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Original Article

Antibacterial *in vitro* screening of *Helminthostachys zeylanica* (L.) Hook. root extracts

[Cribado antibacteriano *in vitro* de extractos de raíz de *Helminthostachys zeylanica* (L.) Hook.]

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Abstract

Context: *Helminthostachys zeylanica* (L.) Hook. has been used as a traditional medicine plant to treat diabetic mellitus and various infectious in China, Malaysia, and Indonesia.

Aims: To determine the antibacterial activity of *H. zeylanica* root extracts against various pathogenic bacteria.

Methods: The extraction was carried out by maceration of fresh roots using methanol, then partitioned to obtain extracts of *n*-hexane, dichloromethane, ethyl acetate, and water. The extracts were tested for antibacterial activity using the agar diffusion method, minimal inhibition concentration (MIC), and minimal bactericidal concentration (MBC).

Results: Dichloromethane and ethyl acetate extracts showed inhibitory activity against *Bacillus subtilis* ATCC 1965, *Staphylococcus aureus* ATCC 6538, *B. cereus* ATCC 10876, *Vibrio parahaemolyticus* ATCC 17802, *V. alginolyticus* ATCC 17749, *Listeria monocytogenes* ATCC 7644, and *Salmonella typhimurium* ATCC 142028. The MIC value of dichloromethane extract against these bacteria was 125-500 µg/mL, while the ethyl acetate extract was 500 µg/mL. Furthermore, the MBC for dichloromethane against bacteria *B. subtilis* and *V. parahaemolyticus* were 250 µg/mL.

Conclusions: The study demonstrated the *in vitro* antibacterial potential of the species extracts against pathogenic bacteria. The dichloromethane and ethyl acetate extracts showed the most significant inhibitory activity, suggesting that this plant could be a potential source for developing new antimicrobial agents. Further research is needed to identify the active compounds responsible for the observed effects.

Keywords: antibacterial; *Helminthostachys zeylanica*; minimal bactericidal concentration; minimal inhibition concentration.

Resumen

Contexto: *Helminthostachys zeylanica* (L.) Hook. se ha utilizado como planta medicinal tradicional para tratar la diabetes mellitus y diversas enfermedades infecciosas en China, Malasia e Indonesia.

Objetivos: Determinar la actividad antibacteriana de los extractos de raíz de *H. zeylanica* contra diversas bacterias patógenas.

Métodos: La extracción se llevó a cabo por maceración de raíces frescas con metanol, y luego se particionó para obtener extractos de n-hexano, diclorometano, acetato de etilo y agua. Se comprobó la actividad antibacteriana de los extractos mediante el método de difusión en agar, la concentración mínima de inhibición (MIC) y la concentración mínima bactericida (MBC).

Resultados: Los extractos de diclorometano y acetato de etilo mostraron actividad inhibidora frente a *Bacillus subtilis* ATCC 1965, *Staphylococcus aureus* ATCC 6538, *B. cereus* ATCC 10876, *Vibrio parahaemolyticus* ATCC 17802, *V. alginolyticus* ATCC 17749, *Listeria monocytogenes* ATCC 7644 y *Salmonella typhimurium* ATCC 142028. El valor MIC del extracto de diclorometano contra estas bacterias fue de 125-500 µg/mL, mientras que el del extracto de acetato de etilo fue de 500 µg/mL. Además, la MBC del diclorometano frente a las bacterias *B. subtilis* y *V. parahaemolyticus* fue de 250 µg/mL.

Conclusiones: El estudio demostró el potencial antibacteriano *in vitro* de los extractos de las especies frente a bacterias patógenas. Los extractos de diclorometano y acetato de etilo mostraron la actividad inhibidora más significativa, lo que sugiere que esta planta podría ser una fuente potencial para desarrollar nuevos agentes antimicrobianos. Se necesitan más investigaciones para identificar los compuestos activos responsables de los efectos observados.

Palabras Clave: antibacteriano; concentración bactericida mínima; concentración mínima de inhibición; *Helminthostachys zeylanica*.

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INTRODUCTION

Antimicrobials, such as antibacterial/antibiotic, antifungal, antiviral, and antiprotozoal medications, are the options for combating infectious disease, which remains a significant problem in society. Antibiotics are increasingly utilized to treat bacterial infections. The use of non-adherent antibiotics can result in resistance to antimicrobials, rendering the drugs used to treat infections ineffective because microorganisms are difficult to treat with either a single antimicrobial (antimycobacterial resistance) or specific antibiotics (multiple drug resistance). According to a report in 2019, 4.95 million deaths were attributable to antimicrobial resistance (AMR), of which 1.27 million were directly attributable to AMR (Murray et al., 2022).

The development of new antibiotics is necessary to treat infectious diseases, as is the investigation of potential resistance mechanisms to slow or stop the spread of antimicrobial resistance. The inability to treat certain diseases with modern medicines has contributed to an increase in the number of cases in which information on herbal medicines is sought after, which in turn has led to an increase in the use of herbal medicines in developed countries. Traditional medicine also has less potential for unwanted side effects, making it a more secure choice for patients (Alksne and Projan, 2000). Consequently, herbal medicines can be used as a viable alternative to conventional antimicrobial treatments by making use of the active substances present in medicinal plants.

Helminthostachys zeylanica (L.) Hook. (family *Ophioglossaceae*) or rawu berkubang (Malay), paku pacar bumi (South Sumatran tribe), jajalakan (Sundanese), manon (Javanese), kamraj in India, and daodi-ugon It thrives in moist, shady soil, along riverbanks, or on shady slopes with a high humus content (Cicuzza, 2020; El Ridhasya et al., 2020; Ridhasya et al., 2019). This species' rhizome is employed in treating dysentery, colds, and early-stage pulmonary tuberculosis. The dried leaves are smoked to treat nosebleeds, while the young leaves are consumed as vegetables (Sarker et al., 2012). This species is used by the Talang Mamak tribe, which lives in the Province of Riau in Indonesia, to treat inflammatory conditions, dysentery, cataracts, early-stage tuberculosis, syphilis, diabetes, and malaria (Ridhasya et al., 2019).

The secondary metabolites present in the species possess a variety of different biological activities, and they have the potential to serve as a source of lead compounds in the development of drug discovery. Ugonin J and K isolated from the root showed potential activity to inhibit α-glucosidase, and six ugonins, including ugonin J, S, L, U, and M, showed high activity to inhibit PTP1B and α-glucosidase (El Ridhasya et al., 2020; Shah et al., 2020). In addition, the roots have the potential as antioxidants (Chen et al., 2003), anti-osteoporosis (Huang et al., 2017), anticancer (Tsai et al., 2021), anti-inflammatory (Chen et al., 2014; 2017), and immunomodulatory (Chen et al., 2014).

H. zeylanica is reportedly one of the treatments for dysentery and infections caused by food-borne bacteria in the eastern regions of India, as stated by a publication that was reported by Mandal and Mondal (2011). In addition, in Bangladesh, this species is utilized in conjunction with *Lygodium flexuosum* in order to treat fever caused by microbial infections (Sarker et al., 2012). However, despite the fact that this plant species is utilized in the treatment of microbial infections in a number of different countries, there has been very little extensive research conducted on the antimicrobial properties of this species, particularly in relation to food-borne microbes. In this study, we describe the details of the species' extraction, including the production of sub extracts ranging from less to more polar, as well as the antibacterial activity. Yenn et al. (2018) found that the MIC and MBC values of the ethanolic extract from the frond of the species with antibacterial activity against *Bacillus cereus* were 6.25 mg/mL and 12.5 mg/mL, respectively. These values were discovered for the ethanolic extract of the species' frond. Even though it has been previously reported that the extract has antibacterial activity, only one pathogenic bacterium was used in this study. In this study, we report the antibacterial activity of various extracts of the roots of this species against a broad spectrum of pathogenic bacteria, including *Vibrio* and *Listeria* species. In relation to the planta pharmacological claims, previous molecular chemotypes derived from this species were also discussed.

MATERIAL AND METHODS

Plant material

The species was collected from the Sungai Kampar in the Indonesian province of Riau, in the town of Kampar (0°09'23.6"N 101°21'54.0"E). A voucher specimen (No. 67/UN19.5.1.1.3/2019) was identified as *Helminthostachys zeylanica* (L.) Hook and deposited by a botanist (Prof. Dr. Fitmawati) with the Department of Biology at Universitas Riau.

Extraction

The species was cleaned, and its roots, stems, leaves, and spores were separated and put in the fridge. Maceration in methanol (1000 mL) of up to 500

g for a total of 24 h was repeated three times until the results of the maceration were no longer green. After the maceration process, the extract was put through a filter, and the filtrate was saved. The macerate was then concentrated with a rotary evaporator set to 40°C and produced crude methanol extract. The liquid-liquid extraction was then used to separate the crude methanol extract in order to obtain *n*-hexane, dichloromethane, ethyl acetate, and water extracts (Afham et al., 2022; Khodijah et al., 2022; Hendra et al., 2020).

Antibacterial activity

Eight microorganisms, namely *Bacillus subtilis* ATCC 1965, *Staphylococcus aureus* ATCC 6538, *B. cereus* ATCC 10876, *Vibrio parahaemolyticus* ATCC 17802, *V. alginolyticus ATCC17749, Listeria monocytogenes* ATCC 7644, *Escherichia coli* ATCC 8739, and *Salmonella typhimurium* ATCC 142028. were used in this study. The microbial isolates were maintained on an agar slant at 4°C in the Laboratory of Biochemistry, Department of Chemistry, Universitas Riau. The strains were subcultured on fresh agar plates for 24 h before any antimicrobial tests were performed.

The antibacterial activity was determined using the Kirby-Bauer technique and chloramphenicol as a positive control. The final concentration of each extract was 500 ppm in 10% DMSO, and the concentration of the bacterial suspension was standardized to 0.1 McFarland. A volume of 10 µL of extract solution, positive control, and negative control was dropped onto paper discs that were placed on the surface of 100 µL of bacterial suspension-supplemented Mueller–Hinton agar (MHA). A 24-h incubation was performed at 37°C. Observations were made by observing and measuring the formed clear zone. The experiment was repeated three times (Hendra et al., 2022).

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

The MIC and MBC of the extracts were done using the method described in Loo et al. (2018) with modification. The MIC test was carried out in a 96-well round bottom microtiter plate using the standard broth microdilution methods, whereas the MBC test was carried out on MHA plates. Inoculums of bacteria were diluted to a concentration of 10^6 CFU per milliliter of solution. For the MIC test, 100 µL of the extracts stock solution (500 µg/mL) was added, and then it was diluted two-fold with the bacterial inoculums in 100 µL of MHB (Mueller–Hinton broth). This was done starting from column 12 and going all the way down to column 3. In the microtiter plate, the highest concentration of the extracts was found in column 12, while the lowest concentration was found in column 3. Column 1 was the control for the negative variable (only medium), and column 2 was the control for the positive variable (medium and bacterial inoculums). After 24 h of incubation at 37°C, the plate was checked to see if there was a change in the density of each well. The minimum inhibitory concentration (MIC) was determined by testing at the lowest concentration of antibacterial agents known to stop the growth of bacteria. The minimal bactericidal concentration, or MBC, refers to the lowest concentration of antibacterial agents that is capable of eradicating bacteria in their entirety. In order to carry out the MBC test, the suspension from each well of the microtiter plates was plated onto the MHA plate. The plates were kept in an incubator at 37°C for 24 h. The MBC value was determined by using the lowest concentration at which there were no visible growths on the MHA plate.

Statistical analysis

All tests were performed in triplicate, and the results are expressed as mean ± SD. Data were analyzed using GraphPad Prism Version 9, and comparisons of the means were determined by one-way analysis of variance (one-way ANOVA) followed by the Tukey test. Values with $p<0.05$ were considered statically significant.

RESULTS

Prior to the extraction of secondary metabolites, it is crucial to treat the species, as this impacts the extracted bioactive compounds. Because the compounds in the sample can be rapidly degraded by oxidative, enzymatic, or polymerization processes, the fresh roots were used in this study to determine antibacterial activity (Yenn et al., 2018). The proper selection of an extraction solvent is necessary in order to isolate the desired bioactive compounds from the plant sample. According to Tong et al. (2014), the type of natural product that can be extracted and, as a result, the biological activity of the crude extract are both impacted by the organic solvents used. In this study, the species were extracted with different polarities of organic solvents (n-hexane, dichloromethane, and ethyl acetate) to separate the hydrosoluble molecules from the liposoluble ones.

The antibacterial activity of various extracts from the root of *H. zeylanica* was tested against eight pathogenic bacteria: *B. subtilis* ATCC 1965, *S. aureus* ATCC 6538, *B. cereus* ATCC 10876, *V. parahaemolyticus* ATCC 17802, *V. alginolyticus ATCC17749, L. monocytogenes* ATCC 7644, *E. coli* ATCC 8739, and *S. typhimurium*

ATCC 142028. Table 1 provides a summary of the disk diffusion assessment of the extracts. For the disk diffusion, the presence of a clear zone around the extracts disk suggested that the extracts possessed various antibacterial activity that was capable of inhibiting the growth of tested bacteria. The antibacterial activity of the extracts revealed that ethyl acetate and dichloromethane extracts possess intermediate susceptibility antibacterial activity against Gram-positive and negative bacteria except for *E. coli*, whereas water extract demonstrates no growth inhibition activity (Table 1).

The disk diffusion method was described as having been used in this study as a preliminary test to determine the antibacterial activity of an antimicrobial agent. As a result, it was necessary to conduct additional research in order to determine the antibacterial activity of the extracts, particularly dichloromethane and ethyl acetate extracts, by calculating their Minimum Inhibitory Concentration (MIC) values (Burt, 2004). Through the use of serial dilution, we were able to determine the minimal inhibitory concentration (MIC), which was defined as the lowest concentration of the antibacterial agent that could inhibit the growth of bacteria. The minimum inhibitory concentration (MIC) of ethyl acetate and dichloromethane extracts against the tested bacteria ranged from 125 to 500 µg/mL, as indicated in Table 2.

The minimum inhibitory concentration (MIC) for dichloromethane extract against *B. subtilis* ATCC 1965 was 250 µg/mL and 125 µg/mL against *V. parahaemolyticus* ATCC 17802, whereas the MIC for ethyl acetate extract was greater than 500 µg/mL for all bacteria. In addition, the extracts were subjected to a test that determined the minimum bactericidal concentration (MBC), which is also referred to as the minimum lethal concentration (MLC). The MBC is the lowest antimicrobial agent concentration required to kill 99.9 percent of the final inoculum following an incubation period of 24 h (Balouiri et al., 2016). In this investigation, the MBC value for *B. subtilis* and *V. parahaemolyticus* was found to be 250 µg/mL when exposed to dichloromethane extract. On the other hand, the MBC value for all bacteria in the ethyl acetate extract was found to be greater than 500 µg/mL.

Based on the inhibition zone category, dichloromethane and ethyl acetate extracts are categorized as intermediate potential in their ability to suppress bacterial growth. Dichloromethane extract inhibited *Staphylococcus aureus* more than any other, while ethyl acetate extract inhibited *Vibrio parahaemolyticus* the most. This demonstrated that the dichloromethane and ethyl acetate extracts of the species' roots contained secondary metabolites with antibacterial properties. Huang et al. (2009) have previously discovered that the roots of the species were abundant in flavonoids such as ugonin, prenylated flavonoids, and quercetin. Our previous study successfully isolated prenylated flavonoids, ugonin J and K, from dichloromethane and ethyl acetate extracts (El Ridhasya et al., 2020). Studies have demonstrated that quercetin possesses broad-spectrum antibacterial properties. It has not only a strong inhibitory effect on bacteria, but also a significant inhibitory effect on fungi. Multiple

Table 1. Antibacterial activity of *H. zeylanica* (L.) Hook. root extracts at 500 µg/disc.

Microorganism	Diameter of inhibition zone (mm)					
	Extracts				DMSO	CFN
	n-Hexane	Dichloromethane	Ethyl acetate	Water		
Gram-positive						
Bacillus subtilis ATCC 1965	0.00 ± 0^{c}	**11.6 ± 0.20^{b}**	**11.6 ± 0.45^{b}**	0.00 ± 0^{c}	0.00 ± 0^{c}	30.6 ± 0.46^{a}
Bacillus cereus ATCC 10876	6.2 ± 0.48^{d}	**10.4 ± 1.48^{b}**	9.0 ± 2.21^{c}	0.00 ± 0^{e}	0.00 ± 0^{e}	26.4 ± 0.20^{a}
Staphylococcus aureus ATCC 6538	0.00 ± 0^{d}	**18.9 ± 1.31^{b}**	9.7 ± 0.71^{c}	0.00 ± 0^{d}	0.00 ± 0^{d}	29.4 ± 0.75^{a}
Listeria monocytogenes ATCC 7644	0.00 ± 0^{d}	**10.6 ± 1.99^{b}**	8.9 ± 0.58^{c}	0.00 ± 0^{d}	0.00 ± 0^{d}	24.1 ± 0.25^{a}
Gram-negative						
Vibrio parahaemolyticus ATCC 17802	0.00 ± 0^{d}	10.1 ± 0.53^{c}	**13.5 ± 1.71^{b}**	0.00 ± 0^{d}	0.00 ± 0^{d}	29.3 ± 0.97^{a}
Vibrio alginolyticus ATCC17749	8.5 ± 0.31^{c}	**11.3 ± 0.58^{b}**	10.2 ± 1.54^{b}	0.00 ± 0^{d}	0.00 ± 0^{d}	22.8 ± 0.58^{a}
Salmonella typhimurium ATCC 142028	7.8 ± 0.92^{d}	**11.0 ± 0.60^{b}**	9.1 ± 1.67^{c}	0.00 ± 0^{e}	0.00 ± 0^{e}	27.1 ± 0.35^{a}
Eschericia coli ATCC 8739	0.00 ± 0^{b}	0.00 ± 0^{b}	0.00 ± 0^{b}	0.00 ± 0^{b}	0.00 ± 0^{b}	29.1 ± 0.48^{a}

$^{a-e}$ Means ± SD in the same row with different letters in superscript indicate statistically significant differences ($p<0.05$). **Bold:** the highest inhibition on each indicator microorganism. CFN: Chloramphenicol (20 µg/disc).

Table 2. MIC and MBC values of dichlormethane and ethyl acetate extracts of *H. zeylanica* (L.) Hook.

Microorganism	MIC (µg/mL)			MBC (µg/mL)		
	Dichloromethane	Ethyl acetate	CFN	Dichloromethane	Ethyl acetate	CFN
B. subtilis ATCC 1965	250	>500	16	250	>500	16
B. cereus ATCC 10876	>500	>500	16	>500	>500	32
S. aureus ATCC 6538	>500	>500	64	>500	>500	64
L. monocytogenes ATCC 7644	>500	>500	32	>500	>500	64
V. parahaemolyticus ATCC 17802	125	>500	8	250	>500	8
V. alginolyticus ATCC17749	>500	>500	8	>500	>500	8
S. typhimurium ATCC 142028	>500	>500	4	>500	>500	8

CFN: Chloramphenicol.

experiments have demonstrated that quercetin inhibits the growth of pathogenic bacteria, including *Staphylococcus aureus*, *Escherichia coli*, *Listeria*, and *Salmonella species*. Quercetin's antibacterial mechanism consists primarily of destroying the bacterial cell wall and altering cell permeability, as well as affecting protein synthesis and expression, reducing enzyme activities, and inhibiting nucleic acid synthesis (Yang et al., 2020). Furthermore, the antibacterial activities of the extract are due to the presence of prenylated flavonoids, as mentioned above. Shah et al. (2020; 2022) reported that six of the ugonins isolated from this species could inhibit bacterial neuraminidase and that Ugonin J could block the biofilm formation of *E. coli* in a dose-dependent manner up to 150 µM without inhibiting the bacteria.

CONCLUSION

This study aimed to investigate the antibacterial potential of various extracts of *H. zeylanica* roots against a wide variety of pathogenic bacteria. According to the findings, the dichloromethane and ethyl acetate extracts displayed significant inhibitory activity against several different bacteria, such as *B. subtilis*, *S. aureus*, and *L. monocytogenes*. It indicates that *H. zeylanica* might be a good candidate for use as a source in the research and development of new antimicrobial agents. However, additional research is required to determine the active compounds accountable for the observed effects.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Army MK	Khodijah RK	Haryani YH	Teruna HY	Hendra RH
Concepts or ideas				x	x
Design					x
Definition of intellectual content				x	
Literature search	x	x			
Experimental studies			x		x
Data acquisition	x	x			
Data analysis	x		x		
Statistical analysis			x		
Manuscript preparation	x				x
Manuscript editing		x		x	
Manuscript review	x	x	x	x	x

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