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Article Eleutherin and isoeleutherin activity against Staphylococcus aureus

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Abstract: Naphthoquinones eleutherin and isoeleutherin have demonstrated promising biological 27 activity. This study investigates their activities against Staphylococcus aureus and Escherichia coli 28 and explores the possible mechanisms of action. The minimum inhibitory concentration (MIC), the 29 concentration that inhibits 50% of microbial growth (IC50) and the Minimum Bactericidal Concen-30 tration (MBC) were determined. Molecular docking was performed to identify protein targets and 31 interaction mechanisms, using PharmMapper server and GOLD software. The docking predic-32 tions were validated by redocking, considering structures with a root mean square deviation 33 (RMSD) lower than 2 Å. Eleutherin and isoeleutherin were moderately active against S. aureus, 34 considered bacteriostatic, but inactive against E. coli. Docking revealed significant affinity of eleu-35 therin for peptide deformylase, transcriptional regulator QacR, and regulatory protein BlaR1, with 36 better interactions with BlaR1 compared to the crystallographic ligand (benzylpenicillin). Isoeleu-37 therin demonstrated specific interactions with methionine aminopeptidase, indicating specificity 38 and affinity. In summary, the difference in naphthoquinones activities may be related to isomer-39 ism. Eleutherin exhibits potential as a therapeutic adjuvant to reverse bacterial resistance in S. 40 *aureus*, suggesting this molecule interferes with the antibiotic resistance mechanism. The inactivity 41 against E. coli may be attributed to the absence of homologous proteins or structural differences in 42 the target proteins. 43

Keywords: Antibacterial activity; *Eleutherine plicata*; Molecular Docking; naphthoquinones.

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1. Introduction

Traditional knowledge about plants is essential for discovery of bioactive com-48 pounds, providing a basis for scientific research. A notable example is Eleutherine bulbosa 49 (Mill.) Urb., which has as a synonym Eleutherine plicata Herb., a native American plant, 50 which occurs in several tropical countries [1,2]. It is widely used in traditional Amazoni-51 an medicine to treat malaria, gastric ulcers, intestinal disorders, amoeba and other para-52 sitic infections, dysentery and diarrhea from bacterial origin, hemorrhoids, and men-53 strual disorders [3-5]. 54

Through phytochemical analyses, several compounds present in this plant were 55 identified, including quinones and terpenes. Chemical studies led to the isolation of: (A) 56 isoeleutherin; (B) eleutherin; (C) eleutherol; (D) eleutherinone; (E) (R) 4-57 hydroxyeleutherin; (F) eleuthone; (G) isoeleuthoside C; (H) eleutherinol-8-O-b-D-58 glucoside (Figura 1) [6-8]. 59



Figure 1. Chemical constituents isolated from *Eleutherine plicata;* (A) isoeleutherin; (B) eleutherin; 61 (C) eleutherol; (D) eleutherinone; (E) (R) -4-Hydroxyeleutherin; (F) eleutherone; (G) isoeleuthoside C; (**H**) eleutherinol-8-O-β-D-glucoside.

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Other studies evaluated the possible binding targets of eleutherin and isoeleutherin, 65 and molecular docking for malaria demonstrated the mechanism of action is similar to 66

atovaquone, interactions with conserved residues in the binding cavity of the cytochrome bc1 complex, a protein found in the parasite's mitochondria [4] and possible interaction with antioxidant defense enzymes in the regulation of oxidative stress [9]. In silico studies showed these naphthoquinones can stabilize the topoisomerase II complex [10] and act in the apoptosis pathway [11], inhibit enzymes involved in the biosynthesis of nucleic acids in the energy metabolism of *Plasmodium falciparum*, in addition to inducing oxidative stress[4].

A study demonstrated through agar diffusion and microdilution tests against 74 Staphylococcus aureus that the Dichloromethane Fraction (DF) was the most active, with a 75 minimum inhibitory concentration (MIC) of 125 µg/mL, indicating strong inhibitory ac-76 tivity. The fraction containing isoeleutherin, obtained from DF, had an MIC of 250 77 μ g/mL. For all samples, the minimum bactericidal concentration (MBC) was greater than 78 1000 µg/mL, suggesting that the substances present in this plant were not able to elimi-79 nate the bacteria, only inhibit them [2]. This activity has been related to the isoeleutherin 80 and eleutherin, however, the targets involved are probably different. Among the targets 81 of S. aureus are the metalloenzyme peptide deformylase (PDF), the transcriptional regu-82 lator QacR, the sensory regulatory protein BlaR1 and the monomeric methionine ami-83 nopeptidase (MetAP). 84

PDF acts in the ribosome translation process (Ferreira, 2016) encoded by the def gene, essential for both bacterial growth and survival [12,13] catalyzing the removal of the formyl group from the N-terminal methionine residue of newly synthesized polypeptides, necessary for proper protein folding and function [14]. The regulator QacR is a repressor protein responsible for controlling the expression of the multidrug efflux pump QacA in *S. aureus*, contributing to bacterial resistance [15].

BlaR1 has been demonstrated to detect β -lactam antibiotics and subsequently 91 transmit this information to the cytoplasm. In the MRSA strain, resistance to β -lactam 92 antibiotics is mediated by this protein [16]. MetAP is a dinuclear metalloprotease that is 93 essential for cell growth in organisms such as S. aureus, which contains cobalt and exhibits catalytic properties in removing the N-terminal methionine from newly synthesized proteins [17]. 96

Thus, the present study investigated the activity of eleutherin and isoeleutherin 97 against *S. aureus* and *E. coli* and the intermolecular interactions between naphthoquinones and protein targets involved in the action. It is expected that this research's results 99 will highlight this plant's therapeutic potential and may contribute to the development 100 of new antimicrobial agents based on natural compounds. 101

2. Results

2.1. Chemical studies

The EE (yield = 10% in relation to the dry material) and its hexane (19.2%), dichloromethane (38.5%), ethyl acetate (19.6%) and methanolic (22.6%) fractions were subjected to phytochemical analysis. Bands suggestive of naphthoquinones were observed 108 in all fractions. From the FrDcm, 35 subfractions were obtained and analyzed in TLC, 109 and grouped by similarity of the bands. 110

The subfraction Fr 22-23 presented a band with Rf equivalent to that of eleutherin, 111 and after recrystallization, yellow/orange crystals were obtained. Analysis of fraction 27 112 indicated the presence of a single band with the same Rf as isoeleutherin, which became 113 purer after recrystallization. The subfractions Fr 22-23 and Fr 27 (after recrystallization) 114 were subjected to 1H NMR analyses. The 1H NMR results demonstrated subfraction Fr 115 22-23 was eleutherin and Fr 27 was its isomer, isoeleutherin. 116

2.2. Antibacterial activity of eleutherin and isoeleutherin

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In the evaluation of the eleutherin and isoeleutherin activity against Gram-positive 119 (Staphylococcus aureus) and Gram-negative (Escherichia coli) bacteria, a relationship be-120 tween bacterial inhibition and the concentration of the compounds was noted. The re-121 sults indicated a greater inhibitory potential in S. aureus when compared to E. coli (Table 122 1). 123

Table 1. Staphylococcus aureus and Escherichia coli inhibition at different concentrations of eleutherin 125 and isoeleutherin. 126 107

129 -		Staphylococcus aureus		Escherichia coli		
		Inhibition (%)				
		Eleutherin	Isoeleutherin	Eleutherin	Isoeleutherin	
2130	1000	99,7	99,4	30,2	30,0	
. 131	500	94,0	94,4	30,0	30,2	
1132	250	59,5	52,5	30,3	29,6	
. 133	125	47,7	48,7	27,0	27,4	
1134	62,5	35,6	28,4	25,8	25,2	
. 135	31,2	12,5	7,8	23,1	22,8	
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When the MIC was determined, both isoeleutherin and eleutherin were inactive for 137 S. aureus and E. coli. A similar fact was observed for the MBC, however, when the IC₅₀ 138 was determined, the naphthoquinones were moderately active against S. aureus (Table 139 2).

Table 2. Antibacterial activity of eleutherin, isoeleutherin and chloramphenicol against Staphylococcus aureus e Escherichia coli.

Compounds	S	Staphylococcus aureus		Escherichia coli		
	MIC	IC50	MBC	MIC	IC50	MBC
	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)
Eleutherin	1000	165.00	1000	>1000	>1000	>1000
Isoeleutherin	1000	172.90	1000	>1000	>1000	>1000
Chloramphenicol	250	78.2	-	125	71.33	-

CI₅₀ - Concentration that inhibits 50% of microbial growth; MIC - Minimum inhibitory concentration; 144 MBC – Minimum bactericidal concentration; active (IC50 < 100 µg/mL), moderately active (IC50 between 145 100 and 500 µg/mL), weakly active (IC₅₀ between 500 and 1000 µg/mL) and inactive (IC₅₀ greater than 146 $1000 \, \mu g/m$) 147

2.3. Target proteins involved in the eleutherin and isoeleutherin action

To determine targets of action involved in the activity against S. aureus, a prelimi-150 nary study was carried out with the *PharmMapper* Program. In the case of eleutherin, the 151 activity may be related to 3 different proteins, while for isoeleutherin the results suggest 152 the involvement of one protein (Table 3). 153

Table 3. Targets of eleutherin and isoeleutherin. PDB – Protein Data Bank.

	Cód PDB	Target Name	Score adjustment
Eleutherin	1Q1Y	Peptide deformylase (PDF)	1.56
	1RKW	Regulator QacR	1.37
	1XA7	Regulatory protein BlaR1	1.07
Isoeleutherin	1QXY	Methionine aminopeptidase (MetAP)	2.48

2.4. Molecular docking of eleutherin and isoeleutherin in S. aureus proteins

For PDF and QacR regulator, the crystallographed ligands (actinonin, pentamidine, 158 respectively) presented CS and GS values higher than those of eleutherin. Likewise, all 159 these ligands demonstrated higher Van der Walls energy related to naphthoquinones 160

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(Table 4). A different behavior is observed for BlaR1, where eleutherin presented CS, GS, 161 Van der Walls energy and ΔG values comparable to those of benzylpenicillin (Table 4), 162 suggesting an analogous binding profile between the two molecules and the target. 163

Table 4. Docking parameter of S. aureus target proteins with naphthoquinones and crystallographic ligands.

167 Staphylococcus aureus target	Compound	CS	ΔG	GS	WdWExt
eptide deformylase (PDF)	Eleutherin	23.00	- 25.47	28.58	20.62
S170	Actinonin*	28.45	- 29.46	62.07	41.12
Regulator QacR	Eleutherin	33.79	-33.86	29.53	21.30
-172	Pentamidine*	36.58	- 36.62	56.31	39.71
Regulatory protein BlaR1	Eleutherin	28.67	- 28.71	35.19	21.68
G 74	Benzylpenicillin *	27.01	- 27.39	35.01	22.72
hMæthionine aminopeptidase (MetAP)	Isoeleutherin	21.79	- 22.33	33.72	33.72
e176	Ketoheterocyclo 618*	23.00	- 24.49	45.79	29.97
m177					

Score; GS – GoldScore; WdWExt – Van der Waals interactions; ΔG – Energy variation; * Reference ligand used for comparison.

In the interactions with MetAp, when comparing isoeleutherin and ketoheterocyclo 181 618, a similar binding profile with close values of CS, GS and Δ G was observed. The Van 182 der Walls energy was higher for isoeleutherin (Table 4). 183

When analyzing the molecular interactions, for PDF, both eleutherin and actinonin184show similar binding patterns in the active site of the protein (Figure 2). Both present185Van der Waals interactions with similar residues, such as VAL A:59, TYR A:147, GLU186A:185, SER A:57, LEU A:61, GLN A:65, GLY A:110, GLY A:58, and LEU A:112, with similar187lar distances (Table 4; Figure 2; Table S1), suggesting they comparably occupy the binding site.188

The two ligands present an unfavorable interaction (eleutherin with GLU A: 155 at 190 2.99 Å and actinonin with HIS A: 154 at 2.67 Å) indicating a possible steric conflict or repulsion. Based on the analysis of Pi-alkyl and Alkyl interactions, eleutherin and actinonin show a balanced distribution of these interactions. Actinonin presents two Alkyl interactions (VAL 59 and VAL 151) and one Pi-alkyl interaction (LEU 112). Eleutherin has 194 two Pi-alkyl interactions (VAL 59 and VAL 151) and one Alkyl interaction (LEU 112; 195 Figure 2; Table S1).



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Figure 2. Molecular interactions of eleutherin and actinonin with peptide deformylase – PDF. (A) 198 Interactions of eleutherin; (B) Interactions with the crystallographic ligand actinonin; RMSD - Root 199 Mean Square Deviation, value in angstrom. 200

For the QacR regulator, eleutherin forms significant interactions with specific residues in the binding site, with a predominance of Pi-pi stacked and Alkyl interactions. Residues such as TYR 93 and TYR 123 show both Pi-pi stacked and Alkyl interactions, 204 indicating a strong and diverse binding with eleutherin and the distances range from 205 approximately 4.80 Å to 6.63 Å. Pentamidine mainly forms Pi-sigma, Alkyl and conven-206 tional hydrogen bond interactions with residues in the protein 2 binding site, with distances ranging from approximately 3.16 Å to 5.24 Å, with emphasis on the formation of 208 a conventional hydrogen bond with LYS 60 (Figure 3; Tab.S.3- supplementary material). 209



Figure 3. Molecular interactions of eleutherin and pentamidine with the regulator QacR . (C) Inter-211 actions of eleutherin; (D) Interactions with the crystallographic ligand pentamidine; RMSD - Root 212 Mean Square Deviation, value in angstrom.

Related to BLAR1, eleutherin apparently forms specific favorable interactions (Pi-Sigma and Alkyl with shorter distance) that may be stronger and better accommodated 216 by the binding site of this target - a Pi-sigma bond at residue TYR 199, indicating a pla-217 nar interaction between the pi group of eleutherin and the sigma system of the tyrosine 218 residue, Alkyl bonds with residues ILE 201 and TYR 206. Benzylpenicillin interacted as 219 Pi-sulfur with PHE 91 and varied distances of Van der Waals interactions with other res-220 idues (Figure 4; Table S1). 221



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Figure 4. Molecular interactions of eleutherin and benzylpenicillin with the transcriptional regula-223 tor BLAR1. (E) Interactions of eleutherin; (F) Interactions with the crystallographic ligand ben-224 zylpenicillin; RMSD - Root Mean Square Deviation, value in angstrom. 225

In the intermolecular interactions of MetAP, isoeleutherin exhibits a variety of more specific interactions, such as Pi-pi T-shaped and Pi-Alkyl, while ketoheterocyclo 618 exhibits mainly Van der Waals and Pi-sulfur interactions. For HIS 76, for example, isoeleutherin exhibits Pi-pi T-shaped interactions in two different conformations and one Alkyl 230 interaction. Ketoheterocyclo 618 interacts with HIS 76 mainly through Van der Waals 231 bonds, indicating a less specific and more distant interaction compared to isoeleutherin 232 (Figure 5; Table S1).



Figure 5. Molecular interactions of isoeleutherin and ketoheterocycle 618 with methionine aminopeptidase – MetAP. (G) Interactions of isoeleutherin; (H) Interactions with the crystallographic ligand ketoheterocycle 618; RMSD - Root Mean Square Deviation, value in angstrom

HIS 175 also exhibits T-shaped Pi-pi interactions in two different conformations, in 239 addition to an Alkyl interaction with isoeleutherin, while with ketoheterocyclo 618, it 240 forms a specific Pi-sulfur interaction of 7.12 Å. LEU 174 interacts mainly through Alkyl 241 and Pi-Alkyl bonds with isoeleutherin, around 5.66 Å and 5.83 Å, indicating close con-242 tact, but also interacts through an Alkyl bond with ketoheterocyclo 618 at 5.00 Å. PHE 243 204 forms an Alkyl interaction with isoeleutherin at a distance of 6.46 Å, which does not 244 occur with ketoheterocyclo 618, which interacts through Van der Walls bonds (Figure 5). 245

The RMSD of the PDF with actinonin interactions (Figure 2), QacR Regulator with 246 pentamidine (Figure 3) and MetAP with ketoheterocyclo 618 (Figure 5) were less than 2 247 Å. However, redocking of BLAR1 with benzylpenicillin had an RMSD greater than 2 Å 248 (Figure 4). 249

3. Discussion

Eleutherin and isoeleutherin are promising molecules in terms of biological activi-251 ties[2,4,10]. The structure of eleutherin and isoeleutherin differs in the presence of a sin-252 gle chiral center in the pyran ring, along with the functionality of the a-methyl group. 253 Isoeleutherin has a pyran ring with an a-methyl group in a specific configuration, while 254 eleutherin has a slightly different structure [18]. These structural changes may be in-255 volved in the differences between the targets in S. aureus, inhibited by eleutherin but not 256 inhibited by isoeleutherin, resulting in different therapeutic potential. 257

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In this study, we investigated their activities against Staphylococcus aureus and Esch-258 erichia coli and explored the possible mechanisms of action. Both naphthoquinones inhib-259 ited S. aureus (Table 2) activity with eleutherin showing greater. When comparing these 260 results with other antimicrobial activity studies carried out with naphthoquinones, a 261 synergistic effect was observed, preventing the development of resistant strains of S. au-262 reus when associated with another antimicrobial [19]. Other studies corroborate these 263 findings, demonstrating the efficacy of extracts and fractions of E. plicata against S. aure-264 us [2,20,21] and the absence of activity against E. coli [22]. 265

The findings indicate the eleutherin activity does not involve a bactericidal effect. 266 Several bacterial signaling pathways may be involved in this effect, and it is necessary to 267 understand how naphthoquinones and crystallographic ligands act on *S. aureus* targets. 268 Eleutherin, which had the greatest antimicrobial activity, interacted with different proteins in S. aureus: PDF, regulator QacR and BlaR1, unlike isoeleutherin, which interacted 270 with a single target, MetAP (Table 3). 271

PDF is involved in the bacterial translation process and eleutherin, when binding to 272 this protein, interferes with this process and can lead to an accumulation of defective 273 proteins, which are functional. PDF inhibitors, such as actinonin, can be bactericidal, as 274 they interfere with essential protein synthesis, leading to cell death [23]. When compar-275 ing eleutherin with actinonin, they had almost compatible stability in the docking study 276 (Table 4; Figure 2). Regarding the distances, they are reasonably close, however actinon-277 in presents some slightly smaller distances, it indicates some advantage in binding effi-278 ciency. Eleutherin and the control showed the same binding pattern in the active site 279 (Figure 2, Table S1), these interactions are important for the stabilization of the protein-280 ligand complex (Table 4). 281

Since actinonin is bactericidal, the same was expected for eleutherin but it was not observed. The bacteriostatic activity observed in the present study may be related to the ATCC 6538 strain characteristics, considered moderately resistant to penicillin. Perhaps the result would be bactericidal if the strain was sensitive to penicillin.

Eleutherin forms more specific and stronger interactions (Figure 3; Figure 4), such 286 as Pi-Pi stacked with QacR, which are considered robust and specific, and the distances 287 suggest a good proximity for specific and stable interactions with amino acid residues in 288 the binding site of the regulator QacR (Figure 3; Tab. S.3 – supplementary material). The 289 QacR regulator is involved in bacterial multidrug resistance because it regulates the ex-290 pression of the QacA efflux pump. Inhibitors of this protein, such as pentamidine, in-291 crease the susceptibility of bacteria to antimicrobials by preventing the expression of the 292 efflux pump [24]. The binding free energy of eleutherin with the QacR regulator also in-293 dicates a spontaneous and thermodynamicalily favorable interaction (Table 4) [25], sug-294 gesting that eleutherin can effectively bind to this protein, inhibiting its regulatory func-295 tion in the expression of the QacA efflux pump. In this context, eleutherin may be an 296 important therapeutic adjuvant, being able to reverse bacterial resistance to different 297 classes of drugs. 298

BLAR1, another target of eleutherin, is a sensor part of a system that regulates re-299 sistance to β -lactam antibiotics in *S. aureus*. It controls the expression of β -lactamases and 300 penicillin-binding proteins, such as PBP2a [26]. In the docking, eleutherin formed more 301 specific and stronger bonds with BLAR1 compared to benzylpenicillin and also demon-302 strates a good ability to interact with the hydrophobic surface of the target through Van 303 der Walls forces (Figure 4; Table 5; Table S1) contributing to the total free energy [27], 304 significantly favoring stability, and high complementarity of the complex's binding sur-305 face [28], suggesting greater efficacy of eleutherin in preventing β -lactamase production 306 (Table 2). 307

The binding of eleutherin to BLAR1 is an interaction that releases energy (negative 308 enthalpy), corroborated by the highest ΔG values for this substance (Table 4). However, 309 the Root Mean Square Deviation (RMSD) between the conformation of eleutherin and 310 BLAR1 (Figure 4) was 3.0064 Å, indicating the structure departs from the conformation 311

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of the reference, the crystallographic ligand, that is, although eleutherin can bind to the target, there is significant conformational variability in the binding position over time. During binding, eleutherin may be inducing conformational changes in BlaR1 [29,30] 314

Inhibition of BlaR1 phosphorylation by synthetic kinase inhibitors reversed the resistance phenotype, restoring the bacteria's susceptibility to β -lactam antibiotics [31]. 316 This inhibition may be related to the eleutherin effect, which may be concentrationdependent, thus the use of higher concentrations of eleutherin can achieve greater effective inhibition of BLAR1 [32]. 319

Isoeleutherin inhibits MetAP, slowing down bacterial growth, a characteristic of bacteriostatic agents. The interference in protein maturation caused by MetAP inhibition prevents newly synthesized proteins from reaching their correct functional conformation, leading to an accumulation of misfolded and dysfunctional proteins. This accumulation can interrupt essential cellular processes and slow bacterial growth but does not cause immediate cell lysis [33]. This mechanism explains the possible bacteriostatic effect on *S. aureus* produced by isoeleutherin. 326

In molecular docking, MetAP and isoeleutherin showed comparable inhibition to 327 the control and both interactions are spontaneous and thermodynamically favorable 328 (Table 4), with slightly better stability for ketoheterocycle 618, reflected in a more nega-329 tive ΔG value (Table 4). Isoeleutherin presents more specific interactions with MetAP 330 and the crystallographic ligand ketoheterocycle 618 exhibits Van der Walls and pi-sulfur 331 bonds. The distances between MetAP residues and the ligands vary significantly be-332 tween the two compounds (Figure 5; Table S1). However, isoeleutherin tends to be clos-333 er to the residues HIS 76, HIS 175, LEU 174 and PHE 204, indicating possible stronger 334 and more specific interactions with this residue (Figure 5; Table S1). 335

In further analysis, the inactivity of eleutherin against *E. coli* could be explained by 336 the absence of homologous proteins or structural differences of the same protein targets 337 in these bacteria. For example, PDF in E. coli have structural variations [34] that reduce 338 the binding affinity of eleutherin, while proteins such as QacR and BlaR1 do not have di-339 rect functional homologs in E. coli [35,36]. The same occurs with isoeleutherin, since 340 MetAP in *E. coli* has structural variations that may reduce the binding affinity of isoeleu-341 therin. The structure of E. coli MetAP reveals a novel structure and a cobalt-dependent 342 active site, making it a new class of proteolytic enzyme [37,38]. 343

Furthermore, the composition and organization of the cell wall in Gram-negative 344 bacteria, such as E. coli, is more complex, due to a peptidoglycan layer between the in-345 ner plasma membrane and the outer membrane. The presence of the outer membrane, 346 with its porin proteins and active transport system, provides an additional barrier, hin-347 dering the entry of many antibiotics and antimicrobial compounds [39,40]. These struc-348 tural differences are crucial to understand the variation in susceptibility of different clas-349 ses of bacteria to antimicrobial agents [41], as observed in biological assays with eleuthe-350 rin and isoeleutherin. 351

4. Materials and Methods

3.1. Chemical studies

The powder from the bulbs of *E. plicata* was subjected to exhaustive maceration 354 with 96% ethyl alcohol for 21 days, with filtration of the extractive solution every 7 days 355 and subsequent addition of fresh solvent. The extractive solutions were concentrated in 356 a rotary evaporator under reduced pressure, obtaining the ethanolic extract of the bulbs 357 (EE). The EE was fractionated under hot reflux and concentrated in a rotary evaporator, 358 this process resulted in four fractions: hexane fraction (FrHex), dichloromethane fraction 359 (FrDcm), ethyl acetate fraction (FrAcOET) and methanolic fraction (FrMeOH; Vale et al., 360 2015). The presence of quinolinic compounds was monitored by thin layer chromatog-361 raphy using silica gel as the stationary phase, and hexane and ethyl acetate 4:1 as the 362 mobile phase. The plates were then visualized under visible and UV light. FrDcm pre-363

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sented a greater quantity of quinolinic compounds and was more selective for them, and this was the fraction chosen to be re-fractionated by an open chromatographic column, using silica gel as the stationary phase and solvent gradients with increasing polarity as the mobile phase. 367

The chromatographic column was monitored by TLC and fractions with similar 368 profiles were pooled. The subfractions that showed precipitates were recrystallized us-369 ing methanol as solvent. The TLCs that presented only one band were taken to NMR, 370 and it was possible to identify the isolation of eleutherin (ELE) and isoeleutherin (ISO). 371 These substances were analyzed by Hydrogen Nuclear Magnetic Resonance (1H NMR) 372 for identification purposes. 1H NMR analyses were performed on a Bruker Ascend 400 373 spectrometer (operating at 400 MHz for hydrogen). Samples were solubilized in deuter-374 ated chloroform (CDCl3). Chemical shifts (δ) were measured in ppm and coupling con-375 stants (J) in Hertz (Hz). Tetramethylsilane (TMS) was used as an internal reference. 376

 $Isoeleutherin - {}^{1}H NMR 400 MHz (CDCl3): d 1.32 (3H, d, J = 8.0 Hz, Me-3), d 1.52 (3H, d, J = 385 8.0 Hz, Me-1), d 2.22 (1H, dq, J = 4.0; 16.0 Hz, H4-ax), d 2.68 (1H, dd, J = 4.0; 16.0 Hz, H-4 eq), d 3.99 (3H, s, OMe-9), d 4.99 (1H, m, H1), d 7.27 (1H, d, J = 8.0 Hz, H-6), d 7.63 (1H, t, J = 8.0; 16 Hz, H-7), d 7.72 (1H, d, J = 8.0 Hz, H-6), d 7.63 (1H, t, J = 8.0; 16 Hz, H-7), d 7.72 (1H, d, J = 8.0 Hz, H-8). 13C NMR 100 MHz (CDCl3): d 19.93 (Me-3), d 21.67 (Me-1), d 388 29.98C-4), d 56.62 (OMe-10), d 62.64 (C-3), d 67.58 (C-1), d 117.99 (C-8), d 119.28 (C-7), d 119.93 (C-380 4a), d 134.26 (C11a), d 134.87 (C-11a), d 139.54 (C-5a), d 148.23 (C-9a), d 159.90 (C-9), d 182.90 (C-390 5), d 184.42 (C-10) (Figure S2).$

3.2. Antimicrobial activity

The microdilution technique (NCCLS, 2003) was performed to evaluate the antimi-394 crobial activity against strains of Staphylococcus aureus ATCC 6538 (gram-positive) and 395 Escherichia coli ATCC 32213 (gram-negative). Initially, the bacteria were cultured for 24 396 hours (prior to the test) in nutrient agar at 37°C. Subsequently, the microbial suspension 397 (inoculum) was prepared; approximately 3 to 4 isolated colonies were transferred to a 4 398 ml tube with Müller Hinton broth and homogenized in a vortex mixer. The inoculum 399 density was measured using a spectrophotometer, and if it was not between 0.08 and 0.1 400 (equivalent to a McFarland value of 0.5) adjustments were made by adding colonies. The 401 final inoculum concentration was 1.0x106CFU/mL. 402

The plates were pre-dosed with 10 μ L of each sample solubilized in methanol at concentrations of 1000, 500, 250, 125, 62.5, and 31.25 μ g/mL. After solvent evaporation (pre-dosing), 180 μ L of Müller Hinton broth and 10 μ L of the inoculum were added for a final volume of 200 μ L/mL in each well (CLSI, 2012). After incubation (35°C/24h), 10 μ L of triphenyl tetrazolium chloride (TTC) was added to all wells and the plates were incubated again for 4 hours. 403 404 405 406 407 408

Subsequently, the plates were visually read to determine the Minimum Inhibitory 409 Concentration (MIC), defined as the lowest concentration without changing the color of 410 the medium. Then, the absorbances were quantified by a spectrophotometer reader 411 (590nm) to determine the concentration that inhibits 50% of microbial growth (IC₅₀). The 412 IC₅₀ determination was performed by linear regression (GraphPad Prism 7.0 program) 413 and expressed as active (IC₅₀ < 100 μ g/mL), moderately active (IC₅₀ between 100 and 500 414 μ g/mL), weakly active (IC₅₀ between 500 and 1000 μ g/mL) and inactive (IC₅₀ greater than 415 1000 µg/mL) [22]. 416

To determine the Minimum Bactericidal Concentration (MBC), 10μ L of the concentration 417 trations equal to or greater than the MIC were removed from the well, seeded in Petri 418

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3.3. Target proteins involved in eleutherin and isoeleutherin activity

concentration without bacterial growth was considered MBC.

Eleutherin and isoeleutherin were designed using the Marvin Js program and pre-423 pared for docking in the BIOVIA® program. The online tool PharmMapper identified 424 potential drug targets of these compounds by reverse pharmacophore matching, com-425 bining the compound with an internal database of pharmacophore models [42]. Then, 426 the targets with the highest scores were selected for molecular docking, through score 427 adjustment to evaluate and classify the potential interactions between the molecules of 428 interest and several target proteins, based on their three-dimensional conformation in-429 volving shape compatibility, binding energy, chemical interactions, and experimental 430 data (crystallography and bioassay data). 431

dishes containing Müller Hinton Agar and incubated at 35°C for 24 hours. The lowest

The targets were obtained from the Protein Data Bank (PBD). Subsequently, the interactions between eleutherin, isoeleutherin and *S. aureus* proteins were explored through molecular docking, based on the preliminary MIC and CBM results.

3.4. Molecular docking

Molecular docking simulations were performed with the naphthoquinones eleutherin and isoeleutherin selected in the Marvin Js program. This study used the GOLD 2020.1 program [43] from the Cambridge Crystallographic Data Center - CCDC, located in Cambridge, United Kingdom. This software employs a genetic algorithm to explore and select conformations of flexible compounds capable of binding to the active site of a protein [43].

The conformations were evaluated using the GoldScore scoring function with a 443 100% effective search. To improve the prediction accuracy, ChemScore and GoldScore 444 were selected for this study. All ligand-receptor interactions were analyzed using BIO-445 VIA ® software. 446

In GoldScore, affinity is evaluated by physical and chemical interactions between 447 the atoms of the ligand and the protein to calculate the score that represents the stability 448 of this complex. The components that form this field are protein-ligand hydrogen bond 449 energy, external Van der Waals energy, internal Van der Waals energy of the ligand, and 450 the intramolecular hydrogen bond energy of the ligand [43]. The ChemScore function is 451 empirically derived from experimental data of known protein-ligand complexes, allow-452 ing parameter adjustments to provide accurate estimates of the binding free energy and, 453 consequently, the affinity of that binding, estimating the total change in free energy that 454 occurs in the binding, using as criteria the hydrogen interactions, hydrophobic interac-455 tion area, unfavorable interactions and binding free energy [41,44]. 456

The higher the GoldScore value, the greater the protein residues binding capacity, 457 indicating greater affinity between the ligand and the protein, and more stability in the 458 complex [45]. In turn, stability is also determined by the binding free energy (ΔG), where 459 a negative ΔG indicates complex formation is thermodynamically favorable and the complex is stable. 460

In this sense, the best ligand choice is related to the complex stability, ligand specificity for the target, general affinity, and specific context of application [26], for example, inhibiting bacteria.

Molecular docking predictions were validated trough redocking, in which the crystallographed ligand was relocated to the protein active site, predicting its conformation. Validation was determined considering only structures whose root mean square deviation (RMSD) was less than 2 Angstroms (Å).

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Eleutherin and isoeleutherin have inhibitory activity against *S. aureus*, their bacteriostatic activity seems to be related to the PDF and MetAP proteins, respectively. In addition, eleutherin interacts with the regulator QacR, involved in bacterial resistance, as it regulates the expression of the efflux pump. Inhibition of this regulator may prevent the bacteria from becoming resistant to different classes of drugs. 475

Another important binding target of eleutherin is BLAR1, a sensor system that regulates resistance to β -lactam antibiotics. Its inhibition is an important tool for preventing resistance to β -lactam class. Based on the docking data, it is the main target of eleutherin action. 476

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