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Research Article

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Induction of p53 and pRb Expression in Cervical Cancer Cells HeLa by Chloroform Extract of *Polyalthia glauca* (Hassk.) Boerl. leaves

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ABSTRACT

The chloroform extract of Polyalthia glauca leaves showed strong cytotoxic activity on cervical cancer cells HeLa. The objective of this study is to determine effects of chloroform extract of P. glauca leaves on expression of tumor suppressor gene p53 and pRb in HeLa cells. Dried leaves was powdered and extracted by maceration using chloroform for 24 hours, filtered, and concentrated. The extract obtained was tested on HeLa cells at a concentration of 8 ug/mL for 15 hours. The expression of both proteins was observed by immunocytochemistry technique using anti-p53 and anti-pRb primary antibodies. Then analyzed semiquantitatively using the Allerd scoring. The results showed that the chloroform extract of Polyalthia glauca leaves able to induce p53 and pRb expression in HeLa cells.

Key words: *Polyalthia glauca*, HeLa, p53, pRb

INTRODUCTION

Cervical cancer is the fourth most common cancer affecting women on the world, after breast, colorectal, and lung cancers; with 528 000 new cases every year. It is also the fourth most common cause of cancer death (266 000 deaths in 2012) in women worldwide[1]. International Agency for Research on Cancer (IARC) reported that 99% cervical cancer was caused infection of *Human Papilloma Virus* (HPV) [2], and the other case caused by p53 mutation [3;4]. HPV types 16 and 18, are responsible for most HPV-caused cancers [5].

HeLa cells have been shown to contain human papillomavirus (HPV) 18 DNA 11

High-risk human papillomavirus (HPV) E6 and E7 oncoproteins are essential factors for HPV-induced carcinogenesis. The abilities of high-risk HPV E6 and E7 proteins to associate with the tumor suppressors p53 and pRB, respectively, have been suggested as a mechanism by which these viral proteins induce tumors [6]. The E6 protein promotes cell proliferation by stimulating degradation of the tumor suppressor p53 protein via the formation of a complex that comprising E6, p53 and the cellular ubiquitination enzyme E6-AP. E6 stimulates degradation of biological functions of p53; thus perturbing the control of cell cycle progression, leading finally to increased tumor cell growth [7].

E7 binds to a region of the Rb protein commonly referred to as the 'pocket domains' [8]. The ability to repress the expressions of replication enzyme genes correlates with the tumor suppression function of Rb. E7 disrupts the interaction between Rb and E2F, resulting in the release of E2F factors in their transcriptionally active forms [9]. E7 oncoprotein of high-risk HPV types functionally inactivates the Rb family of proteins resulting in overexpression of

E2F transcription factor with upregulation of cell cycle genes resulting in DNA replication. Thus inhibition of the activity of E6 and E7 is an appropriate strategy in cancer treatment [10].

many studies have been conducted to find new ovarian cancer drug

Many studies have been conducted to find the new anticancer. Exploration of natural products to find new potentially anticancer was done. Indonesia is one of the richest countries in the world with regard to genetic resources for medicinal plants. The Indonesia plant that has not been widely studied is *Polylthia glauca* (Hassk.) Boerl. Etikawati, *et. al*. [11; 12] research showed that chloroform extract of *P.glauca* leaves has strong cytotoxic activity on cervical cancer Hela cell (IC_{50} 15,6972 µg/mL) and able to repress of the Bcl-2 expression. The objective of the research is to investigate the effect of chloroform extract of *P. glauca* leaves on expression of p53 and pRb in HeLa cells.

EXPERIMENTAL SECTION

Preparation of Chloroform Extract of leaves *P. glauca*

Fresh leaves were collected from Kebun Raya Bogor Indonesia. The plant material was dried in an oven (Binder) at 40 ^{0}C for five days and then powdered. One hundred gram of dried powdered of leaves was maserated in 0,5 L chloroform p.a. overnight and then filtered. After that, the solvent was removed using a rotary evaporator at 50 ^{0}C.

Cell Culture

Cervical cancer cell lines (HeLa) were cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with penicillin G (100 U/mL), streptomycin (100 µg/mL), and 5% Fetal Bovine Serum (Gibco) at 37 °C in a humidifid atmosphere containing 5% CO_2. Chloroform extract of *P. glauca* leaves was dissolved in DMSO at a concentration of 8 µg/mL, while the fial concentrations were prepared in culture medium.

Determination of p53 and pRb expression in HeLa cells

The expression of p53 and pRb were detected with immunocytochemistry tecnique with kit from Daco LSAB-2 System-HRP. HeLa cells at a density of 5 x 10^4 cells/well grown on cover slips were put in mikroplate 24 well, incubated for 24 hours until the cell under normal condition. Furthermore, the extract obtained was tested on HeLa cells at a concentration of 8 ug/mL and incubated in CO_2 incubator at 37 ° C for 15 hours. Furthermore, the medium is taken and a plate containing cells were washed with PBS. Subsequently, cells were fixed with cold absolute ethanol for 20 minutes , then washed PBS, and dehydrated using ethanol with various concentrations 50 , 70 , and 95 % respectively for 5 minutes. After that, cover slips containing the cells is removed and placed on a dish. Furthermore, the specimen was incubated with diluted mouse primary antibody (1:500) : anti-p53 (Bioss) and anti-pRb (SantaCruss), followed by sequential 10-minute incubations with biotinylated link antibody and peroxidase-labelled streptavidin. Staining is completed after incubation with substrate-chromogen solution. After that the specimen was observed with a light microscope and documented with a digital camera (Canon Power Shoot A460 , 16 mega pixels). The observations were made five times. Furthermore, the specimen was analysed semiquantitatively using Allred scoring [13].

Statistical significance

Three independent experiment were performed in triplicate. The data were analysed for statistical significance using analysis of variance (ANOVA) test. P-value less than 0,05 were considered to be significant.

RESULTS AND DISCUSSION

Immunocytochemistry assay was performed to determine the expression of a gene *in vitro* . This test was often done to study the phenomenon of cancer and to see the effect of a drug on the expression of a particular protein. In this study, immunocytochemistry assay results were analyzed using the Allred scoring. The specimen observations were made five times. The calculation of the score is determined by adding the Proportion Score with Intensity Score (Table 1).

The protein expression is declared negative if the value below 2 and positive if the value above 3. In this study using DAB chromogen so that a positive result is indicated by the brown dots/spots .

Table 1. The grade of protein expression using Allert method

Proportion Score (PS)	Observation	Intensity Score (IS)	Observation
0	NONE	0	None
1	1%	1	Weak
2	1-10%	2	Intermediate
3	10-33%	3	Strong
4	33-66%		
5	66-100%		
Total Score			**Interpretation**
Sum of proportion score and intensity score			
0-2			Negative
3-8			Positive

The first result showed on that HeLa cells treated with chloroform extract of *P. glauca* leaves (Figure 1). The figure showed that p53 expression can be detected after treatment and can not detect in untreatment cell. This indicated that the chloroform extract able to induce p53 expression, supported by Allert scoring that showed positive expression (Figure 2).

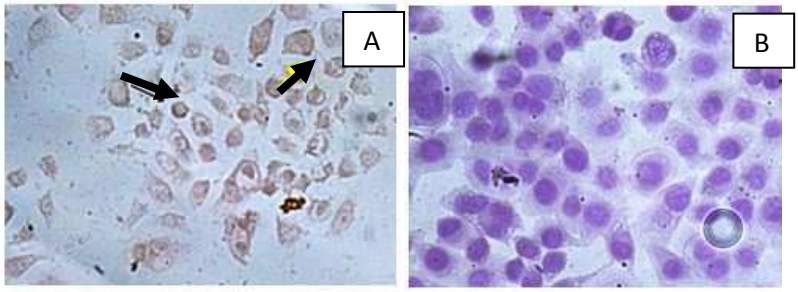

Figure 1. Profile of HeLa cells. A. treatment with chloroform extract of *P. glauca* leaves for 15 hours. B. untreatment cells; The arrow indicated p53 expression

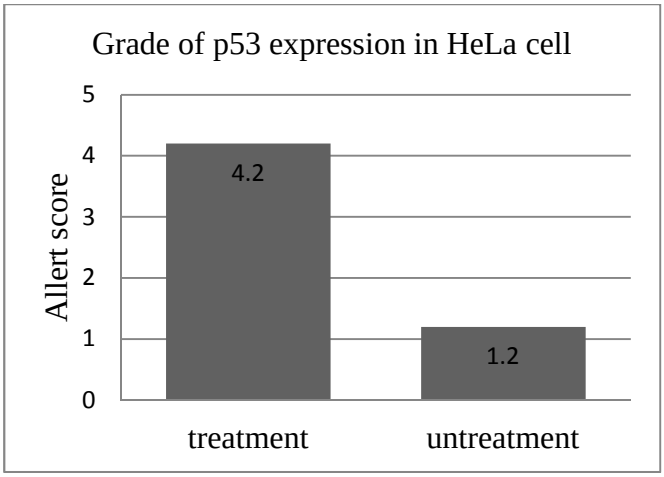

Figure 2. Expression of p53 in HeLa cells after treatment with chloroform extract of *P. glauca* leaves treatment

HeLa cells are the cells that has been inserted 2 oncogene of HPV - 18 E6 and E7. E6 binds to HeLa p53 , thus p53 become inactive and unable to control the cell cycle. The cells will divide continuously because the cell is not able to detect any damage on DNA cell. p53 gene is a tumor suppressor gene, a class of genes which control the cell cycle. p53 gene is an important gene in cancer as it is known about 50 % of cancer cases occur due to mutations in the p53 gene, so that p53 is an anticancer therapeutic targets [14].

In this research showed that p53 expression on HeLa cells will increase. It is suspected that chloroform of *P. glauca* leaves induced p53 expression, so it caused of the return function of cell repair mechanisms and apoptosis. DNA repair mechanisms by p53 occurs in G1 phase of the cell cycle. Under normal conditions, the p53 protein is only slightly expressed, if there is DNA damage p53 expression will increase. p53 will stimulate cell enters cell cycle arrest to repairing DNA and if DNA can not be repaired it will trigger apoptosis. The results of this research showed that the chloroform extract of the leaves of *P. glauca* able to improve p53 expression HeLa cells , thereby increasing cell apoptosis . The recovery of p53 function can recover normal cell function.

The second result showed on Figure 3. that HeLa cells treated with chloroform extract of *P. glauca* leaves. The figure showed that pRb expression can be detected after treatment. This supported that the chloroform extract able to induce pRb expression, strengtened by Allert scoring that showed positive result.

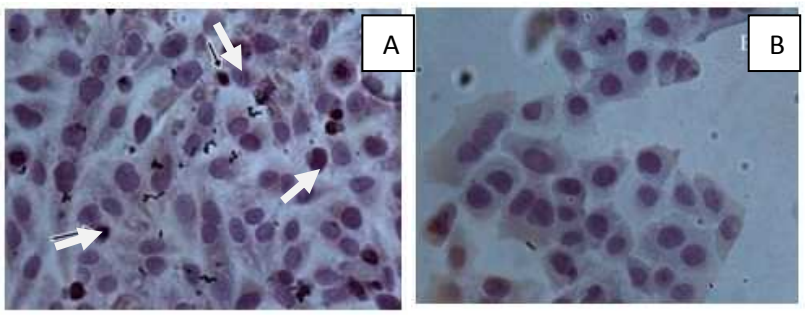

Figure 3. Profile of HeLa cells A. treatment with chloroform extract of *P. glauca* leaves for 15 hours. B. untreatment cells; The arrow indicate pRb expression

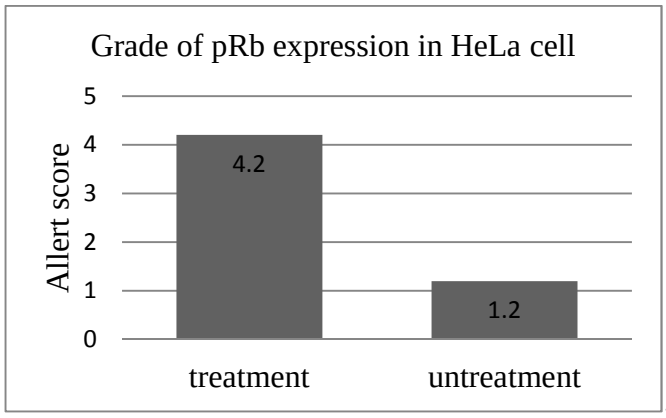

Figure 4. Expression of pRb in HeLa cells after treatment with chloroform extract of *P. glauca* leaves treatment

The retinoblastoma protein pRb is one of the genes that plays a role in the cell cycle, on the HeLa cells HPV E7 proteins interact with pRb, which are negative cell-cycle regulators involved in the G1/S and G2/M transitions. E7 binds to pRb, resulting pRb phosphorylated which causes releasing of E2F transcription factor, and then E2F induces genes that initiate the process of DNA synthesis and mitosis, so the cell cycle become unmanageable [15]. The interaction between E7 and pRb results in enhanced phosphorylation and degradation [16], so this caused uncontrolled proliferation.

Nowadays, the treatment of cancer focused on how to enhance apoptosis and to control the cell cycle. Cervical cancer cell HeLa was cell that loss of cell cycle and apoptosis control. Its caused E6 and E7 oncoprotein from HPV-18 which disturb of p53 and pRb function. The results of this research showed that chloroform extract of *P. glauca* able to induce p53 and pRb expression in cervical cancer cells HeLa, so the extract potentially to be developed as chemotherapeutic agent.

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