Determination of Active Substance Content and Bacteriostasis in *Polygonatum kingianum* at Different Growth Ages

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Abstract: *Polygonatum kingianum* is an important traditional Chinese medicine. In this paper, the correlation between the exploration age and the content of polysaccharide and total flavone, and the bacteriostatic activity of the active ingredient were discussed. The findings demonstrated that in *Polygonatum kingianum* the maximum content of total flavonoids and polysaccharides appeared at plant growth stage of 2nd and 3rd year, respectively. While the polysaccharide, total flavone, and boiled water extract had not shown an obvious inhibitory effect on the 5 indicator bacterial and fungal strains (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Candococcus albicans,* and *Aspergillus niger*) through *in-vitro* bacteriostatic experiment. Therefore, it is suggested that 3-year old plant growth stage is the most suitable for harvesting, and it should be carefully used in the application and development of antibacterial properties.

1. Introduction

Polygonatum kingianum, which was included in the 2020 edition of the Chinese Pharmacopoeia, has received great attention in recent years for its cough-relieving, antioxidant, anticancer, and immune-enhancing effects. Generally, the change of growth age in plants is accompanied by the variation of secondary metabolites, and these alterations of chemical constituents will impact its usefulness. *P. kingianum* used as medicine with rhizomes, and rhizome is extended by one section every year. If the age section of the new rhizomes is 1 year, the age section of the root tubers growing in the previous year is 2 years, and so on, the planting years of rhizome can be known by the age section of the rhizomes. However, there are relatively few studies examining the relationship between the age of *P. kingianum* and its active ingredient content, as well as the active ingredients and its antibacterial properties. This study is based on *P. kingianum*, which is planted in Pu'er, Yunnan and has a lifespan of 2-5 years, as the experimental material. The content of polysaccharides and total flavonoids were used as quantitative measurement indicators to study the correlation between the harvesting period of *P. kingianum* and the content of active ingredients. The *in vitro* antibacterial effect of active ingredients was also studied, which provides an experimental basis for further understanding the rational harvesting and pharmacological effects of *P. kingianum*.

2. Materials and Methods

2.1 Site Selection

Pu'er (Latitude: 22.78, Longitude: 100.97) is located in the southern part of Yunnan Province in China. It also shares borders with Vietnam, Laos, and Burma. It has a warm humid subtropical climate, with average temperature of 19 °C, annual rainfall of 1,487mm, and relative humidity of 80%.

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2.2 Sample collection

The samples (rhizomes) were collected from 2 to 5 years old *P. kingianum* plants cultivated at Lancang County, Pu'er City. The samples were packed in polythene bags and immediately transferred into the ice box and brought to the laboratory for further analyses.

2.3 Main equipment and instruments

UV spectrophotometer UV-2550; ultrasonic washer SK8200LHC; electronic balance CP224C; low-speed Centrifuge SC-3616; stream needle filter 0.22um.

2.4 Extraction and content determination of polysaccharide

The polysaccharide content was measured by phenol-sulfuric acid method[1]. Three-gram powder of each *P. kingianum* (ranging from 2-5 years) was screened with a sieve of 60 mesh and further added to the beaker, and then 60 mL of distilled water was added. The beaker was placed in 80℃ constant temperature water bath for 2h, then the filter was pumped while hot, the filtrate was concentrated to 20 mL, and a certain amount of ethanol was slowly added in, and stirred quickly, centrifuged at 3000 rpm/min for 10 min, filtered, and precipitated. The filter paper was dissolved in 80 mL of deionized water, and all of them were dissolved and filtered. Then the filtrate was poured into a 100 mL of volumetric flask for constant volume. This experiment was done with three replications.

2.5 Extraction and determination of total flavonoids

The content of total flavonoids was measured by the aluminum nitrate-sodium nitrite method. There was 15 mL of 60% ethanol added into centrifuge tube. Furthermore, the extract was filtered with 60% ethanol to 25 mL volumetric flask under the extraction temperature of 60℃ and the extraction power of 400w.

2.6 Preparation of water extract

Five grams of 2-5 years old *P. kingianum* was added into 60mL of hot water (75-80℃) twice for 1h, filtered, combine the filtrate twice, and concentrated to 50 mL, which is equivalent to 0.1g /mL.

2.7 Measurement of antibacterial activity

Five strains *(Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Candococcus albicans, and Aspergillus niger)* were cultured in LB medium (Tryptone 10 g, yeast extract 5 g, NaCl 5 g, agar 18 g, distilled water 1000 mL, and pH 7.0) and PDA medium (Potato (infusion form) 200 g dextrose 20 g, agar 15 g, and distilled water 1000 mL), respectively. The filter paper method was used to observe the effect of active ingredients on the tested bacteria. The bacterial strains were cultured and placed into incubator for 2 days, while fungal strains were placed into an incubator for 5 days of cultivation. The antibacterial zone was measured as the distance between the outer edge of the circular filter paper and the outer edge of the antibacterial zone.

2.8 Statistical analysis

The data were statistically analyzed using analysis of variances (ANOVA) in IBM SPSS Statistics 23, the means were subjected to Duncan's multiple range test at $p \leq 0.05$ to compare the differences in the content of polysaccharides and total flavonoids in *P. kingianum* under different cultivation years. Pearson correlation analysis was used to determine the correlation between age groups, polysaccharides, and total flavonoids.

3. Results and Discussion

3.1 Comparative analysis of polysaccharides and total flavonoids content in *P. kingianum* at various growth ages

P. kingianum prefers cool, damp, and moderate wetness environments, and can be found in the

wild on shrubs, wet grass slopes, or rocks. It has impeller growth with four to ten flowers per round and a lanceolate shape. The inflorescence typically consists of four to ten flowers, and the total blossom stem is pendulous (Figure 1). The medicinal portion of the plant is the rhizome, which has been cultivated for two to five years and is shaped like long, tuberous blocks of varying lengths (Figure 2).

Figure 1 Leaves and flowers of *P. kingianum*

A1: 2 years, **A2:** 3 years, **A3:** 4 years, **A4:** 5 years

Figure 2 The tubular stems of *P. kingianum* of different Ages;

When the polysaccharide content was determined, the regression equation was obtained by using the standard curve of glucose as the standard material: y=9.4421x-0.0058, R^2=0.9992. The polysaccharide content values of age 2-5 were 19.09%, 23.11%, 21.41% and 13.19%, respectively. When the content of total flavonoids was determined, rutin was used as the standard product to make the standard curve, and the regression equation was obtained: y=0.0119x+0.0063, R^2=0.9997. The polysaccharide content values of age groups 2 to 5 were 0.073%, 0.06%, 0.053%, and 0.063%, respectively.

The results of one-way ANOVA showed that the highest polysaccharide content was found in *P. kingianum*, which was aged 3 years and significantly higher than the samples of 2 and 5 years old plants ($P<0.05$). (Figure 3) There was no significant difference in polysaccharide content between *P. kingianum* of 3 and 4 years old plants ($P>0.05$). There was no significant difference in total flavone content among different age segments ($P > 0.05$) (Figure 4), but the results also showed an inconsistent trend in total flavone content under different cultivation years.

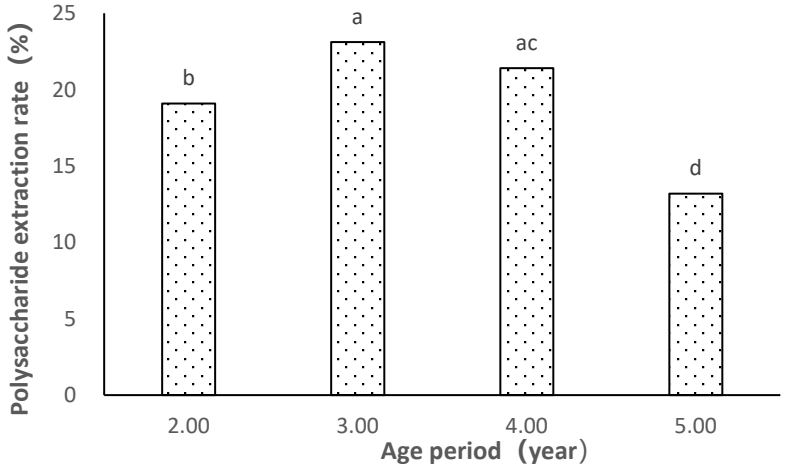

Figure 3 Polysaccharide Content in *P. kingianum* of different ages

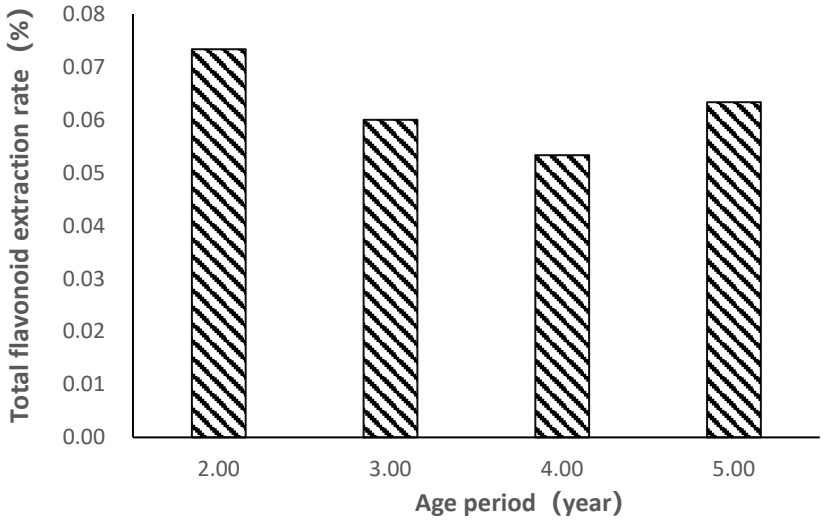

Figure 4 Total flavonoid Content in *P. kingianum* of various growth ages

Correlation analysis was conducted on the content of polysaccharides and total flavonoids in Dianhuangjing under different cultivation years (Table 1). There was no significant correlation between the content of polysaccharides and total flavonoids and the cultivation year of Dianhuangjing ($P>0.05$), but both showed a trend of decreasing component content with the growth of cultivation years.

Table 1 Correlation between age group, polysaccharide and total flavonoid content

	Polysaccharide(%)	Total flavonoid(%)
Pearson correlation	-0.572	-0.376
Significance	0.052	0.228

In the operating procedures for the detection of *Polygonatum* plants in the Chinese Pharmacopoeia, only the determination of polysaccharide content is specified as the quality evaluation index in the content determination. Based on this standard, planting *P. kingianum* in Pu'er for 3 years yields high-quality rhizomes.

3.2 The effect of active components of *P. kingianum* on microbial growth

Many Chinese herbal extracts have shown antibacterial effects during *in vitro* experiments. There are numerous reports published on the antioxidant and diabetes-treating properties of *P. kingianum*

extract, but relatively few on its antibacterial properties. This experiment conducted *in vitro* antibacterial experiments on polysaccharides, total flavonoids obtained in the early stage, and water extracts obtained after boiling by using the filter paper method (Table 2).

Table 2. Effects of *P. kingianum* extract at different ages on the growth culture of five microorganisms

Indicator Microorganisms	**Age section (mm) of *P. kingianum* (year)**											
	2			3			4			5		
	P*	T*	W*	P	T	w	P	T	w	P	T	w
E.coli	1.1	1.0	1.1	1.1	1.1	1.1	1.1	1.1	1.0	1.1	1.1	1.1
S.aureus	1.0	0.8	0.8	0.8	0.8	1.1	0.8	0.8	0.8	0.8	0.8	0.8
P. aeruginosa	—	—	0.8	—	—	0.8	—	—	—	—	—	—
C. albicans	—	0.8	—	—	0.8	—	—	0.8	—	—	0.8	—
A.niger	—	—	—	—	—	—	—	—	—	—	—	—

*Active ingredient of *P. kingianum*. P= Polysaccharides, T= Total flavonoids, W= water extract.— indicates no antibacterial zone. The data in the table refers to the distance between the outer edge of the circular filter paper and the outer edge of the antibacterial ring

This bacteriostasis test includes three kinds of bacteria (two kinds of G -, one kind of G+), one kind of yeast (*Candida albicans*) and one kind of mold (*Aspergillus niger*). In general, no matter polysaccharides, total flavonoids or water extracts obtained after boiling, they have no obvious inhibitory effect on the growth of the five indicator bacteria except *E. coli,* and there are no obvious bacteriostatic circles (Figure 5, B1), and there is almost no inhibitory effect observed on eukaryotic microorganisms such as *Candida albicans* (Figure 5, B2). On the plate inoculated with *A. niger*, the mycelium is covered with filter paper soaked with different extracts (Figure 5, B3); In addition, the extracts obtained from different ages of Dianhuangjing had a relatively consistent effect on the growth of the tested strains. For example, in the test of *E. coli,* the inhibitory effect did not increase or decrease with age, and in the test of *A. niger,* no matter the age was 2 years or 5 years, it had no effect on the growth of *A. niger.*

B1: *E. coli,* B2: *C. albicans,* B3: *A. niger*

Figure 5 Effect of *P. kingianum* polysaccharides on the growth of different microorganisms in three years of age section.

As the most essential active ingredient, polysaccharides have been extensively discussed in extraction methods and efficacy studies. However, the correlation between age and active constituents has been the subject of relatively few studies, which may be due to difficulties in collecting experimental materials. Su Weigeng et al. [2] investigated the effects of various planting modes and age stages on the polysaccharide content of *P. kingianum* under three sample plots in Pu'er, Yunnan. Under the field planting mode, the 5-year-old *P. kingianum* polysaccharide peaked at 13.52 percent, while under the understory planting mode, it peaked at 17.40 percent. The experimental material used in this study is the understory planting mode, which also peaked at 23.11% after three years. Polygonatum is comprised of *P. kingianum*, *Polygonatum sibiricum*, and *Polygonatum cyrtonema* in the Chinese Pharmacopoeia. Pan Defang et al. [3] determined that the optimum age of Polygonatum in Qingyang County, Chizhou, Anhui Province was three years

(approximately 15%). Zhang Puzhao et al. [4] determined that *Polygonatum* in Lueyang County, Shanxi Province, attained a high level at 3 years (approximately 4.5%). *Polygonatum* multiflora in Guizhou, as measured by Chen Yi et al. [5], reached a high value at 1 year of age (14.65%), and then began to decline. In general, the planting location, planting mode and age are all important factors affecting the content of *Polygonatum.* However, when the age is 3 years, the polysaccharide of most *Polygonatum* plants can reach the highest value, which is the most suitable planting period for harvesting. Yunnan Pu'er is very suitable for developing the cultivation of *Polygonatum.*

In vitro antibacterial experiments, Zheng Chunyan [6] and Su Wei [7] have both reported the inhibitory effects of *Polygonatum* polysaccharides on various bacteria and molds, but there are significant differences from the results of this experiment. In the experiment, neither polysaccharides, total flavonoids, nor water extracts showed significant inhibitory effects on the five indicator bacteria. The detailed reasons need further research.

4. Conclusion

It can be concluded that the polysaccharide and total flavonoid content varied with harvest life. In Pu'er, the active ingredient had a mild antibacterial activity that was unrelated to harvest life. Pu'er is an ideal location for the cultivation of *P. kingianum* because of its high polysaccharide content. While three years old plants are better for the harvest period.

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