Postprint: Preparation, Purification, and Anti-inflammatory Activity of Total Flavonoids from Amomum paratsao-ko

Authors: Chai Ling, Chen Mingsheng, Yuan Jiantong, Feng Jun, Liu Buming

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Abstract

Building upon previous research, this study employed the methanol crude extract of Amomum paratsao-ko as raw material to investigate purification methods for total flavonoids from Amomum paratsao-ko and evaluate their anti-inflammatory activity. HP-20 was screened as the optimal macroporous adsorption resin for purifying total flavonoids from Amomum paratsao-ko via static adsorption-elution experiments. Using adsorption rate and desorption rate as evaluation indices, the effects of mass concentration, volume, and flow rate of both the sample solution and eluent on the purification process were examined. The optimal purification parameters were determined as: sample solution mass concentration 0.5 $mg \bullet mL^{-1}$, sample loading flow rate 4 $mL \bullet min^{-1}$, sample loading volume 15 BV, ethanol concentration in eluent 70%, elution flow rate 2 $mL \bullet min^{-1}$, and eluent volume 10 BV, yielding a total flavonoid retention rate of 65.48% under these conditions. Furthermore, by assessing the effect of the obtained total flavonoids on interleukin (IL)-6 levels in lipopolysaccharide-stimulated microglial BV2 cells, the results demonstrated that they significantly downregulated the expression of the inflammatory cytokine IL-6, thereby exhibiting notable anti-inflammatory activity.

Full Text

Purification and Anti-inflammatory Activity of Total Flavonoids from *Amomum paratsao-ko*

Chai Ling, Chen Mingsheng, Yuan Jiantong, Feng Jun, Liu Buming*

Guangxi Institute of Traditional Medical and Pharmaceutical Sciences & Guangxi Key Laboratory of Traditional Chinese Medicine Quality Standards, Nanning 530022, China

Abstract

Building upon previous research, this study investigated purification methods for total flavonoids from the methanol crude extract of *Amomum paratsao-ko* and evaluated their anti-inflammatory activity. Static adsorption-elution experiments identified HP-20 as the optimal macroporous adsorption resin for purifying total flavonoids from *A. paratsao-ko*. Using adsorption rate and desorption rate as evaluation indices, we examined the effects of sample solution concentration, volume, flow rate, and other factors on the purification process. The optimal conditions were determined as follows: sample concentration 0.5 mg · mL^{-1}, loading flow rate 4 mL · min^{-1}, loading volume 15 BV, ethanol elution concentration 70%, elution flow rate 2 mL · min^{-1}, and eluent volume 10 BV. Under these conditions, the retention rate of purified total flavonoids reached 65.48%. Evaluation of the obtained total flavonoids on interleukin-6 (IL-6) levels in lipopolysaccharide-stimulated BV2 microglial cells demonstrated significant downregulation of the inflammatory cytokine IL-6, confirming notable anti-inflammatory activity.

Keywords: *Amomum paratsao-ko*, total flavonoids, macroporous adsorption resin, purification process, anti-inflammatory activity

Introduction

Amomum paratsao-ko, also known as white caoguo or Guangxi caoguo, is a commonly used Zhuang ethnic medicine. It consists of the dried ripe fruits of the Zingiberaceae plant *Amomum paratsao-ko* S.Q. Tong et Y.M. Xia, primarily produced in subtropical regions of Guangxi and Yunnan provinces. In Guangxi, it is mainly cultivated in Napo County, with scattered distribution in Jingxi and Longlin areas. The species is documented in Volume 3 of the Guangxi Zhuang Medicine Quality Standards, where it is used by the Zhuang people as a substitute for *Amomum tsao-ko* for culinary flavoring and in folk medicine to treat nausea, vomiting, abdominal distension, cold pain, phlegm retention, food accumulation, and malaria.

Despite its long history of folk use and extensive cultivation in Guangxi, research on its chemical composition remains limited, and its development and utilization lag behind. Our previous pharmacological studies revealed that *A. paratsao-ko* exhibits significant anti-inflammatory and analgesic effects. Subsequent chemical investigations isolated a substantial series of flavonol compounds, notably rhamnocitrin, from the fruits, leading us to hypothesize that flavonoids constitute the primary anti-inflammatory constituents. Based on these preliminary findings, this study aimed to optimize the purification process for flavonoid components from *A. paratsao-ko* and investigate the in vitro anti-inflammatory activity of its total flavonoids, thereby providing a foundation for the comprehensive development and utilization of this medicinal resource.

Materials and Methods

1.1 Instruments and Reagents

Instruments: UV2550 UV-Vis spectrophotometer (Shimadzu, Japan), KQ-5200B ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd.), RE-5210A rotary evaporator (Shanghai Yarong Biochemical Instrument Factory), HH-S constant temperature water bath (Changzhou Wanke Instrument Technology Co., Ltd.).

Reagents: Rhamnocitrin reference standard (prepared in our laboratory, purity $ $98%); AB-8 and D101 macroporous adsorption resins (Beijing Huideyi Technology Co., Ltd.); HP-20 macroporous adsorption resin (Beijing Green Grass Technology Development Co., Ltd.); ultrapure water; methanol and anhydrous ethanol (analytical grade, Guangdong Guanghua Technology Co., Ltd.); DMEM high-glucose medium, fetal bovine serum, trypsin (Gibco, USA); BD™ Cytometric Bead Array (CBA) Human Inflammation Kit and BD™ Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD, USA).

Plant Material: *A. paratsao-ko* fruits were collected in September 2017 from Napo, Guangxi, and identified by Associate Researcher Huang Yunfeng of the Traditional Chinese Medicine Resources Institute, Guangxi Institute of Traditional Chinese Medicine.

1.2 Experimental Methods

1.2.1 Preparation of Test Solution Coarsely powdered *A. paratsao-ko* (200.3 g) was mixed with 2,000 mL methanol, refluxed in a water bath for 60 min, and filtered. The filtrate was evaporated to dryness under reduced pressure, redissolved in methanol, and diluted to 1,000 mL as a stock solution, stored at 4 °C. One milliliter of the stock solution was diluted to 50 mL with methanol to prepare the test solution.

1.2.2 Standard Curve Preparation Rhamnocitrin reference standard (1.26 g) was accurately weighed, dissolved in methanol in a 10 mL volumetric flask, and diluted to volume to prepare the reference stock solution. Aliquots of 0.4, 0.8, 1.2, 1.6, 2.0, and 2.4 mL were transferred and diluted to 10 mL with methanol to prepare standard solutions at concentrations of 5.04, 10.08, 15.12, 20.16, 25.20, and 30.24 $\mathrm{mg \cdot mL^{-1}}$. The absorbance (A) of these solutions was measured at 359 nm in order of increasing concentration to establish the standard curve.

1.2.3 Total Flavonoid Content Determination The test solution was analyzed according to the method described in Section 1.2.2, and the total flavonoid concentration was calculated using the regression equation.

1.2.4 Resin Pretreatment Three types of macroporous resins (HP-20, AB-8, D101) were soaked in 95% ethanol, stirred to disperse uniformly, and left to

stand for 24 h. The resins were pretreated according to the method described by Fu et al. (2015), stored in 95% ethanol, and washed with distilled water until ethanol-free before use.

1.2.5 Optimization of Macroporous Resin Purification Process The optimal resin type was selected through static adsorption and desorption experiments based on adsorption and desorption rates. Single-factor experiments were conducted to investigate various loading and elution conditions to optimize the purification process.

1.2.5.1 Static Adsorption-Desorption Test for Resin Selection

Pretreated resins (3.0 g each, after removing ethanol and surface moisture) were placed in three 250 mL Erlenmeyer flasks. The stock solution from Section 1.2.1 was evaporated to dryness, redissolved in water, and prepared into three 60 mL portions of 0.975 $mg \cdot mL^{-1}$ loading solution, which were added to the flasks. The flasks were shaken at 30 °C and 120 $r \cdot min^{-1}$ for 24 h. After adsorption saturation, the supernatant was filtered, and the total flavonoid concentration was determined according to Section 1.2.3 to calculate the adsorption rate. The resin was then transferred to a 250 mL flask with 50 mL of 95% ethanol for desorption. After 24 h, the ethanol solution was sampled to determine total flavonoid content and calculate the desorption rate.

1.2.5.2 Effect of Sample Concentration on Dynamic Adsorption

Six portions of HP-20 resin (35 mL wet volume each) were packed into columns. Loading solutions (100 mL each) at concentrations of 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 $mg \cdot mL^{-1}$ were prepared and loaded at 1 $mL \cdot min^{-1}$. The effluents were collected, evaporated to dryness, redissolved in methanol, and diluted to 50 mL. The total flavonoid concentration was measured to calculate the adsorption rate on HP-20 resin.

1.2.5.3 Effect of Loading Flow Rate on Adsorption Rate

Four portions of HP-20 resin (35 mL wet volume each) were packed into columns. Four 100 mL loading solutions at 0.5 $mg \cdot mL^{-1}$ were loaded at flow rates of 1, 2, 3, and 4 $mL \cdot min^{-1}$. The effluent total flavonoid concentration was measured as described in Section 1.2.5.2 to evaluate the effect of flow rate on adsorption rate.

1.2.5.4 Investigation of Loading Volume

HP-20 resin (35 mL wet volume) was packed into a column. A 700 mL (20 BV) loading solution at 0.5 $mg \cdot mL^{-1}$ was continuously loaded at 4 $mL \cdot min^{-1}$. Effluents were collected in 9 fractions of 2–3 BV each. The total flavonoid concentration in each fraction was measured to calculate the leakage percentage.

1.2.5.5 Effect of Ethanol Concentration on Desorption Rate

Four portions of HP-20 resin (35 mL wet volume each) were packed into columns.

A 525 mL loading solution at 0.5 mg · mL^{-1} was loaded at 4 mL · min^{-1}, followed by washing with 350 mL (10 BV) distilled water. Elution was performed with 10 BV of 30%, 50%, 70%, and 90% ethanol at 2 mL · min^{-1}. The eluates were collected, concentrated, and analyzed to calculate the desorption rate.

1.2.5.6 Effect of Elution Flow Rate on Desorption Rate

Four portions of HP-20 resin (35 mL wet volume each) were packed into columns. A 525 mL loading solution at 0.5 mg · mL^{-1} was loaded at 4 mL · min^{-1}, washed with 10 BV distilled water, and eluted with 10 BV of 70% ethanol at flow rates of 1, 2, 3, and 4 mL · min^{-1}. The eluates were collected to determine total flavonoid content and calculate the desorption rate.

1.2.5.7 Investigation of Eluent Volume

HP-20 resin (35 mL wet volume) was packed into a column. A 525 mL loading solution at 0.5 mg · mL^{-1} was loaded at 4 mL · min^{-1}. After washing with distilled water, continuous elution was performed with 525 mL of 70% ethanol at 2 mL · min^{-1}. Eluates were collected in 35 mL (1 BV) fractions. An elution curve was plotted with elution volume (BV) as the x-axis and total flavonoid content (mg · mL^{-1}) in each fraction as the y-axis.

1.2.5.8 Validation Experiment

Based on the optimal loading and elution conditions determined from Sections 1.2.5.2–1.2.5.7, parallel experiments (n=3) were conducted using the retention rate of total flavonoids as the evaluation index to verify reproducibility.

1.2.6 Anti-inflammatory Activity Assay Murine microglial BV2 cells were cultured in DMEM high-glucose medium supplemented with 10% FBS, 1% penicillin, and 1% streptomycin at 37 °C in a humidified incubator with 5% CO_2. The adherent cells were passaged with 0.25% trypsin as needed. For experiments, cells were seeded in 12-well plates at 10^6 cells per mL, incubated for 6–9 h, then treated with LPS or DMSO. After 2 h, compounds at 10 mol · L^{-1} were added and incubated for 24 h. Cell supernatants were collected for CBA analysis. Each sample was tested in triplicate.

Results

2.1 Selection of Optimal Macroporous Resin by Static Adsorption-Desorption Test

Table 1 presents the screening results for three macroporous resins regarding adsorption and desorption of total flavonoids from *A. paratsao-ko*. The HP-20 resin exhibited moderate adsorption capacity but the highest desorption rate among the three resins, making it the optimal choice for subsequent process optimization.

Table 1 Adsorption and desorption capacity of total flavonoids in various macroporous resins

Type of macroporous resin	Polarity	Adsorption capacity (mg · g^{-1})	Adsorption rate (%)	Desorption capacity (mg · g^{-1})	Desorption rate (%)
HP-20	Non-polar				

2.2.1 Effect of Sample Concentration on Dynamic Adsorption Performance

Table 2 shows the changes in total flavonoid concentration in the effluent after loading solutions of different concentrations. The adsorption rate on HP-20 resin gradually increased with increasing sample concentration, reaching a maximum of 98.30% at 0.5 mg · mL^{-1}. Further concentration increases resulted in decreased adsorption rates, likely due to solution saturation and formation of flocculent precipitates that could not be adsorbed and were washed out during water rinsing. Therefore, the optimal sample concentration was determined to be 0.5 mg · mL^{-1}.

Table 2 Adsorption rate of total flavonoids after subjecting with various concentrations of sample solution on HP-20 resin column

Concentration of total flavonoids (mg · mL^{-1})	Adsorption rate (%)

2.2.2 Effect of Loading Flow Rate on Adsorption Rate

Table 3 demonstrates the effect of different loading flow rates on the adsorption rate of total flavonoids on HP-20 resin. The adsorption rate increased with flow rates from 2.0 to 4.0 mL · min^{-1}. The relatively high adsorption rate at 1.0 mL · min^{-1} may be attributed to longer adsorption time or formation of precipitates on the resin surface due to prolonged water residence time, resulting in incomplete washing. Considering both efficiency and time savings, 4.0 mL · min^{-1} was selected as the optimal loading flow rate.

Table 3 Effects of loading flow rate on adsorption rate

Loading flow rate (mL · min^{-1})	Adsorption rate (%)

2.2.3 Effect of Loading Volume on Effluent Total Flavonoid Concentration

As shown in Figure 1 [Figure 1: see original paper], the leakage percentage increased with loading volume, stabilizing at 15 BV. Although leakage percentage gradually decreased at 17 BV, it did not reach the breakthrough point (10%

leakage). Considering the short storage time of aqueous solutions and lack of automated loading equipment, prolonged loading times could affect experimental outcomes. Therefore, 525 mL (15 BV) was determined as the optimal loading volume.

Figure 1 Investigation of the upper column volume

2.2.4 Effect of Ethanol Concentration on Desorption Rate of Total Flavonoids from HP-20 Resin

Table 4 shows that desorption rate increased with ethanol concentration, with minimal difference between 70% and 90% ethanol. To conserve ethanol usage, 70% ethanol was selected as the optimal elution concentration.

Table 4 Effects of ethanol concentration on desorption rate of total flavonoids in HP-20 resin

Concentration of ethanol (%)	Desorption rate (%)

2.2.5 Investigation of Elution Flow Rate

Table 5 presents the desorption effects of different elution flow rates on HP-20 resin. The desorption rate increased slowly and then decreased with increasing flow rate, reaching a maximum at 2.0 $mL \cdot min^{-1}$. Therefore, 2.0 $mL \cdot min^{-1}$ was selected as the optimal elution flow rate.

Table 5 Effects of elution flow rate on desorption rate

Elution flow rate ($mL \cdot min^{-1}$)	Desorption rate (%)

2.2.6 Investigation of Ethanol Eluent Volume

Figure 2 [Figure 2: see original paper] illustrates the effect of ethanol eluent volume on total flavonoid concentration in the effluent. The eluted content increased initially and then decreased, reaching a plateau at 10 BV. Therefore, 10 BV (350 mL) was determined as the optimal elution volume to minimize solvent usage.

Figure 2 Elution curve

2.2.7 Validation Experiment

Using the optimal conditions determined from single-factor experiments (35 mL wet HP-20 resin, 0.5 $mg \cdot mL^{-1}$ sample loaded at 15 BV and 4 $mL \cdot min^{-1}$, washed with 1 BV distilled water, and eluted with 10 BV of 70% ethanol), three parallel experiments yielded total flavonoid retention rates of 67.86%, 64.29%,

and 64.29%, with a mean of 65.48% and RSD of 3.15%, demonstrating good reproducibility.

2.3 Anti-inflammatory Activity Assay

The effect of *A. paratsao-ko* total flavonoids on IL-6 expression is shown in Table 6 . The results indicate that the total flavonoid extract significantly inhibited IL-6 expression, confirming its anti-inflammatory activity.

Table 6 Levels of inflammatory cytokines IL-6 in BV2 cell supernatant ($pg \cdot mL^{-1}$, mean ± SD)

Group	IL-6 level
Control (DMSO)	0.5 ± 2.93
LPS treated group	17067 ± 537**
FLA + LPS treated group	5024 ± 534##

Note: **P < 0.01 compared with the control group (DMSO); ##P < 0.01 compared with the positive control group (LPS).

Discussion

Non-polar and weakly polar adsorption resins exhibit excellent extraction performance for flavonoid compounds. Therefore, we selected three macroporous resins of different types and polarities for adsorption-desorption experiments. The non-polar HP-20 resin demonstrated superior adsorption and desorption rates compared to the other two resins, establishing it as the optimal resin for separating and purifying total flavonoids from *A. paratsao-ko*.

Single-factor experiments determined the optimal purification conditions: sample concentration 0.5 $mg \cdot mL^{-1}$, loading volume 15 BV, loading flow rate 4 $mL \cdot min^{-1}$, ethanol elution concentration 70%, elution flow rate 2 $mL \cdot min^{-1}$, and ethanol elution volume 10 BV. After purification with HP-20 resin, the total flavonoid content increased from 9.735 $mg \cdot g^{-1}$ to an average of 50.526 $mg \cdot g^{-1}$, representing a 5.19-fold enrichment and demonstrating effective purification.

In vitro anti-inflammatory assays revealed that *A. paratsao-ko* total flavonoids significantly inhibited IL-6 expression, confirming notable anti-inflammatory activity. These findings provide experimental data and scientific basis for the development and utilization of *A. paratsao-ko* medicinal resources and enhancement of its application value.

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Note: Figure translations are in progress. See original paper for figures.