Cytotoxic Activity of *Peronema canescens* Jack Leaves on Human Cells: HT-29 and Primary Adenocarcinoma Colon Cancer

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**ABSTRACT**

**Background:** In Indonesia, this species was well known in Sumatera, Kalimantan, Java, and Sulawesi. *Peronema canescens* Jack (Sungkai) was traditionally used as an anti-flatulent, fever, toothache. Sungkai leaves contain many secondary metabolites with potential anticancer activity. The reported anticancer research was still limited to the cytotoxic activity of chloroform extract on the HT-29 colon cancer cell line. However, it was necessary to uncover the underlying mechanism. **Aim:** The purpose of this study was to investigate the mechanism (such as cell cycle inhibition, induces cells apoptosis, and necrosis) of the chloroform subfraction (SF3) from *P. canescens* extract has anticancer activity on HT-29 cells and primary adenocarcinoma (AdenoCa pT3N1cM1) colon cancer cells. **Materials and Methods:** The extraction was by maceration method using methanol solvent, the fractionation process was using column chromatography with polarity gradient eluent. The cytotoxicity of SF3 was measured by MTT assay. **Results:** The cell cycle inhibition, apoptosis induction, and necrosis cells were evaluated with the Flow cytometry method. The inhibition activity of synthesis and mitosis phase in cell cycle demonstrated that the different concentrations of SF3 have inhibition activity on HT-29 (29.614 µg/ml) of 26.79% and 0.16%, AdenoCa cells (14.807 µg/ml) of 10.27% and 19.29%, respectively. For induced apoptosis activity on HT-29 (29.614 µg/ml) and AdenoCa cells (14.807 µg/ml) were 26.58% and 11.50%, successively. Whereas, necrosis activity on HT-29 (29.614 µg/ml) AdenoCa cells (14.807 µg/ml) were 0.02%, and 9.56%, respectively. **Conclusion:** The subfractions chloroform (SF3) of *P. canescens* extract has potential activity on HT-29 and Adenocarcinoma colon cells through cell cycle inhibition, induces apoptosis and necrosis cells. **Key words:** Apoptosis, Cell cycle, Colon cancer cells, Necrosis, *Peronema canescens* Jack.

**INTRODUCTION**

Cancer is a group of cells characterized by uncontrolled and abnormal cell growth. Cancer cells can invade other tissues and perform metastases. Colorectal cancer occurs in the rectum, initially as a polyp in the large intestine or rectum, called adenocarcinoma. In addition to synthetic product cancer drugs, natural products containing secondary metabolites as chemopreventive: anticarcinogenic and anti-mutagenic, which work by inhibiting the development of cancer cells, such as flavonoids, alkaloids, terpenoids, and phenolics. *Peronema canescens* Jack is the only species in the genus Peronema, family Verbenaceae, is a tree that grows in tropical areas: Indonesia, Thailand, and Malaysia. Several studies from the Verbenaceae family are active in colon cancer (HCT-15) cell lines, such as *Vitis Fructus* and *Vitex trifolii*. Phytochemical investigations from *P. canescens* had isolated biologically active compounds including β-sitosterol, phytol, β-amyrin, peronemin A1, A3, B2, B2, B3, C1, and D1, peronemin A3 and C1 are antimarial, also contain secondary metabolites: alkaloids, terpenoids, steroids, flavonoids, phenolics, and saponins. This compound was a cytotoxic component that inhibits cancer. Empirically, in Indonesia, Peronema leaf known as 'Sungkai' are traditionally used, especially the Dayak tribe of East Kalimantan used as medicine toothache, ringworms, anti-flatulence, and fever. Research has reported bioactivity with the Brine Shrimp Lethality Test (BSLT) method using methanol, hexane, and ethyl acetate extracts with shrimp larvae animal models. It was also known to have antibacterial, antioxidant, anti-inflammatory, immune-enhancing, and anti-diabetic activities. In addition, the chloroform subfraction extract was reported to have a cytotoxic effect on HT-29 colon cancer cells. However, it was very important to add further scientific evidence regarding the anticancer activity of the chloroform subfraction of this plant before the active chemical compounds are isolated. Hence, this study continued previous studies with the expansion of testing on primary colon cancer cells and was the first report of *in vitro* anticancer of *P. canescens* extract through inhibition of cell cycle, apoptosis, and necrosis HT-29 cells and primary colon cancer cells AdenoCa pT3N1cM1.

**MATERIALS AND METHODS**

**Materials and Equipment**

The sample of *P. canescens* leaf was obtained in Tanah Merah Sub-District, Samarinda, East Kalimantan, in May 2019. It was stored at Pharmaceuticals Research and Development Laboratory of FARMAKA TROPIS, Faculty of Pharmacy Mulawarman University East Kalimantan, Indonesia. It was determined at the Dendrology and Forest Ecology Laboratory of the Faculty of Forestry, Mulawarman University, Samarinda. The voucher specimen (33/ H17.4.1.08/LL/V1/.2011). In this study, the chemical materials were used included: methanol p.a, hexane p.a, chloroform p.a, ethyl acetate p.a, ethanol p.a.
Annexin V-FITC kit (Biolegend), Flow cytometer (BD FACS Lyric), airflow (Biobase, Indonesia), and ELISA (Model 550, Bio-Rad, USA), modification. Suspension of HT-29 and AdenoCa pT3N1cM1 cells, Yellow tips, and Blue tips (Neptune, USA). Moreover, tools were used, Cytotoxicity test carried out according to the method, 5,21,22 with slight modification. Suspension of HT-29 and AdenoCa pT3N1cM1 cells, which were with a density of 1 x 10^5 cells/100 µl, were added to the well and incubated at 37°C for 24 h in a 5% CO₂ humidified incubator. Cell condition examined by inverted microscopy. If formazan has formed after incubated for four h, supernatant media was removed by adding 100 µl of DMSO (Dimethyl Sulfoxide), and a 5 µl PI kit. Incubation was carried out in a dark room for 10-20 min, suspension with binding buffer, transferred into the FC tube using a nylon filter. Thereafter, the solutions were analyzed by a Flow cytometer (BD FACs Lyric).

**Extraction Preparation**

According to a previous study, the extraction process was carried out using a macerator.18 Briefly, P. canescens leaves were dried and ground into a powder, then extracted with methanol, filtered with filter paper, and evaporated under vacuum to obtain a thick extract. The extract obtained was stored at room temperature until ready for fractionation using vacuum chromatography.

**Fractionation Process**

According to a previous study, the fractionation process was carried out using vacuum column chromatography (VCC)19 with some modification. Briefly, the extracted sample (20 g) was dissolved in hexane solvent, impregnated with 10 g silica gel G 20 - 50 mesh, homogenized. Samples were separated using VCC with silica gel G 70 – 230 mesh, column size 13 cm, height 24 cm, and 1000 ml of polarity gradient eluent, respectively: hexane, chloroform, ethyl acetate, and methanol. Then refraction was carried out with the same treatment as in the fractionation section, 10 g of the chloroform fraction extract, impregnated with 5 g of silica gel G using a vacuum column measuring 4-5 cm, and a polarity gradient ratio of 250 ml of eluent: hexane: ethyl acetate, methanol (hexane, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, methanol), respectively, the refraction was performed three times. Six subfractions were obtained for the detection of the TLC plate, which was visualized with a UV lamp at 254 nm.

**Cell Preparation**

This study used both HT-29 and human primary adenocarcinoma (AdenoCa pT3N1cM1) colon cancer cells. These HT-29 cells were obtained from the Laboratory of Anatomy Pathology, Faculty of Medicine, University of Indonesia, and human primary AdenoCa pT3N1cM1 colon cancer cells obtained from the Cancer Virology and Pathobiology Research Center, Cipto Mangunkusumo National Hospital. The test cells were maintained in DMEM and RPMI 1640 media; the cells were washed three times with 2 ml of PBS, centrifuged at 2000 rpm for 3 min. It was treated with a 100 µl Annexin V-FITC and a 5 µl PI kit. Incubation was carried out in a dark room for 10-20 min, suspension with binding buffer, transferred into the FC tube using a nylon filter. Thereafter, the solutions were analyzed by a Flow cytometer (BD FACs Lyric).

**RESULTS AND DISCUSSION**

**Extraction and Fractionation Process**

Extraction of solutes was based on differences in the chemical and physical properties of the solute (molecular weight, hydrophobic charge, polarity, and volatility).21 Extraction of the maceration method using methanol as a solvent; this method is suitable for use on soft textured samples.6,13 The sample fractionation process was carried out using vacuum column chromatography (VCC)19 with some modification. In this study, the sample was first impregnated with silica gel and then fractionated using a solvent gradient. The fractionation was carried out three times in a row using a gradient eluent ratio (hexane: ethyl acetate, methanol). As a result, six subfractions (SF1-SF6) were obtained (Table 1), which shows that the value of the weight of subfractions (SF) varies based on the difference in solvent gradient.

Polarity gradient elution was used to separate groups of compounds based on their solubility in suitable solvents. This method has the advantage that the separation of compound groups was faster based on their polarity level with the help of vacuum pressure.32

**ANTICANCER ACTIVITY**

**Cytotoxicity Test MTT Method**

MTT assay was used to measure viability and proliferation cells and acts on macrophage-mediated living mitochondria cells.5,23 The principle through the reaction of succinate dehydrogenase the tetrazolium salt was converted into purple formazan, measured by spectrophotometry after dissolving DMSO (Dimethyl Sulfoxide).18 According to a previous study, chloroform subfraction (SF3) extract of P. canescens leaves had cytotoxic activity on colon cancer cells HT-29.18 To develop research on the cytotoxic activity of SF3 extract on primary colon cancer cells. We conducted a follow-up study by testing the cytotoxic activity (IC₅₀) of SF3 extract on AdenoCa pT3N1cM1 colon cancer cells to complete the cytotoxic data SF3 of Sungkai leaves extract. The results of the MTT test showed that as the concentration of SF3 increased, cell viability decreased. Treatment of dose at 50 µg/ml reduces the viability cell by 16.141% of AdenoCa pT3N1cM1 cells. Meanwhile, the control of 5-FU (16 µg/ml) dose reduces of AdenoCa pT3N1cM1 by 16.078% the cell viability; it shows a comparable toxicity effect (Table 2).
The results obtained in this study of the SF3 extract showed cytotoxic activity on AdenoCa pT3N1cM1 cells can be seen in Table 2. The IC_{50} value of SF3 extract and 5-FU against AdenoCa pT3N1cM1 cells can be determined based on the relationship between SF3 concentration and cell viability by linear regression, and obtained values 1.897 μg/ml and 0.122 μg/ml, respectively (Figure 1).

The above results demonstrated that the cytotoxicity effect was categorized as a cytotoxic agent (any IC_{50} value less than 20 µg/ml was considered cytotoxic). The cytotoxicity criteria for the pure compound and crude extract were IC_{50} <20 µg/ml after 24 h of exposure. As a result, SF3 extract showed a significant cytotoxic potential against AdenoCa pT3N1cM1 cells and was weaker than the cytotoxic activity of 5-FU (positive control).

### Cell Cycle Analysis

Plants were sources of herbal medicine and played an important role in the treatment and prevention of cancer. In this study, we developed an anticancer activity assay of SF3 chloroform extract from *P. canescens* leaves to determine the possible mechanism of action of SF3 anticancer on HT-29 and AdenoCa pT3N1cM1 cells using Flow cytometry. This tool analyzed changes in the cell cycle distribution (G0/G1, S, and G2/M) phases. The working method of this tool was fluorescence-activated cell sorting with propidium iodide (PI) marker after 24 h of treatment. We demonstrated that SF3 inhibited the growth of HT-29 cells and AdenoCa pT3N1cM1 cells in a concentration-dependent and time-dependent manner.

Furthermore, our data reveal that SF3 induces termination of G0/G, S, and M/G2 phases in HT-29 cells and AdenoCa pT3N1cM1 cells. The arrest of the S and M/G2 phases may be associated with DNA damage. The data cell cycle distribution and (G0/G1, S, and G2/M) phases in each concentration assay were shown in Figures 2 and 3.

The results of the study at a dose of 29.614 µg/ml SF3 extract tested showed the highest cell cycle inhibitory activity in the synthesis (S) phase of 26.79%, and mitosis (G2/M) phase of 0.16% (Figure 2), successively. Meanwhile, AdenoCa pT3N1cM1 cells (14.807 µg/ml) were 10.27% synthesis (S) phase and 19.29% mitosis (G2/M) phase (Figure 3) compared to control cells, and weaker than 5-FU (positive control).

### Inducing Apoptosis and Necrosis Cell

A key strategy of cancer therapy was based on inducing apoptosis of cancer cells upon drug administration. An essential feature of chemopreventive agents was caused cell death by apoptosis rather than necrosis selectively. Cell morphology and DNA (Deoxyribonucleic acid) fragmentation are the leading indicators of apoptosis induction. Colon cancer cell programmed death can be detected due to exposure to chloroform subfraction (SF3) of *P. canescens* extract; evaluated
Table 3: Effect of different concentrations of SF3 on the distribution value of apoptotic and necrotic cells.

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>LC (μg/ml)</th>
<th>EA (%)</th>
<th>LA (%)</th>
<th>TA (%)</th>
<th>Nec (%)</th>
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<td>Control</td>
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<table>
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<tr>
<th>Concentrations</th>
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LC - Living cells, EA - Early apoptosis, LA - Late apoptosis, TA - Total apoptosis, Nec - Necrosis
apoptosis and necrosis with Flow cytometry method using Annexin V-PI reagent. The study distribution of live cells, early apoptosis, late apoptosis, and necrosis can be seen in Table 3.

Flow cytometry method with Annexin V and PI reagents works selectively to bind intact and fragmented cells so that it can differentiate among living cells, early apoptosis, late apoptosis, and necrosis. With Propidium Iodide (PI) reagent, cell death due to apoptosis or necrosis can be distinguished based on staining by intercalation with DNA. In the study, total apoptosis and necrosis activity value increased after the treatment dose of 2IC$_{50}$ (29.614 µg/ml) SF3 extract was 26.58%, and necrosis was 0.02% on HT-29 cells (Table 3, Figure 4C). Whereas, treatment dose of IC$_{50}$ (14,807 µg/ml) on AdenoCa pT3N1cM1 cells apoptosis and necrosis value were 11.50% and 9.56%, respectively (Table 3, Figure 5A).

Meanwhile, 5-FU (positive control) test, the concentration IC$_{50}$ (0.040 µg/ml) showed apoptotic and necrotic effects of 27.12% and 0.05% on HT-29 cells, respectively, and for AdenoCa pT3N1cM1 cells were 10.51% and 5.84%, successively. (Table 3, Figures 4D and 5D). The data from the analysis of total apoptosis of HT-29 cells representing the mean ± SD of three replicates were analyzed by paired dependent sample T-test, showing a significant percentage value (*P>0.05) for a concentration of 29.614 µg/ml between the treatment control groups and nonsignificant (**P<0.05) with 5-FU (positive control) (Figure 6A). Whereas, AdenoCa pT3N1cM1 cells, the significant (*)P>0.05 was at a concentration of 14,807 µg/ml between the treatment and control groups, and nonsignificant (**P<0.05) with 5-FU (positive control) (Figure 6B).

In this study, 5-FU as a positive control was the primary chemotherapy agent used for colon cancer therapy. It is a prodrug produced by fluorouridine-5′-triphosphate (FUTP) metabolite, which affects the function of Ribonucleic acid (RNA) and fluoro-deoxy uridylate (FdUMP), inhibits Deoxyribonucleic acid (DNA) replication, induces p53 express p21 and pRb so that it can stop cell synthesis by accelerating apoptosis. The results showed that the chloroform subfraction (SF3) extract of *P. canescens* leaves had potential anticancer. This data was the first to be reported from the genus Peronema which inhibits the proliferation, apoptosis, and necrosis of colon cancer cells, which will help further research, especially the search for compounds from this plant as colon anticancer.

**CONCLUSIONS**

This study was the first to show the first to demonstrate the anticancer properties of *P. canescens* leaf chloroform SF3 extract on primary colon cancer cells HT-29 and AdenoCa pT3N1cM1 cells. It shows a lot exhibits multiple anticancer effects, including cell proliferation inhibition, cell cycle arrest, apoptosis, and necrosis cells. Therefore, this sample appears to be a valuable natural source of research and development for the treatment of colon cancer.

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CONFLICTS OF INTEREST
The authors declared no conflicts of interest.

REFERENCES
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