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Research Article

# OPTIMIZED ULTRASONIC-ASSISTED EXTRACTION OF ANTIOXIDANT FROM MULBERRY (*MORUS ALBA* L.) LEAVES USING MULTIPLE LINEAR REGRESSION ANALYSIS

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**ABSTRACT**

Objective: The aim of this study was to optimize mulberry extraction to yield the highest antioxidant activity as functions of ethanol concentration, extracting time and liquid to solid ratio.

Methods: Three factors, ethanol concentration (30–60%), extracting time (20–40 minutes) and liquid to solid ratio (20:1–40:1 v/w), were carried out using software-assisted experimental design. Central composite design with 2 levels was selected and the model was further fitted with multiple linear regression analysis. *In vitro* antioxidant activity, 2,2'-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, was set as a response for the experiment.

Results: The percentage inhibitions of the extracts were ranged 47.67 – 89.88. The model exhibited well fit with correlation coefficient of observed versus predicted values at 0.993. The model also showed high reproducibility at 0.990. The optimal condition with the highest antioxidant activity was consisted of 60% ethanol concentration, 25.5 minutes extracting time and liquid to solid ratio at 40:1.

Conclusion: Ultrasonic-assisted extraction of mulberry leaves together with multiple linear regression analysis was very useful to yield the highest antioxidant activity. Increasing ethanol concentration and liquid to solid ratio subjected to increase the yield, while increasing extracting time showed negative effect at the longer time.

**Keywords**: ultrasonic-assisted extraction, antioxidant, mulberry, central composite design, optimization, multiple linear regression analysis

## INTRODUCTION

Oxidative stress is a cause of various aging disease such as cancers, Alzheimer's disease and cardio-vascular diseases. It can be activated by pollutant, stress, illness and modern life style. Antioxidant compounds could be considered to protect human body from oxidative stress induced aging diseases. Natural antioxidant compounds are usually found in parts of plant, for instance, fruits [1], flower [2], leaves and bark [3] or seeds [4]. Distribution of antioxidant compounds in various parts of plants was significantly different as presented in leaves, stems and roots of *Boerhavia diffusa* L. [5]. It was also found in honey [6]. Antioxidant-rich plants found in everyday life are normally served as food ingredients, side dishes as well as in beverages. However, the amount of antioxidant consuming is not sufficient for disease prevention. Health products in form of concentrated or extracts are considered as supplementary sources of high antioxidant.

Mulberry (*Morus alba* L.) leaves are usually used as silkworm feeds in sericulture. In Thailand, mulberry is also found in market as healthcare products, herbal tea and dietary supplement. It consists of high nutritional values; phenolic compounds [7], flavonoids, ascorbic acid and β-carotene [8], as well as biological activities; antimicrobial [9] and antioxidant activities [10]. Antioxidants can protect many chronic diseases *i.e.* cardiovascular diseases by inhibiting LDL oxidation which protects atherosclerosis [11, 12].

Extraction of plant materials such as heating, refluxing or using soxhlet apparatus could activate oxidation or hydrolysis of bioactive compounds, while maceration and percolation are needed longer extracting time. The uses of non-polar organic solvents should be limited according to carcinogenesis inducing.

Therefore, the most common polar organic solvent, ethanol, can be considered as a reasonable extracting solvent due to less toxicity. Other modern extracting techniques, for example, ultrasonic-assisted extraction, microwave-assisted extraction and supercritical fluid extraction are subjected to investment on high cost instruments. The advantages of ultrasonic extraction are simple, easy to handle and inexpensive comparing to the others. The mechanism of ultrasonic extraction can be explained that shear force produced by ultrasonic cavitation breaks plant cell wall and accelerates material or compound transfer into extracting solvent in shorter time comparing to maceration or percolation [13].

In a conventional method for extraction with various factors, one factor has to vary while the rests are fixed. Then, changing from the former to the latter has been applied until all factors have been investigated. A number of experiments will increase according to the extraction factors. Moreover, the real optimal condition would not be found, because it is impossible to increase degree of each variable continuously. The use of experimental design with statistical techniques results to obtain the real optimal point in the experimental matrix. This technique can be applied to screening process and optimization. These lead to obtain the most important variables and optimal condition of the experiment. Experimental designs also use in many disciplines, for instance, analytical chemistry [14], organic synthesis, engineering process, pharmaceuticals or food production [15].

The aim of this study was to optimize ultrasonic-assisted extraction of mulberry leaves using central composite face-centered design to yield the highest antioxidant activity. The investigated factors, ethanol concentration (30–60%), extracting time (20–40 minutes) and liquid to solid ratio (20:1–40:1 v/w), were evaluated regarding DPPH radical scavenging activity using response surface methodology.

## MATERIAL AND METHODS

### Chemicals, reagents and plant material

All chemicals and reagents were analytical grade. 2,2'-diphenyl-1-picrylhydrazyl (DPPH) from Sigma-Aldrich (St. Louis, MO, USA) was prepared in a concentration of 0.2 mM in ethanol. Mulberry leaves were previously identified by Chirapha Butiman. Authentic sample was collected at Silk Innovation Center, Mahasarakham University and also cultivated in the field. The leaves were harvested in March 2013, then washed and cut into small pieces. The leaves were subsequently dried under hot air condition at 50 °C. Dry plant materials were then ground to fine powder and protected from light under vacuum condition prior to measurement.

**Experimental design**

Effects of ethanol concentration, extracting time and liquid to solid ratio were investigated in ranges of 30-60%, 20-40 minutes and 20:1-40:1, respectively. Antioxidant activity using DPPH radical scavenging method was performed and measured as response of the model. Central composite face centered design with 2 levels was selected for optimization. Number of experiment was calculated as followed.

$$N = 2^n + 2n + 3 \quad (1)$$

where, N is number of experiment.

n is number of factors.

The experimental matrix was generated by software assisted experimental design, MODDE 9.0 (Umetrics AB, Umeå, Sweden). To obtain the optimal point, the model was fitted with multiple linear regression analysis in accordance with MODDE 9.0.

**Ultrasonic-assisted extraction**

Each sample was weighed (1 g) into test tubes and soaked with extracting solvents for 30 minutes prior to extraction. The samples were then placed to ultrasonic bath at frequency of 30 MHz for extraction. Subsequently, the samples were centrifuged and partitioned with hexane to get rid of chlorophyll. The resulted extracts were then filtered through membrane with pore size 0.45 µm before antioxidant activity measurement.

**Antioxidant activity measurement**

To normalize the volume of the resulted extracts, each extract was diluted to obtain the final volume equivalent to the highest liquid to solid ratio, 40:1. For example, an extract at 20:1 liquid to solid ratio was diluted with the same volume of ethanol before measurement. The free radical scavenging activity of extracts were determined on a basis of their ability to react with the stable DPPH (2,2'-diphenyl-1-picryl hydrazyl) free radical. After incubation in dark place at ambient temperature for 30 minutes, bleaching of purple color of DPPH radicals were investigated according to hydrogen atoms or electron donation ability from herbal extracts [1]. The absorbance of each solution was determined at 517 nm [15]. The measurements were carried out using Jasco V530 UV-spectrophotometer (Tokyo, Japan). Percentage inhibitions (%I) were calculated by equation as shown below.

$$\%I = \left[1 - \frac{\left(A_i - A_j\right)}{A_c}\right] \times 100 \quad (2)$$

where, $A_i$ is the absorbance of the test sample mixed with DPPH solution (2 mL sample + 2 mL DPPH); $A_j$ is the absorbance of the sample without DPPH solution (2 mL sample + 2 mL of 95% ethanol); $A_c$ is the absorbance of DPPH solution without sample (2 mL DPPH + 2 mL of 95% ethanol).

**RESULTS AND DISCUSSION**

The yield of antioxidant from mulberry leaves extract certainly affected by extraction parameters such as concentration of ethanol, extracting time and liquid to solid ratio. To optimize the extraction, experimental design with statistical analysis is a useful technique to reduce a number of experiments as well as obtain the real optimized condition.

The effects of ethanol concentration, ratio of white birch bark to solvent, extraction temperature, ultrasonic frequency and extracting time were also investigated using an ultrasonic-assisted extraction of betulin from white birch bark [13].

The experiments were optimized using central composite design. There was also a study that investigated the effect of extraction parameters on extraction of flavonoids from *Prunella vulgaris* [15]. The investigated parameters were ethanol concentration, ratio of white birch bark to solvent, extraction temperature and extracting time using Box-Behnken design for optimization. In addition, an ultrasonic-assisted extraction of flavonoids from *Folium eucommiae* was performed as functions of ethanol concentration, extracting time and solid to liquid ratio combined with 2-level full factorial central composite design [16].

**Table 1: Design matrix of concentration of ethanol (EtOH conc), extracting time and liquid to solid ratio (Liq/sol ratio) versus response (DPPH radical scavenging)**

| Exp Name | Run Order | EtOH conc (%) | Extracting time (min.) | Liq/sol ratio | DPPH radical scavenging (% inhibition) |
|---|---|---|---|---|---|
| N1 | 7 | 30 | 20 | 20 | 57.10 |
| N2 | 1 | 60 | 20 | 20 | 70.73 |
| N3 | 16 | 30 | 40 | 20 | 46.67 |
| N4 | 12 | 60 | 40 | 20 | 65.76 |
| N5 | 2 | 30 | 20 | 40 | 85.33 |
| N6 | 11 | 60 | 20 | 40 | 89.88 |
| N7 | 14 | 30 | 40 | 40 | 83.42 |
| N8 | 17 | 60 | 40 | 40 | 86.02 |
| N9 | 13 | 30 | 30 | 30 | 75.10 |
| N10 | 15 | 60 | 30 | 30 | 82.36 |
| N11 | 10 | 45 | 20 | 30 | 80.40 |
| N12 | 8 | 45 | 40 | 30 | 73.52 |
| N13 | 6 | 45 | 30 | 20 | 60.31 |
| N14 | 4 | 45 | 30 | 40 | 86.58 |
| N15 | 3 | 45 | 30 | 30 | 80.63 |
| N16 | 9 | 45 | 30 | 30 | 79.02 |
| N17 | 5 | 45 | 30 | 30 | 78.34 |

The run order of all experiments was measure randomly to prevent systematic error. The 3 extra experiments at the center point were performed to evaluate reproducibility of the model. The measurements of the factors were optimized using 2-level central composite face centered design resulted to 17 experiments (from equation 1). The mechanism of ultrasonic extraction is generation of shear forces which destroy cell wall as well as accelerate chemical mass transfer to protect from local concentration [17]. The effect of three factors; concentration of ethanol, extracting time and liquid to solid ratio on antioxidant activity were investigated using 2 levels central composite face centered design. Table 1 shows experimental design matrix and corresponding response data of mulberry leaves extraction.

The experimental model was fitted using multiple linear regression analysis in accordance with MODDE 9.0. The model showed good fit with high correlation coefficient ($r^2$= 0.993) of observed versus

predicted values with relative standard deviation, RSD of 1.51. This model also exhibited high reproducibility ($r^2$= 0.990). Cumulative normal probability plot against residuals showed normal distribution with a value close to 1 (0.970). No experiment located beyond the outliers. Summary of fit for the model and scaled & centered coefficients were showed in Fig. 1.

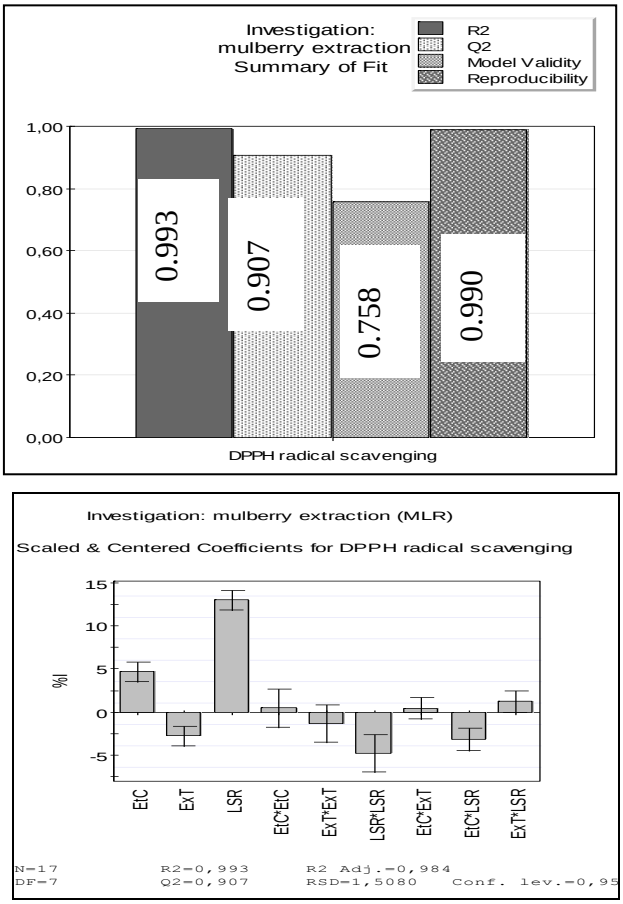


**Fig. 1: Summary of fit for the model and scaled & centered coefficients of DPPH radical scavenging activity against extraction factors (EtC, ethanol concentration; ExT, extracting time; LSR, liquid to solid ratio)**

With MODDE 9.0 software assisted, the generated optimal condition of antioxidant activity using ultrasonic extraction was at 60% ethanol concentration, 25.5 minutes extracting time and liquid to solid ratio at 40:1. The plots of antioxidant activity versus extracting factors were showed as response surface plots in Fig. 2. According to scaled & centered coefficients, increasing liquid to solid ratio and ethanol concentration resulted to obtain higher yield which expressed in positive coefficients. However, extending extracting time resulted to lose antioxidant activity. The scaled & centered coefficient showed in negative value. This phenomenon can be explained that the amount of bioactive compounds had been extracted from plant material at the beginning. Increasing extracting time resulted to the yield of bioactive compounds dissolving in the solvent [16]. At certain time, less antioxidant constituents were diffused into the medium. Hence, degradation of active compounds had been dominated over extraction.

## CONCLUSIONS

A combination of ultrasonic-assisted extraction with central composite face centered design for antioxidant exhibited a simple technique, less time consuming and optimal condition with high antioxidant activity. Increasing extraction time showed higher antioxidant activity at a certain point and then decreased. While increasing concentration of ethanol and liquid to solid ratio exhibited higher antioxidant activity.

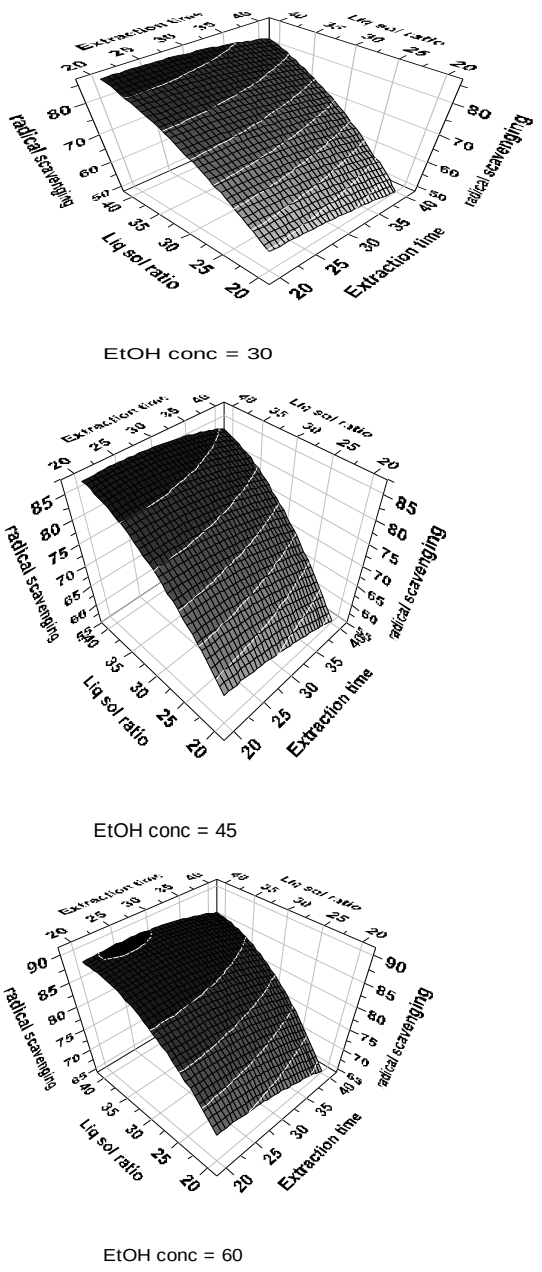


**Fig. 2: Response surface plots of three factors; ethanol concentration, extracting time and liquid to solid ratio against DPPH radical scavenging activity (higher activity shows in dark color)**

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