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DOUTORADO EM DESENVOLVIMENTO E INOVAÇÃO TECNOLÓGICA EM MEDICAMENTOS

FERNANDO GOMES FIGUEREDO

ATIVIDADE ANTIBACTERIANA DE DERIVADOS DO LAPACHOL E NORLAPACHOL E AVALIÇÃO DA INIBIÇÃO DE BOMBAS DE EFLUXOS EM LINHAGENS DE *Staphylococcus aureus*

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ORIENTADORA: Profa. Dra. Marta Maria de França Fonteles
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“Temos o destino que merecemos. O nosso destino está de acordo com os nossos meritos” (Albert Einstein)

RESUMO

Substâncias isoladas e sintetizadas organicamente têm se destacado ao longo dos anos por suas propriedades terapêuticas, incluindo sua atividade antibacteriana. Neste contexto, o objetivo desta pesquisa foi investigar as propriedades farmacológicas de 2-(2-hidroxietilamina)-3-(3-metil-2-butenil)-1,4-di-hidro-1,4-naftalenodiona, 2-(2-hidroxi-etilamina)-3-(2-metil-propenil)-[1,4]naftoquinona e 2-(3-hidroxi-propilamina)-3-(3-metil-2-butenil)-[1,4]naftoquinona por meio de modelos computacionais de predição, avaliar a atividade antibacteriana e moduladora *in vitro* dessas compostos contra cepas bacterianas ATCC e isolados clínicos, além de análisar o efeito inibidor sobre o funcionamento de bomba de efluxo dessas substâncias em cepas de *Staphylococcus aureus* multirresistentes. As substâncias foram sintetizadas a partir de 2-hidroxi-quinonas, lapachol e nor-lapacol obtendo os correspondentes derivados 2-metoxilados via alquilação de sulfato de dimetila em meio básico, estes então reagiram quimiosseletivamente com 2-etanolamina e 3-propanolamina para formar os correspondentes aminoálcoois . A atividade antibacteriana e a atividade moduladora das substâncias foram avaliadas pelo método de microdiluição em caldo para determinação da Concentração Inibitória Mínima (CIM). Para verificar o efeito de redução do CIM do brometo de etídeo e do antibiótico, foi utilizado inóculos obtidos de placas incubadas em estufa bacteriológica por 24h a 37ºC com o repique do estoque. Após esse período foi utilizado inóculos em solução salina de acordo com Mcfarland 0,5 que corresponde a 10^5UFC. Os resultados dos testes foram feitos em triplicatas e como média geométrica. Na análise estatística foi utilizada ANOVA de uma via, seguida do pos teste hoc Tukey usando GraphPad Prism 5.0. As estruturas moleculares foram analisadas usando o banco de dados ChEMBL para prever possíveis alvos e logo após passaram por uma análise virtual de estrutura (*docking*), que apontaram para a molécula 2-(2-hidroxi-etilamina)-3-(2-metil-propenil)-[1,4]naftoquinona como um provável agente antibacteriano para as proteínas Replicative DNA helicase e RecA. As CIMs das substâncias não foram clinicamente significativas, porém, a associação com gentamicina e amicacina reduziu as CIMs desses antibióticos com significância de $p < 0,0001$. Frente a cepas de *Staphylococcus aureus* que carregam o mecanismo de bomba de efluxo NorA, foi observada redução significativa ($p < 0,0001$) de suas CIMs quando as substâncias foram associadas à norfloxacina e ao brometo de etídio, sendo esse efeito atribuído à inibição da bomba de efluxo. Após uma análise virtual baseada em sua estrutura (docking), foram observadas informações sobre a afinidade de novos ligantes para a bomba de efluxo ABC.

As substâncias testadas também demonstraram eficácia em diminuir a CIM de eritromicina, tetraciclina e principalmente do brometo de etídio com redução da CIM em até 16x. Em conclusão, a combinação dessas substâncias com antibióticos pode ser uma alternativa terapêutica para a resistência bacteriana e a redução dos efeitos colaterais

Palavras-chave: Antibacterianos. Estudo in silico. Bomba de efluxo. Modulação. Lapachol. Norlapachol. Derivados.

ABSTRACT

Isolated substances and those organically synthesized have stood out over the years for their therapeutic properties, including their antibacterial activity. In this context, the aim of this research was to investigate the pharmacological properties of 2-(2 hydroxyethylamine)-3-(3-methyl-2-butenyl)-1,4-dihydro-1,4-naphthalenedione, 2-(2-hydroxy-ethylamine)-3-(2-methyl-propenyl)-[1,4] naphthoquinone and 2-(3-hydroxy-propylamine)-3-(3-methyl-2-butenyl)-[1,4]naphthoquinone using computational prediction models, to evaluating the in vitro antibacterial and modulatory activity of these compounds against bacterial ATCC strains and clinical isolates, in addition to analyzing the inhibitory effect on the efflux pump functioning of these substances in strains of multiresistant Staphylococcus aureus. The substances were synthesized from 2-hydroxy-quinones, lapachol and nor-lapachol obtaining the corresponding 2-methoxylated derivatives via dimethyl sulfate alkylation in a basic medium, these then reacted chemoselectively with 2-ethanolamine and 3-propanolamine to form the corresponding amino alcohols. The antibacterial activity and modulatory activity of the substances were assayed by broth microdilution method to determine the Minimum Inhibitory Concentration (MIC). To verify the effect of reducing the MIC of the ethidium bromide and the antibiotic, inoculums obtained from plates incubated in a bacteriological oven for 24 hours at 37ºC with the stock peaking were used. After this period inoculums were used in saline solution according to Mcfarland 0.5 corresponding to 10^5UFC. The results of the tests were done in triplicates and as geometric mean. In the statistical analysis, one-way ANOVA was used, followed by the Tukey hoc post test using GraphPad Prism 5.0.The molecular structures were analyzed using the ChEMBL database to predict possible pharmacological targets and soon after underwent a virtual structure-based analysis (docking)., which pointed to the molecule 2- (2-hydroxy-ethylamine)-3-(2-methyl-propenyl)-[1,4]naphthoquinone as a probable antibacterial agent for the proteins Replicative DNA helicase and RecA. The MICs of the substances were not clinically significant, however, the association with gentamicin and amikacin reduced the MICs of these antibiotics with significance of $p < 0.0001$. against Staphylococcus aureus strains carrying the NorA efflux pump mechanism, a significant reduction ($p < 0,0001$) of their MICs was observed when the substances were associated with norfloxacin and ethidium bromide, with this effect being attributed to efflux pump inhibition. Following a virtual

analysis based on its structure (docking), information regarding the affinity of new ligands for the ABC efflux pump. The tested substances also demonstrated effectiveness at decreasing the MIC of erythromycin, tetracycline and mainly of ethidium bromide with MIC reduction up to 16x. In conclusion, the combination of these substances with antibiotics may be a therapeutic alternative to bacterial resistance and the reduction of side effects
Keywords: Antibacterials. Computer simulation. Efflux pump. Modulation. Lapachol. Norlapachol. Derivative

LISTA DE ABREVIATURAS

ABC	ATP-binding cassette superfamily
BHI	Breat Heart Infusion
CIM	Concentração Inibitória Mínima DHA drug/H+ antiporter
CM	Cellular wall
DMSO	Dimetilsulfóxido
DNA	Deoxyribonucleic acid
EECL	Ethanolic extract of C. leptophloeos;
EUA	Estados Unidos da América
EtBr	Ethidium bromide
HIA	Heart Infusion Agar
IBE	Inibidores de bomba de efluxo
INCQS	National Institute of Health Quality Control
MDR	Multiple drug resistance
MIC	Minimum inhibitory concentration
MMFF94	Merck Molecular Force Field 94
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin sensitive *Staphylococcus aureus*
PAβN	Phenylalanine Arginyl β-Naphthylamide
RND	Resistance-nodulation-cell division
ROS	Reactive oxygen species
Rt	Retention times
AS	Staphylococus aureus SMR Small multidrug resistance
UFC	Unidade Formadora de Colônia
UV	Ultraviolet
WDI	World Drug Index

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1. INTRODUÇÃO

O surgimento de novas cepas microbianas multirresistentes ao longo dos últimos anos provocou o aumento dos casos de mortalidade e morbidade, além de elevação nos custos do tratamento farmacológico (LIMA et al., 2017). Tal quadro, tornou a pesquisa de novos compostos bioativos alvo de grande interesse científico na busca de alternativas terapêuticas para as infecções microbianas (MOURA et al, 2019).

Dentre as bactérias Gram-negativas, *P. aeruginosa* se destaca, pois além de estar relacionada com infecções em pacientes imunocoprometidos, também acometendo pacientes que tenham realizado procedimentos invasivos, queimaduras e feridas operatórias (MATA; ABEGG, 2007). A *Escherichia coli* pertencente à família *Enterobacteriaceae* e é uma bactéria Gram negativa (SANTURIO et al., 2014), responsável por 80 a 90% das infecções do trato urinário. Sua contaminação se faz por via ascendente, proveniente da microbiota intestinal atingindo a uretra, passando em seguida para a bexiga e eventualmente para as vias urinárias (FLORES-MIRELES et al 2015). Dentre as bactérias Gram-positivas mais envolvidas em infecções hospitalares e da comunidade, *S. aureus* destaca-se por sua ampla disseminação ambiental e por estar relacionada a graves quadros de infecções oportunistas (COUTINHO et al, 2009).

O *Staphylococcus aureus*, um importante patógeno humano, possui uma coleção de fatores de virulência e a capacidade de adquirir resistência à maioria dos antibióticos. Esta capacidade é aumentada ainda mais pelo surgimento constante de novos clones, tornando o *S. aureus* uma "superbactéria". O uso clínico de meticilina levou ao aparecimento de cepas de *Staphylococcus aureus* resistente à meticilina resistentes (MRSA). As últimas décadas testemunharam a existência de novos clones de MRSA. Ao contrário dos MRSA tradicionais que residem em hospitais, os novos clones podem invadir ambientes comunitários e infectar pessoas sem predispor fatores de risco (LAKHUNDI AND KUNYAN., 2018).

Para auxiliar na resistência bacteriana as bombas de efluxos são proteínas dependentes de energia que promovem a eliminação de agentes antimicrobianos no meio extracelular mais rapidamente que a difusão da membrana plasmática, de modo que a concentração intracelular do agente permanece insuficiente para bloquear as funções celulares, tornando-os ineficazes, evitando assim a chegada do antibiótico ao seu local de ação (OPPERMAN AND NGUYEN, 2015). NorA é o sistema de efluxo mais bem estudado de *S. aureus* e, portanto, frequentemente usado como modelo para investigar a resistência mediada por efluxo neste patógeno. A atividade de NorA está associada à resistência a fluoroquinolonas, vários anti-sépticos e desinfetantes e vários relatórios

apontaram o papel dos sistemas de efluxo, incluindo o NorA, como uma resposta de primeira linha aos antimicrobianos em *S. aureus* (COSTA *et al*., 2018).

Além disso, há também resistência à tetraciclina que pode ser por dois mecanismos principais: transporte ativo por bomba de efluxo (mediado pelos resultados proteicos dos genes TetK TetL) e por proteção via ribossomos (por meio dos genes TetM e TetO) (PANTOSTI, SANCHINI AND MONACO, 2007). A resistência à tetraciclina é evidenciada, por exemplo, nas cepas de *S. aureus* IS-58 que dispõem da bomba de efluxo TetK incumbido de extrair a tetraciclina do meio intracelular para o meio extracelular ativamente, conferindo proteção à bactéria (PANTOSTI, SANCHINI e MONACO, 2007; TRUONG-BOLDUC, DUNMAN, *et al.*, 2005; LIMAVERDE, CAMPINA, *et al.*, 2017).

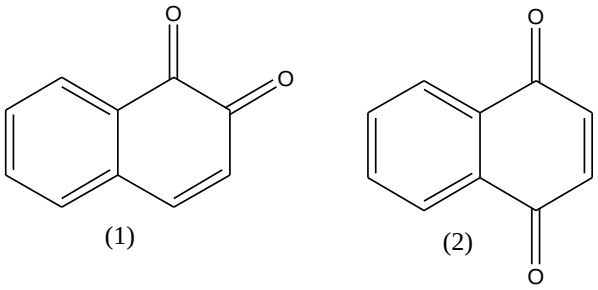
Para combater a resistência combinações de dois ou mais compostos são geralmente superiores ao uso de apenas um composto, principalmente para o tratamento de sérias doenças infecciosas, causadas por resistência bacteriana a antibióticos (EUMKEB; SIRIWOONG AND THUMANU, 2012).

O sinergismo pode ser obtido atráves da combinação de antibióticos com extrato ou substâncias a uma concentração sub-inibitória aplicada diretamente ao meio de cultura (COUTINHO et al., 2008a, 2009 , 2010; FIGUEREDO et al., 2021a). Esta estratégia é chamada de "shotgun ervas" ou "efeito sinérgico de vários compostos " e refere-se a utilização de plantas e drogas em uma abordagem usando mono ou multi-extrato de combinações, o que pode afetar não apenas um alvo único, mas vários alvos, onde os diferentes componentes terapêuticos colaboram de uma forma sinergética-agonísticas. Esta abordagem não é apenas para combinações de extratos; combinações entre os produtos naturais ou extratos e produtos sintéticos ou antibióticos também são possíveis (WAGNER, ULRICH-MERZENICH, 2009; COUTINHO *et al.*, 2010b; BRITO *et al*., 2015; CRISTO *et al* , 2016).

Dessa forma, substâncias de origem vegetal e os seus derivados tornaram-se uma alternativa viável e eficiente, pois a atividade antimicrobiana do fármaco pode ser amplificada ou reduzida pela ação dos produtos naturais (OLIVEIRA-TINTINO *et al*. 2018). Ou que dificultam os mecanismos de resistência antimicrobiana pela complexidade das suas estruturas evitando, consequentemente, adaptações microbianas (DAFERERA *et al.*,2003). Um dos alvos é a bomba de efluxo NorA, presente na cepa 1199B, que já está bem estudado, e demonstrou ser secepitível a produtos naturais e sínteticos (FONTAINE et al., 2014).

E neste contexto, as etapas que antecedem os estudos pré-clínicos e clínicos são importantes para a obtenção do máximo de informações sobre os compostos a serem testadas. Para isso, os modelos *In sílico* podem proporcionar informações importantes para o pesquisador gerando um direcionamento sobre as propriedades físico-químicas e farmacológicas (EKINS et al., 2007) de uma molécula, até a predição de seus possíveis alvos e efeitos adversos (LAUDY et al., 2015).

. Entre as diversas substâncias testadas, as Naftoquinonas (**1**,**2**) (Figura 1) são compostos orgânicos versáteis que fazem parte de uma importante classe de produtos naturais denominadas quinonas. Caracterizam-se, estruturalmente, por se apresentarem como dienonas cíclicas conjugadas, sendo os isômeros 1,4 e 1,2-naftoquinonas os mais comuns (FERREIRA *et al.*, 2010).



(1) (2)

Figura 1: 1,2-naftoquinona; 1,4-naftoquinona

Além de sua origem natural as naftoquinonas são importantes intermediários em síntese orgânica na obtenção de inúmeros compostos naturais ou sintéticos. Os derivados naftoquinonicos naturais (CAVALCANTI *et al*., 2013) representam uma ampla e variada família de metabólitos secundários e são de importância vital para os vegetais, fungos, líquens e algas. A maior ocorrência das quinonas é nos vegetais superiores de família *Bignoniácea*, mais precisamente do gênero *Tabebuia* (*Tecoma*) (BABULA *et al*., 2009).

A importância das quinonas nitrogenadas está relacionada à sua estrutura eletrônica, devido à sua capacidade doadora de elétrons e, consequentemente, modificar o potencial de redução, que induz o estresse oxidativo intracelular., Compostos como a 2-amino-1,4-naftoquinona possuem atividades biológicas como ações antibacterianas, antimaláricas, antitumorais e moluscicidas (BENITES et al., 2010). A participação das

quinolonas é relevante em diversas atividades biológicas, (POWIS, 1989; SILVA; FERREIRA, SOUZA 2003; BARBOSA, *et al*., 2005), entre outras.

O lapachol é uma naftoquinona funcionalizada de origem natural, obtido facilmente através de extração do cerne de árvores da família *Bignoniaceae* (SILVA; FERREIRA e SOUZA 2003; FERREIRA *et al.*, 2010), gênero *Tabebuia*, especialmente a *Tabebuia avellandedae*), conhecida popularmente como ipê, abundante no Brasil e na América do Sul (BARBOSA et al., 2005). O lapachol apresenta diversas atividades biológicas, tais como: ação contra células de câncer de esôfago (SUTHANAN *et al*.,2013), atividade antimicrobiana, tripanocida (FERREIRA et al., 2010), entre outras (FONSECA; BRAGA, SANTANA 2003). O norlapachol, por outro lado, é uma naftoquinona de origem sintética, que possui atividade antitumoral e Trypanocida (JUNIOR,2007).

2. OBJETIVOS

2.1. Objetivos Geral

Este trabalho objetivou realizar estudo sílico de hidroxiaminas derivadas de lapachol e norlachol e avaliar, in vitro, a atividade antibacteriana, moduladora e a capacidade de inibição de bomba de efluxo destes compostos.

2.2 Objetivos Específicos

a. Realizar o estudo in sílico de hidroxiaminas derivadas de lapachol e norlachol, 2-(2-Hydroxyethylamino)-3-(3-methyl-2-butenyl)-1,4-dihydro-1,4-naphthalenedi-one, 2-(2-Hydroxy-ethylamino)-3-(2-methyl-propenyl)-[1,4]naphthoquinone e 2-(3-Hydroxy-propylamino)-3-(3-methyl-2-butenyl)-[1,4]naphthoquinone.
b. Avaliar, *in vitro*, a atividade antibacteriana e moduladora de hidroxi-aminas derivadas do lapachol e norlachol, frente a linhagens bacterianas ATCC e isolados clínicos.
c. Avaliar, *in vitro,* a atividade inibitória de bomba de efluxo de Hidroxi-aminas derivadas do lapachol e norlachol, frente a cepas *Staphylococcus aureus* portadores do mecanismo de bomba de efluxo NorA.
d. Avaliar *in vitro* a capacidade inibitória de bomba de efluxo de Hidroxi-aminas derivadas do lapachol e norlachol, frente as cepas de *S. aureus*, RN4220 portadora do plásmido pUL5054 e IS-58, dotada do plasmídeo PT181.

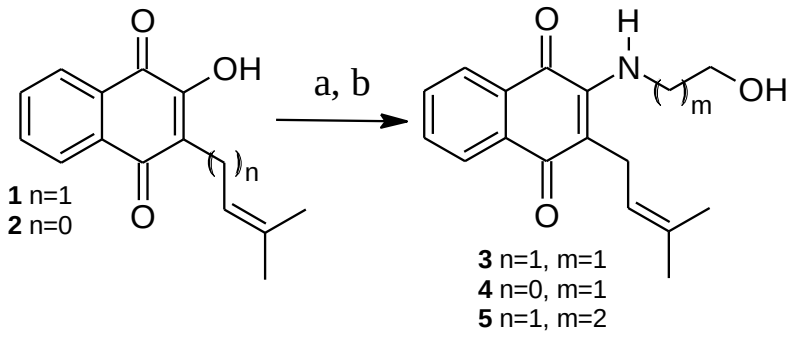
3. MATERIAIS E MÉTODOS

3.1 Síntese de substâncias

Informações gerais

As reações sensíveis ao ar e à umidade foram realizadas sob atmosfera de argônio. Os reagentes foram adquiridos da Sigma-Aldrich, Dinamica ou Vetec e destilados ou usados sem purificação adicional. As reações foram monitoradas por análise de TLC em placas de sílica gel pré-revestidas (Merck, Kieselgel 60 GF_{254}) e os compostos foram visualizados com luz UV. A cromatografia em coluna foi realizada em sílica gel 60 (70-230 mesh, Merck). Os pontos de fusão foram medidos em tubos capilares abertos em um aparelho QUIMIS e não foram corrigidos. Os espectros infravermelhos foram registrados em um espectrofotômetro IFS66 Bruker usando discos KBr ou Varian Mercury 640IR com ATR. As análises de HRMS foram realizadas em um MALDI-TOF / TOF Autoflex III 10, usando o modo refletido positivo. Os espectros de NMR (1H a 400 MHz e 13C a 100 MHz) foram registrados em um espectrômetro Varian Unity Plus-400, Varian Mercury 200 MHz, usando CDCl3 ou DMSO-d6 como solventes e calibrados para o sinal do solvente. Os desvios químicos são expressos em partes por milhão (ppm) e as constantes de acoplamento são dadas em Hz. Os compostos lapachol 1 (CAMARA et al., 2002) e o derivado 2-metoxi correspondente, norlapachol 2 (BARBOSA et al., 2005) e o derivado 2-metoxi correspondente foram obtidos por procedimentos publicados anteriores Figura 1.

Figura 1. Esquema de síntese de derivados de 2-aminoalquil 3-5



Reagents and conditions: a) Me_2SO_4, K_2CO_3, acetone, r.t.; b) 2-amino-ethanol or 3-amino-1-propanol in MeOH, r.t.

1 mmol do derivado 2-metoxi dissolvido em 10 mL de MeOH foi adicionado lentamente a 1,5 mmol da amina apropriada (2-aminoetanol ou 3-amino-1-propanol) no mesmo solvente (40 mL) com agitação contínua. Após a conclusão da reação por inspeção em análise de CCD, o solvente foi removido sob vácuo e o resíduo submetido a cromatografia flash em sílica gel e acetato de etila / hexano com polaridade crescente.

2- (2-Hidroxietilamino) -3- (3-metil-2-butenil) -1,4-dihidro-1,4-naftalenodiona (3)

Obtido com rendimento de 88% como cristais vermelhos, mp 80–81°C; ^{1}H NMR (200 MHz, $CDCl_3$) 1.68 (s, 3H), 1.74 (s, 3H), 2.3 (br, 1H), 3.37 (d, 2H, *J* 5.9 Hz), 3.71 (m, 2H), 3.85 (m, 2H), 5.07 (t, 1H, *J* 5.9 Hz), 6.01 (l, 1H), 7.57 (m, 1H), 7.57 (m, 1H), 7.93 (d, 1H, *J* 7.6 Hz), 8.05 (d, 1H, *J* 7.6 Hz); ^{13}C NMR (50 MHz, $CDCl_3$), 18.2, 23.8, 25.8, 47.2, 62.1, 122.7, 126.1, 126.3, 130.6, 132.0, 132.8, 133.4, 134.5, 146.2, 183.1, 183.2; IR (KBr) (*v* max., cm^{-1}) 3391, 3321, 1678, 1599, 1555, 1513; MS (rel int) m/z 285 (M+, 57), 270 (100), 198 (70). HRMS found: 285.13649. Calcd for $C_{17}H_{19}NO_3$: 285.13649.

2- (2-Hidroxi-etilamino) -3- (2-metil-propenil) - [1,4] naftoquinona (4)

Obtido com rendimento de 87% como cristais vermelhos (p.f. 77-78,5 ° C) com rendimento de 80%. ^{1}H NMR ($CDCl_3$, 200 MHz) 1.47 (d, 3H, *J* 1.0 Hz), 1.89 (d, 3H, *J* 1.6 Hz), 2.46 (br s, 1H), 3.37 (q, 2H, *J* 5.4 Hz), 3.73 (t, 2H, *J* 5.4 Hz), 6.06 (dd, 1H, *J* 1.0/1.6 Hz), 6.25 (br t, 1H, *J* 5.4 Hz), 7.51 (dt, 1H, *J* 1.4/7.6/7.6 Hz), 7.61 (dt, 1H, *J* 1.4/7.6/7.6 Hz), 7.90 (dd, 1H, *J* 1.4/7.6 Hz), 7.99 (dd, 1H, *J* 1.4/7.6 Hz). ^{13}C NMR ($CDCl_3$, 50 MHz) 20.1, 25.4, 46.1, 61.3, 113.6, 117.7, 125.9, 126.1, 130.3, 131.9, 133.3, 134.4, 139.0, 144.8, 182.7, 183.4. IR (KBr) *v* max, 3457, 3349, 3268, 2940, 2874, 1675, 1598, 1563, 1511, 1354, 1335 cm^{-1}. HRMS Calcd for $C_{16}H_{17}NO_2$, 271.1208; found: 271.1169.

2- (3-Hidroxi-propilamino) -3- (3-metil-2-butenil) - [1,4] naftoquinona (5)

Obtido com rendimento de 75% como cristais vermelhos (m.p. 69-70 ^{o}C . ^{1}H NMR ($CDCl_3$, 400 MHz) 1.68 (s, 3H), 1.74 (s, 3H). 1.88 (q, 2H, *J* 6.2, 6.16 Hz), 3.39 (d, 2H), 3.69 (t, 2H, *J* 6,6 Hz), 3.80 (t, 2H, *J* 6.2, 5.5 Hz), 5.08 (t, 2H, *J* 5.8), 7.54 (t, 1H, *J* 7.5

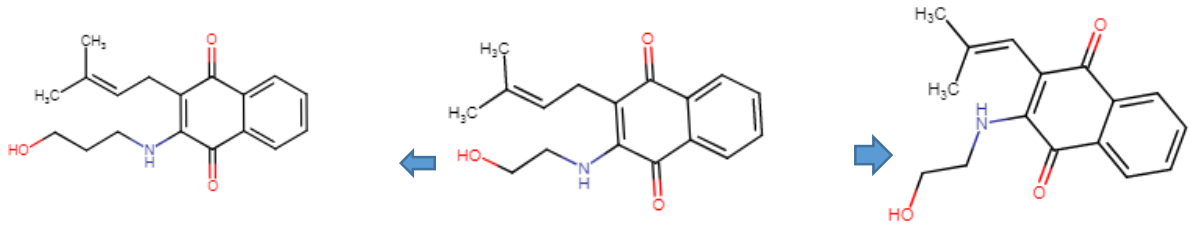
Hz), 7.65 (td, 1H, *J* 7.4 Hz), 7.94 (d, 1H), 8.04 (d, 1H); ^{13}C NMR ($CDCl_3$,100 MHz) 17.8, 23.3, 23.4, 32.8, 42.4, 60.3, 115.3, 122.7, 125.6, 125.9, 130.1, 131.5, 132.1, 133.1, 134.0, 145.6, 182.74, 182.76; IR (KBr) *v* max, 3446, 3334, 1672, 1598, 1557, 1527, 1361, 1276, 1473, 728 cm^{-1}. HRMS Calcd for $C_{18}H_{21}NO_3$, 299.1527; found: 299.1501.

3.2 Predição da atividade farmacológica das substâncias (estudos *in silico*)

3.2.1 Obtenção da estrutura molecular tridimensional das substâncias

As três estruturas químicas que foram objeto deste trabalho foram desenhadas no software ChemSketch®, versão 2015.2.5 livre e sua estrutura tridimensional foi determinada pelo software 3D Viewer, ambos para Windows®, produzidos pela Advanced Chemistry Development, Inc. (ACD/Labs). No software Avogadro, versão 1.1.1. para sistemas MAC OS, foi determinada a conformação mais estável para as moléculas, considerando o campo de força MMFF94 (*Merck Molecular Force Field* 94) utilizando-se um algoritmo de "*steepest descent*" ou "método de Cauchy" como método gradiente para otimização dos comprimentos das ligações e ângulos da molécula (Figura 1 e Tabela 1).

Figura 2. Estruturas químicas dos compostos



5 **3** **4**

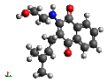
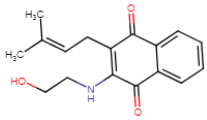
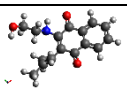
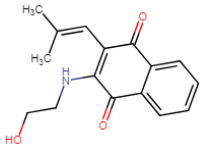
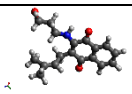
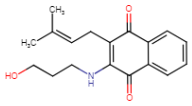
3.2.2 S*creening* farmacológico

As três moléculas passaram por um *screening* farmacológico, com o uso do software ChemProt versão 3.0 da Universidade Técnica da Dinamarca (Chemprot-3.0, 2019) (http://potentia.cbs.dtu.dk/ChemProt/#).

O ChemProt é uma compilação publicamente disponível de recursos de anotações sobre a relação químico-proteína-doença que permite o estudo da farmacologia de sistemas para uma pequena molécula através de múltiplas camadas de complexidade, desde os níveis moleculares até os clínicos. A versão 3.0 do ChemProt disponibiliza análise de mais 1,7 milhão de compostos, com 7,8 milhões de medições de bioatividade para 19.504 proteínas (KRINGELUM et al., 2016).

Para determinação das propriedades das moléculas foi utilizado o software Molinspiration (https://www.molinspiration.com), com o qual as estruturas foram analisadas no tocante a concordância em relação a Regra dos Cinco, dados presentes na Tabela 1 (LIPINSKI et al., 2001).

Tabela 1. Características moleculares das três compostos

Código	SMILE	Observações	Estrutura	Fórmula molecular	Peso molecular
3	CC(C)=CCC1=C(NCCO)C(=O)C2=CC=CC=C2C1=O			$C_{17}H_{19}NO_3$	285.34
4	CC(C)=CC1=C(NCCO)C(=O)C2=CC=CC=C2C1=O			$C_{16}H_{17}NO_3$	271,311
5	CC(C)=CCC1=C(NCCCO)C(=O)C2=CC=CC=C2C1=O			$C_{18}H_{21}NO_3$	299,364

3.2.3 Procedimento de Docking Molecular

Modelagem NorA baseado no procedimento de encaixe da estrutura 3D foi realizada em concordância com o estudo de Dos Santos et al (2018). As simulações de docking molecular foram realizadas pelo SWISSDOCK servidor da web (www. swissdock.ch/) (Grosdidier; Zoete, Michielin, 2011). As coordenadas de estrutura química foram geradas usando CORINA e Gasteiger cargas parciais. A estrutura NoRA 3D foi preparada usando a ferramenta de preparação Dock disponível no pacote de

software livre UCSF quimera (SANTOS et al., 2018). As coordenadas da grade para execuções de encaixe flexíveis foram realizadas por estudos que definem uma região de interesse com 5 Å das coordenadas x = -29,78, y = 49,65, z = 71,78 e tamanho da caixa x = 46,00, y = 38,00 e z = 30,00. A ligação pontuação de energia foi usada para calcular a constante de inibição (valor Ki) usando o equação ki = 10 (Energia de ligação / 1,366) (Onawole et al., 2018), enquanto a eficiência do ligante (LE) foi determinado usando a equação (SILE = ki / N0.3), onde N é o número de átomos pesados (não hidrogênio) presentes no ligante (Nissink, 2009). O encaixe os resultados foram vistos com a ajuda do programa de visualização UCSF Chimera e o Discovery Studio (DS) foi usado para construir o mapa de interação para mostrar como as hidroxiaminas derivadas, clorpromazina e PaβN interagiram com NoRA.

3.3 Meios de culturas

Os meios de culturas utilizados foram os seguintes: Heart Infusion Agar (HIA, Difco laboratorises Ltda.), Brain Heart Infusion (BHI, difco Laboratories Ltda.), e Glicerol.

3.4 Microrganismos

A escolha dos microrganismos para a avaliação da atividade antibacteriana foi efetuada levando em consideração a presença de bactérias Gram-positivas e Gram-negativas, obtidos através do Instituto Nacional de Controle de Qualidade em Saúde (INCQS) da Fundação Oswaldo Cruz, Ministério da Saúde. Foram utilizadas quatro linhagens de bactérias, sendo utilizadas cepas padrão de bactérias *Pseudomonas aeruginosa* ATCC 27853 e *Staphylococcus aureus* ATCC 25923 e linhagens bacterianas multirresistentes (isolados clínicos) de *P. aeruginosa* 31 e *S. aureus* 358.

Tabela 2. Perfil de resistência das bactérias utilizadas nos testes da atividade antibacteriana .

Bactéria	Número	Sitio de coleta	Perfil de Resistência
Staphylococcus aureus	SA 358	Ferida cirúrgica	Oxa, Gen, Tob, Ami, Can, Neo, Para, But, Sis, Net
Staphylococcus aureus	ATCC 25923	-	-
Pseudomonas aeruginosa	PA 31	Secreção nazal	Pol, Cpm, Ctz, Ptz, Ami, Imi, Cip, Lev, Mer
Peudomonas aeruginosa	ATCC 27853	-	-

Perfil de resistência: Ami = amicacina; Cip = ciprofloxacina; Lev = levofloxacina; Ctz = ceftazidima; Pol = polimixina; Imi = imipenem; Mer = meropenem; Ptz = piperacilina; Can = canamicina; Tob = tobramicina; Oxa = oxacilina; Gen = gentamicina; Neo = neomicina; Para = paramomicina; Mas = butirosina; Sis = sisomicina; Net = netilmicina. Os microrganismos utilizados nesta pesquisa foram adquiridos do Laboratório de Micologia da Universidade Federal de São Paulo. Paraíba — UFPB e gentilmente cedido pela Universidade Regional do Cariri — URCA.

Fonte: Elaborada pelo autor

Para a avalição da inibição de bombas de efluxo foram utilizadas cepas de *S. aureus* usadas foram: RN4220 portadora do plásmidio pUL5054, que transporta o gene que codifica referente a proteína MsrA de efluxo de macrolidio; IS-58, dotada do plasmídeo PT181 portadora do gene da proteína de efluxo de tetraciclina TetK; 1199B cepa resistente a fluorquinolonas hidrofílicas via proteína de efluxo NorA e a cepa selvagem 1199 referente a mesma (TABELA 1). As cepas foram gentilmente cedidas pela Prof. S. Gibbons (University of London). Todas as cepas formam inicialmente mantidas em ágar sangue para comprovar o tipo de cepa (Laboratórios Difco Ltda., Brazil), depois foram transferidas para o estoque. Sendo mantidas em dois estoques: um em Heart Infusion Agar slants (HIA, Difco) a 4º C e outro mantido em glicerol em freezer -80 º C. A cepa portadora de plasmídeo foi mantida e meio de cultura sobre condições sub-inibitória de antibiótico com o objetivo do gene do plasmídeo ser expresso e não ser perdido.

Tabela 3- Linhagens utilizadas nos ensaios de efluxo.

Linhagem	Gene	Antibiótico ou classe – Proteína
IS-58	Plasmídeo PT181(Tet(k))	Tetraciclina- Tet(k)
RN 4220	Plasmídeo Pul5054(MsrA)	Eritromicina- MRSA
1199 B	*Nor A*	*Norfloxacina (Nor A)*
1199 (Selvagem)	-	-

Fonte: Elaborada pelo autor

3.5 Origem e preparo dos antibióticos e do brometo de etídio

Para os testes de suscetibilidade bacteriana pelas substâncias em associação com antibióticos, os fármacos utilizados foram da classe dos aminoglicosídeos (amicacina e gentamicina). Foram utilizados também antibióticos específicos para as bombas de cada bactéria: Eritromicina para a bomba MrsA contido na cepa RN4220; Tetraciclina para a bomba Tet k contida na cepa IS-58; e Norfloxacina para a bomba NorA contida na cepa 1199B, com também esse antibiótico foi utilizado para a cepa selvagem. Todos os antibióticos foram inicialmente dissolvidos em DMSO a 10 mg/mL e posteriormente diluídos em água, diminuindo a concentração para 1024 µg/mL. O brometo de etídeo foi diluído em água em uma concentração de 1024 µg/mL. Tantos os antibióticos quanto o brometo de etídio foram obtidas pela da SIGMA Chemical Co. St. Louis, E.U.A.

3.6 Preparo e padronização dos inóculos

Nos ensaios de Concentração Inibitória Mínima (CIM) foram preparados inóculos oriundos a partir dos estoques. Desse estoque foi cultivado novamente em meio sólido Heart Infusion Agar slants e mantido a 37 ºC. A partir desse meio sólido foi feito inóculo utilizando tubos de ensaio contendo solução salina estéril, e esse inóculo teve como base a escala Mcfarland 0.5 que corresponde a 10^5 UFC (Unidades Formadoras de Colônias). Esse método padrão de inoculo foi utilizado tanto nos ensaios de CIM das substâncias quanto na verificação da modulação e da atividade de inibição de bomba de efluxo.

3.7 Ensaios de concentração inibitória mínima

Foi realizado o ensaio de concentração inibitória mínima dos compostos. Também foi realizado com o brometo de etídeo e com os antibióticos para confirmar o nível de

resistência corroborando com a presença da bomba. A partir do estoque em meios sólido HIA foi feito uma repicagem em HIA e mantido em estufa bacteriológica a 37 ºC por 24h. Após esse período foi utilizado inóculos em solução salina de acordo com a escala de Mcfarland 0,5 que corresponde a 10^5 UFC (Unidade Formadora de Colônia). Seguindo os ensaios, foi preparado o meio de distribuição em eppendorfs utilizado 100 µL do inóculo em 900 µL do meio de cultura liquido BHI. Posteriormente o conteúdo do eppendorf foi transferido para placa de microdiluição de 96 poços, em sentido horizontal. Sendo que foi utilizado 100 µL em cada poço, perfazendo 10 poços. Após essa etapa, foi realizada a microdiluição das substâncias sendo 100 µL nesse meio até penúltima cavidade (1:1). Na última cavidade não foi adicionada por ser o controle de crescimento. As concentrações variaram de 1024 µg/mL a 2 µg/mL. Após 24h foi realizada a leitura das placas por visualização de mudança de cor do meio caracterizado pela adição de 20 µL de resazurina (7-hidroxi-3H-fenoxazina-3-ona 10-óxido). A leitura desse experimento tem como característica, a mudança de cor do meio de azul para vermelho indicando à presença de crescimento bacteriano e a permanência em azul, a ausência de crescimento. Todos os experimentos foram realizados em triplicatas.

Figura 3- Ensaio da concentração inibitória mínima

Fonte: Autor

3.8 Ensaios de inibição de bomba de efluxo por redução do CIM do brometo de etídeo

Para verificar o efeito de redução do CIM do brometo de etídeo, foi utilizado inóculos a partir de placas incubadas em estufa bacteriológica por 24h a 37 ºC com o repique do estoque. Após esse período foi utilizado inóculos em solução salina de acordo com Mcfarland 0.5 que corresponde a 105 UFC (Unidades Formadoras de Colônias). Como etapa inicial, foi preparado em *eppendorfs* o meio de distribuição do teste e do controle. No teste foram colocados 150 µL do inóculo, mais substância em concentração sub-inibitória (CIM/8) e completado o volume do eppendorf até o volume de 1,5 mL. Para o controle foi colocado o mesmo volume de inoculo do teste e completado o volume do eppendorf até 1,5 mL. Em seguida foram transferidos para placas de microdiluição de 96 poços, com distribuição vertical, caracterizada pela adição de 100 µL do conteúdo do eppendorf em cada poço. Após essa etapa foi realizada a microdiluição do brometo de etídeo, sendo 100 µL nesse meio até penúltima cavidade (1:1). A solução de brometo de etídeo utilizada neste ensaio foi à citada anteriormente. Na última cavidade não foi adicionada por ser o controle de crescimento. As concentrações variaram de 1024 µg/mL a 0,5 µg/mL. Após 24h foi realizado a leitura das placas por visualização de mudança de cor do meio caracterizado pela adição de 20 µL resazurina (7-hidroxi-3H-fenoxazina-3-ona 10-óxido). Sendo que, a leitura tem como característica a mudança de cor do meio de azul para vermelho indicando à presença de crescimento bacteriano e a permanência em azul a ausência de crescimento. A redução do CIM do brometo de etídeo ou de antibiótico específico, em cepas portadoras de bomba de efluxo, é um indicativo de inibição de bomba de efluxo. Todos os experimentos foram realizados em triplicatas. Esse ensaio com brometo de etídeo foi realizado apenas para o ácido tânico.

3.9 Ensaios de inibição de bomba de efluxo por redução do CIM do antibiótico

Para verificar o efeito de redução do CIM do antibiótico, foi utilizado inóculos a partir de placas incubadas em estufa bacteriológica por 24h a 37 ºC com o repique do estoque. Após esse período foi utilizado inóculos em solução salina de acordo com a escala de Mcfarland 0.5 que corresponde a 10^5 UFC. Como etapa inicial foi preparado em eppendorfs o meio de distribuição do teste e do controle. No teste foram colocados 150 µL do inóculo, mais substância em concentração sub-inibitória (CIM/8) e completado o volume do eppendorf até o volume de 1,5 mL. Para o controle foi colocado o mesmo volume de inóculo do teste e completado o volume do eppendorf até 1,5 mL. Em seguida

foram transferidos para placas de microdiluição de 96 poços, com distribuição vertical, caracterizada pela adição de 100 µL do conteúdo do eppendorf em cada poço. Após essa etapa foi realizada a microdiluição do antibiótico, sendo 100 µL nesse meio até penúltima cavidade (1:1). Na última cavidade não foi adicionada por ser o controle de crescimento. As concentrações variaram de 1024 µg/mL a 0,5 µg/mL. A solução do antibiótico utilizada foi à preparada anteriormente, sendo que para cada cepa foi utilizado um antibiótico específico para uma bomba de efluxo presente. Após 24h foi realizado a leitura das placas por visualização de mudança de cor do meio caracterizado pela adição de 20 µL de resazurina (7-hidroxi-3H-fenoxazina-3-ona 10-óxido). Sendo que, a leitura tem como característica a mudança de cor do meio de azul para vermelho indicando à presença de crescimento bacteriano e a permanência em azul a ausência de crescimento. Todos os experimentos foram realizados em triplicatas. A redução do CIM do brometo de etídeo ou de antibiótico específico, em cepas portadoras de bomba de efluxo, é um indicativo de inibição de bomba de efluxo.

3.10 Análise estatística dos resultados microbiológicos

Os resultados dos testes foram feitos em triplicata e expressados em média geométrica. Na análise estatística foi utilizada ANOVA de uma via, seguida do post hoc Tukey ou Bonferroni usando *GraphPad Prism 5.0*. Em algumas análises foi utilizado o teste T.

4 RESULTADOS E DISCUSSÕES

4.1 ARTIGO 1: IN SILICO EVALUATION OF THE ANTIBACTERIAL AND MODULATORY ACTIVITY OF LAPACHOL AND NOR-LAPACHOL DERIVATES. PUBLICADO (MICROBIAL PATHOGENESIS). **FATOR DE IMPACTO = 3,738.**

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In silico evaluation of the antibacterial and modulatory activity of lapachol and nor-lapachol derivates

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ABSTRACT

The aim of this research was to investigate the pharmacological properties of 2-(2-hydroxyethylamine)-3-(3-methyl-2-butenyl)-1,4-dihydro-1,4-naphthalenedione, 2-(2-hydroxy-ethylamine)-3-(2-methyl-propenyl)-[1,4] naphthoquinone and 2-(3-hydroxy-propylamine)-3-(3-methyl-2-butenyl)-[1,4]naphthoquinone using computational prediction models, in addition to evaluating the *in vitro* antibacterial and modulatory activity of these compounds against bacterial ATCC strains and clinical isolates. The substances were synthesized from 2-hydroxy-quinones, lapachol and nor-lapachol obtaining the corresponding 2-methoxylated derivatives via dimethyl sulfate alkylation in a basic medium, these then reacted chemoselectively with 2-ethanolamine and 3-propanolamine to form the corresponding amino alcohols. The antibacterial activity and modulatory activity of the substances were assayed by broth microdilution method to determine the Minimum Inhibitory Concentration (MIC). The molecular structures were analyzed using the ChEMBL database to predict possible pharmacological targets, which pointed to the molecule 2-(2-hydroxy-ethylamine)-3-(2-methyl-propenyl)-[1,4]naphthoquinone as a probable antibacterial agent for the proteins Replicative DNA helicase and RecA. The compounds had a low molecular weight and a small number of rotatable bonds. The MICs of the substances were not clinically significant, however, the association with gentamicin and amikacin reduced the MICs of these antibiotics. In conclusion, the combination of these substances with aminoglycosides may be a therapeutic alternative to bacterial resistance and the reduction of side effects.

1. Introduction

The emergence of new multiresistant microbial strains over the last few years has led to an increase in mortality and morbidity, as well as increased the pharmacological treatment costs of microbial infections [1]. This scenario has made the research for new bioactive compounds into a target of great scientific interest in the search for therapeutic alternatives for microbial infections [2].

Among the Gram-negative bacteria, *P. aeruginosa* stands out since in addition to being associated with infections in immunocompromised patients, it also affects patients who have had invasive procedures, burns and surgical wounds [3]. *Escherichia coli* belonging to the Enterobacteriaceae family is a Gram-negative bacterium [4], responsible for 80–90% of urinary tract infections. Its contamination ascends from the intestinal microbiota reaching the urethra, passing through to the bladder and eventually the urinary tract [5]. Among the gram-positive bacteria involved in nosocomial and community infections, *S. aureus* is one of the most important, due its wide environmental dissemination and its association with severe opportunistic infections [6].

The combined use of antimicrobials, named poliantibiotc therapy, is commonly used due the possibility that one of the antibiotic agents be active against the microorganism by an additive or synergistic effect,

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reducing the effect of the bacterial mechanisms of antibiotic resistance, being natural products an interesting alternative to this approach [7–9]. Thus, substances with plant origin and their derivatives have become a viable and efficient alternative [10,11], since a drug's antimicrobial activity can be amplified or reduced by the action of natural products [12].

In this context, naphthoquinones are versatile organic compounds that are part of an important class of natural products known as quinones, structurally characterized by their presentation as conjugated cyclic dienones with the 1,4 and 1,2-naphthoquinone isomers being the most common [13,14].

Naturally derived naphthoquinones [15] represent a wide and varied family of secondary metabolites and are of vital importance to plants, fungi, lichens and algae. The highest occurrence of quinones is found in plants from the Bignoniácea family, more precisely from the *Tabebuia* (*Tecoma*) genus [16]. In addition to being important intermediates in organic synthesis for obtaining numerous natural or synthetic compounds, its participation in several biological activities such as antitumor, antifungal, antibiotic, antibacterial [17,18] and molluscicide [19] activities is relevant.

Lapachol is a functional naphthoquinone with natural origin, easily obtained through extracted from a number of species of *Tabebuia* sp. (Bignoniaceae) [13,14], popularly known as "ipê", being abundant in Brazil and South America [19]. Lapachol possesses several biological activities such as: action against esophageal cancer cells [20], antimicrobial activity, trypanocidal [14], including others [21]. Norlapachol is a semi-synthetic derivative of natural lapachol, known to exhibit excellent antitumor and other biological activities [19,22].

Clinical and pre-clinical studies are essential to obtain the necessary informations to the liberation for use of any substance. However, these assays are expensive. By this fact, the usual computational models are useful to give an experimental accurate direction to these assays, informing pharmacological and physico-chemical characteristics of these substances [23].

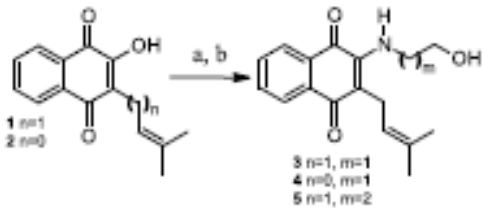
This study aimed to perform an *in silico* analysis of the sampangine alkaloid analogues 2-(2-hydroxyethylamine)-3-(3-methyl-2-butenyl)-1,4-dihydro-1,4-naphthalenedione, 2-(2-hydroxy-ethylamine)-3-(2-methyl-propenyl)- [1,4] naphthoquinone and 2-(3-hydroxy-propylamine)-3-(3-methyl-2-butenyl)- [1,4] naphthoquinone, derived from lapachol and norlapachol, and to evaluate the *in vitro* antibacterial and modulatory activity of these compounds against ATCC bacterial strains and clinical isolates.

2. Materials and methods

2.1. Synthesis of substances

2.1.1. General information

Air- and moisture-sensitive reactions were carried out under argon atmosphere. Reagents were purchased from Sigma-Aldrich, Dinamica or Vetec and distilled or used without further purification. Reactions were monitored by TLC analysis on precoated silica gel plates (Merck, Kieselgel 60 GF_{254}) and compounds were visualized with UV light. Column chromatography was performed on silica gel 60 (70–230 mesh, Merck). Melting points were measured in open capillary tubes in a QUIMIS apparatus and are uncorrected. The infrared spectra were recorded on an IFS66 Bruker spectrophotometer using KBr discs or Varian Mercury 640 IR with ATR. HRMS analyses were performed on a MALDI-TOF/TOF Autoflex III 10, using positive reflected mode. NMR (^{1}H at 400 MHz and ^{13}C at 100 MHz) spectra were recorded on a Varian Unity Plus-400 spectrometer, 200 MHz Varian Mercury, using $CDCl_3$ or DMSO-d_6 as solvents, and calibrated for the solvent signal. Chemical shifts are expressed in parts per million (ppm) and coupling constants are given in Hz. Compounds lapachol [22] and the corresponding 2-methoxy derivative, norlapachol [19] and the corresponding 2-methoxy derivative were obtained by previous published procedures (Fig. 1).



Reagents and conditions: a) Me_2SO_4, K_2CO_3, acetone, r.t.; b) 2-amino-ethanol or 3-amino-1-propanol in MeOH, r.t.

Fig. 1. Schema of synthesis of 2-aminoalquil derivatives 3-5.

2.1.2. Synthesis of 2-aminoalquil derivatives 3-5

1 mmol of the 2-methoxy derivative dissolved in 10 mL of MeOH was slowly added to 1.5 mmol of the appropriate amine (2-aminoethanol or 3-amino-1-propanol) in the same solvent (40 ml) with continuous stirring. After reaction completion by inspection in CCD analysis, the solvent was removed under vacuum and the residue submitted to flash chromatography on silica gel and ethyl acetate/hexane with increasing polarity.

2.1.2.1. 2-(2-Hydroxyethylamino)-3-(3-methyl-2-butenyl)-1,4-dihydro-1,4-naphthalenedione (3). Obtained in 88% yield as red crystals, mp 80–81 °C; ^{1}H NMR (200 MHz, $CDCl_3$) 1.68 (s, 3H), 1.74 (s, 3H), 2.3 (br, 1H), 3.37 (d, 2H, *J* 5.9 Hz), 3.71 (m, 2H), 3.85 (m, 2H), 5.07 (t, 1H, *J* 5.9 Hz), 6.01 (l, 1H), 7.57 (m, 1H), 7.57 (m, 1H), 7.93 (d, 1H, *J* 7.6 Hz), 8.05 (d, 1H, *J* 7.6 Hz); ^{13}C NMR (50 MHz, $CDCl_3$), 18.2, 23.8, 25.8, 47.2, 62.1, 122.7, 126.1, 126.3, 130.6, 132.0, 132.8, 133.4, 134.5, 146.2, 183.1, 183.2; IR (KBr) (ν max., cm^{-1}) 3391, 3321, 1678, 1599, 1555, 1513; MS (rel int) *m/z* 285 (M+, 57), 270 (100), 198 (70). HRMS found: 285.13649. Calcd for $C_{17}H_{19}NO_3$: 285.13649.

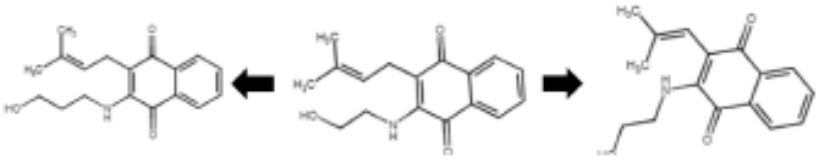
2.1.2.2. 2-(2-Hydroxy-ethylamino)-3-(2-methyl-propenyl)-[1,4] naphthoquinone (4). Obtained in 87% yield as red crystals (mp 77–78.5 °C) in 80% yield. ^{1}H NMR ($CDCl_3$, 200 MHz) 1.47 (d, 3H, *J* 1.0 Hz), 1.89 (d, 3H, *J* 1.6 Hz), 2.46 (br s, 1H), 3.37 (q, 2H, *J* 5.4 Hz), 3.73 (t, 2H, *J* 5.4 Hz), 6.06 (dd, 1H, *J* 1.0/1.6 Hz), 6.25 (br t, 1H, *J* 5.4 Hz), 7.51 (dt, 1H, *J* 1.4/7.6/7.6 Hz), 7.61 (dt, 1H, *J* 1.4/7.6/7.6 Hz), 7.90 (dd, 1H, *J* 1.4/7.6 Hz), 7.99 (dd, 1H, *J* 1.4/7.6 Hz). ^{13}C NMR ($CDCl_3$, 50 MHz) 20.1, 25.4, 46.1, 61.3, 113.6, 117.7, 125.9, 126.1, 130.3, 131.9, 133.3, 134.4, 139.0, 144.8, 182.7, 183.4. IR (KBr) ν max, 3457, 3349, 3268, 2940, 2874, 1675, 1598, 1563, 1511, 1354, 1335 cm^{-1}. HRMS Calcd for $C_{16}H_{17}NO_2$, 271.1208; found: 271.1169.

2.1.2.3. 2-(3-Hydroxy-propylamino)-3-(3-methyl-2-butenyl)-[1,4] naphthoquinone (5). Obtained in 75% yield as red crystals (m.p. 69–70 °C). ^{1}H NMR ($CDCl_3$, 400 MHz) 1.68 (s, 3H), 1.74 (s, 3H), 1.88 (q, 2H, *J* 6.2, 6.16 Hz), 3.39 (d, 2H), 3.69 (t, 2H, *J* 6,6 Hz), 3.80 (t, 2H, *J* 6.2, 5.5 Hz), 5.08 (t, 2H, *J* 5.8), 7.54 (t, 1H, *J* 7.5 Hz), 7.65 (td, 1H, *J* 7.4 Hz), 7.94 (d, 1H), 8.04 (d, 1H); ^{13}C NMR ($CDCl_3$,100 MHz) 17.8, 23.3, 23.4, 32.8, 42.4, 60.3, 115.3, 122.7, 125.6, 125.9, 130.1, 131.5, 132.1, 133.1, 134.0, 145.6, 182.74, 182.76; IR (KBr) ν max, 3446, 3334, 1672, 1598, 1557, 1527, 1361, 1276, 1473, 728 cm^{-1}. HRMS Calcd for $C_{18}H_{21}NO_3$, 299.1527; found: 299.1501.

2.2. Prediction of the pharmacological activity of substances (in silico studies)

2.2.1. Obtaining the three-dimensional molecular structure of substances

The three chemical structures that were the object of this work were designed in free ChemSketch® software, version 2015.2.5 and their three-dimensional structure was determined by 3D Viewer software, both for Windows®, produced by Advanced Chemistry Development,



5 3 4

Fig. 2. Chemical structures of compounds.

Table 1
Molecular characteristics of the three compounds.

Code	SMILE	Observations	Estructure	MF	MW
3	CC(C) = CCC1 = C(N(CCO)C(=O)C2 = CC=CC=C2C1 = O			$C_{16}H_{17}NO_3$	271.311
4	CC(C) = CC2 = C(N(CCO)C(= O)C2 = CC=CC=C2C1 = O			$C_{16}H_{17}NO_3$	271.311
5	CC(C) = CCC1 = C(N(CCO)C(=O)C2= CC=CC=C2C1 = O			$C_{18}H_{21}NO_3$	299.364

MF- Molecular Formula; MW - Molecular weight.

Inc. (ACD/Labs). In Avogadro software, version 1.1.1. For MAC OS systems, the most stable conformation of the molecules was determined considering the force field MMFF94 (Merck Molecular Force Field 94) using a steepest descent algorithm or the Cauchy method as a gradient method for lengths of the bonds and angles of the molecule (Fig. 2 and Table 1).

2.2.2. Pharmacological screening

All three molecules were pharmacologically screened using the ChemProt version 3.0 software from the Technical University of Denmark (Chemprot-3.0, 2019) (http://potentia.cbs.dtu.dk/ChemProt/#) [24].

ChemProt is a publicly available compilation of annotation features on the chemical-protein-disease relationship that enables the study of small-molecule system pharmacology across multiple layers of complexity, from molecular to clinical levels. ChemProt Version 3.0 provides the analysis of over 1.7 million compounds, with 7.8 million bioactivity measurements for 19,504 proteins [25].

The Molinspiration software was used to determine the properties of the molecules (https://www.molinspiration.com), with which the structures were analyzed for agreement with the Rule of Five, data presented in Table 1 [26].

2.3. Preparation of the test solution

To prepare the test solution, the extract was dissolved in Dimethyl sulfoxide (DMSO) in the following proportion: 10 mg of the extract for each 1 mL of DMSO. This solution was diluted in distilled water, obtaining an initial concentration of 2048 μg/mL.

2.4. Microorganisms

The microorganisms used in the tests were provided by the National Institute of Health Quality Control (INCQS) of the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. Four bacterial strains were used, including standard strains of *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 and multiresistant strains (clinical isolates) of *P. aeruginosa* 31 and *S. aureus* 358 (Table 2).

2.5. Culture media

Heart Infusion Agar - HIA (Difco Laboratories Ltd.) and Brain Heart Infusion Broth - BHI (Acumedia Manufacturers Inc.) were prepared according to the manufacturer's specifications. Brain Heart Infusion Broth - BHI at 10% concentration was also used in the assays.

2.6. Preparation and standardization of bacterial inocula

Bacterial cultures were maintained at 4 °C in Heart Infusion Agar (HIA). Prior to the tests, the strains were transferred to the BHI medium and incubated at 35 °C for 24 h. Then, they were diluted in saline to the concentration of 10^5 cells/mL, which equivalent yo the 0.5 in the McFarlan scale. The pre-standard bacterial suspensions were diluted in BHI broth (1:10) to obtain the final concentration of 10^4 cells/mL [27].

2.7. Determination of the minimum inhibitory concentration (MIC)

The determination of the MIC of the compounds was carried out through the Broth Microdilution Method, using concentrations ranging from 1024 to 16 μg/mL [27].

2.7.1. Execution and readings of the assays

The samples were prepared in a folded concentration (2048 μg/mL) relative to the initial concentration and then, serially diluted 1: 2 in 10% BHI broth. Each well was added with 100 μL of the culture medium containing a 1:10 diluted bacterial suspension sample.

Negative controls (culture medium), positive controls (medium + inoculum) and the compounds at concentrations ranging from 1024 to 1 μg/mL were included in the assays. The filled plates were incubated at 35 °C for 24 h [27].

To determine the MIC, a solution of resazurin sodium (Sigma) in sterile distilled water at the concentration of 0.01% (w/v) was prepared. After incubation, 20 μL of the indicator solution was added into each well and the plates were incubated for 1 h at room temperature. All tests were performed in triplicate.

Table 2
Resistance profile of the bacteria used in the tests.

Bacterium	N	Collection Site	Resistance Profile
Staphylococcus aureus	SA358	Surgical wound	Oxa, Gen, Tob, Ami, Can, Neo, Para, But, Six, Net
Pseudomonas aeruginosa	P31	Nose	Pol, Cpm, Ctz, Ptz, Ami, Imi, Cip, Lev, Mer

Resistance Profile: Ami = amikacin; Cip = ciprofloxacin; Lev = levofloxacin; Ctz = ceftazidime; Pol = polymyxin; Imi = imipenem; Mer = meropenem; Ptz = piperacillin; Can = kanamycin; Tob = tobramycin; Oxa = oxacillin; Gen = gentamicin; Neo = neomycin; Para = paramomycin; But = butyrosine; Sis = sisomycin; Net = netilmicin. The microorganisms used in this research were acquired from the Laboratory of Mycology of the Federal University of Paraíba—UFPB and kindly provided by the Regional University of Cariri—URCA.

2.8. Evaluation of the interference of the compounds on the resistance to aminoglycoside antibiotics

To evaluate the antibiotic-modulating activity of the compounds, the MICs of aminoglycoside antibiotics (amikacin and gentamicin) were determined in the presence and absence of the extract in sterile microplates. All antibiotics were obtained from Sigma.

The extract was used at subinhibitory concentration (MIC/8), which was obtained through dilution in 10% BHI broth. The antibiotic solutions were prepared in a folded concentration (2048 μg/mL) relative to the initial concentration with the addition of sterile distilled water. Serial dilutions (1: 2) were performed using in 10% BHI broth. Each well was added with 100 μL of the culture medium containing a 1:10 diluted bacterial suspension sample. The same controls used in the evaluation of MIC of the extract were used [11]. The filled plates were incubated at 35 °C for 24 h and the readings were performed after addition of resazurin sodium as described above.

2.9. Data analysis

The data were obtained in triplicate and expressed as geometric mean. Differences were analyzed by ANOVA (two-way) with Bonferroni's post-test. The results with values of $p < 0.05$ were considered significant.

3. Results

After the preparation of a drug, its active ingredient must be suitable for use by the chosen route of administration. The oral rout is among the preferred routes as it is the most convenient for most drugs and patients, thus drugs must be able to cross a series of obstacles until reaching their target area [28].

For a drug to complete its trajectory to its target, it needs to meet molecular requirements for the viability of its use as a drug to occur. While studying the characteristics of drug molecules, Lipinski [29] identified some characteristics often observed in 2245 new chemical species collected from the World Drug Index (WDI) and presented what became known as the Rule of Five for a molecule to become a drug, these being:

3.1. Molecular mass is less than or equal to 500 da

2. Number of hydrogen bond acceptor groups is less than or equal to 10 (expressed as the sum of N and O atoms);
3. Number of hydrogen bond donor groups is less than or equal to 5 (expressed as the sum of OH and NH in the molecule);

4. Log P is less than or equal to 5

The reason for their denomination as the "Rule of Five" is because each parameter is defined by a value that, coincidentally, is a multiple of five [29]. The molecular properties of the substances were determined using the online software Molinspiration according to the presentation in Table 3

No violation of the rule of five was identified with the three studied molecules. Particularly, the structure of number 4 obtained a lower molecular weight and fewer rotatable bonds than the other structures, which provides better chemical characteristics for structures with pharmacological activities.

After pharmacological screening, possible therapeutic targets for substance 4 were observed, however, no targets were identified for the other substances Table 4. ChemProt version 3.0 software.

These data corroborate with the literature in that the studied compounds are sampangine alkaloid analogues derived from lapachol and norlapachol. According to Muhammad et al., [30], sampangine possesses antimalarial, antifungal and cytotoxic activities, as well as being a potent inhibitor of leukemic cell proliferation [31]. Research conducted through biological assays reveals a great potential for sampangine against human ovarian cancer cell lines, while also possessing activity against human lung cancer cells [32].

The results presented in Table 3 were fundamental for the planning and performance of the assays used to investigate the presence of a possible antibacterial activity in view of the possible targets: RecA and Replicative DNA helicase, which are associated with bacterial DNA maintenance and replication [33]. According to Refs. [34], sampangine possesses an effective action against fungi and mycobacteria.

In the antibacterial activity evaluation of the substances against the *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains, the substances obtained MIC values ≥ 1024 μg/mL, which are clinically irrelevant values, MIC values greater than 1000 μg/mL are considered to lack direct antibacterial activity for clinical practice [35]. The minimum inhibitory concentration (MIC) can be defined as the lowest concentration capable of completely inhibiting growth in microdilution wells [36].

The data in the present study disagree with the work by Oliveira et al. [37], where several 1,4-naphthoquinone derivatives containing a hydrazine group as a side chain were synthesized from 3-diazo-naphthalene-1,2,4-trione and were evaluated as potential antibacterial agents. In the aforementioned study, naphthoquinone derivatives showed higher antibacterial activity at the preliminary disk diffusion test level than lapachol, a 1,4-naphthoquinone well known for its varied biological activities. As for a study on the minimum inhibitory concentration (MIC) of lapachol against *Staphylococcus aureus*, one report showed the 2-[(3-hydroxy-1,4-dioxo-1,4-dihydro-naphthalen-2-yl)-hydrazone] ethyl malonate presented twice as much activity as lapachol. Similarly, an optical density culture study with *S. aureus* and this substance showed an activity similar to that of vancomycin [37].

The activity of several lapachol-derived analogues against sensitive (MSSA) and methicillin resistant *Staphylococcus aureus* (MRSA) strains were verified, where lapachol derivatives presented antibacterial activity against MSSA ATCC [37]. MRSA clinical isolates were susceptible to naphthoquinone derivatives, however, these were resistant to some commercially available antibacterials with the exception of vancomycin. Most naphthoquinone compounds presented MIC values between 30 mg/L and 125 mg/L, while other derivatives obtained MIC values > 500 mg/L. The minimum bactericidal concentrations were > 500 mg/L, demonstrating the tested naphthoquinones exhibited only a bacteriostatic activity against clinical MRSA strains [38].

Table 3
Molecular properties of the compounds obtained from the Molinspiration software (http://www.molinspiration.com/cgi-bin/properties).

Substances	LogP	ALH	DLH	MM	RBN
3 C C(C) = CCC1 = C(NCCO)C(=O)C2 = CC = CC = C2C1 = O	2.6	4	2	285.34	5
4 C C(C) = CC1 = C(NCCO)C(=O)C2 = CC = CC = C2C1 = O	2.45	4	2	271.32	4
5 CC(C) = CCC1 = C(NCCCO)C(=O)C2 = CC = CC = C2C1 = O	2.88	4	2	299.37	6

MM: molecular mass; ALH: Hydrogen bond acceptors; DLH: Hydrogen bond donors; number of rotatable bonds.

Table 4
Possible therapeutic targetsfor substance 4 as identified by the ChemProt software.

Access to UniProt of the target	Possible therapeutic targets	Species
P10253	70 kDa lysosomal alpha-glucosidase	Human
O14727	Apoptotic protease-activating factor 1	Human
Q9UIF8	Bromodomain adjacent to zinc finger domain protein 2B	Human
P42574	Caspase-3 subunit p12	Human
P55211	Caspase-9 subunit p10	Human
P04637	Cellular tumor antigen p53	Human
P83916	Chromobox protein homolog 1	Human
Q13951	Core-binding factor subunit beta	Human
Q9UNA4	DNA polymerase iota	Human
P27695	DNA-(apurinic or apyrimidinic site) lyase	Human
P28563	Dual specificity protein phosphatase 1	Mouse
Q64346	Dual specificity protein phosphatase 6	Rat
Q96KQ7	Histone-lysine N-methyltransferase EHMT2	Human
P02545	Lamin-A/C	Human
Q9Y468	Lethal(3)malignant brain tumor-like protein 1	Human
P08659	Luciferin 4-monooxygenase	*Photinus pyralis*
O75164	Lysine-specific demethylase 4F	Human
B2RXH2	Lysine-specific demethylase 4D-like	Human
P10636	Microtubule-associated protein tau	Human
P28482	Mitogen-activated protein kinase 1	Human
P84022	Mothers against decapentaplegic homolog 3	Human
P30305	M-phase inducer phosphatase 2	Human
Q9NR56	Muscleblind-like protein 1	Human
Q16236	Nuclear factor erythroid 2-related factor 2	Human
P9A5U4	Protein RecA	*Mycobacterium tuberculosis*
O97447	Putative fructose-1,6-bisphosphate aldolase	*Giardia intestinalis*
P71715	Replicative DNA helicase	*Mycobacterium tuberculosis*
P00352	Retinal dehydrogenase 1	Human
Q01196	Runt-related transcription factor 1	Human
Q9GZR1	Sentrin-specific protease 6	Human
Q9BQI6	Sentrin-specific protease 7	Human
Q96LD8	Sentrin-specific protease 8	Human
P11473	Vitamin D3 receptor	Human

Recent studies have shown a series of 12 new 2-hydroxy-3-phenylsulfanylmethyl- [1,4] naphthoquinone analogues have been synthesized by the addition of a thiol group with different substituents to a de-quinone methane using microwave irradiation. The compounds were tested against Gram positive and negative bacteria, where ten compounds presented antimicrobial activity, especially against Gram negative strains, in addition to presenting biofilm formation inhibition [39].

Figs. 3 and 4 demonstrate the aminoglycoside modulatory activity of substances 3, 4 and 5, when associated with gentamicin and amikacin at sub-inhibitory concentrations (1/8 MIC), where a significant ($p < 0.0001$) MIC reduction was obtained against *S. aureus* and *P. aeruginosa* strains, characterizing this as resistance inhibition. However, no antibiotic activity interference was observed against the *E. coli* strain.

The aminoglycoside modulatory activity of substances 3, 4 and 5 may be related to molecular characteristics of these substances, for being sampangine alkaloid analogues. The possible mechanism of action of quinones is not fully known, however two proposals for how this may work exist. The first is associated with the generation of reactive oxygen species (H_2O_2, $O_2^{\cdot-}$, $OH^{\cdot}$) in the intracellular environment, leading to the denaturation of membrane proteins [15], such as the efflux pumps that are essential for bacterial resistance.

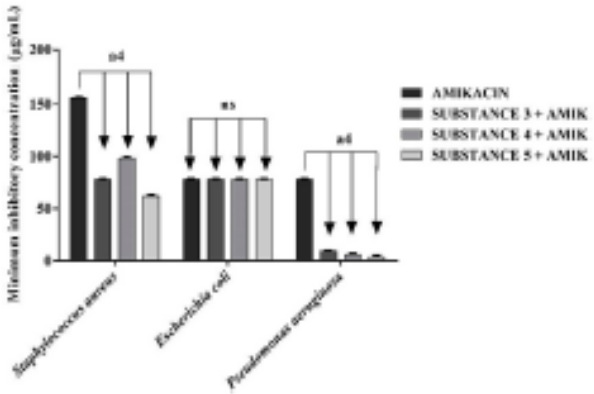


Fig. 3. Effect of substances 3, 4 and 5 on the antibacterial action of amikacin against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* strains.
The values represent the geometric mean ± S.E.M. (standard error of mean). Two-way ANOVA, followed by the Bonferroni test. a4: $p < 0.0001$ vs control of antibiotic; ns: not significant; Amik: Amikacin.

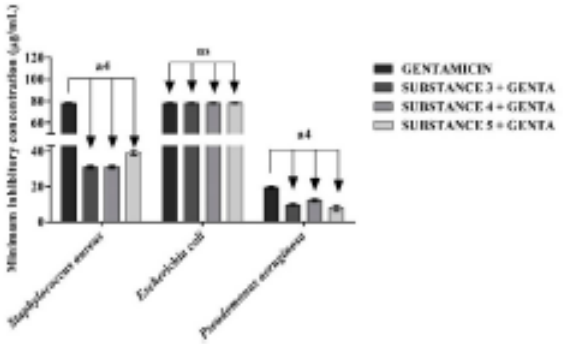


Fig. 4. Effect of substances 3, 4 and 5 on the antibacterial action of gentamicin against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* strains.
The values represent the geometric mean ± S.E.M. (standard error of mean). Two-way ANOVA, followed by the Bonferroni test. a4: $p < 0.0001$ vs control of antibiotic; ns: not significant; Genta: Gentamicin.

The second possibility is through the inhibition of topoisomerase I and II enzymes. These are nuclear enzymes that aid DNA replication and are part of the proper functioning of any cell [15]. In this context, topoisomerases II are usually targets in antibacterial therapy, for being viable options as they are essential for bacterial cell division and replication. These enzymes have a higher specificity/selectivity for prokaryotic enzymes, which is at least three times greater than for eukaryotic enzymes, thus decreasing the likelihood of adverse effects [40] The generation of reactive oxygen species (ROS) due to bioreduction in the region quinolinic by specific enzymes and the interaction with topoisomerase is, until the moment, a possible antibacterial action.

Aminoglycosides presents adverse effects, particularly nephrotoxicity nefrotoxicidade [41,42], ototoxicity and neurotoxicity [43,44], side effects which should be considered before prescribing these antibiotics. In this context, the combination of substances with aminoglycosides may be a therapeutic alternative to bacterial resistance and the reduction of side effects, given that a synergism with significant MIC reduction was observed.

According to Oliveira et al. [45], the combined use of natural products with antibiotics may be an alternative to minimize the adverse effects caused by the use of aminoglycosides, since lower drug concentrations and doses are required for therapeutic effectiveness, especially in cases of multiresistant strain infections.

5. Conclusion

The *in silico* study of the sampangine alkaloid analogues derived from lapachol and norlapachol suggested possible activities for the 2-(2-hydroxy-ethylamine)-3-(2-methyl-propenyl)- [1,4] naphthoquinone molecule as a potential antibacterial agent over Replicative DNA helicase and RecA proteins, highlighting the presence of other targets that could be useful for pharmacological research. The compounds reduced the MICs of gentamicin and amikacin when used in association, against *S. aureus* and *P. aeruginosa* strains. In this context, the combination of these substances with aminoglycosides can be a therapeutic alternative to face the bacterial resistance to antibiotics.

CRediT authorship contribution statement

Fernando Gomes Figueredo: Investigation. **Ingrid T.L. Ramos:** Investigation. **Josinete A. Paz:** Investigation. **Tania M.S. Silva:** Resources. **Celso A. Camara:** Resources. **Cícera Datiane de Morais Oliveira-Tintino:** Formal analysis. **Saulo Relison Tintino:** Formal analysis. **Pablo Antônio Maia de Farias:** Conceptualization. **Henrique Douglas Melo Coutinho:** Conceptualization, Supervision, Project administration. **Marta Maria de F. Fonteles:** Conceptualization, Project administration, Supervision.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2020.104181.

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4.2 ARTIGO 2: EFFECT OF HYDROXYAMINES DERIVED FROM LAPACHOL AND NORLACHOL AGAINST *Staphylococcus aureus* strains CARRYING THE NORA EFFLUX PUMP. PUBLICADO (INFECTION GENETICS AND EVOLUTION). **FATOR DE IMPACTO = 3,342.**

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Effect of hydroxyamines derived from lapachol and norlachol against *Staphylococcus aureus* strains carrying the NorA efflux pump

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ABSTRACT

Isolated substances and those organically synthesized have stood out over the years for their therapeutic properties, including their antibacterial activity. These compounds may be an alternative to the production of new antibiotics or may have the ability to potentiate the action of preexisting ones. In this context, the objective of this study was to evaluate the in vitro antibacterial and efflux pump inhibitory activity of hydroxyamines derived from lapachol and norlachol, more specifically the compounds 2-(2-Hydroxyethylamino)-3-(3-methyl-2-butenyl)-1,4 dihydro-1,4-naphthalenedione, 2-(2-Hydroxyethylamino)-3-(2-methyl-propenyl)[1,4]naphthoquinone and 2-(3-Hydroxypropylamino)-3-(3-methyl-2-butenyl)-[1,4]naphthoquinone, against *Staphylococcus aureus* strains carrying the NorA efflux pump mechanism. The substances were synthesized from 2-hydroxyquinones, lapachol and nor-lapachol, obtaining the corresponding 2-methoxylated derivatives via dimethyl sulfate alkylation in a basic medium, which then reacted chemoselectively with 2-ethanolamine and 3-propanolamine to form the corresponding amino alcohols. All three molecules underwent a virtual structure-based analysis (docking). The antibacterial activity of the substances was measured by determining their Minimum Inhibitory Concentration (MIC) and a microdilution assay was performed to verify efflux pump inhibition using the substances at a sub-inhibitory concentration. The results were subjected to statistical analysis using an analysis of variance (ANOVA) followed by Bonferroni's post hoc test. The substances obtained MIC values ≥ 1024 μg/mL, however, a significant reduction of their MICs was observed when the substances were associated with norfloxacin and ethidium bromide, with this effect being attributed to efflux pump inhibition. Following a virtual analysis based on its structure (docking), information regarding the affinity of new ligands for the ABC efflux pump were observed, thus contributing to the understanding of their mechanism of molecular interactions and the discovery of functional ligands associated with a reduction in bacterial resistance.

1. Introduction

The most commonly used antibacterials in the clinic are those of a natural or semi-synthetic origin, which can be classified into β-lactams, tetracyclines, aminoglycosides, macrolides, cyclic peptides, streptogramins, including others (lincosamides, chloramphenicol, cephalosporins, rifamycin, etc.) (Guimarães et al., 2010). However, despite the existence of these antimicrobial classes, bacterial resistance has become a challenge since microorganisms have been adapting to antibiotics for decades (Tavares, 2000). The increasing advancement in resistance has been intensely reported and its constant change must be taken into account when choosing an antibacterial treatment (Dias et al., 2015). Resistance is considered an ecological phenomenon that occurs as a bacterial response to the widespread use of antibiotics and their presence in the environment (Guimarães et al., 2010).

In this context, *Staphylococcus aureus* is an important human

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pathogen given its collection of virulence factors and ability to acquire resistance to most antibiotics. This capacity is further exacerbated by the constant emergence of new clones, making *S. aureus* a "superbug" (Lakhundi and Zhang, 2018). Due to its great adaptive versatility, *S. aureus* has presented multiple strains resistant to several antibiotics over several decades, such as those resistant to penicillin, which had been identified after approximately a decade of this antibiotic use. These penicillin resistant strains presented in their genetic material the gene penicillinase, which prompted the generation of new antibiotics, such as methicillin, as an option for strains that carried the penicillinase gene. However, in 1961 *S. aureus* strains that integrated mecA in their genetic material emerged, this being a gene that conferred *S. aureus* resistance to methicillin, with the strain thereafter being termed MRSA (Pantosti et al., 2007; Monaco et al., 2017).

Moreover, in recent decades the existence of new MRSA clones has been witnessed. Unlike traditional hospital acquired MRSAs, new clones can invade community environments and infect people without predisposing risk factors (Lakhundi and Zhang, 2018). In this perspective, the mecA gene is essential for resistance not only to methicillin, but also to all β-lactams (penicillins, cephalosporins - except ceftaroline - and carbapenems), since this gene encodes the production of Penicillin 2a binding protein (PBP2a), a protein that catalysis the formation of peptidoglycan, a structural component present in the cellular wall (CW) of *S. aureus*. This protein, which has a lower affinity for β-lactams, catalysis CW production even when in contact with the antibiotics for which the MRSA is resistant to (Pantosti et al., 2017; Taylor and Unaka, 2019).

In addition, resistance to tetracyclines can occur by two main mechanisms: active efflux pump transport (mediated by the protein products from the TetK TetL genes) and by protection via ribosomes (through TetM and TetO genes) (Pantosti et al., 2017). Resistance to tetracycline is evidenced, for example, in *S. aureus* IS-58 strains that possess the TetK efflux pump in charge of actively extruding tetracycline from the intracellular to the extracellular medium, providing protection to the bacteria (Pantosti et al., 2017; Truong-Bolduc et al., 2005; Limaverde et al., 2017).

Efflux pumps are energy-dependent proteins that promote the elimination of antimicrobial agents into the extracellular environment faster than plasma membrane diffusion to aid bacterial resistance, such that the intracellular concentration of the agent remains insufficient to block cellular functions, rendering it ineffective, thus preventing the antibiotic from reaching its active site (Opperman and Nguyen, 2015). In *S. aureus*, NorA, NorB, NorC and Tet38 are chromosome-encoded efflux pumps whose overexpression can confer resistance to multiple drugs (MDR), quinolones and other compounds (Nor pumps) or isolated tetracyclines (Tet38) (Ding et al., 2008). NorA is the best studied efflux system and is therefore often used as a model for investigating efflux-mediated resistance in this pathogen. NorA activity is associated with resistance to fluoroquinolones, various antiseptics and disinfectants, with several reports implicating the role of efflux systems, including NorA, as a first-line response to antimicrobials in *S. aureus* (Costa et al., 2018).

When combating resistance, the combination of two or more compounds are generally superior versus using a single compound, especially for the treatment of serious infectious diseases caused by bacteria resistant to antibiotics (Rumkeb et al., 2012).

Synergism can be achieved by combining antibiotics with extracts or substances at sub-inhibitory concentrations, applied directly to the culture medium (Coutinho et al., 2010; Farias et al., 2015). This approach is not restricted to extract combinations, as combinations between natural products or extracts and synthetic products or antibiotics are also possible (Cristo et al., 2016).

Thus, substances with a plant origin and their derivatives have become a viable and efficient alternative since the antimicrobial activity of a drug may be amplified or reduced by the action of natural products (Oliveira-tintino et al., 2018). Moreover, these hinder antimicrobial resistance mechanisms due to the complexity of their structures, thus avoiding microbial adaptations (Daferera et al., 2003). One such target is the well-studied NorA efflux pump present in the 1199B strain, which has been shown to be susceptible to natural and synthetic products (Fontaine et al., 2014).

Therefore, this study aimed to evaluate the in vitro and in silico efflux pump inhibitory capacity of hydroxyamines derived from lapachol and norlachol, 2-(2-Hydroxyethylamino)-3-(3-methyl-2-butenyl)-1, 4-dihydro-1,4-naphthalenedione, 2-(2-Hydroxy-ethylamino)-3-(2-methyl-propenyl)-[1,4] naphthoquinone and 2-(3-Hydroxy-propylamino)-3-(3-methyl-2-butenyl)-[1,4] naphthoquinone against *Staphylococcus aureus* strains carrying the NorA efflux pump mechanism.

2. Materials and methods

2.1. Bacterial material

The *Staphylococcus aureus* strains used were the SA-1199 wildtype strain and the SA-1199B strain, which expresses the NorA gene encoding the NorA efflux protein and expels hydrophilic fluoroquinolones and other drugs such as DNA intercalating dyes. The strain, courtesy of Prof. S. Gibbons (University of London) was maintained in blood agar base slants (Difco Laboratories Ltd., Brazil). Prior to the assays, the cells were cultivated for 24 h at 37 °C in heart infusion agar (HIA, Difco Laboratories Ltd.).

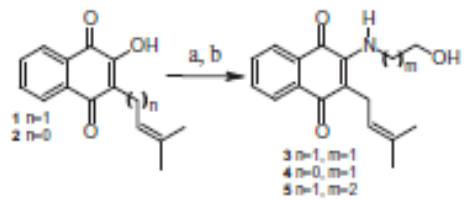
2.2. Synthesis of substances

2.2.1. General Information

Air- and moisture-sensitive reactions were carried out under an argon atmosphere. Reagents were purchased from Sigma-Aldrich, Dinamica or Vetec and distilled or used without further purification. Reactions were monitored by TLC analysis on precoated silica gel plates (Merck, Kieselgel 60 GF_{254}) and the compounds were visualized using UV light. Column chromatography was performed on a silica gel 60 (70–230 mesh, Merck). Melting points were measured in open capillary tubes in a QUIMIS apparatus and are uncorrected. The infrared spectra were recorded on an IFS66 Bruker spectrophotometer using KBr discs or Varian Mercury 640IR with ATR. HRMS analyses were performed on a MALDI-TOF/TOF Autoflex III 10, using positive reflector mode. NMR (^{1}H at 400 MHz and ^{13}C at 100 MHz) spectra were recorded on a Varian Unity Plus-400 spectrometer, 200 MHz Varian Mercury, using $CDCl_3$ or DMSO-d_6 as solvents, and calibrated for the solvent signal. Chemical shifts are expressed in parts per million (ppm) and coupling constants are given in Hz. The compounds lapachol 1 (Camara et al., 2002) and its corresponding 2-methoxy derivative, norlapachol 2 and its corresponding 2-methoxy derivative, were obtained from previously published procedures (Fig. 1).

2.2.2. Synthesis of 2-aminoalquil derivatives 3–5

1 mmol of the 2-methoxy derivative dissolved in 10 mL of MeOH



Reagents and conditions: a) Me_2SO_4, K_2CO_3, acetone, r.t.; b) 2-amino-ethanol or 3-amino-1-propanol in MeOH, r.t.

Fig. 1. Synthesis reaction.

was slowly added to 1.5 mmol of the appropriate amine (2-aminoethanol or 3-amino-1-propanol), in the same solvent (40 mL), with continuous stirring. After the reaction was completed, as per CCD analysis inspection, the solvent was removed under vacuum and the residue submitted to flash chromatography on a silica gel and ethyl acetate/hexane with increasing polarity.

2.2.3. 2-(2-Hydroxyethylamino)-3-(3-methyl-2-butenyl)-1,4-dihydro-1,4-naphthalenedione (3)

This compound was obtained as red crystals with an 88% yield, mp 80–81 °C; ^{1}H NMR (200 MHz, $CDCl_3$) 1.68 (s, 3H), 1.74 (s, 3H), 2.3 (br, 1H), 3.37 (d, 2H, *J* 5.9 Hz), 3.71 (m, 2H), 3.85 (m, 2H), 5.07 (t, 1H, *J* 5.9 Hz), 6.01 (l, 1H), 7.57 (m, 1H), 7.57 (m, 1H), 7.93 (d, 1H, *J* 7.6 Hz), 8.05 (d, 1H, *J* 7.6 Hz); ^{13}C NMR (50 MHz, $CDCl_3$), 18.2, 23.8, 25.8, 47.2, 62.1, 122.7, 1–26.1, 126.3, 130.6, 132.0, 132.8, 133.4, 134.5, 146.2, 183.1, 183.2; IR (KBr) (ν max., cm^{-1}) 3391, 3321, 1678, 1599, 1555, 1513; MS (rel int) *m/z* 285 (M+, 57), 270 (100), 198 (70). HRMS found: 285.13649. Calcd for $C_{17}H_{19}NO_3$: 285.13649.

2.2.4. 2-(2-Hydroxy-ethylamino)-3-(2-methyl-propenyl)-[1,4] naphthoquinone (4) 40.

This compound was obtained as red crystals with an 87% yield, (mp 77–78.5 °C) in 80% yield. ^{1}H NMR ($CDCl_3$, 200 MHz) 1.47 (d, 3H, *J* 1.0 Hz), 1.89 (d, 3H, *J* 1.6 Hz), 2.46 (br s, 1H), 3.37 (q, 2H, *J* 5.4 Hz), 3.73 (t, 2H, *J* 5.4 Hz), 6.06 (dd, 1H, *J* 1.0/1.6 Hz), 6.25 (br t, 1H, *J* 5.4 Hz), 7.51 (dt, 1H, *J* 1.4/7.6/7.6 Hz), 7.61 (dt, 1H, *J* 1.4/7.6/7.6 Hz), 7.90 (dd, 1H, *J* 1.4/7.6 Hz), 7.99 (dd, 1H, *J* 1.4/7.6 Hz). ^{13}C NMR ($CDCl_3$, 50 MHz) 20.1, 25.4, 46.1, 61.3, 113.6, 117.7, 125.9, 126.1, 130.3, 131.9, 133.3, 134.4, 139.0, 144.8, 182.7, 183.4. IR (KBr) ν max, 3457, 3349, 3268, 2940, 2874, 1675, 1598, 1563, 1511, 1354, 1335 cm^{-1}. HRMS found: 271.1169. Calcd for $C_{16}H_{17}NO_3$: 271.1208.

2.2.5. 2-(3-Hydroxy-propylamino)-3-(3-methyl-2-butenyl)-[1,4] naphthoquinone (5) 41

This compound was obtained as red crystals with a 75% yield (m.p. 69–70 °C). ^{1}H NMR ($CDCl_3$, 400 MHz) 1.68 (s, 3H), 1.74 (s, 3H). 1.88 (q, 2H, *J* 6.2, 6.16 Hz), 3.39 (d, 2H), 3.69 (t, 2H, *J* 6,6 Hz), 3.80 (t, 2H, *J* 6.2, 5.5 Hz), 5.08 (t, 2H, *J* 5.8), 7.54 (t, 1H, *J* 7.5 Hz), 7.65 (td, 1H, *J* 7.4 Hz), 7.94 (d, 1H), 8.04 (d, 1H); ^{13}C NMR ($CDCl_3$,100 MHz) 17.8, 23.3, 23.4, 32.8, 42.4, 60.3, 115.3, 122.7, 125.6, 125.9, 130.1, 131.5, 132.1, 133.1, 134.0, 145.6, 182.74, 182.76; IR (KBr) ν max, 3446, 3334, 1672, 1598, 1557, 1527, 1361, 1276, 1473, 728 cm^{-1}. HRMS found: 299.1501. Calcd for $C_{18}H_{21}NO_3$: 299.1527

2.3. Drugs used

Ethidium bromide (EtBr) was obtained from Sigma Aldrich Co. Ltd. The antibiotics used were norfloxacin and erythromycin. The substances were dissolved in dimethyl sulphoxide (DMSO), followed by dilution with sterile water to a concentration of 1024 μg/mL. The ethidium bromide solution (EtBr) was dissolved in sterile distilled water, stored at −20 °C and kept away from light (concentration: 1024 μg/mL). The inhibitors Phenylalanine-arginine β-naphthylamide (PaβN) and chlorpromazine were diluted in distilled sterile water to a concentration of 1024 μg/mL.

2.4. Antibacterial activity test - minimum inhibitory concentration (MIC)

The MIC of the substances was determined with an assay using 100 μL of the bacterial inoculum suspended in saline solution, corresponding to 0.5 of the McFarland scale, followed by the addition of 900 μL of brain heart infusion (BHI) in eppendorfs. The solutions were then transferred to 96-well microdilution plates and a serial dilution of each substance was performed with concentrations ranging from 0.5 to 512 μg/mL. The plates were incubated at 37 °C for 24 h and bacterial growth was evaluated through the use of Resazurin. The MIC was defined as the lowest concentration in which growth was not observed, in accordance with CLSI (2013). The antibacterial assays were performed in triplicates and the results were expressed as the mean of the repetitions.

2.5. Evaluation of efflux pump inhibition by MIC reduction

Efflux pump inhibition was tested using subinhibitory concentrations (1/8 MIC) of substances 3, 4 and 5, with the aim of evaluating the capacity of each substance to decrease the MIC of ethidium bromide (EtBr) and of antibiotics, the substrates for the efflux pump coded for by the MscA gene, present in the plasmid from *S. aureus* 1199B strains. 150 μL of bacterial inoculum suspended in saline solution, corresponding to 0.5 of the McFarland scale, were added to eppendorfs together with 1350 μL of brain heart infusion (BHI) as a control. For the substance evaluation assays, 150 μL of bacterial inoculum suspended in saline solution, corresponding to 0.5 of the McFarland scale, were added to eppendorfs together with 188 μL (1/8 MIC) of the substances, complemented with 1162 μL of brain heart infusion (BHI). The eppendorf solutions were transferred to 96-well microdilution plates and serial dilutions were performed with 100 μL of antibacterial drugs (Oliveira-tintino et al., 2018).

The plates were incubated at 37 °C for 24 h and bacterial growth was evaluated by using Resazurin. The MIC was defined with the tetracycline and ethidium bromide (EtBr) concentrations, which varied between 0.5 and 512 μg/mL, and compared to the chlorpromazine and PAβN standards.

2.6. Molecular docking procedure

2.6.1. NorA modeling and docking procedure

The 3D structure and identification of potential NorA efflux binding pockets are based in concordance with the study by Santos et al. (2018). The molecular docking simulations were performed by the SWISSDOCK webserver (www. Swissdock.ch/) (Grosdidier et al., 2011). All chemical structure coordinates were generated using CORINA and Gasteiger partial charges. The NoRA 3D structure was prepared using the Dock prep tool available in the UCSF chimera free software package (Santos et al., 2018). The grid coordinates for flexible Docking runs were performed by previous studies defining a region of interest with 5 Å from the coordinates x = −29.78, y = 49.65, z = 71.78 and box size x = 46.00, y = 38.00 and z = 30.00. The binding energy score was used to calculate the inhibition constant (K_i value) using the equation $k_i = 10^{(Binding\ Energy/1.366)}$ (Onawole et al., 2018), while ligand efficiency (LE) was determined using the equation ($SILE = k_i/N^{0.3}$), where N is number of heavy atoms (non-hydrogen) present in ligand (Nissink, 2009). The docking results were viewed with the help of the UCSF Chimera visualization program and the Discovery studio (DS) was used to construct the interaction map to show how the derived hydroxyamines, chlorpromazine and PaβN interacted with NoRA.

2.7. Statistical analyses of microbiological results

The results from the assays were performed in triplicates and expressed as geometric means. A one-way ANOVA followed by Bonferroni's post hoc test was used as the statistical analysis test, using the software GraphPad Prism 5.0.

3. Results and discussion

Minimum inhibitory concentration (MIC) values ≥ 1024 μg/mL were obtained (Table 2) in the antibacterial activity evaluation of the substances against *S. aureus* strains with and without the efflux pump resistance mechanism, thus comprising clinically irrelevant values. The MIC was defined as the lowest concentration capable of completely inhibiting growth in microdilution wells. Data from the present study is

in accordance with Figueredo et al. (2020), who did not observe antibacterial activity of these substances against standard and multiresistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains, which obtained MIC values ≥1024 μg/mL.

Recent studies have shown that a series of 12 new 2-hydroxy-3-phenylsulfanylmethyl-[1,4] naphthoquinone analogs have been synthesized by the addition of a thiol, with ten compounds presenting antimicrobial activity, especially against Gram negative bacteria. The in silico study carried out by Figueredo et al. (2020) reported possible activities for the 2-(2-Hydroxy-ethylamino)-3-(2-methyl-propenyl)-[1,4] naphthoquinone molecule as a probable antibacterial agent with action over replicative DNA helicase and RecA proteins.

In addition to the intrinsic antibacterial activity of natural and synthetic products, the potential modulatory action of these products has also been studied by combining them with antibiotics used in the clinic, with the aim of reversing or decreasing bacterial resistance cases (Farias et al., 2015). The combination of natural or synthetic compounds with aminoglycosides has been used, as studies have shown this to be an efficient alternative to reducing the MIC of these antibiotics, by promoting a synergistic action and decreasing bacterial resistance against this class of antibiotics (Siebra et al., 2018). Recent studies have demonstrated the ability of substances 3, 4 and 5 to modulate the activity of aminoglycosides, when associated with gentamicin and amikacin at subinhibitory concentrations, against multidrug resistant *S. aureus* and *P. aeruginosa* strains (Figueredo et al., 2020).

These results guided the assay plans used to investigate a possible efflux pump inhibitory activity by hydroxyamines derived from lapachol and norlachol, against *Staphylococcus aureus* strains that carry the NorA efflux pump mechanism. Following this data analysis, a virtual analysis based on their structures (docking) was performed, which showed promising results.

The lack of an antibacterial activity, as demonstrated by the MIC of these substances, makes them advantageous for use as efflux pump inhibitors. According to Bhardwaj and Mohanty (2012), efflux pump inhibitors should be devoid of antibacterial activity so as not to influence the development of resistance.

The NorA efflux pump inhibitory capacity of substances 3, 4 and 5 at sub-inhibitory concentrations (1/8 MIC), is shown in Fig. 2 when associated with norfloxacin and in Fig. 3 when associated with ethidium bromide, where a significant ($p < .0001$) MIC reduction was obtained, thus demonstrating efflux pump inhibition. The above results are relevant when compared to the MIC of norfloxacin and ethidium bromide in isolation, and their results in association with the tested compounds and standard inhibitors (chlorpromazine and PAβN), against the tested strains (Table 2). According to Oliveira-tintino et al., 2018 a three-fold MIC reduction is associated with an efflux pump inhibition. It is worth noting that SA 1199 and SA 1199B are the same strain, however, the 1199B strain overexpresses the efflux pump, while the 1199 strain has normal expression levels.

According to Olmsted and Kearns (1977) ethidium bromide is a DNA intercalator which severely damages bacterial DNA. As a survival strategy and in the presence of this toxic substance, these microorganisms express efflux pumps, which remove ethidium bromide from inside the bacterial cell (Olmsted and Kearns, 1977; Couto et al., 2008). Ethidium bromide is an indicator of the presence of an efflux pump, therefore, a reduction in ethidium bromide MIC is also indicative of pump inhibition. When efflux pumps are inhibited, the intracellular bromide concentration increases. For this reason, a lower concentration of ethidium bromide poses a toxic effect over the bacterial cell (Viveiros et al., 2008; Patel et al., 2010).

Studies by Kaatz et al. (2003) observed the inhibitory effects of chlorpromazine on *S. aureus* MDR transporters, which exhibited a synergistic or additive action. The authors suggested the possible mechanism of phenothiazine efflux pump inhibition is due to a direct interaction which inhibits efflux driven by the proton motive force (antiport), and which to a lesser extent may lead to a reduction in membrane electrical potential. Phenothiazines may also potentiate the activity of some antituberculosis drugs, medicines against MDR *M. Tuberculosis* strains (Gibbons et al., 2003; Mullin et al., 2004).

Phe-Arg-β-naphthylamide (PAβN) is a cationic dipeptide with a naphthyl moiety, which has been characterized as an efflux pump inhibitor (EPI), since it acts on major efflux transporters associated with multidrug resistance (Lomovskaya et al., 2001) and has often been used to evaluate the efflux phenotype of gram-negative bacteria (Laudy et al., 2015; Schuster et al., 2019). According to Mamelli et al. (2003), PAβN significantly increases the susceptibility of NCTC 11168 erythromycin resistant strains, suggesting the efflux pump is inhibited by PAβN.

In this context, the tested substances are capable of significantly increasing the susceptibility of the *S. aureus* 1199B strain to norfloxacin and ethidium bromide, a result similar to the chlorpromazine and PaβN controls that inhibit the efflux pump.

The importance of nitrogenous quinone studies is associated with their electronic structure, owing to their electron donating capacity and, consequently, their ability to modify reduction potentials, which induce intracellular oxidative stress. Given the above, compounds such as 2-amino-1,4-naphthoquinone possess biological activities which include antibacterial, antimalarial, antitumor and molluscicidal actions (Benites et al., 2010). Therefore, the effect of nitrogenous quinones may have contributed to the reported NorA pump inhibition. The literature reports the mechanism of action of quinones is not fully known, however, two proposals for how this may work exist. The most accepted possibility is associated with the generation of reactive oxygen species (H_2O_2, $O_2^{\cdot-}$, OH-) in the intracellular environment, which trigger programmed cell death, while the other possibility centers around the inhibition of topoisomerase I and II enzymes (Cavalcanti et al.,2013). Moreover, this high instability may provide a great interaction capacity between these substances and the NorA pump, corroborating with the

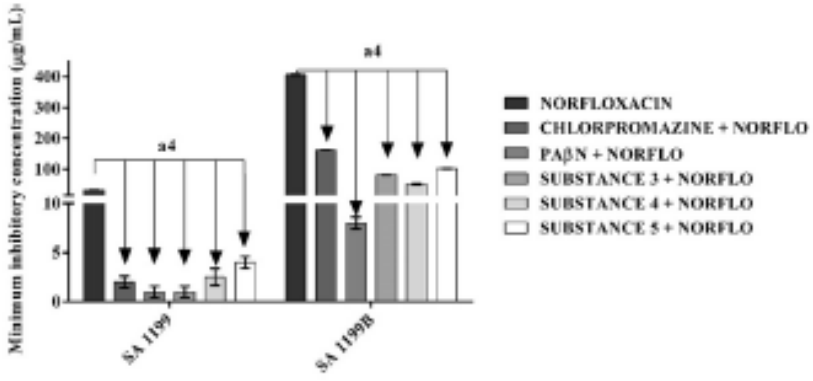


Fig. 2. Ability of inhibition of the NorA efflux pump by the substances 3, 4 and 5, associated with norfloxacin, compared to the chlorpromazine and PAβN standards, against the strains wild type SA 1199 and multiresistant SA 1199B.
The values represent the geometric mean ± S.E.M. (standard error of mean). One-way ANOVA, followed by the Bonferroni test. a4: $p < .0001$ vs control of antibiotic; ns: not significant; Norflo: Norfloxacin; PAβN: Phenylalanine-arginine β-naphthylamide.

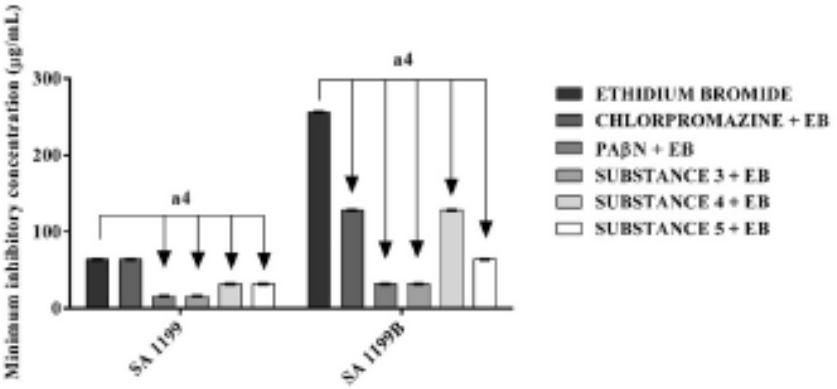


Fig. 3. Ability of inhibition of the NorA efflux pump by the substances 3, 4 and 5, associated with ethidium bromide, compared to the chlorpromazine and PAβN standards against the strains wild type SA 1199 and multiresistant SA 1199B.
The values represent the geometric mean ± S.E.M. (standard error of mean). one-way ANOVA, followed by the Bonferroni test. a4: $p < .0001$ vs control of antibiotic; EB: Ethidium bromide; PAβN: Phenylalanine-arginine β-naphthylamide.

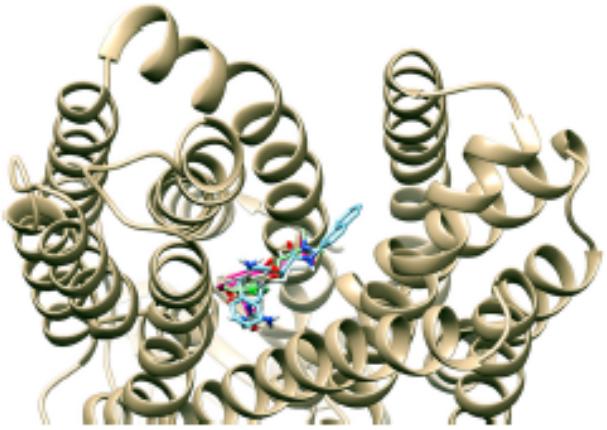


Fig. 4. All docked structure of superimposed complex with NorA.

observed results.

In this study, three new ligands and two NorA inhibitors, Chlorpromazine and PaβN, are used in docking procedures (Fig. 4). The docking simulations were carried out for the most favorable complex binding energy with an energy range between −6.62 Kcal/mol and − 9.1 Kcal/mol (Table 1). The inhibition constant (K_i) and Ligand efficacy calculated corroborate with the experimental activity that shows a compound with inhibitory potential presenting low SILE and Ki values.

The docking results suggested different forces of interactions (Van der Waals, hydrogen bond, Alkyl, π-sulphur, π-π, π-Alkyl) are responsible for stabilization of the NorA complex with different ligands. An important anchorage pocket is formed by the amino acids MET23, GLY56, LEU30, ILE53, TYR55, PHE27, PHE142 and PHE146 that project hydrophobic characteristics, allowing the formation of Van der Waals interactions with all ligands. However, other forces of interactions such as hydrogen bonding with the amino acids GLN345 and GLN59 or sulphur-π interactions with the amino acid MET26, contribute to the stabilization of the NorA/ligand receptor complex. Similar anchoring points in the interaction maps for both the inhibitors and synthetic compounds are shown in the supplementary data.

Table 1
The energy binding of binding of different ligands in NorA and MepA efflux pump.

	NorA		
Compound	Energy of interactions Kcal/mol	Calculated Ki (µM)	SILE
Compound 3	−6.96	8.03	3.222
Compound 4	−6.62	14.25	5.800
Compound 5	−6.73	11.84	4.683
Chlorpromazine	−7.1	6.34	2.545
PaβN	−9.1	0.218	0.075

These results provide further insights into the mechanisms by which new ligand affinities for ABC efflux pumps interact, thereby contributing to the knowledge surrounding mechanisms of molecular interactions and subsequently accelerate the discovery of potent functional ligands with the aim of reducing bacterial resistance in in vivo assays.

Resistant *S. aureus* (MRSA) bacteria possess a series of efflux pumps encoded chromosomally by plasmid transmission, however, for NorA efflux pumps to function a proton mediated antiporter that uses diverse structurally unrelated molecules as its substrates is needed. Several substances have been shown to function as NorA efflux inhibitors in *S. aureus*. Natural products such as silybin (Wang et al., 2018), terpinene (Oliveira- Tintino et al. 2018), tannic acid (Tintino et al., 2016), or polyphenols such as gallic and caffeic acids (Santos et al., 2018), have been shown to inhibit NorA efflux in *S. aureus* and, thereby, restore sensitivity to antibiotics in MRSA.

Discovery approaches involving the NorA pump from Gram-positive bacteria have been functioning, such as with ethidium bromide, as a basis for designing structural analogs with the potential of inhibiting this pump. Product screening and synthesis campaigns have led to the identification of phenothiazines such as chlorpromazine (Rodrigues et al., 2008) and Phenyl-arginine beta naphthylamide (Bohnert et al., 2016), identified as valuable tools for drug discovery with potential antimicrobial agents.

We evaluated the ability of lapachol and norlachol derivatives to interact with the NorA efflux pump using in silico docking simulations. Docking scores demonstrate favorable interactions by Van der Waals, hydrogen bond, Alkyl, π-sulphur, π-π and π-alkyl, with different amino acid residues in the binding site. Similar results were observed with the ligand-based pharmacophore modeling study conducted by Astolfi et al. (2017) using the same NorA site. This result showed the best 3D model obtained was represented by hydrogen-bonding, positive charge, aromatic ring and hydrophobic interactions (Astolfi et al., 2017).

According to Figueredo et al., (2020) the three studied molecules were analyzed with respect to their agreement with the Rule of Five and no violations of the rule were identified, meeting molecular requirements that enable these substances to be potentially viable as medicines.

A series of new amino derivatives and a new partially hydrogenated derivative from the natural naphthoquinone lapachol were analyzed for their molluscicidal activity against *Biomphalaria glabrata*. These derivatives presented low to medium LC_{50} values. The compound 2-(2-Hydroxy-ethylamino) -3-(2-methyl-propenyl)[1,4] described as 3c, did not exhibit toxicity, which according to the authors, is associated with

Table 2
Minimum inhibitory concentration (MIC) values of norfloxacin, ethidium bromide alone and in combination with of Substance 3 through to Substance 5 in sub-inhibitory concentration, against the SA 1199 and SA1199B multiresistant strain.

Compound	MIC of substances (μg/mL)	MIC Norflo + Substance (μg/mL)	MIC Norflo + Substance (μg/mL)	MIC BEr + Substance (μg/mL)	MIC BEr + Substance (μg/mL)
	SA1199	SA1199	SA1199B	SA1199	SA1199B
	SA1199B				
Ethidium Bromide	64 256	–	–	64	256
Norfloxacin	32 406.3	32	406.37	–	–
Substance 3	≥ 1024	1	80	16	32
Substance 4	≥ 1024	2.51	50.8	32	128
Substance 5	≥ 1024	4	101.6	32	64

decreases in the polar character of the tested compounds (Silva et al., 2005).

4. Conclusion

The association of sampangine core alkaloid analogues, derived from lapachol and norlapachol, with norfloxacin and ethidium bromide showed a significant reduction in their MICs, this effect being attributed to an inhibition of the NorA efflux pump by the tested compounds. A correlation was drawn between the interaction of the compounds and the NorA efflux pump through molecular docking, which demonstrated a high affinity.

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Credit author statement

Methodology – F.G.F, I.T.L.R and J.A P.; Resources – T.M.S.S. and C.A.C; Formal analysis – S.R.T. and C.D.M.O.T.; Software analysis – P.A.M.F. and I.R.A.M.; Supervision and project coordination – H.D.M.C and M.M.F.F.

Declaration of Competing Interests

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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4.3 ARTIGO 3: INHIBITION OF *Staphylococcus aureus* TetK and MsrA EFFLUX PUMPS BY HYDROXYAMINES DERIVED FROM LAPACHOL AND NORLACHOL. PUBLICADO (JOURNAL OF BIOENERGETICS AND BIOMEMBRANES). **FATOR DE IMPACTO = 3,342.**

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Inhibition of *Staphylococcus aureus* TetK and MsrA efflux pumps by hydroxyamines derived from lapachol and norlachol

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Abstract
The present study aimed to evaluate the *in vitro* efflux pump inhibitory capacity of hydroxyamines derived from lapachol and norlachol, where compounds 3, 4, and 5 were tested against the *S. aureus* strains: RN4220 carrying the pUL5054 plasmid; and IS-58, endowed with the PT181 plasmid. The substances were synthesized from 2-hydroxy-quinones, lapachol and nor-lapachol obtaining the corresponding 2-methoxylated derivatives via dimethyl sulfate alkylation in a basic medium, which then reacted chemoselectively with 2-ethanolamine and 3-propanolamine to form the corresponding amino alcohols. The antibacterial action of the substances was quantified by determining the Minimum Inhibitory Concentration (MIC), while a microdilution assay was carried out to ascertain efflux pump inhibition of *Staphylococcus aureus* strains carrying the MsrA macrolide and the TetK tetracycline efflux pumps with the substances at a sub-inhibitory concentration. The results were subjected to statistical analysis by an ANOVA test and Bonferroni post hoc test. The MIC from the substances exhibited a value ≥ 1024 µg/mL. However, a significant reduction ($p < 0.0001$) of the erythromycin, tetracycline and ethidium bromide MIC was demonstrated when these were in combination with the substances, with this effect being due to a supposed efflux pump inhibition. The tested substances demonstrated effectiveness at decreasing the MIC of erythromycin, tetracycline and ethidium bromide, potentially by inhibiting the MsrA macrolide and the TetK tetracycline efflux pumps present in the tested *S. aureus* strains.

Keywords Efflux pump inhibition · MsrA · TetK · Hydroxyamines · Lapachol · Norlachol

Introduction

Several microorganisms that make up the human bacterial flora exist and are potentially pathogenic, such as the gram-positive *Staphylococcus aureus*, which is carried by roughly 30 % of humans. *S. aureus* is extremely relevant due to its easy adaptation to different environments, its vast spectrum of infectious diseases (such as bacteremia, infectious endocarditis, septic arthritis and pneumonia), its vast incidence and its high lethality, with complication rates from *S. aureus* infections exceeding 25 % (Wertheim et al. 2005; Shurland et al. 2007; Monaco et al. 2017).

In this context, *S. aureus* bacterial resistance to antibiotics has been shown to be an extremely concerning issue, since bacteremia caused by methicillin-resistant *S. aureus* (MRSA) appears to have greater morbidity and mortality compared to bacteremia caused by methicillin sensitive *S. aureus* (MSSA) (Hal et al. 2012; Vance and Holland 2008).

S. aureus has presented multiple strains that are resistant to several antibiotics over several decades due to its great adaptive versatility. Strains resistant to penicillin were identified following approximately a decade of contact with *S. aureus*, presenting the penicillinase gene in their genetic material. Thus, the need to create new antibiotics, such as methicillin,

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emerged as an option for strains carrying the penicillinase gene. Thereafter, strains integrating the *mecA* gene in their genetic material, a gene that conferred *S. aureus* resistance to methicillin, emerged in 1961, and thus MRSA emerged (Pantosti et al. 2007; Monaco et al. 2017).

In this perspective, the *mecA* gene is essential for resistance not only to methicillin, but to all β-lactams (penicillins, cephalosporins - except ceftaroline - and carbapenems), since this gene encodes the production of Penicillin 2a binding protein (PBP2a), a protein which catalyzes the formation of peptidoglycan, a structural component present in the cellular wall (CW) of *S. aureus* and which has a lower binding affinity to β-lactams. PBP2a catalyzes CW production even when in contact with the antibiotics for which MRSA are resistant to (Pantosti et al. 2007; Taylor and Unaka 2019; Lowy 2019).

In addition, resistance to tetracycline may also occur by two main mechanisms: active transport by efflux pumps (mediated by the protein products from TetK TetL genes) and by protection via ribosomes (through TetM and TetO genes (Pantosti et al. 2007). Resistance to tetracycline is evidenced, for example, in *S. aureus* IS-58 strains that have the TetK efflux pump, tasked with actively extracting tetracycline from the intracellular to the extracellular medium, providing protection to the bacteria (Pantosti et al. 2007; Truong-Bolduc et al. 2005; Limaverde et al. 2017).

It is at this juncture that several therapeutic options for MRSA bacteremia are used, with these ranging from the combination of antibiotics, antibiotics with greater response to a specific species and modification of existing antibiotics, in order to increase treatment effectiveness. While an excellence approach is not yet known, alternatives that demonstrate effectiveness and success exist (Lowy 2019).

The use of vancomycin or daptomycin are good alternatives to monotherapy, where vancomycin is the most commonly used antimicrobial for MRSA bacteremias, since it has an abundance of evidence of success, while daptomycin is a good alternative to vancomycin and has good success rates (Lowy 2019; Murray et al. 2013).

In terms of combined therapy, good results include, for example, the: association between daptomycin and ceftaroline or other β-lactams, association between vancomycin and ceftaroline or other β-lactams, association between daptomycin and trimethoprim-sulfamethoxazole, and association between ceftaroline and trimethoprim-sulfamethoxazole (Lowy 2019; Kullar et al. 2016). Indeed, the mechanisms that provide *S. aureus* with resistance are abundant, diverse and complex, thus urging the need for new therapeutic alternatives, including organically synthesized compounds.

The combination of two or more compounds is generally superior to the use of a single compound, especially for the treatment of serious infectious diseases, caused by bacterial resistance to antibiotics (Hanan et al. 2012). Synergism or additive effect can be obtained by combining antibiotics with extracts or substances at a sub-inhibitory concentration, applied directly to the culture medium, affecting several targets (Wagner and Ulrich-Merzenich 2009; Coutinho et al. 2010; Farias et al. 2015). Between the various targets group, we can cite the efflux pump inhibitors (Van et al. 2006; Piddock 2006).

Thus, plant derived substances and their derivatives have become a viable and efficient alternative (Oliveira et al. 2007, Silva et al. 2007), since the antimicrobial activity of a drug can be amplified or reduced by the action of natural or organically synthesized products (Coutinho et al. 2008; Figueredo et al. 2020a), that hinder antimicrobial resistance mechanisms due to the complexity of their structures, thus avoiding microbial adaptations (Daferera et al. 2003).

In this context, naphthoquinones are important intermediates in the organic synthesis of numerous natural or synthetic compounds (Cavalcanti et al. 2013). Literature reports indicate several biological activities for these compounds, such as antitumor, antifungal, antibiotic, antibacterial, (Powis 1989; Silva et al. 2003) and molluscicide (Barbosa, et al. 2005).

Lapachol is a functionalized naphthoquinone of natural origin, easily obtained by extracting the heartwood from the Bignoniaceae family (Silva et al. 2003; Ferreira et al. 2010). Lapachol has several proven biological activities, such as: an action against esophageal cancer cells (Suthanan et al. 2013), as well as antimicrobial and trypanocide activity (Ferreira et al. 2010). Norlapachol, on the other hand, is a naphthoquinone of synthetic origin, with activity against *Trypanosoma cruzi* (Junior 2007), which can be obtained from a condensation reaction from lausone and isobutyraldehyde, catalyzed by beta-alanine in an acidic medium.

The present study aimed to evaluate the *in vitro* efflux pump inhibitory capacity of the hydroxyamines derived from lapachol and norlachol, 2-(2-hydroxyethylamino)-3-(3-methyl-2-butenyl)-1,4-dihydro-1,4-naphthalenedione, 2-(2-hydroxyethylamino)-3-(2-methyl-propenyl)-[1,4]naphthoquinone and 2-(3-hydroxypropylamino)-3-(3-methyl-2-butenyl)-[1,4]naphthoquinone against the *S. aureus* strains: RN4220 carrying the pUL5054 plasmid; and IS-58 endowed with the PT181 plasmid.

Materials and methods

Bacterial material

The *S. aureus* strains used were: RN4220 carrier of the pUL5054 plasmid, which carries the gene coding for the MsrA macrolide efflux protein; IS-58 with the PT181 plasmid, carrying the TetK tetracycline efflux protein gene. The strains were kindly provided by Prof. S. Gibbons (University of London). Prior to the assays, the cells were cultivated for 24 hours at 37°C in heart infusion agar (HIA, Difco Laboratories Ltda.).

Synthesis of substances

General information

Air- and moisture-sensitive reactions were carried out under an argon atmosphere. Reagents were purchased from Sigma-Aldrich, Dinamica or Vetec and distilled or used without further purification. Reactions were monitored by TLC analysis on precoated silica gel plates (Merck, Kieselgel 60 GF_{254}) and the compounds were visualized using UV light. Column chromatography was performed on a silica gel 60 (70–230 mesh, Merck). Melting points were measured in open capillary tubes in a QUIMIS apparatus and are uncorrected. The infrared spectra were recorded on an IFS66 Bruker spectrophotometer using KBr discs or Varian Mercury 640IR with ATR. HRMS analyses were performed on a MALDI-TOF/TOF Autoflex III 10, using positive reflector mode. NMR (^{1}H at 400 MHz and ^{13}C at 100 MHz) spectra were recorded on a Varian Unity Plus-400 spectrometer, 200 MHz Varian Mercury, using $CDCl_3$ or DMSO-d_6 as solvents, and calibrated for the solvent signal. Chemical shifts are expressed in parts per million (ppm) and coupling constants are given in Hz. The compounds lapachol 1 (Camara et al. 2002) and its corresponding 2-methoxy derivative, norlapachol 2 (Barbosa et al. 2005) and its corresponding 2-methoxy derivative, were obtained from previously published procedures (Fig. 1).

Synthesis of 2-aminoalquil derivatives 3–5

1 mmol of the 2-methoxy derivative dissolved in 10 mL of MeOH was slowly added to 1.5 mmol of the appropriate amine (2-aminoethanol or 3-amino-1-propanol), in the same solvent (40 ml), with continuous stirring. After the reaction was completed, as per CCD analysis inspection, the solvent was removed under vacuum and the residue submitted to flash chromatography on a silica gel and ethyl acetate/hexane with increasing polarity.

2-(2-Hydroxyethylamino)-3-(3-methyl-2-butenyl) -1,4-dihydro-1,4-naphthalenedione (3)

This compound was obtained as red crystals with an 88 % yield, mp 80–81°C; ^{1}H NMR (200 MHz, $CDCl_3$) 1.68 (s, 3H), 1.74 (s, 3H), 2.3 (br, 1H), 3.37 (d, 2H, *J* 5.9 Hz), 3.71 (m, 2H), 3.85 (m, 2H), 5.07 (t, 1H, *J* 5.9 Hz), 6.01 (l, 1H), 7.57 (m, 1H), 7.57 (m, 1H), 7.93 (d, 1H, *J* 7.6 Hz), 8.05 (d, 1H, *J* 7.6 Hz); ^{13}C NMR (50 MHz, $CDCl_3$), 18.2, 23.8, 25.8, 47.2, 62.1, 122.7, 1-26.1, 126.3, 130.6, 132.0, 132.8, 133.4, 134.5, 146.2, 183.1, 183.2; IR (KBr) (ν max., cm^{-1}) 3391, 3321, 1678, 1599, 1555, 1513; MS (rel int) m/z 285 (M+, 57), 270 (100), 198 (70). HRMS found: 285.13649. Calcd for $C_{17}H_{19}NO_3$: 285.13649.

2-(2-Hydroxy-ethylamino)-3-(2-methyl-propenyl)-[1,4] naphthoquinone (4) 40

This compound was obtained as red crystals with an 87 % yield, (mp 77–78.5°C) in 80 % yield. ^{1}H NMR ($CDCl_3$, 200 MHz) 1.47 (d, 3H, *J* 1.0 Hz), 1.89 (d, 3H, *J* 1.6 Hz), 2.46 (br s, 1H), 3.37 (q, 2H, *J* 5.4 Hz), 3.73 (t, 2H, *J* 5.4 Hz), 6.06 (dd, 1H, *J* 1.0/1.6 Hz), 6.25 (br t, 1H, *J* 5.4 Hz), 7.51 (dt, 1H, *J* 1.4/7.6/7.6 Hz), 7.61 (dt, 1H, *J* 1.4/7.6/7.6 Hz), 7.90 (dd, 1H, *J* 1.4/7.6 Hz), 7.99 (dd, 1H, *J* 1.4/7.6 Hz). ^{13}C NMR ($CDCl_3$, 50 MHz) 20.1, 25.4, 46.1, 61.3, 113.6, 117.7, 125.9, 126.1, 130.3, 131.9, 133.3, 134.4, 139.0, 144.8, 182.7, 183.4. IR (KBr) ν max, 3457, 3349, 3268, 2940, 2874, 1675, 1598, 1563, 1511, 1354, 1335 cm^{-1}. HRMS found: 271.1169. Calcd for $C_{16}H_{17}NO_2$: 271.1208.

2-(3-Hydroxy-propylamino)-3-(3-methyl-2-butenyl)-[1,4] naphthoquinone (5) 41

This compound was obtained as red crystals with a 75 % yield, (m.p. 69–70 °C). ^{1}H NMR ($CDCl_3$, 400 MHz) 1.68 (s, 3H), 1.74 (s, 3H). 1.88 (q, 2H, *J* 6.2, 6.16 Hz), 3.39 (d, 2H), 3.69 (t, 2H, *J* 6,6 Hz), 3.80 (t, 2H, *J* 6.2, 5.5 Hz), 5.08 (t, 2H, *J* 5.8), 7.54 (t, 1H, *J* 7.5 Hz), 7.65 (td, 1H, *J* 7.4 Hz), 7.94 (d, 1H), 8.04 (d, 1H); ^{13}C NMR ($CDCl_3$,100 MHz) 17.8,

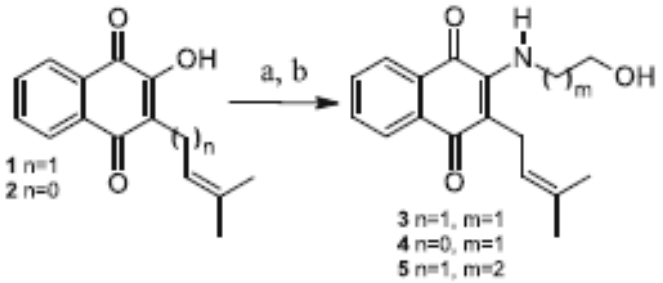

Fig. 1 Synthesis reaction. Figueredo et al. (2020a)

Reagents and conditions: a) Me_2SO_4, K_2CO_3, acetone, r.t.; b) 2-amino-ethanol or 3-amino-1-propanol in MeOH, r.t.

23.3, 23.4, 32.8, 42.4, 60.3, 115.3, 122.7, 125.6, 125.9, 130.1, 131.5, 132.1, 133.1, 134.0, 145.6, 182.74, 182.76; IR (KBr) ν max, 3446, 3334, 1672, 1598, 1557, 1527, 1361, 1276, 1473, 728 cm^{-1}. HRMS found: 299.1501. Calcd for $C_{18}H_{21}NO_3$: 299.1527.

Drugs used

The antibiotics used were specific to the pumps in each bacterium: Erythromycin for the MrsA pump contained in the RN4220 strain; Tetracycline for the TetK pump contained in the IS-58 strain. All antibiotics and compounds were initially dissolved in 10 mg/mL DMSO and subsequently diluted in water, reducing the concentration to 1024 μg/mL. Ethidium bromide was diluted in water to a concentration of 1024 μg/mL. The antibiotics and ethidium bromide were obtained from SIGMA Chemical Co. St. Louis, USA.

Antibacterial activity test - Minimum inhibitory concentration (MIC)

The MIC of the substances was determined with an assay using 100 μL of the bacterial inoculum suspended in saline solution, corresponding to 0.5 of the McFarland scale, followed by the addition of 900 μL of brain heart infusion (BHI) in eppendorfs. The solutions were then transferred to 96-well microdilution plates and a serial dilution of each substance was performed with concentrations ranging from 0.5 to 512 μg/mL. The plates were incubated at 37°C for 24 hours and bacterial growth was evaluated through the use of Resazurin (CLSI 2013). The MIC was defined as the lowest concentration in which growth was not observed, in accordance with CLSI (2013). The antibacterial assays were performed in triplicates and the results were expressed as the mean of the repetitions.

Evaluation of Efflux pump inhibition by MIC reduction

Efflux pump inhibition was tested using subinhibitory concentrations of 128 μg/mL (1/8 MIC) of substances 3, 4 and 5, with the aim of evaluating the capacity of each substance to decrease the MIC of ethidium bromide (EtBr) and of antibiotics, the substrates for the efflux pumps coded for by the genes, present in the pUL5054 and PT181 plasmids from the *S. aureus* strains. 150 μL of bacterial inoculum suspended in saline solution, corresponding to 0.5 of the McFarland scale, were added to eppendorfs together with 1350μL of brain heart infusion (BHI) as a control. For the substance evaluation assays, 150 μL of bacterial inoculum suspended in saline solution, corresponding to 0.5 of the McFarland scale, were added to eppendorfs together with 188 μL (1/8 MIC) of the substances, complemented with 1162 μL of brain heart infusion (BHI). The eppendorf solutions were transferred to 96-well microdilution plates and serial dilutions were performed with 100 μL of the antibacterial drugs (Limaverde et al. 2017).

The plates were incubated at 37°C for 24 hours and bacterial growth was evaluated by using Resazurin. The MIC was defined with the erythromycin, tetracycline and ethidium bromide (EtBr) concentrations, which varied between 0.5 and 512 μg/mL, and were compared to the chlorpromazine and PAβN standards.

Statistical analyses of microbiological results

The results from the assays were performed in triplicates and expressed as geometric means. A one-way ANOVA followed by Bonferroni's post hoc test was used as the statistical analysis test, using the GraphPad Prism 5.0 software.

Results and discussion

The antibacterial action of compounds 3, 4 and 5 was evaluated against the *S. aureus* strains: RN4220 carrying the pUL5054 plasmid; and IS-58 with the PT181 plasmid. The compounds obtained clinically irrelevant results (Houghton et al. 2007), with MIC values ≥ 1024 μg/mL (Table 1), this being the first report of these compounds against these bacterial strains.

These results are in accordance with antibacterial activity tests against standard and multi-drug resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains, which did not show an antibacterial activity for the studied compounds (Figueredo et al. 2020a). According to Figueredo et al. (2020b), when assessing the antibacterial potential of these substances against *S. aureus* strains with and without the NorA efflux pump resistance mechanism, clinically irrelevant results were obtained for both strains.

Figueredo et al. (2020a) performed a pharmacological screening showing the possible antibacterial activity of the molecule 2-(2-hydroxyethylamino)-3-(2-methyl-

Table 1 Minimum inhibitory concentration (MIC) values for the antibiotics, ethidium bromide and substances 3, 4 and 5 in isolation, against the *S. aureus* RN4220 and IS-58 multidrug-resistant strains

Compound and Antibiotics	MIC for SA IS-58	MIC for SA RN4420
Ethidium bromide	32 μg/mL	32 μg/mL
Tetracycline	203 μg/mL	-
Erythromycin	-	≥1024 μg/mL
Substance 3	≥1024 μg/mL	≥1024 μg/mL
Substance 4	≥1024 μg/mL	≥1024 μg/mL
Substance 5	≥1024 μg/mL	≥1024 μg/mL

propenyl)-[1,4]naphthoquinone, with replicative DNA helicase and RecA protein being identified as the possible protein targets. In addition to screening, the properties of the three molecules were analyzed, demonstrating that the substances meet some of the molecular requirements for structures with pharmacological activities, such as low molecular weight and a small number of rotating bonds (Figueredo et al. 2020a). A subsequent study performed a structurally based virtual analysis (docking) showing a possible efflux pump inhibitory activity by hydroxyamines derived from lapachol and norlachol, against *Staphylococcus aureus* strains carrying the NorA efflux pump mechanism (Figueredo et al. 2020b).

Table 2 demonstrate a possible efflux pump inhibitory capacity for substances 3, 4 and 5 against the *S. aureus* IS-58 and RN4220 strains, when in association with tetracycline and erythromycin at sub-inhibitory concentrations (1/8 MIC), an increase being observed in the antibiotic activity against the S. aureus strains reducing the MICs of the antibiotics. Relevant results when compared to the standard inhibitor PaβN were also obtained. However, interference of the erythromycin activity when in association with substance 5 was not observed.

The modulatory activity of the substances in this study were superior to that of the chlorpromazine control. Chlorpromazine has been cited as a potentiator of several antibiotics, such as oxacillin, vancomycin and tetracycline against *S. aureus*, with this antibacterial synergism possibly occurring through the inhibition of efflux pumps by chlorpromazine, given the decrease in MIC of several antibiotics (Kaatz et al. 2003; Couto et al. 2008; Barreto et al. 2014). The exact chlorpromazine mechanism of action for inhibiting efflux pumps is not fully understood, however, it has been previously shown that chlorpromazine impairs the flow of K^+ through the *S. aureus* membrane. Additionally, chlorpromazine causes cell wall structural changes and alterations in bacterial cell divisions, with such alterations being capable of potentiating the action of antibacterial agents (Kaatz et al. 2003 and Kristiansen et al. 1992). Another suggested mechanism is based on the inhibition of the NorA transporter in which chlorpromazine induces cellular damage in systems that provide energy for efflux pumps by inhibiting H^+ dependent transporters, which supply energy, causing a collapse in the energy matrix of this transporter (Kaatz et al. 2003 and Lima et al. 2019).

Phe-Arg-β-naphthylamide (PAβN) is another substance used as a control for efflux pump inhibition in bacteria. PaβN has previously had its efflux pump inhibitory activity described in Gram-negative bacteria with resistance nodulation cell division (RND) type carriers - for example AcrB and MexB -, previously shown in agents such as *Escherichia coli* and *Pseudomonas aeruginosa* (Lomovskaya et al. 2001; Schuster et al. 2019). PAβN's action in repairing the sensitivity of certain bacterial strains that are multiresistant to β-lactam antibiotics such as cefepime and ceftazidime has also been previously demonstrated (Laudy et al. 2015). While PaβN's mechanism of decreasing bacterial resistance to drugs is not fully understood, a double action sensitizing these microorganisms to antibacterial agents has been suggested for PAβN: the permeabilization of the external bacterial membrane, which can lead to the extravasation of enzymes that degrade antibiotics (β-lactamases, for example); and an inhibitory activity over efflux pumps, in which predecessor studies have reported a competitive inhibition of RND by PAβN (Lamers et al. 2013; Laudy et al. 2015; Schuster et al. 2019).

These results are in agreement with Figueredo et al. (2020b) who associated substances 3, 4 and 5 with norfloxacin and ethidium bromide showing a significant reduction in the MIC of the antimicrobials, with this effect being attributed to a NorA efflux pump inhibition by the tested compounds. In this same study, a docking tracing a correlation between the interaction of the compounds with the efflux pump was performed and showed a high affinity. A recent study demonstrated antibiotic activity modulation by hydroxyamines derived from lapachol and norlachol against Gram-positive and -negative bacteria, when these were associated with aminoglycosides at subinhibitory concentrations (Figueredo et al. 2020a).

Table 3 shows the potentiation of the ethidium bromide (EtBr) action by substances 3, 4 and 5 at subinhibitory concentrations (1/8 MIC), where a significant synergistic effect against *Staphylococcus aureus* bacteria expressing efflux

Table 2 Efflux pump inhibitory activity of substances 3, 4 and 5 when associated with erythromycin or tha tetracycline, in comparison to the chlorpromazine and PaβN standards, against the *S. aureus* IS-58 and RN442 strains

	MIC of Strain RN4420 µg/mL		MIC of Strain SA IS58 µg/mL
ERY (Alone)	1024	TET (Alone)	203
Chlorpromazine + ERY	1024	Chlorpromazine + TET	203
PAβN + ERY	16	PAβN + TET	128
Substance 3 + ERY	512	Substance 3 + TET	128
Substance 4 + ERY	512	Substance 4 + TET	128
Substance 5 + ERY	1024	Substance 4 + TET	161

ERY: Erythromycin TET: Tetracycline; PAβN: Phenylalanine-arginine β-naphthylamide

Table 3 Efflux pump inhibitory activity of substances 3, 4 and 5 when associated with ethidium bromide, in comparison to the chlorpromazine and PaβN standards, against the S. aureus IS-58 and RN442 strains

	MIC of Strain RN4420		MIC of Strain SAIS58	
	µg/mL	reduction	µg/mL	reduction
Et Br (Alone)	32	-	32	-
Chlorpromazine+Et Br	32	-	32	-
PAβN+Et Br	8	4x	16	2x
Substance 3+Et Br	8	4x	2	16x
Substance 4+Et Br	16	2x	16	2x
Substance 5+Et Br	20.15	1,59	32	0 x

Et Br: Ethidium bromide; PAβN: Phenylalanine-arginine β-naphthylamide

pumps was observed, decreasing the ethidium bromide MIC by up to 16x. According to DeMarco et al. (2007), a 3x reduction in a MIC value is indicative of efflux pump inhibition. Thus, compound 5 had no influence on EtBr activity against the IS-58 strain.

Ethidium bromide is a DNA intercalant which severely damages bacterial DNA (Olmsted and Kearns 1977; Couto et al. 2008). Some multi-resistant strains have efflux pumps that are very effective at expelling ethidium bromide (EtBr) (Costa et al. 2013). In addition, when exposed to EtBr, *S. aureus* (ATCC 25,923) has been shown to present a greater resistance to many compounds, quinolones, tetraphenylphosphonium and dequalinium via efflux pumps. With this in mind, efflux pump inhibitors such as chlorpromazine, have shown a reduction in these bacteria's resistance capacity against EtBr (Costa et al. 2013). Efflux pump inhibitors have also been effective at reducing the resistance to EtBr in multidrug-resistant strains, with the association of efflux pump inhibitors and EtBr being a viable option (Couto et al. 2008; Viveiros et al. 2008; Costa et al. 2013). It should also be noted that EtBr can be used to identify *S. aureus* strains with efflux capacity by determining the EtBr MIC by a simple microdilution, in which strains with a MIC ≥ 25 mg/L present an efflux phenotype (Patel et al. 2010).

Conclusion

The tested substances did not demonstrate a satisfactory antibacterial effect in terms of their MICs. However, the tested substances were effective at decreasing the MIC of erythromycin, tetracycline and ethidium bromide, by potentially inhibiting the MrsA macrolide efflux protein and the TetK tetracycline efflux pump, present in the *S. aureus* strains.

Declarations

Conflict of interest None.

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4.4 ARTIGO 4: UPLC/QTOF-MS/MS analysis and antibacterial activity of Commiphora leptophloeos (Mart.) J. B. Gillett against multi-drug resistant Staphylococcus aureus and Pseudomonas aeruginosa PUBLICADO (JOURNAL OF HERBAL MEDICINE). **FATOR DE IMPACTO = 3,030.** Este artigo foi resultado de um ensaio piloto para padronização da metodologia.

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Research paper

UPLC/QTOF-MS/MS analysis and antibacterial activity of *Commiphora leptophloeos* (Mart.) J. B. Gillett against multi-drug resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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ABSTRACT

Commiphora leptophloeos is known as "amburana-de-espinhos" or "Imburana" in Brazil. This plant species has been used as a material for combustion and construction, as well as medicinally. Traditional communities use this plant in the treatment of such as flu, urinary diseases and injuries, its pharmacological properties are attributed to the presence of tannins, saponins, flavonoids and alkaloids. Thus, this study aimed to evaluate the antimicrobial and antibiotic-modulating activity of the ethanolic extract of *C. leptophloeos* leaves. The chemical characterization of the extract was performed by UPLC-QTOF-MS/MS, confirming the presence of phenols and flavonoids of recognized pharmacological activity. To evaluate the antibacterial activity and ability of the extract to modulate the antibiotic activity of aminoglycosides towards resistant bacteria, the microdilution method was used in broth with determination of Minimum Inhibitory Concentration (MIC). The microorganisms used in the assays included standard and multi-drug resistant strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The results were analyzed by ANOVA and Bonferroni's posttest. The ethanolic extract of the leaves of *Commiphora leptophloeos* presented no clinically relevant intrinsic antibacterial activity with MIC ≥ 2048 μg/mL. However, modulation tests revealed that the extract significantly reduced the MIC values of the antibiotics, indicating that the extract enhanced the action of the antibiotics against resistant microorganisms. In conclusion, *C. leptophloeos* has antibiotic-modulating potential. However, further research is recommended to better clarify the mechanism of action of the tested plant extract, guiding the development of new alternatives for the treatment of infections resistant to conventional therapies.

1. Introduction

Infections caused by multi-drug resistant microorganisms represent a global health problem. The treatment of patients with resistant bacteria is much more difficult, requiring the use of the latest generation drugs (Terrível et al., 2013).

Before the need to develop novel drugs to combat bacterial resistance, medicinal plants were an important source of new bioactive molecules for the creation of new antibiotics (Duarte, 2006). In fact, the use of medicinal plants has been increasing worldwide, and it is estimated that about 80 % of the population in the developing world rely in one form or another on herbal medicine (Turolla and Nascimento, 2006). This is especially important because for many communities plants are the only effective and accessible therapeutic source

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Fig. 1. The leaves of *Commiphora leptophloeos* and voucher specimen.

(Souza-Moreira et al., 2010).

The therapeutic properties of plants are related to their secondary metabolites, their active ingredients in isolation or in combination (Silva, 2010). In this context, plants may both exhibit antibacterial effects and potentiate the activity of conventional drug. Thus, the synergistic effects obtained by the association of plants with clinically used antibiotics may contribute to overcome the challenge of microbial resistance (Chaves et al., 2011).

Commiphora leptophloeos (Mart.) J. B. Gillett. belongs to the family Burseraceae and is popularly known as "amburana-de-espinhos" or "Imburana". This plant species has been used as a material for combustion and construction, as well as medicinally (Carvalho, 2009). The rural community of Laginhas, municipality of Caicó, Rio Grande do Norte (Northeast Brazil) use this plant in the treatment of many types of infection, such as flu, urinary diseases and injuries (Roque et al., 2010). (Roque et al., 2010). In this context, the pharmacological properties of this plant have been attributed to the presence bioactive secondary metabolites such as tannins, saponins, flavonoids and alkaloids (Clementino, 2014).

The search for new antimicrobial substances, whether natural or synthetic, that have efficacy against multi-drug resistant strains is an important strategy. In fact, several studies have demonstrated that extracts, as well as isolated compounds have the potential to be used in drug development.

In this context, the characterization of the antimicrobial properties of the plant *C. leptophloeos* can provide perspectives for the development of novel drug to treat infections caused by either standard or resistant bacteria. Therefore, the present study has as main objective to evaluate the antimicrobial and antibiotic-modulating activity of the ethanolic extract obtained from the leaves of *Commiphora leptophloeos*.

2. Material and methods

2.1. Plant material and reagents

2.1.1. Plant material

The leaves of *Commiphora leptophloeos* were collected at the "Sítio Riacho", municipality of Vieirópolis, Paraíba, Brazil. The site of collection is an area of "Caatinga", a Brazilian biome. A voucher specimen was deposited in the Herbarium of the Federal Rural University of Pernambuco, under number 53,792 Fig. 1.

2.1.2. Extraction

The leaves of *C. leptophloeos* were dried at room temperature and crushed to provide 614.6 g of the dry powder that was extracted with ethanol in an ultrasound bath at room temperature for 30 min (5 times). The extract was filtered and concentrated using a rotary evaporator to provide the ethanolic extract (78.1 g). This extract (1.0 mg) was dissolved in 1 mL of an acetonitrile:water 0.1 % formic acid (1:1, v/v) solution. This solution was then filtered through a 0.2 μm membrane filter and injected for UPLC-DAD-QTOF-MS/MS analysis.

2.1.3. UPLC-QTOF-MS/MS analysis

A XEVO-G2XSQTOF mass spectrometer (Waters, Manchester, UK) was connected to an ACQUITY UPLC system (Waters, Milford, MA, USA) via an electrospray ionization (ESI) interface. The Waters Acquity PDA analytical detector was used with a wavelength range of 200–400 nm. The chromatographic separation of the compounds was performed on the ACQUITY UPLC device with a conditioned (4 °C) auto sampler, using an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 μm particle size) (Waters, Milford, MA, USA). The column temperature was maintained at 40 °C. The mobile phase, consisting of water containing 0.1 % formic acid in water (solvent A) and acetonitrile with 0.1 % formic acid (solvent B), was pumped at a flow rate of 0.4 mL min^{-1}. The gradient elution program was the following: 0–5 min, 5–10 % B; 5–9 min, 10–95 % B. The injection volume was 10 μL. A MS analysis was performed on a Xevo G2 QTOF (Waters MS Technologies, Manchester, UK) equipment, which is a quadrupole time-of-flight tandem mass spectrometer coupled with an electrospray ionization source in the negative or positive ion mode. The scan range for data acquisition was from 50 to 1200 *m/z*. In addition, MS^E experiments were carried out to allow both precursor and product ion data to be acquired in a single injection. The source conditions were the following: capillary voltage = 3.0 kV; sample cone, source temperature = 100 °C; desolvation temperature = 250 °C; cone gas flow rate = 20 Lh^{-1}; desolvation gas (N_2) flow rate = 800 Lh^{-1}. All analyses were performed using a lockspray to ensured accuracy and reproducibility. Leucine-enkephalin was used as a reference compound to calibrate the mass spectrometers during analysis. All acquisition and analysis of data were controlled using the Waters MassLynx v 4.1 Software (Waters Corporation, Milford, MA, USA).

2.2. Preparation of the test solution

To prepare the test solution, the extract was dissolved in Dimethyl sulfoxide (DMSO) in the following proportion: 10 mg of the extract for each 1 mL of DMSO. This solution was diluted in distilled water, obtaining an initial concentration of 2048 μg/mL.

2.3. Microorganisms

The microorganisms used in the tests were provided by the National Institute of Health Quality Control (INCQS) of the Oswaldo Cruz Foundation, Rio de Janeiro, Brazil. Four bacterial strains were used, including standard strains of *Pseudomonas aeruginosa* ATCC 27,853 and *Staphylococcus aureus* ATCC 25,923 and multi-drug resistant strains (clinical isolates) of *P. aeruginosa* 31 and *S. aureus* 35.

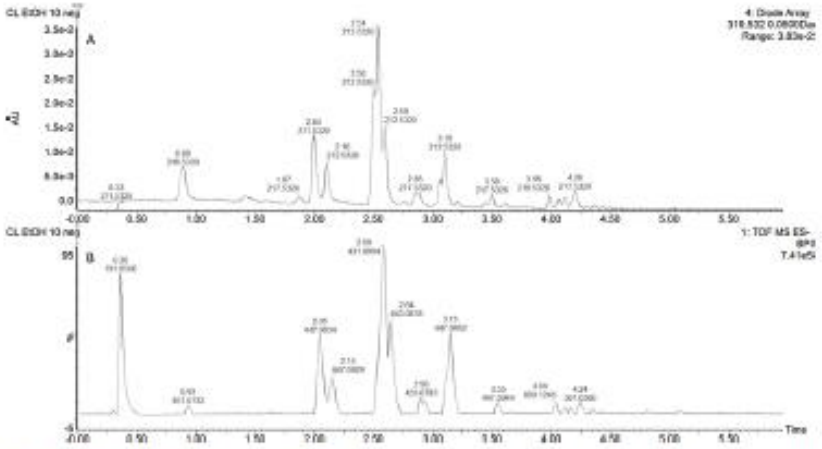

Fig. 2. UPLC-DAD (320 nm) (A) and ESI base peak ion (BPI) (B) chromatogram of the *Cosmiphora leptophloeos* leaves analyzed by UPLC-DAD-QTOF-MS.

2.4. Culture media

Heart Infusion Agar - HIA (Difco Laboratories Ltd.) and Brain Heart Infusion Broth - BHI (Acumedia Manufacturers Inc.) were prepared according to the manufacturer's specifications. Brain Heart Infusion Broth - BHI at 10 % concentration was also used in the assays.

2.5. Preparation and standardization of bacterial inocula

Bacterial cultures were maintained at 4 °C in Heart Infusion Agar (HIA). Prior to the tests, the strains were transferred to the BHI medium and incubated at 35 °C for 24 h. Then, they were diluted in saline to the concentration of 10^5 cells/mL, which is equivalent to the 0.5 in the McFarlan scale (CLSI, 2014). The pre-standard bacterial suspensions were diluted in BHI broth (1:10) to obtain the final concentration of 10^4 cells/mL. (NCCLS, 2000).

2.6. Determination of the minimum inhibitory concentration (MIC)

The determination of the MIC of the extract was carried out through the Broth Microdilution Method, using concentrations ranging from 1024 to 16 µg/mL. (NCCLS, 2003).

2.7. Execution and readings of the assays

The samples were prepared in a folded concentration (2048 µg/mL) relative to the initial concentration and then, serially diluted 1: 2 in 10 % BHI broth. Each well was filed with 100 µL of the culture medium containing a 1:10 diluted bacterial suspension sample.

Negative controls (culture medium), positive controls (medium + inoculum) and the extract at concentrations ranging from 1024 to 16 µg/mL were included in the assays. The filled plates were incubated at 35 °C for 24 h (NCCLS, 2003).

To determine the MIC, a solution of resazurin sodium (Sigma) in sterile distilled water at the concentration of 0.01 % (w/v) was prepared. After incubation, 20 µL of the indicator solution was added into

Table 1
Characterization of compounds from leaves of *Cosmiphora leptophloeos* by UPLC-DAD-QTOF-MS[E].

Comp	TR (Min)	UV λmax (nm)	[M-H]⁻ (m/z)	[M-H]⁻ (m/z)	MS/MS	Identify
1	0.36		191.0564	191.0561	173.0461 [M-H-H_2O]⁻, 127.0403 [M-H-2H_2O-CO]⁻	Quinic acid
2	0.96	249, 323	353.0875	353.0875	191.0565, [M-H-H_2O-caffeic acid]⁻, 174.9565 [M-H-caffeate]⁻, 145.9307 [M-H-caffeic acid-2H_2O-CO]⁻	Caffeoylquinic acid
3	2.08	267, 348	447.0934	447.0932	429.0860 [M-H-H_2O]⁻, 357.0627 [M-H-3CH_2O]⁻, 327.0521 [M-H-4CH_2O]⁻, 297.0420 [M-H-5CH_2O]⁻	Isoorientin*
4	2.15	269, 348	447.0931	447.0932	411.0051 [M-H-2H_2O]⁻, 357.0623 [M-H-3CH_2O]⁻, 327.0511 [M-H-4CH_2O]⁻, 297.0419 [M-H-5CH_2O]⁻	Orientin*
5	2.29	352	609.1463	609.1461	463.0949 [M-H-rhamnose]⁻, 300.0287 [M-H-rutinoside]⁻,	Rutin*
6	2.50	253, 347	463.0885	463.0882	300.0294 [M-H-hexoside]⁻, 271.0263 [M-H-hexoside-CO]⁻, 235.9277	Quercetin-hexoside
7	2.59	265, 344	431.0984	431.0983	341.0706 [M-H-3CH_2O]⁻, 311.0579 [M-H-5CH_2O]⁻	Vitexin*
8	2.64	254, 350	463.0887	463.0882	300.0202 [M-H-hexoside]⁻, 271.0267 [M-H-hexoside-CO]⁻, 235.9276 [M-H-hexoside-CO-2H_2O]⁻	Quercetin-hexoside
9	2.66	265, 347	431.0988	431.0983	413.0876 [M-H-H_2O]⁻, 311.0567 [M-H-4CH_2O]⁻	Isovitexin*
10	2.90	356	433.0775	433.0776	300.0290 [M-2H-xylose]⁻, 271.0273 [M-2H-xylose-CO]⁻,	Quercetin xyloside
11	2.94	356	447.0934	447.0932	284.0338 [M-2H-glucose]⁻	Luteolin glucoside
12	3.15	345	447.0936	447.0932	301.0365 [M-H-rhamnose]⁻, 271.0263 [M-H-rhamnose-CO]⁻	Quercetin rhamnoside
13	3.55	318, 345	447.0930	447.0932	285.0402 [M-H-glucose]⁻, 255.0308 [M-H-glucose-CO]⁻	Luteolin glucoside
14	4.04	318, 345	609.1249	609.1249	463.0902 [M-H-rhamnose]⁻, 301.0304 [M-H-rhamnose-glucose]⁻	Flavone rutinosideo
15	4.12	ND	609.1249	609.1249	463.0886 [M-H-rhamnose]⁻, 301.0350 [M-H-rhamnose-glucose]⁻	Flavone rutinosideo
16	5.08	346	285.0405	285.0404		Luteolin*

* Compared with standard sample. ND Not detected.

each well and the plates were incubated for 1 h at room temperature. All tests were performed in triplicate.

2.8. Evaluation of the interference of the extract on the resistance to Aminoglycoside Antibiotics

To evaluate the antibiotic-modulating activity of the extract, the MICs of aminoglycoside antibiotics (amikacin and gentamicin) were determined in the presence and absence of the extract in sterile microplates. All antibiotics were obtained from Sigma.

The extract was used at subinhibitory concentration (MIC/8), which was obtained through dilution in 10 % BHI broth. The antibiotic solutions were prepared in a folded concentration (2048 μg/mL) relative to the initial concentration with the addition of sterile distilled water. Serial dilutions (1:2) were performed in 10 % BHI broth. Each well was added with 100 μL of the culture medium containing a 1:10 diluted bacterial suspension sample. The same controls used in the evaluation of MIC of the extract were used (Coutinho et al., 2008). The filled plates were incubated at 35 °C for 24 h and the readings were performed after addition of resazurin sodium as described above.

2.9. Data analysis

The data was obtained in triplicate and expressed as geometric mean. Differences were analyzed by ANOVA (two-way) with Bonferroni's post-test. The results with values of $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Chemical profile of ethanolic extract

The ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-PDA-qTOF-MS/MS) in negative mode was used to identify the compounds from the ethanolic extract of *Commiphora leptophloeos*. The UPLC-DAD chromatograms (at 320 nm) and base peak ion (BPI) chromatograms of extract are presented in Fig. 2. The compounds identified in the collections are summarized in Table 1. The identification of the compounds was achieved by matching retention times (Rt), maximum UV absorptions, pseudomolecular ion mass values and MS/MS fragmentation patterns with data published in the literature. A total of 16 compounds were identified, including quinic acid derivatives (**1** and **2**), C-glycosides flavonoids (**3**, **4**, **7** and **9**), glycosylated flavonoid (**5**, **6**, **8**, **10**, **11**, **12**, **13**, **14**, and **15**) and one flavone (**16**).

In the TOF/MS and MS/MS spectra, peak **1** was detected as a deprotonated ion $[M-H]^-$ at *m/z* 191.0564 and fragments ions at *m/z* 173.0461 $[M-H-H_2O]^-$, 127.0403 $[M-H-2H_2O-CO]^-$. On the basis of the fragment interpretation, peak **1** was identified as quinic acid. Compound **2**, gave ion at *m/z* 353.0875 $[M-H]^-$ and yielded three peaks at *m/z* 191.0565, $[M-H\text{-caffeic acid } H_2O]^-$, 174.9565 $[M-H\text{-caffeate}]^-$, and 145.9307 $[M-H\text{-caffeic acid-}2H_2O-CO]^-$ in MS/MS and was identified as caffeoylquinic acid.

The flavonoid C-glycosides showed a series of characteristic fragments at *m/z*$[M-H-18]^-$, $[M-H-60]^-$, $[M-H-90]^-$and $[M-H-120]^-$ due to a cross-ring fragmentation of glucosides molecules. Peaks **3** and **4** were characterized as isoorientin and orientin, with ions at *m/z* 447.0934 $[M-H]^-$, showing the same fragmentation pattern at *m/z* 357.0627 $[M-H-3CH_2O]^-$, 327.0521 $[M-H-4CH_2O]^-$, which corresponds to $[M-H-90]^-$and $[M-H-120]^-$, respectively, from the main fragment. In addition, peak **3** showed an ion at *m/z* 429.0860 $[M-H-H_2O]^-$, which is consistent with the loss of a H_2O molecule. Thus, in the negative mode, a clear loss of H_2O (*m/z* 429) was specific to 6-C glycosides and can be used for its discrimination. These losses were not recorded for the 8-C isomer orientin (Alves et al., 2014). Compound **7** and **9** were identified as vitexin and isovitexin, respectively, by comparing mass spectra and the fragmentation pattern with those of the standards. In the MS^2 spectrum of deprotonated vitexin (*m/z* 431.0984) and isovitexin (*m/z* 431.0988), only two product ions were observed, ion at *m/z* 341.0706 $[M-H-3CH_2O]^-$ and 311.0579 $[M-H-4CH_2O]^-$ both are considered to originate from the cross-ring cleavage of the glucose residue by losing of 90 Da and 120 Da, respectively.

Seven compounds were identified as quercetin glycosides (**5**, **6**, **8**, **10**, **12**, **14** and **15**) and two were identified as glycosides of luteolin (**11** and **13**). In addition, only aglycone luteolin (**16**) was identified. Observation of glycosidic residues pentoside (132 Da), rhamnosyl (146 Da) and glucosyl (162 Da) were cleaved sequentially and generated characteristic aglycone fragments compared to the available literature. Peaks **5**, **14** and **15** were identified as quercetin-rutinoside (*m/z* – 609.1461 $[M-H]^-$ and MS^2 *m/z* 463.0902 $[M-H\text{-rhamnose}]^-$ and 301.0304 $[M-H\text{-rhamnose-glucose}]^-$. The peak **5** was compared to the standard and identified as rutin. The compounds **10** and **12** were identified as quercetin-xyloside *m/z* 433.0775 $[M-H]^-$ and quercetin-rhamnoside 477.0936 $[M-H]^-$ with specific fragmentation at *m/z* 300.0290 $[M-H\text{-xylose}]^-$ and *m/z* 301.0365 $[M-H\text{-rhamnose}]^-$ with the loss of radical from the deprotonated to **10** and **12**, respectively. The **11** and **13** isomers at *m/z* 447.0932 $[M-H]^-$ were identified as luteolin glucosides. The main fragmentation at *m/z* 284.0338 $[M\text{-}2H\text{-glucose}]^-$ and *m/z* 285.0394 $[M-H\text{-glucose}]^-$, resulted from the loss of a glucose moiety, which corresponding to luteolin aglycone. In addition, luteolin (**16**) showed *m/z* 285.0405 $[M-H]^-$ when compared to the standard.

It has been demonstrated that the pharmacological properties of many plants, including antimicrobial and anti-inflammatory activities, may be associated with the presence of quinic acid and caffeoylquinic acid compounds, respectively (Chornobai, 2008). Flavonoids are a class of compounds present in several natural products, especially in plants with antitumor, anti-inflammatory, antiviral and antibacterial properties (Coutinho et al., 2009). These pharmacological activities are determined by the chemical composition in the plant extracts (Huber and Rodriguez-Amaya, 2008).

A Minimum Inhibitory Concentration (MIC) ≥2048 μg/mL was found in the tests that evaluated the antibacterial activity of the ethanolic extract of *C. leptophloeos* against all bacterial strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Table 2). Thus, to be considered as clinically relevant, a given substance must have a MIC ≤ 256 μg/mL, the extract of *C. leptophloeos* is considered to present MIC values clinically irrelevant against bacterial strains (Matias et al., 2010).

According to the study by Chaves et al. (2016), *C. leptophloeos* bark extract presented clinically relevant antimicrobial activity with MIC only for strains of *Staphylococcus aureus*. The same result was observed by Pereira et al. (2017), in which *S. aureus* strains, as well as other Gram-positive strains of *Enterococcus faecalis*, *Bacillus subtilis* and *Micrococcus luteus* were sensitive to the stem extract of this plant.

In addition to the intrinsic antibacterial activity of natural products, these substances have been shown to modulate the action of clinically used antibiotics and therefore can be used to reverse or decrease bacterial resistance. The combination of extracts with aminoglycoside antibiotics has been shown to be an efficient alternative for reducing the MIC of these drugs. Thus, this synergistic action represents a strategy to combat bacterial resistance to this class of antibiotics (Brasil, 2013; Figueredo et al., 2013).

Table 2
Minimal inhibitory concentration (MIC) of the ethanolic extract of *Commiphora leptophloeos* (μg/mL).

Extract	PA ATCC 27853	PA 31	SA ATCC 25923	SA 35
EECL	2048	2048	2048	2048
Amikacin	64	683	32	341
Gentamicin	16	21	8	10.7

EECL: Ethanolic Extract of Commiphora leptophloeos; PA: Pseudomonas aeruginosa; SA: Staphylococcus aureus.

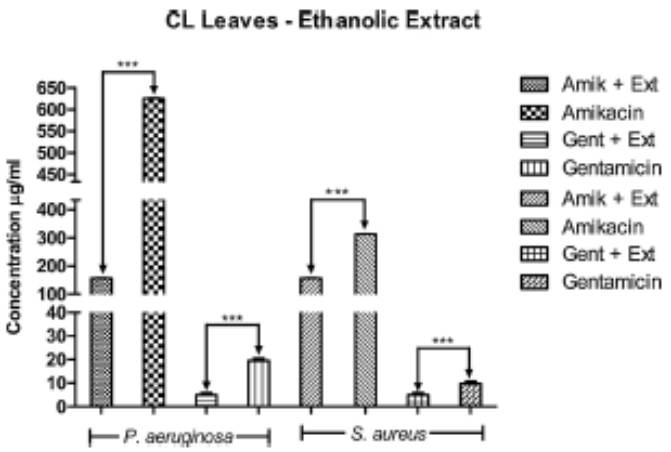

Fig. 3. Antibiotic-modulating activity of the ethanolic extract of *C. leptophloeos* in association aminoglycosides.

Table 3
Antibiotic-modulating activity of the ethanolic extract of *C. leptophloeos* in association aminoglycosides.

	Pseudomonas aeruginosa		*Staphylococcus aureus*	
	MIC	MIC combined	MIC	MIC combined
Amikacin	683	170	341	170
Gentamicin	21	5.3	10.7	5.3

EECL – Ethanolic extract of *C. leptophloeos*; MIC combined- MIC of the aminoglycosides in the presence of ethanolic extract of *C. leptophloeos* at a concentration 256 µg/mL.

Fig. 3 presents the results of the combination of the *C. leptophloeos* ethanolic extract and aminoglycosides (amikacin and gentamicin) against the resistant bacterial strains of *P. aeruginosa* and *S. aureus*. In the modulation tests, it was observed that when combined with the extract in subinhibitory concentration, amikacin as well as the gentamicin presented reduced MIC values. This indicates that the extract had a modulating activity which resulted in a synergistic effect in combination with the antibiotics. The more representative effect was observed with the association of EECL and amikacin, an increase being observed in the antibiotic activity against P. aeruginosa, reducing the MIC of the antibiotic from 683 to 170 µg/mL (Table 3).

The modulatory activity of the ethanolic extract of the leaves of *Commiphora leptophloeos* can be justified by the presence of phenols, including flavonoids that have a synergistic effect against resistant bacterial strains (Cushnie and Lamb, 2005; Matias et al., 2013). Flavonoids have an antimicrobial activity that is related to their ability to inhibit the formation of hydrogen bonds, resulting in inhibition of nucleic acids formation (Ikigai et al., 1993).

Several natural products have presented modulating properties in association with antibiotics with clinically relevant results. In fact, the synergistic effect obtained by the association of plant extracts with antibiotics is a promising option in the fight against the microbial resistance phenomenon (Fernandes et al., 2014; Figueredo et al., 2014; Oliveira et al., 2014; Bitu et al., 2014; Matias et al., 2016).

Antibiotic activity modifiers are substances that modulate or even revert the bacterial resistance phenotype to certain antibiotics. Most of them alter the microbial susceptibility to antibiotics by inhibiting efflux pumps (Chan et al., 2017) In addition, these substances may act on other resistance pathways, acting as antibiotic receptor modifiers, enzyme inhibitors, or increasing membrane permeability (Wagner and Ulrich-Merzenich, 2009; Coutinho et al., 2010)).

Aminoglycosides are potent antimicrobial agents that act by damaging bacterial ribosomes. This class of drugs has been efficiently modulated by the association with one or more types of extracts, with consequent reduction of MIC, which leads to a decrease in the therapeutic dose of the drug (Alves et al., 2014).

4. Conclusion

The ethanolic extract obtained from the leaves of *Commiphora leptophloeos* is constituted by a great diversity of compounds with a wide range of pharmacological activities previously described. Although the intrinsic antibacterial activity of the extract was considered as clinically irrelevant, it presented a significant antibiotic-modulating activity in association with aminoglycosides against resistant bacterial strains.

In conclusion, *C. leptophloeos* has antibiotic-modulating potential. However, further research is recommended to better clarify the mechanism of action of isolated compounds, guiding the development of new alternatives for the treatment of infections resistant to conventional therapies.

CRediT authorship contribution statement

Fernando Gomes Figueredo, Priscilla Ramos Freitas, Johnatan Wellisson da Silva Mendes and Vandbergue Santos Pereira performed the microbiological assays; Sonia Pereira Cabrera, Amanda Lins Bispo Monteiro and Tania Maria Sarmento da Silva performed the chemical characterization; Jaime Ribeiro Filho and Edinardo Fagner Ferreira Matias wrote the manuscript; Henrique Douglas Melo Coutinho and Marta Maria de F. Fonteles supervisioned all steps of the research and revised the work.

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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5 CONCLUSÕES

- O estudo *in silico* de hidroxiaminas derivadas de lapachol e norlachol sugeriram possíveis atividades para o 2-(2-hidroxi-etilamina)-3-(2-metil-propenil)-[1,4]naftoquinona molécula como um potencial agente antibacteriano sobre DNA replicativo heli case e proteínas RecA, destacando a presença de outros alvos que pode ser útil para a pesquisa farmacológica.

- As substâncias testadas não demonstraram um efeito antibacteriano satisfatório em termos de CIMs frente a cepas padrões e multirresistentes. No entanto, os compostos reduziram as CIMs de gentamicina e amicacina quando usadas em associação, contra *S. aureus* e *P. aeruginosa.* Nesse contexto, a combinação das substâncias com os aminoglicosídeos podem ser uma alternativa terapêutica para enfrentar a resistência bacteriana aos antibióticos.

- Os compostos testados quando associados a norfloxacina e brometo de etídio frente as cepas de *S. aureus* 1199B e *S. aureus* 1199, multiressistentes e selvagens respectivamente, apresentaram uma redução significativa em suas CIMs, sendo este efeito atribuído a uma inibição da bomba de efluxo NorA pelos compostos testados. Uma correlação foi traçada entre a interação dos compostos e a bomba de efluxo NorA através de ancoragem molecular, que demonstrou uma alta afinidade.

- As substâncias demonstraram efetividade em diminuir as CIMs da eritromicina, tetraciclina e do brometo de etídio, provavelmente pela inibição da proteína MsrA de efluxo de macrolidio e da bomba de efluxo de tetraciclina TetK, presentes em cepas de *S. aureus*.

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APÊNDICE A – PRODUÇÃO CIENTÍFICA (2017-2021)

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Inhibition of the TetK efflux-pump by the essential oil of *Chenopodium ambrosioides* L. and α-terpinene against *Staphylococcus aureus* IS-58

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JCR: 7.514

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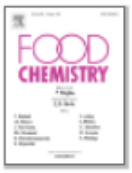
Food Chemistry

Volume 262, 1 October 2018, Pages 72-77



Inhibition of the essential oil from *Chenopodium ambrosioides* L. and α-terpinene on the NorA efflux-pump of *Staphylococcus aureus*

Cícera Datiane de Morais Oliveira-Tintino [a, e], Saulo Relison Tintino [a], Paulo W. Limaverde [a], Fernando G. Figueredo [a], Fábia F. Campina [a], Francisco A.B. da Cunha [a], Roger H.S. da Costa [b], Pedro Silvino Pereira [e], Luciene F. Lima [a], Yedda M.L.S. de Matos [a], Henrique Douglas Melo Coutinho [a] ✉, José P. Siqueira-Júnior [d], Valdir Q. Balbino [c], Teresinha Gonçalves da Silva [e]

JCR:2.315

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Phenolic composition and antioxidant, anticholinesterase and antibiotic-modulating antifungal activities of *Guazuma ulmifolia* Lam. (Malvaceae) ethanol extract

S.M. Morais [a, b], J.T. Calixto-Júnior [a, b], L.M. Ribeiro [a], H.A. Sousa [a], A.A.S. Silva [c], F.G. Figueiredo [d], E.F.F. Matias [d], A.A. Boligon [e], M.L. Athayde [e], M.F.B. Morais-Braga [f], H.D.M. Coutinho [f]

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Original article

Potentiation of antibiotic activity by *Passiflora cincinnata* Mast. front of strains *Staphylococcus aureus* and *Escherichia coli*

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ORIGINAL

CARACTERIZAÇÃO QUÍMICA E ANÁLISE DA ATIVIDADE ANTIOXIDANTE E ANTIFÚNGICA DO EXTRATO ETANÓLICO DE *Amburana cearensis* (ALLEMÃO)

CHEMICAL CHARACTERIZATION AND ANALYSIS OF ANTIOXIDANT AND ANTIFUNGAL ACTIVITY OF ETHANOL EXTRACT OF *Amburana cearensis* (ALLEMÃO)

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Maynara Cavalcante-Figueredo[3]
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Marta Maria Fonteles[5]
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RESUMO

Amburana cearensis (Allemão) A.C. Smith é uma árvore de caule ereto que possui diversas propriedades medicinais, anti-inflamatória, antibacteriana, antitumoral, antiparasitária, dentre outras. As sementes e a casca da árvore são utilizadas na produção de medicamentos populares destinados ao tratamento de asma, tosses, coqueluche e bronquite. O objetivo deste trabalho foi caracterizar os constituintes químicos presentes nas folhas do extrato etanólico de *Amburana cearensis*, além de avaliar a atividade antifúngica e antioxidante. A caracterização química foi realizada por Cromatografia Líquida de Alta Eficiência (CLAE). A atividade antifúngica do extrato foi mensurada através da determinação da Concentração Inibitória Mínima (CIM) e um ensaio de microdiluição foi realizado para verificar as interações entre o produto natural e o fluconazol, utilizando uma concentração subinibitória do extrato. O método Ferric Reducing Antioxidant Power (FRAP), in vitro, demostrou a atividade antioxidante da amostra. Foram identificados polifenóis, flavanóides e lactonas, sendo a cumarina (15,08 mg/g), Quecitrina (8,49 mg/g), Quercetina (7,96 mg/g), Isoquercitrina (7,01 mg/g) e Kaempferol (12 7,14 mg/g) os constituintes majoritários. O extrato demostrou atividade antioxidante apresentando resultado de 2,72 mg

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ANÁLISE MICROBIOLÓGICA DAS MAIONESES DISTRIBUÍDAS EM ESTABELECIMENTOS NO BRASIL: UMA REVISÃO INTEGRATIVA DA LITERATURA

Isaque Barbosa e Silva, Irineu Ferreira da Silva Neto, Rafael de Carvalho Mendes, Annalu Moreira Aguiar, Fernando Gomes Figueiredo

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RESUMO

É muito comum na época atual a alimentação fora de casa, sendo essa constituída, na maioria das vezes, por alimentos de preparo rápido, os chamados *fast-foods*. Juntamente com esses alimentos acompanha-se molhos prontos, como a maionese, especialmente aquelas preparadas no próprio estabelecimento. Tal preparo e armazenamento quando feitos de modo inapropriado tendem a comprometer a

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Artículo original

Potencial antibacteriano e modulador de antibióticos das folhas de *Allamanda puberula A.DC*. (quatro-patacas)

Potencial antibacteriano y modulador de antibióticos de las hojas de *Allamanda puberula* A.DC. (cuatro-patacas)

Antibacterial and modulatory potential of antibiotics from leaves of *Allamanda puberula* A.DC. (quatro-patacas)

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CARACTERIZAÇÃO QUÍMICA E AVALIAÇÃO DA ATIVIDADE ANTIFÚNGICA E ANTIOXIDANTE DO EXTRATO ETANÓLICO DE *Myracrodruon urundeuva* ALL

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Literature Review Volume 4 Issue 5

Primary immunodeficiency diseases: when we should suspect

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Tuberous breast in adolescent: a case report

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Determination of thermotolerant coliforms present in coconut water produced and bottled in the Northeast of Brazil

Determinação da presença de coliformes termotolerantes em águas de coco produzidas e envasadas no Nordeste brasileiro

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Chemical profile, antifungal and modulatory activity of the aqueous and ethanolic extracts of *Libidibia ferrea* (Mart.) L.P. Queiroz (Pau-Ferro)

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ABSTRACT

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PERFIL DE RESISTÊNCIA ANTIMICROBIANA EM AMOSTRAS DE SECREÇÃO DE OROFARINGE NA REGIÃO DO CARIRI CEARENSE NO PERÍODO DE 2018 A 2019.

ANTIMICROBIAL RESISTANCE PROFILE IN OROPHARYNX SECRETION SAMPLES IN THE REGION OF CARIRI CEARENSE IN THE PERIOD FROM 2018 TO 2019.

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RESUMO

Introdução: As vias aéreas superiores são importantes sítios para o surgimento e proliferação de muitos patógenos, devido, principalmente, ao contato direto com o meio externo, presença de oxigênio, umidade, nutrientes e temperatura adequada, promovendo um processo infeccioso, inflamatório e irritativo no local acometido, caracterizando-se como um dos problemas mais frequentes encontrados em serviços de saúde pública. **Objetivo:** Avaliar o perfil de sensibilidade aos antimicrobianos em culturas de secreção orofaringe e de escarros positivas na região do Cariri cearense. **Metodologia:** Trata-se de um estudo epidemiológico, documental, em corte transversal, com coleta retrospectiva dos materiais, no período de 01/07/18 a 30/06/19, a partir da base de dados do Laboratório de Análises Clínicas do Cariri Vicente Lemos, que coleta o material de moradores da região do Cariri. **Resultados:** Foram avaliadas 132.124 amostras positivas de secreção de orofaringe e de escarro com os testes de antimicrobianos, nos quais revelaram 85.691 (64,85%) sensíveis, 43.338 (33,05%) resistentes e 3.099 (2,35%) de caráter intermediário em relação à sua eficácia aos

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