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Original Article

Extraction of *Centella asiatica* leaves using a mixture of subcritical dimethyl ether and ethanol: Optimization of conditions by response surface methodology

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Abstract

This study aimed to develop an effective extraction method to recover triterpenoid saponins from *Centella asiatica* (L.) Urban leaves using a mixture of subcritical dimethyl ether (DME) and ethanol (EtOH) as extractant. The optimal extraction conditions, as suggested by response surface methodology, were a mixture of 2.9 ml EtOH and 5.3 g subcritical DME per g of sample at 48.7ºC. It can provide 18.8% of total triterpenoid saponins (weight/ dry weight of *C. asiatica*) which is 2- to 4-fold that obtained from the extractions with only EtOH (9.3%) or only subcritical DME (4.8%) under the same conditions.

Keywords: *Centella asiatica*, response surface optimization, triterpenoid saponins, dimethyl ether, extraction

1. Introduction

Centella asiatica (L.) Urban is an important medicinal plant belonging to Apiaceae and it has been used as a traditional herbal medicine or in drinks such as tea or juice (Hashim, 2011) in parts of Asia, including China, Sri Lanka, and Thailand, for hundreds of years (Brinkhaus, Lindner, Schuppan, & Hahn, 2000). *C. asiatica* is also known as Bua bok, Gotu kola, water pennywort, etc. (Anonymous, 2007). *C. asiatica* is reported to have many beneficial health effects such as wound-healing activity (Shetty, Udupa, Udupa, & Somayaji, 2006), antioxidant activity (Rahman *et al.*, 2013), anti-inflammatory activity (Abdullah, Mazlan, & Ar Baitee, 2012), memory improvement (Rao, Chetana, & Uma Devi, 2005), antibacterial activity (Taemchuay, Rukkwamsuk, Sakpuaram, & Ruangwises, 2009) and skin protective activity (Kwon *et al.*, 2012). These biological activities are due to active compounds such as triterpenoid saponins, tannins, steroids, alkaloids, phenolic compounds, and flavonoids (Khaw, Parat, Shaw, & Falconer, 2017; M. K. Zainol, Abd-

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Hamid, Yusof, & Muse, 2003; N. Zainol, C. Voo, Sarmidi, & Aziz, 2008; Puttarak & Panichayupakaranant, 2012; Trirattanapikul & Phoungchandang, 2014). Among these compounds, triterpenoid saponins, glycosides including madecassoside and asiaticoside as well as their aglycones madecassic acid and asiatic acid (shown in Figure S1), are the most important bioactive compounds (Bonfill *et al.*, 2006; Inamdar, Yeole, Ghogare, & de Souza, 1996; Rafamantanana *et al.*, 2009). These triterpenoid saponins act as anti-inflammatory (Huang *et al.*, 2011) and anti-oxidant agents (Abdullah *et al.*, 2012) as well as increase the migration rates of skin cells (Lee *et al.*, 2012). *C. asiatica* extracts with high triterpenoid saponin contents are, therefore, in great demand for food, food supplements, and other health products.

For the production of food supplements and health products, extraction should use non-toxic solvents. Therefore, water is generally used for this purpose. However, water is not an effective solvent for recovery of some active compounds, including triterpenoid saponins, because their incompatible polarity (Zhao *et al.*, 2010) results in low yields. Ethanol (EtOH), also considered a green extraction solvent, has been reported to give higher yields of triterpenoid saponins due to its lower polarity (Kim *et al.*, 2009). Alternatively, supercritical fluids, especially supercritical carbon dioxide (SC-CO_2), have been considered safe solvents to extract active compounds from various natural sources. SC-CO_2 is generally more effective than conventional organic solvents because of its high diffusivity into the raw material matrices (Khaw *et al.*, 2017; Lu *et al.*, 2012). To increase its extraction ability, SC-CO_2 is sometimes combined with other solvents to achieve a suitable range of polarity (Liu, Wu, Qian, & Chang, 2013; Wei & Yang, 2015). However, SC-CO_2 requires a high operating pressure leading to high equipment cost, thus making the process uneconomical (Tilly, Chaplin, & Foster, 1990; Wang, Liu, Wei, & Yan, 2012; Zaghdoudi *et al.*, 2016).

Recently, subcritical dimethyl ether (DME) or liquefied DME, considered a safe extraction solvent for the production of foodstuffs and food ingredients (EFSA, 2009), has been reported to be successful in extracting bioactive natural products such as lutein and fucoxanthin from marigold flowers and microalgae, respectively (Boonnoun, Tunyasitikun, Clowutimon, & Shotipruk, 2017). Similar to SC-CO_2 subcritical DME has high diffusivity. However, extraction with subcritical DME is more economical since it requires 20-60 times lower extraction pressure (Goto, Kanda, Wahyudiono, & Machmudah, 2015). Moreover, subcritical DME has potential to be used together as co-solvent with low toxicity solvents including water and EtOH (Billakanti, Catchpole, Fenton, Mitchell, & MacKenzie, 2013; Boonnoun *et al.*, 2017).

In this study, a green extraction process for *C. asiatica* leaves was developed using a mixture of subcritical DME and EtOH. Response surface methodology (RSM) with a spherical central composite design (CCD) model was employed to optimize extraction conditions: the EtOH to sample ratio, the DME to sample ratio, and the extraction temperature. The extract was standardized using triterpenoid saponins (asiaticoside, asiatic acid, madecassoside, and madecassic acid) as markers. The results of total triterpenoid saponins contents obtained from this DME-EtOH system were then compared with conventional extraction using only EtOH and subcritical DME, respectively.

2. Materials and Methods

2.1 Plant material

C. asiatica leaves were supplied from Nakhon Pathom Province, Thailand. The plant was identified by Dr. Pranee Nangngam, an experienced taxonomist from the Department of Biology in the Faculty of Science, Naresuan University, Thailand. A voucher specimen with the catalogue No. 004305 was deposited at the PNU Herbarium in the Faculty of Science, Naresuan University. The method of drying was modified from a previous report (Puttarak & Panichayupakaranant, 2012). After cleaning and rinsing with water, the leaves were dried in a hot air oven at 50°C for 24 hr. The dried sample was powdered and sieved through 60 mesh.

2.2 Chemical reagents

DME (Spray-work air can 420D) was purchased from Siam Tamiya Co., Ltd. (Thailand). Methanol (HPLC grade), acetonitrile (HPLC grade) and 95% ethanol (analytical grade) were purchased from RCI Labscan (Thailand). The suppliers of standard compounds were: asiaticoside (purity 98.5%), product no. 43191 and madecassoside (purity 95%), product no. M6946 from Sigma-Aldrich (USA), asiatic acid (purity ≥98%), product no. SC-233894 and madecassic acid (purity ≥95%), product no. SC-391157A from Santa Cruz Biotechnology (USA).

2.3 Extraction of triterpenoid saponins using a mixture of subcritical DME-EtOH

The extraction of triterpenoid saponins from *C. asiatica* leaves using mixture of subcritical DME and EtOH was carried out using the extraction apparatus previously described by (Boonnoun *et al.*, 2017). Briefly, approximately 5 g of dried plant powder was firstly placed in a 30×100 mm cellulose thimble (CAT No. 2800-300, Whatman). The thimble was then placed into a 100 ml stainless steel extractor and the exact amount of EtOH was added at a required EtOH to sample ratio (ml/g). Then, subcritical DME was placed into the pre-weighed extractor to achieve the required subcritical DME to sample weight ratio (g/g). The extraction temperature was controlled by a control box connected to a thermocouple probe and the extraction took place for 30 min. After extraction, the remaining EtOH in the extract was evaporated at reduced pressure in a rotary evaporator.

2.4 Design of the experiment and process optimization by response surface methodology (RSM)

Response surface methodology (RSM) with spherical central composite design (Spherical CCD) was applied to optimize the extraction process for subcritical DME extraction of *C. asiatica*. The three independent variables were the EtOH to sample ratio (X_1), the subcritical DME to sample ratio (X_2), and the extraction temperature (X_3). Each variable was designated three levels generically represented by -1, 0, and +1 while the α values used in this study were -1.73 and +1.73, corresponding to actual values of X_1: (0.3, 1,

2, 3, and 3.7 v/w_{sample}), X_2: (2.5, 4, 6, 8, and 9.5 w/w_{sample}) and X_3: (31.4, 35, 40, 45, and 48.7°C), respectively. The design suggested a total of 15 extraction conditions. The correlation between the independent variables and the response was described by the following second-order polynomial model (Equation (1)).

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X^2{}_i + \sum_{i=1}^{3}\sum_{i=1}^{3}\beta_{ij} X_i X_j \quad (1)$$

Where Y is the predicted response (total triterpenoid saponins, %w/w), ß$_0$ is a constant; ß*i*, ß*ii*, and ß*ij* are the linear, quadratic and interaction coefficients, respectively, while *Xi* and *Xj* are the levels of the independent variables.

Statistical analysis was performed using free trial Minitab Statistical Software)version 17(. Data were subjected to analysis of variance)ANOVA(, and a p-value lower than 0.05 was considered significant in the response surface analysis.

2.5 Comparison of extraction solvents used for recovery of triterpenoid saponins from *C. asiatica*

To compare the extraction between using subcritical DME-EtOH mixture and conventional extraction using only EtOH or only subcritical DME, the three extractions were conducted same solvent volume, same amount of dried plant powder (5 grams), extraction temperature (48.7 ºC), and extraction time (30 min). The process was executed at the optimal condition following RSM. For EtOH extraction, an experiment was conducted in 125 ml flask placed in an ultrasonic water bath set at 48.7ºC with 58 ml of EtOH as solvent. For subcritical DME extraction, the experiments were carried out as per the procedure described in section 2.3 with 36 g of DME as a solvent (without addition of EtOH). It is noted that the 0.62 g/cm^3 density of DME at T=49 °C and P = 1.4 MPa was used to convert the weight of DME to volumetric measurement (Ihmels & Lemmon, 2007).

2.6 HPLC method for determination of triterpenoid saponins from *C. asiatica*

The HPLC method for determination of madecassoside, asiaticoside, madecassic acid, and asiatic acid was modified from a previous study (Rafamantanana *et al.*, 2009). The HPLC system used in the analysis consisted of a Shimadzu LC-20AD pump equipped with an SIL-20A HT auto sampler and a SPD-M20A photodiode array detector. A phenomenex Luna 5u C-18 column (250 x 4.6 mm) was used in the stationary phase. The mobile phase was a gradient of water / acetonitrile (ACN). The gradient elution conditions applied were: 0.00-5.00 min, linear gradient 23-50% ACN; 5.00-8.00 min, linear gradient 50-100% ACN; 8.00-14.00 min, 100% ACN; 14.00-14.01 min, gradient 100-23% ACN; and final flushing of the column was with gradient 23% ACN for 6 min before reconditioning the column with 23% ACN. The flow rate was 1.0 ml/min, the injection volume was 20 µL and detection was at 210 nm.

The HPLC method was validated to determine the following parameters; precision (intra-day and inter-day), accuracy, specificity, linearity, limit of detection (LOD) and limit of quantitation (LOQ) values. Precision and accuracy were assessed by determination of instrument precision and accuracy of intra-day and inter-day analysis. For intra-day analysis, madecassoside and asiaticoside standards were prepared in final concentrations of 96, 240, and 384 µg/ml while madecassic acid and asiatic acid standards were prepared at 24, 60, and 96 µg/ml (n=3). Then 500 µg/ml of the samples were spiked into each standard solution. Subsequently each solution was analyzed by HPLC. For inter-day analysis, the samples were repeatedly prepared and analyzed in the same way for 3 days. The precision was evaluated by their relative standard deviation (RSD, %) and accuracy was calculated from recovery (%) in the analyses compared to the amount of compounds added to the samples. The RSD and recovery values were within acceptable ranges based on the AOAC Guidelines for Single Laboratory Validation of Chemical Methods (AOAC, 2002) (Table S1). An outstanding specificity was achieved and confirmed by using retention time of chromatogram. The linearity was achieved with coefficient (r^2) exceeding 0.999 and HPLC chromatogram of triterpenoid saponins showed good separation (Figure S1), showing that this analysis method was precise, accurate and sensitive enough for simultaneous quantitative determination of those active compounds in *C. asiatica* extracts.

3.Results and Discussion

3.1 Experiment design and process optimization by RSM

3.1.1 Analysis of variance (ANOVA) of a quadratic regression model

Spherical central composite design suggested extraction conditions for each of the 15 experiments shown in Table 1. The amount of total triterpenoid saponins, expressed as percent weight by dry weight (%w/w) of *C. asiatica* of experimental data in triplicate, and the model predicted data for each experimental run, are also listed in Table 1. The data were then subjected to ANOVA and the results are shown in Table 2. The results show that the quadratic regression model was significant, showing a p-value of 0.0460. The prediction of the model showed good agreement with the experimental data, with an r^2 of 0.8999. Moreover, statistical analysis revealed that the percent of EtOH, significantly affected the recovery of triterpenoid saponins from *C. asiatica*, since the p-value of this parameter was 0.0030 (lower than 0.05). However, the subcritical DME to sample ratio and extraction temperature insignificantly affected total triterpenoid saponins. The quadratic model representing total triterpenoid saponins (Y) as a function of the actual independent parameters, EtOH to sample ratio (X_1), subcritical DME to sample ratio (X_2), and extraction temperature (X_3), is presented in Equation 2:

$$Y = 26.9 + 5.33\,X_1 - 0.17\,X_2 - 0.93\,X_3 - 1.420\,X_1X_1 - 0.036\,X_2X_2 + 0.0105\,X_3X_3 - 0.002\,X_1X_2 + 0.060\,X_1X_3 + 0.0115\,X_2X_3 \quad (2)$$

Table 1. Total triterpenoid saponins (%w/w) in *C. asiatica* extracts from the experimental data and RSM model predictions

Run number	EtOH to sample ratio (v/w)	DME to sample ratio (w/w)	Temperature (°C)	Total triterpenoid saponins (%w/w)		%Error
				Experimental data	Predicted data	
1	1	4	35	13.6±1.31	13.5	0.74
2	3	4	35	16.2±0.84	17.0	4.94
3	1	8	35	13.8±0.37	12.7	7.97
4	3	8	35	15.5±0.88	16.2	4.52
5	1	4	45	14.3±0.44	13.6	4.90
6	3	4	45	17.2±1.05	18.3	6.40
7	1	8	45	14.1±0.47	13.1	7.09
8	3	8	45	17.9±0.24	18.0	0.56
9	0.3	6	40	7.3±0.27	8.8	20.55
10	3.7	6	40	17.5±0.30	15.9	9.14
11	2	2.5	40	17.3±0.46	16.7	3.47
12	2	9.5	40	15.1±1.02	15.7	3.97
13	2	6	31.4	16.7±1.01	16.6	0.60
14	2	6	48.7	18.1±1.25	18.2	0.55
15	2	6	40	16.6±0.71	16.6	0.00

X_1 = EtOH to sample ratio, X_2 = subcritical DME to sample ratio, X_3 = extraction temperature

Table 2. Results of the ANOVA for the response surface quadratic model

Source	df	SS	MS	F-Value	P-Value
Model	9	92.739	10.3043	4.99	0.046
X_1	1	58.334	58.3341	28.26	0.003
X_2	1	1.152	1.1521	0.56	0.489
X_3	1	3.232	3.2321	1.57	0.266
X_1* X_1	1	12.883	12.8828	6.24	0.055
X_2* X_2	1	0.133	0.1326	0.06	0.810
X_3* X_3	1	0.441	0.4408	0.21	0.663
X_1* X_2	1	0.000	0.0001	0.00	0.994
X_1* X_3	1	0.722	0.7221	0.35	0.580
X_2* X_3	1	0.106	0.1061	0.05	0.830
Error	5	10.320	2.0640		
Total	14	103.059			
R^2	89.99				

X_1 = EtOH to sample ratio, X_2 = subcritical DME to sample ratio, X_3 = temperature, SS = Sum of Squares, MS = Mean Square

3.1.2 Effect of extraction parameters on total triterpenoid saponins

The way to visualize the effect of independent variables on a dependent variable is to draw 3D response surfaces or contour plots of the model. The RSM results for the yield of total triterpenoid saponins are given in Equation 3:

$$Y = 0.9055x + 1.4373 \quad (3)$$

where Y is the predicted yield, X is the experimental yield. As shown in Figure 1. The r^2 of the quadratic polynomial model was 0.8971, which indicates that the model adequately fit the real relationships among the selected independent variables.

The effects of extraction parameters on total triterpenoid saponins were assessed from 3D surface and contour plots generated by the quadratic regression model (Equation 2) as shown in Figure 2 (a-f). It can be seen from the results shown in Figure 2 (c, d, e, and f) that an increase in the amount of EtOH from 0.3 to 2.5 ml/g of *C. asiatica* resulted in a corresponding increase in triterpenoid saponin content from 10 to 18%. However, triterpenoid saponin content slightly decreased from 18 to 16% on increasing the amount of EtOH further than 2.5 ml/g of *C. asiatica.* These results could be observed at all extraction temperatures (30 to 50°C) and subcritical DME to sample ratios (2.5 to 9.5 g/g). The explanation of these results might be related to the polarity of the solvent mixture (subcritical DME-EtOH). Subcritical DME has lower polarity than ethanol and it was favorable to extract aglycones (madecassic acid and asiatic acid) more than glycosides (madecassoside and asiaticoside). The presence of ethanol as co-solvent at suitable concentration (suitable solvent polarity) might enhance extraction efficiency for both aglycones and glycosides. Previously, it has been reported that suitable solvent polarity increased the extraction yield (Billakanti *et al.*, 2013).

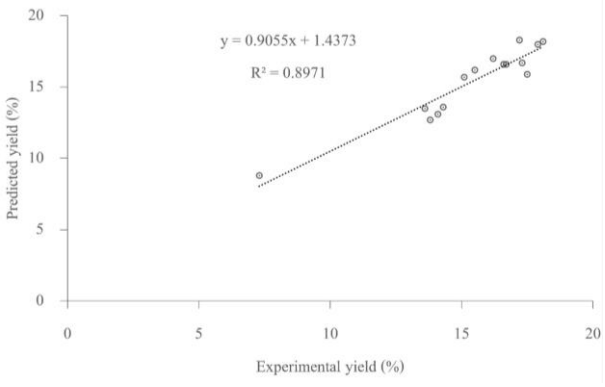

Figure 1. Relationships between experimental and predicted values for the total triterpenoid saponins of *C. asiatica* extracts

The effects of changing the subcritical DME to sample ratio are shown in Figure 2 (a, b, e, and f). The total triterpenoid saponins were insignificantly different when increasing the subcritical DME to sample ratio from 2.5 to 6.0 g/g at all extraction temperatures and EtOH to sample ratios.

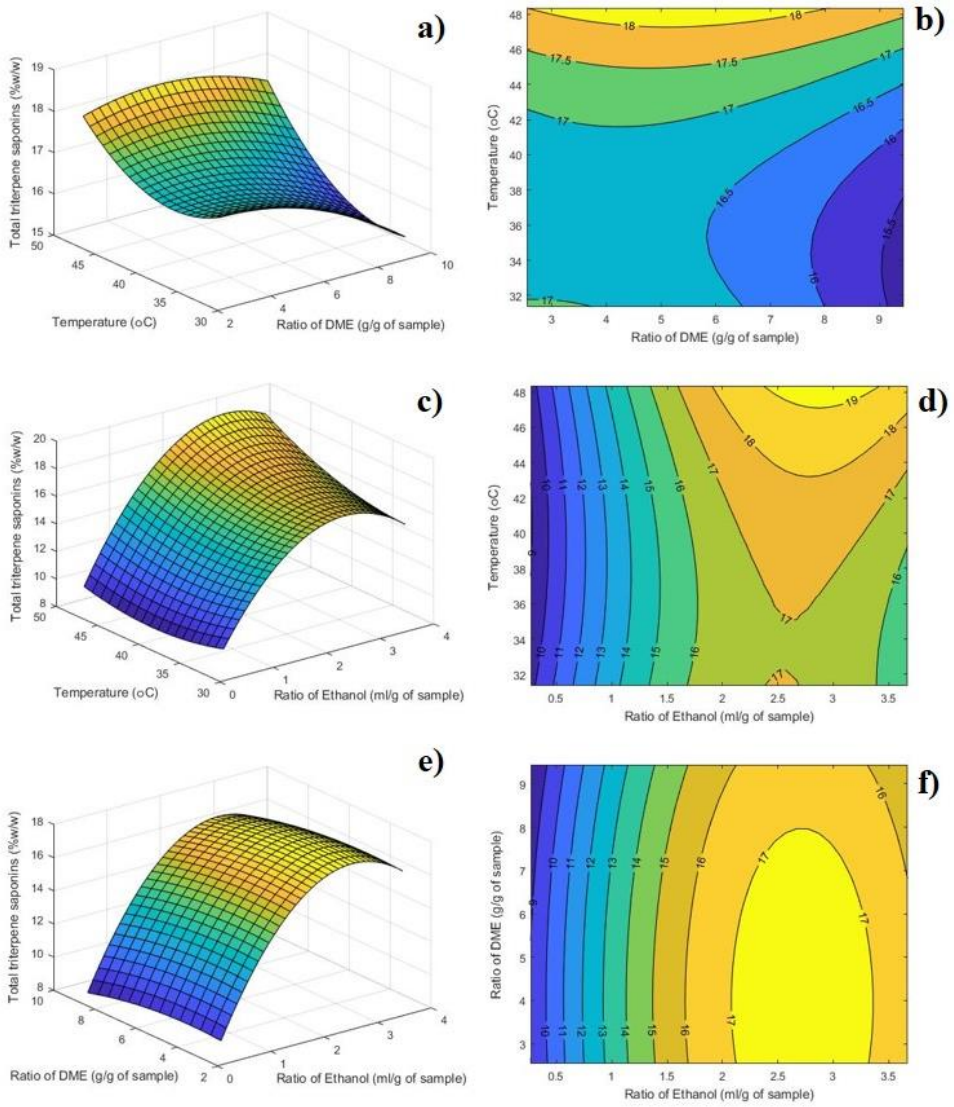

Figure 2. 3D response surface and contour plots for total triterpenoid saponins over independent variables used in extraction of *C. asiatica* : subcritical DME to sample ratio, EtOH to sample ratio, and temperature

However, triterpenoid saponin contents were observed to slightly decrease as the subcritical DME to sample ratio became higher than 6.0 g/g. This also can be explained by the suitability of solvent polarity, as was done earlier. The increase in DME to sample ratio could decrease the concentration of co-solvent (ethanol), lowering solvent polarity, which might not be suitable to extract both aglycones and glycosides. The overall triterpenoid saponins content was therefore decreased.

The effect of extraction temperature on the total triterpenoid saponins is presented in Figure 2 (a, b, c, and d) where total triterpenoid saponins increase slightly with increasing temperature. The maximum total triterpenoid saponins %w/w was obtained at 48.7°C. The increase in temperature increased the solubility of the solute in the solvent (Zhang *et al.*, 2015), resulting in a larger amount of obtained extracts. However, it is worth noting that the active compounds including triterpenoid saponins might be degraded at elevated extraction temperatures (Niamnuy, Charoen chaitrakool, Mayachiew, & Devahastin, 2013).

3.2 Process validation

According to the regression model, the optimal extraction conditions to obtain maximal total triterpenoid saponins %w/w of 19.4% were at 2.9 ml/g of EtOH to sample ratio, 5.3 g/g of subcritical DME to sample ratio, and 48.7°C extraction temperature. To verify the prediction from the model equation, triplicate confirmatory experiments were carried out at these model based optimum conditions. The actual total triterpenoid saponins %w/w obtained experimentally was 18.8%. Thus, the predicted value was very similar to the experimental result, confirming that the regression equation's total triterpenoid saponin prediction was reasonable.

3.3 Comparison of the different extraction solvents

To gain insight into the triterpenoid saponins extraction using a mixture of subcritical DME and EtOH, the system was compared with extraction using only EtOH or only subcritical DME under the same extraction conditions. From the previous results, the optimal EtOH to sample ratio was 2.9 ml/g and subcritical DME to sample ratio 5.3 g/g. This amount of solvent was converted approximately to 36 g of subcritical DME in a subcritical DME only system, and to 58 ml of EtOH in an EtOH only system (Ihmels & Lemmon, 2007). The results shown in Figure 3 reveal that using only subcritical DME gave the least total triterpenoid saponins, only 4.8%, and no glycosides were found in this extract. This was probably due to the much smaller molecule of DME compared with saponin glycosides (asiaticoside and madecassoside). The higher 9.3% content was observed for the system using only EtOH, which indicated that EtOH was more suitable extraction solvent than DME in this case. However, the highest total triterpenoid saponins (18.8%) was achieved by the system using a mixture of subcritical DME and EtOH. The improvement in extraction efficiency is possibly due to subcritical DME improving the diffusivity of the solvent mixture into the matrices of *C. asiatica*, resulting in a large extracts yield.

Compared with the other methods for recovery of triterpenoid saponins (consider only asiaticoside and asiatic acid) from *C. asiatica*, the mixture of subcritical DME and EtOH shows significantly higher content of triterpenoid saponins (7.74%) The extraction using subcritical water or maceration with EtOH reported in a previous study (Kim *et al.*, 2009) provided only 0.88% or 0.72% triterpenoid saponins contents, respectively. The extraction using mixture of subcritical DME and EtOH also requires lower extraction pressure, temperature and time (0.6 MPa, 48.7°C for 30 min.) compared with subcritical water (10 to 40 MPa, 105-250 °C for 5 hours). Although it requires higher extraction pressure and temperature than maceration with EtOH (atmospheric pressure, 25°C), extraction using a mixture of subcritical DME and EtOH needs shorter extraction time (30 min vs 60 min).

4. Conclusions

A green extraction method to recover triterpenoid saponins in *C. asiatica* leaves was successfully developed. The extraction conditions were optimized by response surface methodology (RSM) with a spherical central composite design (CCD) of experiments. The results revealed that the optimal extraction conditions were at 2.9 ml of EtOH/g of sample, 5.3 g of DME/g of sample, and at 48.7ºC, giving 18.8% total triterpenoid saponins. These results greatly improved total triterpenoid saponin content from the 4.8% to 9.3% extracts when using only subcritical DME or only EtOH, respectively. Compared with conventional solvent extraction, this method requires higher operating pressure, leading to a higher equipment cost. However, it provides a significantly higher triterpenoid saponin content with easy separation of solvent from final product by de-pressurization. This separation technique is therefore considered as a preferred alternative process.

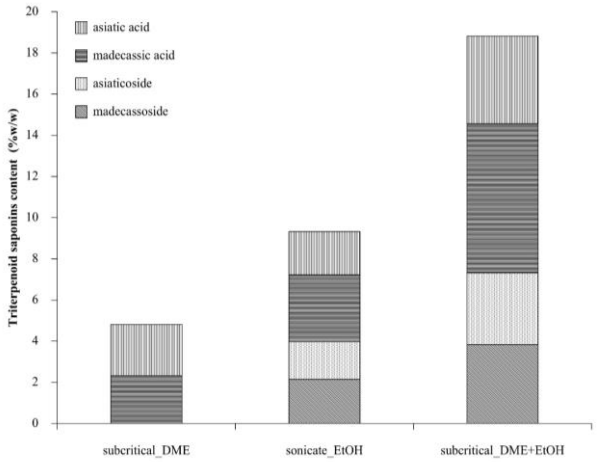

Figure 3. Total triterpenoid saponins obtained from different extraction solvents (subcritical DME, EtOH, and a mixture of subcritical DME and EtOH) using the same conditions

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