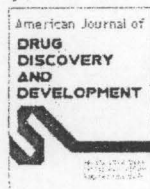




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Cellular Mechanism of the Cytotoxic Effect of Extracts from *Syzygium polyanthum* Leaves

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ABSTRACT

The research objective is to obtain the cellular mechanism(s) of *Syzygium polyanthum* leaves for its development into commercial herbal product for certain degenerative diseases. The approach was to conduct activity-guided fractionation of metabolite extracts based on the antioxidant and the apoptotic modulation activities using cultured cells. Antioxidant activity of the active fractions were screened using 1,1-diphenyl-2-picrylhydrazil (DPPH) free radical assay and thiobarbituric acid (TBA) assay. The cellular effects were investigated using cultures of HB4C5 human hybridoma and mouse colon 26 adenocarcinoma cells. The flavonoids extract which has the lowest IC₅₀ value of 14.84 µg mL⁻¹ for DPPH assay among other metabolites, was found to have significant cytotoxic effect on both HB4C5 and colon 26 cells. Specific assays for apoptosis using caspase 3-gene expression and annexin-V flow cytometry analyses, supported the effect of cell proliferation inhibition which was not due to apoptosis. Subsequent cell cycle analysis indicated that crude extract may induce cell cycle arrest of HB4C5 cells at G1 to S phase, whereas active compound fractions of flavonoids stacked the cell cycle at G2/M phase. These observations suggest that flavonoid extracts of *Syzygium polyanthum* possess active compound(s) with anti-proliferative effect. This is a new finding for therapeutic potential of *Syzygium polyanthum* leaves in Indonesian traditional medicines.

Key words: Antioxidants, anti-proliferative effect, cytotoxic effect, cell cultures, *Syzygium polyanthum*

INTRODUCTION

In Indonesia, various medicinal plants have been used for generations as traditional remedy for variety of degenerative disorders. *Syzygium polyanthum* is evergreen trees which can be found grown wildly in lowland as well as in the highland. The leaves have been used traditionally in many Indonesian dishes and used as a food additive. The leaf infusion of *S. polyanthum* has been used traditionally for cardiovascular problems. This might be due to its antihypertensive activity (Ismail *et al.*, 2013).

Previous study of the *S. polyanthum* leaves reported a high antioxidative activity of the leaves (Lelono *et al.*, 2009) and had no cytotoxicity effect against Vero cell line (Perumal *et al.*, 2012). From the ripened fruit, it was revealed high antimicrobial, antioxidant and cytotoxicity activities (Kusuma *et al.*, 2011). In this study, we determined the cytotoxicity effect of flavonoid extract from

S. polyanthum leaves against the HB4C5 human hybridoma cell line and the colon 26 adenocarcinoma cell line derived from BALB/c mice and obtained its cellular mechanisms. Thus, activity-guided extraction and fractionation is required further to investigate the cellular mechanisms which will strengthened the scientific bases for its development.

The objective of this research is to investigate the antioxidant activity and the cellular mechanism of flavonoid extracts of *S. polyanthum* leaves by which it can exert its therapeutical effect.

MATERIALS AND METHODS

Materials: Plant materials of *S. polyanthum* leaves were obtained from Biopharmaca Research Center Bogor Agricultural University, Bogor, Indonesia. The leaves were collected from ten-year-old trees. Chemicals for plant extraction and phytochemical test were: ethanol, methanol, ethyl acetate, hexane, chloroform, concentrated sulfuric acid, Dragendorf reagent, Meyer reagent, Wagner reagent and Lieberman Burchard reagent. The solvents were of analytical grade and purchased from Merck (Germany). Antioxidant activity of the active fractions were screened using 1,1-diphenyl-2-picrylhydrazil (DPPH) obtained from Wako Chemical (Osaka, Japan). Linoleic acid and other reagents for TBA assay were obtained from Sigma Aldrich (Germany); 1,1,3,3-tetramethoxy-propane (TMP) was purchased from Merck (Germany).

For cytotoxicity studies and apoptotic bioassays, the HB4C5 human hybridoma cell line and the colon 26 adenocarcinoma cell line derived from BALB/c mice were kindly provided by Animal Cell Technology Laboratory of Ehime University (Matsuyama, Japan). The eRDF culture media were purchased from Kyokuto Pharmaceutical Industrial (Tokyo, Japan). The ApopLadder experiment kit was purchased from Takara (Shiga, Japan) for apoptotic modulation activity determination. The human IgM enzyme-linked immunosorbent assay (ELISA) kit was from Circulex (Ina, Japan). The multiwell (96- and 48-wells) microplates were obtained from Nunc. The WST-8 assay kit for cell proliferation assay were purchased from Khisida Chemical Co. (Osaka, Japan).

Preparation of water extracts and qualitative phytochemical analyses: Water extracts and qualitative phytochemical analyses on *S. polyanthum* leaves were prepared using the method reported by Harborne (1998). Briefly, the leaves of *S. polyanthum* was dried in the oven set at 40°C and then pulverized. The water content of the pulverized dry leaves was determined using gravimetry method. These dry leaves were extracted with water (1 g 10 mL⁻¹), macerated at room temperature for 3×24 h. The extracts were concentrated to dryness under reduced pressure using rotary evaporator. The residues were then analyzed qualitatively for its content of alkaloids, saponins, flavonoids, phenolic hydroquinones, triterpenoids/steroids and tannins using methods described by Harborne (1998).

MAIN METABOLITE COMPOUNDS EXTRACTION

Flavonoids and tannins extracts were prepared using the previous method reported by Mabry *et al.* (1970) with slight modification. Hydroquinones was extracted by using Permana *et al.* (2003) method.

Flavonoids extracts: Pulverized dry leaves (100 g) were successively extracted with MeOH/H₂O (1:1) at room temperature for 24 h. Similarly, another 100 g of the leaves powder was extracted with MeOH/H₂O (9:1). Both 50 and 90% methanol-water extracts were mixed and then partitioned with n-hexane and chloroform to yield n-hexane, chloroform and aqueous fractions. The aqueous fraction contained flavonoids.

Tannins extracts: Pulverized dry leaves (each 100 g) were macerated 3 times with methanol for 24 h and then partitioned by n-hexane to obtain methanol and n-hexane fractions. The methanol fraction was evaporated by rotary vacuum evaporator and reconstituted by acetone/H₂O (70:30) with ascorbic acid 1%. The solutions were partitioned with chloroform and then by EtOAc to yield tannin extract.

Hydroquinone extracts: The pulverized samples (each 100 g) were macerated by methanol (three times, 24 h) and then evaporated to yield methanol crude extracts. These extracts were reconstituted by MeOH/H₂O (1:2) and partitioned with EtOAc which gave ethyl acetate fractions. The fractions were evaporated to yield hydroquinone extracts. All extracts were concentrated to dryness under reduced pressure and controlled temperature (40-50°C) and kept in the freezer until further use.

Separation of flavonoid extract and active compound fractionation: To isolate the active compounds, this aqueous fraction was chromatographed on a silica-gel column using (EtOAc: Acetone: Acetic acid: H₂O) as a step gradient solvent and 60 fractions were collected. These fractions were pooled into six fractions (F1 to F6). Guided by the antioxidant activity of each fraction, F1 was then separated by TLC to yield 8 fractions (F1.1 to F1.8). Similarly, based on the antioxidant activity, Fraction F1.5 was selected for further cell culture experiment.

Assay of antioxidant activity

DPPH free radical method: The free radical scavenging activity of each extracts and fractions was determined by DPPH radical method (Salazar-Aranda *et al.*, 2011). Briefly, extracts in ethanol solutions at different concentration were added to 1 mL of the DPPH 1 mM solution in ethanol. The mixture was shaken and allowed to stand in the dark at 37°C for 60 min. The decrease in absorbance of the resulting solution was monitored at 517 nm using a spectrophotometer (Hitachi U2800). Samples were analyzed in triplicate.

Malondialdehyde-thiobarbituric acid determination (MDA-TBA Assay): Lipid peroxidation inhibitory activity of extracts and fractions were carried out using modified method of Kikuzaki and Nakatani (1993). For MDA determination, a standard curve was made from 6 M stock solution of 1,1,3,3-tetramethoxy-propane (TMP). Sample mixtures consisted of 2 mL of phosphate buffer 0.1 M pH 7.2, 2 mL of linoleic acid 50 mM dissolved in ethanol 99.8% and 1 mL of extract at indicated concentration. Control for antioxidant activity was vitamin E in final concentration of 200 ppm. All mixtures except the standards were incubated in the dark using water bath at 40°C for 4 days. The reaction was stopped by adding 2 mL of TCA 20% and 2 mL of TBA 1% (b/v) in acetic acid glacial to each sample and standard. The reaction mixtures was boiled (waterbath 100°C) for 10 min then cooled, centrifuged and the absorbance of supernatant was measured at 532 nm.

CELLS AND CELL CULTURE

HB4C5 human hybridoma cells obtained from American Type Culture Collection (ATCC) were grown in suspension in 100 mm dishes with eRDF growth medium supplemented with 5% Fetal Bovine Serum (FBS). Colon 26 adenocarcinoma cells derived from BALB/c mice provided by the Institute of Development, Aging and Cancer Tohoku University (Sendai, Japan) were maintained in monolayer with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. All cells were incubated at 37°C in humidified atmosphere containing 5% CO₂.

Cytotoxic assay (Sugahara *et al.*, 2006)

Experimental design: HB4C5 cells were inoculated in 96-well culture plates at a cell density of 1.0×10^5 cells mL^{-1} in aliquots of 200 μL well $^{-1}$ serum-free ERDF medium supplemented with ITES (insulin, transferrin, sodium selenite and ethanolamine). The cells were incubated at 37°C for 24 h with or without the *S. polyanthum* extracts either dissolved in ethanol or methanol at indicated concentrations. At the end of cell incubation, the cell's production of human IgM was determined as a measure of cell viability. Aliquots of 50 μL well $^{-1}$ of cell's medium were taken for human IgM production assay using ELISA method.

ELISA method: Prior to the assay described by Sugahara *et al.* (2006), a 96-well ELISA microplate (Nunc™) that has been coated with anti-human-IgM antibody (CAPEL 550732) was washed with 0.05% Tween 20-Phosphate Buffered Saline (T-PBS) 2 times and then blocked with cold 5% skim-milk-PBS by adding 300 μL of it to each well and incubated at 37°C for 2 h or overnight at 4°C. IgM standard solutions were prepared at concentration of 0, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70 ng mL^{-1} in aliquots of 200 μL . ELISA sampling was done by adding 50 μL aliquots of the standards and samples to the well and incubated at 37°C for 1 h. At the end of incubation, plates were anti-human IgM antibody (Biosource International AH10604) that has been diluted 2000× with cold 5% skim-milk was added to each well and followed by incubation at 37°C for 1 h.

Assay of cell proliferation: Cell proliferation was estimated using the WST-8 assay kit (Kishida, Funakoshi Japan) according to the manufacturer's instruction. Briefly, Colon 26 cells were inoculated in 96-well culture microplate at a cell density of 1.0×10^5 cells mL^{-1} in 10% FBS-DMEM. After 3 h-preincubation, cells were incubated with or without extracts at various concentrations for 24 h. Finally, WST-8 reagent (10 μL well $^{-1}$) was added to cell culture system and incubated for 1 h at 37°C. The absorbance at 450 nm was measured by spectrophotometer in microplate reader (SH-8000).

Detection of apoptosis

ApopLadder experiment: Prior to the experiment, HB4C5 cells were cultured in 5% FBS-eRDF medium using 100 mm petri dishes. On the day of the experiment, confluent HB4C5 cells were washed and incubated with 10 mL of serum-free eRDF medium supplemented with ITES at 37°C for 24 h with or without the presence of extracts (50 μg mL^{-1}). The cell DNA were isolated using the ApopLadder detection kit (TAKARA Bio, Shiga, Japan) according to the manufacturer's instructions. Briefly, cells from experimental dishes were collected into 15 mL tube and centrifuged at 1000 rpm 5 min, followed by PBS pellet wash and centrifuged again at 1000 rpm for 5 min. Cell pellets were suspended with 100-200 μL of PBS and transferred into 1.5 mL vials and then centrifuged at room temperature 1600×g for 5 min. To obtain crude cell extracts, cell pellets were added with 100 μL lysis buffer and stirred with vortex and centrifuged again at room temperature 1100×g for 5 min. Supernatants were mixed with TAKARA reagents according to manual instruction to obtain the cell's DNA. The cell DNA samples were kept in the freezer (-80°C) until ready for electrophoresis. Samples of isolated cell DNA were separated by electrophoresis on 1.5% agarose gel for detection of DNA fragmentation. Agarose gel electrophoresis was performed according to the method described by Nishimoto *et al.* (2003).

Annexin-V flow cytometry analysis: The induction of apoptosis was analyzed by the annexin V-PE Detection Kit I according to the manufacturer's instructions. Briefly, after 24 h incubation

with extracts, HB4C5 cells cultured in 24-well plates (2.0×10^5 cell mL^{-1}) were washed twice with ice-cold PBS and resuspended in a binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl_2] at cell density of 1.0×10^5 cells mL^{-1} . The cell suspension (100 μL) was transferred to a 5 mL culture tube to which 5 μL of annexin-PE and 5 μL of 7-AAD were added as fluorescent dyes. After incubation in the dark at 25°C for 15 min, 400 μL of binding buffer was added to the solution. Flow cytometry analysis was performed within 1 hr by a FACSort (FACSCalibur-Becton Dickinson, Sunnyvale CA) equipped with argon ion laser. A minimum of 10,000 cells per sample were analyzed and data analysis was performed with the Cellfit software (Becton Dickinson).

Reverse transcription-polymerase chain reaction (RT-PCR)/caspase 3 gene analysis:

HB4C5 cells and colon 26 were culture in their corresponding FBS-containing medium containing high (20 $\mu\text{g mL}^{-1}$) and low (2 $\mu\text{g mL}^{-1}$) concentration of the *Syzygium* extract/fractions for 6 or 24 h, harvested by centrifugation and washed twice with PBS. Total RNA was isolated by using sepaol-RNA I super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. One microgram of total RNA was used as a template for cDNA synthesis reaction which was performed by using MMLV-reverse transcriptase and an oligo-dT20 primer (Toyobo, Osaka, Japan), The PCR reaction was performed by using Takara Taq DNA polymerase (Takara, Kyoto, Japan) for amplification of human caspase 3- and mouse caspase 3-cDNAs. The resulting cDNA samples was subjected to 30 cycles of PCR amplification performed under the following conditions: denaturation at 98°C for 10 sec, annealing at 60°C for 2 sec and DNA synthesis at 74°C for 30 sec, according to the manufacturer's recommendation. Human GAPDH and mouse β -actin cDNAs were amplified as internal controls. The PCR reaction was carried out using Real Time PCR system (StepONEplus, Applied Biosystem).

CELL CYCLE PHASE ASSAY

HB4C5 cells were seeded 24-well plates with 0.5% FBS-containing eRDF medium at cell density of 1.0×10^6 cells mL^{-1} . Cells were incubated with extracts for 24 h in humidified atmosphere containing 5% CO_2 . At the end of extract incubation, cells were collected and washed twice with assay buffer by centrifugation. The cell pellet were resuspended to a density of 10^6 cells mL^{-1} . The assay was carried out using Cayman's Cell Cycle Phase Determination Kit (Cayman Chemicals, Michigan USA) according to manual's instruction. All samples were analyzed in the FL2 channel of a flow cytometry with a 488 nm extinction laser.

Statistical analysis: Data were analyzed using ANOVA to determine significant differences ($p < 0.05$). Multiple comparisons were done by the Tukey HSD test. All statistical analysis was done with the software SAS from SAS Institute, Inc. (Cary, NC, USA) version 9.1.3 and SPSS from SPSS Inc. (Chicago, IL, USA) version 13.

RESULTS AND DISCUSSION

Phytochemical content and fractionation of potential secondary metabolites:

Standardized sample preparation of *Syzygium* yields pulverized dry leaves with moisture content of 7.9%. Maceration of dry leaves with water gave extracts with 5.9% yield. Qualitative phytochemical analysis reveals that flavonoids, tannins and phenolic hydroquinones are the most dominant secondary metabolites contained in water extracts. Alkaloids, steroid and triterpenoid

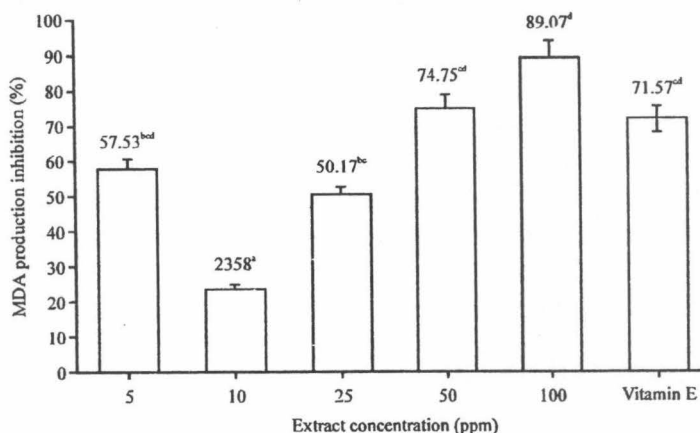


Fig. 1: Inhibition of lipid peroxidation by flavonoid extracts of *Syzygium polyanthum* leaves. Means for homogeneous subsets are displayed ($p < 0.05$)

were also present but in lesser degree. Based on the phytochemical tests, the extraction and fractionation of the flavonoids, tannins and hydroquinones of the leaves were proceeded. The flavonoids extracts of *S. polyanthum* yielded dark brown sticky semi solid residues, weighing 35.9 g (17.4%). Similarly, the tannin and hydroquinone extracts also yielded dark brown sticky semi solid residues, but with less yields: 5.7 g (2.8%) and 4.8 g (4.4%), respectively.

Antioxidant activity of potential secondary metabolites: Upon extraction and fractionation stage, the pulverized dry leaves of *Syzygium* was subjected to antioxidant activity-guided isolation. Evaluation of antioxidative activity with the DPPH method is based on the reduction of DPPH free radical solution to DPPH-H in the presence of a hydrogen-donating antioxidant (Yamaguchi *et al.*, 1998). The antioxidative activity is expressed by Inhibition Concentration index, IC_{50} which is inversely related to antioxidant capacity of a compound, as it expresses the amount of antioxidant needed to decrease the radical concentration by 50%. The lower IC_{50} , the higher the antioxidant activity of a compound is. Using method of Salazar-Aranda *et al.* (2011), the flavonoids extracts of *S. polyanthum* leaves showed the highest antioxidant activity among the 3 selected metabolites as shown by the lowest IC_{50} value of $14.84 \mu\text{g mL}^{-1}$. Nevertheless, the other compounds selected showed relatively very low IC_{50} , thus indicating the presence of very high potency antioxidants (data not shown).

The antioxidant activity of flavonoid extracts was also determined in terms of measurement of % inhibition of lipid peroxidation in linoleic acid system using TBA assay. One of the most important products of lipid peroxidation is malondialdehyde (MDA). This compound reacts with TBA forming a complex MDA-TBA pink fluorescent pigments which can be detected spectrophotometrically at maximum absorbancy of 532-535 nm. The data showed that extracts of *Syzygium* were capable of inhibiting MDA production at various concentrations ($p < 0.05$). The antioxidant potency of the extract at concentration of 50 and 100 ppm also comparable, if not stronger than that of vitamin E at concentration of 200 ppm (Fig. 1). These findings suggest that flavonoids extracts of *Syzygium* leaves were a good source for highly potent antioxidants which is consistent with reports of Wong *et al.* (2006). The antioxidative activities of the barks of *Syzygium* had been reported due to its polyphenol content (Lelono *et al.*, 2009).

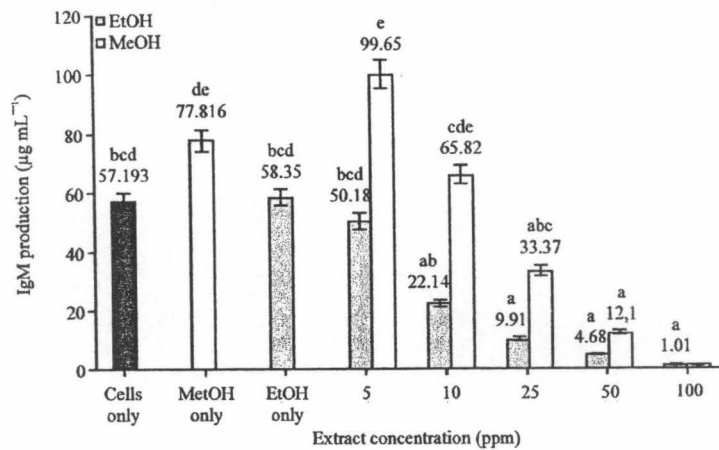


Fig. 2: Effect of flavonoid extracts on HB4C5 hybridoma cells production of human IgM. Means for homogeneous subsets are displayed ($p < 0.05$)

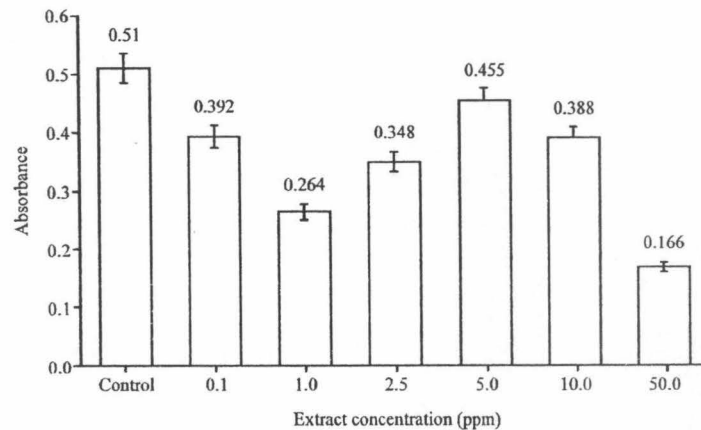


Fig. 3: Effect of flavonoid *Syzygium polyanthum* extracts on colon 26 adenocarcinoma cancer cell's viability

Cytotoxic effect of flavonoids extracts from syzygium leaves

Effect on the IgM production by HB4C5 hybridoma cells: Under normal culture condition, these cultured cells are producing human antibody of IgM (Sugahara *et al.*, 2003). Some bioactive compounds have been reported to modulate the IgM production of these cultured cells (Sugahara *et al.*, 2006). As indicated in Fig. 2, cells incubation with *S. polyanthum* extract dissolved in methanol at concentrations higher than 10 $\mu\text{g mL}^{-1}$ induced apparent reduction in IgM production by HB4C5 cells. This reduction on IgM production usually is attributed to the cytotoxic effect of the bioactive compounds on the cells. These data also reveals the dose-dependent cytotoxic effect of *S. polyanthum*'s extracts on HB4C5 cells. This cytotoxic effects on HB4C5 cells was demonstrated even more strikingly when the flavonoid extracts was added dissolved in ethanol.

Effect on cells viability of colon 26 adenocarcinoma cells: Treatment of cells with flavonoids extract at concentrations of 50 ppm ($\mu\text{g mL}^{-1}$) reduced the amount of water soluble formazan that can be produced through reaction of reagents of WST-8 kit assay with dehydrogenase of colon

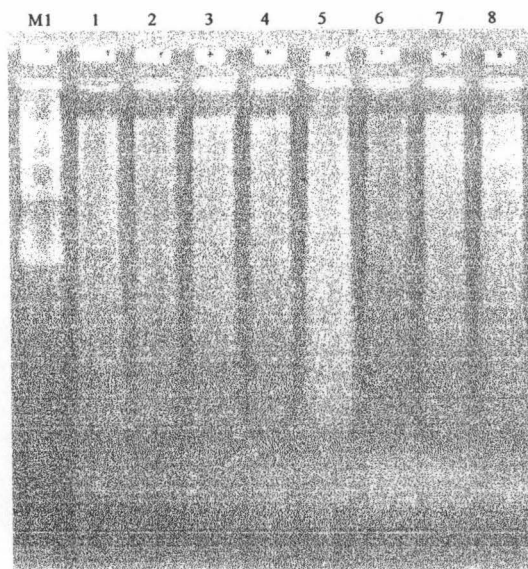


Fig. 4: DNA laddering assay by gel electrophoresis. Lane identification: M1= 1kb DNA marker; lanes 1-8 are HB4C5 cells treated with, *S. polyanthum*/MeOH (1), *Guazuma ulmifolia*/MeOH (2), MeOH alone (3), Cells alone (4, 8), *S. polyanthum*/EtOH (5), *Guazuma ulmifolia*/EtOH (6), EtOH alone (7)

26 cells and measured its absorbance at 450 nm (Fig. 3). Similar observation were found with the F1 and F1.5 fractions of flavonoid extracts (data not shown). The WST-8 assay is a classic approach to assessing metabolic activity which involves the use of tetrazolium salts that are cleaved in the mitochondria of metabolically active cells to form colored, water soluble formazan salt that can be measured by absorbance (Tominaga *et al.*, 1999). The amount of formazan dye produced is directly proportional to the number of metabolically active cells and indicates the reducing potential of the cell. Thus, these observations were consistent with the reduced number of living cells in this experiment and the presence of cytotoxic effect of flavonoid extract of *S. polyanthum* to the cell.

Effect of flavonoids extracts on apoptotic activity: Flavonoids have been reported to induce apoptosis in cancer cells (Ramos, 2007). Apoptosis is associated with the fragmentation of chromosomal DNA into multiples of the 180 bp nucleosomal units, known as DNA laddering. This experiment was carried out to investigate the cellular mechanism by which the flavonoid extracts of *S. polyanthum* exert their cytotoxic effect on HB4C5 human hybridoma cells. Cell death was almost always indicated when there was a reduction of IgM production by HB4C5. The flavonoids extracts of *Guazuma* which did not show significant cytotoxicity against HB4C5 were also included in the experiment as a comparison. Hence, we carried out DNA laddering assay to investigate the nature of cell death on HB4C5 cell caused by the cytotoxic effect of *S. polyanthum* extracts. In DNA laddering assay, small fragments of oligonucleosomal DNA is extracted selectively from the cells, whereas, the higher molecular weight DNA stays associated with the nuclei. The isolated DNA is separated by electrophoresis and visualized using ethidium bromide. Fig. 4 (lanes 1-8) shows the results of the DNA laddering assay on HB4C5 culture cells. No DNA ladders can be seen indicating

there was no apoptosis detected with this assay. In the DNA fragmentation analysis, a DNA smear was detected in cells treated with *Syzygium* extract ($50 \mu\text{g mL}^{-1}$) dissolved in ethanol (Fig. 4, lane 5) which due to random DNA digestion indicating the possibility of necrosis. As a comparison, cells treated with *Guazuma* extracts as well as the solvent controls did not show DNA smears image, indicating that the nucleosomal DNA remain intact in those cells. These observations suggest that the cytotoxic effect exerted by flavonoids extracts of *Syzygium* leaves extract on HB4C5 cells may induced necrosis rather than apoptosis mechanism in the cell. Similar observation has been reported by Cheng *et al.* (2002) on their study of a selective inhibitor for COX-2.

Investigation using more specific methods for apoptotic activity was also carried out using real time-PCR to determine whether transcription of caspase 3 gene, an apoptotic gene marker, is affected. After incubation of cells with crude and fractions of flavonoid ($20 \mu\text{g mL}^{-1}$) for either 6 or 24 h, it was found that caspase 3 gene expression in those cells had not been up-regulated, indicating that the molecular mechanism of the cytotoxic effect may not be related to apoptosis. Experiment using colon 26 cells also gave similar result, that there was lack of effect of flavonoids on the mouse caspase 3 gene transcription (data not shown). Another assay to assess apoptosis activity is by examining the integrity and the asymmetry of the plasma membrane (Schutte *et al.*, 1998). This phenomenon can be detected by hapten labeled annexin V which shows high affinity for phosphatidylserine residues in the presence of millimolar concentrations of Ca^{2+} (Andree *et al.*, 1990). Simultaneous probing of membrane integrity by means of exclusion of the nuclear dye 7-amino actinomycin D (7-AAD) can discriminate apoptotic cells from necrotic cells. The flow cytometry analysis of this kind of experiment using HB4C5 cells indicated that although the cells treated with high concentration ($20 \mu\text{g mL}^{-1}$) of fraction F1 showed higher percentage of dead cells than the untreated cells, no significant amount of apoptotic cells were detected (data not shown).

These data showed that the cytotoxic effect of the flavonoid extract of *S. polyanthum* at $50 \mu\text{g mL}^{-1}$ was affecting the growth of HB4C5 human hybridoma cells as well as the colon 26 adenocarcinoma cells. However, no significant amount of apoptotic activity can be detected using more specific marker for apoptotic activity (caspase 3, annexin V). Another possibility to account for the decrease of cell viability is inhibition of cell proliferation due to disturbance of cell cycle progression. Thus, the effect of extracts on cell cycle distribution in HB4C5 cells was determined by Cayman's cell cycle phase assay. In this assay, when the cells pass through the flow cytometer's laser, a fluorescence pulse is generated that correlates with the amount of dye intercalated in the DNA and thus with the total amount of DNA in the cell. DNA histograms in Fig. 5 showed that the number of cells in G1 phase was increasing compared to control cells (62.0%) but there was a decrease in the number of cells in S phase (control cells 22.7%) and no significant change in G2/M phase of cells treated with flavonoid extract, but increasing in cells treated with flavonoid fraction F1 (control cells 15.3%). Cell cycle analysis revealed that 24 hr treatment with flavonoids extract of *S. polyanthum* at concentrations of $50 \mu\text{g mL}^{-1}$ may inhibit cell cycle progression from G1 phase to S phase, because G1 phase population did not decrease. If the cell cycle were able to proceed from G1 to S phase, G1 population will be decreased. For the effect of the fraction F1, the cell cycle was stacked at G2/M phase. Since only S population was decreased, the same number of population was increased at G2/M population. Li *et al.* (2007) reported that andrographolide, a bioactive compound, induced cell cycle arrest at G2/M phase while studying its cytotoxicity on HepG2 human hepatoma cell lines.

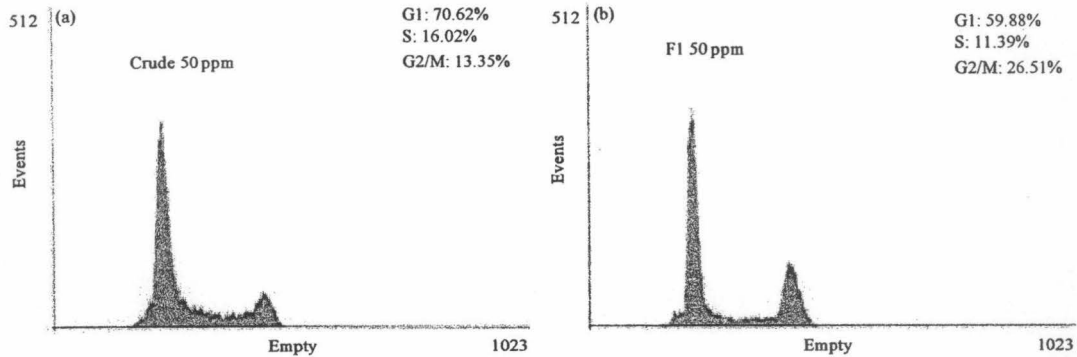


Fig. 5(a-b): Effect of *Syzygium's* (a) Flavonoid extract and (b) F1 fraction on cell cycle distribution

Our findings did not suggest that apoptosis is a major event during the course of growth inhibitory by *S. polyanthum* flavonoid extract/fraction F1 of both HB4C5 and colon 26 cells. Cell cycle arrest may be the cellular mechanism that is responsible for the cytotoxic activity of the flavonoid extract of *S. polyanthum*. Further studies related to protein regulators in cell cycle checkpoints will provide more insight to this mechanism. Nonetheless, in cancer drug development, for example, the goal may not be to cause cell death but simply to knock down the metabolic and proliferative activity of a cell with cell stasis being the desired outcome.

This cellular mechanism provides additional useful information for actions of nutraceuticals, especially natural substances contained in medicinal plant (phytochemicals). As reviewed extensively by Scicchitano *et al.* (2014), various phytochemicals such as carotenoids, flavonoid polyphenolics and non-flavonoid polyphenols, have been reported for their mechanism of actions on several biochemical pathways to prevent dyslipidaemia, a primary risk factor for atherosclerosis. Our findings could provide cellular mechanism related to foam cell formation, the hallmark of early atherosclerotic lesion. The significance of the modulation of apoptosis in the event of atherosclerosis progression and its therapeutic implication has been addressed (Tabas, 2005). Further studies using macrophage cells are needed to investigate the cytotoxic effect on macrophage apoptosis.

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