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Antibacterial efficacy of clove leaf ethanol extract *(Syzygium aromaticum L.)* against *Salmonella enterica* serovar typhi using chloramphenicol as a comparative standard

Efektivitas antibakteri ekstrak etanol daun cengkih (Syzygium aromaticum L.) terhadap Salmonella enterica serovar Typhi dengan menggunakan kloramfenikol sebagai standar pembanding

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ABSTRACT
Bacterial infectious diseases such as typhoid fever caused by Salmonella enterica serovar Typhi remain a serious public health challenge due to rising antibiotic resistance. Utilizing medicinal plants like clove leaves (Syzygium aromaticum L.) represents a promising alternative strategy. This laboratory experimental study evaluated the antibacterial efficacy of 70% ethanol extract of clove leaves obtained via maceration against active efflux pump-resistant S. typhi isolates. Phytochemical profiling via Thin Layer Chromatography (TLC) confirmed active constituents. Antibacterial susceptibility testing was performed using the disk diffusion method across concentrations of 5%, 10%, 15%, and 20%, compared against chloramphenicol as positive control and distilled water as negative control. The mean inhibition zone diameters were 6.59 ± 0.75 mm (5%), 11.21 ± 0.61 mm (10%), 11.06 ± 0.28 mm (15%), and 15.46 ± 1.22 mm (20%), while chloramphenicol exhibited 19.74 ± 0.88 mm. One-Way ANOVA established significant differences among groups ($p = 0.000$, $p < 0.05$). Based on Davis and Stout criteria, the 5% concentration showed moderate inhibition, whereas 10%, 15%, and 20% demonstrated strong inhibitory activity. Clove leaf ethanol extract is proven effective as a concentration-dependent antibacterial agent against S. typhi.
Keywords: *Antibacterial, chloramphenicol, disk diffusion, Salmonella typhi, Syzygium aromaticum L.*

ABSTRAK
Penyakit infeksi bakteri seperti demam tifoid yang disebabkan oleh Salmonella enterica serovar Typhi tetap menjadi tantangan kesehatan masyarakat yang serius karena meningkatnya resistensi antibiotik. Pemanfaatan tanaman obat seperti daun cengkeh (Syzygium aromaticum L.) merupakan strategi alternatif yang menjanjikan. Studi eksperimental laboratorium ini mengevaluasi kemanjuran antibakteri ekstrak etanol 70% dari daun cengkeh yang diperoleh melalui maserasi terhadap isolat S. typhi yang resisten terhadap pompa efluks aktif. Profiling fitokimia melalui Kromatografi Lapis Tipis (TLC) mengkonfirmasi konstituen aktif. Pengujian sensitivitas antibakteri dilakukan menggunakan metode difusi cakram pada konsentrasi 5%, 10%, 15%, dan 20%, dibandingkan dengan kloramfenikol sebagai

kontrol positif dan air suling sebagai kontrol negatif. Diameter zona inhibisi rata-rata adalah 6,59 ± 0,75 mm (5%), 11,21 ± 0,61 mm (10%), 11,06 ± 0,28 mm (15%), dan 15,46 ± 1,22 mm (20%), sedangkan kloramfenikol menunjukkan 19,74 ± 0,88 mm. ANOVA satu arah menunjukkan perbedaan signifikan antar kelompok (p = 0,000, p < 0,05). Berdasarkan kriteria Davis dan Stout, konsentrasi 5% menunjukkan inhibisi sedang, sedangkan 10%, 15%, dan 20% menunjukkan aktivitas inhibisi yang kuat. Ekstrak etanol daun cengkeh terbukti efektif sebagai agen antibakteri yang bergantung pada konsentrasi terhadap S. typhi.
Kata kunci: *Antibakteri, kloramfenikol, difusi cakram, Salmonella typhi, Syzygium aromaticum L.*

1. INTRODUCTION

Infectious diseases continue to represent a major global public health concern. The burden is exacerbated by the alarming disparity between the slow pace of novel antibiotic development and the rapid rate at which pathogenic bacteria acquire resistance [1]. Typhoid fever, a systemic infection caused by the Gram-negative bacterium *Salmonella enterica serovar Typhi*, remains endemic in many developing regions, causing substantial morbidity and mortality [2,3]. *Salmonella* Typhi is a motile, Gram-negative, facultatively anaerobic enteric bacillus transmitted through the fecal–oral route [4], and its virulence depends in part on adhesion proteins that mediate attachment to host cells [5]. In Indonesia, typhoid fever remains a notable public health problem according to national basic health research data [6], and accurate diagnosis, particularly in children, continues to be challenging [7].

The management of typhoid fever relies heavily on conventional antimicrobial therapy, such as chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole [8]. Chloramphenicol acts by binding the bacterial 50S ribosomal subunit and inhibiting protein synthesis [8,9]. However, the widespread emergence of multi-drug resistant (MDR) and active efflux pump-resistant strains of S. typhi has rendered standard treatments increasingly ineffective [10–12]. Active efflux pumps pump therapeutic agents out of the bacterial cell, thereby reducing intracellular drug accumulation and leading to treatment failures.

To combat multidrug resistance, research has increasingly focused on medicinal plants as rich sources of bioactive phytochemicals with potent antimicrobial mechanisms [13,14]. Clove (*Syzygium aromaticum L., family Myrtaceae*) is an aromatic plant widely cultivated in Indonesia and recognized for its medicinal properties [15]. Clove leaves contain significant amounts of essential oil rich in eugenol, eugenyl acetate, and beta caryophyllene, which exhibit strong antibacterial, antioxidant, and anti-inflammatory activities [16,17].

Eugenol acts primarily by disrupting bacterial cell wall integrity and increasing cytoplasmic membrane permeability, leading to the leakage of intracellular constituents and bacterial cell death [16]. Clove leaf extract has previously been reported to inhibit *S. typhi* in vitro [18], and other plant extracts, such as the methanol fraction of red betel (*Piper crocatum*) leaves [19] and tapak liman (*Elephantopus scaber*) leaf extract [20,21], have also shown activity against *S. typhi*. Although several studies have established the antimicrobial activity of clove essential oils, empirical evidence regarding the antibacterial efficacy of crude ethanol extracts derived specifically from

clove leaves against clinical strains of *S. typhi* remains to be fully synthesized across structured concentration gradients.

This study aimed to evaluate the antibacterial efficacy of clove leaf ethanol extract against *S. typhi* using the disk diffusion method across concentrations of 5%, 10%, 15%, and 20%, comparing its performance to chloramphenicol as a standard reference antibiotic. Furthermore, the study incorporated rigorous statistical testing and classification under the Davis and Stout standards to establish its prospective therapeutic viability.

2. METHOD

a) Research Location and Plant Material Collection

This laboratory-scale experimental research was conducted from February to March 2023 at the Pharmaceutical Chemistry Laboratory, STIKES Bhakti Pertiwi Luwu Raya Palopo, South Sulawesi, Indonesia. Healthy, pest-free young clove leaves (S*yzygium aromaticum L.*) were harvested between 09:00 and 09:30 WITA from Uraso Village, North Bassesang Tempe District, Luwu Regency, South Sulawesi. The harvested leaves were thoroughly washed with running water, shade-dried, and powdered. Materials and reagents included 70% ethanol, ethyl acetate, acetone, Dimethyl Sulfoxide (DMSO), distilled water, sterile 6.28 mm Whatman paper disks, chloramphenicol disk (30 µg), Mueller-Hinton Agar (MHA) media, 0.5 McFarland turbidity standard, and active efflux pump-resistant S. typhi bacterial specimens from laboratory collections.

b) Extraction and Thin-Layer Chromatography (TLC)

Clove leaf powder was extracted by cold maceration using 70% ethanol at a 1:10 sample-to-solvent ratio for 3 x 24 hours with periodic agitation [22,23]. The filtrate was collected and concentrated using a rotary evaporator at 40°C followed by water bath evaporation until a dense crude extract was achieved [24]. Extract concentrations of 5%, 10%, 15%, and 20% (w/v) were prepared using DMSO as a solvent.

Phytochemical confirmation was conducted via Thin-Layer Chromatography (TLC) using silica gel 60 F254 plates. The eluent system consisted of ethyl acetate and ethanol [23,25]. Chromatograms were visualized under UV light at 254 nm and 366 nm to evaluate spot presence and profile across tested concentrations.

c) Bacterial Identification and Disk Diffusion Assay

Salmonella typhi isolate identity was confirmed microscopically using Gram staining (gentian violet, Lugol's iodine, 96% alcohol, and safranin) [4]. Confirmed S. typhi colonies were inoculated onto Mueller-Hinton Agar (MHA) plates and incubated at 37°C for 24 hours. Bacterial suspensions were adjusted to a 0.5 McFarland standard (equivalent to approximately 1.5 x 10^8 CFU/mL) [26].

Antimicrobial testing was performed using Kirby-Bauer disk diffusion method [2,26]. Sterile paper disks (6.28 mm) were impregnated with 1 mL of clove leaf extract at 5%, 10%, 15%, and 20% concentrations, chloramphenicol (positive control), and distilled water (negative control) for 15 minutes. MHA plates inoculated uniformly with

S. typhi suspension were fitted with the impregnated disks and incubated at 37°C for 18–24 hours. Clear growth inhibition zone diameters were measured in millimeters (mm) using a digital vernier caliper across four independent replications.

d) Statistical Analysis and Activity Classification

Data normality was tested using the Shapiro-Wilk test, and variance homogeneity was evaluated using Levene's test. Hypothesis testing for significant differences across treatment groups was conducted via One-Way Analysis of Variance (ANOVA) at a 95% confidence level (alpha = 0.05). Inhibitory power was categorized based on Davis and Stout criteria [27]: <= 5 mm (Weak), 5–10 mm (Moderate), 10–20 mm (Strong), and > 20 mm (Very Strong).

3. RESULTS AND DISCUSSION

a) Thin-Layer Chromatography (TLC) Profiling

Phytochemical screening via TLC verified the presence of active chemical constituents in the extract across all concentrations. As detailed in Table 1, increasing extract concentrations produced enhanced spot visibility and abundance on the stationary phase.

Table 1. TLC Spot Visualization of Clove Leaf Extract Across Concentrations

Concentration	**5%**	**10%**	**15%**	**20%**
Spot Visibility	Spot Present	Moderate Spot	Clear Spot	Abundant Spots

Source data: Primer 2023

b) Antibacterial Efficacy and Inhibition Zone Diameter Measurements

Primary antimicrobial susceptibility testing against Salmonella typhi demonstrated clear concentration-dependent inhibition zones. Table 2 presents the individual replication measurements, overall mean values, standard deviations, and corresponding Davis and Stout classifications.

Table 2. Growth Inhibition Zone Diameters of S. typhi Across Experimental Groups

Treatment Group	**R1 (mm)**	**R2 (mm)**	**R3 (mm)**	**R4 (mm)**	**Mean ± SD (mm)**	**Peak (mm)**	**Davis & Stout Category**
Extract 5%	6.87	5.50	6.77	7.20	6.59 ± 0.75	7.20	Moderate
Extract 10%	11.89	10.55	10.86	11.52	11.21 ± 0.60	11.89	Strong
Extract 15%	11.20	10.84	10.80	11.40	11.06 ± 0.30	11.40	Strong
Extract 20%	16.76	16.01	13.89	15.18	15.46 ± 1.22	16.76	Strong

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Chloramphenicol (+)	20.67	19.30	20.25	18.72	19.74 ± 0.89	20.67	Strong/ Very Strong
Aquadest (-)	0.00	0.00	0.00	0.00	0.00 ± 0.00	0.00	No Activity

Source data: Primer 2023

c) Statistical Testing (Normality, Homogeneity, and ANOVA)

To validate the parametric assumptions for Analysis of Variance, statistical testing was conducted on the inhibition zone data. As shown in Table 3, all experimental groups satisfied the assumption of normal distribution under the Shapiro-Wilk test ($p > 0.05$). Furthermore, Levene's test confirmed variance homogeneity across treatment groups ($p = 0.08$, $P > 0.05$).

Table 3. Shapiro-Wilk Normality Test and Homogeneity Test Results

Group	Shapiro-Wilk Normality Test	Homogeneity Test
Clove leaf extract 20%	0.914	
Clove leaf extract 15%	0.339	
Clove leaf extract 10%	0.726	0.08
Clove leaf extract 5%	0.753	
Chloramphenicol	0.707	

Source data: Primer 2023

Subsequent hypothesis testing via One-Way ANOVA established a highly significant difference among treatment groups (F-statistic, $p = 0.000$, $P < 0.05$). This confirms that clove leaf ethanol extract exhibits a concentration-dependent antibacterial efficacy against Salmonella typhi, rejecting the null hypothesis.

Table 4. One-Way Analysis of Variance (ANOVA) Results

Group	N	Mean ± Standard Deviation (mm)	P-value
Chloramphenicol	4	19.75 ± pm 1.01	
Aquadest	4	0.00 ± pm 0.00	
Clove Leaf Extract 20%	4	15.46 ± pm 1.22	
Clove Leaf Extract 15%	4	11.06 ± pm 0.28	0.000
Clove Leaf Extract 10%	4	11.20 ± pm 0.61	
Clove Leaf Extract 5%	4	8.69 ± pm 0.52	

Source data: Primer 2023

B. Discussion

TLC profiling confirmed that the active components within the clove leaf extract were effectively mobilized by the polar ethanol-ethyl acetate system, resulting in the most abundant and distinct spot detection at the 20% concentration [13,23]. Disk diffusion assays revealed that clear zones expanded progressively with increasing extract concentrations. This concentration-dependent pattern is consistent with the greater amount of secondary metabolites present in more concentrated extracts, as also reported for other plant extract bioassays [22,28]. The peak clear zones recorded per group reached 6.87 mm at 5%, 11.89 mm at 10%, 11.40 mm at 15%, and 16.76 mm at 20% concentrations. The positive control yielded the maximum inhibition zone at 20.67 mm, while the negative control remained completely inactive.

Statistical analysis via One-Way ANOVA established a significant difference among the experimental groups ($p = 0.000$, $P < 0.05$), proving that the extract possesses concentration-dependent antibacterial properties. The highest mean efficacy among the herbal treatments belonged to the 20% clove leaf extract (15.46 ± 1.22 mm). These findings demonstrate that all evaluated concentrations of clove leaf extract fall within the strong inhibitory classification based on the criteria established by Davis and Stout [27].

The verified bioactivity is strongly supported by related literature detailing the antimicrobial properties of Syzygium aromaticum. Clove extracts rich in eugenol display potent antibacterial action against Gram-positive pathogens such as Streptococcus mutans, where higher concentrations (up to 80%) show up to 32.3 mm inhibition zones, as well as antifungal actions against Candida albicans [29]. Similarly, Paliling et al. [30] noted strong inhibitory effects (13.01 mm) against Porphyromonas gingivalis. With respect to Gram-negative targets, Kumala and Indriani [31] verified that clove leaf extract achieves strong zones of 17 mm against Escherichia coli and 19 mm against Salmonella paratyphi at a 20% concentration, closely matching the results observed here against Salmonella typhi. A previous in vitro study likewise reported that clove leaf extract inhibits S. typhi [18], and other plant extracts such as red betel [19] and tapak liman [20,21] have also been reported to inhibit this organism, supporting the potential of plant-derived extracts as candidate antityphoid agents.

Mechanistically, the strong bioactivity is driven by the structural damage eugenol inflicts upon the cytoplasmic membranes and macromolecules of the bacteria [16]. The experimental hypothesis is therefore accepted, showing that elevated extract concentrations and sustained contact durations significantly restrict Salmonella typhi growth.

However, despite its strong performance, the extract remains less effective than the purified positive control, chloramphenicol (19.75 ± 1.01 mm). This difference in efficacy is expected because chloramphenicol is a highly optimized, pure single-molecule antibiotic with a specific mechanism of action [8,9]. In contrast, the clove leaf formulation is a crude extract containing a complex mixture of active and non-active secondary metabolites, which inherently dilutes the concentration of the primary antibacterial agents like eugenol. Nevertheless, utilizing such crude extracts offers a distinct advantage: the diverse phytochemical matrix can induce synergistic antibacterial effects and substantially lower the risk of triggering bacterial resistance—a significant drawback commonly associated with single-target synthetic antibiotics. Moreover, certain plant-derived compounds have been reported to inhibit bacterial efflux pumps [14], which is relevant to the efflux pump-resistant isolate tested here. To fully harness this botanical potential, future research should focus on bioassay-guided fractionation

to isolate, purify, and characterize the specific bioactive compounds responsible for this inhibition, thereby paving the way for targeted and optimized therapeutic applications.

4. CONCLUSION

In conclusion, the ethanol extract of clove leaves (Syzygium aromaticum L.) demonstrates significant, concentration-dependent antibacterial efficacy against Salmonella typhi across all evaluated concentrations (5%, 10%, 15%, and 20%), fully validating the experimental hypothesis. The diameter of the growth inhibition zones expanded proportionally with increased extract concentrations, culminating in the highest mean inhibition zone of 15.46 ± 1.22 mm (with a peak replicate of 16.76 mm) at the 20% concentration. According to the Davis and Stout classification, this denotes a strong inhibitory effect. Nevertheless, this crude herbal extract cannot currently substitute for standard synthetic antibiotics, as its antibacterial performance remains statistically lower than that of the purified positive control, chloramphenicol (19.75 ± 1.01 mm).

Recommendations To build upon these findings, this study should serve as a foundational pharmacological reference for advancing clove-based therapeutic formulations. Future research should prioritize in vivo and clinical evaluations utilizing expanded concentration thresholds and prolonged exposure periods, as well as broader screening against other microbial categories, including fungal and viral pathogens. Furthermore, isolating and purifying the active compounds from the crude extract is highly recommended to optimize its efficacy. Ultimately, these findings provide a valuable scientific framework for academic institutions and the wider community seeking to develop alternative, plant-based interventions for typhoid fever management.

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