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PATRÍCIA GONÇALVES PINHEIRO

ATIVIDADE ANTIMICROBIANA E INIBIÇÃO DE BOMBA DE EFLUXO EM *Staphylococcus aureus* PELO ÁCIDO FERÚLICO E DERIVADOS ESTERIFICADOS

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

Orientador: Prof. Dr. José Galberto Martins da Costa

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RESUMO

Introduçao: Bactérias são organismos hábeis em adquirir resistência a antibióticos, estes mecanismos de resistência bacteriana agem de três modos principais: inativação do antibiótico por hidrólise ou modificação química, modificação do alvo antibiótico e diminuição das concentrações intracelulares do antibiótico. *Staphylococcus aureus é* naturalmente encontrada na microbiota da pele e em mucosas, podendo ser associada a infecções que vão desde superficiais cultâneas até pneumonia necrosante grave. **Objetivo:** Avaliar o composto ácido ferúlico, juntamente com quatro derivados sintetizados, por reação de esterificação com os álcoois metanol, etanol, propanol e butanol, a fim de buscar o efeito potencializador de antibióticos, atuando de forma a inibir o mecanismo de bombas de efluxo, mecanismo de resistência bacterina. **Metodologia:** Derivados esterificados do ácido ferúlico foram sintetizados, os compostos, em seguida, foram isolados e caracterizados por RMN 1H e 13C. Para efetivação dos testes, utilizou-se das cepas de *S. aureus*: K2068; RN4220, IS-58, TetK; 1199B, e a cepa 1199. A concentração inibitória mínima foi determinada pelo método de microdiluiçao. Testes de docagem molecular foram realizados. Um artigo de revisão foi elaborado buscando associar a estrutura química a efeitos de reversção da resistência bacteriana. Results: Um sinergismo foi observado quando os esteres ferulato de etila e propila foram combinados com o norfloxaacino. Os estudos de docagem demonstraram energia de interação favorável entre os éteres e NorA. **Conclusão**: A associação do composto AF-etanol, nos testes de concentração mínima inibitória, com norfloxacina e brometo de etídio, não foi sugestiva de um mecanismo de bomba de efluxo, indicando que o ferulado de etila tem efeitos moduladores de antibióticos contra a cepa investigada, sobre a bomba de efluxo ou outros mecanismos de resistência à norfloxacina. Uma correlação foi traçada entre a interação dos compostos e a bomba de efluxo NorA, por meio do acoplamento molecular, que demonstrou boa afinidade. A associação dos compostos esterificados do ácido ferúlico, nos testes de concentração mínima inibitória com os antibióticos de referência tetraciclina e eritromicina e o composto brometo de etídio, mostrou redução significativa nas CIMs, a inibição de um mecanismo de bomba efflux não pôde ser comprovada, assim foi levantada a suposição de danos estruturais e/ou funcionais à membrana citoplasmática. Novos estudos estão sendo realizados para elucidar o mecanismo completo de sinergismo apresentado.

Palavras-chave: *Staphylococcus aureus*. Resistência bacteriana. Estrutura química.

ABSTRACT

Introduction: Bacteria have the ability of developing resistance to antibiotics. Bacterial resistance mechanisms can occur in three main ways: inactivation of the antibiotic by hydrolysis or chemical modification; modification of the target antibiotic; and reduction of intracellular concentrations of the antibiotic. *Staphylococcus aureus* is usually found in the microbiota of the skin and on mucous membranes, and can be associated with infections ranging from superficial skin infections to severe necrotizing pneumonia. **Objective**: To evaluate the compound ferulic acid and its four derivatives synthesized by esterification with methanol, ethanol, propanol, and butanol, in order to search for the potentializing effect of antibiotics to inhibit the efflux pump mechanism, a mechanism of bacterial resistance. **Methodology**: Esterified derivatives of ferulic acid were synthesized. Then, the compounds were isolated and characterized by 1H and 13C NMR. For carrying out the tests, *S. aureus* strains K2068; RN4220, IS-58, TetK; 1199B, and strain 1199 were used. The minimum inhibitory concentration was determined by the microdilution method. Molecular docking tests were performed. A review article was prepared seeking to associate the chemical structure of the compounds with reversion effects of bacterial resistance. Results: Synergism was observed when ethyl and propyl ferulate esters were combined with norfloxacin. Docking studies demonstrated favorable interaction energy between the ethers and NorA. Propylferulate was seen to reduce the minimum inhibitory concentration (MIC) of both the control substance ethidium bromide and the tested antibiotic, indicating that this compound is promising for efflux pump inhibition of IS-58 strains. **Conclusions**: The association of compound AF-ethanol, in the minimum inhibitory concentration tests, with norfloxacin and ethidium bromide was not suggestive of an efflux pump mechanism, indicating that ethyl ferulate has antibiotic modulating effects against the investigated strain on the efflux pump or other norfloxacin resistance mechanisms. A correlation was drawn between the interaction of the compounds and the NorA efflux pump by molecular docking, which showed good affinity. The association of the esterified ferulic acid compounds in the minimum inhibitory concentration tests with the reference antibiotics tetracycline and erythromycin and the compound ethidium bromide showed significant reduction in MICs, although inhibition of an efflux pump mechanism could not be proven, so the assumption of structural and/or functional damage to the cytoplasmic membrane was raised. Further studies are being conducted to elucidate the full mechanism of synergism found.

Keywords: *Staphylococcus aureus*. Bacterial resistance. Chemical structure.

LISTA DE SIGLAS E SÍMBOLOS

AF	Ácido Ferúlico
BHI	*Brain Heart Infusion*
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CCCP	*Carbonyl Cyanide m-ChloroPhenyl-hydrazone*
CIM	Concentração Inibitória Mínima
d	Dubleto
DCC	Diciclo-hexilcarbodiimida
dd	Duplo Dubleto
DMAP	Dimetilaminopiridina
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
DPOC	Doença Pulmonar Obstrutiva Crônica
HIA	*Heart Infusion Agar*
IUPAC	União Internacional de Química Pura e Aplicada
m	Multipleto
MDR	*Multidrug-resistant*
MRSA	Meticilina ou Oxacilina
q	Quarteto
s	Singleto
sl	Singleto Largo
t	Tripleto
TMS	Tetra Metilsilano
TPSA	*Topological Polarity*
UFC	Unidades Formadoras de Colônias
DEPT	*Distortionless Enhancement by Polarization Transfer*
RMN	Ressonância Magnética Nuclear
Ppm	Partes por milhão
Δ	Deslocamento químico
J	Constante de acoplamento
Nor A	Proteína de efluxo para fluoroquinolonas e outras substâncias
MrsA	*Efflux Protein* (proteína de efluxo para macrolídeos)

TetK	Proteína de Efluxo para Tetraciclina

SUMÁRIO

CAPÍTULO 1: OBJETIVOS E QUESTIONAMENTOS

Bactérias são organismos extremamente hábeis em adquirir resistência a antibióticos e agentes antissépticos, elas desenvolvem estratégias para reparar os efeitos tóxicos sofridos, acarretando aumento da taxa de mortalidade (OLUWATUYI; KAATZ; GIBBONS, 2004). Para Christaki, Marcou e Tofarides (2020), bactéria resistente é aquela capaz de desenvolver mecanismos para driblar os efeitos tóxicos causados por drogas usadas contra elas. As bactérias podem desenvolver resistência a antibióticos, por obtenção de genes, geralmente, contidos em plasmídeos e transposons, e por mutações que produzem alterações no local de ação dos antibióticos (BERNARD *et al*., 2004; LIMA *et al*., 2005). Estima-se que setecentas mil mortes anuais em todo o mundo sejam atribuídas à resistência antimicrobiana, o que torna uma ameaça para saúde global (O'NEILL, 2014).

A resistência antimicrobiana pode ser: adquirida, quando a bactéria adquiri resistência, que pode ocorrer pelo processo de reprodução, pelo processo de mutação ou por transferência horizontal de genes como nos processos de transdução, transformação ou conjugação; intrínseca, a resistência é devido às propriedades inerentes à bactéria, cita-se à resitência à vancominica – um glicopeptídeo isolado a partir do fungo *Amycolatopsis orientalis* - por bactérias Gram-negativas, em virtude da impermeabilidade da membrana externa ou adaptável, ou seja, resistência a um ou mais antibióticos, induzida pelo ambiente, em detrimento dos fatores como alteração no pH, concentração de íons disponíveis e nutrientes (CHRISTAKI; MARCOU; TOFARIDES, 2020).

A transferência de genes bacterianos pode ocorrer por: transformação, onde fragmentos de DNA de uma bactéria morta entram em outra bactéria receptora e são incorporados em seu cromossomo; tradução, neste caso, um meterial genético é transferido por um bacteriófago; conjugação, quando ocorre o contato físico direto entre as bactérias, em que o plasmídeo é transferido da célula doadora para a bactéria receptora. Plasmídeos são DNA circulares extracromossomais que podem transferir resistência a múltiplas drogas em um único efeito de conjugação (HOLMES *et al*., 2016; CHRISTAKI; MARCOU; TOFARIDES, 2020).

Infecções causadas por bactérias são diretamente correlacionadas ao uso excessivo de antibióticos, em humanos, agricultura, medicina veterinária e indústria animal (HARBARTH *et al*. 2015). Países com taxas menores de consumo de antimicrobianos relatam baixas taxas de resistência a antimicrobianos (COSTELLOE *et al*., 2010). Os antibióticos são descritos como o principal responsável por promover efeito bacteriano seletivo, resultando no surgimento de cepas multirresistentes, denominadas de MDR (YEVUTSEY *et al*., 2017). Entretanto,

evidências mostram que os genes de resitência ocorrem de forma natural e abundante em populações microbianas (DANTAS *et al*., 2008).

Os mecanismos de resistência bacteriana agem de três modos principais: inativação do antibiótico por hidrólise ou modificação química; modificação do alvo antibiótico, por mutação genética ou pós-tradução; e diminuição da concentrações intracelulares do antibiótico, como resultado de penetração deficiente ou mecanismos de extrusão (BLAIR *et al*., 2015; POOLE, 2002; RUSSELL, 2000).

Um exemplo clássico de inativação do antibiótico são as enzimas β-lactamases, que destroem a ligação da amida do anel B-lactama, tornando o antibiótico ineficaz. O gene que codifica as β-lactamase pode ser encontrado no cromossomo ou em elementos genéticos móveis, como os plasmídeos. Essas enzimas conferem resistência contra antibióticos da classe dos betalactâmicos, como a penicilina e a cefalosporina (CHRISTAKI; MARCOU; TOFARIDES, 2020).

A modificação do alvo do antibiótico é um mecanismo que causa resistência aos antibióticos, diminuindo ou inativando o alvo do antibiótico exemplifica-se pelas enzimas clorofenicol acetiltransferase que atuam sobre o antibiótico clorofenicol (RAMIRES; TOLMASKY, 2010).

A diminuição da concentração de um antibiótico no meio intracelular pode ser devido ao mecanismo de extrusão, ou seja, a expulsão da droga para fora da célula que pode ocorrer por intermédio de processo dependente de energia, utilizando ATP ou gradiente de íons como fonte de energia, chamado efluxo ativo, mediado por bombas de efluxo (HUGHES; ANDERSSON, 2017; VAN BAMBEKE *et al*., 2003). A resistência mediada por efluxo é causada pela expressão aumentada do gene codificador de bombas de efluxo.

As bomba de efluxo são sistemas bacterianos complexos, mediados por proteínas ubíquas, hidrofóbicas e transmembranares, capazes de expulsar ou trocar ativamente compostos tóxicos para fora da célula bacteriana (BLAIR *et al*., 2015). Esses sistemas podem conferir resistência a uma dada droga ou classe de drogas, mas o principal problema é causado pelas chamadas bombas de efluxo multirresistentes (MDR) que podem lidar com grande variedade de compostos estruturalmente não relacionados (VAN BAMBEKE *et al*., 2003; NIKAIDO; PAGÈS, 2012; KABRA *et al*., 2019).

Os sistemas de efluxo são classificados em seis grandes famílias, utilizando para diferenciá-las e classificá-las o tipo de energia usada para expulsar o substrato e a similiaridade na sequência aminoacídica, citam-se: superfamília MFS (Major facilitator), superfamília ABC (ATP *binding cassette*), família RND (*resistance nodulation division*), família MATE

(*multidrug and toxic compound extrusion*), família SMR (*small multidrug resistance*) e superfamília PACE (*proteobacterial antimicrobial compound efflux*) (KABRA *et al*., 2019). A proteína de efluxo MDR melhor caracterizada em *S. aureus* é a Nor A, encontrada em cepas MSSA (sensível a meticilina), MRSA (resistente a meticilina) e linhagens clínicas que superexpressam o gene Nor, responsável pelo transporte de diversos substratos, entre eles fluoroquinolonas hidrofilicas (norfloxacino, ciprofloxacino, lomefloxacina e ofloxacina), brometo de etídeo, acriflavina, rodamina, centrimida, brometo de tetrafenilfosfônio, clorofenicol, além do alcalóide berberina (AESCHIMANN, 1999; CHRISTAKI; MARCOU; TOFARIDES, 2020).

Staphylococcus aureus naturalmente encontrada na microbiota da pele e em mucosas, principalmente nas fossas nasais, em cerca de 30% da população mundial saudável, podendo ser localizada em animais. Geralmente, está associada a infecções que vão desde superficiais cultâneas até pneumonia necrosante grave, leões necróticas graves, abscessos, endocardite e bacteriamia, sendo retratadas como causa de infecções hospitalares graves, especialmente associadas a sítios de implantes de cateteres e válvulas. Cepas de *S. aureus* produzem toxinas que causam uma variedade de sintomas específicos, incluindo a síndrome do choque tóxico (ARORA *et al*., 2010; CASSETTARI; STRABELLI; MEDEIROS, 2005; LI *et al*., 2019).

O uso incorreto e excessivo de antimicrobianos são associados ao aumento da resistência bacteriana, porém poucos novos compostos antimicrobianos estão em processo de desenvolvimento, além disso novas classes de antibióticos poderiam apresentar um pequeno período de efetiva atuação, devido à rápida capacidade de adaptação das bactérias. A terapia de inclusão de compostos associados a antibióticos de referência vem sendo estudada como uma alternativa ao tratamento convencional de doenças desenvolvidas por cepas resistentes (MITRA; KAR, 2015).

Portanto, para modificar a permeabilidade da membrana bacteriana para facilitar a entrada do antibiótico na célula e diminuir o efluxo bacteriano, buscou-se reverter a resistência bacteriana, mediada pelo efluxo, e restabelecer a suscetibilidade aos antibióticos (IAN; MARILYN, 2001). A descoberta de compostos terapeuticamente úteis que são inibidores desses processos ajudariam a mitigar as infecções pertinentes a cepas multirresistentes (BURT, 2004).

A procura por composto que interagem com os sistemas de efluxo, inibindo-os foi iniciada nos anos 1990, em *S. aureus* por Pidoock (2006). Esses compostos poderiam ligar-se diretamente a um sítio de ligação da proteína de efluxo; interferir no gradiente de prótons; interagir na membrana plasmática modificando a conformação da proteína de efluxo ou inibir

a expressão do gene de efluxo. A associação desses compostos aos antibióticos poderia aumentar a concentração intracelular do antibiótico, uma vez que não seriam expulsos pelas bombas de efluxo, dessa forma restaurando sua eficiência. A busca por produtos naturais capazes de ampliar aspectro antimicrobianos, reduzindo a resistência bacteriana vem sendo estudada (PASQUA *et al*., 2019).

O ácido ferúlico é um ácido fenólico presente nas plantas, sendo encontrado em diversas fontes naturais, como milho, arroz, trigo (PAIVA *et al*., 2013; D'ARCHIVIO *et al*., 2007). Ibitoye (2019) avaliou a influência do ácido ferúlico na atividade antibacteriana de antibióticos à base de quinolona contra *Acinetobacter baumannii*, observando que o ácido ferúlico associado a quinolonas potencializam de forma sinérgica e é um composto que pode ser estudado, visando mitigar a resistência bacteriana.

Diante do exposto, elaboraram-se os seguintes questionamentos do estudo: o ácido ferúlico, um composto fenólico com características hidrossolúveis, é capaz de exibir efeito na reversão da atividade antimicrobiana da bactéria *Staphylococcus aureus*, quando associado aos antibióticos de referência? Modificações na cadeia lateral do anel fenólico apresentarão maior atividade microbiana? Qual a relação entre a estrutura química dos compostos e a atividade bacteriana?

Logo, objetivou-se avaliar o composto ácido ferúlico, juntamente com quatro derivados sintetizados, por reação de esterificação com os álcoois metanol (AF-metanol), etanol (AF-etanol), propanol (AF-propanol) e butanol (AF-butanol), a fim de buscar o efeito potencializador de antibióticos, atuando de forma a inibir o mecanismo de bombas de efluxo. Neste trabalho, estes compostos foram analisados como inibidores de bomba, visando mitigar o efeito da resistência à terapêutica de antibióticos das cepas resistentes da *S. aureus* e estabelecer relações entre estruturas e atividades desses compostos.

1.1 ESTRATÉGIAS DE PESQUISA

Com base no problema de pesquisa e nos objetivos propostos, optou-se pela pesquisa experimental. Derivados esterificados do ácido ferúlico foram sintetizados, os compostos, em seguida, foram isolados e caracterizados por RMN 1H e 13C.

Para efetivação dos testes, utilizou-se das cepas de *S. aureus*: K2068; RN4220, portadora do plasmideo pUL5054; IS-58, dotada do plasmídeo PT181, TetK; 1199B, cepa resistente a antibióticos da classe dos fluroquinolonas, via proteína de efluxo NorA; e a cepa 1199, considerada selvagem (GIBBONS; OLUWATUYI; KAATZ, 2003). Grande variedade

de métodos laboratoriais pode ser empregada para medir a susceptibilidade *in vitro* de bactérias a agentes antimicrobianos, entre estes, ensaios bioautográficos, de difusão e diluição (RIOS; RECIO; VILLAR, 1988). Optou-se por trabalhar com o método de diluição, o qual, em meio líquido, é o que apresenta metodologia mais complexa, entretanto, é o mais preciso, dentre as vantagens, tem-se o pequeno tamanho da amostra utilizada e a possibilidade de testar cinco ou seis compostos contra um único micro-organismo de forma simultânea (RIOS; RECIO; VILLAR, 1988).

1.2 ESTRUTURA DA TESE

O capítulo I descreve as principais características da bactéria estudada, os questionamentos e objetivos do trabalho; o capítulo II caracteriza o ácido ferúlico; nos capítulos III e IV, estão expressos dois artigos deste estudo, denominados, respectivamente, de *Antimicrobial activity and inhibition of the NorA efflux pump of Staphylococcus aureus by ferulic acid and its esterified derivatives,* artigo aceito para publicação, Qualis em Ciências Biológicas II: B2 e *Antimicrobial activity and inhibition of the Tet(k) and Msr(A) efflux pump of Staphylococcus aureus by ferulic acid and its esterified derivatives,* artigo submetido para publicação, Qualis em Ciências Biológicas II: B2; o capítulo IV trata-se de um artigo de revisão que buscou correlacionar a estrutura e a atividade bacteriana de compostos, artigo submetido para publicação, Qualis em Ciência Biológicas II: A2**;** o capítulo VI expõe as considerações finais e está dividido em discussão geral, conclusões, perspectivas futuras e anexos.

CAPÍTULO 2: FUNDAMENTAÇÃO TEÓRICA

Os compostos fenólicos são considerados produtos secundários de plantas, isto é, produtos que não são responsáveis pelo crescimento, pelo desenvolvimento e pela reprodução das plantas (ZHAO *et al.*, 2019). O metabolismo secundário oferece vantagens para manutenção e desenvolvimento das plantas que os sintetizam, atuando como herbicidas, ajudando na atração de polinizadores, aumentando a tolerância de temperaturas extremas, estresse hídrico ou deficiências nutricionais que o solo possa apresentar, entre outros benefícios. Os compostos fenólicos estão envolvidos na adaptação a condições de estresse ambiental, seja na defesa contra a radiação ultravioleta ou agressão por patógenos (ZHAO *et al.*, 2019; ERB; KLIEBENSTEIN, 2020). A contribuição dos compostos fenólicos para os mecanismos de defesa dos vegetais e as características sensoriais e nutricionais, atuando principalmente como agentes de defesa, foram apresentadas por Nychas (1995), no entanto, a função da maior parte dos compostos fenólicos, ainda, é desconhecida (SIQUEIRA; HAMMERSCHMIDT; NAIR, 1991).

Esses compostos podem ser sintetizados pela via do ácido chiquímico, ou via do acetato/malonato (CROTEAU, 2006). Os principais compostos fenólicos comumente encontrados em vegetais superiores podem ser categorizados em várias classes, de acordo com tipo e número de anéis fenólicos; e, em subclasses, conforme substituições específicas na estrutura básica, associações com carboidratos e formas polimerizadas. Os compostos fenólicos são subdivididos em três grupos: ácidos fenólicos, flavonóides e taninos (SPENCER *et al*., 2008; D'ARCHIVIO *et al*., 2007).

Ademais, ácidos fenólicos têm um ácido carboxílico funcional e são hidroxilados derivados do ácido benzóico (por exemplo, ácido gálico) e ácidos cinâmicos (por exemplo, cafeína, ácidos p-cumarico e ácido ferúlico) (ROBBINS, 2003; STALIKAS, 2007). Embora o esqueleto básico permaneça o mesmo, os números e as posições da hidroxila, no anel aromático e o tipo de substituintes causam alterações significativas nas propriedades dos produtos fenólicos (ROBBINS, 2003; SROKA; CISOWSKI, 2003; STALIKAS, 2007). Os compostos fenólicos se caracterizam por apresentar o grupos hidroxila ligados ao anel aromático, podendo ter vários grupos substituintes, como carboxilas, metoxilas, estruturas cíclicas não aromáticas, entre outras (NYCHAS, 1995).

O local e o número de grupos hidroxila no grupo fenol são pensados para ser relacionados à toxicidade relativa aos micro-organismos, sendo as principais características estruturais que influenciam a capacidade antioxidante dos fenólicos, com evidências de que

hidroxilação resulta em aumento da toxicidade (PERUMAL SAMY; GOPALAKRISHNAKONE, 2010; LARIT *et al*., 2019).

Os ácidos fenólicos apresentam um grupo funcional carboxila e são divididos em duas classes: os ácidos hidroxibenzoicos e os hidroxicinâmicos (D'ARCHIVIO *et al*., 2007). Os ácidos hidroxicinâmicos são conhecidos pelas múltiplas funções fisiológicas, como antioxidante, anti-inflamatória, antimicrobiana, anticolagenase e antimelanogênica. Essas propriedades justificam o aumento gradual do uso dos ácidos hidroxicinâmicos e dos derivados em formulações cosméticas para cuidados com a pele.

O ácido ferúlico, extraído pela primeira vez em 1866, a partir da planta *Ferula foetida*, é um derivado hidrocinâmico do ácido fenólico. O anel de catecol e os grupos cinâmicos são responsáveis pela ampla atividade farmacológica deste composto (IBITOYE; AJIBOYE, 2018; GRAFF, 1992) .

O ácido ferúlico (Figura 1), denominado de ácido 3- (4-hidroxi-3-metoxifenil)-2-propenóico, pela União Internacional de Química Pura e Aplicada (IUPAC), foi extraído pela primeira vez em 1866, a partir da planta *Ferula foetida*. É um ácido fenólico, pertencente à classe dos hidroxicinâmicos, abundante em plantas e encontrado em feijão, milho, trigo e outros (CHAUDHARY *et al*., 2019). O anel de catecol e os grupos cinâmicos são responsáveis pela ampla atividade farmacológica deste composto (IBITOYE; AJIBOYE, 2018; GRAFF, 1992).

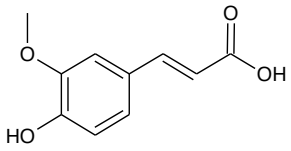
A molécula do ácido ferúlico apresenta isomeria cis-trans, sendo o isômero trans o mais abundante encontrada na natureza. Ambos os isômeros apresentam resultados comprovados na terapêutica de diversas patologias, como câncer, diabetes e doenças neurodegenerativas e cardíacas, além de ações antimicrobiana, anti-inflamatória e, principalmente, atividade antioxidante, responsável pelos principais benefícios e aplicações (SRINIVASAN; SUDHEER; MENON, 2007; GRAFF, 1992; SUNDARAMOORTHY *et al*., 2018). Devido à capacidade de interromper as reações de cadeias radicais por ressonância, seguida de polimerização, o ácido ferúlico oferece proteção contra a radiação UV (SÁNCHEZ *et al*., 1997; KROON *et al*., 1999; SANTOS *et al*., 2008; ERGÜN *et al*., 2011; LARIT *et al*., 2019).

Segundo Takahashi *et al.* (2015), o ácido ferúlico tem atividade antibacteriana sobre *Listeria monocytogenes*, inibindo seu crescimento com risco mínimo de desenvolvimento de resistências ao composto. Uma das hipóteses apresentadas por Campos *et al.* (2009), como possível mecanismo de ação do ácido ferúlico, é a ação inibitória do crescimento bacteriano, primeiro, por meio da promoção do influxo de prótons para o meio intracelular, e, depois, por intermédio do efluxo de íons potássio para o meio extracelular.

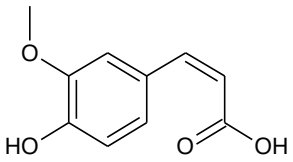
Por outro lado, Borges, Saavedra e Simões (2013) apresentaram uma alternativa para o modo de ação deste composto. Conforme estes autores, o ácido ferúlico, por ser um composto eletrofílico, tem a capacidade de interagir com a superfície membranar bacteriana, de modo a aumentar a solubilidade lipídica e, desta forma, transpor a camada lipídica bacteriana, atuando sobre os constituintes citoplasmáticos.

Borges, Saavedra e Simões (2012) elucidam que o ácido cis ferúlico apresenta atividade antibacteriana sob *Pseudomonas aeruginosa*. Hemaiswarya e Doble (2010) sugerem que a esturtura do ácido ferúlico, com a presença do anel fenólico e do grupo ácido, seja responsável pela atividade ambimicrobiana, esta estrutura parece conseguir atravessar a membrana plasmática.

Figura 1 – Representação estrutural dos isômeros cis e trans do ácido ferúlico.



trans-ferúlic acid



cis-ferúlic ácid

Fonte: Autor

CAPÍTULO 3: ANTIBACTERIAL ACTIVITY AND INHIBITION AGAINST *STAPHYLOCOCCUS AUREUS* NORA EFFLUX PUMP BY FERULIC ACID AND ITS ESTERIFIED DERIVATIVES

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Antibacterial activity and inhibition against *Staphylococcus aureus* NorA efflux pump by ferulic acid and its esterified derivatives

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ABSTRACT

Objective: To evaluate the inhibitory activity of ferulic acid and four of its esterified derivatives (methyl, ethyl, propyl, and butyl) against resistance mechanisms in *Staphylococcus aureus* strains.

Methods: Ferulic acid derivatives were obtained by esterification with methanol, ethanol, propanol, and butanol, and then characterized by hydrogen and carbon-13 nuclear magnetic resonance analysis. The minimum inhibitory concentrations (MIC) of ferulic acid and its esterified derivatives, ethidium bromide, and norfloxacin were obtained using the microdilution test, while the efflux pump inhibition test was conducted by examining reduction in the MICs of norfloxacin and ethidium bromide. Molecular docking was also carried out using the Schrodinger Suite 2015 molecular modeling software. A three-dimensional model of NorA efflux pump was generated using I-TASSER. The best scoring model was used as a receptor for ligand-receptor docking.

Results: The methyl and butyl ester derivatives did not demonstrate significant antimicrobial activity. However, a significant synergic effect was evidenced when norfloxacin was combined with the ethyl and propyl esterified derivatives. The docking study demonstrated favorable energy of interaction between ferulate derivatives and NorA, and amino acid residues TYR57, TYR58, and LEU255 were present commonly in stabilizing all complexes. The PCA analysis corroborated the docking hypothesis that the lipophilic character and hydrogen bond interactions were the most relevant characteristics involved with NorA inhibitors. The pharmacokinetic parameters of ferulic acid derivatives showed good ADMET properties, demonstrating that they can be easily absorbed and have no effect or inhibit the cytochrome P450 enzyme complex, revealing their potential as drug candidates.

Conclusions: This study provides strong evidence that the molecular basis for this activity is potentially due to the NorA efflux pump.

KEYWORDS: Ferulic acid; Esterified derivatives; Efflux pump; *Staphylococcus aureus*; Resistance mechanisms

1. Introduction

The increase in mortality rate as a consequence of bacterial infections is ascribed, in particular, to bacterial resistance, which can be associated with the abusive and indiscriminate use of antibiotics[1−3]. Bacteria become resistant to drugs by obtaining genes, usually contained in plasmids and transposons, and by mutations that produce changes in the active site of antibiotics[4,5].

The mechanisms of bacterial resistance, although not common for all antibiotics, act in three principal ways: antibiotic inactivation, by hydrolysis or chemical modification; antibiotic target modification,

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by genetic mutation or post-translational modification; and reduced antibiotic intracellular concentrations, as a result of poor penetration or efflux mechanisms[6–8].

The decrease in intracellular concentration of an antibiotic may be due to an extrusion mechanism, by which the drug is expelled out of the cell through an energy-dependent process, called active efflux, mediated by efflux pumps[9]. Such pumps are ubiquitous, transmembrane proteins, capable of actively expelling or exchanging toxic compounds out of the bacterial cell[6]. These systems can provide resistance to a given drug or a class of drugs, however, the main problem is caused by so-called multi-resistant efflux pumps (MDR pumps) that can extrude a wide variety of structurally unrelated compounds[9].

The most studied bacteria among the microorganisms that present an efflux pump are the Gram-positive bacteria from the *Staphylococcus* genus, which are associated with infections of the skin, wound, and soft tissues, in addition to being identified as a cause of endocarditis and infections associated with implant devices, such as valves and catheters[10]. The antibiotic resistance severity of this species lies in the fact that these bacterial strains are not only highly virulent but also resistant to commonly available antibacterial drugs[11–13]. Moreover, the number of *Staphylococcus aureus* (*S. aureus*) strains present in clinical isolates resistant to various drugs is increasing[14].

The NorA pump, specific to *S. aureus*, is responsible for the efflux of various drugs such as fluoroquinolones, quinolones, verapamil, and omeprazole, in addition to dyes such as acridine and ethidium bromide[15,16]. Many studies are being carried out to find new substances capable of reversing bacterial resistance from the NorA pump and other pumps promoted by efflux pumps. Various isolated plant compounds, as well as extracts and essential oils, have shown good results in terms of inhibiting this mechanism[17,18].

Phenolic compounds have been widely reported among these substances as promising alternatives in the search for new sources of treatment against infections caused by MDR bacteria[19,20]. Studies indicate that these compounds can contribute satisfactorily as new sources of adjuvant treatment, since they can modify bacteria, impairing their locomotion, as well as surface adhesion, biofilm formation, and the formation of virulence determinants[21].

Ferulic acid is a phenolic compound from the hydroxycinnamic acid class, generated from the metabolism of the phenylalanine and tyrosine amino acids, which can be found in the most diverse natural sources, such as corn, rice, wheat, beet, artichoke, coffee, and red fruits, either in a free form or in conjunction with cell wall proteins and polysaccharides[22–24].

A study evaluated the influence of ferulic acid on the antibacterial activity of quinolone-based antibiotics against *Acinetobacter baumannii* and showed that ferulic acid potentiated the antibacterial activity of quinolones[24]. According to the results obtained by Takahashi *et al.*[25], ferulic acid has a very strong antibacterial activity against *Listeria monocytogenes*, inhibiting its growth with minimal risk of developing compound resistance.

Given the above, the objective of this study was to evaluate the antibacterial activity and possible NorA efflux pump inhibition of ferulic acid and its four esterified derivatives against *S. aureus* strains and to establish relationships between the structures and activities of these compounds.

2. Materials and methods

2.1. Chemical products

Ferulic acid, 4-hydroxy-3-methoxycinnamic acid, *trans*-4-hydroxy-3-methoxycinnamic acid, absolute methyl alcohol content (200, 99.8%), absolute ethyl alcohol content (200, 99.5%), absolute propyl alcohol content (200, 99.7%), *n*-butanol alcohol, absolute butyl alcohol content (200, 99.8%), and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) were used in this study. All chemical substances were obtained from Sigma Aldrich.

2.2. Substance preparation and microorganisms

For the microbiological tests, the substances norfloxacin and ethidium bromide were initially solubilized in dimethyl sulfoxide and then diluted in water. The efflux pump inhibitor used was CCCP, following its dissolution with methylene and concentration adjustment to 1 024 μg/mL. The 1199B strain and all the substances used were carried out according to the study of Santos *et al*[26]. The *S. aureus* strains used in the present study were kindly provided by Prof. Gibbons (University of London).

2.3. Acquisition of the esterified derivatives

The derivatives were synthesized by esterifying ferulic acid with methyl, ethyl, propyl, and butyl alcohols, following the classic Fischer esterification mechanism. Ferulic acid (60 mg, 0.309 2 mmol), was dissolved in the respective alcohols (10 mL: MeOH, EtOH, PropOH, and ButOH) and dicyclohexylcarbodiimide (DCC, 54 mg, 0.262 1 mmol), at catalytic quantities of 4-*N*,*N*-dimethylaminopyridine. The reaction mixture was continuously refluxed, under magnetic stirring with heating at 50 ℃ for 4 h. Afterward, the *N*,*N*-dicyclohexylurea formed was filtered away, the solvent was removed on a rotary evaporator at room temperature, and the crude residue was purified by column chromatography (silica gel, hexane/EtOAc:70/30). Ferulic acid, used as a substrate, and the four esterified derivatives were

characterized by ^{1}H and ^{13}C NMR, including the DEPT 135° technique.

2.4. Structural characterization

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer, operating at the frequency of 300 MHz for ^{1}H and 75 MHz for ^{13}C. The spectra were obtained in CD_3OD solvent and chemical displacements (δ) were expressed in ppm, with tetramethylsilane as the internal standard. The multiplicities of the ^{1}H NMR signals were indicated following the convention: s (singlet), sl (broad singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), and m (multiplet). All derivates obtained were completely used in the NMR ^{13}C and ^{1}H analysis to confirm the reaction, and in the biological assays performed.

2.5. Minimum inhibitory concentration (MIC) assays

Tests for MIC of ferulic acid and its esterified compounds were performed according to the methods of Tintino *et al*[27]. The bacterial strain was incubated at 37 ℃ for 24 h. After that, the procedures for standardization and dilution of the sub-branches were performed according to the base study. After 24 h, the plates were read by visualizing the color change of the medium, characterized by the addition of 20 µL of resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide). The characteristic color change of the medium from blue to red in this experiment indicates the presence of bacterial growth, while its permanence in blue indicates the absence of growth. The above experiments were carried out in triplicate.

2.6. Efflux pump inhibitory study

As previously performed, the methodology of Tintino *et al.*[27] was adopted to assess the antibacterial activity of the compounds, verifying the reduction effect on MICs of ethidium bromide and norfloxacin. In the test, the substance was used in subinhibitory concentration. For the control, the conditions were similar to the test, but without the addition of substances. Then, these substances were transferred to 96-well microdilution plates, with vertical distribution, characterized by the addition of 100 µL of Eppendorf content in each well. After this stage, micro dilutions of ethidium bromide and norfloxacin were performed[27]. After 24 h, the plates were read by visualizing the color change of the medium, as previously performed. The experiments were carried out in triplicates.

2.7. Statistical analysis

Antibacterial assays were performed in triplicates and results were expressed as the average of replicates. The results from the tests were expressed as the mean ± standard error of mean and evaluated by analysis of variance (unidirectional ANOVA), followed by Tukey test using the GraphPad Prism v software. 6.01, with a level of statistical significance set at 5% ($P < 0.05$). Principal component analysis was used to achieve better visualization of the different data sets and a more distinct view of the relationship among the variables. This method is based on matrix linking percentages of the major components to provenance stations and the variability of physicochemical parameters and antimicrobial activity. These multivariate analyses were performed by the Minitab Software.

2.8. NorA structure prediction and molecular docking

All dockings were carried out using the Schrodinger Suite 2015 molecular modeling software. A two-dimensional Rip-B structure was built using Maestro. This structure was converted into its three-dimensional form, including various tautomers, conformers, and ionization states using LigPrep and ConfGen modules. A three-dimensional model of NorA was generated using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The best scoring model was used as a receptor for ligand-receptor docking. The receptor was prepared for docking by employing the Protein Preparation Wizard and generating the binding site grid. Ligand-receptor docking was performed using the Glide module, and flexible docking was carried out for all the conformers to determine the ligand-binding mode. The Glide Extra Precision scoring function was used.

2.9. Measure of drug likeliness, Lipinski rule analysis and ADMET predictions

ADME computational methods are used to predict pharmaceutical potential, optimize new lead candidates, and evaluate pharmacological properties. The therapeutic actions are dependent on pharmacokinetic parameters (Absorption, Distribution, Metabolism, and Excretion). A large number of structural modifications can be an influence of diverse physicochemical parameters. Then, understanding of this helps screen weak candidates in the early stage of drug development that finds potential drug candidates. The prediction of physicochemical descriptors and determination of the pharmacokinetic properties and drug likeliness, and other ADME properties are done by the swissADME (http://www.swissadme.ch) server.

3. Results

3.1. Esterified ferulic acid derivatives

The derivatives (Figure 1) were obtained through the Fischer esterification reaction, where the structural changes occurred specifically in the ferulic acid carboxylic group with the insertion of

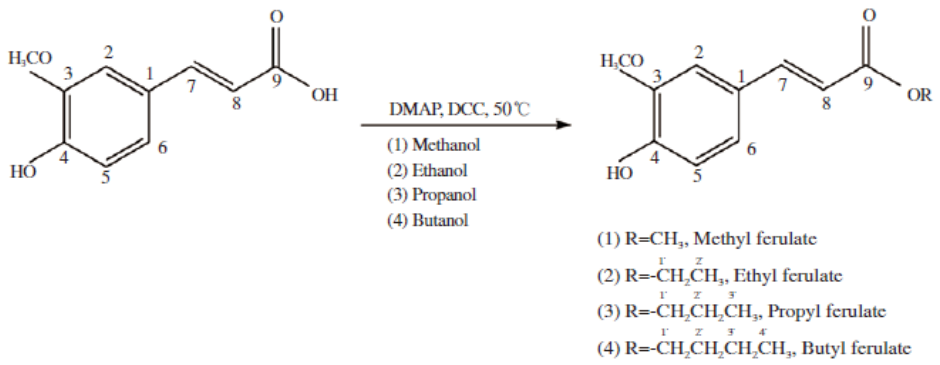


Figure 1. Ferulic acid and esterification reaction products. DCC: Dicyclohexylcarbodiimide, DMAP: 4-Dimethylaminopyridine.

alkyl groups, ranging from carbons 1 to 4, depending on the alcohol used. The compounds were identified by interpreting the respective ^{13}C NMR, ^{1}H NMR, and DEPT 135° spectra.

3.1.1. Ferulic acid (MW = 194.18 g/mol)

White solid: ^{1}H NMR (300 MHz, CD_3OD) 3.78 (s, OCH_3), 6.21 (1H, d, *J*=16.0 Hz, H-8), 6.71 (1H, d, *J*=8.0 Hz, H-5), 6.93 (1H, dd, *J*=8.0 and *J*=1.7 Hz, H-6), 7.06 (1H, d, *J*=1.7 Hz, H-2), 7.49 (1H, d, *J*=16.0, H-7); ^{13}C NMR (75.5 MHz, CD_3OD) 56.56 (CH_3O), 111.83 (C-2), 116.04 (C-5), 116.60 (C-8), 124.12 (C-6), 127.93 (C-1), 147.06 (C-7), 149.48 (C-3), 150.61 (C-4), 171.13 (C=O).

3.1.2. Methyl ferulate (MW = 209.21 g/mol)

Yellow solid (63%): ^{1}H NMR (300 MHz, CD_3OD) 3.75 (s, OCH_3), 3.87 (s, OCH_3), 6.32 (1H, d, *J*=16.0 Hz, H-8), 6.80 (1H, d, *J*=8.0 Hz, H-5), 7.04 (1H, dd, *J*=8.0 and *J*=1.7 Hz, H-6), 7.14 (1H, d, *J*=1.7 Hz, H-2), 7.58 (1H, d, *J*=16.0, H-7); ^{13}C NMR (75.5 MHz, CD_3OD) 52.14 (OCH_3*), 56.56 (CH_3O), 111.82 (C-2), 115.31 (C-5), 116.59 (C-8), 124.20 (C-6), 127.77 (C-1), 146.92 (C-7), 149.46 (C-3), 150.72 (C-4), 169.83 (C=O).

3.1.3. Ethyl ferulate (MW = 223.34 g/mol)

Yellow solid (73%): ^{1}H NMR (300 MHz, CD_3OD) 1.30 (t, $3H^{2'}$), 3.87 (s, OCH_3), 4.21 (q, $2H^{1'}$), 6.32 (1H, d, *J*=16.0 Hz, H-8), 6.80 (1H, d, *J*=8.0 Hz, H-5), 7.04 (1H, dd, *J*=8.0 and *J*=1.7 Hz, H-6), 7.15 (1H, d, *J*=1.7 Hz, H-2), 7.58 (1H, d, *J*=16.0, H-7); ^{13}C NMR (75.5 MHz, CD_3OD) 14.78 ($CH_3^{2'}$), 56.55 (CH_3O), 61.56 ($OCH_2^{1'}$), 111.80 (C-2), 115.75 (C-5), 116.59 (C-8), 124.16 (C-6), 127.82 (C-1), 146.73 (C-7), 149.47 (C-3), 150.69 (C-4), 169.41 (C=O).

3.1.4. Propyl ferulate (MW = 237.27 g/mol)

Yellow solid (47%): ^{1}H NMR (300 MHz, CD_3OD) 0.98 (t, $3H^{3'}$), 1.70 (m, $2H^{2'}$), 3.87 (s, OCH_3), 4.12 (t, $2H^{1'}$), 6.34 (1H, d, *J*=16.0 Hz, H-8), 6.80 (1H, d, *J*=8.0 Hz, H-5), 7.05 (1H, dd, *J*=8.0 and *J*=1.7 Hz, H-6), 7.16 (1H, d, *J*=1.7 Hz, H-2), 7.58 (1H, d, *J*=16.0, H-7); ^{13}C NMR (75.5 MHz, CD_3OD) 10.90 ($CH_3^{3'}$), 23.30 ($CH_2^{2'}$), 56.56 (CH_3O), 67.20 ($OCH_2^{1'}$), 111.78 (C-2), 115.68 (C-5), 116.60 (C-8), 124.19 (C-6), 127.80 (C-1), 146.76 (C-7), 149.48 (C-3), 150.72 (C-4), 169.49 (C=O).

3.1.5. Butyl ferulate (MW = 251.29 g/mol)

Yellow solid (73%): ^{1}H NMR (300 MHz, CD_3OD) 0.95 (t, $3H^{4'}$), 1.42 (m, $2H^{3'}$), 1.65 (m, $2H^{2'}$), 3.87 (s, OCH_3), 4.15 (t, $2H^{1'}$), 6.32 (1H, d, *J*=16.0 Hz, H-8), 6.80 (1H, d, *J*=8.0 Hz, H-5), 7.03 (1H, dd, *J*=8.0 and *J*=1.7 Hz, H-6), 7.14 (1H, d, *J*=1.7 Hz, H-2), 7.56 (1H, d, *J*=16.0, H-7); ^{13}C NMR (75.5 MHz, CD_3OD) 14.22 ($CH_3^{4'}$), 20.35 ($CH_2^{3'}$), 32.06 ($CH_2^{2'}$), 56.55 (CH_3O), 65.41 ($OCH_2^{1'}$), 111.79 (C-2), 115.70 (C-5), 116.58 (C-8), 124.16 (C-6), 127.80 (C-1), 146.72 (C-7), 149.43 (C-3), 150.66 (C-4), 169.45 (C=O).

3.2. Microbiological tests

The microdilution method was used to determine the MIC of ferulic acid and its respective esterified compounds against multi-resistant *S. aureus* strains carrying the NorA efflux systems. All esterified compounds and ferulic acid obtained MIC values ranging between 101.6-128.0 μg/mL against *S. aureus*.

3.3. Effects on S. aureus NorA proteins

Ethidium bromine is used as a marker for the NorA efflux pump activity because the extrusion of this compound is possible only with the action of this pump. The ethyl ferulate compound at subinhibitory concentrations inhibited the NorA efflux pump and reduced the MIC of ethidium bromide, which was similar to that observed when using CCCP, a known efflux pump inhibitor (Figure 2).

Moreover, CCCP, as well as the ethyl and propyl ferulate compounds enhanced the effectiveness of norfloxacin against *S. aureus* 1199B strains that express the NorA pump with reduced MICs (Figure 3).

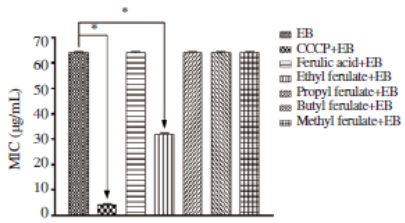


Figure 2. Minimum inhibitory concentration (MIC) of ferulic acid and its four esterified compounds in association with ethidium bromide against *Staphylococcus aureus* 1199B. Statistical significance was determined by one-way ANOVA and Tukey's *post hoc* test. * - indicates statistically significant differences between groups with $P<0.05$. EB: Ethidium bromide, CCCP: carbonyl cyanide *m*-chlorophenyl hydrazone.

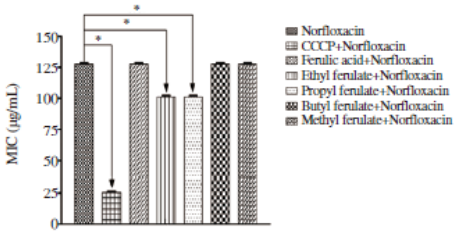


Figure 3. MIC of norfloxacin alone or in the presence of ferulic acid and its esterified compounds against *Staphylococcus aureus* strains. Statistical significance was determined by one-way ANOVA and Tukey's *post hoc* test. * - indicates statistically significant differences between groups with $P<0.05$.

3.4. Docking results and ADMET pharmacokinetic properties predictions

Results from the *in silico* study of the physicochemical properties of ferulic acid derivatives are shown in Table 1. Based on Table 1, the log value of the octanol/water partition coefficient (CLog Po/w) ranged from 1.36 to 2.80 (<5), the number of hydrogen bond donnor ranged from 1 to 2 (≤ 5), and the number of hydrogen bond acceptor was 4 for all derivatives (<10). Thus, all derivatives meet Lipinski's Rules of Five[28].

The docking results suggested different forces of interaction type including hydrogen bonds, van der Waals (VW), Pi-sigma, Pi-Alkyl are necessary to the NorA-ferulate complex formation. These fingerprints present in the active site demonstrated that amino acid residues TYR57, TYR58, and LEU255 were related with the interaction of these derivates with the struture of the efflux pump, mainly due to the effect of hydrophobic interactions and hydrogen bonds.

Based on the principal component analysis using physicochemical property, as shown in Table 1, it can be observed that some physicochemical properties are directly related with the antimicrobial activity. In PC1 axis, positive values indicated the main molecular characteristics of the compounds with action against the NorA efflux pump, as the number of atom donnors of hydrogen bonds and the molar refractivity (Figure 4). In PC2 axis, we observed that MIC activities of ferulate derivatives were influenced by lipophilicity, the rotations bond and the possible hydrogen bonds. Topological polar

Table 1. Physicochemical predictions of ferulic acid derivatives from Swiss ADME and molecular docking results in NorA efflux pump.

Compound	Molecular properties of the ligands									MIC (µg/mL)	Energy of interactions (Kcal/mol)	culated Ki (µM)	SILE*
	NRB	NHba	NHbd	MR	TPSA	CLog Po/w	ASA	Volume Å3	Surface area				
Ethidium bromide	2	0	2	113.02	55.92	2.17	561.66	303.37	326.16	64.0	−78	1.95	0.74
CCCP+Ethidium bromide	2	3	1	53.55	71.97	1.83	449.10	175.45	210.73	4.0	−73	4.53	2.05
Norfloxacin	3	5	2	92.55	74.57	0.98	560.75	264.40	294.75	128.0	−61	34.23	13.36
CCCP+Norfloxacin	2	3	1	53.55	71.97	1.83	449.10	175.45	210.73	25.4	−73	4.53	2.05
Ferulic acid	3	4	2	51.63	66.76	1.36	435.58	176.87	176.87	128.0	−61	34.23	13.36
Methyl ferulate	4	4	1	55.95	55.76	1.76	459.98	193.82	229.49	128.0	−56	79.51	35.29
Ethyl ferulate	5	4	1	60.75	55.76	2.11	488.80	211.00	249.97	101.6	−57	67.18	30.44
Propyl ferulate	6	4	1	65.66	55.76	2.45	519.56	226.78	270.13	101.6	−57	67.18	29.81
Butyl ferulate	7	4	1	70.37	55.76	2.80	547.70	243.62	289.82	128.0	−56	79.51	36.02

*SILE: Size-independente ligand efficiency. MIC: Minimum inhibitory concentration.

Table 2. Pharmacokinetic predictions by Swiss ADME.

Compound	Pharmacokinetic parameters								
	GI	BBB	P-gp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	RL-5
Ferulic acid	Hight	Yes	No	No	No	No	No	No	No
Methyl ferulate	Hight	Yes	No	No	No	No	No	No	No
Ethyl ferulate	Hight	Yes	No	No	Yes	No	No	No	No
Propyl ferulate	Hight	Yes	No	Yes	Yes	No	No	No	No
Butyl ferulate	Hight	Yes	No	Yes	Yes	No	No	No	No

GI = human gastrointestinal absorption (HIA); BBB = blood-brain barrier permeation; RL-5 = Violation of Lipinski's Rule of five.

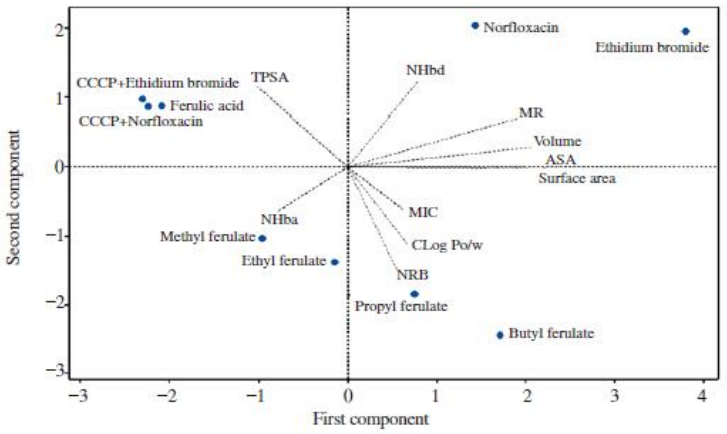
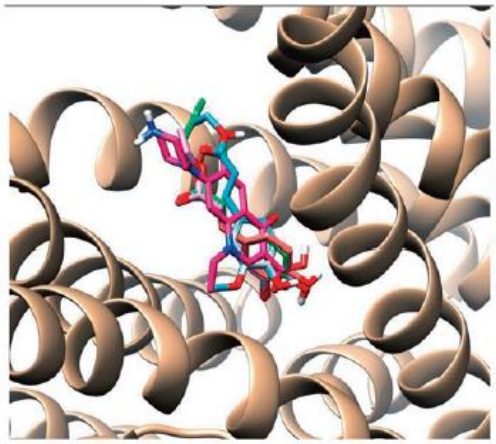


Figure 4. Chemometric multivariate analysis based on physicochemical parameters and antibacterial activity (MIC) using a principal component analysis correlation biplot (loading and score). NHbd: number of hydrogen bond donnor, NHba: number of hydrogen bond acceptor, ASA: water accessible surface area, MR: molar refractivity, MIC: minimum inhibitory concentration, NRB: number of rotatable bonds, CLog Po/w: octanol-water partition coefficient, TPSA: topological polar surface area.



Norfloxacin	VW - THR61, THR90, LEU255, LEU376, MET373, ASP377 Pi-donnor hydrogen bond - TYR58 Pi-Sigma - TYR57 Pi-Alkyl - ILE87, TYR86 Unfavorable - LEU380, ARG94
Ferulic acid	VW - TYR58, LEU255, LEU380, SER176, SER262 Pi-Sigma - ILE258 Pi-Alkyl - ALA179
Methyl ferulic acid	VW - TYR57, TYR58, LEU255, LEU380, SER176, SER262 Pi-Sigma - ILE258 Pi-Alkyl - ALA179
Ethyl ferulic acid	VW - TYR57, TYR58, LEU255, LEU380, SER176, SER262 Pi-Alkyl - ILE258, ALA179
Propyl ferulic acid	VW - TYR57, SER176, SER262 Unfavorable - LEU380 Hydrogen bond - TYR58 Pi-Sigma - ILE258, ALA179 Pi-Alkyl - LEU255
Butyl ferulic acid	VW - TYR57, TYR58, LEU255, LEU380, SER176, SER262 Pi-Sigma - ILE258 Pi-Alkyl - ALA179

Figure 5. Binding pose with the best binding and stability for norfloxacin (red-violet), ferulic acid (white), methyl ferulic acid (pink), ethyl ferulic acid (blue), propyl ferulic acid (coral) and butyl ferulic acid (green) in the NorA efflux pump binding site and the interaction types between the residues.

surface area and accessible surface area were not correlated to NorA inhibition. Therefore, the model showed that not only lipophilia, but also the rotations bond and H-binding pattern are more relevant than efflux size and molecular shape (Figure 4).

In Table 2, it can be seen that almost all ferulic acid derivatives have physico-chemical characteristics that demonstrate high permeability and consequently good absorption capacity without affecting or inhibiting the cytochrome P450 enzyme complex. However, propyl and butyl derivatives can affect or inhibit CYP1A2 enzyme while ethyl, propyl, and butyl derivatives can affect or inhibit CYP2C19 enzyme.

4. Discussion

The ferulic acid esterification reaction with methanol, ethanol, propanol, and butanol produced the esters methyl ferulate, ethyl ferulate, propyl ferulate, and butyl ferulate, respectively, whose structural modifications, with respect to the substrate, occurred in the C-9 carboxylic carbon. The esterified compounds provided moderate to good yields (47%-73%), which were sufficient to carry out the tests, after purification by column chromatography. The synthesized products were characterized by ^{1}H and ^{13}C NMR spectra interpretation, where ferulic acid derivatives were obtained and confirmed by the presence of a pair of doublets assigned at δH 7.49 (J=16.0 Hz; 1H) and δH 6.93 (J=16.0 Hz; 1H), typical of the trans-olefinic system associated with C6-C3 benzene derivatives[29]. The other chemical shifts, associated with the signals corresponding to the benzene ring, showed small differences in the series of esters, according to the insertion of the C_1-C_4 alkyl groups. Taking into account small variations between ferulic acid and the four

derivatives, the signals at δH 7.06 (d, *J*=1.70 Hz), δH 6.93 (dd, *J*=1.70 and 8.10 Hz) and δH 6.71 (d, *J*=8.10 Hz), observed in the ^{1}H NMR spectrum, were associated with the H-2, H-6, and H-5 hydrogens, respectively.

This information suggests the presence of a 1,3,4-trisubstituted benzene ring, thus confirming the basic structure of ferulic acid and its derivatives[29]. The ^{13}C NMR spectrum of ferulic acid showed a similar profile to those observed in the four reaction products, except for the presence of alkyl groups, a consequence of the structural modification caused by the esterification reaction. The hydrogenation pattern of the carbons in the ^{13}C NMR was determined by the DEPT 135° technique, in combination with the variation of the signal multiplicities in the ^{1}H NMR spectrum. The methanol derivative showed a signal at δC 52.14 (OCH_3), confirmed by the appearance of the singlet at δH 3.75 (3H). The product derived from the reaction with ethanol showed two δH signals [1.30 (t) and 4.21 (q)] combined with two δC signals [14.78 (CH_3) and 61.56 (OCH_2)]. The δH signals [0.98 (t), 1.70 (m) and 4.12 (t)], in combination with the corresponding δC [10.90 (CH_3), 23.30 (CH_2) and 67.20 (OCH_2)] confirm the reaction with propanol. An *n*-butyl group, resulting from the reaction with butanol, was evidenced by the appearance of δH signals [0.95 (t), 1.42 (m), 1.65 (m) and 4.15 (t)], associated with δC [14.22 (CH_3), 20.35 (CH_2), 32.06 (CH_2) and 56.41 (OCH_2)]. All the carbons inserted in the ferulic acid structure were justified by monitoring the signals present in the DEPT 135° spectrum.

The literature reports compounds such as tannic acid, a phenolic compound capable of inhibiting the efflux pump effect and reducing bacterial resistance mediated by this mechanism, potentiate the activity of antibiotics against *S. aureus*[30]. Other study showed that ferulic acid esterification resulted in increased lipophilia when compared to ferulic acid[31]. The 24 ferulic acid-related compounds showed experimental partition coefficients ranging from 0.95 to 0.98 in the *n*-octanol/PBS assay, while ferulic acid obtained a value of 0.13. No significant lipophilicity difference was observed between ferulate derivatives, obtaining similar binding interaction values with the NorA efflux pump[32].

This activity may be associated with lipophilicity, assuming that the phenolic acids cross the cell membrane by passive diffusion in their undissociated compound form, or perhaps through binding of the pump substrates, causing a decrease in the drug inhibitory mechanism[33]. However, other efflux pump inhibitory methods, such as pump gene expression effects cannot be ruled out[33].

Ethyl ferulate is a compound whose lipid soluble nature can alter the fluidity of the bacterial membrane, making it more susceptible to antibiotic penetration[33], which demonstrated that ferulic acid has antimicrobial activity against *Cronobacter sakazakii*, affecting the integrity of the bacterial membrane and the synthesis of adenosine triphosphate, reducing the intracelular pH[34,35].

Other effects of the ferulic acid on the cell membrane can be a rupture of the lipid bilayer, causing extrusion of internal cell compounds[36]. Another study demonstrated that derivates of ferulic acid reduced the biofilm formation[37] and potentiated the antibiotic activity against *S. aureus*[38].

The compound methyl ferulate, similar to ferulic acid, did not show any activity due to its small side chain[39]. Another finding showed that an increase in the number of hydroxyl groups in the hydroxybenzoic acid side chain reduced the MIC and that the substitution of the hydroxyl groups with methoxy groups increased the activity of hydroxybenzoic acids, but not hydroxycinnamic acids.

However, the propyl ferulate and butyl ferulate compounds showed a larger side-chain, which does not leave them with unfavorable dynamic binding energy. Lipophilic substances cause disturbances in the bacterial membrane, resulting in damage to the fundamental elements necessary for membrane integrity, such as reduced membrane potential and loss of ions, cytochrome C, proteins, and radicals, followed by the collapse of the proton pump and adenosine triphosphate depletion[38,40].

Lipophilicity is a common characteristic of several compounds referred to as efflux pump inhibitors, as pointed out by Kikuzaki *et al*[31]. The ferulic acid related compounds show partition coefficients in *n*-octanol/PBD assay ranging between 0.95 to 0.98. No significant difference of lipophilicity was observed between ferulate derivatives, resulting in similar values of bindig interactions in NorA efflux pumb. This effect may, because efflux pumps constitute transmembrane proteins, have functions associated with cell membrane structure and fluidity.

The prediction of ADME property is utilized to reduce the fail in identifying candidate molecules in the drug discovery phase. Hence, these ferulic acid derivative compounds can be predicted to be easily absorbed with a low capacity for side effects or toxicity. The pattern fingerprint-based using correlations between molecular descriptors and ADME properties were particularly useful to increase the success rate to identify models from a medicinal chemistry perspective with better therapeutic potential[41].

The principal component analysis demonstrates using those physicochemical properties as hydrophobic properties, hydrogen bonds, and molecular dimensions are directly correlated with compound activity. Other studies suggest that lipophilia and rotating bonds and H-binding patterns may be more relevant than efflux size and molecular shape as the most probable pharmacophore-based model correlated with NorA inhibition. These results corroborate another study that performed a pharmacophore-based model for the NorA biological results and showed that a hydrogen-bond acceptor positive charge and aromatic rings led to the identification of potentially potent NorA inhibitors[42].

A study demonstrated that the antibacterial activity of ferulic acid affected membrane integrity, causing cell membrane hyperpolarization and intracellular pH reduction that could be

correlated with a possible antibacterial mechanism[34]. Another study also demonstrated that significant alterations in membrane properties that correlate with hydrophobicity led to local rupture or pore formation in cell membranes. These results corroborate the hypothesis that lipophilic character is more relevant than efflux size and molecular shape[34,37].

Molecular docking was used to explain their inhibitory activities and the results obtained indicated that hydrogen bond interactions played a very important role in the combination that results in the energy required to form a bond between the ligand and the receptor. In Figure 5, the chemical conformation with the lowest binding energy is represented as the most stable interaction observed in the docking procedure, which shows correlations with the activity. This figure also describes the amino acid residues responsible for stabilizing the ferulate complex derived from NorA through the predominant molecular interactions of hydrogen bonds, van der Waals, Pi-sigma, and Pi-Alkyl interactions. The docking results revealed hydrogen bonds (H-bonds) and hydrophobic interactions that validate the described principal component analysis, through superimposition of the docking poses of all the ferulic acid derivatives and norfloxacin.

The association of the compound ethyl ferulate with norfloxacin and ethidium bromide showed a significant reduction in its MIC, which can be attributed to the inhibition of the NorA efflux pump. A correlation was drawn, through molecular docking, between the interaction of the compounds and the NorA efflux pump, which demonstrated good affinity. Although other derivatives did not modify the activity of the NorA pump, all compounds showed significant ADME parameters that demonstrate the potential as drug-like. Nevertheless, further studies should be carried out to elucidate the complete mechanism of interaction between ethyl ferulate and the 1199B strain.

Conflict of interest statement

The authors declare no conflict of interest.

Authors' contributions

PGP performed the formal analysis and investigation, the writing and the original draft preparation, reviewed, read and agreed to the published version of the manuscript. HDMC performed the supervision. JGMC performed the supervision and the project coordination. GMPS performed the formal analysis of the text. FEFS performed the formal analysis of the text. IRAM performed the docking assays. ACJA and SRT performed the microbiological assays. CRTO performed the chemical synthesis of the ferulic acid derivates. PRF performed the microbiological assays. JER performed the statistical analysis. JBAN performed the chemical synthesis. MMCS performed the chemical synthesis.

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CAPÍTULO 4: FERULIC ACID DERIVATIVES INHIBITING *STAPHYLOCOCCUS AUREUS* TETK AND MSRA EFFLUX PUMPS

Ferulic acid derivatives inhibiting *Staphylococcus aureus* tetK and MsrA efflux pumps[1]

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Declaration of competing interest

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

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ABSTRACT
Bacterial resistance to multiple drugs has recently emerged as a serious health problem. Concomitantly, the characterization of new substances with potential antimicrobial activity has been less frequent in the drug development industry. The overexpression of genes encoding efflux pumps that expel antimicrobial drugs from the intracellular environment, lowering these to subinhibitory concentrations, are among the resistance mechanisms predisposing microorganisms to high drug resistance. *Staphylococcus aureus* is a bacterium found in the normal microbiota of the skin and mucous membranes, and is an opportunistic microorganism capable of causing infections with high rates of morbidity and mortality. TetK is an efflux pump characterized by its ability to provide bacterial resistance to antibiotics from the tetracycline class. This study aimed to evaluate the inhibitory effect of ferulic acid and four of its esterified derivatives against resistant *Staphylococcus aureus* strains. Propylferulate was seen to reduce the minimum inhibitory concentration (MIC) of both the control substance ethidium bromide and the tested antibiotic, indicating that this compound is promising for the use of efflux pump inhibition of IS-58 strains. Further investigations are necessary to prove this hypothesis and elucidate the potentiating mechanism of the modulatory effect.

Keywords:
Ferulic acid
Efflux pump
Staphylococcus aureus

1. Introduction

The *Staphylococcus aureus* bacterium is considered a pathogenic bacterium associated with simple superficial infections (acne, furuncles and cellulite), which can progress to serious infections such as pneumonia, meningitis, endocarditis and others. *S. aureus* is appointed as a causative agent of infections, especially those associated with catheter and valve implant sites, since, for being associated with the skin, can migrate through the catheter until it reaches the blood circulation, becoming one of the main causes of hospital infections [1]. Bacterial strains resistant to methicillin, termed MRSA, are described as one of the pathogens that present the greatest difficulty in treatment in the hospital environment [2].

Studies have demonstrated the development of bacterial resistance to be associated with the indiscriminate use of antibiotics [3,4]. The resistance developed by bacteria to toxic compounds is an inherent characteristic of this microorganism class, however, specific genes and proteins have emerged over the years that have been developed by a direct response to the mechanism of action of antibiotics used in the prophylaxis of bacterial diseases, such as: the appearance of the b-lactamase enzyme when using the B-lactam antibiotic class; cellular target modification by the acquisition of genetic mutations when using fluoroquinolones. A widely studied mechanism of bacterial resistance is the extrusion of a compound mediated by an efflux system [5,6]. In these systems, the bacteria expel drugs against their concentration gradient by using energy, where this extrusion system is mediated by hyphrophobic proteins. The energy

needed to expel these drugs can be obtained from the conversion of ATP or ionic gradients [7,8].

The TetK, NorA [9] and MsrA proteins [10] have been described as proteins from the *S. aureus* bacterium responsible for the efflux of antibiotics. Therefore, *S. aureus* became resistant to tetracycline and erythomycin through an extrusion mechanism, this being the overexpression of efflux proteins located in its plasma membrane [11,12].

Tetracycline is an antibiotic with good oral bioavailability that has low cutaneous reactions as a side effect, where these characteristics are favorable for the treatment of MRSA infections [11,13]. This drug class is capable of inhibiting bacterial protein synthesis by forming a link with the 30S ribosome subunit, which blocks the binding of aminoacyl-tRNA, which normally results in the appropriate addition of new amino acids in the protein chain, thus blocking protein formation [14,15]. Erythromycin, an antibiotic belonging to the macrolide class, acts in the translation and amino acid addition process during bacterial protein synthesis, where the overexpression of the MsrA efflux pump is indicated in bacterial resistance development to this antibiotic [16].

Therefore, studies are searching for a way to reverse bacterial resistance, especially those which are mediated by the efflux of antibiotics, with the aim of reestablishing the susceptibility of these antibiotics [17]. The discovery of compounds that present therapeutically as inhibitors of these antibacterial resistance mechanisms may help in the improvement, containment, treatment and elimination of these strains.

Ferulic acid (AF), 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid, a secondary metabolite, belongs to the phenolic compound class, and is considered one of the most common phenolic acids found in natural species [18,19]. The ferulic acid molecule presents cis-trans isomerism, with the most abundant form in nature being the trans isomer. Both isomers have proven results in the treatment of several pathologies such as cancer, diabetes, neurodegenerative and cardiac diseases, in addition to having antimicrobial, anti-inflammatory and, especially, antioxidant activities, responsible for its main benefits and applications [20,21]. Due to its ability to interrupt radical chain reactions by resonance, followed by polymerization, ferulic acid offers protection against UV radiation [22-24]. The investigation of ferulic acid's antimicrobial potential was proposed after indirect studies showed that ferulic acid had a high antibacterial activity against *L. monocytogenes* [25].

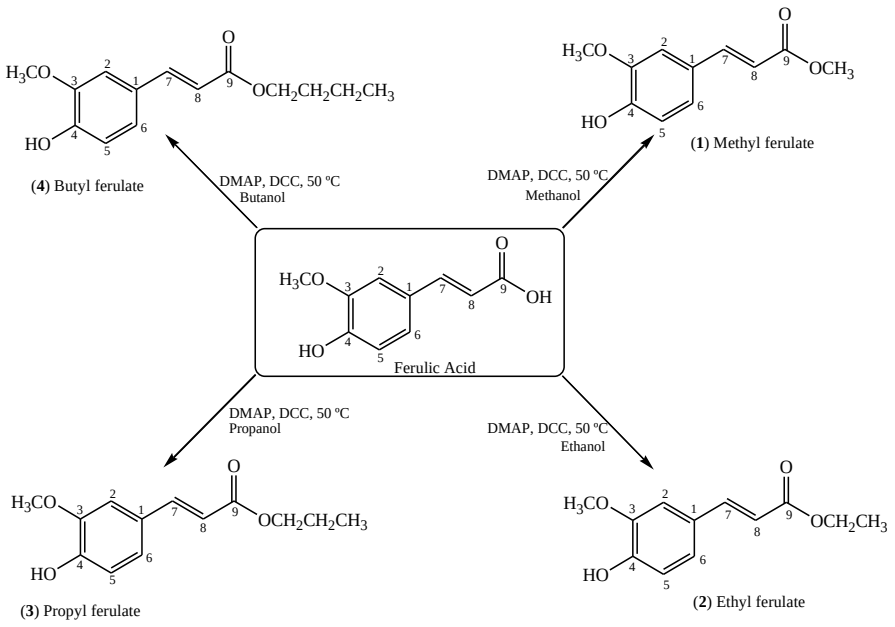
Given the above, this study had as its objective to evaluate the antibacterial activity of ferulic acid and its four derivatives, esterified with the methanol (methyl ferulate), ethanol (Ethyl ferulate), propanol (Propyl ferulate) and butanol (Butyl ferulate) alcohols, in tetK and

MrsA efflux pump expressing *Staphylococcus aureus* strains, with the aim of establishing relationships between the structure of these compounds and their activities through their action on efflux pump mechanisms.

2. Results

2.1. Ferulic acid esters

The synthetic reaction method caused structural changes in the carboxylic group from ferulic acid (Scheme 1) as expected, with ^{1}H NMR (Fig. 1), ^{13}C NMR (Fig. 2) and ^{13}C NMR - DEPT 135° (Fig. 3) analyzes for ferulic acid being in accordance with literature data [26], as seen in Table 1, in which the displacements (δ in ppm) and coupling constants (*J*) confirm the substrate identification. The good quality of the spectra indicates the considerable purity degree of the derivatives, which reflect the efficiency of the synthesis of the derivatives. The hydrogenation pattern of the carbons was determined by the ^{13}C NMR - DEPT 135º technique, in combination with the variation of the signal multiplicities in the ^{1}H NMR spectrum (Fig. 4). The alkyl groups (methyl, ethyl, propyl and n-butyl) incremented in the structure of ferulic acid, resulting from the esterification reactions, were confirmed by the appearance of characteristic signals in the ^{13}C NMR - DEPT 135° spectra (supplementary material) of the derivatives: methyl ferulate (1), ethyl ferulate (2), propyl ferulate (3) and n-butyl ferulate (4), since only carbonyl carbon was been altered.



Scheme 1. Synthesis of esterified ferulic acid derivatives.

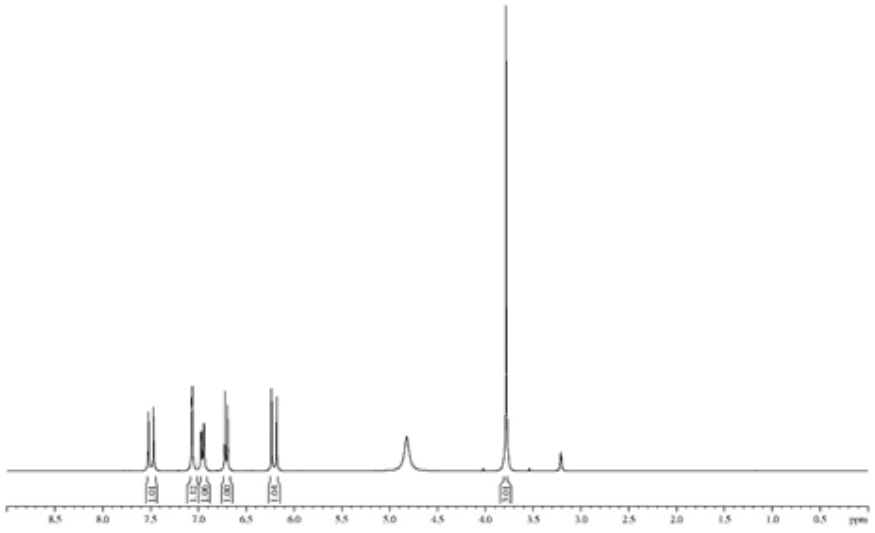


Fig. 1. ^{1}H NMR spectral (300 MHz, MeOD) of the ferulic acid

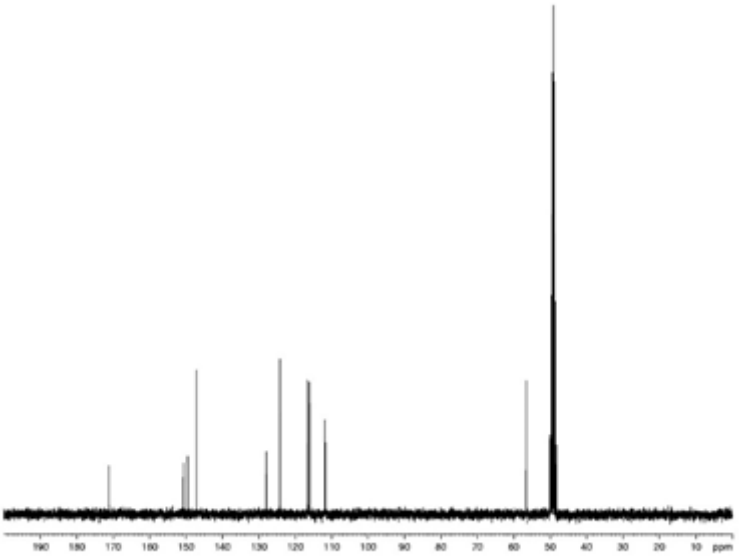


Fig. 2. ^{13}C NMR spectral (75 MHz, MeOD) of the ferulic acid.

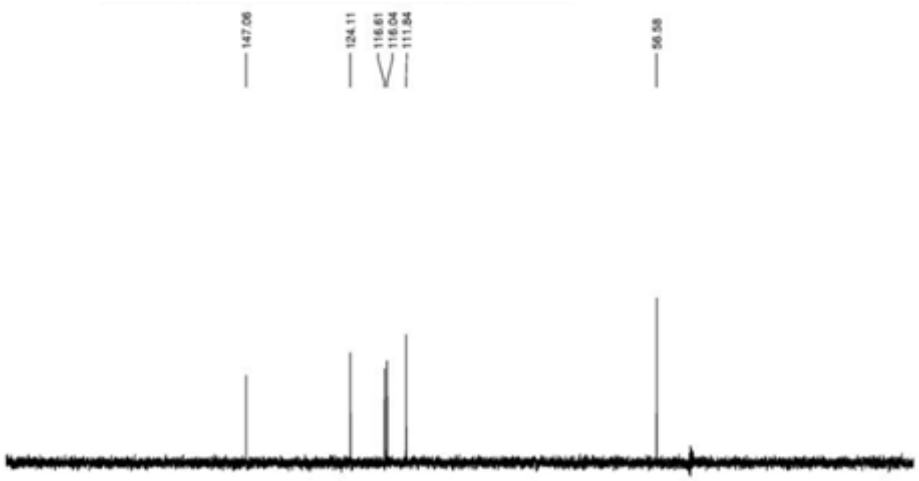
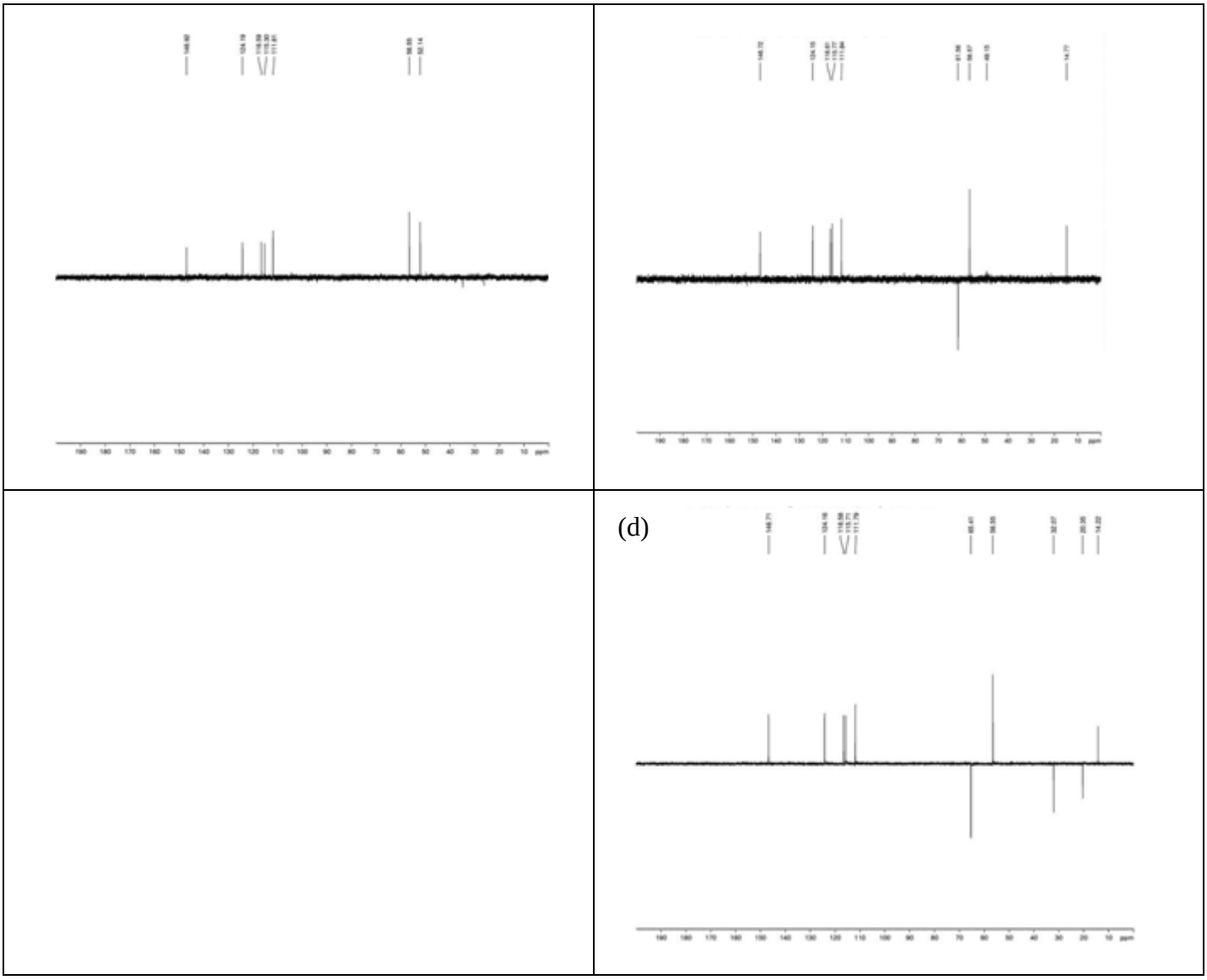


Fig. 3. ^{13}C NMR – DEPT 135 spectral (75 MHz, MeOD) of the ferulic acid.



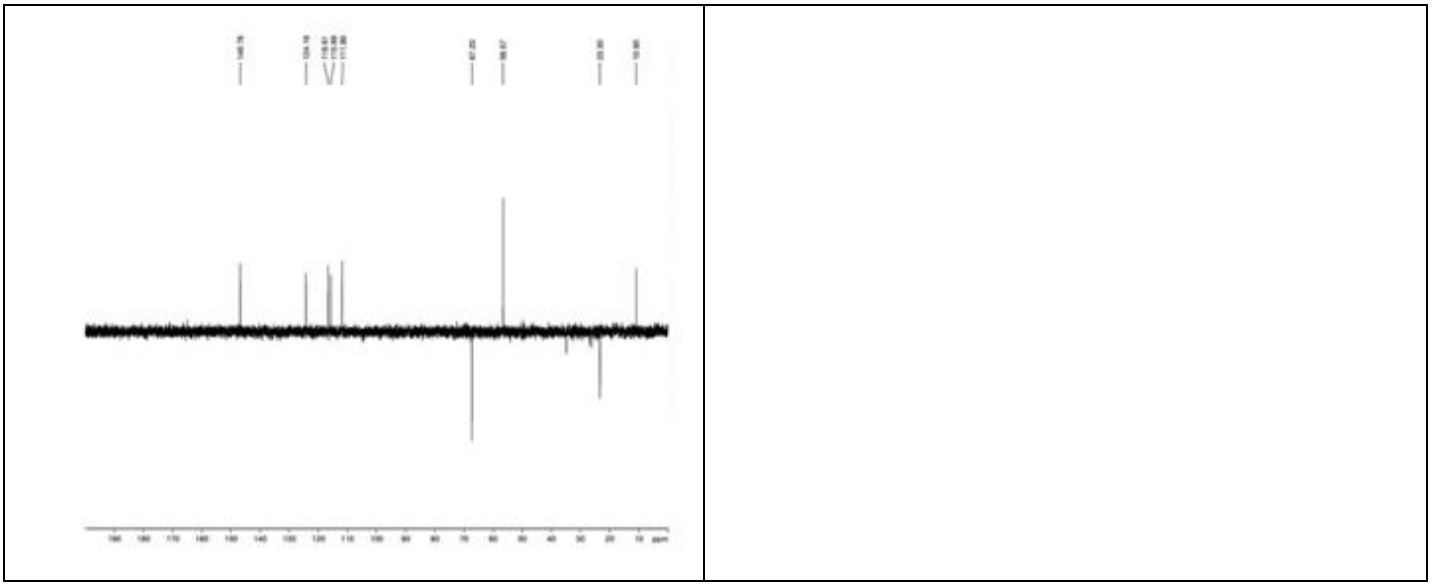


Fig. 4. ^{13}C NMR – DEPT 135 (75 MHz, MeOD). (a) methyl ferulate. (b) Ethyl ferulate. (c) Propyl ferulate. (d) n-butyl ferulate.

Table 1

Spectral data for ferulic acid obtained under the conditions: ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD).

Data		δ_C (ppm)		δ_H (ppm)	
Carbon	**Multiplicity**	**Analysis**	**Yamadio et al**	**Analysis**	**Yamadio et al**
1	C	127.9	127.6	-	-
2	CH	111.8	111.6	7.1 (d, J = 1.7 Hz	7.5 (d, J = 1.7 Hz)
3	C	149.5	150.1	-	-
4	C	150.6	149.6	-	-
5	CH	116.0	116.4	6.7 (d, J = 8.0 Hz)	7.6 (d, J = 8.0 Hz)
6	CH	124.1	123.8	6.9 (dd, J = 8.0 e J = 1.7 Hz)	7.1 (dd, J = 8.0 e J = 1.7 Hz)
7	CH	147.0	149.1	7.5 (d, J = 16.0 Hz)	6.3 (d, J = 16.0 Hz)
8	CH	116.6	115.6	6.2 (d, J=16.0 Hz)	6.3 (d, J=16.0 Hz)
9	C	171.0	171.1	-	-
OCH_3-3	CH_3	56.6	56.3	3.8 (s)	3.9 (s)

2.2. Microbiological assays: minimum inhibitory concentration of the compounds.

Data analysis revealed that all compounds obtained MIC values greater than 1024 μg/mL, indicating these do not present clinically relevant activity against the tested strains

(CLSI) [27]. Demonstrated that thymol, a compound similar to ferulic acid, was able to affect the integrity of the bacterial membrane leading to cell death [28].

2.3. Effects on S. aureus efflux mechanism

The butyl ferulate compound showed a non-significant MIC reduction from 16 µg/mL to 12 µg/mL with the RN-4220 strain. When associated with CCCP, an ethidium bromide MIC reduction from 16 µg/mL to 8 µg/mL was observed. As for the IS-58 strain, only the propyl ferulate compound, at subinhibitory concentrations in association with ethidium bromide, presented a MIC reduction from 16 µg/mL to 12.7 µg/mL; the remaining compounds obtained antagonistic effects when associated with ethidium bromide (Figures 5 and 6).

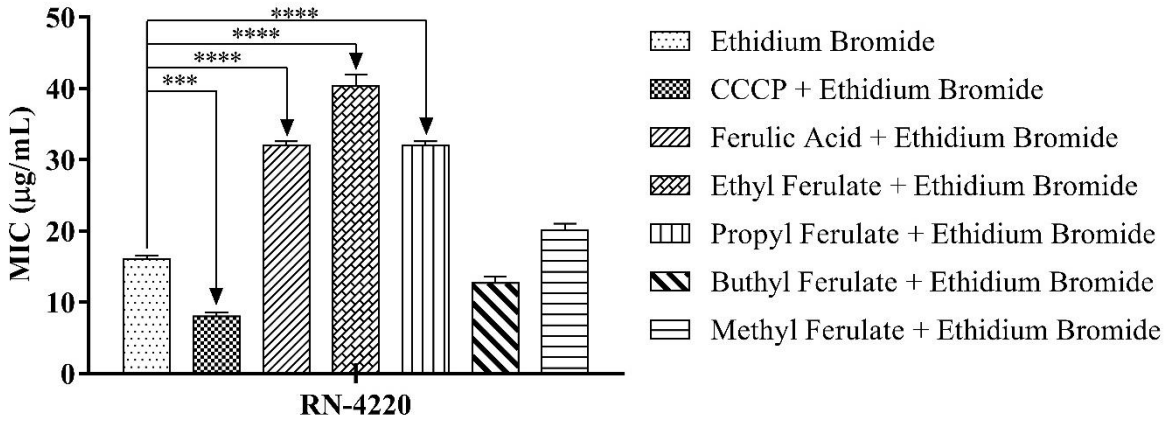


Fig. 5. Minimum Inhibitory Concentration (MIC) of ethidium bromide alone or in association with the compounds under analysis (ferulic acid and its esterified derivatives) against S. *aureus* RN-4220 strains. **** $p < 0.0001$ indicates significant differences between groups. Statistical significance was determined by a one-way ANOVA and Tukey's post-hoc test.

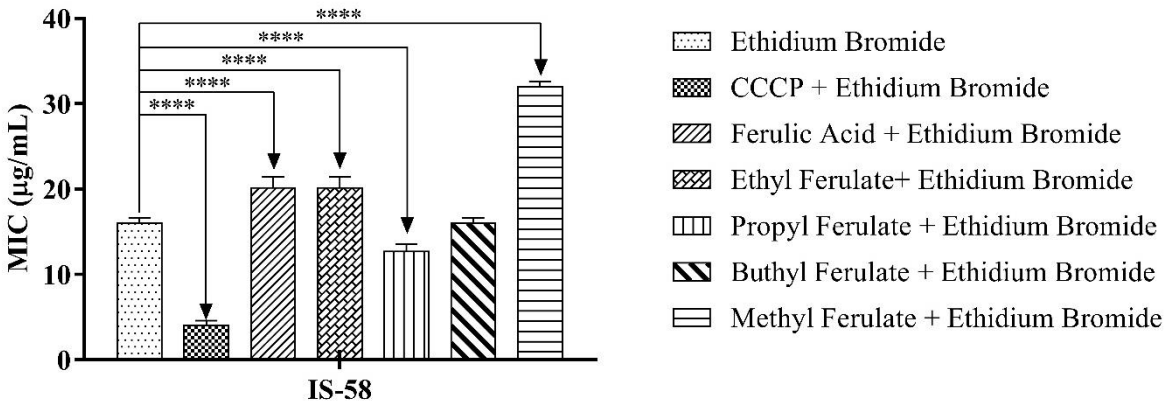


Fig. 6. Minimum Inhibitory Concentration (MIC) of ethidium bromide alone or in association with the compounds under analysis (ferulic acid and its esterified derivatives) against *S. aureus* IS-58 strains. **** $p < 0.0001$ indicates significant differences between groups. Statistical significance was determined by a one-way ANOVA and Tukey's post-hoc test.

2.4. Antibiotic potentiating effects over tetK and MrsA pumps

The ethyl ferulate and propyl ferulate compounds showed, at subinhibitory concentrations in association with erythromycin, MIC reductions from 161 µg/mL to 128 µg/mL against the RN-4220 strain. However, ferulic acid presented greater synergism than the esterified compound series, reducing the MIC from 161 µg/mL to 64 µg/mL. In terms of the IS-58 strain, a MIC reduction from 101.6 µg/mL to 50.8 µg/mL for propyl ferulate, and a reduction from 101.6 µg/mL to 80.6 µg/mL for buthyl ferulate was observed. In both tests, a significant antibiotic MIC reduction occurred when these were in association with CCCP (Figures 7 and 8).

Antibiotic MIC reduction is also a method used to assess pump inhibition, however, it is not as conclusive as using ethidium bromide, since other resistance mechanisms exist when only the antibiotic MIC inhibition is investigated [29].

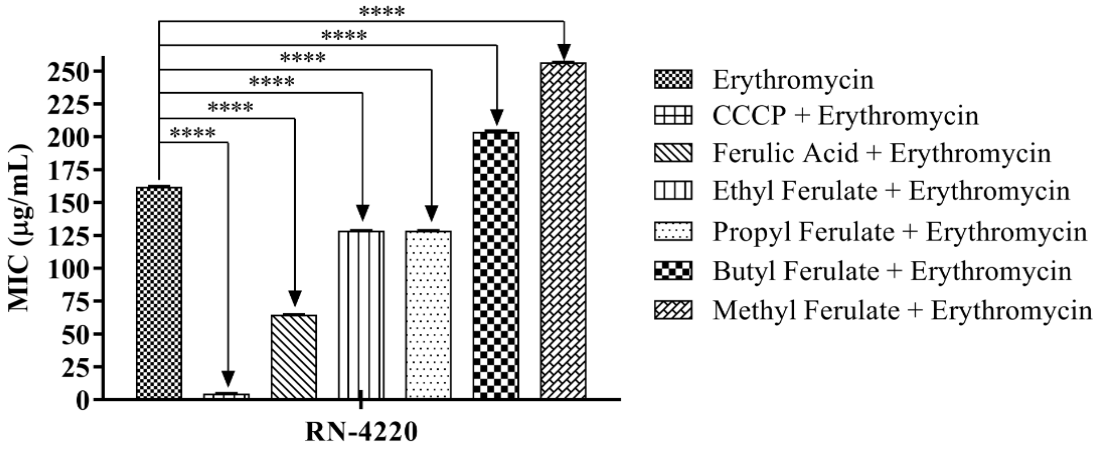


Fig. 7. Minimum Inhibitory Concentration (MIC) of erythromycin in association with the studied compounds (ferulic acid and its esterified derivatives) against the multiresistant RN-4220 *S. aureus* strain **** $p < 0.0001$ indicates significant differences between groups. Statistical significance was determined by a one-way ANOVA and Bonferroni's post hoc test.

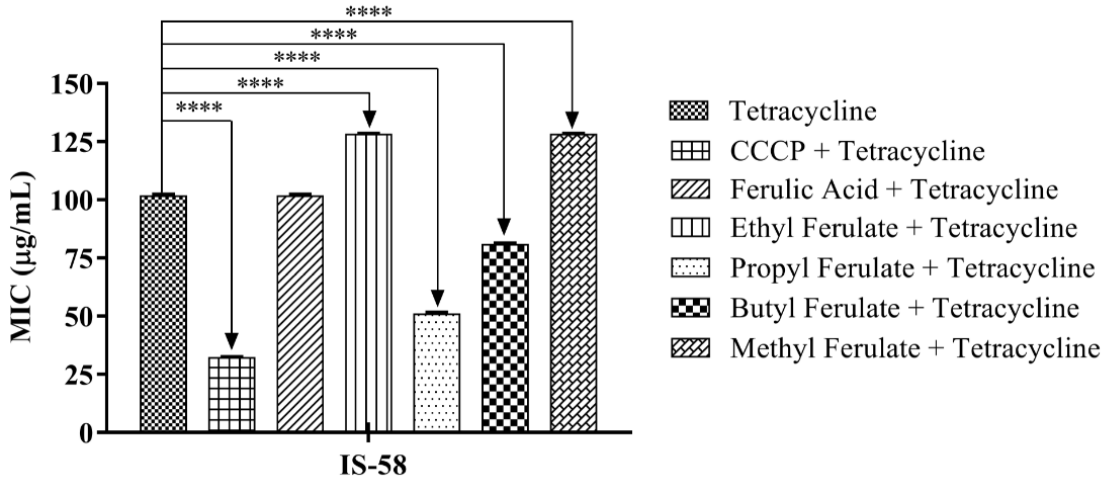


Fig. 8. Minimum Inhibitory Concentration (MIC) of tetracycline in association with the studied compounds (ferulic acid and its esterified derivatives) against the multiresistant IS-58 *S. aureus* strain **** $p < 0.0001$ indicates significant differences between groups. Statistical significance was determined by a one-way ANOVA and Bonferroni's post hoc test.

2.5. Statistical Analysis of Microbiological Results

The results were expressed as the geometric mean ± standard deviation, statistically evaluated through an analysis of variance (ANOVA), followed by Tukey's post hoc test, using the GraphPad Prism 7.0 software. Differences were considered significant when $p < 0.0001$.

3. Discussion

Ethidium bromide is a compound that is extruded from the bacterial cell through the efflux pump mechanism and is, thus, said to be an instrument used to evaluate the efficiency of these proteins [30,31].

A correlation between the MIC reduction of ethidium bromide or antibiotics and the process of inhibition of bacterial resistance, mediated by efflux pump mechanisms, has been known to exist, with studies reporting that a MIC reduction ≥ 3 would be indicative of a resistance mechanism mediated by an efflux pump, when a potential inhibitor is associated with the pump's antibiotic substrate or ethidium bromide [32]. However, the results from this study did not show such an expressive MIC reduction, suggesting the compounds may only be capable of affecting the integrity of the plastic membrane and inhibit bacterial resistance by other mechanisms.

The MIC reduction of tetracycline or ethidium bromide, in the presence of the carbonyl cyanide m-chlorophenylhydrazone proton pump inhibitor (CCCP), is indicative of an efflux pump reversal [33]. The CCCP compound helps to elucidate the efflux system, since it acts as an energy-dependent decoupler preventing antibiotic efflux. Therefore, this is a positive control widely used to prove the existence of an efflux pump in a given strain, as was seen in the present study. However, given the results obtained herein, we believe ferulic acid and its derivatives act less so directly and more so indirectly on efflux proteins, that is, they may act on the bacterial plasma membrane or even secondarily to facilitate antibiotic entry.

Lipidsoluble substances present the possibility of altering membrane permeability, making these more susceptible to penetration by various substances [28]. Changes in membrane permeability may hinder or facilitate the entry of an antibiotic into the bacterial cell [34]. The compounds hypothesized as bacterial resistance inhibitors, ferulic acid and its derivatives, have a polar hydroxyl group, allowing for energy dissolution and favorable entropy, however, further studies are needed to prove this hypothesis, where these may associate with the bacterial plasma membrane, and may be able to enter the bacterial cell, a mechanism that can be further favored by the nonpolar structure of the benzene ring present in the molecule [35]. Signals membrane damage as a likely reason for pump inhibition, since efflux proteins are transmembrane proteins sensitive to changes in this segment [36].

It was observed in this study that despite similar chemical structures, the compounds had distinct biological activities. Synthesized compounds can improve the pharmacological properties and increase the therapeutic benefits of antimicrobial compounds capable of reversing bacterial resistance [37]. However, some compounds presented antagonistic properties to the bacterial resistance reversal process, acting by contributing to bacterial efflux. Moreover, the antagonism was seen to be more pronounced with ethidium bromide, raising the hypothesis that the esterified compounds may protect the bacteria from ethidium bromide toxicity.

4. Materials and Methods

4.1. Chemical products

Ferulic acid, 4-hydroxy-3-methoxycinnamic acid, trans-4-hydroxy-3-methoxycinnamic acid, ferulic acid Sigma Aldrich

absolute methyl alcohol 200, 99.8% Sigma Aldrich

absolute ethyl alcohol 200, 99.5% Sigma Aldrich

absolute propyl alcohol 200, 99.7% Sigma Aldrich
n-butanol alcohol, absolute butyl alcohol 200, 99.8% Sigma Aldrich
dicyclohexylcarbodiimide nickel (ii) carbonate hydroxide tetrahydrate Sigma Aldrich
Carbonyl Cyanide m-ChloroPhenyl-hydrazone Sigma Aldrich

4.2. General procedure for the synthesis of esterified derivatives

The procedure used followed the methodology from Narender et al 2009, for the Fischer esterification of ferulic acid with methyl, ethyl, propyl and butyl alcohols. Ferulic acid (60 mg, 0.3092 mmol) was mixed with the respective alcohols (10 mL: MeOH, EtOH, PropOH and ButOH) and dicyclohexylcarbodiimide (DCC, 54 mg, 0.2621 mmol) in catalytic quantities of 4– N,N–dimethylaminopyridine (DMAP). The reaction mixture was maintained under reflux and magnetic stirring with heating at 50 °C for 4 h. Following this period, the N,N-dicyclohexylurea formed was filtered off, the solvent was removed on a rotary evaporator at room temperature, and the crude residue was purified with a chromatographic column (silica gel, hexane/EtOAc: 70/30).

The synthesized products and ferulic acid were characterized by ^{1}H and ^{13}C NMR, including the DEPT 135º technique. The analyzes were performed on a Bruker Avance DPX 300 spectrometers, operating at the frequency of 300 MHz for ^{1}H and 75 MHz for ^{13}C. The spectra were obtained in a CD_3OD solvent and the chemical shifts (δ) were expressed in ppm, with tetramethylsilane (TMS) being used as an internal standard. The signal multiplicities in ^{1}H NMR were indicated following the convention: s (singlet), sl (broad singlet), d (doublet), dd (double doublet), t (triplet), q (quartet) and m (multiplet).

4.3. Substance preparation

The substances were initially solubilized in dimethyl sulfoxide (DMSO), then diluted in water for the microbiological tests. The DMSO concentration contained in the samples is not considered toxic to cells, this being below 10%. The efflux pump inhibitor Carbonyl Cyanide m-ChloroPhenyl-hydrazone (CCCP) was used, after its dissolution in 50% Methane/Water and concentration adjustment to 1024 µg/mL.

The antibiotic norfloxacin, specific for the NorA pump, was used. This was initially dissolved in DMSO, adjusting the concentration to 10 mg/mL, being subsequently diluted in water, decreasing the concentration to 1024 µg/mL. Ethidium bromide was diluted in water to a concentration of 1024 µg/mL.

4.4. Natural material origin and preparation

First, the substances were solubilized in a small amount of dimethyl sulfoxide (DMSO), only to favor the dissolution mechanism. Thereafter the substances, were diluted in distilled water. Below 10% v/v, DMSO concentrations are not considered toxic to cells (Florão et al., 2007).

4.5. Culture media

Heart Infusion Agar (HIA, Difco laboratorises Ltda.) and Brain Heart Infusion (BHI, difco Laboratories Ltda.) culture media, prepared according to the procedures described by the manufacturers, were used at a concentration of 10% v/v.

4.6. Microorganisms

The RN4220 and IS-58 *S. aureus* strains, resistant to tetracycline and azithromycin by the tetK and Mrs(A) efflux proteins, respectively, were used. All strains were initially kept on agar, then transferred to a stock and kept in Heart Infusion Agar slants (HIA, Difco) at 4 ºC.

4.7. Origin and preparation of the antibiotics and ethidium bromide

The antibiotics tetracycline, specific for the tetK pump, and azithromycin, specific for the Mrs(A) efflux pump, were used. The antibiotics were initially dissolved in DMSO to a concentration of 10 mg/mL and, subsequently, diluted in water, decreasing the concentration to 1024 µg/mL. Ethidium bromide was diluted directly in water, until the 1024 µg/mL concentration was reached. Both the antibiotics as well as ethidium bromide were obtained from Sigma-Aldrich.

4.8. Inoculum preparation and standardization

Test tubes containing sterile saline solution were used to bring a small amount of the inocula to a concentration corresponding to $1.5x10^8$ CFU (Colony Forming Units), which were used both in the MIC test of the substances and the efflux pump inhibition assays.

4.9. Minimum Inhibitory Concentration Assays

The minimum inhibitory concentration of ferulic acid and its esterified compounds were tested to confirm the level of bacterial resistance reversal, with the aim of analyzing whether this mechanism was due to the presence of a pump. Inocula in saline solution with a bacterial concentration corresponding to $1.5x10^8$ CFU (Colony Forming Unit) were distributed in

eppendorfs, using 100 µL of the inoculum and 900 µL of 10% v/v BHI liquid culture medium. The eppendorf content was then horizontally distributed into a standard 96-well microdilution plate, with 100 µL in each well, for a total of 10 wells. Subsequently, the substances were microdiluted (1:1) up until the penultimate cavity. Nothing was added to the last cavity, with this well being defined as the growth control. The concentrations range from 1024 µg/mL to 0.5 µg/mL [29].

After 24h, the plates were read by visualizing the medium color change using a 20 µL aliquot of resazurin (7-hydroxy-3H-phenoxazine-3-one 10-oxide). Resazurin has the characteristic of changing the medium color from blue to red in the presence of bacterial growth and remaining in blue in the absence of bacterial growth. The tests were performed in triplicates.

4.10. Efflux pump inhibition assays by the MIC reduction of ethidium bromide and antibiotics

A preparation similar to that described for the MIC assay was followed, however, 150 µL of the inocula were added to eppendorfs, plus the investigated substance with a volume corresponding to its sub-inhibitory concentration (MIC/8), and the final volume was completed to 1.5 mL. For the control standard, 150 µL of the inocula were added to an eppendorf and its volume was made up to 1.5 mL with 10% BHI solution. Then, eppendorf solutions were vertically distributed in 96-well microdilution plates, with 100 µL of the eppendorf content being transferred to each well. Thereafter, the microdilution (1:1) of ethidium bromide or the antibiotic was performed with 100 µL of the compound, distributed up to the penultimate cavity. No solution was added to the last well, as this was the growth control. Well concentrations ranged from 1024 µg/mL to 0.5 µg/mL. After 24h, the plates were read by visualizing the medium color change, characterized by the addition of 20 µL resazurin (7-hydroxy-3H-phenoxazine-3-one 10-oxide). Experiments were performed in triplicates.

The MIC decrease of ethidium bromide or the specific antibiotic is suggested to be indicative of efflux pump mediated bacterial resistance inhibition, this being a selective test for strains carrying an efflux pump [33].

4.11. Statistical analysis

Each experiment was performed in triplicates, and the results were normalized by calculating their geometric means. Error deviation and standard deviation of geometric means were revealed. Statistical analyses were performed using the GraphPad Prism 5.02 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences between treatments with antibiotics in the absence or presence of derived compounds were examined using a One-way analysis of

variance (ANOVA). Significant differences were analysed by Tukey's post hoc test and were considered statistically significant when $p < 0.0001$.

6. Conclusion

Ferulic acid and some of its esterified derivatives presented a significant capacity for reducing the MIC of the antibiotic, however, the inhibition of an efflux pump mechanism could not be proven, thus the assumption of structural and/or functional damage to the cytoplasmic membrane was raised. Propyl ferulate showed a MIC reduction for both ethidium bromide and the antibiotic against the IS-58 strain, making propyl ferulate a promising compound to be used in efflux pump inhibition. However, further studies are necessary to prove this hypothesis and elucidate the mechanism which led to the restoration and enhancement of the reference antibiotic in this study.

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CAPÍTULO 5: COMPOUNDS FOR INHIBITION OF ANTIBIOTIC RESISTANCE IN *STAPHYLOCOCCUS AUREUS*: A REVIEW

Compounds for inhibition of antibiotic resistance in *Staphylococcus aureus*: a review[1]

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Abstract The discovery of compounds that are able to increase the effectiveness of antibiotics and/or facilitate the processes of reversing bacterial resistance has become necessary in recent times in order to reduce the mortality caused by diseases associated with these microorganisms. Bacterial resistance to various antibiotics and chemotherapeutic agents imposes serious limitations on treatment options for infections, posing a threat to public health. One of the most studied bacteria is *Staphylococcus aureus*, considered pathogenic due to its ability to cause serious infections, such as pneumonia, mainly in hospital environments. The use of compounds with therapeutic characteristics to inhibit antibacterial resistance mechanisms could help in the current treatment performed by antibiotics, in this search phenolic compounds have been widely reported among these substances as promising alternatives in the treatment against infections caused by bacteria, these compounds can modify bacteria impairing its control in the selective permeability of their plasma membranes. Given the above, this study aimed to perform a review study in order to identify the basic chemical structure of compounds and their potential in reversing the resistance developed by *Staphylococcus aureus* strains.

Keywords *Staphylococcus aureus* . bacterial resistance . chemical structure.

[1]Artigo de acordo com as normas da *Phytochem Rev*. Disponível em: https://www.springer.com/journal/11101/submission-guidelines?IFA

Introduction

The development of effective drugs to combat bacterial infections has led to a reduction in mortality caused by diseases associated with these microorganisms. However, bacteria are extremely adept at acquiring resistance to antibiotics and antiseptic agents; they develop strategies to repair the toxic effects caused by antimicrobials (Christaki, Marcou and Tofarides, 2020).

The phenomenon of bacterial resistance to various antibiotics and chemotherapeutic agents imposes serious limitations on the options for treating infections, representing a threat to public health.

The resistance developed by bacteria to toxic compounds is an inherent characteristic of this class of microorganisms, but studies have shown the development of bacterial resistance to be associated with the indiscriminate use of antibiotics (Levy, 2005; Neu, 1992).

Over the years, specific genes and proteins have been developed in direct response to the mechanism of action of antibiotics used in the prophylaxis of bacterial diseases. These include: when β-lactam antibiotics are used, the appearance of the enzyme β-lactamase; when fluoroquinolones are used, the modification of the cellular target by the acquisition of mutations in genes: these are: when β-lactam antibiotics are used, the productio of the enzyme β-lactamase; when fluoroquinolones are used, the modification of the cellular target by the acquisition of mutations in the genes.

A widely studied bacterial resistance mechanism is the efflux system-mediated compound extrusion (Russell, 2000; Poole, 2002). In these systems, the bacteria are able to expel the drugs against the concentration gradient, i.e., with energy expenditure. This extrusion system is mediated by proteins that have hydrophobic properties, and the energy required for the expulsion of these drugs is obtained from ATP conversion or ionic gradients (Schindler et al., 2013; Levy, 2002).

Antimicrobials must be able to overcome bacterial resistance and to do this they need to reach molecular targets, which are intracellular. In other words, the antimicrobial must go beyond the bacterial cell membrane; interact with a target molecule in such a way as to trigger the death of the bacteria; avoid the action of efflux pumps that throw the antimicrobials out of the bacterial cell; avoid inactivation by enzymes capable of modifying the drug in the extracellular environment or inside the cell.

Staphylococcus aureus bacteria are considered pathogenic because they are associated with superficial skin infections, which can evolve into serious infections, such as pneumonia. Being in contact with the skin, it can migrate through the catheter until it reaches the bloodstream, becoming one of the main causes of hospital infections (Tong et al. 2015).

Methicillin-resistant strains, called MRSA, are described as one of the most difficult pathogens to treat in the hospital environment (Carlie et al. 2020).

Studies are searching for a way to reverse bacterial resistance, especially those mediated by the efflux of antibiotics, looking for a way to restore the susceptibility of these antibiotics (Chopra, 2002). The use of compounds with therapeutic properties to inhibit the resistance mechanisms could help in the management of the current antibacterial resistance to therapeuticals (Rampogu et al. 2018; Holmes et al. 2016).

Phenolic compounds have been extensively reported among these substances as promising alternatives in the search for new sources of treatment against infections caused by MDR bactéria. Studies indicate that these compounds can contribute satisfactorily as new sources of adjuvant treatment, since they can modify bacteria, impairing their locomotion as well as their surface adhesion, biofilm formation and formation of virulence determinants. The hydrophobic nature of some components can interact with the double lipid layer of the cell

membrane and affect the respiratory chain and energy production or even make the cell more permeable to antibiotics leading to interruption of vital cellular activity.

This review aimed to identify the basic chemical structure of phenolic compounds and their potential in reversing the resistance acquired by *S. aureus* strains.

Methods

We conducted a literature search in the MEDLINE online database, limiting it to more recent articles on the topic, published from 2015 to 2020. Initially, descriptors were used to search the MEDLINE database:

a) "Phenolic compound" (descritor *Medical Subject Headings* [MeSH]);
b) "*S. aureus*" (descriptor MeSH); e
c) "bacterial resistance" (palavra-chave).
d) "chemical struture"
e) "essential oil"

The searches performed were: 1 AND 2, AND 3, AND 4, NOT 5. In addition to the MeSH descriptors, we chose to include the keyword "*S. aureus*" in the search strategy, since, although it is not included in the list of MeSH descriptors, it is frequently used to describe studies that address the subject of this review. The search strategy and the obtained articles were reviewed on two occasions to ensure adequate sample selection.

A similar search strategy was carried out in the ScienceDirect database, using the aforementioned descriptors and equivalents in Portuguese.

The analysis of the articles followed eligibility criteria with the inclusion system specified below:

a) Articles with at least one combination of the terms described in the search strategy;
b) Publications written in English or Portuguese;
c) Studies that dealt with reversal of bacterial resistance;
d) Original articles with full text accessible through the CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) Periodical Portal (CAPES, 2020), a virtual library linked to the Brazilian Ministry of Education and with content restricted to authorized users; and
e) Experimental studies.

Exclusion criteria were:

(a) other study designs, such as case reports, case series, literature reviews, and commentaries;

b) Non-original studies, including editorials, reviews, prefaces, short communications, and letters to the editor.

Each article in the sample was read in its entirety and the information was entered into a data sheet that included authors, year of publication, chemical structure of the main compound studied, and main findings. Some of the studies found dealt not only with *S. aureus*, but also with other bacteria. Thus, considering that the focus of this study is on *S. aureus*, data related to other bacteria were not recorded or analyzed in the study.

Results and Discussion

Liposoluble nature of the compounds

The bacterial cell membrane is formed by double layer of phospholipids, with protein matrices immersed in this structure. The antimicrobial drug needs to overcome the bacterial cell membrane and interact with a target molecule in order to trigger the death of the bacteria. The

hydrophobic nature of some components allows them to interact with the lipid double layer of the cell membrane and affect the respiratory chain and energy production, or even make the cell more permeable to the antibiotics, leading to interruption of vital cellular activity.

Thus, compounds of liposoluble nature are theoretically effective in causing disruption to the bacterial membrane.

Kim et al. (2019) studied bionionol (Fig.1) being appointed as a bactericidal agent against methicillin-resistant *S. aureus* strains. The molecule showed property of altering the integrity of Gram-positive bacterial membranes by increasing membrane fluidity. Molecular modeling studies indicated that bionionol is initially recruited to the membrane surface by binding polar hydroxyl and chlorine groups, in this way, the compound penetrates the membrane, the interactions between non-polar benzene rings and hydrophobic lipids then contributed to the penetration of the compound inside the cell.

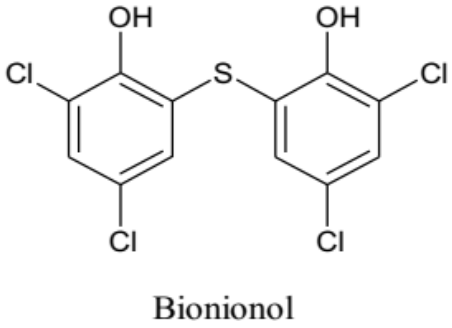

Fig. 1 Chemical structure of Bithionol (Kim et al. 2019)

After entering the cell, bionol is incorporated into the outer leaflet of the lipid bilayer, and simulations show that the polarity of its ramifications and the hydrophobicity of the core rings play important roles in the membrane. Bioionol induces rapid and passive membrane permeabilization that leads to loss of function of bacterial membrane proteins, causing leakage of cellular components and bacterial death. Key elements for membrane activity of bionol are the initial binding to the membrane surface using phenolic hydroxyl groups and chlorinated benzene-induced membrane disruption.

Zhou et al. (2019) pointed in their studies the antibacterial action of phenolic compound correlated to the ability to cause structural and functional damage to the cytoplasmic membrane. The hydrophobic nature of thymol (Fig. 2) was found to have interactions with membrane phospholipids, leading to changes in the cell membrane. Evidence also suggested that thymol could interact with membrane proteins and intracellular targets, which prevents cell recovery after temporary exposure.

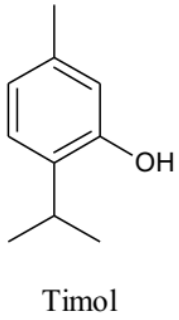

Fig. 2 Chemical structure of thymol (Zhou et al. 2019)

Silva et al. (2016) tested the phenolic acids: ferulic (FA), gallic (GA), caffeic (CA), p-coumaric (p-CA), seric (SyrA) and sinapic acid (SinA) as anti-biofilm agents (Fig. 3), against methicillin-resistant *Staphylococcus aureus* strains, with pH modification. The pH values were shown to play a relevant role in the anti-biofilm activity only.

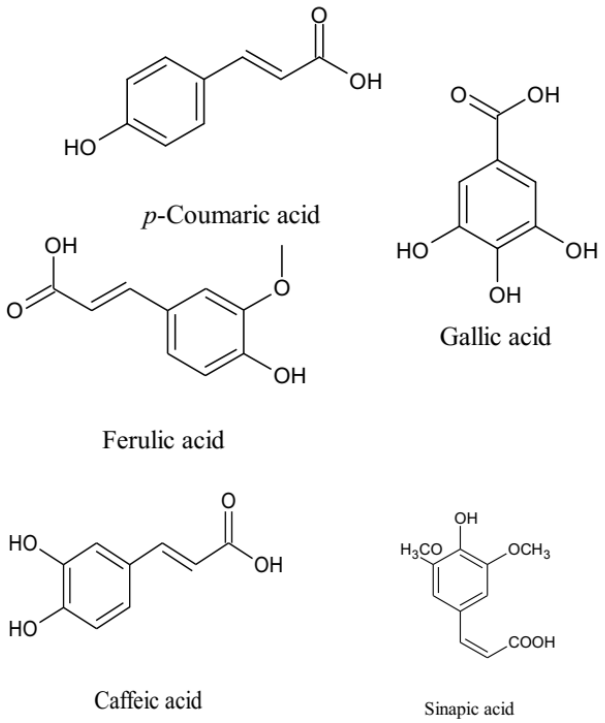

Fig. 3 Chemical structure of (a) p-coumaric acid (b) gallic acid (c) ferulic acid (d) caffeic acid (e) sinapic acid (Silva et al. 2016)

Chanf (2019), studied the compound Tellimagrandin II (TGII), a polyphenol, which by transmission electron microscopy imaging confirmed that the compound destroyed the integrity of the MRSA cell wall and caused the loss of cytoplasm content.

Bogdanov et al. (2020) performed synthesis of quaternary ammonium compounds (Fig. 4) the initial antimicrobial activity study showed that these compounds are highly selective against *S. aureus*. The highest activity (MIC 2.0 μM) was shown by hydzones containing a catechol fragment. These compounds are three times more active against S. aureus than norfloxacin.The results suggest the dependence of the activity associated with the position of the OH group. The presence of phenolic hydroxyl in the ortho position as the C=N group in some compounds resulted in marked increase in activity.

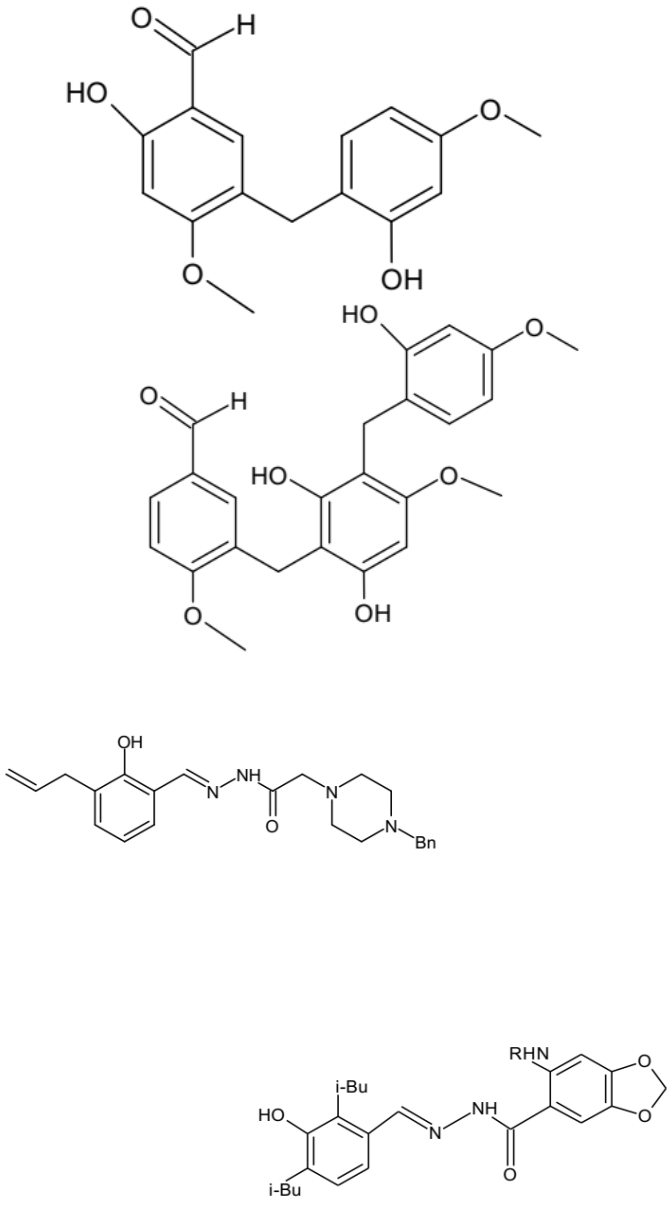

Fig. 4 Chemical structure of ammonium salicyl hydrazones (Bogdanov et al. 2020)

Significant changes in cell membrane permeability and damage to the cytoplasmic membrane of *S. aureus* are described as a possible explanation for antimicrobial action.

Reyes-Melo et al. (2017) synthesized and analyzed thirty-three meso-dihydroguaiaretic acid (meso-DGA) derivatives (Fig. 5) containing esters, ethers and amino ethers. The presence of positively charged group precursors possessing steric and hydrophobic characteristics (e.g., N-ethylpiperidine portions in meso-31) significantly increased the antimycobacterial properties.

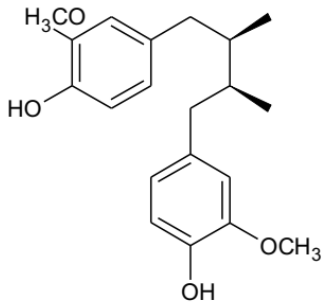

Fig 5 Chemical structure of Meso DGA (Reyes-Melo et al. 2017)

We can also highlight studies demonstrating that liposoluble compounds are cited as modifiers of plasma membrane permeability in bacteria (Pretto et al. 2004; Nicolson et al. 1999). Thus, the liposoluble nature of the compounds can alter the fluidity of the bacterial membrane, making it more susceptible to penetration by various substances, particularly antibiotics (Andrade et al. 2014). The use of fat-soluble compounds, in association with antibiotics, is an interesting alternative to improve antibiotic activity (Dipalma, 1977; 1978).

Efflux pumps

Efflux pumps are proteins that integrate the bacterial plasma membrane to which several cases of drug resistance are attributed, since they are responsible for expelling the antimicrobial drug out of the cell, minimizing its toxic effect on bacteria (Piddock, 2006). Modifiers of antibiotic activity is a term used for substances that modulate or even reverse bacterial resistance to certain antibiotics by altering microbial susceptibility to antibiotics through inhibition of efflux pumps (Gibbons, 2004).

Santos et al. (2018) evaluated the inhibitory action of caffeic acid and gallic acid (Fig. 6) on efflux pumps of resistant strains of S. aureus. In the efflux pump inhibition effect, caffeic acid showed greater efficacy and inhibited the pumps MrsA from strain RN-4220 and NorA from strain 1199B. Caffeic acid showed greater efficacy in the docking model, in agreement with the demonstrated experimental efficacy, the author suggests that phenolic acids act on the efflux pumps by altering not only their permeability to the cell membrane, but also that the mechanism may be due to lipophilic interactions. Amphipathic compounds have been reported as efflux pump inhibitors, the inhibition has been shown to be related, in part, to hydrophobicity (Gibbons et al. 2003). Hydrophobicity is a feature that can reduce recognition and transport by an efflux pump (Piddock et al. 2001) Zhou et al. (2019) and Reyes-Melo et al. (2017) cite the hydrophobic effect as responsible for bacterial reversion in their work.

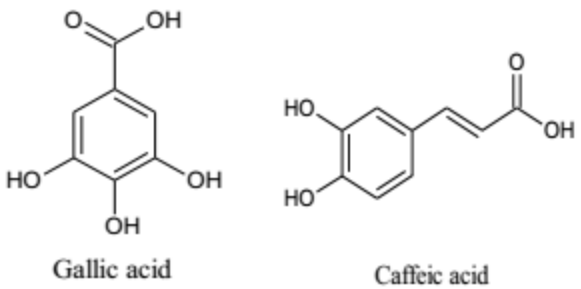

Fig. 6 Chemical structure of (a) gallic acid and (b) caffeic acid (Santos et al. 2018)

Reyes-Melo et al. (2017) studied thirty-three meso-dihydroguaiaretic acid derivatives, concluding that the presence of positively charged groups with hydrophobic characteristics were able to increase bacterial resistance. On the other hand, lipophilic substances cause disturbances in the bacterial membrane, resulting in damage of fundamental elements, necessary for membrane integrity, such as reduced membrane potential and the loss of ions, cytochrome C, proteins, and radicals, followed by proton pump collapse and ATP depletion (Sikkema et al. 1994).

Rampogu et al. (2018) in their research analyzed eight ginger phytochemicals as potential drug candidates that may act as enhancers of antibiotic activity evaluated for potential drugs. Of the eight phytochemicals, shogaol and gingerenone-A were both potent drug candidates, demonstrating higher scores in docking analysis, showing stronger interactions at the active site residue.

Studies signal that efflux pumps preferably transport amphiphilic substrates, it is observed that the chemical structure of various pump inhibitors show common structural features, such as aromatic rings; compounds that have on their surface side chains of ionizable portions, and/or amino acid side chains are prone to establish hydrophobic, aromatic stacking and Van der Waals interactions (Van Bambeke et al. 2010; Yu et al. 2003). The bacterial activity of some compounds associating with the OH group as responsible for this activity, anchoring the studies on the intermolecular bonds that could destabilize the plasma membrane of the bacteria (Murray et al. 2015).

This effect may be due to the fact that efflux pumps are transmembrane proteins, having the function associated with cell membrane structure and fluidity. Lipophilicity is a common feature of several efflux pump inhibitors, it is probably important because it affects the solubility of the bacterial membrane or perhaps by binding to efflux proteins or pump substrates (Zloh et al. 2004), causing inhibition of drug removal (Gibbons et al. 2004).

Inhibition of plasmid conjugation

The widespread and sometimes indiscriminate use of antibiotics results in the selection of bacteria that are resistant. Not only can these bacteria become predominant in a population, they can also transfer genetic material to susceptible bacteria, which then acquire resistance. Antibacterial drug resistance can be encoded on the bacterial chromosome or in plasmids, small extrachromosomal circular genetic elements formed by double-stranded DNA located in the cytoplasm of the bacterial cell that can replicate independently, which facilitates the dissemination of these genes.

Oyedemi et al. (2019) studied a number of compounds with similar chemical structures, the compounds showed different biological activities, and this characteristic is attributed to the binding sites. The common inhibitory activity of capsaicin (Fig. 7), can presumably be related to the difference in the saturation of the alkyl side chain. The antimicrobial activity was attributed to the ability of the compounds to inhibit plasmid conjugation processes. Both capsaicinoids and gingerols showed pharmacologically active effect and also an effect on the conjugative transfer property of the plasmid.

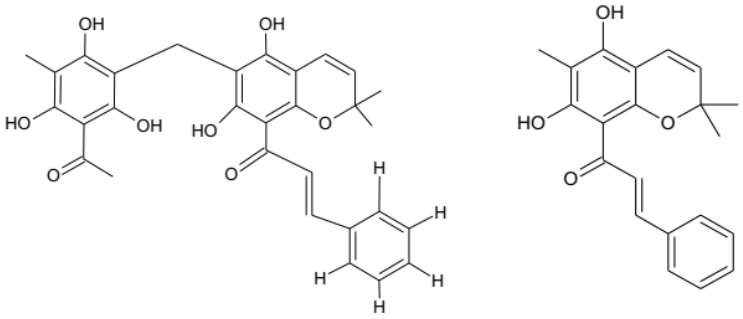

Fig. 7 Chemical structure of capsaicin compounds (Oyedemi et al 2019)

Tang et al. (2019) studied resveratorol (Fig. 8), a natural compound that has been attributed the ability to inhibit alpha-hemolysis, a toxin produced by most *S. aureus* strains associated with virulence of this bacteria. Resveratrol inhibits the extent of alpha-hemolysis when grown on *S. aureus*, due to inhibition of RNAIII transcription. Resveratrol treatment significantly inhibited virulence by *S. aureus in vitro* and *in vivo*.

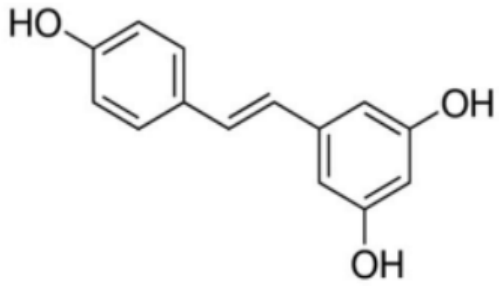

Fig. 8 Chemical structure of resveratrol (Tang et al. 2019)

Transcription is the first step in gene expression; it involves copying the DNA sequence to produce an RNA molecule. The mechanism is carried out by polymerases, which bind nucleotides to produce an RNA strand.

Lipophilic nature of compounds

The interaction of so-called bacterial resistance action modulators produces its effect by binding to receptors located on the membrane or inside the cell, or by altering the biophysical properties of the membrane.

Ouyang et al. (2018), studied compounds isolated from fungi that had similar chemical structure, pointing out the study compound with the most effective antibacterial characteristics was the one that had the side chain with the 3-methylbut-2-enyl group signaling that the side chain could be the group responsible for the activity.

In the quest to reverse bacterial resistance, lipophilic compounds associated with antibiotics have shown promising results.

Tan et al. (2017) studied analogous compounds of acylfloroglucinol (Fig. 9) by modifying only the acyl portion of the side chain, these were tested against MRSA strains. Significant antibacterial activities were observed for most of the acylfloroglucinol analogues. Lipophilicity, rather than the magnitude of the hydrophobic tail, suggests to be more effective in enhancing antibacterial activity.

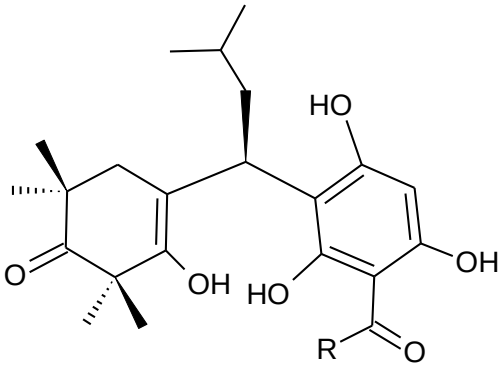

Fig. 9 Basic structure of acylphloroglucinol analogues (Tan et al. 2017)

Studies of the biophysical and morphological mechanism of action revealed that the effects included membrane depolarization and, to a lesser extent, membrane disruption and cell lysis.

Changes in membrane permeability caused by the compounds

The selective permeability of the plasma membrane results firstly from the lipid nature of its basic structure, which facilitates the penetration of non-polar substances and prevents the passage of water-soluble compounds, and secondly from the presence of specific carriers or transporters of protein nature for certain polar substances. The selective permeability of membranes is also dependent on the presence of the chemical element calcium, which, as a cation, is essential to membrane integrity, being involved in the binding process between lipids and proteins (Faria (b) 2000). Compounds that facilitate the entry of the antibiotic into the bacterial cell are being studied, seeking a more effective and efficient action of antimicrobials.

Mandal et al. (2017) studied a series of compounds and reported that the antimicrobial action of the compounds was related to the inactivation of cellular enzymes and the modification of membrane permeability. The antimicrobial activity was shown to be dependent on the chemical structure, in particular the substitution position on the benzene ring and the side chain attached to it.

The MIC values observed for tannic acid, one of the compounds under study, were lower than those observed for epigallocatechin gallate, rutin and eugenol (Fig. 10) tests performed against Staphylococcus aureus. The numbers of hydroxyl, carboxyl, or methyl groups present in the compounds and their lipophilicity have been shown to alter antimicrobial activity by affecting the integrity of the bacterial cell wall, through direct binding to peptidoglycan, inhibiting the function of some bacterial enzymes and membrane transport proteins in the cell envelope, and binding to metal ions.

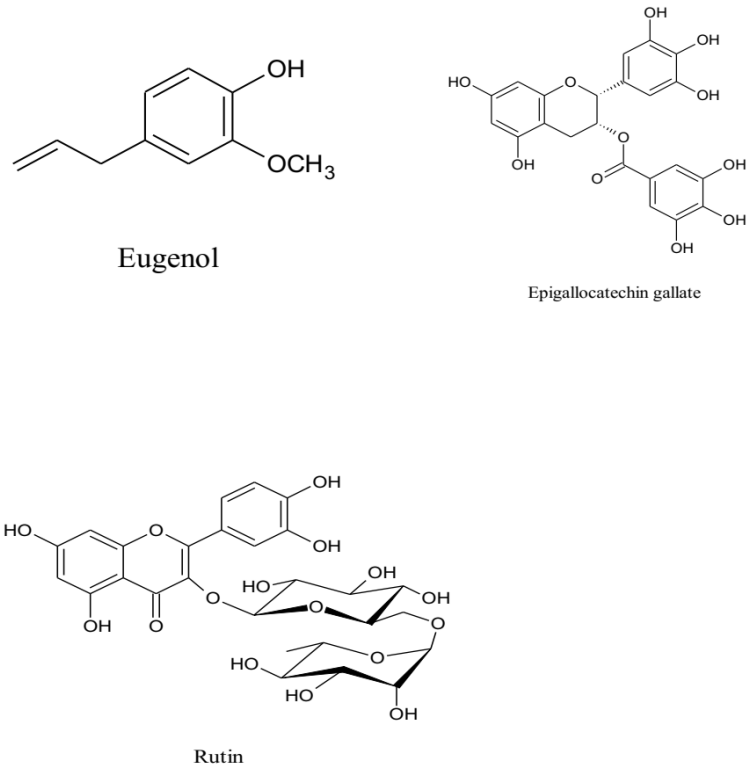

Fig. 10 Chemical structure of (a) Rutin (b) eugenol (c) epigallocatechin gallate (Mandal et al. 2017)

The high efficiency observed in MIC tests for tannic acid was explained by the strong interaction predicted between this compound and net positively charged amino acid residues, such as: Ser70, Lys73, Ser130, Asn132, Glu166, Asn170, Lys234, Ser235, and Arg244.

In vitro and *in vivo* tests showed similar results and concluded that the compounds are potential candidates for antimicrobial action. Among the compounds epicatechin, tannic acid, epigallocatechin gallate, quercetin, rutin, eugenol showed the best results regarding antibacterial activity, biofilm inhibition and b-lactamase inhibition.

Phan et al. (2017) studied the basic framework of benzofuran (Fig. 11), which was cited as a promising and potential group in antibacterial activity against Gram - (+) bacterial strains. The high content of halogen groups (F, Cl and Br) present in the ketones and the occurrence of the substituents -OCH3 / -OH apparently provided the basic characteristics for bacterial activity. Among the ethers studied, a compound with a carboxylic acid group on the ether residue stood out, which showed a significant increase as antibacterial agents. The results indicated that the carboxylic acid group appears to be an essential part of the pharmacophore required for inhibitory activity against bacterial strains, and it is also suggestive that the group maintains an important role for potential hydrogen bonding as hydrogen bond donor functions, thus exhibiting hydrophilic properties. The study further suggested that the position of the heteroatom in the benzofuran heterocycle by substituting bromine atoms at different substituents signaled that bromine atoms may exhibit halogen bond donor functions presenting lipophilic properties.

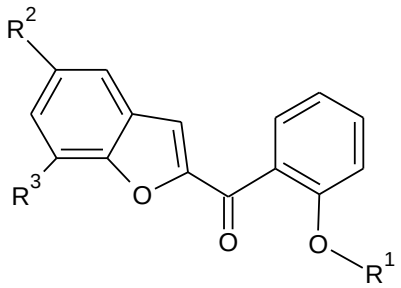

Fig. 11 Chemical structure of 2-salicyloybenzofuram derivatives (Phan et al. 2017)

Howse et al. (2019) analyzed the antibacterial agent triclosan (Fig. 12), which was optimized to provide four new triclosan-α-D-glycopyranoside compounds and three new triclosan-β-D-glycopyranoside compounds, the goal was to increase solubility of the compounds in water in order to increase uptake into bacterial cells via active carbohydrate transport mechanisms. The prepared derivatives showed increased aqueous solubilities compared to free triclosan, demonstrated by calculated logP values, this presents potential opportunities, for triclosan to be used orally for the treatment of systemic infections.

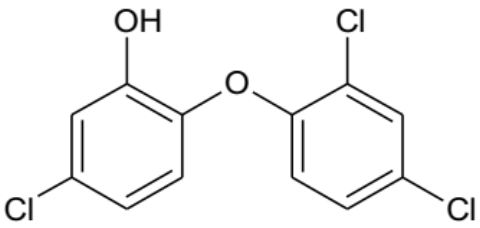

Fig. 12 Chemical structure of triclosan (Howse et al. 2019)

The results therefore demonstrated that glycosidic derivatives of triclosan offer considerable advantages as antibacterial agents compared to triclosan itself, specifically due to the aqueous solubility characteristic.

Other studies

Mikolasch et al. (2016) analyzed the compounds methyl-1,4-hydroquinone and 2,3-dimethyl-1,4-hydroquinone (Fig. 13), these were used as precursors to 14 new cephalosporins, penicillins, and carbacephems, synthesized by amination with amino-β-lactam structures. The compounds were observed to be effective as antimicrobials; the compounds obtained inhibited the growth of several gram-positive bacterial strains, including Staphylococcus aureus. The highest antibacterial activities were exhibited by the compounds: tert-butyl-1,4-hydroquinone, 2,3-dimethyl-1,4-hydroquinone, bromo-1,4-hydroquinone, 2,5-dichloro-1,4-hydroquinone, 3,5-dithert-butyl-1,2 -hydroquinone, 2- (2-chlorophenyl) -1,4-hydroquinone and phenyl-1,4-hydroquinone. The activity of these seven compounds was comparable to the activity of standard β-lactam antibiotics.

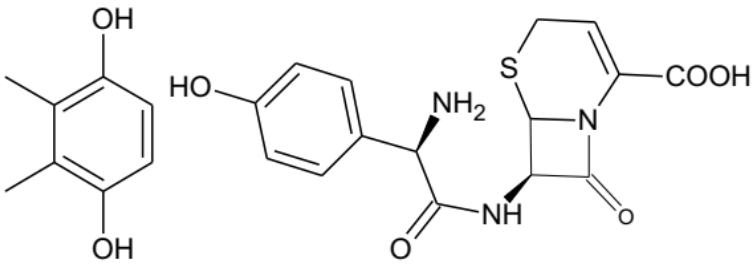

Fig. 13 Chemical structure of 2,3 dimethyhydroquinone 1b and cephalosporin 2ª (Mikolasch et al. 2016)

Xu et al. (2018) studied a compound isolated from the marine fungus *Aspergillus sp*., isolated from sediment sample collected in the Bohai Sea. The chemical investigation resulted in the identification of diphenyl ether named diorcinol (Figure 14), along with three known diphenyl ethers. The compounds obtained significant antibacterial activities against *S. aureus* and methicillin-resistant *S. aureus*, with MIC values ranging from 3.125 to 6.25 µg/ mL, respectively.

Nature of H-bonds and intramolecular interaction

Murray et al. (2015) evaluated the chemical structure and antibacterial activity of some compounds, with Psorothatin C showing the highest activity against methicillin-resistant *Staphylococcus aureus*. This antibacterial activity was associated with the OH groups. Also, the study highlighted that hydrogen bonding and intramolecular interaction are probably responsible for these activities.

In their study Sun et al. (2016) presented the synthesis and biological evaluation of riccardin D derivatives (Fig. 14), a new class of antimicrobial molecules. The structural modifications were achieved by introducing hydroxyl and methoxy groups and by introducing a bromine atom into the aromatic rings of riccardin D. The antimicrobial evaluation of these compounds was performed as *in vitro* assays against clinically isolated bacteria and fungi. The introduction of bromine atom in the riccardin D molecule led to several strongly active antibacterial compounds with MIC value ranging from 0.5 to 4 µg/mL for *Staphylococcus aureus*, methicillin susceptible and methicillin resistant strains. The study suggests that the number and position of hydroxyl groups on the benzene ring could impact the antimicrobial activity. The compounds that featured the bromine showed higher antibacterial activity.

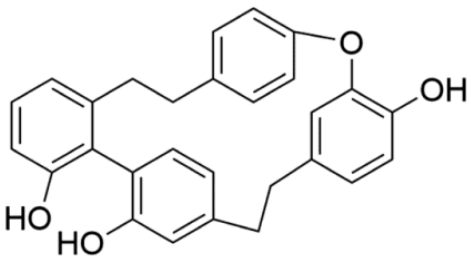

Fig. 14 chemical structure of riccardine D (Sun et al. 2016)

Ding et al. (2016) studied five tyrosol derivatives (Fig. 16), isolated from the fermentation of the fungus species *H. koningii* PF04. All compounds showed weak antibacterial activities against *S. aureus* and methicillin-resistant *S. aureus*.

Zhao et al. (2019) isolated compounds (Fig. 15) that showed antibacterial activity

against methicillin-resistant *S. aureu*s, with MIC values of 12.50-25 µg/mL (ciprofloxacin: 0.78 µg/mL).

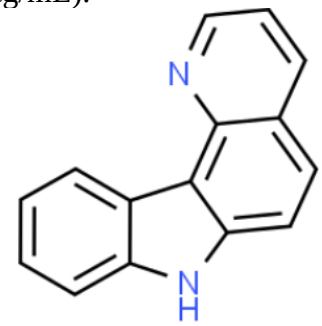

Fig. 15 chemical structure of indoloquinoline (M. Zhao et al. 2015)

Antagonistic nature, chelation effect

Gomes et al. (2018) in their study reported the antibacterial properties of catechins (Fig. 16) against multidrug-resistant strains of *Staphylococcus aureus, Escherichia coli* e *Pseudomonas aeruginosa*. It has been verified that catechins are effective in combination with conventional antibiotics.

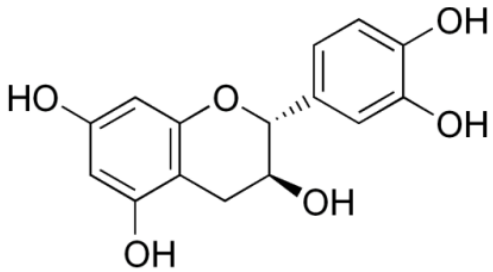

Fig. 16 Chemical structure of catechin (Gomes et al. 2018)

Catechin showed synergistic activity when in combination with norfloxacin and gentamicin against *S. aureus*, increasing the antibiotic activity and an antagonism in association with tetracycline. Regarding the antagonistic effect mentioned, when associated with tetracycline, its action may have occurred due to chelation of the antibiotics.

Chelation is characterized as the binding to the active site of the drug by a certain substance, thus preventing its action, the result presented by catechin may have occurred due to a greater facility for drug penetration into the cell. This usually occurs when the bacterial cell has one of its membranes ruptured.

Mittal et al. (2019) analyzed a series of acylated floroglucinol derivatives (Fig. 17) to determine and evaluate their antibacterial activities. The minimum inhibitory and bactericidal concentrations were determined, as well as their effects as inhibitors of biofilm formation. Out of the 12 compounds tested against MRSA and methicillin-susceptible strains, four showed MIC values ranging from 0.125 to 8 µg ml-1 and all of them showed bactericidal activity. However, none of the compounds were able to eradicate biofilms at the concentrations tested.

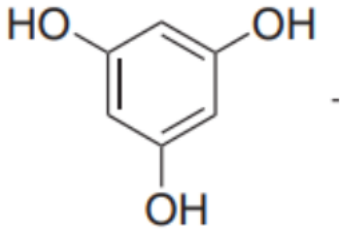

Fig. 17 Chemical structure of phloroglucinol (Mittal et al. 2019)

Three out of these four showed no hemolytic activity under the conditions tested. The results suggest that acylated phloroglucinols have some potential to be investigated as antibacterials. The common structural feature for the four most active compounds may indicate a basic structure for antibacterial activity. The compounds differ only in the chain length of the alkyl group in the anhydride that was used in the acylation step.

Zheng et al. (2019) synthesized a series of new analogues of Cajaninstilbene acid (Fig. 18), electron withdrawing groups such as trifluoromethyl, cyano and trifluoromethoxy were introduced into the chain of the new compounds. The influence of the electron-donating groups with methyl, ethoxyl and t-butyl groups in position 4 of the carbon ring was also analyzed.

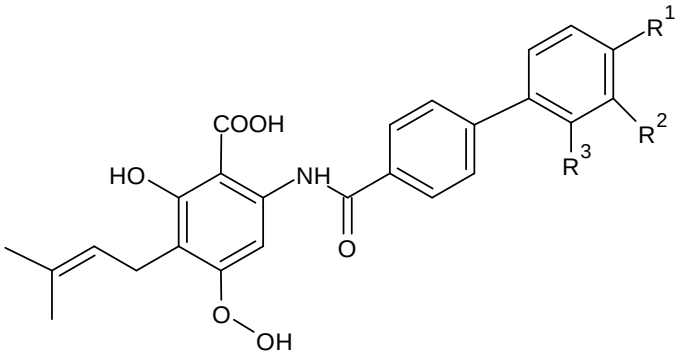

Fig. 18 Chemical structure of 2-hydroxyl-4-methoxyl-3-(3-methylbut-2-en-1-yl)-6-(4-phenylbenzoylamino) benzoic acid derivatives derived from CSA analogs (Zheng et al. 2019)

It was concluded that the addition of two substituents simultaneously at position 2 and 3 of the carbon ring increases the activity against sensitive bacteria, but decreases the antibacterial properties against drug-resistant bacteria. The structure-activity relationships of Cajaninstilbene acid analogues could be summarized as follows: (a) the antibacterial activity of this type of compounds is highly correlated with the position of substituents on the C-ring; (b) substitution of the 4-position of the C-ring is unfavorable for antibacterial activity; (c) 2-position or 3-position substitution of the C-ring is favorable for improving antibacterial activity, where the 2-position is more significant for activity enhancement; (d) the enhancing effects of different substituents at position 2 or position 3 on antibacterial activity are methyl> ethoxyl> halogen; and (e) two substituents at position 3 and position 2 simultaneously increase activity against sensitive bacteria but decrease antibacterial activity against drug-resistant bacteria.

Jiang et al. (2018) from the analysis of a crude extract, synthesized and characterized compounds sorbicillin derivative (1) and diketopiperazine alkaloid (2) (Figure 22), however

only compound 1 showed moderate inhibitory activity against MRSA, and this activity was lower than what was found in the crude extract.

Zhao et al. (2015) screened a series of indoloquinoline analog compounds that were synthesized in order to understand the structure-activity relationship in antibacterial action. The results presented indicate that the position and types of substitution group are relevant to antibacterial activity.

Chen et al. (2017) investigated a class of derivatives of bisbenzimidazole bisamidine compounds (Fig. 19) that was synthesized by keeping the central framework of this compound and replacing the iso-propyl group at the N position with chains and rings of different sizes in order to evaluate the biological effect induced by the steric hindrance caused by the substituents. Evidence signaled that the potent bactericidal action of bisamidines is probably the result of inhibition of DNA synthesis. It was observed that the activity against resistant Gram-positive bacteria was similar to the result presented by sensitive Gram-positive bacteria. They showed that the length and volume of the carbon chain had a great influence on the bacterial activities.

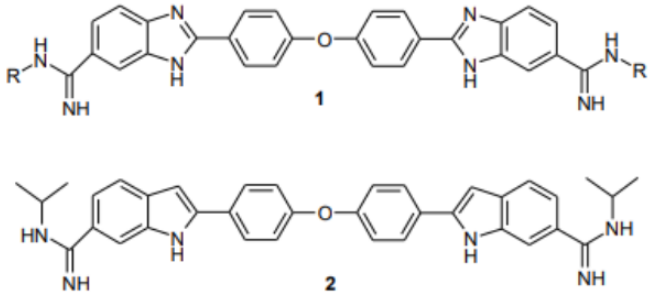

Fig. 19 Chemical structure of Benzimidazole and indole amidine compounds. (Chen et al. 2017)

In a research study by Sanabria-Ríos et al., 2015 the compound named C5-curcumin-2-hexadecynoic acid (Fig. 20) was synthesized and the guiding research question was whether the chemical conjugation of an antibacterial fatty acid with the compound would be able to potentialize its antibacterial activity. It was found that the presence of 2-hexadecnoic acid (2-HDA, 4), increased 4-8 times its antibacterial activity against MRSA strains. The studies indicated that the presence of a C-2 triple bond in the fatty acid portion is important for the antitopoisomerase activity, which could affect the bacterial DNA replication process.

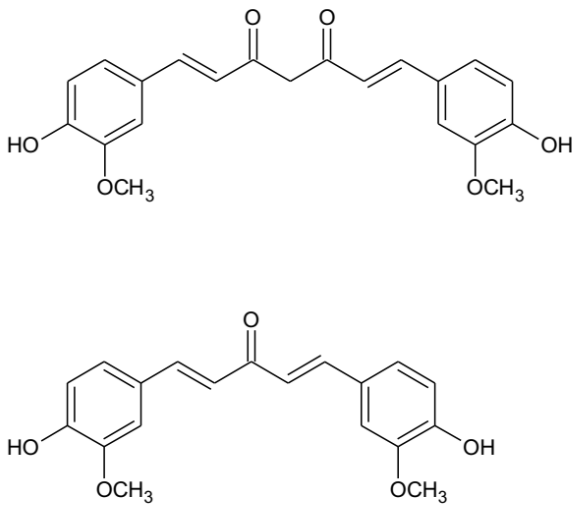

Fig. 20 Chemical structure of crucumim and c5-curcumim (Sanabria-Ríos et al. 2015)

Conclusion

Several bacterial strains may present a variety of genetic elements and mechanisms that favor their survival in their environment. In this context, it is important to understand the mechanisms of adaptation of a given bacterial population before the adoption of antimicrobial drugs in order to avoid failures in the treatment of diseases.

An alternative to mitigate bacterial resistance would be the use of antimicrobials associated with bacterial resistance modulating compounds. In this way, it is observed that the synergistic or antagonistic activity derived from this process seems to be dependent on the chemical structure of the modulator compound, in particular the benzene ring and the chemical substitutions of these rings, according to the results found in this review.

The steric effects were not stated as directly responsible for the antimicrobial activity of phenolic compounds, suggesting that lipophilicity and the possibility of alteration of membrane permeability are responsible for higher MIC values.

The presence of positively charged group precursors, possessing both steric and hydrophobic characteristics, appears to significantly enhance the antimicrobial properties.

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CAPÍTULO 6: CONSIDERAÇÕES FINAIS

6.1 DISCUSSÃO GERAL

S*taphylococcus aureus*, ao longo dos anos, devido ao excessivo e incorreto uso dos antibióticos, adquiriu genes responsáveis por mutações, tornando-se uma bactéria resistente a maioria dos antibióticos de referência. O desenvolvimento de multirresistência oportuniza a interferência no mecanismo de ação dos antibióticos, o que limita a terapêutica e prolonga o tempo de tratamento das patologias e das infecções a ela atibuídas. Entre os mecanismos de resistência, a redução da concentração intracelular do antibiótico do meio intracelular, por meio do processo de extrusão mediado por sistemas de efluxo, vem sendo estudado.

Na busca pela reversão da resistência bacteriana, mediada por sistema de efluxo, conduziu-se a pesquisa na busca de compostos que poderiam alterar e/ou causar danos à estrutura da membrana bacteriana, ou seja compostos com propriedades lipofílicas, anfipáticos, que conjugados com os antibióticos de referência, poderiam alterar e/ou causar prejuízos à estrutura da membrana bacteriana, afetando a solubilidade, integridade, atuação das mecanismos de efluxo e inibição da remoção do fármaco, atuando como inibidor da resistência bacteriana (ZLOH *et al*., 2004; GIBBONS *et al*., 2004).

Neste trabalho, testaram-se os compostos esterificados do ácido ferúlico como inibidores de bomba de efluxo. A reação de esterificação do ácido ferúlico (AF) com metanol, etanol, propanol e butanol produziu os esteres ferulato de metila, ferulato de etila, ferulato de propila e ferulato de butila, cuja modificação estrutural, em relação ao substrato, ocorreu no carbono carboxílico. Após purificação por cromatografia em coluna, os compostos esterificados forneceram rendimentos de moderados a bons (47–73 %), suficientes para realização dos testes.

Os produtos de síntese foram caracterizados por interpretação dos espectros de RMN de ^{1}H e ^{13}C. Todos os carbonos inseridos na estrutura do ácido ferúlico foram justificados por acompanhamento dos sinais presentes no espectro DEPT 135º. Nos resultados obtidos, acredita-se que o AF e os derivados atuem mais indiretamente sobre as proteínas de efluxo, ou seja, sobre a membrana plasmática bacteriana ou até mesmo secundariamente, facilitando a entrada do antibiótico.

O composto ferulato de etila apresenta natureza lipossolúvel, sugestivamente capaz de alterar a fluidez da membrana bacteriana, tornando-a mais suscetível à penetração de antibióticos. Este composto apresentou, nos testes de concentração inibitória mínima com norfloxacina e brometo de etídio redução significativa da CIM, podendo ser atribuído a uma

inibição da bomba efluxo NorA. A correlação foi traçada entre a interação dos compostos e a bomba de efluxo, por meio do acoplamento molecular, que demonstrou boa afinidade. No entanto, outros estudos devem ser desenvolvidos para elucidar o mecanismo completo de sinergismo apresentado.

O composto ferulato de butila apresentou redução não significativa da CIM frente a cepa RN-4220. Quando associado ao CCCP, houve diminuição da MIC do brometo de etídio. Em relação à cepa IS-58, somente o composto ferulato de propila apresentou nas concentrações subinibitórias, associados ao brometo de etídio, redução da CIM; os demais compostos apresentaram efeitos antagônicos, quando associados ao brometo de etídio.Os compostos sintetizados podem melhorar as propriedades farmacológicas e aumentar os benefícios da terapia em derivar compostos antimicrobianos capazes de atuar na reversão da resistência bacteriana (OYEDEMI *et al*., 2019; OUYANG *et al*., 2018). Entretanto, observa-se que alguns compostos apresentaram propriedades antagônicas ao processo de reversão da resistência bacteriana, atuando de forma a contribuir com o efluxo bacteriano. Observa-se que o antagonismo se manifestou de forma mais pronunciada, como o brometo de etídio, levantando a hipótese que os compostos esterificados possam proteger a bactéria da toxicidade do brometo de etídio.

6.2 CONCLUSÕES

A associação do composto AF-etanol, nos testes de concentração mínima inibitória, com norfloxacina e brometo de etídio, não foi sugestiva de um mecanismo de bomba de efluxo, indicando que o ferulado de etila tem efeitos moduladores de antibióticos contra a cepa investigada, sobre a bomba de efluxo ou outros mecanismos de resistência à norfloxacina. Uma correlação foi traçada entre a interação dos compostos e a bomba de efluxo NorA, por meio do acoplamento molecular, que demonstrou boa afinidade. Novos estudos estão sendo realizados para elucidar o mecanismo completo de sinergismo apresentado.

A associação dos compostos esterificados do ácido ferúlico, nos testes de concentração mínima inibitória com os antibióticos de referência tetraciclina e eritromicina e o composto brometo de etídio, mostrou redução significativa nas CIMs, a inibição de um mecanismo de bomba efflux não pôde ser comprovada, assim foi levantada a suposição de danos estruturais e/ou funcionais à membrana citoplasmática.

6.3 PERSPECTIVAS DE INVESTIGAÇÕES FUTURAS

Novos estudos com modelagem molecular serão desenvolvidos.

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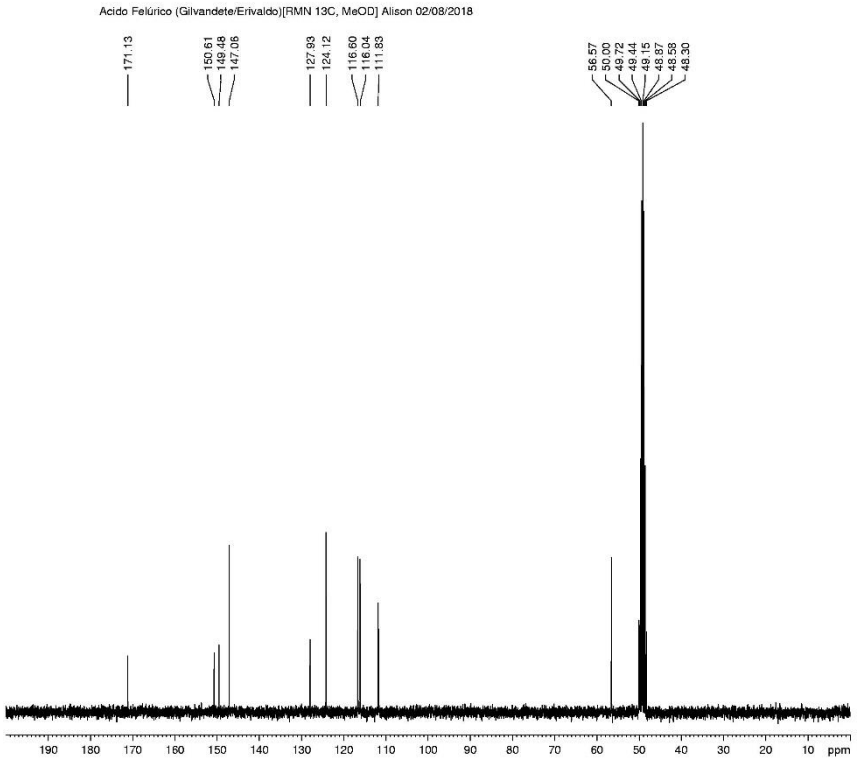
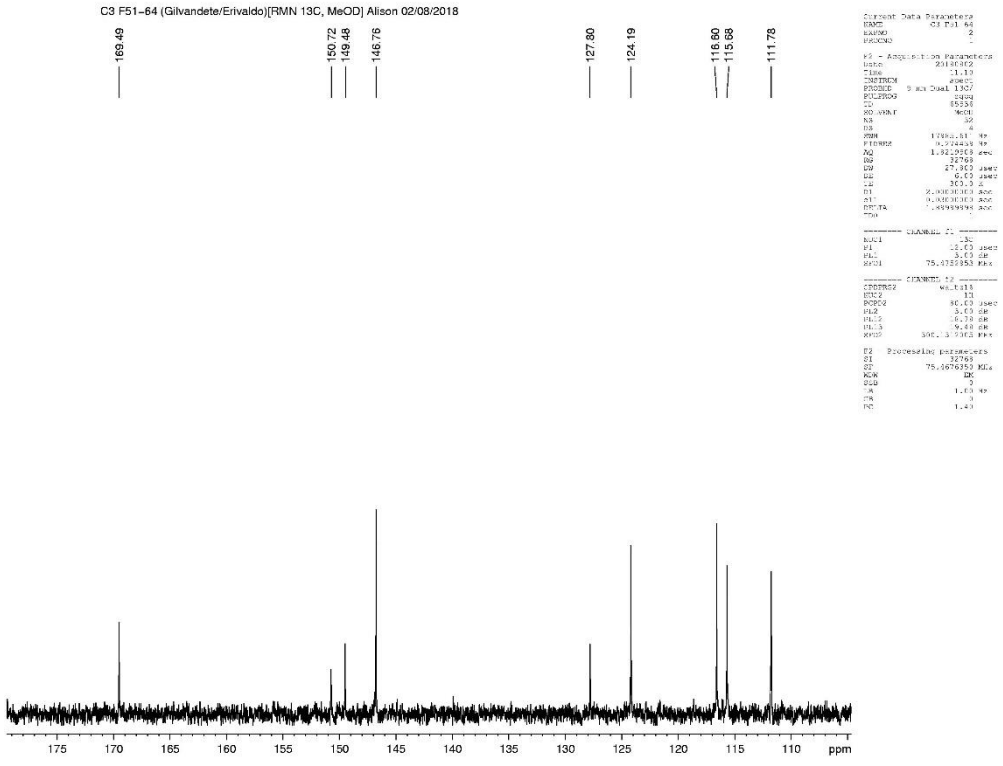
ANEXOS

NEXO A – ESPECTROS DE RMN DOS COMPOSTOS SINTETIZADOS





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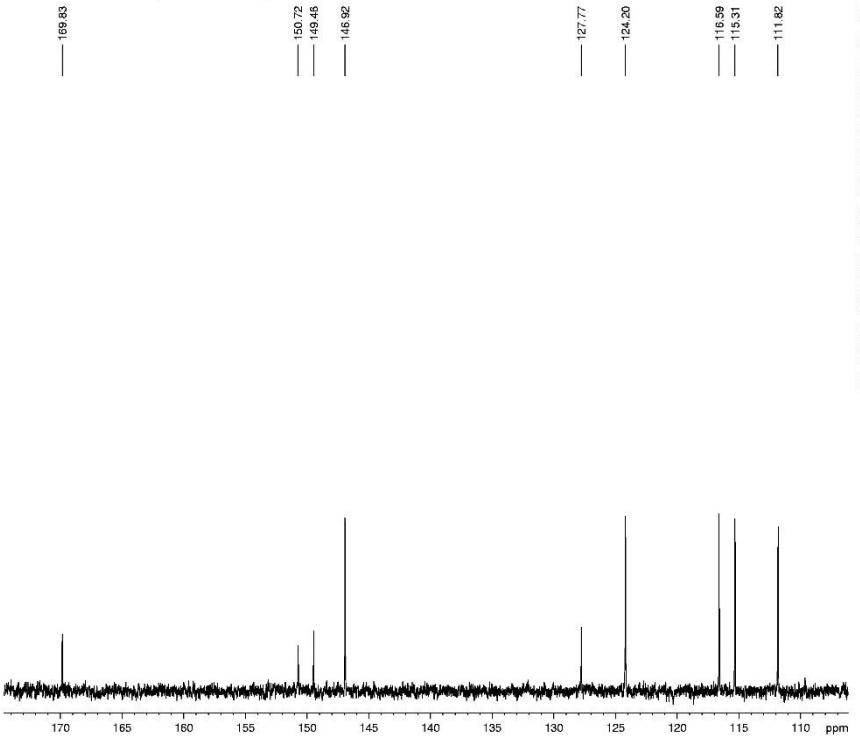

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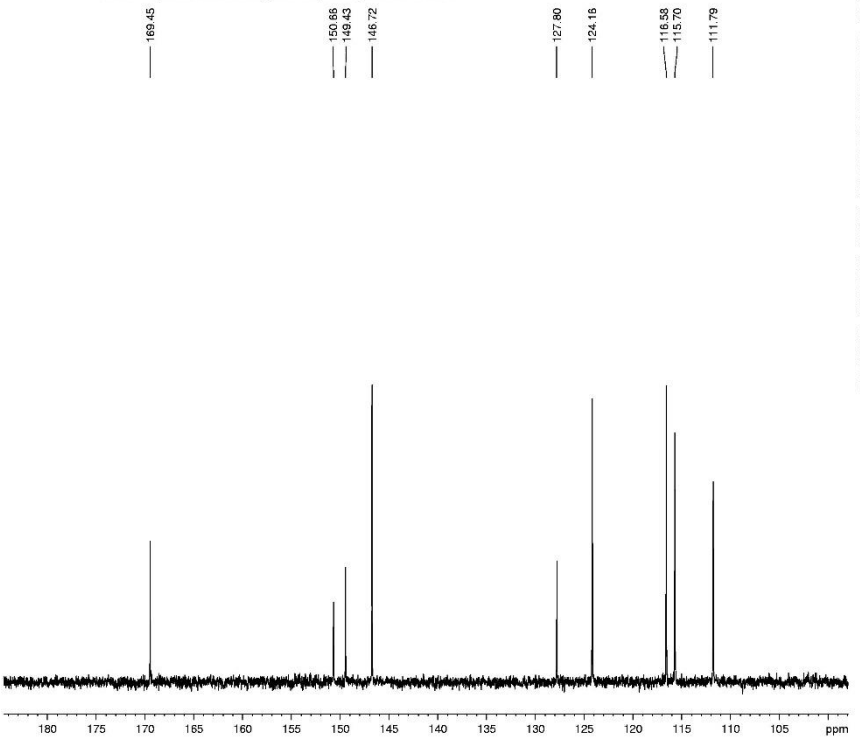

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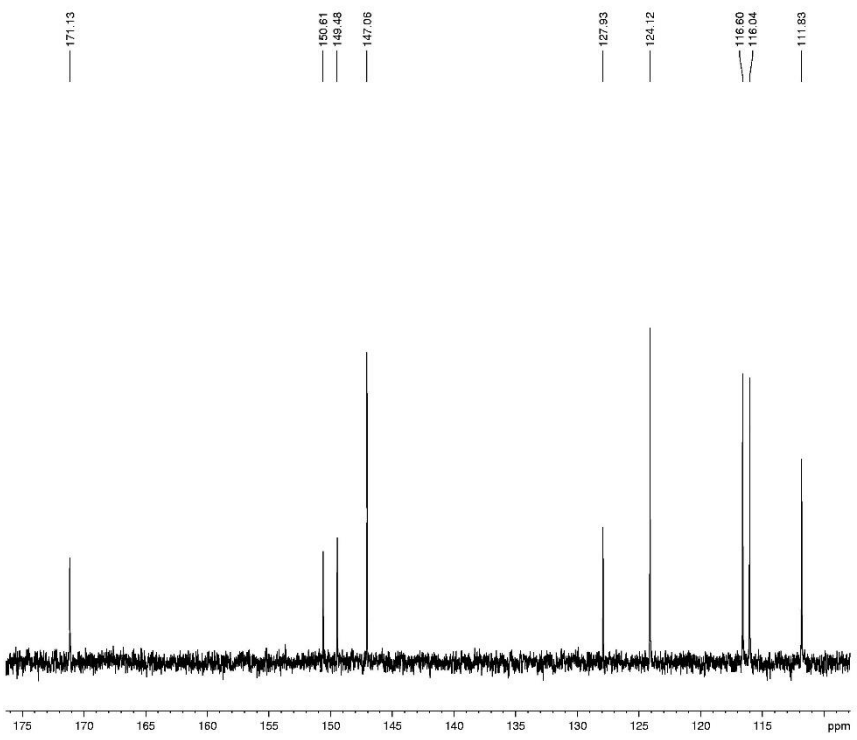

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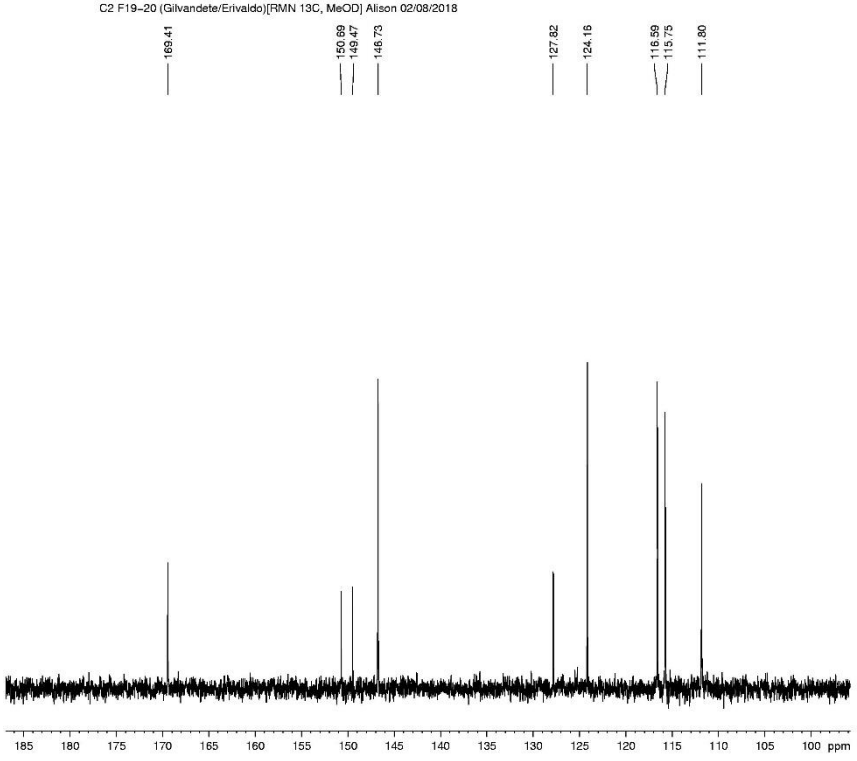

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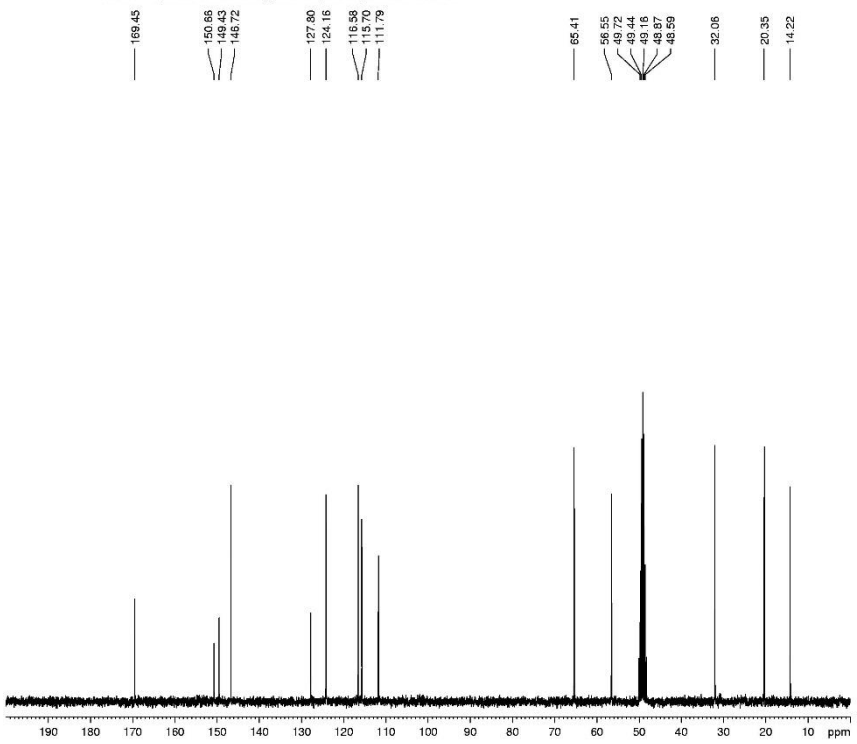

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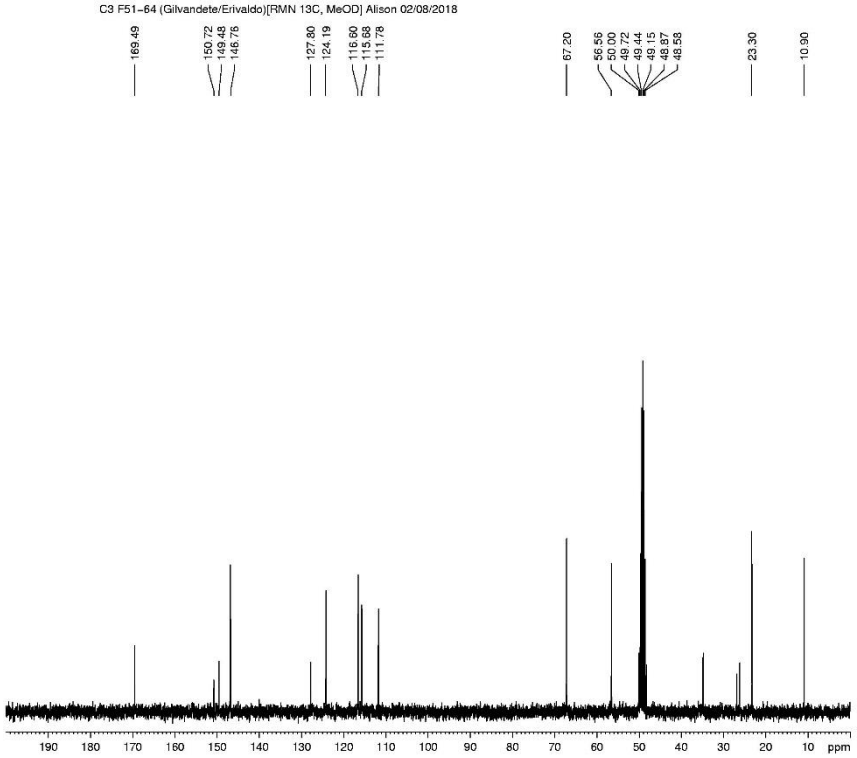

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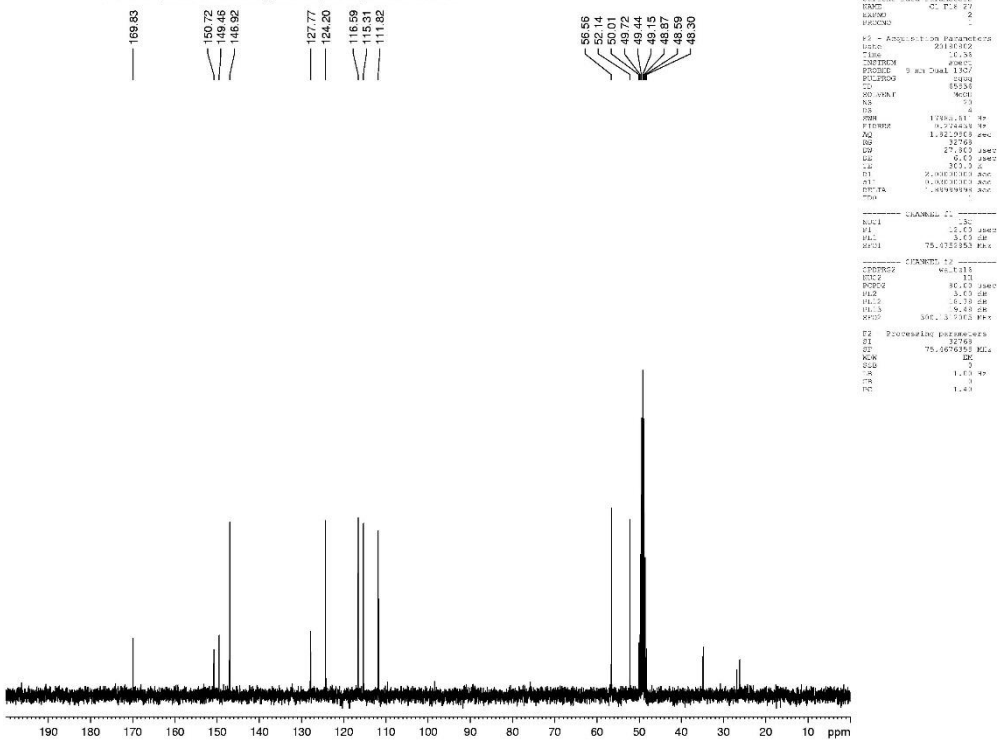

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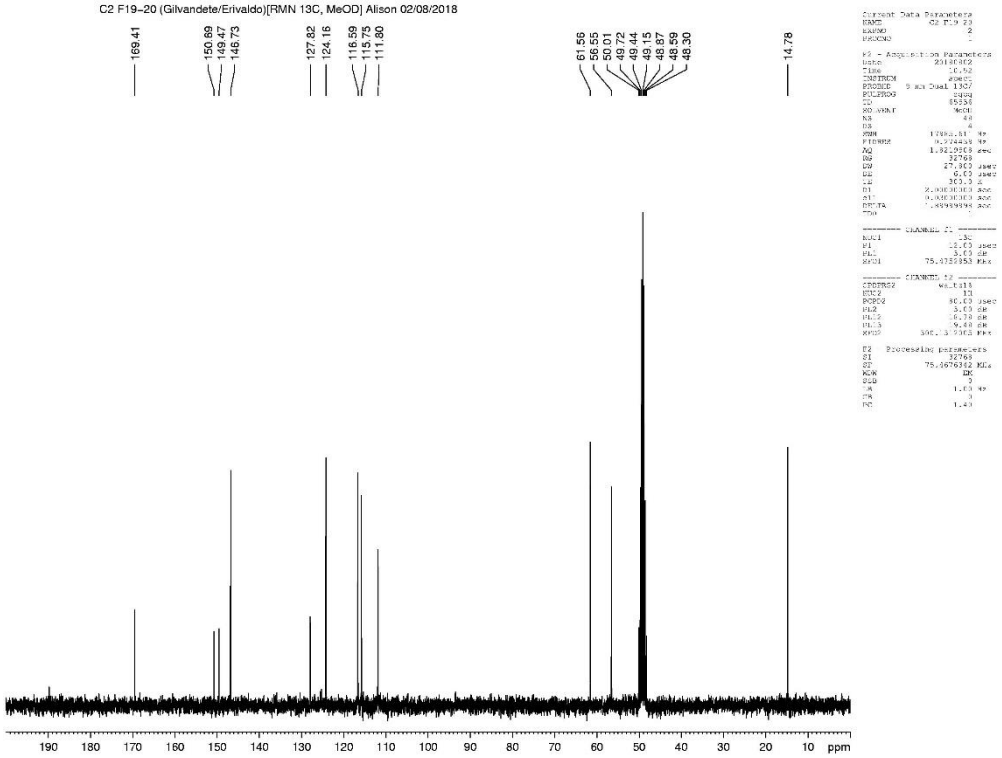

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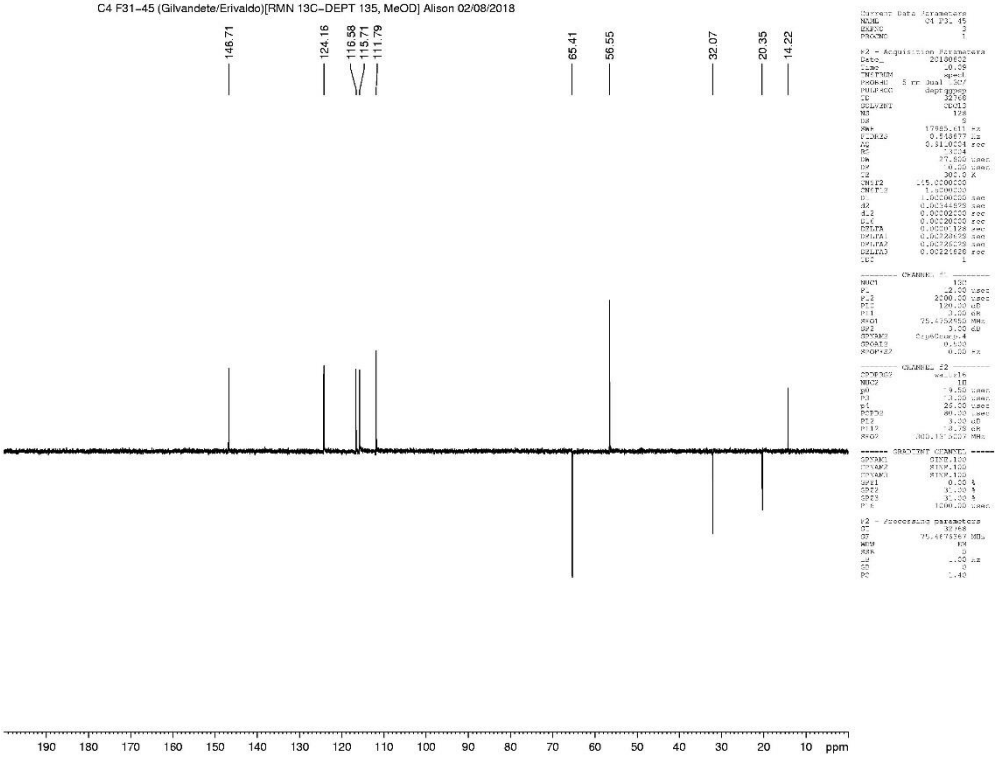

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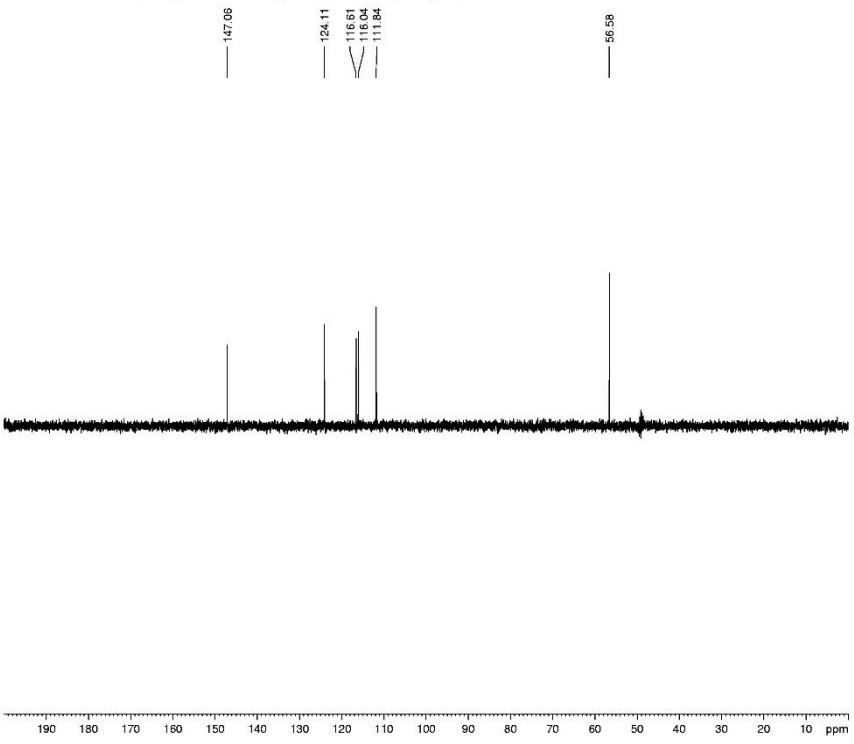

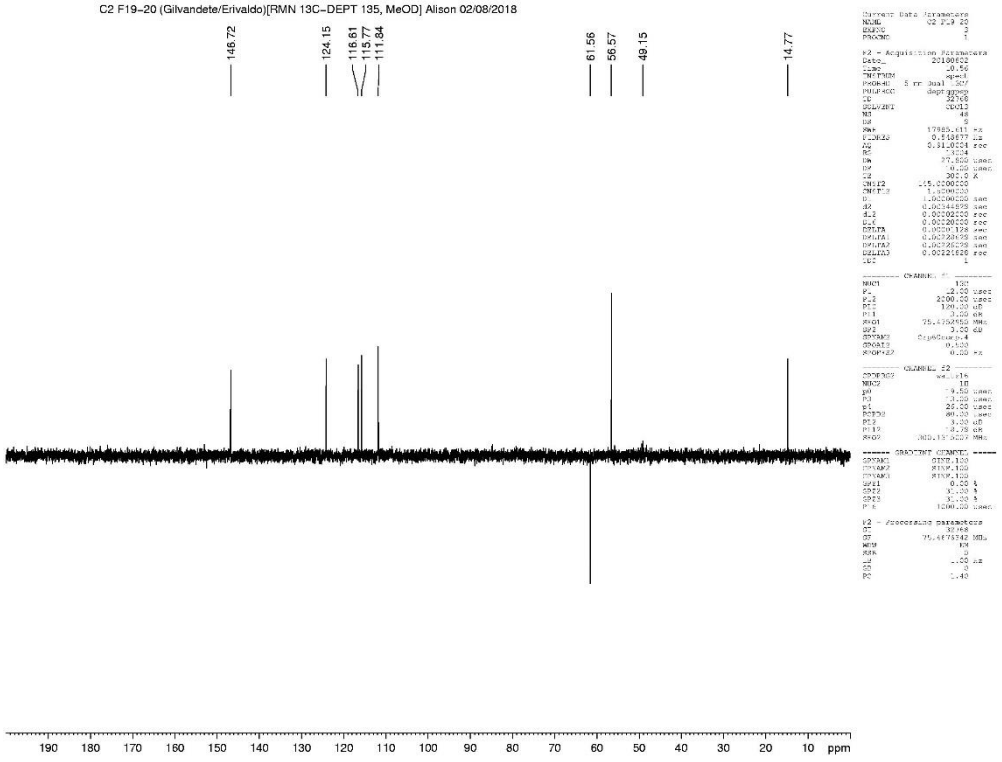
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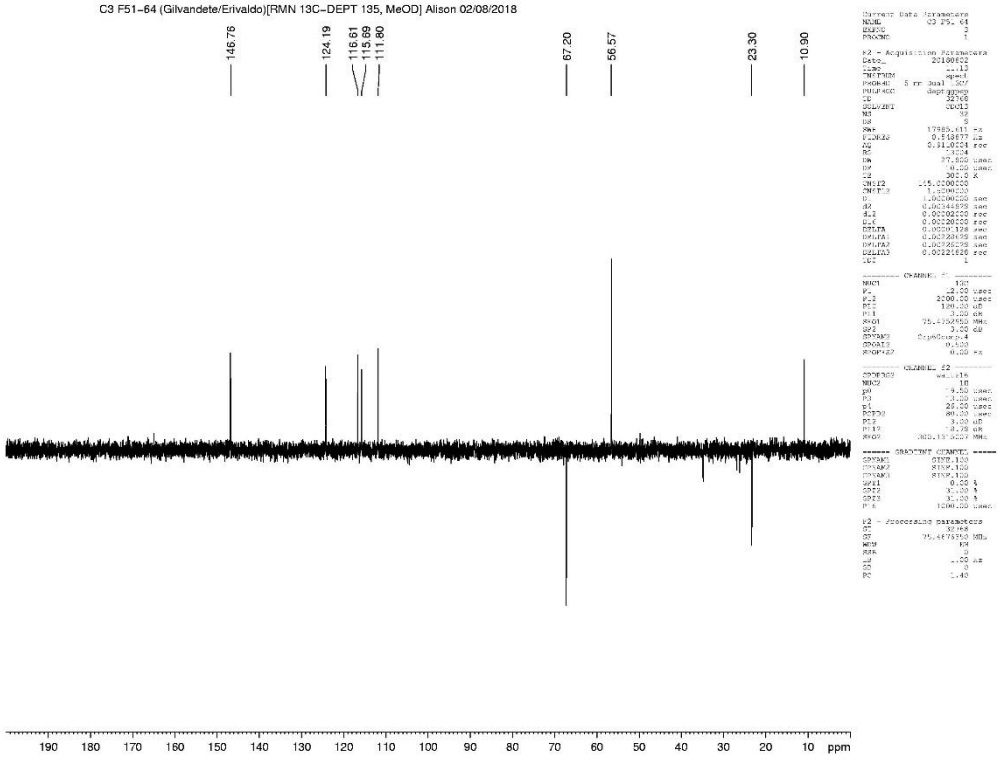
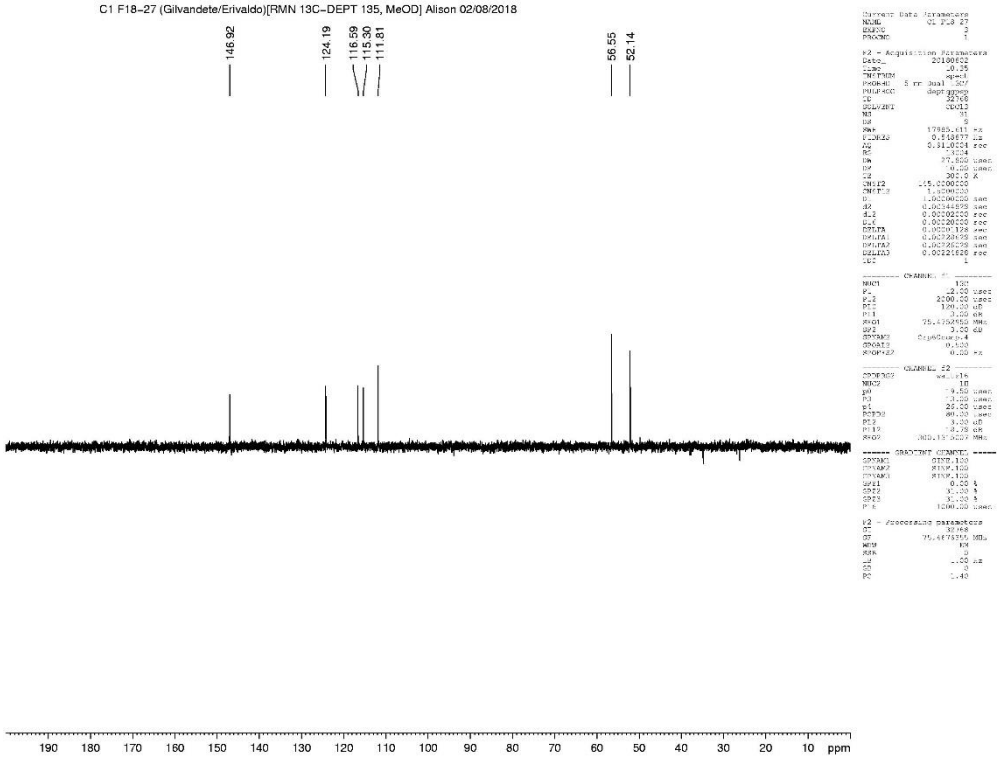
146.72
124.15
116.61
115.77
111.84
61.56
56.57
49.15
14.77
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm


C3 F51-64 (Gilvandete/Erivaldo)[RMN 13C-DEPT 135, MeOD] Alison 02/08/2018
146.76
124.19
116.61
115.69
111.80
67.20
56.57
23.30
10.90
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm


C1 F18-27 (Gilvandete/Erivaldo)[RMN 13C-DEPT 135, MeOD] Alison 02/08/2018
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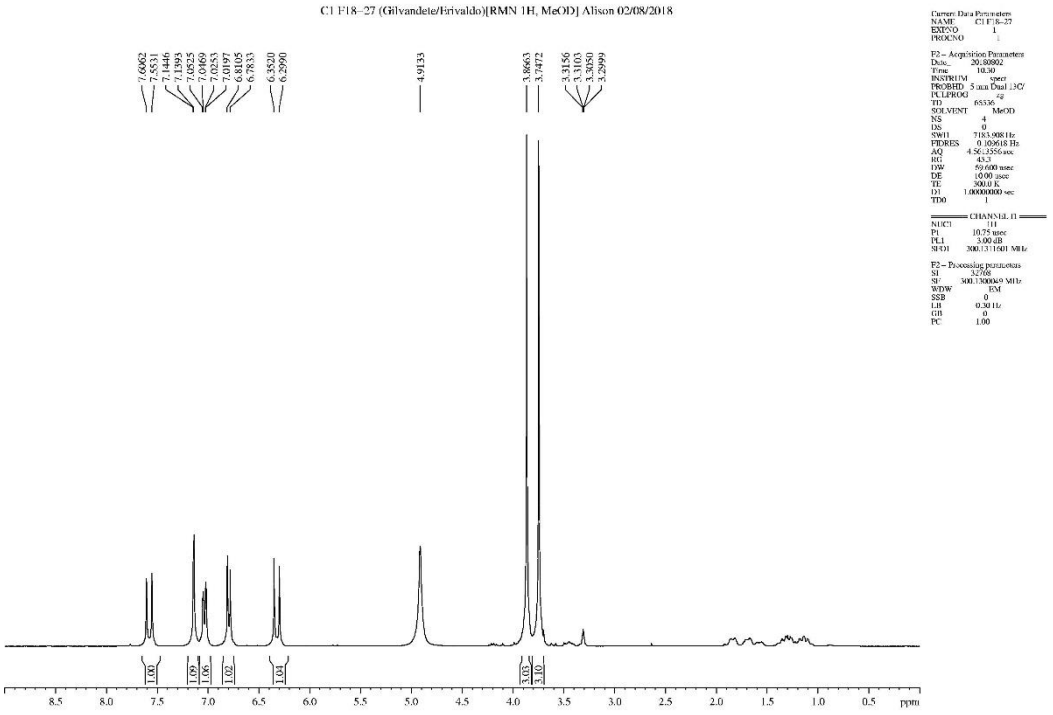
124.19
116.59
115.30
111.81
56.55
52.14
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm


C1 F18-27 (Gilvandete/Erivaldo)[RMN 1H, MeOD] Alison 02/08/2018
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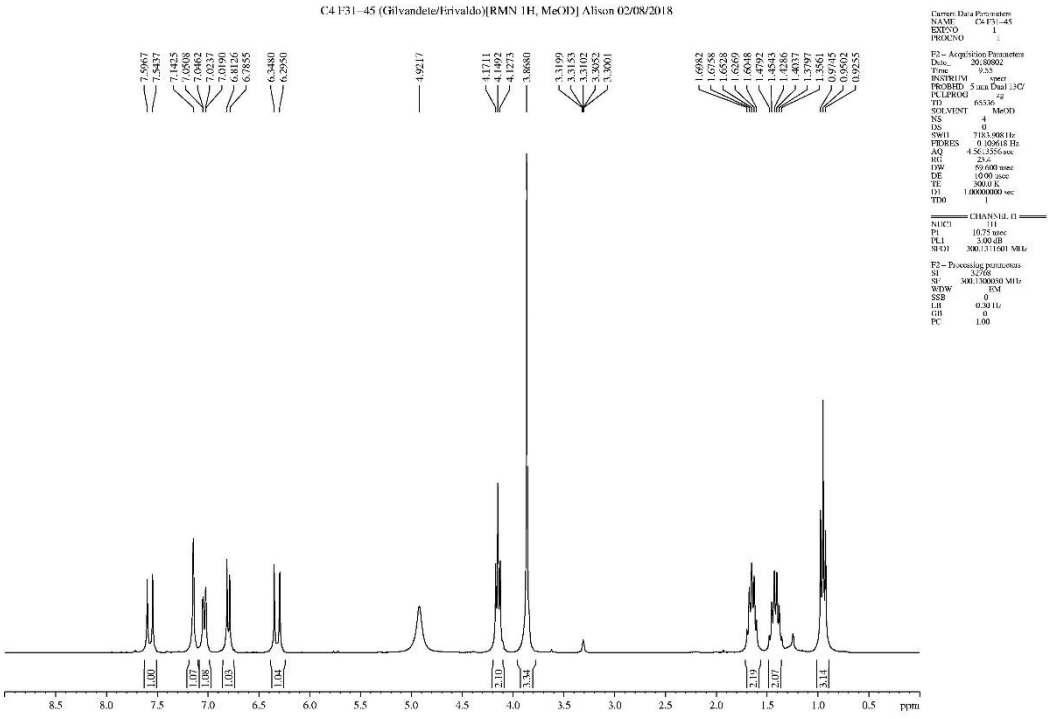
7.5531
7.1446
7.1393
7.0255
7.0469
7.0253
7.0197
6.8105
6.7833
6.3520
6.2990
4.9133
3.8663
3.7472
3.3156
3.3103
3.3050
3.2999
1.00
1.09
1.06
1.02
1.01
3.03
3.10
8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm


C4 F31–45 (Gilvandete/Erivaldo)[RMN 1H, MeOD] Alison 02/08/2018
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7.0237
7.0190
6.8126
6.7855
6.5480
6.2950
4.9217
4.1711
4.1492
4.1273
3.8680
3.3199
3.3153
3.3107
3.3052
3.3001
1.6982
1.6758
1.6538
1.6329
1.6048
1.4792
1.4543
1.4286
1.4037
1.3797
1.3561
0.9745
0.9502
0.9255
1.00
1.07
1.08
1.05
1.04
2.10
3.34
2.19
2.07
3.14
8.5
8.0
7.5
7.0
6.5
6.0
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4.5
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3.5
3.0
2.5
2.0
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1.0
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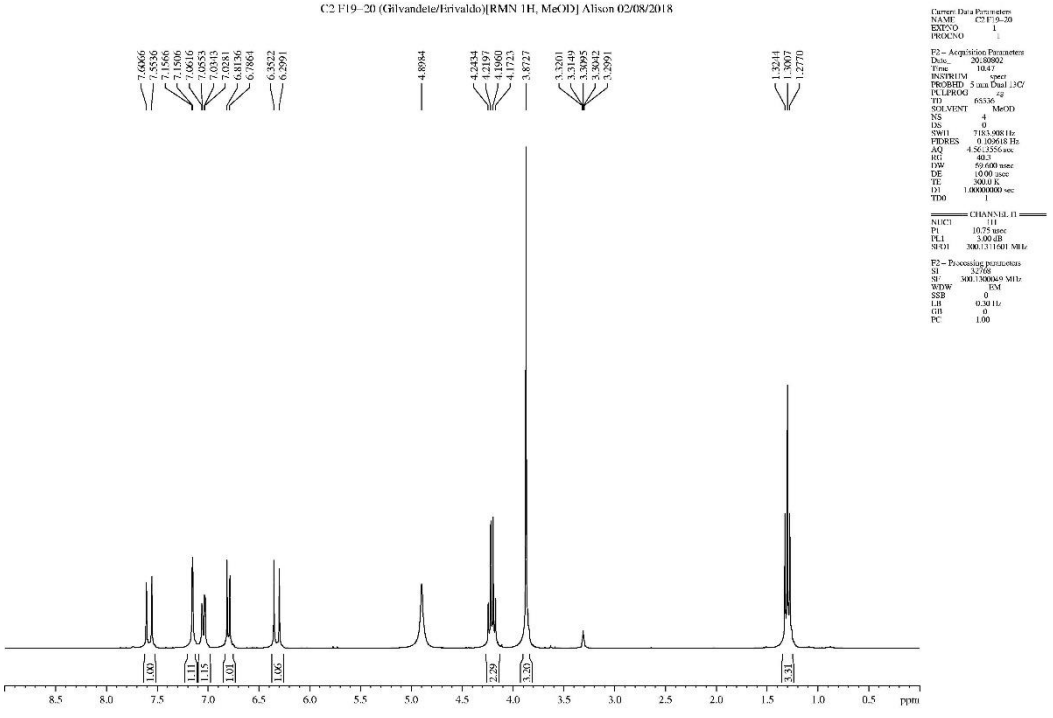
ppm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7.0553
7.0343
7.0281
6.8136
6.7864
6.3522
6.2991
4.8984
4.2434
4.2197
4.1960
4.1723
3.8727
3.3201
3.3149
3.3095
3.3042
3.2991
1.3214
1.3007
1.2770
1.00
1.11
1.15
1.01
1.06
2.29
3.35
3.33
8.5
8.0
7.5
7.0
6.5
6.0
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2.0
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1.0
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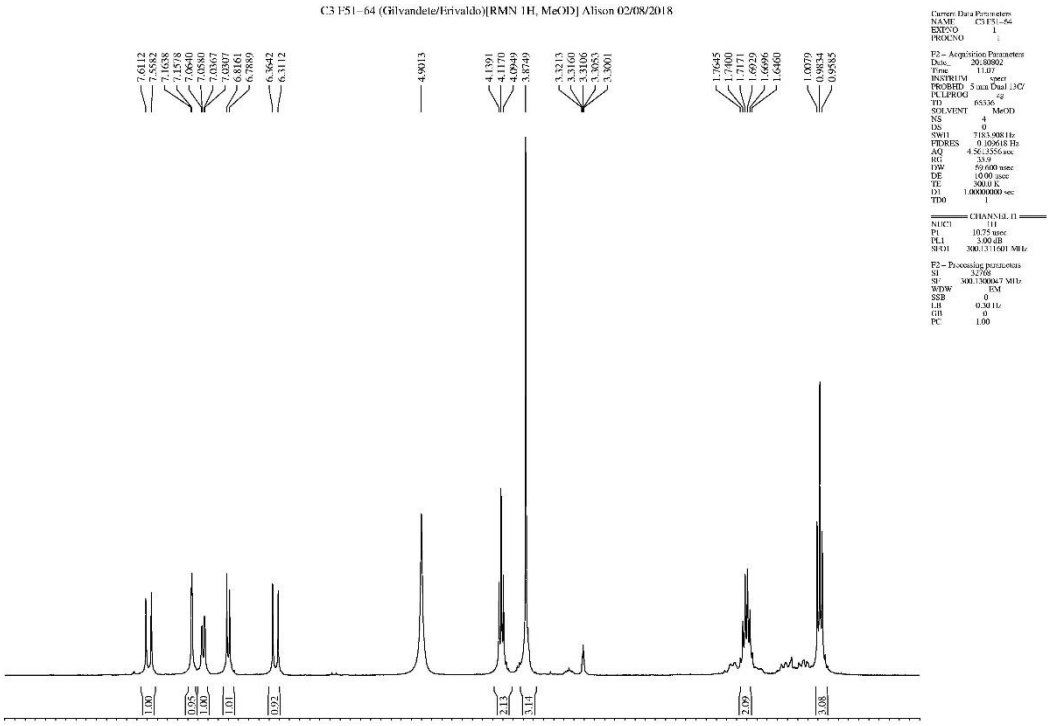
ppm


C3 F51–64 (Gilvandete/Erivaldo)[RMN 1H, MeOD] Alison 02/08/2018
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7.1638
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7.0580
7.0367
7.0307
6.8161
6.7889
6.3642
6.3112
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4.1191
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3.3213
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3.3106
3.3055
3.3001
1.7645
1.7400
1.7171
1.6929
1.6686
1.6460
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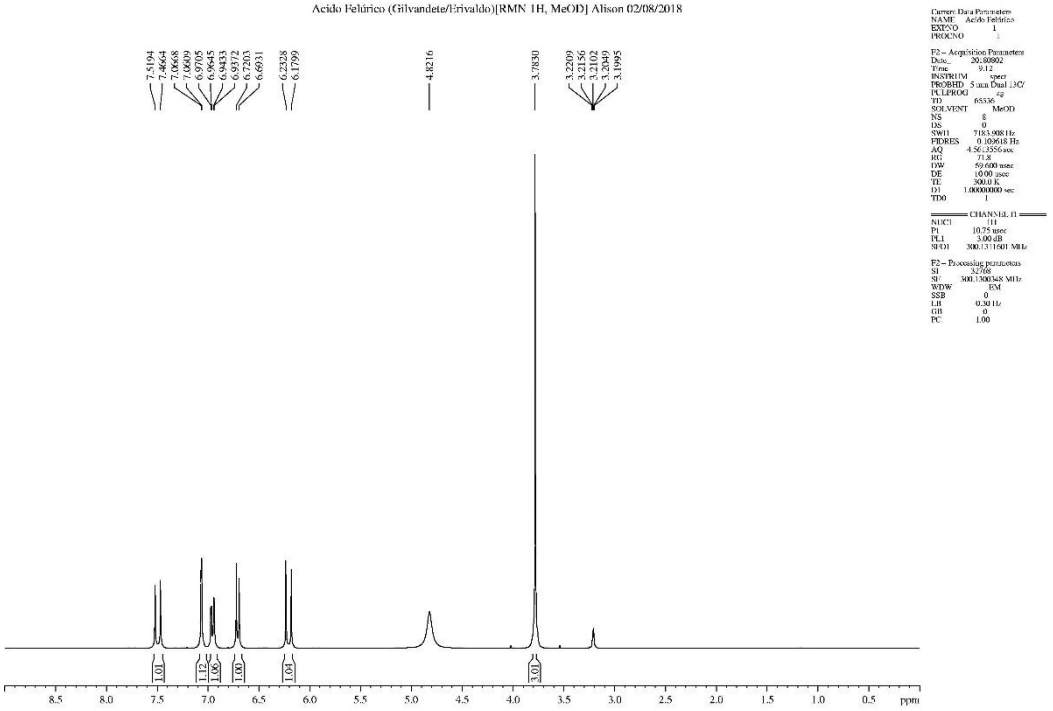
3.08


Acido Felúrico (Gilvandete/Erivaldo)[RMN 1H, MeOD] Alison 02/08/2018
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6.9433
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