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Analytical Methods

Optimisation of Pueraria isoflavonoids by response surface methodology using ultrasonic-assisted extraction

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ABSTRACT

Puerariae Lobatae Radix (PLR) exerts cyto-protective effect against oxidative stress due to its high isoflavonoid content. In this study, the ultrasonic-assisted extraction condition for the maximum recovery of isoflavonoids with high cyto-protective effect was optimised by response surface methodology (RSM). A second-order polynomial fitted the experimental data (R^2: 0.9736; p-value <0.0001). The optimal extraction parameters were determined as: extraction time 16.02 min, ethanol concentration 41.41% and liquid-to-solid ratio 44.35 mL/g. Practical experiments with extraction time 16.00 min, ethanol concentration 41.00% and liquid-to-solid ratio 44.00 mL/g were carried out in triplicate. This subsequently yielded a cell viability of 82.90 ± 0.78% against hydrogen peroxide-induced oxidative stress on EA. hy926, and was comparable to the predicted of 85.60%. Five chemical constituents in the extract were identified to exert cyto-protective effect. Taken together, this method successfully integrated RSM and the partial least squares regression method to optimise the PLR extract with highest cyto-protective activity.

1. Introduction

The dried root of *Pueraria lobata* (Puerariae Lobatae Radix; PLR), a perennial vine native to Southeast Asia, has been used as a functional food and as a medicinal herb for treating fever, dysentery, diarrhoea, diabetes, hypertension, myocardial infraction and arrhythmia. It is commonly known as kudzu root or Gegen. PLR is a rich source of isoflavonoids, and it is widely accepted that the beneficial effects of isoflavonoids are mainly due to their anti-oxidative property (Jin, Son, Min, Jung, & Choi, 2012). However, the bioactive component(s) mediating the effects of the plant remains inconclusive.

Puerarin, as the most abundant component, (Wong, Razmovski-Naumovski, Li, Li, & Chan, 2015a), is recommended by the Pharmacopoeia of the People's Republic of China as the chemical marker for authenticating PLR and is believed to be responsible for most its pharmacological activities, including cytoprotection and anti-oxidant activity (Pharmacopoeia of the People's Republic of China, 2015). However, in our previous study, we have shown that the anti-diabetic effect of PLR extract is not only dependent on puerarin, but also on the minor isoflavonoids present in the extract (Wong et al., 2015a) including 3′-hydroxypuerarin, 3′-methoxypuerarin, mirificin, daidzin, daidzein, genistin and genistein (Wong, Razmovski-Naumovski, Li, Li, & Chan, 2013).

The use of an extract follows the fundamental holistic theory of traditional Chinese medicine as the therapeutic effects are based on the additive and/or synergistic interactions of numerous components. However, the extraction of plant material can be variable, and predetermined conditions would allow consistency in optimising the bioactive constituents in an extract.

Response surface methodology (RSM) is a statistical experimental protocol used in analysing empirical models that describe the effect of several independent variables, such as solvent concentration and extraction time and their interactions, on one or more dependent (response) variables including cyto-protective activity. RSM has successfully been employed in optimising the extraction of polyphenols, polysaccharides and proteins in plants, and the extract has been correlated to the highest biological activity

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(Amado, Franco, Sanchez, Zapata, & Vazquez, 2014; Yang et al., 2015). Compared to conventional and other modern extraction techniques, ultrasound-assisted extraction (UAE) reduces extraction time, extraction temperature, energy input and consumption of organic solvents, and enhances chemical kinetics, solubility and experimental reproducibility (Vilkhu, Mawson, Simons, & Bates, 2008).

Therefore, the aim of this study was to optimise a rapid UAE condition of isoflavonoids from PLR with maximal bioactivity by using a RSM statistical approach and compare the experimental and predicted cell viability values. The extraction parameters (independent variables) included extraction time, ethanol concentration and solid-to-liquid ratio. The pharmacological activity (response variable) was assessed by measuring the cyto-protective effect on human endothelial cells against hydrogen peroxide (H_2O_2)-induced cell death using a cell viability assay. The cyto-protective assay was chosen for correlating the optimised extraction condition and biological activity of PLR due to its simplicity and relevance to its clinical use of PLR in traditional Chinese medicine. In addition, the cytoprotective activity was correlated to antioxidant activity. Subsequently, the bioactive constituents were identified by liquid chromatography-mass spectroscopy. To our knowledge, this is the first study to optimise the extraction processes of Puerariae species based on *in vitro* cyto-protective activity, and confirm the active compounds in the extract.

2. Materials and methods

2.1. Chemicals and solvents

All chemicals, solvents and reagents used were of analytical grade. Hydrogen peroxide and 3-(4,5-dimethylthiazolyl-2)-2,5-di phenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (MO, USA). Reference compounds, including puerarin (>98%), daidzin (>99%), daidzein (>99%), genistin (>98%) and genistein (>99%), were purchased from Tauto Biotech (Shanghai, China). Deionised water was purified by a Milli-Q Water Purification system from Millipore (MA, USA). All other chemicals and solvents were obtained from Thermo Fisher Scientific (NSW, Australia) unless otherwise stated.

2.2. Herbal samples

Seventeen dried PLR samples were purchased from herbal pharmacies in various regions of China and Australia (Table S1). The plant materials were authenticated by Dr. George Li from the Faculty of Pharmacy, The University of Sydney, Australia. The 17 trade samples were authenticated by comparing the macroscopic, microscopic and chemical characteristics described in the PPRC (Pharmacopoeia of the People's Republic of China, 2015) and a previous study (Wong et al., 2015a). Voucher specimens were deposited at the herbarium at the Faculty of Pharmacy, The University of Sydney, Australia.

2.3. Extraction procedure

The PLR samples were oven-dried at 40 °C for 24 h. Each sample was ground into a fine powder and passed through a No. 180 (177 μm) sieve. The powdered sample (1 g) was placed in a glass conical flask and was macerated with a fixed amount (20.00–70.00 mL) of ethanol at a given concentration (15.00–50.00%). The extraction process was performed in an ultrasonic cleaning bath (WUC-A03H, Thermoline Scientific, NSW, Australia) with an output power of 300 W and irradiation frequency of 40 kHz. The mixture was extracted in the sonication bath at 40 °C for a specified time (10.00–30.00 min). The mixture was allowed to cool to room temperature and centrifuged at 10,000*g* for 10 min. The supernatant was filtered through Whatman No. 1 filter paper and evaporated at 40 °C by rotary evaporator under vacuum. The dried residue was considered as the ethanolic extract of PLR and was stored in a glass scintillation vial at −20 °C prior to experiment.

2.4. Response surface methodology

Central composite design (CCD) was used to construct a three-factor, three-level rotatable model consisting of 20 experimental runs, with six replicates at the central point (Table 1). The order of the experiments was randomised to minimise selection bias and the effect of unexplained variability in the observed responses due to systematic errors. The three experimental factors including extraction time (X_1: 10.00, 20.00 and 30.00 min), ethanol concentration (X_2: 15.00, 32.50 and 50.00% v/v) and liquid-to-solid ratio (X_3: 20.00, 45.00 and 70.00 mL/g) were optimised by response surface methodology. The viability of human endothelial cells against H_2O_2-induced cell death (Y_1) was chosen as the response (dependent) variables. The experimental data were fitted to a second-order polynomial model as follows:

$$Y = A_o + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} A_{ij} X_i X_j$$

where Y is the response variable, A_o is the intercept constant, A_i, A_{ii} and A_{ij} are the regression coefficient for the linear, quadratic and cross-product effects of the X_1, X_2 and X_3 factors, respectively. X_i and X_j are coded values of the independent variables, while k equals the number of the test factors (k = 3).

The optimal extraction condition was determined using the "desirability" algorithm described by Mayer and his colleagues (Myers, Montgomery, & Anderson-Cook, 1995). To determine the desirability of various combinations, the "goal category" of the experimental variables was set as "in the range", whereas that of response variables was set as "maximise". The combination of experimental factors yielding the highest desirability was selected as the optimal extraction condition.

2.5. Determination of cell viability against oxidative stress

The human umbilical vein endothelial cell line (EA.hy926) was a kind gift from Dr. Shanhong Ling (Monash University Central Clinical School, Australia). The cells were maintained in an atmosphere of 5% CO_2 at 37 °C in DMEM/Ham's F12 containing 15 mM HEPES and l-glutamine, and supplemented with 10% foetal bovine serum and 100 U/mL of penicillin and streptomycin (Gibco, Australia). EA.hy926 cells were seeded in 24-well plates at a density of 1.0×10^5 cell/mL and allowed to grow until confluent for 24 h. The cells were pre-treated for 4 h with 400 μg/mL of PLR ethanolic extracts, and then exposed to 0.4 mM H_2O_2 for another 20 h. For the control, 41.41% (v/v) ethanol was used to replace the PLR ethanolic extract.

The cell viability of the endothelial cells after exposure to oxidative stress was measured by the MTT dye reduction assay as previously described (Kam et al., 2012). Briefly, MTT solution (5 mg/mL) was added to each well and the plates were incubated for 4 h at 37 °C. After the medium was aspirated, the purple dye crystal formed inside the viable cells was dissolved in DMSO. The optical density of each well was measured at 550 nm using a microplate reader. The experiments were performed in triplicates, and the optical density of formazan in the control was taken as 100% viability.