

CYTOTOXIC AND APOPTOSIS INDUCING ACTIVITY OF ETHANOL AND ETHYL ACETATE EXTRACTS OF BINAHONG LEAVES (*Anredera cordifolia* Tenore Steen) ON CERVICAL CANCER CELL LINES (HeLa)

Dwi Utami, Vivi Januarti, Melly

Faculty of Pharmacy, Ahmad Dahlan University Yogyakarta

Abstract

Background. Cervical cancer is the second largest in the world of all cancers in women in 2012. One of the interesting plants studied as anticancer agent was *Anredera cordifolia* (Tenore) Steenis which was known as Binahong. Binahong leaves are known contains flavonoids, polyphenols, essential oils and triterpenoid saponins. Flavonoids have been shown to have cytotoxic and antiproliferative activity against cervical cancer cells.

Method. Binahong leaf powder had fat leached by petroleum ether, then macerated with 70% ethanol or ethyl acetate to obtain the ethanol extract and ethyl acetate. Cytotoxic activity of extracts were performed by incubating HeLa cells with the extract at a concentration series 250; 125; 62.5; 31.25; 15.63, 7.8125 and 3.906 $\mu\text{g/ml}$ for 24 hours. Cytotoxic IC_{50} parameter determined by calculating the number of viable cells using MTT (3-[4,5-diethylthiazol-2-yl]-2,5-dipheniltetrazolium bromide) method. Apoptosis was detected by acridine orange/ethidium bromide double staining. Statistical analysis was carried out by using one way analysis of variance followed by student's paired t-test with 95 % confident level.

Result. The results showed that ethanol and ethyl acetate extracts of Binahong leaves have cytotoxic activity against HeLa cells with IC_{50} value of each 21.84 $\mu\text{g/ml}$ and 127.93 $\mu\text{g/ml}$. Ethanol and ethyl acetate extracts of Binahong leaves able to induced apoptosis in 21, 84 $\mu\text{g/ml}$ and 58,89 $\mu\text{g/ml}$ concentration.

Conclusion. The ethanol extract is more potential to be developed as cytotoxic agent to cervical cancer cell lines (Hela) than ethyl acetate extract and favorable for exploring its molecular mechanism.

Key words : ethanol extract, ethyl acetate, *Anredera cordifolia* (Tenore) Steenis, cytotoxic, apoptosis, HeLa cells

INTRODUCTION

Cancer is the leading cause of death in economically developed countries and second leading cause of death in developing countries (Jernal et al, 2011). One of the second most frequent cancer among women worldwide is cervical cancer. The average annual incidence of cervical cancer varies widely by geographic area, with highest incidences reported in Latin America, sub-Saharan Africa and South Asia and Southeast Asia (Parkin et al, 2001). In Indonesia, the most frequent and primary cancers are cervix, breast, lymph node, skin and nasopharynx. Among female the most cancers are cervical, breast and ovarian cancers (Tjindarbumi and Mangunkusumo, 2002). There are several approaches used for treating cancer such as surgical excision, irradiation and chemotherapy. Recently, the usage of natural for cancer treatment is being arise based on its chemical constituent.

Anredera cordifolia is a medicinal plant that was originated from China and known as Dhen San Chi or Madeira vine in South America. In Indonesia, this plant is known as Binahong. The recent study showed that Binahong has a lot of pharmacological activities. Some part of the plants are used in traditional medicines preparation and have been proved in laboratory such as wound healing effect (Hammond, 2006) antibacterial effect (Annisa, 2009 ; Rochani, 2009), hepatoprotectif effect (Orbayinah and Kartyanto, 2008), anti-inflammatory effect and antioxidant effect (Yuliani and Wijayanti, 2010 ; Yuswantina, 2009)

Saponin, flavonoid, quinon, steroid, monoterpene and sesquiterpene were reported as the content of *Anredera* leaves. A study had managed to isolate the triterpene saponin from *Anredera* leaves which was known as bousingosida A1 (Lemmens and Bunyapraphatsara, 2003 ; Rahmawati, 2008 ; Hammond, 2006). In other study, it was found that *Anredera* shoot contained flavonoids, such as quercetin (Yang et al., 2008). Flavonoid have been reported delayed the human cervical cancer

cell lines (Hela) proliferation in many ways (Lv W et al, 2008; Najaran T et al, 2009). Flavonoid also induced apoptosis by increased caspase-3 activity and Bax (proapoptotic protein) expression, and decreased Bcl-2 (antiapoptotic protein) (Wenzong et al, 2009).

Based on phytochemistry approach of flavonoid constituent of Binahong (*Anredera cordifolia* (Tenore) Steen) leaves, it is predicted that Binahong leaves has cytotoxic and apoptosis induced activity in Hela cervical cancer cell lines. This research was designed to investigate the cytotoxic and apoptosis induced activity of ethanol and ethyl acetate extract of Binahong leaves.

METHODS

Plant Material

Samples of Binahong (*Anredera cordifolia*, Tenore Steen) leaves were collected from Samigaluh Kulonprogo in 2011, determined and identified in Laboratorium of Biology, Faculty of Mathematics and Science, Ahmad Dahlan University Yogyakarta.

Chemicals material

Petroleum ether, 70 % ethanol and ethyl acetate (technical degree), aquadest HeLa cell lines, RPMI (Roswell Park Memorial Institute) medium, DMSO (Dimethyl sulfoxide), PBS (Phosphate Buffer Saline), 2% Penisillin-Streptomisin, 0,5 % Fungison, 10% FBS (Fetal Bovine Serum), 0,25% Trypsin-EDTA in PBS, 10% SDS (Sodium duodecyl sulphonate) dissolved in HCl 0,01 N, NaHCO₃ and HEPES (N-2-hydroxyethyl piperazin-N-2ethane sulfonic acid).

Extraction

The leaves of plant were dried, grounded and extracted by petroleum ether due to eliminating the greese material. The residu free of petroleum ether then was extracted with 70 % ethanol and ethyl acetate by maceration method.

Both extracts were concentrated to dryness by rotary evaporator.

Cytotoxic Assay

The HeLa cell lines were harvested on 96 well plate with 5×10^3 /well. The serial concentration of ethanol and ethyl acetate extracts were used 250 $\mu\text{g/ml}$; 125 $\mu\text{g/ml}$; 62,5 $\mu\text{g/ml}$; 31,25 $\mu\text{g/ml}$; 15,63 $\mu\text{g/ml}$, 7,8125 $\mu\text{g/ml}$ and 3,906 $\mu\text{g/ml}$. The extract was dissolved in DMSO (dimethyl sulfoxide) as stock solutions and diluted as desired directly in the culture medium. After 24 h incubation, the culture medium was removed and the cells were washed in PBS. The cells were incubated with 100 μL culture medium and 10 μL MTT (3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) 5 mg/mL in each well for 4 h in the dark condition. The MTT reduction reaction was stopped by 100 μL amount of 10 % SDS in 0,01 % hydrochloric acid, followed by overnight incubation. The absorbance was measured by ELISA reader at wave length of 550 nm. The absorbances were correlated with viable cells. The IC₅₀ was determined by probit analysis. A graph was plotted for the percentage of cell viability against concentration of the extract and the cytotoxicity index used was IC₅₀. The percentage of cell viability was determined as comparison OD (optical density) sample and control.

Detection of Apoptotic Cells by double staining method

HeLa cells were treated with 21,84 dan 59,98 $\mu\text{g/ml}$ of ethanol and ethyl acetate extracts of Binahong leaves in 24 well plate for 24 h incubation. Apoptosis was detected by morphological analysis after Acridine Orange/Ethidium Bromide (AO/EB) staining (1:1) as described (Meiyanto et al, 2008). The apoptotic evident observed by fluorescence microscopy equipment.

Statistical Analysis

The viability cells were expressed as the mean \pm SEM. Statistical analysis was carried out by using one way analysis of variance followed by student's paired t-test with 95 % confident level.

RESULT AND DISCUSSION

The result from cytotoxic assay showed that ethanol and ethyl acetate extract of Binahong leaves have cytotoxic effect to HeLa cervical cancer cell lines. The observation after 24 hour incubation (acute toxicity) of HeLa cervical cancer cell lines with both extracts indicated that there was a death cell phenomenon of HeLa cervical cancer cell lines. The morphological of death cell were showed in 62,5 $\mu\text{g/ml}$ treatment of extract (Figure 1). The death cell of extract treatment was higher than cell control.

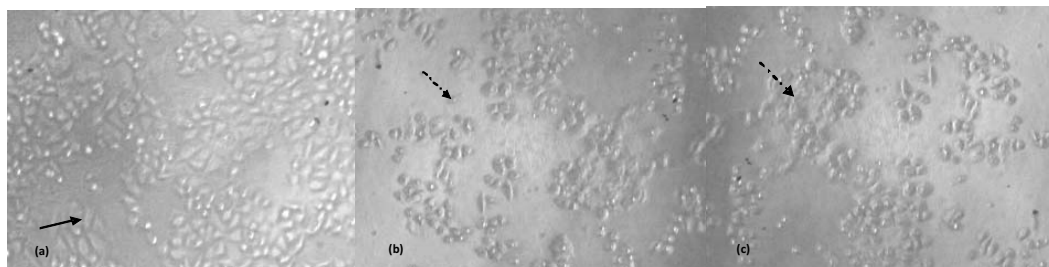


Figure 1. Effect of ethanol and ethyl acetate extract of Binahong leaves on morphology change of HeLa cervical cancer cells (phase-contrast microscopy). a. Untreated cells cultured (DMSO 0,1%). b. Cells were treated with 62,5 $\mu\text{g/mL}$ ethanol extract. c. Cells were treated with 62,5 $\mu\text{g/mL}$ ethyl acetate extract.

The result from MTT assay, showed that ethanol and ethyl acetate extract of Binahong leaves significantly reduced the viability and proliferation of HeLa cervical cancer cell lines in a concentration dependent manner (Table I and Figure 2). Table III showed the IC₅₀ of ethanol extract that was found at 21,84 µg/mL and the IC₅₀ of ethyl acetate extract was 127,93 µg/mL.

Table I. Effect of ethanol and ethyl acetate extract of Binahong leaves on cell viability of HeLa cervical cancer cells by MTT method

Concentration (µg/mL)	Percentage of viability (X ± SD)	
	Ethanol Extract	Ethyl Acetate Extract
250	(39,85 ± 4,09)	(39,42 ± 1,44)
125	(60,92 ± 1,27)	(51,39 ± 1,23)
62,5	(51,36 ± 3,56)	(61,88 ± 3,08)
31,25	(47,41 ± 1,32)	(74,45 ± 1,45)
15,625	(51,72 ± 2,56)	(77,19 ± 0,69)
7,8125	(69,39 ± 5,84)	(86,96 ± 4,02)
3,906	(85,82 ± 3,01)	(93,53 ± 4,64)

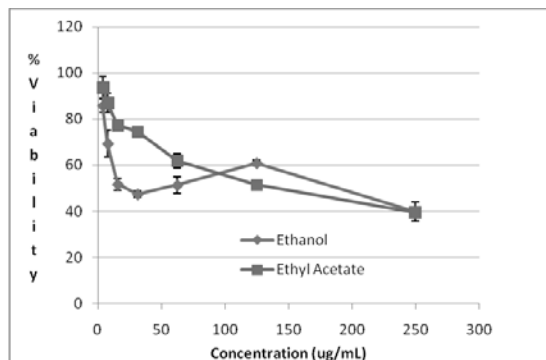


Figure 2. Cytotoxic effect of ethanol extract and ethyl acetate extract on HeLa

Table II. The IC₅₀ value of ethanol extract and ethyl acetate extract of Binahong (*Anredera cordifolia* (Tenore) Steen) leaves on HeLa cervical cancer cells.

Samples	Linear Regression Equation	IC50 Value
Ethanol Extract	Y = -1,272 x + 6,699	21,84 µg/ml
Ethyl acetate extract	Y = -0,963 x + 7,029	127,93 µg/mL

According to the IC₅₀ value, the ethanol extract of Binahong leaves has higher cytotoxic potency than ethyl acetate extract. It was predicted that the cytotoxic activity depend on its bioactive compound. The bioactive compound that has been extracted in ethanol extract is more than in ethyl acetate extract. According to Sulistyani (2011), the ethanol extract of Binahong leaves contains saponin, polyphenol, flavonoid, tannin and alkaloid. While in ethyl acetate extract only contains saponin and polyphenol (Wardani, 2011). In spite of this finding, based on the *American National Cancer Institute* ethanol extract of Binahong leaves is potential to be developed as anticancer agent because its IC₅₀ is equal to 20 µg/mL (Itharat *et al*, 2004).

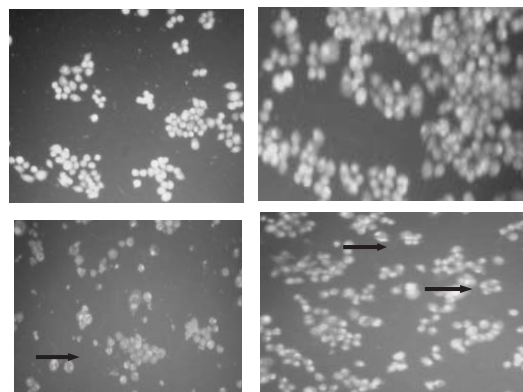


Figure 3. Effect of ethanol and ethyl acetate extract on apoptosis induction of HeLa cervical cancer cell lines, analysis by Acridine Orange/Ethidium Bromide (AO/EB) staining (examined using fluorescent microscopy). A. Untreated cells cultured B. Untreated cells cultured (DMSO 0,1 %). C. Cell were treated with 21,84 µg/ml ethanol extract. D. Cell were treated with 59,98 µg/ml ethyl acetate extract for 24 h. The white arrow indicated the early apoptotic bodies and yellow arrow indicated the late apoptotic bodies.

Figure 3 showed that images of apoptotic and necrotic cells, in 21,84 $\mu\text{g/ml}$ (ethanol extract) and 59,98 $\mu\text{g/ml}$ (ethyl acetate extract) after arcridine orange/ethidium bromide staining. Figure 3-A and B presented the control cells without and with DMSO 0,1%. The live cells have normal nuclei staining which present green chromatin with organized structures. The yellow arrows in figure 3C-D of the extracts treated cells showed late apoptotic cells with condensation and chromatin clumping, while the white arrows showed early apoptotic live cells (green) with chromatin super-aggregation i.e. highly condensed chromatin. This result suggested that ethanol extract and ethyl acetate of Binahong leaves induced apoptosis in HeLa cell lines. For futher study, it is suggested to explore the apoptotis induced activity in various concentration of both extracts and determined by apoptotic index.

The cytotoxic activity of natural product was related to presence of anticancer compound in these plants including Binahong. Yang *et al* (2009) found the quercetin flavonoid from Binahong leaves. Quercetin has been proved may induced apoptosis in Hela cell lines in many ways. Recent study showed that quercetin can significantly inhibit the proliferation of HeLa cells, which may be induced apoptosis of cervical cancer cells via the Ca^{2+} -dependent mitochondrial apoptosis pathway (Huang *et al*, 2009). Priyadasini *et al* (2010) have found that quercetin suppressed the viability of HeLa cells in a dose-dependent manner by inducing G2/M phase cell cycle arrest and mitochondrial apoptosis through a p53-dependent mechanism. This involved characteristic changes in nuclear morphology, phosphatidylserine externalization, mitochondrial membrane depolarization, modulation of cell cycle regulatory proteins and NF- κ B (Nuclear Factor Kappa B) family members, upregulation of proapoptotic Bcl-2 (B-cell lymphoma-2) family proteins, cytochrome C, Apaf-1 (Apoptotic peptidase activating factor 1) and caspases, and downregulation of antiapoptotic Bcl-2 proteins and survival.

Due to the presence of flavonoid quercetin in Binahong leaves, it can be predicted that moleculer mechanism of cytotoxic activity and induced apoptosis of ethanol and ethyl acetate extracts of Binahong leaves is similar with quercetin. However, the chemical constituents of these extracts should be explored further in order to find the definitely anticancer molecular mechanism.

CONCLUSION

The ethanol and ethyl acetate extract of Binahong leaves have cytotoxic activity to Hela cell lines with IC_{50} is 21,84 $\mu\text{g/ml}$ and 127,93 $\mu\text{g/ml}$. Both ethanol extract and ethyl acetate extract could induce apoptosis in Hela cell lines. Based on IC_{50} value ethanol extract is more potential to be developed as cytotoxic agent in cervical cancer cell lines (Hela) than ethyl acetate extract and favorable for exploring its molecular mechanism.

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