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ANTIMICROBIAL ACTIVITY OF SECONDARY METABOLITES PRODUCED BY ENDOPHYTIC FUNGI ISOLATED FROM STEMS OF JATI TREE (*TECTONAGRANDIS* L.F)

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ABSTRACT: *Diaporthephaseolorum* is endophytic fungi isolated from Jatitree (*Tectonagrandis L.f*). Endophytic microbes were microbes that spent part or all of their life live within plant tissue of a host plant. They produce secondary metabolites with potent anti microbial activity. Jatitree (*Tectonagrandis L.f*) was one of herbal plants that act as host plants for endophytic microbes that produce secondary metabolites with anti-microbial activity. The current study focussed on isolation of the endophytic microbes from branches of Jati tree and their secondary metabolites. Isolation of endophytes were performed in PDY (potato dextrose yeast) and further extracted using EtoAC. Four fractions (F I - F IV) was obtained from EtOAc extract through column chromatography (n-Hex: EtoAc, ratio 50:1). Further, agar diffusion method was performed to assess their anti-microbial activity. Results demonstrated that F IV has potent towards *Staphylococcus aureus* (11.10mm). Its GC-MS profile strongly indicated the presence of fatty acid and phenolic related compound. In conclusion, secondary metabolites of endohpytes isolated from Jati tree branches contained predominantly phenol and fatty acid related compound that could be responsible for its potent anti microbial activity.

INTRODUCTION: The spread of infectious diseases were considered as serious problems in many countries, particularly in developing countries. Utilization of secondary metabolites produced by endophytic microbes could be one newly invented alternative development of anti microbial drugs as it was economical and environmentally friendly.

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Using endophytic microbes as source of raw material for developing new drugs from herbal plant was very advantageous because it can be obtained without killing the host plant, hence environmental sustainability can be preserved. Endophytic microbes are microbes that spend part or all of their life lives within plant tissues of a host plant.

They produce secondary metabolites with various potent medicinal activities including anti-microbialactivities, which were beneficial in health and pharamaceutical areas [1]. Jati tree *(Tectonagrandis* L.f) was atimber plant. The woods of this tree was well documented asherbal

medicineto cure fever, cough, laryngitis, cholera, stomachache, purulent dermatitis, and other skin disorders [2].

The aims of this study were to first determine the potency of secondary metabolites as bio product of endophytic fungi isolated from stems of Jati tree *(Tectonagrandis* L.f*),* and then followed by identification of the fungal species isolated using biomolecular analysis techniques.

MATERIALS AND METHODS:

Materials:

Endophytic fungi isolated (fungi A, fungi B, fungi C, and fungi D), media NA (nutrient agar), PDA (potato dextrose agar), PDY (potato dextrose yeast) broth, TSB (tryptic soya broth), distilled water, chloramphenicol, chloroform, methanol (MeOH), ethylacetat (EtOAc). *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, McFarland standard 0.5 (1.5 x 108 CFU/ml), silica gel GF254, spot reagent cerium sulphate, silica gel 60 (0.063-0.200mm), chromatographic column, Gas chromatography-Mass selective (GC-MS) Agilent Technologies with Auto sampler and 5973 Mass Selective Detector, HP Ultra column, capillary columns (30 x 0.25 mm), ID x 0.25p.m thickness layers.

Methods:

Fermentation Process with Shaking Fermentation Method:

Endophytic fungi isolated were grown on PDA medium for 7 days in petri dishes. Five (5) pieces of inoculums were selected using sterile cork punch 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 with rotating velocity of 130 rpm. Supernatant was separated from biomass via centrifugation at -4°C rotating at 2000rpm for 20 min and were extracted with EtOAc [3].

Extraction:

An amount of 100ml supernatant produced from fermentation process was filtered through sieving paper and extracted with 100ml of EtoAc. The extractwas then evaporated till dry until dry solid was obtained and its anti microbial activity was evaluated.

Antimicrobial Activity Test:

The anti microbial activty of the driedEtOAc extract weretested against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 as the test bacteriauisng agar diffusion method using paper disc. Chloramphenicol was used *as* positive control while EtOAc was used for negative control (blank).

Staphylococcus aureus and *Escherichia coli* were inoculated into TSB media and incubated at 35 °C - 37 °C for 18-24 hours. Each of these bacterial suspension were compared to McFarland standard 0.5 (1.5 x 10^8 CFU/ml) for density uniformity andspread onto nutrient agar (NA) evenly using sterile cotton bud in a Petri dish until all surface was covered. Paper discs saturated with EtOAc extract were placed on NA medium surface where bacterial suspension was applied. The discs were incubated at 35 °C -37 °C for 18-24 hours. Clear zone around the disc demontrated microbe inhibition zone area and was measured in millimeter (mm) scale.

Large Scale Bioproduction:

Endophytic fungi isolated were cultivated in PDA media at room temperature for 7-14 days. 20 pieces of the cultivated fungi were picked using sterile cork punch and placed into liquid fermentation media PDY (200 ml media in Erlenmeyer flask 1000 ml). In order to geteven larger scale of endophytic fungi production, 250ml of the content of liquid fermentation PDY was placed into severalelenmeyer flask containing1000 ml of fresh fermentation media to further cultivate the fungi andwere incubated for another 10 days in the shaking incubater with rotating velocity of 130 rpm. The filtrates were separated from biomass by centrifugation with velocity 2000rpm for 20 min at -4°C. The filtrates obtained then were extracted with EtOAc.

Analysis of large scale fermentation product using TLC:

Concentrated EtOAc extract were spotted on t.l.c plate GF_{254} and was placed into a chamber filled with several eluent mixture which were n-Hexane – EtOAc (2:1), n-Hexane – EtOAc (10:1), or CCl_4 : MeoH (5:1). T.L.C analysis was done under UV light at wavelenght 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 min at110^oC. Once components of the fraction were succesfully seperated with good Rf value with appropriate eluent mixture, they were fractionated using column chromatography using solvent mixture n-hexane : EtOAc (50:1).

Fractionation for Ethylacetate Extract by Column Chromatography

100 mg of the extracts were homogenized with Celite 545, dried and fractionated using n-hexane-EtOAc (50: 1 ~ 1: 1) graduated solvent system to yieldsome fractions. Those fractions with same Rf value after t.l.c analysis were pooled together and evaporated till dried fraction (F1-F4) was obtained.

Antimicrobial Activity Test for Fractions from Chromatography Column Analysis:

The anti microbial activity of F1-F4 were evaluated using agar diffusion method. The test bacteria, positive and negative controls employed in anti microbial activity test were the same as those mentioned in the previous section (anti-microbial activity test) .

GC-MS Analysis for Antimicrobial Active Compounds:

Active compound (1mg) obtained from antimicrobial activity test was diluted in analytical grade ethylacetate then injected into GC-MS machine with conditions: Injection temperature: 250 °C

Ion souce temperature: 230°C

Interface temperature: 280°C

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

Identification of Active Endophytic Fungi:

DNA isolation and amplification (PCR) of ITS (internal transcrible spacer) rDNA area were performedusing forward primer. Data sequence was obtained, manually edited and sent to DNA GenBank database by BLAST homology searching program at website :http://vvww.ncbi. nlm.nih. gov/Blast.cgi.

RESULTS AND DISCUSSION:

Fermentation process for endophytic fungi was done using liquid media PDY, because previous studies have demonstrated that PDY was thecontained rich carbon sources from potato extracts and dextrose, and nitrogen source from its yeast extract. The ferments obtained were extracted with EtOAc, evaporateduntil they were completely dried, and tested for their antimicrobial activity.

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 wide spectrum against Gram positive bacteria *Staphylococcus aureus* and Gram negative bacteria *Escherichia coli.*; and also for itscharacter as bacteriostatic agent. Antimicrobial activity test results showed that fungi A from shaking fermentation gave largest inhibition zone around paper disc against *S. aureus* compared to *E. coli.*

We suspected this was caused by the differences in cell wall composition of Gram positive bacteria and Gram negative bacteria. Cell walls of Gram positive bacteria generally consisted only one layer of peptidogly can and more susceptible to antibacterial agent infiltration (e.g. the extracts tested) whileGram negative bacteria have 4 layers cell walls consisted peptidoglycan, lipoprotein, phospholipids outer membrane, and lipopolysacharide [4, 5]. The results of antimicrobial activity test were summarised in **Table 1**.

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.

Large Scale Bioproduction:

Fungi A (*Diaporthe phaseolorum*) isolated that showed antimicrobial activity in previous test were fermented in 1000 ml PDY media at 27 °C for 10 days with agitation velocity 130 rpm. At the

harvest time, the filtrates separated from biomass were extracted with EtOAc. These extracts were evaporated using vacuum rotary evaporator to get 110 mg samples.

TABLE 1: THE RESULTS OF ANTIMICROBIAL ACTIVITY TEST FOR ETHYLACETATE EXTRACTS OF ISOLATED ENDOPHYTIC FUNGI FERMENTS

S. No	Fungi Code	Tested Bacteria	Inhibition Zone Diameter (Mm)						
			Positive Control (Chloramphenicol	Negative Control (Ethylacetate)	Replica 1	Replica 2	Replica 3	Means	Standard Deviation
1	Fungi A	*S.*	24.20	0.00	16.64	16.40	17.38	16.80	031
		E. coli	24.30	0.00	4.66	3.80	7.20	5.22	1.76
2	*Fungi* B	*S.* avreur	*23.51*	0.00	1.80	3.00	3.00	*2.60*	0.69
		E. coli	24.10	0.00	2.86	2.80	2.86	2.84	0.03
3	Fungi C	*S.*	24.11	0.00	8.80	8.70	9.00	• 8.83	0.15
		E. coli	23.22	0.00	6.20	3.00	3.40	4.2	1.74
4	Fungi D	*S.*	15.00	0.00	6.00	9,20	6.20	7.13	1.79
		E. coli	21.90	0.00	5.00	3.40	4.88	4.43	0.89

Note: paper disc diameter 6 mm

Chemical Compound Analysis using TLC:

Thin layer chromatography (TLC) analysis for compounds present within the sample extract resulted in proper eluent for further column chromatography analysis. The eluent is *n*-hexane: EtOAc = 50: 1

Fractionation for Ethylacetate Extract by Column Chromatography

The extracts from fungi a bioproduction with its where fractionated using column chromatography. An amount of 100 mg EtOAc extracts were fractionated with solvent system SiO2; n-hexane: ethylacetate = 50: 1 - 1: 1 to get 4 fractions (fraction I, 10 mg; fraction II, 30 mg; fraction III, 20 mg; and fraction IV, 30 mg).

Antimicrobial Activity Test for Fractions from Chromatography Column Analysis:

The antimicrobial potency of secondary metabolites were investigated in the current study. The four fractions obtained from column chromatography separation, underwent antimicrobial activity test using agar diffusion method against *Staphylococcus aureus.* Chloramphenicol as positive control and n-hexane and ethylacetate as negative controls (blank). The results of the test for each fraction were tabulated in **Table 2**.

TABLE 2: RESULTS OF ANTIMICROBIAL ACTIVITY TEST FOR FRACTIONS OBTAINED FROM COLUMN CHROMATOGRAPHY ANALYSIS

S. No	Fraction	Inhibition Zone Diameter (Mm)						
		Positive Control (Chloramphenicol	Negative Control	Replica I	Replica 2	Replica 3	Means	Standard Deviation
1	Fraction 1	22.30	0.00	0.00	0.00	0.00	0.00	0.00
2	Fraction II	24.12	0.00	0.00	0.00	0.00	0.00	0.00
3	Fraction111	20.15	0.00	0.00	8.90	8.90	8.90	0.00
4	Fraction IV	24.13	0.00	12.20	10.00	11.10	11.10	1.11

Note: paper disc diameter 6 mm

GC-MS Analysis for Antimicrobial Active Compounds:

Identification of chemical compound in the most active fraction (fraction IV) using GC-MS showed some peaks that indicated the presence of compound. Based on database Wiley 275.L we can conclude that these compounds were dominated by phenolic and lipid acids, and need further purification [6].

Identification of Active Endophytic Fungi: [7]

DNA isolation and amplification (PCR) of ITS (internal transcible spacer) rDNA area were done using ITS1 primary. Sequence data obtained were

edited manually and sent to DNA GenBank at website: http://www.ncbi.nlm.nih.gov/Blast.cgi. The nearest species identified was *Diaporthephaseolorum* (Cooke& Ellis, 1878). This species belonged to kingdom Fungi, *phylum Ascomycota*, class *Sordariomycetes*, order *Diaporthales*, family *Diaporthaceae*, genus *Diaporthe*.

CONCLUSION: One of the endophytic fungi isolated from stems of *Tectoniagrandis* L.f. identified as *Diaporthephaseolorum*. The secondary metabolites of this fungi has strong potency as anti microbial activity against *Staphylococcus aureus.*

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