Effects of Extraction Factors on Total Phenolic Compounds and Antioxidant Activity in Mulberry Leaves

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Abstract *The objective of this study is to examine effects of ultrasonic-assisted extraction on bioactive compounds of mulberry leaves in compared with the conventional extraction method. The mulberry leaves used for this research were cleaned and dried by hot air drying technique at 60°C for 5 hours. The ethanol concentration of 50% and 80% with ethanol soaking time of 60 minutes and extraction time of ultrasonic treatments at 20, 30 and 40 minutes were used. The experimental results showed that the ultrasonic-assisted extraction provided highest total amout of phenolic content and DPPH scavenging activity compared with the controlled samples ($p \leq 0.05$). In addition, increasing extraction time could further increase total phenolic content and DPPH scavenging activity; with 50% ethanol and extraction time of 40 minutes provided the highest total phenolic content and DPPH scavenging activity under the tests.*

Keywords:

ultrasonic-assist extraction, mulberry leaves, bioactive compounds

1. Introduction

Mulberry is one of the traditional Thai herbs that is usually used to as a part of drinking releases or as herbal medicines. In most Asian countries, mulberry leaves are also used to feed silkworms (*Bombyx* mori L.) [1]. This is because the mulberry leaves have enrich with proteins and important bioactive compounds such as flavonoids and phenolic compounds; where these compounds help to reduce oxidative stress and provide low blood sugar [2]. It has been reported that mulberry leaves, also the extracts of mulberry leaves, exhibit multiple therapeutic effects such as anti-diabetic, anti-inflammation and anti-cancer effects [3]. Phytochemical investigation has indicated that there are many active constituents, such as flavonoids, alkaloids, polysaccharides, phenolic compounds and steroids in the mulberry leaves [4]. However, these compounds require proper methods to extract them from the leaves for further uses. In fact, the extraction method is the most important factor that affects both quantity and quality of extracted compounds.

There are several extraction methods proposed in the literatures [5]-[7]. However, among those methods few most traditional extraction methods have usually used, which are Soxhlet extraction [8], heating reflux extraction [9], maceration and shaker extraction [10]. The Soxhelt extraction utilizes a laboratory equipment called Soxhlet extractor which is designed to extract a lipid from a solid material. The Soxhlet extraction is typically used when the desired compound has a limited solubility in a solvent whereas the impurity is insoluble in that solvent. However, this method is suitable only for unmonitored and unmanaged operation with less efficient recycling a small amount of solvent to dissolve a larger amount of material [11]. The heating reflux extraction, these procedures have distinct drawbacks, such as the consumption of large volumes of solvent and amounts of energy, low yields and lengthy extraction procedures that can result in the loss or degradation of target compounds [9]. Although, the maceration and shaker extraction methods is the most commonly used method to extract. The shaker extraction is simple and safe, high temperature and long time of maceration and shaker extraction lead to the degradation of bioactive compounds [12]. Unfortunately, it seems that all the aforementioned methods would have some disadvantages about their long extracting time and/or high amount of consuming energy. These lead the traditional extraction methods an inefficient method as well as effects

on deformation of bioactive compounds [13]. Alternatively, the ultrasonic- assisted extraction proposed in [14] that was originally used for the applications of bioactive compound extraction from many kinds of herbs and plants. The acoustic cavitation in ultrasonic assisted extraction can destroy cell walls, then reduce particle sizes and finally decomposition the contacts between solvents and bioactive compounds [15]. Moreover, the ultrasonic-assisted extraction method has also some advantages regarding low energy consumption, low solvent comsumption, high extraction efficiency and high level of automationity [16]. However, the study of effects of extracting bioactive compounds from the mulberry leaves using the ultrasonic-assisted method has not been proposed, which is the objective of this research.

Therefore, this paper presents the experimental results on effects of ultrasonic-assisted extraction on bioactive compounds of mulberry leaves by focusing on the phenolic compounds and antioxidant activity of mulberry leaves.

2. Materials and Extraction Methods

This section describes the processes to prepare the mulberry leaves, explanation of principle and set-up of the ultrasonic-assisted extraction method, the parameters and techniques used to investigate and analyze effects of the ultrasonic-assisted extraction method on bioactive compounds of prepared mulberry leaves.

2.1 Preparation of Mulberry Leaves

Mulberry leaves type CV. Burirum no. 60, the most common type of mulberry leaves in Thailand, were used in this experimental study. The leaves as shown in Fig. 1(a) were harvested during their full growing stages from the Sericulture Research Unit, Mahasarakham University, Thailand. The prepared mulberry leaves were immediately washed and dried at 60 °C for 5 hours, regarding standard proposed in [17]. Then, the sample leaves were ground and sieved with 80 meshes. Finally, they were kept away from light in a disscicator at the controlled room temperature of 25 ^{o}C until they were analytical bioactive compounds, having physical photographs as shown in Fig. 1(b).

(a) harvested full stage leaves (b) final dried-sieved leaves

Fig. 1 physical photographs of the mulberry leaves type CV. Burirum no.60 under study during (a) harvested full stage leaves and (b) final dried-sieved leaves.

2.2 Ultrasonic-Assisted Extraction

The ultrasonic-assisted extraction equipment used for the experimental tests was a rectangular bath model Kj-300 Wuxi Kejie Ultrasonic Electronic Equipment Co., Ltd. The equipment has an inner dimention of 300x240x150 mm with an ultrasonic power and frequency source of 150 W and 60 kHz [18]. The extraction temperature was controled at 30 °C. The sample beakers were immersed into the ultrasonic bath for ultrasonic waves under extraction conditions of 50% and 80% ethanol and solvent to solid ratio of 250 ml per 30 g. The test samples were sonicated at a constant temperature of 30 °C with frequency of 60 kHz for 10, 20 and 40 minutes. In order to validate the experimental results, the conventional solvent extraction was carried out with the same ethanol concentration of 50% and 80%, but with the extraction time of ultrasonic treatment at 20, 30 and 40 minutes, while applying ethanol soaking time of 60 minutes and 50 g of the ground powder was mixed with ethanol for smooth the tests.

After the ultrasonic treatment, the samples were centrifuged with centrifugal speed of 6000 rpm for 20 minutes. The samples then were kept at 4 °C for better separation of compounds. After that the samples were filtered through a 0.45-µm membrane filter. Finally, the filtrates were collected for HPLC analyses.

2.3 Determination of Total Phenolic Compounds

The filtrates obtainded from the untrasonic-assisted extraction then were sent to test quantitative of total phenolic compounds. The total phenolic compounds were analyzed by using a high performance liquid chromatography in comparison to the standard liquid (gallic acid) using LUNA Colum (size of 4.6x250 mm and diameter of 5 mm). The mobile phase A was used with 3% acetic acid while the mobile phase B with 25% acetronotrile per 72% water; under test conditions of diode array detection at 278 nm, velocity of a fluid at 1.2 ml/ minute. Finally, the peak areas were calculated that eventually gave values of total phenolic compounds. The unit of total phenolic compounds were expressed in mg gallic acid equivalent per gram of sample weight (mg GAE/100g) [19].

2.4 DPPH Radical Scavenging Activity

Antioxidant activity of the crude extract was evaluated by DPPH radical scavenging assay [5]. Briefly, 50 µl of the 60% ethanol mulberry leaves extract prepared as described before, 50 µl of 40% ethanol aqueous solution (v/v), and 50 µl of 0.2 M of morpholinoethanesulfonic acid. The mulberry leaves extract was diluted with 60% ethanol aqueous solution. The reaction was intiated by adding 50 µl of 0.1 M DPPH in ethanol. After left standing for 20 minutes at the room temperature of 25 ^{o}C, the reaction

mixture absorbance at 517 nm was measured by the spectrophotometer. The results expressed as a percentage of inhibition that can be calculated using equation (1).

$$\% \text{ radical scavenging} = \frac{A_{control} - A_{sample}}{A_{control}} x100 \quad (1)$$

; where $A_{control}$ and A_{sample} are the absorbance of control and absorbance of mulberry leaves extract, respectively.

2.5 Statisticical Analysis

The triplications were performed for each treatment. The significance of difference of total phenolic compounds and antioxidant activity was calculated though a one-way ANOVA procedure. The results obtained from the HPLC analysis were expressed as the mean value ± standard deviation. Duncant´s multiple range tests were used to determine the significant difference among the treatments with the p-values less than 0.05.

3. Results and Discussions

The moisture contents of the dried mulberry leaves were between 12-14% (dry basis), which would not be significantly different among diferent conditions ($p>0.05$). As shown in Fig. 3, the total phenolic contents of mulberry leaves were analyzed by using a high performance liquid chromatography. The standard material used for the experiment was the gallic acid. The concentration rates of gallic standard were varies between 0, 20, 40, 60, 80 and 100 ppm. The retention time of the gallic standard was at about 5 minutes (Fig.2).

Fig. 3 and Fig. 4 show experimental results obtained from the chromatogrames of the gallic acid in mulberry leaves with ethanol concentration of 50% and 80%, respectively. The extracted mulberry leaves were analyzed by a high performance liquid chromatography. The retention time of the gallic standard at about 5 minutes was used as a reference for comparing with the extracted mulberry leaves. The peak areas were used to calculated the total phenolic compounds for the mulberry leaves and had results as shown in Table 1. The ultrasonic extraction by using ethanol concentration at 50% was found that the total phenolic content higher than the control variable ($p\leq0.05$). The total phenolic compounds increase with increasing of ultrasonic extraction time ($p>0.05$). The use ultrasonic extraction could induce the acoustic cavitation and rupture of plant cell and this facilitates the flow of solvent in to plant cell and enhances the desorption from the matrix of solid sample, and thus would enhance the efficiency of extraction based on cavitation phenomenon. The results agreed with the expectations proposed in sugar beet molasses[12]. However, the increase of extraction time may not affect to the total phenolic contents of mulberry leaves ($p>0.05$).

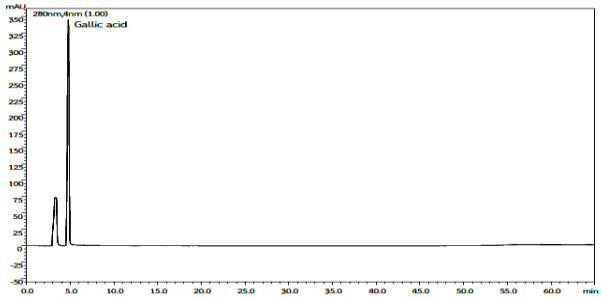

Fig. 2 Experimental result obtaind from chromatogram of gallic acid (Standard) 100 ppm.

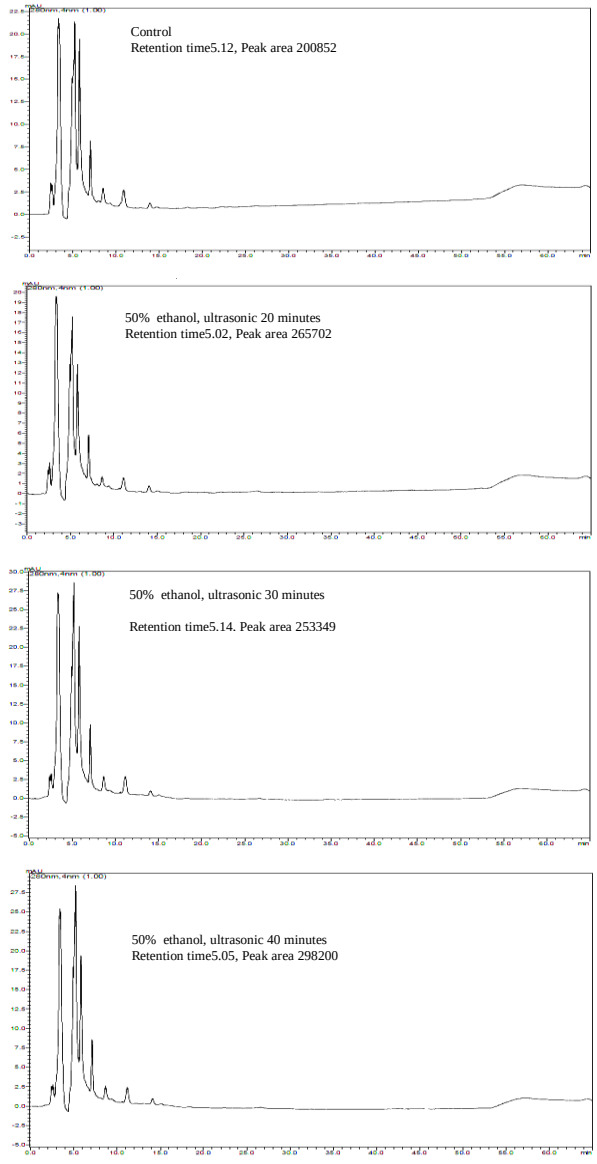

Fig. 3 Experimental result obtaind from chromatogram of gallic acid (Standard) and Ethanol concentration of 50% by used ultrasonic extraction time on total phenolic contents of mulberry leaves.

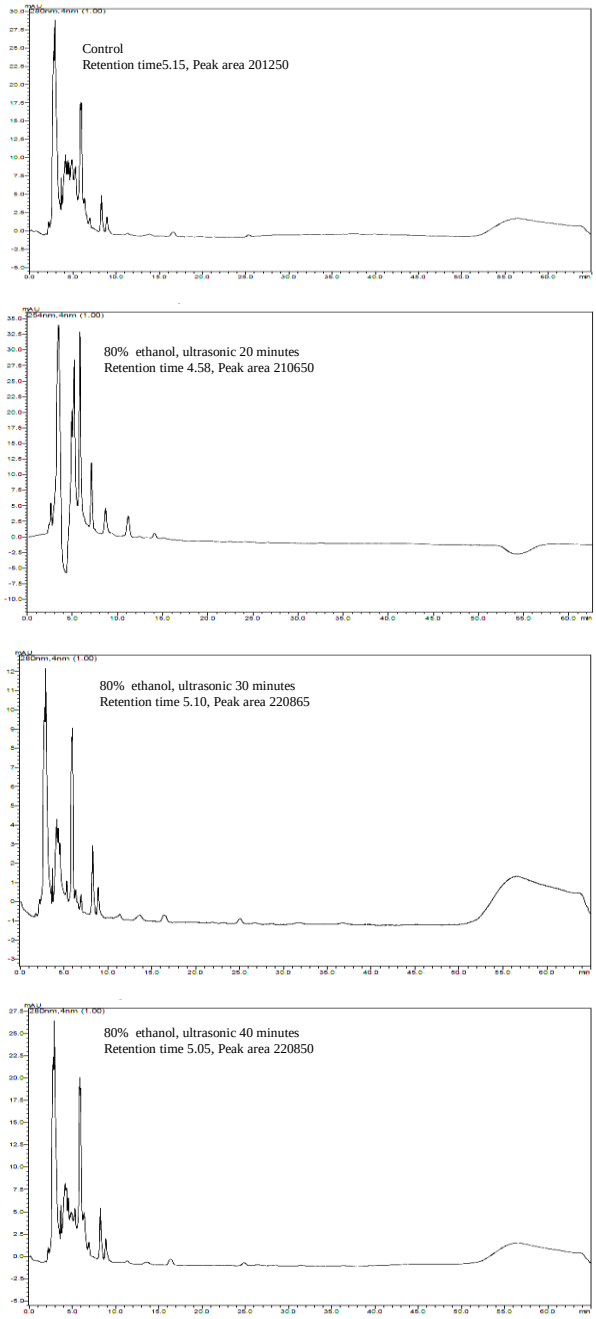

Fig. 4 Experimental Result obtaind from chromatogram of gallic acid (Standard) and Ethanol concentration of 80% by used ultrasonic extraction time on total phenolic content of mulberry leaves

Fig. 3 and Fig. 4 show experimental results obtained from the chromatogrames of the gallic acid in mulberry leaves with ethanol concentration of 50% and 80%, respectively. The extracted mulberry leaves were analyzed by a high performance liquid chromatography. The retention time of the gallic standard at about 5 minutes was used as a reference for comparing with the extracted mulberry leaves. The peak areas were used to calculated the total phenolic compounds for the mulberry leaves and had results as shown in Table 1. The ultrasonic extraction by using ethanol concentration at 50% was found that the total phenolic content higher than the control variable ($p \leq 0.05$). The total phenolic compounds increase with increasing of ultrasonic extraction time ($p > 0.05$). The use ultrasonic extraction could induce the acoustic cavitation and rupture of plant cell and this facilitates the flow of solvent in to plant cell and enhances the desorption from the matrix of solid sample, and thus would enhance the efficiency of extraction based on cavitation phenomenon. The results agreed with the expectations proposed in sugar beet molasses[12]. However, the increase of extraction time may not affect to the total phenolic contents of mulberry leaves ($p > 0.05$).

Condition of extraction	**Total phenolic compounds (mg GAE/100g dry weight)**
Control (shaker extraction)	30.12 ± 0.05^{b}
50% Ethanol, ultrasonic 20 minutes	36.32 ± 3.90^{a}
50% Ethanol, ultrasonic 30 minutes	34.33 ± 0.09^{a}
50% Ethanol, ultrasonic 40 minutes	35.42 ± 0.09^{a}

Table 1 Ethanol concentration at 50% and ultrasonic extraction time on total phenolic content of mulberry leaves.

In Table 2, effects of ethanol concentration of 80% and ultrasonic times on the total phenolic contents were shown. The test results were obtained from the tests with three sets of ultrasonic times (10, 20 and 30 minues). It was found that total phenolic contents will increase with the increasing of ultrasonic extraction time ($p \leq 0.05$). The highest amount of total phenolic content was found when applied ultrasonic time at 40 minutes. Moreover, the total phenolic contents were higher than the control variable ($p \leq 0.05$). The results agreed with the finding [21] who suggested that using ultrasonic cound provide higher total phenolic contents of mulberry leaves compared to the conventional extraction methods.

Condition of extraction	**Total phenolic compounds (mg GAE/100g dry weight)**
Control (Shaker extraction)	27.50 ± 0.08^{b}
80% Ethanol, ultrasonic 20 minutes	27.39 ± 0.05^{b}
80% Ethanol, ultrasonic 30 minutes	29.33 ± 0.25^{ab}
80% Ethanol, ultrasonic 40 minutes	31.41 ± 3.12^{a}

Table 2 Ethanol concentration at 80% and ultrasonic extraction time on total phenolic contents of mulberry leaves.

In this study, the samples were ultrasonic extraction time at 20, 30 and 40 minues. The time of control sample was controlled at the 60 minues. The effect of extraction time on antioxidant activity were shown in Table 3. The highest of antioxidant activity at ultrasonic time at 40 min, then higher than control ($p \leq 0.05$). Moreover, the ultrasonic extraction time was increased from 20 to 40 minues, the increasing of antioxidant activity of mulberry leaves. The ultrasonic-assisted could increase the activity of some enzymes such as pectinase, wich can disintegrate cell wall and membranes and therefore promote the passage of total phenolic contents. Therefore, ultrasonic-assisted technique can provide high antioxidant activity of mulberry leaves.

The effect of ultrasonic time on the antioxidant activity of mulberry leaves under this study was examined with three different ultrasonic times (20, 30 and 40 minues) at 30 °C; with the soaking time of 60 minutes. The experimental results of this test were depicted in Table 4. It can be seen that the highest antioxidant activity was obviously achieved with applied ultrasonic time at 40 minutes. However, when the ultrasonic time was increased from 20 to 40 minutes, the values of antioxidant activity had significant difference. In addition, the antioxidant activity of the samples was higher than one of the control variable ($p \leq 0.05$). As well as research of [22] found that extraction time had effected on antioxidant activity of devils horse whip.

The results shown that 50% ethanol concentration can provide total phenolic contents and antioxidant activity of mulberry leves. Therefore, use 50% ethanol concentration can reduce the cost of the experiment compaired with 80% ethanol concentration.

Condition of extraction	% Redical scavenging
Control(shaker extraction)	24.73 ± 2.34^{c}
50% Ethanol, ultrasonic 20 minutes	26.73 ± 2.56^{b}
50% Ethanol, ultrasonic 30 minutes	26.32 ± 0.34^{b}
50% Ethanol, ultrasonic 40 minutes	43.89 ± 3.96^{a}

Table 3 Ethanol concentration at 50% and ultrasonic extraction time on antioxidant activity of mulberry leaves.

Condition of extraction	% Redical scavenging
Control(shaker extraction)	22.23 ± 2.49^{c}
80% Ethanol, ultrasonic 20 minutes	34.60 ± 1.27^{b}
80% Ethanol, ultrasonic 30 minutes	35.52 ± 0.66^{a}
80% Ethanol, ultrasonic 40 minutes	36.78 ± 0.34^{a}

Table 4 Ethanol concentration at 80% and ultrasonic extraction time on antioxidant activity of mulberry leaves.

4. Conclusions

This research was to investigate effects of using ultrasonic-assisted extraction on bioactive compounds of mulberry leaves in comparison with conventional extraction methods. The experimental results show that the ultrasonic-assisted extraction provides highest total phenolic contents and DPPH scavenging activity of mulberry leaves when compared with the controlled sample galic acid ($p \leq 0.05$). Increasing the extraction time would give result in higher amount of total phenolic contents and DPPH scavenging activity. In addition, the ultrasonic extraction with 50% ethanol concentration for 40 minutes provides the highest total phenolic contents and DPPH scavenging activity.

Acknowledgements

This research was financially supported by the Faculty of Engineering, Mahasarakham University, Thailand**.**

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