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Phytochemical characterization and antibiotic potentiating effects of the essential oil of *Aloysia gratissima* (Gillies & Hook.) and beta-caryophyllene

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ABSTRACT

The traditional knowledge associated with the use of medicinal plants has significantly contributed to drug development research. In this context, *Aloysia gratissima*, commonly known as bee-brush or white-brush, has been widely used in Brazilian folk medicine as an analgesic and antimicrobial species. Phytochemical analysis of *A. gratissima* essential oil has identified sesquiterpene β-caryophyllene (17.3%) as a major component with significant antibacterial activity. Thus, this study aimed to characterize the antibacterial effects of the essential oil obtained from *A. gratissima* (EOAG) and its major compound β-caryophyllene, as well as to evaluate their potential to act as efflux pumps inhibitors in association with conventional antibiotics. The phytochemical characterization of EOAG was performed by gas chromatography coupled to mass spectrometry (GC-MS), while the antibacterial activity against the strains *Pseudomonas aeruginosa* 24, *Staphylococcus aureus* 10, and *Escherichia coli* 06 was assessed using the broth microdilution method. The antibiotic potentiating activity was investigated by analyzing the ability of the natural products to decrease the minimum inhibitory concentration (MIC) of norfloxacin and ciprofloxacin against *S. aureus* strains 1199B and K2068. A reduction in the MIC of ethidium bromide in the presence of essential oil components was interpreted as efflux pump inhibition. The GC-MS analysis identified 30 compounds, including β-caryophyllene as the major component. The antibacterial activity analysis demonstrated that both EOAG and β-caryophyllene both EOAG and β-caryophyllene presented antibacterial effects against *S. aureus* 10 (MIC = 32 μg/mL), in addition to potentiating the activity of norfloxacin against [*S. aureus* 10], [*P. aeruginosa* 24], and [*E. coli* 06]. The isolated compound also reversed the degree of antibiotic resistance observed in strains carrying efflux pumps. In conclusion, the essential oil of *A. gratissima* and β-caryophyllene have the potential to increase the effectiveness of antibiotics and as such can be used in the development of new therapies against bacterial resistance.

1. Introduction

Aloysia gratissima Gilles and Hook Tronc. (Verbenaceae) is a medicinal plant commonly known as bee-brush or white-brush. This species is widely used in Brazilian traditional medicine for the treatment of diseases affecting the respiratory, digestive (Souza and Wiest, 2007), and nervous systems (Silva et al., 2006; Zeni and Bosio, 2011). Due to the high content of compounds such as β-caryophyllene, spathulenol, and β-pinene, the essential oil of this species

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(EOAG) has been largely used in the composition of perfumes, foods, and beverages (Yadegarinia et al., 2006). In addition, studies have shown that both the EOAG and its isolated components have unique pharmacological properties, including antimicrobial (Santos et al., 2015), leishmanicidal (Garcia et al., 2018), anticancer (Yanq et al., 2016), sedative (Goleniowski et al., 2006), and larvicidal (Silva et al., 2014).

Previous research has identified β-caryophyllene among the constituents of essential oils extracted from various plant species such as *Syzygium aromaticum* (clove), *Cinnamomum zeylanicum* (cinnamon), *Zingiber officinale* (ginger) and *Ocimum basilicum* (basil) (Kumphune et al., 2011). Importantly, previous pharmacological studies have demonstrated that this sesquiterpene presents anti-inflammatory, antioxidant, and antibacterial activities (Dorman and Deans, 2000; Dahham et al., 2015; Rodriguez et al., 2015), corroborating the evidence that many essential oils containing β-caryophyllene have antibacterial effects against both Gram-positive and Gram-negative strains (Maia et al., 2010; Neta et al., 2017).

Staphylococcus aureus is a Gram-positive bacterium of public health concern, as it is a major causative agent of both hospital and community infections, being associated with manifestations such as pneumonia, bacteremia, endocarditis, osteomyelitis and a variety of skin diseases (Klevens, 2007; Foster et al., 2014). Evidence has indicated that this bacterium has developed resistance against most conventional antibiotics, impairing the treatment of several bacterial diseases (Gibbons, 2004).

In the group of Gram-negative bacteria, *E. coli* (Trabulsi and Alterthum, 2015) and *P. aeruginosa* (Guedes et al., 2010) stand out for their remarkable pathogenicity and ability to defeat drugs designed to combat them. *E. coli* is the principal cause of enteric urinary tract infections, in addition to being associated with severe conditions such as sepsis and meningitis (Dominguez et al., 2002; Pop-Vicas and Opal, 2014). On the other hand, *P. aeruginosa* causes severe infections in immunosuppressed individuals. In fact, this microorganism has several virulence mechanisms that favor its immune system evasion (Reinhart and Oglesby-Sherrouse, 2016), besides demonstrating resistance to several antibiotics (Morita et al., 2014).

While bacterial resistance can be caused by different genetic factors (Andersen, et al., 2015), efflux pump (EP) expression has been identified as a major resistance mechanism in *S. aureus*. These proteins mediate the active transport of antibiotics, reducing their intracellular concentrations, which results in ineffective therapy (Trabulsi and Alterthum, 2015). In this context, the efflux proteins MepA (Demarco et al., 2007; Correia et al., 2017) and NorA have been identified as important mediators of multidrug resistance in *S. aureus* strains (Kumar and Schweizer, 2005) and therefore are potential therapeutic targets in antibacterial drug development.

Previous studies have shown that some terpenes have the ability to enhance the activity of antibiotics against EP-carrying bacterial strains, indicating that combined therapies associating antibiotics and these natural products might represent a promising strategy to reverse bacterial resistance mediated by efflux pumps (Gill et al., 2015; Espinoza et al., 2019)

Therefore, the present study aimed to characterize the antibacterial and antibiotic-enhancing activity of the essential oil of *A. gratissima* and its major compound β-caryophyllene, as well as to evaluate their potential as efflux pumps inhibitors.

2. Materials and methods

2.1. Botanical Material

The botanical material was obtained from "The Private Reserve of Natural Heritage Butuguara", a segment of the Atlantic Forest located in the Paraná state, with an altitude ranging from 985 to 1,145 m (coordinates: 25° 20.884′ S and 049° 47.258′ W). The soil is classified as lithosol and cambisol. According to the Köppen climate classification, the climate is Cfb (humid temperate climate with moderately hot summer), presenting annual average temperatures around 17°C, with severe and frequent frosts and rainfall of 1,200 mm per year. The plant collection and transport of the plant were made under the license of the Environmental Institute of Parana State (license number 284/11). Approximately 1 Kg of botanical material including cladodes, terminal branches with leaves, and inflorescences were collected from at least 10 plants of each species. Herbarium specimens and photographic records were prepared and identified by Dr. Wanderlei do Amaral using morphologic characteristics and registered at the herbarium of the *Faculdades Integradas Espirita* (registry number 8.822).

2.2. Essential oil extraction and phytochemical analysis

The essential oil of *A. gratissima* was extracted by hydrodistillation in a Clevenger-type apparatus. Briefly, 50 g of dried leaves were crushed and subjected to three cycles of extraction with 1L of distilled water at boiling temperature for 2h (Wasicky, 1963). After extraction, the essential oil was collected and stored under refrigeration (-4°C) for preservation. The yield of the essential oil on a dry basis was expressed as a percentage of the total weight of the dry leaves used in the extraction.

The chemical composition of the essential oil was determined by gas chromatography coupled to mass spectrometry (GC-MS). To this end, the essential oil was diluted in dichloromethane (1:100) and injected with a 1:20 flow rate in an Agilent 6890 chromatograph (Palo Alto, CA) coupled to an Agilent 5973N selective mass detector, operated at 250°C. The separation of the constituents was performed in a HP-5MS capillary column (5% -phenyl-95% -dimethylpolysiloxane, 30m x 0.25m x 0.25 μm). For quantification, the diluted samples were injected into an Agilent 7890A chromatograph equipped with a flame ionization detector (FID), operated at 280°C. The percentage composition was assessed using the electronic integration of the FID signal by dividing the area of each component by the total area.

The identification of the chemical constituents was obtained by comparing their mass spectra with the standards reported in the literature (Wiley, 1994; Nist, 2016) in addition to comparing their linear retention indexes, calculated from the injection of a homologous series of hydrocarbons (C7- C26) with the literature data (Adams, 2007).

2.3. Investigation of antibacterial activity

The multidrug-resistant strains *P. aeruginosa* 24, *S. aureus* 10, and *E. coli* 06 were used for the investigation of the antibacterial activity of *A. gratissima* essential oil and its major constituent. The origin and resistance profile of these strains is shown in Table 1, as reported in the study of Bezerra et al. (2017).

The effects of these natural products on bacterial resistance, as well as their interference with efflux pump activity, were

Table 1
Origin and antibiotic resistance profile of the strains.

Bacterial strain	Origin	Resistance Profile
S. aureus 10	Rectum swab	Amc, Amox, Amp, Asb, Azy, Ca Cef, Cf, Cip, Cla, Clin, Eri, Lev, Mox, Oxa, Pen
E. coli 06	Urine	Asb, Ca, Cep, Cfo, Cmp, Cro
P. aeruginosa 24	Nasal discharge	Ami, Cip, Ctz, Imi, Lev, Mer, Ptz

Legend: Amc - Amoxicillin + Clavulanic Acid, Ami - Amikacin, Amox - Amoxicillin, Amp - Ampicillin, Asb - Ampicillin + Sulbactam, Azi - Azithromycin, Ca - Cefadroxil; Cep - Cephalexin, Cfo - Cefoxitin, Cip - Ciprofloxacin, Cla - Clarithromycin, Clin - Clindamycin, Cmp - Cefepime, Cro - Ceftriaxone, Ctz - Ceftazidime, Ery - Erythromycin, Imi - Imipenem, Mer - Levofloxin, Oxa - Oxacillin, Pen - Penicillin and Ptz – Piperacillin.

investigated using *S. aureus* strains 1199B and K2068, which carry the NorA and MepA efflux proteins, respectively. All strains were initially cultured on blood agar (Laboratorios Difco Ltda., Brazil) and maintained in Heart infusion agar (HIA, Difco) medium at 4°C. Samples were transferred from the solid medium to test tubes containing sterile saline, and turbidity was assessed using a value of 0.5 on the McFarland scale, corresponding to 10^5 Colony-Formed Unit (CFU).

2.4. Drugs

Norfloxacin and ciprofloxacin were used as control antibiotics for the evaluation of sesquiterpene activity against *S. aureus* 1199B and K2068 strains, respectively, which express the NorA and MepA efflux proteins, respectively, conferring thus resistance to the corresponding antibiotic. Ethidium bromide and chlorpromazine (CPZ) were used as control EP inhibitors. Both drugs were dissolved in dimethyl sulfoxide (DMSO) and diluted in water, while ethidium bromide and CPZ were dissolved in water. All drugs were prepared at an initial concentration of 1.024 μg/mL and serially diluted in test tubes. All drugs were purchased from SIGMA Chemical Co. (St. Louis, USA).

2.5. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was determined using the broth microdilution method (CLSI, 2009). Bacterial cultures were kept on agar under refrigeration at -80°C, cultured in brain and heart infusion (BHI) broth, and incubated at 37°C for 24h. Then, each inoculum was prepared with 10 % BHI at a ratio of 1:9. Next, 100 μL of inoculum in medium was placed in wells on a 96-well plate with 100 μL of the substance at concentrations ranging from 1.024 to 8 μg/mL, followed by incubation at 37°C for 24h. Positive controls (medium + inoculum) were included in the last wells of the plate. After incubation, 20μL of a 0,01% sodium resazurin solution in saline (p/v) was added to each well, followed by an additional 1h incubation period at room temperature. A change in the color of the solution (from blue to red), due to the reduction of resazurin, was used as an indicator of bacterial growth. The MIC was defined as the lowest concentration capable of inhibiting bacterial growth. All experiments were carried out in triplicate for all bacterial strains. Wells filled only with culture medium were used as negative controls.

2.6. Analysis of antibiotic resistance modulation

The antibiotic-enhancing activities of EOAG and β-caryophyllene were analyzed by investigating their interference in the MICs of norfloxacin and ciprofloxacin (Sigma, MO, USA) against resistant strains of *P. aeruginosa, E. coli*, and *S. aureus. To this end, these fluoroquinolone antibiotics were combined with* the essential oil or the isolated compound prepared at concentrations equivalent to their MIC/8 (Coutinho et al., 2008). Inoculum preparations and bacterial cultures were carried out as previously described. All experiments were carried out in triplicate for all bacterial strains.

2.7. Efflux pump inhibition analysis

To assess the potential action of EOAG and β-caryophyllene as EP inhibitors, we investigated their ability to reduce the MIC of fluoroquinolones or ethidium bromide against the EP-carrying *S. aureus* strains 1199B and K2068, which express efflux proteins associated with antibacterial resistance to norfloxacin and ciprofloxacin, respectively (Coutinho et al., 2008; Tintino et al., 2018). The bacterial inocula were prepared as described before, and β-caryophyllene was added at a concentration equivalent to its MIC/8. Wells on a 96-well plate were filled with 100 μL of the treatment solution, and then, ethidium bromide or the control antibiotic were added to the wells at concentrations ranging from 1.024 to 0.5 μg/mL. A reduction in the MIC of ethidium bromide or fluoroquinolones was interpreted as EP inhibition.

Alternatively, chlorpromazine (CPZ) was used as a standard efflux pump inhibitor (EPI) to evaluate the antibiotic-enhancing effect of β-caryophyllene (Amaral et al., 2010), as evidence has highlighted the CPZ assay as an important pharmacological tool to investigate the mechanism associated with efflux pump (Almeida et al., 2020; Freitas et al., 2021). All cultures, treatments, and analyses were performed as described above.

2.8. Statistical analysis

Data are expressed as arithmetic means ± standard deviation and were analyzed by analysis of variance (ANOVA), followed by Bonferroni's post-test using GraphPad Prism software version 7.0. Statistical significance was considered when $p < 0.05$

3. Results and discussion

3.1. Chemical Composition of the essential oil of A. gratissima

The extraction of *A. gratissima* essential oil by hydrodistillation presented a yield of 0.33% in relation to the dry weight of the botanical material. The phytochemical analysis of the EOAG by GC-MS identified 91.8% of the total constituents, revealing the presence of 30 different compounds, including β- caryophyllene (17.3%) nerolidol (9.7%), bicyclogemacrene (7.3%), β-pinene (7.2%), α-pinene (6.2%), and caryophyllene oxide (5.9%) as major constituents (Table 2).

A study by Trovati et al. (2009) identified the presence of 14 constituents in the essential oil of the same species, including β-caryophyllene as one of the main components (Santos et al., 2015). In addition, Dambolena et al. (2010) identified β-elemene (35.7%), viridiflorol (33.6%), β-caryophyllene (28%), α-thujone (17.5%), 10-epi-cubebol (13.4%), bicyclogermacrene (12.8%), (E)-nerolidol (11.6%),

Table 2
GC-MS profile of the EOAG.

RI	Compound	%
937	Alpha-pinene	6.2
979	Beta-pinene	7.2
991	Myrcene	0.5
1030	Limonene	0.9
1098	Linalool	0.4
1139	Nopinone+trans-pinocarveol	1.3
1145	Trans-verbenol	1.5
1163	Pinocarvone	1.2
1194	Mythenol	1.5
1336	Delta-elemene	0.5
1417	(E)-beta-caryophyllene	17.3
1433	Trans-alpha-bergamotene	1.1
1441	(Z)-beta-bergamotene	3.3
1449	Alfa-humulene	1.3
1456	(E)-beta-farnesene + alpha-aromadendrene	0.8
1477	Gama-muurolene	5.3
1492	Bicyclogemacrene	7.3
1505	Beta-bisabolene	0.5
1509	Beta-curcumene + cubebol	0.8
1519	Delta-cadinene	0.6
1541	Cis-sesquisabinene hydrate	1.1
1565	(E)-nerolidol	9.7
1575	Spathulenol	3.8
1578	Caryophyllene oxide	5.9
1586	Globulol	0.7
1603	Ledol + rosifoliol	1.3
1636	Epi-alpha-cadinol	2.2
1649	Alpha-cadinol	1.2
1666	Epi-beta-bisabolol	1.1
2175	Sandaracopimaral	5.3
TOTAL		91.8%

Legend: RI = Retention Index.

and germacrene D (10.1%), as the main constituents of this species. On the other hand, Bersan et al. (2014) reported that E-pinocamphone (16.07%) was found as the predominant compound in the essential oil of *A. gratissima*, while β-caryophyllene was not identified among the constituents. These differences are justified by evidence demonstrating that the chemical composition of a given species may vary according to the method of extraction and collection, as well as the influence of seasonal factors (Figueiredo et al., 1997; Santos et al., 2013). Santos et al. (2013) reported that the EOAG composition may be variable according to the region of collection, where different major compounds were found, including iso-pinocamphone (cis-3-pinanone) (25,4%), limonene (15,1%) and guaiol (12,7%) (Trovali et al., 2009).

3.2. Antibacterial activities of the EOAG and β-caryophyllene

The antibacterial activity analysis demonstrated that both EOAG and its major compound β-caryophyllene presented MIC values equivalent to 32 μg/mL against *S. aureus* (Table 3). However, these treatments presented MIC values above 1.024 μg/mL against *E. coli* and *P. aeruginosa*, indicating that under the conditions adopted in the present study, they showed clinically useful antibacterial activity only against the Gram-positive strain.

Our study showed that both EOAG and its β-caryophyllene presented clinically useful antibacterial activities against *S. aureus*, but not against *E. coli* and *P. aeruginosa*. This finding is corroborated by the work of Pérez-Zamora et al. (2018), who demonstrated that an essential oil obtained from *A. gratissima* containing β-caryophyllene as a major compound exhibited antibacterial activity against *S. aureus*. Accordingly, species of the same genus, such as *Aloysia polystachya* and *Aloysia sellowii* showed potent antibacterial actions against *S. aureus*, which may be related to the presence of β-caryophyllene. According to Dahham et al. (2015), this compound is active against both Gram-negative and Gram-positive bacteria. In addition, Maia et al. (2010) demonstrated that the essential oils of *Vernonia remotiflora* and *V. braziliana*, which had approximately 40% of β-caryophyllene in their composition, had antibacterial action against both Gram-negative and Gram-positive bacteria, including *S. aureus*. Finally, evidence has shown that plants of the family Verbenaceae, due to the abundance of phenolic compounds, have excellent antibacterial activity.

3.3. Antibiotic-enhancing effects of the EOAG and β-caryophyllene

Following the antibacterial activity analysis, this study investigated the potential of the essential oil and its major compound to potentiate the activity of norfloxacin against resistant strains of *S. aureus, E. coli* and *P. aeruginosa*. As shown in (Fig. 1), both EOAG and β-caryophyllene significantly ($p < 0.0001$) reduced the MIC of this antibiotic against all strains, indicating an enhanced-antibiotic activity of this fluoroquinolone when combined with the natural products. It is worth mentioning that while the EOAG and β-caryophyllene presented comparable activity against the Gram-negative strains, the essential oil presented a more potent effect against the Gram-positive strain, suggesting that other components could contribute to the antibiotic-potentiating action of the essential oil against *S. aureus*. Since the properties of the different chemical constituents found in the essential oil of this species can vary significantly, the antibacterial activity of its isolated constituents should be carefully evaluated (Lewis, 2001; Mahizan et al., 2019).

Table 3
Minimum Inhibitory Concentrations (MICs) of the EOAG and β-caryophyllene.

Bacterial Strain	EOAG MIC (μg/mL)	β-caryophyllene MIC (μg/mL)
E. coli 6	≥ 1,024	≥ 1,024
S. aureus 10	32	32
P. aeruginosa 24	≥ 1,024	≥ 1,024

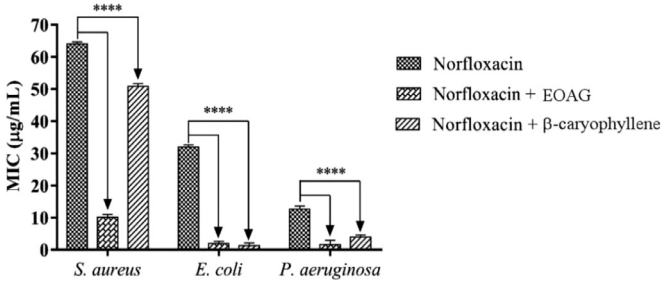

Fig. 1. Minimum Inhibitory Concentration (MIC) of norfloxacin alone or in the presence of *A. gratissima* and β-caryophyllene against *E. coli* 06, *S. aureus* 10 and *P. aeruginosa* 24.
**** $p < 0.0001$ indicates significant differences between groups. Statistical significance was determined by one-way ANOVA and Bonferroni's post-hoc test.

3.4. Effects of β-caryophyllene on the S. aureus NorA and MepA efflux proteins

Since *A. gratissima*-derived natural products exerted antibiotic-enhancing effects in association with norfloxacin, we evaluated the ability of β-caryophyllene to reverse bacterial resistance to fluoroquinolones against EP-carrying *S. aureus* strains (Fig. 2). While the sesquiterpene significantly decreased the MIC of norfloxacin against the NorA-expressing strain 1199B, it was found to increase the MIC of ciprofloxacin against the MepA-carrying strain K2068, suggesting a selective modulation of antibacterial resistance to fluoroquinolones. As expected, the pharmacological control chlorpromazine presented synergistic effects when associated with both antibiotics against these strains.

Fig. 3

Considering the importance of EP-dependent mechanisms in antibacterial resistance, the present study evaluated the action of β-caryophyllene against strains of *S. aureus* carrying the NorA and MepA efflux pumps, which confer resistance to fluoroquinolones (Gibbons et al., 2003). Our data show that although this sesquiterpene has reversed the degree of resistance observed against norfloxacin, the results of EP inhibition using an ethidium bromide assay are inconclusive, since a direct action on these proteins was not observed. However, previous studies have shown that terpenes can act as inhibitors of the NorA pump (Gibbons, 2005).

Interestingly, in the ethidium bromide assay, β-caryophyllene presented opposite effects, increasing the MIC of this drug against the 1199B strain, and decreasing its MIC against the K2068 strain. Such difference may be due to some physicochemical interaction between sesquiterpene and ethidium bromide, leading to a different pattern of modulation when compared to that observed in the tests with fluoroquinolones. Therefore, further research is required to characterize the action of β-caryophyllene as an EP inhibitor in *S. aureus*.

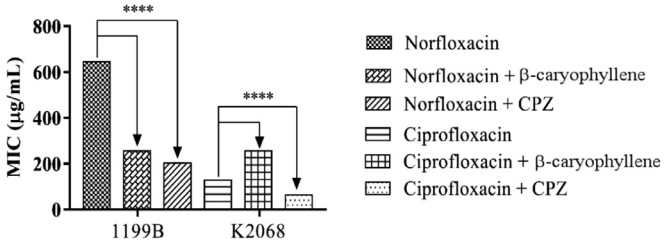

Fig. 2. Modulation of antibiotic resistance by β-caryophyllene and chlorpromazine (CPZ) in association with norfloxacin or ciprofloxacin against *S. aureus* 1199B and K2068 strains. **** $p < 0.0001$ indicates significant differences between groups. Statistical significance was determined by one-way ANOVA and Bonferroni's post-hoc test.

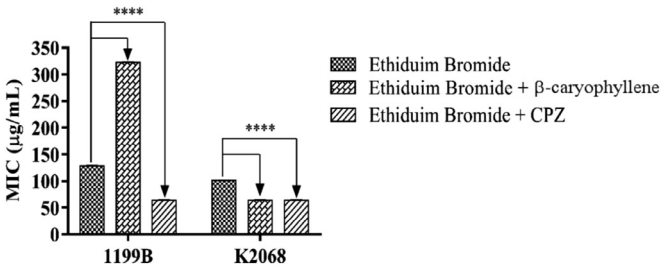

Fig. 3. Minimum Inhibitory Concentration (MIC) of ethidium bromide alone or associated with β-caryophyllene and chlorpromazine (CPZ) against *S. aureus* 1199B and K2068 strains. **** $p < 0.0001$ indicates significant differences between groups. Statistical significance was determined by one-way ANOVA and Bonferroni's post-hoc test.

On the other hand, as expected, the CPZ control demonstrated EP-inhibiting activity against both NorA and MepA proteins. Studies have shown that *S. aureus* species expressing the NorA EP are significantly more sensitive to the action of ethidium bromide. In fact, CPZ causes a collapse in the transmembrane electrical potential, impairing the active transport mechanism required by the pump (Kaatz et al., 2003)

4. Conclusion

The essential oil of *A. gratissima* showed antibacterial and antibiotic-potentiating activities. These activities are, at least partially, mediated by its major constituent β-caryophyllene. It is emphasized that both the essential oil and the isolated compound have antibacterial activity and are capable of potentiating the activity of norfloxacin against MDR strains, which is corroborated by the effectiveness of β-caryophyllene as an EP inhibitor. Nevertheless, direct inhibitory effects in the ethidium bromide assay were not observed.

In conclusion, both EOAG and β-caryophyllene have a notable potential for the development of new therapies against bacterial resistance. However, further research is needed to characterize the molecular mechanisms underlying the pharmacological effects demonstrated by the present study.

Author contribution

Conceptualization: E.L.S; experiments were conducted by: A.C.J.A and P.R.F.; data analysis: H.D.M.C.; formal analysis, T.F.M.; writing—original draft preparation, W.d.A. and C.D.; writing of manuscript: A. C.A.S and J.R.F.; critical review: S.R.T and J.R.F. All authors read and agreed with the final version of the manuscript.

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Declaration of Competing Interest

The authors deny the existence of any conflicts of interest regarding this publication.

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