

# Chemical Profile of Essential oil of *Ocimum gratissimum* L. and Evaluation of Antibacterial and Drug Resistance-modifying Activity by Gaseous Contact Method

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## ABSTRACT

**Introduction:** Plant products with antimicrobial potential and ability to modulate the action of drugs have become the target of several studies. Many studies of the species *Ocimum gratissimum* Lamiaceae have reported edbiological activities in the essential oil and extracts of various parts of the plant. **Objective:** Evaluate the antibacterial and resistance-modifying activity of the essential oil of *O. gratissimum* (OEOg) and its major compound, Eugenol. **Materials and Methods:** OEOg was extracted by hydro-distillation and Eugenol was obtained commercially. Multiresistant strains of *Escherichia coli* and *Staphylococcus aureus* were used to test for drug activity. Antibacterial and resistance-modifying activity of OEOg and Eugenol was evaluated by gaseous contact. **Results:** Chemical analysis of OEOg demonstrated the presence of Eugenol and other compounds such as 1, 8-cineole and  $\beta$ -selinene as well. In the evaluation of the antibacterial activity, both OEOg and Eugenol were unable to form a bacterial growth inhibition zone at any of the concentrations tested. However, the modulation tests with amikacin and erythromycin identified synergism with OEOg and eugenol against *E. coli* and *S. aureus*. Gentamicin combined with OEOg

showed antagonism against *S. aureus* but synergism against *E. coli*. When gentamicin was combined with Eugenol, there was also synergism against *S. aureus*, whereas no effect was seen against *E. coli*. **Conclusion:** Given the results obtained in this study, it is concluded that OEOg and Eugenol may eventually represent a new therapeutic tool against antibiotic-resistant bacteria.

**Key words:** Eugenol, Gaseous contact, Modifying, *Ocimum gratissimum*, Resistance.

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## INTRODUCTION

The family Lamiaceae consists of about 240 genera and 7200 species, which can be found in almost all parts of the world.<sup>1</sup> The genus *Ocimum* L. consists of more than 150 species,<sup>2</sup> which are widely used in folk medicine in some countries and are also great sources of essential oils used in industry.<sup>3</sup>

The species *Ocimum gratissimum* Lamiaceae (*O. gratissimum* L.) is a sub-brush native to Asia and South Africa, but with spontaneous occurrence throughout Brazil.<sup>4</sup> In the Brazilian Amazon is referred to as "alfavacão"<sup>5</sup> and is also much used as a spice in cooking.<sup>6</sup> It is widespread in traditional medicine in several countries, mainly in the form of teas and infusions of its leaves.<sup>7</sup> It is used to treat abdominal pain and cough<sup>8</sup> and colds, pruritus, stress and headache.<sup>9</sup> Also, there are reports of the use of *O. gratissimum* to treat upper respiratory tract infections, pneumonia, conjunctivitis and skin diseases.<sup>10</sup>

Various scientific studies have identified some of the plant's activities, such as antidiabetic,<sup>11</sup> antioxidant,<sup>12</sup> anxiolytic,<sup>13</sup> sedative,<sup>14</sup> anti-inflammatory,<sup>15</sup> hepatoprotective,<sup>16</sup> antitumor,<sup>17</sup> gastroprotective,<sup>18</sup> and hypolipidemic.<sup>19</sup> The antibacterial activity of this plant species has been described in many studies that tested various parts of the plant: extract of leaves,<sup>20</sup> oil from leaves,<sup>21</sup> extract of leaves and stem,<sup>22</sup> oil from aerial parts,<sup>23</sup> and oil from leaves, stem and roots.<sup>24</sup>

Essential oils are compounds found in plants that have different properties related to survival and defense of these plant species.<sup>25</sup> However, the chemical composition of the oil varies according to the genotype of the plant, geographical origin, environmental conditions, season of the year, method of extraction of this oil and its preservation.<sup>26</sup>

Several chemical have been found in the essential oil of *O. gratissimum* (OEOg), such as Eugenol and Thymol,<sup>27</sup>  $\gamma$ -muurolene and

$\beta$ -caryophyllene,<sup>28</sup>  $\alpha$ -bisabolene and  $\gamma$ -terpinene,<sup>29</sup> 1,8-cineole and  $\beta$ -selinene,<sup>30</sup> and Geraniol and p-cymene.<sup>31</sup>

OEOg is grouped into some chemo types according to the major constituents found, such as Eugenol, Thymol and Geraniol.<sup>31</sup>

Eugenol has a molecular formula of  $C_{10}H_{12}O_2$ , and at normal temperatures, it appears as an oil liquid that is viscous, pale yellow, slightly water soluble and easily dissolved inorganic solvents.<sup>32</sup> It is widely used as a sedative in dental surgeries, has an antipyretic action, decreases motor activity, has an anticonvulsant effect, is a general anesthetic, and has antibacterial, antiviral and antifungal activities.<sup>33</sup> It has also been reported to have antioxidant, anti-inflammatory and cytotoxic properties.<sup>34</sup>

Chemicals of natural origin with antimicrobial potential have become targets of study aimed at finding new substances capable of inhibiting the vital process of microorganisms resistant to common drugs.<sup>35</sup> Antibiotic resistance phenomenon refers to the ability of microorganisms to multiply even at higher drug concentrations.<sup>36</sup>

One type of resistance mechanism developed is the production of enzymes that inactivate the antibiotic. This mechanism is developed by the bacterium *Staphylococcus aureus* (*S. aureus*),<sup>37</sup> a Gram-positive bacterium found on the skin and in the nasal passages of healthy people, which can cause simple infections such as pimples to severe infections such as pneumonia.<sup>38</sup> Another mechanism is the change in porin channels, which impedes antibiotic uptake by the cell. This type of resistance is commonly developed by *Escherichia coli* (*E. coli*).<sup>39</sup> *E. coli* is a Gram-negative bacterium commonly found in the intestinal tract of humans, but some serotypes can cause intestinal infections; it is also the most common etiological agent in urinary tract infections.<sup>40</sup>

Considering that *O. gratissimum* is widely used in popular medicine, the investigation of its antibacterial properties and also its combination with other microbial control techniques has become important, since compounds of natural origin may also act as drug resistance modifiers or may potentiate the action of antibiotics against resistant pathogens.<sup>41</sup>

Thus, the aim of this study was to conduct a chemical characterization of the essential oil obtained from *O. gratissimum* L. leaves and to evaluate OEOg and Eugenol for antibacterial and resistance-modifying activity by the gaseous contact method.

## MATERIALS AND METHODS

### Collection of Plant Material

Leaves of *O. gratissimum* L. were collected in Chapada do Araripe, Crato municipality, Ceará, Brazil, and a voucher specimen of the species was deposited in the herbarium Dárdano Andrade Lima, University Regional of Cariri, and given the registration number 3978.

### Extraction of Essential Oil From Leaves of *O. gratissimum* L.

OEOg was extracted from fresh leaves of *O. gratissimum* L. by hydrodistillation using a Clevenger-type apparatus, where the leaves were crushed and placed in a 5.0-L flask along with 2.5 L of distilled water, followed by boiling for 2 h. Anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ) was added to the essential oil obtained, and this was stored under refrigeration ( $-4^\circ\text{C}$ ) until time of analysis.

### Identification of chemical components

The chemical analysis of OEOg was performed by gas chromatography-coupled to mass spectrometry (GC/MS) on a Shimadzu instrument. Identification of the components was done by comparison of their mass spectra with standards reported in the literature.<sup>42</sup>

Eugenol was obtained from Sigma Chemical Co. (St. Louis, MO, USA), and the antibiotic disks were from Labor Clin (Brazil). The culture media were purchased from HIMEDIA (India), and dimethyl sulfoxide (DMSO) was from Merck (Germany).

### Microorganisms

We used clinical isolates *E. coli*<sup>27</sup> and *S. aureus* 358, multidrug resistant bacterial strains, provided by Instituto Oswaldo Cruz. Both strains were maintained in Brain Heart Infusion (BHI), and for testing for resistance-modifying activity, the strains were suspended in a test tube with distilled water to obtain a suspension with turbidity equivalent to 0.5 on the McFarland scale ( $1 \times 10^8 \text{ CFU/mL}$ ).

### Evaluation of Antibacterial Activity

In Petri dishes containing Mueller-Hinton agar medium, seeding was done to obtain a carpet of bacteria. OEOg and eugenol were diluted in DMSO to obtain solutions containing 50, 25, 12.5 and 6.25% of these compounds, and 100% of the substance was also used. An absorbent-paper disk, similar to those used in the antibiogram test, was placed on the lid of each plate, and it was moistened with 10  $\mu\text{L}$  of test substance. The plates were incubated in an oven for 24 h at  $37^\circ\text{C}$ . A millimeter ruler was used to measure the inhibition zones. All tests were performed in triplicate, and only DMSO was used in control plates.

### Evaluation of Resistance-modifying activity by gaseous contact

The evaluation of resistance-modifying activity was performed by the gaseous contact method as modified and reported elsewhere.<sup>43</sup> In Petri-

plates containing Mueller-Hinton agar, seeding was done to obtain a carpet of bacteria. Antibiotic disks with 30 mg amikacin, 10 mg gentamicin and 15 mg erythromycin were placed on the agar. An absorbent paper disk similar to an antibiogram disk was placed on the lid of each plate, and it was moistened with 10  $\mu\text{L}$  of the highest concentration of OEOg or Eugenol, where there was growth in the antibacterial activity test, so that through volatilization, the test substance could interact with the antibiotic disks. Other plates containing the drug disks but without oil or Eugenol were also prepared, and afterwards, all plates were incubated for 24 h at  $37^\circ\text{C}$ . A millimeter ruler was used to measure the inhibition halos of the antibiotics in the presence and absence of the test substances to determine synergism or antagonism. All tests were performed in triplicate, and only DMSO was used in control plates.

### Statistical Analysis

The results were expressed as the arithmetic mean  $\pm$  standard deviation, statistically evaluated by analysis of variance (ANOVA) followed by the Bonferroni post-test, utilizing Graph Pad Prism software. Differences were considered significant when  $p < 0.05$ .

## RESULTS AND DISCUSSION

OEOg obtained by hydrodistillation showed a yield of 0.17%. Other studies have reported yields of OEOg between 0.21 and 0.70%<sup>44-48</sup> These differences may be related to changes in environmental factors to which the plant is exposed, such as light, temperature and humidity, which significantly affect the emission of volatile compounds and thus the yield and chemical composition.<sup>49</sup>

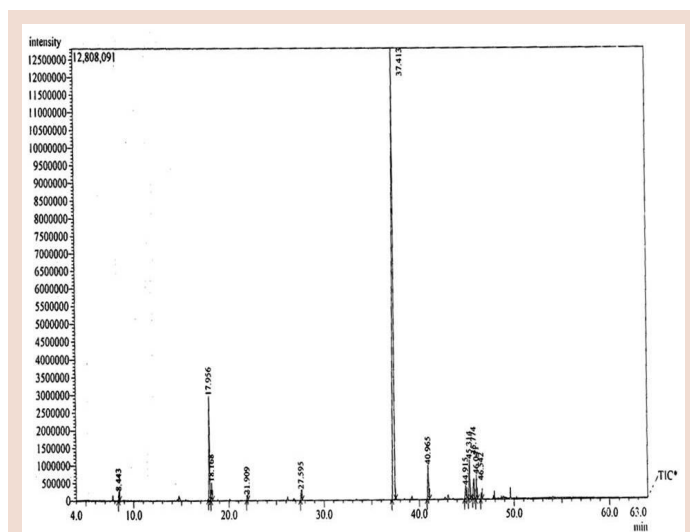
Through phytochemical oil prospecting, it was possible to identify the presence of a wide variety of chemical compounds (Table 1 and Figure 1).

**Table 1: Chemical composition and percentages of essential oil components of *Ocimum gratissimum***

Components	R. Time	Composition (%)
3-Hexen-1-ol	8.443	0.53
1,8-Cineole	17.956	9.69
Cis-Ocimene	18.168	1.49
L-Linalool	21.909	0.42
Linalyl propionate	27.595	0.94
Eugenol	37.413	72.26
$\beta$ -Caryophyllene	40.965	3.80
Germacrene-D	44.915	1.73
$\beta$ -Selinene	45.314	4.25
Bicyclogermacrene	45.774	2.56
Valencene	46.041	1.87
$\gamma$ -Murolene	46.542	0.46

R. Time: Retention Time.

Eugenol is the compound present in greatest quantities in the essential oil obtained from the fresh leaves of *O. gratissimum* collected around 8:00 h. This compound corresponds to 72.26% of the substances found, but the percentage of Eugenol in the essential oil can be as high as 98% at 12:00 h. This compound is responsible for the antimicrobial activity of the plant, so when needed as a local antiseptic, the ideal time to collect the plant is when Eugenol content is highest. Around 17:00 h, Eugenol is only 11%, since the content of this compound in OEOg is affected



**Figure 1:** Chromatogram of the Essential Oil from *Ocimum gratissimum* L. leaves

by sunlight.<sup>50</sup> During the early morning and late afternoon, there is the predominance of the balsamic principle 1,8-cineol, which explains the popular use of the plant in cold medicine preparations when collected at this time.<sup>51</sup>

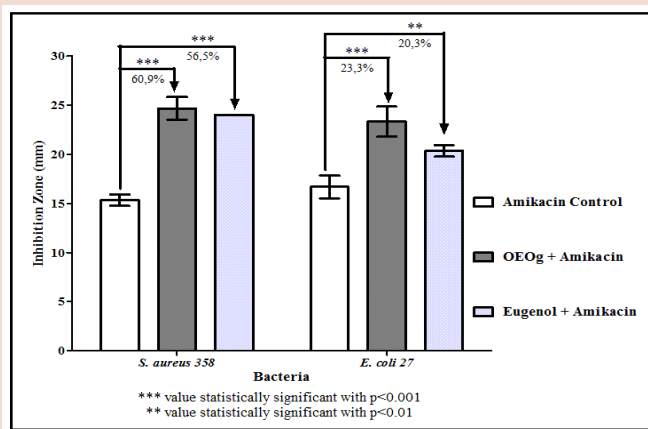
Among the chemical constituents of OEOg analyzed, Eugenol was the main component, followed by 1,8-cineol. Other studies also report these compounds as main components of OEOg and classify it as the Eugenol chemotype.<sup>52-54</sup>

In the evaluation of the antibacterial activity by the gaseous contact method, neither OEOg nor eugenol formed a bacterial growth inhibition zone at any of the concentrations tested. In a pilot trial using only DMSO, no antibacterial activity was observed, indicating that there was no toxicity using this solvent. On the basis of these results, we decided to use 100% OEOg and Eugenol in the resistance-modifying assays.

A study of OEOg evaluating its antibacterial activity against strains of *S. aureus* and *E. coli* by the thin layer chromatography-bioautography method showed inhibition of bacteria growth determined by the formation of inhibition zones around spots corresponding to Eugenol, suggesting that the antibacterial activity of OEOg was due to the action of this compound.<sup>29</sup> The antibacterial activity of OEOg has also been determined by disk diffusion method, where this compound was effective against Gram-negative and Gram-positive bacteria.<sup>21</sup> Tests conducted with Eugenol by microdilution technique in broth, also identified antibacterial activity for this compound.<sup>55</sup> Several studies have reported the antibacterial activity of Eugenol and OEOg. However, in our assays, we did not observe this effect, suggesting that the antibacterial action of these substances is through direct contact with the bacterial cells and not by gaseous contact.

The increasing incidence of bacterial resistance to aminoglycosides is widely recognized as a serious health threat. Thus, natural products of plant origin are increasingly being targeted by studies, and there is evidence that these compounds can alter the effect of antibiotics, either increasing antibiotic activity or reversing resistance.<sup>56</sup>

Aminoglycosides exert their bactericidal effect by binding to the bacterial ribosome. They interact with the cell surface, enter the cell through the cell membrane, and then bind to the 30 S subunit of the ribosome. Cell death occurs due to drug interference with protein synthesis and misreading of messenger RNA, which leads to changes in membrane function, causing the release of constituents essential for cell viability.<sup>57</sup>



**Figure 2:** Modulation activity of Aminoglycoside Resistance (Amikacin)

In the drug-modifying assays with amikacin (Figure 2), there was an interaction of OEOg and Eugenol with the antibiotic, resulting in synergism in both *S. aureus* and *E. coli*. That is, when amikacin was combined with the test substances, there was a statistically significant increase in the inhibition halo.

Resistance-modifying assays performed with the microdilution broth method also showed synergism between extracts of *O. gratissimum* and amikacin against *E. coli* and *S. aureus*.<sup>58</sup> By this same method, a synergistic effect was also observed with the essential oil of *Cordia verbenacea* DC., when combined with amikacin.<sup>59</sup>

A resistance-modifying effect was observed with erythromycin (Figure 3), where there was a synergistic growth inhibitory effect with all combinations tested against the two bacterial strains.

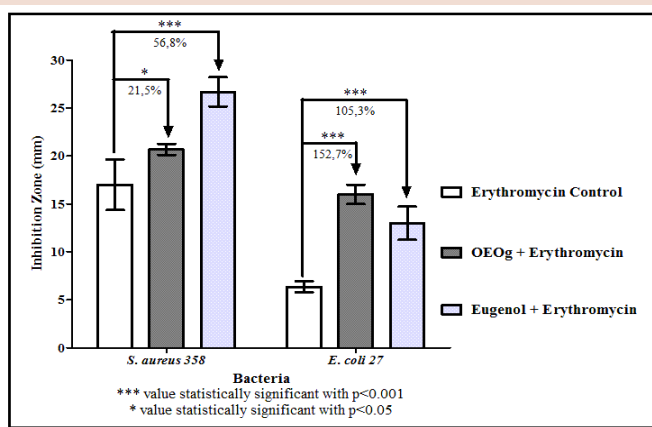
Another study that evaluated the modification of aminoglycosides activity using the gas contact method, reported good results with the essential oil of *Lantana montevidensis* against multidrug-resistant strains.<sup>60</sup>

Figure 4 shows results for the modulation of gentamicin activity, where antagonism was observed when combined with OEOg against *S. aureus*; that is, there was a decrease in the inhibition halo. However, synergism occurred against *E. coli*. When gentamicin was combined with Eugenol, there was also synergism against *S. aureus*, but no effect was seen against *E. coli*, where there was no change in the inhibition halo.

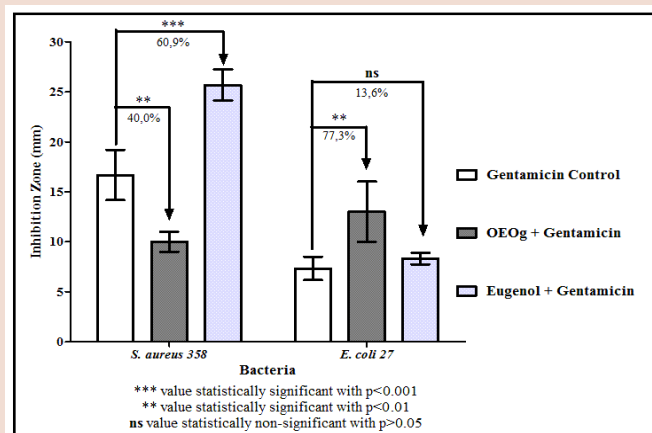
Tests carried out with the isolated compound Carvacrol by the gaseous contact method, also showed a synergistic effect when combined with gentamicin.<sup>61</sup> The lack of drug modulation observed with *E. coli* can be explained by the fact that Gram-negative bacteria are less sensitive to antibiotics compared to Gram-positive bacteria,<sup>62</sup> where the former have a highly complex barrier system including an outer membrane consisting of phospholipids, lipopolysaccharides and proteins, which confers a high degree of impermeability to antimicrobials.<sup>63</sup> In the present study, *E. coli* showed less sensitivity to the gentamicin-eugenol combination than did *S. aureus*. Regarding the results in the literature cited and the data obtained in this study, there was divergence as to the effect of OEOg, where the results were better against the Gram-negative strain.

When comparing the drug-modifying effects of Eugenol and OEOg, the results against *E. coli* were better when amikacin and gentamicin were combined with OEOg. Against *S. aureus*, better results were obtained when gentamicin and erythromycin were combined with Eugenol. For other combinations, the test substances did not show differences statistically significant effects.

Although Eugenol is the major component in OEOg, differences were observed between Eugenol and OEOg in their drug-modifying effect,



**Figure 3:** Modulation activity of Aminoglycoside Resistance (Erythromycin)



**Figure 4:** Modulation activity of Aminoglycoside Resistance (Gentamicin)

suggesting that the presence of other substances leads to interactions that influence the effect of the compounds. Many pharmaceutical companies have invested in the study of plants that produce pharmacologically active principles,<sup>64</sup> most research efforts focus on the isolation of active components, but the pharmacological effect of herbal medicines probably involve synergistic actions between the compounds present.<sup>65</sup>

## CONCLUSION

OEOg and Eugenol showed no antibacterial activity when evaluated by the gaseous contact method, but in the resistance-modifying assays, interactions were observed between the test substances and antibiotics, thus demonstrating promising results. The combination of natural products of plant origin in the form of complex mixtures or isolated compounds with conventional antibiotics can be an alternative to fight against pathogenic bacteria. Therefore, these products associated with the methodology used in this study can eventually represent a new form of drug therapy against resistant bacteria.

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## CONFLICT OF INTEREST

All authors have not any conflict of interest to disclose.

## ABBREVIATION USED

**ANOVA:** Analysis of Variance, **BHI:** Brain Heart Infusion, **CFU:** Colony Forming Unit, **DMSO:** Dimethylsulfoxide, **GC/MS:** Gas Chromatography-Mass Spectrometry, **Na<sub>2</sub>SO<sub>4</sub>:** Anhydrous sodium sulfate, **OEOg:** Essential Oil of *Ocimum gratissimum*.

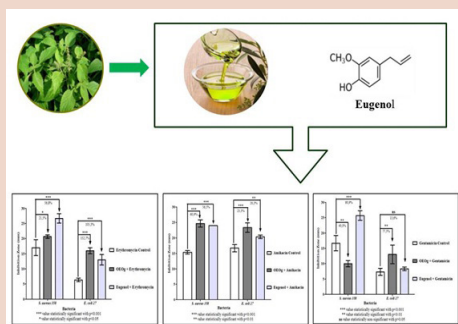
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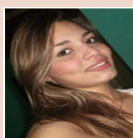
### PICTORIAL ABSTRACT



### SUMMARY

- OEOg obtained by hydrodistillation showed a yield of 0.17% and Eugenol is the compound present in greatest quantities in the essential oil obtained.
- In the evaluation of the antibacterial activity by the gaseous contact method, neither OEOg nor eugenol formed a bacterial growth inhibition zone at any of the concentrations tested.
- In the drug-modifying assays with amikacin, there was an interaction of OEOg and Eugenol with the antibiotic, resulting in synergism.
- Resistance-modifying effect was observed with erythromycin, where there was a synergistic growth inhibitory effect with all combinations tested.
- The modulation of gentamicin activity, where antagonism was observed when combined with OEOg against *S. aureus*. However, synergism occurred against *E. coli*. When gentamicin was combined with Eugenol, there was also synergism against *S. aureus*, but no effect was seen against *E. coli*.

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