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Cytotoxic activity of an octadecenoic acid extract from *Euphorbia kansui* (Euphorbiaceae) on human tumour cell strains

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Abstract

We have investigated the cytotoxic and antitumour activity of an octadecenoic acid extract, mainly containing oleic and linoleic acids, from *Euphorbia kansui* on human gastric (SGC-7901), hepatocellular carcinoma (BEL-7402), and leukaemia (HL-60) tumour cell strains. Significant and dose-dependent antiproliferation effects were observed on tumour cells from the dose of 3.2 μg mL^{-1}, which were comparable with or better than those of the common antitumour agent 5-fluorouracil. Results from the clone formation assay and flow cytometry indicated that the mixture of octadecenoic acids resulted in a dose-dependent reduction in the number of tumour cells and significantly inhibited cell proliferation, with induced apoptosis and G_0/G_1 phase cell cycle arrest. Also, the octadecenoic acids could not only cause cell apoptosis/necrosis but also functionally and structurally damage the tumour cell membrane and cell ultra-structures. These observations encourage further clinical evaluation of the inhibitory effects of octadecenoic acids on various forms of cancer.

Introduction

A fatty acid is a carboxylic acid with a long aliphatic tail, either saturated or unsaturated, derived from natural fats and oils. Fatty acids are used as basic building blocks of biological membranes and can also be used as a source of energy through fatty acid oxidation. Accumulating evidence in man and animal models indicates that fatty acids have distinct, important bioactive properties, but the impact on inflammatory processes and particularly immune function is not easily predictable due to parallel pro- and anti-inflammatory effects (Stulnig 2003; King et al 2006).

Due to the inhibitory effects on tumour cells, including decreased production of inflammatory eicosanoids, cytokines, and reactive oxygen species (Hunt & Dean 1989; Calder 2006; Deckelbaum et al 2006), many fatty acids are used as supplements to anticancer drugs. For example, unsaturated fatty acids have been reported to have not only a large influence on the structure and function of cell membranes (Szachowicz-Petelska et al 2007) but also cytotoxic activity against pancreatic adenocarcinoma cells (Pettersson et al 2002), bovine lens epithelial cells (Nguyen et al 2000), neoplastic cells (Germain et al 1998) and lymphocytes (Kageyma et al 1989). The inhibitory effects on breast, gastric and renal cancer cells were seen in the inhibition of cell proliferation and induction of apoptosis in cultured tumour cells (Mengeaud et al 1992; Palozza et al 2000). However, apparently there have been few biological or phytochemical investigations of the underlying mechanisms by which unsaturated fatty acids such as oleic and linoleic acids affect cell growth and proliferation in human tumour cell strains, such as human gastric (SGC-7901), hepatocellular carcinoma (BEL-7402) and leukaemia (HL-60) tumour cells.

Euphorbia (Euphorbiaceae) is one of a number of plants that have attracted attention as herbal remedies for a variety of diseases (Hohmann et al 2000; Yu et al 2005). *Euphorbia*

extracts (e.g. diterpenes, euphols, triterpenes, sterols, and phenols) have demonstrated nti-inflammatory, antitumour, immuno-modulatory, and antiproliferative activity on multiple cultured tumour cell Lines (Hohmann et al 2000; Yasukawa et al 2002; Yu et al 2005). The promising in-vitro data, coupled with the clinical treatment, prompted us to investigate this plant for its other active constituents, such as unsaturated fatty acids. In this study, an octadecenoic acid extract mainly containing oleic and linoleic acids was isolated from *E. kansui* to investigate the effects on cell proliferation, colony formation ability, and the cell cycle and survival of the SGC-7901, BEL-7402 and HL-60 cell strains.

Materials and Methods

Plant and extracts

The plant *Euphorbia kansui* was collected in Tianshui, Gansu Province, China, in October 2005; the voucher specimen was deposited in our laboratory for future reference. Air-dried roots of *E. kansui* (1.0 kg) were ground and mixed with 3 L petroleum ether and extracted for 12 h at 40°C. The extract was saponified with 200 ml saturated KOH, dissolved in water, and re-extracted with petroleum ether. The petroleum ether extract was then treated with 2.5 M HCl to isolate the mixture of saturated and unsaturated fatty acids. After removal of the saturated fatty acids with ethanol, 937.5 mg (0.0938%) of an oily extract containing unsaturated fatty acids was obtained. The extract was purified by HPLC on a Spherisorb C18 column (250 × 4.6 mm) with acetonitrile–H_2O (50:50) and measured at 225 nm. Components in the extract and their chemical structures were identified by gas chromatography–mass spectrometry (GC-MS).

Tumour cell strains

The human gastric SGC-7901, hepatocellular carcinoma BEL-7402 and leukaemia HL-60 cells were obtained from the laboratory of Lanzhou Army Hospital. The cells were grown overnight in RPMI 1640 media supplemented with 10% fetal bovine serum, 2% penicillin–streptomycin, and 1% L-glutamine in a humidified atmosphere (5% CO_2) at 37°C.

Experimental design

The extract of octadecenoic acids was used at concentrations of 0.2, 0.8, and 3.2 μg mL^{-1} in all treated groups. 5-Fluorouracil (5-FU; 10 μg mL^{-1}) and phosphate-buffered saline (PBS; 5 mg mL^{-1}) were used in positive and negative control groups, respectively. The solvent used throughout this work contained dimethyl sulfoxide (DMSO) at a concentration less than 0.003%.

MTT colorimetric assay

MTT assays were performed to quantify the effect of the extract on cell viability and proliferation. Each cell strain, at a concentration of 2.5×10^8cells mL^{-1}, was plated into a 96-well disposable plate, containing 180 μL growth medium (RPMI 1640) per well. Sixteen individual wells were prepared for each treatment. After 24-h incubation, 20 μL PBS, 5-FU, or the extract at three concentrations were applied to each well. The plate was incubated for a further 48 h, and 10 μL MTT suspended in PBS were then added to each sample. Following 4-h incubation, the MTT crystals or precipitate were thoroughly dissolved by applying 100 μL DMSO and shaking the plate for 10 min. The inhibitory rate (IR) was calculated based on the optical density read at 494 nm (OD_{494}) on an enzyme-linked immunodetector (MULTISKAN MK3, Shanghai, China):

$$\text{Inhibitory rate (IR)} = (1 - \text{average } OD_{494} \text{ of the treated group / average } OD_{494} \text{ of the control group}) \times 100\%.$$

Colony forming efficiency

The effects of the extract on tumour cells were also investigated using a cloning inhibition assay, a standard method of assaying a population of tumour cells for drug sensitivity.

For the assay of the HL-60 cell strain, 10 g agar was mixed with 200 mL PBS and heated at 120°C for 10 min. A 5-ml portion of the agar and PBS mixture at 50°C was added to 45 ml RPMI 1640 and distributed to a 16-well culture plate, 1 ml per well. Then, 20 μL PBS, 5-FU, or the extract at three doses were added to each well, three wells per treatment. Each well was overlaid with 1 ml of a mixture of 47 ml of the cell suspension (1.0×10^5 cells mL^{-1}) and 3 ml of the agar/PBS at 50°C, and the plate was incubated for seven days at 37°C.

For SGC-7901 and BEL-7402 cell strains, 1 ml cell suspension at a concentration of 4.0×10^2cells mL^{-1} was plated into a 16-well culture plate, containing 1 ml RPMI 1640 per well. After incubation for 24 h, 20 μL PBS, 5-FU, or the extract of unsaturated fatty acids at three doses were applied to each well, three wells per treatment. The plates were then incubated for seven days at 37°C.

After seven days, the tumour cells were fixed with methanol for 5 min, stained with 20 μL Giemsa's solution for 10 min, and washed with running water. Colonies containing more than 50 cells were scored as survivors and were counted with an inverted microscope. The colony efficiency of clone formation was calculated as:

$$\text{Colony efficiency} = (1 - \text{number of clones in the treated group / number of clones in the control group}) \times 100\%.$$

Assessment of cell cycle distribution

To quantify the number of apoptotic cells we analysed the cellular DNA content by flow cytometry after propidium-iodide nuclear staining. Each tumour cell strain at 2.0×10^6cells mL^{-1} was inoculated into five 20-mL culture flasks containing 3.5 ml RPMI 1640 medium. The culture flasks were incubated at 37°C in a humidified incubator containing 5% CO_2 for 24 h. After removal of the supernatant, 500 μL PBS, 5-FU, or the diluted extract (0.2, 0.8 and 3.2 μg mL^{-1}) were added to culture flasks. After 48 h, cells were collected by centrifuging at 5000 rev min^{-1} (2000 *g*)

for 5 min, washed with PBS, and fixed with 75% cold ethanol for 24 h at 0–4°C. Following thorough removal of ethanol by centrifuging and decanting, cell pellets were resuspended in PBS containing 0.5 ml RNAse (200 μg mL^{-1}) for 4 h at 4°C, treated with PBS containing 0.5 ml propidium iodide (20 μg mL^{-1}), incubated for 30 min at room temperature, and analysed using flow cytometry (Coulter EPICS XL, US).

Cytotoxicity and morphological evaluation

Antitumour activity of the extract was verified by observing the ultrastructural changes in tumour cells. Each cell strain at a concentration of 2.0×10^6cells mL^{-1} was plated into five 20-mL culture flasks containing 3.5 ml RPMI 1640 medium. Following 24-h incubation at 37°C, 500 μL PBS, 5-FU or the extract at three concentrations was added to culture flasks. After 48-h incubation, the cells were collected by centrifuging (5000 rev min^{-1} (2000 *g*)), washed twice with PBS, and fixed with a solution containing 3% paraformaldehyde and 1.25% glutaraldehyde-PB (pH 7.4) for 24 h. The cell smears were gold plated and examined with a scanning electron microscope (JSM-5600LV, Japan).

Statistical analysis

SPSS10.0 and Dunnett's analysis of variance were used to compare statistical differences between the treated and the control groups in each assay. A difference at the $P < 0.05$ level was considered to be statistically significant.

Results

Three unsaturated fatty acids were isolated from the extract of *E. kansui*, including oleic acid (44.2%), linoleic acid ((Z,Z)-9,12-octadecadienoic acid) (39.1%), and (E)-9-octadecenoic acid (12.1%) (Figure 1A). The chemical structures identified by GC-MS are shown in Figure 1B. Treatment of the SGC-7901, BEL-7402 and HL-60 cell strains with the *E. kansui* extract indicated that the unsaturated fatty acid extract had cytotoxic activity on all tumour cells and could induce apoptosis. The growth inhibitory effects were observed in a dose-dependent manner in all three tumour cell strains.

The MTT assay demonstrated dose-dependent inhibitory effects of the octadecenoic acids on cell survival of these three human cancer cell strains (Table 1). Significant dose-dependent antiproliferation effects were observed on all three tumour cell strains with a dose of 3.2 μg mL^{-1}. The survival rates in SGC-7901, BEL-7402 and HL-60 were decreased by 22–65.7% (IC50 = 1.073 μg mL^{-1}), 22.6–65% (IC50 = 1.49 μg mL^{-1}) and 21.7–56.5% (IC50 = 2.04 μg mL^{-1}), respectively. The extract at a dose of 0.8 μg mL^{-1} elicited the same degree of inhibition as did the 5-FU treatment.

When the cell strains were subjected to a cloning assay, the treatment with the octadecenoic acids resulted in a significant reduction in the number of tumour cells, in a dose-dependent manner (Figure 2). The colony inhibitory effects of the extract at a dose of 0.8 μg mL^{-1} were comparable with those with 5-FU treatment. Maximal proliferation inhibition was observed with a concentration of 3.2 μg mL^{-1}, which inhibited growth by 83.6, 84.7 and 78.8% with SGC-7901, BEL-7402 and HL-60, respectively, compared with those of the PBS controls.

Flow cytometry quantitatively confirmed the effects of the octadecenoic acids on cell cycle progression. As shown in Table 2, treatment with the extract resulted in significant accumulation of cells in the G_0/G_1 phase, which was accompanied by a decrease in cells with G_2/M DNA content. In the treated tumour cells 60.7–63.6% of total cells were in the G_0/G_1 phase, 22.3–26.6% in the S phase and 10.1–15.3% in the G_2/M phase. The unsaturated fatty acids enhanced the cellular apoptotic rates significantly by 16.3–26.3%, 17.7–28.4% and 16.7–24.2% in BEL-7402, SGC-7901 and HL-60, respectively.

In addition, the octadecenoic acids led to ultrastructural changes in tumour cells. Figure 3 shows the ultrastructural changes of BEL-7402 cells. Disruption and lysis of cell membrane and nuclear disintegration were noted in all three treated tumour cell strains (not shown). Some cells disintegrated into small membrane-bound fragments, while others became round and swollen with time. Membrane blebbing was prominent, and many membrane blebs were observed to pinch off from the cell surface, containing redistributed and compacted cytoplasmic organelles. Vacuoles were frequently seen in the cytoplasm, even obliterating the cytoplasm completely.

Discussion

Octadecenoic acids (e.g. oleic, linoleic acids) are widely distributed and abundant unsaturated fatty acids occurring in animal and vegetable oils. It has been observed that they can significantly inhibit cell proliferation and induce apoptosis (Hunt & Dean 1989; Anel et al 1992), and that linoleic, arachidonic and eicosapentaenoic acids are highly effective in killing human breast, lung, prostate and pancreatic tumour cells (Begin et al 1986; Ikushima et al 1990; Pettersson et al 2002). This study has shown that the octadecenoic acids extracted from *E. kansui* exhibited potent cytotoxicity in all human gastric tumour, hepatocellular carcinoma and leukaemia cell strains; the inhibitory and apoptotic rates were significantly increased (Tables 1 and 2).

One of the first observations was that these octadecenoic acids were toxic, decreasing the survival of tumour cells. Results presented in Figure 2 showed that the extract treatment led to a significant reduction in the average number of tumour cells, compared with the control groups in the colony forming assay. The results determined by the MTT assay also showed the significant decrease of tumour cell growth by treatment with the octadecenoic acids. The greatest cellular proliferative inhibitory rates were achieved at a concentration of 3.2 μg mL^{-1}, which were much better than the antitumour activity of 5-FU (Table 1).

Unsaturated fatty acids can affect cell membrane structure and fluidity (Begin et al 1986; Germain et al 1998; Field & Schley 2004), while some, such as palmitoleic, oleic, linoleic and arachidonic acids, are known to be Na^+, K^+-ATPase inhibitors (Swarts et al 1990) or are major components of

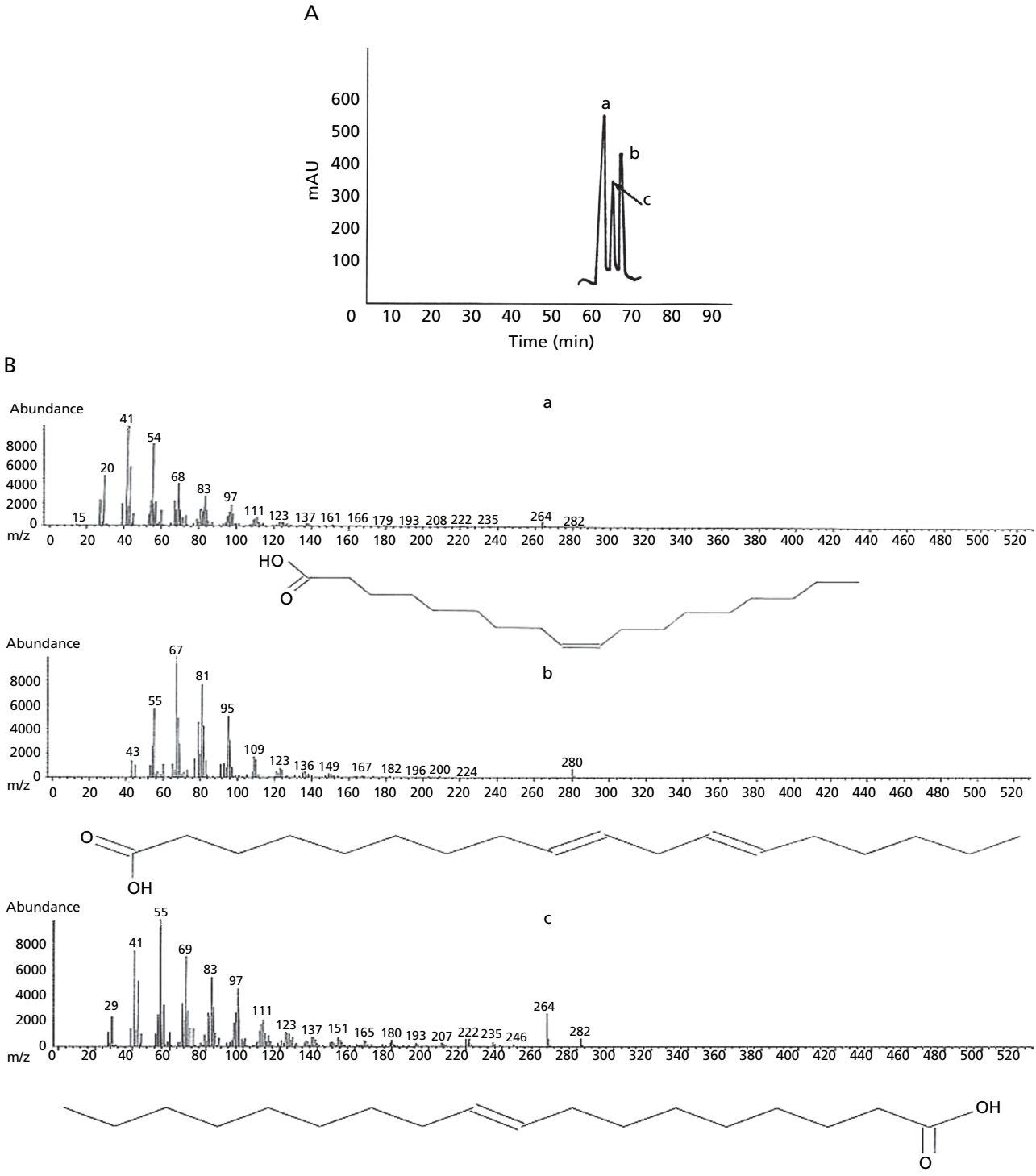

Figure 1 HPLC analysis (A) and chemical structures of the octadecenoic acids isolated from *Euphorbia kansui* (B). a, Oleic acid; b, linoleic acid ((Z,Z)-9,12-octadecadienoic acid); c, (E)-9-octadecenoic acid.

cell membrane phospholipids (Harrell & Stimers 2002). The cytotoxic action of unsaturated fatty acids has been implicated in an increase in free radical formation and generation of lipid peroxidation, which could cause morphological changes of tumour cells (Badway et al 1984; Sravan & Das 1995). The present results imply that reduction in tumour viability could result from changes in biophysical properties and functions of membranes brought about by octadecenoic acids.

Table 1 Effects of the octadecenoic acids on the cell proliferation of human cancer cell strains, assessed by the MTT assay

Group	BEL-7402		SGC-7901		HL-60	
	OD	IR	OD	IR	OD	IR
Control	1.67 ± 0.03		1.67 ± 0.04		1.86 ± 0.03	
5-FU	1.10 ± 0.04*	33.8	1.16 ± 0.03*	30.4	1.20 ± 0.06*	35.4
0.2 μg mL^{-1}	1.29 ± 0.06*	22.6	1.30 ± 0.03*	22	1.46 ± 0.04	21.7
0.8 μg mL^{-1}	1.11 ± 0.03*	33.6	1.12 ± 0.04*	32.5	1.18 ± 0.03*	36.5
3.2 μg mL^{-1}	0.58 ± 0.06**#	65	0.57 ± 0.03**#	65.7	0.81 ± 0.04**#	56.5

*$P < 0.05$ and **$P < 0.001$ compared with control; #$P < 0.05$ compared with 5-FU.

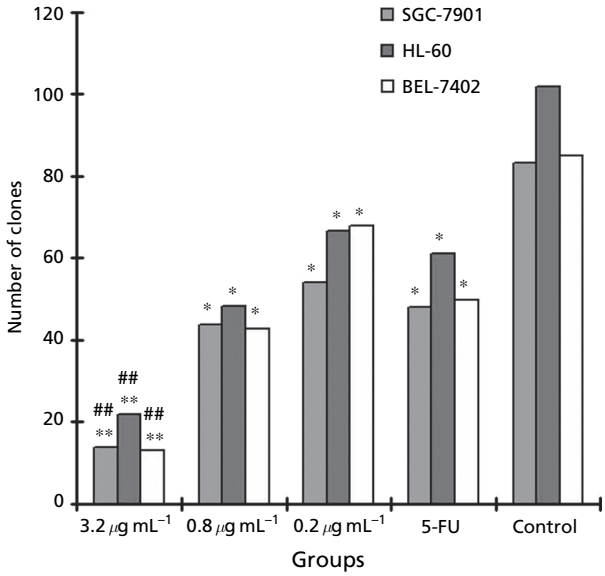

Figure 2 Effects of the octadecenoic acid extract on the cell survival probability of the human cancer cell strains, assessed by the clone formation assay. *$P < 0.05$ and **$P < 0.001$ compared with controls. ##$P < 0.001$ compared with 5-FU.

It is evident from the results that the extract of the octadecenoic acids could result in disruption of membrane integrity. The tumour cells treated with the octadecenoic acids exhibited various ultra-structural changes, such as cell retraction, bleb formation, pycnotic cell nuclei, and eventually cell detachment (Figure 3). Blebbing of cell membranes, pinching off membrane blebs and appearance of apoptotic bodies are typical ultrastructural features of cell apoptosis, whereas the typical ultrastructural changes of cell necrosis include cell swelling, distended or even disrupted organelles, followed by cell death. The ultra-structural changes of cell membrane or organelles reported in this study implied that the octadecenoic acids may have caused not only cell necrosis but cell apoptosis as well.

Apoptosis is a fundamental cellular activity needed to maintain the physiological balance of an organism by eliminating damaged cells or abnormal excess cell proliferation (Hengartner 2000). To determine whether suppression of proliferation of cell strains induced by the octadecenoic acids is associated with cell-cycle arrest, cellular DNA content was measured to estimate the frequency of apoptotic cells within the major phases of the cell cycle (G_0/G_1 vs S vs G_2/M). Our results suggested that all strains were responsive to the octadecenoic acids, as shown by the cell cycle arrest (Table 2). When the tumour cells were incubated with the extract, the octadecenoic acids significantly inhibited cell proliferation and induced G_0/G_1 phase cell cycle arrest. In the cell cycle, G_1 and G_2 play very important roles in proper timing of cell cycle events, monitoring the integrity of the DNA and regulating cells proceeding to S and M phases (Hartwell & Weinert 1989). The increase of cell population in G_0/G_1 phase accompanied by a decrease of cell population in

Table 2 Effects of 5-FU and octadecenoic acids on apoptotic rates (AR) and cell cycle distribution in the BEL-7402, SGC-7901 and HL-60 tumour cell strains

Group	BEL-7402				SGC-7901				HL-60			
	G_0/G_1	S	G_2/M	AR	G_0/G_1	S	G_2/M	AR	G_0/G_1	S	G_2/M	AR
Control	50.4	23.2	26.4		49.8	25.5	24.6		53.2	24.4	22.4	
5-FU	59.6	24.8	15.6	19.6**	60.4	23.6	16	20.4**	61.6	26.3	12.1	18.2**
0.2 μg mL^{-1}	62.2	25.1	13.6	16.3**	61.4	26.6	12	17.7**	60.7	24.2	15.1	16.7**
0.8 μg mL^{-1}	62.8	26.1	11.1	23.4**	63.3	25.8	10.9	24.2**	64.1	23.4	12.5	22.2**
3.2 μg mL^{-1}	63.6	26.3	10.1	26.3**##	62.7	25.4	11.9	28.4**##	62.4	22.3	15.3	24.2**#

**$P < 0.001$ compared with control,; #$P < 0.05$ and ##$P < 0.001$ compared with 5-FU.

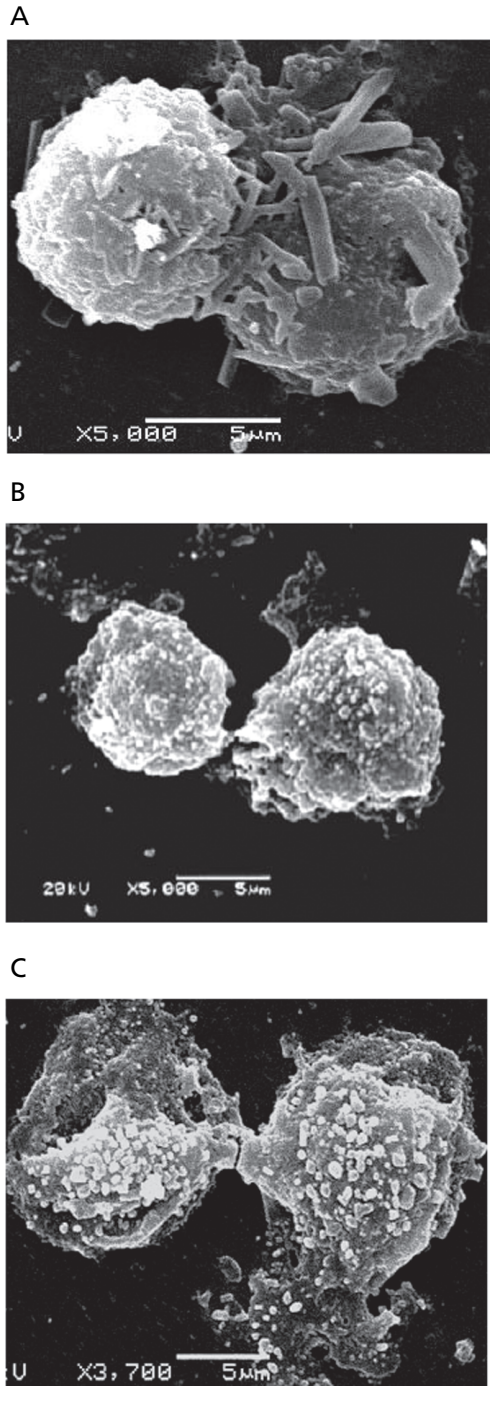

Figure 3 Scanning electromicroscopic observation of ultrastructural changes in BEL-7402 cells treated with the PBS (A), 5-FU (B), and octadecenoic acid extract at a concentration of 3.2 μg mL^{-1} (C) for 48 h.

G_2/M phase of the cell cycle was associated with a significant increase in the proportion of cells undergoing apoptosis.

Cells in the G_1 phase are committed to preparation for mitosis, including transcription, translation (protein synthesis) and growth of cytoplasmic materials. It has been observed that the G_1 phase arrest can either be in the form of a transient pause in cell cycle progression, or a permanent arrest, with the vast majority of cells having lost the capacity for continued proliferation (Di Leonard et al 1994). Previous studies have shown that anti-carcinogenic compounds or extracts from natural products, such as flavonoids (e.g. quercetin and genistein) (Deschner et al 1991; Chen et al 2003), bufalin from the skin and parotid venom glands of toad, CKBM from plants, and benzyl isothiocyanate from cruciferous vegetables (Nasu et al 2004), could inhibit cell cycle progression from the G_0/G_1 and G_2/M phases and induce apoptosis in tumour cells (Artwohl et al 2004; Chiu et al 2004; Finstad 1994; Srivastava & Singh 2004; Luk et al 2005). Our results confirmed that octadecenoic acids could induce cell-growth control and an arrest in the G_1 phase. The colony formation assay showed that long-term survival of the cell lines treated with the octadecenoic acids was significantly reduced (Figure 2).

Conclusions

Our study demonstrated that the extract of the octadecenoic acids from *E. kansui* exhibited antitumour activity and could result in disrupting membrane integrity and inducing tumour cell apoptosis. Although it is still unclear what the effect is of the octadecenoic acids on other cancer cell lines and normal cells, the observed effect is likely to be of clinical significance, and maybe beneficial in evaluating the use of octadecenoic acids in management of various forms of cancer. A further phytomedical and biochemical study on various cancer cell lines will undoubtedly help extend this knowledge to therapeutic approaches.

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