

CYTOTOXIC ACTIVITY OF *ANREDERA CORDIFOLIA* LEAF EXTRACT ON HEЛА CERVICAL CANCER CELLS THROUGH p53-INDEPENDENT PATHWAY

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ABSTRACT

Objective: This study investigated cytotoxic activity and apoptosis induction of *Anredera cordifolia* leaf extract on cervical cancer cells.

Methods: *A. cordifolia* leaf extract was prepared by maceration using 70% ethanol. This study used HeLa cells, a model of stress inducing-p53 cervical cancer cells. Cytotoxic study on HeLa cells was designed by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) test, further apoptotic induction assay was determined by annexin V-FITC method. Molecular mechanism of the extract on HeLa was assayed by p53 immunocytostaining. The MTT data were analyzed using linear regression to get IC₅₀ then apoptosis was conducted by ModFit LT and immunocytochemistry data were qualitatively analyzed.

Results: *A. cordifolia* leaf extract showed cytotoxic activity and promoted apoptosis in HeLa cells with IC₅₀ value 75 µg/mL. The extract did not increase level expression of p53 in cells.

Conclusion: Cytotoxic activity of *A. cordifolia* leaf extract on HeLa cells was through a p53-independent pathway.

Keywords: *Anredera cordifolia*, Apoptosis, Cervical cancer, p53.

INTRODUCTION

Recently, world cancer statistics showed that the incidence of breast and cervical cancer are globally on the rise [1]. Cervical cancer was the second highest diagnosed and the third leading cause of cancer death in females in ASEAN countries. It accounted for 11% of total new cancer cases and 9% the total cancer deaths among female in 2008 respectively [2]. Since cervical cancer also the most leading cause of female death in Indonesia [3], research focus on cervical cancer is an interesting topic.

Cervical cancer is transmitted by sexual intercourse that leads to human papillomavirus (HPV) infection. Oncogenic DNA is found in 95% of invasive HPV caused cervical cancers [4]. Previous studies showed that the E6 oncogenic protein from HPV form a complex with p53, a tumor suppressor gene and, as a result, targets it for rapid proteasome degradation [5,6]. Restoration of p53 function in cervical cancer is proposed to promote a selective therapeutic effect [6]. Moreover, reactivation of p53 is one of the key points in selective anticancer screening against this type of cancer.

Discovering selective anticancer agent against cervical cancer from plants is very challenging. Many compounds previously extracted from plant (vinblastine, vincristine, taxol) have excellent activity against several cancer cells. Molecular targeting approaches were applied to get selective activity against cervical cancer. *Anredera cordifolia* is a promising source of anticancer agent since its leaf contains good antioxidant compound; 8-glucopyranosil 1-4',5,7 trihydroxyflavone with an IC₅₀ of 68.07 µg/mL [7]. The antioxidant activity of *A. cordifolia* is potential to inhibit cervical cancer cells since oxidative stress is one of inducing cervix carcinogenesis. However, antioxidant and cytotoxic activity of an extract is not always has a positive correlation in cervical cancer [8]. Another study performed that *A. cordifolia* leaf had good activity against colon cancer cells HT-29 and breast cancer cells MDA-MB231 [9]. Meanwhile, cytotoxic activity of *A. cordifolia* on cervical cancer cells remained unclear.

This study investigated cytotoxic activity and apoptosis induction by *A. cordifolia* leaf ethanol extract (ALE) on cervical cancer cells. Furthermore, the contribution of p53 reactivation in the molecular pathway of apoptosis was deeply observed through p53-expression levels in HeLa cells. This research was performed using HeLa cells, an *in vitro* model of cervical cancer that could express p53 under genetic stress condition.

MATERIALS AND METHODS

Materials

A. cordifolia leaf was cultivated and harvested from Wirobrajan, Yogyakarta. Dried *A. cordifolia* leaf was extracted using 70% ethanol (Merck) in the Phytochemistry Laboratory of Sanata Dharma University then the solvent was evaporated using a rotary evaporator and a dry extract was obtained by freeze drying. A HeLa cervical cancer cell line was derived in the Parasitology Laboratory, of the Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. It was cultured in Dulbecco's Modified Eagle Media (DMEM) (Gibco) containing fetal bovine serum 10% (v/v) (Gibco) and penicillin-streptomycin 1% (v/v) (Gibco). Cytotoxic assay used 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) (Sigma) while the apoptosis kit used was Annexin V from Roche. Immunocytochemistry used p53 primary antibody purchased from Novus Bio NB 200-103 while the universal secondary antibody was derived from Starr Trek Universal HRP Detection System No.901-STUHRP700-090314. All culture plates used in this study were Iwaki and all tips were supplied by Biologix.

Methods

MTT cytotoxic assay

HeLa cells were seeded until confluent, then 5×10³ cells plated into a 96-well microplate and incubated at 37°C and under 5% CO₂ for 24 hrs. The medium was removed and rinsed by phosphate-buffered saline (PBS) 10%. ALE and cisplatin were dissolved in dimethyl sulfoxide as stock.

The various concentrations of ALE in the medium were poured into 96-well plate at 100 µL each and incubated for 24 hrs. Each concentration was assayed in triplicates (n=3). The medium culture was removed and rinsed by PBS 10%, then 100 µL medium containing 5 mg/mL MTT was added into each well and incubated for 4 hrs. Further, the medium was removed and 100 µL SDS was added to each well to dissolve Formazan crystals. The 96-well microplate was incubated for 24 hrs in a dark room, avoid contact from light. Formazan crystals were measured by ELISA reader at a wavelength of 595 nm.

Apoptosis assay

HeLa was seeded 5×10^5 /100 µL into coverslips in a 6-well plate, then acclimated at 37°C and under 5% CO₂ for 24 hrs. Then, the medium was removed, and the culture was rinsed twice by PBS. The IC₅₀ concentration of ALE in DMEM was added into the well plate and incubated for 24 hrs. At the end of incubation time, cells were detached by 2000 rpm centrifugation, and then it was washed twice with cold PBS. The cells were suspended in 500 µL of Annexin V buffer, and Annexin V and propidium iodide were added for 10 minutes at 37°C. Finally, the cells were subjected to FACS flow cytometry. Bivariate analysis of FITC-fluorescence (FL-1) and PI-fluorescence (FL-3) gave information on the designed cells population where RI quadrant showed viable cells, RII showed early apoptotic cells, RIII showed late apoptosis cells and RIV showed necrotic cells.

Immunocytostaining of p53

HeLa cells in DMEM were seeded 10^5 /100 µL on a 6-well plate and incubated under 5% CO₂ for 24 hrs. Cells were treated with ALE at IC₅₀ concentration and incubated for 24 hrs. After incubation, the cells were collected and washed in PBS. They were resuspended in DMEM and placed in object glass. Later, they were fixed for 5 minutes and incubated in H₂O₂ for 10-15 minutes and then washed again using PBS. Monoclonal antibodies of p53 were added to the cells and incubated at least for an hour and washed three times using PBS. Secondary antibodies of biotinylated goat anti-polyvalent were dropped to the cells and incubated in room temperature for 10 minutes and washed four times in PBS. DAB as chromogen was dropped into the cells and incubated for 3-8 minutes, then washed using distilled water. Finally, hematoxylin solution was added and incubated for 3-4 minutes. These slides were dried and observed under a light microscope. Expression of p53 was indicated by brown cell color.

Analysis

The cytotoxic data of ALE were plotted and analyzed by excel then IC₅₀ was linear regression using Excel MS Office 2010. Immunocytostaining data of p53 were visual qualitatively analyzed by observing p53 expressing cells that showed brown color.

RESULTS AND DISCUSSION

Cytotoxic activity of ALE against cervical cancer has not yet been evaluated. In this study, cisplatin was used as positive control since it has been widely recommended to women with cervical cancer, especially combined with radiotherapy [10,11]. The drug strongly cross-links DNA, form intra- and inter-strand adducts that poorly repaired [12]. Even though cisplatin is an effective anticancer drug, it can cause severe side effect to many tissues namely that of peripheral nerves, renal tubules, the gastrointestinal tract, and bone marrow [13].

The MTT method demonstrated that ALE is a potential anticancer agent against cervical cancer cells with IC₅₀ 75 µg/mL ($R^2=0.9759$) as shown in Fig. 1. Due to compounds complexity in ALE, cytotoxic activity of ALE was weaker than that of cisplatin, as pure compound with IC₅₀ 40 µM or equal to 12 µg/mL ($R^2=0.9908$) (Fig. 2). But, both of ALE and cisplatin reduced HeLa cells viability in a dose-dependent manner and also manipulated the morphology of HeLa cells (Fig. 3). Cytotoxic activity of both of ALE and cisplatin could be related with a molecular mechanism induced apoptosis.

Apoptosis assay was determined using Annexin V that is able to bind phosphatidylserine residue in outer plasma membrane as the

consequences of the apoptosis process. Annexin V specifically bound to this phospholipid that translocate in the surface membrane in the early stage of apoptosis stage [14]. Figs. 4 and 5 show ALE-induced apoptosis (9.44%) compared to untreated HeLa cells (2.31%) and cisplatin (6.23%). ALE triggered apoptosis better than cisplatin against HeLa cells, but the involvement of the molecular mechanism of apoptosis was still questionable. Jordan and Fonseca reported that

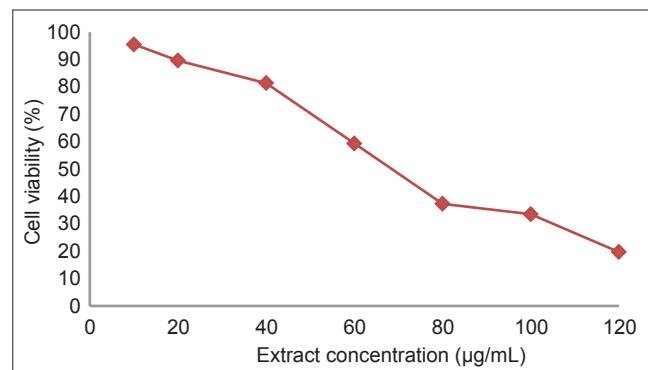


Fig. 1: Effect of *Anredera cordifolia* leaf ethanol extract on HeLa viability after 24 hrs incubation

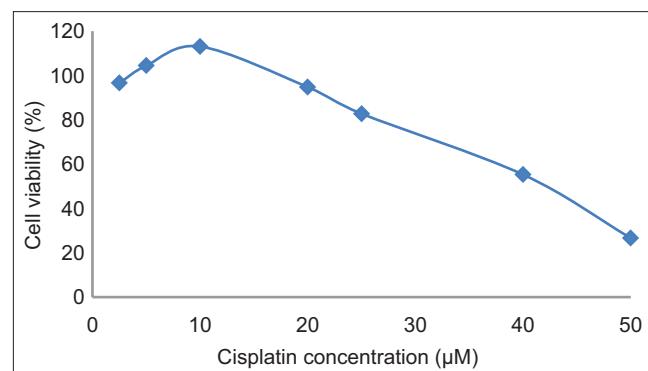


Fig. 2: Effect of cisplatin on HeLa viability after 24 hour incubation.

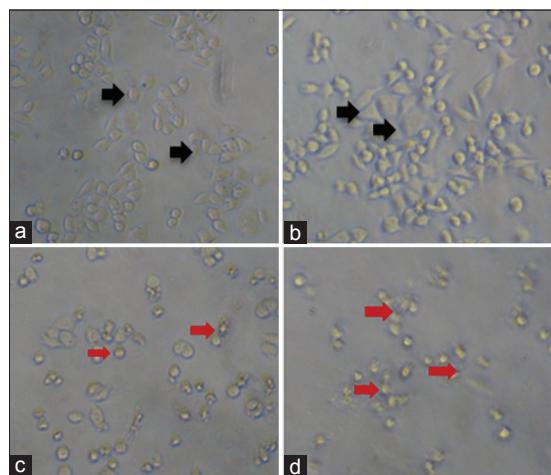


Fig. 3: Effect of *Anredera cordifolia* leaf ethanol extract (ALE) and cisplatin on HeLa cells morphology after 24 hrs incubation
(a) Untreated cells (b) 40 µg/mL, (c) 80 µg/mL ALE treated cells,
(d) 40 µM cisplatin treated cells → Living cells → Death cells

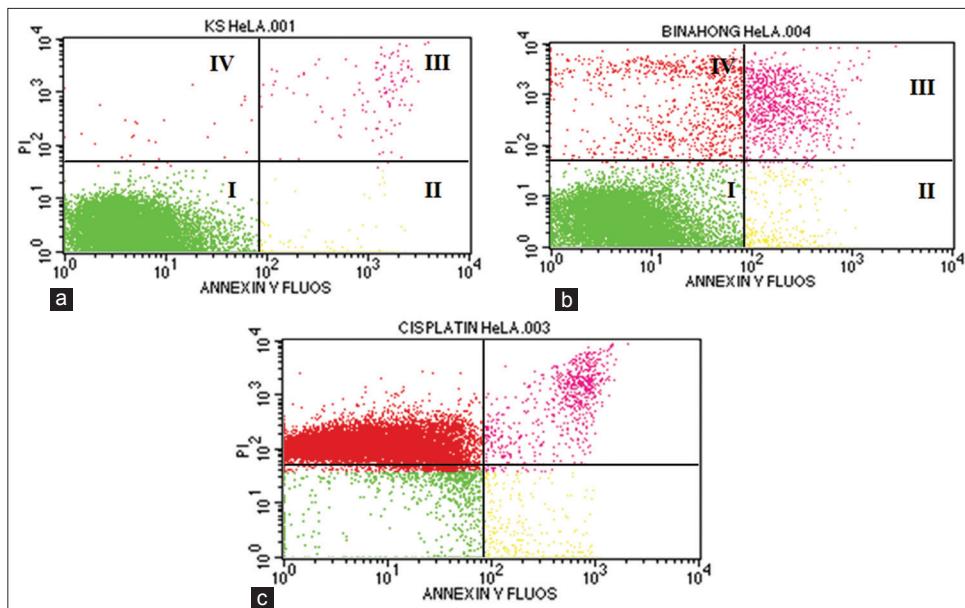


Fig. 4: Apoptotic cells observed using the annexin V/PI method. (a) Untreated cells, (b) 75 µg/mL *Anredera cordifolia* leaf ethanol extract-treated cells, (C) 40 µM cisplatin-treated cells. RI: Living cells, RII: Early apoptotic cells, RIII: Late apoptotic cells, RIV: Necrotic cells

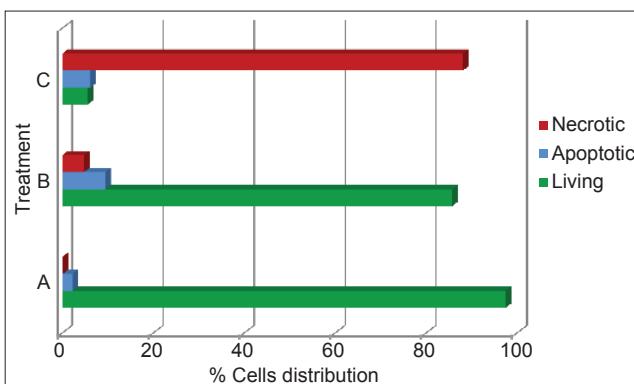


Fig. 5: Cell distribution in apoptosis assay using the annexin V/PI method, (A) Untreated cells, (B) 75 mg/mL *Anredera cordifolia* leaf ethanol extract-treated cells, (C) 40 µM cisplatin-treated cells

p53 and p73-related p53 protein were responsible in the apoptosis process of cisplatin in cervical cancer treatment [12]. Thus, p53 protein is interesting tumor suppressor gene to observe in relation to ALE cytotoxicity and apoptosis pathway.

Cervical cancer cells infected by an oncogenic virus are able to degrade tumor suppressor genes, like p53 by ubiquitination [5,6]. Since the restoration of p53 function promotes a selective therapeutic effect [6], this study focused on p53 role in apoptosis induction pathway of ALE against HeLa cells. ALE-treatment did not increase protein p53 expression in these cells (Fig. 6) compared to untreated cells. On the other hand, cisplatin treatment showed a slight increase in p53 expression. Cytotoxic activity of ALE was not related with p53 restoration in HeLa cells. The molecular mechanism of ALE anticancer activity was p53-independent pathway. Further, study on other proteins involved in the molecular mechanism of anticancer activity must be conducted.

CONCLUSION

This research concludes that *A. cordifolia* leaf extract is promising anticancer agent against HeLa cervical cancer cells with an IC₅₀ value of 75 µg/mL. It definitely triggered apoptosis on HeLa cells through

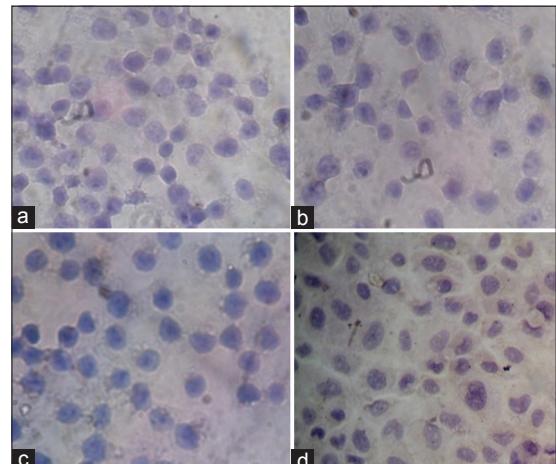


Fig. 6: Effect of *Anredera cordifolia* leaf ethanol extract (ALE) on p53 expression of HeLa cells after 24 hrs incubation (a) Untreated cells with antibody, (b) Untreated cells without antibody, (c) 75 µg/mL ALE, (d) 40 µM cisplatin-treated cells

p-53 independent pathway. Other regulating proteins involved in this molecular mechanism must be investigated.

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REFERENCES

- World Health Organization. International Agency for Research on Anticancer: Global: Latest World Statistics. Press Release. December 12, 2013.
- Kimman M, Norman R, Jan S, Kingston D, Woodward M. The burden of cancer in member countries of the Association of Southeast Asian Nations (ASEAN). *Asian Pac J Cancer Prev* 2012;13(2):411-20.
- Idamardi. *Breast Cancer*. Jakarta: Indonesian Cancer Foundation; 2007.
- Ibeau OA. Molecular pathogenesis of cervical cancer. *Cancer Biol Ther* 2011;11(3):295-306.

5. Thomas M, Pim D, Banks L. The role of the E6-p53 interaction in the molecular pathogenesis of HPV. *Oncogene* 1999;18(53):7690-700.
6. Hietanen S, Lain S, Krausz E, Blattner C, Lane DP. Activation of p53 in cervical carcinoma cells by small molecules. *Proc Natl Acad Sci U S A* 2000;97(15):8501-6.
7. Djamil R, Wahyudi PS, Wahono S, Hanafi M. Antioxidant activity of flavonoid *Anredera cordifolia* (Ten) steenis leaves. *Int Res J Pharm* 2012;3(9):241-3.
8. Sathis M, Niraimathi V, Noorulla KM, Palanimuthu A. *In vitro* antioxidant and cytotoxic activity of isolated compounds in roots of *Clerodendrum phlomidis* Linn. *Asian J Pharm Clin Res* 2015;8(1):196-201.
9. Uddin SJ. Cytotoxic Screening of Bangladeshi Medicinal Plant and Structural Elucidation of Novel Anticancer Compound. PhD Thesis. Queensland : Griffith University; 2010.
10. Rose PG, Bundy BN, Watkins EB, Thigpen JT, Deppe G, Maiman MA, et al. Concurrent cisplatin-based radiotherapy and chemotherapy for locally advanced cervical cancer. *N Engl J Med* 1999;340(15):1144-53.
11. Hong JH. Concurrent chemotherapy for cervical cancer patients primarily treated with radiotherapy: Is it necessary for all? *Chang Gung Med J* 2006;29(6):550-4.
12. Jordan P, Carmo-Fonseca M. Molecular mechanisms involved in cisplatin cytotoxicity. *Cell Mol Life Sci* 2000;57(8-9):1229-35.
13. Boulikas T. Molecular mechanisms of cisplatin and its liposomally encapsulated form, Lipoplatin™ Lipoplatin™ as a chemotherapy and antiangiogenesis drug. *Cancer Ther* 2007;5:349-76.
14. van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. Annexin V-affinity assay: A review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 1998;31(1):1-9.