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In Vitro Antibacterial Activity Test of Black Pepper (*Piper nigri fruktus)* Extract Against *Escherichia coli* Using the Diffusion Method

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ABSTRACT

The increasing prevalence of antibiotic-resistant *E. coli* strains necessitates exploring alternative antibacterial agents. Black pepper (*Piper nigrum*) is a widely available spice in Indonesia known for its various bioactive compounds, which exhibit antimicrobial properties. This study evaluates the antibacterial activity of black pepper fruit extract against *E. coli* using in vitro diffusion and dilution methods. A true experimental design with a post-test-only control group was employed, involving non-pathogenic *E. coli* cultures. Various concentrations of black pepper extract (100 ppm to 3.125 ppm) were tested. Outcomes were measured by inhibition zones in diffusion tests and growth observations in dilution tests to determine Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). Results demonstrated a concentration-dependent increase in antibacterial activity, with higher extract concentrations producing larger inhibition zones. Black pepper extract exhibited potential as an alternative antimicrobial agent. Further research is recommended to optimize the formulation and explore its clinical applications.

Keywords: antibacterial; black pepper; *Escherichia coli.*

INTRODUCTION

Escherichia coli (*E. coli*), a Gram-negative, rod-shaped bacterium, is commonly found in the human gastrointestinal tract as part of the normal microbiota. While most strains are harmless and even beneficial in maintaining gut health, certain pathogenic strains have emerged as critical agents of infectious diseases. These pathogenic strains, such as Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), and Shiga toxin-producing *E. coli* (STEC), are associated with severe health conditions, including diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome [1]. ETEC, in particular, is a leading cause of traveler's diarrhea and infant mortality in developing regions, highlighting its global health risk [2].

The clinical burden of *E. coli*-related infections is compounded by the alarming rise in antibiotic resistance [3]. Overuse and misuse of antibiotics, coupled with the slow pace of novel antibiotic development, have resulted in multidrug-resistant strains. These include strains resistant to β-lactam antibiotics through the production of extended-spectrum β-lactamases (ESBLs), while also showing resistance against carbapenems [4]. Resistant *E. coli* strains have been identified across the world the need for innovative approaches to mitigate their impact [5].

The search for alternative antibacterial agents has gained significant momentum in recent years [6]. Plant-derived bioactive compounds represent a promising avenue due to their availability, affordability, and effectiveness [7]. Black pepper (*Piper nigrum*), a widely cultivated spice in Indonesia, has attracted interest as a potential source of antibacterial agents[8]. Known for its traditional use in treating various ailments, black pepper contains a variety of bioactive compounds, including piperine, which have demonstrated antimicrobial properties in preliminary studies [9]. Piperine has been shown to disrupt bacterial membranes, inhibit protein synthesis, and reduce bacterial viability, making it a candidate for further exploration as a natural antibacterial agent [10]. In addition to piperine, black pepper contains a complex mixture of essential oils and phytochemicals, including tannins, flavonoids, and terpenes, which contribute to its antimicrobial activity. These compounds have demonstrated effectiveness against both Gram-positive and Gram-negative bacteria, including *E. coli*, in vitro [9].

This study aims to evaluate the antibacterial activity of black pepper fruit extract (*Piper nigrum*) against *E. coli* using diffusion and dilution methods in vitro.

Specifically, the study seeks to assess the relationship between extract concentration and antibacterial efficacy, compare the activity of black pepper extract with that of gentamicin, a conventional antibiotic, and provide insights into the viability of black pepper as an alternative antibacterial agent. By addressing these objectives, this research hopes to contribute to the growing body of knowledge on plant-based antibacterials and their role in mitigating the global threat of antibiotic-resistant *E. coli*.

METHODS

The research employed a true experimental approach with a post-test-only control group design. Black pepper (*Piper nigrum*) fruit extracts were prepared using the maceration technique with 96% ethanol, followed by evaporation to obtain a concentrated extract. The study investigated antibacterial effects across different extract concentrations (100 ppm, 50 ppm, 25 ppm, 12.5 ppm, and 6.25 ppm). Mueller-Hinton Agar (MHA) served as the culture medium, and *Escherichia coli* cultures were standardized to 0.5 McFarland (1.5×10^8 CFU/mL). The antibacterial activity was evaluated using the diffusion method methods:

- Wells were prepared on MHA plates.
- Extracts at concentrations of 100 ppm, 50 ppm, 25 ppm, 12.5 ppm, and 6.25 ppm were introduced into separate wells, alongside controls: gentamicin (positive control) and DMSO (negative control).
- Plates were incubated at 37°C for 24 hours, and the diameters of inhibition zones were measured using calipers.

RESULT

In the diffusion method, the black pepper extract demonstrated measurable antibacterial activity, forming zones of inhibition around the wells containing the extract on Mueller-Hinton Agar (MHA) plates. The size of the inhibition zones was directly proportional to the concentration of the extract, indicating a dose-dependent response. At the highest concentration (100 ppm), the extract produced the largest inhibition zone, followed by progressively smaller zones at concentrations of 50 ppm, 25 ppm, 12.5 ppm, and none at 6.25 ppm. When compared to controls, gentamicin (positive control) produced significantly larger inhibition zones, confirming its superior efficacy as a standard antibiotic, whereas DMSO (negative control) produced almost no inhibition zones, confirming that the solvent used to prepare the extract did not interfere with bacterial growth. These results indicate that the observed effects were solely due to the active compounds in the black pepper extract.

TABLE 1: Diffusion Inhibition Zone.

Treatment Group	Replication no. 1(mm)	Replication no. 2(mm)	Replication no. 3(mm)	Average (mm)
100	6.33	6,33	6.73	6,46
50	5,82	6,08	6,18	6,03
25	5,68	5,67	5,87	5,74
12.5	5,64	5,67	5,67	5,66
6.25	0	0	0	0
Control +	20,36	20,44	19,94	20,25
Control -	5,21	5,11	5,20	5,17

The data obtained for the inhibition zone diameters from the diffusion method was subjected to statistical analysis carried out in SPSS to determine the significance of differences among treatment groups.

The first step was to assess the normality of the data distribution using the Shapiro-Wilk test. The Shapiro-Wilk test was selected for its robustness and sensitivity in detecting deviations from normality compared to other normality tests[11].

TABLE 2: Normality Test.

Tests of Normality						
Treatment Group	**Kolmogorov-Smirnov[a]**			**Shapiro-Wilk**		
	Statistic	**df**	**Sig.**	**Statistic**	**df**	**Sig.**
100 ppm	0.385	3		0.750	3	0.000
50 ppm	0.280	3		0.938	3	0.520
25 ppm	0.369	3		0.787	3	0.085
12.5 ppm	0.385	3		0.750	3	0.000
6.25 ppm	-	3	-	-	3	-
Control -	0.353	3		0.824	3	0.174
Control +	0.330	3		0.866	3	0.286

a. Lilliefors Significance Correction.

The results of the Shapiro-Wilk test are presented in Table 2. The significance (p-value) for each treatment group was evaluated to determine whether the data was normally distributed. For the 100 ppm and 12.5 ppm treatment groups, the p-values were less than 0.05, indicating that the data were not normally distributed. Similarly, the 6.25 ppm group showed deviations from normality. Conversely, the 50 ppm, 25 ppm, positive control (gentamicin), and negative control (DMSO) groups displayed p-values greater than 0.05, suggesting normal data distribution for these groups. Since normality was not consistent across all groups, a parametric test such as one-way ANOVA could not be applied [12].

Due to the lack of normal distribution in some groups, the non-parametric Kruskal-Wallis test was used to analyze the data. This test is suitable for comparing more than two independent groups when the assumption of normality is violated [13]. The Kruskal-Wallis test evaluates differences in median ranks among groups, providing a non-parametric alternative to one-way ANOVA. The statistical design was modified to exclude the positive control (gentamicin) and the 6.25 ppm treatment group from statistical analysis due to the inability to correlate their results with the other groups. The positive control consistently produced inhibition zones significantly larger than those observed for the black pepper extract, making direct comparisons unfeasible. Conversely, the 6.25 ppm treatment group showed no measurable inhibition zone, indicating a lack of antibacterial activity at this concentration. As a result, these groups were excluded to ensure the statistical analysis accurately reflected the dose-dependent antibacterial activity of the black pepper extract.

TABLE 3: Kruskal-Wallis Test.

Ranks			Test Statistics[a,b]	
Treatment group	**N**	**Mean Rank**		
100 ppm	3	14.00	Kruskal-Wallis H	13.050
50 ppm	3	10.67	df	4
25 ppm	3	8.00	Asymp. Sig.	0.011
12.5 ppm	3	5.33	a. Kruskal Wallis Test	
Kontrol negatif	3	2.00	b. Grouping Variable: Treatment gorup	
Total	15			

Hypothesis Test Summary

No	Null Hypothesis	Test	Sig.[a,b]	Decision
1	The distribution of variasi ukuran diameter is the same across categories kelompok perlakuan	Independent-Samples Kruskal-Wallis Test	0.011	Reject the null hypothesis.

a. The significance level is .050.

b. Asymptotic significance is displayed.

The test results revealed a significant difference among the treatment groups, with an Asymp. Sig. Value of 0.003. This value is less than the significance threshold of 0.05, indicating that the differences in mean ranks of inhibition zone diameters among the treatment groups were statistically significant. The mean rank scores for each treatment group highlighted a dose-dependent increase in antibacterial activity, with the 100ppm concentration yielding the highest mean rank and the negative control showing the lowest.

To identify specific group pairs with significant differences, a post-hoc Dunn-Bonferroni test was conducted. The results demonstrated that the mean ranks of the negative control group were significantly different from the 100ppm group ($p = 0.010$, adjusted for multiple comparisons). However, no significant differences were observed between several lower concentration groups (e.g., 12.5 ppm and 25 ppm) and between intermediate and higher concentrations (e.g., 50 ppm and 100 ppm), after applying the Bonferroni correction.

The only significance discovered was that only the 100-ppm group had a significant difference over the negative control. These findings highlight that the antibacterial effect was more pronounced at higher concentrations, with diminishing differences between successive higher doses.

Discussion

The findings of this study highlight the antibacterial potential of black pepper (*Piper nigrum*) extract against *Escherichia coli* using the diffusion method. A clear dose-dependent relationship was observed, with higher concentrations of the extract producing larger inhibition zones. This relationship underscores the increasing efficacy of black pepper extract as the concentration increases, though statistical analysis revealed nuanced differences across the treatment groups. While black pepper extract demonstrated measurable antibacterial activity, its efficacy was significantly lower than that of gentamicin. This discrepancy highlights the limitations of black pepper extract as a standalone antibacterial agent.

Antibacterial Activity and Dose Dependency

The results demonstrated that the antibacterial effect of black pepper extract is concentration-dependent. The 100 ppm concentration exhibited the largest inhibition zone, with progressively smaller zones observed at 50 ppm, 25 ppm, and 12.5 ppm. Notably, no antibacterial activity was detected at 6.25 ppm, suggesting a threshold concentration below which the extract is ineffective. The positive control (gentamicin) produced significantly larger inhibition zones compared to all concentrations of the black pepper extract, confirming its superior efficacy as a standard antibiotic. In contrast, the negative control (DMSO) produced no measurable inhibition zones, verifying that the antibacterial activity observed was due to the active compounds in the black pepper extract rather than the solvent.

Statistical Analysis

The Shapiro-Wilk normality test revealed that not all data sets were normally distributed, particularly for the 100 ppm, 12.5 ppm, and 6.25 ppm groups. Consequently, a non-parametric Kruskal-Wallis test was employed to evaluate the differences in antibacterial activity across the treatment groups. The test results showed a significant difference ($p = 0.003$) in inhibition zone diameters among the groups, validating the dose-dependent effect of the extract.

Post-hoc analysis using the Dunn-Bonferroni test provided deeper insights. While the 100 ppm group showed significant differences in mean ranks compared to the negative control ($p = 0.010$), no significant differences were observed between intermediate concentrations (e.g., 50 ppm vs. 100 ppm or 12.5 ppm vs. 25 ppm) after Bonferroni adjustment. This suggests that while higher concentrations generally improve antibacterial activity, the differences become less pronounced at successive higher doses, potentially due to a saturation effect in the antibacterial mechanism.

Mechanism of Action

The observed antibacterial activity of black pepper extract can be attributed to its bioactive compounds, such as piperine, tannins, flavonoids, and terpenes[9]. Essential oils, particularly terpenoids and other bioactive compounds, have been shown to exhibit antibacterial properties. Previous studies, such as those by Zhang et al[14]. indicate that the essential oil content in black pepper fruits ranges from 1.24% to 5.06%, influenced by factors like variety, cultivation region, extraction method, and fruit maturity. The relatively low yield of essential oils in crude black pepper extract necessitates higher concentrations to achieve significant antibacterial effects, which may explain the limited efficacy observed in this study. According to this study, black pepper essential oil disrupts the cell membrane's permeability, causing physical and morphological alterations in the bacterial cell wall and membrane. This disruption leads to the leakage of essential intracellular components, including electrolytes, ATP, proteins, and DNA materials.

These changes result in cellular disorder, decomposition, and eventual bacterial death, corresponding to a reduction in viable *E. coli* cells. The heterogeneous composition of essential oils suggests that these effects are likely due to the synergistic actions of multiple compounds, rather than a single component [14].

Piperine, in particular, is known to disrupt bacterial cell membranes, inhibit protein synthesis, and impair bacterial viability[9] These mechanisms likely underpin the dose-dependent antibacterial effect observed in this study. However, the diminishing differences between higher doses suggest that the active compounds reach a saturation point in their interaction with bacterial cells, limiting additional inhibitory effects.

CONCLUSION

The study demonstrates that black pepper extract exhibits a significant dose-dependent antibacterial effect against *E. coli* in vitro, though its efficacy remains inferior to that of gentamicin. These findings highlight the potential of black pepper as a natural antibacterial agent, warranting further investigation to fully explore its clinical applications and role in combating antibiotic-resistant bacterial infections.

REFERENCES

[1] Estrada-Garcia, T & Tarr, P., "*Escherichia coli* Infection," *Foodborne Infections and Intoxications*, July 13, 2023, 125–63, https://doi.org/10.1016/B978-0-12-819519-2.00018-9.

[2] Fleckenstein, J & Kuhlmann, F., "Enterotoxigenic *Escherichia coli* Infections," *Current Infectious Disease Reports* 21, no. 3 (March 1, 2019), https://doi.org/10.1007/s11908-019-0665-x.

[3] Walsh, T et al., "Antimicrobial Resistance: Addressing a Global Threat to Humanity," *PLOS Medicine* 20, no. 7 (July 3, 2023): e1004264, https://doi.org/10.1371/journal.pmed.1004264.

[4] Ejaz, H et al., "The Rising Tide of Antibiotic Resistance: A Study on Extended-Spectrum Beta-Lactamase and Carbapenem-Resistant *Escherichia coli* and Klebsiella Pneumoniae," *Journal of Clinical Laboratory Analysis* 38, no. 10 (June 17, 2024): e25081, https://doi.org/10.1002/jcla.25081.

[5] Huang, J et al., "Carbapenem-Resistant *Escherichia coli* Exhibit Diverse Spatiotemporal Epidemiological Characteristics across the Globe," *Communications Biology* 7, no. 1 (January 6, 2024): 1–13, https://doi.org/10.1038/s42003-023-05745-7.

[6] Mantravadi, P et al., "The Quest for Novel Antimicrobial Compounds: Emerging Trends in Research, Development, and Technologies," *Antibiotics* 8, no. 1 (January 24, 2019): 8, https://doi.org/10.3390/antibiotics8010008.

[7] Schmitz,M & Havelt, T., "Siginficance and Applicability of Bioactive Compounds from Plants for Innovative Technologies and Processes," *Processes* 12, no. 11 (November 2024): 2539, https://doi.org/10.3390/pr12112539.

[8] Takooree, H. et al., "A Systematic Review on Black Pepper (*Piper nigrum* L.): From Folk Uses to Pharmacological Applications," *Critical Reviews in Food Science and Nutrition* 59, no. sup1 (2019): S210–43, https://doi.org/10.1080/10408398.2019.1565489.

[9] Ashokkumar, K. et al., "Phytochemistry and Therapeutic Potential of Black Pepper [*Piper nigrum* (L.)] Essential Oil and Piperine: A Review," *Clinical Phytoscience* 7, no. 1 (June 9, 2021): 52, https://doi.org/10.1186/s40816-021-00292-2.

[10] Angelini, P., "Plant-Derived Antimicrobials and Their Crucial Role in Combating Antimicrobial Resistance," *Antibiotics* 13, no. 8 (August 2024): 746, https://doi.org/10.3390/antibiotics13080746.

[11] Razali, N. and Wah, Y. *Power Comparisons of Shapiro-Wilk, Kolmogorov-Smirnov, Lilliefors and Anderson-Darling Tests*, *Journal of Statistical Modeling and Analytics*, vol. 2, 2011.

[12] Kwak, S. & Park, S. "Normality Test in Clinical Research," *Journal of Rheumatic Diseases* 26, no. 1 (January 1, 2019): 5–11, https://doi.org/10.4078/jrd.2019.26.1.5.

[13] McKight, P. and Najab J., "Kruskal-Wallis Test," in *The Corsini Encyclopedia of Psychology* (John Wiley & Sons, Ltd, 2010), 1–1, https://doi.org/10.1002/9780470479216.corpsy0491.

[14] Zhang, J. et al., "Antibacterial Activity and Mechanism of Action of Black Pepper Essential Oil on Meat-Borne Escherichia Coli," *Frontiers in Microbiology* 7 (January 4, 2017), https://doi.org/10.3389/fmicb.2016.02094.