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Study on The Contents of Aristololactams in *Piper wallichii* and Their Renal Cytotoxicities

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ABSTRACT

In the study, three aristololactams were isolated from *Piper wallichii* and determined simultaneously by a HPLC method using a C_{18} column with a gradient elution of methanol and water. The content ranges of the target compounds in twelve batches of *Piper wallichii* were 0.100 - 0.110 mg/g dry weight (cepharanone B), 0.198 - 0.208 mg/g dry weight (aristolactam AII), and 0.047 - 0.059 mg/g dry weight (aristololactam AIIIa), respectively. Their anti-proliferative effects in HK-2 cells were determined by MTT assay and cells membrane damage were assessed by LDH release assay. Results indicated that the test compounds showed a time- and dose-dependent cytotoxicity in HK-2 cells, and the values of IC_{50} were cepharanone B > aristolactam AII > aristololactam AIIIa.

Key words: *Piper wallichii*, Aristololactams, HPLC, Cytotoxicities

INTRODUCTION

Aristolochic acid nephropathy (AAN) is a progressive tubulointerstitial renal disease, and is predominantly a result of aristolochic acid (AA) intoxication[1]. Some traditional Chinese medicines containing AA have been observed to be nephrotoxic[2]. Owing to the severe nephrotoxicity of AA, the US Food and Drug Administration declared a prohibition of using the products that contain AA, including *Fructus Aristolochiae, Caulis Aristolochiae Manshuriensis* and other eleven formulations[3,4].

Aristololactam (AL), as the important analog of AA, exists in many medicinal plants. Meanwhile, it was reported that AA could be converted to AL in the metabolism process *in vivo*[5]. Researchers found some ALs showed potential cytotoxicity in the human proximal tubular epithelial (HK-2) cell[6,7].

Piper wallichii is a perennial herb of the genus *Piper*, which belongs to the family *Piperaceae*[8,9], and is widely used for the treatment of dysaemia, pain, sexual disturbance and beriberi disease. In the previous studies, three ALs were isolated from the herb[10,11]. Considering the potential renal cytotoxicities of these ALs existing in *P. wallichii*, it is necessary to study their renal cytotoxicities and establish a quality control system for this herb. In this study, a proposed strategy was developed to determine the contents of ALs in *P. wallichii* by HPLC, and their renal cytotoxicities *in vitro* were investigated in HK-2 cells.

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MATERIALS AND METHODS

I. *Plant Materials*

Twelve batches of *P. wallichii* were collected from Wufeng County, Hubei Province, China, and identified by the Associate Chief Pharmacist Zuhai Zhou, Institute of Drug Control of Wufeng County. A voucher specimen (PW0825) was deposited in the herbarium of College of Pharmacy, Tongji Medical Center, Huazhong University of Science and Technology.

II. *Reference Prepared from P. wallichii*

The reference compounds were isolated from the dried herb of *P. wallichii* in our laboratory[10,11], and were identified by UV, IR, ESI-MS, ^{1}H-NMR and ^{13}C-NMR[12-14]. Among them, cepharanone B, aristolactam AII, and aristololactam AIIIa were included. The purity of each compound was determined to be higher than 98% by HPLC. The

chemical structures of the reference compounds are shown in Figure 1.

III. *Pharmacological Studies in Vitro*

(I) *Materials*

HK-2 cells were obtained from the Cell Culture Center of Wuhan College (Wuhan, China). Dulbecco's modified Eagle's medium/F12 (DMEM/F12) and fetal bovine serum (FBS) were purchased from Ginuo (Hangzhou, China). Dimethyl sulfoxide (DMSO) (99.9%), mercuric chloride ($HgCl_2$) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (MO, USA). LDH kits were provided by Nanjing Jiancheng Biology Engineering Institute (Nanjing, China). The reference compounds were first dissolved in DMSO, and then diluted in cell culture medium. The final concentration of DMSO in all preparations was 0.5% (v/v).

(II) *Cell Culture*

HK-2 cells were maintained in DMEM/F12 supplemented with 10% FBS at 37°C in an atmosphere containing 5% CO_2.

(III) *Cytotoxicity Assay*

Cytotoxicity was determined using MTT assay as described by Mosmann[15] with minor modifications. In brief, HK-2 cells (100 μL) were seeded at 10^5 cells/mL in 96-well plates. After 24 h, the cells were exposed to 100 μL of 0.5% DMSO (vehicle control), culture medium without any test compound, 2.5 mg/L $HgCl_2$, 2.5, 5, 10, 20, 40, 80 and 160 mg/mL of cepharanone B, aristolactam AII or aristololactam AIIIa dissolved in FBS free medium. Each condition was tested in four replicates and three independent experiments were carried out. Following incubation for 24 h, 48 h or 72 h, MTT was added and cells were incubated for additional 4 h. The absorbance was then measured at 570 nm on a microplate reader (SLT, Crailsheim, Germany). Relative cell viability (%) was calculated as (mean absorbance of sample/mean absorbance of vehicle control) × 100. Median inhibition concentration (IC_{50}) was defined as the drug concentration that resulted in 50% of cell viability inhibition.

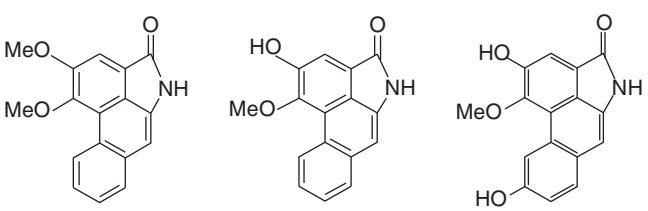

Figure 1. Chemical structures of cepharanone B; aristolactam AII; aristololactam AIIIa.

(IV) *Lactate Dehydrogenase (LDH) Assay*

The integrity of cell membrane was examined by measuring the release of LDH. The cells were treated the same as described in section III(III). 0.5% of DMSO was used as vehicle control. After 24 h of exposure, the release of LDH was measured using the LDH assay kit (Nanjing, China) according to the method described by Lorentz[16]. Each condition was tested in four replicates and three independent experiments were carried out. The LDH release rate (%) was calculated as LDH content in the medium / (LDH content in the medium + LDH content in cell lysis solution) × 100.

IV. *HPLC Analysis*

(I) *Equipment and Chromatographic Conditions*

The HPLC system (Hitachi, Japan) was equipped with a Model L-2130 pump, an on-line solvent vacuum degasser, an auto sampler with 20 μL injection loop, and an L-2400 UV detector. T2000P software was used to record chromatograms and calculate peak areas. Chromatographic separations were carried out on an Agilent TC-C_{18} column (250 mm × 4.6 mm, 5 μm). A binary gradient elution system consisting of methanol (A) and water (B), and was used with the following gradient program: 0 - 35 min, 43 - 68% A; 35 - 50 min, 68 - 100% A; The flow rate was set at 1.0 mL/min. The column temperature was set at 25°C. The injection volume was 20 μL. The detection wavelength was 287 nm.

(II) *Standards Preparation and Calibration Curves*

Cepharanone B (6.6 mg), aristolactam AII (5.3 mg), and aristololactam AIIIa (3.75 mg) were dissolved in a 10 mL volumetric flask with methanol to prepare the mixed standard stock solution. Working standards at the concentration of the calibration range were prepared by stepwise dilution with methanol. The quantitative analysis was performed by the external standard method: the calibration curve for each species was obtained after subjecting a series of standard solutions to the same analytical procedure. The calibration curve was obtained by peak area/corresponding concentration of the injected standard solutions, and the concentrations of target compounds in the samples were calculated based on the calibration curve.

(III) *Sample Preparation*

The herbs of *P. wallichii* were dried at room temperature and crashed into powder (20 meshes). The resulting powder was accurately weighed (about 1 g) and then was extracted with 20 mL methanol in a reflux bath at 85°C for 30 min and then cooled at room temperature. The extract was filtered through a 0.45 μm polytetrafluoroethylene membrane filter (Tianjin Jinteng Instrument Factory, Tianjin, China). The herbal extract was transferred into a 20 mL volumetric flask, and adjusted to volume with methanol.

V. *Statistical Analysis*

In order to classify the target herbal plants, hierarchical clustering analysis (HCA) was performed by using SPSS software (SPSS for windows 11.0, SPSS, USA). In the study on pharmacology, all values are expressed as means ± SD. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

I. *In Vitro Pharmacological Studies*

(I) *Cytotoxicity of ALs in HK-2 Cells*

The cytotoxicity of cepharanone B; aristolactam AII and aristololactam AIIIa in HK-2 cells was determined by MTT assay. The cell viability results for various concentrations of three ALs with 24, 48 and 72 h of incubation are shown in Figure 2 and IC_{50} values are provided in Table 1. $HgCl_2$ (2.5 mg/L), a known nephrotoxic agent, exhibited significant decrease ($p < 0.05$) in HK-2 cells viability, which was similar to the previous study and verified the *in vitro* testing system[17]. As shown in Table 1, the values of IC_{50} were cepharanone B > aristolactam AII > aristololactam AIIIa, indicating Aristololactam AIIIa as the most and Cepharanone B the least toxic in HK-2 cells. Furthermore, the cytotoxicities exhibited in a time- and dose-dependent manner.

(II) *Cell Membrane Injury Caused by ALs*

To investigate the effects of cepharanone B, aristolactam AII and aristololactam AIIIa on the membrane integrity, the release of LDH was examined with 0.5%

Table 1. The median inhibition concentration (IC50) of cepharanone B, aristololactam AIIIa in HK-2 cells following incubation for 24, 48 and 72 h, respectively

Compound	IC_{50} (mg/mL)[a]		
	24 h	48 h	72 h
Cepharanone B	> 160	136.13 ± 4.21	102.04 ± 3.74
Aristolactam AII	97.22 ± 2.21	74.15 ± 2.38	44.06 ± 1.44
Aristololactam AIIIa	58.78 ± 2.54	33.19 ± 1.05	22.28 ± 0.97

[a] Values are means ± SD (n = 3).

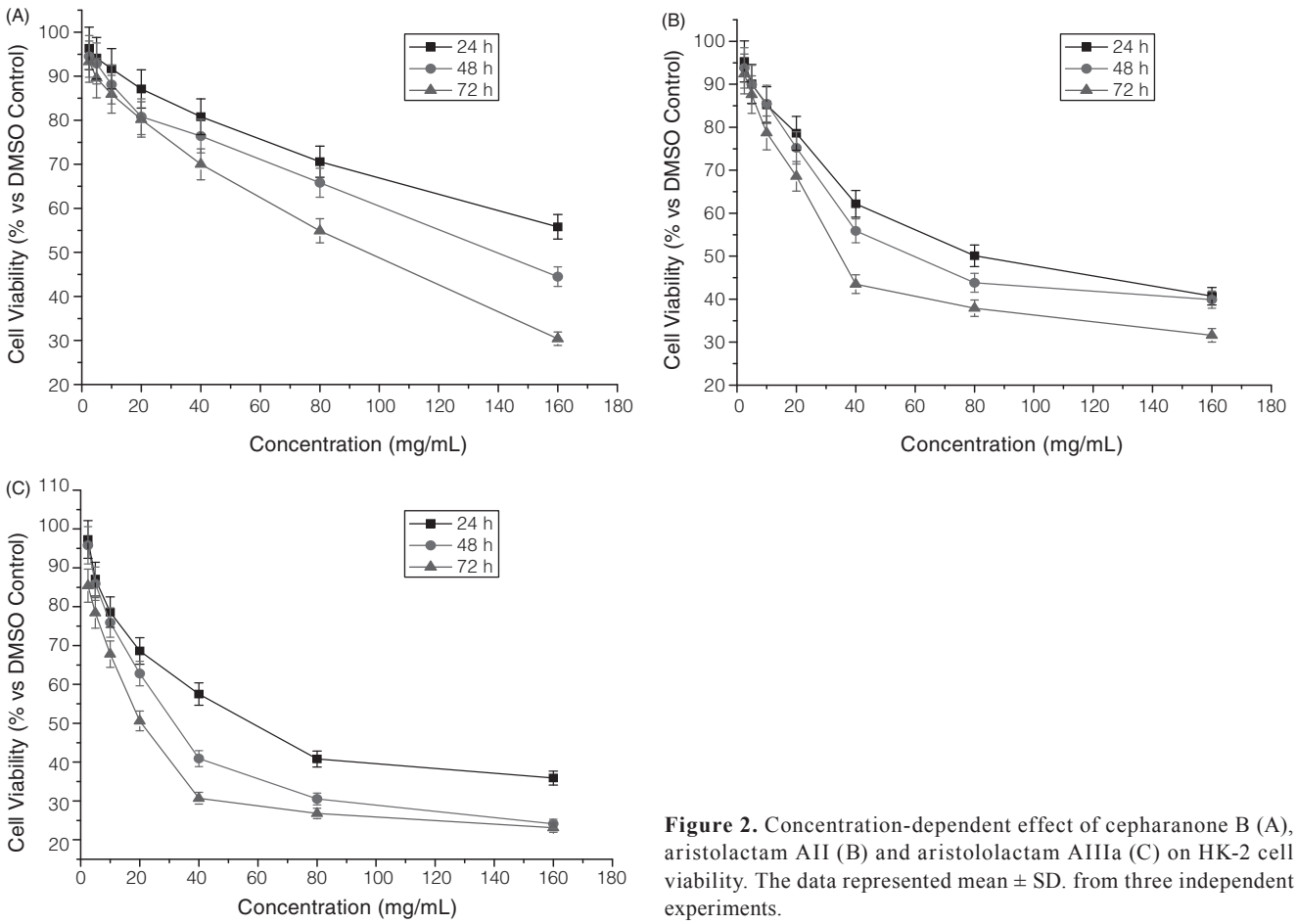

Figure 2. Concentration-dependent effect of cepharanone B (A), aristolactam AII (B) and aristololactam AIIIa (C) on HK-2 cell viability. The data represented mean ± SD. from three independent experiments.

DMSO as vehicle control. The result of the LDH release was similar to that of the MTT assay. As shown in Figure 3, exposure of HK-2 cells to ALs for 24 h led to significant increase of the LDH release ($p < 0.05$) in a concentration-dependent manner. Aristolactam AII and aristololactam AIIIa showed higher LDH release rate than cepharanone B, suggesting that aristolactam AII and aristololactam AIIIa led to greater damage to cell membrane and manifested stronger cytotoxicity than cepharanone B.

II. *Phytochemical Studies by HPLC*

(I) *Optimization of Extraction Methods*

In order to obtain optimal extraction efficiency of *P. wallichii* for HPLC analysis, three relative extraction conditions were designed and evaluated, which involved the following factors and corresponding levels: extraction method (ultrasonication, reflux), extraction temperature (75, 80 and 85°C) and extraction time (0.5, 1 and 2 h). By comparing the sum numbers and areas of target peaks in each chromatogram of different factors, the condition was optimized as follows: 1.0 g powder of dried sample extracted with 20 mL methanol in a reflux bath at 85°C for 1 h.

(II) *Optimization of HPLC Condition*

In order to achieve good resolution of chromatography, the mobile phase, detection wavelength and profiles of gradient elution were systematically investigated. The results indicated that there was no obvious difference between methanol-water and acetonitrile-water. Considering the high-toxicity and price of acetonitrile, the binary mixture of methanol-water was chosen. On the basis of the absorption maxima of the reference compounds in UV spectra acquired by UV-756 MC Spectrophotometer (Shanghai Precision & Scientific Instrument Co., Shanghai, China), the monitoring wavelength was set at 287 nm.

(III) *Method Validation*

The proposed method was validated in terms of linearity, limits of detection (LODs), quantification (LOQs), precision, repeatability and recovery test.

1. *Linearity, Limits of Detection and Quantification*

Six concentrations of the reference compounds solution

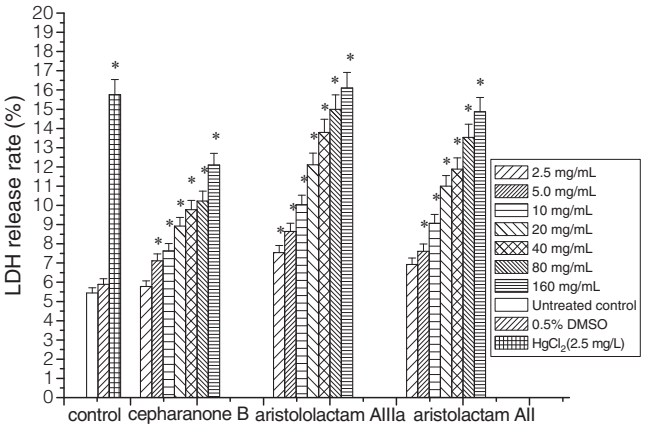

Figure 3. The LDH release rate in HK-2 cells exposed to various concentrations of cepharanone B, aristolactam AII and aristololactam AIIIa, respectively, for 24 h. The data represented mean ± SD. from three independent experiments. *$p < 0.05$, compared with the vehicle control.

Table 2. Calibration curves, detection limits and quantification limits of the target compounds

Compound	Calibration curve[a]	Linear range (μg/mL)	R^2 (n = 5)[b]	LOD (ng)[c]	LOQ (ng)[d]
Cepharanone B	$y = 75186x - 23723$	1.66 - 26.5	0.9997	0.41	1.36
Aristolactam AII	$y = 70599x + 17091$	1.33 - 21.2	0.9996	0.25	1.74
Aristololactam AIIIa	$y = 55145x - 5850$	0.94 - 15.0	0.9999	0.13	0.42

[a] y, peak area; x, the concentration of each reference compound (μg/mL).
[b] R, correlation coefficient of regression equations.
[c] Limit of detection (S/N = 3).
[d] Limit of quantification (S/N = 10).

Table 3. The intra- and inter-day variability and repeatability of the target compounds in *Piper wallichii*

Compound	Intra-day (n = 6)		Inter-day (n = 6)		Repeatability (n = 6)	
	Content (mg/g dry weight)	RSD (%)	Content (mg/g dry weight)	RSD (%)	Content (mg/g dry weight)	RSD (%)
Cepharanone B	0.105	1.8	0.106	2.3	0.104	0.9
Aristolactam AII	0.201	1.4	0.201	2.5	0.203	1.8
Aristololactam AIIIa	0.052	1.7	0.055	2.8	0.050	1.3

Table 4. Recovery of each compound determined by standard addition method (n = 3)[a]

Compound	Original (mg)	Spiked (mg)	Found (mg)	Recovery (%)	RSD (%)
Cepharanone B	0.105	0.200	0.306	100.3	1.8
Aristolactam AII	0.201	0.200	0.403	101.1	2.5
Aristololactam AIIIa	0.052	0.100	0.151	99.1	2.6

[a] The data was present as average of three determinations. Recovery (%) = 100 × (amount found-original amount)/amount spiked.

Table 5. Contents (mg/g dry weight) of the target compounds in different samples (n = 3)

Sample	Cepharanone B	Aristolactam AII	Aristololactam AIIIa
080423	0.103	0.198	0.049
080428	0.105	0.202	0.054
080503	0.103	0.200	0.050
080510	0.108	0.202	0.051
080518	0.104	0.201	0.050
080527	0.105	0.205	0.053
080530	0.102	0.200	0.049
080608	0.110	0.208	0.059
080615	0.106	0.204	0.052
080620	0.100	0.198	0.047
080628	0.105	0.206	0.053
080706	0.107	0.207	0.058

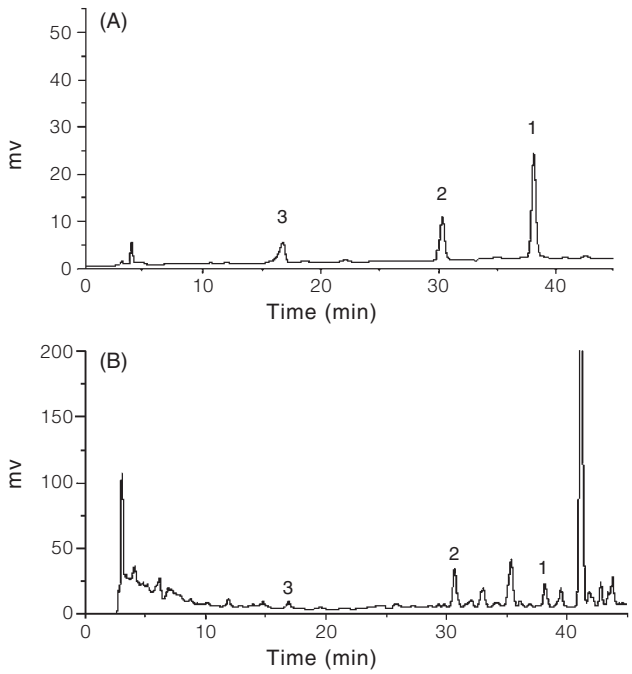

Figure 4. (A) Chromatogram of standard mixture compounds; (B) Chromatogram of *Piper wallichii* extract; (1) cepharanone B; (2) aristolactam AII; (3) aristololactam AIIIa.

were injected in triplicate, and the calibration curves were obtained. LOD and LOQ under the chromatographic conditions were determined at signal-to-noise ratios (S/N) of 3 and 10, respectively. As shown in Table 2, good linearity (coefficient of determination $R^2 > 0.999$) was obtained in the studied ranges for the target compounds.

2. *Precision Test*

Intra- and inter-day variations were utilized to determine the method precision. The intra- and inter-day variations were determined by analyzing six replicates on one day and on three consecutive days. Variations were expressed as relative standard deviations (RSD). As shown in Table 3, the RSD values of the overall intra- and inter-day variations were less than 2.8%.

3. *Repeatability Test*

The repeatability was confirmed by analyzing six different working solutions prepared from the same sample. As shown in Table 3, the RSD values of the repeatability were less than 1.8% for all the target compounds.

4. *Recovery Test*

In order to validate the accuracy of the method, a known amount (low, medium and high levels) of the three standard compounds were spiked into about 1.0 g of the herb of *P. wallichii*, and then subjected to the extraction procedure as described in materials and methods section IV(III). The extracts were analyzed by HPLC and the results are shown in Table 4. The RSD were less than 2.6% and the recoveries of all compounds varied from 99.1 to 101.1%. The results showed good reliability and accuracy.

(IV) *Sample Analysis*

All samples were processed by the optimized extraction method and analyzed under the optimized HPLC conditions. Each sample was analyzed in triplicate to determine the mean content and the results are tabulated in Table 5. The chromatograms of the reference compounds and sample are shown in Figure 4. The content ranges were 0.100 - 0.110 mg/g dry weight, 0.198 - 0.208 mg/g dry weight and 0.047 - 0.059 mg/g dry weight for cepharanone B, aristolactam AII and aristololactam AIIIa, respectively. In addition, *via* the multivariable analysis in SPSS software, the result showed that the contents of 12 batches of samples were different ($p < 0.05$).

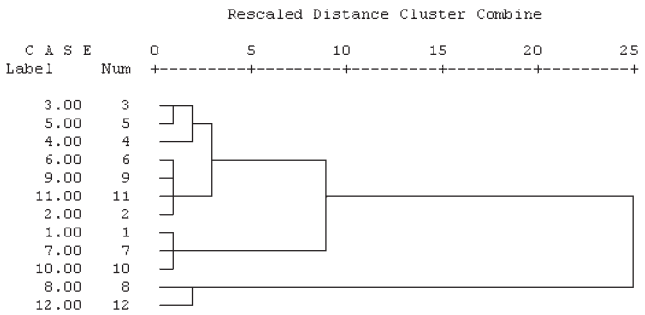

Figure 5. The clustering analyses of chromatograms of 12 batches of *Piper wallichii* samples. (1) 080423; (2) 080428; (3) 080503; (4) 080510; (5) 080518; (6) 080527; (7) 080530; (8) 080608; (9) 080615; (10) 080620; (11) 080628; (12) 080716.

(V) *Hierarchical Clustering Analysis*

In order to classify the different batches of *P. wallichii*, the clustering analysis was operated in SPSS software, and the results are shown in Figure 5. The peak areas of three target compounds were selected as the clustering variable, average linkage between groups was applied and Euclidean distance was selected as measurement. The result showed that 12 batches of the samples could be classified into two categories containing 2 and 10 samples. Accordingly, 10 batches of *P. wallichii* could be classified into 2 categories containing 3 and 7 samples. We supposed that the contents of major constituents in these batches were significantly different and the picking time was a major factor.

CONCLUSIONS

In our study, the potential renal cytotoxicities *in vitro* and the contents of the ALs in *P. wallichii* were analyzed. The overall results provided further evidence to establish the quality and safety control method, but the nephrotoxicity of ALs have not been confirmed, which need more in-depth studies.

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