

## **Structural characterization of the active substances in *Taxus Cuspidata* and their anti-osteosarcoma activity.**

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### **Abstract**

The aim of this paper was to study the active substances in *Taxus uspidate* and their anti-osteosarcoma effect. Preparative chromatography, column chromatography and preparative HPLC were used for the structural characterization of isolated active substances. MTT assay, electron microscopy and flow cytometry were applied to detect the anti-osteosarcoma effect of paclitaxel. Five compounds were isolated. Different concentrations of paclitaxel test solutions all had an inhibitory effect on MG-63 cells. With the increase in action time, the survival rate of tumor cells declined. Under an inverted microscope, MG-63 cells; which were treated with paclitaxel for 24 h, partially turned round and were floating. Cell volumes diminished, after which the cells immediately detached from the adjacent cells, and apoptosis occurred. Osteosarcoma is one of the most common bone tumors in the adolescent group, and *Taxus uspidate* has a significant anti-osteosarcoma activity.

**Keywords:** *Taxus cuspidate*, flow cytometry, MG-63 cell

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### **Introduction**

*Taxus cuspidata* is a plant in the genus *Taxus* of the family Taxaceae, which is an evergreen arbor or shrub mainly found in the northern hemisphere. It has two subspecies, *T. cuspidata* Sieb. et Zucc. and *T. cuspidata* Sieb. et Zucc. var. *nana* Rehder. Compared with other species of *Taxus*, *T. cuspidata* has a relatively higher paclitaxel content. Experimental studies have shown that [1-2], paclitaxel content in various parts of *T. cuspidata* varies; dry bark has the highest paclitaxel content, followed by xylem, while twig and leaf have the lowest content. Because *T. cuspidata* grows slowly, and its resource is scarce, there is a relative shortage of raw materials for obtaining paclitaxel. Although paclitaxel content in the branch and leaf of *Taxus* is only about 1/10 of that in the dry bark, its total production is much larger than the dry bark, so increased usage of twigs and leaves is an important way to ease the serious shortage of paclitaxel production.

In 1922, Kono first reported that *T. cuspidata* contains Taxine, since then, the research on *Taxus* plants has been increasing. So far, taxane diterpenoids, sesquiterpenoids, steroids, lignans, flavonoids, glycosides, and a variety of other compounds have been isolated from *T. cuspidata* [3-8].

Modern pharmacological studies have shown that paclitaxel can promote the irreversible polymerization and synthesis of

tubulin, block the normal dynamic regeneration of microtubule bundles, cells cannot form normal mitotic spindles, thereby inhibiting cell division and proliferation. Clinically, paclitaxel compounds can effectively inhibit the growth of a variety of drug-resistant tumor cell lines, and thereby have relatively significant effects on esophageal cancer, nasopharyngeal cancer, bladder cancer, lymphoma, prostate cancer, malignant melanoma, gastrointestinal cancer and other diseases [9-12].

In this study, the active constituents in *T. cuspidata* are isolated and identified, while its osteosarcoma inhibitory effect is also investigated.

### **Materials and Method**

Bruker AV superconducting NMR, CO<sub>2</sub> incubator (NBS, USA), clean bench (Suzhou Purification Equipment Factory), flow cytometry (BECKMAN-COULTER, USA), low-speed centrifuge (Shanghai Anting Scientific Instrument Factory). 200-300 mesh column chromatography silica gel (Qingdao Haiyang Chemical Factory); Sephadex LH-20 column chromatography media (Pharmacia), RPMI 1640 medium (HyClone); fetal bovine serum (Hangzhou Sijiqing Bioengineering Materials Co., Ltd.), DMSO, MTT (Sigma, USA). The reagents used were all of analytical or chemical grade.

The crude drug was identified as the twigs and leaves of *T. cuspidata* Sieb. et Zucc. in the genus *Taxus* of the family *Taxaceae*. Paclitaxel test solution was prepared in appropriate concentrations as per the experimental requirements. MG-63 cells were purchased from China Center for Type Culture Collection, Wuhan University.

#### Extraction and isolation

Fifteen kilograms of *Taxus cuspidata* twigs and leaves were ground into a coarse powder, and soaked overnight with methanol (medicinal materials were submerged at a depth of 2-4 cm) with three changes. The extracts were then combined, and methanol was removed. The final extract was dissolved with an appropriate amount of water, defatted with cyclohexane, and extracted with dichloromethane. The extractant was then evaporated to give the extract. The extract was loaded on the column, gradient eluted with chloroform-methanol. Similar fractions were combined, and subjected to preparative chromatography, column chromatography and preparative HPLC to isolate 5 compounds.

#### Cell culturing

MG-63 cell lines were cultured in DMEM medium containing 10% fetal bovine serum, placed in a 37°C, 5% CO<sub>2</sub> incubator and subcultured routinely. Cells in logarithmic growth phase were used in the experiment.

#### MTT assay

After digestion with 0.25% trypsin, logarithmic growth phase MG-63 cells were prepared into a cell suspension with a concentration of  $5 \times 10^4$  cells/mL, and seeded in 96-well plates. Three replicate wells were set up with different concentrations of paclitaxel test solutions (concentrations of 300 nmol/L, 600 nmol/L, 900 nmol/L), and in the control group was added an equal volume of PBS. After culturing for 24 h, 36 h and 48 h, in each well was added 20  $\mu$ L of MTT (5 mg/mL) solution, and incubation was continued for another 4 h till culturing was terminated. Supernatant was carefully discarded, 100  $\mu$ L of DMSO was added to each well and after proper shaking, A490 of each well was measured on an ELISA reader, followed by calculation of MG-63 cell inhibition rates by various concentrations of paclitaxel.

Cell inhibition rate = (mean A490 of the control group - mean A490 of the experimental group) / mean A490 of the control group  $\times$  100%

#### Inverted microscopy

After the treated MG-63 cells were grown on slides, they were observed under scanning electron microscope.

#### Flow cytometric detection of cell cycle

The  $5 \times 10^5$ /ml MG-63 cells were seeded in 96 well plates, and treated with the above test concentrations of paclitaxel and PBS as in the experimental groups and the control group. After culturing for 24 h, cells in each group

were collected separately (each group had three wells). The collected cells were stained with propidium iodide (PI), and loaded on the flow cytometer for detection and analysis.

#### Statistical analysis

Data were processed using SPSS 13.0 statistical software, measurement data were expressed as  $\bar{x} \pm s$ , comparison among groups was performed by t test, and  $P < 0.05$  was considered statistically significant.

## Results

#### Structural characterization of active constituents:

##### Compound 1

Colorless crystals, m.p. 258-260°C. <sup>1</sup>HNMR(CDCl<sub>3</sub>, 500MHz):  $\delta$  1.31, 1.25s (Me-16, Me-18), 2.19s, 2.17s, 2.11s, 2.05s (3H, OAc), 2.38, 1.85 (m, H<sub>2</sub>-6), 2.62 (1H, d, H-14  $\beta$ ), 3.11 (1H, dd, H-14), 3.57 (1H, d, H-3), 4.12, 3.58 (1H, H<sub>2</sub>-17), 5.24, 4.42 (1H, d, H<sub>2</sub>-19), 5.62, (1H, H<sub>2</sub>-20), 5.63 (2H, m, H-5, H-7), 5.34 (2H, brs, H-9, H-10), 6.24 (1H, dd, H-2), 7.86 (10H, m, Ph), 8.07, 7.05 (1H, CinnCH=CH), 8.23 (10H, m, Ph).

<sup>1</sup>HNMR spectral data of compound 1 were basically consistent with the reported literature [13], so its structure was 1-hydroxybaccatini.

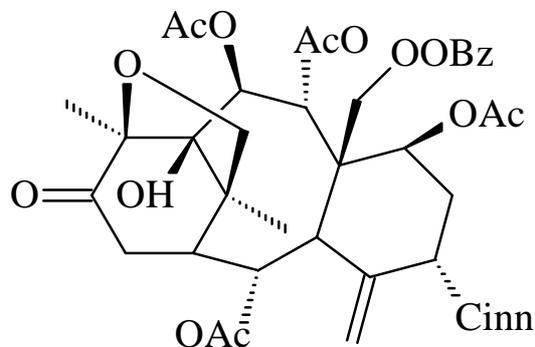


Figure 1. 1-hydroxybaccatini

##### Compound 2

White amorphous powder, m.p. 160-162°C. <sup>1</sup>HNMR (CDCl<sub>3</sub>, 500MHz):  $\delta$  1.92(1H, m, H-6 $\beta$ ), 1.12(3H, s, 16-CH<sub>3</sub>), 1.14(3H, s, 17-CH<sub>3</sub>), 1.74(1H, dd, H-14 $\alpha$ ), 1.83(3H, s, 19-CH<sub>3</sub>), 1.98(3H, s, 18-CH<sub>3</sub>), 2.23(3H, s, OCOCH<sub>3</sub>), 2.25(1H, dd, H-14 $\beta$ ), 2.52(1H, dt, H-6 $\alpha$ ), 3.08(1H, d, H-3), 4.12(1H, d, H-20 $\alpha$ ), 4.25(1H, t, H-7), 4.38(1H, dd, H-9), 4.48(1H, d, H-20 $\beta$ ), 4.52(1H, m, H-10), 4.55(1H, m, H-13), 4.92(1H, d, H-5), 6.11(1H, d, H-2), 7.42(2H, t, Ph-m), 7.63(1H, t, Ph-p), 8.05(2H, d, Ph-o). <sup>13</sup>C-NMR(CDCl<sub>3</sub>, 125MHz):  $\delta$ : 11.6(C-18), 12.5(C-19), 22.2(OCOCH<sub>3</sub>), 25.2(C-17), 27.9(C-16), 37.7(C-6), 39.4(C-14), 42.5(C-8), 44.2(C-3), 67.9(C-1), 68.2(C-10), 68.3(C-2), 72.8(C-7), 74.3(C-20), 76.8(C-15), 77.3(C-13), 80.1(C-4), 80.4(C-9), 84.5(C-5), 128.3(Ph-m), 129.4(Ph-

o), 129.7(Ph-1), 133.2(Ph-p), 137.6(C-11), 146.5(C-12), 166.5(COPh), 171.4(OCOCH).

<sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectral data of compound 2 were basically consistent with the literature [14], so its structure was identified as 7,13-dideacetyl-9,10-didebenzoyl taxchinin C.

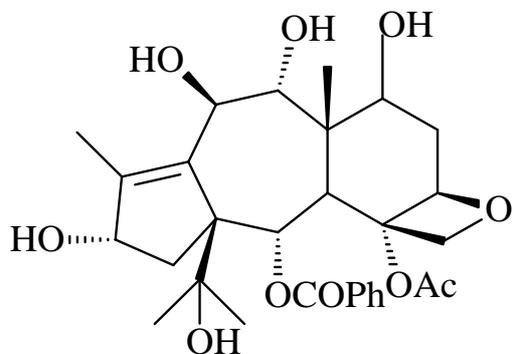


Figure 2. 7, 13-dideacetyl-9,10-didebenzoyl taxchinin C

### Compound 3

White powder, m.p. 170-172°C. <sup>1</sup>HNMR(CDC13, 500MHz): δ 1.13(3H, Me-16), 1.32(3H, Me-18), 2.05(3H, s, OAe), 2.04(3H, s, OAe), 2.12(3H, s, OAe), 2.16(3H, s, OAe), 2.25, 1.82(1H, m, H<sub>2</sub>-6), 2.78(1H, d, H-14 β), 3.05(1H, dd, H-14 α), 3.74(1H, d, H-3), 4.12(1H, d, H<sub>2</sub>-17), 4.48(1H, t, H-5), 5.25(1H, d, H<sub>2</sub>-19), 5.34(1H, d, H-9), 5.37(1H, d, H-10), 5.45, 4.72(1H, s, H-20), 5.58(1H, dd, H-7), 7.83(5H, m, ph), 8.17(1H, dd, H-2).

Structural data of compound 3 were basically consistent with the literature [15], so its structure was 2α-deacetoxy taxinine J.

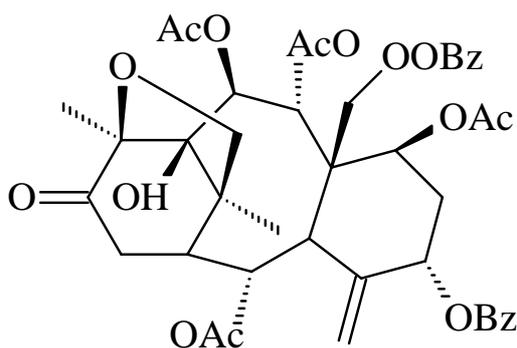


Figure 3. 2α-deacetoxy taxinine J

### Compound 4

White crystals, m.p. 178-180°C. <sup>1</sup>HNMR (CDC13, 500MHz): δ 1.17(3H, s, 16-CH<sub>3</sub>), 1.25(3H, s, 17-CH<sub>3</sub>), 1.30(3H, s, 19-CH<sub>3</sub>), 1.72(1H, dd, H-1), 1.94(1H, d, H-3 α), 1.97(3H, s, 18-CH<sub>3</sub>), 2.01(3H, s, 7-OCOCH<sub>3</sub>), 2.11(2H, m, H-6), 2.18(3H, s, 10-OCOCH<sub>3</sub>), 2.19(3H, s, 13-OCOCH<sub>3</sub>), 2.12(1H, m, H-14 α), 2.59(1H, d, H-3 β), 2.68(1H, ddd, H-14 β), 4.42(1H, m, H-5), 4.62(1H, dd,

H-2), 5.21(1H, t, H-7), 5.38(1H, brd, H-13), 5.74(1H, d, H-20), 6.37(1H, s, H-10). <sup>13</sup>C-NMR(CDCl<sub>3</sub>, 125MHz) δ : 18.9(C-18), 20.2(7-OCOCH<sub>3</sub>), 20.3(10-OCOCH<sub>3</sub>), 20.4 (13-OCOCH<sub>3</sub>), 20.8(C-19), 24.2(C-17), 25.2(C-14), 35.3(C-6), 35.4(C-16), 35.5(C-3), 37.5(C-15), 49.5(C-1), 67.4(C-2), 68.3(C-5), 69.1(C-13), 69.7(C-7), 52.9(C-8), 77.4(C-10), 127.2(C-20), 131.8(C-11), 136.2(C-12), 137.6(C-4), 167.2(7-OCOCH<sub>3</sub>), 169.5(10-OCOCH<sub>3</sub>), 169.8(13-OCOCH<sub>3</sub>), 205.5(C-9).

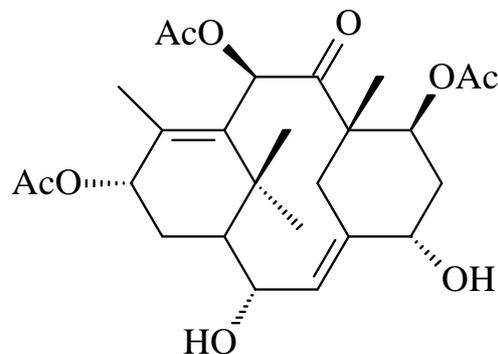


Figure 4. 2-deacetyl taxin B

The above data were basically in line with the reported literature [16], so the structure of compound 4 was 2-deacetyl taxin B.

### Compound 5

Colorless crystals, m.p. 245-251°C, compound 5 and the previously isolated taxayuntin were chromatographed on the same thin layer plate with multiple development systems, the results revealed that their R<sub>f</sub> values were identical, so the structure of compound 5 was taxayuntin. Its <sup>1</sup>HNMR spectral data are as follows: <sup>1</sup>HNMR(CDC13, 500MHz): δ 1.04(3H, s, Me-16), 1.25(3H, s, Me-16), 1.57(3H, s, Me-16), 1.93(3H, OAe), 2.02(3H, OAe), 2.17(3H, OAe), 2.15(3H, OAe), 2.27(1H, m, H-6), 2.58(1H, d, H-14 β), 3.05(1H, dd, H-14 α), 3.45(1H, d, H-3), 4.92(1H, d, H-9), 5.45m(2H, H-7), 5.57(1H, dd, H-2), 7.94, 6.95(1H, d, C1nnCH=CH), 7.85(5H, m, Ph).

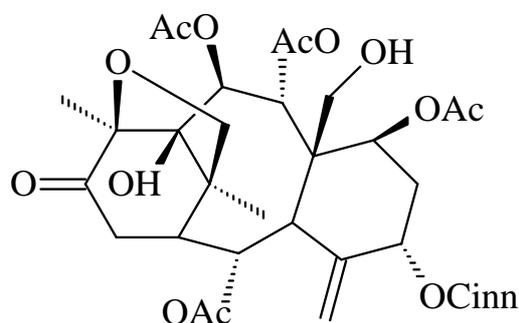


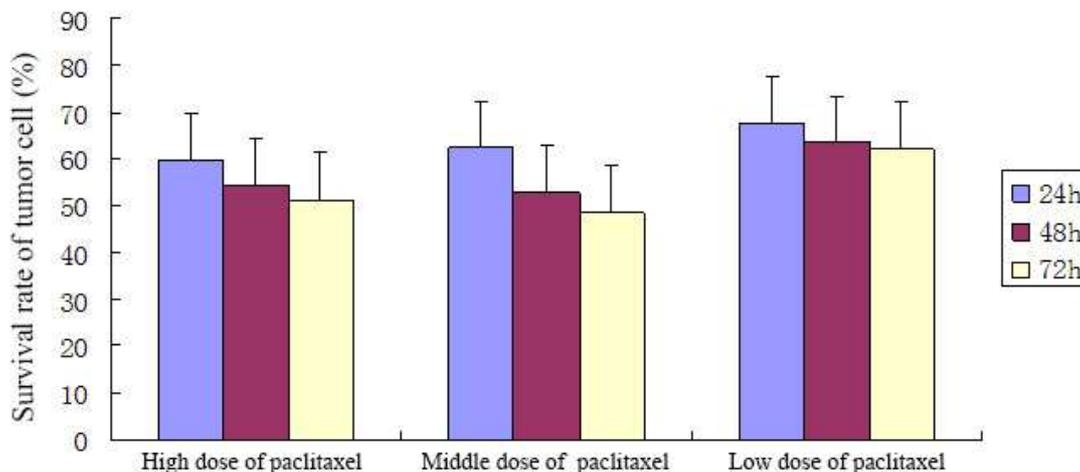
Figure 5. taxayuntin

### Determination of the inhibitory effect of paclitaxel on MG-63 cells by MTT assay.

The experimental results showed that all the different concentrations of paclitaxel test solutions had an inhibitory effect on MG-63 cells. With increase in action

time, the survival rate of tumor cells declined. High concentration group had a significantly higher effect on cell survival rate than the medium and low concentration

groups. It is thus evident that the inhibitory effect of paclitaxel on MG-63 cell survival was in a time-dose-effect relationship.



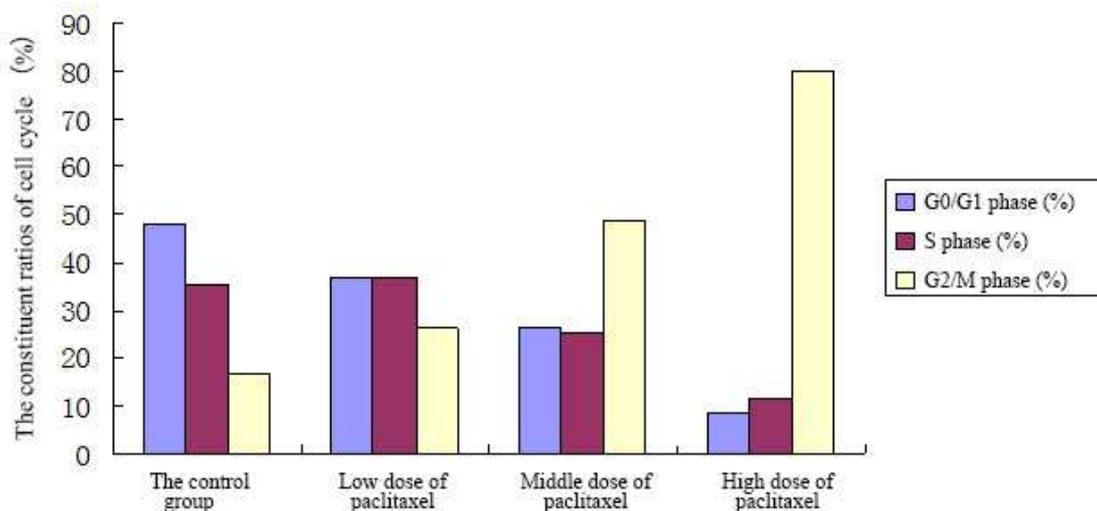
**Figure 6.** Determination of the inhibitory effect of paclitaxel on MG-63 cells by MTT assay

#### Morphological observation

Under an inverted microscope, MG-63 cells which were treated with paclitaxel for 24 h, partially turned round and were floating. Cell volumes diminished and the cells immediately detached from adjacent cells. Loss of microvilli, cytoplasmic concentration, cell shrinkage, poor adherence and karyopyknosis were seen. With the increase in action time, cell volume further diminished, cytoplasm was concentrated, endoplasmic reticulum was expanded to a bubbly form and fused with the cell membrane. Nuclear chromatin were condensed and were in a half-moon shape. Parts of cell nucleoli were fragmented, leading to cell membrane retraction. Cells were self-divided into multiple apoptotic bodies enveloped by cell membranes without overflow of inclusions.

#### Flow cytometry results

After treatment with different drugs (low dose of paclitaxel, middle dose of paclitaxel, high dose of paclitaxel.) for 24 h, the MG-63 cells were stained with PI, and its apoptosis rate was analyzed by flow cytometry. The results are shown in Fig. 7. Apoptosis rate of G2/M phase cells increased significantly, which reached 79.8% in the high-dose group, presenting an obvious G2/M phase arrest. With the increase of action time, apoptosis rates of G0/G1 and S phase cells gradually decreased, where the G0/G1 phase apoptosis rate was only 8.7% in the high-dose group. With increasing concentrations of paclitaxel, apoptosis rate of MG-63 cells markedly increased.



**Figure 7.** Flow cytometry results

## Discussion

*Taxus cuspidata* is a plant in the genus *Taxus* of the family Taxaceae, which is also known as Zishan. At present, there are a total of 11 *Taxus* species worldwide, and China has 4 species and 1 variant, they are: *T. cuspidata* Sieb. et Zucc., *T. wallichiana* Zucc., *T. yunnanensis* Cheng et L. K. Fu, *T. chinensis* (Pilger) Rhd, as well as *T. chinensis* var. *mairei* Cheng et L. K. Fu [17-18]. *Taxus cuspidata* is a plant in the family Taxaceae S. F. Grey, order Taxales, class Coniferopsudec, subdivision Gymnospermae, which is distributed in Heilongjiang, Liaoning and Jilin provinces. It is also found in countries like Japan and Korea.

*T. cuspidata* was recorded in the "Compendium of Materia Medica" to be mainly used for the treatment of cholera, typhoid and detoxification. In the folk medicine, the fruits, twigs and leaves of *Taxus* plants are also taken in decoction, mainly for dispersing accumulations, expelling parasites, moistening dryness, inducing diuresis and stimulating menstrual flow, it can also treat a variety of intestinal parasites.

To fully protect the *Taxus* resources, and find substitutes for traditional medicinal parts of *Taxus*, this experiment was carried out using the extract from 15 kg of the twigs and leaves of *Taxus cuspidata*.

Osteosarcoma is one of relatively common bone tumors, which belongs to primary bone malignancy, it occurs mostly in the adolescent age group bringing a heavy blow to the patients and their families. Currently, osteosarcoma is treated mostly with surgery plus chemotherapy, and cisplatin is used extensively in the clinical setting owing to its excellent anti-tumor effects. Due to its serious side effects and increasingly prominent drug resistance problems, its clinical application is restricted, and the search for effective substitutes is needed urgently.

Paclitaxel is an active substance isolated from a variety of Taxaceae plants, whose anti-tumor activities have already been clinically demonstrated. A study has reported that the important pathway for paclitaxel's inhibition of osteosarcoma growth is that paclitaxel can inhibit tumor cell mitosis, induce and promote tubulin polymerization to form stable microtubule polymers while inhibiting the depolymerization of formed microtubules, making the vascular bundles unable to interconnect with the microtubule organizing center, significantly reducing the number of free microtubules, and arresting cells in the G2 and M phases. It breaks the dynamic balance of microtubule system functionally necessary during cell division, causing apoptosis of tumor cells by blocking their replication.

The characteristics of the research lie in making activity study of anti-osteosarcoma on paclitaxel, extracting and

separating the Terpenoids of *Taxus cuspidata*, and five Terpenoids were gained. Through activity study of pharmacology, it finds that there is good inhibition effect of paclitaxel on MG-63 osteosarcoma cell. For paclitaxel is the featured composition of *Taxus cuspidata*, as well as the Terpenoids, other featured Terpenoids of *Taxus cuspidata* should be separated and five featured Terpenoids Monomers of *Taxus cuspidata* were gained. The content of the five Terpenoids in *Taxus cuspidata* is little, so the gained quantity after separation is little as well. And then the five Monomers should be accumulated to build foundation for the further activity study, for the high value of activity study on these five Terpenoids.

## Conclusions

Through MTT methods, morphological observation and flow cytometry test, it finds that the inhibition effect of paclitaxel on MG-63 osteosarcoma cell shows relation of time-dose-effects. Five Terpenoids were separated from the extraction of *Taxus cuspidata*: 1-hydroxybaccatini, 7,13-dideacetyl-9,10-didebenzoyl taxchinin C, 2 $\alpha$ -deacetoxy taxinine J, 2-deacetyl taxin B, taxayuntin. It makes research on the Terpenoids of *Taxus cuspidata*, to lay material basis for the future activity study of anti-osteosarcoma on Terpenoids of *Taxus cuspidata*.

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