PHYTOTHERAPY RESEARCH
Phytother. Res. (2008)
Published online in Wiley InterScience
(www.interscience.wiley.com) **DOI**: 10.1002/ptr.2496

Involvement of the Bcl-2 Family Members in *Pinus massoniana* Bark Extract induced Apoptosis in HeLa Cells

Hongling Ma[1], Fei Lai[1], Heng Xie[2], Jinfa Wang[1] and Hongbin Wang[1]*

[1]The State Key Laboratory of Biocontrol and The Key Laboratory of Gene Engineering of Ministry of Education, School of Life Science, Sun Yat-sen (Zhongshan) University, Guangzhou 510275, China
[2]Institute of Songzhen Nutritional Resource, Guangzhou 510645, Guangdong Province, China

***Pinus massoniana* bark extract (PMBE) contains a variety of flavonoids whose antioxidant properties have been confirmed *in vitro*. This study was undertaken to evaluate the cytotoxic effects and the mechanism of cell death on the PMBE-treated human cervical cancer cell line, HeLa. PMBE treatment led to cell growth inhibition in a dose- and time-dependent manner, and PMBE-induced apoptosis was confirmed by DAPI staining, TUNEL assays and sub-G1 phase accumulation. Cell cycle was also arrested in G2/M phase. Immunoblotting analysis showed that cytochrome *c* was released, the protein expression of Bax was increased, the protein expression of Bcl-2 was down-regulated and caspase-9 and -3 were activated in PMBE-treated HeLa cells. Taken together, PMBE inhibited proliferation, induces apoptosis and causes cell cycle arrest in HeLa cells, indicating that PMBE may be a potential therapeutic agent for cancer.**

Keywords: apoptosis; Bax; HeLa; mitochondria; *Pinus massoniana.*

INTRODUCTION

In recent years, investigations have focused on natural plant components with potential cancer inhibition effects. Flavonoids are polyphenols that are ubiquitous in fruits, vegetables, grains, bark, roots, stems, flowers, tea and wine (Middleton, 1998), which exert multiple biological effects including vascular protection, anti-inflammatory and antiallergic responses, as well as antiviral and antitumor activities (Di Carlo *et al.*, 1999; Nijveldt *et al.*, 2001). The pine tree contains many flavonoids (Packer *et al.*, 1999). *Pinus massoniana* Lamb of the Pinaceae family is an indigenous tree found throughout China. Its needle, bark, pollen and turpentine have been used in Chinese folk medicine for the treatment of haemorrhage, rheumatism, arthralgia, inflammation and cancer, which have been listed in Shennong's Classic of Materia Medica and the Pharmacopoeia of the People's Republic of China. Our studies have shown *Pinus massoniana* bark extract (PMBE) has strong, dose-dependent antioxidant and radical scavenging activities, compared with well-known antioxidants such as α-tocopherol, butylated hydroxyanisole, butylated hydrosytoluene and quercetin (Cui *et al.*, 2005a). PMBE also selectively induced apoptosis of human liver cancer Bel-7402 cells without impacting the growth of normal liver L-02 cells (Cui *et al.*, 2005a). However, the mechanism by which PMBE induces apoptosis is poorly understood.

This study investigated the effects of PMBE on the proliferation and apoptosis of human cervical cancer cell line HeLa and elucidated the possible molecular mechanism. The experiments showed that treatment of HeLa cells with PMBE significantly reduced cell viability, which correlated with apoptosis and cell cycle arrest. Activations of caspase-9 and -3 were involved in PMBE-induced apoptosis. The results also showed that apoptosis occurred via the mitochondrial pathway, as shown by the translocation of cytochrome *c* from mitochondria into the cytosol, and changes in Bax and Bcl-2 protein expression. These findings suggest that PMBE may be developed into an anticancer therapeutic agent.

MATERIALS AND METHODS

Materials. RPMI1640 media, penicillin-streptomycin and trypsin were obtained from Hyclone (Logan, Utah, USA). Fetal bovine serum (FBS) was purchased from Sijiqing (Hangzhou, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cisplatin (*cis*-diaminedichloroplatinum (II), CP), DAPI (4′-6-diamidino-2-phenylindole) and DMSO reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA). CP was prepared freshly as 50 mg/mL stocks in DMSO. The human caspase-9, Bax and cytochrome *c* antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Human Bcl-2, caspase-3 and actin antibodies were purchased from Neomarker (Fremont, CA, USA).

* Correspondence to: Dr Hongbin Wang, The State Key Laboratory of Biocontrol and The Key Laboratory of Gene Engineering of Ministry of Education, School of Life Sciences, Sun Yat-sen (Zhongshan) University, Guangzhou 510275, China.
E-mail: wanghb@mail.sysu.edu.cn
Hongling Ma and Fei Lai contributed equally to this work.
Contract/grant sponsor: Natural Science Foundation of Guangdong Province, China; contract/grant number: 2004b10401044.

Received 12 October 2007
Revised 19 November 2007
Accepted 14 December 2007

Preparation of extract from *Pinus massoniana* bark. *Pinus massoniana* bark was collected from Chenzhou, Hunan Province, China in May 2005. The plant material was authenticated by an engineer from the Institute of Songzhen Nutritional Recourses (Guangdong, China). A voucher specimen (050500) was deposited in our laboratory for future reference.

PMBE was provided by the Institute of Songzhen Nutritional Recourses (Guangdong, China), and was prepared as described previously (Cui *et al.*, 2004). In brief, after being screened, baked and crushed, the pine bark powder was dissolved in pure water and filtered through a fibre membrane to remove macro-impurities. The liquid was concentrated by filtering to remove saccharides, inorganic salts and water, and the final compound was dried. The extraction yield was approximately 4‰ (w/w). PMBE was standardized by quantification of a reference standard with HPLC system (Hp 1100, Palo Alto, CA, USA). The reference standard was epigallocatechin galloate.

PMBE powder was dissolved in DMSO (dimethyl sulfoxide) to make a 100 mg/mL solution, which was sterilized through a 0.2 μm polytetrafluoroethylene (PTEF) micro-filter (Millipore, Bedford, MA, USA) and stored at –20 °C. For HeLa cell treatment, PMBE solution was diluted to 10 mg/mL with RPMI1640 media containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin.

Cell culture. The human cervical cancer cell line, HeLa, was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). HeLa cells were grown in RPMI1640 media supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 humidified atmosphere at 37 °C.

Cell growth inhibition assay. HeLa cells were seeded in a 96-well plates at 7000–10 000 cells per well and treated with PMBE at concentrations of 0, 40, 80, 120 μg/mL. The cells were treated with 10 μg/mL of CP as a positive control, which was used as an important chemotherapeutic agent for cancer treatment. At 72 h post-treatment, 20 μL of a 5 mg/mL MTT stock solution was added into each well, and the cells were incubated for an additional 4 h. DMSO (150 μL) was added to each well to block the reaction. The OD values were read on a Multiskan MK3 microplate reader (Thermo Labsystems, Helsinki, Finland) with a wavelength of 570 nm. The 50% inhibitory concentration (IC_{50}) value was calculated by regression analysis. To determine the time-dependent effects of PMBE, HeLa cells were treated with 120 μg/mL of PMBE or 10 μg/mL of CP and MTT assays were conducted every 24 h.

Flow cytometry analysis. To determine cell cycle distribution, 2×10^5 HeLa cells were plated in 6-well plates, incubated overnight and allowed to reach 70%–80% confluency. The cells were treated with PMBE (0, 40, 80, 120 μg/mL) for 24, 48 and 72 h. After treatment, the attached cells were trypsinized and collected with the floating cells in the RPMI1640 media supernatant by centrifugation (1000 × **g**, 5 min). The cells were washed in PBS and fixed in 70% ethanol at 4 °C overnight. After fixing, the cells were washed with PBS and resuspended in 200 μL of PBS containing 20 μg/mL RNase A and 50 μg/mL propidium iodide, and incubated in the dark for 30 min at room temperature before analysis on a Coulter Epics Elite flow cytometer (Beckman Coulter Co., Miami, FL, USA). The cell cycle was analysed using MultiCycle software (Phoenix Flow System, San Diego, CA, USA). The proportion of apoptotic cells was measured using WinMDI 2.9 software.

Analysis of apoptotic cells by DAPI staining. HeLa cells seeded on coverslips were treated with or without 120 μg/mL of PMBE for 72 h. Then the cells were rinsed with PBS containing 1.0 M Ca^{2+} and 0.5 M Mg^{2+} and resuspended in 1 mL of 0.1% DAPI prepared in methanol for 15 min. The cells were rinsed with PBS three times. Nuclear morphology was analysed using a fluorescent microscope (Olympus BH-2, Osaka, Japan).

Analysis of apoptotic cells by TUNEL. HeLa cells were treated with 0, 40, 80 or 120 μg/mL of PMBE for 72 h. After treatment, the cells were collected and washed twice with PBS. Then the cells were processed using the *In situ* Cell Death Detection Kit (Roche, Mannheim, Germany). Briefly, the cells were fixed with freshly prepared 2% paraformaldehyde in PBS (pH 7.4) for 60 min at room temperature. After washing in PBS, the cells were resuspended in freshly prepared permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. The cells were washed again and resuspended in a terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL) reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein-dUTP and incubated for 60 min at 37 °C in a humidified atmosphere in the dark. The samples were detected by flow cytometry at an excitation wavelength of 488 nm and a detection wavelength of 515–565 nm.

Protein extraction and immunoblotting analysis. The cellular cytoplasmic fractions were prepared using the method described by Finucane *et al.* (1999) with a minor modification. Briefly, 1×10^6 HeLa cells were seeded in 75 mL flasks, incubated overnight and allowed to reach 70%–80% confluency. Then they were treated with PMBE (0, 40, 80, 120 μg/mL) for 72 h. After treatment, the cells were collected and washed twice with PBS and the cell pellet was resuspended in ice-cold cell extract buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 220 mM mannitol, 68 mM sucrose) with 1% PMSF added before use and incubated for 1 h at 4 °C. The lysates were homogenized with 40 strokes using a homogenizer, and the homogenates were centrifuged at 12 000 × **g** for 5 min at 4 °C. The supernatants were collected and further centrifuged at 100 000 × **g** for 30 min at 4 °C to obtain cytosol. Total protein was extracted with RIPA lysis buffer (PBS containing 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate and 5 mM EDTA) with 1% PMSF added before use. For each sample, 30 μg of protein was separated by 12% or 15% SDS – polyacrylamide gel electrophoresis (Sigma, St Louis, MO, USA) and transferred to polyvinylidene fluoride membranes (Pall Corporation, East Hills, NY, USA). The blots were incubated with the corresponding mouse/rabbit monoclonal/polyclonal IgGs and antibody reactions with anti-mouse or anti-rabbit IgGs-HRP

DOI: 10.1002/ptr

were detected by the ECL western blotting system (Amersham, Piscataway, NJ, USA).

Statistical analysis. All data are expressed as mean ± standard deviation (SD) from three independent experiments, and the level of significance between two groups was assessed with Student's *t*-test. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Effect of PMBE on HeLa cell viability

A dose-dependent decrease in HeLa cell viability was observed after 72 h treatment with 0, 40, 80 or 120 μg/mL PMBE (Fig. 1A) and the IC_{50} value of PMBE was 67.91 μg/mL. To determine the time course, the cells were treated with 120 μg/mL of PMBE or 10 μg/mL CP, and MTT assays were conducted every 24 h. The PMBE or CP-treated HeLa cell viability decreased gradually in a time-dependent manner (Fig. 1B).

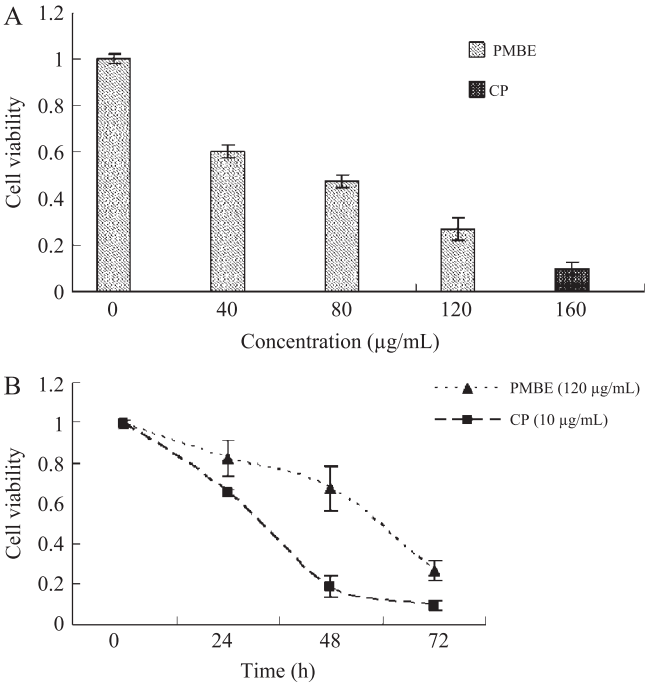

Figure 1. Effect of PMBE on inhibiting growth of Hela cells in a does- and time-dependent manner. (A) Hela cells were treated with 0, 40, 80, 120 μg/mL of PMBE or 10 μg/mL of CP for 72 h and cell viability was detected with MTT assays. (B) Hela cells were treated with 120 μg/mL of PMBE or 10 μg/mL of CP. MTT assays were performed at the 0, 24, 48 or 72 h to detect cell viability. (The values are expressed as means ± SD from three independent experiments.)

Effect of PMBE on cell cycle distribution

In order to investigate whether changes in cell cycle regulation were responsible for the observed antiproliferative effects of PMBE, the cell cycle distribution was evaluated using flow cytometric analysis. Compared with the control, PMBE treatment resulted in an appreciable arrest of cells in G2/M phase of cell cycle (Table 1). In addition, the cells accumulated in the sub-G1 phase with increased time and concentration of PMBE treatment (Table 1). These results indicate that PMBE induces apoptosis and cell cycle arrest in G2/M phase in HeLa cells.

Effect of PMBE on apoptosis induction

To further investigate whether PMBE causes apoptosis in HeLa cells, DAPI staining and TUNEL assays were performed. As shown in Fig. 2A, condensed chromatin and apoptotic bodies were observed in treated HeLa cells, indicating apoptosis. PMBE-treated HeLa cells both had a significant apoptotic index in contrast to the non-treated cells, analysed by TUNEL assay (Fig. 2B), indicating that PMBE induces apoptosis after 72 h treatment.

Effects of PMBE on regulation of apoptosis related proteins

To elucidate the apoptotic pathways involved in PMBE-treatment, the expression of caspase-3 and -9 was

Table 1. Effect of PMBE on cell cycle distribution in HeLa cells

Time (h)	Treatment (μg/mL)	Distribution of sub-G1	Distribution of cell cycle		
			G0/G1	S	G2/M
24	Control	3.2 ± 0.7	67.9 ± 1.2	22.7 ± 1.0	9.5 ± 0.5
	PMBE (40)	6.9 ± 2.8	66.5 ± 1.7	23.3 ± 2.7	10.3 ± 1.6
	PMBE (80)	15.8 ± 6.5[a]	65.6 ± 2.4	24.0 ± 3.8	10.4 ± 1.4
	PMBE (120)	23.2 ± 12.2[a]	63.0 ± 2.6[a]	20.6 ± 3.9	16.3 ± 2.9[a]
48	Control	7.7 ± 5.5	69.2 ± 1.0	22.5 ± 1.2	8.3 ± 1.1
	PMBE (40)	19.6 ± 6.5	64.8 ± 5.7	24.0 ± 1.7	11.1 ± 4.6
	PMBE (80)	27.1 ± 5.9[a]	52.5 ± 4.1[b]	22.1 ± 3.3	25.4 ± 2.0[b]
	PMBE (120)	33.5 ± 10.6[a]	51.0 ± 10.2[a]	21.9 ± 2.1	27.1 ± 9.1[a]
72	Control	7.0 ± 5.1	64.5 ± 1.9	25.5 ± 2.7	10.0 ± 3.5
	PMBE (40)	27.7 ± 5.7[b]	58.6 ± 4.3	31.0 ± 5.3	10.4 ± 4.5
	PMBE (80)	40.0 ± 1.2[b]	41.3 ± 7.4[b]	29.8 ± 4.4	29.0 ± 9.4[a]
	PMBE (120)	47.2 ± 2.8[b]	37.8 ± 9.0[b]	35.3 ± 7.6	26.9 ± 5.7[a]

Numeric data are expressed as mean ± SD from three independent experiments, indicating proportion of the cells in sub-G1 and different cell cycle phases (G0/G1, S, and G2/M). [a] $p < 0.05$ and [b] $p < 0.01$ represent significant differences between the experimental and the control group.

DOI: 10.1002/ptr

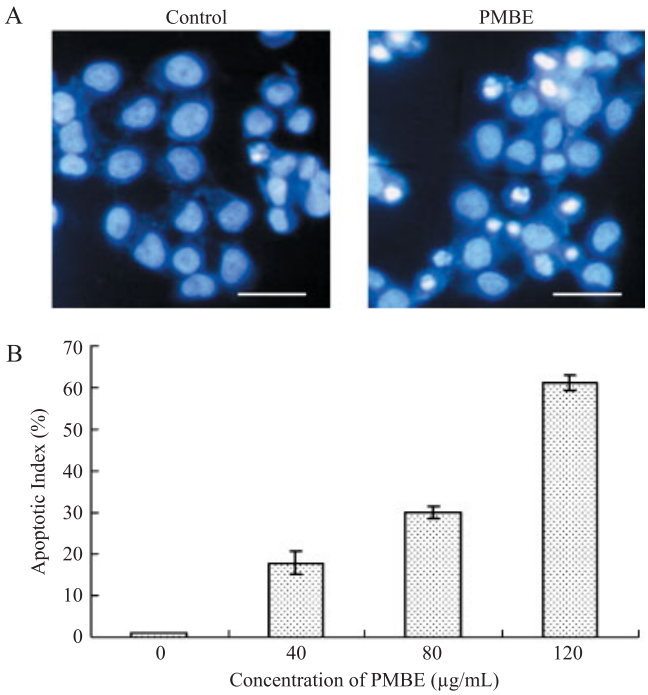

Figure 2. Effect of PMBE on inducing apoptosis on Hela cells. (A) Hela cells were treated with or without 120 μg/mL of PMBE for 72 h and the nuclear morphological changes were detected by DAPI staining. Scale bar: 25 μm. (B) Hela cells were treated with or without PMBE for 72 h and apoptotic indexes were detected with TUNEL assay. (The values are expressed as means ± SD from three independent experiments; each was performed in triplicate.)

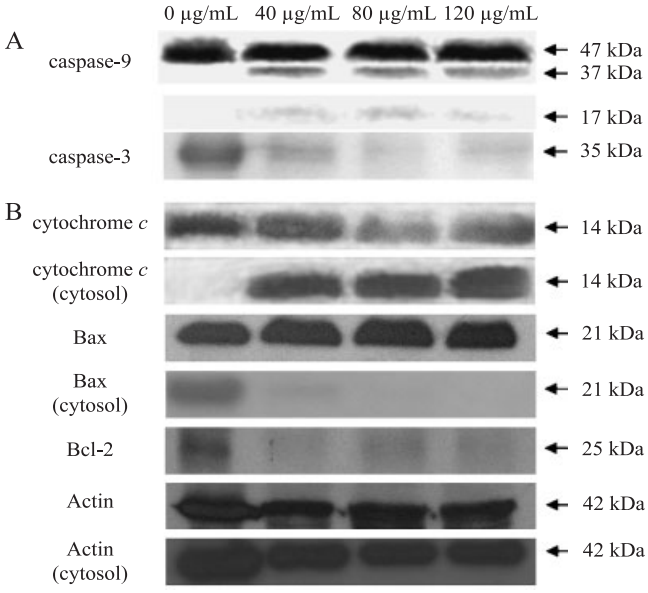

Figure 3. Immunoblotting analysis of effects of PMBE on regulation of apoptosis related proteins. (A) Total extracts were analyzed to examine the caspases activation. (B) Cytosolic extracts were analyzed to examine the subcellular redistribution of cytochrome c and Bax. Total extracts were also used to examine the effect of PMBE on the basal expression level of cytochrome c, Bax and Bcl-2. Actin was used for loading control.

examined. A reduction of the inactive form of caspase-3 was seen 72 h after treatment with 40 μg/mL of PMBE, and the bands almost disappeared completely after 72 h of treatment with 80 μg/mL of PMBE treatment, indicating complete activation (Fig. 3A). The active form (37 kDa or 17 kDa fragments) of caspase-9 was also seen in HeLa cells treated with 40, 80 or 120 μg/mL of PMBE for 72 h, indicating that the 47 kDa procaspase-9 form was activated (Fig. 3A).

To confirm apoptosis via the mitochondrial pathway in PMBE-treated HeLa cells, cytochrome *c* release and Bax translocation were detected. The results of these studies indicated that cytochrome *c* was released from the mitochondria to the cytosol and Bax was translocated from the cytosol to mitochondria in HeLa cells treated with PMBE (Fig. 3B).

The protein expression levels of proapoptotic protein Bax and antiapoptotic protein Bcl-2 were examined in PMBE-treated HeLa cells. The results showed that Bax expression was increased by PMBE treatment at the protein level; however, PMBE treatment greatly down-regulated the expression of Bcl-2 protein (Fig. 3B).

DISCUSSION AND CONCLUSION

Pine bark extract has been reported to have antioxidant and strong free radical-scavenging activities against reactive oxygen radicals (Rice-Evans *et al.*, 1996; Tourino *et al.*, 2005). Huynh and Teel (2000) found that pycnogenol extracted from the bark of *Pinus maritima* selectively induced apoptosis in human mammary cancer cells (MCF-7) but not in normal human mammary MCF-10 cells. Pycnogenol induces differentiation and caspase-3 dependent apoptosis in leukaemia cells HL-60, U937 and K562 (Huang *et al.*, 2005). Many studies of various plant extracts are being undertaken to screen compounds that cause apoptosis of cancer cells (Kwon *et al.*, 2006; Li *et al.*, 2005; Zheng *et al.*, 2005). In this study, it was found that PMBE significantly inhibits cell viability in HeLa cells in a dose- and time-dependent manner (Fig. 1). Moreover, it was found that the growth inhibition of PMBE-treated HeLa cells was associated with apoptosis. Nuclear fragmentation in PMBE-treated HeLa cells were detected. Condensed chromatin and DNA fragmentation indicate that PMBE induces apoptosis in HeLa cells (Fig. 2 and Table 1). Because in certain situations, induction of apoptosis occurs when a cell receives opposing signals regulating proliferation or cycle arrest (Casaccia-Bonnefil, 2000), the cell cycle distribution and apoptosis induced by PMBE in HeLa cells were analysed by flow cytometry. The results showed that cell cycle was arrested in G2/M phase with a decreased G0/G1 population, but notable cell cycle arrest occurred later than apoptosis when treated by the same dose of PMBE (Table 1). These findings indicate that PMBE can directly induce apoptosis, which seems to be primary to G2/M phase arrest to the cell proliferation inhibition in HeLa cells. Although G2/M phase arrest might be the result of apoptosis induced by PMBE in HeLa, the possibility that PMBE directly induces cell cycle arrest in G2/M phase cannot be excluded.

Mitochondria play an important role in apoptosis. Previously, the involvement of mitochondria in PMBE-mediated apoptosis was uncertain. It was found that cytochrome *c* release is involved in PMBE-induced apoptosis and induces subsequent caspase-9 and caspase-3 activation (Fig. 3). Proteins of the Bcl-2 family are

DOI: 10.1002/ptr

important mitochondrial pathway regulators of apoptosis. Bax was identified as the first pro-apoptotic member of the Bcl-2 family and Bax translocation can trigger the release of cytochrome *c* (Eskes *et al.*, 1998; Hsu *et al.*, 1997). The experiments reveal that PMBE not only elevates the Bax protein expression level, but also induces Bax translocation from the cytosol to the mitochondria (Fig. 3B). In contrast, Bcl-2 is an antiapoptotic protein. Bcl-2 overexpression prevents the translocation of cytochrome *c* from the mitochondria to the cytosol (Yang *et al.*, 1997) and can prevent caspase-3 activation and the initiation of apoptosis (Rosse *et al.*, 1998). In this study, PMBE greatly decreased Bcl-2 expression to initiate apoptosis (Fig. 3B). These results strongly indicate that mitochondrial death signaling pathway plays a role in PMBE-mediated HeLa cell apoptosis, and the proapoptotic Bcl-2 family members are also involved in the process of apoptosis. Taken together, the results suggest that PMBE exhibits potential as a therapeutic treatment for cancer.

With improvements in standardizing production and quality control, traditional herbal medicine continues to become more accepted. Certain flavonoid extracts have exhibited great potential as cancer chemopreventive or therapeutic agents by inhibiting the growth of cancer cells and inducing apoptosis. Flavonoids purified from *Rhus verniciflua* Stokes (RVS) are capable of inhibiting proliferation and inducing apoptosis in the human B lymphoma cell line BJAB and osteosarcoma cell line HOS (Jang *et al.*, 2005; Lee *et al.*, 2004). Activation of caspase-3 and Bax, the inhibition of Bcl-2 expression, and the release of cytochrome *c* are involved in the RVS chloroform–methanol fraction (RCMF)-mediated apoptosis of HOS cells (Jang *et al.*, 2005). Abnormal Savda Munziq (ASMq) total flavonoid treatment also significantly up-regulates Bax gene expression and down-regulates Bcl-2 gene expression, similar to PMBE (Yusup *et al.*, 2006). However, more experiments need to be done to identify further anticancer mechanisms of PMBE.

In conclusion, PMBE can inhibit cell viability, and induce apoptosis through the mitochondrial apoptosis pathway, and cause cell cycle arrest in HeLa cells *in vitro*. These results provide a foundation for the future development of PMBE for cancer treatment.

Acknowledgement

This work was supported by a grant from the Natural Science Foundation of Guangdong Province, China (No.2004b10401044).

REFERENCES

Casaccia-Bonnefil P. 2000. Cell death in the oligodendrocyte lineage: a molecular perspective of life/death decisions in development and disease. *Glia* **29**: 124–135.

Cui YY, Xie H, Lai F, Wang JF. 2004. Preliminary study on antioxidative activations of *Pinus massoniana* bark extract (PMBE). *Food Sci* **25**: 179–183 (Chinese).

Cui YY, Xie H, Qi KB, He YM, Wang JF. 2005a. Effects of *Pinus massoniana* bark extract on cell proliferation and apoptosis of human hepatoma BEL-7402 cells. *World J Gastroenterol* **11**: 5277–5282.

Cui Y, Xie H, Wang J. 2005b. Potential biomedical properties of *Pinus massoniana* bark extract. *Phytother Res* **19**: 34–38.

Di Carlo G, Mascolo N, Izzo AA, Capasso F. 1999. Flavonoids: old and new aspects of a class of natural therapeutic drugs. *Life Sci* **65**: 337–353.

Eskes R, Antonsson B, Osen-Sand A *et al.* 1998. Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg^{2+} ions. *J Cell Biol* **143**: 217–224.

Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG, Green DR. 1999. Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. *J Biol Chem* **274**: 2225–2233.

Hsu YT, Wolter KG, Youle RJ. 1997. Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. *Proc Natl Acad Sci USA* **94**: 3668–3672.

Huang WW, Yang JS, Lin CF, Ho WJ, Lee MR. 2005. Pycnogenol induces differentiation and apoptosis in human promyeloid leukemia HL-60 cells. *Leuk Res* **29**: 685–692.

Huynh HT, Teel RW. 2000. Selective induction of apoptosis in human mammary cancer cells (MCF-7) by pycnogenol. *Anticancer Res* **20**: 2417–2420.

Jang HS, Kook SH, Son YO *et al.* 2005. Flavonoids purified from *Rhus verniciflua* Stokes actively inhibit cell growth and induce apoptosis in human osteosarcoma cells. *Biochim Biophys Acta Gen Sub* **1726**: 309.

Kwon HJ, Hong YK, Kim KH *et al.* 2006. Methanolic extract of *Pterocarpus santalinus* induces apoptosis in HeLa cells. *J Ethnopharmacol* **105**: 229–234.

Lee JC, Lee KY, Kim J *et al.* 2004. Extract from *Rhus verniciflua* Stokes is capable of inhibiting the growth of human lymphoma cells. *Food Chem Toxicol* **42**: 1383.

Li WG, Li QH, Tan Z. 2005. Epigallocatechin gallate induces telomere fragmentation in HeLa and 293 but not in MRC-5 cells. *Life Sci* **76**: 1735–1746.

Middleton E Jr. 1998. Effect of plant flavonoids on immune and inflammatory cell function. *Adv Exp Med Biol* **439**: 175–182.

Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA. 2001. Flavonoids: a review of probable mechanisms of action and potential applications. *Am J Clin Nutr* **74**: 418–425.

Packer L, Rimbach G, Virgili F. 1999. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. *Free Radic Biol Med* **27**: 704–724.

Rice-Evans CA, Miller NJ, Paganga G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med* **20**: 933–956.

Rosse T, Olivier R, Monney L *et al.* 1998. Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c. *Nature* **391**: 496–499.

Tourino S, Selga A, Jimenez A *et al.* 2005. Procyanidin fractions from pine (*Pinus pinaster*) bark: radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells. *J Agric Food Chem* **53**: 4728–4735.

Yang J, Liu X, Bhalla K *et al.* 1997. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* **275**: 1129–1132.

Yusup A, Upur H, Tursun X, Berke B, Baudrimont I, Moore N. 2006. The effect of abnormal Savda Munziq total flavonoids on the proliferation, apoptosis and correlative gene expression in human hepatoma (HepG2) cells. *Toxicol Lett* **164**(Suppl 1): S250.

Zheng PW, Chiang LC, Lin CC. 2005. Apigenin induced apoptosis through p53-dependent pathway in human cervical carcinoma cells. *Life Sci* **76**: 1367–1379.