Cytotoxic assay of endophytic fungus 1.2.11 secondary metabolites from *Brucea javanica* (L) Merr towards cancer cell *in vitro*

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Abstrak

Telah dilakukan uji sitotoksik metabolit sekunder kapang endofit 1.2.11 tanaman Brucea javanica (L.)Merr. Sampel tanaman diambil dari cianjur, bagian tanaman yang digunakan adalah buah. Uji sitotoksik dilakukan terhadap sel Raji, NS-1,sel HeLa dan sel Vero. Pengamatan dilakukan selama 24 jam dan 48 jam dengan menghitung sel hidup menggunakan metode tripan biru. Penghitungan IC_{50} dilakukan secara aritmatikal dengan rumus Reed and Muench. Untuk melihat mekanisme kerja pada proses sitotoksik dilakukan teknik pengecatan DNA menggunakan etidium bromida dan acridine orange. Dari penelitian ini diperoleh IC_{50} terhadap sel Raji 58,35 µg/ml, 88,39 µg/ml; IC_{50} sel NS-1 162,09 µg/ml, 66,24 µg/ml; IC_{50} sel HeLa 361,21 µg/ml, 219,97 µg/ml. IC_{50} sel Vero 1075,18 µg/ml, 656,82 µg/ml Pengamatan dilakukan dalam waktu 24 jam dan 48 jam. Mekanisme kerja dari metabolit sekunder kapang endofit 1.2.11 terhadap sel NS-1 cenderung melalui mekanisme apoptosis. ***(Med J Indones 2006; 15:137-44)***

Abstract

Cytotoxic assay of secondary metabolite endophytic fungus 1.2.11 from Brucea javanica (L.)Merr has been carried out. Brucea Javanica fruit collected from Cianjur was used in this experiment. Cytotoxic assay was done on Raji, NS-1, HeLa and Vero cells. The observation was done for 24 hours and also for 48 hours. IC_{50} was calculated using the Rich and Muench theory. To observe the working mechanism of cytotoxic process, DNA staining with etidium bromide and acridine orange was conducted. The cytotoxic assay of endophytic fungi 1.2.11 showed an IC_{50} of 58.35µg/ml, 88.39 µg/ml on Raji cell,; 162.09 µg/ml, 66.24 µg/ml on NS cell; 361.21 µg/ml, 219.97 µg/ml on HeLa cell; and lastly 1075.18 µg/ml, 656.82 µg/ml on Vero cell after 24 and 48 hour incubation respectively. The results of this study showed that secondary metabolite of endophytic fungus 1.2.11 has selective cytotoxic effect towards cancer cell and also showed that it might cause apoptosis in NS-1cell. ***(Med J Indones 2006; 15:137-44)***

Keywords: *Brucea javanica (L.) Merr, endophytic microbe, Cytotoxic assay, endophytic isolate 1.2.11 and apoptosis*

Cancer, beside coronary and cerebrovascular diseases,[1] is one of the main causes of mortality in developing countries. In Indonesia, mortality rate caused by cancer takes the 6th position, and the number of cancer patients' increases annually.[2] This situation makes it is necessary for us to search and develop a potent and selective medicine against cancer cells. One of the efforts is by utilizing natural substances such as extracts of medicinal plants for drug development.

Several plants can be used to develop cancer treatment medicines, such as *Catharathus Roseus* containing anticancer substances Vinblastine and Vincristine.[3] Taxols from pine trees (*Taxus brevifolia* are effective substances for the treatment of ovary and breast cancer. From previous researches, it was discovered that taxol can be generated from endophytic microbe called *Pestaloptiopsis microspora.*[4,5] Endophytic microbes are microbes living in tissues of host plants. Endophytic microbes can produce potential bioactive secondary metabolites, such as growth factor, hormones, antifungal, antibacterial and also anticancers substances.[5,6]

Brucea javanica (L.) Merr (tanaman buah Makassar) is used by local people as traditional medicine. Based on traditional experience, the fruit of *Brucea javanica* is used to cure dysentery, malaria, and cancer.[7,8]

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This study was conducted to evaluate the cytotoxic effect of secondary metabolite towards Raji, NS-1, HeLa and Vero cells. The results hopefully can be used as starting material for anticancer drug development.

MATERIAL AND METHOD

Material

Fruits of *Brucea javanica* (L) (Merr) (tanaman Buah Makassar), were collected from Kebun Obat PT Eisai Indonesia (Cianjur).

Method

Isolation and screening of endophytic microbes using surface sterilization and direct seed based on published methods. [9,10]

Cytotoxic assay of secondary metabolites from endophytic fungus 1.2.11

Cytotoxic assay of endophytic fungus 1.2.11 was conducted to find selective cytotoxic effect on several cancer cells, in turn Raji, NS-1 and HeLa cells. Vero cell was used as normal cell. In this study, fermentation was also conducted to obtain the secondary metabolites.

Fermentation

In order to obtain secondary metabolites potential as anticancer substance, liquid fermentation using PDY medium consisting of potato dextrose broth, 24.0 gL^{-1}; yeast extract 2,0gL^{-1} and $CaC0_3$ 5.0 gL^{-1} at pH 6 of endophytic fungus, was performed 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) incubated using orbital shaker incubator rotating at 130 rpm for 14 days at room temperature. Separation of cell mass from supernatant which contains the secondary metabolites was carried out in cold centrifuge at -4^oC rotating at 2000 rpm for 20 minutes. The pH of the supernatant was adjusted to 3-4 using acetic acid 1 M then was extracted in *n*-butanol. The extraction process was done three times using 100mL, 50 mL and 50 mL *n*-butanol. The *n*-butanol extract was evaporated into small volume using rotary evaporator prior for bioassay (Cheeptan modification).[11]

Cytotoxic assay

In vitro cytotoxic assay was carried out using a number of cell lines, Raji, NS-1, HeLa and Vero cells. To obtain the cytotoxic effect, this study used 5 concentration, and DMSO (dimethyl sulfoxide) concentration ranging from 0,5% to 1% to dilute the extract. Micro plate 96 wells (12x8) was used in this study. The microplate was filled in $2x10^4$ - $5x10^4$ cells /100 µl of cancer cells for each well. Then 100 µl of sample with various concentrations was added. The samples concentration used in this study for Raji and NS-1 were 500, 250, 125, 62.5 and 31.25 µg/ml, respectively. While for HeLa cells, they were 2000, 1000, 500, 250 and 125 µg/ml. And for Vero cells, they were 4000, 2000, 1000, 500 and 250 µg/ml. As negative control, the well was only filled with 100 µl of cancer cells without any samples.

Apoptosis assay of secondary metabolites generated from isolated endophytic fungus 1.2.11

Apoptosis assay was carried out by staining DNA cancer cells. DNA staining was performed with flurochrome etidium bromide and acridine orange (50 mg of etidium bromida, 15 mg of acridine orange) which was dissolved in 1 ml 96% ethanol and 49 ml of distilled water. One ml of the stock solution was taken and diluted with 1:100 PBS (Phosphat buffer saline). The first step to do it was to put the cover slip in the well of microplate, then it was filled with 200 µl cancer cells. Finally, the sample is added with several concentrations. Two concentrations were used in this study : IC50 concentration and below IC50 concentration. Incubation were done for 24 hours at 37^oC with 5% CO_2. After the liquid was discarded from the well, the cover slip was put on the object glass. Then, one or two drops of acridine orange and etidium bromide were added and observed using fluoresence microscope (100–400). The result of the study demonstrates that viable cell has green fluorescent in nucleus while cells that undergoing apoptosis shows orange fluorescent color with chromatin condensation.

Calculation of cell deaths percentage

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

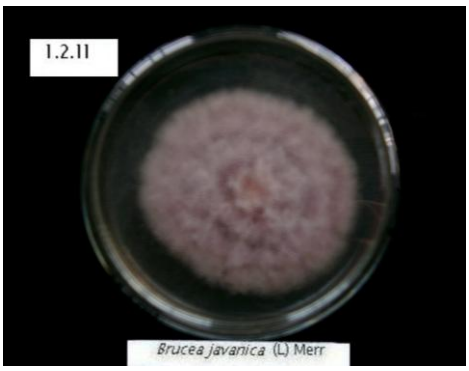
Calculation IC 50

Using Reed and Muench Calculation by arithmetic[12]
log CPE 50 % = log A + P. log 4.

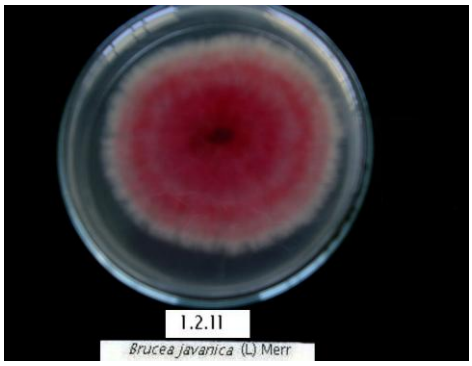
RESULTS

Isolation and screening of endophytic microbes

The endophytic fungus 1.2.11 was obtained from Brucea javanica's fruit. (Figure 1).



A



B

Isolated endophytic fungus 1.2.11 was obtained from *Brucea javanica's* fruit collected in Cianjur. (A) isolated colony (B) reverse colony.

Cytotoxic assay of endophytic fungus secondary metabolites

Cytotoxic assay of isolated endophytic fungus 1.2.11 towards Raji, NS-1 HeLa Vero cells Observation was conducted for 24 hours and for 48 hours.

Cytotoxic data of isolated endophytic fungus 1.2.11 using tryphan blue after incubation 24 hours were shown on table 1A.

Table 1A. Average amount of viable cells and percentage of dead Raji cells after 24 hours incubation with secondary metabolite of endophytic fungus 1.2.11 using tryphan blue

Sample	Concentration (µg/ml)	Amount of viable cell ($x10^4$ ±SD)	Cells Death (% ± SD)
1.2.11	500	0.33 ± 0.58	99.65 ± 0,61
	250	25.00 ± 1.00	73.78 ± 1,05
	125	37.50 ± 1.50	60.66 ± 1,57
	62.5	45.33 ± 1.53	52.45 ± 1,60
	31.25	69.00 ± 1.00	27.62 ± 1,05
Negative control	**0.00**	**95.33 ± 1.53**	**0.00 ±0.00**

Table 1a showed that the higher the concentration, the higher percentage of cell deaths was found. The amount of viable cells in control was larger compare to the sample.

Cytotoxic data for the isolated endophytic fungus 1.2.11 using tryphan blue towards Raji cell after 48 hours incubation were shown on Table 1B.

Table 1B. Average amount of viable cells and the percentage of the dead Raji cells after 48 hours incubation with the secondary metabolite of isolated endophytic fungus 1.2.11 using tryphan blue.

Sample	Concentration (µg/ml)	Viable cell ($x10^4$ ±SD)	Cell death (% ± SD)
1.2.11	500	0.00 ± 0.00	100.00 ± 0.00
	250	10.00 ± 2.00	94.08 ± 1.18
	125	77.00 ± 3.00	54.44 ± 1.78
	62.5	92.00 ± 3.00	45.56 ± 1.78
	31.25	125.00 ± 2.00	26.04 ± 1.18
Negative control	**0.00**	**169 ± 2.00**	**0.00 ±0.00**

Table 1B showed that the higher the concentration, the higher percentage of cell death was found. The amount of viable cells of control was larger compare to those of sample.

Cytotoxic data of isolated endophytic fungus 1.2.11 using tryphan blue toward NS-1 cell after 24 hours incubation were shown on Table 2A.

Table 2A. Average amount of viable cells and percentage of dead NS-1cells of isolated endophytic fungus 1.2.11 after 24 hours incubation using tryphan blue

Sample	Concentration (µg/ml)	Amount of viable cell ($x10^4$ ±SD)	Cell death (% ± SD)
1.2.11	500	0.00 ± 0.00	100.00 ± 0.00
	250	17.67 ± 2.31	63.70 ± 4.75
	125	28.33 ± 0.58	41.78 ± 1.19
	62.5	32.33 ± 1.53	33.57 ± 3.14
	31.25	37.67 ±1.15	22.61 ± 2.37
Negative control	**0,00**	**48.67 ± 1.53**	**0.00 ± 0.00**

Table 2A showed that the higher the concentration, the higher percentage of cell deaths was found. The amount of viable cells in control was larger compare to those of sample.

The data of cytotoxic assay for the isolate fungus 1.2.11 using tryphan blue toward NS-1 cell after 48 hours incubation were shown on Table 2B.

Table 2B. Average amount of viable cells and percentage of dead NS-1 cells of isolated endophytic fungus 1.2.11 after 48 hours incubation using tryphan blue

Sample	Concentration (µg/ml)	Amount of viable cell ($x10^4$ ±SD)	Cell death (% ± SD)
1.2.11	500	0.00 ± 0.00	100.00 ± 0.00
	250	0.00 ± 0.00	100.00 ± 0.00
	125	23.00 ± 1.00	56.87 ± 1.88
	62.5	27.00 ± 2.00	49.37 ± 3.75
	31.25	31.33 ± 0.58	41.25 ± 1.08
Negative control	**0.00**	**53.33 ± 1.53**	**0.00 ± 0.00**

Table 2B shows that the higher the concentration, the higher percentage of cell death was found. The amount of viable cells in control was larger compare to those of sample.

The data of cytotoxic assay for the isolated endophytic fungus 1.2.11 using tryphan blue toward HeLa cell after 24 hours incubation were shown on Table 3A.

Tabel 3A. Average amount of viable cells and percentage of dead HeLa cells of isolated endophytic fungus 1.2.11 after 24 hours incubation using tryphan blue

Sample	Concentration (µg/ml)	Amount of Viable cells ($x10^4$ ±SD)	Cell deaths (% ± SD)
1.2.11	2000	7.7 ± 0.58	87.19 ± 0.96
	1000	18,33 ± 0,58	69.36 ± 0.96
	500	24,83 ± 1,04	58.49 ± 1.74
	250	35,67 ± 0,58	4039 ± 0.96
	125	38,83 ± 1,26	35.09 ± 2.10
Negative control	**0.00**	**59.83 ± 2.02**	**0.00 ± 0.00**

Table 3A shows that the higher the concentration, the higher percentage of cell death was found. The amount of viable cells in control was larger compare to those of sample.

The data of cytotoxic assay for the isolated endophytic fungus 1.2.11 using tryphan blue toward HeLa cells after 48 hours incubation were shown on Table 3B.

Tabel 3B. Average amount of viable cells and percentage of dead HeLa cells of isolated endophytic fungus 1.2.11 after 48 hours incubation using tryphan blue

Sample	Concentration (µg/ml)	Amount of viabel cell ($x10^4$ ±SD)	Cell death (% ± SD)
1.2.11	2000	3.00 ± 2.00	95.78 ± 2.81
	1000	13.83 ± 1.15	80.56 ± 1.62
	500	25.33 ± 1.53	64.40 ± 2.15
	250	33.00 ± 1.00	53.63 ± 1.41
	125	47.00 ± 1.50	33.96 ± 2.11
Negative control	**0.00**	**71.17 ± 0.29**	**0.00 ± 0.00**

Table 3B showed that the higher the concentration, the higher percentage of cell deaths was found. The amount of viable cells in control was larger compare to those of sample.

The data of cytotoxic assay for the isolated endophytic fungus 1.2.11 using tryphan blue toward Vero cell after 24 hours incubation was shown on Table 4A.

Tabel 4A. Average amount of viable cells and percentage of dead Vero cells of isolated endophytic fungus 1.2.11 after 24 hours incubation using tryphan blue

Sample	Concentration (µg/ml)	Amount of viabel cell ($x10^4$ ±SD)	Cell death (% ± SD)
1.2.11	4000	0.83 ± 0.76	90.56 ± 8.65
	2000	0.83 ± 0.76	90.56 ± 8.65
	1000	4.83 ± 0.29	45.26 ± 3.27
	500	6.17 ± 0.29	30.16 ± 3.27
	250	7.67 ± 0.29	13.17 ± 3.26
Negative control	**0.00**	**8.83 ± 1.15**	**0.00 ± 0.00**

Table 4A shows that the higher the concentration, the higher percentage of cell deaths was found. The amount of viable cells in control was larger compare to those of sample.

The data of cytotoxic assay for the isolated endophytic fungus 1.2.11 using tryphan blue toward Vero cells after 48 hours incubation was shown on Table 4B.

Tabel 4B. Average amount of viable cells and percentage of dead Vero cells of isolated endophytic fungus 1.2.11 after 48 hours incubation using tryphan blue

Sample	Concentration (µg/ml)	Amount of viabel cell ($x10^4$ ±SD)	Cell death (% ± SD)
1.2.11	4000	0.17 ± 0.29	98.30 ± 2.94
	2000	0,.7 ± 0.58	93.22 ± 5.87
	1000	3.50 ± 0.00	64.39 ± 0.00
	500	5.83 ± 0.76	40.66 ± 7.77
	250	7.17 ± 0.29	27.09 ± 2.94
Negative control	**0.00**	**9.83 ± 0.29**	**0.00 ± 0.00**

Table 4B showed that the higher the concentration, the higher percentage of cell deaths was found. The amount of viable cells in control was larger compare to those of sample.

IC_{50} of secondary metabolites isolated endophytic fungus 1.2.11 toward Raji, NS-1, HeLa and Vero cells, after 24 hours and 48 hours incubation was shown on Table 5.

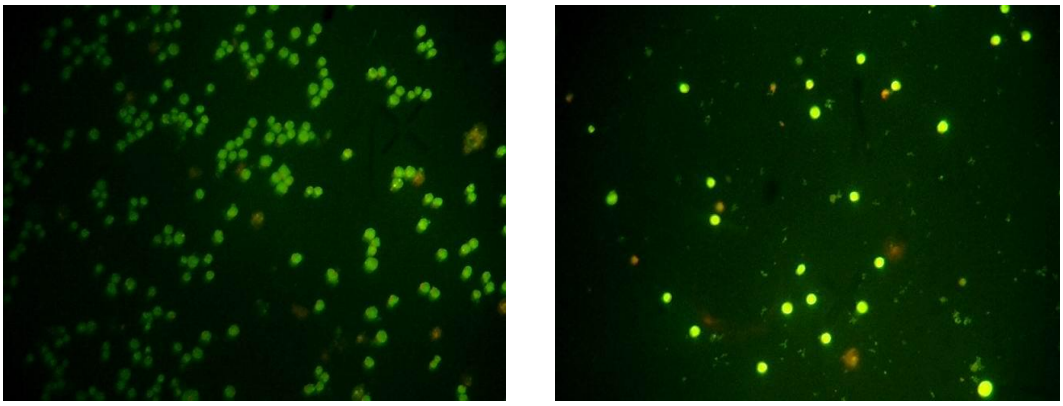
The IC_{50} for secondary metabolite of isolated endophytic fungus 1.2.11 on Raji cells after 24 hours incubation was 58.35 µg/ml, and the IC_{50} increased to 88.39 µg/ml in 48 hours. While the IC_{50} for secondary metabolite of isolated endophytic fungus 1.2.11 on NS-1 cells after 24 hours incubation was 162.09µg/ml and the IC_{50} decreased within 48 hours to 66.24 µg/ml. The value of IC_{50} for secondary metabolite isolated endophytic fungus 1.2.11 toward HeLa cells was 361.21 µg/ml after 24 hours incubation and after 48 hours, it was 219.97 µg/ml. IC_{50} for Vero cell was 1075.18 µg/ml after 24 hours incubation and after 48 hours decreased to 656.82 µg/ml.

Table 5. The IC_{50} of secondary metabolite isolated endophytic fungus 1.2.11 on Raji, Ns-1, HeLa and Vero cells after 24 and 48 hours incubation using tryphan blue

Cancer cells	IC_{50} (µg/ml) Secondary metabolite of isolated endophytic fungus 1.2.11	
	24 hours	48 hours
Raji cell	58.35	88.39
NS-1 cell	162.09	66.24
HeLa cell	361.21	219.97
Vero cell	1075.18	656.82

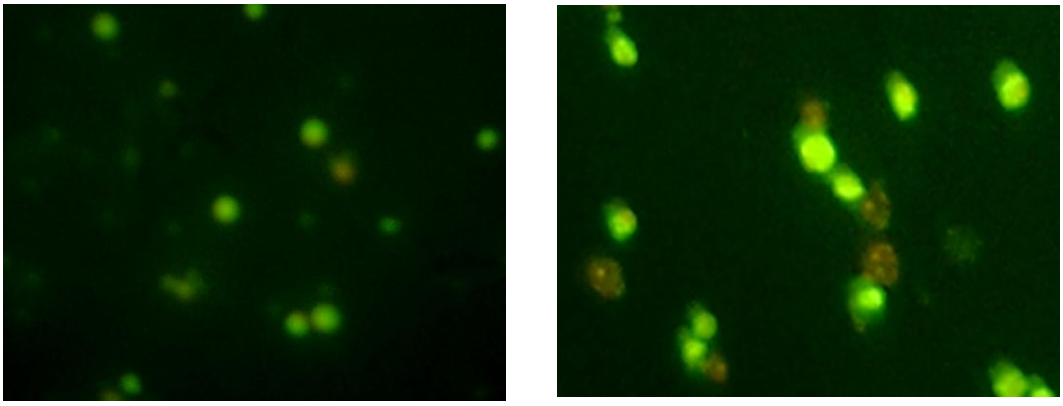
Cytotoxic assay of secondary metabolite of fungus isolate 1.2.11

Raji cells that were exposed to secondary metabolite isolated endophytic fungus 1.2.11 didn't show any apoptosis mechanism. The cells' color change to orange but they didn't show chromatin condensation (Figure 2). While NS-1cells, that were exposed to secondary metabolite of isolated endophytic fungus 1.2.11 showed apoptosis mechanism. The cells' color change to orange and showed chromatin condensation in nucleus (Figure 3).



(A) (B)

Figure 2. Shows Raji Cells after 24 hours incubation (A) viable cell in control and (B) cells exposed to sample didn't show apoptosis. (fluoresence microscope 100x).



(A) (B)

Figure 3. Shows immunoflourescent assay on NS-1 cells after incubation of 24 hours. (A) viable cells in control. (B) apoptosis cells in sample (fluoroscent microscope 100 x).

DISCUSSION

Isolated endophytic fungus 1.2.11 used in this study was isolated from *Brucea javanica* fruits collected in Cianjur This fungus are used because of the result in primary study of cytotoxic assay toward L1210 cell has an IC_{50} 4.05 µg/ml. This fact indicates that the secondary metabolite from isolated endophytic fungus 1.2.11 had a cytotoxic effect because IC_{50} was less than 20 µg/ ml. Swanson et al.[14] stated that secondary metabolite fungi with $IC_{50} \leq 20$ was cytotoxic active, so that they were potential for anticancer drug development.

To count viable cell, both MTT (3-[4,5}-dimethylthiazol] -2yI] -2,5-diphenyltetrazolium bromide) or hemocytometer can be used. In this study, hemocytometer was used although it was not as acurate as MTT method. Viable cells were not able to be stained with tryphan blue. The cell deaths were able to be stained because the cell deaths' membranes were destroyed so that the dye could go inside the cells and made the cells colored. This hemocytometer method was simpler, and cheaper than MTT and it is fairly accurate.[15]

In this study, cytotoxic assay used several cancer cells. Raji cells were cancer cells from *human haematopoetic*,

NS-1 cells were mielomas from bone marrow and HeLa cells were from epithel cervix. The results of cytpotoxic assay can be compared from one cell to another cell. To see the mechanism of cell deaths, apoptosis assay with staining DNA cell was prefered.

The results of cytotoxic assay on Raji cells were shown on Table 1A and 1B. The IC_{50} value was 58.35µg/ml, but at 48 hours incubation the value of IC_{50} was increased to 88.39 µg/ml. This result indicates cytotoxic effect decreases with the increase of period of time. Different from Raji cells case, the value of IC_{50} on NS-1 cell within 24 hours was 169.09 µg/ml and within 48 hours the value decreased to 66.24 µg/ ml. This result indicated that secondary metabolites of isolated endophytic fungus 1.2.11 were more cytotoxic on NS-1 cells with the increase period of time. Data were shown on table 2A and 2B. The value of IC_{50} obtained on HeLa IC_{50} was above 100 µg/ml, and did not change with increase period of time. This result indicated that secondary metabolite of isolated endophytic fungus 1.2.11 did not have cytotoxic effect on HeLa cells. The IC_{50} against Vero cells was 107.18 µg/ml decrease to 656.82 µg/ml after 48 hours. Thus Vero cells were used as normal cell control. Further more, the data indicated that secondary metabolites of isolated endophytic fungus 1.2.11 had selective cytotoxic effect. This result was similar to the result of Sismindari et al.[16] Sismindari results showed that the active compound from *Erythrina fusca* Lour was effective on HeLa cells but not on NS-1. The result of this study showed IC_{50} value still below 100 µg/ml, for Raji cells and NS-1. This indicated secondary metabolites still had cytotoxic effect. In developing substance for anti-cancer agent, it is better to get the substance with lower IC_{50} value to enable the usage of smaller quantity raw materials compare to high IC_{50}. But there was a possibility to explore the substance with the IC_{50} value above 50 µg/ml. The IC_{50} from this study was ranging from 50-90 µg/ml, more than IC_{50} 20 µg/ml but below 100 µg/ml. This result showed weak cytotoxic similar with Choo CY, et al.[17] the cytotoxic effect from *Typhonium flagelliforme* between IC_{50} > 20 - >100 µg/ml, having weak cytotoxic effect.

In order to resolve whether these metabolites caused apoptosis in cancer cells, DNA staining method was performed using acridine orange and ethidium bromide. The result showed that the secondary metabolite of isolated endophytic fungus 1.2.11 might have cause apoptosis on the NS-1 cells, as shown by chromatin condensation and orange color of the cells (Figure 3a and 3b). To get better results, staining method should be combined with gen *P53* method.

CONCLUSION

The results of the study showed:

1. *n*-Butanol extract of secondary metabolite fungus isoalte 1.2.11 from *Brucea javanica* (L.) Merr fruit had a selective cytotoxic toward cancer cells.
2. IC_{50} values of Raji cells, and NS-1, were below 100 µg/ml.
3. There was an impairing mechanism tendency against NS-1 cell by inducing apoptosis mechanism.

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