Chinese Journal of Oceanology and Limnology
Vol. 28 No. 1, P. 122-130, 2010
DOI: 10.1007/s00343-010-9054-x

Anti-proliferative activity of phlorotannin extracts from brown algae *Laminaria japonica* Aresch*

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Received March 24, 2009; revision accepted Aug. 5, 2009

Abstract In this study, we evaluated the anti-proliferative activity of phlorotannins derived from brown algae *Laminaria japonica* Aresch extracts on the human hepatocellular carcinoma cell (BEL-7402) and on murine leukemic cells (P388) by MTT assay. Cells were incubated with 100 μg/mL of the phlorotannin extract (PE) for 48 h. The inhibitory rate of PE on BEL-7402 and P388 cells was 30.20±1.16% and 43.44±1.86%, respectively, and the half-inhibitory concentration of PE (IC_{50}) on P388 and BEL-7402 cells was 120 μg/mL and >200 μg/mL, respectively. Microscopic observation shows that the morphologic features of tumor cells treated with PE and 5-fluorouracil are markedly different from the normal control group. The inhibitory rate of fraction A_2 isolated from PE by sephadex LH-20 for BEL-7402 and P388 cells at the sample concentration of 70.42 μg/mL was 61.96±7.02% and 40.47±8.70%, respectively. The apoptosis peak for fraction A_2 was the most profound of all fractions used in the flow cytometry assay. The results indicate that the anti-proliferative of this algal extract is associated with the total phlorotannin content.

Keyword: *Laminaria japonica* Aresch; phlorotannins; anti-proliferative activity

1 INTRODUCTION

Algae are rich in bioactive materials including polysaccharides, phycocyanin, terpenes and fucosterol because of their unique living environment. Epidemiological data are supported by rodent model studies demonstrating the protective effects of dietary kelps and other red and green algae against mammary (Funahashi et al., 2001; Yamamoto et al., 1987; Teas et al., 1984), intestinal (Lee et al., 2003; Yamamoto et al., 1985) and skin carcinogenesis (Yamamoto et al., 1986; Higashi-Okai et al., 1999).

Fresh algae contain not only labile antioxidants (i.e., ascorbate, glutathione) (Morgan et al., 1980; Indergaard et al., 1991; Kakinuma et al., 2001; Burritt et al., 2002), but also more stable molecules such as carotenoids (Morgan et al., 1980; Okai et al., 1996; Yan et al., 1999), mycosporine-like amino acids (Nakayama et al., 1999) and a variety of polyphenols (Nakamura et al., 1996; Yoshie et al., 2000). Brown algae and some red and green algae are known to release phlorotannins into seawater directly via exudation, indirectly via tissue erosion or through cell damage (Ragan et al., 1986; Carlson et al., 1984; Jennings et al., 1994). In some species of brown algae, such as kelps used in Japanese cuisine [‘Hijiki’ (*Hijikia fusiformis*), ‘Makonbu’ (Sea Tangle, *Laminaria japonica*)] or Chinese cuisine [‘Hai dai’ (*Laminaria sp.*)] and *Sargassum*, phlorotannins account for 20% of the tissue dry weight (Steinberg, 1985, 1989) and tend to be concentrated in the outer cortical cell layers, mitotic meristematic and meiotic sporogenous tissues (Ragan et al., 1986; Tugwell et al., 1989). Phlorotannins (polyphenolics) derived from brown algae have long been considered to be important secondary metabolites playing a role in chemical deterrence (Ragan et al., 1986; Steinberg, 1992). Some researchers have reported the antioxidant activity of phlorotannin-containing extracts from a variety of kelps from China and Spain (Jiménez- Escrig

* Supported by the National Key Technology Research & Development Program of the 11th Five Year Plan of China (No. 2006BAD30B01) and the National Natural Science Foundation of China (No. 30871945)
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et al., 2001; Yan et al., 1996). Moreover, phlorotannins may also have anticarcinogenic and antibacterial effects. Brown *Laminaria* in Japan has been reported to reduce the risk of intestinal cancer in animal studies (Harada, 1997).

The evidence outlined above suggests an inhibitory role for edible seaweeds against cell proliferation. However, few have elucidated the anticarcinogenic effects of phlorotannins from brown algae such as kelp. Therefore, the objectives of the present study were to evaluate the antitumor potential of kelp extracts on the proliferation of human hepatocellular carcinoma cells (BEL-7402 cells) and murine leukemic cells (P388 cells) *in vitro*.

2 MATERIALS AND METHODS

2.1 Materials

Fresh kelp (*Laminaria japonica* Aresch) was purchased from the local aquatic product market in Qingdao between July and October, 2006. BEL-7402 and P388 cells were obtained from the Shanghai Institute of Cellular Biology, Chinese Academy of Sciences.

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluorouracil (5-FU) and dimethyl sulfoxide (DMSO) were purchased from Amresco (USA). RPMI-1640, DMEM, fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were purchased from Gibco (USA). Macroporous resin (AB-8) was purchased from Haiguanghuagong Co. Ltd. (Tianjin, China) and Sephadex LH-20 was purchased from Pharmacia (Sweden). All other chemicals were of analytical grades.

2.2 Preparation of the crude kelp phlorotannin extract (PE)

The kelp was washed with tap water to remove sediment and epiphytes, and homogenized. The resulting algal homogenate was stored at -20°C until use. Samples of homogenate (20 g) were pretreated with 70 mL of 85% ethanol in an ultrasonic chamber (25 kHz) and microwave (2 450 MHz) for 6 min and extracted at 70°C, with centrifugation at 100 r/min for 1 h, filtered, and then the residue extracted again. The two filtrates were combined and ethanol was removed by rotary evaporation (Heidolph, Germany) at 40°C.

2.3 Determination of total phlorotannins content and composition of PE

The total phlorotannin content in the PE was determined by the ferrous tartrate colorimetry method (GB8313-87, 1988), as modified by Zhang (2004). Briefly, aliquots of test samples (5 mL) were mixed with 5 mL of ferrous tartrate and 15 mL of PBS (pH 7.5) and the tube was incubated at room temperature for 15 min before reading the absorbance at 540 nm in a spectrophotometer. Propyl gallate (PG) was used as the standard material and the total polyphenol contents of the kelp extracts are expressed as PG equivalents.

The other major components of PE include polysaccharides and proteins. The polysaccharide content was determined by the phenol-sulfate acid method and the protein content was determined by the Kjeldahl nitrogen method.

2.4 Separation and purification of PE

The PE was placed at 4°C overnight to deposit solids and the supernatant was filtered and concentrated. Then, the extracts were washed with an equal volume of chloroform and petroleum ether. The aqueous phase was extracted with 250 mL of ethyl acetate and separated into two layers to obtain ethyl acetate-soluble phlorotannins (sample A) and water-soluble phlorotannins (sample B) at 50–55°C by rotary evaporation. All separations were performed at room temperature in the dark. Dynamic adsorption experiments with the samples were carried out in a glass column (10 mm inner diameter × 400 mm) wet-packed with AB-8 adsorbents (equal to 5 g of dry resin). The bed volume (BV) was 7 mL and the packing length of the resin bed was 31 cm. The concentrations of samples A and B in the feeder solution were 87.31 and 12.29 μg/mL, respectively, and the leak flow rate was 1 mL/min. After adsorption saturation, the adsorbate-laden column was washed with 2 BVs of deionized water, and desorbed with 85% ethanol at the flow rate of 1 mL/min. The desorbed liquid was applied to column chromatography (15 mm inner diameter × 400 mm) with sephadex gel (LH-20). Elution was carried out with 1 000 mL of 85% ethanol at a flow rate of 1.2 mL/min. The resulting eluate was collected (fraction size 10 mL) into test tubes using a fraction collector. Fractions with the highest absorbance at 280 nm were pooled and concentrated to a small volume to remove ethanol at 40°C by a rotary evaporator. Finally, the original PE was purified to four bioactive fractions (A_1 and A_2 which were obtained from sample A; B_1 and B_2 which were obtained from sample B.) using the isolation methods described above.

2.5 Cell culture

BEL-7402 and P388 cells were seeded at 7.5×10^3 cells/mL in 96-well plates and maintained in RPMI-1640 or DMEM containing 10% (v/v) heat-inactivated FBS. Then 180 μL or 90 μL of cell suspension per well was incubated at 37°C under 5% CO_2 (NUAIR, USA) for 24 h and used for the MTT and apoptosis assays.

2.6 MTT assay

MTT assay (Mossman, 1983; Carmichael et al., 1987) was used to measure the cell growth inhibitory activity of BEL-7402 and P388. Each experiment comprised three groups: a normal control group, a PE-treated group, and 5-FU-treated group with four wells per group. After incubation for 24 h at 37°C in a humidified atmosphere of 5% CO_2, when the cells had adhered to the plates, the kelp extracts or equal volumes of sterile water or the anti-tumor drug 5-FU (100 μg/mL) were added to the control and 5-FU groups, respectively. The mixtures were then incubated at 37°C under 5% CO_2 for a further 48 h, and 5 mg/mL of MTT stock solution (20 μL) was added to each well. After incubating for a further 4 h, the supernatants were aspirated to remove untransformed MTT and 150 μL DMSO was added to the BEL-7402 cells. After the formazan crystals had dissolved, the amount of purple formazan was determined at 570 nm with a reference wavelength of 630 nm using a microplate reader (TECAN, Austria).

P388 cells were added to 50 μL of triplet liquid (10% SDS, 5% isobutanol, 0.012 mol/L HCl (w/v/v)) and incubated at 37°C for 4 h. The plates were then left overnight and the absorbance value (A) was measured using an automated microplate reader at 570 nm. The percentage inhibition of cell proliferation was calculated as follows:

$$\%Inhibition = \frac{Abs.nm\ \text{normal} - Abs.nm\ \text{sample or drug reated}}{Abs.\ \text{normal control}} \times 100$$

For treated cells, viability was expressed as the percentage of that of normal control cells. The IC_{50} was determined to indicate the anti-proliferative activity of the tested extracts as the amount (μg/mL) of extract needed to inhibit 50% of cell growth.

2.7 Microscopic detection of the morphologic characteristics

During MTT assay, we observed the morphology changes in tumor cells (P388 and BEL-7402) by microscopy (CK-40).

2.8 Flow cytometry assay

For flow cytometric analyses, a homogenous sample of half-suspended cells in the logarithmic growth period were seeded in six-well plates at a density of 30×10^4 cells/well (180 μL per well) and grown in DMEM containing 10% FBS. After the cells were fixed, 20 μL of sample and an equal volume of sterile water and 5-FU were added at concentrations of 75 μg/mL and 45 μg/mL, respectively. After incubation for 48 h at 37°C under 5% CO_2, the cells were harvested, centrifuged (25°C, 1300 r/min, 5 min) three times, washed with PBS twice and stained with propidium iodide at 25°C for 30 min. Then, 1×10^4 cells were gated for cell-cycle analysis and apoptosis detection by a flow cytometric analyzer (Becton Dickinson, NJ).

2.9 Scavenging ability of free radicals and carcinogenic agents

The A_2 fraction, which was obtained from PE isolated by sephadex LH-20, was assayed to scavenge ·OH, O_2^-· and NO_2^- (NO_2^- was assumed as a carcinogenic agent) in different systems with tea polyphenol and Vitamin C as controls.

2.10 Statistical analysis

The results are expressed as means ± standard error of the mean and statistical significance was determined by Duncan's method using the DPS data processing system. Values of $p<0.05$ were considered significant and $p<0.01$ considered highly significant.

3 RESULTS

3.1 Composition of the kelp phlorotannin extract (PE)

The components of PE were determined (Table 1). The relative proportions of total phlorotannins, polysaccharides and protein in the kelp extract was 79.77%, 19.78%, and 0.45%, respectively. Accordingly, the main component of PE after extraction is algal phlorotannin.

Table 1 Composition of PE

Composition	Phlorotannins	Polysaccharides	Proteins
Content (μg/mL)	200.06	49.62	1.13

3.2 Effect of PE on growth of BEL-7402 and P388 cells

Hepatocellular carcinoma (HCC) is the most common malignant tumor worldwide, and leukemia is a malignant blood disease, often referred to as blood cancer. We investigated the inhibitory effects of PE on the growth of BEL-7402 and P388 cells, *in vitro* models of HCC and leukemia, and the results are shown in Table 2.

The inhibitory rate of PE on BEL-7402 and P388 cells after treatment for 48 h at the concentration of 100 μg/mL was 30.20±1.16% and 43.44±1.86%, respectively (Table 2). The difference between the PE-treated group and the 5-FU-treated group was significant (p<0.01) for BEL-7402 cells, but not for P388 cells (p>0.05). However, the effect of PE on cell growth was significantly different to that in the control group (p<0.01).

Microscopic observation showed that the morphologic features of tumor cells treated by PE and 5-FU were different to those in the normal control group (Figs.1 & 2). Furthermore, the inhibitory effect of PE and 5-FU increased with extending incubation time.

Moreover, the cytotoxicity of PE on BEL-7402 and P388 cells seems to be dose-dependent and the inhibitory rate of PE on P388 cell proliferation was superior to that on BEL-7402 cells. The dose-effect relationship showed the half-inhibitory concentration (IC_{50}) for P388 cells and BEL-7402 cells was 120 μg/mL and >200 μg/mL, respectively (Figs.3a & b).

Table 2 Inhibitory effects of PE (100 μg/mL)

Cell type	P388 cells			BEL-7402 cells		
Groups	PE-treated group	5-FU-treated group	Normal control	PE-treated group	5-FU-treated group	Normal control group
Dose (μg/well)	2.0	2.0	—	2.0	2.0	—
Absorbance value (A)	0.443 0	0.431 0	0.638 0	0.443 0	0.431 0	0.638 0
	0.439 0	0.425 0	0.639 0	0.439 0	0.425 0	0.639 0
	0.454 0	0.429 0	0.645 0	0.454 0	0.429 0	0.645 0
	0.453 0	0.432 0	0.641 0	0.453 0	0.432 0	0.641 0
Means ± SEM	0.447 3±0.007	0.429 3±0.003 1	0.640 8±0.00	0.447 3±0.007 4	0.429 3±0.003	0.640 8±0.003 1
Inhibition (%)	30.20±1.16^{B}	33.01±0.48^{C}	—^{A}	30.20±1.16^{B}	33.01±0.48^{C}	—^{A}

A, B, C, p<0.01

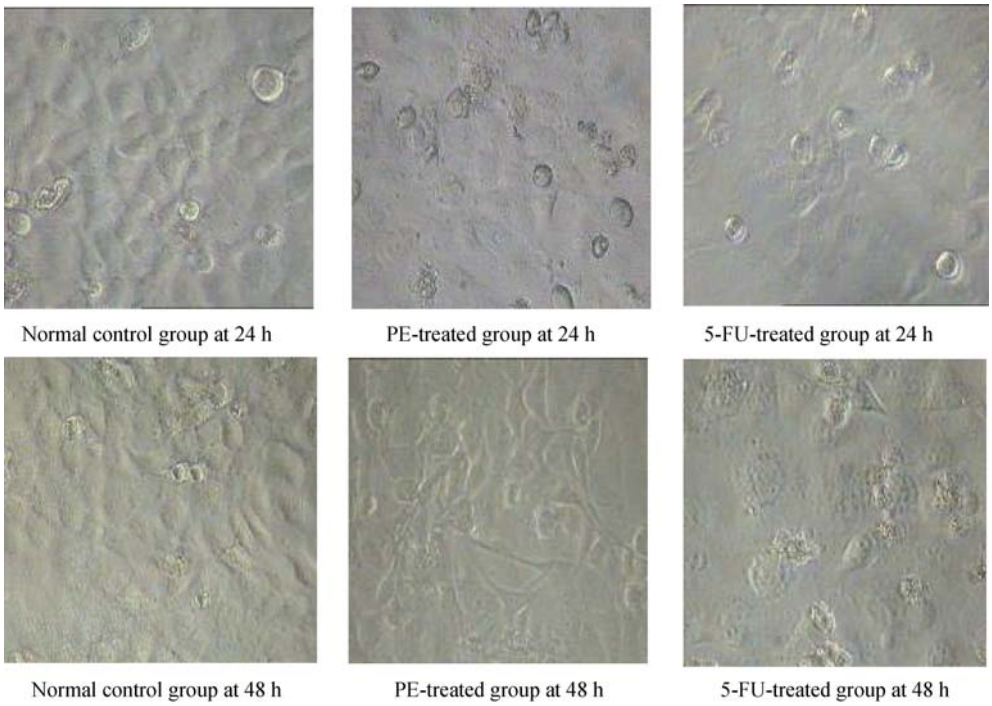

Fig.1 Morphological effects of PE and 5-FU on BEL-7402 cells

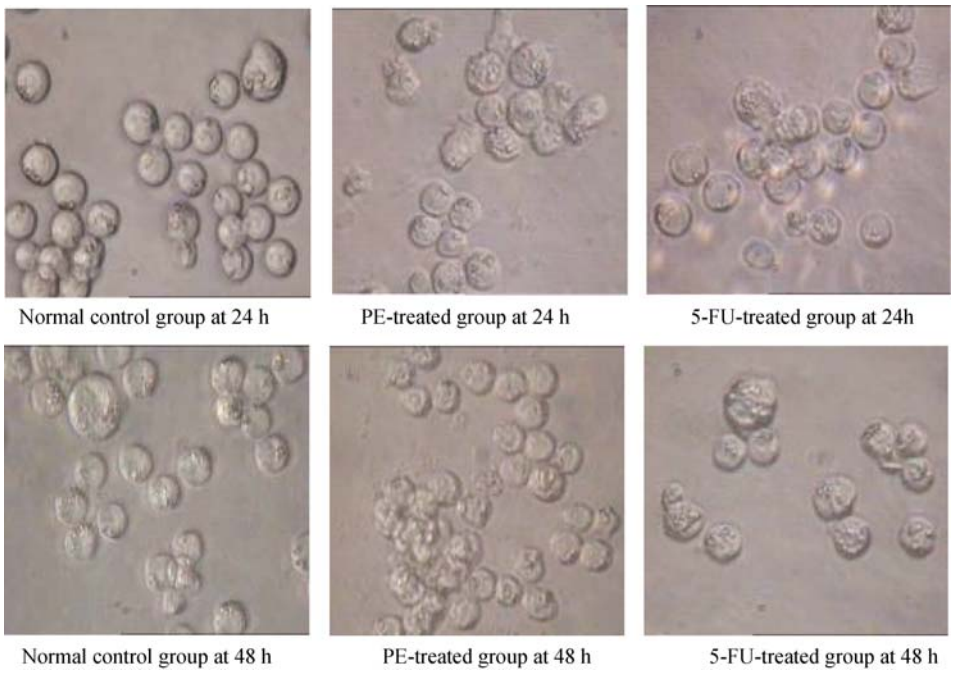

Fig.2 Morphological effects of PE and 5-FU on P388 cells

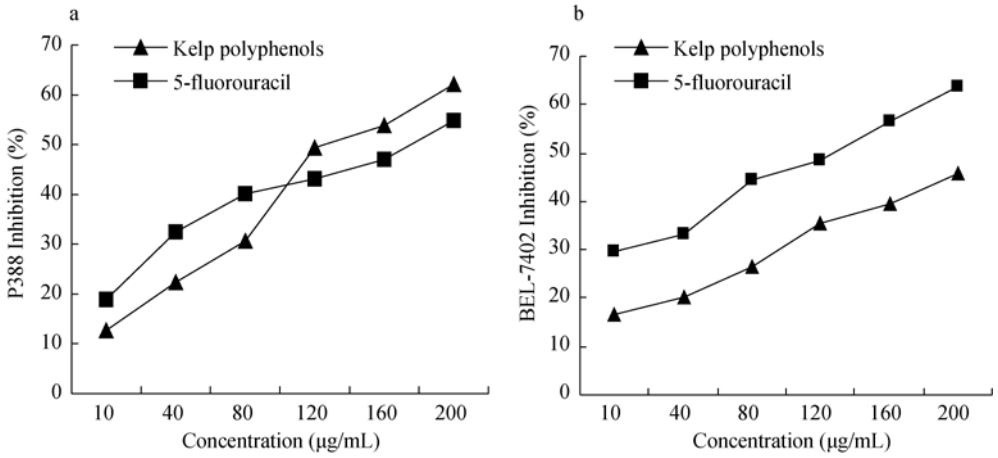

Fig.3 Dose-effect relationship of PE on P388 (a) and BEL-7402 (b) cells

3.3 Anti-proliferative effects of purified PE fractions

The four bioactive fractions (A_1, A_2, B_1 and B_2) of PE were prepared by a series of extractions with organic solvents, adsorption and desorption with AB-8 macroporous resin and column chromatography with sephadex LH-20. The color of the A_1 and B_1 fractions was yellow, and that of the A_2 and B_2 fractions was green. According to the retention time, the molecular weight of collected fractions was $A_1 > A_2$ and $B_1 > B_2$.

The results of anti-tumor experiment showed that the anti-tumor activity was fraction $A_2 > A_1 > B_2 > B_1$ (Fig.4). The inhibitory rate of fraction A_2 (70.42 μg/mL) on BEL-7402 and P388 cells was 61.96±7.02% and 40.47±8.70%, respectively. By contrast, fraction B_1 did not inhibit the proliferation of either cell line. At the same tine, fraction A_2 showed higher activity for both tumor cells than original phlorotannin extract (OPE), but other fractions showed lower activity comparing to OPE.

3.4 Effects of PE fractions on cell morphology

The morphology of the normal P388 cells treated with the A_2 fraction changed from round and regular to wizened and abnormal (Fig.5). The cell surface was rough and the numbers of cells were greatly reduced after treatment with the A_2 fraction or 5-FU for 48 h. Changes in the morphology of BEL-7402 cells were also notable after treatment with the A_2 fraction or 5-FU for 48 h. Cells were abnormal,

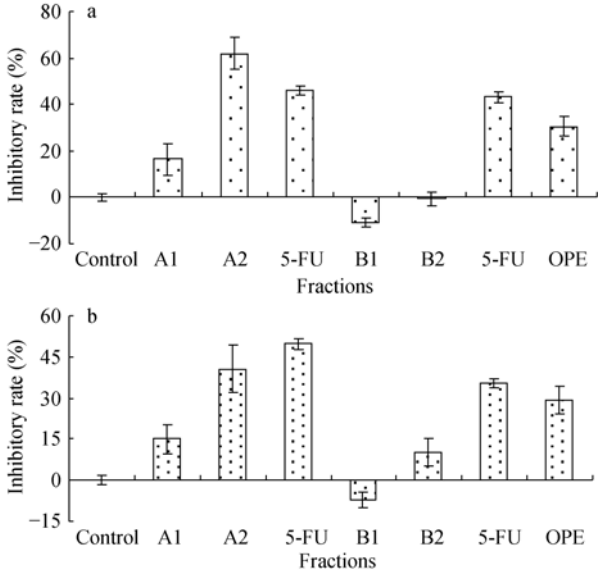

Fig.4 Inhibition activity of fractions on BEL-7402 (a) and P388 (b) cells

Vertical bars represent standard deviation values

unclear, broken up and had shrunk; furthermore, some cells had died (Fig.5).

3.5 Flow cytometry assay

Using Cell Quest software (WinMDI 2.8) analysis, the anti-tumor activity of phlorotannins was found to be related to cell apoptosis and cell proliferation cycle. An apoptotic peak for fraction A_2 was marked and was higher than that for 5-FU (Fig.6). This suggests that fraction A_2 has better anti-proliferative activity than 5-FU, but the molecular mechanism remains unclear.

3.6 Scavenging free radicals and carcinogenic agents

Fraction A_2 derived from kelp phlorotannins was able to scavenge free radicals and carcinogenic agents. The scavenging effects of fraction A_2 against ·OH, O_2^-· and NO_2^- were 75.37±5.68%, 55.48±4.35% and 50.51±6.46%, respectively (Table 3). The ability of kelp phlorotannins to scavenge free radicals and carcinogenic agents was superior to that of Tea polyphenol but inferior to that of Vitamin C at concentrations of 100 μg/mL.

Table 3 Scavenging of free radicals and carcinogenic agents with the A_2 fraction versus Tea polyphenol and Vitamin C

Experimental group (100 μg/mL)	·OH (%)	O_2^-· (%)	NO_2^- (%)
A_2 fraction	75.37±5.68^A	55.48±4.35^A	50.51±6.46^A
Tea polyphenol	80.32±3.96^A	49.27±2.60^B	37.60±2.39^B
Vitamin C	70.19±4.09^B	91.40±2.74^C	73.42±5.00^C

A, B, C, $p<0.01$

4 DISCUSSION

The bioactivities of phlorotannins have been studied widely. Cho et al. (1997) reported that methanol extracts of various kelps, including *L. japonica*, dose-dependently inhibited the growth of human gastric (AGS) and HT-29 colon cancer cells. Similarly, Lin (2005) reported that the anticancer activity of phlorotannins from *Sargassum thunbergii* Kuntze inhibited the growth of BEL-7420 liver cancer cells and A-549 lung cancer cells. The anticarcinogenic and antiproliferative roles of phlorotannins are further supported by the antitumor effect against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor growth in BALB/c 3T3 fibroblasts with 75%–87% inhibition by *Laminariales* sp. and 92% inhibition by *P. tenera* methanol extracts (Okai et al., 1994). Moreover, some researchers in Japan fed rats with algae phlorotannins and found that the phlorotannins were able to prevent cancer. Indeed, several studies *in vitro* and *in vivo* have revealed that dietary polyphenols not only have antioxidant effects, but can also prevent cancer or control the proliferation of various cancers (Funahashi et al., 2001; Yuan et al., 2005, Naasani et al., 1998).

In this paper, we demonstrated that phlorotannins obtained from *Laminaria japonica* Aresch inhibited the proliferation of two tumor cell lines. First, the present study shows the antiproliferative activities of extracts from the edible kelp species *Laminaria japonica* Aresch using HCC (BEL-7402) and murine leukemia (P388) cell lines. The kelp extracts exhibited dose-dependent inhibition against the proliferation of the BEL-7402 and P388 cells, with IC_{50} values after incubation for 48 h of 120 μg/mL and >200 μg/mL, respectively. Moreover, the anti-proliferative effects of the kelp extracts were positively correlated with the total phlorotannin content in the extracts.

The phlorotannin profile of the soluble ethyl acetate crude extracts seems to include various constituents; therefore, we purified the extract and obtained four bioactive fractions (A_1, A_2, B_1 and B_2) using organic solvent, adsorption and desorption with an AB-8 macroporous resin and column chromatography with sephadex LH-20. Fraction A_2 showed the highest activity for both tumor cells comparing to OPE and other fractions. Furthermore, the isolated fractions displayed different cytotoxity profiles. The anti-tumor activity of the ethyl acetate-soluble phlorotannins (A_1, A_2) from sample

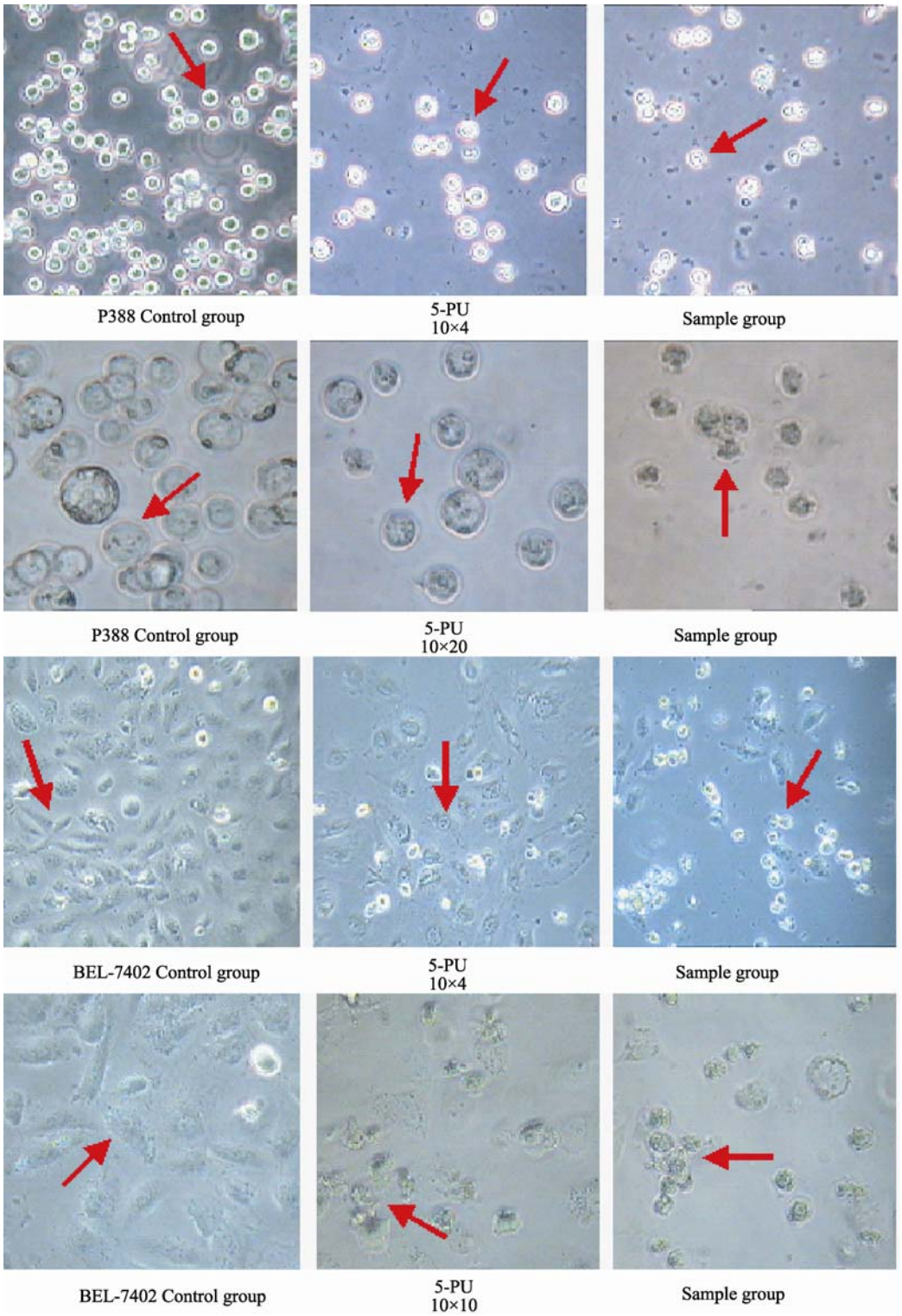

Fig.5 Microscopic observation of the effects of the A_2 fraction and 5-FU on P388 cells and BEL-7402 cells

Red arrows indicate cell morphology including shape, size and evenness

A was greater than that of the water-soluble phlorotannins (B_1, B_2) from sample B. Interestingly, the growth rate of BEL-7402 and P388 cells was promoted by fraction B_1. Similar results have been reported elsewhere (Bai, 2008), but the reasons for the effects on growth are still unknown. The molecular weight of fractions was A_1>A_2 and B_1>B_2 and the inhibitory rate of the fractions was A_2>A_1 and B_2>B_1. Because fractionation using LH-20 is based on molecular size, it is possible that the molecular weight of involved in the cytotoxicity of phlorotannins. Indeed, other researchers have already reported a relationship between molecular weight and the bioactivity of brown algal extracts (Fan et al., 1999;

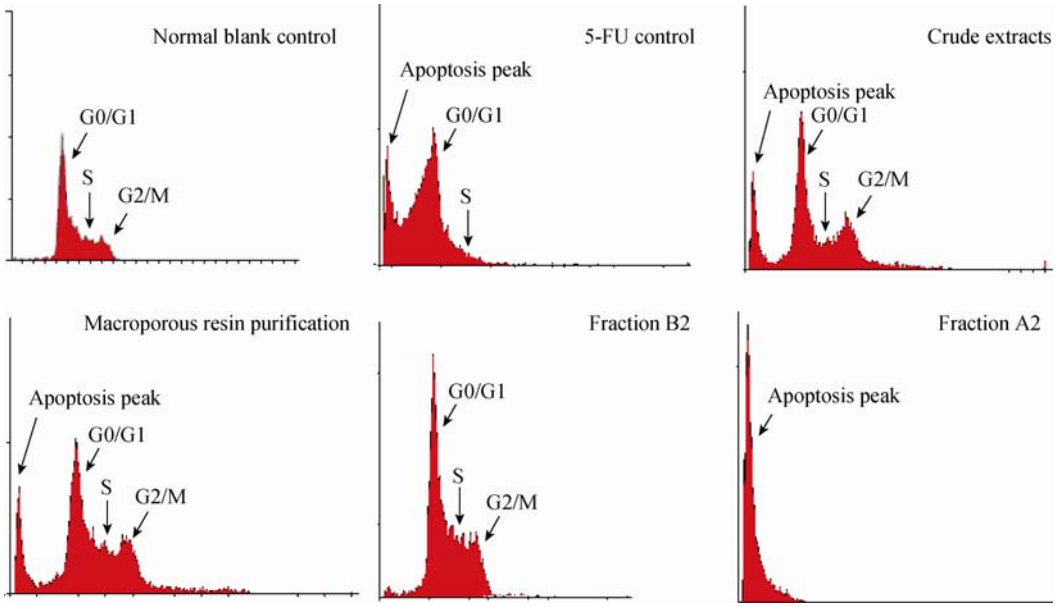

Fig.6 Detection of P388 cells by flow cytometry

Normal blank control: G0/G1, 42.62%; S, 54.85%; G2/M, 2.53%; APO, 3.85%; 5-FU: G0/G1, 72.38%; S, 27.62%; G2/M, 0%; APO, 46.76%; Crude extracts: G0/G1, 46.66%; S, 32.29%; G2/M, 21.05%; APO, 17.94%; Macroporous resin purification: G0/G1, 36.12%; S, 51.85%; G2/M, 12.04%; APO, 15.57%; Fraction B2: G0/G1, 37.59%; S, 57.03%; G2/M, 5.38%; APO, 5.60%; Fraction A2: G0/G1, 0%; S, 0%; G2/M, 0%; APO, 93.36%

Wei et al., 2003). According to the infrared spectrum, the structures of fraction A_1 and A_2 are broadly similar to those of PG and pyrogallol (data not shown). Therefore, further studies are needed to elucidate the relationship between bioactivity and molecular weight or structure, which could help to identify the anti-proliferative molecules contained in kelp extracts.

In this study, the anti-proliferative activity of phlorotannins extracted from the brown algae *Laminaria japonica* Aresch was preliminarily determined. Future studies are needed to investigate the effect of this extract and phlorotannins in particular on many other tumors before clinic application. However, the extraction of pure phlorotannins from algae is technically demanding and expensive. Fortunately, the crude extract itself is effective. Microscopic observation of P388 and BEL-7402 cells treated with PE indicate that the PE inhibits growth by disrupting the cell membrane. This effect might be a characteristic of phlorotannins for the inhibition of growth or inducing cell death of cells. On the other hand, the antitumor activity of phlorotannins might be related to its ability to scavenge free radicals and carcinogenic agents. Nevertheless, the mechanisms involved in the anti-proliferative activity of phlorotannins remain to be elucidated.

Laminaria japonica Aresch is widely used as foodstuff in some regions in Asia, particularly in coastal regions of China. Therefore, the use of *Laminaria japonica* Aresch may offer a safe method to utilize the anti-proliferative activity of phlorotannins to reduce the incidence of cancer. Studies of the bioactivity of algae phlorotannins will contribute to the knowledge of marine-derived compounds and their use in the processed and functional food industries.

5 ACKNOWLEDGEMENT

The authors thank Mr. ZHU Tianjiao and Mr. ZHAO Yuanhui for their technical support.

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