African Journal of Biotechnology Vol. 10(40), pp. 7766-7771, 1 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.194
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Procyanidins from *Pinus koraiensis* bark inhibits HeLa cell growth by inducing apoptosis and reducing survivin protein expression

Kun Li[1], Qingwang Li[1,2]*, Tao Zhang[3], Zengsheng Han[1], Jian Li[1], zhiwei Liu[1] and Fulu Zheng[4]

[1]College of Environment and Chemical Engineering, Yanshan University, No. 438, Hebei street, Qinhuangdao 066004, P. R. China.
[2]College of Animal Science, Northwest A&F University, No. 22, Xinong Street, Yangling 712100, P. R. China.
[3]College of Basic Medicine, Jiamusi University, No.148, Xuefu street, Jiamusi 154007, P. R. China.
[4]Renmin Hospital of Qinhuangdao, No. 22, Wenhua Road, Qinhuangdao 066004, P. R. China.

Accepted 5 May, 2011

Recently, the development of herbal medicine is an important advancement in anticancer therapy. *Pinus koraiensis* bark procyanidins extract (PKBPE) has been used in traditional Chinese medicine for thousands of years. Our study demonstrated that *P. koraiensis* bark procyanidins extract had antitumor effects. In some of our recent experiment, we further explored the inhibiting Hela activity of the procyanidins from *P. koraiensis* bark. In this study, the effect of procyanidins from *P. koraiensis* bark on inhibiting Hela cells growth has been investigated by many approaches which are MTT method, agarose gel electrophoresis and Western blot method *in vitro*. The results showed that Marron powder were extracted from *P. koraiensis* bark with the extract purity of 21.04%, which was identified as procyanidins. In HeLa cell experiments, the IC_{50} values of PKBPE was 196.38 µg/ml. PKBPE could make the agarose gel electrophoresis of HeLa cells appear with "ladder" zone in a dose-dependent manner, increase the expression of Bax, reduce Bcl-2 and survivin protein expressions. These results showed that the mechanism of PKBPE inducing apoptosis might be associated with increasing the expression of Bax protein, reducing Bcl-2 and survivin protein expressions.

Key words: *Pinus koraiensis* bark, procyanidins, HeLa cell, apoptosis.

INTRODUCTION

Cervical cancer, after the breast cancer, is the second malignant tumor which threatens the health of women. But the current use of chemical synthetic drugs such as cyclophosphamide, not only kill tumor cells, but also damage blood cells, resulting in decreased immune function, or even death which results from complications caused by serious side-effects. Indeed, worldwide efforts were made to discover new anticancer agents from plants. Pharmacologic study extract not only contain favourable nutrition, but also has the activities of antitumor, antioxidant, antiaging and antimutation. *Pinus koraiensis* bark has been extensively used in traditional Chinese medicine not only domestically for thousands of years, but also in foreign countries such as Korea, Japan and Russia.

The purpose of this study was to investigate the effect of PKBPE on cervical cancer inhibition and provide strong scientific evidence as a new and effective antitumor herbal medicine. In the previous paper, we reported that alkaloid from *Angelicae Dahuricae* inhibited HeLa cell growth by inducing apoptosis, increasing Caspase-3 activity, and procyanidins from *P. koraiensis*

*Corresponding author. E-mail: jmsmulk@yahoo.com.cn.

Abbreviaton: PKBPE, *Pinus koraiensis* bark procyanidins extract.

bark might have an anti-cervical cancer activity.

MATERIALS AND METHODS

Chemicals

RPMI-1640 medium was obtained from Gibco Co, fetal bovine serum (FBS) was purchased from Hyclone CO, DMSO, trypsin, EDTA, MTT, Bcl-2, Bax and survivin antibodies were obtained from Sigma Chemical Co. (St. Louis, USA). All other chemicals were of analytical grade and supplied by Sigma Chemical Co. (St. Louis, USA).

Cell lines

Hela cell line was obtained from Institute of Materia Medica, Chinese Academy of Medical Sciences.

Preparation of extracts and fractions

The bark of *P. koraiensis* was collected in Heilongjiang province, China in March 2009. Authentication of plant material was carried out by Dr Li at the College of Animal Science, Northwest Agriculture and Forestry University, China, where the herbarium voucher has been kept.

The fresh collected *P. koraiensis* bark was first air-dried (30 ± 2°C) and then minced. The 300 g of minced sample was exhaustively extracted with 10 times volume of 95% ethanol by maceration for 7 days and heat circumfluence for 2 h, twice. Dry ethanol extracts (6.852 g) were obtained after removing the solvent by evaporation under reduced pressure. Concentrated leaching liquor was filtrated and the residue was removed after centrifugation at 956 g for 10 min. Acetone (1:2) was added in concentrated solution for precipitating dopant. Then dopant was filtrated and removed. The filtrate was put in drying oven to cryodry (Fei et al., 2005; Huynh and Teel, 1999; Jianhua et al., 2004).

The qualitative analysis of procyanidins

Following the earlier mentioned process of sample preparation, the sample was prepared as a concentration of 0.1 g/L. To determine the concentration of procyanidins, 6 ml of n-butanol/acid hydroc (volume ratio 95:5) was added to 1 ml of the sample solution. The mixture was agitated for uniformity, refluenced and condensed in 95°C aqueous bath for 40 min, cooled quickly to room temperature in cold water. The absorbance (Abs) in 550 nm wavelength was used to determine procyanidin concentration based on the standard curve of procyanidins.

Cell culture

HeLa cell lines were cultured in RPMI-1640 medium supplement with 10% FBS, 100 u/µl of penicillin and 100 u/µl of streptomycin at 37°C and in a humidified incubator with 5% CO_2. During the experiment time, the medium was replaced by medium containing 125 and 250 µg/ml PKBPE, which were dissolved in a small qualities ethanol vehicle (50%), then RPMI-1640 medium were supplied according to the contraction of PKBPE. The final ethanol concentration of ethanol in each well of 6 wells plate was 0.1%v/v. However, the vehicle (only the control) received ethanol (0.1% v/v).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The capacity of PKBPE to interfere with the growth of Hela cells was determined using the MTT dye assay. Cells were seeded in RPMI-1640 medium at a density of 5 x 10^4 cells/ml in 96-well microplates for 12 h at 37°C, washed free with phosphate-buffered saline (PBS, pH 7.4), then the medium was removed and incubated with a fresh medium with PKBPE (the final concentration of 125 and 250 µg/ml) for an additional 24 h, respectively. Then MTT 20 µl was added to the medium for incubation for addition 4 h, then the medium was removed. MTT was converted to a blue formazan product by mitochondrial succinate dehydrogenase. This product was eluted from cells by addition of 150 µl of DMSO, and absorbance at 540 nm, was determined using an autoreader (model EL310 EIA), and the values for each PKBPE concentration tested represent the average from eight replicate wells (one row of a 96-well plate).

The rate of cell growth inhibition (IC_{50}) was calculated using the formula: the rate of cell growth inhibition = (C-T) / C × 100, where “C” and “T” mean average OD value of control group and average OD value of treated group. Regressive curve was determined as the rate of HeLa cell growth inhibition to the drug acted concentration.

DNA fragmentation of HeLa cells assay

Both detached and attached HeLa cells were harvested by scraping and centrifugation. After washing with PBS three times, the cells were resuspended in 0.5 ml lysis buffer (10 mmol/L Tris-cl, 0.1 mol/L EDTA, 0.5% SDS; pH 8.0) for 45 min. Fragmented DNA in the supernatant fraction after centrifugation at 2500 rpm was extracted twice with phenol: chloroform : isoamyl alcohol (25:24:1, v/v/v) and once with chloroform, and then, precipitated with ethanol and 5 M NaCl overnight at -20°C. The DNA pellet was washed once with 70% ethanol and resuspended in Tris–EDTA buffer (pH 8.0), and treated with 100 mg/ml RNase A for 2 h at 56°C. The DNA samples were subjected to electrophoresis in a 1.5% agarose gel. The DNA bands were stained with ethidium bromide and visualized under UV light after staining with ethidium bromide (0.5 mg/ml).

Western-blot analysis

Cells were cultured in six-well plates and incubated overnight. After 48 h, the cells were harvested and washed with PBS and lysed in buffer containing 50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS) and 5% b-mercaptoethanol, and then lysed by sonication. Total protein in cell lysates was measured in triplicate using a Bio-Rad colorimetric kit. 50 mg protein lysate were separated by 12.5% SDS-PAGE and transferred onto nitrocellulose. After blocking in a 5% nonfat dry milk solution in washing buffer containing 10 mmol/l Tris (pH 7.5), 150 mmol/l NaCl and 0.05% Tween-20, membranes were incubated overnight at 4°C with a mouse monoclonal IgG1 of anti-Bcl-2, anti-Bax and anti-survivin antibodies, respectively, washed three times and further incubated for 2 h with a goat anti-mouse horseradish peroxidase-coupled secondary antibodies at room temperature, respectively. Signals were detected using the ECL kit.

Statistical analysis

Data were expressed as mean ± S.D. Statistical analysis was performed by one-way analysis of variance, and differences between means were tested using Duncan’s multiple range tests. *P*-values of less than 0.05 were considered significant.

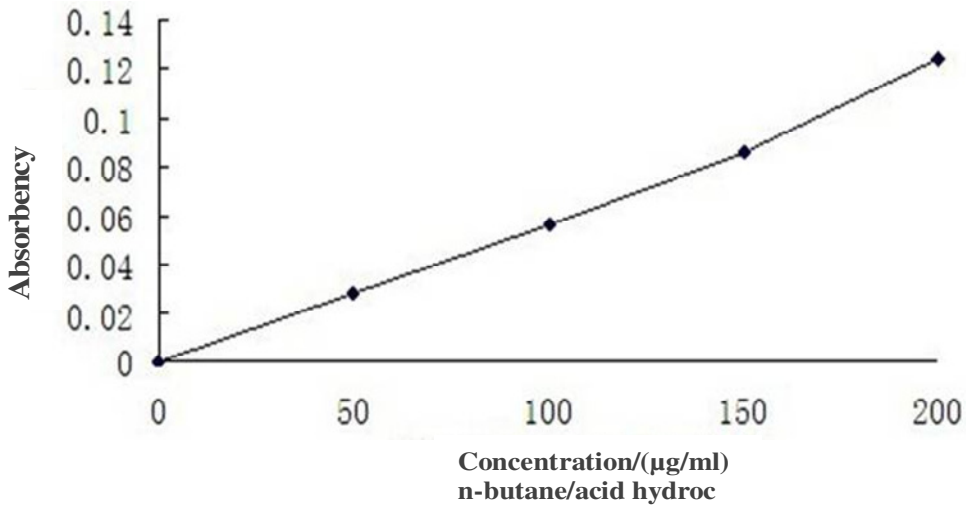

Figure 1. Standard curves of procyanidins.

Table 1. Inhibitory effect of PKBPE on HeLa cells growth (24 h) ($\bar{x}$ ±s).

Group	Density (μg/ml)	OD value	Inhibition rate (%)
Control	0	0.81±0.02	—
PKBPE	150	0.66±0.03**	18.51
PKPPE	250	0.58±0.03**	28.40

** P<0.01 when compared with control group.

RESULTS

The qualitative analysis of procyanidins

We first determined the concentration of procyanidins in the air-dried bark of *P. koraiensis* based on the standard curves generated with commercial pure procyanidins (Figure 1). The extract purity of procyanidins was 21.04%.

MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

HeLa cells were treated with PKBPE of various concentrations (150 and 250 μg·ml^{-1}) for 24 h and the cell viability were determined by MTT assay. As shown in Table 1, PKBPE could inhibit the growth of HeLa cells in a dose-dependent manner. It was noted that PKBPE at 250 μg·ml^{-1} had an inhibitory effect of more than 25% on the HeLa cell growth in 24 h treatment. The IC_{50} value of PKBPE was 196.38 μg/ml.

Determination of DNA fragmentation

In HeLa cell experiments, PKBPE (150 and 250 μg·ml^{-1}) could make the agarose gel electrophoresis of HeLa cells appear with “ladder” zone in a dose-dependent manner, but in the control group, there was no such appearance. The cells treated with 150 μg·ml^{-1} of PKBPE for 48 h could increase proportion with DNA, as indication of apoptosis. With 250 μg·ml^{-1} of concentration, the addition of PKBPE caused an increase in the DNA fragments proportion. The results illustrated that the exposure of PKBPE for 48 h induced DNA fragmentation in HeLa cells, showing the characteristic ladder of DNA fragments (Figure 2).

Effect of PKBPE on expression of Bax, Bcl-2 and survivin

Here, we performed Western blot to determine the expression of Bax, Bcl-2 and survivin at protein level, respectively. At the concentration of 250 μg/ml, the effects of PKBPE on Bcl-2, Bax and survivin protein expression after 72 h in the HeLa cells are shown in Figures 3 and 4. Bcl-2 and survivin expression was decreased in the HeLa cells treated with PKBPE, and Bax expression was slightly increased with PKBPE. Western blotting assays were repeated at least three times (Figures 3 and 4). These results showed the molecular basis of PKBPE inducing HeLa cells apoptosis.

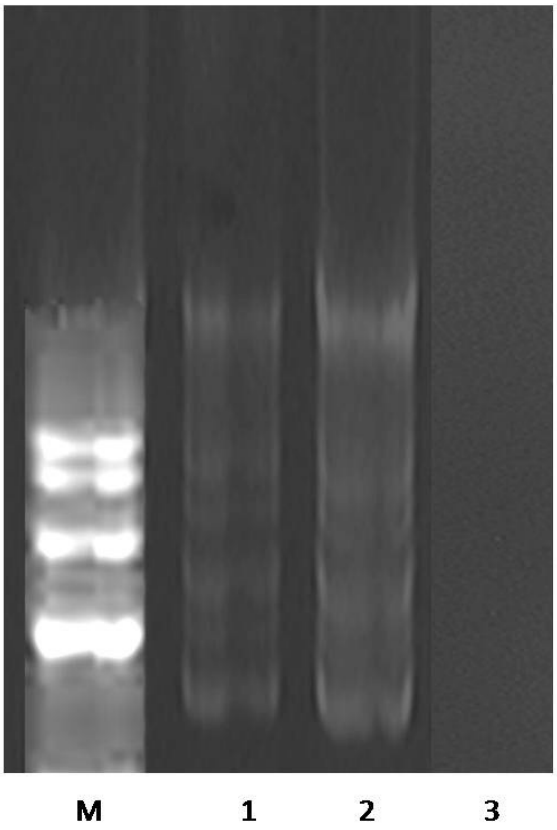

Figure 2. Effect of PKBPE on the induction of DNA fragmentation in HeLa cells after 48 h. M: DNA marker; 1: high density PKBPE; 2: light density PKBPE; 3: control group.

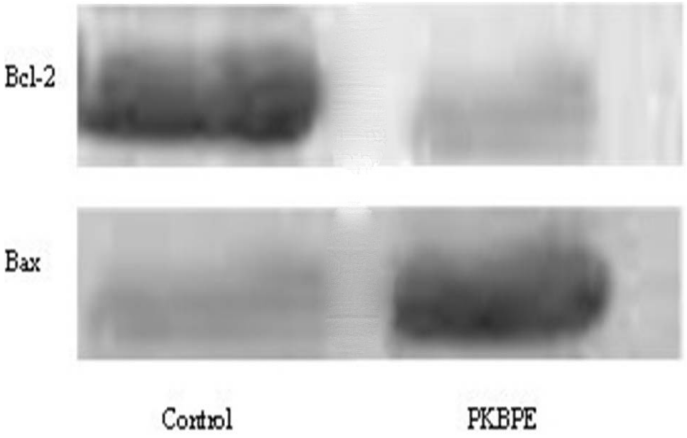

Figure 3. Expression of Bcl-2 and Bax protein induced by PKBPE in HeLa cells after 72 h.

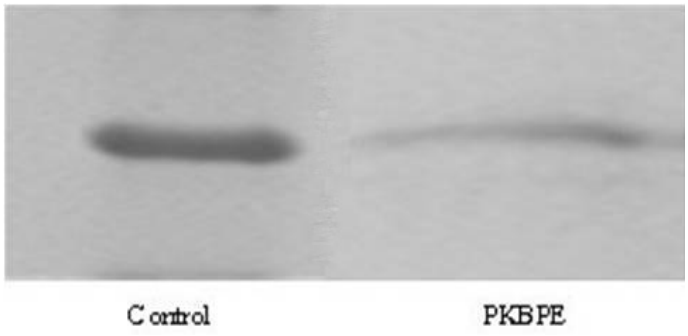

Figure 4. Effect of PKBPE on expression of survivin in HeLa cell after 72 h.

DISCUSSION

It is well established that plants have been a useful source of clinically relevant antitumor compound. *P. koraiensis* belongs to naked seed plant sect and the pine family (Pinus). The procyanidins has been reported to have effects of removing free radical, antitumor (Xiaofen and Bijun, 2005), antioxidant, antiaging and antimutation activities (Debasis et al., 2000; Packer et al., 1999). Due to these bioactivities, PCA has been extensively used in traditional Chinese medicine and food supplements. But there was no systematic study on pharmacologic activity of PKBPE. In some of our recent experiments, we further explore the inducing apoptosis activity of the procyanidins from *P. koraiensis* bark. We examined PKBPE activities on the expression of survivin, Bcl-2 and Bax proteins and the relationship between Bcl-2, survivin and Bax were shown here by Western blotting. The purpose was to shed some light on the mechanism of how PKBPE induces cell apoptosis activity.

Apoptosis, also called programmed cell death, is an evolutionarily conserved genetic program of cellular characteristics. It is well recognized that an alteration of the cellular homeostasis occurs in cancer, which disrupts the balance between cellular proliferation and cell death (apoptosis). Apoptosis is also a form of cell death which is characterized morphologically by extreme chromatin condensation and formation of apoptotic bodies, and biochemically by inter-nucleosomal DNA fragmentation (Kerr and Harmon, 1991; Wyllie et al., 1980).

Our results showed that PKBPE (150 and 250 $\mu g \cdot ml^{-1}$) could inhibit the growth of HeLa cells in a dose-dependent manner by MTT assay and make the agarose gel electrophoresis of HeLa cells appear with “ladder” zone. The results illustrated that PKBPE could inhibit HeLa cell growth and induce apoptosis.

Apoptosis is also an active process of cell destruction and an important mode of cell death that occurs in response to a variety of agents including anticancer chemotherapeutic drugs or ionizing radiation (Fisher, 1994). It involves the balanced transcription of anti-apoptotic and proapoptotic genes such as Bax, Bcl-2 and survivin (Zengli et al., 2004). Studies demonstrated that the apoptosis process requires the coordinated regulation of specific genes and is often dependent on RNA and protein synthesis. Moreover, these genes are known to be crucial regulators of apoptosis in HeLa cell lines

(Whitte, 1996; Levy et al., 2003). Among the proteins that have been identified, are member of the Bcl-2 gene family. The Bcl-2 family of proteins plays an important role in the regulation of apoptosis of many cellular systems by either inhibiting or promoting apoptosis (Bhathena and Velasquez, 2002; Reed, 1998; Gross et al., 1999). The Bcl-2 family of proto-oncogenes encodes specific proteins which regulate programmed cell death in different physiological and pathological conditions (Davies, 1995; Greenlund et al., 1995; Reed, 1994). Bcl-2 protects a variety of cell types from program-med cell death, whereas Bax promotes apoptosis. Therefore, Bax can be regarded as pro-apoptotic, whereas Bcl-2 is perceived as anti-apoptosis. This family consists of the pro-apoptotic (Bax, Bad, etc.) and anti-apoptotic (Bcl-2, Bcl-XL, etc) family members. Interac-tions between these pro- and anti-apoptotic members integrate diverse upstream signals to determine the cellular response (Korsmeyer, 1992). Bcl-2 is localized in the mitochondrial membrane, nuclear envelope and endoplasmic reticulum (Hockenberry et al., 1990; Krajewski et al., 1993), and it promotes cell survival (Korsmeyer, 1992; Vaux et al., 1988). Bax is also localized in the mitochondria, nuclear envelope and endoplasmic reticulum, and it accelerates apoptotic cell death.

Survivin, a member of the “inhibitor of apoptosis” (IAP) family, has been reported to promote cell proliferation and play a major role in antagonizing mitochondrial-depen-dent apoptosis, mitosis regulation and apoptosis suppression, but its exact function remains unclear (Jiru et al., 2010; Altieri, 2003). Therefore, the anti-apoptotic protein, survivin, deserves attention as a target for cancer therapy due to its high expression levels in most human cancers which correlates with unfavorable prognosis, resistance to therapy, and accelerated rates of recurrences (Altieri, 2001). Survivin expresses in several subcellular compartments like cytosol, mitochondria and nucleus (Altieri, 2008). The anti-apoptotic mechanism of survivin during PKBPE–induced apoptosis is still not investigated.

Here, we investigated the HeLa cell apoptotis induced by PKBPE. It was shown that Bcl-2 and survivin expres-sion decreased in the HeLa cells treated with PKBPE, and Bax expression was slightly increased with PKBPE in a dose-dependent manner.

We studied whether survivin overexpression could influence Bax activation after PKBPE treatment. In HeLa cell experiments, the activity of survivin protein over-expression was remarkably down-regulated by PKBPE and promoted apoptosis. To help elucidate the molecular mechanism involved in PKBPE-induced apoptosis, we analyzed Bcl-2 and Bax at protein levels in HeLa cells treated with PKBPE for 72 h. We demonstrated that PKBPE induced apoptosis through the down-regulation of Bcl-2 and up-regulation of Bax is one of the main mole-cular mechanisms responsible for apoptosis induced by PKBPE in HeLa cells, because survivin prevented the accumulation of pro-apoptotic Bax at mitochondria during DNA-damage induced apoptosis.

In summary, PKBPE induced apoptosis through the overexpression of Bax and the down-regulation of Bcl-2 and survivin protein in HeLa cells. It is concluded that PKBPE could induce apoptosis and inhibit the growth of HeLa cells which make it possible for it be used for the treatment of cervical cancer, which continues to be a major public health problem in the health of women. Further investigations are in progress to elucidate its direct targets and signal transduction pathways, which it might regulate.

ACKNOWLEDGEMENTS

This study was support by Technology Committee of Qinhuangdao City. The authors would like to thank Zhangtao (Jiamusi Medical University, China) for providing the HeLa cell line in the present study.

REFERENCES

Altieri DC (2001). The molecular basis and potential role of surviving in cancer diagnosis and therapy. J. Trends Mol. Med., 7: 542-547.

Altieri DC (2003). Survivin, versatile modulation of cell division and apoptosis in cancer. Oncogene, 22: 8581-8589.

Altieri DC (2008). Survivin, cancer networks and pathway-directed drug discovery. Nat. Rev. Cancer, 8: 61-70.

Bhathena SJ, Velasquez MT (2002). Beneficial role of dietary phytoestrogens obesity and diabetes. Am. J. Clin. Nur., 76: 1191-1201.

Davies JC (1995). The Bcl-2 family of protein and the regulation of cell survival. TINS, 18: 355-358.

Debasis B, Manashi B, Sidney JS (2000). Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. Toxicology, 148: 187-189.

Fei L, Heng X, Jinfa W (2005). Study of the biology and prevention disease of Pycnogenol. Chin. Herbal Med. 36(1): 127-130.

Fisher DE (1994). Apoptosis in cancer therapy: crossing the threshold. Cell, 78: 539-542.

Greenlund LSJ, Korsmeyer Jr. SJ, Johnson EM (1995). Role of bcl-2 in the survival and function of developing and mature neurons. Neuron, 15: 639-664.

Gross A, McDonnell JM, Korsmeyer S (1999). Bcl-2, Bcl-2 family member and the mitochondria in apoptosis. Genes Dev., 13: 1899-1911.

Hockenberry D, Nunez G, Milliam C, Schreiber RD, Korsmeyer SJ (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature, 48: 334-346.

Huynh HT, Teel RW (1999). Effects of intragastrically administered *Pycnogenol* on NNK metablism in F_{344} rats. Anticancer Res., 19(3A): 2095-2099.

Jianhua Hu, Yiliang Wei, Yan L (2004). Study of Anthocyanin from rapeseed skin. China Grease, 29(4): 26-28.

Jiru CHU, Shengnan Wu, Xing D (2010). ROS/cdc25c/CDK1 signaling pathway during tumor cell apoptosis induced by high fluence low-power laser irradiation. Cancer Lett., 297: 207-219.

Kerr JFR, Harmon BV (1991). Definition and incidence of apoptosis: an historical perspective, In: Tomei LD, Cope FO (Eds), Apoptosis: The Molecular Basis of Cell Death. Cold Spring Harbor Laboratories Press. pp. 1-29.

Korsmeyer SJ (1992). Bcl-2 initiates a new category of oncogene regulators of cell death. Blood, 80: 879-886.

Krajewski S, Tanaka S, Takayama S, Sxhibler MJ, Fenton W, Reed JC

(1993). Investigation of the subcellular distribution of the Bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res., 53: 743-753.
Levy P, Robin H, Bertrand F, Kornprobst M, Capeau J (2003). Butyrate-treated colonic Caco-2 cells exhibit defective integrin-mediated signaling together with increased apoptosis and differentiation. Cell Physiol., 197: 336-347.
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(5/6): 704-724.
Reed JC (1994). Bcl-2 and the regulation of programmed cell death. Cell Biol., 124: 4-6.
Reed JC (1998). Bcl-2 family proteins. Oncogene, 17: 3225-3236.
Vaux DL, Corry S, Adams JM (1988). Bcl-2 gene promotes hematopoietic cell survival and cooperates with c-myc to immortalize pro-B cells. Nature, 335: 440-442.
Whitte E (1996). Life, death, the pursuit of apoptosis. Genes Dev., 10: 1-15.
Wyllie AH, Kerr JF, Currie AR (1980). Cell death the significance of apoptosis. Int. Rev. Cytol., 68: 251-306.
Xiaofen D, Bijun X (2005). Study of Anthocyanin on anticancer. Study and Exploitation of savageness production. Future Med., 17(6): 822-824.
Zengli Yu, Wenjie Li, Fuyun L (2004). Inhibition of proliferation and induction of apoptosis by genistein in colon cancer HT-29 cells. Cancer Lett., 215: 159-166.