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Isolation of Endophytic Fungi from *Brucea javanica* L. (Merr.) and Cytotoxic Evaluation of their *n*-butanol Extract from Fermentation Broth

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Abstract: Isolation of endophytic fungi from *Brucea javanica* L. (Merr) and leukemia cell L1210 cytotoxic evaluation of their *n*-butanol extract of the fermentation broth were carried out. Twigs, leaves and fruits of *B. Javanica* were collected randomly from three locations in Indonesia: Bogor, Cianjur and Tawangmangu. All specimens were cut and surface sterilized by using ethanol and sodium hypochlorite solution. Direct seed inoculating technique and Corn Meal Malt were used. For potential secondary metabolites evaluation was conducted by liquid fermentation using potato dextrose-yeast extract broth (PDY) and extracted in *n*-butanol. In this study, endophytic fungi were isolated; eleven isolates from Bogor, sixteen isolates from Cianjur and eighteen isolates from Tawangmangu. Cytotoxic assay were performed on *n*-butanol extract generated from the fermentation broth. The IC₅₀ of the *n*-butanol extracts from 18 endophytic fungi isolates ranged from 3.29-15.90 ug mL⁻¹. This results demonstrated that secondary metabolites of the *n*-butanol extract of those endophytic fungi may act as a potent anti cancer substance.

Key words: Endophytic fungi, secondary metabolite, cytotoxicity assay, *n*-butanol extract, *Brucea javanica* L. (Merr)

INTRODUCTION

Endophytic, in general, refers to a situation where one organism lives in another. Endophytes reside in the tissues between living plant cells, forming a mutually beneficial relationship with plants from symbiotic to bordering pathogens. In this case, endophytic fungi lives a symptomatically within the living tissue of the host plant and form mutualistic symbiosis. There could be more than one type of endophytic fungi found within one plant (Petrini *et al.*, 1992).

Studies by Carroll (1988) demonstrated that endophytic fungi caused no harmful effects to the living host plant and they can be isolated from various part of the plant with a careful selection and screening process. The isolation method may affect the species composition of the endophyte collection within a given host plant. Large number of isolates can be obtained from host species, however, only few host specific strains are dominant (Petrini *et al.*, 1992). Different endophytic fungi species are found in different part of a plant; this represents an adaptation mechanism of endophyte against microecology and physiology of given host plant. Various studies demonstrated that endophytic fungi produce secondary metabolites such as enzyme and growth hormone which are useful for treatment of various

diseases. These bioactive metabolites demonstrated potent anti-bacterial, anti-arthritis and anti-cancer activity as well as immunosuppressive activity (Petrini *et al.*, 1992; Carroll, 1988; Strobel and Long, 1998).

In developing countries, cancer has been a major cause of death apart from cardiovascular and cerebrovascular disease (Ueda *et al.*, 2002). Currently, Indonesia is in the 6th rank according to survey result by Cancer Registration Board of the association of Indonesian Pathologist and this status may shift up from year to year (Agency for Health Research and Development, 1997; Department of Health, 1977; DR Cipto Mangunkusurno, Cancer in DR Mangunkusurno National Center of Public Hospital, 1999). This evidence encourages scientist to intensively search for newer and more effective anti-cancer agent to minimize this problem. As an alternative, one strategy is to develop new herbal medicine which would improve the traditional means of treating cancer to the patient with high selectivity and ability to suppress cancer cells metastasis, whenever possible (Filler and Kripke, 1997).

One of the herbal plant found in regional area of Indonesia is a Simaroubaceae plant, *Brucea javanica* L. (Merr) with its local name buah makasar. This plant, empirically, has been reported to be used as traditional medicine for the treatment of diseases like dysentery,

malaria, leukemia and other cancers, including lung, cervical and skin cancer, by the local society (Department of Health, 1977; Tang and Eisenbrand, 1992; Dalimartha, 2002), however, very few studies were conducted to investigate that the effectiveness of secondary metabolites produced by its endophytes.

The current study was focussed on the isolation of endophytic fungi from buah makasar [*Brucea javanica* L. (Merr)] plant and the cytotoxic effect of secondary metabolites in *n*-butanol extract isolated in leukemia cell L1210.

MATERIALS AND METHODS

Materials: Eagle's MEM media, leukemia cell L1210 were a kind gift from Prof Dr. Yasuo Fujimoto, College of Pharmacy, Nihon University, Chiba, Japan for research work carried out in BATAN (National Atomic Energy Agency), Indonesia Chloramphenicol and Glutamine were purchased from Indonesian registered drug store. Ethanol and sodium hypochlorite were purchased from Kimia Farma. Foetal calf serum was purchased from Flow laboratories. Sodium hydrogen carbonate (NaHCO_3) was purchased from Kimia Farma.

Endophytic fungi and bacterial culture media: Endophytic fungi culture media is composed of Corn Meal Malt (CMM) from DIFCO (USA) agar. Potato dextros broth from Oxoid (England). Potato Dextrose Agar (PDA) DIFCO and Potato Dextrose Yeast Extract (PDY) were used in bacterial culture. 76% ethanol and 5.3% sodium hypochlorite were used for surface sterilization.

Sampling of *Brucea javanica* L. (Merr) plant: Fresh and healthy *Brucea javanica* L. (Merr) plant were sampled from three locations in West Java: Kebon Raya Bogor, Kebun obat P.T. Eisai Indonesia Cianjur and Balai Penelitian Tanaman obat (BPTO) Tawangmangu using random technique from June until August in dry season. Parts of plant used in the study are twigs (diameter 0.8-10 mm), leaves and fruits. The plant samples were characterised in Herbarium Balitbang Botani, Puslitbang Biology Department of LIPI, Bogor, West Java, Indonesia.

Endophytic fungi isolation: Endophytic fungi isolations were carried out *via* direct seed method. Surface sterilization methods were adapted from Petrini *et al.* (1992) and Carroll (1988). Briefly, samples were washed under running water for about 10 min to remove dust and dirt, and were cut into small pieces and air-dried at room temperature. The twigs and fruits were cut into dimension of 1 cm pieces; while the leaves were cut into square 0.5×0.5 cm dimension. Then, surface sterilization was conducted as follows: specimens were soaked in 76%

ethanol for 1 min, followed by hypochlorite solution 5.3% for 5 min and finally in ethanol 76% for 30 sec. The surface-sterilized pieces of twigs and fruits were aseptically sliced lengthwisely. The cut surface of the twigs and fruits and the squared pieces leaves were immediately placed on the surface of CMM medium which contained chloramphenicol (0.005% w/v). They were then incubated at temperature of 27-29°C for 5-7 days. Then, a selection process based on fungi morphology were applied to differentiate endophytic fungi from other microbes grown in the culture. The selected fungi were transferred and were cultured in PDA media (Tomita, 1995).

Purification of isolated fungi: Colony identification was done macroscopically by observing colony characteristic such as growth rate, color and colony morphology. Colonies with same morphology from one isolates were pooled together, on the other hand, the unidentified colonies were separated and grouped according to their morphological status. The pure fungi is then cultured in slant agar. Each of the isolated fungi was culture in two different types of culture media (stock and work culture). Stock culture was kept in freeze dried while work culture was kept in slant agar.

Liquid fermentation: In order to obtain secondary metabolites which have the potential as anti cancer substance, liquid fermentation using PDY medium, which consists of potato dextros broth 24.0 g L⁻¹, yeast extract 2.0 g L⁻¹ and CaCO_3 5.0 g L⁻¹ at pH 6.0 was conducted. Endophytic fungi was cultured in Petri dish., for 5-7 days. Liquid fermentation was carried out in a 250 mL Erlenmeyer flask, containing 50 mL PDY medium and 5 pieces of endophytic fungi (using cor bock) using orbital shaker incubator rotating at 130 rpm for 14 days at room temperature. Separation of cell mass from supernatant which contain the secondary metabolite of interest was carried out in cold centrifuge at -4°C rotating at 2000 rpm for 20 min. pH of the supernatant was adjusted to pH 3-4 using acetic acid 0.1 M and then extracted in *n*-butanol. This butanol extract was evaporated down to small volume, using rotary evaporator for bioassay.

Preparation for Leukemia L1210

Preparation of Eagle's MEM: 4.7 g of Eagle's MEM (Nissui) was dissolved in 475 mL water to make up solution A and then, 1.3 g of NaHCO_3 together with 0.3 g of glutamine were dissolved in 50 mL of water to give solution B. Then, 25 mL of solution B was added to solution A to give a final volume of 500 mL medium. Medium was filtered aseptically through millipore filter and stored in the fridge until use. 15% (v/v) foetal bovine serum/MEM was used in leukemia cell (L1210) culture and for bioassay (Sumatra, 1998).

L1210 leukemia cell culture method: Frozen stock of L1210 was thawed and transferred into centrifuge tube which contained 4 mL of MEM and were centrifuged at 1000 rpm. Supernatant was removed and cell pellet was washed three times with 2 mL of MEM. After washing, cell pellet was resuspend in 10 mL MEM, transferred into tissue culture flask for 48 h incubation in an incubator filled with 5% CO₂.

Following the incubation periods, cell were harvested and counted using heamocytometer to get cell density of 2x10⁶ cells mL⁻¹ used in the bioassay (Sumatra, 1998).

Bioassay using leukemia cell L1210: Anti cancer activity of endophyte fungi secondary metabolite was measured based on its 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 over 48 h. As a negative control, cells were treated with only Eagle's MEM with 10 µL metanol. Each treatment was done in triplicate.

To calculate IC₅₀ values of secondary metabolite butanol extract from each of 18 endophytic fungi isolates following formula is used:

$$\% \text{ Cell proliferation inhibition} = 1 - \frac{\sum \text{ treated cells}}{\sum \text{ control cells}} \times 100\%$$

RESULTS

Endophytic fungi isolation: Endophytic fungi which is isolated from the pieces of twig, fruit and leaf of Buah Makasar (*Brucea javanica* L. (Merr)) plant, was cultured in PDA medium treated with 0.005% chloramphenicol to isolate the endophytic fungi. The isolation result yields 45 endophytic fungi, where 18 of the total 45 isolates were used in cytotoxic assay (Table 1).

Endophytic fungi with cytotoxic activity: In this study, a selection process of endophytic fungi was performed to produce secondary metabolite which has the cytotoxic activity. Anti proliferative test conducted L1210 cell showed that the isolates have IC₅₀ concentration range from 3.29 to 15.90 µg mL⁻¹ (Table 2).

DISCUSSION

This study is the first to isolate and to demonstrate the effectiveness of endophytic fungi from *B. javanica* in leukemia cells (L1210). Furthermore, this preliminary results may serve as useful template for future study in finding new anti-cancer substance from endophytic fungi.

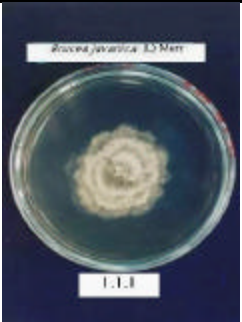
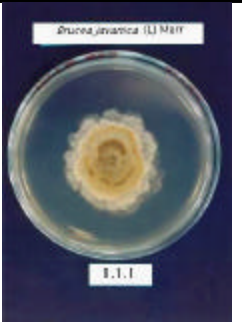

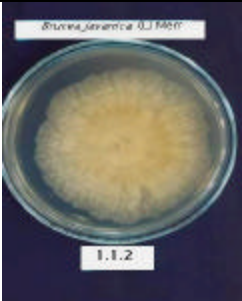
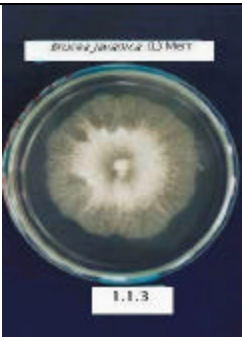

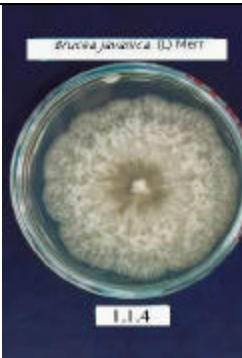

The endophytic fungi isolation procedure used in this study generated 45 fungal endophyte. The effectiveness of endophytic fungi, as anti cancer substance was assessed using cytotoxic assay on 18 of the total 45 endophytic fungi isolated. *B. javanica* was chosen in this study for isolation of fungal endophyte because, of its traditional use for treatment and cure of malaria, lung cancer and leukemia Samples were selected randomly from 3 locations (Bogor, Cianjur and Tawangmangu) to obtain variety of endophytic fungi based on different geographical condition, as different environmental condition may result in different strain of endophytic fungi (Song, 1998). Interestingly, the samples collected from three parts of the plant twigs, leaves and fruits, variety of endophytic fungi strains were found in a given host plant. This results confirmed previous finding by Petrini *et al.* (1992).

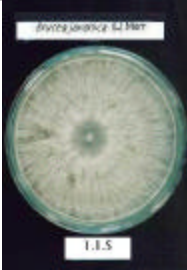


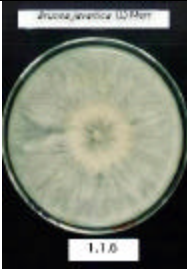

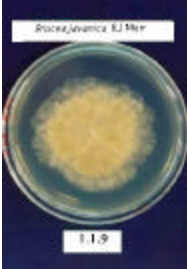
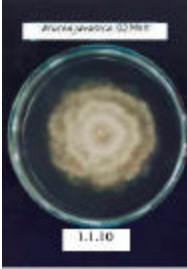
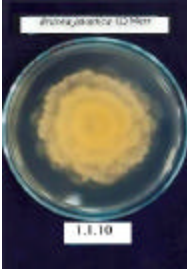
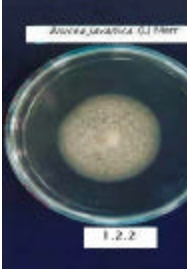
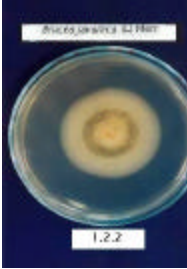
Furthermore, liquid fermentation on endophytic fungi isolates were performed in our study for two weeks in order to test the ability of the these endophytes to produce secondary metabolites, as describe previously (Stanbury and Whitaker, 1987).

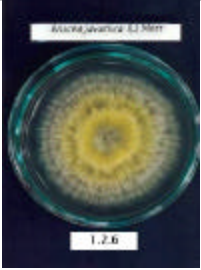
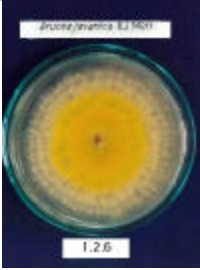
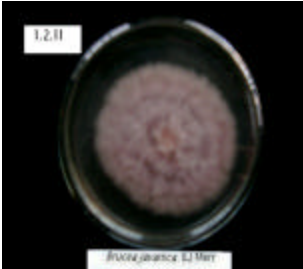
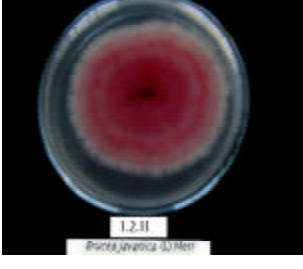

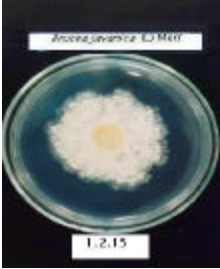
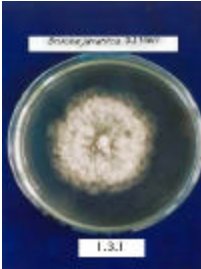

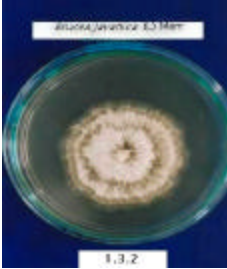
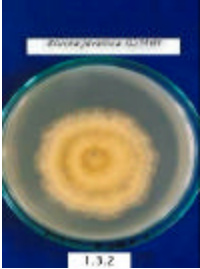
Microscopic identification has been done in our study, however almost all of endophytic fungi were sporeless, so that the microscopic identification was not able to be performed; the identification was done only by macroscopic morphology.

In our fermentation procedure, secondary metabolite produced by the fungi were extracted using non-polar (hexane), semi-polar (Dichlormetane) and polar solution (butanol). Our fermentation results demonstrated that *n*-butanol fraction has strong anti-cancer activity against L1210 cells. The secondary metabolites were extracted from polar solvent such as *n*-butanol in order to obtain polar substance. It is likely that bioactive substances extracted using *n*-butanol will be more water-soluble. The extraction result using *n*-butanol solution was tested by anti proliferation test against leukemia cell. Leukemia cancer cell were selected as a model for anti-cancer activity test in our study. This is in accordance with previous evidence on consumption of *B. javanica* extract as anti cancer agent in leukemic patient. Additionally, this is also important to justify whether endophyte fungi produce bioactive substance that are similar to that in buah makasar plant extract (Strobel and Long, 1998). Cytotoxic assay other than the MTT assay were used in this study, because direct cell counting by haemocytometer is one of the simplest method, inexpensive, accurate measure of cell number to assess the cytotoxic effect of secondary metabolites of endophytic fungi. The result of the cytotoxic assay shows IC₅₀ range of 3.29 to 15.90 µg mL⁻¹, suggesting that

Table 1: Macroscopic morphology of endophytic fungi from *B. javanica*

No.	Code isolate	Source of sample	Part of sample	Morphology and color of colony
		Bogor		
1	1.1.1			Twig Colony of PDA at 27-29°C attaining a diameter 4 cm within 5 days. White, edge of colony undulate. Reverse white yellowish to brownish.
2	1.1.2			Colony of PDA at 27-29°C attaining a diameter 6,5 – 7 cm within 5 days. Edge of colony undulate. Reverse whitish, yellowish.
3	1.1.3			Fruit Colony of PDA at 27-29°C attaining a diameter 6 cm within 5 days. White, edge of colony undulate. Reverse white-yellowish to brownish.
4	1.1.4			Colony of PDA at 27-29°C attaining a diameter 7 cm within 5 days. White edge of colony undulate. Reverse yellow to brownish.

5	1.1.5				Colony of PDA at 27-29°C attaining a diameter 9-10 cm within 5 days. whitish, Reverse white.
6	1.1.6				Colony of PDA at 27-29°C attaining a diameter 9 cm within 5 days. Aerial mycellium sparse, concentris circle white velvety. Reverse cream- white.
7	1.1.9.			Leaf	Colony of PDA at 27-29°C attaining a diameter 5,8 - 6 cm within 5 days. White, edge of colony endulate, smooth hyphae, reverse yellowish.
8	1.1.10				Colony of PDA at 27-29°C attaining a diameter 5 cm within 5 days. Edge of colony entire, smooth. Reverse white-yellowish.
Cianjur					
9	1.2.2			Twig	Colony of PDA at 27-29°C attaining a diameter 4 cm within 5 days. Edge of colony entire withish. Reverse yellowish.

10	1.2.6			Fruit	Colony of PDA at 27-29°C attaining a diameter 7 cm within 5 days. Edge of colony sharp yellow to white milk. Reverse concentric circle yellow to cream white.
11	1.2.11				Colony of PDA at 27-29°C attaining a diameter 6 cm within 5 days. Reddish edge of colony entire, reverse pinky.
12	1.2.15			Leaf	Colony of PDA at 27-29°C attaining a diameter 10 cm within 5 days. Center area white hyphae. Edge of colony undulate, reverse yellowish.
Tawangmangu					
13	1.3.1			Twig	Colony of PDA at 27-29°C attaining a diameter 5-5,5 cm within 5 days. Center white, edge of colony undulate. Reverse white-yellowish.
14.	1.3.2.				Colony of PDA at 27-29°C attaining a diameter 4,8 - 5 cm within 5 days. White color, edge of colony undulated. Reverse concentric circle cream white.

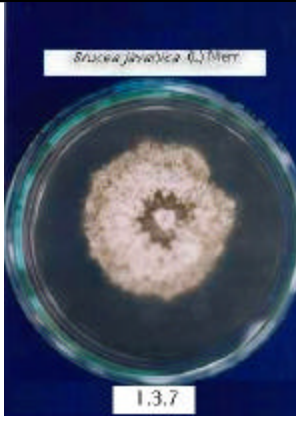
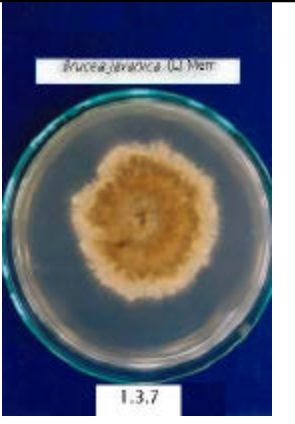
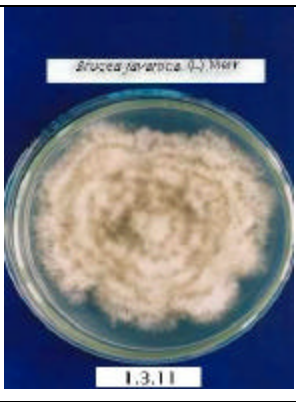
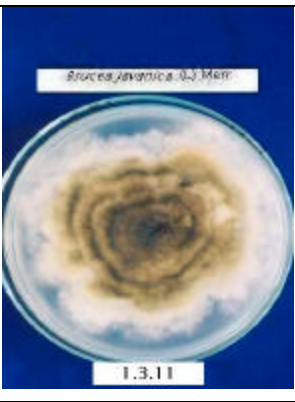
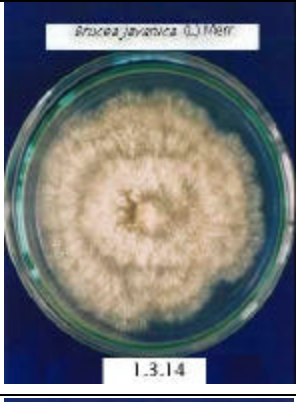

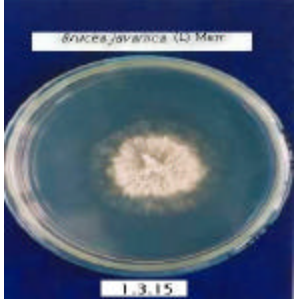
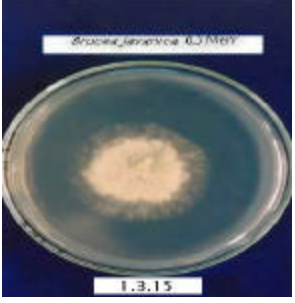
15	1.3.7			<p>Colony of PDA at 27-29°C attaining a diameter 5 cm within 5 days. White, reverse yellow-brownish.</p>
16	1.3.11			<p>Fruit Colony of PDA at 27-29°C attaining a diameter 9 cm within 5 days. White green-brownish. Edge of colony undulate. Reverse black.</p>
17	1.3.14			<p>Leaf Colony of PDA at 27-29°C attaining a diameter 8 cm within 5 days. White-greenish, edge of colony undulate. Reverse yellowish.</p>
18	1.3.15			<p>Colony of PDA at 27-29°C attaining a diameter 4 cm within 5 days. White, edge of colony entire. Reverse white.</p>

Table 2: Cytotoxic assay result for *n*-butanol extract of endophytic fungi from *B. javanica* collected in Bogor, Cianjur and Tawangmangu

No.	Code isolate	Source of sample	Part of sample	IC ₅₀ (µg mL ⁻¹)
Bogor				
1	1.1.1		Twig	5.93
2	1.1.2			6.72
3	1.1.3		Fruit	3.29
4	1.1.4			4.98
5	1.1.5			15.90
6	1.1.6			4.16
7	1.1.9		Leaf	6.00
8	1.1.10			4.50
Cianjur				
9	1.2.2		Twig	9.92
10	1.2.6		Fruit	6.28
11	1.2.11			4.05
12	1.2.15		Leaf	6.52
Tawangmangu				
13	1.3.1		Twig	9.73
14	1.3.2			9.66
15	1.3.7			4.32
16	1.3.11		Fruit	5.04
17	1.3.14		Leaf	6.28
18	1.3.15			8.51

endophytic fungi from buah makasar plant generated secondary metabolites with anti-cancer activity. The bioactivity of these secondary metabolites in the *n*-butanol extracts was considered to be active when the IC₅₀ of less than 20 µg mL⁻¹ as described by Swanson (1990). In attempt to search for a new potent bioactive substance, crude extract of metabolites with IC₅₀ of less than 20 µg mL⁻¹ is potential for further purification phase and IC₅₀ that is less than 4 µg mL⁻¹ for pure substance isolated from natural sources has potential as anti-cancer substances.

In this preliminary study, *n*-butanol extract of secondary metabolite from endophytic fungi has potent anti-cancer activity and will be further purified to isolate the bioactive compound.

Several endophytic fungi such as *Taxomyces andreanae*, *Pestalotiopsis*, *Altenaria* sp., had been isolated from *Taxus brevifolia*. These fungi can produce taxol (Petrini *et al.*, 1992; Strobel, 2002). In accordance to this study, the endophytic fungi from *B. javanica* may also produce secondary metabolites, such as anti-cancer bruceantin which has been isolated in *B. javanica* as the host plant.

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