International Journal of Agriculture and Biosciences 2025 14(1): 1-10.
https://doi.org/10.47278/journal.ijab/2024.187

RESEARCH ARTICLE

eISSN: 2306-3599; pISSN: 2305-6622

Effect of Ultrasound-assisted Aqueous Two-phase Extraction on Phenolic Compounds from Nymphaea Pubescens Willd. and its Antioxidant and Antimicrobial Properties

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ABSTRACT

This work aims to study the effect of ultrasound-assisted aqueous two-phase extraction (UAATPE) parameters, including temperature (30, 50, and 70°C), time (10, 15, and 20min), and frequency level (12, 24, and 36kHz) on the extract yield and its activity. The experimental results exhibited that total phenolic content (TPC) and total flavonoid content (TFC) had the highest content when using a temperature of 70°C for 20min which was 77.24mgGAE/g and 81.08mgQE/g, respectively. Conversely, DPPH radical scavenging activity decreased with increasing temperature. Moreover, the frequency level of 12kHz could improve the TPC yield which was 95.88mgGAE/g. Thus, to evaluate the best extraction conditions, the Fuzzy Analytical Method (FAM) was performed to obtain the overall performance index of each condition. The TPC, TFC, DPPH radical scavenging activity, and Fe2+chelating activity were used as criteria with the weights of 30, 40, 20, and 10%, respectively. The best extraction condition was an extraction temperature of 70°C for 20min with an ultrasonic frequency of 12kHz, showing the highest overall performance index of 6.774. The extracts also exhibited antimicrobial activity against *Staphylococcus aureus* TISTR 746 (MIC 62.5mgGAE) and *Escherichia coli* TISTR 117 (MIC 1000mgGAE), with both showing MBC as 2000mgGAE/mL. It can be pointed out that UAATPE is an alternative powerful technique for bioactive compound extraction from red water lily.

Keywords: Ultrasound-assisted extraction; Aqueous two-phase extraction; Phenolic compounds; Red water lily; Fuzzy analytical method

Article History
Article # 24-773
Received: 23-Aug-24
Revised: 28-Sep-24
Accepted: 08-Oct-24
Online First: 10-Nov-24

INTRODUCTION

Red water lily (*Nymphaea pubescens* Willd.) belongs to the Nymphaeaceae family. It is an aquatic herb plant with interesting pharmacological properties (Sathasivampillai and Rajamanoharan, 2021). The flower of Nymphaeaceae is distinguished and is cultivar for tourist attraction. It is widely distributed, and can be found in Nongkhai province, Thailand, especially in the winter time. There contained the high number of phenolic compounds with different 74 flavonoids and phenolic acid compounds. In addition, it has been reported that the important substances in the flavonoid, phenolic, and saponin groups were found in Nymphaeaceae flower extracts (Debnath et al., 2013). Narkprasom et al. (2017) studied the effect of microwave assisted extraction condition for bioactive compound from the petal of *Nelumbo nucifera* Gaertn and found that the microwave power of 300watts for 15min using 34% (v/v) ethanol was the optimal condition. This condition yielded the highest total phenolic compounds (134.58±0.20mgGAE/gDW) and showed the DPPH scavenging activity of 24.02%. Furthermore, Pokhrel et al. (2022) found that the extracts of *Nymphaea lotus* L. var. pubescens (Willd.) using an ethanolic fermentation for 3 days showed the highest antioxidant activity and IC50 was in the range of 31.43±0.08 to 14.30±0.43µg/mL. Moreover, Chaisri et al. (2019) studied the effect of petal and pollen water lily (*Nymphaea lotus* L.) extract on antimicrobial activity and found that the ethyl acetate petal extracts and hexane pollen extracts showed the highest inhibition of gastrointestinal bacteria, *E. coli* ATCC 25922, *S. epidermidis* ATCC 35984, *V. cholerae* and *S. aureus* ATCC 29213.

Cite this Article as: Rueangsri K, Lasunon P, Kwantrairat S and Taweejun N, 2025. Effect of ultrasound-assisted aqueous two-phase extraction on phenolic compounds from *Nymphaea pubescens* Willd. and its antioxidant and antimicrobial properties. International Journal of Agriculture and Biosciences 14(1): 1-10. https://doi.org/10.47278/journal.ijab/2024.187

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While, hexane pollen extracts showed the inhibition of *E. coli* ATCC 25922, *S. epidermidis* ATCC 35984 and *V. cholerae*. Therefore, the water lily is one of interesting material which can be found in many areas in Thailand and has a potential for flavonoid compound extraction.

The extraction yield of phenolic compound is influenced by many factors, for example extraction condition including temperature and time, solvent type, material size, and material -solvent ratio (Irakli et al., 2018; Goltz et al., 2018). Conventional solvent extraction such as maceration, soxhlet extraction has some disadvantages for instance low yield, high solvent and energy consumption, long extraction times and high temperatures requirement, which may affect to phenolic compound structure and also its antioxidant activity (Wen et al., 2018; Ozdemir et al., 2024). Therefore, the novel extraction techniques have been studied in order to enlarger the extraction yield, however, lesser the structure degradation. Ultrasound assisted extraction (UAE) is widely used method for bioactive compound extraction from many plants due to the simplicity, high extraction yield and quality with less time and low temperature consumption, less expensive with less energy and solvent demand, and also capability to scale up (Carrera et al., 2012; Khoddami et al., 2013; Kumar et al., 2021). However, extraction yield of phenolic compound is depended on many factors for example temperature, frequency, power, and time, solvent/solid ratio (Golmohamadi et al., 2013; Shaidi, 2022; Zampar et al., 2022; Ozdemir et al., 2024). Consequently, the UAE condition should be optimized for application and efficiency of the technologies.

Aqueous two-phase system (ATPS) is composed of water together with short chain alcohols and inorganic salts. Comparing ATPS with polymer-based ATPSs, inorganic salts ATPS may exhibit lower extraction costs and faster phase separation (Pratiwi et al., 2015; Du et al., 2018; Zhu et al., 2019). Additionally, the ATPS is a technique which combines pre-concentration, partial purification, and separation in a single step, it is frequently used to separate bioactive chemicals (Guo et al., 2015; Iqbal et al., 2016). However, the combination of ATPS with other technique such as microwave-assisted extraction or ultrasound-assisted extraction could improve the extraction efficiency (Xu et al., 2017; Zhao et al., 2021). Xu et al. (2017) studied the ultrasound-assisted and ATPS for phenolic compound extraction conditions from *Aronia melanocarpa* pomace (AMP) and found that the performing using ultrasonic power of 200W for 52min with 0.324g/mL of ammonium sulfate and ethanol-water ratio of 0.69 was the optimum extraction conditions for total phenolic compound and using ultrasonic power of 200W for 50min with 0.320g/mL of ammonium sulfate and ethanol-water ratio of 0.71 was the optimum extraction conditions for total flavonoid content. Wang et al. (2021) studied the effect of ultrasound-assisted with ATPS extraction for polyphenols extraction from olive leaves and found that the optimum extraction condition was 29% (w/w) ammonium sulfate, 35% (w/w) ethanol, pH 6.7, and 45°C with extraction yield of 34.06 mg/g. Moreover, they also reported that this extraction method gave higher radicals scavenging ability and reducing power. Zhu et al. (2022) reported the extraction condition for flavonoids condition from jujube peels using ultrasound-assisted -ATP extraction was a green and clean method by using ATPS composition of 35% (w/w) potassium phosphate dibasic and 20% (w/w) ethanol with ultrasonic power of 200W and solid: liquid ratio 1:30g/mL (w/v) for 50min.

Fuzzy Analytical Method (FAM) is a decision method which using a mathematic theory. It generated the value by transforming the data into a fuzzy scale [0 to 10] which make all data to be the same scale. The fuzzy scale will be integrated to the fuzzy grade matrix by the relative weights and give the overall index of each condition. The index value can be applied for making a judgement. FAM has been used for sensory evaluation and to make decisions about the best conditions (Lasunon and Sengkhamarn, 2016; Lasunon et al., 2021; Lasunon et al., 2022a). Lasunon et al. (2022b) studied the effect of soaking conditions for producing the Quick-Cooking Black Jasmine Rice and applied fuzzy analytical method to judge the best soaking conditions. They exhibited that the soaking with 0.1% baking powder for 30min at room temperature was the best soaking conditions with the highest overall performance index (6.52).

However, so far, no research has been reported the effect of ultrasound-assisted extraction and also using aqueous two-phase system as a solvent for phenolic compound extraction from red water lily. Therefore, the aims of this study were to investigate the effect of ultrasound-assisted and ATPS extraction (UAATPE) parameters on phenolic compound content and also on its bioactive activity. Moreover, the best UAATPE condition was evaluated by FAM. The knowledge from this work will provide a green extraction technique to develop the red water lily extracts with higher content of active ingredients and higher activity.

MATERIALS & METHODS

Materials

The red water lily (Nymphaea pubescens Willd.) from the growing area were collected in Muang District, Nong Khai Province. The red water lily was washed and divided from the petal. The clean petal was steamed for 3min and then dried at 65°C for 24hr. The dried petal of red water lily was mashed to a smaller size and was kept in the aluminum foil bag and desiccator for further analysis.

Conventional Aqueous Two-Phase Extraction (C-ATPE) and Classical Water Extraction (C-W)

For the comparison, C-ATPE and C-W were applied on water lily petal and pollen powder. A sample of 1g of petal red water lily powder and 50mL of prepared ATPS was placed into a beaker and covered with aluminum foil. The extraction was conducted for 2hr at room temperature on a magnetic stirrer. After extraction, the extract was filtered with filter paper. The upper phase was for C-ATPE, which was rich in phenolic compounds. The total volumes were recorded and used for other analysis.

Ultrasound-Assisted-Aqueous Two-Phase Extraction (UAATPE) procedures

To study the effect of extraction temperature and time on bioactive compound, One g of red water lily petal powder and 50mL of prepared ATPS containing 26% of ammonium sulfate and 28% of ethanol according to our previous studied were put into bottle and placed in an ultrasonic processor (Elmasonic S70H, ElmaHans Schmidbauer Gmbh & Co. KG, Germany) with the variation of temperature (30, 50 and 70°C), and time (10, 15 and 20min) at ultrasonic frequency level of 37kHz. The best extraction temperature and extraction time was performed in next experiment.

Consequently, to study the effect of ultrasonic frequency level on bioactive compound, one g of petal red water lily powder and 50mL of prepared ATPS were put into bottle and extract at the best extraction temperature and extraction time with ultrasonic frequency level of 12, 24 and 36kHz using an ultrasonic bath (GT SONIC P6, GuangDong GT Ultrasonic Co.,Ltd, China).

After all ultrasonic treatment, the extracts were cooled at room temperature to rest for at least 30min to form aqueous two-phase systems. The extracts were filtered through filter paper. The volumes of the top phases of the system were recorded and the total phenolic content and total flavonoid content yields were determined. The extracts were stored at 4°C in the refrigerator.

Bioactive Compound Content

The total phenolic content (TPC) and total flavonoid content (TFC) of the upper phase extract was determined according to İşçimen and Hayta (2021) and Lasunon et al. (2022a), respectively, with some modification. The TPC was demonstrated as milligrams of gallic acid equivalent (GAE) per gram of sample (mg GAE/g) while the TFC was demonstrated as milligrams of quercetin acid equivalent (QAE) per gram of sample (mg QE/g).

Antioxidant Activity

The DPPH radical scavenging activity of all of the extracts was measured by DPPH using the modified method according to Jalali-Jivan and Abbasi (2020). The results expressed as DPPH radical scavenging activity (%). The Fe^{2+}chelating activity of the upper phase extract was determined according to İşçimen and Hayta (2021). The results expressed as % for 3mg/mL.

Antibacterial Activity

In the study, theminimum inhibitory concentration (MIC) was determined using the Agar disc diffusion method, corresponding to the approach by Konaté et al. (2012). Both bacterial testing strain, gram-positive bacteria *Staphylococcus aureus* ITSTR 746 and gram-negative bacteria *Escherichia coli* ITSTR 117, were obtained from the Thailand Institute of Scientific and Technological Research (TISTR). Each single colony of testing strain on nutrient, at 37°C for 24hr, were diluted with sterile normal saline to 0.08-0.1 optical density at 600nm or equal turbidity to McFarland No. 0.5 (1.5x10^8 CFU/mL). This solution was then spread on Mueller Hinton Agar (MHA). Plant extracted discs were two-fold serial diluted with aqueous two-phase system (ATPS) from 2000 to 31.125µg/disc. Plant extracted disc were placed on MHA plate after applied with bacterial test. Then, all of plates were incubated at 37°C for 18hr. The antibacterial properties of MIC values were determined from the clear zone of inhibition. An aqueous two-phase system (ATPS), used along extract solution, and 10mg of gentamicin was used as negative and positive control, respectively. All experiments were performed in triplicate.

Theminimum bactericidal concentration (MBC) was determined using the Microdilution techniques, in alignment with Balouiri et al. (2016). In this test, plant extract underwent a two-fold serial dilution ranging from 2000 to 31.125µgGAE/mL in 96 well plate with Mueller Hinton broth (MHB), with total volume of 150mL. Next, 1.5 x 10^8 cell/ml of bacterial suspended were added at 15µL per well. Incubation at 37°C was performed for 18hr. After that, 100mL was spread onto MHA and incubated at 37°C for 18hr. The MBC value was defined as the lowest concentration at which no bacterial growth occurs.

Statistical Analysis

The experimental design was performed by factorials for 2 factors and 3 levels for studying the effect of extraction temperature and time, and by CRD for studying the effects of ultrasonic frequency level. All experiments were done in triplicate and Duncan's New Multiple Range Test at significance level of $P<0.05$ was applied to compare between treatment.

Fuzzy Analytical Method (FAM)

The best ultrasonic-assisted and aqueous two-phase extraction condition was achieved using the overall performance index according to the Lasunon (2016). The phenolic content, the flavonoid content, DPPH radical scavenging activity, and Fe^{2+}-chelating activity were used as a criterion with the weights of 30, 40, 20 and 10%, respectively.

RESULTS & DISCUSSION

Effects of Temperature and Time on Bioactive Compound Content

The total phenolic content (TPC) and total flavonoid content (TFC) of extracts obtaining from different temperature and time extraction were determined and the results are given in Fig. 1. The results showed that TPC and TFC were significantly raised as temperature increased from 30 to 70°C. This probably explained by the high extraction temperature could enhance the solubility of target compound into the solvent. Moreover, the cavitation bubble, which generated during ultrasound assisted extraction (UAE), and its micro-jet on the surface and also a high extraction temperature would strengthen the release of bioactive compound. Moreover, the high extraction temperature would decrease the viscosity of solvent resulting in the higher mass transferring of solute to solvent (Kumar et al., 2021). Our result showed that the extraction temperature of 70°C gave the highest of TPC and TFC which was similar to Dobrincčić et al. (2020) who reported the optimum temperature for phenolic extraction from olive leave was of 60°C and to Irakli et al. (2018) who extracted phenolic compound from olive leave using UAE in the range of 25 to 60°C and reported that the optimum temperature was of 60°C. However, Al-Dhabi et al. (2017) found that the increased in UAE temperature caused the reduction of total phenolic compound from spent coffee grounds and noted that the optimum UAE temperature was of 40°C (Kumar et al., 2021). The extremely high temperature extraction may degrade the phenolic compounds (Khemakhem et al., 2017). Moreover, the higher extraction temperature would decrease the cavitation effect due to the evaporation of solvent resulting in the decrease of the differentiation between pressure inside and outside solvent (Kumar et al., 2021). However, the difference of extraction temperature may be affected by many factors such as solvent type (boiling point of solvent), planet material and also type and composition of phenolic compound in the plant matrix.

Considering the effects of ultrasonic time on bioactive compound content, the result exhibited that the TPC and TFC significantly raised when ultrasonic time increased from 10 to 20min. This can be explained that the prolonged time could improve the extraction efficiency by more rupturing the material cell. However, the longer extraction time may degrade the phenolic compound due to the high temperature, light, or oxygen exposure during extraction (Naczk and Shahidi, 2006). Moreover, the longer extraction time may cause the ethanol evaporation (Wang et al., 2021). From our results, the maximum TPC and TPC was found when the extraction was 20min. This result was similar to Dobrincčić et al. (2020) who reported the optimum time for phenolic extraction from olive leave which was 21min and Wang et al. (2021) who stated extraction time of 25min was an optimum time for UAATPE. However, our result was shorter than the Hannachi et al. (2019) who reported the UAE time was 28.69min for polyphenols extraction from olive leaves and Zhu et al. (2022) who found that the maximum TPC extraction time was found to be 40min using UAATPE from jujube peel. The difference of extraction time was probably due to the polyphenol composition and stability in material.

Effects of Temperature and Time on Antioxidant Capacity

In studies, the effects of temperature and time on antioxidant activity are shown in Fig. 2. The results exhibited that the DPPH Radical Scavenging Activity was significantly decreased as extraction temperature increased from 30 to 70°C. This is probably due to the cavitation

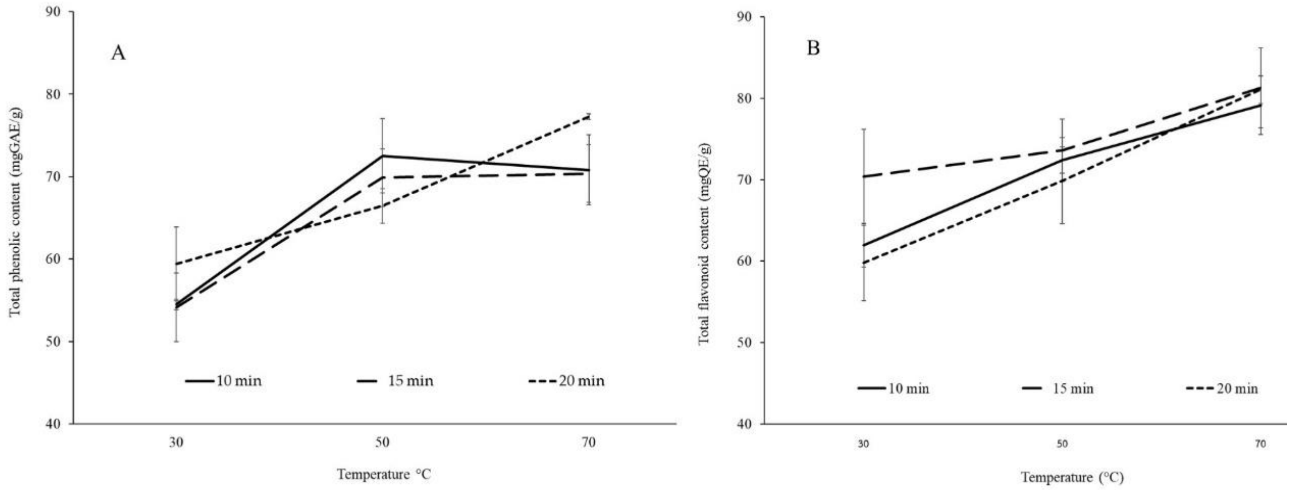

Fig. 1: Total phenolic content (A) and total flavonoid content (B) of the extracts at different temperature and time.

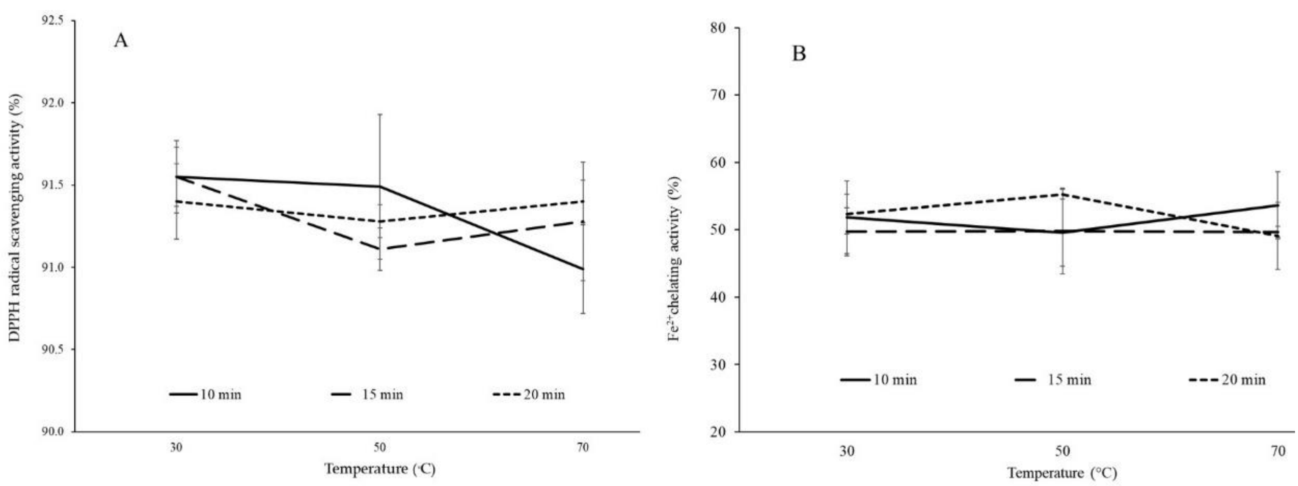

Fig. 2: DPPH radical scavenging activity (A) and Fe^{2+}-chelating activity (B) of the extracts at different temperature and time.

effects during extraction. The cavitation effect was a phenomenon of cavitation bubble which was generated and continually collapsed on the surface of material. These cavitation effects may generate reactive hydroxyl radicals. In addition, the solvent in this study contained high amount of water which may affect to the high reactive hydroxyl radicals and also the decrease of DPPH radical scavenging activity of the extract even though high TPC and TFC was found. Furthermore, the results revealed that the extraction temperature showed more affect on DPPH Radical Scavenging Activity than extraction time. This result was in accordance with the research conducted by Lasunon et al. (2022c) who studied the effect of ultrasonication on carotenoid content in tomato extract and found that at high extraction temperature, the DPPH radical scavenging activity of extract was decreased. Moreover, this was also found in the research conducted by Tomšik et al. (2016) who studied the effect of UAE condition including extraction temperature, time, ultrasonic power and ethanol concentration on bioactive compounds from wild garlic (*Allium ursinum* L.). They found that the antioxidant activity (IC50) of the extracts was positively affected by ultrasonic power and temperature. Moreover, our results were in accordance with the research conducted by Weremfo et al. (2022) who studied the effect of UAE on phenolic compound from Turkey berry fruit and its antioxidant and reported that the temperature has a high positive impact on both DPPH and ABTS scavenging activity. However, no significant difference was found for Fe^{2+}chelating activity values with increasing temperature and longer time extraction. This can be observed that the efficiency of ultrasound-assisted extraction of bioactive compounds has a limit, due to sonication degradation and thermo-degradation or oxidization of phenolic compounds (Pingret et al., 2013; Dranca and Oroian, 2016; Li et al., 2017; Goltz et al., 2018).

Selection of Temperature and Time Extraction

The extraction condition can be affected on both bioactive content of extract and also its bioactive activity. Therefore, the quality and quantity of extract should be concerned in order to gain the suitable extraction condition. The Fuzzy Analytical Method was applied to calculate the overall performance index to determine the best of total phenolic compounds extraction conditions. The criteria for decision were the quantity factor namely phenolic content, the flavonoid content, and also quality factor namely DPPH radical scavenging activity and Fe^{2+}chelating activity. The lowest and highest value was used as lower and upper bounds, respectively, for converting the results into a fuzzy score from 0 to 1. The weighing value has been decided to be 30, 40, 20 and 10 for the TPC, TFC, DPPH radical scavenging activity, and Fe^{2+}chelating activity, respectively. The overall performance index was calculated by an online program developed by Lasunon (2016) and the results were shown in Table 1. The results demonstrated that the condition of 70°C for 20min gave the highest overall performance index (7.450). Therefore, an extraction temperature of 70°C for 20min was selected as the optimal extraction condition for the following experiments. This condition was similar to the research reported by Weremfo et al. (2022), who found the optimum UAE temperature and time for phenolic compound extraction from Turkey berry fruit, which was 80°C for 17.3min. This condition gave the highest TPC, TFC, DPPH and ABTS scavenging activity. Moreover, our results were in accordance with the research conducted by Dobrincčić et al. (2020) who reported the optimum UAE temperature and time for polyphenol extraction from olive leaves polyphenols was 60°C for 21min.

Table 1: The overall performance index from each temperature and time condition.

Extraction condition		Overall performance index
Temperature (°C)	Time (min)	
30	10	3.313
	15	3.957
	20	3.114
50	10	5.945
	15	5.252
	20	5.315
70	10	6.178
	15	6.573
	20	7.450

Effects of Ultrasonic Frequency Level on Bioactive Compound Content and its Activity

The effects of ultrasonic frequency on bioactive compound content are shown in Fig. 3 which exhibited that TPC yield significantly decreased as ultrasonic frequency increased. Nevertheless, no significant difference was found for TFC yield values with increasing ultrasonic frequency. The high frequency of ultrasonic may reduce oscillation cycle of cavitation bubbles resulting in small bubble generated. This effect affects the pressure generated by the ultrasound waves, resulting in lower power and cavitation effects (Zhang et al. 2021). The results are consistent with Cravotto et al. (2008) who studied the effect of ultrasonic frequency on vegetable oil from soyabean germ and found that the low frequency gave the high oil yield, and the best ultrasonic frequency was 19kHz which gave the best yield. Furthermore, these results are consistent with Bin Mokaizh et al., (2024) who studied the effect of ultrasonic frequency (10-50kHz) and noted that the optimum ultrasonic frequency was 20kHz resulting in the highest yields of TPC and TFC form *C. gileadensis* leaves. On the other hand, our result was found to be lower than the research conducted by Liu et al. (2014) who found that ultrasonic frequency 45kHz gave higher flavonoid yield values from *Adinandra nitida* leaves compared to 80 and 100kHz. However, the suitable of ultrasonic frequency was depended on the type of material (Shen et al., 2023).

The results of the effects of ultrasonic frequency on antioxidant activity are given in Fig. 3. As the frequency of the ultrasonic increased, the antioxidant activity dropped for Fe^{2+}chelating activity, however, no significant difference was found for DPPH scavenging activity values. This probably explained by the effects of high ultrasound power. The ultrasound wave caused unstable bubbles forming and rapid cavitation. In aqueous solutions, these unstable bubbles rupture and release hydrogen atoms (H) and hydroxyl radicals (OH*), which are probably going to be involved in the breakdown of polyphenols, polymerization, and solvent and solute decomposition (Masuda et al., 2015; Fernando and Soysa, 2015; Dzah et al., 2020). Dzah et al. (2020) stated that the high frequency ultrasound may built the high amounts of free radicals causing the degradation of polyphenols and reducing their biological activity. The results were consistent with Altemimi et al. (2016) who reported the highest scavenging rate of phenolic free radicals and DPPH in pumpkin and peach extracts were obtained by using a frequency of 37kHz, and there was a significant statistical difference between 37 and 80kHz.

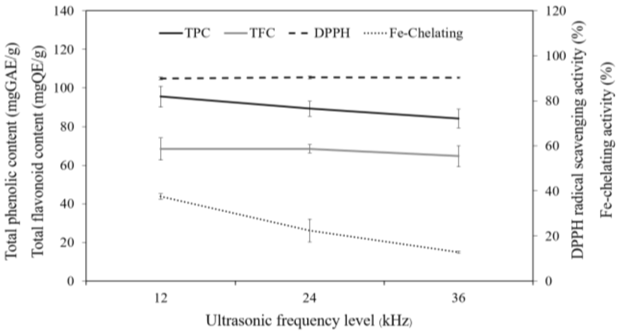

Fig. 3: Bioactive compound content (Total phenolic and total flavonoid content) and antioxidant activity at different ultrasonic frequency.

Decision of Suitable Conditions for Extraction

The overall performance index of each condition was calculate using 4 parameters with the weights of 30, 40, 20, and 10% for TPC, TFC, DPPH radical scavenging activity, and Fe^{2+}-chelating activity respectively, as shown in Table 2.

Table 2: The overall performance index from each ultrasonic frequency condition.

Ultrasonic frequency level (kHz)	Overall performance index
12	6.774
24	5.058
36	2.968

The results showed that the ultrasonic frequency of 12kHz gave an overall performance index of 6.774, which was higher than the ultrasonic frequencies of 24 and 36kHz. Thus, the best condition for extraction condition for phenolic compounds from red water lily petal was the temperature of 70°C for 20min with an ultrasonic frequency of 12kHz. This condition would give a high quality and quantity of extract. This condition was similar to UAE condition for phenolic compound from *C. gileadensis* leaf which was at the ultrasonic frequency of 20kHz for 15min with solvent: sample ratio of 1:20g/mL, and 40% (v/v) ethanol (Bin Mokaizh et al., 2024). However, our condition was different from the UAE condition for phenolics compound from the jaboticaba peels which was the extraction time of 20min at 25kHz (Fernandes et al., 2020) which gave highest yield of anthocyanins and phenolics. The difference of UAE condition was probably due to many factors such as the type of material and the solubility of the bioactive substance in the solution, the type of ultrasonic device with different sizes or wave energies, and also the type of solvent for bioactive compound extraction.

Comparison with Conventional ATPE (C-ATPE) and Classical Water Extraction (C-W)

The conventional ATPE (C-ATPE) and classical water extraction for phenolic compound extraction from red water lily petal was compared. The TPC and TFC content in extracts are shown in Table 3. It was found that at the same extraction condition including temperature and time, the TPC and TFC of C-ATPE was higher than C-W. This result was consistent with the research reported by İşçimen and Hayta (2021) who compared ATPE and water extraction for phenolic compound from green lentils, chickpeas, and dry beans. This can be pointed that ATPE was a high efficiency solvent for phenol compound extraction from petal of red water lily.

Table 3: The total phenolic and flavonoid content of the extracts at different extraction method from petal and pollen of water lily.

Extraction method	Total phenolic content (mgGAE/g)	Total flavonoid content (mgQE/g)
C-ATPE	58.03±1.38	41.37±1.14
C-W	7.52±0.45	7.37±0.27
UAATPE	77.24±0.35	81.08±1.72

Moreover, many research has been reported that UAE is an efficiency technique which can improve the bioactive yield. Therefore, the C-ATPE and UAATPE for phenolic compound extraction from red water lily petal was compared and the results as shown in Table 3. The results showed that the TPC and TFC in UAATPE extract was higher than C-ATPE extracts. This result could point that UAE can improve the phenolic and flavonoid compound yield from red water lily petal.

Antibacterial Activity

In a study examining the antibacterial activity through agar disc diffusion technique, it was observed that ATPS extracts from petals had the inhibitory effects on both *S. aureus* TISTR 746 and *E. coli* TISTR 117, as shown in Fig. 4. The triplicate disc, each containing 2000µgGAE/disc of extract, were subjected to agar diffusion testing against *S. aureus* TISTR 746 and *E. coli* TISTR 117. The results showed clear inhibition zones compared to those observed with ATPS and 10µg/disc of gentamicin, which served as the negative and positive controls, respectively. Moreover, it was found that the antimicrobial activity of extracts was higher against *S. aureus* TISTR 746 than *E. coli* TISTR 117. According to the size of clear zone at the concentration of 2000 µgGAE/ml, the size of clear zone against *S. aureus* TISTR 746 was 1.417cm which was bigger than size of clear zone against *E. coli* TISTR 117 which was about 1.063cm. This can be point that the extracts have a stronger inhibitory effect on Gram-positive pathogenic bacteria (*S. aureus*) than on Gram-negative pathogenic bacteria (*E. coli*). Chaisri et al. (2019) reported that ethyl acetate petal extracts and hexane pollen extracts showed high antibacterial activity.

The results of agar disc diffusion test revealed that the MIC value for *S. aureus* TISTR 746 was determined to be 62.5µgGAE, while MIC for *E. coli* TISTR 117 was found to be 1000µgGAE. The percentage of inhibition compared to the positive control was 8.26 and 9.63%, respectively, as shown in Fig. 5. The results of the microdilution technique reveal

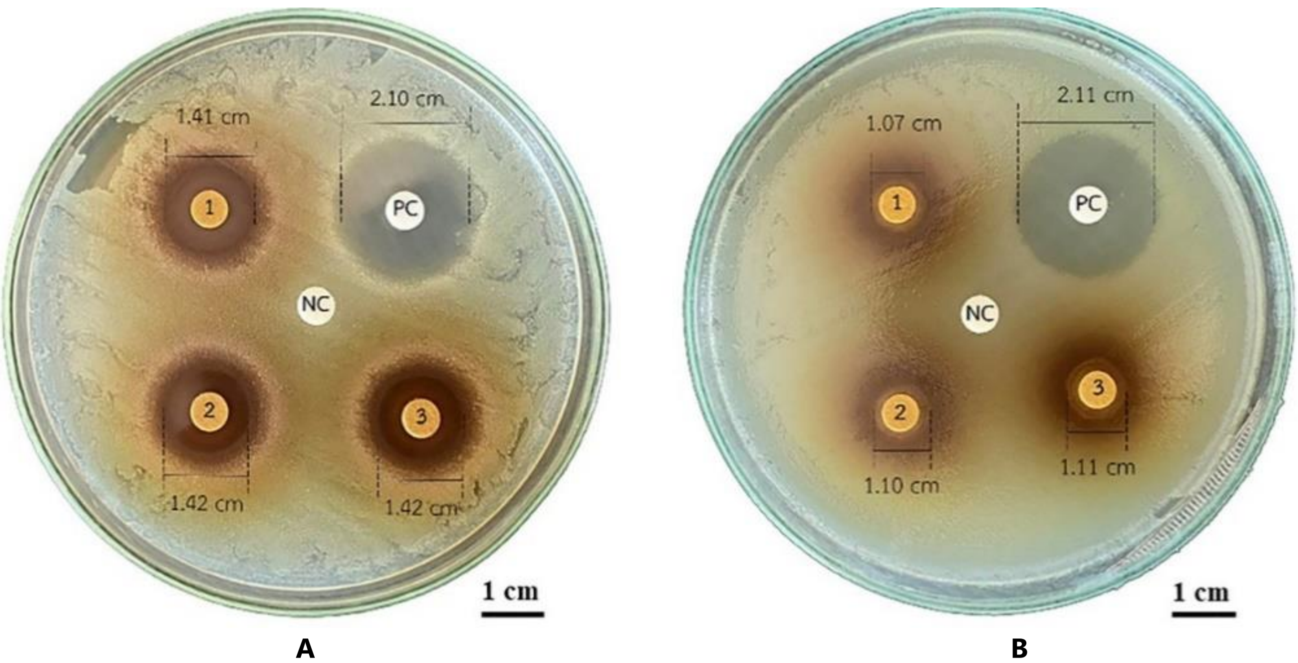

Fig. 4: Shows the inhibition clear zone of triplicate discs containing 2000mgGAE/disc of petal red water lily extracts using the agar disc diffusion technique. A) against *S. aureus* TISTR 746 and B) *E. coli* TISTR 117. Positive control (PC)=10µg/disc from gentamicin, Negative control (NC)=ATPS solvent (250µL, equivalent to the sample volume per disc).

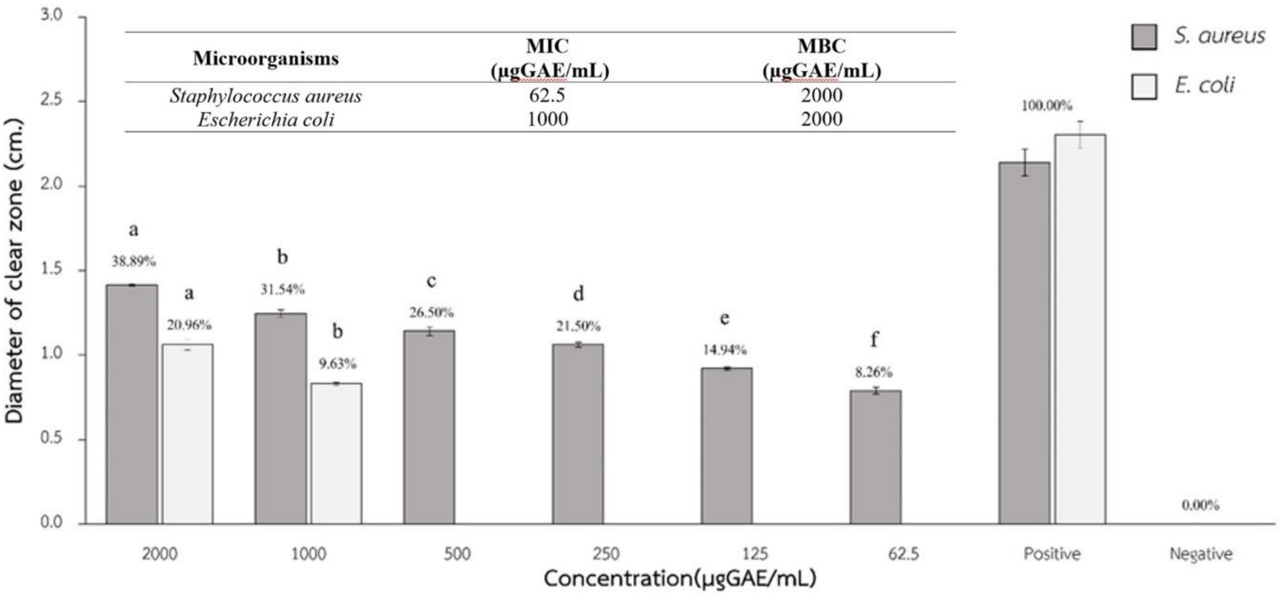

Fig. 5: Shows the diameter of inhibition zone for 2-fold serial dilution range of petal red water lily extracts, from 2000 to 62.3µgGAE/mL, using the agar disc diffusion technique. The percentage values above each graph indicate the inhibition percentage compared to 10µg/disc of gentamicin. The letter above each graph represents significant difference within group at a 95% confidence level. (Different letter in each bar shows the significant ($P<0.05$) difference.

that the MBC value of the extracts for both *S. aureus* TISTR 746 and *E. coli* TISTR 117 are 2000µgGAE/mL, as shown Fig. 5. In accordance with Vamanu et al. (2011), the ethanol extract of *Cynara scolymus* demonstrated MIC values for *E. coli*, *B. cereus* and *S. aureus* were 15, 5, and 5mg/mL, respectively. The petal red water lily extract exhibited a stronger inhibitory effect on Gram-positive bacteria, *S. aureus* TISTR 746, compared to Gram-negative bacteria, *E. coli* TISTR 117. This difference may be caused by their dissimilar cell wall compositions (Salman et al., 2022; Gündüz et al., 2023). The higher antimicrobial activity against *S. aureus* than *E. coli* was also found in many extracts. Chaisri et al. (2019) studied the antimicrobial of water lily extract from petal and found that the ethyl acetate petal extracts showed the better effect to inhibit gastrointestinal bacteria, *S. aureus* ATCC 29213 than *E. coli* ATCC 25922. Sruthi et al. (2023) found that the phenolic fractions of cashew nut showed higher antimicrobial activity against *S. aureus* than *E. coli*. Moreover, Salman et al. (2022) investigated the antimicrobial activity of tea extracts against five pathogenic bacteria and found no antimicrobial activity against *E. coli*. Chen et al. (2024)

stated that the antimicrobial activity mechanism of polyphenols can be categorized into three main types: 1) altering the function of the cytoplasmic membrane; 2) interfering with intracellular processes; and 3) causing cell death akin to apoptosis. The tolerance of Gram-negative bacteria on phenolic was probably due to the lipopolysaccharides of its membrane which act as barrier (Sharma et al., 2012; Reygaert, 2018).

Conclusion

Ultrasound-assisted aqueous two-phase extraction (UAATPE) condition was developed for phenolic compound extraction from red water lily petal. The results evidence that the temperature, time, and frequency of extraction influenced the bioactive compound content as well as its activity. The yield of TPC and TFC increased with increasing temperature and time. However, temperature and time may have opposite effects on antioxidant activity (DPPH and Fe^{2+}chelating activity). In addition, higher frequency levels can cause more destruction of substances than lower frequency levels. The determination of the best extraction conditions was evaluated using FAM. The overall performance index showed that the most suitable extraction condition extraction condition was an extraction temperature of 70°C for 20min with an ultrasonic frequency of 12kHz. Furthermore, this extract also could inhibit the growth of microorganisms for against *S. aureus* TISTR 746 and *E. coli* TISTR 117. UAATPE can be an efficient technique for bioactive compound extraction from plants. The knowledge gained from this study should be useful for future utilization and application.

Authors' Contribution: Khajornyod Rueangsri: Investigation, Methodology, Data curation, Writing - Original Draft. Sutthiluck Kwantrairat: Investigation, Methodology, Data curation, Writing - Original Draft. Patareeya Lasunon: Investigation, Methodology, Data curation, Writing - Original Draft. Nipaporn Taweejun: Supervision, Investigation, Conceptualization, Validation, Writing - Review & Editing. The final manuscript has been approved by all listed authors.

Conflicts of Interest: The authors declare no conflict of interest.

Acknowledgment: The researchers gratefully thank the National Research Council of Thailand (NRCT) through the Research Team Promotion Grant/Senior Research Scholar (Grant No. N42A650552) and to the Faculty of Interdisciplinary Studies for supporting instruments.

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