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Full Length Research Paper

Kenaf seed oil from supercritical carbon dioxide fluid extraction shows cytotoxic effects towards various cancer cell lines

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Hibiscus cannabinus **(Kenaf) from the family of Malvaceae is a valuable fiber source and medicinal plant. It has long been prescribed as traditional folk medicine in Africa and India to treat various diseases. Nevertheless, little research has been carried out on the potentials of this plant as treatment for cancer. This study was designed to determine the cytotoxicity of kenaf seed oil from two varieties (Quiping 3 and V36) extracted by supercritical carbon dioxide fluid extraction (SFE) with different combinations of pressure (bars) and temperature (°C) towards breast cancer (MCF-7, MDA-MB-231, 4T1), cervical cancer (HeLa), lung cancer (A549) and leukemic (MOLT-4) cell lines. Even though kenaf seed oil from both varieties were cytotoxic to all the cancer cells, kenaf seed oil variety V36 extracted by SFE at 600 bars 40°C (V600/40) was the strongest towards MOLT-4 and MDA-MB-231, with the IC_{50} values of 153.26 and 483.35 µg/ml, respectively. MOLT-4 and MDA-MB-231 cells treated with V600/40 exhibited typical characteristics of apoptosis such as blebbing, chromatin condensation and nuclear fragmentation as viewed under an inverted light microscope and a fluorescence microscope. In conclusion, V600/40 was the most cytotoxic towards the MOLT-4 and MDA-MB-231 cells in a dose-dependent manner possibly via the induction of apoptosis.**

Key words: Kenaf (*Hibiscus cannabinus*), supercritical carbon dioxide fluid extraction, cytotoxicity, apoptosis.

INTRODUCTION

Natural products play an important role in the current cancer treatment with substantial numbers of anticancer agents used in the clinic being either natural or derived from natural products such as plants (Nobili et al., 2009). The advantage of using plant-derived anticancer agents is that, the agents destroy the cancer cells without harming healthy cells. In fact, the produced adverse effects by the natural anticancer agents are lesser, something these other drugs do not do (Kinghorn et al., 2003). *Hibiscus cannabinus* (Kenaf) from the family of Malvaceae is a natural product and good source of fiber and it is native to India and Africa (Mohamed et al., 1995). Kenaf is also one of the allied fibers of jute and shows similar characteristics. The plant is being cultivated in Malaysia with the aim to replace tobacco. This plant is composed of various active components including tannins, saponins, polyphenolics, alkaloids, essential oils and steroids, and has long been prescribed in traditional folk medicine in Africa and India (Agbor et al., 2005; Kobaisy et al., 2001). However, only few studies have been reported with regards to the anticancer effect of kenaf (Ghafar et al., 2010; Moujir et al., 2007; Yazan et al., 2010).

Kenaf seed contains various bioactive constituents such as fatty acids, phenolic acids, phytosterols and tocopherols (Coetzee et al., 2008; Nyam et al., 2009). Kenaf seed oil can be extracted conventionally by using the organic solvents such as *n*-hexane or petroleum either. Neverthe-

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less, the oil is always doubted for the safety of consumption due to the residue of the solvents. SFE is considered as the most favorable extraction technique as it is non-toxic, non-explosive, environmental friendly, cost effective, time saving and selectivity-adjustable solvent (supercritical carbon dioxide fluid) in the extraction (Araújo and Sandi, 2007; Vaquero et al., 2006). SFE has indeed been extensively studied for the separation of active compounds from herbs and other plants including kenaf seeds (Chan and Ismail, 2009; Reverchon and De-Marco, 2006). This study was designed to determine the cytotoxic effects of kenaf seed oil from two varieties (Quiping 3 and V36) extracted by SFE with different combinations of pressure (bars) and temperature (°C) towards breast cancer (MCF-7, MDA-MB-231, 4T1), cervical cancer (HeLa), lung cancer (A549) and leukemic (MOLT-4) 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) 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 Quiping 3 and V36) were purchased from the Agricultural Research and Development Institute (MARDI), Serdang, Selangor, Malaysia and National Tobacco Board, Pasir Putih, Kelantan, Malaysia, respectively. Kenaf seeds were cleaned, soaked in water at ambient temperature for 24 h and were dried at constant temperature (50°C) overnight in an oven (FD 115, Fisher Scientific, Germany). The moisture content of the dried seeds was measured by moisture analyzer (MLB 50-3, Kern and Sohn GmbH, Germany). The seeds were stored at 4°C until further use.

The detailed procedures for kenaf seed oil extraction was previously reported in by Chan and Ismail (2009). For SFE, kenaf seeds were extracted by using the supercritical carbon dioxide fluid extractor (Thar 1000 F, USA) at 3 different combinations of pressure (bars) and temperature (°C). The combinations were V600/40, V600/60 and V600/80. Briefly, kenaf seeds were ground in a stainless steel Waring blender for 1 min and 100 g of kenaf seeds were placed into a 1 L extraction vessel. After the extraction vessel was tightly sealed, the desired temperature and pressure were set. The flow rate of carbon dioxide (CO_2) was set at 25 g/min and was regulated by an automated back pressure regulator. The SFE extraction was initiated after the desired temperature and pressure were achieved. The whole extraction lasted for 150 min and the yield was measured.

For Soxhlet extraction, 25 g of the kenaf seeds were ground by a stainless steel Waring blender for 1 min and was transferred into an extraction thimble. The thimble was then transferred into a Soxhlet extractor (Witeg-Labortechnik GmbH, Germany). Prior to extraction, 300 ml of *n*-hexane was added into the round bottom flask. After extraction was initiated, the solvent flow rate was manually adjusted to 7 min/cycle and the extraction was terminated after 100 cycles (classic Soxhlet extraction, SOX/L).

Cell culture

The breast cancer (MCF-7, MDA-MB-231, and 4T1), cervical cancer (HeLa), lung cancer (A549) and leukemic (MOLT-4) cell lines purchased from the American type culture collection (ATCC, USA) were grown in RPMI 1640, supplemented with 10% of fetal bovine serum and were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Treatment

Cells at the concentration of 1 x 10^5 cells/ml were seeded in a 96-well flat-bottomed tissue culture plate and were treated with the different concentrations of kenaf seed oil ranging from 78 to 5000 µg/ml for 72 h (Manosroi et al., 2006).

Determination of cytotoxicity (MTT assay)

Following the treatment, cytotoxicity was evaluated using the MTT assay. Briefly, 20 µl of the medium with 5 mg/ml of MTT in PBS was added into each well. The plate was incubated at 37°C for 4 h. Next, the medium was totally removed and 200 µl of Tris-DMSO solution was added to each well. The absorbance, which was proportional to cell viability, was measured at 570 nm and at 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. The concentration that gave 50% inhibition of the cell viability (IC_{50}) was determined.

Morphological changes of cells treated with kenaf seed oil

The treated and untreated cells were viewed for morphological changes, and for 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, USA) and 1 mg/ml of PI (Sigma, USA) at 1:1 mixture after treatment with kenaf seed oil. The suspension was placed onto a clean microscopic slide and was viewed under a fluorescence microscope at 400X magnification. A minimum of 150 cells was counted in every sample. The number of viable, apoptotic and necrotic cells was counted and expressed as a proportion of the total cell number (%).

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

Moisture content

The final content of the seeds was less than 5%.

Yield of kenaf seed oil

Table 1 shows the yield of kenaf seed oil extracted by the

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

Extraction technique	Yield (%)	
	Quiping 3	V36
SFE 600/40	13.87 ± 0.41[a]	11.21 ± 0.84[1]
SFE 600/60	14.21 ± 0.49[a]	11.98 ± 0.32[1]
SFE 600/80	14.74 ± 0.86[a]	12.65 ± 0.73[1]
Soxhlet	21.51 ± 0.38[b]	18.26 ± 0.42[2]

Values were the mean of three independent experiments ± SD. [a], [b], 1 and 2 were significantly different ($p < 0.05$).

SFE and *n*-hexane (Soxhlet). The yield of kenaf seed oil extracted by SFE ranged from 11 to 15% (w/w). The yield from both kenaf seed varieties slightly increased ($p > 0.05$) in correspondence to the rise in extraction temperature. The yield from Soxhlet extrac-tion was significantly higher when compared with SFE ($p < 0.05$).

Cytotoxicity of kenaf seed oil towards the cancer cell lines

In general, kenaf seed oil of both varieties (Quiping 3 and V36) from all the extraction procedures (SFE and Soxhlet) exhibited dose-dependent cytotoxic effects towards all the cancer cell lines. Oil from SFE was more cytotoxic when compared with the one from Soxhlet (Table 2). SFE oil from variety V36 at 600/40 (V600/40) showed stronger cytoto-xicity than Quiping 3 especially towards MOLT-4 and MDA-MB-231, with the IC_{50} values of 153.26 and 483.35 µg/ml, respectively (Figure 1). Amongst all the cell lines, 4T1 was the least sensitive to all the extracts.

Morphological changes of MOLT-4 and MDA-MB-231 treated with V600/40

The cell number of MOLT-4 and MDA-MB-231 reduced with the increase in the concentration of V600/40. The affected cells showed some features of apoptosis such as cellular shrinkage, membrane blebbing, nuclear com-paction and fragmentation and formation of apoptotic bodies (Figure 2).

Fluorescence analysis of mode of cell death

Fluorescence analysis following staining with AO/PI distinguished viable, apoptotic and necrotic cells. Some features of apoptosis such as membrane blebbing, chromatin condensation, nuclear margination and fragmentation were also clearly noticed (Figure 3). The percentage of viable, apoptotic and necrotic cells for V600/40-treated-MOLT-4 and MDA-MB-231 at the IC_{50} dose was significantly different ($p < 0.05$) from the control. Higher percentage ($p < 0.05$) of apoptotic cells compared with necrotic cells was observed in the treated samples (Figure 4). The percentage of apoptotic and necrotic cells at the IC_{50} dose of MDA-MB-231 for 72 h was 27.35±2.31 and 11.42±1.73%, respectively. For MOLT-4, the percen-tage of apoptotic and necrotic cells at the IC_{50} dose after 72 h was 29.24±1.76 and 12.55±2.24%, respectively.

DISCUSSION

Not only because the cytotoxic effects of kenaf seed oil extracted by SFE towards cancer cells have not been well investigated yet, but it is also the advantages offered by the extraction procedure over others (the use of liquid solvents) (Reverchon and De-Marco, 2006) that war-ranted the conduct of this study. Even though the yield of the oil extracted using the liquid solvent (*n*-hexane) was obviously higher ($p < 0.05$) (Table 1), SFE is still a better extraction technique because it is free from any residues of the solvents (Pourmortazavi and Hajimirsadeghi, 2007). SFE at 600 bar was selected to extract the oil in the study due to its higher production of the oil when compared with other pressures (200 and 400 bars) (Chan and Ismail, 2009). Nevertheless, the yield obtained from our experiment was lower, possibly due to the differences in the batch of the plant, cultivation climate, ripening stage and harvesting time of the seeds (Nyam et al., 2009). Data in Table 1 shows that, temperature influenced the yield of kenaf seed oil ($p > 0.05$). For SFE, pressure and temperature were the important factors that contributed to the yield of kenaf seed oil. An increase in the pressure at certain temperature resulted in an increase in the CO_2 density, thus, enhanced the solvent power and increased the yield. Meanwhile, 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 the components to be extracted passing through the supercritical fluid will increase (Reverchon and De-Marco, 2006).

IC_{50} was used as the index of cytotoxicity of kenaf seed oil. As kenaf seed oil showed cytotoxic effects towards MOLT-4 and MDA-MB-231 cells with the IC_{50} value between 125 and 5000 µg/ml, it could be developed as the cancer therapeutic agents (Manosroi et al., 2006). 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 and linoleic acid are speculated to be responsible for the cytotoxic effects of kenaf seed oil in this study. 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

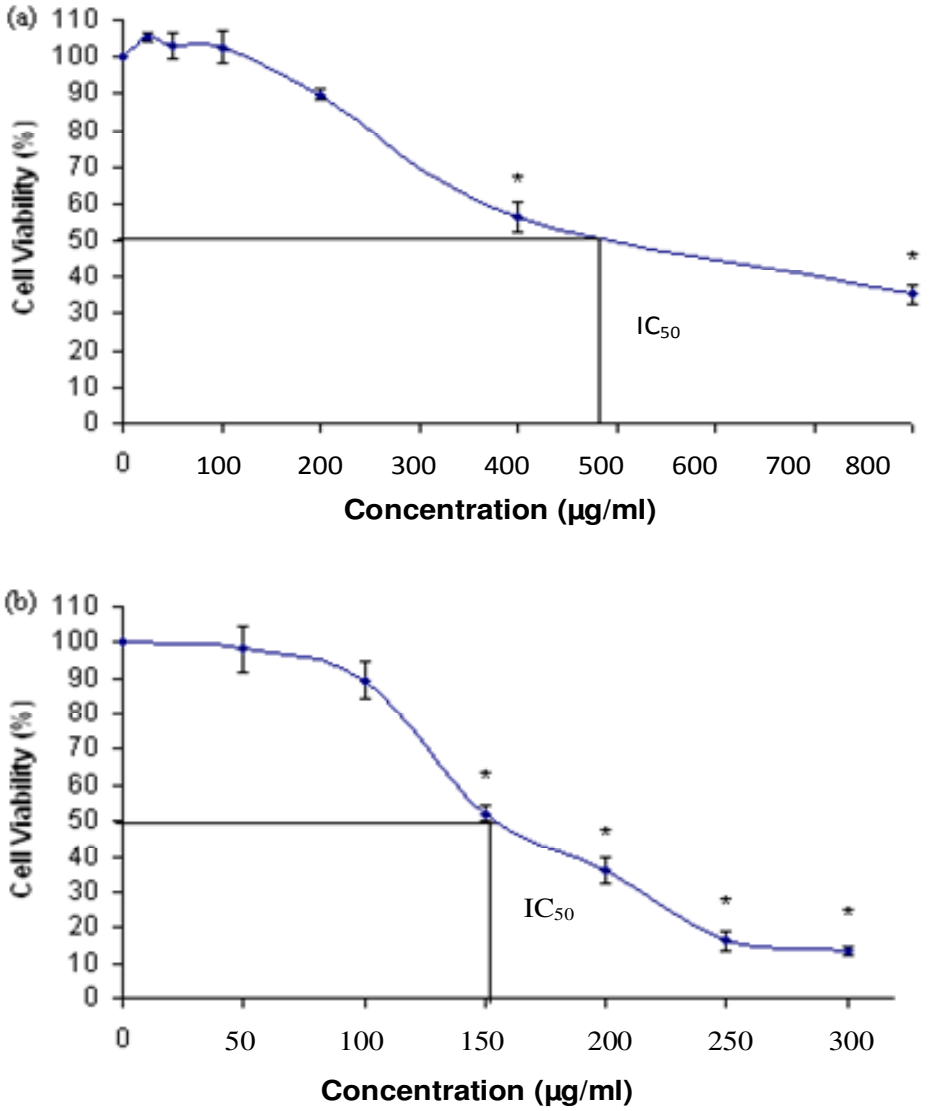

Figure 1. Effect of V600/40 on the viability of (a) MDA-MB-231 and (b) MOLT-4 cell lines after 72 h as determined using the MTT assay. The oil was cytotoxic to the cells in a dose-dependent manner. Each data point represents the mean of three independent experiments ± SD. *, were significantly different from the control ($p < 0.05$).

2005), ovarian (McCann et al., 2003), stomach (De-Stefani et al., 2000) and prostate cancer (McCann et al., 2005) cell lines. Linoleic acid inhibits the proliferation of human skin, breast, colon, stomach, as well as leukemia *in vitro* and *in vivo* (Hubbard et al., 2000; Kritchevsky, 2000; MacDonald, 2000; Phoon et al., 2001). It should be noted that, the increase in the temperature of SFE resulted in the increase of the IC_{50} values (Table 2). It was postulated that higher temperature may denature some of the heat sensitive compounds that exist in kenaf seed oil (Cossuta et al., 2008), making it less cytotoxic to the cells.

It is interesting that the anchorage-independent cell line (MOLT-4) was more sensitive (lower IC_{50} value) to kenaf seed oil when compared with other tested anchorage-dependent cell lines (Table 2) (Ghafar et al.,2010; Yazan et al., 2010). The findings were consistent with those of previous reports (Ali et al., 2000; Ishak et al., 2010). This phenomenon is due to many factors in the microenvironment of a solid tumor that are responsible for non-uniform and insufficient levels of anti-cancer agents being delivered. For instance, the extracellular matrix (ECM) of a solid tumor is made up of proteo-glycans, collagens and additional molecules, which are produced and assembled by stromal and tumor cells (Mow et al., 1984; Wang and Yuan, 2006). This great collagen content in the ECM obstructs the transpo-tation of anti-cancer agent from reaching the target tumor cells (McGuire et al., 2006).

Kenaf seed oil was also studied for the mode of cell

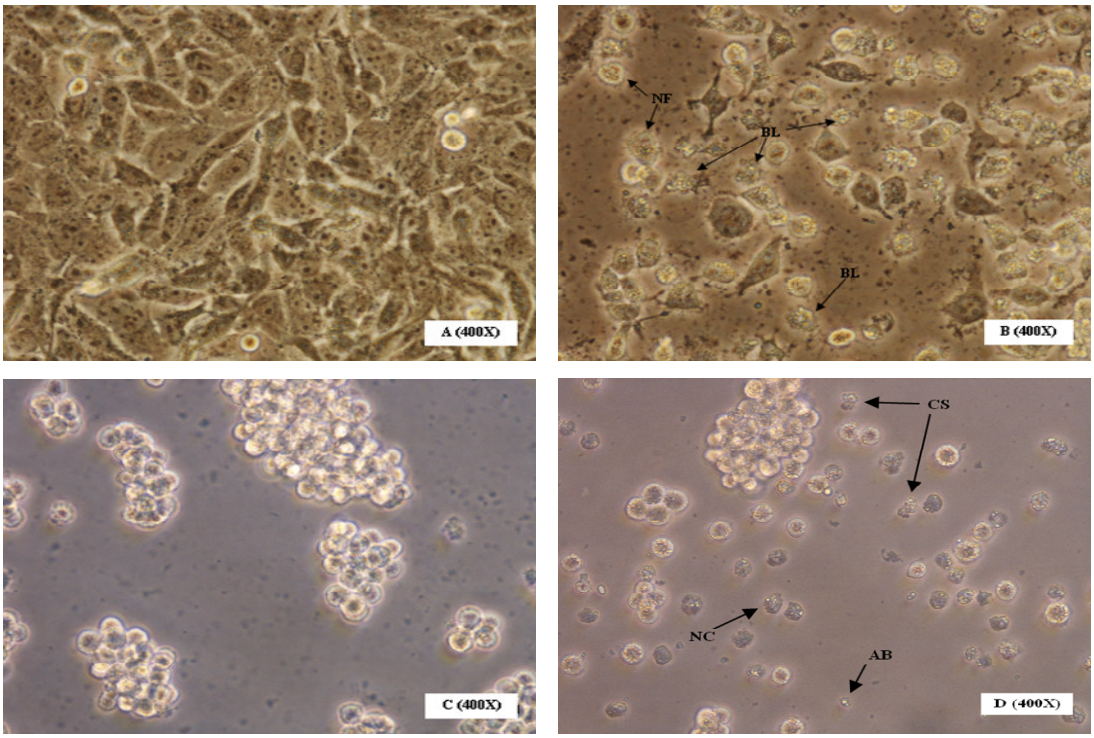

Figure 2. Morphological changes of the MOLT-4 and MDA-MB-231 treated with V600/40 for 72 h. A and B, Untreated and IC_{50}-treated MDA-MB-231; C and D, untreated and IC_{50}-treated MOLT-4. Reduced cell population was observed in the treated sample (B and D). The affected cells showed some features characteristic of apoptosis such as cellular shrinkage (CS); blebbing (BL); nuclear compaction (NC); nuclear fragmentation and apoptotic bodies (AB) (arrows) (400× magnification).

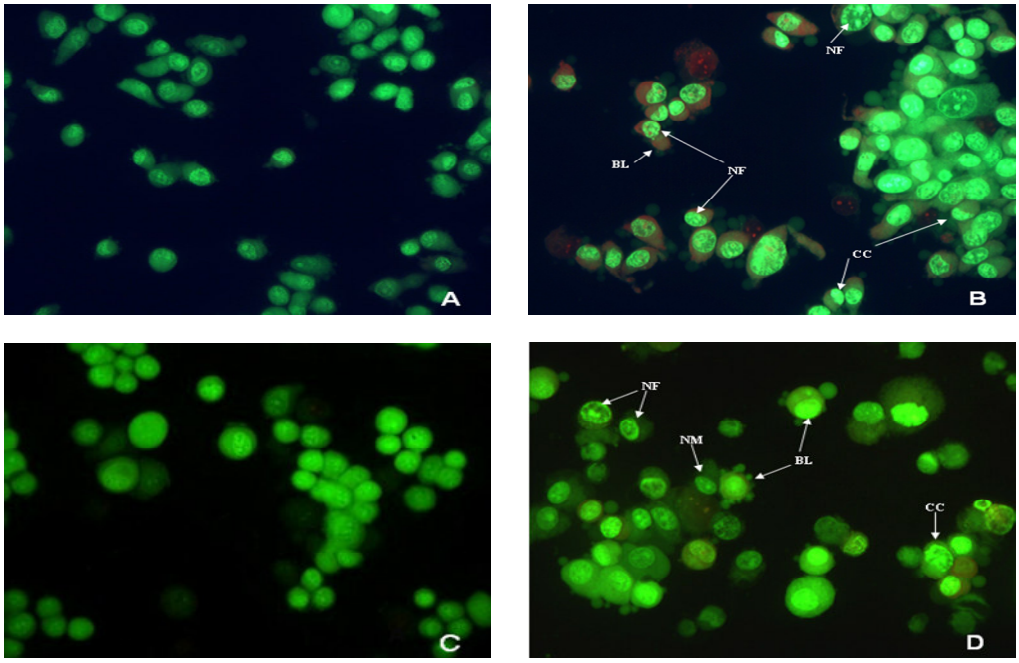

Figure 3. Fluorescence micrograph of AO/PI double-stained MOLT-4 and MDA-MB-231 cells treated with V600/40 at the IC_{50} dose for 72 h. A and B, Untreated and IC_{50}-treated MDA-MB-231; C and D, untreated and IC_{50}-treated MOLT-4. Treated cells (B and D) showed typical characteristics of apoptosis such as membrane blebbing (BL); chromatin condensation (CC); nuclear fragmentation (NF) and nuclear margination (NM) (400× magnification).

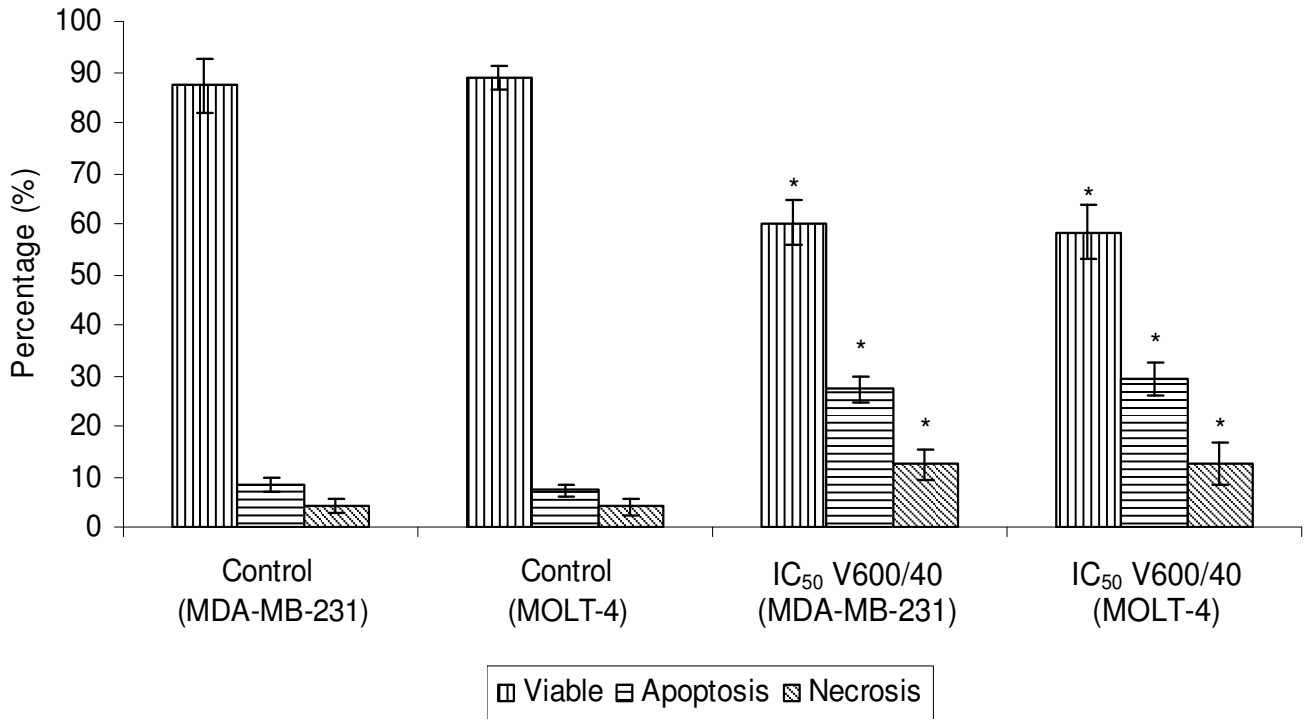

Figure 4. Percentage of viable, apoptotic and necrotic cells of MOLT-4 and MDA-MB-231 cells treated with V600/40 at the IC_{50} for 72. Each data point represents the mean of three independent experiments ± SD. * Significantly different from the control ($p < 0.05$).

death it induced in the cells; whether apoptotic or necrotic. It was found that majority of the V600/40-treated MOLT-4 and MDA-MB-231 cells showed features of apoptosis such as cellular shrinkage, membrane blebbing, nuclear compaction and the formation of apoptotic bodies as viewed under an inverted light microscope (Figure 2). The induction of apoptosis by V600/40 was further confirmed by staining the cells using AO/PI fluorescence dye. AO is a cell-permeable DNA-binding dye, whereas PI is a plasma membrane-impermeable DNA-binding dye. AO and PI excite a green and red fluorescence, respectively, when they are intercalated into DNA. AO is taken up by both viable and non-viable cells, while PI is excluded by cells with intact membrane (viable and apoptotic). PI fluoresce red predominantly for necrotic cells. In the fluoresces analysis following staining with AO/PI, the control cells without treatment for both cell lines looked healthy with green color of intact nucleus. Almost all the control cells for both cell lines appeared to be round in shapes with similar sizes (Figure 3a, c). In contrast, the nucleus of majority of the treated cells for both cell lines was also green but fragmented. The cells were of irregular shape and smaller in size when compared with the control. In addition, the treated cells also showed other features of apoptosis such as chromatin condensation, nuclear fragmentation and nuclear margination (Kerr et al., 1972). It was wise to conclude that the cells treated with kenaf seed oil died primarily due to apoptosis since the number of apoptotic cells was higher than necrotic cells ($p < 0.05$). Since kenaf seed oil induced apoptosis in the cells, it has the potential to be used for anti-tumor therapy (Saraste and Pulkki, 2000). Apoptosis is of advantage and it is more favorable when compared with necrosis, since it does not trigger inflammatory responses (Lin et al., 2007).

It is also interesting to note that, there was a discrepancy between the MTT results (Figure 1) and the percentage of cell death determined using the AO/PI double staining method (Figure 4). The viability of V600/40-treated-MDA-MB-231 and MOLT-4 cells at the IC_{50} dose was approximately 60% in AO/PI double staining but was 50% from the MTT assay. This phenomenon may have occurred as a result of the different end points and the principles of the two different methods (Chan et al., 2006). In conclusion, this study showed that V600/40 kenaf seed oil from supercritical carbon dioxide fluid extraction was more cytotoxic especially towards the human leukemic (MOLT-4) and human breast cancer (MDA-MB-231) cell lines in a dose-dependent manner possibly via the induction of apoptosis.

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Table 2. IC_{50} values of the kenaf seed oil towards various cancer cell lines after 72 h as determined by using the MTT assay.

Cell line	IC_{50} (µg/ml)							
	SFE 600/40		SFE 600/60		SFE 600/80		Soxhlet	
	Quiping 3	V36	Quiping 3	V36	Quiping 3	V36	Quiping 3	V36
MCF-7	1406.67±53.35	>5000	1803.15±12.23	>5000	>5000	>5000	>5000	4188.47±78.89
MDA-MB-231	1645.30±23.13	483.35±31.97	2030.46±56.35	>5000	>5000	>5000	4203.27±97.87	4863.47±87.11
4T1	>5000	>5000	>5000	>5000	>5000	>5000	>5000	>5000
HeLa	2209.45±21.34	>5000	3849.12±33.14	>5000	>5000	>5000	>5000	>5000
A549	2325.34±13.21	>5000	3608.45±84.32	>5000	>5000	>5000	>5000	>5000
MOLT-4	817.33±12.12	153.26±25.43	1236.38±43.34	1657.42±72.83	>5000	>5000	2302.57±53.33	1957.62±89.36

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

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