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# 土木香内酯与异土木香内酯的提取与纯化工艺

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**摘　要**:本文报道了一种从土木香药材中提取以及纯化倍半萜内酯的方法。乙醇回流提取的方法采用正交试验设计方法[$L_9(3^4)$],考察了4个因素对产率与内酯浓度的影响,最终确定了乙醇回流提取的最佳方法:药材加10倍量的95%乙醇,加热回流提取1次,2 h。而硅胶柱层析的最佳纯化方法为:以100~200目的硅胶,1∶3的上样量,径高比为1∶2。其洗脱剂成分为石油醚∶丙酮(100∶3,v/v),洗脱流速为35 mL/min。采用该方法,异土木香内酯与土木香内酯的提取率大于70%,其纯度大于90%。

**关键词**:土木香内酯;异土木香内酯;正交试验;硅胶柱层析;土木香

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# Extraction and Purification of Alantolactone and Isoalantolcatone from Root of *Inula helenium*

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**Abstract**: The extraction and purification method of sesquiterpene lactones from the root of *Inula helenium* L. was optimized. The tests of orthogonal array design [$L_9(3^4)$] were performed to analyze the effect of single factors, namely ethanol concentration, extraction duration and times of extraction, on the extraction yield of isoalantolcatone and alantolactone. As the result, the optimal extraction conditions were found as follows: the dried roots of *I. helenium* were extracted by reflux with 95% ethanol in solid/liquid ratio of 1∶10 (w/v) for 2 hrs. The optimized purification process of ethanol extract was achieved by a pressurized silica gel (100-200 mesh) column chromatography with diameter to height ratio of 1∶2. The ratio of mixed sample to silica gel was 1∶3 (w/w), the eluent composed of petroleum ether (30-60 ℃) and acetone (100∶3, v/v), flowed at the rate of 35 mL/min. The extraction yield of the lactones under the optimal method was above 70%, and the purity was above 90%.

**Key words**: Isoalantolcatone; alantolactone; orthogonal array design; silica gel column chromatography; *Inula helenium* L.

## Introduction

*Inula helenium* L. (Compositae) is a perennial plant, distributed in many areas of China, Europe and North America. The root of *I. helenium* is rich in eudesmane-type sesquiterpene lactones, such as isoalantolcatone and alantolactone[1]. In recent years, extensive investigations on active constituents of *I. helenium* roots proved that sesquiterpene lactones were responsible for various biological activities, such as antibacterial, antifungal, hepatoprotective and antitumor activities[2-4]. Therefore, it is necessary to find an efficient method for extraction and purification of sesquiterpene lactones.

The current study was mainly focused on the ultrasonic-assisted extraction of phenolic and microwave-assisted extraction of flavones[5,6], but the purity was not reported. In this study, we report an improved extraction method for sesquiterpene lactones by ethanol-reflux and purification by silica gel column chromatography. The extraction yield of the lactones obtained by the optimal method was above 70%, and the purity was above 90%.

## Materials and methods

### Plant material and reagents

Dried roots of *I. helenium* were purchased from the

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herbal market in Sichuan Province, China. The plant material was ground and kept in a cool and dark place. Alantolactone and isoalantolcatone standards were internally isolated and characterized by spectral methods (IR, NMR and MS)[7,8]. The purity of each reference substance was above 98% as determined by UPLC. All the solvents used in the experiments were of analytical grade.

**Instruments**

UPLC (Agilent 1290), Ultraviolet detector (G4212-60008), Rotovapor, Electric heating blast drying chamber, Electric balance, *etc*.

**Standard preparation**

Each reference substance was dissolved in methanol to the concentration of 0.115, 0.230, 0.345, 0.460, 0.575 and 0.690 mg/mL of isoalantolactone, and 0.114, 0.228, 0.342, 0.456, 0.570, and 0.684 mg/mL of alantolactone. These samples of reference substance were injected in triplicate (injection volume 1 μL). The retention times (RTs) of isoalantolactone and alantolactone were 2.864 min and 3.039 min, respectively. Linear regression equations of isoalantolactone and alantolactone were $y = 3736.8x + 21.674$ ($R^2 = 0.9997$) and $y = 4176x + 25.284$ ($R^2 = 0.9996$), respectively.

**Sample preparation**

The sample extracts of *I. helenium* roots were dissolved in methanol for UPLC analysis. 1 μL solution of each sample extract was injected into UPLC system. Contents of isoalantolactone and alantolactone in the examined samples were calculated by linear regression equations and expressed as milligram per milliliter solutions (mg/mL).

Each experiment was performed in triplicate. All the data values were expressed as means with standard deviation.

**Quantitative determination of isoalantolactone and alantolactone**

The determination of isoalantolactone and alantolactone content was carried out on an Agilent Zorbax Eclipse Plus $C_{18}$ column (2.1 ×50 mm, 1.8 μm) with a mobile phase consisting of 40% acetonitrile and 60% water, at a flow rate of 0.6 mL/min, and the detection wavelength was set at 210 nm. The column oven temperature was set at 35 ℃. The injection volume was 1 μL. Quantitative data were obtained from the peak areas of each sample using an external standard method.

**Ethanol-reflux extraction**

To optimize the ethanol-reflux extraction conditions, 4 parameters were selected and investigated, namely: ratio of solid to liquid (A), solvent concentration (B), extraction duration (C) and times of extraction (D). The extraction process was further optimized using the orthogonal array design shown in Table 1. Dried roots of *I. helenium* (20 g) and ethanol were put into flask for ethanol-reflux extraction with designed ratio of plant material to ethanol (solid/liquid, w/v) and various conditions of experiments. The extract was filtered, and solvent was removed under vacuum. The obtained ethanol extract was dried and concentrated under vacuum. All experiments were performed in duplicate.

The yield ($X_1$) of extracts were calculated based on the weight of obtained extract and plant material, while the extraction yield ($X_3$) was defined as the ratio of the weight of lactones in extracts to the total weight of lactones in the plant material. The weight of lactones in extracts was calculated based on the purity ($X_2$) of the extracts, the ratio of the weight of lactones to the extracts.

The method of weighed score ($Y$) comparison was used to evaluate the effects of every factor on the yield and purity, and $Y$ was calculated using the following expression: $Y = 0.8X_2/6.55 - 0.2X_1/66.37$, where 6.55 was the average purity of the extracts in group 9 (Table 2), which was the best in purity ($X_2$) of the extracts, while the 66.37 was the average of the yield ($X_1$) of the group 4, which was the best in yield. A single factor analysis of variance (ANOVA) was adopted to investigate the effect of each factor in the process of extraction.

**Purification by silica gel column chromatography**

The extracts obtained from the *I. helenium* roots by an optimized ethanol-reflux process were further purified by silica gel column chromatography. The chromatographic column was packed by dry column-packing. The mixed sample was composed of the ethanol-extracts

**Table 1 Levels and factors of orthogonal design**

| Levels | Factors | | | |
|---|---|---|---|---|
| | (A) Ratio of solid to liquid (g/mL) | (B) EtOH concentration (%) | (C) Extraction duration (h) | (D) Extraction times (n) |
| 1 | 8 | 65 | 1 | 1 |
| 2 | 10 | 75 | 2 | 2 |
| 3 | 12 | 95 | 2.5 | 3 |

and chromatographic silica gel (1∶1.5, w/w), and the following factors were investigated in the process of purification: granularity of chromatographic silica gel, loading amount, ratio of diameter to height, ingredients of eluent and flow rate. The purified ethanol-extract was dried, concentrated under vacuum and further dissolved in methanol for UPLC analysis.

The method of weighted score ($Y$) comparison was used to evaluate every effect of factors on the yield and purity as well, according to extraction yield of lactones ($X_3$), purity ($X_2$) and the amount of eluent ($X_4$): $Y = 0.2X_3 + 0.4X_2 - 0.4X_4$, where the amount of eluent ($X_4$) (mL/g) was defined as the ratio of the amount of eluent used for purifying ethanol extracts to the weight of plant material for producing the ethanol extract. Each experiment was performed in triplicate. All the data values were expressed as means with standard deviation.

The experimental designs of every factor were as follows:

*Granularity of chromatographic silica gel*

Three types (60-100 mesh, 100-200 mesh and 200-300 mesh) of granularities of chromatographic silica gel were tested. The ratio of diameter to height of column chromatography was 1∶5, and the ratio of mixed sample to silica gel was 1∶3 (w/w). The eluent of column chromatography was composed of petroleum ether (30-60 ℃) and acetone (100∶3, v/v), at a flow rate of 5.5 mL/min.

*Loading quantity*

The experiment was performed using silica gel (100-200 mesh) column chromatography by eluent composed of petroleum ether (30-60 ℃) and acetone (100∶3, v/v), at a flow rate of 5.5 mL/min. Different loading quantities of mixed sample were tested. The ratios of mixed sample to silica gel were 1∶6, 1∶4, 1∶3, 1∶2, 1∶1 (w/w), respectively.

*Ratio of diameter to height*

The experiment was performed by silica gel (100-200 mesh) column chromatography. Different ratios of diameter to height were studied: 1∶0.5, 1∶2, 1∶5, 1∶16. The ratio of mixed sample to silica gel was 1∶3 (w/w) and the eluent was composed of petroleum ether (30-60 ℃) and acetone 100∶3 (v/v). The eluent flowed at the rate of 5.5 mL/min.

*Ingredient of eluent*

The experiment was performed by silica gel (100-200 mesh) column chromatography whose diameter to height was 1∶5. The ratio of mixed sample to silica gel was 1∶3 (w/w). Different ingredients of eluent were studied: petroleum ether (30-60 ℃)/acetone 100∶2, 100∶3, 100∶4, 100∶5 (v/v). The eluent flowed at the rate of 5.5 mL/min.

*Flow rate*

The experiment was performed by silica gel (100-200 mesh) column chromatography whose ratio of diameter to height was 1∶5. The ratio of mixed sample to silica gel was 1∶3 (w/w) and the eluent was composed of petroleum ether (30-60 ℃) and acetone 100∶3 (v/v). Three different flow rates were studied: 5.5, 9.0, 35 mL/min. The low flow rate between 5.0 and 9.0 mL/min was adjusted by the switch on the column chromatography, while the high flow rate of 35 mL/min was achieved by pressurized column chromatography.

Each experiment was performed in triplicate. All the data values were expressed as means with standard deviation.

## Results and Discussion

### Extraction by ethanol-reflux

*Orthogonal array design*

The results of each experimental group, yield and purity of extract, and data analysis were shown in Table 2. According to the $R$ value, the ethanol concentration was found to be the most important factor, the order of the effects of all factors was B > D > A > C. This was also demonstrated by $F$ and $P$ value of ANOVA (Table 3). The ethanol concentration (factor B) and reflux times (factor D) were markedly correlative with yield of isoalantolactone and alantolactone. The optimal method of ethanol-reflux extraction of sesquiterpene lactones was determined as: the ratio of solid to liquid was 1∶10 (w/v, g/mL) with 95% ethanol by reflux extraction for 2 hours, once ($A_2B_3C_2D_1$).

**Table 2 Experimental results and data analysis**

| | Factors | | | | Results ($n=2$) | | | | $Yi_n$ ($n=1,2$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | A | B | C | D | Yield (%) $X_{11}$ | Purity (%) $X_{21}$ | Yield(%) $X_{12}$ | Purity (%) $X_{22}$ | $Yi_1$ | $Yi_2$ | $Yi=\sum Yij$ |
| 1 | 1 | 1 | 1 | 1 | 47.23 | 3.52 | 43.77 | 3.44 | 28.76 | 28.83 | 57.59 |
| 2 | 1 | 2 | 2 | 2 | 53.02 | 3.05 | 49.91 | 2.96 | 21.27 | 21.11 | 42.39 |
| 3 | 1 | 3 | 3 | 3 | 33.73 | 4.69 | 30.03 | 4.51 | 47.12 | 46.03 | 93.15 |
| 4 | 2 | 1 | 2 | 3 | 69.11 | 2.99 | 63.63 | 2.75 | 15.69 | 14.41 | 30.11 |
| 5 | 2 | 2 | 3 | 1 | 41.87 | 3.88 | 35.78 | 3.66 | 34.77 | 33.92 | 68.69 |
| 6 | 2 | 3 | 1 | 2 | 25.33 | 5.18 | 22.76 | 5.00 | 55.63 | 54.21 | 109.84 |
| 7 | 3 | 1 | 3 | 2 | 69.65 | 3.00 | 62.87 | 2.93 | 15.65 | 16.84 | 32.49 |
| 8 | 3 | 2 | 1 | 3 | 44.98 | 2.83 | 40.08 | 2.72 | 21.01 | 21.14 | 42.15 |
| 9 | 3 | 3 | 2 | 1 | 20.03 | 6.61 | 18.99 | 6.49 | 74.70 | 73.54 | 148.24 |
| K1 | 193.13 | 120.19 | 209.58 | 274.52 | | | | | | | K=624.66 |
| K2 | 208.64 | 153.23 | 220.74 | 184.73 | | | | | | | W=28148.96 |
| K3 | 222.89 | 351.24 | 194.34 | 165.41 | | | | | | | P=21677.75 |
| k1 | 32.19 | 20.03 | 34.93 | 45.75 | | | | | | | |
| k2 | 34.77 | 25.54 | 36.79 | 30.79 | | | | | | | |
| k3 | 37.15 | 58.54 | 32.39 | 27.57 | | | | | | | |
| Rj | 4.96 | 38.51 | 4.40 | 18.18 | | | | | | | |
| Uj | 21751.61 | 26882.38 | 21736.28 | 22807.75 | | | | | | | |
| Qj | 73.87 | 5204.63 | 58.53 | 1130.00 | | | | | | | |

Note: $K=\sum Yi$, $W=\sum Yi^2$, $P=1/18\times K^2$, Rj = Max (Ki)-Min (Ki), $Uj=1/6\sum(Ki^2)$, Qj = Ui-P

**Table 3 Variance analysis of experiments results (ANOVA)**

| Source Corrected model Intercept | Sum of squares Qj | Degree of freedom $f$ | Mean square | $F$ value | Significant level |
|---|---|---|---|---|---|
| A | 73.87 | 5204.63 | 36.94 | 3.93 | |
| B | 5204.63 | 2 | 2602.32 | 276.91 | * * |
| C | 58.53 | 2 | 29.27 | 3.11 | |
| D | 1130.00 | 2 | 565.00 | 60.12 | * * |
| Error | 4.18 | 9 | 2.09 | | |
| Total | 6471.21 | 17 | | | |
| | | $F0.01(2,9)=8.02$; $F0.05(2,9)=4.26$ | | | |

Note: $Q_T$ = W-P, $Qe=Q_T-Q_A-Q_B-Q_C-Q_D$, $Fj=(Qj/fj)/(Qe/fe)$

*Verification experiment*

Toverify the above the results, three experiments of ethanol-reflux extraction were performed using the optimal method: ratio of solid to liquid was 1∶10 (w/w, g/mL); ethanol concentration was 95%; extraction time was 2 hours, once. The result was shown in Table 4. The extraction yields of 3 groups were above 75% and their purities were around 7%. As a result, the optimized method was stable and reliable.

**Table 4 Results of verification experiment**

| No. | Plant material (g) | Yield (%) | Content (%) | | | Extraction yield (%) |
|---|---|---|---|---|---|---|
| | | | Isoalantolactone | Alantolactone | Total lactones | |
| 1 | 653.0 | 24.86 | 4.79 | 2.37 | 7.16 | 75.16 |
| 2 | 650.0 | 24.10 | 4.48 | 2.69 | 8.17 | 82.38 |
| 3 | 650.8 | 24.21 | 4.94 | 2.20 | 7.94 | 81.09 |

**Purification by silica gel column chromatography**

*Effects of granularity of chromatographic silica gel*

Three types of granularities of chromatographic silica gel 60-100 mesh, 100-200 mesh and 200-300 mesh were tested for comparing their effects on extraction yield ($X_3$), purity ($X_2$) and amount of eluent ($X_4$), and the results were showed in Fig 1. The experimental results showed that the increasing of granularity of chromatographic silica gel would result in lower extraction yield, in a little higher purity, and remarkably higher amount of eluent. According to the comparative equation ($Y = 0.2X_3 + 0.4X_2 - 0.4X_4$). The optimal granularity of chromatographic silica gel was 100-200 mesh (Fig. 1).

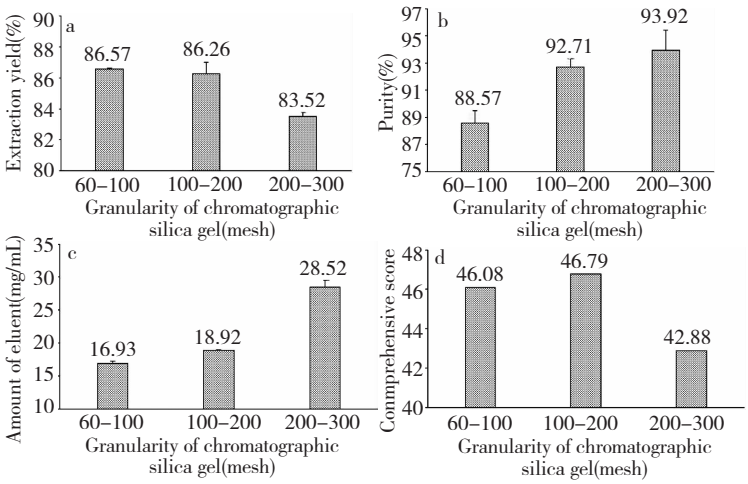


**Fig. 1 Effect of granularity of chromatographic silica gel on extraction yield (a), purity (b), amount of eluent (c) and comprehensive score (d) of isoalantolactone and alantolactone ($n = 3$)**

*Effects of loading quantity*

A granularity of chromatographic silica gel 100-200 mesh was used to test the effect of loading quantity, by eluent composed of petroleum ether (30-60 ℃) and acetone (100∶3, v/v) at a flow rate of 5.5 mL/min. The effects of different loading quantities of mixed sample on the extraction yield ($X_3$), purity ($X_2$) and amount of eluent ($X_4$) were shown in Fig. 2. The results demonstrated that with the increasing of loading quantity, the extraction yield would get lower. In addition, the increasing of loading quantity would result in a little higher purity, and remarkably higher amount of eluent. According to the comparative equation ($Y = 0.2X_3 + 0.4X_2 - 0.4X_4$), the optimal method was achieved when the ratio of mixed sample to silica gel was 1∶3 (w/w, g/mL).

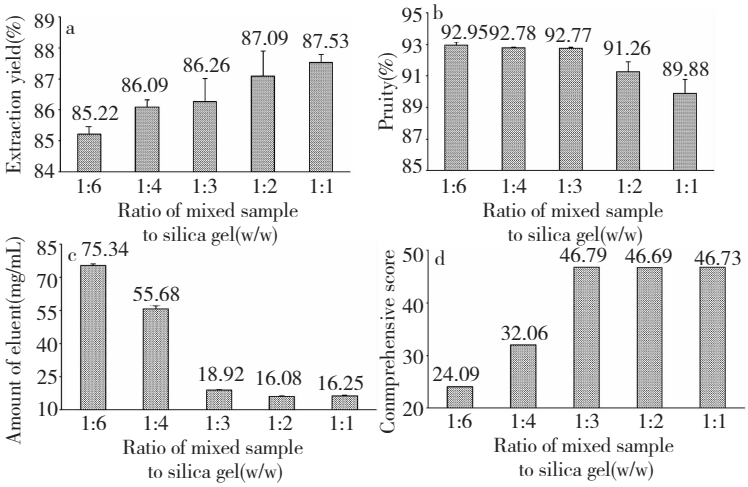


**Fig. 2 Effect of loading quantity on extraction yield (a), purity (b), amount of eluent (c), and comprehensive score (d) of isoalantolactone and alantolactone ($n = 3$)**

*Effects of the ratio of diameter to height*

Based on the results of 2.1 and 2.2, different ratios of diameter to height (1∶0.5, 1∶2, 1∶5 and 1∶16) were tested. According to the results, the increasing of the ratio of diameter to height would result in a little lower extraction yield, and higher , purity of lactones. However, the amount of eluent was remarkably increased with the increasing of the ratio of diameter to height (Fig. 3). Judged by the comparative equation ($Y = 0.2X_3 + 0.4X_2 - 0.4X_4$), the optimal ratio of diameter to height was 1∶2.

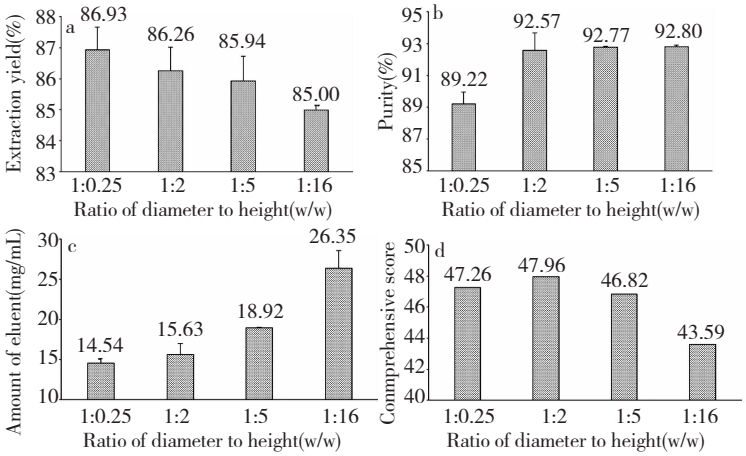


**Fig. 3 Effect of the ratio of diameter to height on extraction yield (a), purity (b), amount of eluent (c), and comprehensive score (d) of isoalantolactone and alantolactone ($n = 3$)**

*Effects of ingredient of eluent*

As shown in Fig 4, the results indicated that increasing acetone would result in higher extraction yield, lower , purity of lactones, and significantly less amount of eluent. Therefore, the optimal method was achieved when the eluent was composed of petroleum ether (30-60 ℃) and acetone (100/3, v/v) according to the comparative equation ($Y = 0.2X_3 + 0.4X_2 - 0.4X_4$).

*Effects of flow rate*

Three different flow rates (5.5, 9.0, 35 mL/min) were tested using silica gel (100-200 mesh) column chromatography with ratio of diameter to height 1∶5, ratio of mixed sample to silica gel 1∶3 (w/w) and the eluent composed of petroleum ether (30-60 ℃) and acetone

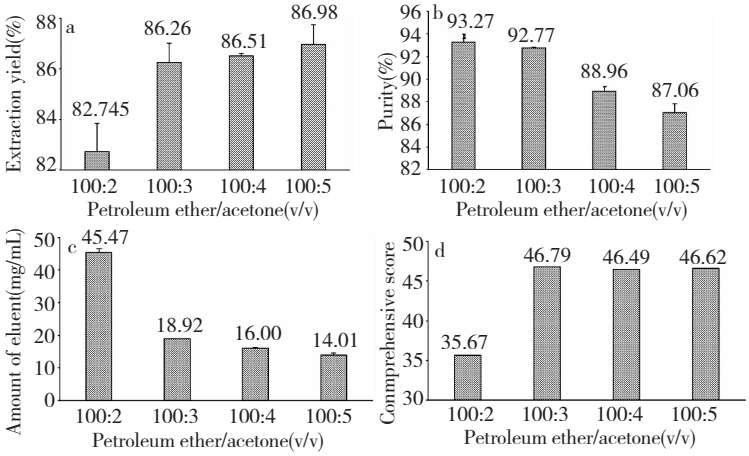


**Fig. 4 Effect of ingredient of eluent on extraction yield (a), purity (b), amount of eluent (c) and comprehensive score (d) of isoalantolactone and alantolactone ($n = 3$)**

100∶3 (v/v). The results demonstrated that the pressurized column chromatography did not have significant effects on the extraction yield, , purity of lactones, and the amount of eluent (Fig. 5). However, it shortened the time of experiment significantly. As a result, the pressurized column chromatography can be adopted in the manufacturing process.

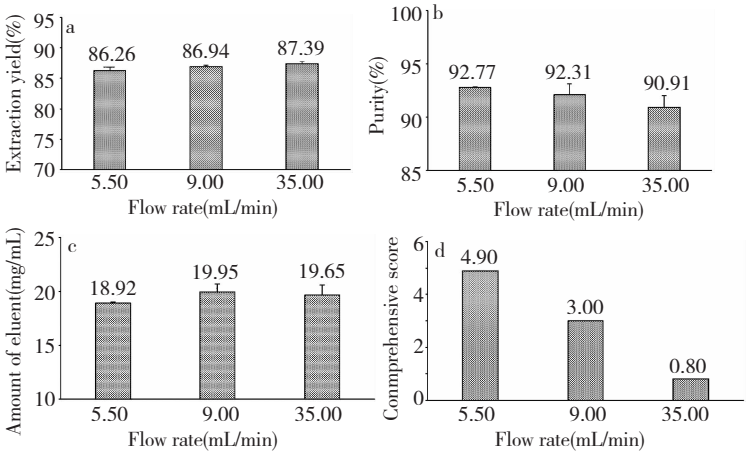


**Fig. 5 Effect of flow rate on extraction yield (a), purity (b), amount of eluent (c) and purification duration (d) ($n = 3$)**

*Verification experiment*

To verify the results of single factor experiments, 3 experiments were performed according to the optimal condition: the ratio of mixed sample to silica gel was 1∶3 (w/w); granularity of chromatographic silica gel was 100-200 mesh; ingredient of eluent was petroleum ether (30-60 ℃)/acetone (100∶3, v/v); the ratio of diameter to height was 1∶2, at a flow rate of 35 mL/min. As shown in Table 5, the extraction rates ($X_3$) of the obtained lactones among the 3 groups were above 85%, and their purities were above 90%. As a result, the optimized method of purification was stable and reliable.

**Table 5 Results of verification experiments of purification process**

| No. | Mixed sample (g) | Yield (%) | purity (%) | | | Amount of eluent (mL/g) | Extraction yield (%) |
|---|---|---|---|---|---|---|---|
| | | | Isoalantolactone | Alantolactone | Total lactones | | |
| 1 | 15.097 | 6.53 | 73.65 | 17.94 | 91.59 | 14.2 | 85.81 |
| 2 | 15.127 | 6.67 | 72.70 | 19.07 | 91.77 | 15.1 | 87.82 |
| 3 | 15.063 | 6.61 | 73.29 | 17.83 | 91.13 | 15.8 | 86.42 |

## Conclusion

The results showed that the method of ethanol-reflux extraction and purification by silica gel column chromatography was stable and reliable for producing isoalantolactone and alantolactone from *I. helenium* roots. The optimal ethanol-reflux extraction was achieved when the plant material was extracted by reflux with 95% ethanol in solid/liquid ratio of 1∶10 (w/v) for 2 hrs. The optimal purification method was performed by pressurized silica gel (100-200 mesh) column chromatography whose ratio of diameter to height was 1∶2. The ratio of mixed sample (ethanol extract/silica gel, 1∶1.5, w/w) to silica gel was 1∶3 (w/w), and the eluent was composed of petroleum ether (30-60 ℃) and acetone (100∶3, v/v) which flowed at the rate of 35 mL/min. By this producing method, the extraction yield of sesquiterpene lactones from *I. helenium* roots was above 70%, and their purities were above 90%.

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