Determination of some phenolic compounds from fermented unpolished black rice with anti-tyrosinase activity

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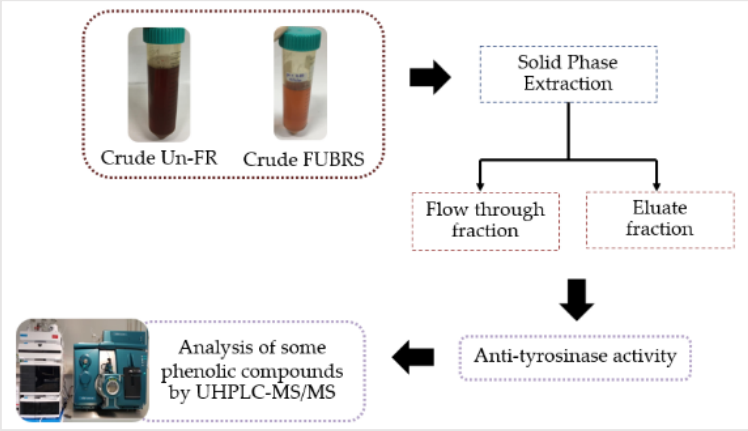
Abstract: Tyrosinase is a key rate-limiting enzyme in melanin synthesis. The phenolic compounds are beneficial to human health that have been reported as tyrosinase inhibitors. Our previous study found that the fermented unpolished black rice sap (FUBRS) could reduce melanin synthesis in B16F10 cell, while such activity could not be detected in the unfermented rice (Un-FR). This study aimed to investigate some phenolic compounds previously reported as tyrosinase inhibitors in the FUBRS by using ultra high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). The results showed that the FUBRS sample showed higher tyrosinase inhibitory activity than Un-FR sample. Analysis of some phenolic compounds by UHPLC-MS/MS revealed top three compounds including protocatechuic acid, vanillic acid and ferulic acid in both FUBRS and Un-FR. However, the level of these compounds was much higher in FUBRS than those in Un-FR. Taken together, we demonstrated that the fermentation of unpolished black rice yielded some phenolic compounds containing anti-tyrosinase activity. The potential compounds in FUBRS having anti-tyrosinase activity will be further investigated.

Graphical abstract:



Keywords: fermented unpolished black rice; phenolic compounds; anti-tyrosinase activity; UHPLC-MS/MS

1. Introduction

Tyrosinase is a key rate-limiting enzyme that can catalyze melanin synthesis. Tyrosinase catalyzes two different reactions: the hydroxylation of monophenols to o-diphenols (monophenolase activity) and the oxidation of o-diphenols to o-quinones (diphenolase activity). Then, o-quinone is transformed into melanin in a series of non-enzymatic reaction [1,2]. The use of tyrosinase inhibitors is the most promising method for inhibiting melanin synthesis [3]. Phenolic compounds are natural components of plant including plant-based food. Recently, polyphenols have been of interesting because of their biological activities which may be beneficial in preventing various diseases, such as ophthalmic disease, cardiovascular disease, diabetes, and aging. Plant secondary metabolites can be divided into three chemically groups. Phenolic compounds is largely secondary product from plant [4]. Fermentation is metabolic process, mainly with the involvement of yeasts, bacteria, and fungi. This process increases the physiological and biochemical activities of biological substrates by modifying their naturally occurring molecules. It has been associated with nutrition-promoting effects for food and is also beneficial for increasing level of the content and biological activity of polyphenolic components in food materials [5, 6]. Rice wine is alcoholic beverage made from rice through fermentation process with unique strain of microorganisms [7]. Fermentation has been accepted that has potential to produce new beneficial compounds or increase bioactive compounds, resulting in the enhancement of biological functions [8]. The enhancement of the bioactive compound production in fermented products has also been observed in recent studies. Soybeans incubated with *Aspergillus oryzae* at 30 ℃ for 48 hr. resulted in a 23-fold increase in genistein aglycone when compared to the content found in unfermented soybean flour [9]. In addition, the amount of aglycones was also found to be higher in solid-state fermentation of soybean with *Rhizopus sp*. compared to unfermented soybeans [10]. Fungal fermentation of whole grain was observed on increased in the sum of phenolic compounds with up to a 382% increase in ferulic acid after fermentation [11]. A similar trend of increase in investigated phenolic compounds and total phenolic compounds during the fermentation of whole grain with *Rhizopus oryzae* RCK2012 had been reported [12]. So, this process has been used in many applications including biotherapeutics, biological materials, and ethanol production [13]. Recently, phenolic compounds have received considerable attention because they play a significant role in the prevention of many chronic diseases due to their antioxidant, anti-inflammatory, anti-aging, antimicrobial and anti-carcinogenic properties [14]. Therefore, phenolic compounds are used in numerous sectors of the food and cosmetic industry as natural additives (natural coloring agents, conservative agents, natural anti-oxidants, nutritional additives) [15]. In our previous study, the fermented unpolished black rice sap (FUBRS) was determined for the inhibitory activity on melanogenesis in B16F10 melanoma cells. Results showed that the expression levels of tyrosinase, tyrosinase relate protein 1 (TYRP-1), TYRP-2 and microphthalmia-associated transcription factor were reduced by FUBRS, while in the unfermented rice did not contain melanogenesis inhibition activity [16]. Therefore, the aims of this present study were to determine chemical compositions in FUBRS with tyrosinase inhibitory activity and identify some phenolic compounds, previously been reported as tyrosinase inhibitors in the FUBRS by using ultra high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS).

2. Materials and Methods

2.1 Chemicals & Reagents

The chemicals were obtained: acetonitrile (HPLC-MS grade from Supelco®), formic acid analytical grade (from Fluka), HCl analytical grade (from Applichem). Nine phenolic compound standards were purchased from Sigma-Aldrich (MO, USA): gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid,

syringic acid, ferulic acid and quercetin. Potassium dihydrogen phosphate and dipotassium hydrogen phosphate trihydrate were analytical grade (from Ajax Finechem Pty Ltd.), mushroom tyrosinase from Sigma-Aldrich (MO, USA), 3-(3,4-Dihydroxyphenyl)-L-alanine (L-DOPA) and kojic acid were analytical grade (from Tokyo Chemical Industry Co., Ltd).

2.2 Preparation of samples

The preparation of FUBRS was perfomed following Sangkeaw et al., 2020. In brief, raw materials used in fermentation process consisted of unpolished black rice (purchased from Green Niche Rice, Thailand.) and the defined microbial starter (received from Dr. Orrarat Sangkeaw Department of Microbiology, Faculty of Science, Chulalongkorn University, Thailand.). For fermented unpolished black rice, 40 g of unpolished black rice was mixed with 80 mL of distilled water and autoclaved at 121℃ for 15 min. Then, the defined starter culture was mixed with cooked unpolished black rice and incubated at 30℃ for 12 days in a closed sterilized bottle. The liquid from the rice fermentation (FUBRS) was collected and kept at -20℃ for further study. For unfermented unpolished black rice, 10 g of unpolished black rice was boiled in 100 mL of distilled water for 10 min. The sample was then clarified by centrifugation at 11000 ˣ g for 15 min. The supernatant (Un-FR) was kept at -20℃ for further study.

2.3 Procedure for Solid Phase Extraction (SPE)

SPE have been the most widely used in phenolic compounds separation [17]. The samples were submitted to SPE using Sep-Pak Vac 1 cc (50 mg) C18 cartridges (purchased from Waters Corporation, Milford, MA, USA). The cartridges were conditioned with 1 mL of methanol and 1 mL of milliQ water, respectively. After that 1 mL of sample of either crude Un-FR or crude FUBRS were loaded into the cartridges. Then the cartridges were washed with 5 mL of milliQ water. This part was collected as first fraction (flow through). Ultimately, the interested analytes were eluted with 5 mL of 0.1% HCl in methanol and collected as second fraction (eluate). Both fractions were evaporated to dryness by rotary evaporator at 40℃ and kept at -20℃ until use [18].

2.4 Anti-tyrosinase activity

The samples of Un-FR and FUBRS were examined for tyrosinase inhibiting activity by using mushroom tyrosinase on dopachrome method. The reaction mixture consists of 80 μL of 50 mM potassium phosphate buffer (pH 6.8), 40 μL of a test sample and 40 μL of mushroom tyrosinase (50U mL^{-1}) Sigma-Aldrich (MO, USA) was mixed and incubated at room temperature for 10 min. Then 40 μL of 1.5 mM L-DOPA was added and incubated at room temperature for 10 min. The amount of dopachrome in the reaction mixture was measured for optical density at 492 nm. Kojic acid was used for positive control [19].

The percent inhibition of tyrosinase activity was calculated as follows:

$$\%\text{inhibition} = (A-B)/A\times 100$$

A = absorbance at 492 nm without test sample, B = absorbance at 492 nm with test sample.

2.5 Optimization of UHPLC-MS/MS conditions and sample analysis

Stock solution of each phenolic compounds was prepared in methanol at 1000 mg/L. These solutions were stored at -20℃ in refrigerator until use. Working standards, a mixture of nine phenolic compounds were prepared by diluting these stock solutions in methanol in a range of 10-400 ng mL^{-1}. LC-MS/MS analysis was performed using an Agilent 1290 Infinity II LC System (Agilent Technologies, USA) connected to a 4500 QTRAP Mass spectrometer with electrospray ionization interface. Chromatographic separation was carried out using a Zorbax Eclipse XDB-C18 column (50 × 2.1 mm, 1.8 μm, Agilent Technologies, USA) at 30℃. A gradient elution of two mobile phases of A and B was used, where A was 0.1%(v/v) formic acid in milliQ water and B was 0.1%(v/v) formic acid in acetonitrile. The suitable gradients profile was applied as the following: 0 min 5% B, 8 min 60%,

8.1-10 min 5% B. The flow rate was set to 0.3 mL min^{-1} and the injection was 3 μL [20]. For optimization, a standard solution at 10 ng L^{-1}, the MS/MS condition was performed with the following setting: certain gas (CUR), 20; IonSpray voltage (IS), -4500V; temperature (TEM), 500℃; ion source gas 1 (GS1), 60; ion source gas 2 (GS2), 50. Some phenolic compounds in Un-FR and FUBRS were determined.

2.6 Statistical analysis

All measurement were carried out in 3 replicate and the data were expressed as mean ± SD. Statistic were performed with one-way ANOVA tests using GraphPad prism 8.0.1 for tyrosinase inhibitory activity and sample analysis.

3. Results

3.1. Effect of fermented unpolished black rice on anti-tyrosinase activity

Tyrosinase is the major enzyme in melanin production. The anti-tyrosinase activity assay was used to determine the inhibitory effect of Un-FR and FUBRS on tyrosinase activity as shown in Figure 1. Both Un-FR and FUBRS were subjected to fractionation using C18 cartridge, resulting in flow through and eluate fractions. Mushroom tyrosinase and each sample were incubated with L-DOPA and measured for the formation of dopachrome. The results showed that the samples from FUBRS (crude and flow through) showed higher tyrosinase inhibitory activity than those from Un-FR (crude and flow through). Among samples from FUBRS, crude and flow through showed the highest tyrosinase inhibitory activity 94.4 ± 0.8% and 95.2 ± 1.7%, respectively. Whereas sample from Un-FR, crude, flow through and eluate showed lower tyrosinase inhibitory activity as 78.2 ± 1.9%, 56.0 ± 1.9% and 70.9 ± 3.9%, respectively. In addition, the results revealed that flow through fraction of FUBRS did not show significant difference from those of crude. Eluate fraction of Un-FR were found to inhibit the tyrosinase activity higher than that of FUBRS.

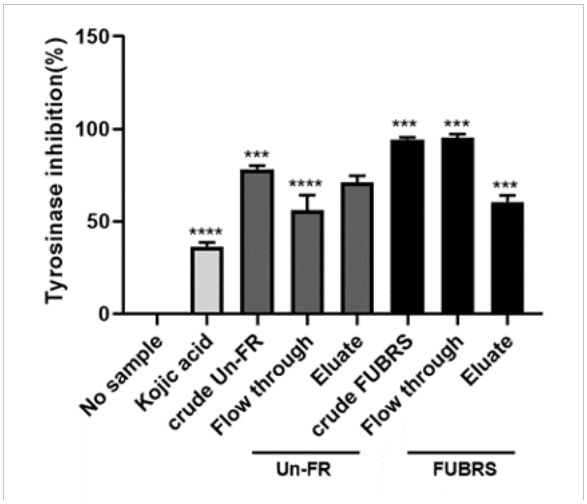


Figure 1. Anti-tyrosinase activity of Un-FR, FUBRS and their chromatographic fractions. The sample of FUBRS, Un-FR and their fractions (flow through and eluate) were determined on anti-tyrosinase activity. Kojic acid was used as a positive control. Data were shown as the mean ± SD from three independent experiments performed in triplicate. Statistically significant differences compared with Un-FR are indicated by ****$p < 0.0001$, *** $p < 0.001$.

3.2. Optimization of MS/MS

The suitable parameters for MS/MS analysis of some phenolic compounds were optimized to obtain product ion in MRM negative mode as shown in Table 1.

Table 1. Overall optimal MS/MS parameters

Compounds	Molecular ion		Q1/Q2	Product ion	Time (msec)	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
	From	m/z							
Gallic acid	$[M+H]^-$	169.0	Q1	124.2	50	-33.1	-12.1	-29.25	-8.07
			Q2	78.9					
Protocatechuic acid	$[M+H]^-$	152.9	Q1	108.0	50	-63.68	-7.38	-29.23	-6.54
			Q2	108.0					
p-Hydroxybenzoic acid	$[M+H]^-$	137.0	Q1	92.9	50	-45.9	-2.85	-35.13	-6.06
			Q2	64.9					
Vanillic acid	$[M+H]^-$	166.9	Q1	107.9	50	-66.34	-12.0	-24.1	-9.0
			Q2	91.0					
Caffeic acid	$[M+H]^-$	178.9	Q1	133.8	50	-78.04	-8.7	-38.8	6.07
			Q2	109.0					
Syringic acid	$[M+H]^-$	196.9	Q1	167.0	50	-4.09	-4.59	-29.89	7.86
			Q2	123.2					
p-Coumaric acid	$[M+H]^-$	162.9	Q1	119.1	50	-16.09	-7.67	-38.09	-12.01
			Q2	93.2					
Ferulic acid	$[M+H]^-$	192.9	Q1	133.1	50	-42.15	-4.09	-37.88	-12.0
			Q2	89.2					
Quercetin	$[M+H]^-$	300.69	Q1	150.9	50	-107.0	-9.0	-31.0	-12.0
			Q2	121.0					

DP: Declustering potential; EP: Entrance potential; CE: Collision energy; CXP: Collision exit potential

3.3. Quantitative analysis of some phenolic compounds in the Un-FR and FUBRS using UHPLC-MS/MS.

Ultra-high performance liquid chromatography (UHPLC) is a technique used for fast separation of the components in the mixture as well as detection of the analytes at low levels. UHPLC was combined with high resolution tandem mass spectrometer (MS/MS) [21]. For quantification, calibration curve was plotted between signals and concentrations. The amounts of some phenolic compounds in the crude Un-FR and crude FUBRS under optimal conditions of MS/MS were listed in Table 2. Gallic acid could not be detected in both Un-FR and FUBRS analyzed. Three most abundant compounds in the FUBRS were protocatechuic acid of 7,507 ± 10.3 ng mL^{-1}, vanillic acid of 766.7 ± 88.1 ng mL^{-1} and ferulic acid of 167 ± 8.5 ng mL^{-1}, respectively. The same three most abundant compounds were also detected in crude Un-FR but with much lower amounts; protocatechuic acid of 4,631 ± 173.0 ng mL^{-1}, vanillic acid of 305 ± 32.5 ng mL^{-1} and ferulic acid of 23 ± 0.5 ng mL^{-1}, respectively. Other compounds including *p*-hydroxybenzoic acid, caffeic acid, syringic acid, *p*-coumaric acid were found in both crude Un-FR and crude FUBRS. However, caffeic acid, syringic and quercetin were not found in crude Un-FR. For using C18 cartridge for the isolation of the phenolic compounds in Table 3, it was found that gallic acid could not be detected in Un-FR, FUBRS and their fractions. It was noteworthy that quercetin could be found in eluate fraction of FUBRS. However, ferulic acid was found in eluate of Un-FR (1,569 ± 24.5 ng mL^{-1}) much higher than that in the eluate of FUBRS (90 ± 3.8 ng mL^{-1}). In addition, the level of phenolic compounds (except for gallic acid and quercetin) in flow through fraction of Un-FR was higher than those in the flow through of the FUBRS.

Table 2. The amounts of some phenolic compounds in the crude Un-FR and crude FUBRS.

Compounds	**Amounts (ng mL^{-1})**	
	Crude	
	Un-FR	**FUBRS**
Gallic acid	nd	nd
Protocatechuic acid	4,631 ± 173.0	7,507 ± 10.3****
p-Hydroxybenzoic acid	12 ± 1.2	106 ± 2.2****
Vanillic acid	305 ± 32.5	766.7 ± 88.1****
Caffeic acid	nd	153 ± 9.4
Syringic acid	nd	11 ± 0.6
p-Coumaric acid	13 ± 0.3	7.0 ± 0.1****
Ferulic acid	23 ± 0.5	167 ± 8.5****
Quercetin	nd	nd

Data were shown as the mean ± SD from three independent experiments. Statistically significant differences compared with Un-FR are indicated by ****$p < 0.0001$; nd: not detected

Table 3. The amount of some phenolic compounds in the Un-FR and FUBRS using solid phase extraction

Compounds	**Amounts (ng mL^{-1})**			
	Un-FR		**FUBRS**	
	Flow through	**eluate**	**Flow through**	**eluate**
Gallic acid	nd	nd	nd	nd
Protocatechuic acid	34,726 ± 667.1	5,622 ± 140.8	105.4 ± 3.7****	14,642 ± 203.5****
p-Hydroxybenzoic acid	33 ± 0.6	14 ± 1.3	nd	193 ± 0.5****
Vanillic acid	204 ± 26.1	nd	11 ± 1.8****	305 ± 51.0
Caffeic acid	20 ± 0.3	nd	nd	159 ± 3.1
Syringic acid	104 ± 4.5	13 ± 1.1	nd	237 ± 23.6****
p-Coumaric acid	26 ± 1.3	10 ± 0.1	13.5 ± 0.1****	337 ± 4.57****
Ferulic acid	42 ± 2.3	1,569 ± 24.5	nd	90 ± 3.8****
Quercetin	nd	nd	nd	12 ± 0.4

Data were shown as the mean ± SD from three independent experiments. Statistically significant differences compared with flow through and eluate of Un-FR are indicated by ****$p < 0.0001$; nd: not detected

4. Discussion

In this study, FUBRS, Un-FR and their fractions were tested on anti-tyrosinase activity. The result indicated that the fermentation led to produce more bioactive compounds resulting in enhancement of various biological activities responsible for the inhibition of tyrosinase activity [22] and the level of some phenolic compounds were decreased or increased during black rice fermentation using *Saccharomyces cerevisiae* ATCC 9763 [23]. The Sep-pak C18 cartridge contains silica-base bonded phase that used to adsorb analytes of weak hydrophobicity from aqueous solution [24]. Thus, high polarity compounds such as sugar, protein and amino acid could not be bound on its surface. Overall data revealed that the fermentation process of FUBRS caused an increased anti-tyrosinase compounds with high polarity.

A variety of some phenolic compounds was previously reported as tyrosinase inhibitors (gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid and quercetin) [25, 26]. Analysis of some phenolic compounds of Un-FR, FUBRS and their fractions using UHPLC-MS/MS has shown that three most abundant compounds in the FUBRS were protocatechuic acid, vanillic acid and ferulic acid, respectively same as Un-FR. The level of bioactive compounds was reported to increase in fermentation process including alcohols, sugars and enzymes depended on several factors [27]. Quercetin-3-O-galactoside and quercetin-3-O-rhamnoside were enriched by C18 cartridge [28]. The level of phenolic compounds in FUBRS eluate was higher than that in the eluate of Un-FR suggesting that fermentation process could enhance the production of phenolic content by changing the free phenolic acids composition [29]. Ferulic acid was found in eluate of Un-FR much higher than that in the eluate of FUBRS. It has been reported that rice bran fermented with *Aspergillus oryzae* or *Aspergillus awamori* could increase the ferulic acid content, reached maximum on the third day and decreased on the fourth day, the fungal enzymes remained release to synthesize ferulic acid from rice polysaccharides during initial 3 days after that it was decomposed to insoluble polysaccharides [30]. Therefore, the ferulic acid composition in FUBRS after fermentation for 12 days might be decreased. However, pH of Un-FR and FUBRS were approximately 3 and 6 respectively, this might affect the chromatographic efficiency of C18 sorbent because optimal pH of sample being separated by C18 cartridge should be at a pH of 3 [31].

Several studies have already reported that the phenolic compounds possess tyrosinase inhibitory activity. Result in this study demonstrated that the eluate fraction of FUBRS contained lower anti-tyrosinase activity than that of Un-FR (Fig 1). This result was inconsistent with the amount of total phenolic compounds in FUBRS which showed higher level than that in Un-FR (Table 3). This may be influenced by the strong tyrosinase inhibitory activity of ferulic acid [32] that was higher amount in the eluate fraction of Un-FR than that of FUBRS (Table 3). Conversely, the anti-tyrosinase activity in the flow through fraction of FUBRS was found higher than that in the flow through fraction of Un-FR (Fig. 1). This might possibly due to the increase in high polarity compounds, such as sugar and a variety of secondary metabolites being converted from starch by the microorganisms in the starter during fermentation, which contributed to increase anti-tyrosinase activity [33].

5. Conclusions

In this study, FUBRS and Un-FR were fractionated and the anti-tyrosinase activity was monitored using mushroom tyrosinase. Moreover, some phenolic compounds previously been reported as tyrosinase inhibitors were detected using UHPLC-MS/MS. The results indicated that the fermentation process not only increased the level of phenolic compounds (gallic acid, protocatechuic acid, *p*-Hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, *p*-Coumaric acid, ferulic acid) but also produced more kinds of phenolic compounds in FUBRS that could not detected in Un-FR (ie. quercetin). These phenolic compounds in FUBRS may lead to the increase in anti-tyrosinase activity. In further study, all compounds in FUBRS involved in melanogenesis inhibition will be investigated.

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