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Potential Antibacterial Effects of Ethanol Extract and Essential Oil of *Origanum vulgare* on *Klebsiella pneumonia* and *Staphylococcus aureus*

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ABSTRACT

*Klebsiella pneumoni*a (*K. pneumonia*) and *Staphylococcus aureus* (*S. aureus*) are pathogenic bacteria causing various infectious diseases in humans and animals. Currently, herbal ingredients are widely used as antibacterial agents to combat bacterial infections due to their lower side effects, compared to chemical drugs. One such plant with medicinal promise as an antibacterial agent is the oregano plant (*Oregano vulgare*). It contains substances, such as tannin, flavonoids, carvacrol, thymol, and saponin. Therefore, the current study was conducted to regularly compare the *in vitro* antibacterial potential of ethanol extract essential oil oregano (*Oregano vulgare*) on *K. pneumonia* and *S. aureus*. In this research, the diffusion method using discs was employed to observe the inhibition zones, while the dilution tube method was utilized to determine the minimum inhibitory concentration (MIC) of the ethanol extract and essential oil of oregano against the test bacteria. The bacterial treatment group received the test material at concentrations of 100%, 50%, 25%, and 12.5%. The obtained data were analyzed descriptively in terms of zone inhibition and MIC values. According to the disc diffusion test, the essential oil of *oregano* demonstrated greater efficacy as an antibacterial agent against *K. pneumoniae* at a concentration of 100%, resulting in an average inhibition zone of 18 mm. Conversely, for *S. aureus*, a concentration of 1.5% of the essential oil exhibited higher effectiveness, yielding an average inhibition zone of 30 mm. Based on the MIC values, the essential oil was more effective as an antibacterial for *K. pneumonia* at a concentration of 0.2% (2 mg/mL), while for *S. aureus* it was more effective at a concentration of 0.19% (1.9 mg/mL).

Keywords: Antibacterial, Ethanol Extract, Essential Oil, *Oregano vulgare*

INTRODUCTION

Natural ingredients are increasingly used as an alternative medicine against bacterial infection. Natural ingredients have low side effects and are easy to obtain compared to chemical drugs. Oregano plant (*Oregano vulgare*) is one of the natural ingredients commonly used to process food and is also used as medicine (Milagres De Almeida et al., 2023). Recently, the oregano plant has gained significant recognition as an herbal medicine, supported by robust scientific evidence. Oregano plant is very influential against Gram-positive and Gram-negative bacteria (Zhong et al., 2023). The most common compounds in the oregano plant are carvacrol, thymol, tannins, phenols, and flavonoids (Chevallier, 2016). Carvacrol and thymol disrupt the phospholipid layer to inhibit protein synthesis, which can damage bacterial cell membranes. This leads to an increase in cell membrane permeability. Carvacrol can inhibit the movement of the flagellum due to a decrease in ATP production (Walczak et al., 2021). The mechanism of phenol compounds will react with cell membrane phospholipid compounds. These processes lead to alterations in fatty acid compositions, phospholipid levels, and disruptions in the bacterial cell walls, resulting in cellular damage. Tannins function by attaching to proteins located on the surface of bacterial cells, preventing them from allowing water to enter. Flavonoids operate similarly, with the potential to induce cell or bacterial cell wall leakage (Chevallier, 2016).

Klebsiella (K.) pneumonia is a short rod-shaped Gram-negative bacterium with no spores and a thick and non-motile capsule with a size of 0.5-1.5 µ. *Klebsiella pneumonia* is a significantly opportunistic and gram-negative bacteria

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that causes infections in the respiratory tract, circulatory system, urinary system, and wounds in individuals with underlying disease (Ranjbarian et al., 2023). *Staphylococcus (S.) aureus* is a Gram-positive bacteria (clustered like grapes) and easily grows on various deep bacterial media aerobic states. *Staphylococcus aureus* is a prevalent pathogen in human infections, often contributing to various diseases, including skin and soft tissue infection, infectious arthritis, and septicemia (Zhao et al., 2022). Therefore, the current study aimed to investigate and compare the antibacterial potential of essential oil (EO) and extract ethanol (EE) of *Oregano vulgare* against *K. pneumonia* and *S. aureus*.

MATERIALS AND METHODS

Ethical approval

The current study was conducted according to the guidelines of the University Brawijaya, Puncak Dieng Eksklusif Malang, East Java, Indonesia.

Extract preparation

Oregano vulgare was obtained from the local farmers in Malang, Indonesia. The leaves were dried in an open and shady place for 7 days. Then, the dried *Oregano vulgare* leaves were powdered to obtain 500 grams. *Oregano vulgare* powder was soaked for 3 days with a maceration procedure using 3 L of 96% methanol at room temperature for 24 hours and stirred once daily for 3 days. The extract was evaporated using a rotary evaporator (Sarmira et al., 2021).

The EO was extracted through the hydro-distillation process using a Clevenger's type apparatus, as described by Clevenger in 1928. The distillations were conducted by subjecting aerial plant parts to boiling for 3 hours. The resulting EO was then transferred to airtight, sealed vials and subsequently stored at a temperature of 4°C until it was to be utilized for subsequent purposes.

Bacterial culture and preparation

The bacteria used are *K. pneumonia* and *S. aureus*. These isolates were obtained from the Laboratory of Microbiology, Faculty of Veterinary Medicine, University Brawijaya, Malang, Indonesia. The *K. pneumonia* was reidentified using Mac Conkey Agar *(*MCA) media. The confirmation tests were carried out in the form of biochemical tests and Gram staining with a solution of crystal violet, acetone alcohol, Lugol and safranin with the results of bacteria in the form of Gram-negative bacilli (Riedel et al., 2019). At the same time, *S. aureus* reidentification tests were carried out in the form of mannitol fermentation tests, gram staining, catalase tests, and coagulase tests. The confirmed bacteria were then propagated using the Nutrient Broth (NB) media on *K. pneumonia* and *S. aureus* and incubated at 37°C for 24 hours.

Antibacterial testing tube dilution

In tube dilution, 13 reactions were provided for EE and EO. Each tube was filled with 0.5 ml of Mueller hilton broth (MHB) and 0.5 ml of the test material. It was then put into the second test tube, and 1 ml of bacterial suspension was added so that each test tube would be filled with 2 ml and incubated at 37^{O}C for 24 hours. The turbidity was observed, and the MIC value was determined on the media. Tubes tested for MIC were subculture on MHA media and incubated at 37^{O}C for 18-24 hours. The growth of bacteria from incubation was observed with the presence or absence of colony growth (Aiemsaard et al., 2023).

Antibacterial testing disc diffusion

The disc method was carried out by inoculating a bacterial suspension on MHA media as much as 0.1 ml using a sterile cotton swab and left to dry. Disc paper soaked in EE and EO common oregano for 15 minutes. The soaking time of disc paper could affect the absorption of the active ingredients contained in the extract into the paper disc because a longer soaking time results in increased absorption of active materials. For the agar disk diffusion method, the sterile filter paper disk was saturated with 100%, 50%, 25%, and 12.5%. The soaked paper discs were aseptically placed on the surface of the MHA media and incubated at 37°C for 24 hours. The bacterial growth activity was determined by measuring the diameter of the zone of inhibition (Nordin et al., 2019). Dimethyl sulfoxide (DMSO) 5% was used as a negative control because it is a solvent with no antibacterial properties (Adiwibawa Prasetya et al., 2019).

Data analysis

Research data on the antibacterial potential of EE and EO of *Oregano vulgare* was analyzed descriptively by looking at the Minimum Inhibitory Concentration (MIC) and inhibition zone results. The obtained data were analyzed using SPSS software (version 29 IBM) with one-way ANOVA and a follow-up Tukey when the p-value was significant ($p < 0.05$).

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RESULTS AND DISCUSSION

Antibacterial activity test

The disc diffusion method was used in this study, where EE and EO of *Oregano vulgare* were tested for their antibacterial activity against *K. pneumonia*. The inhibition diameter of the bacterial activity was measured as a result of the disc diffusion method. According to Sarmira et al. (2021), the strength of the antibacterial activity can be determined based on the diameter of the inhibition. Therefore, very strong activity is for a diameter of inhibition > 20 mm, strong activity has an inhibition zone diameter of 10-20 mm, moderate activity corresponds to an inhibition zone diameter of 5-10 mm, and inhibition zone diameter < 5 shows a weak activity.

The results obtained after measuring the average diameter of the clear zone formed against the test bacteria *K. pneumonia* and *S. aureus* with EE and EO of *Oregano vulgare* are shown in Tables 1 and 2.

Table 1. The antibacterial activity of ethanol extract of *Oregano vulgare*

Bacteria	Concentration (%)	Inhibition Zone (mm)
Klebsiella pneumonia	DMSO 5%	0 ± 0^{a}
	100%	6.4 ± 0.5^{c}
	50%	4.7 ± 0.5^{b}
	25%	3.74 ± 0.5^{b}
	12.5%	0.0 ± 0^{a}
Staphylococcus aureus	DMSO 5%	0 ± 0^{a}
	100%	14.3 ± 0.9^{d}
	50%	12.3 ± 0.5^{c}
	25%	9 ± 0.5^{b}
	12.5%	7.3 ± 0.5^{b}

Values represent mean ± SD. ^{abcd}Means with different superscripts in a column differ significantly among different concentrations ($p < 0.05$)

Table 2. The antibacterial activity of the essential oil of *Oregano vulgare*

Bacteria	Concentration	Inhibition Zone (mm)
Klebsiella pneumonia	DMSO 5%	0 ± 0^{a}
	100%	18 ± 0^{c}
	50%	16.3 ± 0.9^{c}
	25%	13 ± 0.8^{b}
	12.5%	12 ± 1.4^{b}
Staphylococcus aureus	DMSO 5%	0 ± 0^{a}
	100%	30.3 ± 0.5^{d}
	50%	28.3 ± 0.5^{d}
	25%	18.6 ± 0.9^{c}
	12.5%	14 ± 1.4^{b}

Values represent mean ± SD. ^{abcd}Means with different superscripts in a column differ significantly among different concentrations ($p < 0.05$)

The study indicated that EE and EO of *Oregano vulgare* had antibacterial activity against *K. pneumonia* and *S. aureus* (Tables 1 and 2). The average value of the diameter of the bacterial inhibition zone *K. pneumonia* with ethanol extract test material *Oregano vulgare* at a concentration of 100% is 6.4 mm, which is with a medium inhibition, and *S. aureus* concentration of 100% was 14.33 mm with strong inhibition. Table 2 indicates that *Oregano vulgare* EO to *K. pneumonia* at a concentration of 100% was 18 mm, which is a strong inhibition. Notably, a concentration of 100% *Oregano vulgare* EO demonstrated a very strong inhibition against *S. aureus*, evidenced by an inhibition zone diameter of 30.33 mm. *Oregano vulgare* EO indicated a more powerful antibacterial effect against bacteria than EE, which was likely to inhibit Gram-positive bacteria more than Gram-negative bacteria. The *Oregano vulgare* EE antibacterial activity of 100% against *K. pneumonia* and *S. aureus* showed a medium and strong inhibition (6.4 ± 0. 5 and 14.3 ± 0.9 mm, respectively; Table 1). Moreover, the antibacterial activity of EO of 100% *Oregano vulgare* against *K. pneumonia* and *S. aureus* showed a strong and very strong inhibition (18 ± 0 and 30.3 ± 0.5 mm, respectively; Table 2). It was found that EO had more powerful antibacterial effects than the EE of *Oregano vulgare*, especially in this research using *K. pneumonia* (Gram-negative) and *S. aureus* (Gram-positive bacteria). The antibacterial activity against gram-positive *S. aureus* was stronger than that against gram-negative *K. pneumonia*. The reason could be the fact that Gram-negative bacteria are usually more resistant to antibacterial compounds since their cell walls are more complicated (Nurhayati et al., 2020).

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Essential oils exhibit potent antibacterial properties due to their capacity to enhance membrane permeability and induce cytoplasmic membrane depolarization. These actions effectively inhibit the production of microbial toxins and the formation of biofilms while also reducing fimbriae production (Băicuș et al., 2022). Carvacrol and thymol, the main components of oregano EO, are antibacterial and antioxidant (Coccimiglio et al., 2016).

The EE of oregano leaves contains several secondary metabolite compounds, such as phenolics, tannins, flavonoids, saponins, triterpenoids, and steroids (Cortés-Chitala et al., 2021). Ethanol extract of oregano leaves contains secondary metabolite compounds that have various mechanisms of action, such as antibacterial. The mechanism of action of phenolic compounds involves the denaturation of proteins, leading to the disruption of their tertiary structure and the subsequent loss of their original properties. Phenolic compounds can denature protein; thus, the main structure of the protein will be damaged (Coccimiglio et al., 2016). The cell wall of bacteria can be easily damaged by the antibacterial properties of *Oregano vulgare*, namely carvacrol and thymol (Can Baser, 2008). However, the mechanisms by which thymol and carvacrol cause cell death in bacteria have not been thoroughly investigated. Koparal and Zeytinoglu (2003) demonstrated that carvacrol is a very potent inhibitor of cell growth in A549 cell line, as evidenced by the concentration-dependent decreases in cell number, degeneration of cell morphology, and a decrease in total protein amount (Koparal and Zeytinoglu, 2003). Based on Hsu et al. (2011), thymol induces cell death in human osteosarcoma and astrocytes and may involve apoptosis via mitochondrial pathways.

Minimum inhibition concentration

The MIC is defined as the smallest concentration of an antimicrobial agent that prevents the visible growth of test microorganisms in a tube. The first with no visible growth after the incubation period is taken as a MIC. Minimum inhibition concentration (MBC) is a minimal concentration of antimicrobials that kills inoculum and can be determined by the MIC assay (Ngadino et al., 2018). The tube dilution method was used to determine MIC for the antibacterial potential of *Oregano vulgare* EE and EO against *K. pneumonia* and *S. aureus*.

Table 3. Determination of minimum inhibition concentration

Bacteria	*Oregano vulgare*	Concentration of *Oregano vulgare* (%)												
		100	**50**	**25**	**13**	**6.3**	**3.1**	**1.6**	**0.8**	**0.4**	**0.2**	**0.1**	**0.05**	**0.02**
Klebsiella pneumonia	Ethanol extract	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
	Essential oil	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)
Staphylococcus aureus	Ethanol extract	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
	Essential oil	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)

(+): Growing; (-): Not growing

Tube dilution test results using EE and EO against bacteria *K. pneumonia* turbidity at each concentration can be observed after incubation (Table 3). In the ethanol extract that had been given a bacterial culture of 1 ml, the observed MIC results were at a concentration of 50% or the equivalent of 500,000 µg/mL (500 mg/ml). The observed MIC results were at a concentration of 0.2% or equivalent to 2,000 µg/mL (2 mg/ml) found on *S. aureus* tube dilution test using ethanol extract. The smallest concentration was found in the tube where there was no growth of bacterial colonies, as indicated by the clarity of the tube. In EO, the lowest MIC value at 12.5% was equivalent to 125 mg/mL, and in EO, at a concentration of 0.19% or 1.9 mg/mL.

Essential oil *Oregano vulgare* has a greater antibacterial potential than ethanol extract *Oregano vulgare*, as measured by the MIC values. The literature explains that a drug substance or test material can be classified as antibacterial if it has bacteriostatic and bactericidal properties. Bacteriostatic refers to a drug's ability to inhibit bacterial growth at a specific concentration. In contrast, bactericidal refers to the capacity of a drug or test substance to kill bacteria at a particular concentration. In this study, bacteriostatic activity was indicated by the variation in turbidity levels and the reduction in the number of bacteria growth in response to the administration of the extract at varying concentrations. In contrast, bactericidal activity was indicated by the presence or absence of bacterial growth on the medium supplemented by *Oregano vulgaris* extract in various concentrations.

CONCLUSION

This study revealed that *Oregano vulgare* EE and EO is a potential alternative natural antibacterial agent against *Klebsiella. pneumonia* and *S. aureus*. Compared to ethanol extract, EO has a greater antibacterial potential, and both are more effective against gram-positive bacteria.

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DECLARATIONS

Authors' Contributions

Indah Amalia Amri, Nurul Fitri Ramadani, Farah Hamidah, Fidi Nur Aini EPD, and Sruti Listra Adrenalin contributed manuscript writing, data analysis, and study design. All authors contributed review data from this research and approved the final draft of the manuscript.

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Ethical consideration

The authors confirm that all authors have reviewed and submitted the manuscript to this journal for the first time.

Availability of data and materials

The original contributions presented in the study are included in the article/supplementary material. For inquiries, please contact the corresponding author/s.

Conflict of interests

The authors have not declared any conflict of interest.

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