—Regular Articles—

Antitumor Activity of the Procyanidins from *Pinus koraiensis* Bark on Mice Bearing U14 Cervical Cancer

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Pinus koraiensis Bark Procyanidins Extract (PKBPE) has been used in traditional Chinese medicine for thousands of years. In this study, we determined PKBPE effect on tumor weight, SOD (superoxidate dismutase) activity, the content of MDA (malondialdehyde) through colorimetric analysis antigenic, and expression of Ki-67, p53 and Bcl-2 on mice bearing U14 cervical cancer. Treatment with PKBPE (158 and 250 mg/kg body weight, *p.o.*) could inhibit U14 cervical carcinoma growth up to 47.68 and 58.94%. In addition, PKBPE enhance the activity of SOD ($p<0.01$) and decrease MDA content. Furthermore, we also observed that PKBPE treatment significantly inhibited the expression of Ki-67, mutant p53 and Bcl-2 protein ($p<0.01$). The results suggested that PKBPE showed antitumor activities on U14 cervical carcinoma mice. The mechanism of PKBPE antitumor activity might be associated with free radical production inhibition and regulation of the expression of Ki-67, mutant p53 and Bcl-2 protein.

Key words——antitumor activity; Procyanidins; *Pinus koraiensis* Bark; U14 cervical cancer

INTRODUCTION

Pinus koraiensis is a valuable tree, which distributes in Small Xing An Ling and Long White Mountain area of China. It belongs to naked seed plant sect, pine family, pinus. *Pinus koraiensis* has been used in traditional Chinese medicine for thousands of years. Pharmacologic study revealed that *pinus bark* extract not only contains favourable nutrition, but also has the activities of antitumor,[1] antioxygen, antiaging and antimutation.[2,3] Biochemistry analysis indicated that *Pinus bark* extract contains affluent polyphenolics such as catechin, epicatechin, taxifolin and procyanidins (PCA). The major active ingredient in the extract is oligomerization procyanidins and accounts for more than 95% in content. It has very high bioactivity, which can remove superfluous free radical *in vivo*,[2,3] enhance immunity and has powerful antioxygen function. It has been often considered as a native antioxidant to prevent cancer, mutation and cardiovascular. Due to these bioactivities, PCA has been extensively used in traditional Chinese medicine and food supplements. Previous studies indicated that PCA may have anti-carcinogenic activity without NNK and prevent lung cancer induced by smoking.[4]

The purpose of this study was to investigate the antitumor activity of the PKBPE and to provide a strong scientific evidence for implication of *pinus koraiensis* bark as a new and effective antitumor herbal medicine.

MATERIAL AND METHODS

Chemicals Cyclophosphamide was purchased from Taisheng Pharmacy Inc. (shanxi province, China). Hematoxylin and eosin were obtained from Sigma Chemical Co. (St.Louis,USA). Mouse antiproliferating cell nuclear antigen (Ki-67) and anti-p53, anti-Bcl-2 monoclonal antibodies and Streptavidin-Peroxidase immunohistochemical staining kit were obtained from Maixin Biotechnology Inc. (Fujian province, China). The kits used to analyze SOD and MDA were purchased from Nanjing Jiancheng Biotechnology Inc. All other chemicals used were of analytical reagent grade.

Cell Lines U14 cervical cancer cell line was obtained from Institute of Material Medica, Chinese Academy of Medical Sciences.

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Animals Fifty Female Kunming mice (6 weeks old, 18–22 g) were provided by the Experimental Animal Center of the Chinese Academy of Medical Sciences. The mice were randomly divided into five groups and kept in plastic cages in a temperature-controlled room at 20±2℃. The animals were fed with a standard pellet diet and provided with water *ad libitum*.

Preparation of Extracts and Fractions The bark of *Pinus koraiensis* was collected in Heilongjiang province, China in March 2006. 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 *Pinus koraiensis* Bark was first air-dried (30±2℃) and then minced. The 500 g of minced sample was exhaustively extracted with 95% ethanol of 10 time volumes by maceration for 7 days, heat circumfluence for 2 hours twice. Dry ethanol extracts (114.2 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.[4,5] The process was summaried as in Fig. 1.

The Qualitative Analysis of Procyanidins Following the process of sample preparation above, 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 ℃ 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.

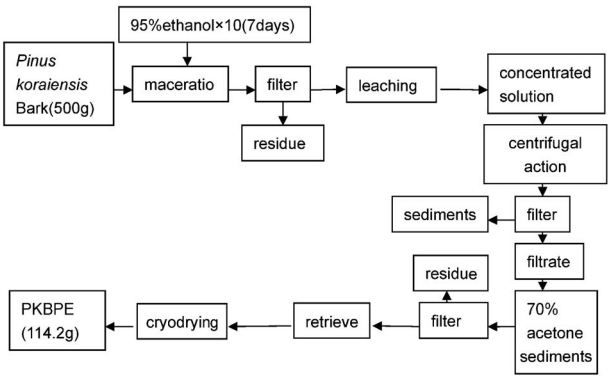

Fig. 1. Flow Chart of PKBPE

Effect of PKBPE on Solid Tumor Growth Inhibition Mice were randomly divided into five groups, and each group was ten animals. The five groups were designated as tumor source mice group, tumor control group, CTX group, PKBPE low dose and high dose groups. To generate tumor mice were injected into the left fore limb (*s.c.*) with 1.60×10^6 U14 cervical cancer cells in sterile physiological saline (prepared from 7 days old U14 ascites tumor inoculation in mice) in 0.2 ml/mouse. After 24 h of tumor inoculation, PKBPE was administered orally at a dose of 158 mg/kg and 250 mg/kg body weight and continued for 15 consecutive days. The group administered with vehicle alone (distilled water, *p.o.*) was maintained as control. Cyclophosphamide (CTX, 25 mg/kg body weight, *i.p.*) was used as the standard reference drug. On day 16, all animals were executed for determination of mice weight and tumor size. The rate of tumor inhibition was calculated by the formula: (C-T)/C×100, where "T" and "C" mean average tumor weight of treated groups and control group.[6]

Histopathological and Morphological Examination of Liver and Kidney Liver and kidney of executed mice administrated with PKBPE (250 mg/kg body weight, *p.o.*) were collected and processed as slides for histopathological analysis with microscope.

Histopathological and Morphological Examination of Solid Tumor The tumors collected from all groups were fixed in 12% acidic formalin at 4℃, embedded in paraffin and sectioned. The sections were stained with HE staining method and then examined with light microscope.[6]

Effect of PKBPE on SOD and MDA in Serum All animals were subjected to collect blood samples via eyeball before executed. The blood samples were kept at 4℃ for 1h and centrifugated at 956 g for 5 min at room temperature to prepare serum. The content of serum SOD and MDA were determined with commercial kits by following manufacturer's instruction.

Expression of Ki-67, Mutant p53 and Bcl-2 Tumor sections were prepared as mentioned above and used to examine the expression of ki-67, mutant p53 and Bcl-2 proteins. The tumor slides were stained

with standard immunohistochemical SP (streptavidin peroxidase conjunction) method and examined with light microscope. The distinctly brown nucleus stain suggested positive cells and blue nucleus stains indicated negative cells counterstained by hematoxylin. The numbers of positive cells were counted for statistical analysis.

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

RESULTS

The Qualitative Analysis of Procyanidins We first determined the concentration of procyanidins in the air-dried bark of *Pinus koraiensis* based on the standard curves generated with commercial pure procyanidins (Fig. 2). The analysis indicated that the procyanidins concentration was 105.2 g and the extract purity was 21.04%.

Effect of PKBPE on Solid Tumor The administration of PKBPE and standard reference drug (CTX) both inhibited tumor growth in a dose dependent manner. Comparing to the control group (1.51±0.16 g), the tumor weight was reduced to 0.79 ±0.09 g in the low dose PKBPE group, 0.62±0.04 g in the high dose PKBPE group, and 0.49±0.03 g in CTX group respectively. The corresponding tumor inhibition rate was calculated for 47.68, 58.94 and 67.55%, respectively (Table 1). The weight of tumor in the low dose PKBPE group, the high dose PKBPE group and the CTX group was all significantly lower than that of the control group ($p<0.01$).

Pathological Analysis of Effect of PKBPE on Liver and Kidney We examined the color, luster and texture of liver and kidney with eyes. There was no toxic effect of PKBPE observed on liver and kidney tissues. The microscopic examination of the slides of liver and kidney showed clear central vein and hepatic lobule. The cells looked health from liver samples and the renal tubular from kidney samples appeared normal (Fig. 3). All these data suggested that administration of PKBPE on both dose did not affect liver and kidney growth.

Effect of PKBPE on Tumor Cell Morphology By microscopic examination of tumor tissue, we found that, in contrast to control group, the administration of PKBPE (250 mg/kg body weight, *p.o.*) and CTX (25 mg/kg body weight, *i.p.*) significantly reduced the numbers of tumor cells and inhibited the malignant phenotype of tumor cells such as heter-

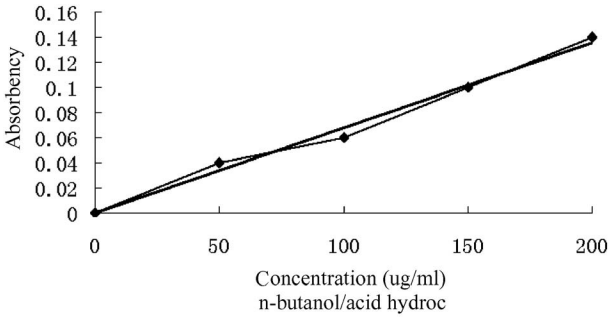

Fig. 2. Standard Curves of Procyanidins

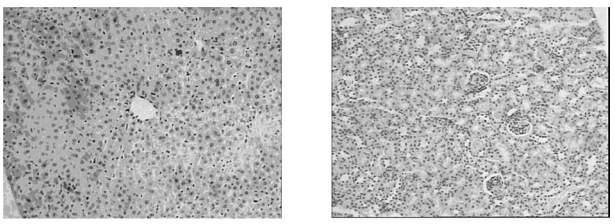

Fig. 3. Pathology Sections of Liver and Kidney (H.E. ×100)

Table 1. Effect of PKBPE Treatment on the Tumor Inhibition

Groups	Treatment (mg/kg)	Animal number		Body weight (g)		Tumor weight (g)	Inhibition (%)
		Beginning	End	Beginning	End		
Control	Vehicle	10	8	20.73±1.95	26.67±2.54	1.51±0.16	
CTX	25	10	8	19.35±1.76	22.38±2.06	0.49±0.03*	67.55
PKBPE	158	10	8	20.68±1.96	26.55±2.31	0.79±0.09*	47.68
	250	10	8	20.81±1.38	23.87±2.66	0.62±0.04*	58.94

* $p<0.01$ as compared with control group. Values are mean±S.D.

omorphism, heteropyknosis and decrease of nucleus/plasma. In PKBPE and CTX groups, the tumor cells appeared scattered monolayer distribution and putrescence such as nucleus atrophy disintegrating region (Fig. 4).

Effect of PKBPE on SOD and MDA in Serum In contrast to the tumor control group, administration of the PKBPE increased the activity of SOD and reduced the content of MDA. The activity of SOD in the tumor control group was 284.71±10.84 U/ml. However, the administration of PKBPE (158 and 250 mg/kg body weight, *p.o.*) and CTX (25 mg/kg body weight, *i.p.*) significantly increase the activity of SOD to 292.49±11.91 U/ml 451.62±15.58 U/ml and 322.62±11.19 U/ml (Table 2). Furthermore, the content of MDA was found to be 5.06±0.22 nmol/ml in the tumor control group, which was reduced to 4.15±0.27 nmol/ml, 1.91±0.06 nmol/ml and 3.16±0.13 nmol/ml in the group of animals treated with PKBPE and CTX (Table 2).

Effect of PKBPE on Expression of Ki-67, Mutant p53 Protein and Bcl-2 We finally examined the effect of PKBPE on expression of the gene ki-67, mutant p53 and Bcl-2. In contrast to the tumor control group, administration of the PKBPE reduced the expression of Ki-67 and mutant p53 protein in a dose dependent manner. The percent of Ki-67 positive cells in the tumor control group was 81.46±6.49%. However, the administration of PKBPE (250 mg/kg body weight, *p.o.*) and CTX (25 mg/kg body weight, *i.p.*) significantly reduced the percentage of positive cells to 21.34±1.69 and 42.31±3.46% (Table 3, Fig. 5). Similarly, the percentage of the mutant p53 protein positive cells was found to be 76.46±5.39% in the tumor control group, which was reduced to 33.25±2.46% and 51.39±4.23% in the group of animals treated with PKBPE and CTX (Table 3, Fig. 6). Furthermore, the percentage of Bcl-2 protein positive cells was 89.26±7.67% in the tumor control group, which was reduced to 6.96±0.84% and 67.56±5.47% in the group of animals treated with PKBPE and CTX (Table 3, Fig. 7).

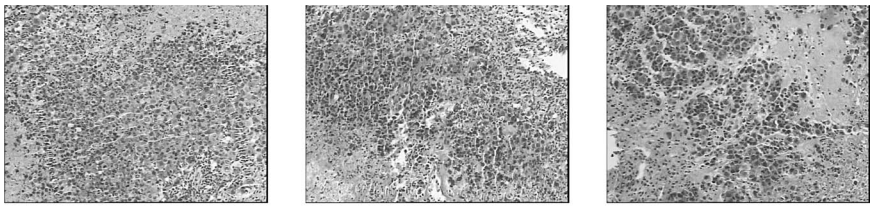

Fig. 4. Sections of H_{22} Hepatocellular Carcinoma Cell (H.E. ×100)

Table 2. Effect of PKBPE on SOD and MDA in Serum

Groups	Treatment (mg/kg)	Animal number	SOD (U/ml)	MDA (nmol/ml)
Control	Vehicle	10	284.71±10.84	5.06±0.22
CTX	25	10	322.62±11.19*	3.16±0.13*
PKBPE	158	10	292.49±11.91*,‡	4.15±0.27*,‡
	250	10	451.62±15.58*,‡	1.91±0.06*,‡

* $p<0.01$ as compared with control group. ‡ $p<0.01$ as compared with CTX group. Values are mean±S.D.

Table 3. Effect of PKBPE on Expression of Ki-67, Mutant p53 and Bcl-2

Groups	Treatment	Animal	Ki-67	Mutant p53	Bcl-2
Control	Vehicle	10	81.46±6.49	76.46±5.39	89.26±7.67
CTX	25	10	42.31±3.46*	51.39±4.23*	67.56±5.47*
PKBPE	250	10	21.34±1.69*	33.25±2.46*	6.96±0.84*

* $p<0.01$ as compared with control group. Values are mean±S.D.

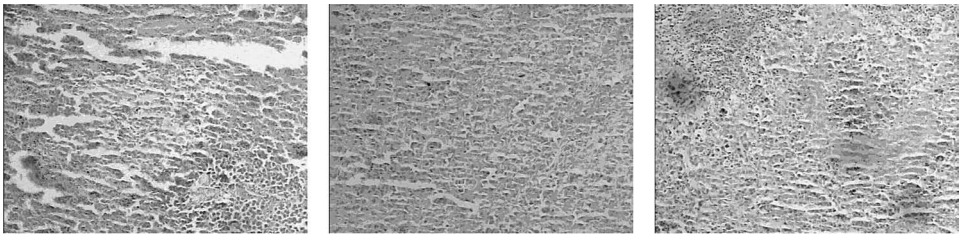

Fig. 5. Expression of Ki-67 in Nucleus (H.E. ×100)

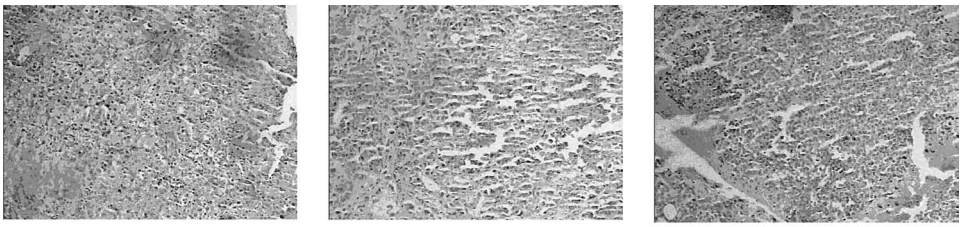

Fig. 6. Expression of Mutant P53 Protein in Nucleus (H.E. ×100)

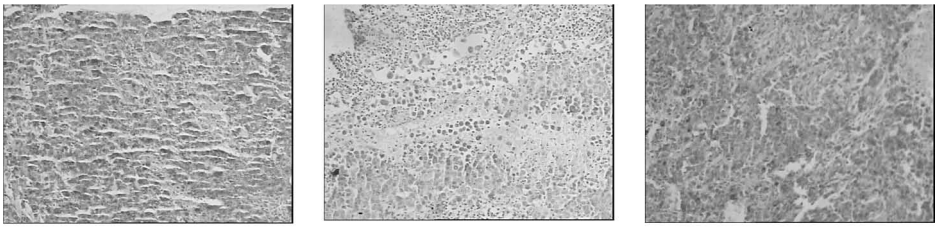

Fig. 7. Expression of Bcl-2 Protein in Nucleus (H.E. ×100)

DISCUSSION

In recent years, deaths from heart diseases are improving while cancer has taken over as the top killer in the worldwide. The current cancer therapeutic approaches include surgery, chemotherapy, and radiotherapy. However, none of these cancer treatments can cure cancers and even some of these treatments have strong side effects. Therefore, discovery and development of effective cancer therapeutic medicines are becoming a big challenge to the life science.

It is well established that plants have been a useful source of clinically relevant antitumor compound.The Procyanidins has been reported to have the removing free radical and antitumor activities. But there was no systematic study on pharmacologic activity of PKBPE. In the present studies, we examined PKBPE activities on its antitumor, anti-oxidantion and its effect on the expression of ki-67, Bcl-2 and p53. The purpose was to shed some light on the mechanism of PKBPE anti-tumor activity.

The results showed that antitumor activity of the PKBPE is in a dose dependent manner. When the dose of PKBPE was raised up to 250 $mg \cdot kg^{-1}$ body weight, we did not observe PKBPE effect on mice weight and toxicity on kidney and liver. The microscopic examination of tumor tissue showed that the number of tumor cell decreased and tumor cell nucleus appeared putrescence such as nucleus atrophy, disintegrating and dissolving. It indicated that PKBPE possessed direct effect of killing and wounding tumor cell.

Many studies indicated that the occurrence, development and metastasis of tumor cells were tightly

correlated with free radical content and metabolism.[7] Free radical molecule is very unstable and always results in major injury to organs by lipid peroxidation. The recent studies indicated that it also involves in cancer development. Similarly, oxygen free radical attacks unsaturated fatty acid in cell membrane and initiates lipid peroxidation and results in LPO (include MDA). Lipid peroxidation crosslinks with protein to affect cell membrane and mitosis. The effect of antioxidation of the antioxidants can implement their activities through inhibition or removing free radical, even enhance endogenous antioxidizing material. SOD is the most important antioxidase in body, which can exclusively eliminate adverse free radical to relieve body damage. Our studies found that the content of SOD in mice serum can increase significantly in PKBPE administration than those of control group. However, the content of MDA in serum decreased. Therefore, PKBPE had the effect of anti-lipid peroxidation due to inhibiting free radical.[8]

Ki-67 is a cell nuclear protein and expressed in cell cycle G1, S, M, and G2 is closely correlated with DNA replication. Ki-67 is connected with the proliferation of tumor cells and reflects cell proliferation. The wild p53 protein is tumor-suppressor gene, however the wild p53 protein can prevent DNA impaired cells from cell cycle and maintain genomic integrality. Bcl-2 belongs to proto-oncogene and its over expression can inhibit apoptosis. The expression of Ki-67, mutant p53 and Bcl-2 in PKBPE administration groups were lower than those of the tumor control admin group in our study. It showed the antitumor mechanism of PKBPE might be related with the low expression of Ki-67 and Bcl-2, mutant p53 protein.[9–27]

In summary, our study suggests that PKBPE can inhibit tumor growth through increasing SOD content and decreasing MDA content by reducing free radical lesions. Similarily, PKBPE enhanced immunity and suppressed expression of Ki-67, p53 and Bcl-2 genes. Therefore, we concluded that *Pinus koraiensis* Bark Procyanidins Extract have antitumor activity. Further investigations are in progress to elucidate its direct targets and signal transduction pathways, which it might regulate.

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