Walisongo Journal of Chemistry
Walisongo Journal of Chemistry Vol. 7 Issue 2 (2024), 211-220
ISSN: 2621-5985 (online); 2549-385X (print)
DOI: https://doi.org/10.21580/wjc.v7i2.23333

ANTIBACTERIAL ACTIVITY OF LIQUID-LIQUID EXTRACTION FRACTIONS FROM TEMULAWAK (*Curcuma Xanthorrhiza Roxb*) RHIZOME

Dante Alighiri[1*], Indah Putri Firnanda[1], Ravely Adhitya Abdullah[1], Naufaldi Bani Widodo[1], Apriliana Drastisianti[2]

[1]Chemistry Department, Faculty of Science and Mathematics, Universitas Negeri Semarang, Indonesia
[2]Department of Chemistry Education, Faculty of Science and Technology, Universitas Islam Negeri Walisongo Semarang, Indonesia

***Corresponding author:** dante_alighiri@mail.unnes.ac.id

Abstract

The rhizome of temulawak (Curcuma xanthorrhiza Roxb.) has been traditionally used to treat various ailments due to its rich content of curcumin, demethoxycurcumin, and bisdemethoxycurcumin. These compounds exhibit notable antioxidant and antibacterial properties. Hence, this study aimed to evaluate the antibacterial efficacy of temulawak rhizome extracts obtained using n-hexane, ethyl acetate, and methanol, specifically against Escherichia coli and Staphylococcus aureus. The extraction process involved successive maceration with each solvent. Antibacterial activity was assessed using well-diffusion and microdilution methods to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Phytochemical screening revealed the presence of flavonoids, alkaloids, phenolics, steroids, and triterpenoids in the extracts. The results indicated that the n-hexane and ethyl acetate fractions exhibited significant antibacterial activity against S. aureus, while all fractions demonstrated potent activity against E. coli. The MIC values ranged from 39.0625 to 625 ppm for S. aureus and from 39.0625 to 78.125 ppm for E. coli, with MBC values generally higher than the MIC values. These findings highlighted the potential of temulawak rhizome extracts as effective antibacterial agents.

Keywords: *temulawak, antibacterial activity, minimum inhibitory concentration, minimum bactericidal concentration.*

Introduction

The rhizome of temulawak (Curcuma xanthorrhiza Roxb.) has long been used as a traditional remedy to address various ailments, including constipation, acne, diarrhea, hemorrhoids, seizures, kidney diseases, muscle pain, liver diseases, rheumatism, arthritis, canker sores, vaginal discharge, and loss of appetite. This plant, belonging to the ginger family, is prevalent in tropical regions and thrives in loose, dry soil, home gardens, and fields at altitudes ranging from 5 to 1500 meters above sea level (Rahmat et al., 2021; Syamsudin et al., 2019).

The primary constituents of temulawak rhizome include curcumin,

demethoxycurcumin, and bisdemethoxycurcumin. These compounds play a significant role in neutralizing free radicals, highlighting temulawak's potential as an antioxidant (Rosidi et al., 2017; Simamora et al., 2024). Beyond its antioxidant properties, temulawak rhizome also exhibits antibacterial potential. Research conducted by Mustikaturrokhmah and Risanti (2020) demonstrated that a 70% ethanol extract of temulawak could inhibit the growth of Staphylococcus epidermidis and Salmonella typhi at extract concentrations ranging from 5–45%. Another study revealed that temulawak essential oil could impede the development of Streptococcus viridans at concentrations between 25–100%, with inhibition zone diameters averaging between 8.22 and 16.1 mm (Khotimah et al., 2021; Mirza et al., 2023; Yogiara et al., 2020).

While numerous studies have explored the antibacterial activity of temulawak, research on the antibacterial efficacy of temulawak rhizome extracts in different solvents, such as n-hexane, ethyl acetate, and methanol, remains limited. This gap intrigued further investigation into the antibacterial activity of both polar and non-polar solvent fractions.

This antibacterial activity can be assessed through several approaches, notably diffusion and dilution methods. The diffusion method is commonly applied to analyze the antibacterial activity of a compound, whereby the test compound diffuses in a solid medium inoculated with bacteria. A clear zone around the compound indicates inhibition of bacterial growth (Akarchariya et al., 2017; Helen et al., 2012; Nurhayati et al., 2020). In contrast, the dilution method determines a compound's minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), identifying the lowest concentration required to inhibit or kill bacterial growth.

Therefore, the present study aimed to compare the effectiveness of antibacterial testing methods—diffusion and dilution—on temulawak rhizome extract fractions obtained using n-hexane, ethyl acetate, and methanol as solvents. Additionally, this research sought to identify the phytochemical contents of each fraction and evaluate their antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*.

Materials and Method

Materials

The materials used in this research included temulawak (Curcuma xanthorrhiza Roxb.), sourced from Semarang, Central Java, Indonesia. Chemicals such as n-hexane, ethyl acetate, methanol, ammonia, iron(III) chloride, and NaOH were purchased from Merck, Germany. Additional materials included distilled water, chloroform, an alcohol burner, filter paper (Whatman), disc paper (Whatman No. 42), Mueller-Hinton Agar medium (Oxoid), Mueller-Hinton Broth (Himedia), Nutrient Agar (Himedia), Nutrient Broth (Oxoid), tetracycline (Sigma-Aldrich), DMSO (Emsure), Staphylococcus aureus bacteria (ATCC 6538), Escherichia coli bacteria (ATCC 8739), acetic anhydride (Fission), Liebermann-Burchard reagent (composed of concentrated sulfuric acid and acetic anhydride), and silica gel 60 F254 (Sigma-Aldrich), which were purchased from Brataco, Indonesia. The tools utilized included glassware (Pyrex), a rotary evaporator (Buchi Rotavapor R-124), round-bottom flasks (Buchi), evaporation flasks (Buchi), TLC plates and chambers (Camag), an ultraviolet lamp with wavelengths of 254 and 336 nm (Camag), a digital scale (Quantix 224-1S), micropipettes (Thermo Scientific Finnpipette F2), an oven-shaking incubator (Lab Tech LSII6002M), an autoclave (Lab Tech LAC 51100SD), a laminar airflow cabinet (Lab Tech LCB-1502V), a hot plate (DLAB MS7-H550-S), and a vortex mixer (DLAB MX-S).

Temulawak Powder Preparation

The *temulawak* rhizomes were washed with water and dried at temperatures between 50°C and 60°C for 60 minutes. Subsequently, the dried rhizomes were ground using a grinder machine. The resulting powder was sifted through a 100-

mesh sieve, dried at 60°C until a constant weight was achieved, and then designated for further experimentation.

Extraction of *Temulawak* Rhizomes

Initially, 400 grams of finely powdered dried *temulawak* rhizomes were extracted using three solvents: n-hexane, ethyl acetate, and methanol. Each extraction cycle lasted 24 hours and was repeated three times. The resulting macerated extracts were concentrated using a rotary evaporator. After concentration, the weights of the extracts from the n-hexane, ethyl acetate, and methanol fractions were recorded, and their yields were calculated as percentages.

Phytochemical Assay

Alkaloid Compound Test

The alkaloid test employed Dragendorff's reagent. A 10 mg sample of the extract was mixed with 5 mL of ethanol in a test tube, followed by the addition of two drops of Dragendorff's reagent to the filtrate. Positive results were indicated by the formation of reddish-orange precipitates (Habibi et al., 2018; Harjono et al., 2020; Masturi et al., 2019; 2020; 2021; Musman, 2017).

Test for Steroid and Triterpenoid Compounds

The Liebermann-Burchard reagent was utilized for this test. A 10 mg sample of the extract was combined with a few drops of Liebermann-Burchard reagent. A green or blue color indicated the presence of steroids, cholesterol, or related compounds, while a reddish-purple hue indicated the presence of terpenoids (Habibi et al., 2018; Musman, 2017).

Flavonoid Compound Test

To detect flavonoids, a 10 mg sample of the extract was mixed with 5 mL of ethanol. The filtrate was treated with 1 mL of hot methanol, magnesium metal, and 0.5 mL of concentrated HCl. A red or orange solution confirmed the presence of flavonoids (Habibi et al., 2018; Harjono et al., 2020; Masturi et al., 2019; 2020; 2021; Musman, 2017).

Phenolic Compound Test

For the phenolic compound test, a 10 mg sample of the extract was mixed with 5 mL of ethanol. Then, 1 mL of iron(III) chloride ($FeCl_3$) was added to the filtrate. A purple-blue color confirmed the presence of phenolic compounds (Habibi et al., 2018; Harjono et al., 2020; Masturi et al., 2019; 2020; 2021; Musman, 2017).

Tannin Test

For the tannin test, 10 mg of the methanol extract was mixed with 15 mL of hot water and boiled for 5 minutes. A few drops of 1% $FeCl_3$ solution were then added to the sample. The appearance of a violet-green color indicated the presence of tannins (Habibi et al., 2018; Harjono et al., 2020; Masturi et al., 2019, 2020, 2021; Musman, 2017).

Saponin Test

In the saponin test, 10 mg of the extract was vigorously shaken with 10 mL of hot water in a test tube and allowed to cool. The formation of stable foam within 1–10 minutes, persisting after the addition of 2N HCl, indicated a positive result (Habibi et al., 2018; Harjono et al., 2020; Masturi et al., 2019, 2020, 2021; Musman, 2017).

Antibacterial Activity Test Using the Well Diffusion Method

Fifty microliters of n-hexane, ethyl acetate, and methanol fractions at 5000 ppm, along with a positive control (tetracycline 200 ppm) and a negative control (DMSO), were pipetted into wells on sodium agar medium inoculated with test bacteria. The plates were incubated at 37°C for 24 hours. Antibacterial activity was assessed by measuring the diameters of the inhibition zones.

Minimum Inhibitory Concentration (MIC) Test

A sterile 96-well microplate, arranged in 8 vertical rows (A–H) and 12 horizontal rows (1–12), was prepared and filled with 100 µL of Mueller-Hinton Broth (MHB). Samples, positive control (tetracycline), and negative control were added to well A. Serial dilutions were performed from well A to well H. The test bacteria were prepared and diluted in sterile 0.85% saline solution to match the 0.5 McFarland standard (approximately 1×10^8 CFU/mL). The diluted

bacteria (100 µL) were then added to each well. The microplate was incubated at 37°C for 24 hours. MIC was determined visually as the lowest concentration that produced a clear zone of inhibition (Cho et al., 2023; Khotimah et al., 2021; Simamora et al., 2024).

Minimum Bactericidal Concentration (MBC) Test

The lowest concentration of the sample that effectively halted bacterial growth underwent additional plating on Mueller-Hinton Agar (MHA). Subsequently, 5 µL of this plated solution was streaked onto Petri dishes containing MHA. The dishes were incubated at 37°C for 24 hours. The Minimum Bactericidal Concentration (MBC) was identified as the lowest concentration, showing no bacterial growth (Cho et al., 2023; Khotimah et al., 2021; Simamora et al., 2024).

Results and Discussion

Extraction of *Temulawak* Rhizome

The *temulawak* rhizome was fractionated using the liquid-liquid partition extraction method. This fractionation process employed n-hexane (non-polar), ethyl acetate (semi-polar), and methanol (polar) to separate chemical compounds based on their polarity levels (Masturi et al., 2021).

The powdered *temulawak* rhizome underwent successive maceration extractions, beginning with a non-polar solvent and progressing to a polar solvent. Initially, n-hexane was used for its affinity with non-polar active compounds. The extraction process lasted for three cycles of 24 hours each, yielding 17.3501 grams of concentrated n-hexane fraction, with a yield of 4.3375%. The residue was then re-macerated with ethyl acetate under similar conditions, producing 27.3553 grams of concentrated ethyl acetate fraction with a yield of 6.8388%. Lastly, the residue from ethyl acetate extraction was subjected to re-maceration with methanol for three cycles of 24 hours each, resulting in a methanol fraction weighing 5.0543 grams, with a yield of 1.2636%.

Phytochemical Screening

Qualitative phytochemical screening was conducted to identify secondary metabolite groups in the n-hexane, ethyl acetate, and methanol fractions of the *temulawak* rhizome. The screening targeted flavonoids, alkaloids, phenolics, saponins, steroids, and triterpenoids. The results are summarized in Table 1.

Antibacterial Activity Test Using the Well Diffusion Method

The antibacterial activity of the *temulawak* rhizome fractions was tested using the well diffusion method, allowing bioactive compounds to diffuse into the agar medium. The tested bacteria included gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli*. The results of the antibacterial activity test are presented in Table 2.

Table 1. Phytochemical screening of temulawak rhizome extract fractions: n-Hexane, Ethyl Acetate, and Methanol.

Parameter	Reagent	Fraction Extract		
		n-hexane	ethyl acetate	methanol
Flavonoid	Mg powder + 10 drops of concentrated HCl	-	+	+
Alkaloid	Dragendorff	-	+	+
Phenolic	$FeCl_3$	-	+	+
Saponin	Shaken + 1 mL concentrated HCl	-	-	-
Triterpenoid	Liebermann-Burchard	+	+	-
Steroid	Liebermann-Burchard	+	+	-

Information:
+: Positive / Detected;
-: Negative / Undetected

The temulawak rhizome fractions were tested at a concentration of 5000 ppm. Against Staphylococcus aureus, the n-hexane fraction exhibited inhibition zone diameters of 10.0, 10.5, and 9.625 mm, while the ethyl acetate fraction showed diameters of 10.25, 10.75, and 9.5 mm. The methanol fraction displayed diameters of 9.75, 9.5, and 9.625 mm. Against Escherichia coli, the n-hexane fraction showed inhibition zone diameters of 14.25, 12.0, and 11.875 mm, the ethyl acetate fraction exhibited diameters of 12.25, 11.75, and 11.875 mm, and the methanol fraction displayed diameters of 12.0, 11.75, and 11.875 mm. The positive control, tetracycline at 200 ppm, produced inhibition zone diameters of 14.5, 14.0, and 14.25 mm against S. aureus and 12.5, 12.25, and 12.375 mm against E. coli. The negative control, DMSO, showed no antibacterial activity, serving primarily to validate the test system.

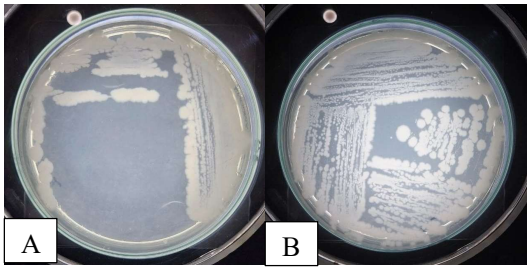

Figure 1. Staphylococcus aureus (A) and Escherichia coli (B) bacteria

Figure 2 illustrates the antibacterial activity of temulawak rhizome extract fractions from n-hexane, ethyl acetate, and methanol in comparison to the positive control, tetracycline (4), and the negative control, DMSO, against Staphylococcus aureus (SA) and Escherichia coli (EC). The antibacterial activity was categorized as strong (≥20 mm), moderate (10–20 mm), and weak (<10 mm). The n-hexane and ethyl acetate fractions exhibited strong antibacterial activity against S. aureus, whereas the methanol fraction showed moderate activity. Meanwhile, against E. coli, all three fractions demonstrated strong antibacterial potential.

The antibacterial activity was affected by the metabolite compounds present in each fraction. Phytochemical screening of the *temulawak* rhizome fractions revealed the presence of

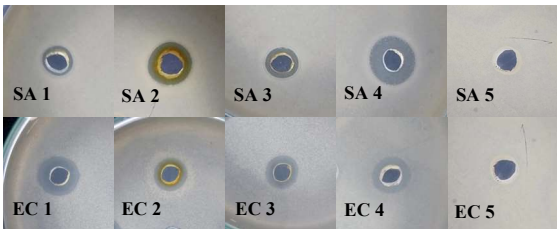

Figure 2. Antibacterial activity of temulawak rhizome fraction extracts: (1) n-hexane fraction, (2) ethyl acetate fraction, (3) methanol fraction, (4) positive control, and (5) negative control against Staphylococcus aureus (SA) and Escherichia coli (EC).

flavonoids, alkaloids, phenolics, triterpenoids, and steroids. Flavonoid compounds inhibit nucleic acid synthesis, plasma membrane function, and energy metabolism in bacteria, thereby hindering bacterial growth. Alkaloids disrupt peptidoglycan components in bacterial cells, leading to imperfect bacterial cell wall formation and eventual cell death (Rini et al., 2017). Additionally, phenolic compounds disrupt plasma membrane function and bacterial enzyme systems while damaging bacterial cell proteins.

Furthermore, the presence of steroids and triterpenoids also interferes with bacterial growth. Steroids exhibit permeable properties to lipophilic compounds, causing a decrease in cell membrane integrity and leading to cell lysis and brittleness (Kumalasari et al., 2020). Triterpenoids can interact with transmembrane proteins on the outer membrane of bacterial cells, forming strong polymers that damage porins (Cowan, 1999).

The differences in inhibitory effects between Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* were due to the differences in the composition and structure of their cell walls. Gram-positive bacteria are more vulnerable to chemical compounds compared to Gram-negative bacteria. Gram-positive bacteria have a simpler cell wall structure with a single layer and low lipid content (approximately 1–4%), making it easier for bioactives to penetrate the cell. Conversely, Gram-negative bacteria have a

more complex cell wall structure with three layers: an outer lipoprotein layer, a middle lipopolysaccharide layer that blocks the entry of bioactive, and an inner peptidoglycan layer with a higher lipid content (approximately 11–12%).

Table 2. Diameter of clear zones of temulawak rhizome fractions against gram-positive Staphylococcus aureus and gram-negative Escherichia coli

Sample	Concentration (ppm)	Diameter of Clear Zones (mm)	
		Staphylococcus aureus	*Escherichia coli*
Fraction Extract of n-hexane	5000	9.75	14
		10.25	14.5
		10	14.25
Fraction Extract of ethyl acetate	5000	10.25	12.25
		10.75	11.75
		10.5	12
Fraction Extract of methanol	5000	9.75	12
		9.5	11.75
		9.625	11.875
Positive Control of Tetracycline	200	14.5	12.5
		14	12,25
		14.25	12.375
Negative Control of DMSO	p.a	0	0

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the antibacterial activity of temulawak rhizome fraction extracts

Sample	*Staphylococcus aureus*		*Escherichia coli*	
	MIC (ppm)	MBC (ppm)	MIC (ppm)	MBC (ppm)
Fraction extract of n-hexane	39.0625	> 39.0625	78.125	> 78.125
Fraction extract of ethyl acetate	625	625	78.125	> 78.125
Fraction extract of methanol	156.25	> 156.25	39.0625	> 39.0625
Positive control (tetracycline)	0.390625	> 0.390625	0.390625	> 0.390625
Negative control (DMSO)	24.975×10^4	$> 24.975 \times 10^4$	24.975×10^4	$> 24.975 \times 10^4$

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Testing

The MIC was determined using the microdilution method, with observations conducted either through turbidimetry or visual assessment of turbidity. Concentrations ranging from 5000 ppm to 39.0625 ppm were tested. Tetracycline served as the positive control at concentrations ranging from 50 ppm to 0.390625 ppm, while DMSO was used as the negative control, with concentrations ranging from 49.95×10^4 ppm to 0.1951×10^4 ppm. Turbidity levels were observed in each well after 24 hours of incubation, as indicated by distinct changes in well color. The turbidity results reflected the antimicrobial potential of *temulawak* fractions against Gram-positive and Gram-negative bacteria. The MIC results are summarized in Table 3.

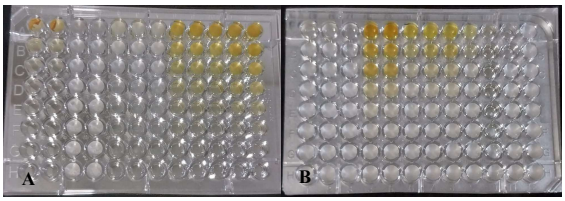

Figure 3. MIC assay of antibacterial activity of temulawak rhizome fraction extracts: n-hexane fraction, ethyl acetate, methanol, positive control, and negative control against Staphylococcus aureus (A) and Escherichia coli (B).

The MIC values for the n-hexane, ethyl acetate, and methanol fractions against *Staphylococcus aureus* were 39.0625 ppm, 625 ppm, and 156.25 ppm, respectively. Against *Escherichia coli*, these values were 78.125 ppm, 78.125 ppm, and 39.0625 ppm, respectively. The positive control, tetracycline, inhibited bacterial growth at 0.390625 ppm. In contrast, the negative control, DMSO, exhibited bacterial inhibition at a concentration 24.975×10^4 ppm, as evidenced by turbidity observations in the tubes/wells. Research by Gallardo-Villagrán et al. (2020) indicated that DMSO at a 0.5% concentration exhibited high toxicity, killing up to 25% of cells within 24 hours. Following the MIC determination, bacterial cultivation was conducted by incorporating inhibitory samples at their respective concentrations into agar media.

The MBC is defined as the lowest concentration of an antibacterial agent capable of killing bacteria. The determination is indicated by the absence of microorganism growth on nutrient agar, signifying cell death due to the applied concentration. In this study, MBC values were derived from the two lowest concentrations identified in the MIC test. The MBC results were visually analyzed by observing growth on the petri dishes. For *Staphylococcus aureus*, the MBC value for the ethyl acetate fraction matched its MIC value, while the n-hexane and methanol fractions had MBC values greater than their MIC values. For *Escherichia coli*, the MBC values for all three fractions were higher than their MIC values.

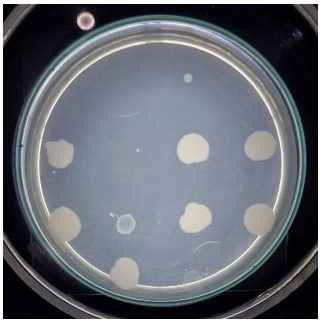

Figure 4. MBC value assay of antibacterial activity of temulawak rhizome fraction extracts: n-hexane fraction, ethyl acetate, methanol, positive control, and negative control against Staphylococcus aureus and Escherichia coli.

Conclusion

This study demonstrated that rhizome extracts of *temulawak* (*Curcuma xanthorrhiza* Roxb.) possessed significant antibacterial properties against *Escherichia coli* and *Staphylococcus aureus*. The n-hexane and ethyl acetate fractions exhibited strong activity against *S. aureus*, while all fractions showed notable effects on *E. coli*. Phytochemical analysis revealed the presence of bioactive compounds such as flavonoids, alkaloids, phenolics, steroids,

and triterpenoids, which likely contributed to the observed antibacterial effects. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values varied based on the solvent used for extraction, with the n-hexane and methanol fractions generally exhibiting lower MIC values. Specifically, the MIC for *Staphylococcus aureus* was 39.06 ppm for the n-hexane fraction, 625 ppm for the ethyl acetate fraction, and 156.25 ppm for the methanol fraction. For *Escherichia coli*, the MIC values were 78.13 ppm for the n-hexane and ethyl acetate fractions and 39.06 ppm for the methanol fraction. Additionally, the average MBC values were higher than the MIC values, further underscoring the extracts' effectiveness in inhibiting bacterial growth. These findings suggested that *temulawak* rhizome extracts had the potential as effective natural antibacterial agents, highlighting their applicability in treating bacterial infections.

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 | ISSN 2621-5985 (online) | ISSN 2549-385X (print)
Volume 7, Nomor 2, 2024