CYTOTOXIC EFFECTS OF COMMERCIAL WHEATGRASS AND FIBER TOWARDS HUMAN ACUTE PROMYELOCYTIC LEUKEMIA CELLS (HL60)

NOORJAHAN BANU ALITHEEN*, CHUAH LI OON, YEAP SWEE KEONG, TAN KEE CHUAN, HO KET LI AND HO WAN YONG

Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia

ABSTRACT

Cytotoxicity, the possible selective activity upon HL60 as well as the anti-proliferation effect of local health supplement wheatgrass and mixture of fibers were investigated in vitro using various cancerous cell line and normal blood cell culture. The IC_{50} of wheatgrass-treated HL60 (17.5 ± 1.1, 12.5 ± 0.3, and 16 ± 0.5 microgram/ml for 24, 48 and 72 h, respectively) and fibers-treated HL60 (86.0 ± 5.5, 35.0 ± 2.5, and 52.5 ± 4.5 microgram/ml for 24, 48 and 72 h, respectively) showed that both extracts possessed optimum effect after 48 hours of treatment. No significant cytotoxic effect was observed on other type of cells. For trypan blue dye exclusion method, wheatgrass reduced the number of viable cells by 13.5% (±1.5), 47.1% (±3.6), and 64.9% (±2.7) after 24, 48 and 72 h exposure, respectively. Mixture of fibers reduced the number of viable cells by 36.4% (±2.3), 57.1% (±3.1), and 89.0% (±3.4) after 24, 48 and 72 h exposure, respectively, indicated that necrosis is also an alternative to the apoptotic mechanism of cell death. Annexin-V/propidium iodide staining revealed that both extracts induced apoptosis where early apoptosis had been detected concurrently with the reduction of percentage of cell viability. Cell cycle analysis revealed that in HL60, the percentage of apoptosis increased with time (wheatgrass: 16.0% ± 2.4, 45.3% ± 3.4 and 39.6% ± 4.1; mixture of fibers: 14.6% ± 1.8, 45.4% ± 2.3 and 45.9% ± 1.2) after exposure for 24, 48 and 72 h, respectively at the concentration of 100 microgram/ml and showed optimum effect at 48 hours. Thus, these health products can be a potential alternative supplement for leukaemia patients.

Keywords: Cytotoxicity, HL60, fiber, wheatgrass.

INTRODUCTION

Wheat (*Triticum aestivum*) belongs to the Kingdom Plantae, Division Spermatophyta, Class Angiosperma, Sub-class monocotyledone, Order Poales, and family Poacea (Porter, 1959). It is one of the finest cereals among barley, rye and rice. Wheat was a staple food crop for Egyptian as early as 5000 BC (Wilson, 1961). Wheatgrass is the young stage of the common bread wheat plant (*Triticum aestivum*). It is grown from the wheat berries. Indoor, tray-grown wheatgrass is leafy and has a deep green colour and a strong sweet taste. It only takes 5 days to a week to sprout and get ready to harvest (Seibold, 1990).

Wheatgrass juice is a complete food that can be taken orally with no toxic side effects. It contains most of the vitamins and minerals needed for human maintenance. It is also a complete protein with about 30 enzymes and has approximately 70 % crude chlorophyll. One ounce (280ml) of wheatgrass juice is equivalent in vitamins, minerals, and amino acids that are found in 2.5 pounds (1.1 kg) of green leaf vegetables (Wigmore, 1982).

Wheatgrass juice, which is a rich source of chlorophyll, has many therapeutic purposes. This sweet green juice was first applied to human health by Dr. Ann Wigmore, founder of the Hipocrates Health Institute, over 30 years ago (Wigmore, 1985). It was shown that chlorophyll was a powerful cleanser and may start an immediate reaction with toxins and mucus in the stomach, possibly causing nausea (Humsberger and Loeffler, 1975). Chlorophyll also produces an unfavourable environment for bacteria growth and thus arrest growth and development of unfriendly bacteria (Wigmore, 1982). It is the active factor in wheat sprout extract that inhibits the metabolic activity of carcinogens. A twenty-year study of 2000 telephone company workers had found that a natural ingredient in carrots and leafy vegetables significantly reduced the risk of lung cancer in cigarette smokers. Cruskin (1940) stated that chlorophyll neutralized strep infection, healed wounds, hastened skin grafting, cured chronic sinusitis and etc. Chlorophyll rich diet (wheatgrass) also increased the survival of experimental animals undergoing lethal doses of radiation (Wigmore, 1985).

Fiber is a food material not digestible by the human small intestine and is only partially digestible by the large intestine. Whole grains, green, leafy vegetables, nuts, fruits and unrefined foods such as bran and sprouted seeds are all good sources. Fiber is also known as roughage.

**Corresponding author*: e-mail: noorjahan@biotech.upm.edu.my

Fiber is beneficial in diet because it relieves and prevents constipation, appears to reduce the risk of colon cancer, and reduces plasma cholesterol levels and therefore the risk of heart disease. Fiber also slows gastric emptying and contributes to satiety.

One working hypothesis recently proposed was a complementary approach in which multiple antioxidant supplements together with a low fat, high fiber diet and lifestyle modifications, including physical exercise, might markedly improve the efficacy of standard and experimental cancer therapies (Norman *et al*., 2003). Estrogen, insulin, and insulin-like growth factor (IGF)-1 had been identified as independent risk factors for the development of breast cancer. A study carried out by Barnard *et al*. (2004) showed a low-fat, high-fiber diet and exercise intervention resulted in major reductions in recognized risk factors for breast cancer.

In addition, Glei *et al*. (2004) carried out a study on high intake of bread which is associated with lower fecal water (FW) genotoxicity and decreased DNA damage in lymphocytes of humans. Higher bread consumption which caused higher fiber intake (22g/d versus 37g/d) resulted in markedly reduced FW genotoxicity in both smokers and non-smokers after intervention with bread. By using various tissue targets of the body, their data showed that a high consumption of bread and fiber was associated with a decrease of cancer risk factors.

With the value of this two food material, lot of local health food companies have come out with daily supplement of these food materials. However, the effectiveness and the safety of this product especially for cancer patients were still unclear. Thus, the study was carried out to check on the cytotoxicity and the safety of the methanol extract from these local health supplements.

MATERIALS AND METHODS

Cells

The cancerous and non-cancerous cell lines (suspension and anchorage-dependent cells) were obtained from the American Type Culture Collection (ATCC), the National Cancer Institute (NCI) and the RIKEN Cell Bank (RCB). The cancerous cell lines used were HL-60 (Human acute promyelocytic leukemia), anchorage-dependent MCF-7 (human breast adenocarcinoma) cell line and suspension K562 (human chronic myeloid leukemia) cell line. The non-malignant human peripheral blood mononuclear cells (PBMC) was used as normal cell control. PBMC was prepared by using the gradient centrifugation where venous blood was collected aseptically from healthy donors in preservative free heparin tubes. The blood was diluted with phosphate buffered saline (PBS), pH 7.4 and layered onto Ficoll plus (Amersham). After centrifugation at 400 x g for 50 min, the lymphocytes were collected at the interface and washed three times with PBS. The cells were resuspended in DMEM supplemented with 10% foetal bovine serum and antibiotics. PBMC was used as a normal cell control as this study focused on evaluating the effects of natural pure compounds on several leukemia cell lines.

Extracts

The supplement of 2 g of organic wheatgrass and mixture of fiber in powder form were kindly supplied by Mr Lim Phoey Keat (salesperson of one of the local supplement company) and were soaked in 250 mL of methanol (J.T. Baker, USA) for 72 h. The extracts were filtered with Whatman filter paper no. 1 and evaporated to dry under reduced pressure by using Aspirator A-3S (EYELA, Japan) at 40 °C. The process was repeated three times (yield 27.3%, w/w). Briefly, the concentrated extracts were dissolved in dimethylsulphoxide (DMSO) (Sigma, USA) to obtain a stock solution of 10 mg/mL. The substock solution of 0.2 mg/mL was prepared by diluting 20 µL of the stock solution into 980 µL serum-free culture medium, RPMI 1640 (the percentage of DMSO in the experiment should not exceed 0.5). The stock and substock solutions were both stored at 4 °C.

MTT (Tetrazolium) colorimetric cytotoxicity assay

Varying concentrations of 1.5625, 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL of methanol extracted wheatgrass and fibers were prepared from the substock solutions by serial dilution in RPMI-1640 to a volume of 100 µL in each microtitre plate well as described by Shier (1991). Each well was then added with 100 µL of 5×10^5 cells/mL cells in complete growth media (RPMI 1640). Untreated control that contained only the cells were also prepared for each sample. The assay for each concentration of extracts was performed in triplicate. The plate was then incubated at 37^0C, 5% CO_2, 90% humidity for 72 h. Cytotoxicity of the tested extracts were determined by the non-radioactive, colorimetric assay system using tetrazolium salt, MTT (Sigma, USA) as reported by Mosmann (1983). MTT was dissolved in phosphate buffer saline (PBS) (pH 7.5) at 5 mg/mL. The MTT stock solution was added directly to all appropriate microtitre-plate wells (20 µL for each well). The plate was then incubated for 2 to 4 h at 37°C and 5% CO_2. After incubation, MTT were reduced to insoluble purple formazan crystals by metabolically active cells in the wells. Subsequently, the supernatant was aspirated and 100 µL of dimethylsulfoxide (Sigma, USA) was added and mix thoroughly to dissolve the dark blue formazan crystals. The optical density (OD) was measured on an automated spectrophotometric EL 340 multiplate/ microelisa reader (Bio-Tek instruments Inc) using test and reference wavelength of 570 nm. The cytotoxic dose that killed cells by 50% (IC_{50}) was determined from an absorbance versus concentration curve.

Cell viability trypan blue dye exclusion assay

Trypan blue dye exclusion assay is a simple way to evaluate cell membrane integrity (and thus assume cell death). Cell viability was investigated by means of trypan blue exclusion. Cells were plated in 48-well plates in 0.5 ml of medium, and were treated with extracts and drug. Trypan blue was added to same volume of cells and the number of blue (dead) cells and transparent (live) cells were counted using a haemocytometer. The cell viability percentage was calculated using the following formula:

Percentage of cell viability = (viable cell count / total cell number) x 100%

Annexin-V/Propidium Iodide (PI) dual staining assay

One of the hallmarks of apoptotic cells is the externalization of phosphatidylserine (PS). In principle, Annexin-V detects cell surface phosphatidylserine while PI stains cellular DNA of cells with compromised cell membranes. This allows the discrimination of viable cells (unstained with either fluorochrome) from early apoptotic cells (stained only with FITC-conjugated Annexin-V) and late apoptotic and necrotic cells (stained with both FITC-conjugated Annexin-V and PI). In this study, mode of cell death was examined using the BD Biosciences Annexin V-FITC Apoptosis Detection Kit. The extracts were tested at the concentration that possessed the best cytotoxic effect (100 microgram/ml) and incubated for respective time (24, 48 and 72 h). Then, the samples were transferred to different 15ml centrifuge tubes, pelleted down and resuspended in binding buffer. Cells were then stained with with Annexin V-FITC and PI for 15 min in dark and diluted with binding buffer to a final volume of 500 µl before flow cytometry analysis. A total of 10,000 events per sample were acquired. Data acquisition and analysis were performed on FACS-Calibur flow cytometer (BD Biosciences, USA). Results are expressed as the mean±SEM of at least three separate experiments.

Cell cycle flow cytometry PI analysis

To support the results for cytotoxic effect on human acute promyelocytic cells (HL60) in Annexin-V/PI dual staining, flow cytometer was used. The human acute promyelocytic leukemia cells (1ml) was treated with same volume of extract (1ml). The extracts were tested at the concentration that possessed the best cytotoxic effect. The HL60 was than incubated for respective time (24 hours, 48 hours, and 72 hours). Then, the samples were transferred to different 15ml centrifuge tubes. The cells were pelleted and fixed with 80% cold ethanol and incubated at $4^{o}C$ for 2 h. Then, the cell were pelleted again and washed with PBS buffer supplemented with 2% BSA, 0.1% azide and 2mg/ml EDTA (PBS-BSA-Az-EDTA) buffer for twice. The cell pellet was finally dissolved and stained in PBS buffer consisted of 0.1% triton X-100, 10mM EDTA, 50 microgram/ml RNase and 2 microgram/ml PI. This process was done in dark because PI is sensitive to light. The cells were then incubated for 30 min at $4^{o}C$ and then read with COULTER EPICS ALTRA flow cytometer (Beckman Coulter, USA) at Laboratory of Biologic at UPM Faculty of Veterinary Medicine under 610BP within 24 hours.

RESULTS

MTT cytotoxicity assay of methanol extract of wheatgrass and fibers towards human cancerous cells lines and normal cells

Basically, MTT assay is based on the ability of viable cells with active mitochondrial to produce succinate-dehydrogenate enzyme which cleave the tetrazolium rings of MTT (Mosmann, 1983) where the optical density (OD) obtained was proportional to the number of healthy viable cells. In this study, HL60, MCF-7, K562 and PBMC were treated with organic wheatgrass and mixture of fiber at different concentrations for 24, 48, and 72 hours. Based on the graph plotted, IC_{50} value was determined.

In order to establish the concentration of wheatgrass and mixture of fibers necessary to produce inhibitory effects on cell proliferation, the cells were incubated with the extracts at concentrations varying from 1.5625 to 100 microgram/ml. Based on data collected (table 1) from experiments in four replicates; the IC_{50} of wheatgrass-treated HL60 could be estimated as 17.5 (±1.1), 12.5 (±0.3), and 16 (±0.5) microgram/ml for 24, 48 and 72 h, respectively. Even though the IC_{50} value was lower at 48 h, however in overall the cell viability decreased with the increase of wheatgrass concentration with optimum effect at 72 h at 100 microgram/ml. Besides, wheatgrass showed anti-proliferation effect on HL60 in time- and concentration-dependent manner. The effect was more significant at high concentrations (100 and 50 microgram/ml) at which the readings of absorbance after 48 hours remain constant (almost no proliferation) (result not showed). For MCF7, no IC_{50} value obtained for 24 hours period indicating that organic wheatgrass extract only exhibits cytotoxic effects at longer incubation time (48 and above). This observation was supported by a study that shows wheatgrass possesses good antioxidant activity (Kulkarni *et al*., 2006) which could modify the redox environment of cancer cells and their behavior (Schafer & Buettner, 2001), hence, inhibiting the development of cancer cell. Besides, percentage of cell viability of MCF-7 treated with wheatgrass extract of concentration less than 80 µg/ml incubated for 48 hours was lower than 72 hours treatment time, indicating that cytotoxic effects of organic wheatgrass is dose-dependent. Although cytotoxic effect was observed in both HL60 and MCF7 after treating with wheatgrass, it did not affected K562. For normal cell control, there was no cytotoxic effect being detected on PBMC treated with wheatgrass extract.

Based on data collected from four replicates, the IC_{50} of mixture of fibers-treated HL60 could be estimated as 86.0 (±5.5), 35.0 (±2.5), and 52.5 (±4.5) microgram/ml for 24, 48 and 72 h, respectively. In overall, the cell viability decreased with the increase of concentration of mixture of fibers with optimum cytotoxic effect at 72 h at 100 microgram/ml. On the other hand, the mixture of fibers showed anti-proliferation effect as lower absorbance value were obtained with longer incubation time and higher concentration (result not showed). However, the mixture of the fibers only showed cytotoxic effect on HL60 but not on other type of human cancerous cell lines. For normal cell control, wheatgrass extract showed no cytotoxicity on PBMC without any IC_{50} was obtained.

Trypan blue dye exclusion assay

Cultures were scored for nonviable cells at several time points by trypan blue dye exclusion to determine whether necrosis was a quantitatively important form of cell death. A reduction of cell viability percentages was observed after 24, 48 and 72 h of incubation in all the wheatgrass and mixture of fibers-exposed cells (fig. 1). These lowering were always statistically significant ($P<0.05$) towards the corresponding values obtained with untreated cell. After 24 h of incubation, the trypan blue dye exclusion test showed slight membrane damage in HL60 exposed to both wheatgrass and mixture of fibers; these

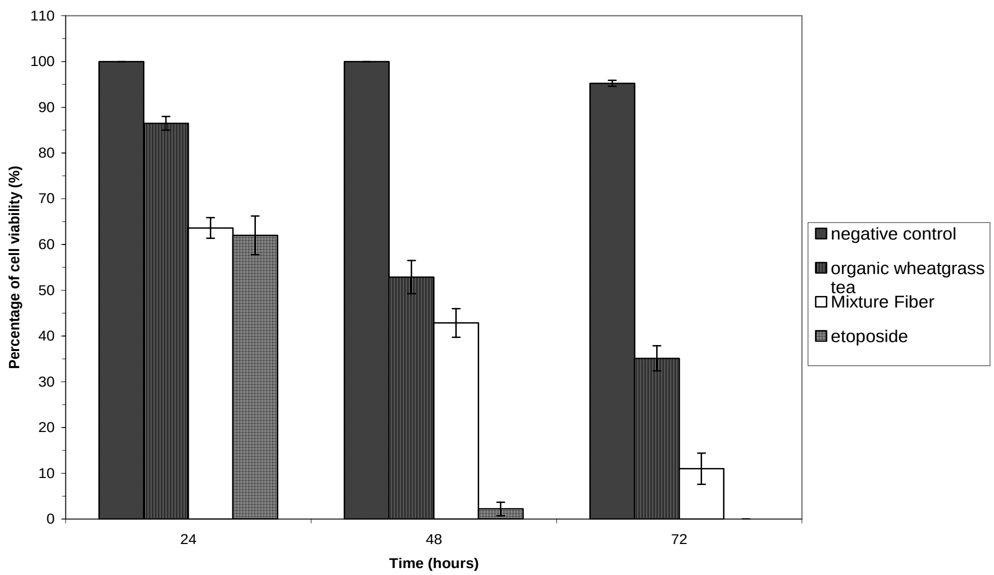

Fig. 1: Percentage of cell viability of HL60 with different treatments (untreated, wheatgrass 100microgram/ml, mixture of fibers 100microgram/ml and etoposide 30 microgram/ml). Data represent means ± SD of triplicate determinations from four independent experiments. The differences between the control group and treated group were determined by one-way ANOVA (*P≤0.05).

Table 1: Effects of methanol extract of wheatgrass and fibers towards several human cancerous cell lines and normal cell at their respective IC_{50}. Data represent means ± SD of triplicate determinations from four independent experiments.

Compounds / Cell lines	IC_{50} value (µg/mL)			
	HL-60	K562	MCF-7	PBMC
Wheatgrass				
24h	17.5 ± 1.1	>100	>100	>100
48h	12.5 ± 0.3	>100	38 ± 2.2	>100
72h	16 ± 0.5	>100	72 ± 6	>100
Fibers				
24h	86 ± 5	>100	>100	>100
48h	35 ± 2	>100	>100	>100
72h	52.5 ± 4	>100	>100	>100

Table 2: HL 60 Annexin V-FITC/PI apoptosis study after treated with different treatments (wheatgrass, mixture of fibers and etoposide) for 24, 48 and 72 hours by flow cytometer. Each value represents the means ± S.E.M. for three assays in triplicate each. The differences between the control group and treated group were determined by one-way ANOVA (*P≤0.05).

Compounds / Time	Annexin V-FITC /PI apoptosis assay		
	Early Apoptosis	Late Apoptosis/Necrosis	Viable
Untreated control			
24h	2.6 ± 0.5	0.8 ± 0.1	96.3 ± 0.3
48h	1.8 ± 0.4	1.0 ± 0.1	97.2 ± 0.4
72h	5.0 ± 2.9	60.5 ± 2.4	95.8 ± 0.6
Wheatgrass			
24h	37.4 ± 2.7*	24.6 ± 1.0*	38.1 ± 3.2*
48h	22.3 ± 0.1*	51.1 ± 0.7*	26.7 ± 0.4*
72h	6.0 ± 0.1	78.6 ± 0.4*	15.4 ± 0.3*
Fibers			
24h	13.0 ± 0.4*	4.8 ± 0.4	81.7 ± 0.4
48h	23.5 ± 0.5*	38.0 ± 2.2*	38.2 ± 0.9*
72h	10.4 ± 3.5*	81.7 ± 0.7*	10.5 ± 0.3*
Etoposide			
24h	0.9 ± 0.1*	94.8 ± 0.2*	4.0 ± 0.2*
48h	0.4 ± 0.1*	98.3 ± 0.2*	1.2 ± 0.1*
72h	0.5 ± 0.1*	98.0 ± 0.2*	1.5 ± 0.1*

results were in agreement with those previously obtained in MTT assay. For trypan blue dye exclusion assay of HL60 incubated at a concentration of 100 microgram/ml, wheatgrass reduced the number of viable cells by 13.5% (±1.5), 47.1% (±3.6), and 64.9% (±2.7) after 24, 48 and 72 h exposure, respectively. Light microscopy showed that the concentrations at 100 microgram/ml caused cytolytic effect (necrosis) after 24, 48 and 72 h with the integrity of cytoplasmic membrane of the wheatgrass-treated HL60 cells damaged and stained with trypan blue.

Similarly, process of lysis or necrosis in the mixture of fibers-treated HL60 was observed, but at a higher rate at the same concentration (100 microgram/ml). For trypan blue dye exclusion assay of HL60 incubated at a concentration of 100 microgram/ml, mixture of fibers reduced the number of viable cells by 36.4% (±2.3), 57.1% (±3.1), and 89.0% (±3.4) after 24, 48 and 72 h exposure, respectively.

In fact, the percentage of necrotic HL-60 leukemia cells increased from 0 % in control to 38.0% (±4.2) after 24 h of 30 microgram/ml etoposide exposure, exhibiting highest cytotoxic effect, compared to the other two extracts. After 48 h, the tested concentration of etoposide had already shown an acute cytotoxic effect manifested by the 97.8% (±1.5) of cells took up the dye, and the decrease in cell viability did not change significantly thereafter. The number of nonviable cells measured by the uptake of Trypan blue reached 100% (±0.0) after 72 h of incubation with the tested concentration of etoposide.

Cell death occurred naturally in control cells after 72 h of culture, but was never higher than 5 % of the total cell number. In contrast, significant levels of cell death occurred in HL60 after incubation with wheatgrass, mixture of fibers and etoposide, leading to a time-dependent decrease in cell viability. HL60 displayed a maximum necrotic response to 100 microgram/ml of the two extracts and 30 microgram/ml of etoposide at 72 h, where 67.9% (±2.7), 89.0 % (±3.4) and 100.0% (±0.0) of the cells were dying by necrosis induced by wheatgrass, mixture of fibers and etoposide, respectively.

Annexin-V/Propidium Iodide (PI) dual staining assay

In order to determine whether this cytotoxic effect was due to apoptosis, Annexin-V/PI staining was carried out. The mean values of the early apoptotic populations in organic wheatgrass-treated HL60 cells (Annexin-V^+/PI^-) were 37.4% ± 2.7, 22.3% ± 0.1 and 6.0% ± 0.1 after incubation of 24, 48 and 72 h, respectively (table 2). Concomitantly, percentage of late apoptotic and necrotic populations increased in time-dependent manner (23.0% ± 1.0, 49.0% ± 0.7 and 78.1% ± 0.4). For mixture of fibers-treated cells, the early apoptotic populations were 13.0% ± 0.4, 23.5% ± 1.3 and 7.1 % ± 1.3 after incubation of 24, 48 and 72 h, respectively (table 2). The percentage of late apoptotic and necrotic cells increased in time-dependent manner also (4.8% ± 0.4, 37.6% ± 2.2 and 82.0% ± 0.7, respectively). More significant cytotoxic effect was observed at etoposide-treated HL60 where

Table 3: HL60 cell cycle distribution (in percentage) after treated with different treatments (wheatgrass, mixture of fibers and etoposide) for 24, 48 and 72 hours by flow cytometer. Each value represents the means ± S.E.M. for three assays in triplicate each. The differences between the control group and treated group were determined by one-way ANOVA (*P≤0.05).

Compounds / Time	Cell cycle			
	Apoptosis	G_0/G_1	Synthesis	G_1/Mitosis
Untreated control				
24h	7.74 ± 2.4	48.12 ± 1.1	35.62 ± 3.1	8.52 ± 1.3
48h	6.98 ± 3.1	57.99 ± 2.7	25.24 ± 3.8	9.79 ± 2.6
72h	4.97 ± 2.9	60.49 ± 2.4	11.13 ± 3.4	23.41 ± 3.2
Wheatgrass				
24h	15.95 ± 2.4*	49.67 ± 3.9	11.11 ± 2.5*	23.27 ± 2.4*
48h	45.3 ± 3.4*	36.9 ± 3.1*	8.34 ± 2.8*	9.46 ± 1.7
72h	39.6 ± 4.1*	34.77 ± 2.8*	10.98 ± 2.4	14.65 ± 1.9*
Fibers				
24h	14.55 ± 1.8*	57.24 ± 4.1*	9.37 ± 1.7*	18.84 ± 2.9*
48h	45.43 ± 2.3*	35.36 ± 3.8*	6.4 ± 1.2*	12.81 ± 1.8
72h	45.9 ± 1.2*	39.26 ± 3.4*	11.69 ± 1.3	3.15 ± 1.3*
Etoposide				
24h	10.39 ± 3.5*	81.26 ± 4.8*	5.93 ± 1.5*	2.42 ± 1.2*
48h	24.88 ± 2.9*	70.11 ± 4.2*	3.21 ± 0.9*	1.8 ± 1.4*
72h	55.86 ± 1.7*	39.95 ± 2.6*	2.69 ± 1.1 *	1.5 ± 1.9*

percentage of late apoptotic and necrotic cells were 94.8% ± 0.2, 97.7% ± 0.2 and 97.4% ± 0.2, respectively.

Effects of wheatgrass and mixture of fibers on HL60 by cell cycle analysis

Flow cytometry is a rapid method to study the cell cycle and DNA content of thousands of individual cells by measurement of light scattering and fluorescence (Shapiro, 2003). This method is based on using specific DNA staining dye such as propidium iodide (PI), which was applied in this study to monitor physiological status of the cells. Thus, flow cytometry is a well established method in cancer drug evaluation and prognosis of tumour (Vanparys *et al*., 2006).

In the first part of this study, method routinely used in the in vitro cytotoxicity testing has been applied: the MTT test. The result was further verified by the flow cytometry assay and the trypan blue dye exclusion test. Cell cycle analysis of drug-treated cells, using flow cytometry, revealed the presence of a distinct cell cycle region below the G_0/G_1 region. This "sub-G_1" peak, displaying particles with lower DNA content, represents apoptotic bodies with their characteristic reduced volume and nuclear condensation (Dimas *et al*., 1999).

As shown in table 3, the percentage of apoptotic bodies in HL60 increased with the time of incubation with both the wheatgrass and mixture of fibers. Cell cycle analysis revealed that in HL60, the percentage of apoptosis increased from 16.0% (±2.4), 45.3% (±3.4) and 39.6% (±4.1) after exposure to 100 microgram/ml wheatgrass for 24, 48 and 72 h, respectively. While the percentage of apoptosis after exposure to 100 microgram/ml mixture of fibers for 24, 48 and 72 h increased from 15.6% (±1.8), 45.4% (±2.3) and 45.9% (±1.2), respectively. More significant increase of apoptosis was observed in cells treated with etoposide with an increase from 10.4% (±3.5), 24.9% (±2.9), and 55.9% (±1.7).

DISCUSSION

There is a growing interest in identifying plant-based anti-cancer drugs ever since their possible use in modern medicine was suggested (Sharma *et al*., 1994 and Lee *et al*., 1995). In this paper, the in vitro cytotoxic and anti-proliferation activities of the two extracts–the wheatgrass and the mixture of fibers, were tested. It should be noted that MTT assay can only detect formation of tetrazolium salt (presumably due to mitochondrion activity) but cannot differentiate the modes of death (Mosmann, 1983). Although the morphology of the dying cells closely resembles the classical changes attributed to an apoptotic mode of death, but there are no literature review that clearly stated the mode of death of the cells. For this reason, the mode of cell death was determined by examining well-characterized apoptosis markers by flow cytometry and by trypan blue dye exclusion. Annexin-V/PI dual staining detected phosphatidyl serine externalization, single PI staining with Triton-X detected the DNA contents of the cells while trypan blue dye exclusion examined the membrane integrity.

The two extracts used showed a time- and dose-dependent cytotoxic effect on HL60 cells. Dose-dependent regulation of cellular processes is one of the important features of signaling molecules naturally occurring in cells. A similar phenomenon is observed in many extracts tested as potential anticancer drugs (Czyz *et al*., 2005). Depending on the concentration used, many different cellular processes may be influenced (Bezerra *et al*., 2007). In an attempt to determine the pathway responsible for cell death for anti-proliferative effects of the extract tested, induction of apoptosis or necrosis by the two extracts using the concentration of 100 microgram/ml were assessed, with 30 microgram/ml of etoposide as positive control. High-dose and time-related responses suggested a selectivity by wheatgrass and mixture of fibers against viability of HL60. In contrast, treatment of PBMC did not exert cytotoxicity, indicating cytotoxic effect of the two extracts were selective for malignant cells especially HL60, but spared the normal cells.

The findings outlined above had demonstrated that both the wheatgrass and mixture of fibers possessed a potent cytotoxic and anti-proliferation action at high concentration (100 microgram/ml). Higher doses of the extracts were found to exhibit pronounce cytotoxic and anti-proliferation effect as assessed by the MTT reading. However, cytotoxic indexes obtained using the MTT assay were super imposable to those obtained with the trypan blue dye exclusion assay and Annexin-V/PI staining at which the percentage of viable cells decreased dramatically in time-dependent manner for both extracts at 100 microgram/ml. Based on MTT result, viability percentage of HL60 treated with mixture of fibers was higher, compared to wheatgrass. However, the trypan blue dye exclusion test showed lower percentage of viability when treated with mixture of fibers. The MTT reading in fibers-treated HL60 might be caused by the spared mitochondria that should actually be dysfunction after the treatment (Scott *et al*., 2003).

In the present study, direct growth inhibitory effect of the wheatgrass and mixture of fibers at different time point (24, 48 and 72 h) were studied. Growth inhibition was seen in short duration meaning cytotoxic effect including necrosis and apoptosis play an important role (Dartsch *et al*., 2002). This finding associated with Annexin-V binding, increased DNA fragmentation and loss of mitochondrial transmembrane potential and plasma membrane integrity greatly suggested that in HL60, the two extracts caused both apoptosis and necrosis. The cell cycle analysis revealed only 16.0% ± 2.4 in apoptosis peak, after 24 hours of incubation. At the same time, however, the trypan blue test revealed that 36.4% (±2.3) of the cells treated with mixture of fibers were unable to exclude the dye. When genome was intact at a time when the plasma membrane integrity had already sustained serious damage, it indicates as death through necrosis (Dartsch *et al*., 2002). Thus, necrosis was the main form of cell death occurring in fiber-treated HL60 with only 13.0% (±0.4) early apoptotic cells being detected after 24 h. In fact, after a short exposure (24, 48 and 72 h), similar cytotoxic effects were obtained for HL60 treated with both wheatgrass and mixture of fibers, showing that both extracts possessed similar efficiency in induction of apoptosis. However, apoptosis was the preferred mode of cell death in wheatgrass-treated HL60 with 37.4% (±2.7) of early apoptotic populations being detected at 24 h.

At 72 hours, the percentages of cells undergoing apoptosis were lower than non-viable cells obtained from trypan blue staining. Besides, percentages of apoptosis in cells treated with both extracts at 72 hours decreased, compared to the percentages obtained at 48 hours. The decrease might be due to the cells losing their DNA repairing ability at late apoptosis and underwent necrosis, and was being washed away during the washing steps before the PI staining.. Consequently, in this system, the distinction between programmed and necrotic cell death becomes impossible after a certain length of incubation, because even the cells undergoing programmed cell death will eventually lose their membrane integrity (Silva *et al*., 1996). Therefore, it is mandatory to determine the shortest possible incubation period that is sufficient to elicit a cytotoxic effect (Dartsch, 2002).

Despite that some authors consider the distinction between apoptosis and necrosis difficult and controversial, the morphological definition of apoptosis and necrosis is based on membrane integrity (Masquelier *et al*., 2004). According to Dartsch *et al* (2002), there are two crucial points for the distinction between apoptosis and necrosis: the former is characterized by the occurrence of DNA and nuclear fragmentation with concomitant maintenance of membrane integrity, and the latter involves the externalization of phosphatidyl serine with loss of membrane integrity. Thus, more accurate methods such as Annexin-V staining can be applied at which the necrotic cells will take up the Annexin-V with concomitance of PI uptake while apoptotic cells will uptake Annexin-V with no concomitance uptake of PI (Hawley and Hawley, 2004).

Wheatgrass showed more promising anti-proliferative and apoptotic activity against HL60 cells, compared to mixture of fibers. Besides, the inhibition of proliferation of HL60 was roughly constant over a wide concentration range (100-25 microgram/ml) for wheatgrass. Since extracts which regulate apoptosis and overcome apoptosis deficiency of cancer cells are of high medical significance (Sanhueza et. al., 2006), further chemical and biochemical studies are currently under way.

This current study has effectively demonstrated that these food supplements have a good anti-leukemia potential with less or no toxic effects towards healthy immune system. Wheatgrass and fiber methanol extract have successfully exhibited to be cytotoxic towards HL60 cell lines without causing toxicity towards normal human PBMC. Further assay on the extracts' pharmacokinetics and toxicology study should be carried out to support this in vitro assay before *in vivo* or clinical test is being carried out.

REFERENCES

Barnard RJ, Liva M and Tung H (2004). Effect of a low-fat, high-fiber diet and exercise intervention on breast cancer risk factors and tumor cell growth. *Journal of Nutrition*, **134**: 3521-3547.

Bezerra DP, Milit GCG, Oliveira de Castro F, Pessoa C, Odorico de Moraes M, Silveira ER, Lima MAS, Elmiro FJM and Costa-Lotufo LV (2007). Piplartine induces inhibition of leukemia cell proliferation triggering both apoptosis and necrosis pathways, *Toxicology in Vitro,* **21**: 1-8.

Cruskin S (1940). Chlorophyll – its therapeutic place in acute suppurative disease. *American Journal of Surgery,* **49**: 49-55.

Czyz M, Szulawska A, Bednarek AK, Duchler M (2005). Effects of anthracycline derivatives on human leukemia K562 cell growth and differentiation. *Biochemical Pharmacology*, **70**: 1431-1442.

Dartsch DC, Schaefer A, Boldt S, Kolch W and Marquardt H (2002). Comparison of anthracycline-induced death of human leukemia cells: programmed cell death versus necrosis. *Apoptosis*, **7**: 537-548.

Dimas K, Demetzos C, Vaos B, Marselos M and Kokkinopoulos D (1999). Cytotoxic and anti-proliferative effects of heptaacetyltiliroside on human leukemic cell lines. *Leukemia Research*, **23**: 1021-1033.

Glei NHM, Osswald K, Persin C, Jahreis G and Pool-Zobel BL (2004). High intake of bread is associated with lower fecal water genotoxicity and decreased DNA damage in lymphocytes of humans – results of a multi-tissue biomarker approach. *Journal of Nutrition*, **134**: 3521S-3547S.

Hawley TS and Hawley RS (2004). Flow cytometry: An introduction. Methods in molecular biology: Flow cytometry protocols. volume 263. 2nd edition. Humana Press Inc., Totowa, New Jersey, pp.1-33.

Humberger EM and Loeffler C (1975). How I conquer cancer naturally. San Diego Production House, p.320.

Kulkarni SD, Acharya R, Rajurkar NS, Reddy AVR (2006). Evaluation of bioaccessibility of some essential elements from wheatgrass (*Triticum aestivum* L.) by *in vitro* digestion method. *Food Chem*, **103**: 681-688.

Lai C, Butler M and Matney T (1980). Anti-mutagenic activities of common vegetables and chlorophyll content. *Journal of Mutation Research*, **77**: 245-250.

Lee GI, Young HJ, Rakmin J, Nakagawa H, Tsurufuji S and Chang IM (1995). Inhibitory effects of oriental herbal medicines on IL-8 induction in lipopoly-saccharide-activated rat macrophages. *Planta Med*, **61**: 26-30.

Masquelier M, Zhou QF, Gruber A and Vitols S (2004). Relationship between daunorubicin concentration and apoptosis induction in leukemic cells. *Biochemical Pharmacology*, **67**: 1047-1056.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunology Methods*, **65**: 55-63.

Norman HA, Butrum RR, Feldman E, Heber D, Nixon D, Picciano MF, Rivlin R, Simopoulos A, Wargovich MJ, Weisburger EK and Zeise SH (2003). The Role of Dietary Supplements during Cancer Therapy, *Journal of Nutrition*, **133**: 3794-3799.

Porter CL (1959). Taxonomy of flowering plants. W.H. Freeman Company, San Francisco and London, pp.45.

Sanhueza CA, Mayato C, Garcia-Chicano M, Diaz-Penate R, Dortaa RL and Vazqueza JT (2006). Anti-proliferation and apoptosis induced by C-glycosides in human leukemia cancer cells, *Bioorganic and Medicinal Chemistry Letters*, **16**: 4223-4227.

Schafer FQ and Buettner GR (2001). Redox environment of the cell as viewed bioaccessibility of some essential elements from wheatgrass (*Triticum aestivum* L.) by *in vitro* digestion method. *Food Chem*, **103**: 681-688.

Scott S V, Cassidy-Stone A, Meeusen SL and Nunnari J (2003). Staying in aerobic shape: how the structural integrity of mitochondria and mitochondrial DNA is maintained. *Cell Biology*, **15**: 482-488.

Seibold, R C (1990). Cereal grain: What's it for you! *Wilderness Community Education Foundation Lawrence, Kansas,* p.141.

Shapiro HM (2003). Practical Flow Cytometry, forth edition, Wiley-Liss, New Jersey. pp.1-35

Sharma ML, Rao CS and Duda PL (1994). Immu-nostimulatory activity of *Picrorhiza kurroa* leaf extract. *Journal of Ethnopharmacology*, **41**: 185-192.

Silva C, Oliveira C R and Lima M C P (1996). Apoptosis as a Mechanism of Cell Death Induced by Different Chemotherapeutic Drugs in Human Leukemic TMLymphocytes. *Biochemical Pharmacology*, **51**: 1331-1340.

Vanparys C, Maras M, Lenjou M, Robbens J, Van Bockstaele D, Blust R and DE Coen M (2006). Flow cytometric cycle analysis allows for rapid screening of estrogenicity in MCF-7 breast cancer cells. *Toxicology in Vitro*, **20**: 1238-1248.

Wigmore A (1982). Be your own doctor. Avery Publishing Group Inc., Wayne, New Jersey, p.190.

Wigmore A (1985). The wheatgrass book. Avery Publishing Group Inc., Wayne, New Jersey, p.129.

Wilson C (1961). Grass and people. University of Florida Press, p.204.