The potency of flavonoid n-hexane, chloroform, and ethanolic fraction from *Scurrula atropurpurea* (Blume) danser on proliferation and apoptosis through methylation of p16 gene on HeLa cells

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The Potency of Flavonoid N-hexane, Chloroform, and Ethanolic Fraction from *Scurrula atropurpurea* (Blume) Danser on Proliferation and Apoptosis Through Methylation of p16 Gene on HeLa Cells

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Abstract. Human Papilloma Virus (HPV) infection suggested could induced gene hypermethylation. Hypermethylation occurs on the p16 tumor suppressor gene promoter resulted in silencing tumor suppressor gene so that the gene is inactive and contributes to the malignancy of cancer cells. Tea parasites or Loranthaceae are known to be a plant that has an anticancer potency, correlated to flavonoids content. Flavonoid from Scurrula atropurpurea (Blume) Danser (SAD) suggested have a role in inhibiting methylation towards p16. This research objective was to identify the potency of Lawang - East Java SAD flavonoids compound towards Hela cells. In this study, we examine flavonoids from SAD that been extracted in n-hexane, chloroform, ethanol solvent, using TLC Chromatogram, and LCMS. We also examined the cells proliferation using MTT assay, the apoptotic level was examined by Tunnel, and then completed with methylation level detection using bisulfite direct sequencing method. The result showed that flavonoids that found were: flavanone, dihydro flavonol, and flavon, dihydro flavonol, flavanone and catechin, and flavonol, flavon, and EGCG, respectively n-hexane, chloroform and ethanol fraction. Flavonoids have proliferation inhibitor and apoptotic inductor potency via inhibition of p16 promoter of HeLa cells methylation. SAD flavonoids in the n-hexane and chloroform fraction shown the most potent in methylation inhibition.

INTRODUCTION

The last decades showed that cancer incidence and mortality levels were so variable in the whole world. Cancer is a disease that not only related to genetic but also epigenetic such as DNA methylation can induce changes in gene expression[1–3]. In patients with cancer, particularly cervical cancer, tumor suppressor genes such as p16, DAPK, MGMT, APC, HIC-1, and E-cadherin become silence due to hypermethylation. P 16, which is one of the Tumor suppressor genes (next will stated as p16) known to have the highest hypermethylated rate towards other tumor suppressor genes on cancer incidences [1]. On cervical carcinoma, hypermethylated often found on many essential tumor suppressor genes, so they are silenced [4,5]. p16, especially on cervical carcinoma, are playing proliferation and apoptotic control simultaneously with the other two genes, p53, and pRb. There was a study that reported that p16 expression was a sign that found in almost every state of squamosa dan glandular cervix lesson, which those expressions closely related to the HPV infection risk [6–8]. Nowadays, people are known that eating fruits and vegetables that contain bioactive compounds could provide significant protection against carcinogenesis[9–11].

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Bioactive compounds such as flavonoids from the tea parasite have potential as an anticancer drug through the mechanism of reactivation of silencing tumor suppressor genes because of hypermethylation

Recently, plant parasite species that showed great potential for cancer treatment are tea plant parasite, Scurrula atropurpurea. Flavonoid SAD that have been found from the previous study were known as flavanone, dihydro flavonol, flavon, catechin, flavonol, and EGCG, respectively [12]. Almost all parts of this plant have been reported could be used as a cancer treatment. Species of this tea plant parasite is spreading in almost all Indonesia region and several Asian countries. Herein we reported an in vitro study about the effect of tea plant parasite extract, Scurrula atropurpurea (Blume) Denser, on the culture of HeLa cells through observation of several apoptosis marker expressions and apoptosis index.

EXPERIMENTAL DETAILS

Cell Culture

HeLa cells were obtained from the Center of Assessment and Application Technology (BPPT) Serpong. Cells were grown on tissue culture in media: RPMI medium, supplemented with 1% penicillin-streptomycin and 10% Fetal Bovine Serum (FBS). Cultured cells were incubated in temperature 37 °C and 5% CO_2. If culture cells already confluent, the passage was done using trypsin-based dissociation.

Plant parasite (Scurrula atropurpurea (Blume) Danser) extract

Tea plant parasite (SAD) were collected from Lawang Jawa Timur Indonesia. SAD was obtained based on previous methods. The procedure of plant parasite extract was divided into three steps: drying process, maceration using N-Hexane solution, and continued with maceration using chloroform and ethanol solution. The dose that used to examine apoptotic and methylation was the IC50 dose from n-hexane fraction, which was 500 µg/mL, 100 µg/mL for chloroform fraction, 300 µg/mL from ethanol fraction and 30 µg/mL for EGCG. The stock solution was made by diluting the SAD using 1% DMSO and distilled water. Further dilution for making proper doses was used using the media of cultured cells.

DNA isolation

The cell culture was digested using a trypsinization protocol. The supernatant was digested with 3 mL of TE buffer and 100 µL 20% SDS, supplemented with 20 mg/mL Proteinase-K and overnight incubation at 55 °C was performed, followed by DNA ethanol precipitation using 1 mL of saturated NaCl and 10 mL of absolute ethanol. The sample was overnight incubated with constant agitation. The DNA was air-dried and suspended in 100-300 µL of water, and DNA concentration determined using a fluorometer.

Apoptosis analysis

Apoptosis analysis was measured using TUNEL. In addition, the apoptosis index was also measured using the TUNEL method. All measurement was conducted based on the manufacture's procedures.

MTT analysis

HeLa cells with 80% confluency were harvested, cell number was counted and transferred into 96 well plates with 100 µL per well. The cell distribution was observed using an inverted microscope. HeLa cells were overnight incubated and exposed to varying treatment. After removing the medium of each well and washing twice by PBS, 10 mL of MTT solution (0.5 mg/mL) and 10 mL of complete medium were introduced. After incubation for another 2-4 hours, the result (formazan crystals) were dissolved with 10% SDS in 0.1 N HCl. The plate was wrapped and overnight incubated in the dark. Absorbance intensity measured by ELISA reader at 550-600 nm with 595 nm as a reference wavelength.

Methylation analysis

Genomic DNA isolated from HeLa cells and subjected to methylation analysis. Briefly, two µg of DNA was denaturated by incubation with 0.3 M NaOH. About ten mM fresh quinol and saturated sodium bisulfite pH six were added and incubated at 55 °C for 4-16 hours. treated DNA was purified using Milli-Q Water and desulphonate with 0.3 M NaOH. tRNA (10 mg/mL) was added and supplemented with 3 M ammonium acetate to neutralize. DNA was precipitated by added cold absolute ethanol, and overnight incubation was performed. DNA pellet was resuspended with TE buffer or water.

Statistical analysis

The analysis was performed using One Way ANOVA followed by Post Hoc Tukey test with significance $p < 0.05$. All analyses were conducted by SPSS for windows.

RESULTS AND DISCUSSION

MTT analysis was used to observe the effect of flavonoid extract on the proliferation of HeLa cells. From viability measurement (Fig. 1) using the lowest dose from each fraction, it showed that the lowest viability level was obtained from treatment with n-hexane compare to other fractions, with a percentage 81%. The viability level of N-hexane fraction was similar to EGCG as a positive control. Still, it could be suggested that n-hexane had a better effect, since the concentration of N-hexane (2.12 ppm) fraction less than half of EGCG concentration (5 ppm) in the lowest dose. The viability levels were decreased based on a dose-dependent manner. Higher doses were resulted in the lowering of viability levels in each fraction and also EGCG. Different from the result from the lowest dose, at the highest dose, the lowest level of cell viability was obtained from chloroform fraction (13 %). Although there was a different result of effectivity between the lowest and highest dose, If we compare the decreasing of viability levels from each fraction in every concentration increment, chloroform fraction was showed a better effect than other fractions.

TABLE 1. Percentage of the methylated level of p16 gene promoter area at 13 predictive methylation position in each treatment

Treatment	Methylated level (%)
Control	53.84
N-hexane fraction	7.69
Chloroform fraction	15.38
Ethanol fraction	23.08
EGCG	23.08

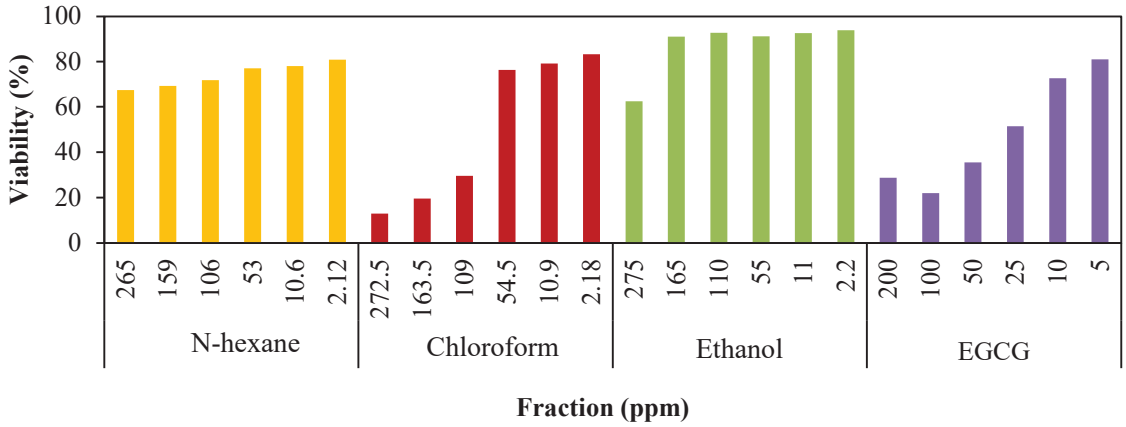
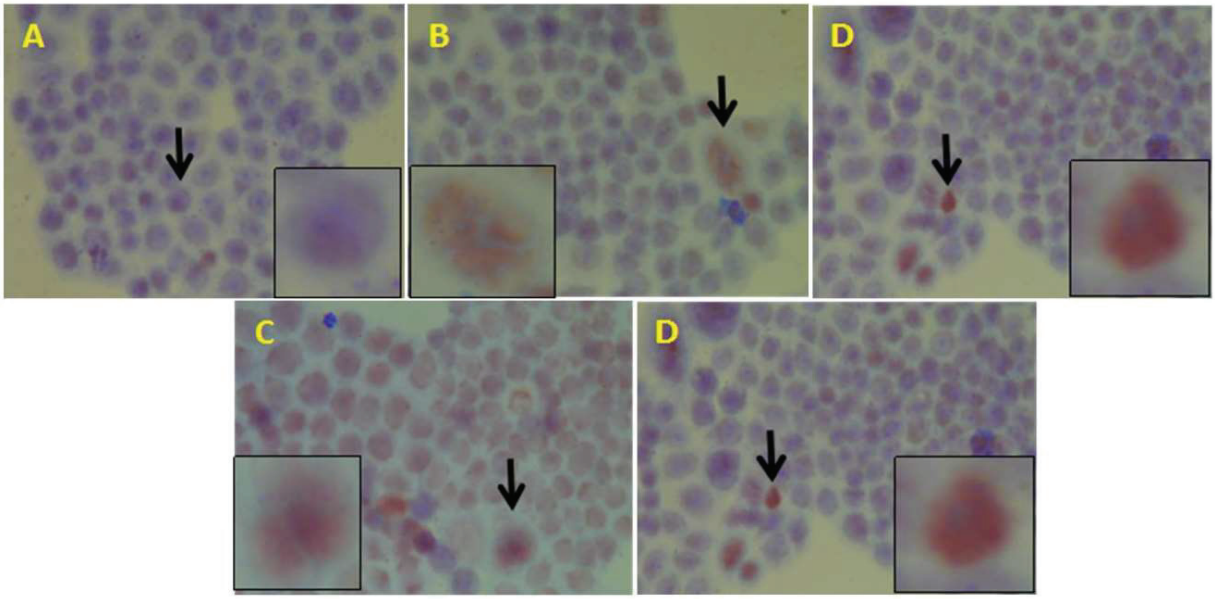


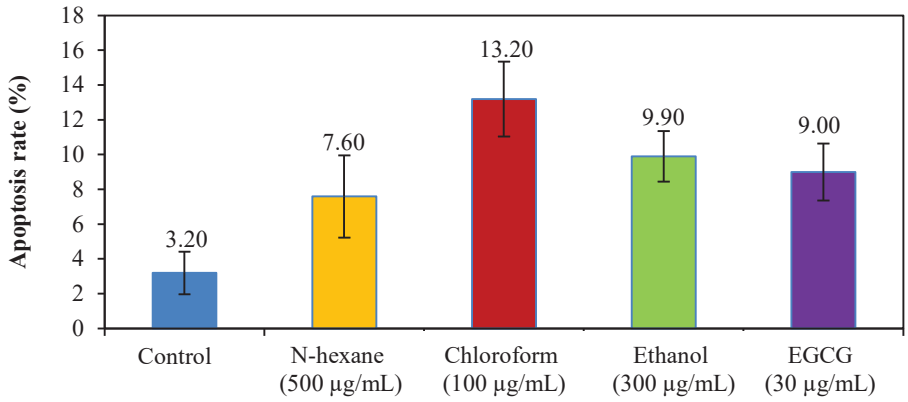
FIGURE 1. MTT assay from fractions of SAD and EGCG. The average cell viability was treated with six different concentrations of n-hexane, chloroform, ethanol, and EGCG. On Hela cells that treated with chloroform fraction have the lowest viability rate compared to other fraction treatment results

In this study, the effectiveness of each fraction was also determined based on the apoptosis level. The apoptosis level after treatment using each fraction and EGCG were confirmed using TUNEL assay. Cells that were undergoing apoptosis process was characterized by red-brown color at the nucleus (Fig. 2A). After quantification (Fig. 2B), the apoptosis level of treatment using chloroform was significantly higher compared to control, other fractions, and EGCG as a positive control, with a percentage 13.2%.

Methylation analysis using a direct sequencing method was conducted to observe the possible effect of a SAD fraction on the methylation process of p16 gene as one of an important cancer-related gene. Methylation was observed in the promoter area of p16 gene (DQ406745.1) at nucleotides position between 1490 – 1659 bp. There were 13 positions that suggested having a methylation process. The percentage of methylated positions was showed in Table 1. The lowest methylated level was obtained from treatment using N-hexane fraction (7.69%), then followed by chloroform (15.38%), ethanol (23.08%), and EGCG (23.08%) respectively.



(A)



(B)

FIGURE 2. Apoptosis analysis of cells that treated with the SAD fraction. (A). Morphology of cells after treatment with SAD. Example cells that were undergoing apoptosis process were shown in black narrow. (B). Apoptotic rate on Hela cells. The highest apoptotic rate was on chloroform fraction treatment with the apoptotic rate was 13.2%. All SAD fraction is known to have a significant effect on cells apoptotic increasing ($p<0.05$)

All SAD flavonoids from n-hexane, chloroform, and ethanol fraction showed the methylation inhibiting potency towards p16 on HeLa cells. This decrease will result in an increase in p16 activity. The highest methylation decrease was resulting from n-hexane SAD treatment. Flavonoid compounds on SAD will bind at the active site of DNMT and give it DNA methylation inhibiting potency. Previous in silica study has shown that all SAD flavonoid compounds can bind to DNMT active site via specific different amino acids and have a different affinity. EGCG has the highest bind affinity with DNMT active site and then respectively followed by catechin, dihydro flavonol, and flavon. The binding affinity was respectively were: -10.4 Kcal/mol, -8.4 Kcal/mol, -8.2 Kcal/mol, and -8.0 Kcal/mol, for EGCG, catechin, dihydro flavonol and flavon. The lowest bind affinity of flavonoid compound towards DNMT will increase the potency of it inhibiting DNMT activity [12]. On n-hexane fraction were found: dihydro flavonol, flavon, and flavanone, and those compound interactions were predicted to have a synergetic effect on DNA inhibiting methylation towards He La cells.

DNA methylation is a mechanism that plays a role in eliminating gene transcription process, regulating gene expression that will be print, arrange the number of genes associated with tumor suppressor and suppress the gene expression on the inactive X chromosome [13]. Generally, methylation analysis conducted at CpG island on promotor [14]. Cytosine methylation at CpG island from a gene closely related to the loss of gene expression via transcription mechanism suppression. Several studies show that the gene of tumor suppressor and DNA repair, often through hypermethylation process and affect to inactivation process. p16/CDKN2A is one of the tumor suppressor gene [13]. p16 gene inactivation closely related to severity level and stadium increase on cervical cancer [15]. Decrease expression of p16 caused by mutation and has been shown in several studies related to cancer and plays a role of CDK4/6/cyclin D and p16 inhibitor imbalance [16].

SUMMARY

In summary, SAD was effective as a cytotoxic agent for cancer cells. The fraction from SAD was showed an effect at the cellular and molecular levels. At cellular levels, it has resulted in the decreasing cell viability and increase of apoptosis levels. At the molecular level, it resulted in the decreasing of methylated levels on the promoter area of p16 gene. At least in part, from all analyses, chloroform had the highest effect compare to other fractions. Further analysis still needs to conducted to observe the complete molecular mechanism of SAD fraction and those effects on other cancer genes.

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