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Evaluation of antibiotic efficacy of *Ocimum gratissimum* L. essential oil against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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***Ocimum gratissimum* essential oil (EOGT) has been evaluated for its antibacterial efficacy, and its combinational therapy with antibiotics may enhance the therapeutic efficiency against infection-causing bacteria. Herein, we evaluated the chemical composition of EOGT and its antibiotic efficacy against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. GC-MS and GC-FID analyzed EOGT. The antibiotic efficacy was determined by the agar diffusion method, microdilution, minimum inhibitory concentration (MIC), and fractional inhibitory concentration index (FICI). Eugenol (74.2%) was the main component of OGT. Using the agar diffusion method, the action of rifampicin, ciprofloxacin, and tetracycline was evaluated against *S. aureus*, while the action of cefepime and ciprofloxacin was evaluated against *P. aeruginosa*. FICI showed a reduced MIC of ciprofloxacin and tetracycline associated with EOGT. In the presence of EOGT, MIC of ciprofloxacin reduced from 0.6 to 0.0006 µg/mL and of tetracycline decreased from 0.028 to 0.0018 µg/mL against *S. aureus* and from 4 to 0.12 µg/mL against *P. aeruginosa*. EOGT enhanced the antibacterial efficacy of the antibiotics suggesting a synergistic effect, thereby enhancing the efficacy in treating infection against *S. aureus* and *P. aeruginosa*.**

Key words: *Ocimum gratissimum*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, essential oil, synergism, antibiotics, checkerboard.

INTRODUCTION

Infectious diseases constitute a significant threat to human health and are responsible for high morbidity and mortality. These diseases are often treated with antibiotics, which have led to the emergence of microbial resistance and the development of adverse side effects (Brower, 2018). The emergence of antibiotic resistance problems with severe clinical and economic consequences for health and government health agencies (Brower, 2018; Aljeldah, 2022). Among the several strains that have emerged and spread in hospitals

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and communities in different countries, methicillin-resistant *Staphylococcus aureus* (MRSA), which causes meningitis and pneumonia, and *Pseudomonas aeruginosa*, which affects individuals with compromised immunity (AIDS, cancer, transplant or cystic fibrosis), have been a worldwide public health problem that needs new treatments (Reddy, 2022). Furthermore, from an epidemiological point of view, MRSA is constantly changing, and circulating clones and their antibiotic resistance profiles vary considerably by region and country (Romero and Cunha, 2021). Considering these aspects, natural products are one of the most promising sources of antibacterial agents that can be associated with antibiotics to enhance the therapeutic potential by inhibiting infectious mechanisms (Qadri et al., 2022; Ramata-Stunda et al., 2022).

Ocimum gratissimum L. belonging to the family Lamiaceae is popularly known as basil and is a shrub found in tropical and subtropical regions of Asia, Africa, and South America (Padalia et al., 2013). It is used as a condiment in cooking and as a sedative and anxiolytic, and in traditional medicines for headache and abdominal pain, cough, cold, and bronchitis (Silva et al., 2015a; Ashokkumar et al., 2020). Its essential oil has been used as an anesthetic (Silva et al., 2012; Silva et al., 2015a), antinociceptive (Rabelo et al., 2003; Sahouo et al., 2003; Paula-Freire et al., 2013), anti-inflammatory (Sahouo et al., 2003), antioxidant (Pereira and Maia, 2007; Vasconcelos et al., 2021), insecticide (Nguemtchouin et al., 2013), and antimicrobial against bacteria and fungi (Vasconcelos et al., 2021; Franco et al., 2007; Matasyoh et al., 2007; Adjou et al., 2013; Dambolena et al., 2010).

The essential oil of *O. gratissimum* (EOGT) is composed of eugenol as a major constituent (Ashokkumar et al., 2020; Vasconcelos et al., 2021; Matasyoh et al., 2007). Eucalyptol (1.8-cineol), methyl eugenol, germacrene D, β-ocimene, and caryophyllene, among others, have been described in the literature (Ashokkumar et al., 2020; Vasconcelos et al., 2021; Matasyoh et al., 2007). The antibacterial effect of EOGT has been attributed to eugenol, which can inhibit the growth of Gram-negative and Gram-positive bacteria (Catherine et al., 2012; Marchese et al., 2017; Ulanowska and Olas, 2021). However, even in lower concentrations, the other components can contribute to antimicrobial activity (Hendry et al., 2009; Mak et al., 2019).

EOGT has been identified as a promising antibacterial agent, and its association with antibiotics can increase therapeutic efficacy. This study evaluated the chemical composition and efficacy of the antibiotic effect induced by EOGT against *S. aureus* and *P. aeruginosa*.

MATERIALS AND METHODS

Plant and essential oil extraction

O. gratissimum was cultivated in the medicinal garden of the Faculty of Pharmacy of the Federal University of Juiz de Fora (UFJF), Minas Gerais state, Brazil (latitude 21° 41' 20" S and longitude 43° 20' 40" W). A voucher (CESJ 48248) was deposited at the Herbarium Leopold Krieger/UFJF. After collecting the fresh leaves (400 g) on 10 February, 2014, the essential oils were extracted by hydro-distillation in a Clevenger apparatus for 2 h.

Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS analyses of EOGT were conducted on a Perkin Elmer Turbomass Quadrupole mass spectrometer (PerkinElmer, Shelton, USA) fitted with a DB-5 capillary column (30 m × 0.32 mm, 0.25 µm film thickness; Supelco Bellefonte, PA, USA). The oven column temperature was programmed from 60 to 240°C with a heating rate of 3°C/min. Helium was used as carrier gas with a 1.0 mL/min flow. The injection volume was 1 µL (1% EO/dichloromethane v/v) with a split ratio of 1:50. The injector and ion source temperatures were 250°C. The ionization energy was 70 eV (EI), with a mass scan range of 40 to 400 m/z, at a sampling speed of 1.0 scan/s. The compounds were identified by comparing the experimental mass spectra with the Pherobase database and their linear retention index (LRI), which were calculated following Van Den Dool and Kratz (1963) after injecting a homologous series of n-alkanes (C7–C26), under the conditions mentioned earlier and compared with the literature (Adams, 2007).

Gas chromatography with a flame ionization detector (GC-FID) method

EOGT was analyzed using a GC-FID with a capillary column of fused silica and 5% phenyl dimethyl polysiloxane (HP-5MS) with a size of 30 m × 0.25 mm × 0.25 µm. Hydrogen was used as carrier gas with a flow of 1.5 mL/min. The temperature program and injection procedures were the same as the GC-MS analysis (60-240°C at 3°C/min). The percentage composition of the compounds was determined by normalization in triplicate.

Microorganism standards

S. aureus (ATCC 6538) and *P. aeruginosa* (ATCC 25619) were the bacterial species used in this study. These microorganisms were provided by the Instituto Nacional de Controle de Qualidade em Saúde (INCQS) of the Fundação Oswaldo Cruz.

Determination of minimum inhibitory concentration

The minimal inhibitory concentration (MIC) was determined using the microdilution method described by the Clinical and Laboratory Standards Institute Standard M7-A6 (CLSI, 2006). EOGT (solubilized in 0.2% polysorbate 80 in BHI) was diluted in sterile BHI broth, followed by serial dilutions in a sterile 96-well U microplate (100 µL per well). Then, 100 µL of bacterial inoculum (2×10^6 CFU) in 0.9% NaCl was added to each test well. Two positive controls (BHI and BHI containing 0.2% polysorbate 80) were used with bacteria, while the negative control was prepared without bacteria, and the EOG concentration ranged from 58.6 to 7500 µg/mL. The microplate was incubated for 16 to 20 h in an oven at 35 ± 2°C. After incubation, inhibition of bacterial growth was evidenced by the addition of 20 µL of triphenyltetrazolium chloride (TTC) (5% v/v in distilled water) with a change in color to red, indicative of bacterial growth. The MIC was defined as the lowest concentration of EOG without visible bacterial growth.

Evaluation of effectiveness of antibiotics associated with EOGT

The study of the effectiveness of antibiotics associated with EOGT

was carried out according to the methodology described by Oliveira et al. (2006) with some modifications. Petri dishes with 150 mm in diameter and 20 mm in height were prepared with two different layers of TSA (lower layer with 36 mL of sterile agar and the upper layer containing 12 mL of agar inoculated with 1% of the inoculum containing 10^8 CFU/mL). Antibiogram multi discs (Laborclin®, soaked with 10 µL of the MIC obtained from the EOGT) were inserted onto the surface of the inoculated medium. After incubation for 24 h at 35 ± 2°C, bacterial growth inhibition was measured using a caliper.

The effectiveness of the association between antibiotics and EOGT was classified as synergistic effect (when the zone of inhibition of the association has a diameter ≥ 2 mm compared to the antibiotic alone), antagonistic effect (when the zone of inhibition of the association is smaller than that of the isolated antibiotic), and indifferent effect (when the zone of inhibition of the association is the same with the combined or isolated antibiotic) (Oliveira et al., 2006).

Determination of the fractional inhibitory concentration index

The antibiotic effectiveness data selected those with promising results to determine the fractional inhibitory concentration index (FICI). For this, two microplates were used in each bacterial strain. The BHI medium (50 µL per well) was added to the first microplate with successive dilutions of EOGT and the second with successive dilutions of the promising antibiotics. In the first microplate, after serial dilution, 50 µL of the MIC of the antibiotic was added, and the MIC of the EOGT in combination with the antibiotic was verified. In the second microplate, 50 µL of EOGT MIC was added to obtain the MIC result of the antibiotic in combination with EOGT. After plate preparation, 100 µL of the inoculum suspension (2×10^6 CFU) was added, followed by incubation for 24 h. Bacterial growth was evidenced by adding 20 µL of 2% TTC. The effect of the combination was calculated using FICI, which is equal to the sum of the Fractional Inhibitory Concentration (FIC) of EOGT and the FIC of the antibiotic. The FIC is defined by the MIC of the substance in combination divided by the MIC of the isolated substance (antibiotic). The result is considered a synergistic effect when FICI ≤ 0.5; an additive or indifferent effect when FICI is > 0.5 and < 1; and an antagonistic effect when FICI > 1 (Malik et al., 2011).

RESULTS AND DISCUSSION

Identification of compounds

After hydro-distillation, the fresh leaves of *O. gratissimum* produced 0.5% essential oil. The GC-MS and GC-FID analyses showed that eugenol (74.2%), cis-β-ocimene (17.2%), germacrene D (2.3%), and trans-β-ocimene (1.6%) were the major components of EOGT (Table 1). Our results showed a higher eugenol content (74.2%) in EOGT when compared with the studies published by Ribeiro et al. (2016) and Chimnoi et al. (2018). This compound is essential for EOGT chemotype and is a basis for its antibacterial activity (Silva et al., 2015b; Melo et al., 2019; Rodrigues et al., 2020). Like eugenol, other compounds, such as terpineol-4, γ-muurolene, (*Z*, *E*)-α-farnesene, α-trans-bergamotene, 1,8-cineole, β-selinene, γ-terpinene, p-cymene, thymol, and myrcene, have also been identified in the essential oil of *O. gratissimum* (Matasyoh et al., 2007; Chimnoi et al., 2018; Rodrigues et al., 2020; Yayi et al., 1999).

Effectiveness of antibiotics associated with EOGT against *S. aureus*

In the microdilution test, EOGT showed a MIC of 468.8 µg/mL against *S. aureus* and *P. aeruginosa*, highlighting a promising antibacterial activity against the tested strains, according to the criterion described by Aligiannis et al. (2001). Using the same strain (*S. aureus*, ATCC 6538), Melo et al. (2019) the MIC was 1000 µg/mL, twice as high as the value obtained in the present study. This difference may be related to the variations in the identified components.

As expected, the tested antibiotics inhibited the growth of *S. aureus* (Table 2). However, antibiotics associated with EOGT potentiated the antibacterial action of rifampicin, tetracycline, and ciprofloxacin showing a synergistic effect. Furthermore, when associated with EOGT, there was an antagonistic effect of cefepime and penicillin G, while with the other antibiotics, there was an indifferent effect (Table 2). The synergistic effect induced by EOGT may be related to different mechanisms against *S. aureus* since eugenol is capable of causing membrane rupture by inhibiting ATPase activity, inhibiting efflux pump, and reducing several virulence factors at sub-inhibitory concentrations (Langeveld et al., 2014).

Effectiveness of antibiotics associated with EOGT against *P. aeruginosa*

Considering the data in Table 3, the antibiotics ampicillin, amoxicillin-clavulanic acid, cephalothin, and cefoxitin showed no growth inhibition zone against *P. aeruginosa* (ATCC 25619), which demonstrates the presence of resistance mechanisms for these drugs. This resistance phenomenon is common in the genus *Pseudomonas* since it is a Gram-negative bacteria, and the major mechanisms involved include low permeability of the outer membrane, expression of efflux pumps, and production of antibiotic-inactivating enzymes (Pang et al., 2019).

The antibiotic action of cefepime and ciprofloxacin was evaluated in the presence of EOGT, demonstrating a synergistic effect (Table 3). The activity against the growth of *P. aeruginosa* has been attributed to eugenol (Lou et al., 2019) that may be associated with the competitive binding of this compound to a QS receptor (LasR), which promotes significant repression of genes associated with QS in addition to VF genes (Rathinam et al., 2017). Furthermore, the synergistic effect is related to the ability of eugenol to increase cell membrane permeability (Hemaiswarya and Doble, 2009).

In addition to the synergistic effect, amikacin, ceftazidime, cefuroxime, gentamicin, meropenem, and sulfazotrim had an indifferent effect since the growth

Table 1. Chemical composition of EOGT obtained by hydro-distillation.

LRI	Compound	Group	%
931	α-Thujene	Monoterpene hydrocarbons	0.4
938	α-Pinene	Monoterpene hydrocarbons	0.4
982	β-Pinene	Monoterpene hydrocarbons	0.5
1036	cis-β-Ocimene	Monoterpene hydrocarbons	17.2
1048	trans-β-Ocimene	Monoterpene hydrocarbons	1.6
1059	γ-Terpinene	Monoterpene hydrocarbons	0.5
1183	Terpinen-4-ol	Oxygenated monoterpene	0.6
1360	Eugenol	Phenylpropanoid	74.2
1431	β-Caryophyllene	Sesquiterpene hydrocarbons	1.0
1499	Germacrene D	Sesquiterpene hydrocarbons	2.3
Total			98.7

LRI: Linear retention index.
Source: Authors 2023

Table 2. Effectiveness of antibiotics associated with EOGT against *S. aureus*.

Antibiotic	Growth inhibition zone (mm)		Effect
	Antibiotic	Antibiotic + EOGT	
Cefepime	31.5 ± 2.1	30.0 ± 2.8	A
Oxacillin	23.5 ± 2.1	24.5 ± 0.7	I
Penicillin G	43.5 ± 2.1	42.5 ± 0.7	A
Erythromycin	21.5 ± 0.7	21.5 ± 0.7	I
Clindamycin	24.5 ± 0.7	24.5 ± 0.7	I
Vancomycin	18.0 ± 0.0	18.5 ± 0.7	I
Rifampicin	**33.5 ± 0.7**	**35.5 ± 0.7**	**S**
Tetracycline	**28.0 ± 0.0**	**31.0 ± 0.0**	**S**
Sulfazotrim	20.0 ± 0.0	20.0 ± 0.0	I
Ciprofloxacin	**23.5 ± 2.1**	**28.5 ± 0.7**	**S**
Gentamicin	13.5 ± 0.7	14.5 ± 0.7	I
Chloramphenicol	25.0 ± 0.0	25.5 ± 0.7	I

The values represent mean ± SD (n = 3). S: synergistic effect; A: antagonistic effect; I: indifferent effect.
Source: Authors 2023

inhibition zone was the same with the combined or isolated antibiotic (Oliveira et al., 2006). Because they are ineffective in inhibiting growth, ampicillin, amoxicillin-clavulanic acid, cephalothin, and cefoxitin were considered resistant to *P. aeruginosa*.

Fractional inhibitory concentration and fractional inhibitory concentration index

From the results of Tables 2 and 3, ciprofloxacin and tetracycline were used to evaluate the fractional inhibitory concentration (FIC) and the Fractional Inhibitory Concentration Index (FICI) using the MIC of these antibiotics and their combination with EOGT (Table 4). Thus, ciprofloxacin reduced MIC to 0.6 and 0.03 µg/mL for *S. aureus* and *P. aeruginosa*, respectively, while the MIC of tetracycline was 0.028 µg/mL (*S. aureus*) and 4 µg/mL (*P. aeruginosa*). Using the Checkerboard method to obtain FICI values, the synergistic effect of EOGT was confirmed for ciprofloxacin and tetracycline on the growth of *S. aureus* and tetracycline against *P. aeruginosa* (Table 4).

As demonstrated in Table 4, in the presence of EOGT, ciprofloxacin, and tetracycline, the MIC was reduced by 99.9 and 92.8%, respectively, against *S. aureus* and *P. aeruginosa*, which confirmed the synergistic effect shown in Tables 2 and 3. In addition, tetracycline decreased MIC by 97% against *P. aeruginosa* (synergistic effect), but ciprofloxacin showed an antagonistic effect after

Table 3. Effectiveness of antibiotics associated with EOGT against *P. aeruginosa*.

Antibiotic	Growth inhibition zone (mm)		Effect
	Antibiotic	Antibiotic + EOGT	
Ampicillin	*	*	I
Amikacin	17.5 ± 0.5	18.5 ± 0.5	I
Amoxicillin–clavulanic acid	*	*	I
Ceftazidime	13.5 ± 0.5	14.5 ± 0.5	I
Cephalothin	*	*	I
Cefepime	**21.0 ± 0.0**	**23.5 ± 0.0**	**S**
Cefoxitin	*	*	I
Cefuroxime	17.5 ± 1.0	17.5 ± 0.5	I
Ciprofloxacin	**32.0 ± 0.0**	**34.5 ± 0.0**	**S**
Gentamicin	13.5 ± 0.9	13.5 ± 0.5	I
Meropenem	27.0 ± 1.0	28.0 ± 1.0	I
Sulfazotrim	16.0 ± 1.0	15.0 ± 0.5	I

The values represent mean ± SD (n = 3). S: synergistic effect; I: indifferent effect; * antibiotic resistant strain.
Source: Authors 2023

Table 4. Fractional inhibitory concentration and fractional inhibitory concentration index.

Microorganism	Test substance	MIC (µg/mL)		FIC	FICI	Effect
		Isolated	Combined			
S. aureus ATCC 6538	EOGT	468.8	3.5	0.0075	0.009	Synergistic
	Ciprofloxacin	0.6	0.0006	0.0010		
	EOGT	468.8	3.5	0.0075	0.072	Synergistic
	Tetracycline	0.028	0.002	0.0643		
P. aeruginosa ATCC 25619	EOGT	468.8	3.5	0.0075	5.008	Antagonist
	Ciprofloxacin	0.03	0.15	5.00		
	EOGT	468.8	3.5	0.0075	0.038	Synergistic
	Tetracycline	4	0.12	0.03		

Synergistic: FICI ≤ 0.5; Indifferent: > 0.5 and < 1; Antagonist: FICI > 1.
Source: Authors 2023

combination with EOGT (Table 4). As a major compound with antibacterial action, eugenol should contribute to the potentiation of antibiotic inhibitory activity (Yadav et al., 2015).

Conclusions

EOGT consists of eugenol as the major component and potentiates the antibiotic efficacy of ciprofloxacin and tetracycline against *S. aureus* and *P. aeruginosa* via growth inhibition and reduction of MIC, which may serve as a promising agent for the treatment of infections caused by these pathogens.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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