Antibacterial activity and antibiotic modulating potential of the essential oil obtained from Eugenia jambolana in association with led lights


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ABSTRACT
Bacterial resistance has risen as an important health problem with impact on the pharmaceutical industry because many antibiotics have become ineffective, which has affected their commercialization. The Brazilian biodiversity is marked by a vast variety of natural products with significant therapeutic potential, which could bring new perspectives in the treatment of infections caused by resistant microorganisms. The present study aimed to evaluate the antibacterial effect of the essential oil obtained from Eugenia jambolana (EjEO) using the method of microdilution method to determine the Minimum Inhibitory Concentration (MIC). The modulatory effect of this oil on antibiotic activity was determined using both the broth microdilution and gaseous contact methods. The antibacterial effect of the association of the gaseous contact and the use of a LED unit with red and blue lights was also determined. The chemical components of the EjEO were characterized by HPLC, which revealed the presence of α-pinene as a major constituent. The EjEO presented a MIC ≥ 128 μg/mL against S. aureus and ≥ 1024 μg/mL against E. coli. The combination of the EjEO with antibiotics presented synergism against E. coli and antagonism against S. aureus. An antagonistic effect was obtained from the association of EjEO with amikacin and erythromycin by the method of gaseous contact. On the other hand, the association of EjEO with ciprofloxacin presented a synergistic effect against S. aureus and E. coli exposed to LED lights. A similar effect was observed in the association of the EjEO with norfloxacin presented synergism against S. aureus in the same conditions. In conclusion, our results demonstrated that the essential oil obtained from Eugenia jambolana interferes with the action of antibiotics against bacteria exposed to LED lights. Thus, further researches are required to elucidate the mechanisms underlying these effects, which could open new perspectives in the development of new antibacterial therapies.

1. Introduction

Medicinal plants have represented an important source of new substances for drug development since the beginning of the XIX century [1]. Currently, natural products stand out in the pharmaceutical industry, accounting for about 45% of all pharmaceutical products [2].

In recent years, the increase in bacterial resistance to conventional antibiotics has stimulated the development of research aimed at the development of new antibiotics. In addition, in the face of the side effects caused by conventional drugs, natural products represent a promising source of novel molecules for the development of antimicrobial drugs [3]. In this context, essential oils (also known as volatile oils), which can be obtained from various plant structures, including flowers, leaves, seeds, fruits and roots, have demonstrated significant pharmacological activity in association with antibiotics [4,5].

In addition to the use of medication, it has been demonstrated that the use of the Light Emitting Diodes (LED) apparatus promotes a beneficial effect in the management of cutaneous infections and tissue healing. However, the mechanisms underlying the antimicrobial effects caused by conventional drugs, natural products represent a...
activity of the LED lights alone or in combination with drugs remain to be elucidated [6].

Therefore, the aim of this study was to evaluate the antibacterial and modulatory activity of the essential oil obtained from Eugenia jambolana (EjEO) in association with antibiotics against bacteria exposed to LED lights in vitro.

2. Materials and Methods

2.1. Collection and Identification of the Plant

The leaves of Eugenia jambolana were collected in the city of Crato, Ceará state – Brazil. The material was identified by comparison with a sample of the Herbarium Caririense Dárdivo de Andrade-Lima n° 3107.

2.2. Extraction of the Essential Oil From the Leaves of Eugenia jambolana (EjEO)

The essential oil was extracted from fresh leaves of Eugenia jambolana by hydrodistillation, using a Cleverger type device. Briefly, the leaves were crushed and placed in a 5 L flask with 2.5 L of distilled water to boil for 2 h. Then, the essential oil was added with Anhydrous sodium sulphate (Na₂SO₄) and stored under refrigeration (− 4 °C) until analysis.

2.3. Chemicals

Antibiotics (soluble and disc) and Resazurin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), culture media were purchased from HIMEDIA (India) and Dimethyl sulfoxide (DMSO) was purchased from Merck (Germany).

2.4. Identification of the Chemical Components

2.4.1. Gas Chromatography (GC)

Gas chromatography (GC) analyses were carried out using an Agilent Technologies 6890N GC-FID system, equipped with a DB-5 capillary column (30 m × 0.25 mm; film thickness 0.25 mm) and connected to a FID detector. The injector and detector temperatures were set to 280 °C. The carrier gas was helium, at a 1.3 mL/min concentration, equipped with a split/splitless injector (250 °C). The transfer line Technologies AutoSystem XL) operating in the EI mode at 70 eV, with a thickness 0.25 mm) and HP Innowax (30 m × 0.32 mm i.d., Agilent Technologies 6890N GC-FID system, equipped with a DB-5 capillary column (30 m × 0.25 mm; film thickness 0.25 mm) and con-

2.4.2. Gas Chromatography–Mass Spectrometry (GC-MS)

GC-MS analyses were performed using a GC-MS system (Agilent Technologies AutoSystem XL) operating in the EI mode at 70 eV, equipped with a split/splitless injector (250 °C). The transfer line temperature was 280 °C. Helium was used as carrier gas (1.3 mL/min) and the capillary columns used were HP 5MS (30 m × 0.25 mm; film thickness 0.25 mm) and HP Innowax (30 m × 0.32 mm i.d., film thickness 0.50 mm). The temperature program was the same of the GC analyses. The injected volume was 1 μL of the essential oil diluted in hexane (1:1).

2.5. Evaluation of Antimicrobial Activity

2.5.1. Bacterial Strains

Standard bacterial cultures of Escherichia coli ATCC 9027, Staphylococcus aureus ATCC 25923 and Pseudomonas aeruginosa ATCC25853 and multiresistant strains from clinical isolates of Escherichia coli EC 06, Staphylococcus aureus SA 358 and Pseudomonas aeruginosa PA 03 (Table 1) were supplied by the Oswaldo Cruz Institute. For the agar disc-diffusion tests, the strains were suspended in a test tube with distilled water to obtain a turbidity equivalent to 0.5 of the McFarland scale (1 × 10⁵ CFU/mL).

2.5.2. Irradiation

This test was carried out using the LED apparatus (Dermaled®), with semiconductor diodes, which has red, blue and amber spectra and allows a combination of these colors. The lights used were blue (with a wave length of 470 nm) and red (at 625 nm), pre-determined by the apparatus. Each plate received irradiation for 10 min with blue and red lights.

2.5.3. Determination of the Minimum Inhibitory Concentration (MIC) and Modulation of the Activity of Aminoglycosides

The MICs were determined using the broth microdilution technique in sterile 96-well plates with serial dilutions (1:1) [8]. Microbial cultures kept under refrigeration in agar medium were transferred to brain heart infusion broth (BHI) medium and incubated at 35 °C for 24 h. Then, the inoculum was standardized by preparing a suspension in BHI at a concentration capable of completely inhibiting microbial growth in the McFarland scale (1 × 10⁵ CFU/mL).

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Table 1

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Source</th>
<th>Profile of resistance</th>
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<tbody>
<tr>
<td>Staphylococcus aureus ATCC 25923</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC 25853</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Escherichia coli ATCC 9027</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Staphylococcus aureus SA358</td>
<td>Surgical wound</td>
<td>Oxa, Gen, Tob, Ami, Neo, Para, But, Sel, Net</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa PA 03</td>
<td>Nasal discharge</td>
<td>Cf, Cpf, Cld, Caz, Lm Mero, Pip</td>
</tr>
<tr>
<td>Escherichia coli EC 06</td>
<td>Urine</td>
<td>Cf, Ca, Cx, Amp, Nor, Ln, Cip, Ls, Of, Amp</td>
</tr>
</tbody>
</table>

Amp – ampicillin; Ampsol – ampicilina-sulfactam; Ami – amikacin; Amox – amoxicilina; Ca – cefadroxil; Clc – cefaclor; Cf – cefotaxim; Cip – cefazolin; Cmp – cefepimo; Cld – cefradinm; Cip – ciprofloxacin; Im – imipenem; Can – canamicina; Lm – lonfloxacin; Lx – levofloxacín; Tob – tobramycin; Of – ofloxacin; Oxa – oxacilina; Gen – gentamicin; Mero – meropenen; Nor – norfloxacín; Neo – neomicina; Para – paramomicin; Pip – pipércilina; But – butiroxina; Sis – sisomicin; Net – netilimicin.

In this study, all tests were performed in triplicates. The plates were incubated at 35 ± 2 °C for 24 h. Then, a solution of Resazurin 0.01% (w/v) was prepared in sterile distilled water and was used as an indicator. Following incubation, 20 μg/mL of the indicator were added to each well, and 1 h later the readings were performed in a room temperature. Thus, the wells in blue indicated absence of microbial growth, whilst those in red indicated presence of microbial growth [9]. Culture medium containing the inoculum was used as positive control. The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration capable of completely inhibiting microbial growth in the microdilution wells, as detected macroscopically due to the change of the colour of the resazurin, an indicator dye to detect the bacterial growth.

The modulation of the activity of antibiotics was evaluated using aminoglycosides (gentamicin and amikacin) against multiresistant bacterial strains in a concentration of 1.024 μg/mL. The antibiotics
were serially diluted into the wells in a volume of 100 μL containing 10% BHI, the suspension of the multiresistant strain, and the EjEO in a sub inhibitory (MIC/8) concentration [8]. The final concentrations of the antibiotics in the culture medium ranged from 512 to 0.5 μg/mL. The plates were incubated at 35 ± 2 °C for 24 h and the readings were performed using Sodium Resazurin as indicator.

2.5.4. Evaluation of Antibacterial and Modulatory Activities by the Method of Gaseous Contact

To seed the microorganisms, petri dishes containing BHI-agar were used. The essential oil was diluted in DMSO to obtain solutions containing 50; 25; 12.5 and 6.25% of the EjEO. Solutions containing 100% of the oil were also used. Antibiotic discs with antibiotics and blank discs containing 10 μL of the oil were used in the tests. The halos of inhibition were determined using millimetric rulers after incubation at 37 °C for 24 h. The tests were performed in triplicates and dishes containing only DMSO were used as control.

The analysis of the modulatory activity was also performed using the methodology of gaseous contact in petri dishes containing BHI, as described by Inouye and colleagues [10] with adaptations. The following antibiotic discs were used: amikacin (10 mg), gentamicin (30 mg) and erythromycin (15 mg). The plates were inverted and 10 μL of the EjEO (we used the highest concentration at which bacterial growth was observed) were added to the lids to interact with the discs through volatilization. The remaining discs were prepared without addition of the oil and incubated at 37 °C for 24 h. The effect of the antibiotics, in the presence or absence of the compounds, was analyzed by measuring the halos of inhibition with a millimetric ruler. The tests were performed in triplicates and DMSO was used as control.

2.5.5. Evaluation of Antibacterial and Modulatory Activities Through Gaseous Contact and Exposure to LED

In this test, we used the methodology described in the last session. However, here we used quinolone class antibiotics (ciprofloxacin [5 mg] and norfloxacin [10 mg]). The petri dishes were subdivided into three groups. The first group was exposed to blue LED light; the second group was exposed to red LED light for a period of 10 min for each dish. The third group was not exposed to LED lights. The dishes were incubated at 35 ± 2 °C, for 24 h before the readings.

2.6. Statistical Analysis

The data were analyzed using the GraphPad Prism 5.0. software. The differences were determined the Two-Way ANOVA test, followed by Bonferroni’s post hoc test.

3. Results and Discussion

3.1. Identification of the Compounds

The constituents were identified according to their retention indexes (RI), with reference to the homologous series of n-alkanes, C7-C30, under identical experimental conditions, comparing with the mass spectra library search (NIST and Wiley), and with the mass spectra literature. The relative amounts of the individual components were calculated based on the CG peak area (FID response). As shown in Table 2, we identified 26 constituents in the EjEO, representing 98.93% of the composition, being the α-pinene (48.09%) and Nerolidol (8.73%) the main phytocompounds observed.

3.2. Determination of the Minimum Inhibitory Concentration

In the microdilution test we demonstrated that EjEO presented MIC = 128 μg/mL against S. aureus and ≥ 1024 μg/mL against E. coli, demonstrating that this in vitro treatment was more effective against S. aureus, whose value is considered clinically relevant and promising for in vivo tests.

A previous study demonstrated that an essential oil obtained from the leaves of Eugenia jambolana effectively inhibited the growth of Vibrio cholerae at a dilution of 1:500 and presented caused a moderate inhibition at 1:1000 [11]. In another study, the essential oils obtained from 17 species of the family Myrtaceae were tested against six microorganisms, including bacteria and fungi. It was demonstrated that the treatments presented best results against Staphylococcus aureus and S. epidermidis, supporting the data found in the present study [12]. Noteworthy results were also obtained with an essential oil obtained from Lippia sidoides and the constituent thymol, which presented a MIC of 128 μg/mL against S. aureus. On the other hand, extracts obtained from other plant species, such as Cordia verbenacea [14] presented significant inhibitory effects against multiresistant strains of Escherichia coli, in contrast with the EjEO, which was not effective against this bacterium.

3.3. Modulation of Antibiotic Activity

In the method of microdilution by direct contact (Fig. 1), the combination of EjEO with amikacin or gentamicin caused a reduction in the MIC of these antibiotics against E. coli, indicating that the association between these treatments presented a synergistic effect. However, the combination of EjEO with the same antibiotics caused an increase in their MIC against S. aureus indicating that the association between these treatments presented a synergistic effect.

The resistance of E. coli to the treatments performed in this work might be justified by the presence of an external membrane in this Gram-negative bacterium, which forms an envelope and thus, difficult the action of natural products and other antimicrobial drugs [15,16]. Although essential oils usually present higher efficacy against gram-positive bacteria, we demonstrated that the EjEO improved the activity

<table>
<thead>
<tr>
<th>Table 2</th>
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<tr>
<td>Compounds</td>
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<tr>
<td>Hexanol</td>
</tr>
<tr>
<td>α-Pinene</td>
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<tr>
<td>β-Pinene</td>
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<tr>
<td>β-Mycarene</td>
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<tr>
<td>α-Terpineine</td>
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<tr>
<td>Limonene</td>
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<td>δ-Terpineine</td>
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<tr>
<td>Nonanol</td>
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<tr>
<td>Linalool</td>
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<tr>
<td>Isoeugenol</td>
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<tr>
<td>Borneol</td>
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<tr>
<td>α-Terpineol</td>
</tr>
<tr>
<td>Tetradecane</td>
</tr>
<tr>
<td>Nerol</td>
</tr>
<tr>
<td>Geraniol</td>
</tr>
<tr>
<td>(E,Z)-2,4-decadienal</td>
</tr>
<tr>
<td>Eugenol</td>
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<tr>
<td>Geranyl acetate</td>
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<tr>
<td>Ionone</td>
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<tr>
<td>Damascena</td>
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<tr>
<td>Caryophyllene</td>
</tr>
<tr>
<td>α-Humulene</td>
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<tr>
<td>Nerolidol</td>
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<tr>
<td>Caryophyllene oxide</td>
</tr>
<tr>
<td>Globulol</td>
</tr>
<tr>
<td>α-Cadinol</td>
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<tr>
<td>Total identified (%)</td>
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</table>

<sup>a</sup> Retention indices experimental (based on homologous series of n-alkane C7-C30).

<sup>b</sup> Retention indices from literature.

Relative proportions of the essential oil constituents were expressed as percentages.

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of the antibiotics against the gram-negative bacteria, which could be justified by the role that essential oils play in defending plants from phytopathogenic bacteria and fungi [17].

According to the research performed by Siani and collaborators [18] the EjEO is also effective in the control of the delayed reaction triggered by stimuli of bacterial origin, suggesting that this oil could be effective in the control inflammatory responses associated with bacterial infections.

In the modulation assays by the method of gaseous contact, the combination of the EjEO with amikacin or erythromycin against P. aeruginosa decreased the halos (Fig. 2), indicating that these associations presented synergistic effects. However, no noteworthy modulator effect was obtained from the association of EjEO with gentamicin. The association of the EjEO and ciprofloxacin and norfloxacin through gaseous contact against S. aureus and E. coli under exposure to red and blue LED lights caused an increase in halo, indicating that these treatments presented synergism. However, no difference was shown between this result and the addition of the EjEO to these treatments (Figs. 3 and 4).

Phototherapy can be considered an alternative to reduce the abusive use of antimicrobials, with impact on microbial resistance. The effectiveness of this modality of therapy is possibly associated with the following mechanisms: alteration of cellular homeostasis, modulation of DNA and RNA synthesis, modifications of the membrane permeability, alkalization of the cytoplasm and cell membrane depolarization [19,20]. In this study, we demonstrated that the exposure to blue LED lights had similar effects against gram-positive or gram-negative bacteria. Even though the gram-negative bacteria are considered (due to their physical characteristics) harder to penetrate by the photons of the light
therapy [21]. In addition, the synergistic effects obtained with the use of blue LED light may be due to a bactericidal action via induction of oxidative stress. In fact, it was previously reported that the light caused inactivation of Propionibacterium acne by inducing the removal of the external layer of electrons (especially of the oxygen atoms) of the molecules that form the cytoplasmic membrane of the bacteria [22].

4. Conclusion

The results obtained in this study demonstrated that the essential oil obtained from Eugenia jambolana as well as the exposure to LED lights interfere with the action of antibiotics. These findings represent promising results in the search for new therapies for antibiotic-resistant bacterial infections. Finally, further research elucidating the
modulatory effects of the oil upon association with the LED lights is suggested.

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Competing interest

None of the authors had any conflict of interest to disclose.

References


