# Effect of procyanidins from *Pinus koraiensis* bark on growth inhibition and expression of PCNA and TNF-α in mice with U14 cervical cancer

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**Background:** An important recent advance in anticancer therapy was the development of specifc herbal medicines. Pharmacologic studies revealed that pinus bark extract not only contains favorable nutrition, but also has antitumor, antioxidant, anti-aging and antimutation activity. **Aim:** The aim of the study was to identify the potential antitumor effects of *Pinus koraiensis* bark procyanidins extract (PKBPE) on tumor weight, the content of IL-8 and TNF-α by ELISA in serum and expression of proliferating cell nuclear antigen (PCNA) and Bcl-2 protein on mice with U14 cervical cancer. **Participants:** A total of 50 female Kunming mice were provided by the Animal Department of Beijing Institute of Traditional medical and Pharmaceutical Sciences. **Results:** A dose of PKBPE (163 and 262 mg/kg body weight, *per os*) could inhibit U14 cervical carcinoma growth. In addition, PKBPE increases the content of TNF-α and decrease the content of IL-8 in mice bearing U14 cervical cancer ($p < 0.01$). Furthermore, PKBPE treatment significantly inhibited the expression of PCNA and Bcl-2 protein ($p < 0.01$). **Conclusion:** The results suggested that PKBPE showed antitumor activities in U14 cervical carcinoma mice. The mechanism of PKBPE antitumor activity might be associated with immune-modulation activity and regulation of the expression of PCNA and Bcl-2 protein.

**Keywords:** antitumor activity, Bcl-2 protein, PCNA, procyanidins from *Pinus koraiensis* bark, U14 cervical cancer

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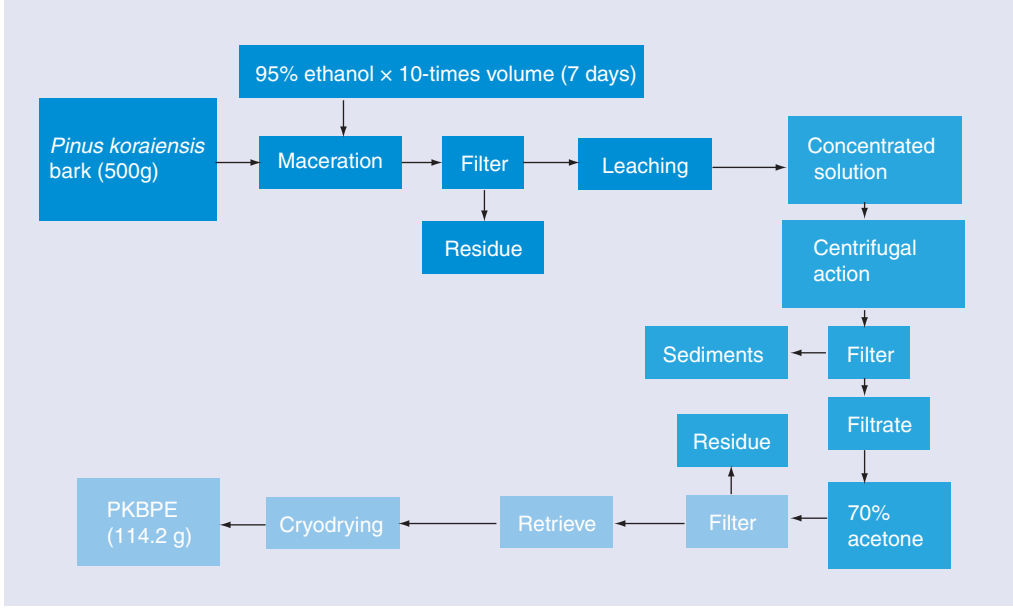
It has been established that plants have are a useful source of clinically relevant antitumor compounds [1]. Indeed, worldwide efforts were made to discover new anticancer agents from plants that would prevent, slow and/or reverse the cancer induction and its subsequent development [2]. There are many approaches for the selection of a plant that may contain biologically active compounds [3,4]. *Pinus koraiensis* is found in the North Mountain area of China and has had lots of successful applications in pharmacologic studies for thousands of years. For example, it has antitumor [1] and antimutation effects [5,6]. The major active ingredients are oligomerization procyanidins, which have very high bioactivity and can remove superfluous free radicals *in vivo*, enhance immunity and have a powerful antioxidant function [5,6].

Recently, our study demonstrated that alkaloids from *Oxytropis ochrocephala* had antitumor effects [7]. In recent experiments, we further explored the antitumor activity of the procyanidins from *P. koraiensis* bark.

## Methods

A total of 50 female Kunming mice (aged 6 weeks), weighing 18–22 g, were provided by the Animal Department of Beijing Institute of Traditional Medical and Pharmaceutical Sciences and fed on a standard pellet diet. The mice were kept in plastic cages in an isolated room at a controlled temperature (18–22°C) and ambient humidity (50–80%). The mice were randomly divided into five groups, with ten animals in each group. The five groups were designated the tumor source mice group, tumor control group, cyclophosphamide group and *P. koraiensis* bark procyanidins extracts (PKBPE) low-dose (163 mg/kg body weight, orally) and high-dose groups (262 mg/kg body weight, orally). To generate the tumor-expressing mice, mice in the tumor source group were injected (intraperitoneal) with $1.60 \times 10^6$ U14 cervical cancer cells at a dose of 0.2 ml/body weight [7]. After 7 days, ascites were aspirated and injected into the left forelimb (subcutaneous) of the rest, with $1.60 \times 10^6$ U14 cervical cancer cells dissolved in normal saline in 0.2 ml/mouse. At 24 h after injection, PKBPE was treated by oral infusion with a dose of 163 mg/kg and 262 mg/kg body weight; mice in the control group received normal distilled water for 15 days. Cyclophosphamide was injected at a dose of 25 mg/kg body weight as the standard reference drug. For determination of mouse weight and tumor size, all animals

**Figure 1. Flow chart of *Pinus koraiensis* bark procyanidins extract.**




PKBPE: *Pinus koraiensis* bark procyanidins extract.

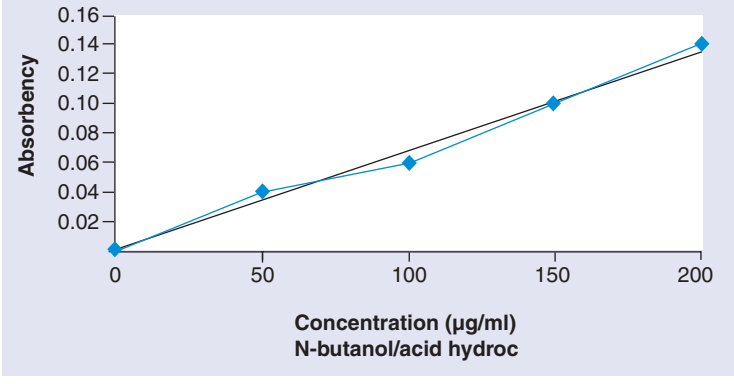
were executed on day 16. The rate of tumor inhibition was calculated using the formula:

$$\frac{C - T}{C \times 100}$$

Where T and C represent average tumor weight of treated groups and control groups, respectively [7].

The fresh collected bark of *P. koraiensis* (collected in Heilongjiang province, China, in September 2006) was first air-dried (30 ± 2°C) and then minced. The 500 g minced sample was exhaustively extracted with 95% ethanol of 10-times volume by maceration for 7 days, heat circumfluence for 2 h twice. Dry ethanol extracts (106.8 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 the dopant was filtrated and removed. The filtrate was put in a drying oven to cryodry [4–6]. The process is summarized in **Figure 1**.

**Figure 2. Standard curves 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 a 95°C aqueous bath for 40 min and cooled quickly to room temperature in cold water. The absorbance in 550 nm wavelength was used to determine procyanidin concentration based on the standard curve of procyanidins.

With PKBPE (262 mg/kg body weight, *per os*), the liver and kidney of executed mice were collected and processed as slides for histopathological analysis with microscope.

The tumors collected from all groups were fixed, embedded and sectioned, then stained with hematoxylin and eosin and observed using a light microscope [7].

Blood samples were collected from all animals via the eyeball, before they were executed. The blood samples were kept at 4°C for 1 h and

**Table 1. Effect of *Pinus koraiensis* bark procyanidins extract treatment on tumor inhibition.**

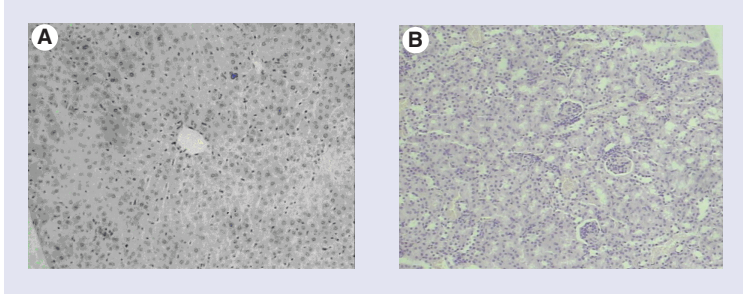
| Groups | Treatment (mg/kg) | Animal number | | Body weight (g) | | Tumor weight (g) | Inhibition (%) | *p-value |
|---|---|---|---|---|---|---|---|---|
| | | *Beginning* | *End* | *Beginning* | *End* | | | |
| Control | Vehicle | 10 | 8 | 20.73 ± 1.95 | 26.67 ± 2.54 | 1.51 ± 0.16 | | |
| CTX | 25 | 10 | 8 | 19.35 ± 1.76 | 22.38 ± 2.06 | 0.49 ± 0.03 | 67.55 | p <0.01 |
| PKBPE | 158 | 10 | 8 | 20.68 ± 1.96 | 26.55 ± 2.31 | 0.79 ± 0.09 | 47.68 | p <0.01 |
| | 250 | 10 | 8 | 20.81 ± 1.38 | 23.87 ± 2.66 | 0.62 ± 0.04 | 58.94 | p <0.01 |

**Value for the tumor weight of CTX, PKBPE group compared with control group. Values are mean ± standard deviation.*
*CTX: Cyclophosphamide; PKBPE:* Pinus koraiensis *bark procyanidins extract*

centrifuged at 956 g for 5 min at room temperature to prepare serum. The concentration of serum TNF-α and IL-8 with commercial ELISA kits by following the instructions of manufacturer.

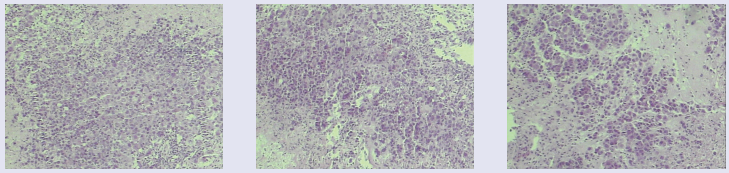
Tumor sections were prepared as previously mentioned and used to examine the expression of proliferating cell nuclear antigen (PCNA) and Bcl-2 proteins. Using the standard immunohistochemical streptavidin peroxidase conjunction method and light microscopy, the tumor slides were stained and examined. Counterstained by hematoxylin, positive and negative cells were the cells of the distinctly brown nucleus and blue nucleus stains, respectively. The numbers of positive cells were counted for statistical analysis.

**Figure 3. Pathological analysis of PKBPE effect on the liver and kidney.**




Pathology Sections of **(A)** liver and **(B)** kidney (hemotoxin and eosin ×100).

**Figure 4. Effect of PKBPE on tumor cell morphology.**




Sections of U14 cervical cancer Cell (hemotoxin and eosin ×100).
CTX: Cyclophoshamide; PKBPE: *Pinus koraiensis* bark procyanidins extract.

Data were expressed as mean ± standard deviation (SD). 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.

## Results

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 2**). The analysis indicated that the procyanidin concentration was 106.8 g and the extract purity was 21.3%.

The administration of PKBPE and standard reference drug (cyclophosphamide) both inhibited tumor growth. Compared with the control group, the tumor weight was reduced in the low- and high-dose PKBPE groups and cyclophosphamide group, respectively. The corresponding tumor-inhibition rate was calculated (**Table 1**). The weight of tumor in the low-dose PKBPE group, the high-dose PKBPE group and the cyclophosphamide group were all significantly lower than that of the control group ($p < 0.01$).

The microscopic examination of the slides of liver and kidney samples showed clear central vein and hepatic lobule. The cells from liver samples looked healthy and the renal tubular from the kidney samples appeared normal (**Figure 3**). These data suggested that administration of PKBPE did not affect liver and kidney growth.

As shown in **Figure 4**, microscopic examination of the tissue sample revealed that PKBPE (262 mg/kg body weight, *per os*) and cyclophosphamide significantly inhibited the numbers of tumor cells and the maglignant phenotype compared with the control group. PKBPE significantly increased the content of TNF-α and reduced the content of IL-8 compared with the control group ($p < 0.01$) (**Table 2**).

**Table 2. Effect of *Pinus koraiensis* bark procyanidins extract on superoxide dismutase and malondialdehyde in serum.**

| Groups | Treatment (mg/kg) | n | SOD (U/ml) | MDA (nmol/ml) | *p-value |
|---|---|---|---|---|---|
| Control | Vehicle | 10 | 284.71 ± 10.84 | 5.06 ± 0.22 | |
| CTX | 25 | 10 | 322.62 ± 11.19 | 3.16 ± 0.13 | $p < 0.01$ |
| PKBPE | 158 | 10 | 292.49 ± 11.91 | 4.15 ± 0.27 | $p < 0.01$ |
| | 250 | 10 | 451.62 ± 15.58 | 1.91 ± 0.06 | $p < 0.01$ |

*Value for CTX, PKBPE group compared with control group. Values are mean ± standard deviation.
CTX: Cyclophosphamide; MDA: Malondialdehyde; PKBPE:* Pinus koraiensis *bark procyanidins extract; SOD: Superoxide dismutase*

Finally, we examined the effect of PKBPE on the expression of the *PCNA* gene and Bcl-2 protein. The expression of PCNA was inhibited in a dose-dependent manner in the PKBPE group compared with the control groups. The percentage of PCNA and Bcl-2 protein-positive cells was lower with the administration of the PKBPE (262 mg/kg body weight, *per os*) and cyclophosphamide compared with the control group ($p < 0.01$) (**Table 3; Figures 5A & B**).

## Discussion & conclusion

Cancer is a major threat to humans today. Over the last few years, cancer has become regarded as the top killer and cervical cancer is the third most common cancer among women worldwide, promoting a higher emphasis on research into the condition. There are many therapy methods, such as surgery, chemotherapy, radiotherapy and gene therapy. In general, these cancer treatments are not effective enough and have unpleasant side effects. Therefore, searching for effective cancer therapeutic medicines is very significant.

Plant drugs are effective enough to cure cancer clinically. There were reports that the procyanidins had antitumor activity, but there are no previous studies on cervical cancer. In the present study, we examined PKBPE activitiy, including its antitumor and immunomodulation effects and its effect on the expression of PCNA and Bcl-2 protein.

The results demonstrate that the antitumor activity of PKBPE occurs in a dose-dependent manner. When the dose of PKBPE was raised up to 262 mg/kg body weight, no effects on mice weight or kidney and liver toxicity were observed.

TNF is secreted by mononuclear macrophages and is an important regulatory factor of immunoreactions and inflammatory reactions. It has anti-infection and antitumor properties and promotes the healing of impaired tissues. The cancer-inhibiting effects of TNF-α are due to killing and wounding tumor cells directly and indirectly. IL-8 is produced by activated mononuclear macrophages or T cells. It attracts neutrophilic leukocytes and T lymphocytes to inflamed areas to participate in the inflammatory reaction [8]. In the PKBPE administration group, the content of TNF-α increased to induce T cells and other killer cells to kill and wound tumor cells; therefore, it inhibited tumor cell secretion of IL-8 and decreased the amount of IL-8.

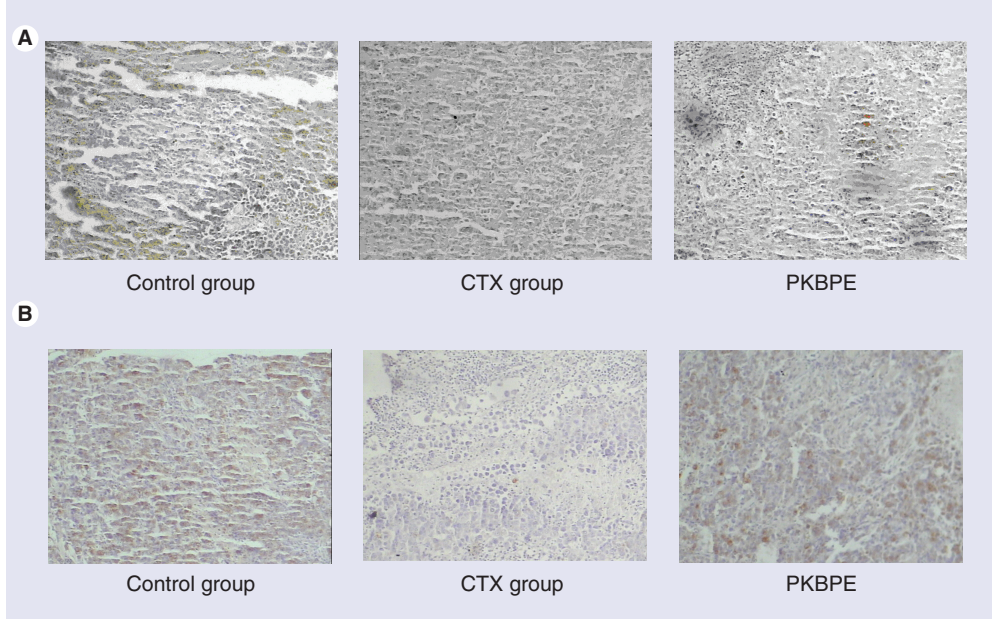
PCNA is a nuclear cell protein expressed in cell cycle G1, S, M, and G2, which is closely correlated with DNA replication. PCNA is connected with the proliferation of tumor cells and

**Table 3. Effect of *Pinus koraiensis* bark procyanidins extract on expression of Ki-67, mutant p53 and Bcl-2.**

| Groups (mg/kg) | Treatment | Animal | Ki-67 | Mutant p53 | Bcl-2 | *p-value |
|---|---|---|---|---|---|---|
| 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 | $p < 0.01$ |
| PKBPE | 250 | 10 | 21.34 ± 1.69 | 33.25 ± 2.46 | 6.96 ± 0.84 | $p < 0.01$ |

*Value for CTX,* Pinus koraiensis *bark procyanidins extract group compared with control group. Values are mean ± standard deviation.
CTX: Cyclophosphamide; PKBPE:* Pinus koraiensis *bark procyanidins extract.*

**Figure 5. Effect of PKBPE on expression of PCNA and Bcl-2 protein.**




Expression of **(A)** PCNA and **(B)** Bcl-2 in nucleus (hemotoxin and eosin ×100).
PCNA: Proliferating cell nuclear antigen; PKBPE: *Pinus koraiensis* bark procyanidins extract

reflects cell proliferation. Bcl-2 is a proto-oncogene and its overexpression can inhibit apoptosis. The expression of PCNA and Bcl-2 protein in the PKBPE administration groups were lower than those of the tumor control group in our study. It showed the antitumor mechanism of PKBPE might be related to the low expression of PCNA and Bcl-2 protein [9–25].

Therefore, our study demonstrated that PKBPE can inhibit tumor growth through increasing TNF-α content and decreasing IL-8 content. Similarly, PKBPE suppressed expression of PCNA and Bcl-2 protein. We showed that PKBPE can inhibit tumor growth and has an antitumor effect. Further studies are now required now to investigate its mechanism.

## Executive summary

- The procyanidins from *Pinus koraiensis* bark extract not only have antioxidant, anti-aging and antimutant properties, but also has antitumor activity.
- The procyanidins from *Pinus koraiensis* bark can inhibit tumor growth by increasing TNF-α content and decreasing IL-8 content.
- The procyanidins from *Pinus koraiensis* bark can inhibit tumors by suppressed expression of *PCNA* and *Bcl-2* genes.

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