

Antibacterial Effects of Basil (*Ocimum sanctum*) Leaf Extract in Combination with Meropenem against Extended Spectrum Beta-Lactamase (ESBL) Producing *Klebsiella pneumoniae*

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ABSTRACT

Introduction: Antibiotic usage for bacterial infections causes antibiotic resistance in bacteria. One is extended-spectrum beta-lactamase (ESBL), which produces *Klebsiella pneumoniae*, a pathogen responsible for increased antibiotic resistance. Basil (*Ocimum sanctum*) is a candidate for combination therapy. It has been proven to have antibacterial effects. However, its combination with antibiotics is rarely researched. This study evaluated the antibacterial effects of *O. sanctum* leaf extract and meropenem combination against ESBL-producing *K. pneumoniae*.

Methods: This study used the disk diffusion method. The extract was tested for each experiment at 6%, 4%, 2%, 1%, and 0.5% concentration. The research was divided into two experiments to evaluate the antibacterial effects of *O. sanctum* leaf extract (n=18) and its combination with meropenem against ESBL-producing *K. pneumoniae* (n=18). The data was analyzed using the International Business Machines Corporation (IBM) Statistical Package for the Social Sciences (SPSS) version 26.0 for Windows. A probability $p < 0.050$ was considered significant.

Results: The first experiment showed that the extract had the largest antibacterial effect at 0.5% concentration (n=18, $p=0.007$). Meanwhile, the second experiment showed that the combination of the extract and meropenem did not have significant antibacterial effects (n=18, $p=0.597$).

Conclusion: *Ocimum sanctum* leaf extract has viable antibacterial effects, but its combination with Meropenem does not significantly improve its antibacterial effects against Extended Spectrum Beta-Lactamase (ESBL) producing *K. pneumoniae*.

Highlights:

- Ocimum sanctum* leaf extract does not significantly increase its antibacterial effects when combined with meropenem against ESBL-producing *K. pneumoniae*.
- Ocimum sanctum* leaf extract has different antibacterial effects depending on geological and environmental factors.

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Introduction

Antibiotics are medicines used to prevent or treat bacterial infections. However, resistance towards antibiotics has been developing due to misuse of antibiotics and other socioeconomic factors. This can be seen with penicillin. Several years after penicillin use, most *Staphylococcus aureus* isolates have resisted the antibiotic. However, the issue of antibiotic resistance did not gain attention since other antibiotics that could be used to treat the infection were discovered. The continued use of antibiotics throughout the years led to the development of antibiotic resistance against multiple classes of antibiotics.¹

Infection caused by bacteria with multiple antibiotic resistance is a health threat with high mortality.² An example of this is catheter-associated urinary tract infection (CAUTI), the most common healthcare-associated infection in the United States (US), which is often caused by multiresistant pathogenic bacteria.³ Urinary tract infections (UTIs) caused by gram-negative bacteria also contribute to infections in pregnant women.⁴ Maternal infections during pregnancy may contribute to other factors causing low birth weight and preterm birth.⁵ Therefore, the World Health Organization (WHO) set a list of the most threatening families of bacteria. Among those families, bacteria with the highest priority are *Acinetobacter*, *Pseudomonas*, and several Enterobacteriaceae, such as *Escherichia coli* and *Klebsiella pneumoniae*.¹ *K. pneumoniae* can produce an enzyme called extended-spectrum beta-lactamase (ESBL), thus gaining resistance towards beta-lactam antibiotics. The number of ESBL-producing *K. pneumoniae* in a hospital can reach up to 70.9%.⁶ The common therapy for ESBL-producing *K. pneumoniae* is Carbapenem, such as meropenem. However, the use of Carbapenems can also cause resistance by the production of Carbapenemase enzymes that can degrade carbapenems. *K. pneumoniae* is also the most common microbe with carbapenem resistance and is the most responsible for carbapenem resistance worldwide. For example, there has been a steady increase in imipenem resistance in China. This can be attributed to the increased use of imipenem in the population. Therefore, increased Carbapenem use will lead to increased resistance to that antibiotic in the population.⁷

Many efforts have been conducted to suppress the development and spread of antibiotic resistance. One of those efforts is the use of plant extracts. Plant extracts, such as *Averrhoa bilimbi* and *Zingiber officinale*, have been shown to have antibacterial effects.^{8,9} Some plant extracts can also reduce bacterial biofilms, which are known to give resistance to pathogenic bacteria. Cocoa pod husk extract is an example of this.¹⁰ However, the biggest potential of plant extracts lies in their ability to form synergy with antibiotics. The phytochemicals inside plants can form synergy with antibiotics, thus preventing the development of antibiotic resistance. Furthermore, a combination of antibiotics and phytochemical substances showed stronger antibacterial effects than either antibiotic or phytochemical.¹¹

Basil (*Ocimum sanctum* or *Ocimum tenuiflorum*) is one of the plants that can be used as a combination therapy. *Ocimum sanctum* has been found to have antibacterial effects against various bacteria, such as *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *K. pneumoniae*.¹² *O. sanctum* extracts also have antifungal effects.¹³ It can also synergize with other antibiotics, like penicillin and amoxicillin-clavulanic acid.¹⁴ Therefore, *Ocimum sanctum* extract has the potential to be used as a combination therapy for antibiotic-resistant bacteria.

The antibacterial effects of *Ocimum sanctum* extract have been researched against various bacteria. However, research regarding the antibacterial effects of *Ocimum sanctum* extract combined with antibiotics is rarely conducted, especially in Indonesia. Therefore, this study aimed to evaluate the antibacterial effects of *O. sanctum* extract and meropenem combination against ESBL-producing *K. pneumoniae*. Furthermore, this study will also try to explain the suspected mechanism of the antibacterial effects previously mentioned.

Methods

This was an experimental study with a randomized controlled method and a post-test control group design. Data collection was conducted only at the end of the study period after administering treatments. This study had received ethical clearance from the Ethics Committee for Health Research, Faculty of Medicine, Universitas Airlangga, Surabaya.

Extraction of *Ocimum sanctum* leaves

Ocimum sanctum leaves were extracted using the maceration method with ethanol.¹⁵ The extraction was performed at the Faculty of Pharmacy, Universitas Airlangga, Surabaya. The obtained extract was then dissolved using 100% dimethyl sulfoxide (DMSO) to obtain working solutions with the concentration of 6%, 4%, 2%, 1%, and 0.5% each.

Antibiotic Susceptibility Test

The antibacterial effects of the extract and the combination were evaluated using an antibiotic susceptibility test using the disk diffusion method.¹⁵ In this study, two experiments were conducted. The first experiment was the antibiotic susceptibility test of ESBL-producing *K. pneumoniae* against the *Ocimum sanctum* leaf extract. Meanwhile, the second experiment was the antibiotic susceptibility test of ESBL-producing *K. pneumoniae* against the combination of *Ocimum sanctum* leaf extract and meropenem.

The First Experiment

In the first experiment, six 6 mm blank disks and a 10 µg meropenem disk were placed on a Mueller-Hinton agar inoculated with ESBL-producing *K. pneumoniae*. After that, five of the blank disks were impregnated with 25 µL of the extract solution with the concentration of 6% (E6), 4% (E4), 2% (E2), 1% (E1), and 0.5% (E0.5). Meanwhile, the last blank disk was impregnated by 25 µL 100% DMSO. The

last blank disk was the negative control, while the meropenem disk was the positive control. The medium was then incubated at 37°C for 24 hours. After incubation, the zone of inhibition on the medium would be measured using a caliper in units of mm. The experiment was performed 3 times (n=18).

The Second Experiment

In the second experiment, a 6 mm blank disk and six 10 µg meropenem disks were placed on a Mueller-Hinton Agar inoculated with ESBL-producing *K. pneumoniae*. After that, five of the meropenem disks were impregnated by 25 µL of the extract solution with the concentration of 6% (M6), 4% (M4), 2% (M2), 1% (M1), and 0.5% (M0.5). The last meropenem disk was not given any extract. The blank disk was then impregnated by 25 µL 100% DMSO. The blank disk was the negative control, while the meropenem disk was the positive control. The medium was then incubated at 37°C for 24 hours. After incubation, the zone of inhibition on the medium would be measured using a caliper in units of mm. The experiment was performed three times (n=18).

Data Analysis

The data collected from this study were analyzed using a normality test and homogeneity test, as well as a comparison test using the one-way analysis of variance (ANOVA) or Kruskal-Wallis’s test. A probability $p < 0.050$ was considered significant. If there were a significant difference in the results, the data would be further analyzed using a post-hoc test. The data were analyzed using the International Business Machines Corporation (IBM) Statistical Package for the Social Sciences (SPSS) version 26.0 for Windows with a 95% confidence level.¹⁶

Results

This study was conducted from January to August 2023 at the Laboratory of Medical Microbiology, Faculty of Medicine, Universitas Airlangga, Surabaya.

Table 1. Zone of inhibition diameter of *Ocimum sanctum* leaf extract solution against extended spectrum beta-lactamase-producing *Klebsiella pneumoniae*

Group	Average Zone of Inhibition Diameter (mm)	p-value
E6	7.08	0.007
E4	7.25	
E2	7.65	
E1	8.11	
E0.5	8.38	
K+	28.19	
K-	0	

Source: Research data, processed

Since the data in Table 1 were not homogenous, the data was analyzed using the Kruskal-Wallis’s test. Based on Table 1, the p-value was 0.007 ($p < 0.050$).¹⁶ This meant a significant difference in the diameter of the zone of inhibition due to the extract. After that, the data were analyzed using the Mann-Whitney U test. Based on the test, there was no significant difference among each group.¹⁶

Table 2. Zone of inhibition diameter of *Ocimum sanctum* leaf extract solution combined with Meropenem against extended spectrum beta-lactamase-producing *Klebsiella pneumoniae*

Group	Average Zone of Inhibition Diameter (mm)	p-value
M6	30.43	0.597
M4	30.12	
M2	30.18	
M1	29.88	
M0.5	29.98	
K+	28.94	
K-	0	

Source: Research data, processed

The data in Table 2 were tested using one-way ANOVA. Based on Table 2, the p-value was 0.597 ($p \geq 0.050$).¹⁶ This meant that the extract combined with meropenem did not significantly change the diameter of the zone of inhibition. Since the results of the one-way ANOVA were not significant, the data were not further analyzed using a post-hoc test.

Discussion

This study found that the *Ocimum sanctum* leaf extract had significant antibacterial effects against ESBL-producing *K. pneumoniae*. The antibacterial effects of *Ocimum sanctum* leaf extract against *K. pneumoniae* have been researched before. This study confirms the previous study by Khanifah, *et al.* (2023).¹⁷ However, the result that differentiates this study from the previous studies was the inverse correlation between the concentration of the extract solution and the diameter of the zone of inhibition. In the previous study, the largest zone of inhibition was observed at the 100% concentration, while the 25% concentration had the smallest zone of inhibition. This showed a positive correlation between the concentration of the extract and the diameter of the zone of inhibition. Meanwhile, this study showed the opposite. The largest diameter zone was observed at 0.5% concentration, while the smallest zone of inhibition was observed at 6% concentration. Another difference was the inhibition power of the extract in both studies. The inhibition power of an antibiotic is categorized by the diameter of the zone of inhibition. A zone of inhibition with >20 mm diameter correlates with very strong inhibition. A zone of inhibition with a 10-20 mm diameter correlates with strong inhibition. A zone of inhibition with a 5-10 mm diameter correlates with moderate inhibition. Lastly, a zone of inhibition with <5 mm diameter correlates with weak inhibition.¹⁷ In the previous study, regardless of concentration, the extract showed a weak inhibition against *K. pneumoniae*. Meanwhile, in this study, the extract, regardless of concentration, showed moderate inhibition against ESBL-producing *K. pneumoniae*. The differences in both studies are suspected to be caused by the difference in composition of antibacterial substances in the *Ocimum sanctum* used. It is thought that the concentration of antibacterial substances in the *Ocimum sanctum* extract in this study was higher than in the previous study. This is because the geological and environmental factors of each region affect the chemical composition of *Ocimum sanctum*.¹⁸ Since this study and the previous study were

conducted in different regions, the differences in geological and environmental factors could explain the differences in both studies.¹⁸

The phytochemicals in the *Ocimum sanctum* extract are responsible for the observed antibacterial effects. Essential oils mainly cause an antibacterial effect in the extract.¹⁹ Eugenol is the main constituent of all the essential oils in the extract.¹² It can exert antibacterial effects by damaging bacterial biofilms and membranes. Reactive oxygen species (ROS) production increases when a cell is exposed to eugenol. The increase in ROS decreases the intracellular concentration of glutathione. After the glutathione is depleted, ROS will react with deoxyribonucleic acid (DNA), ribonucleic acid (RNA), lipids, and protein in the cell. When ROS reacts with lipids in the cell membrane, lipid peroxidation will occur. Lipid peroxidation of the cell membrane then will cause damage to the cell membrane, thus causing leakage and killing the cell.²

Other phytochemicals responsible for the antibacterial effects are flavonoids, tannins, alkaloids, and saponins. Flavonoids inhibit cell wall synthesis, nucleic acid synthesis, bacterial motility, adenosine triphosphate (ATP) production, bacterial toxin, biofilm formation, bacterial enzymes, and efflux pumps. Flavonoids can also disrupt the cell membrane, causing it to rupture.²⁰ Tannins work by inhibiting cell wall synthesis, damaging the cell membrane, and inhibiting bacterial virulence factors such as biofilm, enzyme, toxin, and motility.²¹ Saponins destroy the biofilm through the surfactant effect and binding of sterol on the cell membrane.²² Meanwhile, alkaloids work by inhibiting the production of nucleic acid and protein, damaging cell walls and membranes, inhibiting efflux pumps, and inhibiting bacterial enzymes.²³

The previously explained mechanisms explain the antibacterial effects of the *Ocimum sanctum* leaf extract. Meanwhile, the synergy between antibiotics and plant extract can occur through three mechanisms: the inhibition of active site modification, drug-modifying enzymes, and efflux pumps.¹¹ It should be known that *K. pneumoniae* can produce ESBL and has efflux pumps.²⁴ Therefore, the phytochemicals contained in the extract, flavonoids, tannins, and alkaloids, can form synergy with meropenem because they can inhibit bacterial enzymes and efflux pumps.^{20,21,23}

Although *Ocimum sanctum* leaf extract should be able to form synergy with meropenem, it was found that the combination of the extract and meropenem did not yield a significant increase in antibacterial effects against ESBL-producing *K. pneumoniae*. This finding differs from a previous study by Srichok, *et al.* (2022).¹⁴ The previous study found that *Ocimum sanctum* extract could form synergy with penicillin or amoxicillin-clavulanic acid against several bacteria. Although the previous study used different antibiotics, penicillin and amoxicillin are beta-lactam antibiotics, similar to meropenem.¹⁴

Therefore, the mechanism of action for those antibiotics should be similar. The other differences between this study and the previous study that could explain the differing results are the bacteria used and the *Ocimum sanctum* extract. Since both studies used different bacteria, the bacteria might possess different resistance. Meanwhile, the difference in *Ocimum sanctum* extract refers to the difference in geological and environmental factors previously mentioned.¹⁸ Since those factors can affect the chemical composition of *Ocimum sanctum*, it is possible that the *Ocimum sanctum* extract in the previous study had more phytochemicals that can form synergy with the antibiotics than the *Ocimum sanctum* extract in this study.¹⁸

The last finding in this study was the difference in concentration for the strongest antibacterial effects. In the first experiment, the largest zone of inhibition was produced by the 0.5% extract solution. Meanwhile, the largest zone of inhibition in the second experiment was produced by the 6% extract solution combined with meropenem, although not statistically significant. The difference in phytochemicals responsible for both experiments could explain this. The first experiment evaluated the antibacterial effects of the extract only. Hence, the phytochemicals responsible were essential oils, mainly eugenol.^{12,19} Eugenol works by increasing the production of ROS in the cell, damaging the membrane of the exposed cell.² Meanwhile, the second experiment evaluated the antibacterial effects of the extract combined with meropenem. Therefore, the phytochemicals responsible were flavonoids, tannins, and alkaloids. Those phytochemicals can inhibit ESBL and efflux pumps of the bacteria, increasing the effect of antibiotics.^{11,20,21,23} Since the phytochemicals work differently, the optimal concentration of the specific phytochemicals may also be different. This explains the difference between the first and second experiments.^{11,20,21,23}

Strength and Limitations

The strengths of this study were using easily reproduced methods and providing mechanisms of the antibacterial effects of the *O. sanctum* leaf extract and its combination with meropenem. The limitations of this study were not using a higher concentration of the extract solution to better measure the scale of antibacterial effects and not measuring the minimum inhibitory concentration (MIC) using the dilution method.

Conclusion

In conclusion, *Ocimum sanctum* leaf extract has significant antibacterial effects against ESBL-producing *K. pneumoniae*. However, combining the extract with meropenem does not significantly improve antibacterial effects. Therefore, further research must be conducted on combining *Ocimum sanctum* extract with other antibiotics.

For example, *Ocimum sanctum* extract can be combined with Meropenem, but the combination is used against a Carbapenemase-producing Enterobacteriaceae (CPE) to evaluate the effect of the combination against antibiotic resistance.

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Conflict of Interest

The authors declared there is no conflict of interest.

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Ethical Clearance

This study had received ethical clearance from the Ethics Committee for Health Research, Faculty of Medicine, Universitas Airlangga, Surabaya (186/EC/KEPK/FKUA/2022) on 10/24/2022.

Authors' Contributions

Designing the study by BYL. Collecting data by BYL and NW. Performing statistical analysis by BYL. BYL is responsible for writing the draft of the manuscript. Revising by NW and DNI. BYL, NW, and DNI were responsible for writing the results and conducting the discussion. All authors reviewed and approved the final version of the manuscript.

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