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Chemical Composition, Antibacterial and Inhibitory Activity of the Efflux Pump of Essential Oils from *Croton piauhiensis* Müll.

Beatriz Gonçalves Cruz [1], Thiago Sampaio de Freitas [1], Maria do Socorro Costa [1], Ana Raquel Pereira da Silva [1], Henrique Douglas Melo Coutinho [1], Selene Maia de Morais [2], Emmanuel Silva Marinho [2], Alexandre Magno Rodrigues Teixeira [1,2] and Hélcio Silva dos Santos [1,2,3,*]

[1] Graduate Program in Biological Chemistry, Department of Biological Chemistry, Regional University of Cariri, Crato 63105-000, CE, Brazil; bgcruz3@gmail.com (B.G.C.); thiagocrato@hotmail.com (T.S.d.F.); corrinha_live@yahoo.com.br (M.d.S.C.); anaraquel_ar@hotmail.com (A.R.P.d.S.); hdmcoutinho@gmail.com (H.D.M.C.); alexandremrodriguesteixeira@gmail.com (A.M.R.T.)
[2] Center for Science and Technology, Graduate Program in Natural Sciences, State University of Ceará, Fortaleza 60714-903, CE, Brazil; selenemaiademorais@gmail.com (S.M.d.M.); emmanuel.marinho@uece.br (E.S.M.)
[3] Science and Technology Centre, Course of Chemistry, State University Vale do Acaraú, Sobral 62040-370, CE, Brazil
* Correspondence: helciodossantos@gmail.com

Abstract: As the spread of bacterial resistance to clinically available antibiotics has become a global public health problem, the scientific community has intensified its studies in the search for natural compounds and their derivatives to combat bacterial resistance. In this work, a circadian study of the essential oil extracted from the leaves of *Croton piauhiensis* (EOCP) was carried out. We also sought to evaluate its antibacterial activity, modulatory potential and if it acts as a possible inhibitor of the efflux pump by determining the minimum inhibitory concentration (MIC) and the association of the oil in subinhibitory concentrations with the antibiotic ciprofloxacin and with ethidium bromide (EtBr) against the strain of *Staphylococcus aureus* K2068 strain. The assays used to obtain the MIC of the EOCP were performed by broth microdilution, while the efflux pump inhibitory test was performed by the MIC modification method. According to the results, the circadian study showed differences in the chemical composition and percentage of oils collected at different times of the day, which can be attributed to environmental conditions. The main components of the EOCP were β-caryophyllene (6 h—21.23%; 12 h—22.86% and 18 h—16.95%), followed by D-Limonene (6 h—13.27% and 18 h—15.95%) and γ-Elemene (12 h)—12.61%). The EOCP collected at 12 h had a better profile in reducing MIC, presenting antibacterial activity for *Staphylococcus aureus* and *Escherichia coli*. In the efflux pump test, it was observed that the oil was able to potentiate the action of ethidium bromide against the *S. aureus* K2068 strain, which can contribute to the prevention or treatment of infectious diseases caused by multidrug-resistant (MDR) strains.

Keywords: antibiotic resistance; essential oil; circadian rhythm; efflux pump inhibitors; MepA

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1. Introduction

Epidemiological studies point to the importance of further research to discover new drugs or compounds with therapeutic properties [1] since bacterial resistance to clinically used antibiotics and their spread throughout the world has become a global issue of public health concern [2–5]. Thus, the search for efficient natural compounds with antimicrobial activity and low toxicity has increased; therefore, plant secondary metabolites have become a promising alternative to combat microbial infections [6–9].

Due to the increase in microbial resistance, traditionally used antibiotics have been losing their effectiveness [10]. The *Staphylococcus aureus* is a worldwide worrying strain, responsible for a high incidence of infections in hospital environments and which presents

cross-resistance to other antibiotics [11]. Several mechanisms are involved with the emergence and dissemination of resistance, making its control difficult; among them, an important mechanism is the presence of efflux pump proteins in bacterial membranes, and examples of resistance to multiple drugs (MDR pumps) are NorA and MepA [12].

Efflux pumps act by active transport, causing the extrusion of one or several types of antibiotics from the bacterial cytoplasm [13]. *S. aureus* bacteria have efflux pumps that likely act as important factors in the development of high-level antibiotic resistance, not only causing this resistance, but impacting virulence and even biofilm formation [14–16]. Thus, efflux pump inhibitors become an important therapeutic alternative in the treatment of infectious diseases.

In view of these facts, the scientific community has intensified studies in the search for more new effective drugs with a lower level of toxicity. Alternative ways as in vitro methods in antimicrobial tests to evaluate the potential of extracts, essential oils and isolated compounds have been widely discussed by science, revealing potential antimicrobial agents, or even potentiating the activity of antibiotics used in clinical practice [17–20]. Thus, the combination of products may provide antimicrobial efficacy at doses that were previously ineffective due to the antimicrobial resistance presented by some strains [20–22].

Products of natural origin are important sources in the search for molecules with antibacterial activities [23–25]. Furthermore, the combination of a natural compound with an antibiotic could potentialize the antibacterial activity and/or reduce the dose necessary for therapeutic success [24,26].

The Northeast region of Brazil has a botanical potential of high diversity, with emphasis on *Croton* (Euphorbiaceae), the second largest genus of the family, with approximately 1300 species distributed throughout the tropical and subtropical region [27]. Studies of chemical and biological characterization of the activities of essential oils extracted from *Croton* species have already been carried out [28,29].

In view of the diversity of species of this genus, we highlight the *Croton piauhiensis* Müll., popularly known as "velame", an endemic species of the Brazilian Northeast frequently found in the Caatinga biome. It is used in folk medicine for stomachache, nausea, vomiting and diarrhea [30].

In this context, the objective of this work was to carry out a circadian study of the essential oils extracted from the leaves of *C. piauhiensis* (EOCP), determining their chemical composition. Additionally, it will present, for the first time, a study of the direct antibacterial and antibiotic-modifying activity of the EOCP against strains of *Staphylococcus aureus* and *Escherichia coli*, as well as the evaluation of the oil in the inhibition of the efflux pump against the strain of *S. aureus* K2068 (MepA overexpresser).

2. Materials and Methods

2.1. Experimental Design

The essential oil was extracted from the leaves of *C. piauhiensis* by hydrodistillation and its constituents were identified by GC-MS, then the microbial resistance modulator and inhibition of the efflux pump of the essential oils were verified (Figure 1).

2.2. Plant Material and Essential Oil Extraction

C. piauhiensis leaves were collected in Sobral, Ceará, Brazil at the flowering stage in June 2017 at the experimental farm of the Acaraú Valley State University (03°36′44″ S 40°18′37″ W). Plant authentication was performed by Professor Daniela Santos Carneiro-Torres, and a voucher specimen was deposited at the Universidade Estadual de Feira de Santana (HUEFS) with the identification number #14989. The fresh leaves of *C. piauhiensis* were subjected to hydrodistillation in a Clevenger type apparatus with 2 L of water for 2 h. After being filtered and dried over anhydrous sodium sulfate, the essential oils were stored in sealed glass vials, which were maintained under refrigeration at 4 °C until GC-MS. The following nomenclature was used for essential oils extracted from *C. piauhiensis*: EO1 = Essential oil extracted at 6 h; EO2 = Essential oil extracted at 12 h; EO3 = Essential oil

extracted at 18 h. GC-MS for the quantitative analysis was carried out on a Shimadzu GC-17A gas chromatograph using a dimethylpolysiloxane DB-5 fused silica capillary column (30 mm × 0.25 mm, film thickness 0.25 m). H_2 was used as the carrier gas at a flow rate of 1 mL/min and 30 psi inlet pressure; split, 1:30; temperature program: 35–180 °C at 4 °C/min, then heated at a rate of 17 °C/min to 280 °C and held isothermal for 10 min; injector temperature, 250 °C; detector used in flame ionization detector (FID), detector temperature, 250 °C.

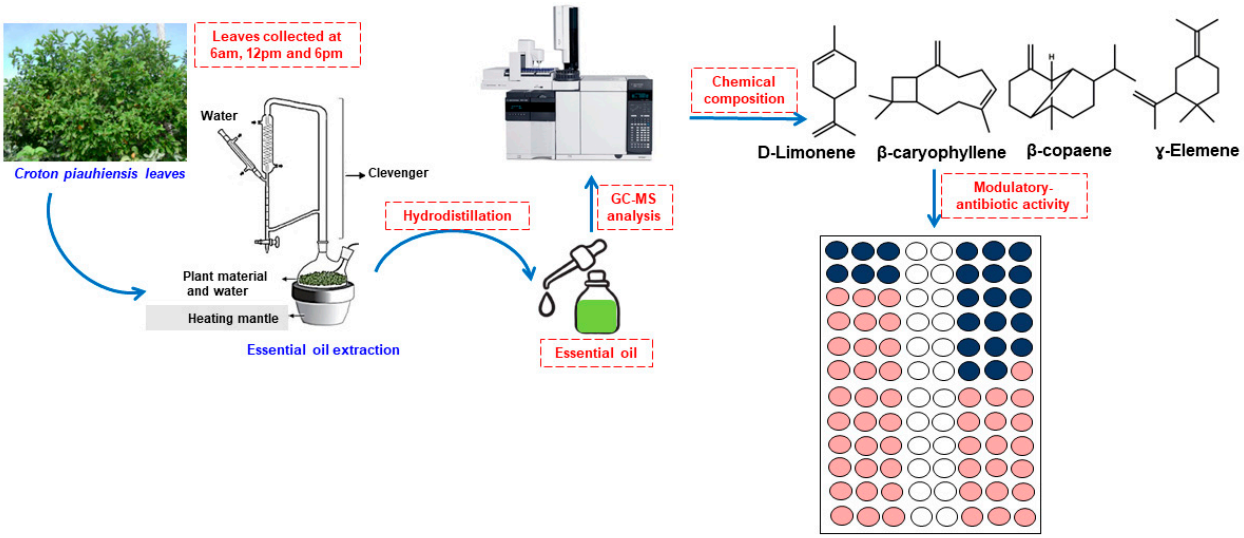

Figure 1. Extraction, characterization and antibacterial activity of the essential oils from *C. piauhiensis.*

2.3. Bacterial Strains

In the studies related to the modification of antibiotic activity, the standard bacterial strains used were *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, while the resistant strains were *E. coli* 06 and *S. aureus* 10. These strains were acquired from the microbiology and molecular biology laboratory of the Regional University of Cariri-URCA. In the efflux pump tests, the bacterial strain *S. aureus* K2068 strain, which overexpresses the MepA efflux pump, provided by Professor S. Gibbons (University of London, London, UK) and kept on blood agar (Laboratorios Difco Ltd.a., São Paulo, SP, Brazil). All strains before the experiments, were grown for 24 h and kept in a bacteriological incubator (SANYO, model MOC-17AC, Osaka, Japan) at 37 °C in a solid brain and heart infusion—BHI agar (microMED—ISOFAR, Duque de Caxias RJ, Brazil) prepared at a concentration of 10%.

2.4. Drugs

To evaluate the potentiating activity, the antibiotics used were ampicillin (β-lactams) (SIGMA-ALDRICH, St. Louis, MO, USA), norfloxacin (fluroquinolones) (SANDOZ, Cambé, PR, Brazil) and gentamicin (aminoglycosides) (SIGMA-ALDRICH, St. Louis, MO, USA). A total of 10 mg of each of the compounds used in the tests were weighed. To find out if the EOCP has an efflux pump indicator, the pump inhibitor chlorpromazine (CPZ) (SANOFI, São Paulo, SP, Brazil) and the beta-lactamase inhibitor sulbactam (SIGMA-ALDRICH, St. Louis, MO, USA) were used. In tests with efflux pumps, the antibiotic ciprofloxacin (GEOLAB, Anapolis, GO, Brazil) was used, which acts as a substrate for the bacterial strain *S. aureus* K2068. In addition to antibiotics, ethidium bromide (EtBr) was used to check for efflux pumps. EtBr when associated with standard pump inhibitors is a technique used in several studies to investigate the presence or absence of efflux pumps [31,32]. Carbonyl-m-chlorophenyl hydrazone cyanide (CCCP) (SIGMA-ALDRICH, St. Louis, MO, USA) and EtBr (SIGMA-ALDRICH, St. Louis, MO, USA) were obtained from Sigma Aldrich Co. Ltd. The antibiotic ciprofloxacin and EOCP were initially diluted in 0.5 mL of dimethyl sulfoxide (DMSO) (LABSYNTH, Diadema, SP, Brazil) and then in sterile water. CPZ and

EtBr solutions were dissolved in distilled and sterile water, and kept protected from light. The CCCP was dissolved in a 1:1 methanol/water solution. All the solutions were prepared on the basis of recommendations established in [33] and diluted in sterile water to reach a final concentration of 1024 μg/mL.

2.5. Antibacterial Activity

Minimum inhibitory concentration (MIC) was determined for EOCP according to the broth microdilution method proposed by with adaptations. The bacterial inoculum was suspended in saline, corresponding to 0.5 of the McFarland scale, approximately 1.5×10^8 (CFU)/mL. Eppendorfs® microtubes (CRALPLAST, Cotia, SP, Brazil) were then filled with 900 μL of BHI and 100 μL of the inoculum and the microdilution plates (CRALPLAST, Cotia, SP, Brazil) were filled with 100 μL of the final solution with serial dilutions up to the penultimate well of the plate (1:1), the latter being used as a growth control. The final concentration of the EOCP samples ranged from 512 to 8 μg/mL. After 24 h of incubation, readings were performed by adding 20 μL of resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one). Resazurin reagent (VETEC, Rio de Janeiro, RJ, Brazil) was oxidized in the presence of the acid medium caused by bacterial growth, promoting the color change of blue to pink [34]. The MIC was defined as the lowest concentration in which no growth can be observed [35]. The tests were performed in triplicate.

2.6. Antibiotic Resistance Modulation Test

To evaluate the EOCP as a microbial resistance modulator, the MIC values of antimicrobials (ampicillin, norfloxacin and gentamycin) against multidrug-resistant bacterial strains were determined in the presence of the compound in a sub-inhibitory concentration (MIC/8), based on the methodology by Freitas and collaborators [36], and to evaluate a possible efflux pump mechanism, was also used to control the antibiotics chlorpromazine and sulbactam in a subinhibitory concentration. The distribution medium was prepared in Eppendorf® tubes, each containing 10% BHI, 150 μL of the bacterial suspension of *S. aureus* and *E. coli* and the oil, totaling 1.5 mL of solution. For the control, 1.5 mL of this solution contained only 10% BHI and 150 μL of the microbial suspension. The microdilution plate was filled with 100 μL of the solution followed by series microdilution (1:1) with the antibiotic until the penultimate well is filled. The plates were incubated at 37 °C for 24 h and were analyzed in the same way as in the MIC test.

2.7. Evaluation of MepA Efflux Pump Inhibition

The inhibition of the efflux pump was tested using a sub-inhibitory concentration (MIC/8) of the EOCP and using effluent pump inhibitors (EPI) to verify the effect on the tested pump, following the methodology proposed by the Clinical and Laboratory Standards Institute—CLSI [32]. The comparative study between the effects of the standard inhibitors of the efflux pump was used, evaluating the ability of both to decrease the MIC of EtBr and the antibiotic ciprofloxacin. The standard CCCP inhibitors and CPZ were used to provide the expression of the MepA pump by the strain *S. aureus* (SA-K2068). In the tests, 170 μL of each bacterial inoculum suspended in saline, corresponding to 0.5 of the McFarland scale, approximately 1.5×10^8 (CFU)/mL, was added together with the inhibitors and oil (MIC/8) and completed with BHI. These were then transferred to 96-well microdilution plates to which 100 μL of antibiotic or EtBr were added in serial dilutions (1:1) ranging from 512 to 0.5 μg/mL. The plates were incubated at 37 °C for 24 h and bacterial growth was evaluated with resazurin.

2.8. Statistical Analysis

The tests were performed in triplicate, and the results were expressed as the geometric mean. Central data and standard deviations were obtained according to the methodology used by Ribeiro and collaborators [37], in microbiological analysis in microdilution plates. Antibiotic potentiating activity data were analyzed using the GraphPad Prisma 6.01 statisti-

cal program via a one-way ANOVA test. Then, a post hoc Bonferroni test was performed considering statistically values significant (**** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$ or * $p < 0.05$), and - non-significant values (ns) represented by $p > 0.05$.

3. Results

3.1. Chemical Composition of Essential Oil

The essential oils collected from the leaves of *C. piauhiensis* at 6 h, 12 h and 18 h were analyzed by GC/MS (Table 1).

Table 1. Chemical composition of essential oils extracted from *Croton piauhiensis* leaves at 6 h, 12 h and 18 h.

Compounds	KI *	Percent Composition (%)		
		6 h	12 h	18 h
α-Thujene	930	0.40	0.19	0.49
α-Pinene	939	2.95	1.59	2.84
Camphene	954	0.12		0.13
β-Pinene	979	0.28		
β-Myrcene	990	3.55	2.19	3.00
α-Phellandrene	1002	0.44	0.77	0.77
2-Carene	1002	1.16		
(+)-4-Carene	1011		0.91	
α-terpinene	1017	0.27	0.22	0.36
p-Cymene	1024	4.04	2.08	2.70
β-Phellandrene	1029	0.34	0.56	1.00
D-Limonene	1029	13.27	9.14	15.95
Eucalyptol	1031	2.91	2.77	1.98
β-Ocimene	1037	1.12	0.72	1.08
γ-Terpinene	1059	5.56	3.68	4.60
Terpinolene	1088			1.45
Linalool	1096	1.18	1.16	1.24
Terpinen-4-ol	1177	0.63	0.74	1.01
α-Terpineol	1188	0.35	0.37	0.38
Isoamyl tiglate (E)	1192	0.14	0.15	0.15
Hexenyl valerate (Z)	1281	0.39	0.20	0.27
Bornyl acetate	1285			0.11
Cycloisolongifolene	1319		0.34	0.18
α-Terpinyl acetate	1349	0.20	0.18	0.27
Cyclosativene	1371			0.11
α-Copaene	1376	1.06	1.01	0.82
Isoledene	1376	0.22	0.27	0.20
β-Damascenone	1384	0.42		0.34
b-Cubebene	1388			0.13
b-Elemene	1390	2.10	2.43	1.66
Cyperene	1398			0.18
β-Caryophyllene	1419	21.23	22.86	16.95
β-copaene	1432	8.28	10.07	7.70
β-Gurjunene	1433	0.62	0.61	0.48
γ-Elemene	1436	6.59	12.61	9.59
Aromandendrene	1441	0.40	2.55	1.41
α-Humulene	1454	2.70	2.85	2.17
γ-Muurolene	1479	0.46	0.36	0.51
γ-Himachalene	1482			0.12
α-Amorphene	1484	0.11	0.59	0.19
Valencene	1496			0.06
α-Muurolene	1500	0.37	0.57	0.42
Cuparene	1504	0.16		
δ-Cadinene	1523	2.18	2.83	2.13
Cadina-1,4-diene (trans)	1534	0.16	0.29	0.17

Table 1. *Cont.*

Compounds	KI *	Percent Composition (%)		
		6 h	12 h	18 h
Germacrene B	1561	0.23	0.32	
Palustrol	1568	0.25	0.21	0.27
Spathulenol	1578	3.08	2.5	2.59
Caryophyllene oxide	1583	0.11		0.10
Gleenol	1587		0.09	
Viridiflorol	1592	0.79		
Ledol	1602	0.28	0.35	
α-epi-Muurolol	1642	0.15	0.67	4.03
Cubenol	1646	0.32	0.33	0.34
α-Cadinol	1654	3.92	3.95	1.22
Selin-11-en-4-α-ol	1659			0.14
Cembrene	1938			0.23
Elemol	1549	0.11		
Pentadecanone	1697	0.17	0.19	0.19
Phytol	1943	0.23	0.35	0.30
Total		96.04	96.82	96.25

*: The most common index is the Kovats Index.

Analysis of the chromatograms (Supplementary Materials) allowed the identification of 48 constituents (94.04%) in the essential oil extracted at 6 h. To the essential oil extracted at 12 h, 43 constituents (96.82%) were identified. While in the oil extracted at 18 h, 51 essential constituents (96.25%) were identified. With the analysis of the circadian variation in essential oils of *C. piauhiensis* leaves, a production of different chemical constituents in different proportions was observed, which was probably affected by climatic factors. The percentage of the main components of the EOCP were D-Limonene (6 h—13.27%, 12 h—9.14% and 18 h—15.95%), β-caryophyllene (6 h—21.23%; 12 h—22.86% and 18 h—16.95%), followed by β-copaene (6 h—8.28%, 12 h—10.07% and 18 h—7.70%) and γ-Elemene (6 h—6.59%, 12 h—12.61%, 18 h—9.59%) (Figure 2).

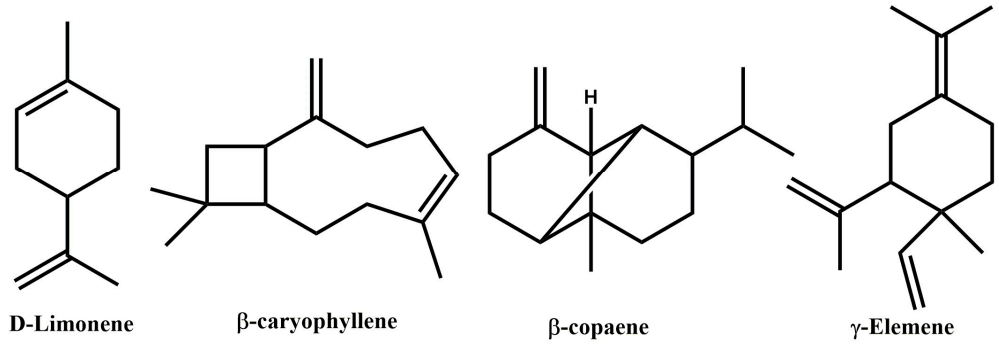

Figure 2. The main constituents of the essential oil from the leaves of *C. piauhiensis*.

3.2. Direct Antibacterial Activity by Minimum Inhibitory Concentration (MIC)

Using this value as a cutoff point, the essential oil of *C. piauhiensis* (EOCP) showed antibacterial activity by reducing the concentration of MIC against some standard and multidrug resistant strains of *Staphylococcus aureus* (SA) and *Escherichia. coli* (EC). The MIC obtained for each strain is indicated in the table below (Table 2).

As verified in our study, the EOCP collected at 6 h showed MIC = 813 μg/mL to *S. aureus* 10 e MIC = 406 μg/mL to *E. coli* ATCC. On the other hand, tests performed with the EOCP 12 h showed greater bacterial sensitivity with a significant reduction in the MIC value for all *S. aureus* and *E. coli* strains tested. This result is probably related to the major constituent β-caryophyllene, which at this time showed a chemical percentage (22.86%).

The oil collected at 18 h had a subinhibitory concentration of 645 µg/mL for *S. aureus* ATCC and 16 µg/mL for *E. coli* ATCC.

Table 2. Minimum Inhibitory Concentration (MIC) of the circadian cycle of *Croton piauhiensis* essential oil (EOCP) against standard and multidrug-resistant bacterial strains.

Strains	EOCP (µg/mL)		
	6 h	12 h	18 h
	MIC	MIC	MIC
Staphylococcus aureus 10—SA10	813	256	≥1024
Staphylococcus aureus ATCC 25923—SA ATCC	≥1024	25	645
Escherichia coli 06—EC06	≥1024	323	≥1024
Escherichia coli ATCC 25922—EC ATCC	406	128	16

MIC: Minimum inhibitory concentration.

3.3. Modulatory-Antibiotic Activity of EOCP

In the antibiotic activity modification test, three different classes of antibiotics were used to test the modulating effect: ampicillin (beta-lactam), norfloxacin (fluoroquinolone class) and gentamicin (aminoglycoside). The effect was tested in two models using multidrug-resistant bacteria, Gram-positive (*S. aureus*) and Gram-negative (*E. coli*). The antibiotics were used in the presence and absence of the EOCP, we also tested the association of the antibiotic ampicillin with the efflux pump inhibitor sulbactam, and for the antibiotics norfloxacin and gentamicin, the efflux pump inhibitor chlorpromazine.

In the test with ampicillin (Figure 3) when the antibiotic is associated, the substance EOCP is observed to potentiate antibiotic activity with statistically significant values with $p < 0.0001$ for the three oils tested against *S. aureus* and *E. coli* strains.

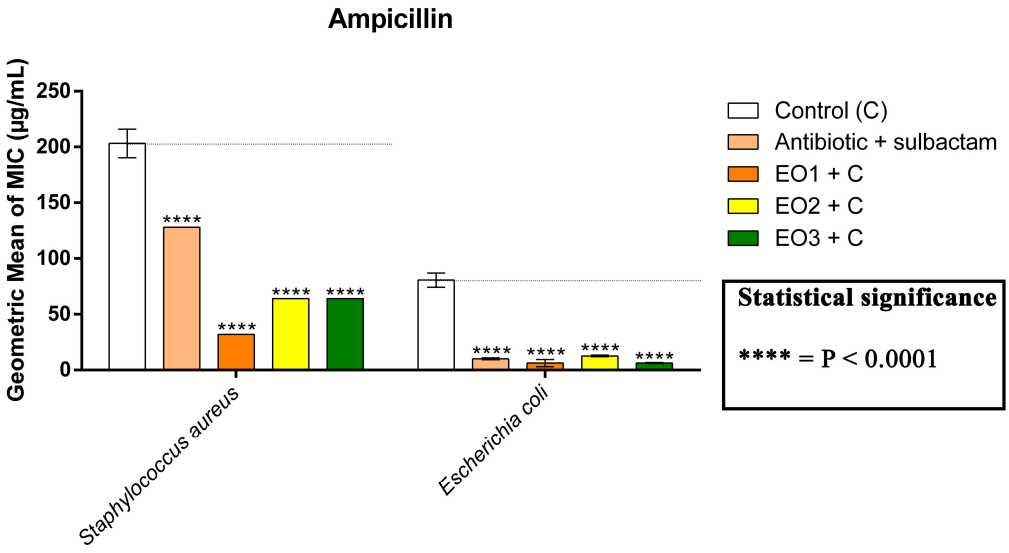

Figure 3. Minimum inhibitory concentration (MIC) of ampicillin in the presence and absence of the substance EOCP collected 6 h (EO1); 12 h (EO2); 18 h (EO3), and the inhibitor sulbactam, against *S. aureus* 10 and *E. coli* 06. Statistically significant values **** $p < 0.0001$—Non-significant values (ns) with $p > 0.05$.

The antibiotic norfloxacin (Figure 4) with the EOCP showed to potentiate the antibiotic effect against *S. aureus*, for the 6 h and 18 h oil with statistically significant values $p < 0.0001$ and for the 12 h oil $p < 0.05$.

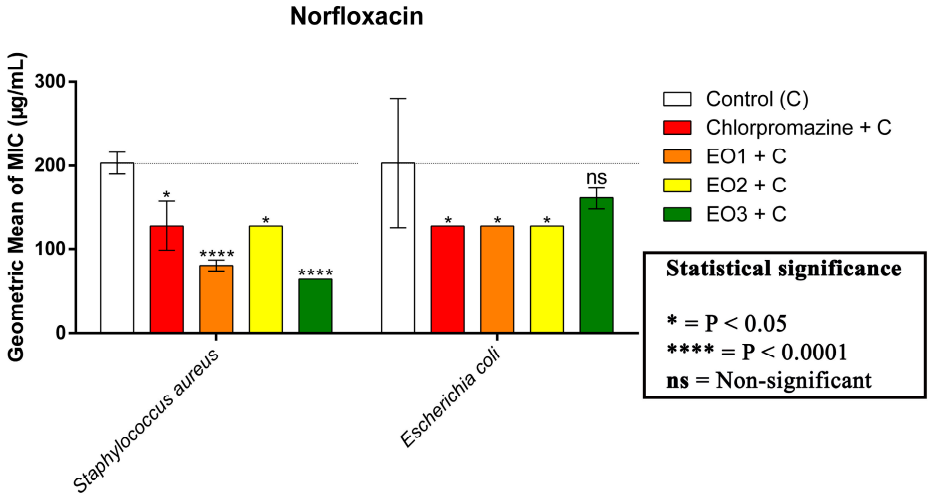

Figure 4. Minimum inhibitory concentration (MIC) of norfloxacin in the presence and absence of the substance EOCP collected 6 h (EO1); 12 h (EO2); 18 h (EO3), and the inhibitor chlorpromazine, against *S. aureus* 10 and *E. coli* 06. Statistically significant values **** $p < 0.0001$—Non-significant values (ns) with $p > 0.05$.

When the antibiotic used was gentamicin (Figure 5), we observed the relevant results only in association with EOCP collected at 12 h with a potentiation of the antibiotic action against the *S. aureus* bacteria of $p < 0.0001$.

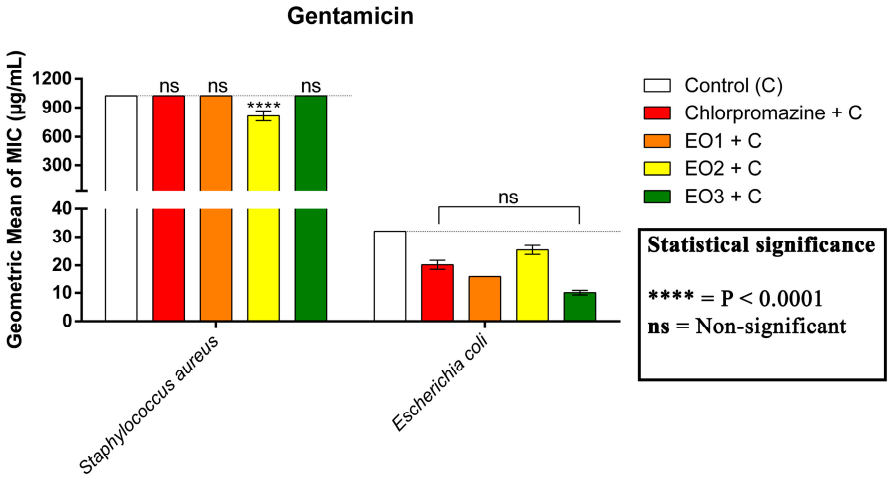

Figure 5. Minimum inhibitory concentration (MIC) of gentamicin in the presence and absence of the substance EOCP collected 6 h (EO1); 12 h (EO2); 18 h (EO3), and the inhibitor chlorpromazine, against *S. aureus* 10 and *E. coli* 06. Statistically significant values **** $p < 0.0001$—Non-significant values (ns) with $p > 0.05$.

3.4. Efflux Pump in Staphylococcus aureus K2068, Carrier of the MepA Gene

To carry out the efflux pump tests, the OECP collected at 12 h (EO2) was used. At this time of collection, the highest concentration of the majority constituent, βcaryophyllene, was obtained, in addition to showing a significant reduction in the MIC value in bacterial activity tests, proving to be effective against all strains tested.

EOCP did not have an antibacterial effect on the tested strain SA K2068 when associated with the antibiotic ciprofloxacin (Figure 6). However, there was a reduction in the MIC of the antibiotic when used in conjunction with the standard efflux pump inhibitors, CCCP and CPZ, both with statistically significant values $p < 0.0001$, thus suggesting that the oil in question may be acting on the resistance mechanism of the bacterium.

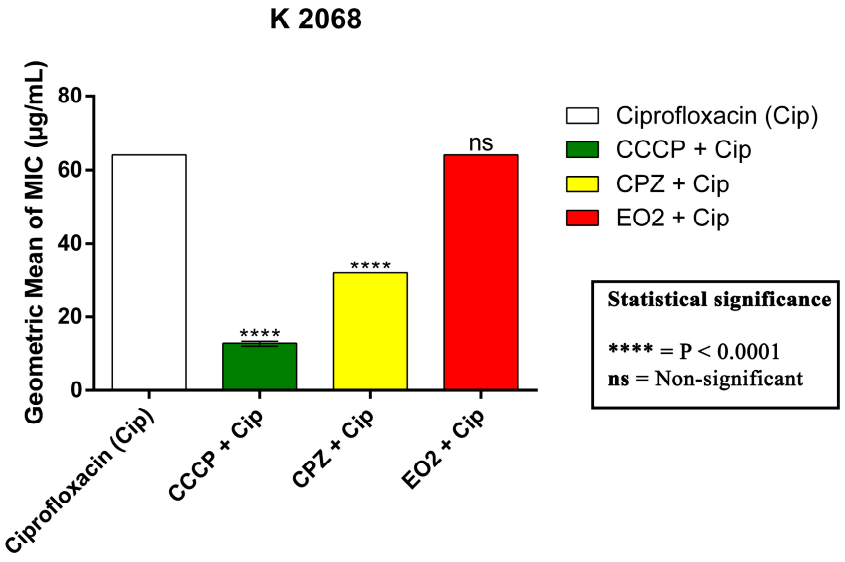

Figure 6. Evaluation of the MIC of ciprofloxacin alone and in association with standard inhibitors (CCCP and CPZ) and EOCP collected 12 h (EO2) against the strain *S. aureus* K2068 (SA-K2068) that overexpresses MepA.

When the oil was used in association with EtBr (Figure 7), it caused a reduction in MIC showing that EOCP potentiated the action of bromide against SA-K2068 (reduction from 323 µg/mL to 256 µg/mL). A similar result was also observed when using the standard efflux pump inhibitors CCCP and CPZ, which potentiated the action of bromide with $p < 0.0001$ and $p < 0.001$, respectively. The importance demonstrated by the reduction of the MIC of EtBr in association with the standard inhibitors indicates the expression of the MepA efflux pump by the strain tested.

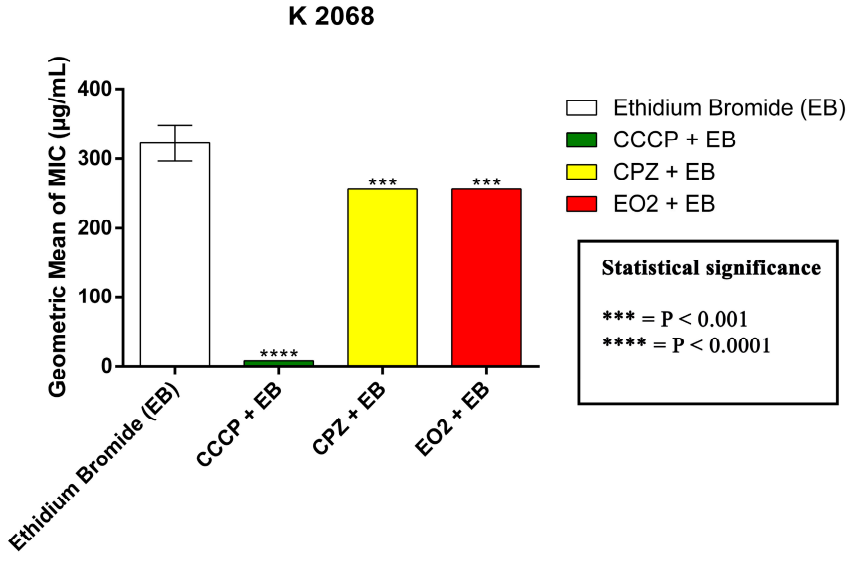

Figure 7. Evaluation of MIC of ethidium bromide (EtBr) alone and in association with standard inhibitors (CCCP and CPZ) and EOCP collected 12 h (EO2) against strain *S. aureus* K2068 (SA-K2068) that overexpress MepA.

4. Discussion

Essential oils are acquired from the secondary metabolism of plants, being composed mainly of monoterpenes, sesquiterpenes and phenylpropanoids [38]; its chemical composition is determined by genetic factors, however, other factors can change its composition. Factors such as temperature, rainfall, type of soil, fertilization, use of pesticides, seasons of

the year, etc., act with varying degrees of intensity, mediating the quantity and nature of the substances produced [39,40]. Radiation is an important factor that can directly interfere with plant growth and development, through photosynthesis, modulation of photoperiod and light quality. Variations in light intensity and temperature may occur throughout the day, acting directly on primary processes, such as photosynthesis and respiration, and may indirectly influence the production of secondary metabolites, including the constituents of essential oil, whose synthesis depends on products of primary metabolism. The intensity of light can also alter the production of essential oil through the activation of photosensitive enzymes involved in the mevalonic acid pathway, the precursor of terpenes, which are chemical constituents of essences. In general, essential oil content tends to increase with increasing temperature, due to the increase in the number of oil glands per unit of leaf area; however, in some cases, it may decrease due to losses, which, in turn, are due to volatilization [40].

Studies carried out with β-caryophyllene showed bactericidal activity against the strain of *Bacillus cereus* [41]. In order for minimum inhibitory concentration (MIC) values to be considered clinically relevant, they cannot present concentrations above 1000 μg/mL, as it may be impractical to extrapolate the dose from in vitro activity to that which would be equivalent to adult human size [42].

In the scientific literature, the *C. piauhiensis* species is rarely reported, but some studies have already begun to be carried out and corroborate our results. Studies carried out to evaluate the development of the biofilm formed by *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 10145 showed that the essential oils of *C. piauhiensis* and *Vitex gardneriana* were able to inhibit microbial growth in both forms of bacterial life and also have antioxidant action action [26].

The essential oil of *C. piauhiensis* showed larvicidal activity against *Aedes aegypti* with CL_{50} = 336.8 μg/mL (oil extracted 8 h), CL50 = 283.9 μg/mL (oil extracted 12 h), and CL50 = 252.5 μg/mL (oil extracted 17 h). O flavonoid kaempferol 7-O-β-D-(6″-O-cumaroyl)-glucopyranoside isolated from *C. piauhiensis* leaves showed a synergistic effect potentiating the action of the aminoglycosides gentamicin and amikacin against strains of *Staphylococcus aureus* 10 e *Escherichia coli* 06 [43].

From the analysis of ampicillin with sulbactam we can infer that both *S. aureus* and *E. coli* have an enzymatic mechanism against ampicillin expressing significant values of $p < 0.0001$. Ampicillin, as β-lactam, has a mechanism of action that acts outside the cell, preventing cross-bridge formation in the cell wall. Norfloxacin and gentamicin antibiotics that act inside the bacterial cell were analyzed with chlorpromazine control, as the bacterial resistance mechanism for these antibiotics is different. While ampicillin is tested with sulbactam to see the enzymatic mechanism, norfloxacin and gentamicin are tested with chlorpromazine to see if the bacteria are shedding the antibiotics.

To *E. coli* bacteria, we also observed the potentiation of the antibiotic except for the 18 h oil, which was not significant. Analyzing the norfloxacin test in the presence of the inhibitor chlorpromazine, it is observed that *S. aureus* and *E. coli* strains may present an efflux pump mechanism for this antibiotic with $p < 0.05$.

The antibiotic norfloxacin, fluoroquinolones, inhibit the bacterial DNA gyrase enzyme, which cuts double-stranded DNA, in Gram-positive bacteria; the main target of action is topoisomerase IV, which cuts and separates the daughter strand of DNA after DNA replication. A higher affinity for this enzyme may confer greater potency against Gram-positive bacteria [44].

However, we did not obtain significant results for the *E. coli* bacteria. The loss of efficacy in this bacterial model can be explained by structural differences between Gram-positive and Gram-negative bacteria, which alter permeability and by drug interaction with their targets [45]. We also had no significant results for the pump inhibitor chlorpromazine in combination with gentamicin.

Aminoglycosides, such as gentamicin, are potent bactericides that inhibit protein synthesis by binding to the 30S subunit of the bacterial ribosome. Several resistance

mechanisms have been observed in microorganisms against this class of molecules [46,47]. In the general analysis of the activity with the inhibitor sulbactam, the *S. aureus* and *E. coli* strains showed an enzymatic mechanism against ampicillin.

The antibiotic norfloxacin in the presence of chlorpromazine indicates a possible efflux pump mechanism against the strains tested, but gentamicin with chlorpromazine did not show significant results. Efflux pump inhibitor evaluation was performed against the mutant multidrug resistant (MDR) strain *S. aureus* K2068 (SA-K2068), which features the MepA efflux pump. To assess efflux pump reversal, subinhibitory concentrations (MIC/8) of EOCP and standard pump inhibitors, the carbonyl-m-chlorophenyl hydrazone cyanide (CCCP) and chlorpromazine (CPZ) were used, each with its own inhibition mechanism, and it was verified if they were able to modulate the action of ciprofloxacin and ethidium bromide (EtBr), substrate for the mentioned efflux pump.

The CCCP acts by causing a disturbance in the electrochemical potential due to a decrease in the production of adenosine triphosphate (ATP) leading to inhibition of the efflux pump, while chlorpromazine acts via competitive inhibition of antibiotics interacting directly with the efflux pump, inhibiting them [48]. Thus, the presence of the efflux pump will be visualized when the minimum inhibitory concentration of EtBr associated with a standard inhibitor is lower than the MIC of the bromide control, indicating the inability of the bacterial cell to utilize the efflux mechanism. Efflux pump studies were performed with the essential oil of *Piper caldense* (OEPC); this work corroborates our research, showing that it is possible to observe the presence of an efflux pump using essential oil even if it is a mixture of chemical compounds. OEPC presented caryophyllene oxide (11.9%) as the main constituent found, followed by δ-cadinene (9.6%) and spathulenol (9.1%). When the oil was combined with norfloxacin and ethidium bromide, it reduced the MIC values against *S. aureus* strains (SA1199B, K2068 and K4100), acting as inhibitors of NorA, MepA and QacC [49].

We showed that both CCCP and CPZ associated with ethidium bromide had lower MICs than the control, showing the presence of efflux pumps. Because the only mechanism used by bacteria to resist the action of EtBr is the efflux pump with distinct mechanisms of expulsion of intracellular bromide [50]. Bromide has antibiotic activity due to its characteristic of intercalating DNA and has been used to evaluate the potential inhibitory activity of the effux pump of natural products, isolated phytochemicals and synthetic compounds [51].

Thus, considering the role of efflux pumps in innate and evolved resistance, they have been targets for the discovery and development of antimicrobial adjuvants being of great importance to research new substances that have pump inhibitors and are able to reduce the incidence of infectious diseases. Thus, we can conclude that EOCP is a source of phytochemicals that act as efflux pump inhibitors in the multidrug-resistant strain of SA-K2068 (MepA overexpresses).

5. Conclusions

The circadian study of *C. piauhiensis* essential oils allowed us to conclude that the volatile composition of the vegetable was influenced by environmental variables (light, temperature and humidity) throughout the day, varying its chemical composition. The main components found were β-Caryophyllene, D-Limonene, γ-Elemene. In the activity test, the EOCP collected at 12 h showed a better profile in the reduction of MIC showing antibacterial activity for *Staphylococcus aureus* and *Escherichia coli*. When the modulating power of the oil was evaluated in association with different classes of antibiotics, it was possible to observe a potentiation of the activity with ampicillin and norfloxacin, both for *S. aureus* and *E. coli* and in the tests carried out with gentamicin only the EOCP 12 h showed potentiation of the antibiotic against *S. aureus* and non-significant results for the bacterium *E. coli*. In addition, EOCP was able to modulate ethidium bromide resistance against the bacterium SA-K2068, and indicates the presence of compounds acting as MepA inhibitors. These data provide information for a potential use of the oil as an efflux pump inhibitor

of the studied strain, which may contribute to the prevention or treatment of infectious diseases caused by multidrug-resistant *S. aureus*.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/nutraceuticals3040042/s1. Figure S1: chromatogram of *Croton piauhiensis* essential oil collected at 6:00 a.m., Figure S2: chromatogram of *Croton piauhiensis* essential oil collected at 12:00 a.m., Figure S3: chromatogram of *Croton piauhiensis* essential oil collected at 18:00 p.m.

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