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High-Purity Calycosin-7-O-β-D-glucopyranoside Recovered from *Radix Astragali* by Extraction, Fractionation and Recrystallization

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Abstract: Calycosin-7-O-β-D-glucopyranoside (CG), one major isoflavonoid in *Radix Astragali* with promising pharmacological effects, was separated with a low-cost process in the present study. The sequential separation and purification procedures established involved extraction with 90% (v/v) aqueous ethanol at 75°C for 2 h twice followed by partition with ethyl acetate, elution with water, and 40% (v/v) aqueous ethanol on a styrene-based resin column and recrystallization at 4°C for 12 h with methanol. These conditions resulted in recovery of 81.6% of total CG with over 97% purity.

Keywords: Calycosin-7-O-β-D-glucopyranoside, extraction, fractionation, *Radix Astragalis*, recrystallization, resin

INTRODUCTION

Radix Astragali, the dried root of *Astragalus membranaceus* (Fisch.) Bge. or *Astragalus membranaceus* var. *Mongholicus* (Bge.) Hsiao (family Leguminosae), known as 'Huangqi' in China, is one of the most popular herbal medicines known worldwide to reinforce 'Qi' (vital energy). The roots have been reported to contain triterpene saponins, isoflavonoids,

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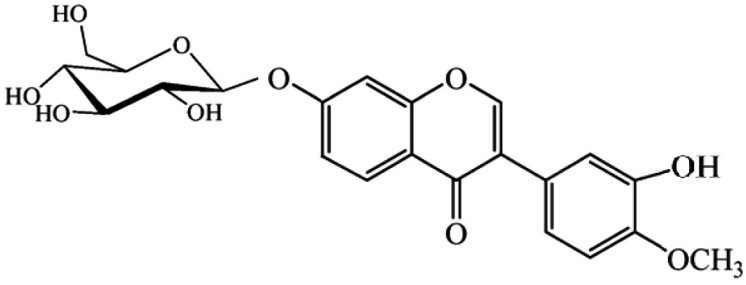

Figure 1. Chemical structure of calycosin-7-O-β-D-glucopyranoside.

and polysaccharides (1–3). Calycosin-7-O-β-D-glucopyranoside (3′-hydroxy-4′-methoxyisoflavone-7-O-β-D-glucopyranoside, CG; Fig. 1), one of the major isoflavonoids in *Radix Astragali* (4), has demonstrated various pharmacological effects, such as inhibiting the oxidative modification of low-density lipoprotein (5), inhibiting COX-2 activity (6), scavenging activity to 1,1-diphenyl-2-picrylhydrazyl radicals (7) and inhibitory effect on myocardial ischemia (8). It also showed the anti-coxsackie virus activity (9) and alleviation of osteoarthritis in rabbit osteoarthritis (OA) model (10).

There are a few reports on the extraction of CG from *Radix Astragali* such as soxhlet extraction for analytical purpose (11,12) and high-speed counter-current chromatography for preparative separation in laboratories (13). However, low-cost processes for recovering CG from *Radix Astragali* which are applicable to large-scale production have not yet been studied.

This study developed a cheap and easy process encompassing the efficient extraction, fractionation, and recrystallization to obtain high-purity CG from *Radix Astragali*, which could improve the pharmacodynamic research for this isoflavonoid and the chemical evaluation or standardization of *Radix Astragali* and its relative products.

EXPERIMENTAL

Extraction of CG from *Radix Astragali*

Twenty grams of completely air-dried *Radix Astragali* bought from Beijing Tong Ren Tang Technology Development Co., Ltd were ground and sieved through 10 mesh screen. The powder was extracted with aqueous alcohol solution, under reflux with agitation at 60 rpm. Chemical reagents used in this study were purchased from the Beijing Chemical

Plant (Beijing, P.R. China) except HPLC grade chemicals which were from Fisher Scientific International Inc., USA.

The extraction temperature, aqueous ethanol concentration, time and solvent to material ratio were chosen in the ranges 35–75°C, 60–100% (v/v), 0.5–3.0 h, and 5–25 ml/g respectively. The reflux rate was above 90%. After the extraction, the extracted slurry was filtered off to collect the extract alone. The above procedures were repeated twice for each sample, and the extracts obtained from the first and second extractions were combined to be concentrated into 2000 ml syrup at 60°C under vacuum.

Fractionation of the Extract

For the fractionation of CG from the *Radix Astragali* extract obtained with the optimal extraction procedure, the syrup obtained above was dissolved in water by sonication and partitioned with ethyl acetate. The ethyl acetate solution was vacuum evaporated at 50°C. About 55 g residue of ethyl acetate was obtained from 4 kg dry *Radix Astragali* material, and 11 g residue of ethyl acetate was then loaded on NKA macroporous resin column (40 cm × 4.6 cm, NKA, Chemical Plant of Nankai University Tianjin, China) and eluted with 2000 ml of distilled water and aqueous ethanol. The elution fraction was then concentrated and freeze dried.

Recrystallization of the Extract

One gram of 40% ethanol fraction was dissolved into 20 ml of methanol at 50°C, and the solution was left for 12 h at 4°C to induce the crystallization of CG contained in the residue. The extract solution was then centrifuged for 15 min at 4200 g to recover recrystallized CG, and the CG crystals were air-dried. The supernate was concentrated repeating the above procedure to recrystallization.

Concentration/Purity Determination and Identification of the Crystal

The concentration and final purity of the obtained CG was determined by following the HPLC protocol for analyzing *Radix Astragali* flavonoids reported previously (14). Chromatographic separations were carried out using a Supelocosil^{TM}C18 column (250 mm × 4.6 mm, 5 μm, Supelco USA). The calibration of CG was: $y = 3.6 \times 10^{-5}x + 3.6$, $R^2 = 0.999$. The crystal was identified by HPLC with the authentic

standard (National Natural Product Standard Lab, Beijing, China) and electrospray ionization mass spectrometry (ESI–MS, Agilent 1100 Series LC–MS Trap VL, USA).

Statistical Analysis

The one-way ANOVA test was used to calculate the significance of the differences of flavonoids. The results of HPLC analysis were expressed as means of yield ± SD, and the means were compared using Duncan's significant difference test. The p-values <0.05 are considered significant.

RESULTS AND DISCUSSION

Effect of Ethanol Concentration

Heat reflux extraction with ethanol, which is widely applied in industrial production, was studied to develop an easy and safe approach for the extraction of CG from *Radix Astragali*.

The concentration of aqueous ethanol varied from 60% to 100% to investigate the effect of ethanol concentration. Figure 2 shows that the extraction yield of CG increased obviously ($p < 0.05$) with increase of ethanol concentration and reached maximum yield (47.6 mg of CG from 100 g of material) with 90% ethanol. When extracted with anhydrous alcohol i.e. 100% (v/v) ethanol, the extraction decreased sharply

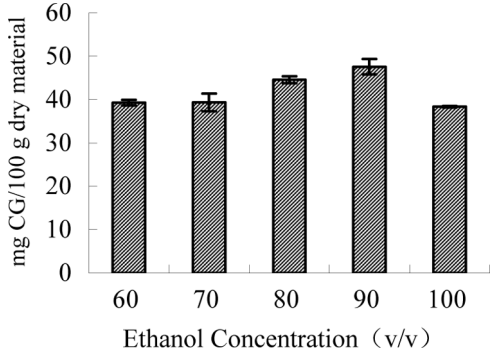

Figure 2. Effect of ethanol concentration on CG yield. Extraction conditions under heat reflux: extraction temperature, 75°C; extraction time, 2.0 h; solvent to material ratio, 10 ml/g.

($p < 0.05$). From these results, it is clear that the addition of some amount of water enhances the extraction efficiency. One possible reason for the increased efficiency with a presence of some water might be due to the increase in swelling of plant material by water, which increased the contact surface area between the plant matrix and the solvent (15–16). So 90% (v/v) aqueous ethanol concentration was used in the following experiments.

Effect of Extraction Temperature

To establish an optimal extraction temperature, the extraction was performed with 90% (v/v) ethanol at temperatures of 35–75°C. As shown in Fig. 3, the extraction yield of CG increased with the increase of temperature and reached its maximum at 75°C. Increasing temperature favored extraction by enhancing both the solubility of the solute and the diffusion coefficient and heating also might soften the plant tissue (17). However, the temperature can not go higher than 75°C as it is over the solvent boiling point and will cause serious solvent loss. Therefore, 75°C was considered optimal for achieving a high recovery of CG.

Effect of Extraction Time

In this experiment, under the optimal conditions determined from Sections 3.1 and 3.2 (90% ethanol at 75°C), extraction was performed for varying times to find the optimal extraction time. As demonstrated in Fig. 4, the extraction yield increased markedly when extending the

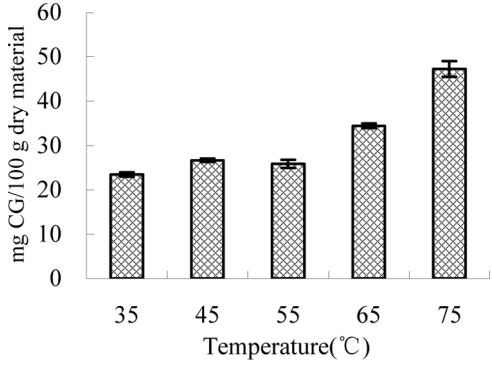

Figure 3. Effect of temperature on CG yield. Ethanol concentration, 90%; extraction time, 2.0 h; solvent to material ratio, 10 ml/g.

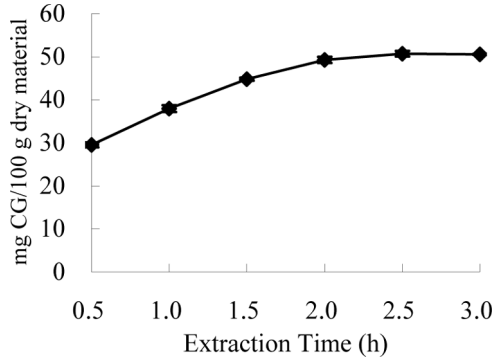

Figure 4. Effect of extraction time on CG yield. Ethanol concentration, 90%; extraction temperature, 75°C; solvent to material ratio, 10 ml/g.

extraction time from 0.5 to 1.5 h. No increase of CG yield was observed with time extending from 2.0 to 3.0 h ($p < 0.05$) which indicated the time of 2.0 h was enough for the flavonoids to diffuse from the material. Therefore, the optimal extraction time was considered to be 2.0 h.

Effect of Solvent to Material Ratio

Under the optimal conditions determined above (90% ethanol at 75°C for 2.0 h), extraction was performed for varying ratios of solvent to material to establish an optimal solvent volume. As demonstrated in Fig. 5, the extraction yield of CG increased with solvent to material ratio until reaching its maximum (53.9 mg of CG from 100 g of material) at the ratio

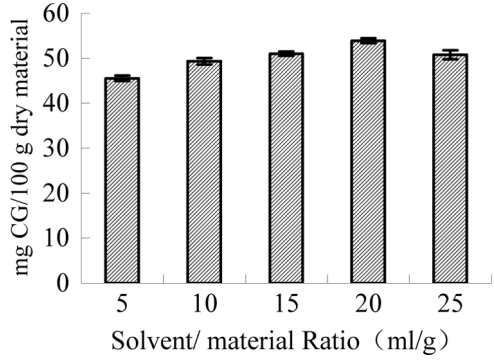

Figure 5. Effect of solvent to material ratio on CG yield. Ethanol concentration, 90%; extraction temperature, 75°C; extraction time, 2.0 h.

of 20 ml/g. This is consistent with mass transfer principles. Further increases of solvent to material ratio resulted in a slight decrease in the yield ($p < 0.05$). That might be due to excessive solvent causing the resolving of other polymer matrix which resulted in the absorption of flavonoid. Similar results about the effect of solvent to solid ratio on the extraction of flavonoid compounds were reported for *Saussurea medusa* (18). Therefore, the optimal solvent to material ratio was considered to be 20 ml/g.

Fractionation of Extract Using a Macroporous Adsorbent

Crude CG was extracted from 4 kg Radix Astragali under the optimal conditions determined in the Sections 3.1–3.4 (90% ethanol 20 ml/g at 75°C for 2.0 h), and the extract solution was concentrated. The syrup obtained was partitioned with ethyl acetate and about 55 g residue of ethyl acetate was obtained. Then 11 g residue of ethyl acetate was loaded on a NKA macroporous resin column. The column was washed with 2000 ml of water and then eluted with 4000 ml of 40% (v/v) ethanol and 70% (v/v) ethanol successively. The elution fraction was then concentrated and freeze dried and the content of each part was determined by HPLC. The results in Table 1 showed that 40% ethanol preferentially dissolved the highest proportion of CG: eluting with 40% ethanol yielded 99.2% (w/w) of total CG while the elution of CG by 70% ethanol after 40% ethanol gave only 0.8% of total CG. Water preferentially dissolved the proportion of hydrophilic fraction relative to CG and eluting with 40% aqueous ethanol yielded crude CG with 39.5% of purity while the CG concentration of crude extract was only 3.4%.

The 55 g residue of ethyl acetate was loaded on the resin column for 5 times, 11 g each time. The elution fraction was then concentrated and

Table 1. Fractionation of CG on NKA resin column with water and aqueous ethanol

Elution solvent	Total dry solids weight (g)	CG concentration (%)	CG recovery yield (%)
Water	29.8 ± 2.7	—*	—
40% ethanol	4.9 ± 0.7	41.0 ± 3.2	99.2
70% ethanol	16.5 ± 1.2	0.1 ± 0.0	0.8

*Not detected.

freeze dried and 4.9 g dried solid with the purity of 39.5% was yielded from the elution of 40% aqueous ethanol.

The enrichment of total flavonoid of *Radix Astragali* with NKA macroporous adsorbent was reported (19) while the successful fractionation of CG with NKA from *Radix Astragali* extract was studied for the first time.

Recrystallization of Fractionated Extract Concentrate

Recrystallization is a conventional simple method for the purification of flavonoid (20). One gram of fractionated extract concentrate containing CG with 39.5% purity was completely dissolved in 20 ml methanol at 50°C. The solution was then left at 4°C for 12 h to recrystallize CG. The recrystallization step yielded 360 mg (dry weight) of CG through centrifugation (4200 g for 15 min) followed by air-drying steps. HPLC analysis (Fig. 6) revealed that the purity of the CG crystals was 97.9%, with the final recovery yield of CG for the overall separation and purification processes involving extraction, fractionation, and recrystallization steps being 81.6% (Table 2). The whole roadmap can be described as Fig. 7.

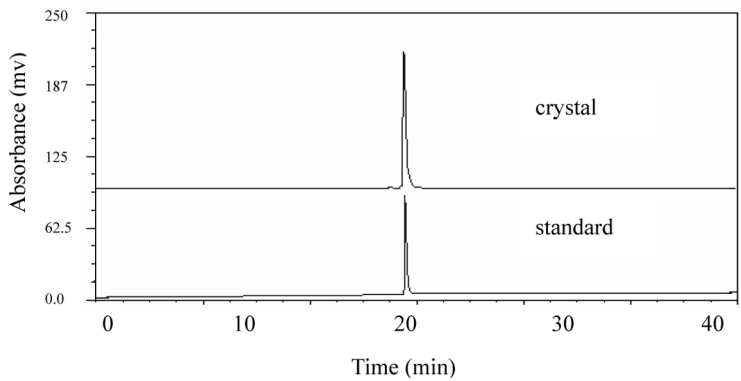

Figure 6. HPLC chromatograms of CG standard and the obtained crystal. Column: LC-18 (250 mm × 4.6 mm, 5 μm, Supelco, USA). Mobile phase: water and MeCN (MeCN: 0–15 min, 0–28%, flow rate 1.2–1.0 ml/min; 15–30 min, 28–38%, flow rate 1.0 ml/min; 30–40 min, 38%, flow rate 1.0 ml/min). Column temperature was kept constant at 40°C. UV detection: 230 nm.

Table 2. Total recovery of CG

Radix astragali		CG crystal		
Weight (kg)	Extractable CG (mg/100 g)	Weight (mg)	Purity (%)	Total CG recovery (%)
4.0	53.9	1 759	97.9	81.6

Identification of the Recrystallized Fraction

Identification of the recrystallized fraction was based on retention time comparing to standard (Fig. 6) together with electrospray ionization mass spectrometry (ESI-MS). The retention time of the crystal was in

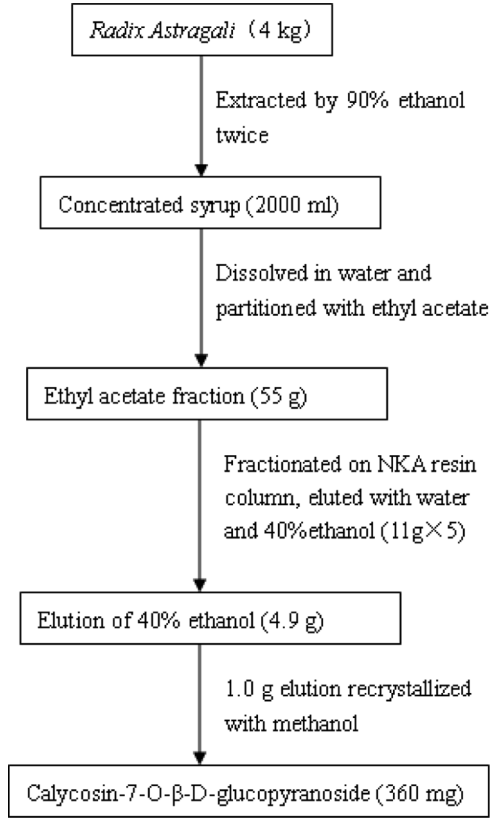

Figure 7. Roadmap of CG extraction and separation.

good accordance with the standard. The MS data are as follows: positive ESI-MS, m/z 469 (M+Na), 447 (M+H), 285 (M+H-162); negative ESI-MS, m/z 481 (M+Cl), 283 (M-H-162). Compared with the data given in reference (21) the compound was calycosin-7-O-β-D-glucopyranoside (CG).

CONCLUSIONS

This study determined the optimal extraction, fractionation, and recrystallization conditions for recovering CG with high purity from *Radix Astragali*. Under the optimal conditions established, 81.6% of total CG in *Radix Astragali* was obtained in crystal form with over 97% purity through relatively low-cost sequential processes only requiring water, ethyl acetate, ethanol, methanol, and polystyrene-based resin.

This economically-feasible process can be readily applied in those industries demanding high-purity CG such as pharmacological evaluation and standardization of *Radix Astragali* and its relative products. It also can promote the economic utilization of *Radix Astragali* with low value.

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