CYTOTOXIC ACTIVITY OF *Anredera cordifolia* LEAF EXTRACT ON HELA CELLS

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ABSTRACT

Cervical cancer is the second most frequently occurring cancer among females all over the world. Since the best strategy against cancer is to kill the cancer cells without endangering the normal cells, discovering a potentially selective anticancer agent from plants has become very challenging for researchers worldwide. A previous study on *Anredera cordifolia*, known as binahong in Indonesia, revealed its cytotoxic activity on HeLa cervical cancer cells with IC₅₀ 75 μg/mL. However, the selectivity of the chemical agent and its molecular target was not investigated. Thus, this study was aimed at determining the selectivity of ethanolic extract of *Anredera cordifolia* leaf (EAC) on Vero cells and its molecular target on HeLa cells. The extracts were prepared by macerating *A. cordifolia* leaf powder in 70% ethanol. The viability of Vero cells was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. While the cell cycle of HeLa cells was analyzed using the flow cytometry, the molecular target of the extract was investigated by using the immunocytochemical staining. The results exhibited the selective cytotoxicity of EAC on HeLa cells compared to Vero cells with a Selectivity Index (SI) of 17.36. It arrested the G1/S phase of the cell cycle and suppressed the Bcl-2 expression, the anti-apoptotic protein, which also regulates the cell cycle. These findings confirmed the use of *A. cordifolia* leaf extract as a promising anticancer agent against cervical cancer, particularly the HeLa cells.

Keywords: *Anredera cordifolia*, cytotoxic, HeLa, selective

INTRODUCTION

Cervical cancer is one of the most common type of cancers among women all over the world and ranks second in the female cancer incidence in the ASEAN (Jan et al. 2012; IARC 2013, 2014). In Indonesia, it is one of the major causes of deaths among female cancer patients, with an addition of 20,928 new cervical cancer cases annually (Bruni et al. 2017). Its pattern of occurrence has gradually shifted from developed to developing countries (Torre et al. 2015). Since the number of cervical cancer patients has alarmingly gone up, several treatment strategies have been administered for treating cervical cancer, such as chemotherapy, radiation, and their combination (Hong 2006).

Nowadays, cisplatin; cis-[Pt(II)(NH₃)(2)Cl(2)] ([PtCl₂(NH₃)₂] is considered one of the most widely used chemotherapeutic agents against cervical cancer (Hu et al. 2012). Cisplatin is applied as first-line chemotherapy in cervical cancer in single or combination with other chemotherapeutic agents (Tharavichitkul et al. 2016; Zhu et al. 2018). It has also been clinically tested for other types of cancer, such as bladder cancer (Drayton & Catto 2012), head and neck cancer (Pendleton & Grandis 2012), lung cancer (Ahmadzadeh et al. 2015) and gastric cancer (Ahmadzadeh et al. 2015; Huang et al. 2016). However, severe side effects were observed among patients associated with cisplatin such as nephrotoxicity and bone marrow suppression resulting in hematologic toxicity as well as increased resistance in cancer cells (Drayton & Catto 2012; Huang et al. 2016; Florea & Büsselberg 2011; Hu et al. 2012; Prasaja et al.

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This setback can be attributed to its limited selectivity between cancer cells and normal cells. Consequently, extensive studies have been conducted in order to discover and develop a novel selective anticancer agent for use in anticancer therapies.

Indonesia has natural products that are potentially active against cervical cancer cells (Larasati et al. 2014) including Anamdera cordifolia (Ten) Steenis which is commonly known as binahong in Indonesia. Its leaves contain the compound 8-glucopyranosil 1-4,5,7 trihydroxyflavone that has previously been demonstrated as an active antioxidant (Jamil 2012). Natural antioxidant compounds can selectively inhibit tumor cell proliferation and have the potential to be explored as chemopreventive agent on cervical cancer (Di Domenico et al. 2012). In previous studies, A. cordifolia extracts successfully exhibited cytotoxic activity as well as apoptosis induction in HeLa cells with an IC50 value 75 μg/mL without interfering with p53, a tumor suppressor protein (Yuliani et al. 2015). However, its selective cytotoxic effect on HeLa cells has yet to be ascertained.

The present study evaluated the selective cytotoxic effects of ethanolic extracts of A. cordifolia leaves (EAC) towards Vero cells and also determines the molecular targets of EAC in HeLa cervical cancer cells. Vero cells represent a mammalian cell line derived from the kidney of the African green monkey (Cercopithecus aethiops) that are recommended for in vitro screening of its chemical toxicity (International Standard ISO 2009; Menezes et al. 2013). The cytotoxic effect of A. cordifolia extracts and cisplatin on Vero cells was measured by MTT assay. By using the Selectivity Index, the current study was conducted to explore the selective cytotoxic effect of ethanolic extracts of A. cordifolia leaves (EAC) towards Vero cells, as a model system of normal cells. Furthermore, the molecular pathway of EAC on HeLa cells was also determined by cell cycle analysis using the flow cytometric method, particularly the immunocytochemical staining.

**MATERIALS AND METHODS**

**Extract Preparation**

The A. cordifolia leaves were collected from the Herbal Garden of the Faculty of Pharmacy, Sanata Dharma University, Maguwoharjo, Depok, Yogyakarta. The extracts were prepared according to that of Yuliani et al. (2015). First, the leaves were dried and extracted using maceration method with 70% ethanol (Merck Milipore Cat No.1.00983.2511). The extract was soaked in 70% ethanol in erlenmeyer flask for 72 hours and repeat it until get clear macerat.

The extract was collected and concentrated using a rotary evaporator (Bucchi, Rotavapor R-300) followed by freeze drying in a lyophilizer (VirTis B'TK, SP Scientific, Gardiner, NY, USA). All these processes were carried out at the Phytochemistry Laboratory, Sanata Dharma University. The extract was stored in tightly amber bottles in refrigerator 4°C (Samsung Type RT32FARCDSA). For longer use, the extract was kept in -20°C.

**Vero Cells and HeLa Cervical Cancer Cells**

The Vero cells and HeLa cervical cancer cells used in the current research were procured from the Parasitology Laboratory, Faculty of Medicine, Universitas Gadjah Mada, Indonesia. The cells were cultured and maintained in Roswell Park Memorial Institute (RPMI) as medium (Gibco, USA Cat.No.11875093 supplemented with Fetal Bovine Serum (FBS) 10% (v/v) (Gibco, GI, USA Cat.No.16000044) and 1% (v/v) penicillin-streptomycin (Gibco, USA Cat. No. 15070063). Dimethyl sulfoxide (DMSO) (Merck, Germany Cat. No. 1.02952.2500) was used to dissolve stock solution of extract and cisplatin (Cisplatin Kalbe 10 mg/10 mL) was used as a positive control. The cytotoxic effect was determined using the 3-[4,5-dimethylthiazol–2-yl]–2,5 diphenyl tetrazo- lium bromide (MTT) (Sigma, Missouri, USA Cat. No.M2003-1G) and the formazan complex was dissolved in 10% sodium duodecyl sulphate (SDS) (Sigma Cat. No. L3771). The flow cytometric analysis was carried out using propidium iodide kit (BD Bioscience Cat. No.556463), 0.1% Triton X solution (Sigma-Aldrich, Cat. No. T9284), 0.2% RNase solution (Sigma, MO, USA Cat. No. R4642) and 5% propidium iodide solution (Sigma-Aldrich, Cat.No. P4170) in Phosphate Buffer Saline (PBS) (Gibco Cat No.70011044). For the Immunocytochemical staining, the BCl-2 primary antibodies were purchased from Novus Biologicals (Cat. No. NB100–56098, Littleton
USA), while the secondary antibody was diluted from Starr Trek HRP universal detection system (Control No. 901-STUHRP700–090314, USA). The 96-well plate and 6 well plate used in this study were purchased from Iwaki®, and micropipette tips of all sizes are Axygen®. Fluorescence-Activated Cell Sorting (FACS) tubes used in study were BD Biosciences Falcon (Cat.No.552054).

MTT Cytotoxicity Assay on Vero Cells

The cytotoxic effect of *A. cordifolia* extracts and cisplatin on Vero cells was measured by MTT assay based on the studies of Yuliani *et al.* (2015) and Setiawati (2016). Vero cells were cultivated in a culture flask up to 80% confluence, and then 5 × 10⁵ cells in 100 μL medium were seeded in a 96-well microplate. The cells were cultured in an incubator at 37°C and 5% CO₂. The medium was then discarded and the cells were soaked twice using phosphate buffered saline (PBS). The initial stock solution of EAC and cisplatin were prepared by dissolving them into dimethyl sulfoxide (DMSO) and later diluted with Roswell Park Memorial Institute (RPMI) to obtain different concentrations. The medium was replaced with 100 μL medium containing EAC and cisplatin concentration into each well of the 96-well microplate and analyzed three times per set. The range concentration of EAC was 500, 1000, 1500, 2500, 3000 and 4000 μg/mL while the range of cisplatin concentrations were 20, 30, 40, 60, 70 and 80 μM. The treated cells were incubated at the same condition as the previous step. The medium was then eliminated and replaced with the medium containing 10% 3-[4,5-dimethylthiazol–2-yl]–2,5 diphenyl tetrazolium bromide (MTT) in each well. The reaction between MTT and succinate hydrogenase of cells led to the formation of formazan crystals within 3 to 4 hours. After 4 hours of incubation, 100 μL of 10% SDS solution was added to each well to dissolve formazan crystals. The microplates were wrapped with aluminum foil to avoid light exposure and incubated for 12–24 hours. Finally, the formazan complex was determined in 595 nm visible wavelength by using ELISA reader (Bio-Rad).

Flowcytometric Assay of HeLa Cells

The molecular pathway of EAC on HeLa cells was also determined by cell cycle analysis using the flow cytometric method, particularly the immunocytochemical staining. In applying the flow cytometric assay procedure to analyze the cell cycle of HeLa cells, the cells were first cultured in a 6 well plate at the density of 1 × 10⁵ cells in 2000 μL medium per treatment and incubated at 37 °C under 5% CO₂ for 24 hours (Setiawati 2016). The extracts were prepared at a concentration of 75 μg/mL, showing the IC₅₀ towards HeLa cells (Yuliani *et al.* 2015). They were poured into the precise flask and incubated for 12 and 24 hours. The cells were separated from the flask by pouring trypsin solution and eventually were washed and collected by centrifugation at 2000 rpm for 5 min. Finally, the cell pellets were rinsed with phosphate buffer saline (PBS) solution three times at 5 °C. The cells were suspended in a propidium iodine solution (10 μg/mL) containing 300 μg/mL RNase and incubated for 10 min in a water bath. The cells were then transferred into a flowcytovial and analyzed by FACSflow cytometer (Becton, Dickinson and Company).

Immunocytochemistry Assay of HeLa Cells

At a density of 10⁵ per 1000 μL, the HeLa cells were cultured on a coverslip inside 24-well plate and incubated at 5% CO₂ and 37 °C for 24 h. Subsequently, the cells were rinsed with PBS three times. The solution of EAC and cisplatin at IC₅₀ concentration were added to the cells and incubated for 24 hours and rinsed with PBS solution three times (Yuliani *et al.* 2015). The cells on the coverslips were then added with cold methanol and incubated for 10 minutes after which the methanol was removed and the cells were washed three times using PBS solution. The coverslips containing cells were then transferred to the object glass. Hydrogen peroxidase was added to the object glass and the cells were incubated at room temperature for 10–15 min. The PBS solution was gently dropped onto the coverslips to wash the cells. The monoclonal antibody of Bcl–2 was added to the coverslips and incubated for 1 hour at room temperature. The cells were rinsed three times with PBS and following this, the secondary antibody was also added to the coverslips, incubated at room temperature for 15 minutes. The traces of secondary antibody were removed by pouring PBS solution three times. The cells were stained with the chromogen solution of 3,
3'-diaminobenzidine (DAB) for 8 minutes. Finally, the cells were gently rinsed with distilled water and stained with hematoxylin-eosin for 4 min. The intensity of Bcl-2 expressions was observed under an inverted microscope (Axiovert 40 CFL, Zeiss).

**Data Analysis**

The viability of Vero cells was calculated from MTT data using the following formula:

\[
\text{Sample treatment absorbance} - \text{Medium absorbance} \times 100\%
\]

\[
\text{Untreated cells absorbance} - \text{Medium absorbance}
\]

The cell viability data were analyzed using regressions fit on Microsoft Excel 2013 to calculate IC\textsubscript{50} of EAC and cisplatin. Furthermore, the Selectivity Index (SI) was used to calculate and determine the differential selectivity of extracts and cisplatin towards cancer cells and normal cells. This SI was calculated by the ratio of IC\textsubscript{50} towards Vero cells and IC\textsubscript{50} towards HeLa cells (Calderón-arancibia et al. 2015). The number of cells, out of 10,000 HeLa cells in each phase, was determined using ModFit LT 3.0\textsuperscript{TM} software using FACS caliber. Lastly, the number of Bcl-2 expressing cells was qualitatively analyzed.

**RESULTS AND DISCUSSION**

The leaf extract of *Anredera cordifolia* manifested its cytotoxic effect starting at a concentration level above 1000 \( \mu \text{g/mL} \) and its cytotoxic profile followed the polynomial order 4 with IC\textsubscript{50} value at 1302 \( \mu \text{g/mL} \) (Fig. 1) while cisplatin demonstrated strong cytotoxic effects with IC\textsubscript{50} value at 64 \( \mu \text{M} \) on Vero cells (Fig. 2).

![Figure 1 Effect of EAC on vero cell viability](image1.png)

![Figure 2 Effect of cisplatin on vero cell viability](image2.png)
The leaf extract of *A. cordifolia* (EAC) had a significantly higher selectivity to the HeLa cells (SI 17.36) than cisplatin (SI 1.60). EAC manifested its enormous potential as an anticancer agent for the treatment of cervical cancer. Nevertheless, the lower selectivity shown by cisplatin than EAC may be attributed to the formation of active species that interacts primarily with DNA via formation of DNA crosslinks (Hassan *et al.* 2014), so that cisplatin interferes with DNA both in normal as well as cancer cells. However, this selective effect of *A. cordifolia* extracts on the molecular target in cervical cancer cells shows remarkable results.

The calculated Selectivity Index (SI) was used to determine the selective cytotoxic effect of EAC and cisplatin. Higher SI value refers to a higher selective cytotoxic effect of an extract or a compound; a selective extract or compound has an SI higher than 3 (Mahavorasirikul *et al.* 2009). SI was calculated based on IC₅₀ value of extract (75 μg/mL) and cisplatin (40 μM or equal to 11.9 μg/mL) on HeLa cells (Yuliani *et al.* 2015; Setiawati 2016).

In addition, flow cytometric method was employed in this study to assess the cell cycle of HeLa cells during EAC treatment. The cells accumulated more in G0/G1 phase due to the extract treatment (67.91 ± 6.43%) compared to the untreated cells (59.72 ± 3.38%) (Fig. 3).

The regulation of the Human Papilloma Virus (HPV) cell cycle is an important molecular target in cervical cancer therapy (Tulay & Serakinci 2016). The study results demonstrated that EAC caused a delay in the G0/G1 phase of HeLa cells, while another study presented that cisplatin induced HeLa cells accumulation in the G2/M phase (Setiawati 2016). Furthermore, the molecular effect triggered by the extract that was further revealed by the immunocytochemical staining method on Bcl–2. Bcl–2 protein, was significantly over-expressed in the cervical cancer cells than in the normal cells (Eisler *et al.* 2014; Zhou & Wang 2015). The Bcl–2 protein family plays an important role in the regulation of the mitochondrial apoptotic pathway, Bcl–2 expressing cells showed brown color while non-expressing cells showed purple color staining (Fig. 4). Untreated HeLa cells were stained brown color, while EAC and cisplatin treated cells were stained in blue color. This result indicated that Bcl–2 expression was suppressed by the extract and cisplatin.

![Figure 3](image)

**Figure 3** Cell cycle analysis of HeLa cells: (A) Untreated cells, (B) EAC 75 μg/mL treated cells
The results showed that both EAC and cisplatin suppressed the Bcl–2 expression in HeLa cells. The down-regulating effect of Bcl–2 in cisplatin-treated HeLa cells indicated that the cells are not resistant to cisplatin (Leisching et al. 2015). The Bcl–2 protein family contains two functional subfamilies of proteins: pro-apoptotic proteins (Bax and Bid) and anti-apoptotic proteins (Bcl–2 and Bcl-xL). The subfamily of Bcl–2 proteins binds to apoptosis protein and neutralizes its activity and induces apoptosis (Terrano et al. 2010). The downregulation of Bcl–2 contributes to the escape of cancer cells from death (Koff et al. 2015) and play an important role in cell cycle regulation (Naser et al. 2015). However, anti-apoptotic activity of Bcl–2 is prevented by Bax activity, a dominant pro-apoptotic protein of Bcl–2 family (Weinberg 2007). Bax protein induced the release of cytochrome C from mitochondria and caused apoptosis. Therefore, the comprehensive assessment of both Bcl–2 and Bax protein expression gave a clearer understanding on the molecular mechanism of the extract. The ratio of Bax/Bcl–2 expression indicated the intrinsic pathway of apoptosis induction through the release of cytochrome C from mitochondrion. This ratio is a critical determinant of a cell’s threshold for undergoing apoptosis (Zhang et al. 2014). The higher value of this ratio indicates a compound or an extract with an apoptotic induction activity through the intracellular mitochondrial pathway (Peng et al. 2015). However, the ratio of Bax/Bcl–2 expression was not determined in this study.

Figure 4  The expression of Bel–2 in HeLa cells detected by immunohistochemistry method under a light microscope at 400X magnification. Brown color (arrow) in cells refers to a positive result. (A) Untreated cells, (B) EAC 75 µg/mL treated cells, (C) Cisplatin 40 µM treated cells.

CONCLUSION

The leaf extract of Anredera cordifolia showed selective cytotoxic activity on HeLa cells than on the Vero cells by arresting the cell cycle at G1/S phase through down regulating Bcl–2 expression. However, further studies on the molecular dynamics of the active compound in Anredera cordifolia is recommended.

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REFERENCES


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