Jurnal Sains dan Teknologi
Volume 12 Number 2, Tahun 2023, pp. 430-438
P-ISSN: 2303-3142 E-ISSN: 2548-8570
Open Access: https://doi.org/10.23887/jstundiksha.v12i2.53577

Antimicrobial Activity of Basil (*Ocimum basilicum L.*) Against Gram-Negative Bacteria Involved in Pneumonia Infection

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ARTICLE INFO

Article history:
Received October 30, 2022
Revised November 11, 2022
Accepted July 13, 2023
Available online July 25, 2023

Kata Kunci:
Resistensi Antimikroba, Penyakit Menular, Ocimum Basilicum, Fitokimia, Pneumonia

Keywords:
Antimicrobial Resistance, Infectious Diseases, Ocimum Basilicum, Phytochemical, Pneumonia

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ABSTRAK

Penyakit infeksi masih menjadi permasalahan dunia, contohnya, infeksi pernapasan akut. Resistensi pada kasus pneumonia yang melibatkan infeksi bakteri Gram negatif dapat menurunkan efektifitas penggunaan antibiotik, serta meningkatkan durasi perawatan di rumah sakit. Penelitian ini bertujuan menganalisis aktivitas senyawa aktif tanaman kemangi terhadap isolat bakteri Gram negatif yang diisolasi dari pasien pneumonia. Penelitian ini merupakan penelitian eksperimental melalui beberapa tahapan yaitu ekstraksi kemangi, penapisan awal senyawa bioaktif, isolasi dan identifikasi bakteri dari sputum, dan pengujian aktivitas antimikroba ekstrak kemangi. Sampel dahak dikumpulkan dari 43 pasien pneumonia (laki-laki dan perempuan) yang melibatkan Klebsiella pneumoniae, Acinetobacter baumannii, Enterobacter aerogenes, Pseudomonas aeruginosa, Serratia marcescens, Proteus mirabilis, dan Escherichia coli. Aktivitas penghambatan bakteri patogen berbanding lurus dengan konsentrasi ekstrak kemangi. Penapisan awal senyawa fitokimia menunjukkan bahwa ekstrak daun dan batang kemangi mengandung senyawa aktif tanin dan flavonoid. Konsentrasi 25% ekstrak kemangi merupakan konsentrasi hambat minimum terhadap bakteri uji. Konsentrasi ekstrak kemangi 100% menghasilkan zona hambat terbesar yaitu 10,93 mm. Temuan keseluruhan dari penelitian ini memberikan informasi dasar untuk kemungkinan penggunaan ekstrak Ocimum basilicum dalam pengobatan pneumonia yang melibatkan bakteri Gram negatif.

ABSTRACT

Infectious diseases are still a global problem, for example, acute respiratory infections (SARI). Resistance in pneumonia involving Gram-negative bacterial infection reduces the effectiveness of antibiotic use and increases the length of hospital stay. This study examines the potential of antimicrobial compounds from basil plants against isolates of Gram-negative bacteria involved in pneumonia infection. This research is an experimental study through several stages: basil extraction, initial screening of bioactive compounds, isolation and identification of bacteria from sputum, and testing for antimicrobial activity of basil extracts. Sputum samples were collected from 43 pneumonia patients (male and female) involving Klebsiella pneumoniae, Acinetobacter baumannii, Enterobacter aerogenes, Pseudomonas aeruginosa, Serratia marcescens, Proteus mirabilis, and Escherichia coli. The inhibitory activity against pathogenic bacteria was directly proportional to the basil extract concentration. Initial screening for phytochemical compounds showed that the extracts of basil leaves and stems contained active compounds of tannins and flavonoids. The 25% concentration of basil extract was the minimum inhibitory concentration (MIC) against the test bacteria. The 100% basil extract concentration produced the largest inhibition zone i.e., 10.93 mm. The overall findings of this study provide baseline information for the possible use of the Ocimum basilicum extract in the treatment of pneumonia involving Gram-negative bacteria.

1. INTRODUCTION

Infectious diseases are still a global problem, for example, acute respiratory infections (SARI). This lung disease is also known as a wet lung because it causes swelling and inflammation of the air sacs. According to WHO, acute respiratory tract infections characterized by difficulty breathing, a history of fever up to 38^0C, and cough require hospitalization (Aman et al., 2021; Melloni et al., 2018). In humans, this disease can cause immunosuppression, hospitalization, and even ventilator use in a patient. Lung infection with a diagnosis of pneumonia is one of the dangerous diseases that cause mortality in Indonesia. According to WHO, pneumonia is the leading cause of child mortality each week, 16% in children under five years of age. Indonesia was ranked 10^{th} in the world in terms of pneumonia cases in 2015, which was 554,650

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(Akram et al., 2018; Rustam et al., 2020). Infections such as viruses and bacteria can cause pneumonia. Gram-negative bacteria that cause pneumonia include *Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumanii,* and *Stenotrophoma maltophilia.*

Prescribing antibiotics is still an option in the treatment of infectious diseases. Pneumonia is the most common lung infectious disease receiving antibiotic therapy worldwide. Previous study reported as many as 19% of patients treated for pneumonia were given pre-antibiotics, namely penicillin (29%); amoxicillin (16%) and piperacillin (8%), third-generation cephalosporins (ceftriaxone 16%); followed by administration of fluoroquinolones (14%) (Versporten et al., 2018). On the other hand, this condition is feared to be able to increase cases of antibiotic resistance in Gram-negative bacteria, such as multi-drug resistant (MDR), extensively drug-resistant (XDR), and pan-drug resistant (PDR) (Martin-Loeches et al., 2018).

Antibiotic resistance is a natural phenomenon in bacteria that is difficult to stop. Resistance to pneumonia involving Gram-negative bacterial infection reduces the effectiveness of antibiotic use and increases the length of hospital stay (Lemos et al., 2016; Windels et al., 2019). An effort is needed to explore and develop alternative antimicrobial compounds made from natural ingredients. Therefore, measures to slow the resistance rate of development must be implemented. Traditional medicinal ingredients use is an alternative therapy for treating lung infections in the long term, one of which is basil (Indonesia's local name: Kemangi). In Indonesia, basil is more often used as fresh food. Few data were presented on the antimicrobial effect of *Ocimum basilicum* against Gram-negative bacteria.

People often use basil (*Ocimum basilicum*) to treat respiratory disorders, including asthma, bronchitis, and coughs. Basil is also reported potentially as an anti-inflammatory, immunomodulatory, and antioxidant because it contains bioactive ingredients such as saponins, tannins, alkaloids, flavonoids, and polyphenols (Aminian et al., 2022; Eftekhar et al., 2019). Previous study reported basil essential oil containing linalool (35.1%), eugenol (20.7%), and 1,8-cineole (9.9%) (Salleh et al., 2021). Linalool compounds have antimicrobial activity against Gram-positive and negative bacteria, including *Mycobacterium tuberculosis, Mycobacterium bovis*, *Staphylococcus aureus* and *Klebsiella pneumoniae* (Gaio et al., 2015; Mittal et al., 2018). Therefore the use of bacterial isolates from lung infection patient specimen samples is very important to be studied more in research related to antimicrobial compounds. The novelty and urgency of this study used local resources for traditional medicine, namely basil from Banyumas, and the use of lung infection patient specimen isolates from the distribution area of Banyumas Regency, especially at Prof. Hospital. Dr. Margono Soekardjo, who had never been tested on basil extract compounds, was the priority for the research feasibility study. This study examines the potential of antimicrobial compounds from basil plants for medicinal raw materials formulation against Gram-negative bacteria involved in lung infections.

2. METHOD

This research was conducted experimentally with one-time analysis of data measurement during the study during June-August 2022. The study used a purposive sampling method based on specific criteria to obtain a specific target, involving all pnuemonia infection patients with treatment who had examined bacterial culture and meet the criteria. The object population used in this study was basil extract with variant concentrations and the subjects were isolates of Gram-negative bacteria from pneumonia patients treated at Hospital. Prof. Dr. Margono Soekardjo. This research is a laboratory-scale experimental study through several stages, namely basil extraction, initial screening of bioactive compounds, isolation and identification of bacteria from sputum, and testing for antimicrobial activity of basil extracts.

Parts of the basil plant that have been dried (leaves and stems) and mashed, every 50 g of the sample is put in a separate Erlenmeyer flask, added 100 mL of ethanol, the sample is incubated for 6 hours in a water bath with a temperature of 55-60^{0}C (Rezzoug et al., 2019)**.** Separation extraction of the residue mixture was carried out with Whatman No. 1 filter paper, each plant residue was re-extracted with ethanol three times. After filtration, the two halves were mixed and the residual solvent in the ethanolic extract was removed at low pressure at 48-49^{o}C, using a rotary evaporator (Rotavapor IKA VB 10, Germany). The filtration results were made of preparations with concentrations of 0%, 25%, 50%, 75%, and 100%.

The initial screening of *Ocimum basilicum* extract for various phytochemical compounds followed (Yadav et al., 2014). Sputum samples were collected at RSUD Prof. Dr. Margono Soekardjo, Purwokerto, Banyumas, Central Java. The sampling technique is based on the purposive sampling method. Patient criteria were patients diagnosed with pulmonary infection. The sample of specimens taken was 43 samples calculated based on cross-sectional, with the population size (N) (Masturoh & Anggita, 2018). A total of 1 mL of sputum was grown on MacConkey Agar media and incubated at 37^{0}C for 24 hours. The growing bacterial colonies were purified and characterized based on Gram type, colony morphology, and

physiological and biochemical tests. This study used the bioMeriueux Vitek 2 compact system version 07.01 to identify the bacteria.

This study used the paper disc diffusion method to evaluate the antimicrobial activity of basil extracts. Isolates of Gram-negative bacteria were grown on MacConkey Agar media. The paper discs with a diameter of 3 mm were immersed in the basil extract liquid filtrate for 2 minutes (concentrations were 100%, 75%, 50%, and 25%, respectively). The paper discs were then placed on the bacterial growth medium and incubated at 36^0C for 2x24 hours. The positive control was the antibiotic ampicillin, while the negative control was disc paper without any inhibitors. The calculation of antimicrobial activity follows (Ariani et al., 2020; Nurhayati et al., 2020). The data were analyzed using ANOVA and Turkiye HSD analysis.

3. RESULT AND DISCUSSION

Result

Sputum samples were collected from 43 pneumonia patients (male and female). Bacterial isolates were characterized by Gram staining as show in Figure 1. The Gram-negative bacteria obtained were *Klebsiella pneumoniae* (27 samples), *Acinetobacter baumannii* (6), *Enterobacter aerogenes* (3), *Pseudomonas aeruginosa (1), Serratia marcescens* (1), *Proteus mirabilis* (2), and *Escherichia coli* (3). These results explained that *K. pneumoniae* (79.41%) was the most dominant bacteria in pneumonia patients, followed by *A. baumannii* (17.64%).

Figure 1. Isolated Gram-Negative Bacteria from Sputum Samples of Pneumonia Patients. (a) *Klebsiella Pneumoniae* ss Pneumoniae; (b) *Acinetobacter Baumannii*; (c) *Klebsiella Pneumoniae*

Base on Figure 1 Basil (*Ocimum basilicum*) is an aromatic plant of the Lamiaceae family of great tradition in Indonesia and is widely used in cooking. The antimicrobial activity of basil extract (leaves and stems) was tested against isolated Gram-negative bacteria. The results showed that basil leaf extract with a concentration of 25% produced an inhibition zone of 6.82 mm. Meanwhile, basil stem extract with a concentration of 25% has an inhibition zone of 8.86 mm. These data also showed that the 25% concentration of basil extract was the minimum inhibitory concentration (MIC) against the test bacteria. The 100% basil extract concentration produced the largest inhibition zone i.e., 10.93 mm as show in Figure 2.

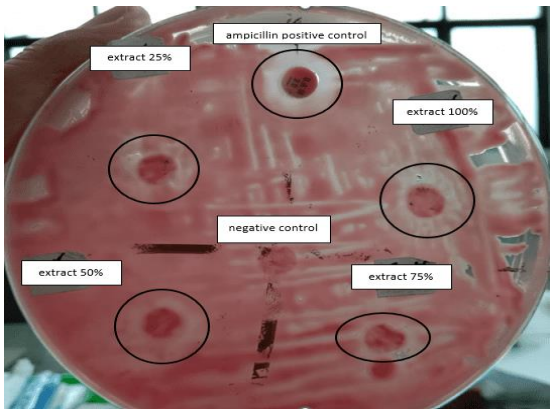

Figure 2. Inhibitory Zone of Basil Extract (Stem and Leaves) Against *Klebsiella Pneumoniae* Clinical Isolate (Extract Concentrations: 25%, 50%, 75%, and 100%). Positive Control: Ampicillin; Negative Control: *Blank disc*

ANOVA analysis showed a significant difference between treatments for the concentration of basil extract. Meanwhile, the Post-Hoc test on the inhibition zone of 25% basil extract in leaf and stem samples had a significance value of $p<0.05$. It explains that between treatments have the same or not significant results. Antibacterial testing using Turkey HSD analysis showed the similarities and differences of several treatments given. There are treatments with leaf extract concentrations of 25%, 50%, 75%, 100%, and stem extract concentrations of 25 to 75%. It shows that the average treatment did not significant as show in Table 1.

Table 1. Data Analysis Using Turkey HSD Among Treatments

Treatments	N	Subset for alpha = 0,05	
		1	2
Leaves extract (25% concentration)	7	6.821	
Leaves extract (50% concentration)	7	8.214	8.214
Leaves extract (75% concentration)	7	8.500	8.500
Leaves extract (100% concentration)	7	8.857	8.857
Stem extract (25% concentration)	7	9.429	9.429
Stem extract (50% concentration)	7	9.571	9.571
Stem extract (75% concentration)	7	9.750	9.750
Stem extract (100% concentration)	7		10.929
Positive control	7		11.000
Sig.		0.136	0.182

To screen the *Ocimum basilicum* extract for various phytochemical compounds, various tests and procedures can be performed. In this present study, the initial phytochemical screening is perfomed with simple methods. The screening results presented that leaf and stem samples of basil contained several phytochemical compounds, such as tannins and flavonoids. Meanwhile, terpenoids, saponins, steroid, and alkaloids were not detected in leaf nor basil's stem.

Discussion

This present study found that *Klebsiella pneumoniae* (79.41%) was the most dominant bacteria in pneumonia patients, followed by *Acinetobacter baumannii* (17.64%). A total of seven clinical isolate of Gram-negative bacteria group were isolated in this study, accordance with the report of World Health Organization (WHO). Globally, the prevalence of Gram-negative bacteria in pneumonia patients ranges from 49.7-95.3%. WHO reported that *Klebsiella pneumoniae* is one of the most common Gram-negative bacteria causing hospital-acquired pneumonia. *Acinetobacter baumannii* to be one of the dominant organisms (48%) causing hospital-acquired pneumonia (HAP) and ventilator-acquired pneumonia (VAP). Meanwhile, the most commonly found Gram-positive bacteria causing pneumonia is *Streptococcus pneumoniae* (Ikram et al., 2021; Purba et al., 2019).

Our data showed that *Enterobacter aerogenes, Pseudomonas aeruginosa, Serratia marcescens, Proteus mirabilis,* and *Escherichia coli* coinfected the respiratory tract. It may worsen the condition of pneumonia patients. Previous study that bacterial coinfections harm the severity of pneumonia and increase patients morbidity and mortality (Lim et al., 2019). Other study reported that Gram-negative bacteria are the most common pathogens causing secondary lung bacterial infection in COVID-19 patients in Indonesia (Santoso et al., 2022). MDR pathogens were discovered in high concentrations among isolates, which was associated with a considerable risk of mortality. Previous research in Brazil looked at bacterial infections in patients with severe COVID-19 and found multi-drug resistance in 96% of *A. baumannii* and 57% of *K. pneumoniae*, which was related with a longer ICU stay and increased mortality.

The new thing found in this study is that *Klebsiella pneumoniae* in addition to *Acinetobacter baumannii* in clinical specimens of sputum, dominates as pathogenic bacteria that cause respiratory infections. The discovery of pathogenic bacteria causing infection in Tertiary Hospital Inpatient Care requires special attention. Previous study found that *K. pneumoniae* and *A. baumannii* are commonly infect

the respiratory tracts in Tertiary Hospital (Tsakiridou *et al*., 2014). Similarly other study found that *K. pneumoniae* and *A. baumannii* carried a high risk for multidrug resistance of community-acquired pneumonia in Tertiary Referral Hospital in Indonesia (Purba et al., 2019).

The use of antibiotics is still the main line in the prevention of diseases caused by bacteria. All guidelines concur that at least one empiric antibiotic is required particularly for hospitalized patient with pneumonia (da Costa et al., 2022; Purba et al., 2019). Pneumonia is the most common lung infectious disease receiving antibiotic therapy worldwide. Previous study reported as many as 19% of patients treated for pneumonia were given pre-antibiotics, namely penicillin (29%); amoxicillin (16%) and piperacillin (8%), third-generation cephalosporins (ceftriaxone 16%); followed by administration of fluoroquinolones (14%) (Martin-Loeches et al., 2018; Versporten et al., 2018). Less restrictions and inappropriate use of antibiotics can increase cases of antibiotic resistance in Gram-negative bacteria, such as multi-drug resistant (MDR), extensively drug-resistant (XDR), and pan-drug resistant (PDR).

Several recent studies reported that the dominant multidrug resistance of Gram-negative bacteria causing pneumonia was *A. baumannii, K. pneumoniae,* and *P. aeruginosa*. Previous stydy reported that infections with *Acinetobacter baumannii* linked with community-acquired pneumonia lead to multidrug resistance (MDR) and increased mortality in Asia Pacific nations (Ong et al., 2009). A study at Dr. Soetomo in Surabaya (Indonesia) found an increased prevalence of *Klebsiella pneumoniae* infection producing beta lactamase enzyme (Yamasaki *et al*., 2021). Bacterial resistance to antibiotics can lead to failure in response to treatment including; extension of illness (prolonged illness); the increased risk of death (greater risk of death) and the longer length of stay in the hospital which will become a financing burden (Assefa, 2022; Harapan et al., 2018).

Bacterial infections caused by Gram-negative bacteria are usually more resistant to antibiotic than Gram-positive. The molecular components and membrane morphology of Gram-positive bacteria are fundamentally different from those of Gram-negative bacteria. Gram-negative bacteria have two membranes: the cell membrane and the outer membrane. The outer layer of the membrane contains lipopolysaccharide (LPS) as the main lipid component, a lipid species unique to Gram-negative bacteria. Gram-negative bacteria are less susceptible to antibacterial agents because of their lipopolysaccharide outer membrane, which restricts the diffusion of hydrophobic compounds. Meanwhile, these membranes offer antimicrobials. The target bacteria modify it for resistance by providing antimicrobials (Epand et al., 2016; Loutet & Valvano, 2011). Gram-negative bacterial resistance mechanisms can occur due to the outer membrane as a permeability barrier for many substances including antibiotics. The low permeability of the outer membrane of bacteria to certain antibiotic agents is responsible for the intrinsic resistance of some Gram-negative bacteria to antibiotics (Christaki et al., 2019; Favour et al., 2014). Changes in the permeability of the outer membrane can contribute to the development of acquired resistance. The overexpression of efflux pumps is reported as the major resistance mechanism in bacteria.

The Gram-negative pathogenic isolates found in this study were mostly obtained from respiratory specimens, especially sputum samples. *K. pneumoniae* infection in the lungs cause destructive changes, the appearance of necrosis, and imflammation discharge resulting in a viscous, bloody and slimy sputum. It makes sputum specimens as a target in the examination of lung infection cases. Morphology-based phenotypic identification of *K. pneumoniae* as the dominant pathogen has identical special features on MacConkey media featuring colonies of large, smooth, slimy, convex and pinkish shapes (data not showen). *K. pneumoniae* are capable of producing thick capsules that are important for pathogenicity. A capsule is a bacterial cell wrapper in the form of a polysaccharide layer. Previous study state the function of capsules in bacteria in general is as protection, and their main function in pathogenic bacteria provides protection from the host's immune system (Dunstan et al., 2021). Polysaccharide capsules in *K. pneumoniae* can act as an important virulence factor because of their ability to block phagocytosis, and have an impact on increasing the severity of a disease. Capsule serotypes *K. pneumoniae* K1 and K2 are associated with cases of bacterimia with high mortality rates in Taiwan, Europe, and North America (Hasani et al., 2020; Opoku-Temeng et al., 2019).

Traditional medicine has long been an alternative for treating various diseases in the community. For them, traditional medicine is more affordable than buying antibiotics. In LMICs, infection prevention is challenging due to low awareness and high transmission between patients and the population. Therefore, researchers are increasingly exploring the potential of traditional medicine in treating infectious diseases. Basil (*Ocimum basilicum*) is an aromatic plant of the Lamiaceae family of great tradition in Indonesia and is widely used in cooking. Basil contains flavonoids, tannins, carbohydrates, oils, and proteins. Those active compound are reported to have antibacterial activity against various pathogens, such as *S. aureus, E. faecalis, E. coli, P. aeruginosa*, and the yeast *Candida albicans* (Khair-ul-Bariyah et al., 2012; Pushpalatha. et al., 2021).

In this study, we studied the antimicrobial activity of basil extract against Gram-negative bacteria involved in lung infections. The treatment with a concentration of 25% basil leaf extract and a 100%

concentration of basil stem extract showed minimal and maximum inhibition results. Meanwhile, the average of other treatments gave the same results. The concentration difference treatment variable only significantly affects the average treatments of basil leaf extract with a concentration of 25% and stem extract with a concentration of 100%, compared to the inhibition zone by positive control. The results showed that the extracts of basil leaves and stems produced a zone of inhibition that is broader with the increasing extract concentration. We assumed that the inhibitory activity of pathogenic bacteria was directly proportional to the basil extract concentration. It is supporting the study which tested the inhibition of basil extract against *Klebsiella pneumoniae* and *Staphylococcus aureus* (Mittal et al., 2018). Research on basil with an MBC (micro bactericidal concentration) at 2,500 mg/mL resulted in an extract that was most effective against Gram-positive and Gram-negative bacteria, especially *Staphylococcus aureus, Escherichia coli,* and *Salmonella typhimurium*. Similarly other study found that basil extract could inhibit the growth of *Pseudomonas aeruginosa* with the highest concentration of 10 mg/mL (Ikram et al., 2021).

Ocimum basilicum is known to contain a variety of phytochemicals, including phenolic acids, flavonoids, terpenoids, alkaloids, and tannins, which are responsible for its medicinal properties. Active compounds of plant extract have one or more functions and act on specific sites in the cell. Since these compounds have different chemical properties, they can affect the cellular metabolism of microorganisms in various mechanisms. Initial screening for phytochemical compounds showed that the extracts of basil leaves and stems contained active compounds of tannins and flavonoids. The target of action of flavonoid compounds is destroying the function of bacterial membranes, formation of biofilms, and efflux pumps (Salleh et al., 2021; Song et al., 2022). Inhibition of nucleic acid synthesis, energy metabolism, inhibition of porins in cell membranes causes changes in permeability and weakening of pathogenicity. According to literature studies conducted by various countries, basil leaf essential oil contains the following constituents: methyl chaviol, linalool, eugenol, methyl eugenol, fenchyl alcohol, limoenene, α-pinene, β-pinene, β-caryophyllene, thymol, camphene, α-bergamonete, geranial, geranial acetate, 1,8 – cineol, estragole, cineol, α-cubebene, nerol, methyl cinnamate, linalil acetate (Pandey et al., 2014; Song et al., 2022). The presence of prenyl functional groups with hydrophobic substituents inhibits the function of bacterial membranes.

We want to emphasize the limitations of the study. The first is a specimen study subject with a small sample size and comes from one hospital for a short period of time. Second, our focus is limited to testing basil extract on isolate specimens from sputum samples. Third, we demonstrated the diversity of bacterial isolates from sputum samples, which were not analyzed for their virulence genes, therefore we do not have data on the virulence ability of these bacteria against pneumonia cases. Fourth, we do not have data on the bioactive compounds contained in the basil extract, and only have data on the results of the analysis of the chemical. In addition to these tests, other methods such as Thin Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), and Gas Chromatography-Mass Spectrometry (GC-MS) can also be used to identify and quantify specific phytochemical compounds in the extract. We also recommend conducting further studies with more isolates, examining the content of virulence genes and focusing on patients, as well as characterizing the content of bioactive compounds in basil extract, where currently available data are limited.

4. CONCLUSION

Isolates of pathogenic bacteria, *K. pneumoniae,* were found predominate in clinical specimens of sputum, followed by *Acinetobacter baumannii, Enterobacter aerogenes, Pseudomonas aeruginosa, Serratia marcescens, Proteus mirabilis,* and *Escherichia coli.* The bacterial growth can be inhibited by the extract of the basil plant with a maximum inhibitory zone at an extract concentration of 100%. The inhibitory activity against pathogenic bacteria was directly proportional to the basil extract concentration. Initial screening for phytochemical compounds showed that the extracts of basil leaves and stems contained active compounds of tannins and flavonoids. The overall findings of this study provide baseline information for the possible use of the *Ocimum basilicum* extract in the treatment of pneumonia involving Gram-negative bacteria.

5. ACKNOWLEDGE

Thank you Ministry of Education, Culture, Research and Technology for funding the Research Grant for Beginner Lecturers for Fiscal Year 2022 based on Decree No. 158/E5/PG.02.00.PT/2022, and the Institute for Research and Community Service (LPPM) Nahdlatul University Purwokerto.

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