Research Article

Ethanol Extraction of Active Ingredients and Antioxidants from Germinated Sangyod Rice

Nirana Chairerk, Prukraya Pongyeela and Juntima Chungsiriporn
Department of Chemical Engineering, Faculty of Engineering, Prince of Songkla University, Songkhla, Thailand

Nirattisai Rakmak*
Department of Chemical and Process Engineering, School of Engineering and Resources Management, Walailak University, Nakhon Si Thammarat, Thailand

* Corresponding author. E-mail: nirattisai.ra@wu.ac.th DOI: 10.14416/j.asep.2019.03.003
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Abstract
Sangyod rice is a special red rice variety, originally planted in Pattalung province of Thailand. This type of rice is high in nutrients and antioxidants. This research aimed to produce a rice extract by solvent extraction of germinated Sangyod rice. For the extraction, ethanol at the concentrations 0, 50, or 95% (v/v) was used. Various extraction times and rice-to-solvent ratios, as well as solvent reuse and recovery were studied. The results show that the extract from germinated Sangyod rice has higher TPC, TFC, and GABA contents and higher antioxidant activity than not germinated or Riceberry rice. An 0.5 h extraction time with rice-to-solvent ratio 1:15 (g/mL) were optimal for extracting germinated Sangyod rice. The 50% ethanol solvent gave the highest TPC, TFC, GABA and DPPH antioxidant activities, at 1,627.00 ± 53.08 mg GAE/100 g rice, 559.50 ± 17.06 mg QE/100 g rice, 9.97 ± 0.17 mg GABA/100 g rice, and 0.16 ± 0.01 mg/mL, respectively. Regarding stability of the rice serum, temperature affected stability of the extracts. The results of this study can provide new opportunities to promote Sangyod rice to farmers and add value to rice products. The extract has potential as an active ingredient in cosmetics.

Keywords: Antioxidant activity (IC_{50}), GABA, Germination, Sangyod rice, Solvent extraction, Total Flavonoid Content (TFC), Total Phenolic Content (TPC)

1 Introduction

Sangyod rice is a source of many bioactive compounds including phenolics, γ-aminbutyric acid and oryzanol that tend to reduce the chances of getting cancer. Phenolic compounds are commonly present in whole grains as phenolic acids and flavonoids [1]. Gamma-aminobutyric acid (GABA) is a non-protein amino acid that acts as the main inhibitory neurotransmitter in the mammalian cortex, and it was first discovered as an integral part of the mammalian central nervous system. Furthermore, GABA has other physiological functions in humans and other vertebrates, such as blood vessels strengthening, modulation of insulin secretion, prevention of blood cholesterol amplification, alleviation of emotional unrest, and protection from chronic alcohol-related diseases [2].

Sangyod rice is cultivated especially in the Phatthalung province of Thailand. In 2006, Sangyod rice was certified by Geographical Indications Protection Act 2003 under the name of "SANGYOD MUANG PHATTHALUNG". In 2012, the cultivation

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of Sangyod rice decreased because the average selling price and yield per rai were lower than for other types of rice. Sangyod rice is rich in nutrients, therefore value-added agricultural commodity products should be developed from it. Germination of rice is a metabolic process that induces changes in the bioactive components [3] and gives a high content of phytochemicals. Germinated brown rice has comparatively short cooking time, soft texture and high Nutrition [4], with possibly elevated GABA and phenolic contents. However, many factors affect the apparent total phenolic content, such as extraction method including choice of solvent, growth conditions, and germination time [5].

Solvent extraction has low production costs, and is simple and easy to operate compared with other separation methods. It is recommended as a suitable method for extracting active ingredients from rice with various solvents, such as methanol, ethanol, acetone, or the aqueous phase of a solvent mixture. Mixtures of ethanol and water at various ratios are commonly used to study the extraction of antioxidants from plant materials, especially rice [6]. Several parameters may influence the extraction yield, including extraction time, sample-to-solvent ratio, solvent type and particularly its polarity [7]. The extraction process is temperature controlled to around 50°C because too high temperature causes chemical degradation that reduces extraction yield.

Therefore, the aim of this research was to create potential value-added products from Sangyod rice by preparing germinated rice and extracting from it. Solvent extraction was selected for producing an extract possibly suitable for pharmaceutical and cosmetic products. Ethanol and water were used as extraction solvents and were evaporated in a rotary evaporator to produce concentrated extract. To evaluate the effects of extraction time, rice-to-solvent ratio, and concentration of ethanol on extract properties, total phenolic, total flavonoid, GABA content, and antioxidant activity were measured. The recovery and reuse of solvent in the extraction was studied to reduce chemical consumption costs.

2 Materials and Methods

2.1 *Materials*

Sangyod paddy rice was harvested from Amphoe Singhanakhon, Songkhla, Thailand in 2016. After sun drying to a moisture content below 14%, the grains were put in a tight plastic bag kept at room temperature for about 3 months before germination. The chemical reagents used in this work were purchased from U&V Holding (Thailand) CO., LTD. Ethanol, Folin-Ciocalteu reagent, sodium carbonate, GABA reagent, gallic acid, Quercetin, sodium nitrite, aluminum chloride, sodium hydroxide, sodium hypochlorite, phenol reagent and borate buffer were used.

2.2 *Preparation of germinated Sangyod rice*

Germinated Sangyod paddy rice was prepared by using the method reported by [8] with modifications. The paddy rice was washed with distilled water and soaked in distilled water for 24 h to saturated moisture content. Then, the soaked rice was sprouted for 60 h in the dark at room temperature. After germination, the germinated Sangyod rice was dried at 40°C for 24 h until the moisture content was below 14%. After that, to prepare rice flour, the germinated Sangyod rice grains were de-husked and ground in a mill and passed through a 40 mesh sieve. All samples were stored in a desiccator prior to analysis.

2.3 *Rice extraction*

Rice flour from Sangyod rice and from germinated brown rice were used as alternative raw materials to extract. 100 g of rice flour was extracted with solvent. For each extraction the mixture of rice and solvent was kept on a mechanical shaker at 200 rpm at room temperature. After centrifuging at 4,000 rpm for 5 min, the supernatants obtained from each extraction were collected and combined together. Concentrating the extracts was performed using a rotary evaporator at 40°C. The extracts were kept at 4°C for analysis, and extraction yield was estimated as:

%Extraction yield = (The extract obtained by extraction (g)/Rice flour used (g)) × 100

2.4 *Optimization of solvent extraction*

By fixing the extraction time and the rice to solvent ratio at 1 h and 1:15, respectively, rice flour was extracted using binary solvents of ethanol and water.

Three concentrations of ethanol (0, 50, and 95%, v/v) were used as alternative extraction solvents. The best ethanol concentration was selected to maximize the yield.

2.5 *Optimization of extraction time*

The rice flour was extracted with the optimal choice of solvent determined in the first step above, with extraction times 0.5, 1, 2, or 4 h, and with fixed 1 : 15 rice to solvent ratio. The optimal extraction time was selected based on the four responses.

2.6 *Optimization of rice to solvent ratio*

Using the optimal ethanol concentration and extraction time determined above, samples were extracted at the alternative rice-to-solvent ratios 1 : 10, 1 : 15, 1 : 20, or 1 : 25 (g/mL). The best rice-to-solvent ratio was selected according to the responses.

2.7 *Determination of Total Phenolic Content (TPC)*

TPC in the mixture was evaluated by using the Folin–Ciocalteau method [9]. 0.5 mL of diluted extract was mixed with 9.5 mL of distilled water and 0.5 mL of Folin–Ciocalteau reagent. After reaction for 5 min, 2 mL of 10% sodium carbonate was added. Reaction was allowed to continue for 2 h, then absorbance of the mixture was measured at 730 nm. The results of TPC determinations are expressed in milligrams of Gallic Acid Equivalent (GAE) per 100 g of dry rice flour (mg GAE/100 g). Three replicates determinations were carried out for each extract.

2.8 *Determination of Total Flavonoids Content (TFC)*

TFC was determined by a colorimetric method with slight revisions [8]. 150 µL of 5% $NaNO_2$ was mixed with 500 µL of sample extract or standard solution in a 15 mL tube, and then incubated for 5 min. After adding 150 µL of 10% $AlCl_3 \cdot 6H_2O$ and allowing 5 min of reaction, 1 mL NaOH (1 M) and 3 mL twice distilled water were added. The absorbance was measured at 510 nm after thoroughly mixing and reacting for about 10 min. TFC is expressed in milligrams of quercetin equivalent per 100 g of dry rice flour (mg QE/100 g rice flour). Three replicates determinations were carried out for each extract.

2.9 *Determination of GABA content*

The analysis of GABA content was performed by the method reported by [10] with modifications. 0.4 mL of diluted extract was mixed with 0.4 mL of borate buffer (pH 9.0) and 2 mL of 6% phenol. Then, the blend was mixed thoroughly and cooled in a cooling bath for 5 min. Then 0.8 mL of 7.5% sodium hypochlorite was added. After intensive shaking the mixture was put in ice bath water for 10 min, and then put in boiling waterbath for 10 min. Finally, the sample was put in ice bath water for 5 min, and the sample was analyzed for absorbance at 630 nm wavelength, with ethanol as the blank. GABA content of the sample was determined by a calibration curve for absorbances of standard GABA solutions.

2.10 *Determination of DPPH radical scavenging activity (DPPH)*

The DPPH radical scavenging activity of extracts was determined according to the method reported by [11] with modifications. Briefly, 500 µL of the diluted extract was mixed with 500 µL of 0.1 mm DPPH solution. After incubating for 30 min in the dark, the absorbance was measured at 517 nm. Results are expressed as IC_{50}, meaning the concentration giving 50% inhibition of the DPPH radical. This activity is given as the percent of DPPH radical scavenged, which is calculated from:

$$\% \text{ Inhibition} = (A_{control} - A_{sample} / A_{control}) \times 100\%$$

Here $A_{control}$ is absorbance of DPPH radical + ethanol and A_{sample} is absorbance of DPPH radical + sample.

2.11 *Stability test*

Heating-cooling tests of storing the crude extract in varying conditions were done. The tests were performed on samples kept at 4°C for 24 h and at 45°C for 24 h. pH, viscosity and antioxidant activity were tested for 6 cycles.

2.12 *Statistical analysis*

All determinations were done in triplicate, and are presented on a dry basis as mean ± Standard Deviation

(SD). Data analysis was performed with SPSS program, using ANOVA, followed by Duncan multiple comparison tests. Statistical significance was defined as $p < 0.05$.

3 Results and Discussion

3.1 *The germination of rice*

The total phenolic and GABA contents in extracts obtained with 50% ethanol solvent are shown in Table 1, for Sangyod rice, Riceberry, and germinated Sangyod rice. The results show that germinated rice had the highest 6.56% yield. Total phenolic, total flavonoid and GABA contents significantly increased when germination was allowed. The germinated Sangyod rice gave total phenolic and total flavonoid contents of 1,627.00 ± 53.08 mg of GAE/100 g of rice and 559.50 ± 17.06 mg QE/100 g rice, respectively. The GABA content in germinated Sangyod rice was up to 9.97 ± 0.17 mg GABA/100 g rice, which matches [6], according to whom both phenolic and gamma aminobutyric acid (GABA) in germinated brown rice were increased 1–4 fold compared with brown rice. The antioxidant activity of germinated Sangyod was the highest among the cases tested, at 0.16 ± 0.01 mg/mL. Moreover, the ethanolic extract of Riceberry had higher total phenolic, total flavonoid, GABA contents and antioxidant activity than that of Sangyod rice, but lower than germinated Sangyod rice.

Germination can increase GABA and total phenolic contents in Sangyod rice sprouts by the changes in chemical compounds and bioactive compounds in rice during germination [12]. During germination, the total phenolic and GABA contents increased: as the grains were soaked, imbibition begun, respiration accelerated, and this stimulated the metabolism of amino acids, resulting in the formation of enzyme systems. Amino acid and glucosidase such as GABA and phenolic compounds are also synthesized. Therefore, germinated Sangyod rice is the most valuable source of total phenolic, total flavonoid and GABA, for extraction. This study suggests that Sangyod rice after germination is a potential source of natural antioxidants, especially phenolic and flavonoid compounds, and could contribute to the income of rice farmers.

Table 1: Total phenolic, total flavonoid, GABA and antioxidant activity profiles (mean ± SD) of Sangyod and Riceberry rice cultivars. Each data point is mean of 3 replicates

Rice Cultivar	Yield (%)	TPC (mg GAE/ 100 g rice)	TFC (mg QE/ 100 g rice)	GABA (mg GABA/ 100 g rice)	IC_{50} (mg/mL)
Sangyod	2.25	689.71± 4.95^{b}	171.48± 10.72^{b}	1.76± 0.03^{a}	0.54± 0.02^{c}
Riceberry	2.65	711.45± 3.34^{b}	234.73± 30.57^{b}	1.72± 0.01^{b}	0.32± 0.01^{b}
Germinated-Sangyod	6.56	1,627.00± 53.08^{a}	559.50± 17.06^{a}	9.97± 0.17^{a}	0.16± 0.01^{a}

Different small letters within one column indicate a significant difference ($p < 0.05$)

3.2 *Effects of solvent concentration*

The effects of ethanol concentration on germinated rice extraction yield, total phenolic, total flavonoid, GABA content and antioxidant activity were assessed at extraction time of 1 h and rice to solvent ratio of 1 : 15. Water (0% ethanol) gave the significantly highest yield of 10.26% whereas 95% ethanol showed the lowest 2.77% yield. 50% ethanol showed 6.26% yield, which is higher than that with 95% ethanol; this is consistent with the study of [13]. These results show that the yield of extraction decreased with ethanol concentration in the aqueous solvent. The mixture of ethanol and water can facilitate dissolving and extracting various compounds.

The phenolic, flavonoid, GABA, and antioxidant activity profiles in extracts of the germinated Sangyod rice at various ethanol concentrations are presented in Figure 1(a)–(d). The optimal 50% ethanol case gave the highest total phenolic and flavonoid contents at 1,469.60 ± 32.95 mg GAE/100 g rice and 417.23 ± 7.14 mg QE/100 g rice. The 50% ethanol has higher polarity than 95% ethanol but below that of water, and the phenolic and flavonoid compounds show polarity, so these can be effectively dissolved in an ethanol-water mixture. In contrast the 95% ethanol gave the highest GABA content of 21.52 ± 0.84 mg GABA/100 g rice. This may be attributable to the higher solubility of GABA in ethanol than in water. According to the principle of "like dissolves like", a solvent should mainly extract those compounds that have similar polarity to the solvent [14]. The antioxidant activity was highest with 50% ethanol at 0.21 ± 0.01 mg/mL.

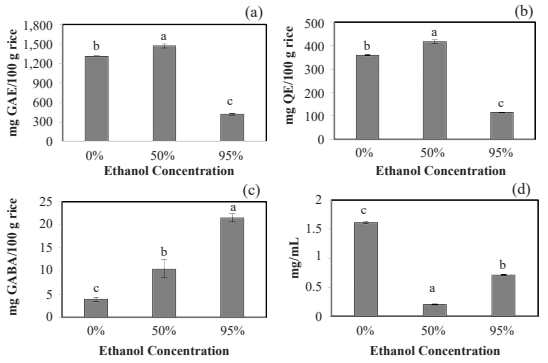

Figure 1: Bioactive contents with various ethanol concentrations: total phenolic content (a); total flavonoid content (b); GABA content (c); and antioxidant activity (d) (error bars represent SD).

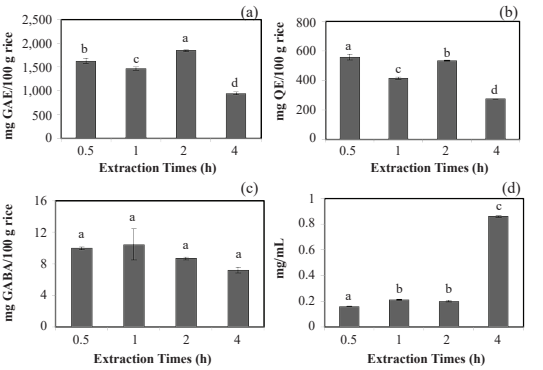

Figure 2: Bioactive content for various extraction times: total phenolic content (a); total flavonoid content (b); GABA content (c); and antioxidant activity (d) (error bars represent SD).

These results show that the concentration of ethanol affected the total phenolic compound extraction. The results regarding polarities of the solvent and extracted compounds match the studies of [15]. Using 50% ethanol for extraction gave more phenolic compounds than those using water or 95% ethanol. Therefore, a solvent with high polarity is suitable for phenolic and flavonoid extractions. The selection of an efficient solvent is of great importance to extraction results.

3.3 *Effects of extraction time*

The effects of extraction time on yield, total phenolic, total flavonoid, and GABA contents and on antioxidant activity of germinated rice extract were determined with 50% ethanol and the ratio of rice to solvent fixed at 1 : 15 (g/mL). The results are presented in Figure 2(a)–(d). The extraction time directly affected the yield. The extraction time of 2 h gave significantly highest 7.75% yield. If the extraction time is too long (4 h), some constituents in the rice grain will decay [16]. The optimum extraction time 0.5 h gave the highest total flavonoid content of 559.50 ± 18.88 mg QE/100g rice and high total phenolic and GABA contents of 1,627 ± 53.08 mg GAE/100 g rice and 9.97 ± 0.17 mg GABA/100 g rice, respectively. The extraction time affected the ability of the grain constituents [15]. The antioxidant activity was lowest at 0.16 ± 0.01 mg/mL for 0.5 h extraction time, which is associated with high total flavonoid content, total phenolic content, and high antioxidant activity [17].

3.4 *Effects of rice to solvent ratio*

The effects of rice-to-solvent ratio in germinated Sangyod rice extraction were studied with 50% ethanol and 0.5 h extraction time, fixed based on previous tests. The results in Table 2 show that the ratio 1 : 25 got the highest 7.09% extract yield. The highest total phenolic, total flavonoid, and GABA contents, and antioxidant activity were observed for this same case.

Table 2: Total phenolic, total flavonoid, GABA, and antioxidant activity profiles (mean ± SD) of germinated rice extraction with varied rice to solvent ratio. Each data point is derived from 3 replicates

Rice to solvent ratio (g/mL)	Yield (%)	TPC (mg GAE/ 100 g rice)	TFC (mg QE/ 100 g rice)	GABA (mg GABA/ 100 g rice)	IC_{50} (mg/ mL)
1 : 10	6.15	1,162.72± 8.95^{d}	403.69± 1.41^{d}	6.09± 0.09^{d}	0.53± 0.01^{d}
1 : 15	6.56	1,627.00± 53.08^{b}	559.50± 17.06^{c}	9.97± 0.17^{b}	0.16± 0.01^{b}
1 : 20	6.43	1,533.81± 22.04^{c}	617.02± 18.88^{b}	9.00± 0.04^{c}	0.22± 0.03^{c}
1 : 25	7.09	2,093.18± 35.69^{a}	785.86± 2.66^{a}	12.34± 0.09^{a}	0.13± 0.02^{a}

Different small letters in the same column indicate a significant difference ($p < 0.05$)

When the amount of solvent is too low for extraction, the total phenolic, total flavonoid, and GABA were

not effectively extracted. The rice-to-solvent ratios 1 : 15 and 1 : 25 (g/mL) gave the best results, without significant difference between these alternatives. However, the ratio 1 : 15 is more economic. Therefore, the extraction conditions 50% ethanol for 0.5 h at a rice to solvent ratio of 1 : 15 were chosen for the extraction of bioactive compounds, as with these choices the extracts also displayed a high antioxidant activity. Furthermore, consuming less extraction solvent is practical from an economic point of view. For this reason, the rice-to-solvent ratio 1 : 15 g/mL was used in validation tests.

3.5 *Stability of physico-chemical properties of the serum formulations*

The extracts were kept at 4°C for 24 h and at 45°C for 24 h in each cycle, for a total of 6 cycles. The pH, viscosity, and antioxidant activity were tested at each cycle, as well as before this heating–cooling test. The serum ingredients and the results are shown in Tables 3 and 4, respectively. The antioxidant activity decreases with the cycle counts becuase temperature affects the chemical structures by breakdown of antioxidants [18]. The serum ingredients formula 3 contains the highest amount of witch hazel that exhibited strong antioxidant as the witch hazel compost of tannins and polyphenols such as gallic acid, which using as a positive control [19]. Subsequent analysis revealed that formula 3 has the highest antioxidant activity from rice extract and witch hazel with suitable viscosity and pH range of 5.25–5.40. Then formula 3 is not susceptible to oxidation and shown more stable for antioxidant than other formulas. This result is related to the study of [20], in which high temperature (maximum 35°C) reduced the active ingredients content to less than half of that in the control berries (maximum 25°C)

Table 3: The main ingredients of serum

Ingredients	Formula 1	Formula 2	Formula 3	Formula 4
Methylcellulose	✓	-	-	✓
Hydroxyethyl cellulose	✓	✓	✓	-
Aloe vera	✓	✓	-	
Witch hazel	✓	✓	✓	-
Rice extract	✓	✓	✓	✓

Table 4: pH, viscosity, and antioxidant activity profiles (mean ± SD) of serum. Each data point is derived from 3 replicates

Formula	pH		Viscosity (cPs)		Antioxidant Activity (%)	
	Before T	After T	Before T	Aftert	Before T	After T
1	5.79± 0.07^{a}	5.94± 0.05^{a}	1,444.33± 1.53^{a}	1,430.00± 6.08^{a}	82.77± 2.05^{c}	79.35± 3.08^{b}
2	5.54± 0.09^{b}	5.65± 0.10^{b}	1,018.22± 1.53^{c}	1,018.00± 13.11^{c}	83.13± 1.12^{b}	79.89± 1.90^{b}
3	5.25± 0.02^{c}	5.40± 0.05^{c}	1,159.67± 2.08^{b}	1,144.00± 9.54^{b}	89.44± 00^{a}	87.28± 1.63^{a}
4	5.20± 0.02^{c}	5.33± 0.06^{d}	605.33± 1.53^{d}	612.67± 1.53^{d}	73.93± 2.19^{d}	68.71± 3.30^{c}

T = Temperature cycle
Different small letters in the same items indicate a significant difference ($p < 0.05$)

4 Conclusions

Germination of Sangyod rice increased contents of biologically active compounds. Total phenolic, total flavonoid, and GABA in extracts increased with germination of rice grains before extraction. The optimal conditions for maximal solvent extraction of bioactive compounds from germinated Sangyod rice were 50%ethanol for 30 min at rice-to-solvent ratio 1 : 15 g/mL. Total phenolic, total flavonoid, and GABA contents and antioxidant activity of the extract were 1,627 ± 53.08 mg GAE/100 g rice, 559.50 ± 17.06 mg QE/100 g rice, 9.97 ± 0.17 mg GABA/100 g rice and 0.16 ± 0.01 mg/mL, respectively. Storage temperature variations affected stability of the extracts. This study suggests that germinated Sangyod rice is a potential sources of natural antioxidants, especially phenolic and flavonoid compounds. This value-added agricultural product could contribute to incomes of rice farmers.

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