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Modulatory Effect of *Croton heliotropiifolius* Kunth Ethanolic Extract on Norfloxacin Resistance in *Staphylococcus aureus*

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Abstract: The high frequency of infectious diseases has spurred research into effective tactics to combat microorganisms that are resistant to several drugs. The overproduction of the transmembrane efflux pump protein NorA, which may export hydrophilic fluoroquinolones, is a common mechanism of resistance in *S. aureus* strains. This work evaluated the antimicrobial activity of the ethanolic extract from the leaves of *Croton heliotropiifolius* (EECH) against different bacterial and fungal strains, as well as investigating its modulating effect on the resistance to norfloxacin in a *Staphylococcus aureus* SA1199B overproducing the NorA efflux pump. Microdilution assays were used to assess the EECH's antibacterial efficacy. The MIC of norfloxacin or ethidium bromide (EtBr) against the SA1199B strain was determined in the presence or absence of the EECH in order to assess the modifying influence on drug resistance. The EECH showed no activity against the Gram-positive and Gram-negative bacterial strains tested. The EECH also showed no antifungal activity against *Candida albicans* ATCC 10231. On the other hand, the extract reduced the MIC values for norfloxacin against SA1199B at subinhibitory concentrations. In addition, the EECH also reduced the MIC values of EtBr at subinhibitory concentrations, suggesting the occurrence of phytochemicals that inhibit efflux pumps. Molecular docking showed that retusin, a flavonoid found in the extract, could compete with norfloxacin at the orthosteric site of the NorA, indicating that it could be a potential efflux pump inhibitor. However, isolated retusin did not enhance the activity of norfloxacin or EtBr and it did not inhibit the EtBr efflux, showing that it is not a NorA inhibitor. Even though *C. heliotropiifolius* is a source of phytochemicals that function as adjuvants for norfloxacin, isolated retusin cannot be used in conjunction with norfloxacin to treat infections brought on by *S. aureus* that overproduces NorA.

Keywords: medicinal plants; fluoroquinolone; bacterial resistance; efflux pump inhibitors

Citation: Brito, S.B.d.; Alcântara, F.A.d.O.; Leal, A.L.A.B.; Veloso, K.H.d.S.; Sousa, L.d.R.; Oliveira, A.P.d.; Santos, A.D.d.C.; Dutra, L.M.; Almeida, J.R.G.d.S.; Nogueira, C.E.S.; et al. Modulatory Effect of *Croton heliotropiifolius* Kunth Ethanolic Extract on Norfloxacin Resistance in *Staphylococcus aureus*. *Drugs Drug Candidates* **2024**, *3*, 1–12. https://doi.org/10.3390/ddc3010001

Academic Editors: Fatma Sezer Senol Deniz and Duygu Sevim

Received: 25 August 2023
Revised: 6 December 2023
Accepted: 13 December 2023
Published: 22 December 2023

1. Introduction

Infections caused by multidrug-resistant *Staphylococcus aureus* pose a major problem and are considered as a serious health risk problem [1]. Resistance mediated by the overproduction of proteins that extrude antibiotics out of the bacterial cell, known as efflux pumps, is a common mechanism verified in *S. aureus* strains [2]. NorA is a proton motive force-dependent efflux pump of *S. aureus* that extrudes hydrophilic fluoroquinolones such as norfloxacin and ciprofloxacin, as well as ethidium bromide and ammonia quaternary compounds [3].

Several approaches have been put forth to combat bacterial resistance, such as the use of artificial or natural compounds that can increase the effectiveness of antibiotics by inhibiting resistance processes [4,5]. This method keeps the antibiotic effective even against resistant organisms by co-administering it with an inhibitor that neutralizes the resistance [6,7].

Several natural or synthetic compounds have been characterized as efflux pump inhibitors (EPIs), including alkamides [8], terpenoids [9], flavonoids [10,11], chalcones [12–14], imidazolidines [15], 1,8-naphthyridines sulfonamides [16], 2-phenylquinoline derivatives [17], pyridine-3-boronic acid derivatives [18], and indole-based derivatives [19], among others. Clinically approved drugs for other clinical indications, such as nilotinib and azelastine, have shown good inhibitory activity against NorA [20]. Reserpine, verapamil, omeprazole, prochlorperazine, and paroxetine, which are also drugs already in clinical use for other indications, have been suggested as prototypes for the development of NorA inhibitors [3].

Croton heliotropiifolius Kunth (Euphorbiaceae) is a shrub, popularly known as velame, that is found in different Brazilian biomes, including the Amazon Forest, Atlantic Forest, Cerrado, and Caatinga [21,22]. Rural communities from the Brazilian northeast have cited its medicinal use, especially for wound healing and blood purification [23].

The ethanolic extract from the stem bark of *C. heliotropiifolius*, as well as several phytochemicals isolated from it, were able to inhibit the activity of acetylcholinesterase [24]. Moreover, the aqueous extract from the leaves of *C. heliotropiifolius* inhibited the growth of *Ralstonia solanacearum* and was able to inhibit the formation of biofilm by this phytopathogen [25]. The essential oil from the leaves of *C. heliotropiifolius* showed activity against *Aedes aegypti* larvae [26,27], as well as repellent, ovicide, and larvicide activities against *Tribolium castaneum* [28]. Furthermore, it was also verified that the essential oil of this species showed weak or moderate activity against Gram-positive and Gram-negative bacteria [29]. The antibacterial activity of the essential oil of the leaves and stem of *C. heliotropiifolius* was also evidenced against *Staphylococcus aureus* and *Bacillus subtilis* [30].

Although the intrinsic antimicrobial activity of natural products extracted from *C. heliotropiifolius* has been reported, there are few studies addressing the evaluation of the modulating effect on resistance to traditionally used antibiotics. The objective of this research was to evaluate the antimicrobial activity of the ethanolic extract from the leaves of *Croton heliotropiifolius* (EECH) against different microorganisms, as well as investigate the effect of the EECH on the activity of norfloxacin against a *Staphylococcus aureus* strain overproducing the NorA efflux pump.

2. Results and Discussion

The ^{1}H NMR spectrum of the ethanolic extract of *C. heliotropiifolius* leaves showed signals throughout the spectral region (Figure 1 and Table 1). The resonance related to the aliphatic hydrogens revealed the presence of doublets at δ 0.86 (J = 6.5 Hz, ^{1}H,) and 1.04 (J = 6.5 Hz, ^{1}H) characteristic of C-17 methyl hydrogens at δ 22.7 and 28.6 of the clerodane diterpene skeleton, respectively. Moreover, the singlet at δ 1.59 at C-18 was determined to have a direct ^{1}H-^{13}C correlation from HSQC with the carbon at δ 16.0 and a long-range ^{1}H-^{13}C correlation from HMBC with the carbons attributed at δ 39.8 (C-5), 124.4 (C-3), and 135.0 (C-4), whereas the signal at δ 5.11 (m, ^{1}H) displayed a direct ^{1}H-^{13}C correlation from HSQC with the carbon at C-3 and long-range ^{1}H-^{13}C correlation from HMBC with the carbons C-5 and C-18, confirming the presence of clerodane diterpene in the extract of

the *C. heliotropiifolius*. This diterpene type has been highlighted as constituting the main secondary metabolites in *Croton* species; however, the intense overlapping of signals makes it difficult to identify them completely [31–33]. However, phytochemical investigations have also confirmed the presence of tigliane, kaurane, crotofolane, labdane, cembrane, abietane, casbane, halimane, pimarane, cleistanthane, atisane, phytane, and laevinane diterpenes [31].

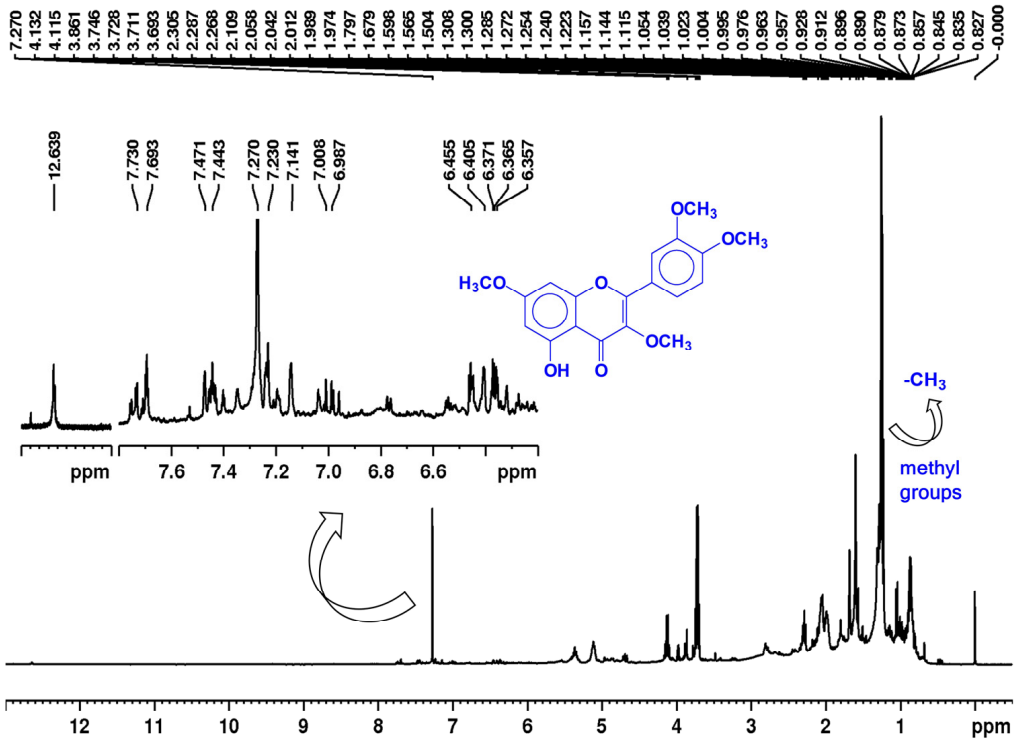

Figure 1. ^{1}H NMR spectrum of the ethanolic extract of *Croton heliotropiifolius* leaves.

Table 1. Resonance assignments of the metabolites based on 1D and 2D NMR analysis of the EEHC.

Metabolites	Assignment	δ ^{1}H (mult., *J* in Hz)	δ ^{13}C	HMBC Correlations ^{13}C (ppm)
Retusin	C-6	6.36 (*d*, 2.4)	98.1	92.3 (C-8), 106.2 (C-10), 156.8 (C-10), 165.6 (C-7)
	C-8	6.45 (*d*, 2.4)	92.3	98.1 (C-6), 106.2 (C-10), 165.6 (C-7)
	C-2′	7.69 (*d*, 2.3)	111.7	122.2 (C-5′), 151.1 (C-2), 151.4 (C-4′)
	C-5′	6.99 (*d*, 8.8)	110.8	122.9 (C-1′), 149.2 (C-3′)
	C-6′	7.74 (*dd*, 8.8 and 2.3)	122.2	111.7 (C-2′), 151.4 (C-4′)
	OCH_3-3	3.86 (*s*)	60.3	139.1 (C-3)
	OCH_3-7	3.87 (*s*)	56.0	165.6 (C-7)
	OCH_3-3′	3.97 (*s*)	55.9	149.2 (C-3′)
	OCH_3-4′	3.98 (*s*)	55.8	151.4 (C-4′)
	OH-5	12.64 (*s*)	-	161.9 (C-5)
Clerodane diterpenes	CH-3	5.11 (*m*)	124.4	16.0 (C-18), 39.8 (C-5)
	CH_3-17	0.86 (*d*, 6.5)	22.7	22.8 (C-7), 28.1 (C-8), 39.1 (C-9)
	CH_3-17	1.04 (*d*, 6.5)	28.6	20.3 (C-7), 27.8 (C-8), 29.6 (C-9)
	CH_3-18	1.59 (*s*)	16.0	39.8 (C-5), 124.4 (C-3), 135.0 (C-4)

In the present study, the expansion and increase in intensity of the aromatic hydrogen region allowed the identification of the flavonoid 5-hydroxy-3,7,3′,4′-tetramethoxyflavone, known as retusin (Figure 1). This flavonoid was identified due to the existence of the aromatic hydrogens at δ 6.36 (d, J = 2.4 Hz, ^{1}H) and 6.45 (d, J = 2.4 Hz, ^{1}H,) assigned to ring A, whereas the hydrogens at δ 6.99 (d, J = 8.8 Hz, ^{1}H,), 7.69 (d, J = 2.3 Hz, ^{1}H), and 7.74 (dd, J = 8.8 and 2.3 Hz, ^{1}H,) are representative of the ring B of that flavonoid. Four signals at δ 3.86 (s, ^{3}H), 3.87 (s, ^{3}H), 3.97 (s, ^{3}H), and 3.98 (s, ^{3}H) are for the C-3, C-7, C-3′, and C-4′ methoxy groups, respectively, and the singlet at δ 12.64, typical of a hydroxyl,

chelated at C-5 with the carbonyl group. In addition to this flavonoid, other characteristic signals of phenolic compounds were observed in the region at δ 6.00–13.00 ppm. Although in a smaller extension, the flavonoids are frequently reported in *Croton* species [34–36]. The metabolite and signals identified were established based on the direct and long-range maps from the ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC NMR experiment and comparison with the literature data.

Plant extracts presenting MIC values higher than 1000 µg/mL have been considered clinically irrelevant [37]. Taking this criterion into account, the results found in the present study showed that the EECH was inactive against the Gram-positive bacterial strains *S. aureus* SA1199B, *S. aureus* ATCC25923, and *S. pyogenes*, as well as against the Gram-negative bacterial strains *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, and *Salmonella* Typhimurium ATCC14028. It was also not active against the yeast strain *C. albicans* ATCC10231 (Table 2). These results were different from a previous study that reported antimicrobial activity for the methanol–chloroform extract from both the stem bark and roots of *C. gratissimus*, which showed activity against *S. aureus* and *Bacillus cereus* [38]. The methanol extract from the leaves of *C. megalobotrys* was active against *S. aureus* and *Enterococcus fecalis*, and the methanol extract from the leaves of both *C. megalobotrys* and *C. steenkapianus* was able to inhibit the growth of *P. aeruginosa* [39]. The essential oil from the leaves of *C. zehntneri* also showed antimicrobial activity against *S. aureus* and *P. aeruginosa* [40].

Table 2. Minimum inhibitory concentration (MIC) values for the ethanolic extract from the leaves of *C. heliotropiifolius* (EECH) against different microorganisms. MIC values for chlorpromazine (CPZ), norfloxacin, and ethidium bromide (EtBr) were determined for the SA1199B strain only, aiming to determine subinhibitory concentrations for use in the modulation assays.

	MIC (µg/mL)			
CEPAS	**EECH**	**CPZ**	**NOR**	**EtBr**
Staphylococcus aureus SA1199B	16,384	128	64	32
Staphylococcus aureus ATCC 25923	8192	-	-	-
Streptococcus pyogenes ATCC 19615	4096	-	-	-
Escherichia coli ATCC 25922	4096	-	-	-
Pseudomonas aeruginosa ATCC 27853	16,384	-	-	-
Salmonella Typhimurium ATCC 14028	16,384	-	-	-
Candida albicans ATCC 10231	16,384	-	-	-

A previous study verified that the ethanolic extract from the stem bark of *C. heliotropiifolius* collected in the northeast coastal region of Brazil showed good activity against *Candida albicans* [24]. On the other hand, the ethanol extract obtained in the present study was prepared from a plant that is located 1131 km from the coast, thus under different environmental conditions. Variations in genotype and habitat, as well as soil type, collection duration, extraction technique, and extract drying temperature, can all have an impact on the final phytochemical composition found in medicinal plants [29,41]. Thus, the absence of antifungal activity found in the extract analyzed in the present study may be related to quantitative and/or qualitative differences in its chemical composition.

Additionally examined was the inhibitory action of the EECH/norfloxacin combination against the *S. aureus* SA1199B strain, which overexpresses the *norA* gene that codes for the NorA efflux pump [42]. Overproduction of efflux pumps such as NorA is one of the fluoroquinolone resistance mechanisms found in some multidrug-resistant *S. aureus* strains [3]. As shown in Figure 2a, when subinhibitory concentrations of EECH were added to the growth medium, the MIC values for norfloxacin dropped. The modulating effect verified for the EECH was similar to that caused by chlorpromazine (CPZ), a known efflux pump inhibitor (EPI). According to this finding, EECH could contain phytochemicals that can modify fluoroquinolone resistance in *S. aureus* overexpressing NorA. However, no modulatory effect was observed for retusin in combination with norfloxacin, indicating that it is not a NorA inhibitor (Figure 2a).

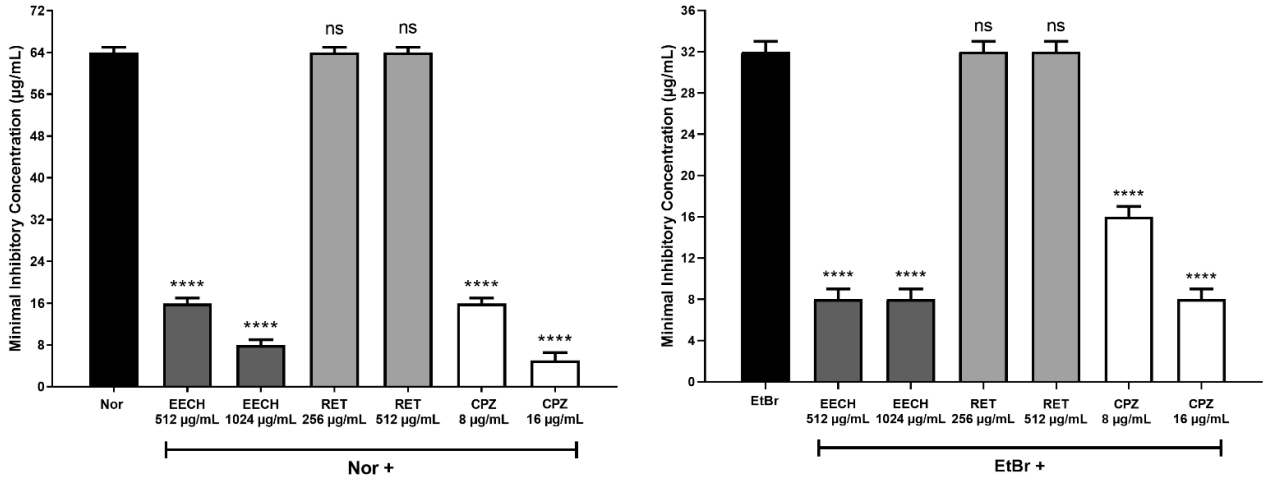

Figure 2. MIC values for norfloxacin (Nor) (**a**) and ethidium bromide (EtBr) (**b**) against *S. aureus* SA1199B in the presence or absence of the ethanolic extract from the leaves of *C. heliotropiifolius* (EECH), retusin (RET), or chlorpromazine (CPZ). Each result represents the geometric mean of three simultaneous experiments. One-way ANOVA followed by Dunnett's post-hoc test (**** $p < 0.0001$; ns = not significant).

To determine whether the confirmed modifying effect of EECH was related to efflux pump inhibition, assays were conducted using EtBr instead of norfloxacin. As previously reported, only efflux pumps such as NorA can mediate bacterial resistance to EtBr [43]. As verified in Figure 2b, the EtBr resistance was also attenuated in the presence of subinhibitory concentrations of the extract, indicating the modulating effect could be due to NorA inhibition by phytochemicals present in the EECH.

A molecular docking was performed between retusin and the NorA efflux pump, and the best position of the flavonoid docked on the binding site of the NorA model is shown in Figure 3A. The binding score found for retusin with the NorA model was −7.6 kcal/mol, and the flavonoid was able to make short contacts with several residues, in particular Phe140, Glu222, and Arg310 (Figure 3B). The orthosteric binding site of the NorA protein was described as consisting mainly of the residues Ile23, Phe140, Glu222, Tyr225, Ile244, Phe303, and Arg310 [44]. In the present study, we verified that retusin interacts roughly with the same residues that make up the orthosteric binding site.

Based on the results obtained from the molecular docking analyses, one could argue that the tetramethoxylated flavonoid retusin could be a competitive inhibitor of the NorA efflux pump. However, in vitro assays performed in the present study showed that retusin did not reduce the MIC values of norfloxacin or EtBr against *S. aureus* SA1199B (Figure 2b), indicating that this phytochemical is not a NorA inhibitor. These findings were supported by results obtained via fluorometry assays, which showed that retusin did not increase the intracellular accumulation of EtBr in the *S. aureus* SA1199B strain (Figure 4), confirming that retusin is not a NorA inhibitor. These results indicate that retusin cannot be used in combination with norfloxacin in the treatment of infections caused by NorA-producing strains of *S. aureus*.

Prior research has demonstrated that methoxy groups are commonly found in a variety of NorA inhibitors, including phyllanthin [45], and riparin B [8]. Other work verified that increasing the number of methoxy groups enhanced the performance of chalcones as NorA inhibitors, with the best performance presented by the 3,4,4′-trimethoxy-chalcone [46]. A further investigation verified that 4,5,6,7-tetramethoxyflavone, the most methoxylated molecule, exhibited the highest modulating agent performance, reducing the minimal inhibitory concentration of norfloxacin against *S. aureus* SA1199B by a factor of 16 [47]. However, quercetin, which is a flavonoid analogue of retusin without methoxy groups,

has been reported as a TetK and NorA inhibitor [48], suggesting that the mere presence of methoxyls in a compound is not a determining factor for its action as a NorA inhibitor.

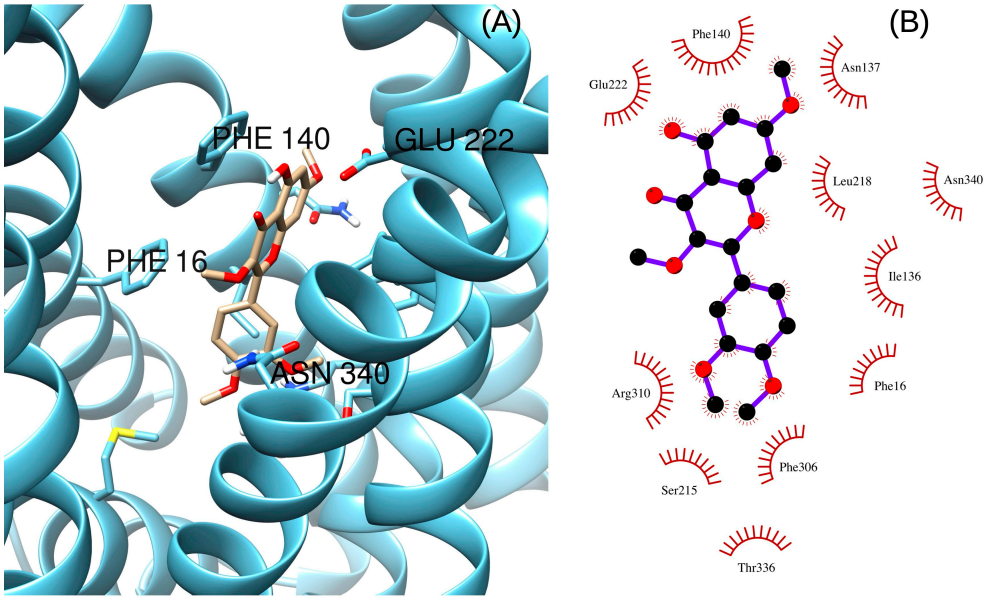

Figure 3. Best pose of the flavonoid (brown) on the binding site of the NorA model (**A**). Two-dimensional ligand–protein interaction diagram of the flavonoid and the NorA protein model (**B**).

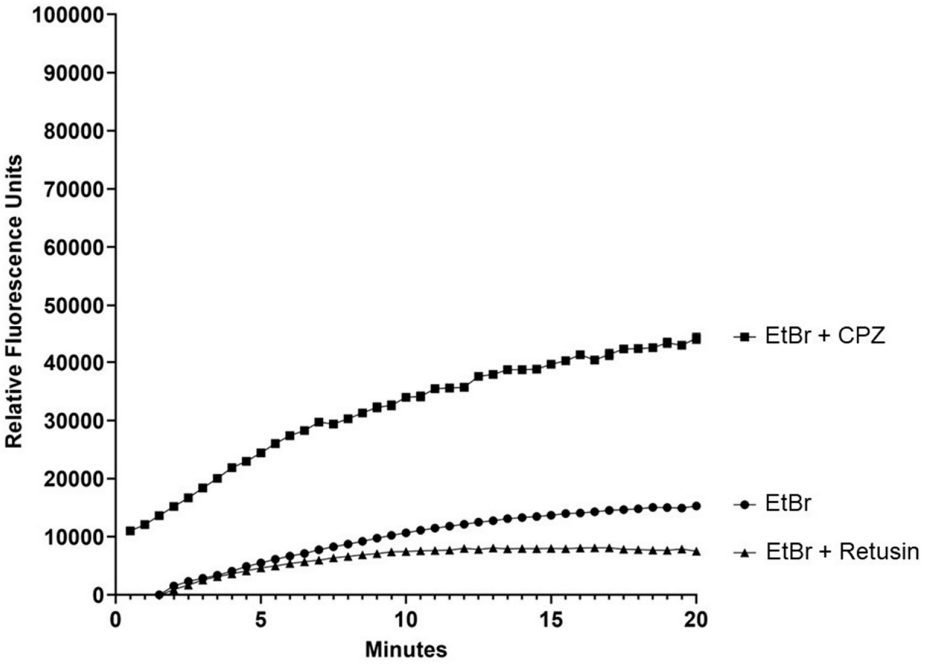

Figure 4. EtBr accumulation is affected by retusin in *S. aureus* SA1199B overexpressing *norA*. The bacterial suspension was incubated for 20 min at 37 °C with EtBr (3 µg/mL) added either with or without subinhibitory doses of retusin (128 µg/mL) or CPZ (8 µg/mL). The calculation of relative fluorescence units involved removing each point by the corresponding blank control. Efflux inhibition is indicated by variations in the relative fluorescence units obtained with or without CPZ or retusin.

Attenuation of bacterial drug resistance has been reported for different plant species [49,50]. For *Croton* species, it has been demonstrated that volatile components from the essential oil of *Croton zehntneri* potentiate the gentamicin activity against *Pseudomonas aeruginosa* [40], as well as the norfloxacin activity against *S. aureus* SA1199B [51]. Another study verified that an ethanolic extract from the leaves of *C. campestres*, as well as its hexane partition fraction, enhanced the

norfloxacin activity against *S. aureus* SA1199B [52]. The ethanolic extract from the leaves of *C. campestres*, as well as their hexane and dichloromethane fractions, attenuated the resistance to amikacin, gentamicin, and neomycin against multidrug-resistant *S. aureus*, *E. coli*, and *P. aeruginosa* [53].

Although this study has the limitation of not being able to identify NorA-inhibiting compounds in EECH, the results obtained showed that the flavonoid retusin can be ruled out as a NorA inhibitor. There is therefore a need for future studies to elucidate which phytochemicals present in EECH are related to its modulating effect on norfloxacin resistance in this *S. aureus* strain.

3. Materials and Methods

3.1. Plant Material and Extraction

Leaves of *C. heliotropiifolius* were collected in Itaueira (latitude 7°35′33″ S and longitude 43°00′54″ W), Piauí, Brazil in March 2013. Plant material was identified, and a voucher specimen was deposited at the Herbarium Graziela Barroso with the number TEB29158. Leaves were dried in a forced-circulation oven at 60 °C for five days. Then, 500 g of the dried material was pulverized in a knife mill (MA680, Marconi Equipamentos Laboratório, Piracicaba, SP, Brazil). The leaf powder was submitted to an exhaustive maceration process in 95% ethanol at a sufficient volume for total immersion for 72 h in a covered glass bottle at room temperature. The filtered ethanolic extract was concentrated under reduced pressure on a rotary evaporator at 60 °C (Laborota 4000, Heidolph Instruments, Schwabach, BY, Germany), yielding 30 g of dried ethanolic extract (EECH).

3.2. Nuclear Magnetic Resonance (NMR) Analysis

The extract was dispersed in 600 µL of deuterated chloroform ($CDCl_3$) and analyzed using NMR. An AVANCE III 400 NMR spectrometer (Bruker, Karlsruhe, Germany) running at 9.4 T was used to collect 1D and 2D NMR data at 298 K. The spectrometer observed ^{1}H and ^{13}C at 400 and 100 MHz, respectively. A 5 mm z-gradient direct detection probe (BBO) was included with the NMR spectrometer. The average coupling constants 1J(C,H) and LRJ(C,H) of 140 and 8 Hz, respectively, were optimized for ^{1}H-^{13}C HSQC (one-bond) and HMBC (long-range) NMR correlation experiments. The coupling constants (J) in Hz and all ^{1}H and ^{13}C NMR chemical shifts (δ) in ppm were provided in relation to the TMS signal at 0.00 as an internal reference.

3.3. Strains and Chemicals

Evaluation of the intrinsic antimicrobial activity of the EECH was performed against standard microbial strains, including *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 19615, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella* Typhimurium ATCC 14028, and *Candida albicans* ATCC 10231. The antimicrobial activity of the EECH was also evaluated against the *S. aureus* SA1199B strain overexpressing the *norA* gene (which encodes the NorA efflux pump [54]), which was kindly provided by Dr. Glenn Williams Kaatz (Department of Internal Medicine, Division of Infectious Diseases, Wayne State University, Detroit, MI, USA). Assays for the evaluation of the modulating effect on drug resistance were performed only with the *S. aureus* SA1199B strain. The bacterial strains were maintained on brain heart infusion agar (BHIA, Himedia, India) slants at 4°C. The yeast strain was maintained on Sabouraud dextrose agar (SDA, Himedia, India) slants at 4 °C.

Before the assay, the bacterial strains were grown overnight at 37 °C in brain heart infusion (BHI, Himedia, India). The yeast strain was grown for 24 h at 37 °C in Sabouraud dextrose broth (SDB, Himedia, India). The following chemicals were acquired from Sigma Chemical Corp. in St. Louis: norfloxacin (Nor), ethidium bromide (EtBr), retusin (CAS 1245-15-4), and chlorpromazine (CPZ). A solution of 1 M NaOH and sterile distilled water (1:9 ratio) was used to dissolve norfloxacin. Sterile water was used to dissolve retusin, CPZ, and EtBr.

3.4. Assays for Evaluation of the Intrinsic Antimicrobial Activity

Dimethyl sulfoxide (DMSO) was used to generate a stock solution of EECH, which was then diluted to a final concentration of 16,384 µg/mL in sterile distilled water. The 96-well plate micro-dilution method was used to find the minimal inhibitory concentrations (MICs) in BHI broth. To prepare bacterial suspensions, the bacterial growth on slant agar was seeded by multiple streaks in petri dishes containing nutrient agar (Himedia, India), followed by incubation at 37 °C for 24 h. Following this time, the bacterial growth was moved to a falcon tube with 3.0 milliliters of BHI (Himedia, India), which was incubated at 37 °C for 24 h. A bacterial solution was made from this culture in sterile saline (0.85% NaCl), standardized to a density that corresponded to 0.5 on the McFarland scale (approximately 1.5×10^8 CFU/mL, colony-forming units/mL). In order to get the final inoculum in each well to approximate 10^5 CFU/mL, this suspension was diluted in BHI. The solution that was diluted with BHI (100 µL) was placed in the microtiter plate wells in alphabetical order (A–H). After that, wells A through G were serially diluted with 100 µL of the EECH (at 16,364 µg/mL) at concentrations ranging from 8182 to 8 µg/mL, making sure to homogenize the solution three times before moving on to the next well. The test product was left out of the last well (H), which acted as a positive control for bacterial growth. As a negative growth control, a row of wells that were merely filled with BHI broth (without the test product or inoculum) was utilized (control of contamination of the medium and/or microplates). Microtiter plates were incubated at 37 °C for 24 h. Subsequently, 20 µL of resazurin (0.01% *w*/*v* in sterile distilled water) was introduced into each well to detect bacterial growth via the color change caused by the reduction of resazurin (blue) to resorufin (pink) in the presence of viable cells [55]. All tests were performed in triplicate.

Yeast suspensions were made in sterile saline, which was diluted to produce a cell population of roughly 1.0×10^5 CFU/mL after being standardized to 0.5 on the McFarland standard scale (or roughly 1.0×10^6 CFU/mL). As previously mentioned, antifungal assays were carried out using the microdilution method in double-concentrated Sabouraud dextrose broth (SDB) containing a 10^5 CFU/mL yeast suspension and EECH solutions ranging from 8182 to 8 µg/mL. The microtiter plates were incubated at 37 °C for 24 h. By transferring an aliquot from each well of the MIC test microtiter plate to a Petri dish containing Sabouraud dextrose agar (SDA) and assessing cell viability following a 24 h incubation at 37 °C, the inhibition of fungal growth was verified. Every test was run in triplicate.

3.5. Assays for Evaluation of the Modulating Effect of Antibiotic-Resistance

To evaluate if EECH or retusin were able to modulate antibiotic resistance in the *S. aureus* SA1199B strain, MIC values for norfloxacin and EtBr were determined in the presence or absence of sub-inhibitory concentrations of the EECH or retusin (MIC 1/32 or MIC 1/16). Antibiotic or EtBr concentrations ranged from 0.125 to 128 µg/mL. Microtiter plates were incubated at 37 °C for 24 h and readings were performed with resazurin as previously described. Additionally, control experiments were conducted using CPZ, a recognized efflux pump inhibitor, in place of EECH [42].

3.6. Statistical Analysis

The geometric mean values were calculated to standardize the data; tests were carried out in triplicate throughout the experiments. The standard deviation and error deviation of the geometric means were disclosed. The statistical analysis was carried out with GraphPad Prism 5.02. ANOVA was used to compare treatment with norfloxacin (or EtBr) alone, in conjunction with EECH, or in conjunction with CPZ. Dunnett's post-test was used to examine the differences, and a value of $p < 0.05$ considered to be statistically significant.

3.7. Docking Procedure

The following is how the NorA model for the docking process was built. First, the Universal Protein Resource database (Uniprot, Entry Q03325) was searched to find the

NorA sequence of the *S. aureus* SA1199B strain. The homology model was then constructed using the SWISS-MODEL [56] service. Among the 50 templates produced, the *E. coli* YajR transporter's (PDB-ID: 3wdo) structure served as the basis for the template as it had the highest GMQE (global model quality estimation) score. The model was subsequently solvated and submitted to a 0.5ns molecular dynamics run utilizing the AMBER force-field via the MDWeb [57] service. The grid box for the docking process was defined as a 30 × 30 × 30 Å box surrounding the geometric centroid of the model. This was conducted using the Autodock Vina 1.1.2 program (La Jolla, CA, USA) [58]. All other parameters were left at their default levels, with the exception of mixing non-polar hydrogen atoms and adding partial Gasteiger charges to ligand atoms. The binding score was used to choose the best outcomes.

3.8. Fluorometry Assays for Evaluation of the Inhibition of Ethidium Bromide Efflux

Using a semi-automated EtBr technique, the putative efflux pump inhibitory activity of retusin was evaluated following a modified version of the protocol [59]. The strain *S. aureus* SA1199B was cultured in BHI until an optical density at 600 nm of 0.6 was achieved, followed by centrifugation at 5000× *g* for 5 min. Pellets were again suspended in two milliliters of saline. Bacterial suspensions were vortexed and then transferred to 96-well plates followed by the addition of a saline solution containing EtBr (3 μg/mL) and retusin (128 μg/mL) or chlorpromazine (16 μg/mL). As a positive control, a bacterial solution containing solely EtBr (3 μg/mL) was employed. Saline solution was used to produce blank controls for every test. After 60 min, the plates were put in an StepOnePlus Real-Time PCR SystemTM Heat Cycler (Applied Biosystems, Waltham, MA, USA) and the relative fluorescence, which represents the amount of EtBr that the bacteria have accumulated, was evaluated. Values for the corresponding blank controls were subtracted in order to compute the relative final fluorescence (RFF). The ability of the compound to prevent EtBr efflux was indicated by differences in the RFF between the test and positive control experiments.

4. Conclusions

In conclusion, the EECH did not present activity against the Gram-positive strains *S. aureus* SA1199B, *S. aureus* ATCC25923, and *S. pyogenes* or against the Gram-negative strains *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, and *Salmonella* Typhimurium ATCC14028. It was also not active against yeast strain *C. albicans* ATCC10231. On the other hand, the EECH potentiates the norfloxacin activity against the *S. aureus* SA1199B strain overproducing NorA. In addition, EECH also reduced the MIC values of EtBr at subinhibitory concentrations, suggesting the occurrence of phytochemicals that inhibit efflux pumps. Although the molecular docking results suggested the potential interaction of retusin with NorA, this flavonoid did not reduce the MIC values of norfloxacin or EtBr against the *S. aureus* SA1199B strain. Moreover, retusin did not inhibit the EtBr efflux in the *S. aureus* SA1199B strain, indicating that it is not a NorA inhibitor. However, this study opens the perspective for future research aiming at the isolation and characterization of other phytoconstituents from EECH acting as modulators of drug resistance. These potential efflux pump inhibitors could be used as enhancers of norfloxacin activity in the therapy of infections caused by NorA-overproducing *S. aureus*.

Author Contributions: Conceptualization, H.M.B., D.D.R.A., N.C.-M., J.S.N.d.S. and J.R.G.d.S.A.; Resources, H.M.B., C.E.S.N. and J.R.G.d.S.A.; Formal analysis, A.P.d.O., A.D.d.C.S. and L.M.D.; methodology, S.B.d.B., A.L.A.B.L., F.A.d.O.A., K.H.d.S.V. and L.d.R.S.; Software, C.E.S.N.; Writing—review and editing, D.D.R.A., N.C.-M., L.M.D. and J.R.G.d.S.A.; Supervision, H.M.B. and N.C.-M.; project administration, H.M.B. and D.D.R.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Fundação de Amparo à Pesquisa do Estado do Piauí (Grant 050/2019), Conselho Nacional de Desenvolvimento Científico e Tecnológico, and Federal University of Piauí.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All material and data are stored at the Department of Parasitology and Microbiology, Federal University of Piauí, Piauí, Brazil and may be shared upon a request directed to the corresponding authors.

Acknowledgments: The authors thank Glenn W. Kaatz for kindly providing the *S. aureus* SA1199B strain.

Conflicts of Interest: The authors declare no conflicts of interest.

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