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Cytotoxic Secondary Metabolites from Fermentation Broth of *Brucea javanica* Endophytic Fungus 1.2.11

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Abstract: Endophytic fungus 1.2.11 was isolated from the fruits of *Brucea javanica* (L.) Merr plant collected from Cianjur, West Java. The purpose of this study was to investigate the secondary metabolites from the fermentation broth of endophytic fungus 1.2.11 as anticancer potential. Liquid fermentation with Potato Dextrose Yeast extract (PDY) broth was carried out followed by incubation over 14 days in a shaking incubator. *In vitro* cytotoxic assay was performed using leukemia cell L1210. Viable cells were counted using Trypan blue method. The IC_{50} was obtained arithmetically using the Reed and Muench standard. Purification and isolation of cytotoxic secondary metabolites of the n-butanol extract were performed using gravity column chromatopgraphy and semi-preparative high performance liquid chromatography (HPLC) led to the F4-fraction as the active fraction. This fraction showed an IC_{50} of 4.29 μg mL^{-1}. The LC-MS analysis of F4 showed a total ion chromatogram having five peaks, peak 1-5 as the secondary metabolites. The MS data of peaks 3 and 5 had molecular ions of m/z $[M]^+$ 487 and 252, respectively. Peak 3 was proposed as a derivative of bruceocin and peak 5 was a canthin-6-one, respectively. Bruceocin and canthin-6-one were previously reported as the cytotoxic constituents from the *B. javanica* leaves. The current study demonstrated the possible endophytic fungi living symbiotically within the host plant *B. javanica* to produce cytotoxic secondary metabolites. One of the isolated fungi was fungus 1.2.11, identified as Fusarium chlamydosporum.

Key words: *Brucea javanica* (L.) Merr, endophytic fungus 1.2.11, *Fusarium chlamydosporum*, IC_{50}, cytotoxic

INTRODUCTION

Microorganisms such as bacteria, fungi or yeast, can live symbiotically within the host plant for more or less long period of their life, colonize symptomless in the living plant tissues of their host. These microorganisms are named as endophytic microbes. Endophytic microbes can produce secondary metabolites, such as, enzyme, growth hormone, anti-microbial, anti-fungi or as anti-cancer substance (Petrini *et al*., 1992; Strobel and David, 1998). Strobel and Daisy (2003) demonstrated that these secondary metabolites were potential drugs for treatment of newly developing disease in humans, also in plants and animals. Lacking in advance knowledge on endophytes and their medicinal activity has encouraged researchers to investigate the importance of endophytic microorganisms particularly the secondary metabolites produced by these microorganisms as potential anti-cancer agents.

Brucea javanica (L.) Merr was well known by the Indonesian people as Tanaman Buah Makassar. This plant has been used as traditional medicine for various diseases including leukemia and cervical, skin and lung cancer (Sudarsono *et al*., 2002). Extracting plant for isolating the potential bioactive substances was the most common techniques used in many studies. This technique needs

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an ample quantity of extracts and from environmental perspective; It was harmful to the plant. A more advance technique was developed recently, by isolating endophytic microbes from parts of the plant such as fruits, leaves or twigs (Petrini *et al*., 1992). Previous studies on *Brucea javanica* (L.) Merr plant led to the isolation of quassinoids, bruceocin, bruceosides D, E, F and alkaloids canthin-6-one which were demonstrated to have potent cytotoxic effect against leukemia, colon, melanoma and ovary cancer cells (Kim *et al*., 2004; Ohnishi *et al*., 1995). Interestingly, up to now, to best our knowledge, there was no study on the isolation of bioactive secondary metabolites from endophytic microbes of *B. javanica*. The current studies were focused on (I) isolation and identification of endophytic microbe from *B. javanica* and cytotoxic evaluation against leukemia cell L1210 of the secondary metabolites generated from the isolated microbe and (ii) purification of the active secondary metabolites.

MATERIALS AND METHODS

Materials

Fresh and healthy *B. javanica* was collected from Cianjur. Parts of plant used in the study were fruits. The plant voucher specimen was deposited at the Bogor Herbarium, Indonesian Institute of Sciences. The Leukemia cells were obtained from National Agency of Atomic Energy, BATAN, Pasar Jumat, Jakarta.

HPLC Semi Preparative and LCMS Analysis

Semi preparative HPLC was a Shimadzu 6AD, C-18 column, uv-detector λ 254 and 366 nm, solvent system a mixture of Methanol-water 70%. The LC-MS was ESI TOF Biosystem model Mariner Biospectrometry Workstation system, C-18 capillary column, MS detector, solvent system Methanol-water 70%.

Endophytic Fungi Culture Media

Endophytic fungi culture medium was composed of corn meat malt agar (CMM) from DIFCO USA. Potato dextrose broth was from Oxoid (England). Potato dextrose agar (PDA) DIFCO, 76% ethanol and 5.3% sodium hypochlorite were used for surface sterilization.

Methods

Endophytic Fungi Isolation

Endophytic fungi isolation was carried out via direct seed plant and Surface sterilization methods, adapted from Song (1998) and Strobel and Daisy (2003). Identification of endophytic fungi (Domsch and Gams, 1980).

Endophytic fungi were identified by observation of the hypae and conidia-using a microscope. Media used for this study were (I) MEA which composed of Malt extract (20 g), Agar (20 g) and (ii) MIURA medium containing glucose (1 g), KH_2PO_4 (1 g), $MgSO_4$. $7H_2O$ (0.2 g), $NaNO_3$ (2 g), yeast extract (0.2 g) and agar (20 g). Both MEA and MIURA media were made using distilled water to final volume of 1000 mL. Endophytic fungi were cultured in Petri dishes, for 5 days. Hypae were collected using needles and cultured on the MEA and MIURA media at 27-29°C for 5-7 days in an incubator. The development of hypae and conidia were observed using a microscope at a magnification of 400 x.

Liquid Fermentation

In order to obtain secondary metabolites as anti-cancer potential, liquid fermentation was performed using potato-dextrose-yeast (PDY) medium: (potato dextrose broth (24 g L^{-1}), yeast extract (2 g L^{-1}) and $CaCO_3$ (5 g L^{-1}) at pH 6.0). A liquid fermentation of the isolated cultured endophytic fungus was carried out for 14 days, in a 250 mL Erlenmeyer flask, containing 50 mL PDY medium in an orbital shaker incubator rotating at 130 rpm, at room temperature (Kumala *et al*., 2005).

Purification of Isolate Fungus 1.2.11

Extraction

Separation of cell mass from supernatant containing the secondary metabolites of interest was carried out in a cold centrifuge at -4°C rotating at 2000 rpm for 20 min. The pH of supernatant was adjusted to 3-4 using acetic acid 1 M and then extracted by n-butanol three times. Then, Na_2SO_4 anhydrous was added to remove the last trace of water from the n-butanol extract. This extract was evaporated to small volume at 60°C using rotary evaporator.

Fractionation

Secondary metabolites of the extract produced by fungus 1.2.11 were further purified by silica gel short column chromatography using non polar solvent mixtures of Hexane: Ethyl acetate, in turn, (100:0; 90:10; 80:20; 70:30; 60:40; 50:50; 40:60; 30:70; 20:80; 10:90; 0:100 and followed by semi polar solvent mixture of ethyl acetate: methanol (99:1; 97:3; 95:5; 93:7; 90:10; 85:15; 80:20; 75:25; 70:30; 65:35; 60:40; 55:45; 50:50; 45:55; 40:60; 35:65; 30:70; 25:75; 15:85; 5:95; 0:100). This fractionation was monitored by TLC (Thin Layer Chromatography) and purified using semi preparative HPLC and analyzed using LC-MS.

Isolation and Purification of Fractions and Cytotoxic Assay Towards Leukemia Cell L1210

The cytotoxic effect of these fractions was evaluated using *in vitro* leukemia cell L1210 the cytotoxic assay. Then, an LC-MS instrument was selected to identify the active constituents using an established purification procedure.

Bioassay Using Leukemia Cell L1210

Cytotoxic evaluation of secondary metabolites generated by the endophytic fungus was measured based on its *in vitro* cytotoxic effect on leukemia cell (L1210). One thousand microliter of L1210 cell suspension were added into each of the 24 multi-well plate followed by the addition of 10 μL methanolic solution of fermentation product at three different concentrations, 2.5, 5 and 10 μg mL^{-1} and incubated for 48 h. As the negative control, the cells were treated with only Eagle's MEM containing 10 μL methanol. Each treatment was done in triplicate (Sumatra, 1998).

Calculation of Cell Deaths Percentage

$$\text{celldeath\%} = \frac{K - P}{K} \times 100\%$$

K = Control, viable cell without any treatment.
P = amount of viable cell with treatment.

Calculation IC_{50}

The IC_{50} was calculated according to Reed and Muench Calculation by an arithmetic method (Robert and Turner, 1972).

log CPE 50% = log A + P. log B

RESULTS

Identification of Isolated Endophytic Fungus

Nine fungi isolates consisting of various endophytic fungi were obtained from the fruits of *B. javanica*. Identification of fungi morphologically was done by macroscopic observation of the

colonies. Fungi colonies grew in the PDA medium at 27-29°C attained a diameter 6 cm within 5 days. It was rapid growth. Colonies woolly, frequently growing in blooms, initially white, becoming pink to red and brown centrally. The reverse colony was brown to carmine red. This fungus colony, fungus 1.2.11, was isolated and identified as *Fusarium chlamydosporum* (Fig. 1).

From all the nine microbial isolates, the isolated endophytic fungus 1.2.11 was selected for further study.

Isolation and Purification of Secondary Metabolite Generated from Endophytic Fungus Isolate 1.2.11 and Cytotoxic Assay of Fraction F4 on Leukemia Cell L1210

Secondary metabolites from the fermentation broth extract produced by fungus 1.2.11 were purified by silica gel short column chromatography using various non-polar solvent mixtures of hexane: Ethyl-acetate followed by semi polar solvent mixtures of ethyl acetate and methanol, to yield 10 fractions, divided based on the similarity of the thin layer chromatography (TLC) patterns.

Furthermore, a semi preparative HPLC was performed to purify of these 10 fractions and as a result, 4 fractions (F1-F4) was generated further. F1-F4 fractions were dried and the mass each fraction was 3.7, 0.5, 2.5 and 60 mg, respectively. F4 (60 mg) was chosen for the cytotoxic assay. It was incubated with L1210 cells for 48 h and showed an IC_{50} of 4.29 μg mL^{-1}.

Considering the potent cytotoxic effect of F4 on L1210, purification of this fraction using a semi preparative HPLC was conducted. The HPLC profile of this fraction showed only two peaks. Then, further purification was carried out using LC-MS. Identification of the fractions using LC-MS the secondary metabolites having protonated molecular ions $[M^+]+1$, m/z 487 and 252, respectively.

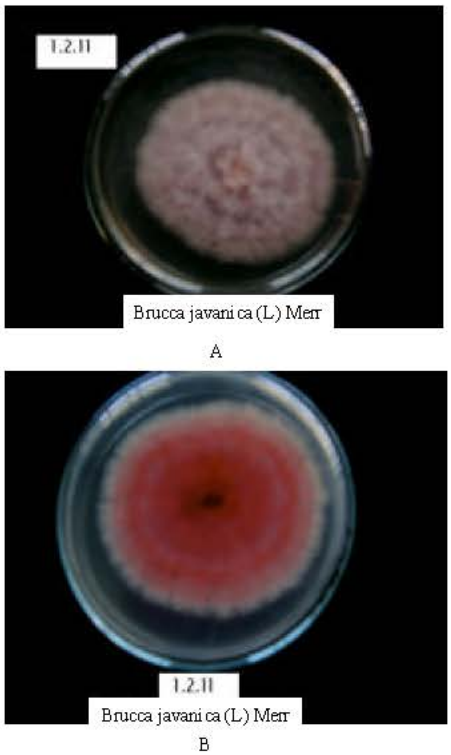


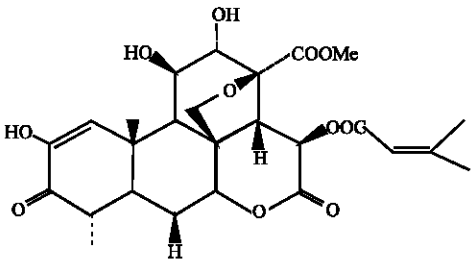
Fig. 1: Isolated endophytic fungus 1.2.11 was obtained from *Brucea javanica's* fruit in Cianjur location. (A) isolated colony and (B) reverse colony

DISCUSSION

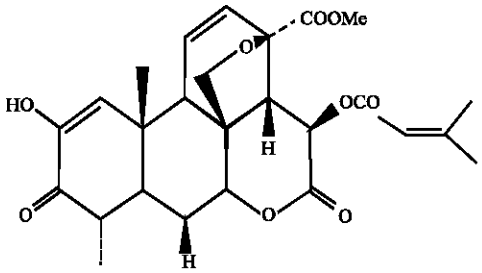
The current study was to isolate endophytic microbes from *B. javanica* and to evaluate the secondary metabolites generated by these microbes for anti-cancer activity using the cytotoxic bioassay against Leukemia cells L1210. The technique performed in this study might be more efficient compared to common technique using the plant extraction. The secondary metabolites can be extracted from microbe fermentation products for bioactive screening against various biological activities including anti-cancer activity. Moreover, from environmental perspective, these techniques cause no harm to the plants as only parts of the plants were used in the experiment.

Endophytic fungus isolate (1.2.11) produced secondary metabolites with potent cytotoxic activity (IC_{50} of 4.05 μg mL^{-1}) against leukemia cells (L1210) on primary study (Kumala *et al*., 2006). This evidence suggested that secondary metabolites generated by *Fusarium chlamydosporum* fungus isolated from this plant might be potential for anti-cancer substances. n-Butanolic extract containing secondary metabolites generated by fermentation of this fungus was purified using various chromatography techniques to isolate the active constituents.

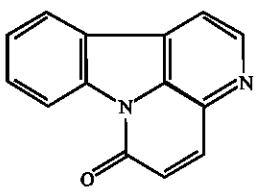
Fractionation F4 consisted of two compounds having molecular weight of 486 and 252, respectively. F4 was cytotoxic active showing an IC_{50} of 4.29 μg mL^{-1} (Swanson and Pezzuto, 1990). It is likely that the first secondary metabolite, having molecular weight of 486, was a derivative of Bruceosin by loosing of 2 hydroxyl groups. Based on this evidence, the derivative compound was proposed as 11, 12-Dedihydroxybruceosin. Bruceosin, the known secondary metabolite from *B. javanica* might have lost 2 hydroxyl groups (predicted the OH functional groups of C11 and 12). The second secondary metabolite, having molecular weight of 252, was proposed as Dihydroxy-canthin-6-on, resulted from an enzymatic hydroxylation process of canthin-6-on, the common alkaloid found in Simarobaceae plant.



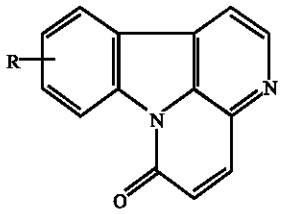
Bruceosin $C_{26}H_{32}O_{11}$ M = 520.532



11,12-Dedihydroxybruceosin. $C_{26}H_{30}O$, Molecular Weight (MW): 486.19



Canthin-6-one $C_{14}H_8N_2O$ BM: 220, 06



R = 20H

Dihydroxy canthin-6-one MW: 252, 05

Further purification of fraction F4 followed by NMR and elemental analysis are required to identify these secondary metabolites. Bruceosin and Canthin-6-one are natural substances found in *Brucea javanica* (L.) Merr with a well-known cytotoxic effect. LC-MS analysis demonstrated that substances identified were likely to be derivatives of these naturally occurring substances, suggesting that fungus 1.2.11 produce secondary metabolites having similar cytotoxic effect to the host plant, *B. javanica*. The current study also demonstrated clearly that endophyte fungi from *B. javanica* produced similar substances as the host plant. This is in line with the findings of (Stierle and Strobel, 1995) that showed endophytic fungi *Taxomyces andreane* isolated from the *Taxus brevifolia* produce secondary metabolites, the anti neoplastic taxol, similar to that produced by the host plant.

REFERENCES

Domsch, K.H. and W. Gams, 1980. Compendium of soil fungi. Vol. 1. Academic Press, pp: 310-311, 378-380.

Kim, I.H., S. Takashima, Y. Hitotsuyanagi, T. Hasuda and K. Takeya, 2004. New quassinoids, javanicolides C and D and Javanicosides B-F, from seeds of *Brucea javanica*. Nat. Prod., 67: 863-868.

Kumala, S., W. Mangunwardoyo and P. Budiarti, 2005. Stated and shake fermentation of endophytic fungi isolate from *Brucea javanica* (L.) Merr and anti-microbial assay. Ind. J. Pharm. Sci., 3: 12-15.

Kumala, S., R. Utji, P. Sudarmono and L.B.S. Kardono, 2006. Isolation of endophytic fungi from *Brucea javanica* (L.). Merr and cytotoxic evaluation of their n-butanol Extract from Fermentation Broth. Pak. J. Biol. Sci., 9: 825-832.

Ohnishi, S., N. Fukamiya, M. Okano, K. Tagahara and K.H. Lee, 1995. Bruceosides DE and F three new citotoxic quassinoid glucosides from *Brucea javanica*. J. Nat. Prod., 58: 1032-1038.

Petrini, O., T.N. Sieber, L. Toti and O. Viret, 1992. Ecology metabolite production and substrate utilization in endophytic fungi. Nat. Toxins, 1: 185-196.

Robert, A. and Turner, 1972. Screening Methods in Pharmacology. New York, Academic Press, pp: 60-67.

Song, Y., 1998. Isolation and cultivation of endophytic fungi. Proceeding of International Conference on Asian network on Microbial Researches. Feb 1998, Gajah Mada University, Yogjakarta, Indonesia, pp: 255-258.

Stierle, A. and G. Strobel, 1995. The search for a taxol producing microorganism amomg the endophyte fungi of the pacific yew, *Taxus brevifolia*. J. Nat. Prod., 58: 1315-1324.

Strobel, G. and B. Daisy, 2003. Bioprospecting for microbial endophytes and their natural products. Microbiol. Mol. Biol. Rev., 67: 491-502.

Strobel, G.A. and D.M. Long, 1998. Endophytic microbes embody pharmaceutical potential. ASM News, 64: 263-268.

Sudarsono, G., Didik, W. Subagus, A.D. Imono and Purnomo, 2002. Medicinal Plant II Research product, characteristic and usage. 1st Edn., Jogyakarta. Center of traditional medicine, University of Gajah Mada, pp: 28-31.

Sumatra, M., 1998. *In vitro* Bioassay of Leukenmia L1210, Screening method of antitumor agent from natural product: Seminar Proc. Indonesian Marine Biotechnology. Jakarta, pp: 14-15: 183-188.

Swanson, S.M. and J.M. Pezzuto, 1990. Bioscreening technique for cytotoxic potential and ability to inhibit macromolecule biosynthesis. In: Drug Evaluation Techniques in Pharmacology. Thompson, E.B. (Ed.), New York, WCH Publisher Inc., pp: 273-297.