Cytotoxic Effects of *Vitex agnus-castus* Fruit Extract Against Human Cultured Uterine Cervical Fibroblast, Breast Cancer and Ovarian Cancer Cells, and its Biochemical Mechanism

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Abstract

From dried ripened *Vitex agnus-castus* fruit growing in Israel a crude extract was prepared with methanol and ethanol. Cytotoxicity of this extract against human uterine cervical canal fibroblast (HCF), and breast and ovarian cancer cells (SKOV-3 and MCF-7) was then examined. After these cells were cultured on a 96 well micro-culture plate for 24 hr (logarithmic growth phase), various concentrations of Vitex extract were added to each well and incubated for 48 hr. Viability of these cells was measured by XTT-dye reduction assay. It was shown that at the logarithmic growth phase, growth activity of these cells were higher, and cytotoxic activity of Vitex extracts against these cells was more effective. These cells were cultured for 72 hr (at the stationary growth phase) and treated with various concentrations of Vitex extracts for 48 hr. At the stationary phase of these cells, cytotoxicity of Vitex extracts was the most effective against SKOV-3, and the activity against MCF-7 was higher than that against non-cancer cells (HCF). Analysis by agarose gel electrophoresis showed that DNA fragmentation was revealed only with SKOV-3 cells treated with Vitex extract at the stationary growth phase. It is concluded that the cytotoxic activity of Vitex extract may be attributed to growth activity of respective cells, that cytotoxic activity against cancer cells at the stationary phase was significantly higher than that of non-cancer cells, and that the cytotoxicity against SKOV-3 cells may take place through apoptosis.

INTRODUCTION

Vitex agnus-castus Kurz. (Vitex) is a shrub belonging to the Verbenaceae family, and grows naturally along the shores of the Mediterranean Sea. Vitex fruits have been used as a folk medicine for a long time for alleviation and/or improvement of symptoms from obstetric and gynecological disease. However, its pharmacological efficacy and active mechanism have not been reported scientifically. Hobbs has stated some history, directions and efficacy for Vitex as a folk medicine in his writing, "Vitex: The Women's Herb" (Hobbs, 1996). It has also been described that Vitex is effective as a remedy for emmeniopathy, including amenorrhea, oligomenorrhea and menorrhagia, and that through estrogen hypersecretion before menstruation in premenstrual tension, long-term use of Vitex reduced these symptoms and prevented a relapse of the diseases. It also appears that the function of corpus luteum in corpus luteum deficiency syndrome was improved by Vitex through the crinogenic effect of its luteinizing hormones.

It has previously been reported that an anti-tumour effect results from the extract of ripened Israeli grown Vitex fruits as studied with Chinese hamster lung carcinoma

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cells, V-79, and that the methanol extract of Vitex revealed higher anti-tumour activity (Hirobe et al., 1994). Furthermore, analysis of the components of the Vitex extracts showed 4 kinds of new flavonoid compounds and a further 4 kinds of known flavonoids were found (Hirobe et al., 1997). The preliminary investigation showed that seven of the eight compounds demonstrated anti-tumour activity, and that several other un-identified components also revealed higher anti-tumour activity, and that the anti-tumour activity of the crude extract was higher than that of each individually separated compound.

Accordingly, we investigated the cytotoxicity of the Vitex extract, prepared by the same method as that used for folk medicine, against human cultured non-cancer cells, human uterine cervical fibroblast (HCF), ovarian cancer cells (SKOV-3) and breast carcinoma cells (MCF-7).

MATERIALS AND METHODS

Cells and Media

Human uterine cervical fibroblast cells (HCF) were cultured in MEM with 10% calf serum (Ojima et al., 1989). Ovarian cancer cells (SKOV-3) were provided from American Type Culture Collection (Rockville, MD, USA) and cultured in McCoy 5a with 15% calf serum (Ohyama et al., 1997). Breast carcinoma cells (MCF-7) were, as reported previously (Kosano and Takatani, 1988), cultured in RPMI-1630 with 10% calf serum.

Preparation of Methanol and Ethanol Extracts from Dried Ripened Fruit of *Vitex agnus-castus*

Dried ripened fruit of Israeli grown *Vitex agnus-castus* was triturated, and a crude extract from the triturate (1 g) was prepared with methanol and ethanol (10 mL) using reflux extraction for 2 hr at 70^{o}C. After the extract was cooled, filtered and then evaporated, it was dried in a vacuum desiccator. Methanol and ethanol extracts were named as Vitex-1 and Vitex-2, respectively, and both yields were almost the same (0.08-0.1 g from 1 g dried fruit).

Treatment of Cultured Cells with Vitex Extracts and Cell Viability Assays

Two different methods were used to investigate the cytotoxicity of Vitex-1 and -2. In order to investigate cytotoxic activity of Vitex extract against logarithmic growth phase cells, 0.1 mL of three kinds of cell suspensions were inoculated into 96-well micro-culture plates at 7500, 10000, 25000 and 50000 cells per mL in the respective culture media. Both Vitex-1 and -2 were dissolved in DMSO at 50 mg/mL and diluted to the appropriate concentrations with the respective culture medium. After 24 hr culture of cells, 0.1 mL of each Vitex-1 and -2 solution was added at 30, 70 or 100 mg/mL (the final concentrations) to quadruple culture wells. After these cultures had been incubated for 48 hr, the viability of these cells was determined by the XTT-dye reduction assay according to the method described previously (Scudiero et al., 1988). A control assay was set up by using treatment cells with the respective medium containing 0.05% DMSO without the Vitex extract. To investigate cytotoxicity of Vitex extract against stationary phase cells, each cell suspension was prepared at 1x106 cells per mL of appropriate medium, and 0.1 mL of each suspension was inoculated into a 96-well micro-culture plate. After these cells were cultured for 72 hr, 0.1 mL of Vitex-1 or -2 solution was added to each well at 1, 5, 10, 30, 70 or 100 mg/mL (at final concentration) by triplicate assay. After these cultures had been incubated for 48 hr, the XTT-dye reduction assay, as a control, was conducted according to the method described above.

Preparation of DNA and Analysis with Agarose Gel Electrophoresis

Preparation and agarose gel electrophoresis of DNA were carried out according to a method previously reported. (Ohyma et al. 1989) Harvested cells were suspended in a lysis buffer (50 mM Tris-HCl buffer, pH 7.8, containing 10 mM EDTA-2Na, 0.5% sodium-N-lauryl sarcocinate). The suspension was then incubated for 10 min at room

temperature after addition of 50 mL of 10% sodium dodecyl sulfate. The lysate was incubated successively at 50°C for 90 min with Proteinase K (Sigma) (1 mg/mL), at 50°C for 30 min with RNase A (Sigma) (1 mg/mL) and at 60°C for 15 min after addition of the same volume of NaI solution (6.0 M NaI in 26mM Tris-HCl buffer, pH 8.0, containing 13 mM EDTA-2Na and 10 mg/mL of bovine glycogen). DNA was precipitated by addition of an equal volume of isopropanol. DNA pellets were successively washed with 50% isopropanol, 100% isopropanol and 70% ethanol. The DNA sample was dispersed in a suitable volume of lysis buffer, and about 20 mg/20mL of DNA was loaded onto 2% agarose (Agarose X, Nippon gene, Tokyo) gel electrophoresis. Gels were stained with ethidium bromide (Sigma) and viewed under UV light.

Cell Stage Determination with Flow-Cytometer

The culture supernatant of SKOV-3 cells cultured for 48 hr in a 25 cm2 bottle ($3x10^6$ cells) was changed to a new medium containing Vitex-1 at 1, 10, 30, 70 or 100 mg/mL, and then these cells were cultured for 48 hr. Cells were harvested using trypsin and washed at once with PBS. These cells were suspended in pre-chilled 70% ethanol at $1\text{-}5x10^6$ cells/mL and fixed for 4 hr at 4°C. These fixed cells were washed with PBS two times and then resuspended in PBS containing 10 mg/mL of RNase, and the suspension was incubated for 30 min at 37°C. After washing with PBS at once, these cells were suspended in PBS at $1\text{-}5x10^6$ cells/0.5 mL, and 0.5 mL of 0.2% Triton X-100-PBS solution and 50 mL of 1 mg/mL propidium iodide solution were added to the cell suspension. After 30 min, these cells were used for cell-cycle distribution analysis with a flow-cytometer "FACS Calibur" (Becton Dickinson Immunocytometry Systems, CA, USA). Basic establishment of measurement and data acquisition conditions of the flow-cytometer were according to the instruction manuals provided by Phenix Flow System, Inc. and Becton Dickinson Immunocytometry System. The data obtained were analysed using software called "CELL Quest" (Becton Dickinson Immunocytometry System) for Apple Macintosh computers.

RESULTS AND DISCUSSION

Cell Growth Activity and Cytotoxic Effects of Vitex Extracts

The relationship between cell growth activity and the cytotoxic effects of Vitex extracts were investigated. HCF, SKOV-3 and MCF-7 cells were cultured for 24 hr in micro-culture plates and treated with appropriate concentrations of Vitex-1 and Vitex-2 for 48 hr. The viability of each cell type was examined by the XTT-dye reduction assay method. We have previously examined the relationship between culture cell density and its ability to form formazan, and we were able to show the linear range between them (data not shown). The growth rate in Table 1 shows a ratio for control cell growth for the treatment period (48 hr), which was calculated from the control cell population for 24 hr culture and that for 72 hr. The inhibition rate in Table 1 displays the cytotoxicity of Vitex extract at various concentrations after 48 hr, which was estimated from the cell population of the control and cell populations after treatment for 48 hr with Vitex extract. It was shown that with every cell line, the initial cell density was higher, and the formazan formation (as the growth rate) after 24 hr culture was higher, and that the growth rate and inhibition effect (as the cytotoxic activity with Vitex extract) were higher. It was also shown that the cytotoxic activity of Vitex extracts depended on their concentration. Furthermore, it was demonstrated that this effect was shown with both Vitex-1 and -2, that the effect of Vitex-2 was relatively higher than that of Vitex-1, and that the cytotoxic effect of Vitex extracts against the three kinds of cells was almost at the same level. These results suggest that the cytotoxicity of Vitex extract against these cells is closely related to the growth activity of these cells.

The Cytotoxic Effect of Vitex Extracts against these Cells at the Stationary Growth Phase

Next we investigated the cytotoxic effects of the Vitex extracts against the stationary phase cells of the growth. The initial cell density for the culture was prepared at 1 x 10^6 cells/mL, and these cells were cultured in micro-culture plates for 72 hr. After culture, various concentrations of Vitex-1 or -2 (1-100 mg/mL) were added into each culture well and the cell viability after treatment for 48 hr was measured by the XTT-dye reduction assay. Fig. 2 (a) shows that at every concentration of the extracts, the viability of HCF cells decreased 10-15% compared with that of the control (without extracts); however, dose dependency of the extract was hardly shown. The cytotoxic effect against SKOV-3 cells is shown in Fig.2 (b). With both Vitex extracts, the SKOV-3 cells' viability decreased 75% compared with that of the control. The effect against SKOV-3 cells at low concentration of these extracts (-30 mg/mL) was more rapid than at a high concentration (40-100 mg/mL). As shown in Fig. 2 (c), the cytotoxic effect of these extracts against MCF-7 cells was similar to that against SKOV-3 cells. The viability of the cells decreased to 50% till 30 mg/mL, while a further decrease in the viability was not revealed with more than 40 mg/mL. These results showed that the cytotoxic activity of both Vitex extracts against the stationary stage cells was more effective for cancer cells (SKOV-3 and MCF-7 cells) than for non-cancer cells (HCF), and that the effect was relatively higher against SKOV-3 cells than against MCF-7 cells.

Possibility of Apoptotic Cell Death

In order to study the mechanism of cell death with Vitex extract, cells at the stationary growth phase were treated with various concentrations of Vitex-2 and then the extracted DNA fractions from these cells were analysed by agarose gel electrophoresis. As shown in Fig.1, the HCF cell DNA fraction did not show any fragmentation nor degradation on a gel, while DNA fragmentation was shown with SKOV-3 cells at the lower Vitex-2 concentration. The electrophoresis pattern of DNA extracted from MCF-7 DNA was quite different from the other two DNA fractions and showed smear patterns but not fragmented ladder DNA. These results were consistent with those, shown in Fig.2, which show that HCF cells at the stationary phase were hardly affected by the Vitex extracts; however, SKOV-3 and MCF-7 cells died with Vitex treatment. Furthermore, it is suggested that the cytotoxicity of Vitex extract against SKOV-3 cells was due to apoptosis; however, that against MCF-7 cells may be from necrosis.

Relation of Vitex-2 Cytotoxicity to Growth Stages in the Cell Cycle

The relationship between the cytotoxic effects of Vitex-2 against SKOV-3 cells and growth stages in the cell cycle were investigated. SKOV-3 cells were treated with 70 and 100 mg/mL of Vitex-2 for 48 hr and stained with propidium iodide, after which the relative cell population in each growth stage in the cell cycle was analysed with a flow-cytometer. Histograms of the fluorescence intensity for SKOV-3 cells treated with 0, 70, and 100 mg/mL of Vitex-2 are shown in Fig. 3 (a), (b) and (c), respectively. A fluorescence intensity peak at 240-260 shown in each figure means G0/G1 stage cell population and the peak at intensity 480-500 reveals the G2/M stage cell population having a double dose of DNA content. The S stage cell population was shown between G0/G1 and G2/M stage cells. Dead cells appeared at a lower intensity than the G0/G1 cells' fluorescent intensity. From these analytical results, we estimated ratios of the cell population distributed to each cell stage. As shown in Table 2, the ratio of dead cells increased when treatment exceeded 30 mg/mL of Vitex-2, and the ratio was above 15% when treated with 100 mg/mL. The cell population at the G0/G1 stage in the cell cycle decreased depending on the Vitex-2 concentration. While the G0/G1 cell population of the control was 90% of the total cell population, the population decreased to 64% by treatment with 100 mg/mL of Vitex-2. On the other hand, both S and G2/M stage cells increased slightly with treatment, depending on the concentration. Furthermore, dead cells, which were not detected in control cell groups, significantly increased to 15.7%

when treated with 100 mg/mL of Vitex-2.

Hirobe et al., (1997) have reported previously that the methanol extract of Vitex contained 8 kinds of flavonoids, and that 4 of the 8 were new compounds. Furthermore, the cytotoxicity of 7 flavonoids, including the 4 new compounds, against mouse lymphocytic leukemia cells P388 was found, and a higher cytotoxic activity was found with patuletin-3, 6, 7, 3'-tetra-methylester and luteolin (preliminary results). While the cytotoxicity of both Vitex extracts against these cells may be attributed to these flavonoids, other unknown components in Vitex methanol extract also showed high cytotoxic activity. Furthermore, their results showed that the cytotoxic activity of the crude extract was significantly higher than that of any of the separated compounds. Phenomena like the cytotoxic effects of Vitex extracts have often been observed in cases investigating biological activity of natural resources. Accordingly, in this experiment we used the crude Vitex extract to investigate cytotoxic activity against human cancer cells.

At the logarithmic growth phase three kinds of cultured cells, HCF (non-cancer cells), SKOV-3 and MCF-7 (cancer cells) were treated for 48 hr with Vitex extracts, Vitex-1 (extract with methanol) and Vitex-2 (that with ethanol), and then the viability of these cells was examined by the XTT-dye reduction assay method. This method has been characterized by a good relationship between the cell density and the degree of formazan formation. In our case, we have previously confirmed the relationship between the formazan formation ability of the culture and its cell density. The results in Table 1 show that the higher the rate of cell growth, the higher the effect of cytotoxic activity was with both Vitex extracts against every cell line. This suggests a selective cytotoxicity for cancer and non-cancer cells could not be detected at the logarithmic growth phase.

On the other hand, under a confluent condition (a stationary phase) of cell growth it was shown that both Vitex extracts could not reduce formazane formation activity of HCF (non-cancer cells), but significantly reduced SKOV-3 and MCF-7 activities, and that this effect was dependent on concentrations in the lower range. It is worth notice that, at the stationary phase, the cytotoxicity of Vitex extracts was greater against cancer cells than against non-cancer cells, and that the cytotoxic effect was different against the two types of cancer cells.

It is well known that cancer cells may be characterized by an extremely high growth activity compared to normal (non-cancer) cells and by growth out of inhibition control. We consider that the efficacy of Vitex extracts specific to cancer cells and the difference of the effect on the three cell species may be attributed to the differences in their growth activity after the confluent stage. Accordingly, our results suggest that the cytotoxicity of Vitex extracts may be significantly relevant to the growth rate of cell species, but not to non-selective cytotoxicity. In other words, the cytotoxic effect of Vitex extracts against some cells, such as normal cells where growth stops after the confluent stage, is lower than that against other cells, like cancerous ones. Furthermore, the result that the effect of Vitex extracts against SKOV-3 cells at the stationary phase was higher than that against MCF-7 cells, as shown in Fig. 2, suggests that the cytotoxicity of the Vitex extract may be different towards cancer cell species. Thus, it is possible to say that Vitex has a selective cytotoxicity on cancer cell species, as well as being dependent on cell growth activity.

We investigated two possibilities to study the mechanism of cell death with Vitex extracts. One was cell death through apoptosis, and the other was the relation between the growth stage in the cell cycle and cell death. DNA fragmentation analysis of Vitex extract treated cells revealed that the DNA ladder on agarose gel appeared only with SKOV-3 cell DNA but not with that of MCF-7 cells. The biochemical phenomenon of apoptosis is characterized by DNA fragmentation (Wyllie, 1980). Thus, the results suggest that SKOV-3 and MCF-7 cells may die through apoptosis and necrosis with Vitex extracts, respectively, and that the mechanism of cell death was quite different between cancer cell species. Flow cytometric analysis results showed that the cell density at the G0/G1 stage of SKOV-3 cells decreased depending on Vitex extract concentration, while those at S and G2/M stages increased with the treatment. Thus, it is possible to consider that SKOV-

3 cells stagnating at the G0/G1 stage may be proceeded by Vitex extract stimulus, or that the cell population at the G0/G1 stage decreased from the cell death effect of Vitex extracts. However, any direct evidence indicating that the appropriate cell population of SKOV-3 cells decreased at certain cell stages in the cell cycle could not be detected. While it has been previously reported that the cell stage in the cell cycle was significantly related to apoptotic cell death (King- Cidlowski, 1998), it hardly can be said that apoptotic cell death of SKOV-3 stimulated with Vitex extracts begins at a certain cell stage.

We recognize that the results in this paper could be obtained with an *in vitro* system using cultured human cells, and that these anti-tumour effects could not directly induce the possibility of clinical application of Vitex extracts. Nevertheless, in a living body, the growth activity of cancer cells seems to be extremely higher than that of normal cells, and the functional mechanism of the anti-tumour materials may be because the materials have cytotoxic effects against certain cancer cells whose growth rate is considerably higher than that of normal cells. From this point of view, it can be presumed that the cytotoxic activity of anti-cancer components contained in Vitex extracts may be relatively low against normal cells in the living body, so that these components are applicable to clinical medical treatment with minimized adverse reaction against normal cells.

Vitex extracts, Vitex-1 and Vitex-2, used in this paper were prepared with methanol and ethanol, respectively. It was shown that the cytotoxic activity of Vitex-2 was relatively higher than that of Vitex-1. The fact that using ethanol extracted effective components from Vitex fruits with higher yield can be recommended for clinical application in respect of its safety.

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Table 1. The cytotoxic activity of Vitex extracts against human cultured cells at the logarithmic growth phase - Human cervical uterine cells (HCF), ovarian cancer cells (SKOV-3) and breast cancer cells (MCF-7) at the logarithmic growth phase were treated with various concentrations of Vitex methanol and ethanol extracts (Vitex-1 and Vitex-2, respectively) after 24 hr cultivation.

Cell number *1 means the initial cell density for the culture, growth rate *2 was estimated from the cell density of the control cultivated for 24 hr and that cultivated further for 48 hr, and inhibition rate *3 calculated from the cell density treated for 48 hr with Vitex extracts and that of appropriate control at the period. Each value represents the mean of 4 measurements.

Cell	Cell number *1 (cells/ml)	Vitex-1			Vitex-2		
		Concentration (μg/ml)	Growth rate*2	Inhibition rate*3	Concentration (μg/ml)	Growth rate*2	Inhibition rate*3
HCF	7500	0	1.03	1.00	0	0.98	1.00
		30		1.01	30		1.08
		70		1.03	70		1.20
		100		1.12	100		1.32
	10000	0	1.24	1.00	0	1.37	1.00
		30		0.94	30		0.91
		70		0.94	70		0.90
		100		1.02	100		1.00
	25000	0	1.80	1.00	0	2.54	1.00
		30		0.98	30		0.76
		70		0.82	70		0.55
		100		0.74	100		0.48
	50000	0	3.26	1.00	0	3.36	1.00
		30		0.85	30		0.89
		70		0.74	70		0.39
		100		0.50	100		0.33
SKOV-3	7500	0	1.26	1.00	0	1.26	1.00
		30		1.24	30		1.20
		70		1.22	70		1.19
		100		1.03	100		1.20
	10000	0	1.70	1.00	0	1.61	1.00
		30		1.00	30		0.90
		70		0.75	70		0.89
		100		0.80	100		0.95
	25000	0	2.78	1.00	0	2.53	1.00
		30		1.04	30		0.76
		70		0.67	70		0.68
		100		0.54	100		0.60
	50000	0	2.05	1.00	0	1.94	1.00
		30		1.16	30		1.16
		70		1.12	70		1.09
		100		0.83	100		0.90
MCF-7	7500	0	1.25	1.00	0	1.22	1.00
		30		0.74	30		0.76
		70		0.62	70		0.69
		100		0.71	100		0.75
	10000	0	1.50	1.00	0	1.96	1.00
		30		0.64	30		0.58
		70		0.59	70		0.51
		100		0.65	100		0.50
	25000	0	2.57	1.00	0	3.30	1.00
		30		0.72	30		0.61
		70		0.48	70		0.38
		100		0.45	100		0.33
	50000	0	3.57	1.00	0	3.57	1.00
		30		0.92	30		0.83
		70		0.51	70		0.44
		100		0.37	100		0.29

Table 2. Relative cell density of Vitex-2 treated SKOV-3 at each stage in the cell cycle. Relative cell population at each stage in the cell cycle of Vitex-2 treated SKOV-3 cells were estimated from the results shown in fig.3 by using the software "CELL Quest" (Becton Dickinson Immunocytometry System). Total cell population used for the analysis was 30000 cells.

Concentration of Vitex-2 (μg/ml) Stage in the cell cycle	0	1	10	30	70	100
			Relative cell population (%)			
Dead cells	0.6	0.7	0.7	1.0	3.9	15.7
G0/G1	90.3	90.9	89.6	89.3	81.3	83.6
S	4.0	3.0	3.6	3.6	6.9	9.3
G2/M	4.9	5.6	6.3	8.0	8.0	11.7

Figures

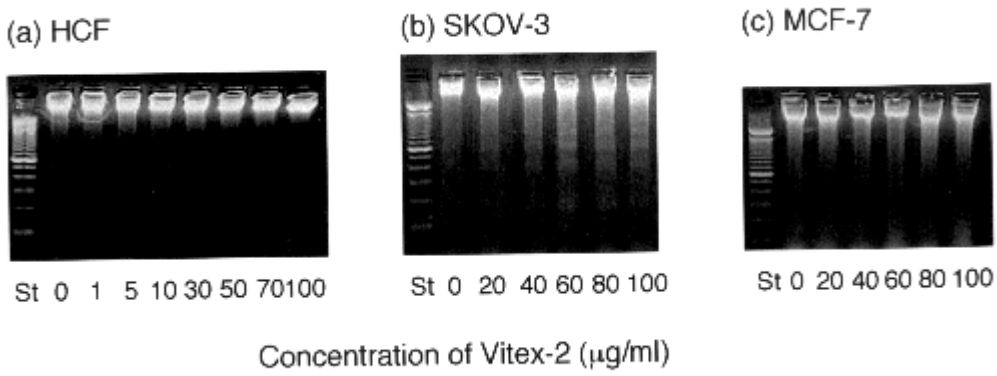

Fig.1. DNA fragmentation analysis of Vitex-2 treated human cultured cells by agarose gel electrophoresis - (a) Human cervical uterine cells (HCF), (b) ovarian cancer cells (SKOV-3) and (c) breast cancer cells (MCF-7) at the stationary growth phase were treated with various concentrations of Vitex-2 for 48 hr. DNA fractions extracted from these cells were analyzed by agarose gel electrophoresis in the presence of ethidium bromide.

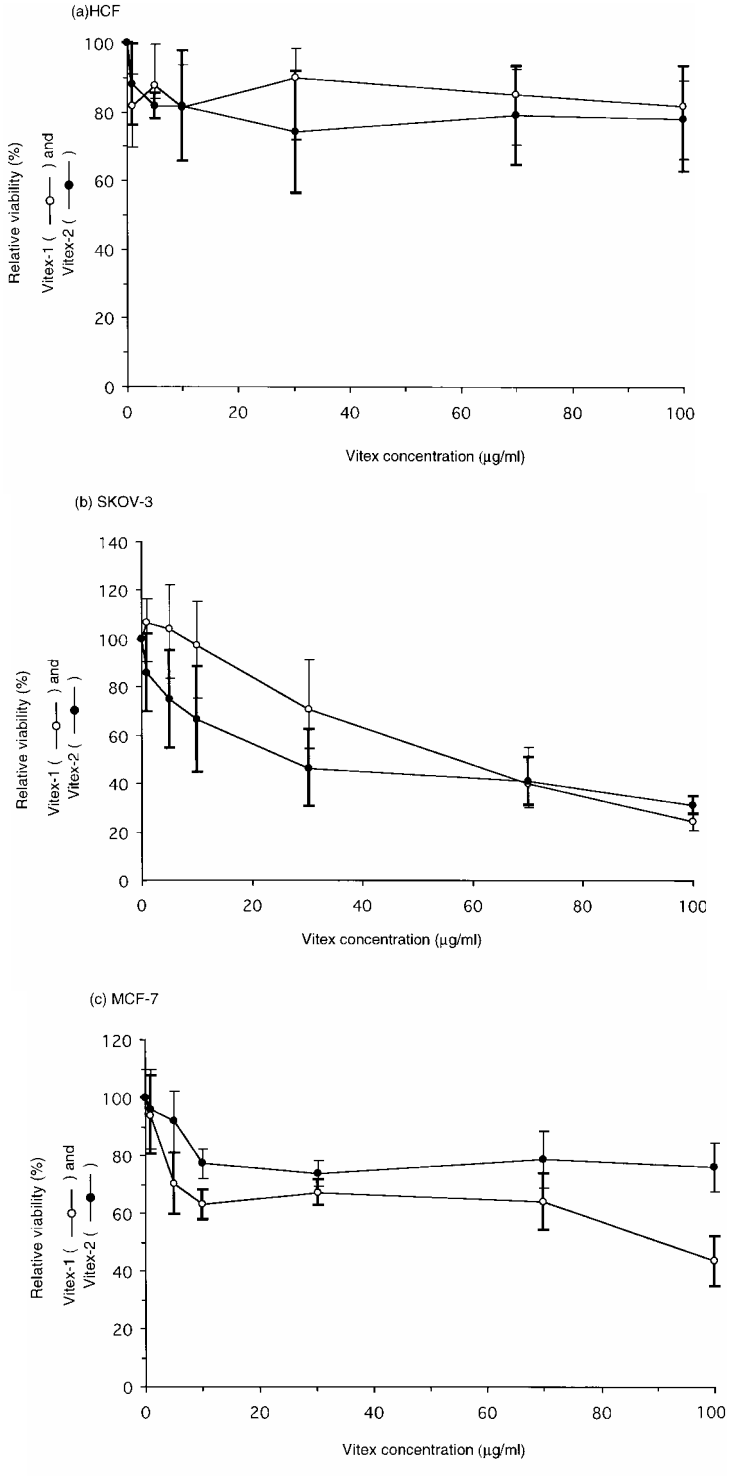

Fig.2. Dose dependent cytotoxic activities of Vitex-extracts against human cultured cells at the stationary growth phase - (a) HCF, (b) SKOV-3 and (c) MCF-7 at stationary growth phase were treated with various concentrations of Vitex-1 and Vitex-2. Relative viability of these cells was estimated by the XTT-dye reduction method. Each value represents the mean of 4 measurements with SD% of the Y axis.

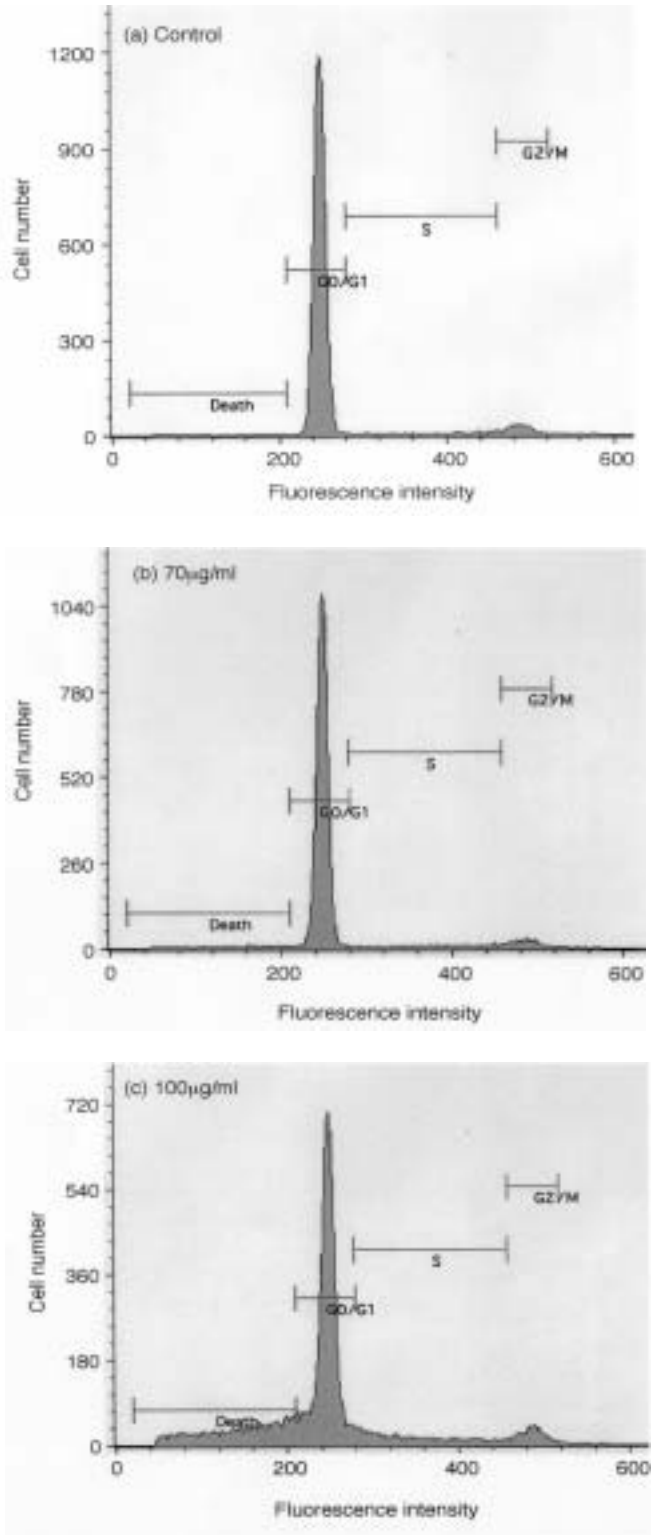

Fig.3. Analysis of growth stages in the cell cycle of Vitex-2 treated SKOV-3 cells with flow-cytometer - Stationary phase cells of SKOV-3 were treated with (a) 0 (control), (b) 70 mg/mL and (c) 100 mg/mL of Vitex-2 for 48 hr. These cells were fixed, treated with Triton X-100 and then stained with propidium iodide. The details of analysis with a flow-cytometer were described in "Materials and Methods".