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Enhancement of the antibiotic activity mediated by the essential oil of *Ocotea odorifera* (VELL) ROWHER and safrole association

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ABSTRACT

In a recent study, our research group demonstrated that the essential oil of *Ocotea odorifera* (EOOO) and its major compound safrole potentiated the action fluoroquinolones, modulating bacterial resistance possibly due to direct inhibition of efflux pumps. Thus, in the present study, we investigated whether these treatments could enhance the activity of gentamicin and erythromycin against multidrug-resistant (MDR) bacteria. The EOOO was extracted by hydrodistillation, and the phytochemical analysis was performed by gas chromatography coupled to mass spectrometry (GC–MS). The antibiotic-enhancing effect of the EOOO and safrole against MDR strains of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* was analyzed by the broth microdilution method. The chemical analysis confirmed the presence of safrole as a major component among the 16 compounds identified in the EOOO. Both the essential oil and the isolated compound showed clinically relevant antibacterial activities against *S. aureus*. Regarding the modulation of antibiotic resistance, the EOOO was found to enhance the activity of erythromycin against the strains of *P. aeruginosa* and *S. aureus*, as well as improving the action of gentamicin against *S. aureus*. On the other hand, safrole potentiated the activity of gentamicin against the *S. aureus* strain alone. It is concluded, therefore, that the EOOO and safrole can enhance the activity of macrolides and aminoglycosides, and as such are useful in the development of therapeutic tools to combat bacterial resistance against these classes of antibiotics.

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Introduction

Brazil is known for its great biodiversity, accounting for about 20% of all plant species of the planet [1], many of which are used in folk medicine [2]. Essential oils are volatile substances synthesized by the plant secondary metabolism, composed mainly of monoterpenes, sesquiterpenes and phenylpropanoids [3]. Importantly, several studies have demonstrated that this class of compounds present anti-inflammatory, antitumor, antioxidant and antimicrobial properties [4–6].

Ocotea odorifera (Vell.) Rohwer (Lauraceae) is a species native to Brazil, where it is traditionally used to treat infected wounds [2]. This species is phytochemically characterized by the presence of flavonoids such as kaempferol and quercetin [7], as well as for producing an essential oil containing safrole as a major component [8]. Safrole is a phenylpropanoid widely used in the production of fragrances and inseticides [9]. Previous research has demonstrated that both the essential oil of *O. odorífera* (EOOO) and safrole have antibacterial [10,11], antileishmania [8] and insecticidal [12] activities, justifying their use in drug development research.

Bacterial resistance is a global health problem with significant impact on mortality rates and health care costs [13]. Evidence has indicated that multi-drug resistant (MDR) bacterial strains can become increasingly virulent, hindering the treatment of several infections [14,15]. Bacteria such as *Staphylococcus aureus* [16–18], *Escherichia coli* [19–21] and *Pseudomonas aeruginosa* [22–24] have been recognized as highly pathogenic microorganisms responsible for a significant number of serious infections. Accordingly, the emergence of multidrug-resistant strains in these species has hampered the treatment with most conventional antibiotics and therefore, the development of new drugs capable of combating bacterial resistance has become a priority in the search for new antimicrobial agents.

Our group has recently demonstrated that the EOOO and safrole presents clinically relevant activity against *S. aureus* and that safrole modulates antibiotic resistance to quinolones in the *S. aureus* SA1119B and K2068 strains, possibly through direct inhibition of the NorA and MepA efflux pumps, respectively [25]. However, to date it is unknown if these products are capable of modulating antibacterial resistance to other classes of antibiotics.

Therefore, this study aimed to investigate the antibiotic-enhancing activity of the EOOO and safrole in association with macrolides against MDR bacterial strains.

Materials and methods

Collection, extraction and analysis of the botanical material

The botanical material was collected, identified, extracted, and analyzed as described by Almeida et al. [25]. Briefly, the essential oil was extracted from terminal branches and inflorescences of plants collected in a segment of Atlantic Forest in the State of Paraná, Southern Brazil (coordinates: S 25° 19.862′ W 49° 48.338′). A voucher specimen was prepared and registered at the Herbarium of "Faculdades Integradas Espirita" (HFIE) (registry number 9.000).

The extraction was carried out by hydrodistillation in a Clevenger type apparatus using 50 g of dry leaves in 1 L of distilled water. The chemical composition of the essential oil was determined by gas chromatography coupled to mass spectrometry (GC–MS) and compounds were identified by comparing their mass spectra with the standards reported in the literature.

Table 1
Origin and antibiotic resistance profile of the strains.

Bacterial strain	Origin	Resistance profile
S. aureus 10	Rectum swab	Amc, Amox, Amp, Asb, Azi, Cefa Cef, Cf, Cip, Cla, Clin, Ery, Lev, Mox, Oxa, Pen
E. coli 06	Urine	Asb, Cefa, Cef, Cfo, Cpm, Ctx
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, Cefa – cefadroxil; Cef — cephalexin, Cfo – cefoxitin, Cip –— ciprofloxacin, Cla – clarithromycin, Clin — clindamycin, Cpm — cefepime, Ctx — ceftriaxone, Ctz – ceftazidime, Ery — erythromycin, Imi — imipenem, Lev — levofloxacin, Mer — meropenem, Mox — moxifloxacin, Oxa — oxacillin, Pen — penicillin and Ptz — piperacillin.

Bacterial cultures

The origin and resistance profile of the bacterial strains used in the present study [26] is shown in Table 1.

All strains were preserved on blood agar (Laboratorios Difco Ltda., Brazil) and in Heart Infusion Agar (HIA, Laboratorios Difco Ltda., Brazil) 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 CFU. The antibiotics erythromycin and gentamicin were obtained from SIGMA Chemical Co. (St. Louis, USA). The EOOO, safrole and both antibiotics were dissolved in DMSO (10 mg/mL) and diluted in distilled water to 1024 μg/mL.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was determined in sterile 96-well microplates by serial dilution. Each bacterial inoculum was prepared by transferring a sample of the bacterial culture to BHI broth, followed by incubation at 37 °C for 24 h. After this period, a 1:10 solution was prepared in test tubes, by adding 100 μL of inoculum and 900 μL of the BHI medium. A total of 100 μL of this solution was transferred to each well of the plate. Then, each well was added with 100 μL of the corresponding treatment serially diluted (1:2) at concentrations ranging from 1024 to 1 μg/mL Positive controls (medium + inoculum) were added in the last well of the plate. The plates were incubated at 35 ± 2 °C for 24 h [27].

Following incubation, each well was added with 20 μL of an aqueous solution of sodium resazurin (0.01% w/v, SIGMA, USA) and 1 h later, bacterial growth observed by the change of color from blue to pink due to the reduction resazurin [28,29]. The MIC was defined as the lowest concentration capable of inhibiting bacterial growth.

Analysis of antibiotic resistance modulation

The analysis of antibiotic resistance modulation was performed by assessing the MICs of gentamicin and erythromycin (aminoglycoside and macrolide, respectively) against *P. aeruginosa* 24, *E. coli* 06 and *S. aureus* 10 were determined in the presence or absence of these natural products at concentrations equivalent to their MIC ÷ 8 (subinhibitory concentration). A reduction in the antibiotic MIC was interpreted as enhanced antibiotic activity (antibiotic resistance modulation) [30].

Statistical analysis

Data are expressed as arithmetic means ± standard error of the mean and were analyzed by analysis of variance (ANOVA), followed

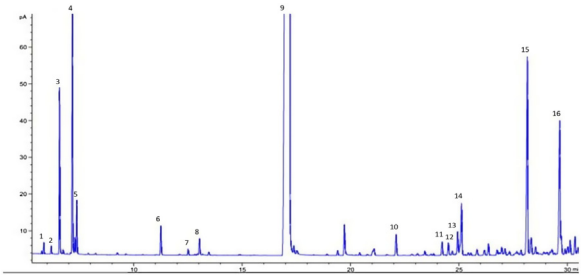

Fig. 1. Chromatogram corresponding to the phytochemical analysis of the EOOO by GC–MS. 1 = α-pinene; 2 = camphene; 3 = β-pinene; 4 = α-felandrene; 5 = ortocymene 6 = 1,8-cineole; 7 = camphor; 8 = α-terpineol; 9 = safrole; 10 = eugenol; 11 = (*E*)-caryophylene; 12 = γ-muurolene; 13 = δ-selinene; 14 = bicyclogermagrene; 15 = spathulenol; 16 = 11-selinen-4-α-ol.

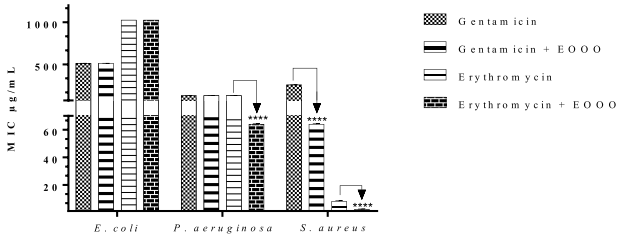

Fig. 2. Modulation of antibiotic resistance by the EOOO in association with gentamicin or erythromycin against MDR *E. coli* 06, *S. aureus* 10 and *P. aeruginosa* 24. **** $p < 0.0001$ indicates significant differences between groups.

by Bonferroni's post-test. Statistical significance was considered when $p < 0.05$.

Results

Chemical composition of the EOOO

Fig. 1 shows the chromatogram corresponding to the phytochemical analysis (GC–MS) of the EOOO [25], which revealed the presence of safrole (77.9%) as a major component, in addition to α-pinene (0.3%), camphene (0.2%), β-pinene (0.1%), α-felandrene (1.9%), ortho-cymene (3.0%), 1.8-cineole (0.9%), camphor (0.4%), α-terpineol (0.3%), eugenol (0.6%), (E)-caryophylene (0.4%), γ-muurolene (0,3%), δ-selinene (0.5%), bicyclogermagrene (1.1%), spatulenol (4.0%) and 11-selinen-4-α-ol.

Antibiotic-enhancing activity of the EOOO

The antibacterial activity analysis of the EOOO found MIC values above 1024 µg/mL against *P. aeruginosa* 24 and *E. coli* 06. On the other hand, the essential oil presented a MIC of 512 µg/mL against *S. aureus* 10, indicating that the EOOO only has clinically relevant antibacterial activity against the Gram-positive strain. However, when a subinhibitory concentration of the essential oil was associated with conventional antibiotics, synergistic effects were obtained with erythromycin against *S. aureus* and *P. aeruginosa,* as well with gentamicin against the Gram-positive strain. Nevertheless, none of these antibiotics had the antibacterial activity against *E. coli* modulated by the essential oil (Fig. 2).

Effects of safrole on antibiotic resistance to gentamicin and erythromycin

The analysis of the antibacterial activity of safrole found the same MIC values as those obtained for EOOO against all bacterial strains. However, the compound alone did not affect the antibacterial activity of antibiotics against none of the Gram-negative strains.

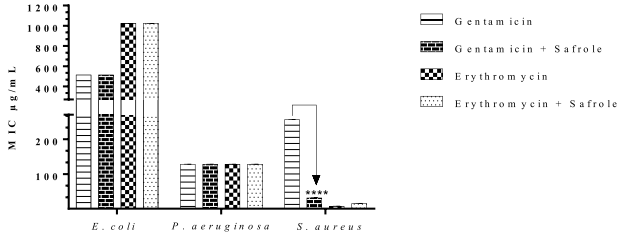

Fig. 3. Modulation of antibiotic resistance by safrole in association with gentamicin or erythromycin against MDR *E. coli* 06, *S. aureus* 10 and *P. aeruginosa* 24. **** $p < 0.0001$ indicates significant differences betsween groups.

On the other hand, the combination of safrole with gentamicin resulted in a significant reduction in the MIC of this antibiotic against *S. aureus*, indicating potentiation of antibacterial activity (modulation of resistance to gentamicin) (Fig. 3).

Discussion

Evidence demonstrates that essential oils represent an important source of active ingredients with the potential to combat bacterial resistance [31–33]. In this context, the chemical composition of these substances was shown to directly influence their pharmacological properties. Importantly, essential oils obtained from the same species, under different conditions of collection and extraction, can undergo significant variation concerning their major components [34–36], as demonstrated by analyzes using gas chromatography coupled with mass spectrometry, one of the main methods used in phytochemical analyses [37].

The present work evaluated the effects of the *O. odorifera* essential oil on the modulation of bacterial resistance to gentamicin and erythromycin. As in a recent analysis performed by Almeida et al. [25], we found safrole as the major component of this oil, the activity of this compound was also evaluated. A similar chemical profile was reported by Mossi et al. and Alcoba et al. [38,39]. The later study identified safrole (36.3%), γ-cadinene (6.6%), camphor (6.5%), and α-copaene (6.0%) as major components in the essential oil of *O. odorifera*. However, a study using gas chromatography coupled to a mass spectrometer with a selective detector found significant differences in the composition of an essential oil obtained from the same species, which consisted mostly of methyl-eugenol (81.2%), followed by safrole (10.6%) [40].

The antibacterial analysis revealed that both the EOOO and safrole showed clinically relevant activity only against the Gram-positive strain, although a different phenomenon was observed in the antibiotic resistance modulation tests. Betim et al. [41] compared the antibacterial activity of the essential oils obtained from *O. odorifera* and *Ocotea nutans* and concluded that the former was more effective than the later against strains of *S. aureus*, *P. aeruginosa*, and *E. coli*. Besides, this oil was more effective against the Gram-positive strain, corroborating the data obtained in the present study. Accordingly, Leporatti et al. [42] demonstrated that the essential oil of *Ocotea puchury-major* exerted clinically relevant activity against *S. aureus*, while no significant activity was verified against *P. aeruginosa* and *E. coli*. On the other hand, the essential oil of *Ocotea bicolor* presented MIC values greater than 1000 µg/mL against each of the strains evaluated in this study [43], indicating a variation in the pharmacological effect between species of the same genus. Interestingly, Cansian et al. [44] demonstrated that the EOOO showed more potent antibacterial activity against Gram-negative strains, which may have occurred due to differences in the chemical composition of the oil as a consequence of seasonal variation.

Considering the evidence that *O. odorifera* components could modulate antibacterial resistance in vitro [25], this study evaluated the antibiotic-enhancing activities of the EOOO and safrole against

MDR strains treated with standard aminoglycoside (gentamicin) and macrolide (erythromycin) antibiotics. A subinhibitory concentration of the essential oil (MIC ÷ 8) was found to significantly potentiate the erythromycin against *S. aureus* and *P. aeruginosa,* as well as the activity of gentamicin against the Gram-positive strain. On the other hand, the isolated compound was shown to enhance the activity of gentamicin against *S. aureus*. However, neither the EOOO nor safrole modulated the activity of the antibiotics tested against *E. coli*. Together, these findings indicate that these compounds differentially modulate antibiotic resistance in MDR strains, suggesting that other constituents of the EOOO might influence its antibiotic-enhancing properties.

While safrole presented little antibiotic-enhancing effect in most conditions analyzed by the present study, it was found to potentiate the activity of norfloxacin against *S. aureus, P. aeruginosa* and *E. coli* [25]. Thus, further research is required to determine the spectrum of action of this compound as a modulator of antibiotic resistance. The modulation of antibiotic resistance by a given compound is highly influenced by its chemical structure, as well as by the three-dimensional arrangement of the atoms in the molecule. Studies indicate that Gram-positive bacteria are more susceptible to the action of essential oils since their less complex cell wall structure compared to that of Gram-negative bacteria. Rios and Recio and Cos et al. [45,46] favors the penetration of chemical constituents that can cause ruptures in the cell membrane [47].

Since gentamicin and erythromycin have intracellular action, the potential action of essential oil components on the bacterial membrane could increase the antibacterial activity of these drugs. With regard to the mechanism of action, both antibiotics act as protein synthesis inhibitors by binding the 30S and 50S ribosomal subunits, respectively [48].

Studies have shown that resistance to these antibiotics in MDR strains of *S. aureus* and *P. aeruginosa* is significantly associated with the expression of efflux pumps [49,50]. Thus, based on evidence obtained from previous work by our group [25], it is suggested that EOOO and safrole may be modulating antibacterial resistance to these antibiotics, at least in part, by directly interfering with drug extrusion mechanisms in these strains.

In conclusion, this study brought new evidence with regard to the action of EOOO and its major compound safrole as modulators of bacterial resistance against MDR strains. The differential action observed in the tests with the EOOO and the isolated compound strongly suggests that other compounds present in the essential oil could be contributing to its antibiotic-enhancing properties and therefore, future research using isolated components of this species can bring important contributions in the development of new molecules useful in the combat of bacterial resistance.

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Competing interests

None declared.

Ethical approval

Not required.

Informed consent

Informed consent was not necessary for this study.

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