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Research article

Comparison of Different Extraction Methods for Bioactive Compounds from Kao-Kum Doi-Saket (*Oryza sativa* L.)

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Abstract: Kao-Kum Doi-Saket is one type of purple rice known for its high content of anthocyanin and its ability to resist free radicals. Anthocyanin extracts can be utilized in various applications, including food coloring, nutritional supplements, natural medicine, and cosmetics. There are several methods for extracting anthocyanin from Kao-Kum Doi-Saket. This research aims to compare the effects of extraction methods, including Conventional extraction (CE), Pulse electric field extraction (PEF), and Ultrasonic assisted extraction (UAE) on total anthocyanin content and antioxidant activity in Kao-Kum Doi-Saket. It was found that the UAE method resulted in the highest total anthocyanin content of 57.05 ± 4.27 mg/L, which was significantly different from the other two methods ($p<0.05$). Kao-Kum Doi-Saket extracts from the UAE method also showed the highest DPPH inhibition (88.32 ± 1.83%) compared to the CE and PEF methods. Therefore, these findings suggest that UAE is the most effective method for extracting anthocyanin and antioxidant activity from Kao-Kum Doi-Saket and could be beneficial for developing processed products from Kao-Kum Doi-Saket in the future.

Keywords: Purple rice; Pulse electric field; anthocyanin; antioxidant activity

1. Introduction

Kao-Kum (*Oryza sativa* L.) is commonly grown in Thailand, particularly in the northern region of Thailand. Kao-Kum Doi-Saket is the name of planting area of this rice since it is popularly cultivated in Doi Saket District, Chiang Mai Province. Kao-Kum Doi-Saket is red to dark purple due to anthocyanin pigments in the rice grain. Kao-Kum is, therefore, called purple rice. Base on the study of Leelawat et al. [1] found that Kao-Kum Doi-Saket contains cyanidin-3-glucoside and peonidin-3-glucoside and Zhang et al. [2] also found that anthocyanin in purple rice has antioxidant properties.

In general, anthocyanin compounds can be extracted using water as a conventional method (CE). However, this method has limitations regarding low anthocyanin yield and lengthy extraction time [3]. Therefore, there have been developments in extraction techniques that consider the stability of anthocyanins under heat, light, and pH conditions [4]. The current popular

extraction techniques include Pulse electric field extraction (PEF), Ultrasonic-assisted extraction (UAE), and Microwave Extraction (ME) [5]. Apart from the extraction methods, the choice of solvent used for extraction is also a factor to be considered. Solvents for anthocyanin extraction include methanol, ethanol, hydrochloric acid, and water. Water-based extraction methods are currently preferred due to the potential health and environmental impacts of other solvents on consumers [6].

Using new techniques for extracting bioactive compounds from plant promotes environmentally friendly extraction methods, known as "Green extraction," following the plan of the United Nations for 2030. These techniques are based on six principles as follows: (1) Sourcing diverse raw materials that can be reused; (2) Reducing the use of organic solvents for extraction by utilizing alternative solvents; (3) Minimizing energy consumption during the extraction process; (4) Achieving efficient and high-value yields (5) Decreasing extraction processes and enhancing safety (6) Focusing on environmentally friendly and toxin-free extraction methods [7].

The PEF method is widely used for extracting anthocyanins from various plant sources because it is an un-thermal extraction process in which technology helps reduce environmental impacts and amounts of solvent and enhances the energy efficiency of the processes [8]. This method utilizes electroporation, which applies electrical pulses to the plant cell walls. The pore from electric pules on the plant cell wall allows extraction even at room temperature (Figure 1). PEF extraction has gained popularity for anthocyanin extraction because it can effectively overcome the limitations associated with the heat sensitivity of anthocyanins [9]. A study by Drosou et al. [10] found that PEF extraction significantly increased the quantity of anthocyanins in grape wine compared to conventional extraction methods.

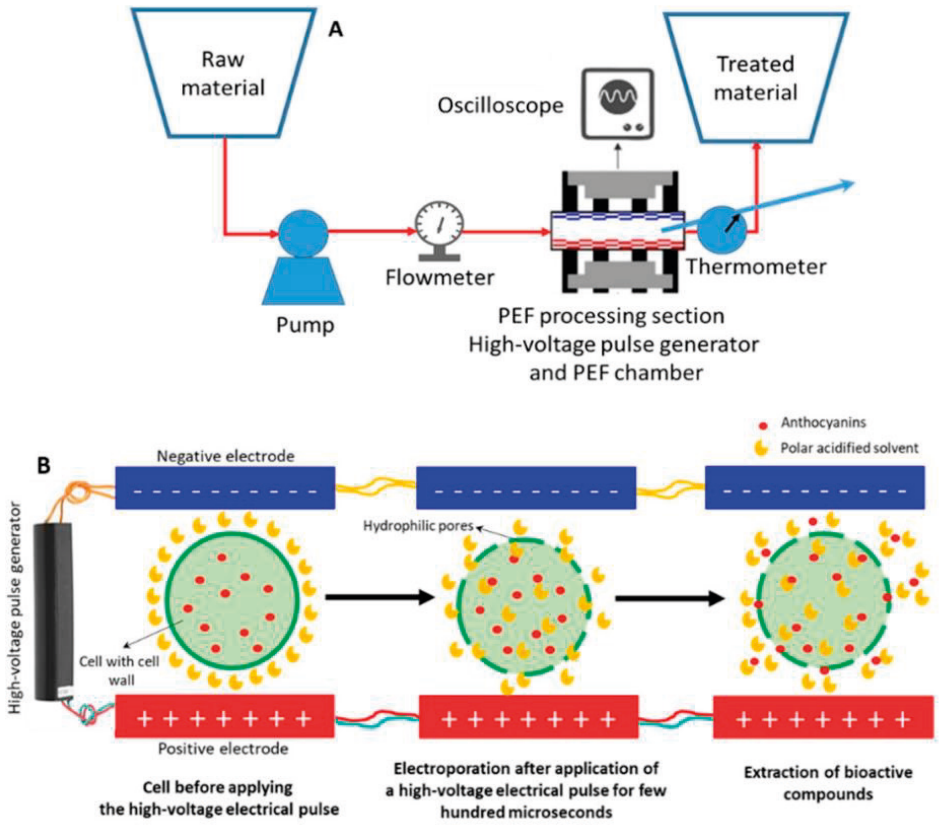

Figure 1. The diagram and general principles of the pulsed electric field extraction process [11].

The UAE method is another popular extraction technique used nowadays. It is a novel, clean, and safe method for extracting biomaterial molecules such as polysaccharides, essential oils, proteins, and pigments

[12]. The UAE principle involves applying ultrasound waves that induce vibration, resulting in a cavitation phenomenon. The cavitation phenomenon leads to the formation of air bubbles within the liquid, and when these bubbles collapse, they cause cell wall disruption in plants [13] (Figure 2). This extraction technique has proven highly efficient, cost-effective, and environmentally friendly. It is suitable for extracting bioactive compounds from plants, including anthocyanins, phenolic compounds, rutin, quercetin, and others [14-16].

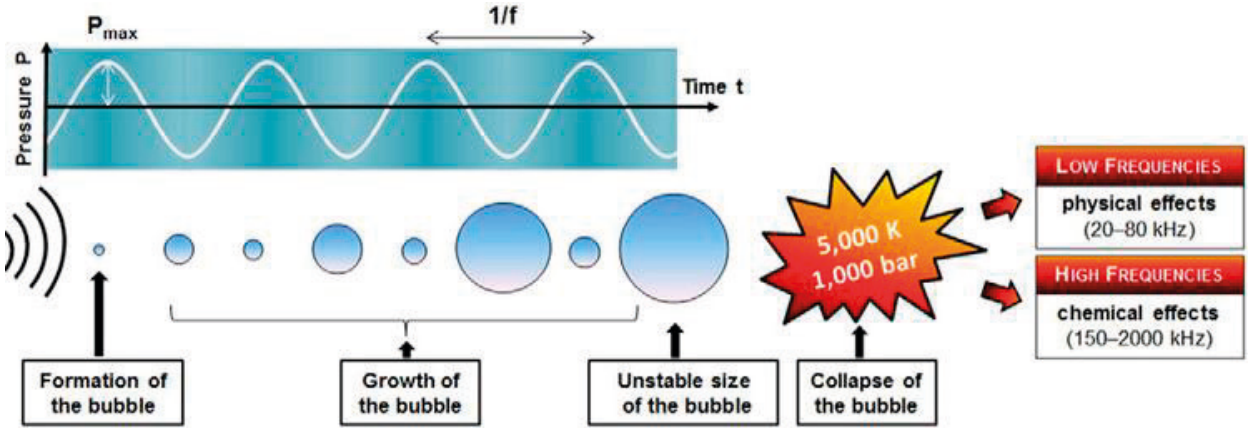

Figure 2. The cavitation phenomenon in the Ultrasonic process [17].

Based on the aforementioned nutritional properties of Kao-Kum Doi-Saket with high nutritional value, there has been an interest in studying extraction methods to utilize Kao-Kum Doi-Saket to produce health products. No suitable extraction technique can preserve essential compounds for this particular Kao-Kum strain. Therefore, this research aims to compare the extraction techniques of Kao-Kum Doi-Saket in terms of CE, PEF, and UAE methods for their ability to extract anthocyanins and antioxidant properties.

2. Materials and Methods

2.1 Materials

The local farmers from Doi Saket Chiangmai, Thailand, supplied the purple rice (*Oryza sativa* L.) or Kao-Kum Doi-Saket. The harvesting period was from November to December, vacuum-sealed using air-vacuum plastic bags to prevent moisture and stored at room temperature in a controlled humidity environment. The chemicals were purchased from Northern Chemical Co., Ltd. and Union Science Co., Ltd. All are analytical grade.

2.2 Extraction method

2.2.1. Conventional extraction method

CE method of Kao-Kum Doi-Saket was performed by soaking Kao-Kum Doi-Saket in distilled water with a ratio of 1 kg of rice to 2 L of water and left overnight for 24 hours at room temperature without stirring. The extracts were filtered using a white cloth, and the samples were stored in an amber glass vial for further analysis.

2.2.2 Pulse electric field extraction method

PEF method was used following the technique of Rajchasom et al. [18]. The extraction ratio was 1 Kg of rice to 2 L of water. The electric field level was generated at 6 kV/cm and the number of pulses was varied at 1,000, 3,000, 4,000, and 5,000 pulses (each pulse lasting 0.078 seconds). The extracts were filtered using a white cloth, and the samples were stored in amber glass vials for further analysis.

2.2.3 Ultrasonic assisted extraction method

The extraction method of Kao-Kum Doi-Saket using the UAE method was adopted from the study conducted by Rajchasom et al. [19]. Kao-Kum Doi-Saket samples were extracted using an ultrasonic device

(Model GT Sonic-D6, China) with a fixed frequency of 40 kHz and electrical power of 150 W at a ratio of 1 kg of rice to 2 L of water. The extraction process was conducted at 30, 50, and 70 °C for 60 minutes (UAE1, UAE2, and UAE3, respectively). The extracts were filtered using a white cloth, and the samples were stored in amber glass vials for further analysis.

2.3 Chemical analysis of Kao-Kum Doi-Saket extraction

2.3.1 Determination of total anthocyanin content

The total anthocyanin content of Kao-Kum Doi-Saket extracts was analyzed using the pH differential method. A sample of Kao-Kum Doi-Saket extract (1 mL) was diluted with distilled water to a volume of 10 mL. Then, the solution was shaken using a centrifuge device (Hettich zentrifugen, EBA 20) at 6,000 rpm for 8 minutes. Afterward, 3 mL of the supernatant solution was pipetted and mixed with buffer solutions, including potassium chloride pH 1 and sodium acetate pH 4.5, to a final volume of 30 mL. Afterward, the sample was left to incubate for 30 minutes in the darkroom conditions and at ambient temperature. Each mixture sample's light absorption (A) was measured using a UV-Vis spectrophotometer at 510 and 700 nm (Spectrum Instruments, SP-UV 200 spectrophotometer). Equation (1) was used to calculate the total anthocyanin content [20] .

$$\text{Total anthocyanin content (mg/L)} = \frac{A_{diff} \times M_w \times df \times 1000}{\varepsilon} \quad (1)$$

Where A_{diff} represents the absorbance value (510 nm -700 nm) pH1 - (510 nm -700 nm) pH4.5, M_w is the molecular weight of Cyanidin-3-glucoside (449.2 g/mol), ε is the molar absorptivity (26,900 $M^{-1}cm^{-1}$), df represents the dilution factor, which is 10.

2.3.2 Determination of antioxidant activity

The antioxidant activity of Kao-Kum Doi-Saket extracts was analyzed using the DPPH radical scavenging assay. A sample of Kao-Kum Doi-Saket extract volume of 200 µL was taken and mixed with 1.8 mL of methanol. Then, 2 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution with a concentration of 0.12 mM was added to the sample. For the control sample, 2 mL of methanol and 2 mL of DPPH solution were added. The blank sample was prepared using 4 mL of methanol [21]. The antioxidant activity was then calculated using the following Equation (2).

$$\text{\% DPPH inhibition} = \frac{(A517_{control} - A517_{sample}) \times 100}{A517_{control}} \quad (2)$$

Where $A517_{control}$ represents the absorbance value at 517 nm of the control sample (Control DPPH), $A517_{sample}$ represents the absorbance value at the nanometer of the experimental sample.

2.4 Statistical analysis

Statistical analysis was performed on the experimental data using MINITAB statistic software version 20. The data was analyzed by calculating the mean ± standard deviation (mean ± SD). The data was further compared using Analysis of Variance (ANOVA) and the mean values were compared using Duncan's test at a confidence level of 95%. The entire experiment was conducted in triplicate.

3. Results and Discussion

3.1 Effect of various extraction methods on total anthocyanin content of Kao-Kum Doi-Saket extract

The study was conducted to compare the effects of different extraction methods,, CE, PEF, and UAE methods, on the total anthocyanin content of Kao-Kum Doi-Saket extract. The results of the total anthocyanin content of the extracted solutions conducted using various extraction techniques are shown in Table 1.

Table 1. Total anthocyanin content (mg/L) and % DPPH inhibition of Kao-Kum Doi-Saket extracts using various extraction methods.

Extraction methods	Conditions	Total anthocyanin content (mg/L)	%DPPH inhibition
PEF1	6 kV/cm, 1,000 pulses	16.09 ± 0.77^{f}	67.74 ± 1.88^{b}
PEF2	6 kV/cm, 3,000 pulses	24.99 ± 0.92^{e}	60.97 ± 0.64^{bc}
PEF3	6 kV/cm, 4,000 pulses	27.66 ± 1.09^{de}	43.50 ± 1.26^{de}
PEF4	6 kV/cm, 5,000 pulses	32.84 ± 1.84^{cd}	33.24 ± 0.43^{e}
UAE1	30°C, 60 min	38.02 ± 1.21^{c}	83.82 ± 9.22^{a}
UAE2	50°C, 60 min	57.05 ± 4.27^{a}	88.32 ± 1.83^{a}
UAE3	70°C, 60 min	47.65 ± 1.90^{b}	83.20 ± 3.67^{a}
CE	24 hr.	9.30 ± 1.11^{g}	51.27 ± 8.60^{cd}

Note: Values are the means of three replicates experimental (mean ± SD). Different superscript letters indicate statistically significant differences at $p \leq 0.05$ for values within the same column**.**

It was found from Table 1 that the extraction using the UAE method yielded a higher total anthocyanin content of the extracts compared to the PEF and CE methods, and the UAE and PEF produced 6 and 2 times anthocyanin contents in the extracts higher than the CE method. The UAE2 (50°C, 60 min) yielded the significantly highest anthocyanin content of the extracts (57.05±4.27 mg/L), and there was a significant difference ($p<0.05$) among them. This was because the low temperature of the UAE1 condition (30°C, 60 min) did not effectively promote extraction efficiency, and the high temperatures of the UAE3 condition (70°C, 60 min) caused the breakdown of phytochemicals. Similar to the results of He et al. [22] and Figueiredo et al. [23] suggested that anthocyanin could be oxidized at exceeding 50°C of extraction temperature. On the other hand, UAE1 (30°C, 60 min) and UAE3 (70°C, 60 min) produced lower anthocyanin content in the extracts (38.02±1.21 and 47.65±1.90 mg/L, respectively) because the exceeded extraction temperature had a damaging effect on the total anthocyanin content of Kao-Kum Doi-Saket extracts [24] and at low extraction temperature with short period of extraction time could not provoke the releasing of total anthocyanin content from Kao-Kum Doi-Saket extracts [25]. The total anthocyanin content of Kao-Kum Doi-Saket extracts using the PEF method increased with an increasing number of pulses, with values of 16.09±0.77, 24.99±0.92, 27.66±1.09 and 32.84±1.84 mg/L for 1,000, 3,000, 4,000 and 5,000 pulses, respectively [18]. However, these values of anthocyanin extracted using the PEF method were lower than those of the UAE method. The temperature of the PEF method in this study was between 50 - 60°C, similar to that of the UAE method. It can be assumed that the temperature of the process was not the main effect on the anthocyanin content when compared between these 2 methods. Still, the extraction mechanism may have more influence. On the other hand, the CE method provided the lowest total anthocyanin content of the extracts (9.30±1.11 mg/L) when compared with the other 2 methods. The reason for this is that the PEF and UAE methods can disrupt the cell walls, which promote the release of anthocyanin pigment by transferring it from the moisture mass within the cellular structure of the sample. As a result, the dissolution of phytochemical compounds in the extract of Kao-Kum Doi-Saket is enhanced without affecting its essential components. Similarly, Zhou et al. [26] investigated the extraction of anthocyanins from the remaining extracts of blueberries using PEF and UAE methods. They found that PEF and UAE methods were more effective in extracting anthocyanins than the CE method. Likewise, Manzoor et al. [27] studied the extraction of anthocyanins from almond seeds (*Prunus dulcis*) using PEF and UAE methods. They found that PEF and UAE methods yielded higher anthocyanin content in the extracts. The UAE yielded the highest total anthocyanin content, 6.14 times greater than the CE method. The result aligns with the findings reported by Lertkaeo et al. [28], who conducted a comparative study on the extraction of anthocyanins from Hom Mali black glutinous rice using CE and UAE methods. The study found that the UAE method resulted in a significantly higher amount of anthocyanins than the CE method, with an increase of 15.49%. Moreover, from the results of this study on the Kao-Kum Doi-Saket extraction, another reason can explain the experimental results that the UAE extraction method resulted in higher anthocyanin content than the PEF method was because a high concentration of anthocyanin compounds was found on the surface of the Kao-

Kum Doi-Saket rice grain. The UAE method utilizes sound waves, which create alternating high-pressure and low-pressure cycles within the sample. This phenomenon, known as cavitation, leads to tiny bubbles' formation and rapid collapse. The collapse of these bubbles generates intense local pressures and temperatures, disrupting cell walls or breaking molecular bonds, facilitating the release of desired compounds [13-16, 29]. Whereas the PEF method involved the use of electric current, caused structural changes in the cell membranes, and induced severe cracking in the cell walls of the Kao-Kum Doi-Saket. The electric field disrupts the cell membranes, creating temporary pores or openings, causing the pigment to be extracted and more starch extracted compared to the UAE method [9-10]. Regarding energy and cost consumption, it was found that the UAE method can reduce extraction time, energy, and operating costs than the PEF method, which was suggested to be more suitable for community enterprises. Furthermore, it was found that the total anthocyanin content of Kao-Kum Doi-Saket or purple rice was higher than other rice species. Das et al. [30] extracted the anthocyanin content of purple and black rice using the UAE method of 30.40 and 35.56 mg C3G/L, respectively. Likewise, the report from Yamuangmorn and Prom-u-Thai [31] found that Kao-Kum Doi-Saket had total anthocyanin content higher than KJ CMU 107, Mamihunger, Kao Hom Nill, Kao Malinil Surin and (10, 3.5, 2 and 1.5 times, respectively).

3.2 Effect of various extraction methods on antioxidant activity of Kao-Kum Doi-Saket extract

Not only was the anthocyanin content of the extracts from Kao-Kum Doi-Saket, but antioxidant activity was also evaluated to compare the extraction efficiency of each method. The antioxidant activity of Kao-Kum Doi-Saket extracts using three different extraction methods, including CE, PEF, and UAE methods, was analyzed using the radical scavenging activity (% of DPPH inhibition) and shown in Table 1. The results show that the UAE method provided a higher percentage of DPPH inhibition (83.20±3.67 - 88.32±1.83%) [19] of Kao-Kum Doi-Saket extracts than that of the extracts using the PEF and CE methods. These values showed a statistically significant difference ($p<0.05$) compared to other extraction methods, but there was no significant difference among them in the UAE method. The highest percentage of DPPH inhibition of the extracts was determined by the UAE method at the condition of 50°C, 60 min. The PEF method produced a lower rate of DPPH inhibition than the UAE method, with values between 33.24±0.43 and 67.74±1.88%. Table 1 shows that the percentage of DPPH inhibition of the extracts using the PEF method decreased with an increase in the number of pulses. The PEF1 and PEF2 were acceptable extraction conditions by providing the extracts with the high antioxidant value of 67.74±1.88% and 60.97±0.64%, respectively. However, the other 2 PEF conditions (PEF3 and PEF4) yielded a deficient antioxidant activity (43.50±1.26% and 33.24±0.43%, respectively), which was lower than that of the CE method (51.27±8.60%) but insignificantly different. These experimental results were consistent with Madalão et al. [32], who studied the effect of extraction methods, CE and UAE methods, on the antioxidant capacity of anthocyanin extracts from palm (*Euterpe edulis M.*). The result showed that the UAE method yielded a significantly higher antioxidant capacity of the palm extracts than that of the CE method. It was also found from the results of this study (Table 1) that the antioxidant activity of the extracts using the PEF method did not correlate with the total anthocyanin content results. Increasing the pulse intensity resulted in a higher anthocyanin content while decreasing the antioxidant capacity. This can be explained by increasing the pulse number during extraction, resulting in more heat generation in the solution and effect in lower ability of antioxidant of the extracts since antioxidant activity was sensitive to high temperature [33]. This was also consistent with the research study on using the PEF method to extract *Moringa oleifera*, which indicated that increasing the pulse intensity led to a decrease in the antioxidant capacity of *Moringa oleifera* extracts since high pulse intensity not only produced high temperature but also consumed longer extraction time. Consequently, there was sufficient time for the antioxidant impact to decay [34]. Nevertheless, the high temperature in the UAE technique did not meaningfully yield any discernible effect on antioxidants. Although the temperatures of the UAE and PEF extraction conditions were similar, there was no effect on the extract's antioxidant content when extracted using the UAE method because the UAE method produced significantly higher amounts of extracts and anthocyanins. Therefore, it was possible to explain that other bioactive compounds in the extracts from the UAE method may result in a high level of antioxidant activity. Furthermore, a previous study on rice berry bran indicated additional antioxidative compounds were

present in purple rice besides anthocyanin, suggesting that anthocyanin may not be the primary antioxidative compound in purple rice [35]. Therefore, it was necessary to consider the extraction time, extraction temperature, and the mechanism of each method for enhancing the major bioactive compounds from Kao-Kum Doi-Saket.

4. Conclusions

In conclusion, it can be concluded that the UAE method was the most effective method for Kao-Kum Doi-Saket extraction. The UAE2 extraction condition (50°C, 60 min) yielded the highest total anthocyanin content (57.05±4.27 mg/L) and antioxidant capacity (88.32±1.83%). These values significantly differed from the CE and PEF methods ($p<0.05$). Therefore, it can be suggested from this study result that this extraction method and condition can be used as a guideline for extracting phytochemical compounds from Kao-Kum Doi-Saket and may be applied in other purple rice grains for developing nutritious commercial products.

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