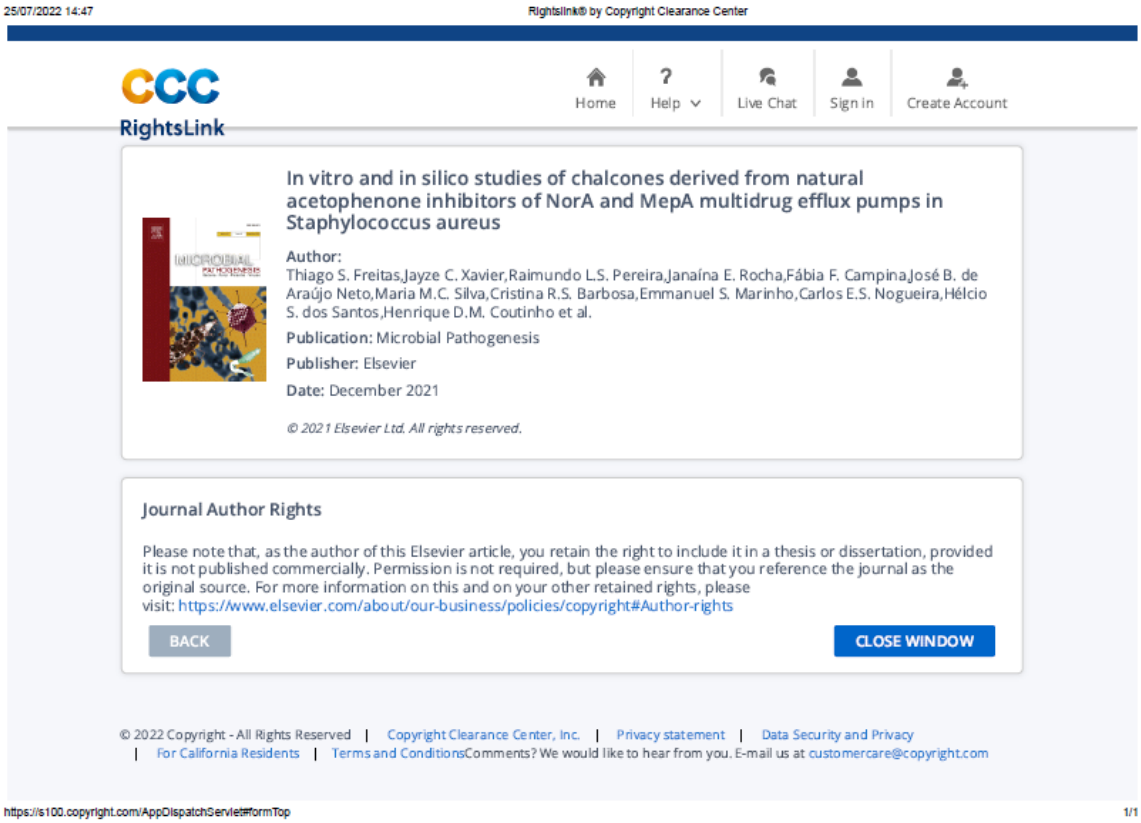

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