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Submission date: 29-Oct-2019 07:21PM (UTC-0700)
Submission ID: 1203230975
File name: Isolation_of_antibacterial_compounds_from_endophyte_fungal.pdf (947.07K)
Word count: 3773
Character count: 18158

Yuyun Yuniati et al /J. Pharm. Sci. & Res. Vol. 10(2), 2018, 260-264

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Isolation of antibacterial compounds from endophyte fungal of fusarium sp. In phyllanthus niruri linn. Leaves

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Abstract

Endophyte fungal was alternative source of antibacterial metabolites derived from plant. Diethyl ether fraction of ethyl acetate extract of endophyte fungal genus Fusarium sp. of meniran leaves (Phyllanthus niruri Linn.) proved have antibacterial activity. This study aims to explore the active antibacterial compounds. IR, LC-MS, 1H-NMR, 13C-NMR, DEPT, and HMQC spectra showed 1.1 and 2.2 are aliphatic carbonyl substituted compounds. Isolate 1.1 has antibacterial activity against Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus mutans, and Salmonella typhi with IC50 values of 1.45 each: 2.19; 2.33; 0.98; 0.56; 1.34 µg/mL and MBC values of each 20.00; 20.00; 40.00; 40.00; 10.00; 10.00 µg/mL. Isolate 2.2 has antibacterial activity against Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus mutans, and Salmonella typhi with IC50 values of 3.38; 2.45; 2.98; 1.35; 2.88; 2.62 µg/mL and MBC values of each 40.00; 40.00; 40.00; 40.00; 20.00; 20.00 µg/mL.

Keywords: Endophytic fungi, Fusarium sp., Structure elucidation, IC_{50}, MBC

Introduction

Antibiotic resistance causes modification in pathogen development so that being resistant to one or more kind of antibiotics. For example, the development of *Salmonella typhimurium* and *Salmonella kentucky* is being resistant to cephalosporin [1], *Staphylococcus aureus* that was resistant to methicillin or MRSA (*Methicillin resistant Staphylococcus aureus*) [2], and a resistance of *Pseudomonas aeruginosa* to antibiotic products such as gentamicin, tobramycin, and amikacin or MARPA (*multiple antibiotic-resistant Pseudomonas aeruginosa*) [3]. Therefore, a study to find new antibiotic substance through chemical and biochemical synthesis or a discovery of active isolate from microorganism was needed.

Endophyte fungal was kind of fungi that live inside the plant tissue without causing any negative effect to its host. The association between Endophyte fungal and its host creates a mutualistic interaction such as a protection from herbivores, insects, and pathogens [4]. Endophyte fungal produces a lot of bioactive substances such as alkaloid, terpenoid, fenolic, etc. [5]. For example, endophyte fungal *Chaetomium globosum* produces apigenin, an antioxidant substance, that is also produced by its host, *Cajanus cajan* (L.) Millsp [6]. Ginkgolide B, an antiplatelet agent, was produced by endophyte fungal *Fusarium oxysporum* and *Ginkgo biloba* (the host for *Fusarium oxysporum*) [7].

The result of a study conducted by Rollando *et al* [8] stated that fungal isolate from *Fusarium* sp. genus had an antibacterial activity to *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella thypi*, dan *Pseudomonas aeruginosa*. The researcher also reported that diethyl ether fraction contains active metabolites from phenylpropanoid or polyketide class that have ketone group and ortho hydroxy group or ortho hydroxy carbonyl and triterpene steroid compound. That result could be used as the foundation for isolation and identification of active compound as an antibacterial derivate from endophyte fungal *Fusarium* sp. through the determination of IC_{50}, IC_{90}, dan LC value of the active compound.

Thus, this research was hoped to be the base in the utilization of *Fusarium oxysporum* endophyte fungal as an alternative natural antibiotic.

Experimental Section

Materials

The ingredients were endophyte fungal from *Fusarium* sp. genus, fungal growth media such as PDA (*Potato Dextrose Agar*), PDB (*Potato Dextrose Broth*), Muller Hinton, NA (*Nutrient Agar*), and NB (*Nutrient Broth*). Tested microbes included *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella thypi*, *Streptococcus mutans*, *Pseudomonas aeruginosa* and streptomycin as positive control. Silica gel F_{254} plate (E. Merck, Jerman), dan Silica gel 60 PF_{254} were used for preparative TLC. Extraction solvent, fermented extract, and eluent for separation and purification of aquadest, methanol, n-hexane, chloroform and ethyl acetate (Dannstat, Jerman).

Instrumentation

Column chromatography, chamber (Sigma), *vacuum rotary evaporator* (*Janke & Kunkel*), *inverted microscope*, *elisa reader*, vortex (*Janke & Kunkel*), *waterbath* (labo-tech, Heraeus), hemocytometer, *cell counter*, polietilsulfon filter, *tissue culture flask*, Eppendorf tubes, autoclave (AC-300AE, Tiyoda Manufactering Co. Ltd), aseptic box, petri dish (Pyrex), inoculating loops, plug, *paper disc*, *microtiter plate 96-well* (Bio-Rad), incubator (Sakura), oven, Erlenmeyer (Pyrex), TLC plate (Merck), TLC chamber (Camag), *Laminar Air Flow cabinet* (FARRco), FTIR (FTIR-100 Parkin Elmer) MS (Manner Bio-spectrometry System HRESIMS) and spectrometer NMR (Delta 2 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR).

Procedure

Extraction

Metabolites were produced through the fermentation of Fusarium sp. for 14 days. Fermented filtrate was separated by separating funnel using diethyl ether in 1 : 1 proportion and was replicated 3 times. The collected fraction was dried in the fume hood. Diethyl ether fraction was fractionated with preparative thin layer chromatography (PTLC) method using eluent that consist of methanol : ethyl acetate : chloroform (2:5:1).

Active Fraction Screening

The antimicrobial activity test was conducted with disc diffusion method (Kirby-Bauer Test). Testing microbes consist of *E. coli*, *S. aureus*, *B. subtilis*, *S. typhi*, *P. aeruginosa*. A concentration series of fraction were made 20; 10; 5; 2,5; and 1,25 µg/µL. As much as 10 µL of each testing substances were dropped on the paper disc so that the number of isolate on each paper disc were 200; 100; 50; 25; and 12,5 µg. Before annealing the disc to bacteria culture media, the discs were dried. It was done to remove every solvent from the discs. Positive control was made using 10 µL streptomycin 10 mg/mL and control solvent used 10 µL absolutely sterile ethanol. Bacteria cultures were incubated at 37°C for 18 –

24 hours, the inhibition zone around paper discs were spotted, and the active fraction was achieved.

Isolation of Active Compound Using Preparative TLC

Isolation process on fraction 1 was done by using eluent consists of chloroform : ethyl acetate (1:2,v/v), while the isolation process on fraction 2 was done by using eluent consists of chloroform : ethyl acetate (1:4,v/v). Chromatogram was detected by using visible rays, UV_{254}, UV_{366}, and anisaldehid sulfuric acid. Each detected spot was scraped and collected, then being dissolved in chloroform:methanol (1:1). The solution was filtered using Millipore and being dried.

Determination of Isolate Purity

Isolate 1 was tested using the 5 mixtures of eluent with different polarity that consist of chloroform : n-hexane (2:3, v/v), n-hexane : ethyl acetate (1:3,v/v), washensin : ethyl acetate (2:1,v/v), methanol : chloroform (1:2,v/v), and ethyl acetate : methanol (5:2, v/v). Isolate 2 was tested using 5 mixtures of eluent that consist of ethyl acetate : washensin (4:1,v/v), ethyl acetate : chloroform (3:1,v/v), ethyl acetate : methanol (5:1, v/v), n-hexane : methanol (3:2,v/v), and n-hexane : ethyl acetate (2:5,v/v). If the result gave 1 spot on each plate, then the isolate was pure in TLC test.

A small amount of isolate was filled into a electrotheranal *capillary tubes*. Then, the tubes were inserted into melting point apparatus to identify the melting process of the crystal and temperature range from the start of melting process to the temperature where every crystal were melted.

High-performance liquid chromatography was used to analyze the isolate purity. If it was shown that there was only 1 peak on the chromatogram at a specific time retention, then the isolate was pure in HPLC test.

Determination of Isolate Activity

IC_{50} value was determined through microdilution method. Into microtiter plate 96-well was added 50 Muller Hinton media, 50 µL, microbial suspension was made by diluting the suspension that is equivalent to McFarland Standard 0,5 with the proportion 1:10, and 100 µL active isolate concentration series: 40; 20; 10; 5; 2,5; 1,25; 0,63; and 0,31 µg/mL so that the final concentration became 20; 10; 5; 2,5; 1,25; 0,63; 0,31; and 0,16 µg/mL. An isolate control was made by adding 100 µL isolate into wells containing 100 µL media, bacterial growth control was made by adding 200 µL bacterial suspension into the wells, and positive control was made using 100 µL streptomycin solution 10 mg/mL and 100 mL microbial suspension.

Observation was done after incubating the testing object on the temperature 37°C for 18 – 24 hours. Cell density was measured using *microplate reader* under UV exposure on 595 nm wavelength to get absorbance value from bacteria cell with or without any treatment. IC_{50} value was got by making graph between isolate concentration (abscissa) and bacterial growth inhibition percentage (ordinate), then analyzed it using Litchfield and Wilcoxon method (probit analysis).

Determination of LC was done by taking 3 µL liquid from each microtiter plate 96-wells and scratched it in a sterile NA media with neither microbe nor isolate being added. The clear scratch on the NA media after incubation process stated the LC value.

Identification of Antibacterial Compound

Structural approach was done with sprayed reagent consists of vanillin-sulfate, 4-DNPH, anisaldehyde-sulfate, cerium-sulfate, Dragendorff and $FeCl_3$. A structural approach also was done by using Fourier-transform infrared spectroscopy, mass spectroscopy, and NMR spectroscopy (1H-NMR, 13C-NMR, and HMQC).

Results And Discussion

Fractionation and Antibacterial Activity Screening

The yield of extraction process was 245,98±2,99 mg per 2 liter media PDB. Fractionation was done using preparative TLC method with an eluent consisted of methanol : ethyl acetate : chloroform (2:5:1) and an addition of 1 drop of glacial acetic acid. The chromatogram resulted by previous process was detected under UV_{254}, UV_{366}, and sprayed reagents anisaldehyde-sulfate, then being classified into 4 fractions. Those 4 fractions were tested through disc diffusion method to screen an antibacterial activity on *E. coli*, *S. aureus*, *B. subtilis*, *S. typhi*, and *P. aeruginosa*. The result of this process was fraction 1 and 2 that showed a good antibacterial activity and were worth enough to be processed on the isolation process and being tested using microdilution method.

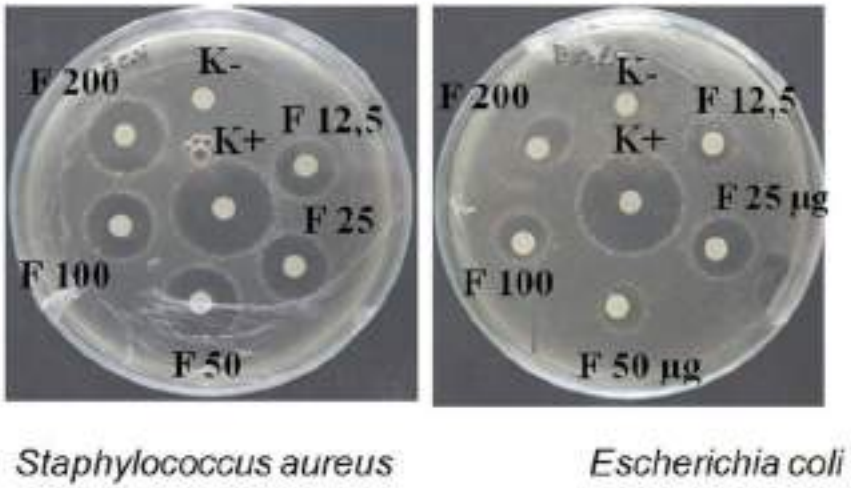

Figure 1. The Result of Disc Diffusion Test on *S.aureus* and *E.coli*

Table 1. The Result of Observation on Disc Diffusion Test

Fraction	Loading (µg)	Growth Inhibition Zone (mm)						
		S. aureus	*B. subtilis*	*E.coli*	*S. mutans*	*P. aeruginosa*	Positive Control	Negative Control
1	12.5	ND	ND	ND	11±0,54	ND	22±0,53	ND
	25	ND	7±0,87	9±0,88	10±0,53	ND		
	50	12±0,47	11±0,64	11±0,12	9±0,16	ND		
	100	13±0,76	10±0,88	13±0,54	10±0,77	ND		
	200	8±0,94	8±0,54	15±0,76	9±0,91	ND		
2	12.5	18±0,73	20±0,34	19±0,63	11±0,72	12±0,98	20±0,98	ND
	25	17±0,93	18±0,67	17±0,91	13±0,11	14±0,55		
	50	18±0,31	21±0,11	19±0,65	16±0,64	12±0,34		
	100	15±0,98	18±0,41	17±0,51	19±0,98	18±0,83		
	200	12±0,49	23±0,73	15±0,72	20±0,54	21±0,73		
3	12.5	ND	ND	ND	ND	ND	19±0,35	ND
	25	ND	ND	ND	ND	ND		
	50	12±0,11	18±0,84	ND	ND	ND		
	100	8±0,41	17±0,74	ND	ND	ND		
	200	9±0,76	8±0,88	18±0,93	ND	ND		
4	12.5	ND	7±0,13	ND	ND	ND	23±0,41	ND
	25	7,5±0,42	9±0,87	ND	ND	ND		
	50	ND	16±0,43	7±0,83	ND	ND		
	100	11±0,25	6±0,98	ND	ND	ND		
	200	10±0,39	15±0,14	ND	ND	17±0,13		

* ND: Not Detected

Table II. IC_{50} and LC Value of Isolate 1.1 and 2.2

BACTERIA	COMPOUNDS			
	ISOLATE 1.1		ISOLATE 2.2	
	IC_{50} (µg/mL)	LC (µg/mL)	IC_{50} (µg/mL)	LC (µg/mL)
B.subtilis	1,45	20,00	3,88	40,00
E.coli	2,19	20,00	2,45	40,00
P.aeruginosa	2,33	40,00	2,98	40,00
S.aureus	0,98	40,00	1,33	40,00
S.mutans	0,56	10,00	2,88	20,00
S.typhi	1,34	10,00	2,62	20,00

Tabel 3. NMR Data (400 MHz, $CDCl_3$) Compounds 1.1 and 2.2

Position	1.1		2.2	
	δC, type	δH, (J in Hz)	δC, type	δH, (J in Hz)
1	163.5 , C		162.8 , C	
2	101.4 , CH	6.44 s	100.9 , CH	6.52 s
3	160.9 , C		159.3 , C	
4	112.3 , C		110.8 , C	
5	134.6 , C		132.7 , C	
6	103.8 , CH	6.63 s	102.2 , CH	6.70 s
7	178.3 , C		180.4 , C	
8	115.8 , C		114.8 , C	
9	130.7 , C		134.2 , C	
10	178.3 , C		180.2 , C	
11	160.4 , C		162.2 , C	
12	116.3 CH	6.60 s	106.8 , CH	6.57 s
13	160.2 , C		162.1 , C	
14	120.3 , CH	7.04 s	108.2 , CH	6.98 s
15	13.8 , CH_3	2.05 s		
16	40.3 , CH_2	3.16 s	40.2 , CH_2	3.24 s
-OH		5.08		5.06

Isolate Purification

Fraction 1 and fraction 2 were purified in order to obtain an antibacterial compound. The purification was done by using preparative thin layer chromatography (PTLC) method. Eluent was optimized and applied on PTLC method for each fraction. Isolation process on fraction 1 resulted 2 separated spots with hRf value were 35 (UV_{366}) and 55 (UV_{254}). Meanwhile, isolation process on fraction 2 resulted 2 separated spots with hRf value were 38 (UV_{366}) and 63 (UV_{254}).

The main compound with high yield from fraction 1 was fraction 1.1, while from fraction 2 was fraction 2.2. On those fractions were conducted a purity test using TLC method with silica gel as the stationary phase and several eluents with different polarity. Fraction 1.1 was tested using 5 kind of eluents and each test showed a single spot with hRf value were 20, 35, 50, 60, 67, and 74. Fraction 2.2 was also tested using 5 different eluents and resulted a single spot on each test with hRf value of 20, 25, 55, 70, and 80. Based on those data, both fraction 1.1 and 2.2 showed a single spot on TLC test so that could be stated as a relatively pure compound.

Melting point determination was conducted to ensure the purity of isolated compounds. The test was done to isolate powders showed that isolate 1.1 had a melting point range between 196,34 – 196,77°C. That result showed a 0,43°C difference between the initial melting point to the final melting point. The test was also conducted on isolate 2.2 and showed a melting point range between 197,11 – 197,34°C so that causing a difference by 0,23°C. The melting point range for each isolate were close indicating that each isolate was relatively pure. The purity test was continued with liquid chromatography method using LC-MS instrument. Chromatogram of fraction 1.1 had a retention time of 3,4 minutes with 100% purity. Meanwhile, Chromatogram of fraction 2.2 had a retention time of 3,8 minutes with 99,88% purity. Those percentage value were obtained by comparing the peak area RT 3,8 with the total area of peak RT 1,2 (impurities) and peak RT 4,7. Based on those results, it could be concluded that fraction 1.1 and 2.2 had been relatively pure in TLC, melting point test, and liquid chromatography so that they could be called isolate 1.1 and 2.2.

Antibacterial Activity Test (Microdilution)

The antibacterial test was conducted by using microdilution method on *B. subtilis, E.coli, P. aeruginosa, S.aureus, S.mutans,* and *S. typhi* with parameter of IC_{50} value and also minimum lethal dose with the parameter of LC. Antibacterial test through microdilution method showed that isolate 1.1 was more active than isolate 2.2 on *B.subtilis, E.coli, P.aeruginosa, S.aureus* and *S.typhi*. That was showed through IC_{50} value analysis on both isolates. Isolate 1.1 had less IC_{50} value than isolate 2.2. But, isolate 2.2 had a better antibacterial activity than isolate 1.1 on *S.mutans*. Isolate 1.1 had a capability to eliminate bacteria or a minimum lethal dose better that isolate 2.2 on *B.subtilis, E.coli, S.mutans,* and *S.typhi*, but having a worse LC value on *P.aeruginosa* and *S.aureus* (Table 2).

Identification Isolate Structure

Isolate 1.1 and 2.2 were recrystallized and analyzed to determine their physical and spectroscopy characteristic. Isolate 1.1 was known as a compound with white crystalline powder, melting point 196,34 – 196,77°C, specific rotation $[\alpha]_D^{25}$ = -148 (chloroform); soluble in dichloromethane and chloroform. Isolate 2.2 was orange colored crystal; melting point of 197,11 – 197,34°C, specific rotation $[\alpha]_D^{25}$ = +151 (chloroform); soluble in dichloromethane and chloroform. Spectral analysis result with IR, MS, ^{1}H-NMR, ^{13}C-NMR, and DEPT confirmed an existence of aliphatic compound with substituted carbonyl group.

Identification of isolate 1.1 and 2.2 used UV spectroscopy on the wave length between 200 – 500 nm. The result showed that isolate 1.1 had a maximum absorbance in dichloromethane on a certain wave length (λ) (log ε) 233 (3.73); 286 (4.22); 462 (4.12) nm. Isolate 2.2 had a maximum absorbance in dichloromethane on a certain wave length (λ) (log ε) 235 (3.16); 287 (4.76); 458 (4.23) nm. Identification of isolate 1.1 and 2.2 using IR (KBr) showed ν-maximum 3332 (-OH group), 3014 (aromatic compound), 2900 (-C-H group), 1725 (–C=O group), 1664 (-C=C- group), 1128 (-C-O-C- group) cm^{-1} (Pavia *et al*, 2014). The measurement of isolate 1.1 using mass spectroscopy showed HRESIMS data had m/z 285.0653 $[M + H]^+$ (being calculated $C_{16}H_{13}O_5$ 284.0346). The measurement of isolate 2.2 showed HRESIMS data had m/z 287.0421 $[M + H]^+$ (being calculated $C_{15}H_{12}O_6$ 286.0465).

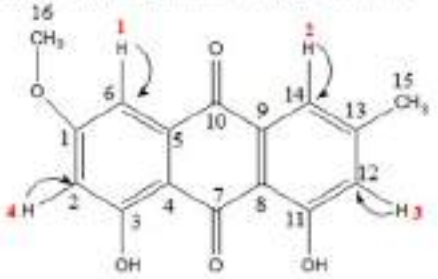

Figure 2. Structure of Isolate 1.1, (HMQC =

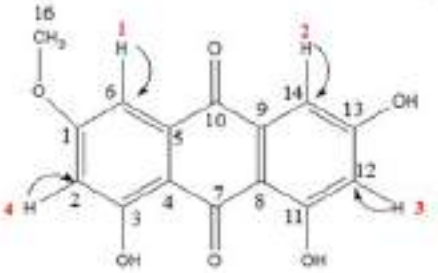

Figure 3. Structure of Isolate 2.2, (HMQC =

H-NMR data on table 3 for isolate 1.1 showed a signal from 4 protons of benzene group (δ_H 7.04 - 6.44), two methyl groups (δ_H 2.05 and 3.16), and two identical hydroxy groups (δ_H 5.08). C-NMR data showed that isolate 1.1 had 16 carbon atoms, including two carbonyl groups (δ_C 178.3). The structure of compound 1 was also ensured by the data from HMQC that was a method to detect proton coupling that bond to carbon atom directly (^{1}H-^{13}C). Correlation data could be spotted on H-1 (δ_H 6.44) with C-6 (δ_C 103.8); H-2 (δ_H 7.04) with C-14 (δ_C 120.3), H-3 (δ_H 6.60) with C-12 (δ_C 116.3), and H-4 (δ_H 6.60) with C-2 (δ_C 101.4). Based on those data, it could be concluded that compound 1.1 was *1,8-dihydroxy-3-methoxy-6-methylanthracene-9,10-dione* (Figure 2).

Isolate 2.2 showed a signal from 4 protons of benzene group (δ_H 6.98 - 6.52), one methyl group (δ_H 3.24), and three identic hydroxy group (δ_H 5.06) (Table III). C-NMR data showed that isolate 2.2 had 15 carbon atoms, with two identical hydroxy carbon atoms (δ_C 180.3- 180.4). Data analysis on HMQC showed that isolate 2.2 had 4 protons were bond directly with 4 carbon atoms, that were H-1 (δ_H 6.70) with C-6 (δ_C 102.2), H-2 (δ_H 6.98) with C-14 (δ_C 108.2); H-3 (δ_H 6.57) with C-12 (δ_C 106.8); and H-4 (δ_H 6.52) with C-2 (δ_C 100.9). Based on those data, it could be concluded that compound 2.2 was *1,3,8-trihydroxy-6-methoxyanthracene-9,10-dione* (Figure 3).

Conclusion

The result of this research showed that isolate 1.1 was a naphthoquinone compound named *1,8-dihydroxy-3-methoxy-6-methylanthracene-9,10-dione* and isolate 2.2 was compound *1,3,8-trihydroxy-6-methoxyanthracene-9,10-dione*. Both of them had an antibacterial activity on *B. subtilis, E.coli, P.aeruginosa, S.aureus, S.mutans,* and *S.typhi*.

Acknowledgements

We gratefully acknowledge the financial support from Program of Chemistry, Faculty of Science and Technology, Ma Chung University. This study was supported by Microbiology laboratory, Faculty of Medicine, Brawijaya University.

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