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Research Article

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Cytotoxic activities of chloroform extract from leaves of *Polyalthia glauca* (Hassk.) Boerl. on HeLa cell line

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ABSTRACT

A member of genus Polyalthia, which has not been studied extensively is Polyalthia glauca. This study investigated chloroform extract from leaves of P. glauca with anticancer activities on human cervical adenocarcinoma cell line (HeLa) in vitro. The chloroform extract of P. glauca leaf was obtained by maceration. Further, the extract was tested using MTT assay against HeLa cell line. The cell cycle analysis was later conducted using flow cytometry, while the measurement of apoptosis was carried out through DNA fragmentation in HeLa cell treatment. The cytotoxicity test results showed that the IC_{50} values of chloroform extract of P. glauca leaf against HeLa cells was 15.697 µg/mL. Based on results of flow cytometry analysis, the extract was able to increase the distribution of cells in phase G0 / 1. In addition, the extract was also capable of inducing late apoptosis evidenced by DNA fragmentation. These findings suggest the potential use of P. glauca as anticancer agent.

Keywords: *P. glauca*, cytotoxic, cell cycle, apoptosis, HeLa

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INTRODUCTION

There are 120 species constituting genus *Polyalthia* [1]. Members of this genus have been proven to have potential as anticancer and antiviral. Bark ethanol extract of *P. cheliensis* has cytotoxic activity against cervical cancer cells (KB) with IC_{50} of 1.59 µM [2], while the ethanol extract of leaves of *P. evecta* has cytotoxic activity against hepatoma cells (HepG2) with IC_{50} of 70 ug/ml, yet inactive in normal cells Vero [3]. The chloroform fractions of *P. longifolia* leaf have been shown to have cytotoxic activity against leukemia cells (HL-60) with IC_{50} of 6.1 mg/mL [4], while the chloroform and petroleum ether fractions of leaves and roots of *P. laui* are also cytotoxic on lung cancer cells (SPC-A-1) and liver cancer cells (BEL-7402) with IC_{50} values ranging from 0.98 to 58 µM [5]. These data indicate that genus *Polyalthia* are potential to be developed as a chemoprevention agent and antiviral.

A species, which bioactivities have not been widely studied is *Polyalthia glauca*. *Polyalthia glauca* plants spread across Southeast Asia, including Indonesia. They are mid canopy trees, which are up to 36 m tall. Referring to the results of a study on some members of *Polyalthia* [], exploration on cytotoxic activity of *P. glauca* needs to be done, so that the potential for drug efficacy, especially as a new chemoprevention agent could be proved and explored. The objective of this research is to investigate the cancer activity of *P.glauca* against human cervical adenocarcinoma cell line (HeLa) *in vitro*.

EXPERIMENTAL SECTION

Preparation of chloroform extract of *P. glauca* Leaves

Fresh leaves were collected from Kebun Raya Bogor Indonesia. The plant material was dried in an oven (Binder) at 40 °C for five days and then powdered. A thirty gram of the dried, powdered leaves was macerated in 100 mL of chloroform p.a. overnight and then filtered. After that, the solvent was removed using a rotary evaporator at 50 °C.

Cytotoxicity assay

Cytotoxicity assay was performed at the Laboratory of Medicinal Plant and Traditional Medicine Research and Development Centre, Tawangmangu, Jawa Tengah. The Hela cell line was obtained from the laboratory. Cytotoxicity tests were carried out using *3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromida* (MTT). Hela cancer cell lines were cultured and maintained in Dulbeco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), antibiotics (100 U/ml penicillin, Sigma), and 100 µg/ml streptomycin (Sigma) at 37 °C in humidified atmosphere containing 5 % CO_2 in incubator. The cells were seeded at a density of 6 x 10^5 cells in 96-well plates with DMEM medium and incubated for 24 h. The cell were treated with chloroform extracts of various concentrations of 2, 4, 8, 16 and 32 µg/ml in 0,1% DMSO within a 24-hour exposure time. After 24 h, solution of MTT in DMEM medium was added to each well of culture plates. The culture plates were gently shaken and incubated at 37 °C for 4 h. After that, the MTT was removed carefully and then the stop solution (HCL in isopropanol) was added and shaken well. The absorbance was measured at 595 nm in an automated plate reader (ELISA Reader, Biorad) and the percentage of growth inhibition was calculated using the following standard formula:

Inhibitory rate % = Absorbation control – Absorbation test/Absorbation test x 100

Statistical significance

Three independent experiments were performed in triplicate. The data were analyzed to obtain statistical significance value using analysis of variance (ANOVA) test. P-values less than 0.05 were considered as significant. Linear regression equation was used to determine IC_{50} value.

Cell cycle analysis by flow cytometry

HeLa cells with a quantity of 1 x 10^6 were seeded in 60-mm dishes and subjected to 8 µg/ml chloroform extract for 24 h in humidified atmosphere containing 5% CO_2. Floating and adherent cells were trypsinized and then washed three times with PBS. Cells were incubated in 70% ethanol at -20 °C overnigth, treated with 20 µg/mL of propidium iodide. Finally the stained cells were analyzed and studied by flow cytometry at wave length of 488 nm (ACS Calibur instrument) equipped with Cell Quest software.

DNA fragmentation analysis

DNA fragmentation analysis was carried out according to the method of Paris *et al*[6] . Cells were treated with extract at 8 µM/mL concentration and were lysed in 250 mL DNA digestion buffer containing 50mM Tris-HCl, pH 8.0, 10mM EDTA, 0.1M NaCl and 0.5% SDS. The lysate was incubated with 0.5 mg/ml RNase A at 37 ^{0}C for 1h. Phenol extraction of this mixture was carried out and the DNA in the aqueous phase was precipitated using 25 ml of 7.5 M ammonium acetate and 250 ml isopropanol. The isolated DNA was subjected to electrophoresis in 1.8% agarose gel containing 1 % silver stain at 70 volt and bands were visualized by exposing the gel to the UV light and photographed using GelDoc (BioRad).

RESULTS AND DISCUSSION

Cytotoxicity of chloroform extract of *P. glauca* leaf

Cytotoxic effect of the extract against HeLa cell line is shown in Figure 1. There was morphology difference observed on the untreated cells and the treated cells. HeLa cells treated with chloroform extract of *P. glauca* leaf showed many apoptosis cells marked by changes on cells' shape and their nucleus. Apoptosis cells displayed circular shaped and with fragmentation in their nucleus. Treated cells were examined after 24-h exposure and completed with MTT assay. On viable cells, mitochondria were able to form blue purple formazan crystal while the dead or apoptosis cells were not.

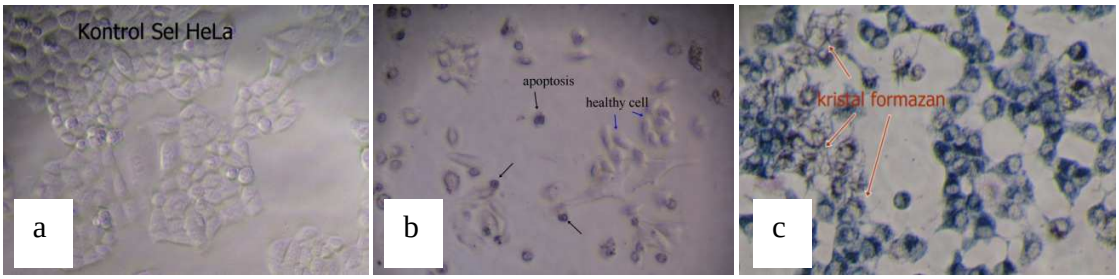


Figure 1. Cytotoxic effect of chloroform extract of *P. glauca* leaf against HeLa cell line. a. Untreated cells (control), b. HeLa cells treated with chloroform extract of *P. glauca*, c. MTT assay on HeLa cell treated

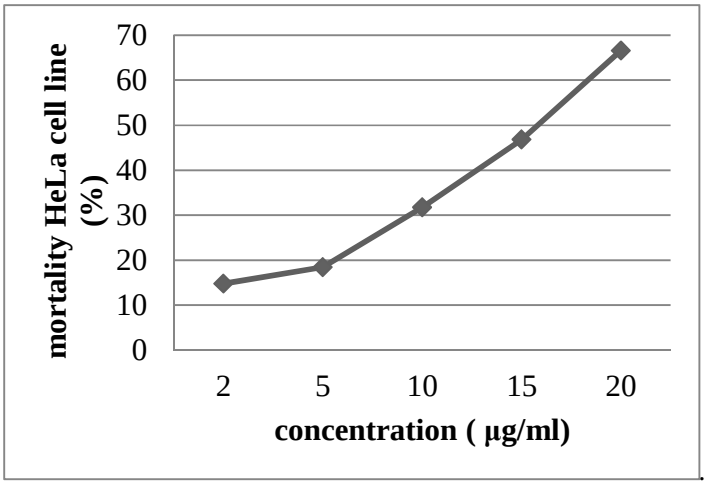
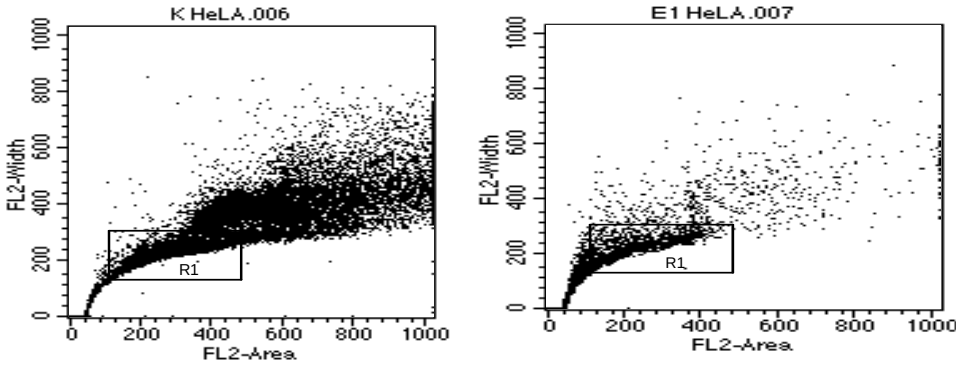


Figure 2. Diagram of cytotoxicity of chloroform extract from leaves of *P. glauca* on HeLa cell line

The HeLa cells were treated with 2, 5 ,10 ,15 , and 20 µg/mL chloroform extract *P. glauca* leaf for 24 h in humidified atmosphere containing 5 % CO_2 incubator. Treatment with the chloroform extract resulted in a significance reduction on viability as shown by MTT assay result (Figure 2). Based on ELISA reader data, the IC_{50} of the extract against HeLa cell line was 15.697 µg/mL. The data suggested that the chloroform extract of *P. glauca* has a cytotoxic effect on HeLa cell line.

Effect of chloroform extract of *P. glauca* leaves on cell cycle

Based on the MTT assays used to determine the effects of extract on viability, doses of ½ IC_{50} were selected for further *in vitro* mechanistic studies required to conduct cell cycle analysis.



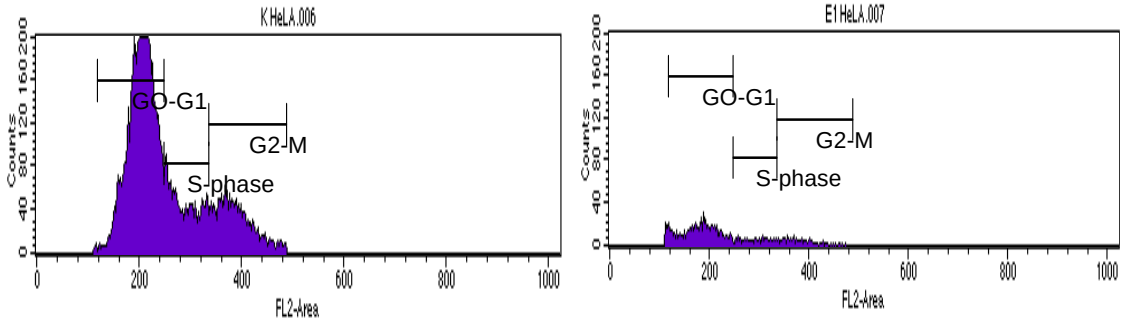


Figure 3. The DNA histogram showing distribution and the percentage of cells in particular phases of HeLa cell cycle. A. Untreated cells. B. Treated cells

Cell cycle analysis results conducted using flow cytometry showed that the chloroform extract of *P. glauca* leaves increased apoptosis at G0/G1 cell population. Figure 3 showed altered cell cycle phase distribution in HeLa cells caused by the extract.

Table 1. Effect of chloroform extract of *P. glauca* leaves on cell cycle phase distribution in HeLa cell line

	Quantity of HeLa cells (%)		
	G0-G1	S	G2-M
Untreated cells	63.28	18.34	18.75
Treated cells	69.66	13.32	12.68

The leaves extract is known external signal that influence many receptors on plasma membrane. Various exogens factors act to stimulate the cell through activation of their respective receptors location on plasma membrane of the cells. In response to certain external signals, a cell will enter the active cell cycle from a resting G0 state [7].

G1 is a critical point at which the cell assesses whether it should enter another full round division. It is perhaps that the proteins involved in G1 progression are frequently mutate in human cell cancer like p53 and pRb on HeLa cell line. It is now recognized that these proteins are among the most attractive targets for the development of new therapeutic agents for the treatment of cancer. The progression from G0 to G1 is triggered by the binding of extracellular growth factors to specific cell-surface receptors. Signal transduction cascades downstream of these receptors, then activate various intracellular processes required for proliferation, including the transcription and translation of many cell-cycle-dependent factors [7].

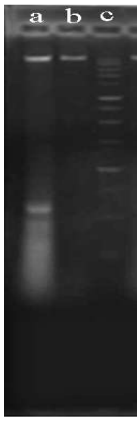


Figure 4. Effect of extract chloroform of *P. glauca* leaves on the DNA fragmentation (24 hours exposure). a. HeLa cell treated with extract chloroform (15 µg/mL), b. Untreated cells (control), c. DNA ladder 100 bp

Effect of chloroform extract of *P. glauca* leaf on apoptosis

Effects of natural products on the DNA ladder pattern are shown in Figure 4. DNA of untreated control cells did not show any ladder pattern (line b), but DNA of treated cells with extract produced DNA ladder formation (line a). This shows late apoptosis. A main characteristic of apoptosis is the fragmentation of DNA.

Degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptotic cell death. It occurs in response to various apoptotic stimuli in a wide variety of cell types. DNA fragmentation is a secondary consequence of apoptosis. Endonuclease involved might be like to DNaseI, a potential indication that DNA fragmentation might occur after the release of enzymes from the plasma membrane lysis, an event that would potentially occur only after the final lytic event in the apoptotic sequence. More recently, data have shown that specific proteases residing in the cytoplasm mediate the terminal events of apoptosis, including those of nuclear morphology.

CONCLUSION

P. glauca is a potential plant with cytotoxic activities. Further isolation of pure compound needs to be performed.

Acknowledgements

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