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Enrichment and separation of quercetin-3-O-β-D-glucuronide from lotus leaves (*nelumbo nucifera* gaertn.) and evaluation of its anti-inflammatory effect

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ABSTRACT

This work aimed to establish a systematic strategy to enrich and separate quercetin-3-*O*-β-D-glucuronide (Q3GA) from lotus leaves with macroporous resin and semi-preparative HPLC. Six resins were tested, and LX-5 was chosen as the appropriate resin for Q3GA based on the adsorption and desorption performances. After one-step enrichment, the content of Q3GA increased from 2.15% in crude extract to 52.25% in 30% ethanol fraction with yield of 11.97%. The Q3GA was then isolated from the 30% ethanol fraction by semi-preparative HPLC, and the purity of Q3GA was above 98.00% with yield of 19.76%. These results suggested that the aforementioned strategy was a useful and economic method to enrich and separate Q3GA from lotus leaves. Additionally, the anti-inflammatory effect of Q3GA was evaluated in lipopolysaccharide-treated RAW264.7 macrophages, and the result demonstrated that Q3GA could significantly inhibit LPS-induced NO release *in vitro* in a dose-dependent manner compared with control group.

1. Introduction

Lotus (*Nelumbo nucifera* Gaertn.), a large and perennial aquatic herb, is extensively planted in China [1], and has been used as a dietary staple, traditional Chinese medicine, and even as an ornamental plant for hundreds of years [2]. The leaves of lotus are aromatic and blue-green in color, usually utilized for the treatment of hemorrhage, hyperlipidemia, obesity, human immunodeficiency virus, sweating, fever, and inflammatory skin conditions [3–6]. Due to the aforementioned advantages, the use of lotus leaves is becoming more and more popular in China as a main dietary ingredient, in some herbal formulations, and as "tea" for losing body weight, reducing blood lipids and as an oxidant. The active constituents in lotus leaves and their promising activities have not yet been fully explored.

It is reported that lotus leaves are rich in flavonoids [7], a type of ubiquitously present natural plant-derived phenolic compounds possessing robust antioxidant activities and reducing the formation of free radicals [8,9]. Until now, there were ten flavonoids isolated and identified from lotus leaves including rutin, quercetin, isoquercitrin, hyperoside, astragalin, (+)-catechin, quercetin 3-*O*-β-D-glucuronide, quercetin 3-*O*-β-D-xylopyranosyl-(1→2)-β-D-galactopyranoside and quercetin 3-O-α-arabinopyranosyl-(1→2)-β-D-galactopyranoside [6,7]. Quercetin-3-*O*-β-D-glucuronide (Q3GA), a quercetin glycoside, exhibits antioxidant, antiarteriosclerotic and antiviral activities in both *in vivo* and *in vitro* models [10]. Q3GA could also promote the anti-inflammatory properties of M2a macrophages and modulate immune response effects with pro-inflammatory stimuli [11]. Macrophage could be a target of Q3GA in human atherosclerotic arteries [12]. What is more, Q3GA is reported to possess inhibitory activity against influenza A virus (FLUAV) [13], and even can pass through the blood-brain barrier and accumulate in macrophages, thereby acting as anti-inflammatory agents in the brain [14]. Its chemical structure is shown in Fig. 1. However, Q3GA is difficult to be enriched and/or purified, and to our knowledge, the reports about its separation and purification are limited. Moreover, its anti-inflammatory effect assessment using RAW264.7 macrophages is yet to be reported. These two reasons informed the areas of our current study.

As already known, it is particularly difficult to enrich promising candidates from herbs due to many other constituents with varying physicochemical properties [15]. Traditional separation methods, like liquid-liquid extraction and silica gel column chromatography with many disadvantages such as long tedious separation cycles, high cost, hazardous and non-environmental friendliness, make

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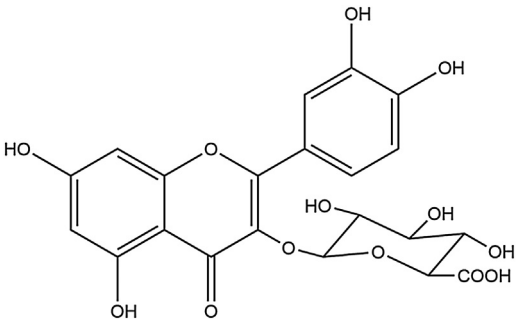

Fig. 1. The chemical structure of quercetin-3-*O*-β-D-glucuronide.

them inappropriate for industrial application. Recently, high speed counter-current chromatography was applied for separation and purification, but the two-phase solvent system was difficult to optimize due to the limited theoretical directions [16]. An appropriate and economic separation and purification method for Q3GA is therefore warranted. Macroporous adsorption resins (MARs), with large surface areas, optimal pore structures, unique adsorption properties and surface functional groups, are widely used for removing most of the impurities and enrich the bioactive substances from natural complex mixtures. Moreover, the use of MARs have other advantages such as procedural simplicity, excellent selectivity, high recovery and efficiency, lower operational cost, easier scale-up and regeneration than other methods [15,17–19]. However, single enrichment technology is unable to achieve the high purity compound requirement. Therefore, the combination of MARs and chromatography has received extensive attention.

The aim of the present study was to develop a simple and efficient combined method for preparation of Q3GA, a major constituent in lotus leaves, and evaluate its anti-inflammatory potential. In the present study, macroporous resin combined with semi-preparative HPLC were applied. The purity of Q3GA obtained was above 98% as determined by HPLC and confirmed by MS and NMR analyses. The anti-inflammatory effect of Q3GA was evaluated in RAW264.7 macrophages.

2. Materials and methods

2.1. Materials and chemicals

HP-20 was purchased from Qinshi Tech. Co. (Zhengzhou, China), LX-5 and LX-60 were obtained from Lanxiao Tech. Co. (Xi'an, China), X-5, HPD722 and AB-8 were purchased from Cangzhou Baoen Tech. Co. (Hebei, China), ethanol and methanol (analytical grade) were purchased from Nanjing Hanbon Chemical Reagent Company (Nanjing, China). Those chemical reagents for high performance liquid chromatography analysis were of HPLC grade.

2.2. Plant materials

The dried lotus leaves (*Nelumbo nucifera* Gaertn.) were purchased from Bozhou medicinal materials market (Anhui, China), and identified by Professor Lian-Wen Qi, School of Traditional Chinese Medicine, China Pharmaceutical University. The voucher specimen (No. 8201503) has been deposited in the State Key Laboratory of Natural Medicines, China Pharmaceutical University.

2.3. Samples extraction and sample solution preparation

The lotus leaves (0.9 kg, 40-mesh) were extracted with 50% ethanol (v/v, 3 × 9000 mL, 1 h each time) using heat reflux extraction, and the obtained extracts were concentrated under partial vacuum to get concentrated liquid (900 mL). The concentrated liquid was lyophilized to obtain the crude extracts of lotus leaves. The mass content of Q3GA in the crude extracts of lotus leaves was about 7.0% (g/g).

The crude extract (4.24 mg/mL) was dissolved with deionized water before use, and then centrifuged at 4000 rpm for 10 min to obtain the sample solution.

2.4. Pretreatment of macroporous resins

The pretreatments of six resins were carried out to remove the undesired substances trapped inside the resin pores as follows: The resin was soaked in 95% (v/v) ethanol for 24 h, and then washed with deionized water till the ethanol was completely removed. The physical properties of each resin are summarized in Table 1.

2.5. HPLC analysis of Q3GA

Agilent 1290 series HPLC system (Agilent Scientific, Co., Santa Clara, California, USA) equipped with an Agilent Zorbax Eclipse Plus C18 column (2.1 mm × 150 mm, 3.5 μm) was applied to analyze the concentration and purity of Q3GA at 254 nm. The HPLC analytical condition for Q3GA was as follows: mobile phase consisted of acetonitrile (phase B) and deionized water with 0.1% (v/v) formic acid (phase A). The elution program was as follows: 0–30 min, 11% phase B, 30–35 min, 11–100% phase B, and 35–40 min, 100% phase B; the flow rate and column temperature were set as 0.4 mL/min and 35 °C, respectively; the sample injection volume was 2 μL.

2.6. Static adsorption and desorption experiment for screening of MARs

The static adsorption and desorption experiments were carried out to select the appropriate MARs for Q3GA separation as follows: six pretreated resins (2.5 g each) were accurately weighed and added to 200 mL flasks containing 20 mL sample solution (the initial concentration of Q3GA was 4.24 mg/mL), respectively. Subsequently, the flasks were sealed tightly and shaken (100 rpm) for 12 h at 25 °C during the adsorption process. After reaching adsorption equilibrium, those six resins were filtered and washed thoroughly with distilled water to remove the remaining sample solution. The six resins were then desorbed with 100 mL 95% (v/v) ethanol in the flasks by shaking (100 rpm) for 12 h at 25 °C.

The concentration of Q3GA in the initial sample solution and desorbed solutions were determined by HPLC. Each experiment was triplicated, and the absorption capacity (Q_e, Eq. (1)), and absorptioning equations:

Absorption capacity (Eq. (1)):

$$Q_e = \frac{(C_0 - C_e)V_i}{W}$$

Absorption ratio (Eq. (2)):

$$R_a\,(\%) = \frac{C_0 - C_e}{C_0} \times 100\%$$

Desorption ratio (Eq. (3)):

$$R_d\,(\%) = \frac{C_d V_d}{(C_0 - C_e)V_i} \times 100\%$$

where Q_e (mg/g) is the absorption capacity of resin at absorption equilibrium; C_0 (mg/mL) and C_e (mg/mL) are the initial and absorption equilibrium concentrations of Q3GA in the sample solutions, respectively; V_i (mL) is the volume of initial sample solution; W (g) is the weight of dry resin. Q_d (mg/g) is the desorption capacity after desorption equilibrium; C_d (mg/mL) is the concentration of Q3GA in the desorption solutions; V_d (mL) is the volume of the