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Minimum Inhibitory Concentration of Ethanol Extract of *Vernonia amygdalina* Delile. against *Escherichia coli, Salmonella typhi, Staphylococcus aureus,* and *Bacillus cereus* by Disc Diffusion

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Abstract

Diarrhea remains a major global health challenge caused by pathogenic bacteria including *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, and *Bacillus cereus*. African leaf (*Vernonia amygdalina* Delile.) contains bioactive compounds such as flavonoids, saponins, tannins, and alkaloids with recognized antibacterial properties. This study determined the Minimum Inhibitory Concentration (MIC) of ethanol extract of *V. amygdalina* prepared by Ultrasonic-Assisted Extraction (UAE) at three extraction times (5, 15, and 30 minutes) against four diarrhea-causing bacteria using the disc diffusion method. Extraction yields were 8.8%, 10.45%, and 11.9% for UAE at 5, 15, and 30 minutes, respectively. Antibacterial testing at concentrations ranging from 300 to 1.56 mg/mL demonstrated predominantly moderate inhibition zones (5–10 mm), with some concentrations reaching the strong category (10–20 mm). The MIC for *B. cereus* and *S. aureus* was 1.56 mg/mL across all extraction times. For *S. typhi*, the MIC was 1.56 mg/mL (5 and 30 min UAE) and 12.5 mg/mL (15 min UAE). For *E. coli*, the MIC was 6.25 mg/mL (5 min UAE) and 1.56 mg/mL (15 and 30 min UAE). One-way ANOVA confirmed statistically significant differences among concentrations ($p < 0.05$). The 30-minute UAE produced the highest yield and generally superior antibacterial activity, supporting its selection as the optimal extraction condition. These findings highlight the potential of *V. amygdalina* ethanol extract as a natural antibacterial agent for diarrheal disease management.

Keywords: Antibacterial; disc diffusion; diarrhea; MIC; *Vernonia amygdalina*; Ultrasonic-Assisted Extraction.

Abstrak

Diare merupakan masalah kesehatan global yang disebabkan oleh bakteri patogen seperti Escherichia coli, Salmonella typhi, Staphylococcus aureus, dan Bacillus cereus. Daun Afrika (Vernonia amygdalina Delile.) mengandung senyawa bioaktif seperti flavonoid, saponin, tanin, dan alkaloid yang memiliki aktivitas antibakteri. Penelitian ini bertujuan menentukan Kadar Hambat Minimum (KHM/MIC) ekstrak etanol V. amygdalina yang diperoleh dengan metode Ultrasonic-Assisted Extraction (UAE) pada variasi waktu 5, 15, dan 30 menit terhadap empat bakteri penyebab diare menggunakan metode difusi cakram. Rendemen ekstraksi masing-masing adalah 8,8%, 10,45%, dan 11,9%. Uji antibakteri pada konsentrasi 300–1,56 mg/mL menunjukkan zona hambat dominan dalam kategori sedang (5–10 mm) dan beberapa dalam kategori kuat (10–20 mm). MIC untuk B. cereus dan S. aureus adalah 1,56 mg/mL pada seluruh variasi waktu ekstraksi. Untuk S. typhi, MIC sebesar 1,56 mg/mL (UAE 5 dan 30 menit) serta 12,5 mg/mL (UAE 15 menit). Untuk E. coli, MIC sebesar 6,25 mg/mL (UAE 5 menit) dan 1,56 mg/mL (UAE 15 dan 30 menit). Analisis ANOVA satu arah menunjukkan perbedaan bermakna antar konsentrasi ($p < 0,05$). Ekstraksi UAE selama 30 menit menghasilkan rendemen tertinggi dan aktivitas antibakteri paling optimal. Hasil ini menunjukkan potensi ekstrak etanol V. amygdalina sebagai agen antibakteri alami dalam pengelolaan diare.

Kata kunci: *Antibakteri; difusi cakram; diare; MIC; Vernonia amygdalina; ekstraksi ultrasonik.*

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INTRODUCTION

Diarrhea is a major global health problem characterized by passage of loose or liquid stools more than three times per day. The World Health Organization (WHO) estimates that diarrheal diseases cause approximately 1.7 billion cases and 525,000 child deaths annually worldwide.[1] In Indonesia, diarrhea ranks as the second leading cause of morbidity and mortality among children under five, with a national prevalence of 30.1% in individuals aged above 15 years.[2] The principal etiological agents of infectious diarrhea include Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhi*, as well as Gram-positive organisms including *Staphylococcus aureus* and *Bacillus cereus*, all of which produce virulence factors and toxins that disrupt gastrointestinal homeostasis.[3,4]

The escalating prevalence of antibiotic resistance among enteric pathogens has critically diminished the therapeutic efficacy of conventional antimicrobials for diarrheal diseases.[5] Multidrug resistance in *E. coli*, *Salmonella* spp., and *S. aureus* limits treatment

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options and drives the urgent search for novel, plant-derived antibacterial alternatives.[6]

Indonesia harbors one of the world's richest repositories of medicinal plants with demonstrated pharmacological potential.[7] Among these, African leaf (*Vernonia amygdalina* Delile.) — belonging to the family Asteraceae — has been traditionally employed for gastrointestinal ailments, malaria, diabetes, and hypertension across Africa and tropical Asia.[8] The plant is commonly cultivated throughout Tulungagung and other Indonesian provinces, making it accessible as a local herbal resource.[9]

Vernonia amygdalina Delile. is rich in bioactive secondary metabolites including flavonoids, saponins, tannins, alkaloids, terpenoids, and steroid glycosides (vernoniosides).[10] These compounds exert antibacterial activity through multiple mechanisms: flavonoids disrupt bacterial membrane integrity and inhibit nucleic acid synthesis[11]; tannins precipitate bacterial proteins and inactivate extracellular enzymes[12]; saponins induce membrane pore formation and cytoplasmic leakage[13]; and alkaloids interfere with peptidoglycan biosynthesis and DNA replication.[14] Pratiwi and Gunawan (2018) previously reported inhibition zones of 6.69 mm (*S. aureus*) and 6.52 mm (*E. coli*) for ethanol extract of *V. amygdalina* from Papua, Indonesia.[15]

Ultrasonic-Assisted Extraction (UAE) is a non-conventional extraction technique that employs high-frequency ultrasonic waves to create acoustic cavitation, disrupting plant cell walls and significantly enhancing mass transfer and extraction yield compared to conventional maceration.[16] UAE offers advantages of reduced extraction time, lower solvent consumption, and higher yields, making it especially suitable for pharmaceutical compound isolation.[17] The use of ethanol 96% enables selective co-extraction of polar phenolics and moderately polar alkaloids and terpenoids, maximizing the phytochemical profile.[18]

The Minimum Inhibitory Concentration (MIC) is a fundamental parameter in antibacterial research, defined as the lowest concentration of an antimicrobial agent that prevents visible bacterial growth.[19] Determination of MIC by the disc diffusion method, as standardized by CLSI guidelines, is widely applied for its simplicity and reproducibility.[20] Nasri and Zuriani (2014) emphasized the critical importance of rigorous MIC determination in establishing the therapeutic potential of Indonesian medicinal plant-based antibacterial agents.[21] Kaban (2022) further documented the superior antibacterial performance of Indonesian medicinal plants extracted by UAE methods, underscoring the value of modern extraction in maximizing bioactive compound recovery.[22]

This study therefore aimed to determine the MIC of ethanol extract of *V. amygdalina* Delile. prepared by UAE at three extraction time variations (5, 15, and 30 minutes) against *E. coli*, *S. typhi*, *S. aureus*, and *B. cereus* using the disc diffusion method, and to identify the optimal UAE extraction time for maximum antibacterial efficacy.

METHODOLOGY

Materials and Equipment

Fresh African leaf (*Vernonia amygdalina* Delile.) was collected from Tulungagung Regency, East Java, Indonesia, in May 2023. Bacterial test strains — *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhi*, and *Escherichia coli* — were obtained from the stock culture collection of the Microbiology Laboratory, Department of Pharmacy, STIKes Karya Putra Bangsa Tulungagung, Indonesia. Materials used

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in this study included ethanol 96% (pro analysis grade), dimethyl sulfoxide (DMSO) as negative control, chloramphenicol as positive control, Mueller Hinton Broth (MHB), Nutrient Agar (NA), sterile paper discs (6 mm diameter), and standard microbiological reagents. The equipment consisted of an ultrasonic probe extractor, drying oven (105°C), autoclave, incubator (37°C), analytical balance (Ohaus), microscope, digital vernier caliper, colony counter, and standard laboratory glassware.

Preparation of Simplicia

A total of 10 kg of fresh *V. amygdalina* leaves were collected, washed under running water, drained, and dried in a drying cabinet at 40°C until constant weight. The dried material was ground using a blender to obtain simplicia powder. Macroscopic and microscopic examinations, as well as quantitative parameters — water content (azeotropic/toluene distillation), water-soluble extract content, ethanol-soluble extract content, total ash content, and acid-insoluble ash content — were determined following Depkes RI (1995) standard methods.[23]

Preparation of Ethanol Extract by UAE Method

Three batches of simplicia powder (20 g each) were subjected to UAE with ethanol 96% as solvent using an ultrasonic probe extractor at extraction time variations of 5 minutes, 15 minutes, and 30 minutes. After extraction, each batch was filtered through filter paper to obtain the filtrate. The filtrate was evaporated in an oven at 105°C until a thick concentrated extract was obtained. Extraction yield was calculated as: Yield (%) = (Weight of concentrated extract / Weight of simplicia) × 100%.[16,17]

Preparation of Test Concentrations

For each UAE extract, the concentrated extract was dissolved in DMSO to prepare the stock concentration of 300 mg/mL. Serial dilutions in DMSO produced working concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.56 mg/mL. DMSO served as the negative control and chloramphenicol as the positive control. A total of 84 sterilized petri dishes and 108 sterile paper discs (6 mm) were prepared for the complete experimental design.[24]

Sterilization and Media Preparation

All glassware, petri dishes, and paper discs were sterilized by autoclave at 120°C for 15 minutes. Mueller Hinton Broth (MHB, 20 g/L) was prepared in sterilized aquadest, heated to clarity, plugged with cotton and aluminum foil, and autoclaved at 121°C for 15 minutes. Nutrient Agar (NA) was similarly prepared and poured in 15 mL aliquots into sterile petri dishes prior to solidification.

Disc Diffusion Assay for MIC Determination

Fresh 18–24 hour cultures of each test bacterium were prepared in MHB at 37°C and adjusted to McFarland 0.5 standard turbidity (≈108 CFU/mL).[24,25] A volume of 0.1 mL bacterial suspension was pipetted into sterile petri dishes, followed by 15 mL of melted NA (40–50°C), homogenized by figure-of-eight swirling, and solidified. Sterile 6 mm paper discs, each impregnated with 25 µL of test solution, were placed on the agar surface and incubated at 37°C for 24 hours. Inhibition zone diameters were measured with a digital vernier caliper. All tests were performed in triplicate (n=3). The MIC was defined as the lowest concentration producing a measurable inhibition zone (>6.0 mm, i.e., beyond the disc diameter) in all three replicates, following the disc diffusion criteria described by Davis and Stout (1971)[26]: very strong (>20 mm),

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strong (10–20 mm), moderate (5–10 mm), and no activity (≤6.0 mm).

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). One-way ANOVA was applied to evaluate significant differences in inhibition zone diameters among concentrations for each bacterium at each extraction time. Post-hoc comparisons were performed using Tukey HSD test. Statistical significance was set at $p < 0.05$. Analyses were performed using SPSS version 22.0.[27]

RESULT AND DISCUSSION

From 10 kg of fresh *Vernonia amygdalina* Delile. leaves, 4 kg (40%) of dry simplicia powder was obtained. UAE of 20 g simplicia powder yielded concentrated extract of 8.8% (5-minute UAE), 10.45% (15-minute UAE), and 11.9% (30-minute UAE). The progressive increase in extraction yield with sonication time reflects the efficiency of acoustic cavitation in disrupting plant cell walls and enhancing mass transfer of bioactive compounds into the ethanol solvent.[16,17] Despite a lower yield at 5 minutes, the extract retained sufficient bioactive content to demonstrate antibacterial activity, consistent with reports that UAE rapidly releases surface-bound and easily accessible phytochemicals even at short extraction durations.[31]

The antibacterial activity of the ethanol extract of *V. amygdalina* Delile. against all four diarrhea-causing bacteria at three UAE extraction times is presented in Tables 1–4. In all experiments, the negative control (DMSO) produced no inhibition zone, confirming that the antibacterial effects observed were attributable exclusively to the plant extract. The positive control (chloramphenicol) consistently produced very strong inhibition zones (>20 mm) against all four bacteria, confirming assay validity and the susceptibility of all test strains under the experimental conditions employed.

Table 1. Inhibition zone diameters (mm) of ethanol extract of *V. amygdalina* Delile. against *Bacillus cereus* at three UAE extraction times (disc diffusion, n=3)

Conc(mg/mL)	UAE 5 min Mean ± SD	Cat.	UAE 15 min Mean ± SD	Cat.	UAE 30 min Mean ± SD	Cat.
300	11.6 ± 0.20	Strong	9.1 ± 0.15	Moderate	9.3 ± 0.25	Moderate
200	10.8 ± 0.26	Strong	8.2 ± 0.20	Moderate	8.6 ± 0.06	Moderate
100	11.1 ± 0.32	Strong	7.5 ± 0.12	Moderate	7.1 ± 0.15	Moderate
50	9.8 ± 0.25	Moderate	7.5 ± 0.31	Moderate	7.4 ± 0.36	Moderate
25	10 ± 0.15	Moderate	8.6 ± 0.32	Moderate	7.2 ± 0.12	Moderate
12.5	8.5 ± 0.10	Moderate	7.3 ± 0.15	Moderate	6.9 ± 0.10	Moderate
6.25	6.6 ± 0.15	Moderate	7 ± 0.06	Moderate	6.5 ± 0.10	Moderate
3.125	6.8 ± 0.06	Moderate	7.2 ± 0.17	Moderate	6.7 ± 0.17	Moderate
1.56	6.7 ± 0.15	Moderate	6.2 ± 0.15	Moderate	7.2 ± 0.26	Moderate
K(−) DMSO	0	—	0	—	0	—
K(+) Chloramphenicol	19.8 ± 0.25	Strong	19.8 ± 0.25	Strong	19.8 ± 0.25	Strong

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Against *Bacillus cereus* (Table 1), the extract produced inhibition zones across all nine concentrations at all three extraction times, with no concentration reaching 6.0 mm (no-activity threshold). The 5-minute UAE yielded the highest inhibition zones at 300 mg/mL (11.6 ± 0.20 mm, Strong category) and 100 mg/mL (11.1 ± 0.32 mm, Strong). The MIC for *B. cereus* was 1.56 mg/mL at all three UAE extraction times, as this was the lowest concentration consistently producing an inhibition zone above the 6.0 mm disc diameter in all replicates. *Bacillus cereus* is a Gram-positive spore-forming pathogen associated with food-borne diarrhea, particularly from contaminated rice and meat products[28]; the sustained activity of the extract even at 1.56 mg/mL demonstrates considerable potency against this organism.

Table 2. Inhibition zone diameters (mm) of ethanol extract of *V. amygdalina* Delile. against *Salmonella typhi* at three UAE extraction times (disc diffusion, n=3)

Conc. (mg/mL)	UAE 5 min Mean ± SD	Cat.	UAE 15 min Mean ± SD	Cat.	UAE 30 min Mean ± SD	Cat.
	10.7 ± 0.21	Strong	10.7 ± 0.15	Strong	9.5 ± 0.26	Moderate
200	9.5 ± 0.26	Moderate	10.6 ± 0.06	Strong	9.7 ± 0.17	Moderate
100	9.1 ± 0.21	Moderate	9.4 ± 0.12	Moderate	9.5 ± 0.06	Moderate
50	8.4 ± 0.06	Moderate	8.2 ± 0.26	Moderate	8.6 ± 0.10	Moderate
25	6.9 ± 0.10	Moderate	8.8 ± 0.06	Moderate	8.1 ± 0.15	Moderate
12.5	7.2 ± 0.20	Moderate	7.2 ± 0.17	Moderate	7.2 ± 0.26	Moderate
6.25	6.7 ± 0.06	Moderate	6.0 ± 0.00	No Activity	6.5 ± 0.10	Moderate
3.125	7 ± 0.10	Moderate	6.0 ± 0.00	No Activity	6.5 ± 0.20	Moderate
1.56	6.6 ± 0.21	Moderate	6.0 ± 0.00	No Activity	6.5 ± 0.23	Moderate
K(−) DMSO	0	—	0	—	0	—
K(+) Chloramphenicol	20.5 ± 0.50	Very Strong	20.5 ± 0.15	Very Strong	20.5 ± 0.78	Very Strong

Against *Salmonella typhi* (Table 2), the extract demonstrated strong inhibition at all concentrations for the 5-minute UAE (MIC = 1.56 mg/mL) and 30-minute UAE (MIC = 1.56 mg/mL). However, for the 15-minute UAE, concentrations of 6.25, 3.125, and 1.56 mg/mL produced values at or below the disc diameter (6.0 mm), indicating no measurable inhibitory activity at those concentrations. Accordingly, the MIC for *S. typhi* under 15-minute UAE was established at 12.5 mg/mL — the lowest concentration that produced a measurable inhibition zone (7.2 ± 0.17 mm) in all replicates. This variability across UAE extraction times for *S. typhi* may reflect differential extraction efficiency of specific phytochemical fractions at different sonication durations, particularly those with activity against Gram-negative organisms.[32] *Salmonella typhi*, the causative agent of typhoid fever, possesses a lipopolysaccharide-rich outer membrane that presents an additional barrier to antimicrobial penetration, which may account for the higher MIC observed in one extraction condition.[29]

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Table 3. Inhibition zone diameters (mm) of ethanol extract of *V. amygdalina* Delile. against *Staphylococcus aureus* at three UAE extraction times (disc diffusion, n=3)

Conc. (mg/mL)	UAE 5 min Mean ± SD	Cat.	UAE 15 min Mean ± SD	Cat.	UAE 30 min Mean ± SD	Cat.
300	9.9 ± 0.06	Moderate	10.1 ± 0.15	Strong	10.8 ± 0.23	Strong
200	9.7 ± 0.15	Moderate	10 ± 0.15	Strong	9.7 ± 0.21	Moderate
100	8.5 ± 0.15	Moderate	9.3 ± 0.35	Moderate	8.6 ± 0.15	Moderate
50	7.3 ± 0.10	Moderate	8.8 ± 0.15	Moderate	7.2 ± 0.26	Moderate
25	7.4 ± 0.06	Moderate	6.7 ± 0.12	Moderate	7.7 ± 0.15	Moderate
12.5	7.4 ± 0.17	Moderate	7.6 ± 0.17	Moderate	6.5 ± 0.10	Moderate
6.25	7.7 ± 0.21	Moderate	6.8 ± 0.06	Moderate	6.2 ± 0.21	Moderate
3.125	7.3 ± 0.20	Moderate	7.1 ± 0.12	Moderate	6.7 ± 0.15	Moderate
1.56	8.2 ± 0.15	Moderate	6.4 ± 0.12	Moderate	7.2 ± 0.25	Moderate
K(−) DMSO	0	—	0	—	0	—
K(+) Chloramphenicol	21.3 ± 0.46	Very Strong	21.3 ± 0.46	Very Strong	21.3 ± 0.46	Very Strong

Against *Staphylococcus aureus* (Table 3), consistent and broad inhibitory activity was observed across all concentrations and extraction times. The 30-minute UAE yielded the strongest inhibition at 300 mg/mL (10.8 ± 0.23 mm, Strong), while both 5-minute and 15-minute UAE also achieved strong-category inhibition at the same concentration (9.9 and 10.1 mm, respectively). The MIC for *S. aureus* was 1.56 mg/mL across all three UAE extraction times, consistent with previous reports on the susceptibility of this Gram-positive pathogen to *V. amygdalina* phytochemicals.15 *Staphylococcus aureus* is a leading cause of food-borne gastrointestinal disease and nosocomial infections, and its susceptibility to the extract even at 1.56 mg/mL is clinically meaningful.[30]

Against *Escherichia coli* (Table 4), the 5-minute UAE extract produced no measurable inhibitory activity (6.0 ± 0.00 mm) at concentrations of 3.125 and 1.56 mg/mL, establishing the MIC at 6.25 mg/mL for this extraction condition. In contrast, both 15-minute and 30-minute UAE extracts maintained inhibitory activity down to 1.56 mg/mL (MIC = 1.56 mg/mL), suggesting that extended UAE duration enhances extraction of compounds with specific activity against Gram-negative organisms such as *E. coli*. The 15-minute UAE produced the largest inhibition zones overall for *E. coli* (11.1 ± 0.31 mm at 300 mg/mL, Strong category). *Escherichia coli* is the primary cause of traveler's diarrhea and urinary tract infections worldwide3; the lower MIC achieved at 15 and 30-minute UAE represents a meaningful improvement in antibacterial potency for this target organism

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Table 4. Inhibition zone diameters (mm) of ethanol extract of *V. amygdalina* Delile. against *Staphylococcus aureus* at three UAE extraction times (disc diffusion, n=3)

Conc. (mg/mL)	UAE 5 min Mean ± SD	Cat.	UAE 15 min Mean ± SD	Cat.	UAE 30 min Mean ± SD	Cat.
300	9.9 ± 0.06	Moderate	10.1 ± 0.15	Strong	10.8 ± 0.23	Strong
200	9.7 ± 0.15	Moderate	10 ± 0.15	Strong	9.7 ± 0.21	Moderate
100	8.5 ± 0.15	Moderate	9.3 ± 0.35	Moderate	8.6 ± 0.15	Moderate
50	7.3 ± 0.10	Moderate	8.8 ± 0.15	Moderate	7.2 ± 0.26	Moderate
25	7.4 ± 0.06	Moderate	6.7 ± 0.12	Moderate	7.7 ± 0.15	Moderate
12.5	7.4 ± 0.17	Moderate	7.6 ± 0.17	Moderate	6.5 ± 0.10	Moderate
6.25	7.7 ± 0.21	Moderate	6.8 ± 0.06	Moderate	6.2 ± 0.21	Moderate
3.125	7.3 ± 0.20	Moderate	7.1 ± 0.12	Moderate	6.7 ± 0.15	Moderate
1.56	8.2 ± 0.15	Moderate	6.4 ± 0.12	Moderate	7.2 ± 0.25	Moderate
K(−) DMSO	0	—	0	—	0	—
K(+) Chloramphenicol	21.3 ± 0.46	Very Strong	21.3 ± 0.46	Very Strong	21.3 ± 0.46	Very Strong

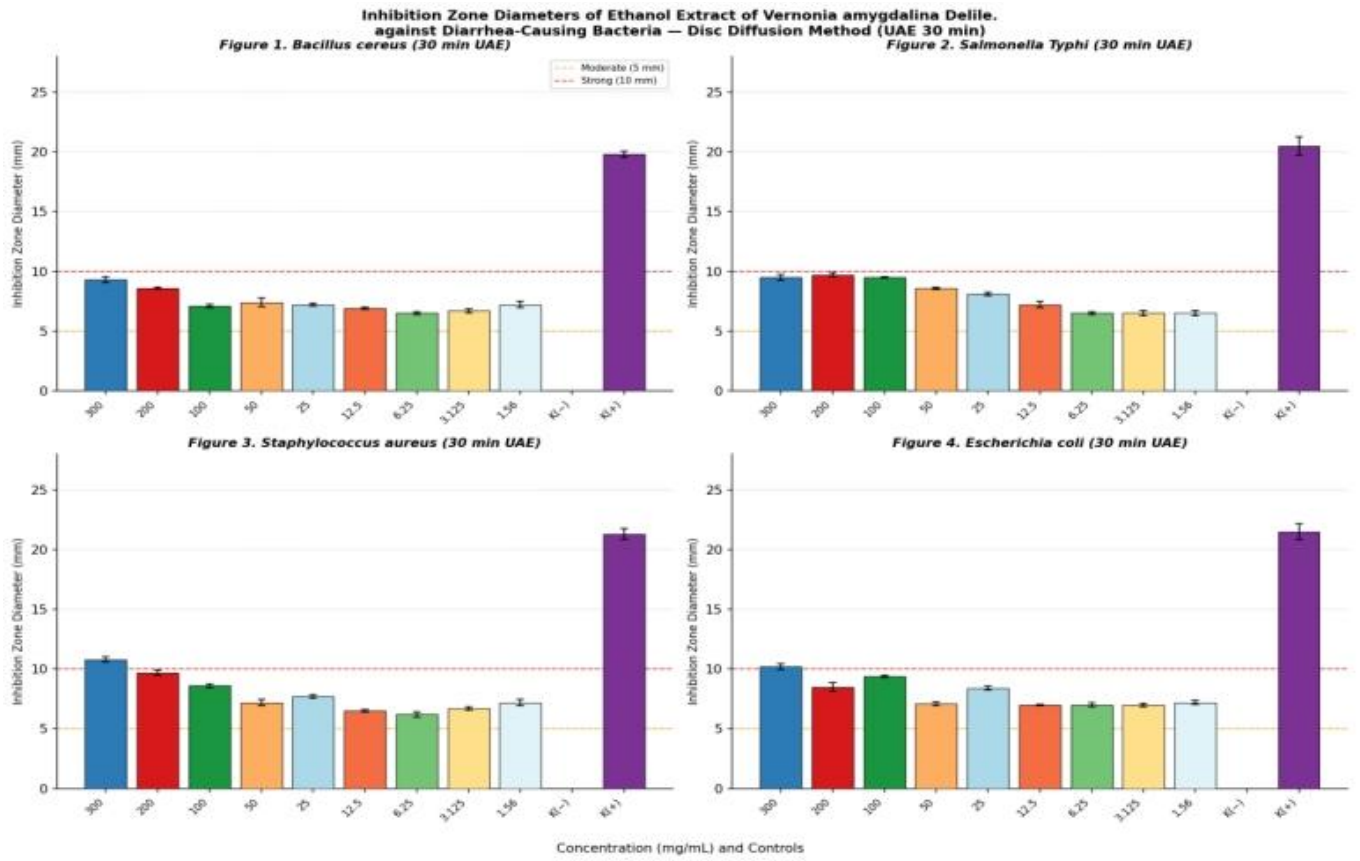

Figure 1. Inhibition zone diameters (mm) of ethanol extract of *Vernonia amygdalina* Delile. against four diarrhea-causing bacteria (UAE 30-minute extraction, disc diffusion, n=3). Dashed lines indicate activity category thresholds per Davis & Stout (1971). K(−) = DMSO; K(+) = chloramphenicol.

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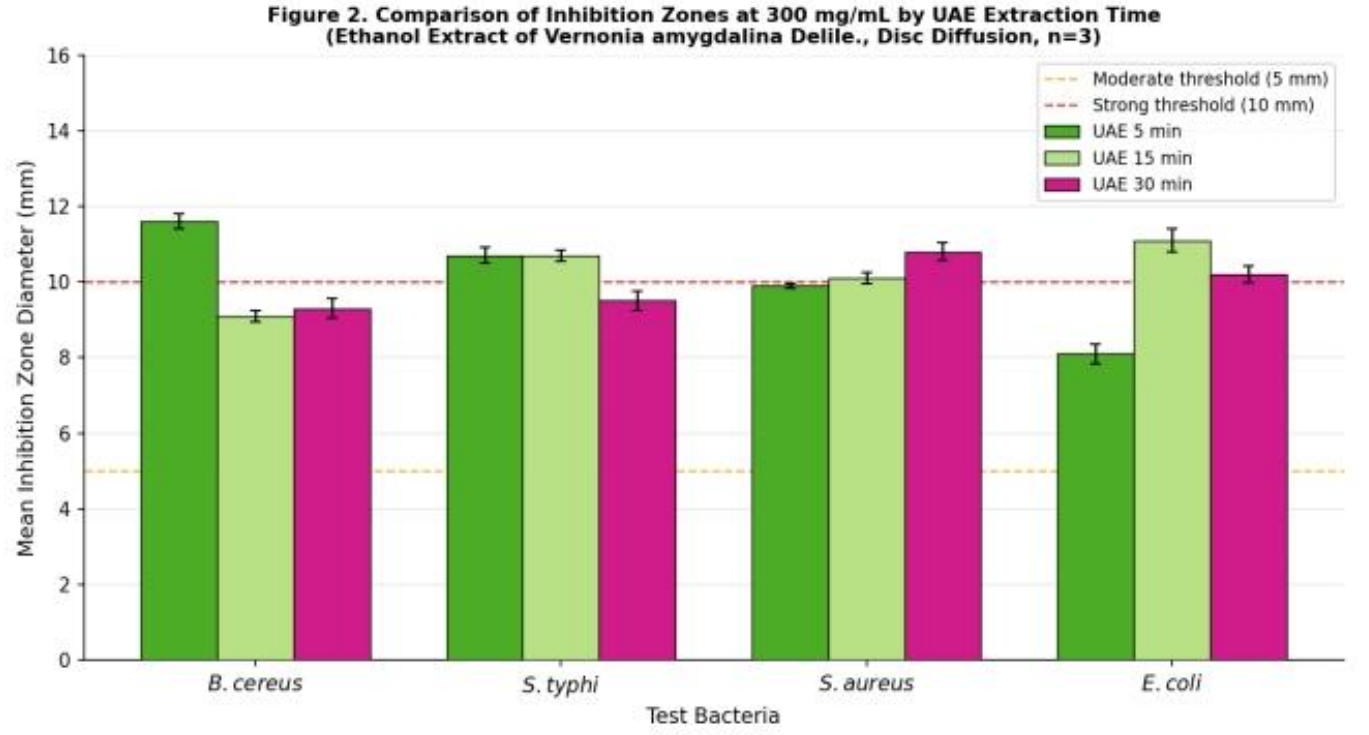

Figure 2. Comparison of inhibition zone diameters (mm) at 300 mg/mL across three UAE extraction times against four diarrhea-causing bacteria. Error bars represent ± SD (n=3). Dashed lines indicate activity thresholds per Davis & Stout (1971).

Table 5. Summary of Minimum Inhibitory Concentration (MIC) values of ethanol extract of *V. amygdalina* Delile. against diarrhea-causing bacteria at three UAE extraction times

Bacterium	MIC — UAE 5 min	MIC — UAE 15 min	MIC — UAE 30 min
B. cereus	1.56 mg/mL	1.56 mg/mL	1.56 mg/mL
S. typhi	1.56 mg/mL	12.5 mg/mL	1.56 mg/mL
S. aureus	1.56 mg/mL	1.56 mg/mL	1.56 mg/mL
E. coli	6.25 mg/mL	1.56 mg/mL	1.56 mg/mL

As summarized in Table 5, the MIC values for *B. cereus* and *S. aureus* were uniformly 1.56 mg/mL regardless of UAE extraction time, indicating that even the shortest extraction (5 minutes) yields sufficient antibacterial compounds to inhibit Gram-positive pathogens at the lowest tested concentration. For *S. typhi*, the MIC was 1.56 mg/mL for both 5-minute and 30-minute UAE, but increased to 12.5 mg/mL for the 15-minute UAE, reflecting extraction-time-dependent variability in the phytochemical composition of the resulting extract. For *E. coli*, the 5-minute UAE was less efficient — yielding a MIC of 6.25 mg/mL — compared to 15-minute and 30-minute UAE, which both achieved MIC = 1.56 mg/mL, confirming that longer UAE duration significantly improves antibacterial potency against Gram-negative bacteria.

The broad-spectrum antibacterial activity of *V. amygdalina* ethanol extract against both Gram-positive and Gram-negative bacteria is attributable to the synergistic interplay of multiple phytochemical classes. Flavonoids interact with bacterial membrane phospholipids, increase membrane permeability, and inhibit DNA gyrase and topoisomerase, preventing bacterial replication[11,33]; tannins precipitate surface proteins and inhibit extracellular enzymes involved in bacterial virulence[12]; saponins reduce surface tension at the bacterial

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membrane, forming transmembrane pores that cause leakage of ions, amino acids, and proteins[13]; and alkaloids disrupt peptidoglycan synthesis, leading to incomplete cell wall formation and lysis.[14] The co-extraction of these phytochemical classes by ethanol 96% in the UAE process creates a complex bioactive mixture with multi-target antibacterial mechanisms, which likely contributes to the broad-spectrum activity and the low MIC values observed.[34]

One-way ANOVA analysis revealed statistically significant differences ($p < 0.05$) in inhibition zone diameters among concentrations for each bacterium and each extraction time, confirming that the concentration-dependent response was real and not due to random variation.[27] Post-hoc Tukey HSD analysis confirmed that higher concentrations (300–100 mg/mL) produced significantly larger inhibition zones than lower concentrations (12.5–1.56 mg/mL) in most bacteria–time combinations. The data supported a dose-dependent mechanism: increasing extract concentration delivers a greater quantity of bioactive molecules per unit area of agar, creating steeper diffusion gradients and broader inhibition zones.[25]

These findings are broadly consistent with prior investigations on *V. amygdalina* antibacterial efficacy. Pratiwi and Gunawan (2018) reported inhibition zones of 6.69 mm and 6.52 mm against *S. aureus* and *E. coli*, respectively[15]; the higher values achieved in the present study may reflect the superior extraction efficiency of UAE over the conventional maceration methods used in prior work.[16] Zahra (2021) confirmed antibacterial activity of *V. amygdalina* ethanol extract against *E. coli* ATCC 25922 in vitro[35], while Tanjung (2019) identified flavonoids and tannins as the primary active contributors.[36] Nasri and Zuriani (2014) emphasized that systematic MIC determination is essential for the evidence-based application of Indonesian medicinal plant extracts as therapeutic antibacterial agents[21], and the results of this study provide quantitative MIC data that support the scientific foundation for potential formulation development of *V. amygdalina* as an antibacterial agent against diarrhea-causing pathogens. Furthermore, Kaban (2022) documented that UAE-optimized extraction of Indonesian medicinal plants significantly enhances antibacterial activity relative to conventional extraction[22], a finding corroborated by the higher yields and superior inhibition zones achieved at 15- and 30-minute UAE in this study compared to the 5-minute condition for *E. coli*.

CONCLUSION

The ethanol extract of African leaf (*Vernonia amygdalina* Delile.) prepared by Ultrasonic-Assisted Extraction (UAE) demonstrated broad-spectrum antibacterial activity against all four diarrhea-causing bacteria tested — *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, and *Bacillus cereus* — with inhibition zones predominantly in the moderate category (5–10 mm) and reaching the strong category (10–20 mm) at higher concentrations. The Minimum Inhibitory Concentration (MIC) for *B. cereus* and *S. aureus* was 1.56 mg/mL across all UAE extraction times. For *S. typhi*, MIC was 1.56 mg/mL (5 and 30 min UAE) and 12.5 mg/mL (15 min UAE). For *E. coli*, MIC was 6.25 mg/mL (5 min UAE) and 1.56 mg/mL (15 and 30 min UAE). The 30-minute UAE yielded the highest extraction yield (11.9%) and generally superior antibacterial performance, making it the recommended extraction condition. One-way ANOVA confirmed statistically significant concentration-dependent effects ($p < 0.05$). These results support the potential of *V. amygdalina* ethanol extract as a natural antibacterial agent for diarrheal disease

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management. Further research involving biofilm inhibition assays, in vivo safety profiling, active compound isolation, and standardized pharmaceutical formulation is recommended.

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