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Full Length Research Paper

Kenaf seed oil from supercritical carbon dioxide fluid extraction induced G1 phase cell cycle arrest and apoptosis in leukemia cells

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This study was designed to determine the cytotoxic effects of kenaf seed oil (*Hibiscus cannabinus*) variety V36 extracted using supercritical carbon dioxide fluid extraction (SFE) with different combinations of pressure (bars) and temperature (°C). Extracted oils were tested on human promyelocytic HL-60, murine myelomonocytic WEHI-3B and human chronic myelogenous K562 leukemic cell lines. The yield of kenaf seed oil extracted by SFE ranged from 11 to 13% (w/w). Oils were found to be cytotoxic towards all the leukemia cell lines in a dose-dependent manner with no effects on normal cells (3T3). Oil from SFE at 600 bar 40°C (V600/40) was more cytotoxic towards HL-60, WEHI-3B and K562 when compared with other kenaf seed oils (extracted with different parameters) with the IC_{50} values of 178.78±10.52, 189.43±11.63 and 213.33±15.45 µg/ml, respectively. V600/40-treated leukemia cells exhibited typical characteristics of apoptosis such as nuclear fragmentation, chromatin condensation, nuclear margination, membrane blebbing and cellular shrinkage, as viewed under inverted light microscope and fluorescence microscope. Cell cycle analysis using flow cytometry revealed that, V600/40 induced G1 phase cell cycle arrest and significantly increased ($P < 0.05$) the sub-G1 apoptotic population in the leukemia cells. In conclusion, kenaf seed oil V600/40 induced apoptosis via G1 phase cell cycle arrest in HL-60, WEHI-3B and K562 leukemia cell lines.

Key words: Kenaf (*Hibiscus cannabinus*), supercritical carbon dioxide fluid extraction (SFE), leukemia, cytotoxicity, apoptosis, cell cycle arrest.

INTRODUCTION

Leukemia is a group of heterogeneous neoplastic disorder of white blood cells that multiply uncontrollably and unable to differentiate into mature cells (Lee et al., 2007). As of 2010, leukemia is diagnosed 10 times more often in adults than in children and more common in males than females (American Cancer Society, 2010). People with leukemia have many options of treatment such as chemotherapy (main treatment), antibiotic, blood transfusion, radiation therapy and bone marrow transplantation. Although, these treatments have prolonged the survival rate for leukemia patients, the adverse effects of these treatments are difficult to handle (Chiang et al., 2004). Thus, there is a need to seek for other remedies in treating leukemia.

Plants are an excellent source of bioactive components possessing a wide variety of biological activities and having great potential therapeutic values (Chiu et al.,2006; Deng

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et al., 2006). Crude extracts isolated from these plants are important source to be developed as anticancer agents and natural healthy foods for the management of cancer (Lin et al., 2007). Kenaf (*Hibiscus cannabinus*) is a valuable fibre and medicinal plant native to India and Africa (Mohamed et al., 1995). Recently, it has gained an important position in Malaysia as a potential plant to replace tobacco (Mariod et al., 2010). Kenaf is composed of various bioactive components including tannins, saponins, polyphenolics, alkaloids, fatty acids, phospholipids, tocopherol and phytosterols (Mohamed et al., 1995). This plant has been reported to be anodyne, aperitif, aphrodisiac, fattening, purgative, stomachic, and has long been used in traditional medicine to treat bilious conditions, bruises and fever (Agbor et al., 2005; Coetzee et al., 2008; Kobaisy et al., 2001; Mohamed et al., 1995; Nyam et al., 2009). However, not many studies have been documented with regards to its anticancer properties, in particular leukemia (Ghafar et al., 2010; Moujir et al., 2007; Yazan et al., 2010). Our preliminary study (unpublished data) showed that, kenaf seed oil was cytotoxic towards human acute lymphoblastic leukemia MOLT-4 cells; therefore, raising the possibility that kenaf seed oil might have some cytotoxic effects towards myelogenous leukemia cells.

Kenaf seed oil can be extracted conventionally by using the organic solvents such as *n*-hexane or petroleum ether. Nevertheless, the oil is always doubted for the safety of consumption. Recently, the supercritical carbon dioxide fluid extraction (SFE) technique for solid materials was introduced. SFE is the non-toxic, non-explosive, environmental friendly, cost effective, time saving and selectivity-adjustable solvent (supercritical carbon dioxide fluid) extraction (Araújo and Sandi, 2007; Vaquero et al., 2006). SFE has been extensively studied for the separation of bioactive compounds from herbs including kenaf seeds (Chan and Ismail, 2009; Reverchon and De Marco, 2006). In this study, we investigated the cytotoxicity of kenaf seed oil extracted by SFE towards myelogenous leukemia cell lines.

MATERIALS AND METHODS

RPMI-1640 with L-glutamine, fetal bovine serum and penicillin–streptomycin were purchased from PAA, Austria. Acridine orange (AO), propidium iodide (PI), RNaseA and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich, USA.

Kenaf seed oil extraction

Kenaf seeds variety V36 were purchased from the National Tobacco Board, Pasir Putih, Kelantan, Malaysia. Kenaf seeds were cleaned, soaked in water at ambient temperature for 24 h and dried at constant temperature (50°C) overnight in an oven (FD 115, Fisher Scientific, Germany). The final moisture content of the dried seeds was less than 5%. Kenaf seeds were extracted by using supercritical carbon dioxide extractor (Thar 1000 F, Thar Technologies, Inc., USA) at different combinations of pressure and temperature (pressure (bar)/temperature (°C):600/40; 600/60; 600/80) as reported previously (Yazan et al., 2010).

Cell culture

The human promyelocytic leukemia HL-60, murine myelomonocytic leukemia WEHI-3B, human chronic myelogenous leukemia K562 and normal mouse embryonic fibroblast 3T3 cell lines were purchased from the American type culture collection (ATCC, USA). Cells were grown in RPMI 1640, supplemented with 10% of fetal bovine serum and maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Treatment

All the kenaf seed oils were dissolved in DMSO. Cells at the concentration of 1 x 10^5 cells/ml were seeded in a 6-well flat-bottomed tissue culture plate and treated with different concentrations of kenaf seed oil ranging from 50 to 800 µg/ml for 72 h. The final concentration of DMSO in the treated wells was kept at 0.5%. DMSO only (0.5%, v/v) was included as the control.

Determination of cytotoxicity (MTT assay)

Following the treatment, cytotoxicity was evaluated using the MTT assay. Briefly, 10 µl of MTT solution (5 mg/ml) with 50 µl of cell suspension was added into a 96-well flat-bottomed tissue culture plate and incubated at 37°C for 4 h. Next, 150 µl of Tris-DMSO solution was added into each well (Hsu et al., 2005). The absorbance which was proportional to cell viability was measured at 570 nm and a reference wavelength of 630 nm by using an ELISA plate reader (Bio-Rad, USA). A graph of percentage of cell viability versus concentration of kenaf seed oil was plotted and the concentration that gave 50% inhibition of the cell viability (IC_{50}) when compared with the control was determined.

Morphological changes of cells treated with kenaf seed oil

The treated and untreated cells were viewed for morphological changes, characteristic of apoptosis or necrosis under an inverted light microscope (Olympus, USA).

Determination of mode of cell death

The cells were stained with 1 mg/ml of AO (Sigma-Aldrich, USA) and 1 mg/ml of PI (Sigma-Aldrich, USA) at the ratio of 1:1 after treatment with kenaf seed oil. The suspension was placed onto a clean microscopic slide and viewed under a fluorescence microscope (Leica, Germany) at 400X magnification.

Cell cycle analysis

The cells were harvested and washed twice with phosphate-buffered saline (PBS), fixed in ice-cold 70% ethanol and incubated at -20°C for 2 h. Prior to analysis, the cells were washed once again with PBS, suspended in 425 µl of PBS, 25 µl of PI (1 mg/ml) (Sigma-Aldrich, USA) and 50 µl of RNaseA (1 mg/ml) (Sigma-Aldrich, USA) and incubated at 4°C for 20 min. DNA content was analyzed by flow cytometer (CyAn ADP, USA) and the population of cells in each cell-cycle phase was determined by using the submit v3.4 software (CyAn ADP, USA).

Table 1. Yield of kenaf seed oil extracted by SFE and Soxhlet.

Extraction technique	Yield (%)
SFE 600/40	11.88 ± 0.63[a]
SFE 600/60	12.29 ± 0.52[a]
SFE 600/80	12.76 ± 0.46[a]
Soxhlet	19.16 ± 0.72[b]

Values with the mean of three independent experiments ± SD. a and b were significantly different (P < 0.05).

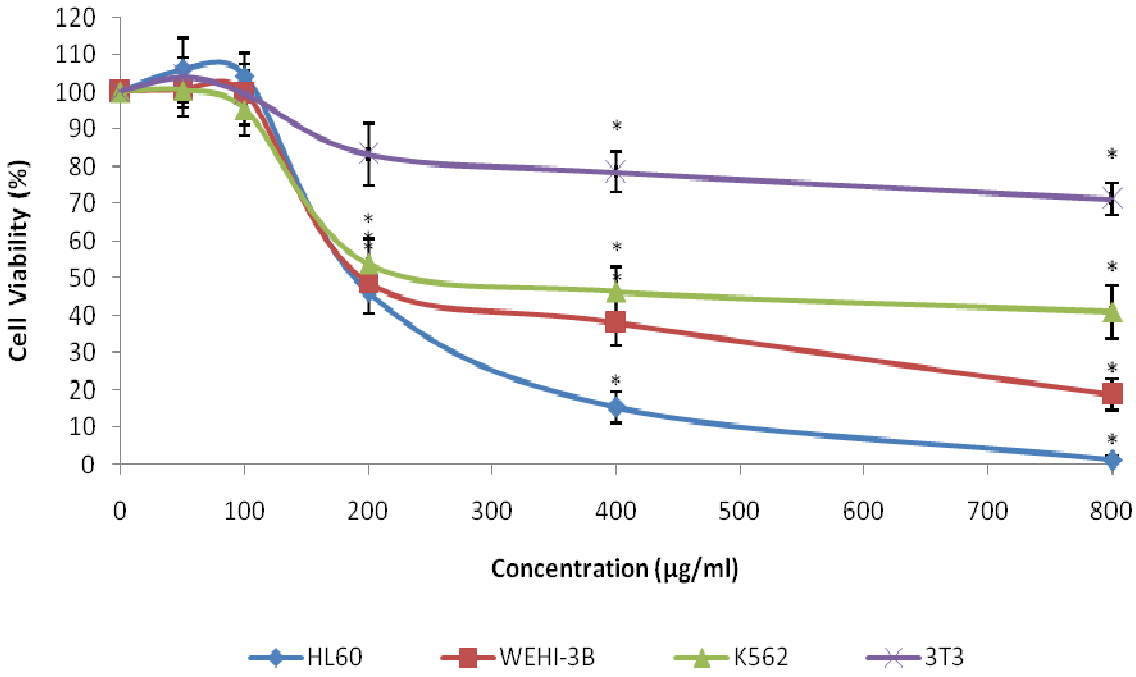

Figure 1. Effect of V600/40 on the viability of leukemia (HL-60, WEHI-3B and K562) and 3T3 normal cell lines after 72 h as determined using the MTT assay. The oil was cytotoxic to the cells in a dose-dependent manner. Each data point represents the mean of three independent experiments ± SD. *significantly different from the control (P < 0.05).

Statistical analysis

Statistical analysis was performed using the statistical package for Social Science (SPSS) version 16.0. Results were analyzed by one-way analysis of variance (ANOVA). Data were expressed as mean ± standard deviation (mean ± SD). A difference was considered to be significant at P < 0.05.

RESULTS

Yield of kenaf seed oil

Table 1 shows the difference in the yield of kenaf seed oil extracted by SFE and *n*-hexane (Soxhlet). The yield of kenaf seed oil extracted by SFE ranged from 11 to 13% (w/w). The yield slightly increased (p > 0.05) in correspondence to the rise in extraction temperature. The yield from Soxhlet extraction was significantly higher when compared with SFE (P < 0.05).

Cytotoxicity of kenaf seed oil towards leukemia and normal 3T3 cell lines

Kenaf seed oil from SFE was more cytotoxic than the one from Soxhlet towards all the leukemia cell lines in a dose-dependent manner (Figure 1). Increase in the temperature of SFE resulted in the increase of the IC_{50} values of kenaf seed oil. Oil from SFE at 600 bar 40°C (V600/40) was more cytotoxic towards HL-60, WEHI-3B and K562 when compared with others with the lowest IC_{50} values of 178.78±10.52, 189.43±11.63 and 213.33±15.45 µg/ml, respectively (Table 2). K562 was the least sensitive towards the extracts (oil). The IC_{50} value of kenaf seed oil

Table 2. IC_{50} values of kenaf seed oil variety V36 with different ways of extraction towards leukaemia (HL-60, WEHI-3B and K562) and 3T3 normal cells after 72 h as determined by using MTT assay.

Cell line	IC_{50} (µg/ml)			
	V600/40	V600/60	V600/80	Soxhlet
HL-60	178.78 ± 10.52	320.48 ± 11.35	>800	>800
WEHI-3B	189.43 ± 11.63	380.32 ± 15.21	>800	>800
K562	213.33 ± 15.45	472.34 ± 13.12	>800	>800
3T3	>800	>800	>800	>800

Each data point represents the mean of three independent experiments ± SD.

against 3T3 normal cells was not obtained even at the treatment of 800 µg/ml of the oil (Table 2). Since V600/40 was the most cytotoxic towards these leukemia cell lines, further analyses were carried out using this extract.

Morphological changes of HL-60, WEHI-3B and K562 cell lines treated with V600/40

Number of HL-60, WEHI-3B and K562 cells reduced with increase in the concentration of V600/40. Affected cells showed some features of apoptosis such as cellular shrinkage and membrane blebbing (Figure 2).

Fluorescence analysis of mode of cell death

Fluorescence analysis following staining with AO/PI distinguished viable, apoptotic and necrotic cells. As shown in Figure 3, the untreated control cells exhibited intact, round and large green nuclei. The number of viable cells reduced with increase in the concentration of V600/40. Nucleus of the cells undergoing apoptosis was green but fragmented. Other features of apoptosis were also noted such as chromatin condensation and nuclear margination. At the highest treatment of the oil (800 µg/ml), majority of the cells were necrotic (red nucleus) (Figure 3).

Cell cycle analysis

DNA content (Figure 4) and changes in the cell cycle distribution (Table 3) showed a dose-dependent accumulation of the leukemia cells in the sub-G1 phase after treatment with V600/40 for 72 h. Cell cycle arrest at the G1 phase was observed in all the three leukemia cell lines (Table 3).

DISCUSSION

SFE has been documented as a more favorable extraction technique due to the advantage over the use of liquid solvents (Reverchon and De Marco, 2006). Even though the yield of the oil extracted using the liquid solvent (Soxhlet) was obviously higher ($P < 0.05$) (Table 1), the products from SFE are still of preference because they are free from any residues of the solvents since CO_2 is easily separated from the oil at the end of extraction (Pourmortazavi and Hajimirsadeghi, 2007). Besides the production of non-toxic products, SFE is also claimed to be non-explosive, environmental friendly, cost effective, time saving and selectivity-adjustable solvent in the extraction (Reverchon and De Marco, 2006). SFE at 600 bar was chosen in this study due to higher oil yield when compared with other pressures (200 and 400 bars) (Chan and Ismail, 2009; Yazan et al., 2010). Nevertheless, the yield obtained from our study was lower (by -5%) possibly due to the differences in the variety, batch of the plant, cultivation climate, ripening stage and harvesting time of the seed (Nyam et al., 2009). From Table 1, it seems that temperature influenced the yield of kenaf seed oil even though the differences are not significant. For SFE, pressure and temperature are the two important factors that contribute to the yield of kenaf seed oil. Elevation in pressure at certain temperature results in an increase in the CO_2 density, thus, enhancing solubility of the solutes and increasing the yield. Temperature affects the volatility of the solute. At constant pressure, the density of CO_2 decreases with the increase in temperature, and becomes more pronounced as the compressibility increases (Pourmortazavi and Hajimirsadeghi, 2007). On the other hand, increase in temperature will increase the vapor pressure of analytes. Therefore, the tendency of components to be extracted passing through the super-critical fluid will increase (Reverchon and De Marco, 2006).

In this study, the IC_{50} was used as an index of cytotoxicity of kenaf seed oil towards leukemia cells. The cell viability of the leukemia cells treated with V600/40 reduced with the increase in the concentration of the oil indicating that the cytotoxic effect was in a dose-dependent manner (Figure 1). Since the oil was more cytotoxic towards HL-60 when compared with WEHI-3B and K562 with IC_{50} value ranging between 0.125 and 5 mg/ml, it has a good potential for the treatment of leukemia (Manosroi et al., 2006). HL-60 has been reported to be very sensitive to apoptosis upon anti-cancer agents' treatment and it is a good *in vitro* model for testing anti-leukemic agents (Suh et al., 1995; Yoshida et al., 1996).

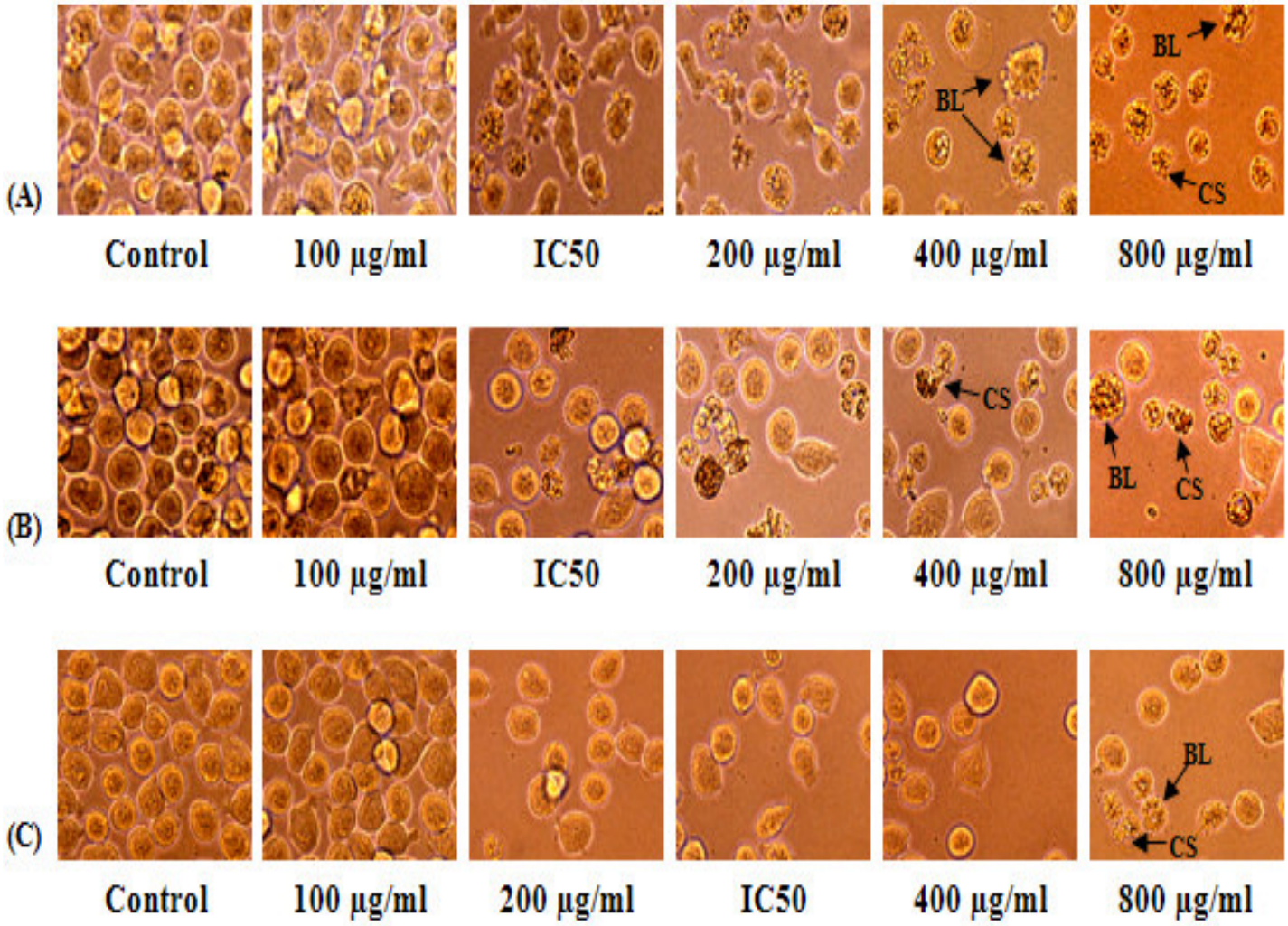

Figure 2. Morphological changes of (A) HL-60, (B) WEHI-3B and (C) K562 cells treated with V600/40 for 72 h viewed under an inverted light microscope. Affected cells showed some features characteristic of apoptosis such as cellular shrinkage (CS) and membrane blebbing (BL) (400X).

Interestingly, it is also of advantage that the oil was least cytotoxic to the normal 3T3 cell line. Recent study revealed that, kenaf seed oil was cytotoxic towards the ovarian cancer cells (CaOV3) (Yazan et al., 2010). In addition, lignans from kenaf core and bark of acetone extract were cytotoxic towards cervical cancer (HeLa), epithelial cancer (Hep-2) and lung cancer (A-549) cell lines (Moujir et al., 2007). Kenaf seed oil contains various active compounds such as fatty acid, phenolic acids, phytosterols and tocopherols (Mohamed et al., 1995; Nyam et al., 2009). Phytosterols showed growth inhibitory effects on breast (Awad et al., 2007), leukemia (Moon et al., 2008; Park et al., 2007), lung (Mendilaharsu et al., 1998; Schabath et al., 2005), ovarian (McCann et al., 2003), stomach (De Stefani et al., 2000) and prostate cancer (McCann et al., 2005). Linoleic acid inhibited the proliferation of human skin cancer, breast cancer, colon cancer, stomach cancer,as well as leukemia *in vitro* and *in vivo* (Hubbard et al., 2000; Kritchevsky, 2000; MacDonald, 2000; Phoon et al., 2001). Thus, phytosterols and linoleic acid are speculated to be responsible for the cytotoxic effects.

It is obvious that increase in temperature influences the cytotoxicity of kenaf seed oil. The higher the extraction temperature of SFE, the higher the IC_{50} value (Table 2). Similar condition was observed for Soxhlet (the use of heat during extraction) where the IC_{50} value was not obtained. It is speculated that high extraction temperature might denature some of the heat sensitive active components in the oil (Cossuta et al., 2008), thus, making it less cytotoxic to the cells.

Kenaf seed oil was also investigated for the mode of cell death, whether it is apoptosis or necrosis. This is important as mode of cell death especially apoptosis has

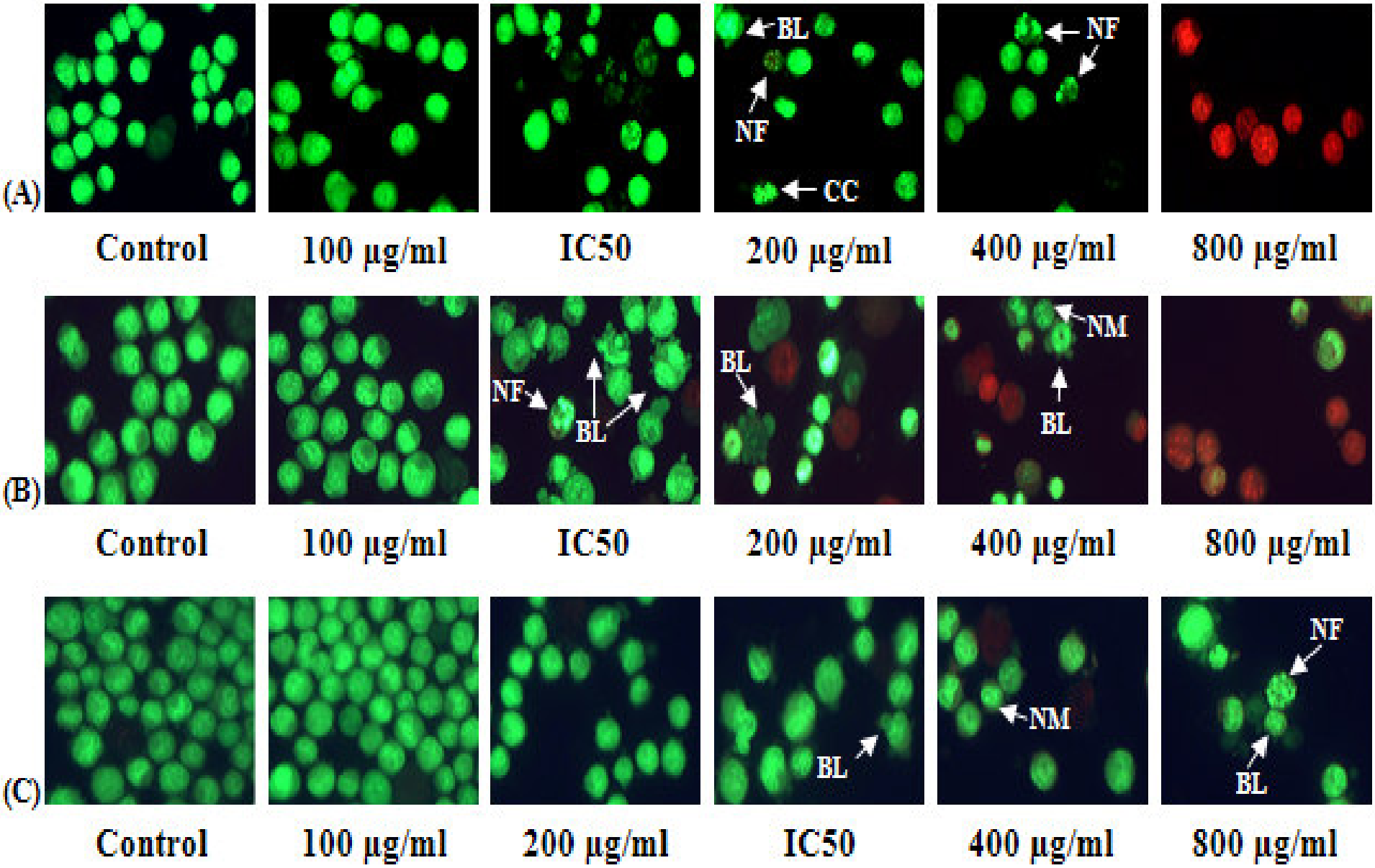

Figure 3. Fluorescence micrograph of AO/PI double-stained of (A) HL-60, (B) WEHI-3B and (C) K562 cells treated with V600/40 for 72 h. Affected cells showed typical characteristics of apoptosis such as membrane blebbing (BL), chromatin condensation (CC), nuclear margination (NM) and nuclear fragmentation (NF) (400X).

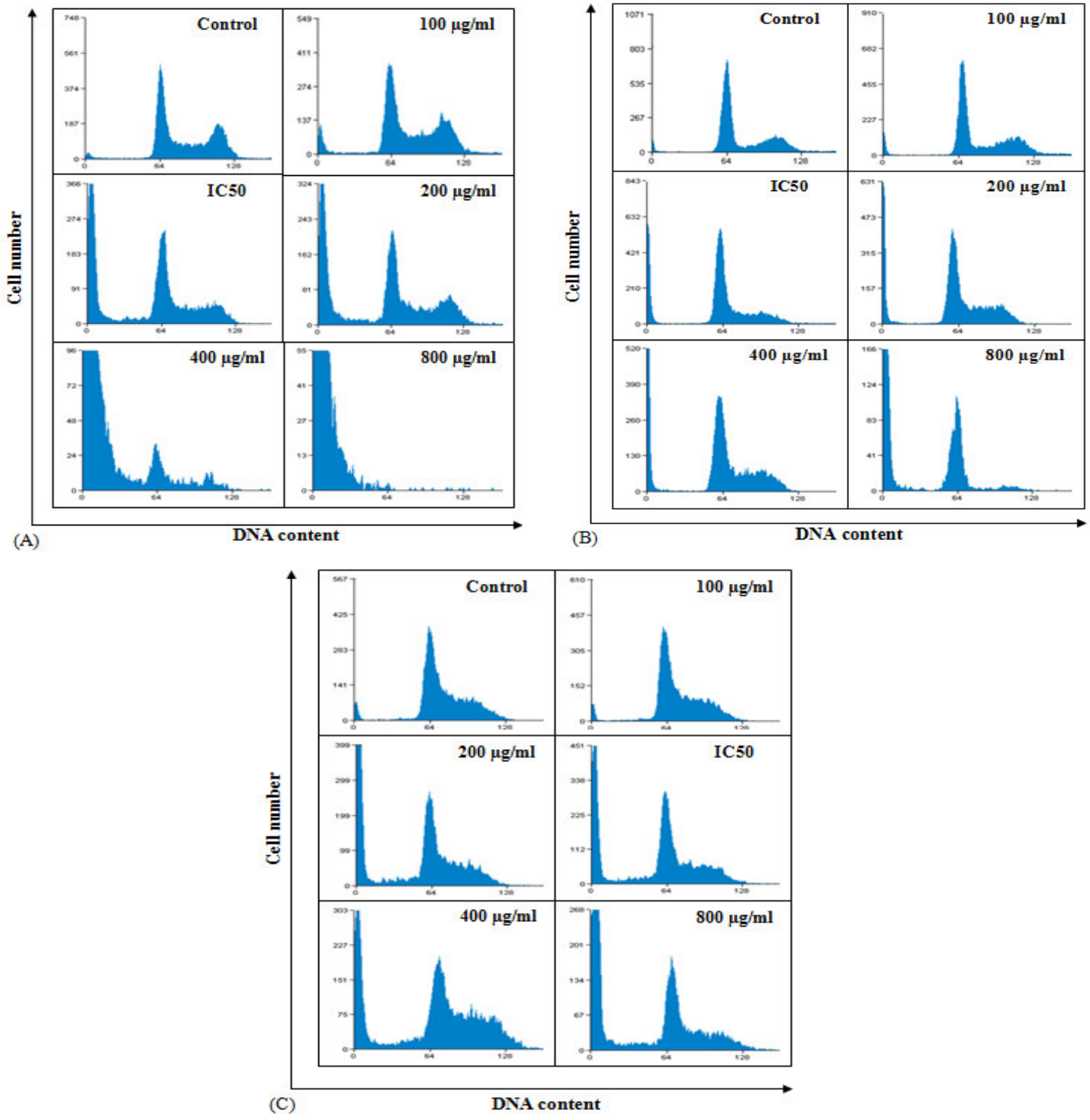

Figure 4. Cell cycle analysis of (A) HL-60, (B) WEHI-3B and (C) K562 cells treated with V600/40 for 72 h. Accumulation of cells at the sub-G1 phase was observed with the increase in concentration of V600/40.

great implication in cancer therapy. Apoptosis is of advantage and a more favorable one since it plays an important role in elimination of damaged cells or tumor cells without causing inflammation (Hou et al., 2005). It is found that the leukemia cells treated with V600/40 died primarily via apoptosis as viewed under an inverted light microscope (Figure 2) and the findings were further supported by fluorescence analysis following staining with AO

Table 3. Changes in the cell cycle distribution (%) of HL-60, WEHI-3B and K562 cells treated with V600/40 for 72 h.

Cell line	Concentration (µg/ml)	Apoptotic cell (%)	Non-apoptotic cell (%)		
		Sub-G1	G1	S	G2/M
HL-60	Control	3.71 ± 2.22	44.84 ± 1.16	11.24 ± 1.22	43.98 ± 0.95
	100	8.01 ± 4.32	46.78 ± 2.31	11.77 ± 0.23	41.55 ± 2.51
	178 (IC_{50})	48.52 ± 2.31*	52.78 ± 2.31*	13.96 ± 1.78	33.26 ± 1.64*
	200	53.36 ± 1.21*	47.57 ± 0.46	14.09 ± 1.23*	38.35 ± 1.24*
	400	92.82 ± 0.56*	49.66 ± 2.68*	14.69 ± 0.97*	35.65 ± 1.74*
	800	99.60 ± 0.99*	57.52 ± 1.69*	7.5 ± 0.54*	35.14 ± 0.95*
WEHI-3B	Control	5.12 ± 2.13	46.77 ± 1.45	9.39 ± 4.33	44.84 ± 0.45
	100	3.86 ± 1.24	50.72 ± 2.34	13.73 ± 3.76	34.55 ± 0.78
	189 (IC_{50})	16.22 ± 1.45*	62.20 ± 1.25*	13.81 ± 1.44	23.99 ± 1.14*
	200	19.30 ± 2.26*	55.83 ± 2.21*	12.02 ± 1.87	32.15 ± 1.31*
	400	26.31 ± 0.94*	53.71 ± 0.79*	11.99 ± 0.96	30.15 ± 2.24*
	800	75.29 ± 3.45*	80.93 ± 0.78*	3.62 ± 0.45*	15.45 ± 3.34*
K562	Control	3.99 ± 0.95	41.81 ± 1.22	16.78 ± 2.33	41.41 ± 3.46
	100	5.33 ± 1.32	42.15 ± 2.13	15.84 ± 1.98	42.15 ± 1.21
	200	29.64 ± 2.34*	48.01 ± 0.97*	18.75 ± 2.11	38.64 ± 2.13
	213 (IC_{50})	39.69 ± 1.23*	49.33 ± 2.78*	19.03 ± 3.24	31.65 ± 0.91*
	400	35.95 ± 3.45*	52.65 ± 2.89*	16.43 ± 1.64	31.74 ± 1.47*
	800	43.32 ± 2.11*	48.61 ± 1.56*	13.46 ± 1.23*	37.98 ± 0.79

Each data point represents the mean of triplicate ± SD. * is significantly different from control ($P < 0.05$).

and PI (Figure 3). In the analysis, almost all the control cells (without treatment with V600/40) for the three cell lines looked healthy, round in shape with similar size and green color of intact nucleus. In contrast, majority of the treated cells were apoptotic. They were smaller in size with green but fragmented nucleus. Other features of apoptosis such as membrane blebbing, chromatin condensation and nuclear margination were also observed (Kerr et al., 1972) (Figure 3).

The induction of apoptosis was then confirmed by the accumulation of population of the cells at sub-G1 phase ($P < 0.05$) from the cell cycle analysis. Appearance of sub-G1 cells is the marker of cell death by apoptosis (Park et al., 2007). Treatment with V600/40 also caused an arrest at the G1 phase in the three leukemia cell lines. The mechanisms on how kenaf seed oil caused leukemia cells to undergo apoptosis at the G1 checkpoint are still unclear. A study (Hsu et al., 2005) drives a hypothesis that kenaf seed oil degraded cdc25A which is a phosphatase associated with CDK4 and CDK2 activities, following by down-regulation of cyclin D2 and cyclin E and up-regulate $p15^{INK4b}$ and $p27^{Kip1}$ genes. These series of events impose a blockade of mid G1-late G1-S transition thereby causing G1 phase cell cycle arrest. It will be followed by a series of typical morphological changes such as cellular shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation, as being observed in Figures 2 and 3. In conclusion, kenaf seed oil V600/40 induced apoptosis via G1 phase cell cycle arrest in HL-60, WEHI-3B and K562 leukemia cell lines. Interestingly, V600/40 was less cytotoxic towards the normal mouse embryonic fibroblast 3T3 cell line. Kenaf seed oil will be a candidate for the development of anti-cancer agent. A further analysis is needed to investigate the anti-tumor property of kenaf seed oil in leukemia mice.

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