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INFLUENCE OF pH AND EXTRACTION CONDITIONS ON ANTIOXIDANT PROPERTIES FROM PURPLE STICKY RICE (*ORYZA SATIVA.L.*GLUTINOSA)

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ABSTRACT

This study aimed to determine the effect of different extraction conditions on antioxidant activity of purple sticky rice *(Oryza sativa* L. Glutinosa). The extractions conditions were ultrasonic 30, 40 and 50 minutes and microwave extraction at 300, 400 and 500 W with various pH 3, 4 and 5. The antioxidant activities were determined in terms of phenolic compound, total monomeric anthocyanins, flavonoid, ABTS and DPPH assays. The results showed that microwave extraction at pH 3 and the power 500 W had the highest extraction yield 69.85% (DW), total phenolic compound 1318.92 mg GAE/100 g, flavonoid 627.74 mg QE/g. DPPH scavenging ability was 85.97% and ABTS 413.36 mg/g. Cyanidin-3-glucoside was the main anthocyanin that found in purple sticky rice in the range of 72.18% to 90.21%. The highest percentage of cyanindin-3-glucoside was microwave extraction at 500 W. These results suggested that microwave extraction at pH 3 and 500 W was the best condition of extraction.

Keywords: anthocyanin, ultrasonic extraction, microwave extraction, antioxidant activity, purple sticky rice (*Oryza sativa L.* Glutinosa)

INTRODUCTION

Rice is the most important cereal crop and the stable food source being consumed by over half of the world's population **(Gomez, 2001)**.In the traditional growing areas of Asia, various colors of rice are red, purple, black, brown, yellow, and the green. They have known the color of rice has been preferred in the past for their special features such as medicinal value and exclusive taste **(Ahuja *et al.*, 2007)**. Several varieties of rice contain pigment particularly in brown, red and black rice have been widely cultivated in Thailand and generally used as an ingredient in snack, dessert, wine, food supplement, pharmaceuticals and cosmetic **(Phengrat and Jearakongman, 2009; Tananuwong and Terawuth, 2010)**. *Oryza sativa* L. glutinosa (Purple sticky rice) composes of 80% endosperm which consists of storage carbohydrate and its bran is rich in the source of Vitamin B and E, mineral, oil, and phytochemicals **(Zhang *et al.*, 2004)**. Purple sticky rice (*Oryza saitva* L. var. glutinosa) is an indigenous Thai glutinous rice strain characterized by purple pigments in the husk and the pericarp. The color is determined by a number of distinct anthocyanins **(Abdel-Aal *et al.*, 2006)**.
Anthocyanin which belongs to flavonoid is responsible for the attractive colors of flowers, fruits, and grains in nature **(Kong *et al.*, 2003)**. It has been widely reported that anthocyanin plays an important role in reducing a risk of oxidative damage and is a kind of the potential drug candidates to treat cancer and cardiovascular diseases **(Chen *et al.*, 2012)**. Anthocyanins undergo structural alterations, depending on the pH and ionic strength of the aqueous environment and adjustment to low pH is found to enhance the efficiency of the anthocyanin extraction.
Extraction yield of anthocyanins is depending on the value of pH and in an acidified solvent with strong acid media have a high yield of extraction because acid can change the native form of anthocyanins, the glucoside bond of monoside anthocyanins could be destroyed **(Kapasakalidis *et al.*, 2006)**. Its antioxidant activity decreases as pH increases from pH 2 to 7 **(Sukhapat *et al.*, 2004)**.
In Microwave accelerate extraction, the process acceleration and high extraction yield may be the result of a synergistic combination of two transport phenomena: heat and mass gradients **(Chemat *et al.*, 2009)**. Microwave power and temperature are interrelated because high microwave power can bring up the temperature of the system and result in the increase in the extraction yield until it becomes insignificant or declines **(Chemat *et al.* 2009; Hu *et al.*, 2008; Xiao *et al.*, 2008)**. In addition, when microwave extraction is performed in closed vessels, the temperature may reach far above the boiling point of the solvent, leading to better extraction efficient by the desorption of solutes from actives sites in the matrix **(Eskilsson and Björklund, 2000)**. However, **Routray and Orsat (2012)** reported that the efficiency increases with the increase in temperature until an optimum temperature is reached and then starts decreasing to the further increase in temperature: this happens because the selection of ideal extraction temperature is directly linked with the stability. Ultrasound-assisted extraction could be used as a tool to overcome the drawbacks of conventional solvent extraction methods and to improve some benefits of the solvent extraction process. The application of ultrasound generates cavitation, which is the generation of bubbles in the system **(Lieu and Le, 2010)**.
In this study was designed to find the effect of different extraction condition of ultrasonic extraction and microwave extraction in various pH on antioxidant activity, anthocyanins, phenolic compound and flavonoid of purple sticky rice (*Oryza sativa* L. Glutinosa).

MATERIAL AND METHODS

Sampling collection

The sample of purple sticky rice has been purchased from the local market from Phitsanulok province, Thailand. The rice was ground and and sieved 0.3 mm. All the samples were stored at -18 ± 2 ^{0}C in aluminium bag until analyzed.

Rice extraction

The purple sticky rice (*Oryza sativa* L. glutinosa) powder was extracted by ethanol extraction method. One gram of rice powder was extracted by combining with 8 mL of ethanol absolutes in ratio 1:8 w/v **(Abdel- Aal *et al.*, 2006)** and divided into two part as:

Ultrasonic extraction

The purple sticky rice was placed into sonication bath for extraction in 30, 40 and 50 min at room temperature and then filter in a vacuum in Whatman paper No. 1. Finally, evaporate at temperature 40 ºC in 15 min and stored at temperature -18 ± 2 °C in the dark bottle color.

Microwave extraction

The purple sticky rice was placed into microwave oven at powers 300, 400 and 500 W for 30 minutes at ambient temperature and then filter in a vacuum in Whatman paper No. 1. Finally, evaporate at temperature 40 ºC in 15 min and stored at temperature -18 ± 2 °C in the dark bottle color.

Extraction yield

The method was determined by **Leung *et al.* (2006)** which the yield was calculated from the following equation

$$Percent\ Yeild = \frac{Weight\ of\ \ purple\ sticky\ rice\ extract}{Weight\ of\ \ purple\ sticky\ rice} \times 100$$

Phenolic compound content

The total phenolic content of each extracted rice was determined by using the colorimetric method described by **Singleton and Rossi (1965)**. First, crude extracts 0.1 mL was added with 2 mL H_2O were reacted with Folin-ciocalteu reagent 1 mL in the ratio of water (1:1) for 5 min. Secondly, neutralized with 10% w/v sodium carbonate 1mL with 1 mL water. It was kept at dark room temperature 60 min and measured the absorbance at 765 nm. Gallic acid was used as the standard and total phenolic content was expressed as mg of Gallic acid equivalents (GAE) per 100 g of dry weight (DW) of the sample.

Flavonoid content

Flavonoid was determined by sodium nitrite aluminum dichloride system **(Zhishen *et al.*,1999)**. Firstly, mix extracted rice with 2 mL distilled water, with 1 mL sodium nitrate 5% and kept 5 min. Then add 0.15 mL of aluminum chloride 10% and kept 5 min. After that, added 1 mL of sodium hydroxide 1 M and kept 5 min before measured absorbance 415 nm. The results were expressed as mg of quercetin (QE) per 1 g of dry weight (DW).

Total monomeric anthocyanin (TAC)

The total anthocyanin content (TAC) was determined by the pH-differential method **(Giusti and Wrolstand, 2005)**. First, transfer 1 mL extracted solution into 10 mL volumetric flask for preparing two dilutions of the sample, one adjusts volume with potassium chloride buffer, pH 1.0, and the other with sodium acetate buffer, pH 4.5. Let these dilutions equilibrate for 15 min. Measure the absorbance of each dilution at the 510 and 700 nm (to correct for haze), and a blank cell filled with distilled water. Calculate the absorbance of the diluted sample (A) as follows:

$A = (A_{510} - A_{700})\ pH_{1.0} - (A_{510} - A_{700})\ pH_{4.5}$

Calculate the monomeric anthocyanin pigment concentration in the original sample using the following formula:

Monomeric anthocyanin pigment (mg/l) = (A x MW x DF x 1000)/ (ε x 1)

Where MW is the molecular weight MW = 449.2, DF is the dilution factor, and ε is the molar absorptivity
calculate pigment content as cyanidin-3-glucoside, ε = 26,900

Antioxidant activity

ABTS

For ABTS assay, the procedure followed the method of **Re *et al.*, (1999)** with some modifications. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution and kept 12 h at room temperature in dark. The solution was diluted methanol to obtain an absorbance of 0.70 ± 0.02 units at 734 nm using the spectrophotometer. Extracted rice 0.1 mL were allowed to react with 0.9 mL of the ABTS+ solution for 2 h in the dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer. The standard curve was linear between 25 and 600 μM Trolox. Results were expressed in mg Trolox equivalents (TE)/g.

DPPH

For DPPH assay was performed based on the method described by **Brand-Williams *et al.*, (1995)**. A 1 mL volume of the sample extract was added to 1 mL of the DPPH solution (dissolved in ethanol), and the mixture was placed in the dark at room temperature for 30 min. Subsequently, the absorbance at 515 nm was recorded. The percentage of radical scavenging ability was calculated by using the formula:

Activity (%) = Ac-At/Ac x 100

Where: At was the absorbance of samples and Ac was the absorbance of ethanol DPPH solution

Anthocyanin detection

Anthocyanin content was determined by reverse phase HPLC. Anthocyanin separation was achieved by gradient elution using 0.1% formic acid as solvent A, and methanol as solvent B. The elution scheme was modified due to differences in instrumentation, column selection and to improve on peak resolution: isocratic 40% B, 0-30 min; linear gradient from 40% B to 90% B, 40-45 min; isocratic 90% B, 45-50 min; gradient to 98% B, 50-55 min; to 100% B, 55-60min; flow rate 0.5 mL/min; injection volume 20 μl and the column that have been used a Zorbax SB-C18 column (150 mm x 4.6 mm, 3.5 mm, Agilent Technologies, Santa Clara, CA, USA) **(Hao *et al.*, 2015)**.

Data analysis

All experiment had been conducted triplicate and expressed as mean standard. Data were analyzed by program with deference mean value by using an analysis of the variance (ANOVA). Significant differences ($P<0.05$) between means were identified using Duncan range test procedures.

RESULTS AND DISCUSSION

Percentage Yield

Extraction efficiency is affected by the chemical nature of phytochemicals, the extraction method used, sample particle size, the solvent used, as well as the presence of interfering substances **(Stalikas, 2007)**. The yield of extraction depends on the solvent with varying polarity, pH, temperature, extraction time, and composition of the sample. Figure 1 showed that the extraction yields range between 69.36 to 69.64% of dry weight for ultrasonic extraction and 69.63 to 69.84% of dry weight for microwave extraction. The highest extraction yield was found using microwave extraction with 500 W at pH 3 ($p<0.05$). In this figure reported that increasing microwave power the percent yeild could be increased because the microwave power could also influence the yield of antioxidants in the microwave extraction process. On the one hand, an increase in microwave power could accelerate the solvent's movement, cell rupture and diffusion of extractives into the solvent, thereby increasing the extraction efficacy. Moreover for ultrasonic extraction, increasing duration time could be increase the percent yeild of extraction beacuse ultrasound also exerts a mechanical effect, allowing greater penetration of solvent into the tissue, increasing the contact surface area between the solid and liquid phase and ultrasonic extraction time could affect the extraction as well, the yield of extraction will be increased by increasing the time for extraction **(Galhiane *et al.*, 2006)**. As a result, the solute quickly diffused from the solid phase to the solvent **(Rostagno *et al.*, 2003)**. The highest extraction yield was found using microwave extraction with 500 W at pH 3 ($p<0.05$).

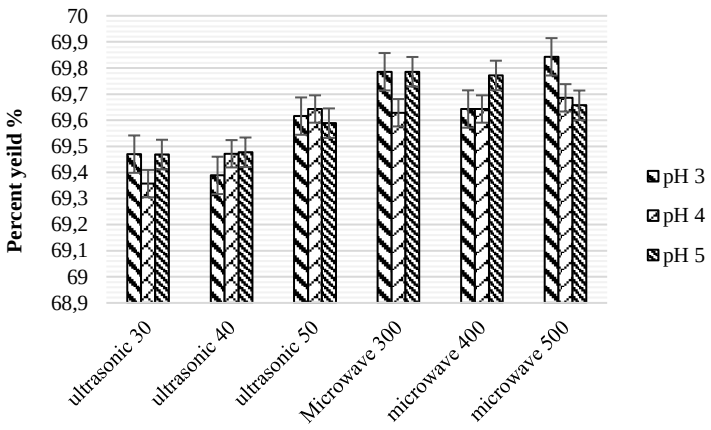

Figure 1 Percent yield of purple sticky rice (*Oryza sativa* L**.** glutinosa) extract using different extraction condition

Total Phenolic compounds (TPC)

According to **Adom and Liu, (2002)** reported that phenolic compounds in rice has been existing as both free and bound forms. Bound phenolic acids are typically involved in cell wall structure. They are conjugated or cross-linked with lignin components and are mainly found in bran fraction **(Zhou *et al.*, 2004)**. Total phenolic content of different extraction condition of purple sticky rice (*Oryza sativa* L**.** Glutinosa) are showed in Figue 2.Total phenolic content has

been in the range of 691.89-1318.92 mg GAE/100 g for ultrasonic extraction and 691.89-2389.19 mg GAE/100 g for microwave extraction. As shown in Figure 2, an increase in exposure power there was significant increase in total phenolic compound in purple sticky rice. Maximum total phenolic compound 2389.19 mg GAE/100 g was recorded at microwave power at 500 W. The highest concentration was pH 3 with microwave extraction ($p<0.05$). Microwave power can have the influence on the total phenolic content when the power of microwave extraction has increased. In microwave extraction, microwave power was a key variable affecting the release of phytochemicals from different matrices by rupturing cell the wall, and also had the ability to modify equilibrium and mass transfer conditions during extraction. Increasing the microwave power accelerated purple sticky rice extraction **(Ghasemzadeh *et al.*, 2017)**. Most phenolics present in plant tissues are soluble in polar solvents and can be extracted using methanol containing a small amount of hydrochloric or formic acid. These results similar to **Seawan *et al.*, (2014)** reported that the power level of microwave effected the total phenolic content of Homnin black rice and Munpu red rice.Moreover, for ultrasonic extraction, increasing duration time the total phenolic compound could be increase because of the structural destruction and the decomposition of polyphenols (**Carrera *et al.*, 2012; Makris *et al.*, 2007; Sun *et al.*, 2011)**. When extraction time increased the variance of extraction yield is relatively rapid and reaches a maximum at 20 min. Ultrasound with mechanical agitation effect, cavitation effect, and thermal effect can improve the mass transport and facilitate the release of compounds from the extracted materials **(Carrera *et al.*, 2012)**. On one hand, the low acid pH value of the extraction solution can stop the oxidation of phenolics, while the use of small temperatures may preserve anthocyanin stability **(Li and Jiang, 2007, Laleh *et al.*, 2006)**. Microwave extraction showed the highest extraction efficacy of total phenolic compound followed by ultrasonic extraction ($p<0.05$). The highest concentration was pH 3 with microwave extraction ($p<0.05$).

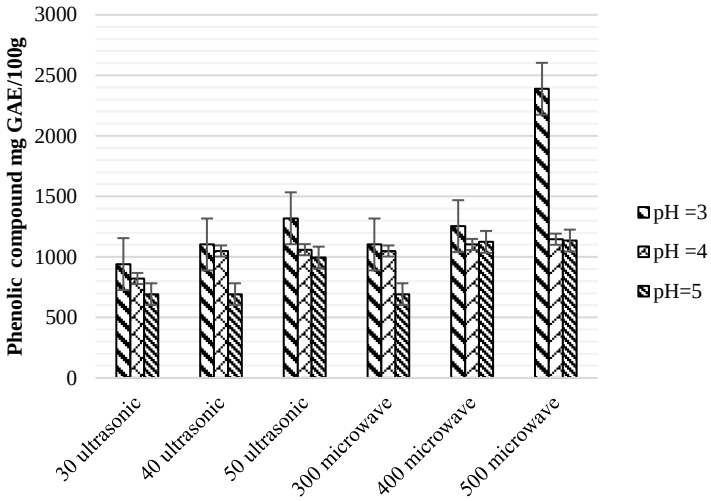

Figure 2 Total phenolic content of purple sticky rice (Oryza sativa L. glutinosa) extract using different extraction condition

Flavonoid content

Flavonoid consist of a 15-carbon skeleton that was organized in two aromatic rings (A- and B-rings) interlinked by a three-carbon chain (structure C6-C3-C6) and are recognized for both their ability to donate electrons and to stop chain reactions. These activities are attributed to the phenolic hydroxyls, particularly in the 3′OH and 4′OH of the three-carbon chain **(Ramarathnam *et al.*, 1989; Hudson *et al.*, 2000; Kim *et al.*,2010; Cho *et al.*, 2013)**. Flavonoid contents in purple sticky rice with different extraction were shown in Figure 3. For ultrasonic extraction, the flavonoid content were between 90.32-464.52 mg QE/g and the highest flavonoid condition content was found by using ultrasonic extraction time of 50 min with the extraction at pH 3 microwave, extraction has been between 489.68-627.74 mg QE/g and the highest concentration is pH 3 in microwave extraction 500 W which was 627.74 mg QE/g.Similar to phenolic acid, flavonoids are synthesized by the phenylpropanoid metabolic pathway. The high concentration flavonoid was depending on low pH because of stability effect on both flavilium salt and anhydrobase **(Goto *et al.*, 1991)**. **Zhou *et al.*, (2004)** reported the high yield of extraction of flavonoid were depending on extraction time. In the present study, the accelerated extraction of flavonoids by increasing microwave power was related to the direct effects of microwave energy on bio-molecules by ionic conduction and dipole rotation which result in power dissipated inside the solvent and plant material and then generate molecular movement and heating **(Gfrerer *et al.*, 2005)**.

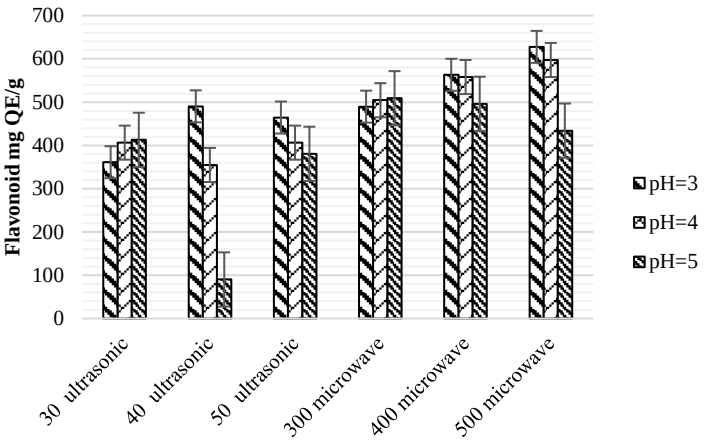

Figure 3 Flavonoid content of purple sticky rice (*Oryza sativa* L. glutinosa) extract using different extraction condition

Total monomeric anthocyanin

Total monomeric anthocyanin in ultrasonic extraction and microwave extraction is shown in figure 4. For ultrasonic extraction total monomeric anthocyanin has been in the range of 98.99-183.24 mg/g and the highest concentration was pH 3 in ultrasonic 50 min which 183.24 mg/g. For microwave, extraction has been in the range between 90.17-614.52 mg/g and the highest concentration was pH 3 in microwave extraction 500 W. Acidity of the solvent had greater effects on total monomeric anthocyanin contents. At the similar extraction duration, acidic solvent provided the extract with significantly higher amount of total monomeric anthocyanin than neutral solvents ($p<0.05$). This could be due to the higher stability of anthocyanin in acidic solution **(Abdel-Aal and Hucl, 2003)**. **Dranca *et al.*, 2016** has been reported that high yield of total monomeric anthocyanins could be the effect of extraction time of ultrasonic extraction, long period of extraction more effect of high yield of extraction. **Abdel-Aal *et al.*, 2014**, has been shown that increasing of microwave power could increase the yield of extraction. This observation is in agreement with **Golmohamadi *et al*, (2013)** where they discovered that total phenolic content and total monomeric anthocyanin were significantly increased by 11.97% and 12.6% after 30 and 20 min sonication at 20 kHz and 400 W ultra-sonication conditions.

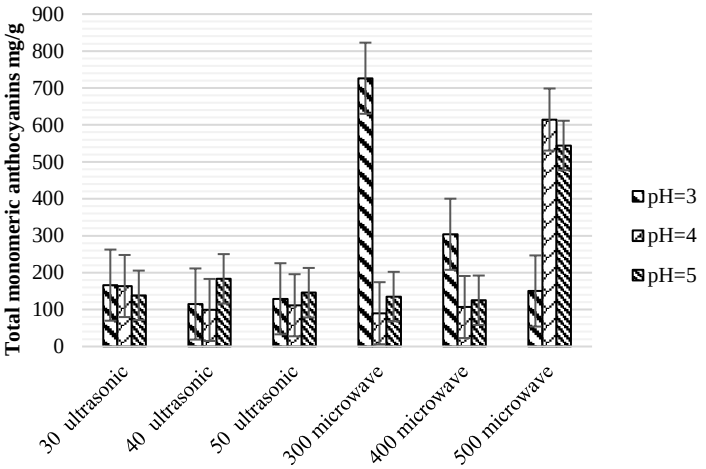

Figure 4 Total monomeric anthocyanin content of purple sticky rice (*Oryza sativa L*. glutinosa) extract using different extraction condition

Antioxidant of DPPH and ABTS

The DPPH free radical (DPPH) does not require any special preparation, while the ABTS radical cation ABTS must be generated by enzymes or chemical reactions (**Arnao, 2000**). Another important difference is that ABTS can be dissolved in aqueous and organic media, in which the antioxidant activity can be measured, due to the hydrophilic and lipophilic nature of the compounds in samples. In contrast, DPPH can only be dissolved in organic media, especially in ethanol, this being an important limitation when interpreting the role of hydrophilic antioxidants. Both radicals show similar bi-phase kinetic reactions with many antioxidants.The antioxidant activity of purple sticky rice has been determined using the ABTS and DPPPH assay and showed very strong antioxidant activity. Figure 5 showed the antioxidant for by using ultrasonic and microwave extraction and value of ABTS express as Trolox/g. The trends seen in antioxidant yields were reiterated in these activities. The increasing with

extraction time for ultrasonic extraction and the power for microwave extraction increased ABTS. For ultrasonic extraction the ABTS value has been in the range of 240.41-348.61 mg/g of ABTS. The highest was found at pH 3 with 50 min of extraction time and for microwave, extraction ABTS value has been in the range of 246.51-413.36 mg/g of ABTS and the highest concentration was pH 3 with microwave extraction 500 W. These results matches well our observations on anthocyanin and total phenolic contents. The DPPH scavenging ability highest of purple sticky rice showed in Figure 6. Ultrasonic extraction has been in the range of 44.70-81.4% and the highest concentration was pH 3 in ultrasonic 50 min and for microwave extraction and DPPH scavenging ability has been in the range of 58.06-85.79% and the highest concentration was pH 3 in microwave extraction 500 W.The power level of microwave affected the ABTS and DPPH activities. The antioxidant activities of purple sticky rice were increased with the increase of microwave power ($p<0.05$). The different of effect of microwave power level might due to different active compound in sample. (**Seawan, *et al*., 2014**).

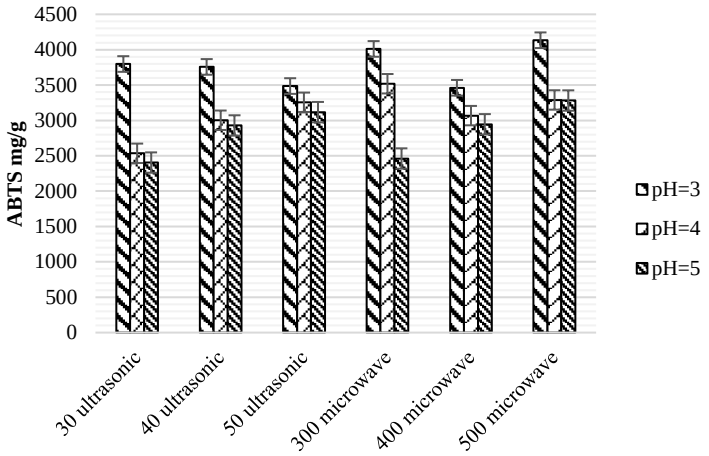

Figure 5 ABTS of purple sticky rice (*Oryza sativa* L. glutinosa**)** extract using different extraction condition

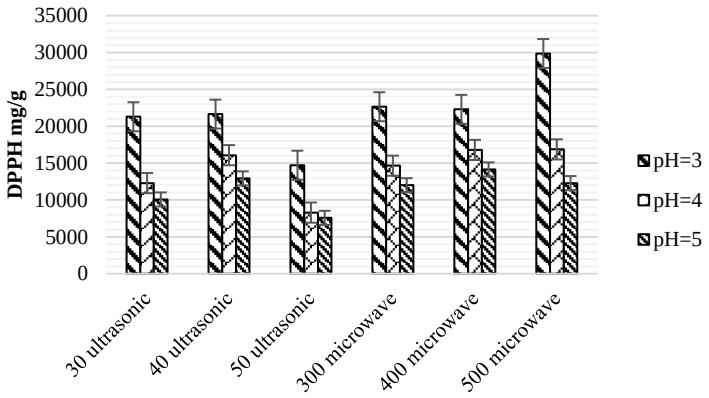

Figure 6 DPPH scavenging activity of purple sticky rice **(***Oryza sativa* L. Glutinosa) extract using different extraction condition

Anthocyanins

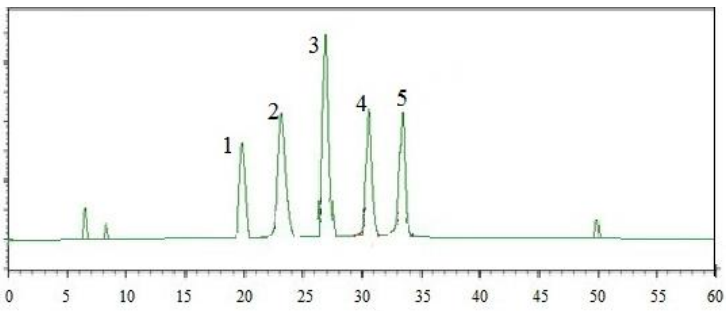

Figure7 Typical chromatogram of anthocyanin from purple sticky rice by using difference retention time condition

Peak 1: Malvidin -3-glucoside, peak 2: Delphinidine -3-glucoside, peak 3: Cyanidin -3-glucoside, peak 4: pelagonidin -3-glucoside, peak 5: peonodin -3-glucoside

Table 1 Anthocyanin profile of purple sticky rice extract using difference conditions, expressed as percentage % of five anthocyanins in purple sticky rice.

Percentage	MvGL	DpGL	CyGL	PtGL	PnGL
pH 3 300 W	9.17	8.40	72.18	3.02	7.23
pH 3 400 W	1.96	5.02	85.67	0.99	6.36
pH 3 500 W	0.25	0.48	98.46	0.16	0.65
pH 4 300 W	5.89	9.28	71.04	4.24	9.54
pH 4 400 W	3.66	5.33	82.24	2.02	6.75
pH 4 500 W	3.84	3.81	89.31	0.20	2.83
pH 5 300 W	7.49	11.11	75.09	2.20	4.10
pH 5 500 W	2.89	7.08	83.11	1.95	4.98
pH 5 500 W	0.40	4.95	87.44	0.54	6.67
pH 3 30 min	14.99	3.82	76.33	1.14	3.71
PH 3 40 min	2.93	4.59	90.43	0.62	1.41
pH 3 50 min	3.08	5.02	90.21	1.66	0.03
pH 4 30 min	6.41	8.04	77.17	1.10	7.27
pH 4 40 min	4.72	6.64	80.62	0.79	7.22
pH 4 50 min	1.42	4.04	87.91	0.73	5.89
pH 5 30 min	4.76	4.66	87.64	0.14	2.80
pH 5 40 min	4.45	4.26	87.59	0.18	3.50
pH 5 50 min	2.19	4.61	89.91	0.23	3.05

MvGL: Malvidin-3-glucoside, DpGL: Delphinidin-3-glucoside, CyGL: Cyanidin-3-glucoside, PtGL: petunidin-3-glucoside and PnGL: Peonidin-3-glucoside.

Table 1 showed anthocyanin profile of each sample, expressed as percentage of five anthocyanins in purple sticky rice. The major of anthocyanin in purple sticky rice is cyaniding-3- glucoside that contains between 70-90% of total amount of anthocyanins. According to **Escribano-Bailón *et al,* (2004)** and cyanidin-3-glucoside is the major anthocyanin, ranging from 80-100% of total contents in pigmented rice cultivars. **Yawadio *et al,* (2007**) identified cyanidin-3-glucoside and peonidin-3-glucoside in black rice, while **Sompong *et al,* (2011)** confirmed that cyanidin-3-glucoside and peonidin-3-glucoside are the most abundant anthocyanins in black rice extracts. For microwave, extraction has been shown that cyaniding-3-glucoside was in the range of 72.17-98.45% and the highest percent was pH 3 in power 500 W that contains 98.45%. For ultrasonic extraction have been shown that cyaniding-3-glucoside is in the range of 76.32-90.20% and the highest percent was pH 3 in 50 min that contain 90.20%. The highest amount of cyaniding-3-glucoside is pH 3 in power 500 W. Many studies report that black and red rice have 2 main compounds of anthocyanin such as cyanidin-3-glucoside and peonidin-3-glucoside in which cyaniding-3-glucoside contain 93% of the quantified anthocyanin. These generally found a pigment of fruits, vegetables, and colored rice have important roles in reducing the risk of cancer and other chronic diseases because of their free radicals scavenging capacities **(Wang and Stoner, 2008; Elisia and Kitts, 2008)**. In acidified solvent extractions, strong acid media should be avoided because the acid may change the native form of anthocyanins, the glycoside bonds of 3-monoside anthocyanins could be destroyed, and acylated anthocyanins might be degraded by hydrolysis reaction **(Kapasakalidis *et al.,* 2006)**.

CONCLUSION

Experiments demonstrate that low pH has influenced the extraction of antioxidant activities of purple sticky rice (*Oryza sativa* L. Glutinosa) and the high yield of extraction in this study was pH 3. Moreover, duration time is effected by ultrasonic extraction and high antioxidant of extraction will be increased when increasing duration time of extraction. On the other hand, microwave power can effect in yield of extraction by increasing high power of microwave. Microwave extraction in power 500 W in 30 min composes of the highest yield of extraction and the greatest amount of phenolic content, flavonoid, total monomeric anthocyanins, ABTS, DPPH and anthocyanin detection. It is the best conditions for extraction.

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