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Antimicrobial and cytotoxic activities of endophytic fungi isolated from *Piper crocatum* Ruiz & Pav

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ABSTRACT

Objective: To investigate antimicrobial and cytotoxic activities of endophytic fungi isolated from *Piper crocatum* Ruiz & Pav (*P. crocatum*).

Methods: Ethyl acetate extracts were obtained by liquid-liquid partition of fermentation broth of endophytes followed by evaporation. The antimicrobial activities were determined by diffusion techniques followed by thin layer chromatography-bioautography. Cytotoxicity studies were conducted using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay. Data generated were used to plot a dose-response curve, of which the concentration of extract required to kill 50% of cell population (IC_{50}) was determined.

Results: Two endophytes were isolated from leaves and stem of *P. crocatum*, designated as DS1 and BS1. Ethyl acetate extracts of BS1 was found to inhibit the growth of *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* at minimum dose of 31.25, 125 and 250 μg, respectively. thin layer chromatography-bioautography of this extract resulted in at least two inhibition zones. BS1 extract also inhibited the growth of WiDr and T47D cell lines with IC_{50} of 120.38 μg/mL and 37.43 μg/mL, respectively.

Conclusions: BS1 extract produced by endophytic fungi of *P. crocatum* is potential to be developed as source of novel bioactive compounds.

1. Introduction

Natural products produced by plants, animals or microbes are sources of bioactive compounds potential to be developed as medicine. Endophytes are microbes which reside in living plant tissues without causing injury or diseases to the hosts[1,2]. Most of endophytes are capable of producing active metabolites and some of these compounds are proven to have medical values[3-6]. Various types of compounds produced by endophytes includ terpenoids, alkaloids, phenylpropanoids, aliphatic compounds, polyketides and peptides that have been reported to have antimicrobial activities[7-9] and many compounds showed bioactivity against various types of cancer cell lines[10-12].

Piper crocatum (P. crocatum) is a traditional medicinal plant which is traditionally used by local people at Yogyakarta palace for routine ceremonial such as "ngadi saliro". It is widely used by Javanese to cure various diseases such as diabetes, hepatitis, kidney failure, stroke, hypertension, candidiasis *etc*[13]. However, there are only limited studies on bioactivity of this plant to be developed as pharmaceutical agent. Safithri and Fahma in 2008 reported the ability of *P. crocatum* decoctions to function as antihyperglycemic agent in alloxan-induced diabetic rats[14]. Methanol extract of *P. crocatum* inhibited the growth of T47D cells through inhibition of p44/p42 phosphorylation[15]. Despite progress towards understanding phytochemical constituents and pharmacological activities of *P. crocatum* plants, bioactivity analysis of endophytic

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fungi associated with this medicinal plant has not been widely explored. Medicinal plants are considered as the source of endophytic fungi which are suggested to be associated with production of compounds with medical values[16]. This study described endophytic fungi isolated from *P. crocatum* and reported the antimicrobial and cytotoxicity activities.

2. Materials and methods

2.1. Materials

RPMI1640, fetal bovine serum, penicillin-streptomycin, fungizon and sodium bicarbonate, were supplied from Gibco. HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (Invitrogen), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (Sigma-Aldrich) were used as well. Nutrient agar, potato dextrose agar (PDA) and dextrose were obtained from Oxoid. Silica gel F254, dimethylsulfoxide, *n*-hexane and ethyl acetate (Merck) were obtained.

2.2. Source and isolation of endophytic fungi

Plant materials were collected from Medicinal Plant Garden, Faculty of Pharmacy, Universitas Gadjah Mada. The parts of plant were taken and isolation of endophytic fungi was carried out by washing healthy plant tissues in running tap water for 10 min and processed as follows: samples were surface-sterilized by immersing in 70% ethyl alcohol for 1 min, then soaked in 5% sodium hypochlorite for 3 min, drained and immersed with 70% ethyl alcohol for 30 seconds. The samples were rinsed three times in sterile distilled water and surface-dried with sterile filter paper.

After surface sterilization, the samples were cut aseptically into 1 cm long segments and placed on the surface of PDA plates supplemented with streptomycin (30 μg/mL). The plates were incubated at 25 °C for 10-15 d and the fungal colonies growing out from the segments were transferred into new PDA plates free of antibiotics. The pure cultures were grown on PDA plates without antibiotics and maintained for culture collection of Pharmaceutical Biology Department, Faculty of Pharmacy Universitas Gadjah Mada. The endophytes were also cultivated for 10 d on potato dextrose broth for investigations of biological activity.

2.3. Semipolar extraction of fermentation cultures

The endophytic fungi isolates were cultured in 200 mL potato dextrose broth in Erlenmeyer flasks and incubated at 25 °C and 160 r/min for 14 d. The crude fermentation broth were filtered with Whatman filter paper and further centrifuged at 4 000 r/min for 5 min. Liquid supernatant was extracted with an equal volume of ethyl acetate thrice. The ethyl acetate fractions were then evaporated under reduced pressure to yield an ethyl acetate extract. The ethyl acetate extracts were dissolved in dimethylsulfoxide for antimicrobial and cytotoxicity testing.

2.4. Antimicrobial testing

Screening for antimicrobial agent producing fungi was conducted using hole-plate assay[17]. The 5 mm holes were made on the agar plates and replaced with isolated fungi. Antimicrobial agent producing fungi were screened based on the appearance of inhibition zone surrounding fungi following 24 h incubation at 37 °C. Five pathogenic bacterial strains [*Bacillus subtilis (B. subtilis)*, *Staphylococcus aureus (S. aureus)*, *Escherichia coli (E. coli)*, *Pseudomonas aeruginosa (P. aeruginosa)*, *Salmonella typhi (S. typhi)*] were used as testing microorganisms. The antimicrobial testing was carried out by using disc diffusion method[18]. Graded doses of extracts (31.25-500 μg/disc) were prepared with each consecutive disc containing half amount of the consecutive dose. Kanamycin (10 μg/disc) was used as standard, and ethanol was used as negative control. The test microorganisms were mixed with sterile nutrient agar and transferred into Petri dishes to give a solid plate. The discs containing extracts were deposited on the surface of agar plates and then were incubated for 24 h at 37 °C. Diameters of clear zone of inhibition were measured at the end of incubation. Contact bioautography was carried out to characterize the bioactive compounds within the extracts. thin layer chromatography (TLC) plates containing separated compounds of extracts were placed onto surface of agar plates containing testing microorganisms for 30 min. The plates were removed and the agar plates were incubated for 24 h at 37 °C. Zone of inhibition appeared in the place where the antimicrobial agents contacted with agar[17].

2.5. Cytotoxic activity

T47D (Human ductal breast epithelial tumor cell line) and WiDr (human colon carcinoma cell line) were cultured in RPMI1640 supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin/streptomycin. Cultures were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO_2. About 100 μL of media containing 5×10^3 cells was added to 96-well plate and incubated for 48 h until 70%-80% confluent. Extracts at various concentrations were added. Following 24 h of incubation, cells were gently washed with 1× phosphate buffer solution, and 100 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (0.5 mg/mL) was added to the well. The cells were incubated for 4 h at 37 °C and the reaction was stopped by adding 100 μL sodium dodecyl sulfonate (10%). The plates were incubated overnight and read in microplate reader (Bio-Rad) at 595 nm. Data generated were used to plot a dose-response curve, of which the concentration of extract required to kill 50% of cell population (IC_{50}) was determined.

3. Results

3.1. Isolation and primary screening of endophytic fungi

Two fungal endophytes were obtained from the *P. crocatum* plant collected from Medicinal Plant Garden, Faculty of

Pharmacy, UGM; one was isolated from the stem (DS1) and the other from the leaves (BS1). All isolates were screened for antimicrobial activities and the results showed that one isolate (BS1) inhibited the growth of *B. subtilis*, *E. coli* and *S. aureus* among five testing microorganisms (Table 1). This isolate was selected for further experiments.

Table 1

Diameters of inhibition zone of endophytic fungi isolated from *P. crocatum* against testing microorganisms.

Code	Diameters of inhibition zone (mm)				
	B. subtilis	*E. coli*	*S. aureus*	*P. aeruginosa*	*S. typhi*
DS1	0	0	0	0	0
BS1	7	7	15	0	0

3.2. Antimicrobial activity and TLC bioautography analysis

The fermentation broth of BS1 was partitioned using ethyl acetate and the ethyl acetate extract was screened for antimicrobial activity against *B. subtilis*, *E. coli* and *S. aureus* by disc diffusion method (Table 2). The extract showed greater activity against *B. subtilis* with minimum dose of 31.25 μg compared to that against *E. coli* and *S. aureus* which need 125 μg and 250 μg respectively to show inhibition. At least 500 μg of extract was required to inhibit the growth of *B. subtilis* and *E. coli* comparably to positive control kanamycin.

Table 2

Diameters of inhibition zone by BS1 ethyl acetate extract as determined by disc diffusion method.

Dose	Diameters of inhibition zone (mm)		
	B. subtilis	*E. coli*	*S. aureus*
Kanamycin 10 μg (control+)	19	20	24
Extract 500 μg	20	20	16
Extract 250 μg	14	17	14
Extract 125 μg	14	11	0
Extract 62.5 μg	9	0	0
Extract 31.25 μg	8	0	0
Solvent (control−)	0	0	0

Data is representative of at least two independent experiments.

TLC-bioautography was conducted to further characterize the active compounds within the extract. The chromatographic profiles of the extract under visible light showed the presence of three compounds with hR_F of 38, 55 and 66 on TLC plate which also absorb 254 nm UV light to give dark spots against green fluorescence (Figure 1). Detection using vanillin-sulphuric acid reagent showed the presence of three major compounds with hR_F of 9, 24 and 60. Based on TLC-bioautography, there were minimum two inhibition zones against three testing bacteria. The first inhibition zone laid between hR_F of 50-60 and based on TLC profiles. It suggested that at least two compounds were responsible for the antibacterial activities. These compounds and the other bioactive compounds which laid on hR_F of 70-80 (second inhibition zone) were unable to be detected with other spray detection reagents ($FeCl_3$, Dragendorff, 2,4-Dinitrophenylhydrazine, $SbCl_3$, citroboric acid). Comparative analysis of TLC profiles revealed that the bioactive compounds were not present in the host plant (Figure 2).

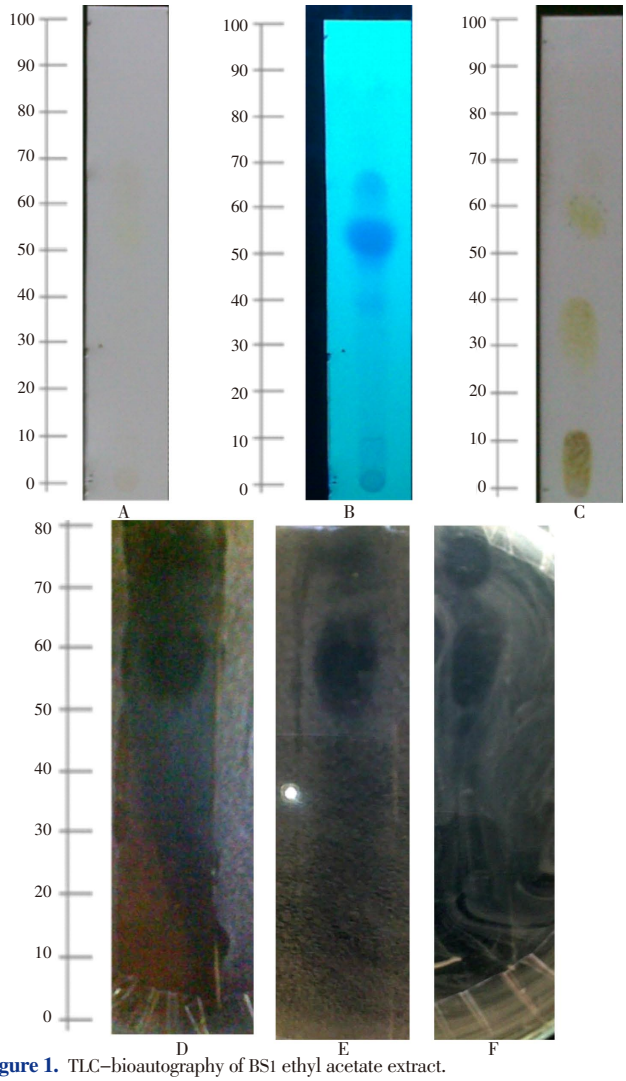

Figure 1. TLC-bioautography of BS1 ethyl acetate extract.

BS1 ethyl acetate extract was separated by TLC using silica gel F254 as stationary phase and *n*-hexane: ethyl acetate: acetic acid glacial (10:89:1) as mobile phase. Inhibition zones were examined following 24 h incubation of the diffused compounds from TLC plates to agar plate at 37 °C.

(A): TLC profile before spraying with vanillin-sulphuric acid detection reagent; (B): TLC profile detected using UV254 lamp; (C): TLC profile after spraying with vanillin-sulphuric acid detection reagent, heated at 110 °C; (D): Zone inhibition of TLC plates against *B. subtilis*; (E): Zone inhibition of TLC plates against *E. coli*; (F): Zone inhibition of TLC plates against *S. aureus*.

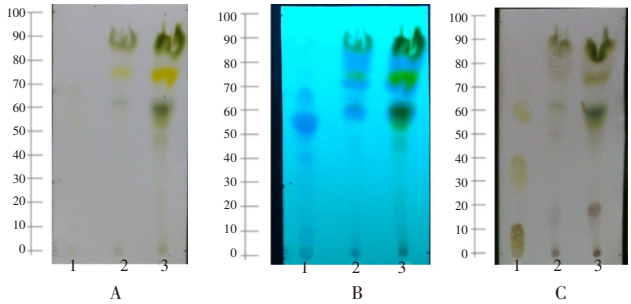

Figure 2. TLC profiles of ethyl acetate extract of BS1 endophytic fungi and *P. crocatum* extract.

(A): Visible light; (B): UV254 lamp; (C): vanillin-sulphuric acid detection reagent. Stationary phase: silica gel F254. Mobile phase: *n*-hexana: ethyl acetate: acetic acid glacial (10:89:1). 1): ethyl acetate extract of BS1; 2): stem extract; 3): leaves extract.

3.3. Cytotoxic activity

Cytotoxicity of the BS1 extract against T47D and WiDr cell lines is shown in Figure 3. The extract was found to inhibit the growth of cancer cell lines in dose dependent manner. The extract was found to be more toxic against T47D than WiDr with IC_{50} of 37.43 μg/mL and 120.38 μg/mL, respectively.

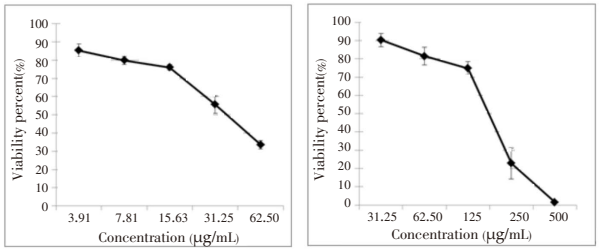

Figure 3. Cytotoxic effect of BS1 ethyl acetate extract against T47D and WiDR cells.

(A): T47D cells; (B): WiDR cells. Cell viability was determined as percentage of the control. Each bar represents the means±SD (n=4). This data is representative from at least two independent experiments.

4. Discussion

Endophytes are ubiquitously present in plant tissues with populations dependent on host species and location[6]. Endophytic fungi have been known as the source of secondary metabolites, some of which exhibited biological activities[19-22]. Medicinal plants have been considered to be potential source for endophytes producing associated plant natural products[22], as in the case of deoxypodophyllotoxin producing endophytic fungi which has been isolated from *Juniperus communis*[23]. *P. crocatum*, a kind of traditional medicinal plant, has been widely used by Indonesian to treat various diseases and used as components in some ethnics' ceremonial. This plant has been reported to contain essential oils, alkaloid, tannin and steroids[13,14]. Despite the progress of its phytochemical and pharmacological screenings, there is little information about bioactivity of endophytic fungi associated with this plant.

In this study, two endophytic fungi have been isolated, and one of them designated as BS1 isolated from the stem of the plants, has been shown to produce compounds having antibacterial and cytotoxic activities. BS1 ethyl acetate extracts exhibited different ability in inhibiting the growth of testing microorganisms. At dose of 500 μg this extract showed similar diameters of inhibition zones to kanamycin control (10 μg) against *B. subtilis* and *E. coli*. Fewer doses is needed to exhibit growth inhibition against *B. subtilis* compared to that against other testing organisms, indicating differential components or target of actions responsible for antibacterial activities. Further analysis of antibacterial components within the extract by TLC bioautography revealed that at least two inhibition zones were observed against three testing microorganisms. TLC bioautography allow us to localize the active compounds on chromatogram so that it provides quicker method for screening and isolating bioactive compounds within the extract[24].

TLC analysis of active compounds using spray detection reagents suggested that the active compounds may be those that have conjugated double bonds, aromatic rings or some unsaturated compounds as shown by its ability to quench fluorescent indicator within the solid phase of chromatogram[25]. These compounds also showed positive interaction with vanillin-sulphuric acid reagent which suggested the presence of terpenoids or phenylpropanoids[26]. Comparative analysis of TLC profiles of BS1 extract with extracts from the stem and leaves of host plant showed different profiles, indicating that secondary metabolites produced by the endophytic fungi may be different from the host plant. The differences in secondary metabolites production may be explained by the uncertainty of products secreted by endophytes in culture and in nature. *In situ*, production may be influenced by its biological function such as protection of host from pathogens. In addition, the biology of the endophytes may be influenced by many factors such as localization, season, age and environment that could affect the host plant[22].

Many studies reported the ability of antimicrobial compounds to be toxic to human cancers[3,19] and anthracyclines were an example of a well-known antibiotics used as anticancer drugs[27]. In this study, the cytotoxicty of BS1 were tested against two human cancers, T47D and WiDr. BS1 extract was found to be cytotoxic to the cancer cell lines with growth inhibition to be more effective against T47D compared to WiDr. These data demonstrated the potential of BS1 extract to be explored for drug candidates.

This finding suggested the presence of bioactive compounds within BS1 extract which are potential for development as pharmaceutical agent.

Conflict of interest statement

We declare that we have no conflict of interest.

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