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Abstract: The effect of temperature and solvent on the extraction of *Artemisia asiatica* using pressurized liquid extraction was examined. These two parameters were operated according to central composite design followed by response surface analysis to obtain the highest extraction efficiency. Determination of eupatilin was carried out by high performance liquid chromatography coupled with a diode array detector. While the rise in temperature could increase the yield of the total extract, the content of eupatilin was decreased in high temperature. In addition, high ethanol proportion maximized the contents of eupatilin in the total extract.

Keywords: *Artemisia asiatica*, ethanol proportion, eupatilin, pressurized liquid extraction, temperature

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INTRODUCTION

Plants have been utilized as medicines for thousands of years. In more recent times, the use of plants as medicines has involved the isolation of active compounds (1). Isolation and characterization of pharmacologically active compounds from medicinal plants continue today. More recently, drug discovery techniques for the development of standardized herbal medicines with proved efficacy, safety, and quality have drawn great attention. Extraction of medicinal plants with a high content of active constituents is very important for both the isolation of drug leads and development of standardized herbal extracts. Thus extraction efficiency should be considered when using medicinal plants.

Extracts of *Artemisia asiatica* has been used in traditional Oriental medicine for the treatment of microbial infections and inflammatory diseases (2). Recently, the anti-gastritis effect of the standardized extract of *A. asiatica* has been pronounced (3). Eupatilin (5,7-dihydroxy-3,4,6-trimethoxyflavone) is known to be the pharmacologically active constituent of the extract of *A. asiatica* (4). This compound has also been reported to be therapeutically useful in cases of cerulean-induced pancreatitis, dextran sulfate sodium-induced colitis, reflux esophagitis (5), and IgE-induced hypersensitivity (6).

Conventionally, this plant has been extracted with 95% ethanol in a cold extraction process (7). The main disadvantages of cold extraction include long extraction time and large consumption of solvent.

Pressurized liquid extraction (PLE) is a solid-liquid extraction process performed at elevated temperatures at high pressures. Extraction is carried out under pressure to maintain the solvent in its liquid state at high temperature (8). High temperatures and pressures increase the penetration of solvent into the plant material and improve constituent solubilization, enhancing extraction speed and yield (9). Moreover, PLE is usually superior in terms of recovery, extraction time, solvent consumption, and reproducibility (10).

Thus, the PLE method was applied for the extraction of *A. asiatica* to investigate the effects of high temperature and pressure on the efficiency of the extraction in the present study. Moreover, the extraction conditions such as temperature and ethanol proportion were optimized by the maximization of the yield of extracts or eupatilin. The relationship between the extraction conditions and the yield of eupatilin was studied by running an experimental design, constructing a mathematical model, and investigating the relationship by response surface analysis (RSA).

EXPERIMENTAL

Plant Materials and Chemicals

Dried leaves of *A. asiatica* were purchased from Kyungdong traditional herbal market (Seoul, Korea). Dried leaves were ground and sieved. Particles with the size between 0.85 mm and 2.8 mm i.d. were collected for the study. Eupatilin was isolated from *A. asiatica* leaves and identified by spectral analysis (11).

High performance liquid chromatography (HPLC) grade solvents (acetonitrile, water, and ethanol) and reagents were obtained from BDH chemicals (Pooles, UK). Formic acid (analytical grade) was purchased from Merch (Darmstadt, Germany). Triply deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

Extraction Procedure

The extraction of *A. asiatica* was carried out using a Dionex ASE 200 (Sunnyvale, CA, USA) system equipped with a 24 sample carousel. Dried powder of *A. asiatica* (1.0 g) were mixed with diatomaceous earth (2.0 g) and placed into a 22 mL stainless steel extraction cell containing a cellulose paper filter at the bottom, respectively. The sample and solvent were preheated for 5 min, and 1 min of static extraction was then performed. The cell was thereafter rinsed with fresh extraction solvent and purged with a flow of nitrogen (1500 psi). The extraction cells were placed into the carousel and the samples were extracted under the extraction conditions.

Experimental Design and Response Surface Modeling

A central composite design (CCD) with two variables was used to determine the response pattern and then to establish a model. Two variables used in this study were temperature (X_1) and ethanol proportion (X_2) with five levels of each variable, while the dependent variable was the yield of eupatilin, respectively. All variables were taken at a central coded value considered as zero. In general, CCD is constructed in such a way that $2^k + 2k + 1$ experiments are required where k represents the number of factors to be studied. Therefore, the nine experiments listed in Table 1 were performed in triplicate. The experiments were randomly assigned to avoid unobserved error. The yield of

Table 1. The CCD matrix in the original and coded form[a] of the independent variables (X_1: temperature and X_2: ethanol) and experimental results from the response variables

No.	X_1 (°C)	X_2 (%)	Total ex. (mg)	EtOAc fr. (mg)	Eupatilin μg/ *A.asiatica* g	Eupatilin μg/ Total ex. mg	Eupatilin μg/ EtOAc fr. mg
1	130.0 (0)	0.0 (−1.5)	276.0	33.8	84.7	0.31	2.51
2	90.0 (−1)	15.0 (−1)	211.4	31.0	120.8	0.57	3.90
3	170.0 (+1)	15.0 (−1)	362.5	37.5	153.1	0.42	4.08
4	70.0 (−1.428)	50.0 (0)	221.3	36.5	182.4	0.82	5.02
5	130.0 (0)	50.0 (0)	275.5	28.5	141.7	0.51	4.97
6	190.0 (+1.428)	50.0 (0)	414.1	46.5	123.5	0.30	2.66
7	90.0 (−1)	85.0 (+1)	138.8	33.0	97.5	0.70	2.95
8	170.0 (+1)	85.0 (+1)	298.9	68.3	141.7	0.47	2.08
9	130.0 (0)	100.0 (+1.5)	158.8	48.6	148.2	0.93	3.05

[a]Values in parenthesis are the coded forms of variables.

eupatilin was estimated by quadratic response surface model, which have the following form:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

where Y is the predicted response, X_1 and X_2 are the coded values of the factors; β_0, β_i ($i = 1, 2$), β_{ii} ($i = 1, 2$) and β_{ij} ($i = 1, 2$; $j = 1, 2$, $i \neq j$) are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively.

All analyses were done in SAS 9.2 (SAS Institute, Cary, NC, USA).

Quantitative Analysis

Since it was difficult to detect eupatilin in the PLE extracts due to the low contents of eupatilin, the ethylacetate (EtOAc) fraction was used for the quantitative analysis. The extracts were filtered and evaporated in vacuum. These extracts were then suspended in 50 mL of water and fractionated with the same volume of EtOAc three times. The EtOAc fractions were evaporated in vacuum and then suspended in 50% methanol. This sample solution was filtered through 0.45 μm membrane filter (Millipore, Nylon, 170 mm) and analyzed with HPLC. The HPLC system consisted of a chromatographic pump (P680, Dionex, Germany) and an injector (7725i, Rheodyne, USA) equipped with a photodiode array (UVD, 340U, Dionex). The output signal of the detector was recorded using a Dionex Chromelon™ Chromatography Data System. Chromatographic separation was achieved on a Shiseido CAPCELL PAK C18 (5 μm, 4.6 mm i.d. × 150 mm) at 20°C and with a flow rate of 1.0 mL/min. The mobile phase was a multistep linear solvent gradient system consisting of (A) acetonitrile and (B) 0.1% formic acid (aq.). The optimal elution profile turned out to be: 0 min 30% A, 5 min 35% A, 9 min 37% A, 15 min 40% A, 20 min 70% A, 25 min 30%. Quantification of eupatilin was performed by HPLC with UV detector using a five point regression curve on the basis of eupatilin standard and determination at 220 nm.

RESULTS AND DISCUSSION

Quantitative Determination of Eupatilin

For the determination of eupatilin in *A. asiatica*, the chromatographic condition was first investigated. Various mixtures of water and acetonitrile in combination with formic acid were tested as a mobile phase. Acid is known to achieve better separation for phenolic compounds by

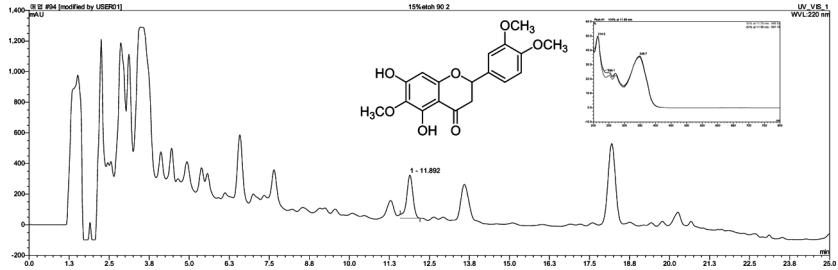

Figure 1. HPLC chromatogram of the EtOAc fraction of *A. asiatica* extracted by PLE, and the chemical structure and the UV spectrum of eupatilin.

reducing the tailing of the peaks (12). Under our chromatographic condition, the addition of 0.1% formic acid in water increased the resolution of the peaks. Strong UV absorption were observed at both 220 and 350 nm. The calibration curve at 220 nm was more linear than at 350 nm. HPLC profile of the EtOAc fraction of the PLE extract from *A. asiatica* was shown in Fig. 1. Specificity was determined by the calculation of peak purity facilitated by diode array detector (DAD). The peak purity was evaluated using DAD and its corresponding computer software, which confirms the singularity of the peak component. Calibration curve of eupatilin was linear in relatively wide range of concentrations ($0.0014\,\mu g/\mu L \sim 0.3481\,\mu g/\mu L$) and showed good linearity regressions ($y = 78.8690x + 1.7471$) with high correlation coefficient value ($R^2 = 0.9998$) between peak area (y) and concentrations of eupatilin (x, $\mu g/\mu L$).

Selection of Variables

The main reasons for the enhanced performance of PLE are the higher solubility of analytes in solvent at higher temperatures and higher diffusion rate as a result of higher temperatures. Therefore, the extraction yield is generally considered to increase with increasing temperature. However, since some flavonoid families are thermosensitive, one must keep the extraction temperature below a certain limit (13). Hence, the impact of temperature on eupatilin extraction was investigated in the range from 70°C to 190°C using the CCD.

In PLE, the solvent is a key factor affecting the recovery of analytes. Flavonoids are soluble in polar solvents and are commonly extracted from plant materials with aqueous methanol, ethanol or acetone (14). Ethanol is appropriate because it is an organic solvent permitted in the pharmaceutical and the food industry. Therefore, the effect of ethanol

proportion in aqueous ethanol extractant on eupatilin yield was evaluated. The proportion of ethanol in extractant was varied in the range from 0 to 100% (v/v).

Optimization of Extraction by RSA

A CCD was used for optimization of PLE parameters, temperature and ethanol proportion. Generally, pressure has not significantly influenced the extraction efficiency during extraction of plant material (15–17). Based on the effect of pressure within the range permitted, 1500 psi as the default level was selected. Multiple linear regressions using the second-order polynomial model (Equation (1)) were performed to fit the results of the CCD. Thus, a mathematical regression model was given as following;

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \qquad (1)$$

where Y was the predicted response, that was eupatilin yield, and X_1 and X_2 were the coded values of factors, temperature and ethanol proportion, respectively; β_0, β_i ($i = 1, 2$), β_{ii} ($i = 1, 2$) and β_{ij} ($i = 1, 2$; $j = 1, 2$, $i \neq j$) are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively.

The significance of regression tells how influential each observation is to the fit of the regression model. In statistics, the coefficient of determination R^2 is the proportion of variability in a data set that is accounted for by a regression model. R^2 is a statistic which gives some information about the good of fit of a model. In regression, the R^2 coefficient of determination is a statistical measure of how well the regression model approximates the real data points (18). An R^2 of 1.0 indicates that the regression model perfectly fits the data. Generally, a regression model can be considered as adequate when its R^2 value is bigger than 0.700. As shown in Table 2, except the value of R^2 for the model of eupatilin μg/*A. asiatica* g, the R^2 values for the others were large (>0.700), implying that the quadratic models are adequate. The influence of the extraction temperature and ethanol proportion on eupatilin yield is shown in the three-dimensional response surface graphs in Fig. 2. According to these graphs, both temperature and ethanol proportion seem to influence the eupatilin yield.

The amount of the total extract of *A. asiatica* and its EtOAc fraction were maximized in relatively high temperature. Whereas the high yields of the total extracts were accomplished in relatively low ethanol proportion, the yields of the EtOAc fractions were increased according to the increment of ethanol. The content of eupatilin in the total extract was maximized in low temperature and high ethanol proportion. However, the content of eupatilin

Table 2. Regression equations of the predicted quadratic model for the response variables

Response	Regression equation[a]	R^2
Total ex. (mg)	$Y = 273.71 + 70.64X_1 - 37.57X_2 + 16.86X_1^2 - 30.86X_2^2 + 2.25X_1X_2$	0.987
EtOAc fr. (mg)	$Y = 28.77 + 6.68X_1 + 6.67X_2 + 6.06X_1^2 + 6.59X_2^2 + 7.20X_1X_2$	0.892
Eupatilin μg/*A.asiatica* (g)	$Y = 140.86 - 1.39X_1 + 6.93X_2 + 4.11X_1^2 - 13.50X_2^2 + 2.98X_1X_2$	0.265
Eupatilin μg/Total ex. (mg)	$Y = 0.50 - 0.14X_1 + 0.13X_2 + 0.02X_1^2 + 0.05X_2^2 - 0.02X_1X_2$	0.799
Eupatilin μg/EtOAc fr. (mg)	$Y = 4.95 - 0.50X_1 - 0.26X_2 - 0.52X_1^2 - 1.09X_2^2 - 0.26X_1X_2$	0.713

[a]Y is response, and X_1 and X_2 are the coded values for temperature and ethanol proportion (v/v%).

in the EtOAc fraction was optimized in higher temperature and lower ethanol proportion compared to the condition for eupatilin in the total extract. The content of eupatilin in 1 g of *A. asiatica* was not adequate for the quadratic regression model by RSA. According to the calculation, it was optimized when the temperature and the ethanol proportion were relatively low. Table 3 shows the optimized condition for each response variable and the predicted value in each condition.

These results imply that both the temperature and the ethanol proportion contribute significantly to the extraction of eupatilin from *A. asiatica*. Rise in temperature could increase the yield of the total extracts and the EtOAc fractions. However, at the same time, the content of eupatilin in total extracts and the EtOAc fractions were decreased. It can be explained, at least in part, by the non-selective extraction of the various compounds and/or the degradation of eupatilin in high temperature. While the yield of the total extract and the content of eupatilin in the EtOAc fraction were maximized in low or medium ethanol proportion, the increment of ethanol increases the amounts of EtOAc fractions and the contents of eupatilin in the total extracts.

Taken together, an extraction condition should be chosen based on the purpose of the extraction such as the optimizations of the amounts of the total extracts and the EtOAc fractions, and the contents of eupatilin in the total extracts and the EtOAc fractions.

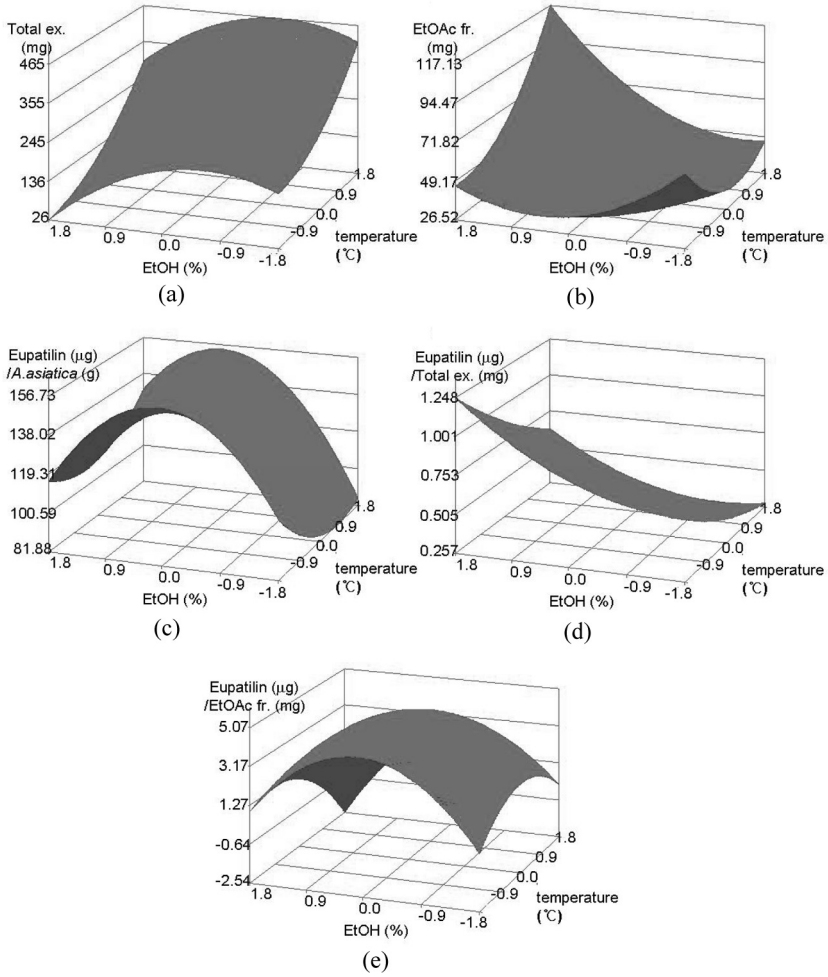

Figure 2. Response surface plots showing effects of temperature and the ethanol proportion on the extaction of *A. asiatica* using PLE. Effects on the yield of the total extracts (mg) (a) the yield of the EtOAc fractions (mg) (b) the contents of eupatilin (μg)/*A. asiatica* (g) (c) the contents of eupatilin (μg)/total extract (mg) (d) the contents of eupatilin (μg)/EtOAc fraction (mg) (e).

Experimental Validation of the Optimal Conditions

By prediction of computing program, the optimal conditions to obtain the highest response variables were determined in the range of experimental conditions as shown in Table 3. They were validated after extraction of

Table 3. Comparison between the predicted values and observed values for the response variables

Response	Optimized condition[a]		Predicted value	Observed value
	X_1 (°C)	X_2 (%)		
Total ex. (mg)	190.0	30.8	427.1	446.8
EtOAc fr. (mg)	190.0	99.0	89.8	75.3
Eupatilin μg/*A. asiatica* (g)	72.0	53.5	151.6	83.0
Eupatilin μg/Total ex. (mg)	72.0	99.0	1.13	1.38
Eupatilin μg/EtOAc fr. (mg)	111.7	47.7	5.07	3.30

[a] X_1 and X_2 are the coded values for temperature and ethanol (%).

eupatilin under these optimal conditions. The results were not significantly different from the predicted values within the 90% confidence interval except the contents of eupatilin in 1 g of *A. asiatica* and in the EtOAc fractions. The disagreements of the results might be caused by the inadequacy of the regression models in part.

CONCLUSION

The effects of the extraction temperature and ethanol proportion on the yield of extract or eupatilin from the leaves of *A. asiatica* were studied using RSA coupled with CCD. Rise in temperature could increase the yield of total extracts and the EtOAc fractions. However, the contents of eupatilin in total extracts and the EtOAc fractions were decreases by the non-selective extraction of the various compounds and/or the degradation of eupatilin in high temperature. While the yield of the total extract and the content of eupatilin in the EtOAc fraction were maximized in low or medium ethanol proportion, the increment of ethanol increases the amounts of EtOAc fractions and the contents of eupatilin in the total extracts. The results showed that both temperature and ethanol proportion significantly influenced the effectiveness of the extraction. Thus, an extraction condition should be chosen based on the purpose of the extraction. In addition, more adequate regression models should be developed and validated for the practical application of them.

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