



CYTOTOXIC EFFECT OF SECONDARY METABOLITES PRODUCED BY ENDOPHYTIC FUNGI 1.3.11, 1.1.6 AND 1.2.6 ISOLATED FROM THE FRUIT OF "TANAMAN BUAH MAKASSAR" (*BRUCEA JANVANICA* (L.) MERR) ON IN-VITRO T47D AND MCF-7 INTACT CELLS AND IDENTIFICATION OF THE FUNGUS 1.3.11 BY RIBOSOMAL DNA SEQUENCE ANALYSIS.

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ABSTRACT

The cytotoxic effect of secondary metabolites extract of endophytic fungi (1.3.11, 1.1.6, and 1.2.6) isolated from the fruit of "Tanaman Buah Makassar" on in-vitro T47D and MCF-7 intact cells and identification of the fungus 1.3.11 by ITS regions of ribosomal DNA sequence were carried out. The cytotoxic effect of these extract (IC₅₀) was assessed on T47D and MCF-7 cell (concentration 5-200 µg/ml) over 24 and 478 hr in CO₂ using MTT methods on ELIZA reader at 595 nm. The results shows IC₅₀ of fungi 1.3.11 and 1.2.6 were 7 and 50 µg/mL respectively. Extracts of fungi 1.1.6, however, showed weak or no cytotoxic effect toward T47D. Incubation of MCF-7 cells with extract of fungi 1.3.11, it showed an IC₅₀ of 31 µg/mL. Furthermore, both T47D and MCF-7 cells showed pronounced morphological changes after 48 hour incubation with extracts of fungi 1.3.11 at 0.5 and 5 µg/mL. Moreover, T47D, but not MCF-7 cells showed morphological changes when incubated with extract of (i) fungi 1.2.6 (40 µg/mL) and (ii) fungi 1.1.6 (232 µg/mL). The results of identification of the fungus based on ribosomal DNA sequence show that it has highest DNA sequence similarity with *Botryosphaeria parva*.

Keywords: Endophytic fungi, Secondary metabolites, Cytotoxic effect, *Brucea javanica* L (Merr), ITS regions of ribosomal DNA sequence, *Botryosphaeria parva*.

INTRODUCTION

Endophytic microbes are microbes living in tissues of host plants. Several researches on endophytic microbes show the importance of these microbes in the production of potentially bioactive substances having effect like enzymes, such as plant growth inducing substance, anti-fungal, antibiotics, anti tumor and anti cancer substances, which is advantageous in industry as well as in the medical field, especially in the production of medicine^{1,2}. A lot of researches have been made on extracting substances from plants, but researches on microbes that can produce anti cancer substance are limited, so that it is necessary to find a plant of which the contained substance is easy to extract, without damaging the plant itself. Endophyte microbes that can produce secondary metabolites with cytotoxic effects, raw material for medicines can be developed.

The fruit of tanaman buah Makassar was consumed by the Indonesian society as traditional medicine for treatment of dysentery, malaria, and cancer. The current study evaluated the cytotoxic effect of secondary metabolites extract of endophytic fungi (1.3.11, 1.1.6, and 1.2.6) isolated from this plant on in vitro T47D and MCF-7 intact cell model, and identification fungus by ribosomal DNA sequence analysis.

MATERIALS AND METHOD

Material

Isolate of endophytic fungi (1.3.11; 1.1.6 and 1.2.6 from fruit of *Brucea javanica* L (Merr), PDY fermentation medium, primer ITS 4 and ITS 5.

Methods

Isolation and screening of endophytic microbes using surface sterilization and direct seed method was performed based on published methods². Liquid fermentation which was followed by cold centrifugation was performed on endophytic fungi to obtain supernatant containing secondary metabolites. Extractions were carried out on the supernatant to give dried n-butanol extracts Cheeptan modification³. The cytotoxic effect of these extracts (IC₅₀) was assessed on T47D and MCF-7 cells (concentration 5-200 µg/ml) over 24 and 48 hr in CO₂ filled incubator at 37°C, followed by

MTT assays on ELIZA microplate reader at 595 nm according to Mossman method⁴. Lastly, morphological studies using light microscopy techniques were carried out both cells after and before treatment with the secondary metabolites extract of fungi mentioned above.

1.3.11 Endophytic fungi Identification

Endophytic fungi culture

Pure and fresh endophytic fungi culture in PDA which has been incubated for 7-14 days in room temperature.

DNA Extraction

Boiling method

Endophytic fungi hypha was suspended into 500 µl of nuclease free water in an eppendorf tube using a sterile toothpick. Boiled the hypha suspension for 30 min then centrifuged at 13000 rpm for 15 min⁵. The supernatant is used as template DNA.

PCR reaction mixture for final volume reaction 12.5µl

Primer ITS 5 (10 µM), 0.5 µl, Primer ITS 4 (10 µM), 0.5 µl, Template DNA 5.0 µl, Ready to Go. Suspension 6.5 µl and Primer ITS 4 and ITS 5⁶.

PCR condition

The PCR condition was begin with denaturation at 95°C for 2 min, then the denaturation at 95°C for 15 sec, the annealing at 58°C for 30 sec and followed by 40 cycles of the extension at 68°C for 1 minute. Final extension at 68°C for 40 sec then stored the PCR product at 16°C.

Measurement of DNA Quality and Quantity

Measurement of DNA Quality and Quantity was performed by Nanodrop Spectrophotometer. The measurement of the quality and quantity of DNA was done at λ= 260 nm/280 nm.

Gel Electrophoresis

Gel electrophoresis was performed by using agarose 2 % and electrophoresis was run at 100 V for 25 min then the gel agarose was soaked in EtBr solution for 10 min. One µl of blue/orange loading dye was added to each sample and DNA molecular weight (MW) marker (100 bp DNA ladder or DNA marker 1 Kb). Two µl of DNA MW marker was mixed into 1 µl of 6x blue/orange loading dye, 5 µl of PCR product was added into 1 µl of 6x blue/orange loading dye. Then electrophoresis result was visualized by UV transilluminators gel documentation system.

Cycle-sequencing

PCR reaction mixture for cycle-sequencing (100 µl)

Big dye terminator ready reaction mix V 3.1 (2.0 µl), 0.5 µl of Primer ITS 5 or ITS 4 (10 µM), 1.0 µl of template DNA (> 60 ng/µl), Sequencing buffer (6.0 µl), and Nuclease free water (0.5 µl).

Cycle sequencing condition

The PCR amplification condition begun with denaturation step at 96°C for 1min and 30 cycles of denaturation step at 96°C for 10 sec, then followed by annealing step at 50°C for 5 sec and extension step at 60°C for 1½ min. Finally, PCR product was stored at 16°C.

Purification of pcr cycle-sequencing product

The PCR cycle-sequencing product was transferred into a new eppendorf tube followed by addition of 2 µl of EDTA 125 mM and 50 µl EtOH 100%. Once mixture was mixed gently, leave at room temperature for 15 min and centrifuge at 5000 rpm for 30 min. The supernatant was carefully removed by pipette followed by addition of 70 µl of EtOH 70% and another centrifugation at 5000 rpm for 15 min. Once, supernatant was removed, samples were either kept inside an incubator at 50°C for 30 min or left at room temperature for air-drying.

Sample denaturation for sequencing

15 µl of Hi-Di formamide was added into sample, mixed well by pipetting up and down and a "spindown" using micro centrifuge. These sample mixture was transferred into a new PCR tube, heated at 95°C in the PCR machine, then transferred to ice as soon as possible and finally, stored in the freezer until time for sequencing.

1. Sequence Analysis by ABI 310 Automated Sequencer
2. 13 µl of sample is transfer into a new sequencing tube then seal the tube. The tube is loaded to sample tray in ABI 310 automated DNA sequencer.
3. Homology Search Using BLAST (Basic Local Alignment Search Tool)
4. The nucleotides sequence data were compared to nucleotides data bank in www.ncbi.nlm.nih.gov by using BLAST program

RESULTS AND DISCUSSION

Following 48 hr incubation of T47D cells with extracts of fungi 1.3.11 and 1.2.6, number of viable T74D cells decreased indicating the effectiveness of these extract as potent cytotoxic substance. The IC₅₀ values, which were 7 and 50 µg/ml respectively, clearly demonstrated the potency of these extract in the intact cell assay model. Extracts of fungi 1.1.6, however, showed weak or no cytotoxic effect.

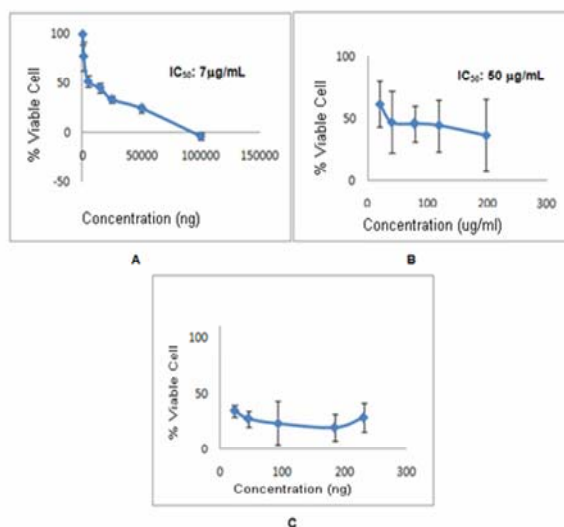


Fig. 1: Profile of viable T47D cells after treatment with (A) 1.3.11,(B) 1.2.6 and (C) 1.1.6. Cells were incubated with extracts over 48 hour as described in the material and method section. IC₅₀ of extracts 1.3.11 and 1.2.6 were 7 and 50 µg/mL respectively while 1.1.6 shown weak or no cytotoxic effect

Incubation of MCF-7 cells with extract of fungi 1.3.11, it showed an IC₅₀ of 31 µg/mL. Furthermore, both T47D and MCF-7 cells showed pronounced morphological changes after 48 hr incubation with

extracts of fungi 1.3.11 (0.5 and 5 µg/mL respectively). T47D, but not MCF-7 cells showed morphological changes when incubated with extract of (i) fungi 1.2.6 (40 µg/mL) and (ii) fungi 1.1.6 (232 µg/mL).

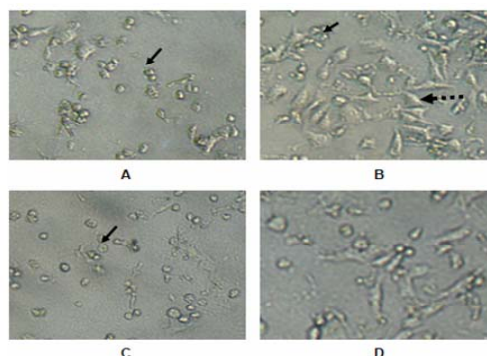


Fig. 2: T47D cell morphology after treatment with (A-B) 1.3.11 at 5 and 0.5 µg/mL, (C) 1.2.6 at 40 µg/mL, and (D) 1.1.6 at 232 µg/mL dosage concentration.

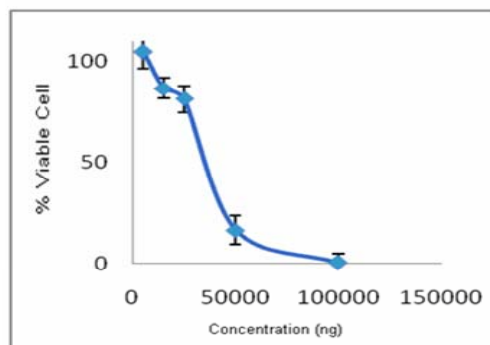


Fig.3: Profile of viable MCF7 cells after treatment with 1.3.11 with an IC₅₀ of 31mg/ml was observed. Cells were incubated over 48 hr with 1.3.11 as described in the material and methods.

Morphological changes were observed, treated cells become round when compared to control. This might be due to an apoptotic mechanism via membrane blebbing. Arrows showed possible apoptotic cells ◀▪▪▪ (Normal cells)

Studies were then continued for extract of fungi 1.3.11 and 1.2.6 on MCF-7 cells under the same condition. Incubation of extract of fungi 1.3.11 with MCF-7 cells, yield an IC₅₀ of 31 µg/ml. Furthermore, both T47D and MCF-7 cells showed pronounced morphological changes after 48 hr incubation with extracts of fungi 1.3.11 (0.5 and 5 mg/ml respectively). T47D, but not MCF-7 cells showed morphological

changes when incubated with extract of (i) fungi 1.2.6 (40 µg/ml) and (ii) fungi 1.1.6 (32 µg/ml). Moreover, based on the morphological studies, Dosage of extract of fungi 1.3.11; (ii) extract of fungi 1.2.6 and fungi 1.1.6 which were 5, 0.5 and 32 µg/ml caused a change in the T47D cells after 48 hr incubation. Observation clearly illustrated that T47D cells shape becomes round after treatment with these extracts when compared to control. Similar observation was seen in MCF-7 cells which had been treated with 5 µg/ml of extract of fungus 1.3.11. The change in morphology of MCF-7 cells was a good indication of apoptosis with membrane blebbing.

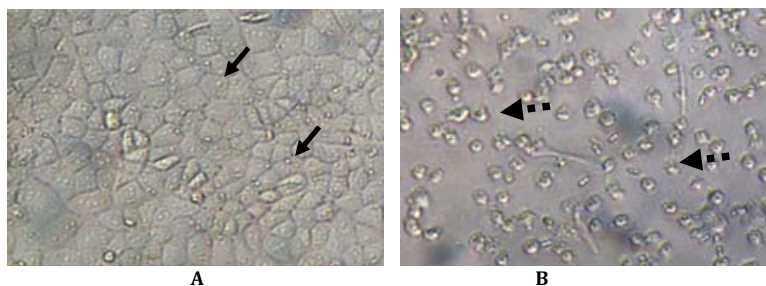


Fig. 4: MCF-7 Cells morphology after treatment with 1.3.11 (5µg/mL) (B) when compared to control (A). This showed a possible apoptotic mechanism via membrane blebbing. Arrows on photos showed ◀▪▪▪arrows showed possible apoptotic cells ◀ (normal cell)

Furthermore, it was very interesting to note the potent cytotoxic effect of 1.3.11 on both cell lines (T47D and MCF-7). Although T47D and MCF-7 cells were both mammalian breast cancer cell and were originated from ductal epithelial cells, they have different molecular characteristics, hence respond differently to treatment. Moreover, this could also suggest two possible different apoptotic mechanisms in the two cell lines. Further studies however, are required to these apoptotic mechanism explorations.

1.3.11 endophytic fungus's DNA was isolated by using 2 methods, boiling method and direct method. Boiling method was used in the current study. Products were amplified by PCR using ITS 5 and ITS 4 primer. The PCR products generated in the current study was tested by electrophoresis gel and nanodrop was conditionally acceptable.

Electrophoresis gel result

NA concentration : 429.6 ng/µl (condition 150-300 ng/µl)

After that, the next step is PCR amplification for cycle-sequencing process. This process using ABI 310 automated DNA sequencer.

After that, the nucleotides sequence data were entered into BLAST program through www.ncbi.nlm.nih.gov for checking and comparison.

Homology search result

1.3.11 endophytic mould has highest DNA sequence similarity with **Botryosphaeria parva to 95%**

Based on BLAST program, 1.3.11 endophytic fungi have highest DNA sequence similarity with *Botryosphaeria parva* to 95%. *Botryosphaeria parva* from *Botryosphaeriaceae* family is use to in stem of tree and it can cause certain diseases such as canker to the stem which infected. The culture characteristics are very solid grayish, brownish white mycelium. The reverse color is getting darker by time.

CONCLUSION

Secondary metabolites product of fungi 1.3.11 was the most potent cytotoxic substance in intact MCF-7 and T47D cell assay model.

The results of DNA sequence of 1.3.11 endophytic fungi have highest DNA sequence similarity with *Botryosphaeria parva* to 95%.

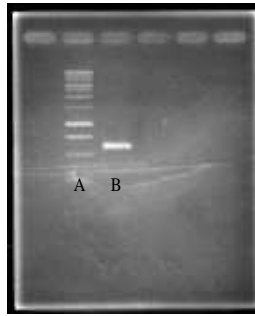


Fig. 5: Profile of electrophoresis gel. A refer to DNA marker 100 bp and B refer to band of 1.3.11 endophytic fungus DNA

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