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Modulation of antibiotic resistance by the essential oil of *Ocimum gratissimum* L. in association with light-emitting diodes (LED) lights

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Abstract: This study aimed to evaluate the antibacterial and antibiotic-enhancing effects of the essential oil obtained from *Ocimum gratissimum* L. (OEOg) alone or in association with light-emitting diodes (LED) lights. The essential oil was obtained by hydrodistillation and its chemical composition analysed by gas chromatography coupled to mass spectrometry. The antibacterial and antibiotic-enhancing activities against multiresistant strains of *Staphylococcus aureus* and *Escherichia coli* were evaluated by the gaseous contact method. The analysis of the photoinductive effect on the antibacterial activity of the OEOg and antibiotics was assessed through exposure to different LED lights (red, blue and yellow). The phytochemical analysis identified five

compounds, including eugenol, as the major constituent. The OEOg caused a significant inhibition of the halo, indicating a direct antibacterial effect. Exposure to the LED lights significantly enhanced the activity of the OEOg against *E. coli*. On the other hand, the action of the essential oil against *S. aureus* was enhanced by exposure to both blue and yellow lights. The effects of LED light exposure on the activity of conventional antibiotics varied significantly according to the drug and the bacterial strain. However, most combinations of LED lights and the OEOg presented synergistic effects against resistant bacterial strains, indicating enhanced antibacterial activity. Thus, these *in vitro* findings suggest that both OEOg and LED lights have promising antibacterial effects. Nevertheless, further research is required to evaluate *in vivo* the potential of these therapies for the treatment of infectious skin diseases.

Keywords: bacterial resistance; enhanced antibiotic activity; LED lights; *Ocimum gratissimum*.

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1 Introduction

The use of plants in folk medicine constitutes a practice that is passed down from generation to generation. Through this ancient practice, empirical knowledge has been shared, learned and internalised among different cultures [1], contributing significantly to the use of extracts and isolated components in scientific research. Nevertheless, therapeutic applications involving bioactive compounds require studies proving their effectiveness and safety [2–4].

Ocimum gratissimum L. is a plant popularly known as ‘alfavaca’. This species is traditionally used for the treatment of rheumatism, mental illness, influenza, cough, pruritus, stress, indigestion, flatulence, headache, fatigue, as well as sedative and expectorant [5, 6]. Previous research has demonstrated that biologically active extracts

and essential oils obtained from *O. gratissimum* present anti-inflammatory, antiseptic, antifungal and antibacterial activities, indicating that this species has the potential to be used in the treatment of infections [7, 8]. While essential oils play a crucial role in the protection and survival of plants, increasing evidence indicates that these substances have remarkable therapeutic potential [2–4].

Antibiotic resistance represents a significant threat to human health [9]. Resistant bacteria are capable of causing a spectrum of manifestations ranging from mild infections to severe diseases. Nevertheless, skin infections represent a major health problem due to their impact on the quality of life of patients [10]. Additionally, according to the Brazilian Society of Dermatology (SBD), skin diseases are among the leading conditions requiring the use of health services [11]. As the emergence of resistant bacteria has limited the treatment of skin infections with conventional antibiotics, the search for new therapies to combat bacterial resistance have a significant impact on public health research. In this context, plant derived natural products, including extracts, essential oils and isolated secondary metabolites have demonstrated the ability to enhance the activity of antibiotics against resistant bacterial strains [9].

In addition to the use of natural products and new synthetic drugs, studies have reported the therapeutic potential of light-emitting diodes (LED). LED devices have been commonly used as a phototherapeutic tool in the treatment of dermatological lesions. Accordingly, previous research has reported that, in addition to presenting antimicrobial properties, LED lights can optimise the penetration of systemic drugs due to a vasodilator action [12]. Moreover, both experimental and clinical studies have demonstrated that due to their photoinductive effects, LED lights have the potential to be used in a wide variety of clinical conditions [13, 14].

Therefore, this study aimed to evaluate the antibacterial and antibiotic-enhancing effects of the essential oil obtained from *O. gratissimum* L. (OEOg) alone or in association with LED lights.

2 Methodology

2.1 Collection and identification of the botanic material

The leaves of *O. gratissimum* L. were collected in a private property located at a region known as ‘Chapada do Araripe’ in the municipality of Crato, Ceará, Brazil. A voucher specimen was prepared and registered at the Herbarium Dárdano Andrade Lima of the Regional University of Cariri (registry number 3978).

2.2 Essential oil extraction

The essential oil of *O. gratissimum* L. was extracted by hydrodistillation in a Clevenger type apparatus. Briefly, 500 g of leaves was crushed and subjected to extraction with 2.5 L of distilled water at boiling temperature for 2 h. After extraction, the essential oil, which presented a yield of 0.2%, was added with anhydrous sodium sulphate (Na_2SO_4) and stored under refrigeration (-4°C) for preservation [15].

2.3 Identification of chemical components

The analysis of the chemical constituents of the essential oil was carried out using a GCMS System (TQ8030 Shimadzu) in the Laboratory of Chemistry of Natural Products of the Federal University of São Carlos (UFSCar). Separation was performed on a fused-silica capillary column RTX-5MS (30 m \times 0.25 mm id, 0.25 μm film thickness, Restek) using ultra-high purity helium as a carrier gas at a flow rate of 3.0 mL/min. The mass spectrometer was operated in the electron impact mode (EI) at 70 eV, scanning at a range of 43–550 m/z . The ion source temperature was set at 230 $^\circ\text{C}$. The separation data were analysed using the GCMS Real Time Analysis[®] Software. The temperature was initially maintained at 60 $^\circ\text{C}$ for 3 min, followed by an increase of 3 $^\circ\text{C}/\text{min}$ until reaching 200 $^\circ\text{C}$. Next, temperature was programmed to increase 15 $^\circ\text{C}/\text{min}$ until reaching 280 $^\circ\text{C}$, which was maintained for 1 min. The apparatus settings were as follows: Injection temperature: 230 $^\circ\text{C}$, detection temperature: 300 $^\circ\text{C}$, injection pressure: 57.4 KPa, Splitless ratio: 150, detection range of the mass spectrometer: 43–550 m/z ; start time (cut time of the solvent): 3.0 min and flow 3 mL/min. The identification of the oil components was based on the Kovats retention index, calculated in relation to the retention times of a homologous series of n-alkanes (C-7 to C-40) and based on the fragmentation pattern observed in mass spectra by comparing them with the literature data and the Nist spectra [16].

2.4 Materials

Amikacin (30- μg disks), gentamicin (10- μg disks), norfloxacin (10- μg disks), ciprofloxacin (5- μg disks), penicillin G (10- μg disks) and oxacillin (1- μg disks) were used as standard antibiotics and purchased from Sigma[®]. Agar heart infusion (HIA) and Brain Heart Infusion (BHI) agar culture media were purchased from HIMEDIA. The LED device (brand NEW Estética[®]) was used in the photoinduction protocols. This device can emit the red, blue and

yellow light spectra, as well as combinations of these lights. The following lights were used in the experiments: blue (with a wavelength pre-determined by the apparatus of 415 nm), red (620 nm) and yellow (590 nm). These lights were applied at a distance of 1.5 cm from the plates.

2.5 Microorganisms

This study used multidrug resistant strains of *Escherichia coli* 06 and *Staphylococcus aureus* 358, which were obtained from clinical isolates. Both strains were maintained in heart infusion agar (HIA) medium and, for testing, 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 10^8 CFU/mL. The resistance profile of the bacterial strains is shown in Table 1.

2.6 Evaluation of the antibacterial and antibiotic-enhancing activities by gaseous contact

The bacteria were sown in Petri dishes containing BHI agar. Disks of antibiogram-like filter paper were placed in the centre of each plate, and 10 μ L of the essential oil was added to the plate lid. For determination of the inhibition halos, the plates were incubated in the oven at 37 °C for 24 h. The tests were performed in triplicate, and a millimetre ruler was used to determine the halos (Table 2).

The evaluation of the antibiotic-enhancing activity by gaseous contact was performed in Petri dishes containing BHI as previously described [17] with adaptations. The plates were inverted, and then 10 μ L of the essential oil was added in the lids allowing the interaction with the antibiotic disks from the volatilisation. Alternatively, plates were prepared in the absence of the essential oil for further comparison between plates containing antibiotics

Table 1: Bacterial origin and antibiotic resistance profile.

Bacteria	Origin	Resistance profile
<i>Staphylococcus aureus</i> SA358	Surgical wound	Oxa, Gen, Tob, Ami, Neo, Para, But, Sis, Net
<i>Escherichia coli</i> EC 06	Urine	Cf, Ca, Clx, Amp, Nor, Lm, Cip, Lv, Of, Ampisul

Amp – Ampicillin; Ampisul – Ampicillin-sulbactam; Ami – Amikacin; Ca – Cefadroxil; Cf – Cephalothin; Clx – Cephalixin; Cip – Ciprofloxacin; Lm – Lomefloxacin; Lv – Levofloxacin; Tob – Tobramycin; Of – Ofloxacin; Oxa – Oxacillin; Gen – Gentamycin; Nor – Norfloxacin; Neo – Neomycin; Para – Paramomycin; But – Butyrosine; Sis – Sisomicin; Net – Netilmicin.

Table 2: Chemical composition of essential oil from *Ocimum gratissimum* leaves.

Compounds	Retention time (min)	(%)
1,8-cineole	8.60	35.61
(E)- β -Ocimene	8.68	2.89
Eugenol	21.86	52.02
(E)-Caryophyllene	24.42	4.37
α -Guaiene	27.07	5.11
Total	–	100.00

exclusively and the plates containing antibiotics and the essential oil. For determination of the inhibition halos, the plates were incubated in an oven at 37 °C for 24 h. The tests were performed in triplicate, and a millimetre ruler was used to determine the inhibition halos.

2.7 Evaluation of the antibiotic-modulating activity in association with LED light exposure

In this experimental protocol, bacterial cultures and treatments were performed as described for the evaluation of antibacterial and antibiotic-enhancing activities by gaseous contact. To evaluate the effects of LED light exposure, the plates were exposed to red, blue or yellow light for 20 min and then incubated at 37 °C for 24 h. The tests were performed in triplicate and results were analysed as described above.

2.8 Statistical analysis

Data are expressed as arithmetic means \pm standard deviations and were analysed by analysis of variance (ANOVA), followed by Bonferroni's post-test using GraphPad Prism software. Statistical significance was considered when $p < 0.05$ [18].

3 Results

The chemical analysis of the essential oil of *O. gratissimum* revealed the presence of five major compounds: (E)- β -ocimene (2.89%; Hydrocarbon monoterpene), (E)-caryophyllene (4.37%; Hydrocarbon sesquiterpene), α -guaiene (5.11%; Hydrocarbon sesquiterpene), 1,8-cineole (35.61%; Oxygenated sesquiterpene) and eugenol (52.02%; Phenylpropanoid).

The analysis of the antibacterial effect of OEOg by gaseous contact demonstrated a significant reduction of the halo, indicating that the chemical compounds of the essential oil volatilised and were absorbed by the filter paper and inhibiting bacterial growth.

Exposure to LED lights significantly enhanced the antibacterial effects of the OEOg against *E. coli*. Blue, red and yellow lights caused inhibitions of bacterial growth corresponding to 35.4, 28.5 and 53.4%, respectively, compared to the control. On the other hand, the experiments with *S. aureus* revealed that while exposure to the red light improved the antibacterial effect of the essential oil, exposure to blue and yellow lights increased the halo in 42.6 and 29.4%, respectively, demonstrating an antagonistic effect (Figure 1).

Following the promising antibacterial effects demonstrated by the OEOg alone, or in combination with some LED lights, this study evaluated the ability of these treatments to modulate bacterial resistance in association with standard antibiotics. The OEOg and amikacin treatments caused similar inhibition of the growth of both strains. Exposure to LED lights did not increase the antibacterial effect of amikacin. However, when this antibiotic was simultaneously combined with the OEOg and red light, a significant increase of the halo in *E. coli* plates was observed. On the other hand, in experiments with *S. aureus*, combination with each of the three lights and the essential oil potentiated the antibacterial effect of amikacin, indicating a synergistic effect (Figures 2 and 3).

The analysis of the antibiotic-enhancing activity using gentamicin (Figures 4 and 5), demonstrated that the association of OEOg with LED lights significantly enhanced the

antibacterial effect of this antibiotic, when compared with the group exposed to gentamicin alone against *S. aureus*. No significant differences were observed between treatments with the antibiotic alone and the treatment with the antibiotic associated with the oil. On the other hand, in the tests with *E. coli*, the association with OEOg and LED did not affect the activity gentamicin.

The experiments using ciprofloxacin (Figures 6 and 7) revealed that this standard drug caused more significant inhibition of bacterial growth in comparison with the OEOg against both bacterial strains. No significant difference between the combined treatments against *E. coli* was found. However, against *S. aureus*, the association between the essential oil and yellow or red light decreased the antibacterial effect of ciprofloxacin, indicating an antagonistic effect as attested by a decrease of the inhibition halo. No relevant difference between other treatment groups was found.

As shown in Figures 8 and 9, the inhibition halo observed in the norfloxacin group was significantly higher than the halo of the OEOg against both strains, indicating a more potent antibacterial activity by the antibiotic. The test with *E. coli* revealed that the simultaneous association with the oil and LED lights did not change the antibacterial effect of ciprofloxacin. Similar findings were obtained for the association of the oil with blue and yellow lights against *S. aureus*. However, when the oil was associated with the red light against this strain, an antagonistic effect was observed. An antagonistic effect was also observed when the antibiotic was associated with the OEOg in the absence of LED light exposure. No relevant difference between other treatment groups was observed.

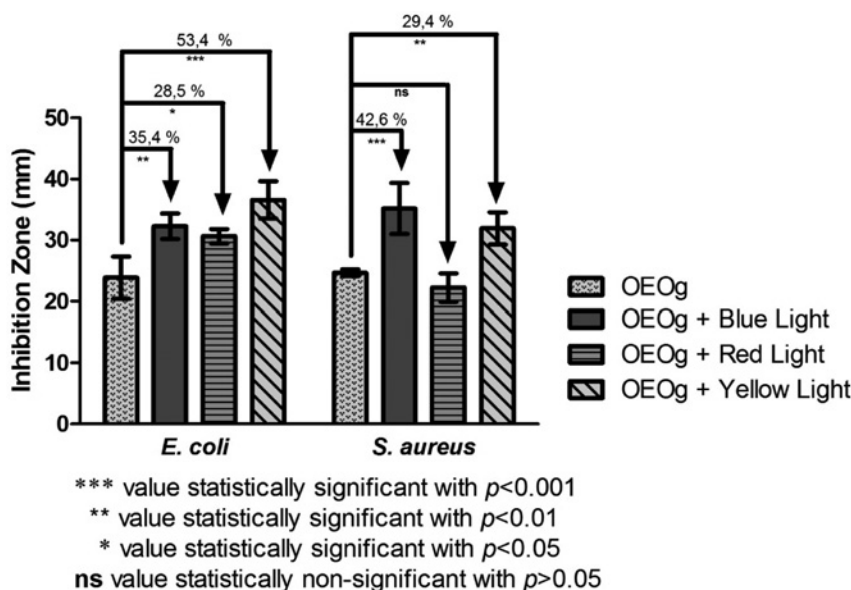


Figure 1: Effect of the association between OEOg and light-emitting diodes (LED) lights on bacterial growth.

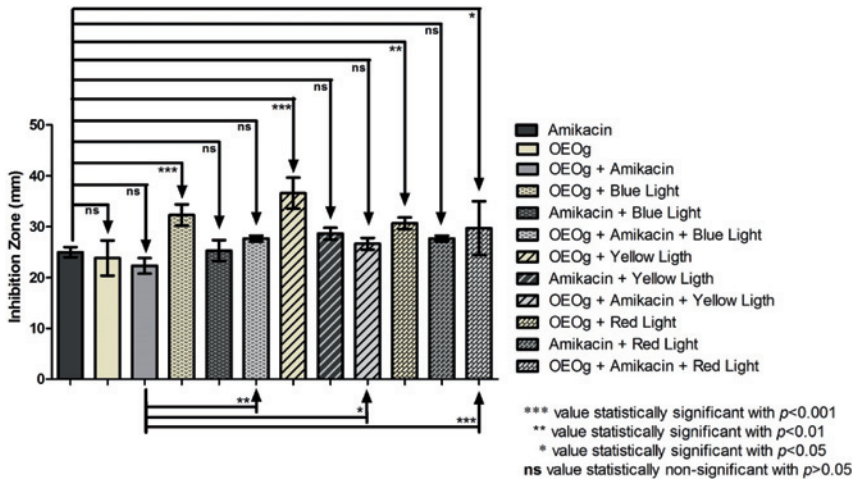


Figure 2: Association of OEOg with amikacin and light-emitting diodes (LED) lights against *Escherichia coli*.

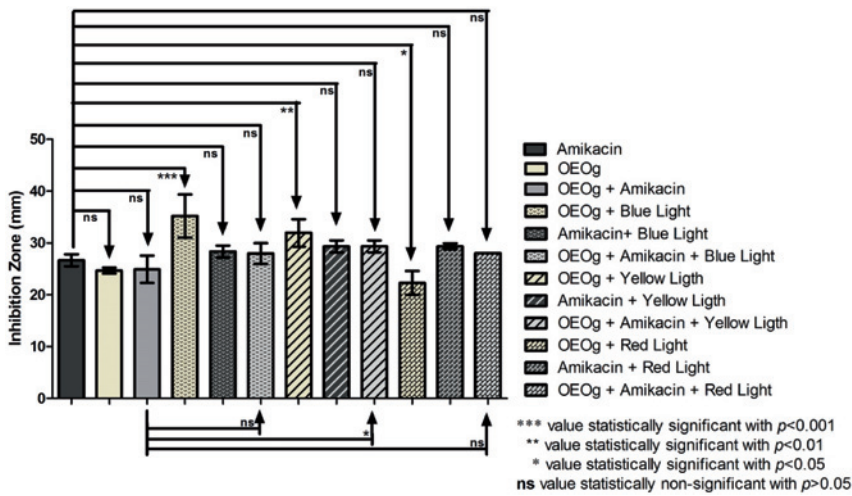


Figure 3: Association of OEOg with amikacin and light-emitting diodes (LED) lights against *Staphylococcus aureus*.

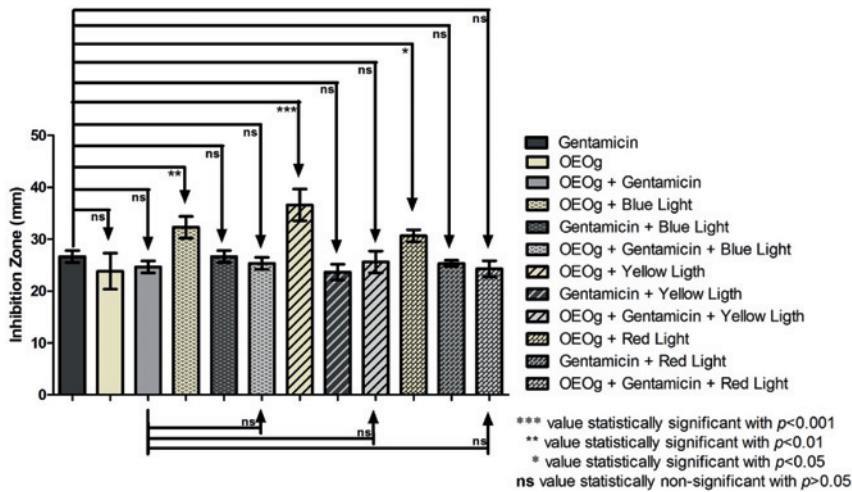


Figure 4: Association of OEOg with Gentamicin and light-emitting diodes (LED) lights against *Escherichia coli*.

Figures 10 and 11 show the effect of different treatment associations on bacterial resistance to penicillin G. The inhibition halo observed in the group treated with the antibiotic was more significant than that demonstrated in

the group treated with the OEOg against *E. coli*. However, no difference between these groups against *S. aureus* was found. The combination of the essential oil and LED lights did not affect the activity of the antibiotic against *E. coli*.

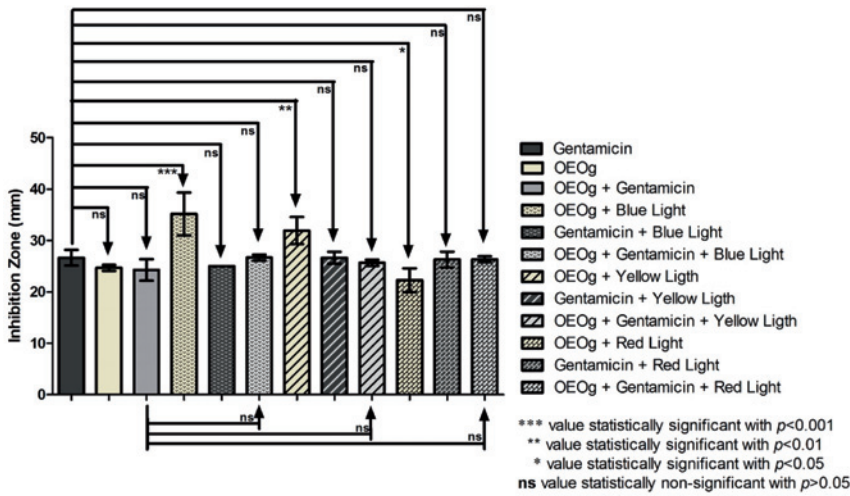


Figure 5: Association of OEOg with Gentamicin and light-emitting diodes (LED) lights against *Staphylococcus aureus*.

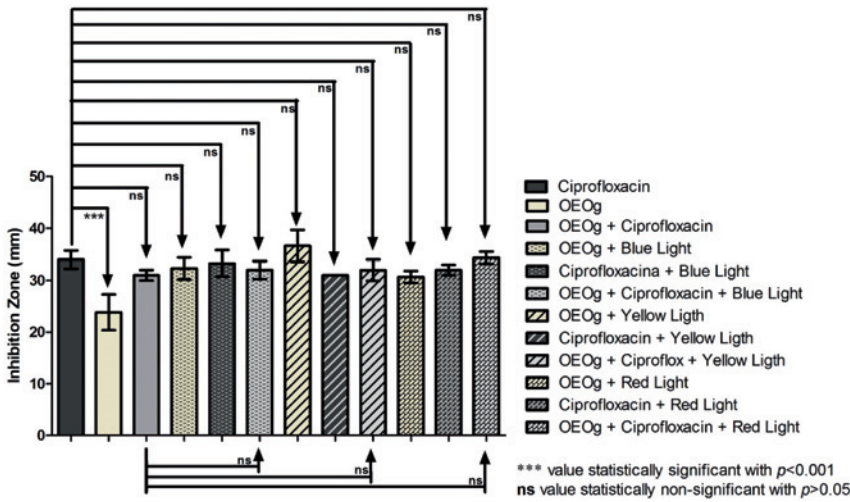


Figure 6: Association of OEOg with Ciprofloxacin and light-emitting diodes (LED) lights against *Escherichia coli*.

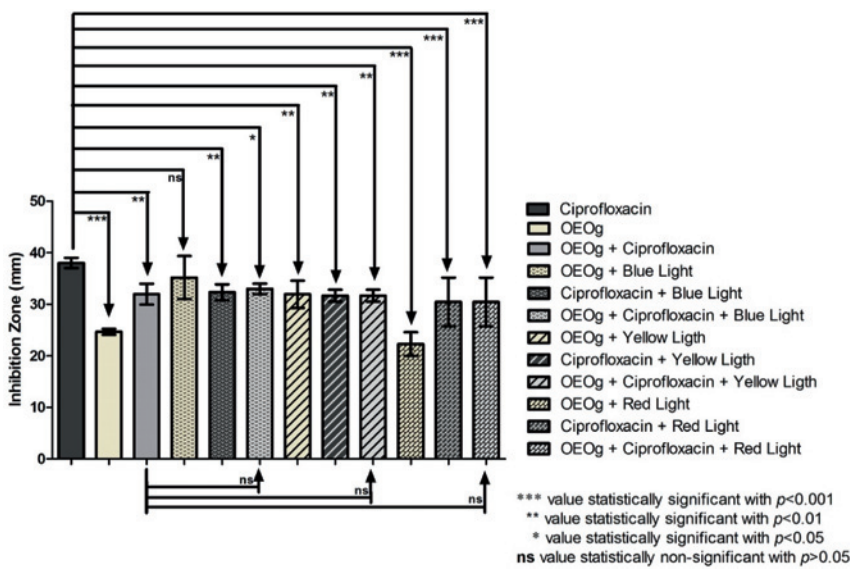


Figure 7: Association of OEOg with Ciprofloxacin and light-emitting diodes (LED) lights against *Staphylococcus aureus*.

However, the combination of the OEOg with blue or yellow lights significantly enhanced the activity of penicillin G against *S. aureus* compared with the drug alone. Interestingly, while the association with the essential oil did not affect the action of the antibiotic, the association with each of the three LED lights decreased the activity of this drug against *E. coli*. Similar findings were observed for the association with the blue light against *S. aureus*.

Finally, the antibacterial analysis using oxacillin as a standard antibiotic found no differences between its antibacterial activity and the effect of the OEOg and against *E. coli* and *S. aureus* (Figures 12 and 13). A comparison between the effect of the antibiotic alone and the antibiotic simultaneously associated with the essential oil and LED lights demonstrated that the combined treatment between the oil and blue or yellow lights enhanced the activity of the antibiotic against broth strains. The association with OEOg alone did not affect the action of oxacillin against both strains. While the association with LED lights did not affect the effectiveness of this antibiotic to *E. coli*, association with yellow and red lights enhanced the antibiotic activity against *S. aureus*. No significant differences between the other treatment groups were found.

4 Discussion

The chemical analysis of the OEOg revealed the presence of eugenol and 1,8-cineole as major constituents. Previous research has demonstrated that both compounds present antibacterial effects. Studies have suggested that due to its hydrophobic properties, eugenol can cause a separation of the lipids in both the cell and mitochondrial membranes, affecting the structure and permeability of

bacterial membranes. Moreover, it has been suggested that the antibacterial action of this compound is also associated with proton-pump inhibition, leading to disturbed electron flow and impaired active transport, which could lead to coagulation of the cell content [19]. Accordingly, Sokovic and collaborators reported that 1,8-cineole exerts remarkable antimicrobial activities, acting as a bacteriostatic and bactericidal compound [20]. These findings support the hypothesis that the essential oil of *O. gratissimum* could constitute a source of bioactive compounds with the potential to combat infections caused by resistant bacteria.

The evaluation of the antibacterial action of the OEOg by gaseous contact demonstrated that the volatilised essential oil induced the formation of a significant inhibition halo formation against resistant strains of *S. aureus* and *E. coli*, which could be attributed to the presence of the major compounds eugenol and 1,8 cineole, whose antibacterial properties have been previously reported [20, 21]. According to the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), a daily dose of eugenol of 2.5 mg/kg of body weight is considered safe, non-carcinogenic and non-mutagenic for humans [22]. The findings of the present study are corroborated by previous research reporting the antibacterial effects of four different extracts obtained from *O. gratissimum* against *E. coli* using the disc diffusion method [23].

The tests evaluating the interaction between the OEOg and different LED lights demonstrated that the activity of the oil was potentiated by exposure to most-light types. Some evidence has suggested that LED light therapy promotes a photoinduced effect that is more effective against Gram-positive strains, since in Gram-negative bacteria, the penetration of photons may be hampered the intrinsic

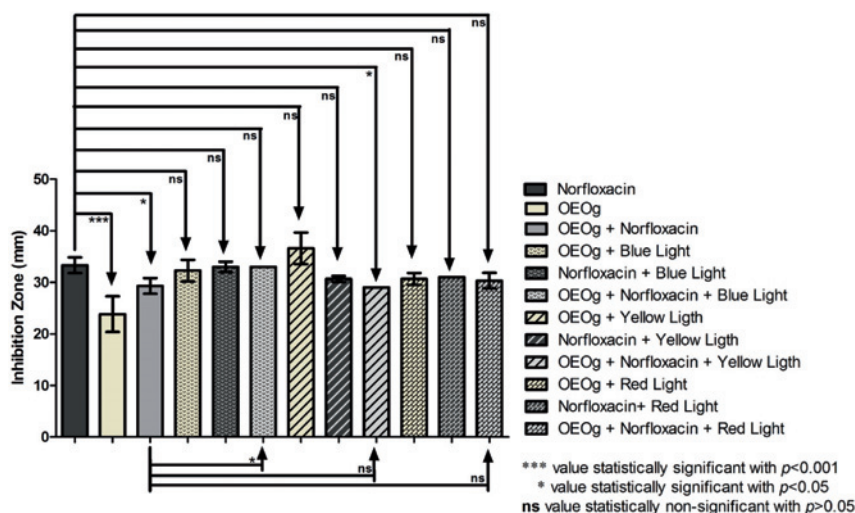


Figure 8: Association of OEOg with Norfloxacin and light-emitting diodes (LED) lights against *Escherichia coli*.

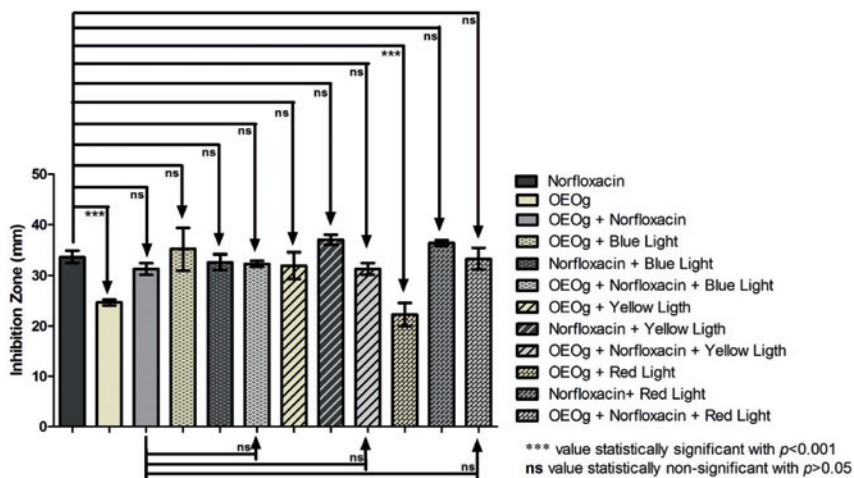


Figure 9: Association of OEOg with Norfloxacin and light-emitting diodes (LED) lights against *Staphylococcus aureus*.

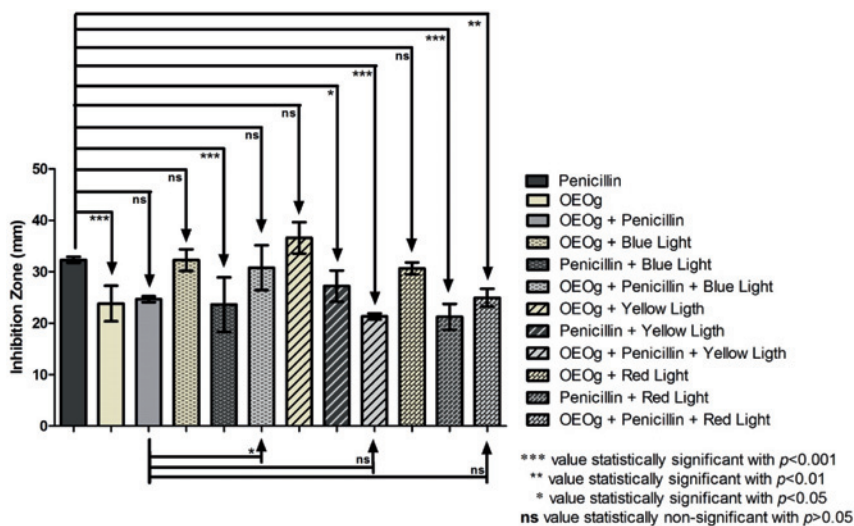


Figure 10: Association of OEOg with Penicillin G and light-emitting diodes (LED) lights against *Escherichia coli*.

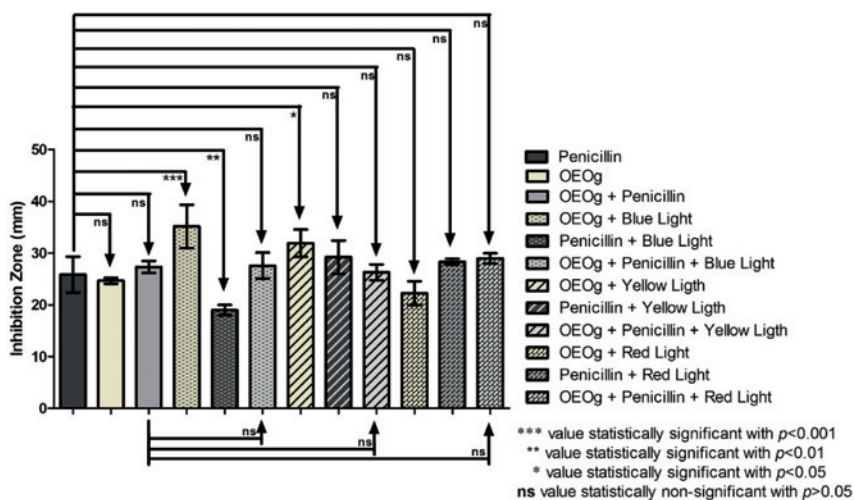


Figure 11: Association of OEOg with Penicillin G and light-emitting diodes (LED) lights against *Staphylococcus aureus*.

characteristics of the cell wall and membrane [24]. In contrast, the results of the present study showed that LED lights exerted promising antibacterial effects on *E. coli*, a

Gram-negative bacterium. Nevertheless, Bevilacqua and collaborators demonstrated that the association of LED lights with blue toluidine presented an excellent activity

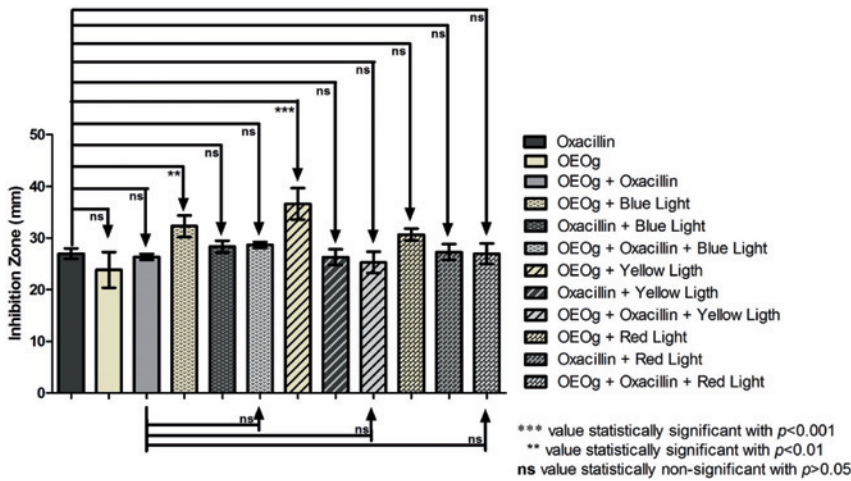


Figure 12: Association of OEOg with Oxacillin and light-emitting diodes (LED) lights against *Escherichia coli*.

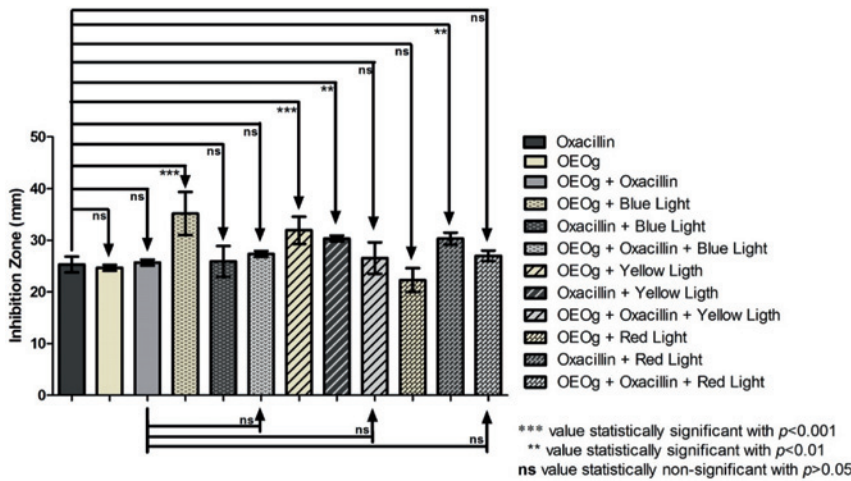


Figure 13: Association of OEOg with Oxacillin and light-emitting diodes (LED) lights against *Staphylococcus aureus*.

against *Streptococcus mutans*, reaching 100% mortality in these microorganisms [25].

Aminoglycosides are drugs with a broad spectrum of action against Gram-negative bacteria that have been widely used to treat severe infections [26]. However, an increasing number of bacterial strains have developed resistance to this class of antibiotics, mainly through the enzymatic inactivation mechanism [27]. The data of the present research indicated that the OEOg, alone or in combination with LED lights, presented antibacterial effects that were comparable in magnitude to those observed for amikacin and gentamicin. This finding suggests that the essential oil, as well as phototherapy, have the potential to be used in the development of new therapies to treat infections caused by bacteria resistant to aminoglycosides. Additionally, the simultaneous association of amikacin with the oil and LED lights potentiated the action of this drug, suggesting that the combined treatment reverted, at least partially, the degree of observed resistance.

Ciprofloxacin and norfloxacin are antibacterial drugs belonging to the class of fluoroquinolones, which have a broad spectrum of action against both Gram-positive and Gram-negative microorganisms. Therefore, they are widely employed in the treatment of infections caused by bacteria resistant to other classes of antibiotics [28]. The resistance to fluoroquinolones occurs mainly through mechanisms that lead to changes in the access to target enzymes, preventing the drug from crossing the bacterial cell wall [29]. In the present study, the inhibition halos observed in the groups treated with both ciprofloxacin and norfloxacin were significantly larger than the halo formed by OEOg against the strains used in this research. However, the simultaneous association of these drugs with the oil and led lights varied considerably, suggesting that these combined therapies might not have a significant impact on the effectiveness of fluoroquinolones. In contrast, another study, using this same methodology, demonstrated that blue and red LED lights increased the inhibition halo of bacterial cultures treated with these drugs [30].

Nevertheless, these differences might be justified due to the use of different wavelengths and LED devices.

Accordingly, researchers evaluated the bactericidal action of LED devices with wavelengths ranging from 415 to 455 nm against *S. aureus* and *E. coli*, and concluded that the wavelength of 415 nm was more effective [31]. It has been demonstrated that the photoinduced antibacterial effect may involve the following mechanisms: Induction of biochemical changes in the bacterial cell membrane; inhibition of the cell respiration through a direct effect on the mitochondria; and induction of DNA damage, which could prevent cell division and inactivate enzymes associated with the cell metabolism [32].

The production of penicillinases is reported as the primary mechanism of resistance to penicillins. These enzymes catalyse the cleavage of the β -lactam ring of the structure of penicillin, inhibiting their antibacterial action [33]. While penicillin G is highly-active against Gram-positive cocci, it is susceptible to the effect of penicillinase. On the other hand, oxacillin has less potent antibacterial activity against penicillin G-sensitive bacteria but is resistant to penicillinase [34]. This study demonstrated that the activity of the OEOg was as potent as the action of these antibiotics, emphasising the antibacterial potential of this essential oil. Additionally, some combinations of LED lights and the OEOg significantly enhanced the activity of these antibiotics, confirming that combined therapies represent a promising alternative to combat bacterial resistance.

5 Conclusion

In conclusion, the essential oil obtained from *O. gratissimum* has an antibacterial effect whose potency varies according to the type of bacteria. The action of this oil can be enhanced by the association with LED lights and, both oil and LED lights potentiated the action of antibiotics under variable conditions, representing a promising alternative in the combat of bacterial resistance.

Nevertheless, further research is required to evaluate the safety and effectiveness of this combined therapy, as well as to its effects on bacterial cell physiology and structure. Moreover, these *in vitro* results encourage *in vivo* testing to investigate the potential use of modulation with OEOg and LED lights in the treatment of infectious skin diseases.

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Conflicts of interest statement: The authors declare no conflicts of interest regarding this article.

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