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Research Article

IDENTIFICATION AND CHARACTERIZATION OF POTENT ANTIMICROBIAL SECONDARY METABOLITE GENERATED FROM ENDOPHYTIC FUNGI ISOLATED FROM JAMBLANG PLANT (*EUGENIA CUMINI* L.)

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ABSTRACT

Objective: *Colletotrichum capsici* endophytic fungi were isolated from Jamblang plant (*Eugenia cumini* L.). The secondary metabolites of this plant have potent antibacterial efficacy as well as antidiarrheal and antidiabetic. This research focused on isolation of the endophytic microbes from branches of Jamblang plants and their secondary metabolites.

Methods: Isolation of endophytes was performed in potato dextrose agar using direct seed plant. Endophytic fungi isolates with strongest antimicrobial activity against the bacteria *Staphylococcus aureus* and *Escherichia coli* were fermented in potato dextrose yeast to produce large scale of the metabolites. Supernatant was extracted with ethyl acetate (EtOAc) solvent. EtOAc extract fractionated by column chromatography (SiO_3, *n*-hexane:EtOAc=50:1~1:1) and obtained three fractions. Further, agar diffusion method was performed to assess their antimicrobial activity.

Results: Antibacterial test results indicated that fraction III had potent antibacterial activity *S. aureus* with inhibition zone diameter of 10.7 mm but no observed activity against *E. coli*. Furthermore, identification by gas chromatography-mass spectrometry showed that compounds present in fraction III were mainly fatty acid and phenolic compounds.

Conclusion: Secondary metabolites isolated from Jamblang plants branches contained predominantly fatty acid and phenol-related compounds that could be responsible for its potent antimicrobial activity.

Keywords: Endophytic fungi, Jamblang (*Eugenia cumini* L.), Antimicrobial activity.

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INTRODUCTION

Based on the history of drug development, medicinal plants can be a useful source of various types of drugs. Jamblang plant [1] was one of the many herbaceous plants where its exudate and parts of the plant (twigs and leaves) were regularly used for the treatment of diseases such as diarrhea and diabetes by the laymen in Indonesian society. Previous studies have documented that leaves of Jamblang plants were used for dental hygiene treatment (strengthening the gums and teeth) while the branches were useful as antimicrobial agent [2], antigenotoxic, antiulcerogenic, antiallergic, and hepatoprotective effect [3]. Furthermore, the extract of this plant contained tannins, essential oils, resins, glucoside, and gallic acid [4].

Herbaceous plants are very limited in source for used in scientific studies within the herbal industry. Uncontrolled harvesting and cultivation might endanger plants to extinction. Thus, alternative sources such as marine life and endophytic microbes were utilized. Endophytic microbes were one type of microbes that live within plants associated with the host plant, without causing any harm to the host. They produced secondary metabolites with proven medicinal potential such as antimicrobial, antiviral, anti-inflammatory, and antioxidant [5,6]. Utilization of endophytic microbes in scientific studies was an advantage since extraction of this microbe can be done on pilot scale, and mass production of secondary metabolites through fermentation process can be done in the laboratory without the need of harvesting massive quantity of the herbal plant. The current study focused on pilot scale isolation of endophytic fungi *Colletotrichum capsici* followed by mass production of its secondary metabolites through fermentation process. Ultimately, identification of chemical composition of the secondary metabolites fraction with the most potent antimicrobial activity was performed using chromatography and gas chromatography-mass spectrometry (GC-MS) analysis.

METHODS

Material

Endophytic fungi isolated EcVil(A), EcVfl(B), EcVjl(C), EcVel(D), and EcVll(E). Media nutrient agar (NA), potato dextrose agar (PDA), potato dextrose yeast (PDY) broth, tryptic soy broth (TSB) chloramphenicol, chloroform, *n*-hexane, methanol (MeOH), and ethyl acetate (EtOAc). *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923, *Escherichia coli* ATCC 25922, McFarland standard 0.5 (1.5×108 CFU/ml), silica gel GF254, spot reagent cerium sulfate, and silica gel 60 (0.063-0.200 mm).

Instruments

Chromatographic column, GC-mass selective Agilent Technologies with Auto sampler and 5973 Mass Selective Detector, HP Ultra column, capillary columns (30 mm×0.25 mm), and ID×0.25 p.m thickness layers.

The principle research

Endophytic fungi isolated from branches or also known as twigs of Jamblang tree (*Eugenia cumini* L.) which showed inhibitory action toward glucosidase enzyme was incubated using fermentation media. The pilot scale fermentation resulted in five endophytic isolates. Supernatant of each isolate was extracted using EtOAc and was concentrated down using rotary evaporator. The dry extract was then

tested for its antibacterial activity against *E. coli* and *S. aureus,* using agar diffusion method with paper disc in the presence and absence of positive control chloramphenicol. Isolate with highest antibacterial activity was selected for the second stage fermentation process to produce isolates of interest in large quantity. Finally, chromatography and GC-MS analysis were performed to identify chemical substance in the isolate responsible for the potent antibacterial activity.

Isolation and identification of endophytic microbes

Part of plants, such as twigs, was used in the experiment as samples. These samples were chopped by knife into 1-1.5 cm length, then washed with running water (approximately 10 minutes) to remove all soils and dirts from the surface. These samples were then sterilized using 75% ethanol for 1 minute, soaked in NaOCl 5.3% w/v for 5 minutes and then back into 75% ethanol for another 30 seconds. Once sterilized, samples were rinsed with distilled water 1-2 times and were then dried on filter paper. Dried sterilized samples were then placed on sterile microscopic slides for horizontal slicing into two halves. Each half was placed onto two different types of media which were NA containing nisatin (0.01% w/v) and PDA in the presence of chloramphenicol (0.005% w/v). The purpose of nisatin in the NA was to inhibit the growth of fungi while chloramphenicol was an antibacterial in PDA. The cut sample was placed with the "cutting surface" directly touch the surface of medium.

Media with samples were then incubated for 5-7 days at temperature ranged from 27°C to 29°C. The growth of fungal culture was observed. Successful culture endophytic fungi culture would show morphological character and transferred to PDA media [7].

Fermentation process with shaking fermentation method

Endophytic fungi isolated were grown on PDA medium for 7 days in petri dishes. Five pieces of inoculums were selected using sterile cork borer and placed into liquid fermentation media PDY (50 ml media in Erlenmeyer flask 250 ml). This was further fermented on shaker for the next 10 days at room temperature (27°C) with rotating velocity of 130 rpm. Supernatant was separated from biomass through centrifugation at −4°C rotating at 2000 rpm for 20 minutes and was extracted with EtOAc [8].

Extraction

Supernatant produced from the fermentation process was separated from the biomass through centrifugation at −4°C rotating at 200 rpm for 20 minutes. Then, an amount of 100 ml supernatant was filtered through sieving paper and extracted with 100 ml of EtOAc. The extract was then evaporated until dry solid was obtained and its antimicrobial activity was evaluated.

Antimicrobial activity test

The antimicrobial activity of the dried EtOAc extract was tested against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 using agar diffusion method with paper disc. Chloramphenicol was used as positive control while EtOAc was used for negative control (blank).

S. aureus and *E. coli* were inoculated into TSB media and incubated at 35-37°C for 18-24 hrs. Each of these bacterial suspensions was compared to McFarland standard 0.5 (1.5×10^8 CFU/ml) for density uniformity and spread onto NA evenly using sterile cotton bud in a petri dish until all surfaces were covered.

Paper discs saturated with EtOAc extract were placed on NA medium surface where bacterial suspension was applied and then were incubated at 35-37°C for 18-24 hrs. Clear zone around the disc represented microbe inhibition zone area and was measured in millimeter (mm) scale. Extract with largest diameter zone of inhibition would be fermented further in large scale (1 L) and was extracted again using EtOAc [9,10].

Large scale bioproduction

Endophytic fungi isolated with largest diameter of zone inhibition were cultivated in PDA media at room temperature for 7-14 days. 20 pieces of the cultivated fungi were picked using sterile cork borer and placed into liquid fermentation media PDY (200 ml media in Erlenmeyer flask 1000 ml). The same fermentation process was repeated using 250 ml of the isolated obtained from the first fermentation process in 1 L fresh fermentation media to further cultivate the fungi, hence produced large scale of the endophytic fungi. The fungi isolate was incubated for another 10 days in the shaking incubator with rotating velocity of 130 rpm. The filtrates were separated from biomass by centrifugation with velocity of 2000 rpm for 20 minutes at −4°C. The filtrates obtained then were extracted with EtOAc.

Analysis of large scale fermentation product using thin-layer chromatography (TLC)

Concentrated EtOAc extract was spotted on TLC plate GF_{254} and was placed into a chamber filled with several eluent mixtures. They were *n*-hexane:EtOAc (2:1), *n*-hexane:EtOAc (10:1), and CCL4:MeOH (5:1). TLC analysis was done under UV light at wavelength of 254 nm or 366 nm. Then, plate was sprayed with a mixture containing 1% $Ce(SO4)_2$ and 10% H_2SO_4 and heated for 5 minutes at 110°C. Once components of the fraction were successfully separated with good Rf value with appropriate eluent mixture, they were fractionated using column chromatography using solvent mixture *n*-hexane:EtOAc (50:1).

Fractionation for EtOAc extract by column chromatography

An amount of 100 mg extracts were homogenized with Celite 545, dried and fractionated using *n*-hexane:EtOAc (50:1~1:1) graduated solvent system to yield some fractions. Those fractions with same Rf value after TLC analysis were pooled together and evaporated till dried fraction (F1-F3) was obtained [11].

Antimicrobial activity test for fractions from chromatography column analysis

The antimicrobial activity of F1-F3 was evaluated using agar diffusion method. The test bacteria, positive and negative controls employed in antimicrobial activity test, were the same as those mentioned in the previous section (antimicrobial activity test).

GC-MS analysis for antimicrobial active compounds

Active compound (1 mg) obtained from antimicrobial activity test was diluted in analytical grade EtOAc then injected into GC-MS with conditions:

- Injection temperature: 250°C
- Ion source temperature: 230°C
- Interface temperature: 280°C.

Oven temperature: Temperature 70°C for 0 minute. Temperature was increased to 200°C for 1 minute, then increased again for other 20°C for 28 minutes.

Identification of active endophytic fungi

DNA isolation and amplification (PCR) of internal transcribed spacer (ITS) rDNA area were performed using forward primer. Data sequence was obtained, manually edited and sent to DNA GenBank database by BLAST homology searching program.

RESULTS

From twigs of the Jamblang plant, five isolates of endophytic fungi were successfully isolated. They were EcVil(A), EcVfl(B), EcVjl(C), EcVel(D), and EcVll(E).

Antimicrobial activity test results showed that fungi EcVll(E) from shaking fermentation gave largest inhibition zone around paper disc against *S. aureus* when compared to *E. coli.* The results of antimicrobial activity test were summarized in Table 1.

Antimicrobial activity test for fractions from chromatography column analysis was summarized in Table 2. Test results demonstrated that fraction III had inhibition activity toward *S. aureus* but not to *E. coli*.

Identification of endophytic fungi using ITS1 primary. This identification was performed to investigate the fungi producing the highest antimicrobial activity. The result displayed that the nearest species identified was *C. capsici* (Fig. 1).

Large scale bioproduction

Fungi E (EcVll) showed antimicrobial activity in the previous test was fermented in 1000 ml PDY media at 27°C for 10 days with agitation velocity 130 rpm. At harvest time, the filtrates were separated from biomass and extracted with EtOAc. These extracts were evaporated using vacuum rotary evaporator to get 110 mg samples.

Chemical compound analysis using TLC

TLC analysis for compounds presents within the sample extract resulted in perfect eluent mixture (*n*-hexane:EtOAc=50:1) for further column chromatography analysis.

Fractionation for EtOAc extract by column chromatography

An amount of 100 mg EtOAc extracts were fractionated with solvent system SiO_2; *n*-hexane:EtOAc=50:1~1:1. This process resulted in isolation of three fractions (fraction I, 40 mg; fraction II, 10 mg; and fraction III, 120 mg).

Fig. 1: *Colletotrichium capsici* endophytic fungi [isolate code EcVII(E)] isolated from Jamblang plant (*Eugenia cumini* L.)

Antimicrobial activity test for fractions from chromatography column analysis

The antimicrobial potency of secondary metabolites was investigated in the current study. The three fractions obtained from column chromatography separation, underwent antimicrobial activity test using agar diffusion method against *S. aureus* and *E. coli.* Chloramphenicol was the positive control while EtOAc was the negative controls (blank).

DISCUSSION

The surface sterilization method employed in the current study used 70% EtOH, NaOCl 5.3% w/v followed by rinsing with distilled water. In previous study Purwantini *et al*. [12] used chlorinated water instead of NaOCl 5.3%w/v. Both of these solution can be used for surface sterilization.

Fermentation process for endophytic fungi was done using liquid media PDY because previous studies have demonstrated that PDY was the contained rich carbon sources from potato extracts and dextrose, nitrogen source from its yeast extract as well as $CaCO_3$ as mineral needed by the microorganisms. This was in supported by the work of Lestari *et al*. [13].

The antimicrobial activity test was performed using agar diffusion method with chloramphenicol as positive control and EtOAc as negative control (blank). Chloramphenicol was chosen as positive control because of its strong protein synthesis inhibition activity and its broad spectrum against Gram-positive bacteria *S. aureus* and Gram-negative bacteria *E. coli*. Furthermore, it was also popular for its character as bacteriostatic agent. Antimicrobial activity test results showed that fungi EcVll(E) from shaking fermentation has the largest inhibition zone around paper disc against *S. aureus* when compared to *E. coli.* We suspected that the differences in cell wall composition of Gram-positive bacteria and Gram-negative bacteria explained the difference in antimicrobial activity. Cell walls of Gram-positive bacteria generally consisted only one layer of peptidoglycan, teichoic acid, and low lipid (1-4%) and more susceptible to antibacterial agent infiltration (e.g., the

Table 1: Antimicrobial activity test results for EtOAc extracts of isolated endophytic fungi ferments

No	Fungi code	Bacteria	Inhibition zone diameter (mm)						
			Test					Means	Standard deviation
			Positive control (chloramphenicol)	Negative control (ethyl acetate)	Replica 1	Replica 2	Replica 3		
1	Fungi EcVil(A)	*S. aureus*	22.00	0.00	6.58	0.00	5.78	6.18	0.57
		E. coli	24.00	0.00	0.00	0.00	10.6	10.6	0.00
2	Fungi EcVfl(B)	*S. aureus*	15.00	0.00	0.00	2.76	0.00	2.76	0.00
		E. coli	20.00	0.00	6.20	4.40	11.24	5.3	1.27
3	Fungi EcVjl(C)	*S. aureus*	22.00	0.00	0.00	0.00	0.00	0.00	0.00
		E. coli	16.00	0.00	0.00	0.00	0.00	0.00	0.0021
4	Fungi EcVel(D)	*S. aureus*	21.00	0.00	0.00	0.00	0.00	0.00	0.00
		E. coli	16.00	0.00	0.00	0.00	0.00	0.00	0.00
5	Fungi EcVll(E)	*S. aureus*	20.00	0.00	8.00	8.4	8.00	8.1	0.23
		E. coli	16.00	0.00	11.90	17.80	11.50	11.70	0.28

EtOAc: Ethyl acetate, *S. aureus: Staphylococcus aureus, E. coli: Escherichia coli*. Note: Paper disc diameter 6 mm

Table 2: Results of antimicrobial activity test against *Staphylococcus aureus* for fractions obtained from column chromatography analysis

No	Fraction	Inhibition zone diameter (mm)					
		Positive control (chloramphenicol)	Negative control (ethyl acetate)	Replica 1	Replica 2	Replica 3	Means±standard deviation
1	Fraction I	25.00	0.00	0.00	0.00	0.00	0.00±0.00
2	Fraction II	25.00	0.00	0.00	0.00	0.00	0.00±0.00
3	Fraction III	20.00	0.00	11.50	11.50	9.20	10.70±1.33

Note: Paper disc diameter 6 mm

extracts tested) while Gram-negative bacteria have four layers cell walls consisted peptidoglycan, lipoprotein, phospholipids, outer membrane, and lipopolysaccharide [14,15]. The results of antimicrobial activity test were summarized in Table 1.

Test results demonstrated that fraction III had inhibition activity toward *S. aureus* but not to *E. coli*. These results were summarized in Table 2, where faction III had a zone inhibition of 10.70 mm in scale toward *S. aureus* but no inhibition toward the *E. coli*. This, in fact, was a very interesting result because the current study has successfully shown endophytic fungi secondary metabolites from twigs of Jamblang tree have selective antimicrobial activity. Furthermore, the selectivity in the antimicrobial activity might be due to the chemical substance presented in fraction III. This compound of interest might be able to penetrate easily through the single layered bacterial cell wall but no penetration through multilayered bacterial cell wall.

Results of antimicrobial activity test showed that shaking fermentation gave better production of antimicrobial compounds compared to static fermentation. Agitation and aeration process in the shaking fermentation led to better oxygen supply efficiency and homogenization of heat distribution on all parts of the substrates that needed by microorganisms [8].

Identification of chemical compound in the most active fraction (fraction III) using GC-MS showed some peaks that indicated the presence of compound. Based on database Wiley275.L, we can conclude that these compounds were dominated by phenolic and lipid acids and need further purification.

Phenolic compounds were well documented to have potent antimicrobial activity because it has self-defense mechanism toward microorganism attack [16]. This bioactive compound damaged cell membrane by binding to the bacterial cells. This resulted in the loss of cations and macromolecules which disturbed the cell growth and eventually led to bacterial cell death [17].

This was consistent with the current study finding. The GC-MS analysis has successfully proven that the present of phenol bioactive components within secondary metabolites produced by endophytic fungi isolate fraction III. This fraction was the most potent isolate obtained from the twigs of Jamblang tree against *S. aureus.*

Identification of endophytic fungi

C. capsici was endophytic fungi isolated from Jamblang plants. Principle identification of endophytic fungi was macroscopic and microscopic ways. Macroscopic character of fungi such as shape and color of the colony must be observed. Furthermore, in a successful fungal culture, usually, they produce spores, and this can be included as additional identification method for endophytic fungi. It was interesting to know, however, successful endophytic fungi culture obtained in the current study did not form any kind of spores. Hence, additional method of identification performed was molecular analysis such as partially based on genetic analyzes at the locus of ITS of ribosomal DNA of fungi. This step was not previously conducted by Perwantini *et al*. [12]; however, it was a crucial step in the current study.

DNA isolation and amplification (PCR) of ITS rDNA area were done using ITS1 primary. Sequence data obtained were edited manually and sent to DNA GenBank database by BLAST homology searching program at: http://www.ncbi.nlm.nih.gov/Blast.cgi. The nearest species identified EcVl (E) were *C. capsici*. This species belonged to Kingdom: Fungi, Rank: *Phylum,* Scientific name: *Ascomycota*, Class: *Sordariomycetes*, Order: *Phyllachorales,* Family: *Phyllachoracecae*, and Genus: *Colletotrichum*.

CONCLUSION

One of the endophytic fungi isolated from Jamblang plants branches (*E. cumini* L.) identified as *C. capsici*. The secondary metabolites of this fungus have strong potency as antimicrobial activity against *S. aureus.* Identification of chemical compounds by GC-MS showed that the fraction III is dominated by compound fatty acids and phenolic groups.

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