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Full Length Article

Antibacterial Activity of Methyl Cinnamate, Cinnamic Acid and Cinnamic Acid Derivatives Derived from *Alpinia malaccensis* Rhizomes

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Abstract

Alpinia malaccensis L. contains the largest phytochemical component, methyl cinnamate. The base hydrolysis of methyl cinnamate resulted cinnamic acid compound. In this study, were carried out isolation of methyl cinnamate, hydrolysis of methyl cinnamate into cinnamic acid and modification cinnamic acid through esterification, amidation, and substitution on the benzene ring. The synthesized compounds were characterized using ^{1}H-NMR and ^{13}C-NMR. The ester of 2-methoxyphenyl cinnamate, the amide of 1-cinnamoylpyrrolidine and methyl-4-nitro cinnamate, as well as methyl-2-nitro cinnamate were identified. These compounds were tested for antibacterial activity against five bacterial strains: *E. coli, P. aeruginosa, B. substilis, S. aureus*, and *MRSA*. The antibacterial activity test determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the characterised compounds. Compound 1-cinnamoylpyrrolidine exhibited the strongest MIC and MBC values of 0.5 mg/mL against all bacterial strains. © 2023 Friends Science Publishers

Keywords: *A. malaccensis*; Methyl cinnamate; Cinnamic acid; 1-cinnamoylpyrrolidine; MIC; MBC

Introduction

Infectious diseases caused by microorganisms such as bacteria and fungi have become a major global health problem, especially in developing and low-income countries (Bygbjerg 2012; Christaki *et al.* 2020). Excessive of drugs, inappropriate consumption of drug and lack of hygiene are causes of bacterial resistance (Australian Government 2022). Bacteria are increasingly developing resistance to drugs, causing a problem in medicine. This rise in resistance microbial species to synthetic antimicrobial agents has emphasized the importance of further investigating new antimicrobial agents.

Cinnamic acid is a natural aromatic carboxylic acid that has many potential activities, which is one antibacterial (Cai *et al.* 2018; Deng *et al.* 2018; Yilmaz *et al.* 2018; Yue *et al.* 2021). Studies on cinnamic acid or cinnamic acid derivatives and their antibacterial activity have been widely reported (Deng *et al.* 2017; Doria *et al.* 2019; Liang *et al.* 2021; Manogaran *et al.* 2023). Cinnamic acid can be derived from natural plant isolation (Tawata *et al.* 1995; Nakazono *et al.* 2006; Elzaawely *et al.* 2007). The isolation cinnamic acid from natural plants result in small yield, only 2.2% (Julianus and Luckyvano 2014). However, research (Ernawati *et al.* 2013) reported result of isolating cinnamic acid from the hydrolysis methyl cinnamate found in *A. malaccensis* L. rhizomes with large yield, this is 85%. according to (Azah *et al.* 2005; Ernawati *et al.* 2011; Sirat *et al.* 2011), stated that the methyl cinnamate is the largest component in *Alpinia malaccensis* and *Alpinia galangal* (Rao *et al.* 2010). Methyl cinnamic is also found in *Ocimum Basilicum* (Sitrallah and Merza 2016; Azhari *et al*. 2022), *Ocimum gratissimum* L. (Prakash *et al.* 2011).

Cinnamic acid derivatives can be obtained by modifying the chemical structure of cinnamic acid. This can be done through esterification, amidation, and substitution on the benzene ring. The purpose of this modification is to increase the activity of the cinnamic acid derivative. This study aims to determine the antibacterial activity of methyl cinnamate, cinnamic acid and cinnamic acid derivatives derived from *Alpinia malaccensis* L. rhizomes.

Materials and Methods

Alpinia Malaccensis L., NaOH, H_2SO_4, HNO_3, $SOCl_2$, Guaiacol, pyrrolidine 99%, organic solvents (CH_3OH, C_2H_5OH, $CH_3COOC_2H_5$ and C_6H_{14}), Na_2SO_4 anhydrous, H_2O, DMSO, silica gel, TLC plat GF_{254}, Strain of bacteria *E. colli, P. aeruginosa, B. substilis, S. aureus* dan *Methicillin-Resistant Staphylococcus aureus.* Tetrasiklin (sigma), NaCl (E-Merck), BHI Agar HIMEDIA, standar Mc-Farland HIMEDIA. Instruments, the equipment utilized comprised a

fume hood, spherical flask, stirrer, hot plate, column chromatography, rotary evaporators, UV light, Eppendorf pipette, 96-well plate, ^{1}H-NMR (500 MHz) and ^{13}C-NMR spectrometer (500 MHz), as well as other chemical glassware.

Isolation of methyl cinnamate

The isolation of the methyl cinnamate (crystal) was conducted according method in patent number: P000070292 (Ernawati *et al*. 2013). Methyl cinnamate was obtained from *Alpinia malaccensis* oil through steam distillation. The *Alpinia malaccensis* oil was separated using ethanol as a solvent at 20°C and atmospheric pressure, the separation process involves a gradual crystallization. The methyl cinnamate crystals are then isolated from the solvent and other impurities through filtration. Finally, the methyl cinnamic compound dried and formed needle-shaped crystals.

Hydrolysis of methyl cinnamate to cinnamic acid

Methyl cinnamate (61.7 mmol) was dissolved in 100 mL of ethanol in the reaction flask. 3 M NaOH solution 20 mL was added, and heated at 80°C for 1.5 h. After the reaction is complete, it is then neutralized with 2N HCl. After neutralization, it was extracted thrice with a mixture of water and ethyl acetat in a 1:1 ratio (300 mL). The ethyl acetate extract was separated from the aqueous extract, and then dried using a rotary evaporator to obtain crystals of cinnamic acid. The formation of cinnamic acid was preliminarily identified using TLC with a ethyl acetat and hexane eluent in a ratio of 2:3.

Synthesis of the ester compound, 2-methoxyphenyl cinnamate (E) and the amide compound 1-cinnamoylpyrrolidine (A)

Cinnamic acid (3 mmol) was added to $SOCl_2$ (10 mL, 10 mmol) and reacted on an oil bath at 50°C with reflux method for 4 h. After reaching room temperature, Guaiacol (3.6 mmol) for esterification and pyrrolidine (3.6 mmol) for amidation was added. The TLC was used to monitor the progress of the reaction and the reaction was stopped when the cinnamic acid spot had completely reacted. The resulting reaction product was extracted three times using a 1:1 ratio of ethyl acetate to water (100 mL each time). The ethyl acetate fraction was then evaporated using a rotary evaporator and *subsequently purified using a silica gel chromatography column with a gradient method employing hexane and ethyl acetate as mobile phases. TLC was utilized to check every fraction and monitor the elution of the targeted compound. The pure compound was analyzed using ^{1}H-NMR and ^{13}C-NMR, to determine its structure.

Synthesis of methyl 4-nitrocinnamate and methyl 2-nitrocinnamate

Methyl cinnamate (30 mmol) was added to concentrated H_2SO_4 (60 mmol) and stirred at room temperature for 5 min. HNO_3 (60 mmol) was slowly added. The reaction product was extracted three times with a mixture of ethyl acetate and water in a 1:1 ratio (100 mL). The ethyl acetat fraction was evaporated using a rotavaps and purified using a silica gel chromatography column, followed by preparative TLC to separate the two compounds resulting from the silica gel column chromatography. The pure compound was characterized using ^{1}H-NMR and ^{13}C-NMR, to determine its structure.

Antibacterial analysis

Bacterial preparation: The bacterial strains were obtained from the Microbiology and Biochemistry laboratory at the Research Center for Pharmaceutical Ingredients and Traditional Medicine, BJ Habibie sains and technology area, Serpong. These strains *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 9027), *B. subtilis* (ATCC 6633) and *S. aureus* (ATCC 25923). Additionally, clinical isolates of *methicillin-resistant Staphylococcus aureus* bacteria were obtained from the central general hospital (RSUP) dr. Kariyadi, Semarang. Prior to testing, the bacteria were cultured on nutrient agar media and incubated for 24 h.

MIC and MBC determined

The sample was dissolved in dimetil sulfoksida (DMSO) solvent to achieve a concentration of 40 mg/mL. In hole A of wellplate 96, 20 μL of the sample and 180 μL of MHB (Mueller Hinton Broth) media were added. Holes B to H were each filled with 100 μL of MHB media. The concentration is dilluted from hole A to hole H, so that the concentration in each hole is half and 100 μL of sample solution in well H was discarded. The same procedure was followed for the positive control tetracycline and negative control DMSO. The bacteria were cultured on *Nutrient Broth* media for 24 h in a shaking incubator at 120 rpm to prepare each bacterium. The bacterial culture was then dissolved in 0.85% NaCl solution and adjusted to the Mc-Farland 0.5 standard, which is equivalent to 1 x 10^8 CFU/mL. Next, the bacterial solution diluted to reach a concentration of 1×10^6 CFU/mL. Subsequently, 100 μL of this bacteria solution was placed into each of the holes, making the total test volume in the holes 200 μL. The wellplate is sealed, it was incubated and shaken for 24 h at 37°C. To determine the MBC value, 10 μL of the sample with a MIC value were cultured on MHA media and incubated at 37°C for 24 h (Priyanto *et al*. 2022).

Results

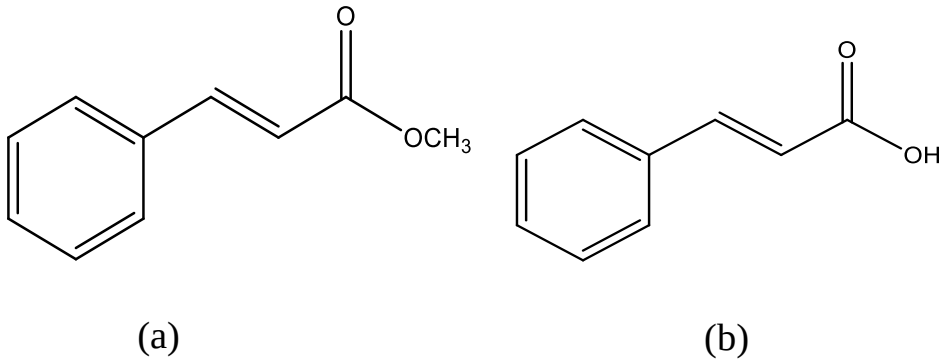
Isolating of methyl cinnamate and hydrolysis methyl cinnamate to cinnamic acid

The crystallization process of *Alpinia malaccensis* oil resulted in a 55% production of methyl cinnamate compounds. The pure cinnamic acid compound from the

Table 1: Minimum inhibitory (MIC) and bactericidal (MBC) concentrations of different characterized compounds

Compound	MIC (mg/mL) / MBC(mg/mL)				
	E.coli	*P. aeruginosa*	*S. aureus*	*B. substilis*	*MRSA*
MC	>4 / >4	>4 / >4	>4 / >4	2 / >4	>4/ >4
CA	1 / > 2	1 / > 2	**0,5 / >1**	**0,5 / >1**	1 / >2
E	2 / >2	2 / >2	2 / >2	2 / >2	1 / 2
A	**0,5 / >0,5**	**0,5 / 0,5**	**0,5 / 0,5**	**0,5 / 0, 5**	**0,5 / 0,5**
S1	1 / >2	1 / >2	1 / >2	1 / >2	1 / >2
S2	1 / >2	1 / >2	1 / >2	1 / >2	1 / >2
Tetrasiklin	0,0039 / 0,0039	0,0039 / 0,0039	0,0039 / 0,0039	0,0039 / 0,0039	0,0039 / 0,0039
DMSO	-	-	-	-	-

Note: "-" has no antibacterial activity



(a) (b)

Fig 1: The chemical structure of methyl cinnamate (a) and cinnamic acid (b) (Meilawati *et al.* 2013)

hydrolysis of methyl cinnamate was obtained as a white powder with a yield of 90%.

Characterization of isolated and synthesized compounds (Fig. 1)

(E)-Methyl cinnamate: White solid δH (500 MHz, $CDCl_3$) 7.70 (d, *J*=16, 1H), 7.53 (d, *J*=6, 2H), 7.38 (t, *J*=6.5, 3H), 6.44 (d, *J*=16, 1H), 3.81 (s, 3H). δC (500 MHz, $CDCl_3$) 167.59, 117.95, 145.03, 134.52,130.45, 129.04, 128.23, 51,86 ppm.
Cinnamic acid: White solid δH (500 MHz, $CDCl_3$) 7.81 (d, *J*=16, 1H), 7.56 (d, *J*=7.5, 2H), 7.41 (t, *J* =7, 3H), 6.47(d, *J*=16, 1H). δC (500 MHz, $CDCl_3$) 172.61, 117.49, 147.29, 134.22, 130.95, 129.16, 128.56 ppm.
2-Methoxyphenyl cinnamate (E): White solid δH (500 MHz, $CDCl_3$) 7.89 (d, *J*=11.5, 1H), 7.59 (m, *J*=6.5, 2H), 7.42 (t, *J*=4.5, 3H), 7.23 (t, *J*=5.5, 1H), 7.125 (dd, *J*=7, 1H), 7.06 (m, *J*=4, 2H), 6.68 (d, *J*=11.5 1H), 3.85(s, 3H). δC (500 MHz, $CDCl_3$) 165.17, 151.46, 146.71, 139.98, 134.47, 130.78, 129.14, 128.48, 127.06, 123.13, 120.99, 112.66, 56.08 ppm.
1-cinnamoylpyrrolidine (A): Yellow oily. δH (500 MHz, $CDCl_3$) 7.69 (d, *J*=15.5, 1H), 7.53 (d, *J*=6.5, 2H), 7.35 (m, *J*=6, 3H), 6.73 (d, *J*=15.5, 1H), 3.63 (tt, 2H), 3.58 (tt, 2H), 2.00 (p, 2H), 1.89 (p, 2H). δC (500 MHz, $CDCl_3$) 164.90, 118.93, 141.90, 135.46, 129.70, 128.93, 128.0, 47.77, 46.24, 26.29, 24.51 ppm.
Methyl 4-nitrocinnamate (S1): White solid**. (**δH (500 MHz, $CDCl_3$) 8.25 (d, *J*=8.5, 2H), 7.72 (d, *J*=16, 1H), 7.67 (d, *J*=8.5, 2H), 6.56 (d, *J*=16, 1H), 3.84 (s, 3H). δC (500 MHz, $CDCl_3$) 164.90, 142.11, 128.84, 128.37, 124.37, 123.88, 122.26, 52.78 ppm.
Methyl 2-nitrocinnamate (S2): White solid**.** δH (500 MHz, $CDCl_3$) 8.25 (dd, *J*=8.1H), 8.11 (d, *J*=16, 1H), 7.67 (d, *J*=8.5, 1H), 7.63 (m, *J*=8.5, 2H), 7.54 (m, *J*=8, 1H), 6.36 (d, *J*=15.5, 1H), 3,82 (s, 3H). δC (500 MHz, $CDCl_3$) 166.39, 148.49, 140.34, 133.71, 129.74, 125.09, 123.25, 52.19 ppm.

As presented in Table 1, the methyl cinnamate compound has a MIC value of > 4 mg/mL against *E. coli and P. aeruginosa* bacteria (gram-negative bacteria) and *S. aureus* and *MRSa* (gram-positive bacteria), except for *B. substilis* with a MIC of 2 mg/mL. The MBC value of methyl cinnamate against all bacterial strains is more 4 mg/mL. Its mean, at a concentration of 4 mg/mL, did not exhibit bactericidal activity against the test bacteria. Cinnamic acid can inhibit the growth of *E. coli* and *P. aeruginosa* bacteria as well as *MRSA* bacteria at a concentration of 1 mg/mL and effectively kill these bacteria at concentrations more 2 mg/mL. Meanwhile, for *B. substilis* and *S. aureus* bacteria, the MIC of cinnamic acid is 0.5 mg/mL and concentrations above 1 mg/mL can kill these bacteria.

Compound A has a strongest inhibit among all compounds, with an MIC value of 0.5 mg/mL and an MBC value at a concentration of 0.5 mg/mL against all bacterial strains, except for *E. coli*, which had a MBC value at a concentration more than 0.5 mg/mL. Compound A has stronger antibacterial activity compared to cinnamic acid

and methyl cinnamate. Compound E showed the strongest MIC only against *MRSA* strains, with an MIC of 1 mg/mL and a MBC of 2 mg/mL. Compounds S1 and S2 inhibited all bacterial strains at a minimum concentration of 1 mg/mL and could kill all bacterial strains at concentrations above 2 mg/mL. In terms of antibacterial activity, compounds S1 and S2 has the same effectiveness as cinnamic acid against *E. coli, P. aeruginosa* and *MRSA* bacteria, but were weaker against *B. subtilis* and *S. aureus* bacteria.

Discussion

The process of isolating methyl cinnamate crystals was achieved through extraction, crystallization and fragmentation of *A. malaccensis* L. oil. The crystallization of *A. malaccensis* oil involved the supercooling method, which creates a low temperature in the oil collection. Supercooled crystallization is a simple and effective cooling method for producing high-quality crystals (Hojjati and Rohani 2005). As the solutions temperature decreases, components with higher freezing points will freeze first, while other substances remain soluble and can be separated through filtration. The filtered substances will dissolve and collect as the filtrate, while the solid residue remains on the filter. Other substances will dissolve and pass through as filtrate, while the solids remain on the filter as residue. In this process, ethanol serves as the solvent to reduce viscosity (Ahmadi and Estiasih 2011). Low viscosity causes mass transfer to be easy so that the crystallization process is efficient. The hydrolysis of methyl cinnamate was conducted using NaOH as a catalyst and ethanol 95% as a solvent. This reaction proceeded smoothly for 150 min. Initially, qualitative identification of the hydrolysis reaction products was performed using TLC (Thin Layer Chromatography) with ethyl acetat and n-hexane eluents in a 2:3 ratio.

The antibacterial activity of a compound is affected by several factors, including the molecular structure of compound, the solvent and the type of test bacteria (Parekh *et al.* 2005). The molecular structure of compound is related with its lipophilicity and steric effect. Compounds with high lipophilicity can easily penetrate the bacterial cell membrane, potentially causing cell lysis or damage (Kepa *et al.* 2018). Compound A has a higher lipophilicity value compared to cinnamic acid and methyl cinnamic, suggesting stronger inhibition of bacterial growth with a lower MIC value. However, this is not true for compound E, which has a higher MIC value than cinnamic acid. It is assumed that the low activity of compound E is not due to its lipophilic value. because based on the prediction of chemdraw ultra 12.0 the logP value of compound E is greater than compound A and cinnamic acid. Compounds S1 and S2 have logP values greater than methyl cinnamate, in line with their MIC values, the activity of compounds S1 and S2 is stronger than the methyl cinnamate. (LogP of compound E: 3.73; compound A: 2.07; compound S1 and S2: 2.39; Cinnamic acid: 1.93; Methyl cinnamate c: 2.2).

The addition of a nitro group to the methyl cinnamate enhances its antibacterial effectiveness, acording research (Tonari *et al.* 2002) stated that substituting the benzene group in cinnamic acid derivatives affects antibacterial activity. Nitrocinnamate compounds exhibit MICs of 89.1 and 79.4 μg/mL against *B. substilis* and *E. coli* bacteria, whereas cinnamic acid has MICs of 89.1 and 100 μg/mL.

The study examined different bacteria types, Gram negative bacteria (*E. coli* and *P. aeruginosa*) and Gram positive bacteria (*B. subtilis, S. aureus,* and *Methicillin-resistant Staphylococcus aureus)*. Differences in bacterial cell wall structure might affect the MIC and MBC values. Cinnamic acid exhibited stronger inhibition against Gram-positive bacteria than Gram-negative bacteria, as evidenced by its MIC value against *S. aureus* and *B. subtilis*, at 0.5 mg/mL and MBC > 1 mg/mL. whereas for *E. colli* and *P. aeruginosa* bacteria, cinnamic acid can inhibit bacterial growth 1 mg/mL and can kill bacteria at concentrations > 2 mg/mL. Gram-positive bacteria have a cell wall is composed of peptidoglycan and teichoic acid. In contrast, Gram-negative bacteria contain small amounts of peptidoglycan, lipoprotein, phospholipids, and lipopolysaccharide. Differences in the structure of the bacterial cell wall, cause differences in resistance to antibacterial compounds. Gram-positive bacteria are more susceptible to antibacterial compounds (Rini and Rochmah 2020). According to Hamidah *et al.* (2019), the cell wall of *E. coli* is more complex, making it harder for antibacterial compounds to penetrate. On the other hand, the cell wall structure of *S. aureus* bacteria is relatively simpler, allowing the penetration of antibacterial compounds into cells is easily. Compound E has stronger inhibitory activity against *MRSA* bacteria compared to *E. coli* and *P. aeruginosa* bacteria.

In this study, Tetracycline was used as the standard for antibacterial testing. Tetracycline exhibited minimal inhibition at a concentration of 0.0039 mg/mL, effectively eliminating all bacterial strains at that concentration. On the other hand, DMSO, being a solvent, does not have any inhibitory effect on bacteria. This indicates that DMSO does not effect on the antibacterial activity.

Conclusion

Methyl cinnamate was successfully isolated from the *Alpinia malaccensis* rhizomes resulting in a high yield. Base hydrolysis has been carried out to obtain cinnamic acid compounds and derivatization of cinnamic acid has been carried out to produce derivatives. Cinnamic acid has stronger antibacterial activity than methyl cinnamate compounds. The derivative 1-cinnamoyl pyrrolidine, which is a cinnamic acid derivative, exhibit inhibitory activity against *E. coli, P. aeruginosa, S. aureus, B. subtilis*, and *methicillin-resistant S. aureus* bacteria with MIC and MBC values of 0.5 mg/mL.

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Author Contributions

All authors contributed equally to this work and have given permission to publish it.

Conflict of Interest

All authors declare no conflicts of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable in this paper.

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