

Chemical Characterization and Biological Potential of the Essential Oil of *Eucalyptus globulus* Labill

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Abstract: Oxidation of lipids in various products, along with the growth of medically important pathogens, has led to a search for medicinal plants with antioxidant and antimicrobial activities. As a result, the aim of this study was to evaluate the antioxidant and antibacterial activity of the essential oil of *Eucalyptus globulus* (EO-Eg). Antioxidant activity was assessed by using the 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) assay method. Existing components were identified through gas chromatography-mass spectrometry (GC-MS) analysis. Antibacterial activity and minimum inhibitory concentration (MIC) were assessed by using the broth microdilution method with standard multidrug-resistant bacterial strains. The main EO-Eg compounds identified by GC-MS were isopulegol, citronellal, and citronellol, which are primarily used in the industrial sectors. EO-Eg demonstrated excellent antioxidant activity with an effective concentration (EC50) of 4.48 μ L/mL owing to the presence of phenolic compounds. Regarding antibacterial activity, the EO-Eg displayed a broad antimicrobial spectrum of antimicrobial activity across the different resistance phenotypes analyzed. The most notable antibacterial activity was observed against *Staphylococcus aureus* 169 MRSA (MIC = 0.0625%). As a result, our findings suggest that EO-Eg has antioxidant and antibacterial potential against hospital-acquired multidrug-resistant pathogens, which may be correlated with its major components.

Key words: Essential oil, antioxidant, chemical identification, multidrug resistance, Eucalyptus globulus.

1. Introduction

Over the past years, there has been a growing interest in finding antioxidants and antimicrobial compounds from natural sources for the control of human diseases. The market constantly directs its attention to secondary metabolites produced by plants to verify their properties and assess their possible use in the industry, which is growing owing to microorganisms developing resistances to currently used antimicrobial agents [1] and the increase of various diseases associated with oxidative stress such as cancer and cardiovascular and inflammatory diseases [2].

Eucalyptus is a shrub belonging to the Myrtaceae family, native to Australia and Tasmania, currently found in tropical and subtropical regions. The *Eucalyptus* genus contains approximately 700 species with over 300 containing volatile oils in their leaves, which possess various pharmaceutical properties, and are used for toiletries, cosmetics, and in the food industry. *Eucalyptus globulus* Labill is the most important species as it is the most used one according to the *International Pharmacopoeia*, having a wide range of applications such as antiseptic, hypoglycemic,

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anti-inflammatory, food preserving, flavoring, and antioxidant applications, owing to molecules present in its oils [3].

Essential oils are volatile products from the secondary metabolism of aromatic plants that are transformed into specialized cells, and are found in the leaves, flowers, seeds, stems, and roots. In general, they are complex mixtures of lipophilic substances, usually odoriferous and liquid. Owing to their diverse compound contents, ranging from terpene hydrocarbons, simple alcohols, aldehydes, ketones, phenols, ethers, oxides, peroxides, furans, organic acids, lactones, and coumarins to sulfur compounds, they demonstrate antimicrobial and antioxidant biological potential [2, 3].

The most used natural antioxidants in the food industry are the phenolic and tocopherol compounds owing to their ability to inhibit lipid peroxidation [3]. Antioxidants of natural or synthetic origin can prevent lipid oxidation; however, the use of synthetic substances has been questioned across industries because of toxic and mutagenic effects in animals. As a result, various studies worldwide focus on the natural antioxidants found in foods rich in saturated fats, as these bioactive compounds have a wide variety of nutrients, vitamins, and chemical compounds formed by the secondary metabolism of plants and can reduce the oxidative reaction rates of lipid compounds present in some products, inhibiting the activity of reactive species [4, 5].

Regarding the problem of bacterial resistance, extra efforts are required for tracing medicinal plants with potential antimicrobial characteristics. Several secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids have been shown to have antimicrobial properties. Many plant materials used in traditional medicine are usually more effective and cheaper than modern medicine used against certain diseases, in addition to showing fewer side effects that are often associated with synthetic antimicrobial agents [6]. Thus, the aim of this study was to evaluate the chemical composition of essential oils from the leaves of *Eucalyptus globulus*, which are bioproducts of economic interest in a municipality of Alagoas and evaluate their antioxidant and antimicrobial biological potentials against multidrug-resistant bacteria.

2. Materials and Methods

2.1 Botanical Material and Essential Oil Sample Collection

Eucalyptus globulus leaves were collected on the outskirts of the municipality of São Sebastião, located in the state of Alagoas. The leaves were dried at room temperature and the extraction of essential oil (EO-Eg) was done by hydrodistillation, using a Clevenger-type apparatus for 4 h. The sample was stored at -20 °C until analysis. The identification of the exsicata is in registry number 896, deposited in the Herbarium of Embrapa Forest.

2.2 Gas Chromatography-Mass Spectrometry (GC-MS) Compound analysis

EO-Eg was analyzed using а Shimadzu GCMS-QP2010 Ultra gas chromatograph coupled to an electron ionization mass spectrometer and an ion trap analyzer (GC-MS-EI-Ion trap), using a slightly modified version of methodology originally proposed by Ghaffar et al. [7]. Helium was used as the carrier gas in the GC-MS with a 1 mL/min column flow and injector port temperature of 200 °C through the splitless injection method, using J&W DB-B5 (30 m) stationary phase capillary columns (15 m \times 0.25 mm) with a density of 0.25 mm (5% phenyl in 95% dimethylpolysiloxane), exerting а maximum temperature of 350 °C. The column's temperature ranged between 50 °C and 250 °C. In the mass spectrometer, the temporary ion source temperature and interface temperature were 200 °C and 250 °C, respectively. One-microliter aliquots of the diluted samples (1 μ L of sample diluted in 99 mL of hexane) were added. The mass spectrum from each of the EO-Eg components was analyzed and compared to the spectral values found in the NIST085.LIB and Wiley libraries.

2.3 In Vitro Analysis of Antioxidant Activity: Reduction of the 2,2-Diphenyl-1-Picrylhydrazyl Hydrate (DPPH) Radical

Antioxidant activity (AOA%) was determined through the ability of the antioxidants present in EO-Eg samples to scavenge the stable DPPH radical, using a slightly modified version of methodology originally described by Sanchez-Moreno et al. [8].

2.4 Qualitative Analysis of AOA%

The analysis of the AOA% of the EO-Eg was performed using thin-layer chromatography (TLC) [9]. The EO-Eg was applied to silica-gel chromatoplates and eluted in the suitable chloroform. Then, the plates were immersed in 0.3 μ mol DPPH. The presence of yellow spots against the purple background indicated antioxidant activity [10].

2.5 Quantitative Analysis of AOA%

To monitor the rate of DPPH consumption by the EO-Eg, the decreases in absorbance values at different concentrations were measured [8, 11]. The measurements were performed on an SP 2000 UV spectrophotometer ($\lambda = 518$ nm). Dilutions of 4, 5, 6, 7, 8, 9, 10, 11, and 12 μ L/mL (v/v) were obtained in triplicate from the 1 mL/mL ethanolic EO-Eg solution. For each 2.5 mL EO-Eg solution, 1 mL of 0.3 µmol DPPH ethanolic solution was added. To the blank solution, 2.5 mL of EO-Eg (for each concentration) and 1 mL of ethanol was added, and 2.5 mL of ethanol and 1 mL of 0.3 µmol DPPH were added to the negative control. After 30 min at room temperature and in the absence of light, the absorbance readings were taken. The absorbance values were converted into AOA% using the formula 100 {[(ABSS - ABSB) \times 100] / ABSC} [12]. ABSS is the mean absorbance value of the sample, ABSB is the mean absorbance of the blank,

and ABSC is the mean absorbance of the negative control [10]. A trolox solution at 2 μ L/mL was used as a positive control.

2.6 EC₅₀ Calculation

 EC_{50} being defined as the concentration required to reach 50% antioxidant activity and was calculated using the straight-line equation, when your R^2 (coefficient of determination) was approximately equal to 1 ($R^2 = 0.9025$) [12].

2.7 AOA% Statistical Analysis

The data were subjected to regression analysis in Microsoft Excel to determine the straight-line equation and the R^2 , and to determine the best antioxidant potential for the sample.

2.8 Bacterial Species Used in This Study

aureus **Staphylococcus** ATCC 29213 (methicillin-resistant Staphylococcus aureus, MRSA), Enterobacter aerogenes ATCC 13048, Klebsiella pneumonia ATCC BAA-1705 (Klebsiella pneumoniae carbapenemase, KPC), Pseudomonas aeruginosa ATCC 27853, and Acinetobacter baumannii ATCC 17978 were the standard strains provided by the LACEN (Central Laboratory of Alagoas). The multidrug-resistant clinical isolates of hospital origin Staphylococcus aureus 169 (MRSA), Enterobacter aerogenes 22 (KPC), Klebsiella pneumoniae 97 (extended-spectrum beta-lactamase, ESBL), Pseudomonas aeruginosa 112 (metallo-beta-lactamase, MBL), and Acinetobacter baumannii 45 (MBL) were provided by Alagoas General Hospital. The clinical specimen and the sensitivity profile to antimicrobials were acquired together with the clinical isolates.

2.9 In Vitro Antibacterial Activity

The EO-Eg used in the antibacterial test was initially diluted in sterile distilled water and Tween 80 to obtain an emulsion at a 16% concentration(v/v), and then added to sterile glass tubes: 0.8 mL of essential oil, 50

µL of Tween 80, and 4.2 mL of sterile distilled water. The solution was mixed for 5 min in a Vortex mixer [13]. The screening tests for determining the EO-Eg antimicrobial activity and minimum inhibitory concentration (MIC) were performed in 96-well microplates according to the methodology described by Ferreira de Lima et al. [14]. The antibacterial activity was measured from an inoculum containing 5×10^5 UFC/mL, obtained from fresh colonies of the bacteria that were tested. To all 96 wells of the microplate, previously inoculated with 100 μL of а double-concentrated Müeller-Hinton broth, 10 µL of the bacterial suspension was transferred. Then, 100 µL of the 16% EO-Eg emulsion (v/v) was added in triplicates to the wells. The microplates were incubated at 37 °C for 20 h. To visualize the results, 10 µL of a solution containing 1 mg/mL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoli um bromide (MTT) was added to each of the 96 wells, and incubated at 37 °C for 30 min. The presence of violet coloration indicated metabolic activity and, as a result, that the essential oil failed to inhibit bacterial growth. Amikacin (5 μ g/mL) was used as the positive control, and 10 µL of the emulsified agent as the negative control, in addition to the growth and sterility controls. EO-Eg underwent serial dilutions in a microdilution plate, starting from a concentration of 8% down to 0.03125% (v/v). The MIC corresponded to the last dilution, in which bacterial growth was not observed in the culture medium after the incubation

period.

3. Results and Discussion

3.1 GC-MS Analysis of the Chemical Constituents Present in Essential Oil

Through GC-MS analysis of the EO-Eg, three primary components were identified by comparing the retention and mass spectra index with those from the NIST085.LIB and Wiley online libraries. Isopulegol (2-isopropenyl-5-methylcyclohexanol), citronellal (3,7-dimethyl-6-octenal), and citronellol (3,7-dimethyl-6-octen-1-ol) were observed as major compounds. These compounds are shown in Figs. 1-4 and in Table 1.

1,8-Cineol has been reported as the major and most important compound found in various *Eucalyptus* spp, even though this compound can be altered depending on the state of the leaves (fresh or dry). A large amount of 1,8-cineol is found in fresh leaves of *Eucalyptus*, as opposed to dry leaves, where large amounts of isopulegol, citronellal, and citronellol are found [5].

The major components of EO-Eg are oxygenated monoterpenes; this may indicate that the secondary metabolism of E. globules may involve specific cyclases and dehydrogenases that result in the biosynthesis of aromatic rings after the formation of terpene precursors [5]. The monoterpene molecules account for up to 90% of the essential oils composition [15], as observed in this study through the detection of isopulegol, citronellal, and citronellol in the EO-Eg.







Fig. 2 A. Isopulegol mass spectra, EO-Eg; B. data obtained from the NIST085.LIB library; C. data obtained from the Wiley library.



Fig. 3 A. Citronellal mass spectra, EO-Eg; B. data obtained from the NIST085.LIB library; C. data obtained from the Wiley library.

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Fig. 4 A. Citronellol mass spectra, EO-Eg; B. data obtained from the NIST085.LIB library; C. data obtained from the Wiley library.

Table 1 Retention indexes, molecular formulas, and molar masses of the major EO-Eg components.

| Peak | Components | Retention index | Molecular formula | Molar mass (g/mol) |
|------|-------------|-----------------|-----------------------------------|--------------------|
| 1 | Isopulegol | 14.006 | C ₁₀ H ₁₈ O | 154.25 |
| 2 | Citronellal | 14.254 | $C_{10}H_{18}O$ | 154.25 |
| 3 | Citronellol | 16.288 | $C_{10}H_{20}O$ | 156.27 |

EO-Eg: essential oil of Eucalyptus globules.

Isopulegol is a 3-oxygenated monoterpene alcohol of the p-menthane family and is found in the essential oils of various aromatic plants such as *Cymbopogon winterianus* and *Zanthoxylum schinifolium*. Owing to its aromatic properties, isopulegol has been used in the manufacturing of perfumes containing flower scent components. However, studies conducted on its biological properties have shown gastroprotective activity and antidepressant, anxiolytic, and anticonvulsant-like effects on the central nervous system [16, 17].

Citronellal is an acyclic monoterpene aldehyde capable of forming isopulegol from cyclization in an acidic medium. Furthermore, it is also one of the compounds responsible for the aroma of Cymbopogon, a plant known for its ability to repel insects. Citronellal also displays larvicidal, anti-inflammatory, antinociceptive, antioxidant [18, 19], antimicrobial [20], and bronchodilator effects [4].

Citronellol is a monoterpene alcohol present in essential oils of Pelargonium, Geranium, Cymbopogon, Rosa and Lippia, and is widely used in perfumes and as an insect repellent [5]. Studies have shown citronellol to have anti-hypertensive, antinociceptive, anti-inflammatory [21], anticonvulsant, antioxidant [22], and antimicrobial activity [20] as evidenced in this study with multidrug-resistant bacteria.

The monoterpenes identified in the EO-Eg in this

study were also found in E. citriodora [23] revealing the similarity between the two species of the Myrtaceae genus. However, these monoterpenes were not found in E. globules sampled from another geographical region [23], demonstrating that the chemical composition of an essential oil can be significantly influenced by and depend on several factors, from plantation and cultivation techniques to the extraction method used. According to Kaur et al. [5], climatic factors are highly responsible for some of the variabilitoes found in the production of terpenoids. Another factor that influences essential oil production is genetic variability. Environmental factors such as soil fertility. temperature, humidity, and altitude can also exert considerable influence on the secondary metabolism of these species.

3.2 Qualitative and Quantitative Analysis with DPPH Radical

The preliminary qualitative evaluation of EO-Eg by TLC revealed that the EO-Eg displays antioxidant activity resulting from the reduction of the DPPH free radical. The quantitative analysis of the EO-Eg showed a significant AOA% of 100% at 9 μ L/mL. EO-Eg antioxidant behavior was described by the straight-line equation model with an R² of approximately 1

 $(R^2 = 0.9025)$, indicating that the generated equation effectively reproduces the antioxidant behavior of the sample. The EC50 of the EO-Eg was 4.48 μ L/mL (Fig. 5).

According to Kaur et al. [5] this activity can be attributed to the presence of monoterpenes, the main component of EO-Eg. Various species of *Eucalyptus*, such as *E. polyanthemos, E. camaldulensis, E. tereticornis*, and *E. oleosa*, also display antioxidant potential owing to the presence of other major compounds such asp-cymene, 1,8-cineol, and 1-(S)- α -pinene [5], which are different from those identified in this research. According to Basak and Candan [24], the antioxidant potential of essential oils can be attributed to the complex mixture of its components.

3.3 Antibacterial Activity

EO-Eg showed broad antibacterial activity against gram-positive and gram-negative bacteria (Table 2), including multidrug-resistant bacteria with an MIC ranging from 0.0625% to 4% (v/v).

Several studies have attributed the antimicrobial characteristics of *E. globules* to the various compounds found in its oil, such as 1,8-cineol, isopulegol, citronellol, and citronellal [25], which are similar to those found in the EO-Eg leaves.



Fig. 5 Antioxidant activity of EO-Eg.

| Bacteria analyzed | MIC of EO-Eg (%) (v/v) | |
|-----------------------------------|--------------------------|--|
| S. aureus ATCC 29213 (MRSA) | 0.0625 | |
| S. aureus 169 (MRSA) | 0.125 | |
| K. pneumoniae ATCC BAA-1705 (KPC) | 1 | |
| K. pneumoniae 97 (ESBL) | 0.5 | |
| E. aerogenes ATCC 13048 | 0.25 | |
| E. aerogenes 22 (KPC) | 2 | |
| P. aeruginosa ATCC 27853 | - | |
| P. aeruginosa 112 (MBL) | - | |
| A. baumannii ATCC 17978 | 0.5 | |
| A. baumannii 45 (MBL) | 4 | |

 Table 2
 Minimum inhibitory concentration (MIC) of EO-Eg in resistant bacteria.

MRSA: methicillin-resistant *Staphylococcus aureus*; KPC: *Klebsiella pneumoniae* carbapenemase; ESBL: extended-spectrum beta-lactamase; MBL: metallo-beta-lactamase; (-): no activity.

The lowest MIC of the EO-Eg was observed in *S. aureus* MRSA according to Ghaffar et al. [7]. Among them were the clinical isolates of *S. aureus* ATCC 29213 (MIC = 0.0625%) and *S. aureus* 169 (MIC = 0.125%) obtained from a cerebral abscess, which were sensitive only to teicoplanin, a glycopeptide used in cases of severe infections by *S. aureus* MRSA. Studies conducted by Tohidpour et al. [26] have demonstrated the action of several essential oils against multidrug-resistant *S. aureus*, which suggests that essential oils have the potential to serve as an alternative therapy for various diseases caused by *S. aureus* MRSA.

In this study, EO-Eg showed activity against ESBL and KPC-producing bacteria. These results are of great value as the beta-lactamase, ESBL and KPC-producing *K. pneumoniae* and *E. aerogenes* are among the main multidrug-resistant enterobacteriaceae that cause infections that result in high mortality rates. Currently, one of the major problems caused by gram-negative bacteria that cause nosocomial infections stems from the production of ESBL and KPC enzymes by these bacteria, which hydrolyze beta-lactam antibiotics, including the carbapenems and the KPC-producing ones, which are special antibiotics used particularly to treat infections by these bacteria [27].

EO-Eg worked against *A. baumannii*, which is highly resistant to different antimicrobial classes (carbapenems, quinolones, aminoglycosides, and

cephalosporins), creating expectations as an alternative source for the development of new drugs for the treatment of serious infections caused by multidrug-resistant bacteria [25]. In recent years, the increasing incidence of A. baumannii nosocomial infections has raised concerns. This is mainly due to its characteristics, which include rapid development of resistance to most antibiotic classes, high adaptability to selective pressure due to extensive use of antibiotics, rapid spread of resistance in clinical settings leaving fewer treatment options for physicians, and increased morbidity and mortality rate in immunocompromised patients [28]. This, in turn, shows the need for the discovery of therapeutic sources. Studies performed with the essential oil of Cymbopogon flexuosus (lemon grass) have shown a bactericidal effect in A. baumannii resistant to carbapenems [28], a promising finding that corroborates with our study.

No evidence of activity against P. aeruginosa was found in our study, which may be explained by the ability of these bacteria to metabolize a large number of organic compounds, inhibiting the components present in the EO-Eg and explaining its high resistance to antibiotics [29].

Data from the literature describe gram-negative organisms as being less susceptible to the action of essential oils, suggesting that the additional membrane that covers the cell wall restricts the diffusion of hydrophilic compounds through the lipopolysaccharide layer of these bacteria [30], as observed in our study, in which EO-Eg showed better antibacterial activity against *S. aureus*.

Hydrophobicity is an important characteristic of essential oils as it allows interaction with the lipids of the cell membrane and mitochondria, changing the membranes' permeability and disturbing these structures. The constituents of essential oils can also bind to ions and molecules, changing some biological functions. Considering the large number of chemical constituents present in the oils, their antibacterial effects may not be attributed to specific mechanisms of action [5].

4. Conclusions

This study showed that the EO-Eg showed efficient antioxidant activity through the DPPH assay, which may be related to the presence of isopulegol, citronellal, and citronellol monoterpenes. The analysis of the results showed that the EO-Eg has considerable antioxidant activity and could be used as a source of natural antioxidant compounds in the food and pharmaceutical industries.

EO-Eg displayed antibacterial activity in both gram-positive and gram-negative bacteria, showing a broad spectrum of action in different inhibitory concentrations in multidrug-resistant hospital pathogens.

Therefore, through data obtained in this study, the importance of EO-Eg as a natural resource that can be used in various industrial sectors can be observed, in producing new products that can inhibit or retard oxidative reactions and proliferation of bacteria, improving existing preventative actions against pathogens, and consequently maintaining human health.

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