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Comparative Evaluation of Different Extraction Techniques and Solvents for the Assay of Phytochemicals and Antioxidant Activity of Hashemi Rice Bran

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Abstract: Secondary metabolite contents (total phenolic, flavonoid, tocopherol, and tocotrienol) and antioxidant activities of Hashemi rice bran extracts obtained by ultrasound-assisted and traditional solvent (ethanol and 50:50 (*v/v*) ethanol-water) extraction techniques were compared. Phenolic and, flavonoid compounds were identified using ultra-high performance liquid chromatography and method validation was performed. Significant differences ($p < 0.05$) were observed among the different extraction techniques upon comparison of phytochemical contents and antioxidant activities. The extracts obtained using the ethanol-water (50:50 *v/v*) ultrasonic technique showed the highest amounts of total phenolics (288.40 mg/100 g dry material (DM)), total flavonoids (156.20 mg/100 g DM), and total tocotrienols (56.23 mg/100 g DM), and the highest antioxidant activity (84.21% 1,1-diphenyl-2-picrylhydrazyl (DPPH), 65.27% β-carotene-linoleic bleaching and 82.20% nitric oxide scavenging activity). Secondary metabolite contents and antioxidant activities of the rice bran extracts varied depending of the extraction method used, and according to their effectiveness, these were organized in a decreasing order as follows: ethanol-water (50:50 *v/v*) ultrasonic, ethanol-water (50:50 *v/v*) maceration, ethanol ultrasonic and ethanol maceration methods. Ferulic, gallic and chlorogenic acids were the most abundant phenolic compounds in rice bran extracts. The phytochemical constituents of Hashemi rice bran and its antioxidant properties provides insights into its potential application to promote health.

1. Introduction

Rice (*Oryza sativa* L.) is the, most important cereal crop in the world and, is the staple food for about half of the world's population. Like other cereal grains rice is rich in nutrient components such as carbohydrates, proteins, certain fatty acids, and micronutrients (vitamins and trace minerals) [1,2]. In addition, rice is a source of many, bioactive compounds and phytochemicals, known as antioxidants, including phenolic compounds [3–5]. During rice milling, rice bran, is produced as a by-product which is reported to be an excellent source of minerals and vitamins [6]. Rice bran has a, high nutritive value and beneficial health effects such as blood cholesterol lowering, laxative effect, and reducing the incidence of atherosclerosis disease [7,8]. Polyphenols are the most important group of phytochemicals [9,10] as they exhibit health-promoting properties, including protective effects against cardiovascular diseases [11], antioxidant properties [12,13], and anti-inflammatory [14] and anticancer activities [15,16]. Blood lipid and glucose reduction, and enhanced human immunity due to intake of flavonoids have been reported in several studies [17,18]. Rice is a good source of phenolic, compounds [19,20]. The phenolic compounds commonly present in whole grains are phenolic acids and flavonoids. In whole grains, gallic, caffeic, ferulic, vanillic, syringic, cinnamic, and protocatechuic acids were reported as common phenolic acids [21]. Gallic [22], ferulic [23], caffeic [24], and cinnamic acids [25] have been reported to be natural antioxidants, which are free radical scavengers and protect the human body from the effectives of oxidative stress. In a recent study, ferulic and *p*-coumaric acids were identified as the most abundant phenolic acids in bran of most rice varieties [26]. Isolation and identification of polyphenols from plants, herbs, and spices among others is mostly dependent on the extraction solvent and technique used. Several extraction techniques have previously been reported in order to extract phenolic, compounds from plant materials such as microwave [27] and ultrasound-assisted methods [28], supercritical fluid extraction methods [29], the shake-flask, technique [29], reflux [30] and Soxhlet extractions [31]. Hashemi rice is a popular rice variety in the north of Iran where most people use this rice for cooking. To the best of our knowledge, there, is little information regarding the phenolic compounds found in Hashemi rice bran and their potential antioxidant activity. Furthermore, extraction and ultra-high performance liquid chromatography (UHPLC) analysis techniques have not been developed for this rice variety. Thus, the aim of this study was to investigate the extraction efficiency of phenolic compounds and flavonoids from Hashemi rice bran, validate the corresponding UHPLC method, and characterize their antioxidant activity.

2. Results and Discussion

2.1. Total Phenolic and Flavonoid Contents

The results from the present study showed that aqueous ethanol extracts had higher total phenolic (TP) and flavonoid (TF) contents than absolute ethanol extracts. Significant differences ($p < 0.05$) were observed depending on the solvent and extraction technique used for TP and TF contents. As shown in

Table 1, ultrasonic rice bran extraction using ethanol-water (50:50 *v/v*) gave the highest content of phenolic compounds (288.40 mg/100 g dry material (DM)), followed by the ethanol-water (50:50 *v/v*) maceration (270.51 mg/100 g DM), ethanol ultrasonic (246.34 mg/100 g DM), and ethanol maceration (221.06 mg/100 g DM) extractions. The total flavonoid contents of Hashemi rice bran ranged from 156.20 to 108.50 mg/100 g DM. Like the TP content, the highest TF content was observed when the ultrasonic-assisted rice bran extraction using ethanol-water (50:50 *v/v*) was used. This may be related to the fact that polyphenols are more soluble in more polar solvents such as aqueous ethanol than in less polar solvents such as absolute ethanol [32,33]. In addition, the mixture viscosity decreased because of the presence of water, which might have improved the mass transfer. The result of a recent study showed that Hashemi rice bran represented the highest contents of phenolic and flavonoid compounds, with respective values of 3.95 and 0.85 mg/g DM, compared to Ali Kazemi, Neda, Binam and Shirodi varieties [34].

Table 1. Total phenolic, total flavonoid and total tocopherol content of Hashemi rice bran extract with different extraction technique.

Extraction Solvent/Technique	TPC (mg/100 g DM)	TFC (mg/100 g DM)	Total Tocopherol (mg/100 g DM)	Total Tocotrienols (mg/100 g DM)
Ethanol maceration	221.06 ± 10.63 [d]	108.50 ± 10.01 [c]	38.11 ± 2.04 [a]	46.54 ± 2.92 [c]
Ethanol-water (50:50) maceration	270.51 ± 11.47 [b]	137.15 ± 12.89 [b]	36.93 ± 2.26 [a]	55.83 ± 1.85 [a]
Ethanol ultrasonic	246.34 ± 12.26 [c]	112.60 ± 13.65 [c]	37.08 ± 2.21 [a]	51.28 ± 2.80 [b]
Ethanol-water (50:50) ultrasonic	288.40 ± 14.35 [a]	156.20 ± 10.69 [a]	37.51 ± 2.05 [a]	56.23 ± 2.37 [a]

TPC: total phenolic content; TFC: total flavonoid content; Data are means of triplicate measurements ± standard deviation. Means not sharing a common single letter for each measurement were significantly different at $p < 0.05$.

The use of ultrasonic ethanol-water (50:50 *v/v*) extraction, due to the effect of the solvent properties on cavitational bubbles which provide force to collapse plant tissues during rice bran extraction, can result in significant differences in phenolic compound contents compared with other extraction methods. In the extraction method using the ethanol-water (50:50 *v/v*) mixture, ethanol (higher vapor pressure) produces more bubbles than water (lower vapor pressure). Moreover, the surface tension of the liquid is another feature that contributes to the formation of cavitational bubbles. In liquids with lower surface tension, cavitational bubbles are created more easily because the ultrasonic energy applied can more easily exceed the surface tension. Thus, the ethanol-water (50:50 *v/v*) mixture was more effective in phenolic compound extraction and can apply a greater force to plant tissues. If we consider liquid viscosity, liquids with low viscosity are more effective because the ultrasonic energy can more easily overcome the molecular forces of low viscosity liquids. In addition, low viscosity liquids can also easily penetrate plant tissues due to their low density and high diffusivity. Therefore, in the ultrasonic extraction the ethanol and water mixture can help to extract more phenolic contents from Hashemi rice bran, so ethanol-water (50:50 *v/v*) ultrasonic extraction was selected for future experiments for the isolation of phenolic acids and flavonoids.

2.2. Total Tocopherol and Tocotrienol Contents

Tocopherol and tocotrienol are commonly known as vitamin E and are the main antioxidants present in rice bran [35]. Tocopherol and tocotrienol constitute a series of related benzopyranols that are produced in plant tissues and are powerful lipid-soluble antioxidants. Considering total tocopherol content, ethanol maceration was the most effective extraction method (38.11 mg/100 g DM) when compared with other extraction methods, including ethanol-water (50:50 *v/v*) ultrasonic (37.51 mg/100 g DM), ethanol ultrasonic (37.08 mg/100 g DM), and ethanol-water (50:50 *v/v*) maceration (36.93 mg/100 g DM) extractions (Table 1). However, there were no significant differences in total tocopherol contents among the various extraction methods used. A high content of total tocotrienols (56.23 mg/100 g DM) was observed when the ethanol-water (50:50, *v/v*) ultrasonic extraction method was used. Significant differences ($p < 0.05$) were observed when the effectivity of the different extraction methods and solvents was assessed regarding tocotrienol extraction. A recent study showed that tocotrienols (specifically γ-tocotrienol) are more abundant in rice bran than tocopherols [36]. In addition, higher vitamin E contents were found in rice bran than in whole rice and milled rice [35,37]. The extraction procedure, especially the extracting solvent, contributes to the amount of phytochemicals that can be recovered from different samples [32,33]. The results of the present study showed that solvent polarity has an important role in the extraction of total tocotrienols. Thus, the more polar solvents, such as the ethanol-water (50:50 *v/v*) mixture, extracted more vitamin E compared with absolute ethanol, a result that is consistent with those from previous studies [38,39].

2.3. Antioxidant Activity of Hashemi Rice Bran Extracts

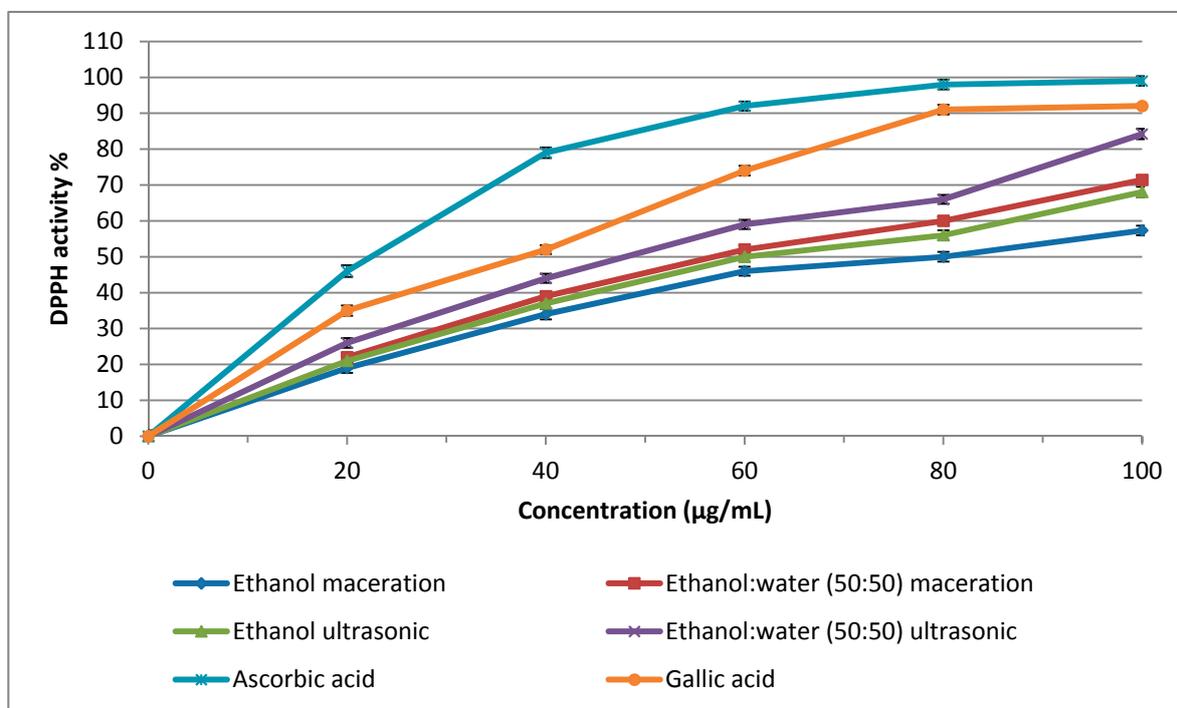### 2.3.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Assay

The DPPH radical scavenging activities of Hashemi rice bran extracts obtained through different extraction methods are shown in Table 2 and Figure 1. Significant differences ($p < 0.05$) were observed among the extracts obtained through different extraction methods for DPPH radical scavenging activity. The DPPH radical scavenging activity of the extracts was ranked in the following order: ethanol-water (50:50 *v/v*) ultrasonic (84.21%), ethanol-water (50:50 *v/v*) maceration (71.41%), ethanol ultrasonic (68.05%), and ethanol maceration (57.33%) methods. No significant difference was observed between the ethanol ultrasonic and the ethanol maceration methods. As shown in Figure 1, a half maximal inhibitory concentration (IC_{50}) of 47.0 μg/mL was observed for the ethanol-water (50:50 *v/v*) ultrasonic extraction technique. The IC_{50} values for the ethanol ultrasonic, ethanol-water (50:50 *v/v*) maceration, and ethanol maceration extraction methods were 56.2, 60.8, and 64.0 μg/mL, respectively. Gallic acid and ascorbic acid, with IC_{50} values of 37.2 and 24.6 μg/mL, respectively, were used as a positive control. As shown in Figure 1, the radical scavenging DPPH activities of the Hashemi rice bran extracts were lower than those of gallic acid and ascorbic acid. In the current study, by using the ultrasonic extraction method and ethanol-water (50:50 *v/v*) solvent, the IC_{50} value of Hashemi rice bran (47.0 μg/mL) was improved in comparison to a previous study which reported an IC_{50} value of 169.86 μg/mL using a different extraction technique (reflux) and solvent (methanol) for extraction [34]. Previous studies have reported that the reducing power of phenolic compounds is higher than that of α-tocopherol [40]. Furthermore, phenolic compounds exhibit four times higher antioxidant activities than γ-oryzanol [41]

and α-tocopherol [42,43]. γ-oryzanol has also been reported to have a higher antioxidant activity than tocopherols (almost 10 times higher), while tocopherols show antioxidant activities approximately 40–60 times lower than those of tocotrienols [41,44]. In the present study, the highest contents of tocotrienols and phenolic compounds were observed when the ethanol-water (50:50 *v/v*) ultrasonic extraction technique was used, and the high antioxidant activity of the Hashemi rice bran extracts obtained with this technique could be related to the high levels of phenolics and tocotrienols. The DPPH radical scavengi.ng assay is an important method to determine the antioxidant activity of plant extracts, but it cannot provide enough information regarding the antioxidant activities of phenolic extracts in food items. Consequently, the antioxidant activity of the extracts was measured using the β-carotene bleaching assay.

Table 2. Effects of extracting solvent/technique on the antioxidant activity of Hashemi rice bran extracts (100 µg/mL), using three different methods.

Extraction Solvent/Technique	DPPH Assay (%)	β-Carotene-linoleic Acid Bleaching Assay (%)	Nitric Oxide Scavenging Activity (%)
Ethanol maceration	57.33 ± 3.51 [d]	47.23 ± 2.55 [c]	46.20 ± 3.78 [d]
Ethanol-water (50:50) maceration	71.41 ± 2.84 [b]	53.67 ± 1.94 [b]	74.50 ± 2.56 [b]
Ethanol ultrasonic	68.05 ± 1.55 [c]	54.76 ± 3.17 [b]	58.30 ± 2.44 [c]
Ethanol-water (50:50) ultrasonic	84.21 ± 3.84 [a]	65.27 ± 2.73 [a]	82.20 ± 2.69 [a]

Data are means of triplicate measurements ± standard deviation. Means not sharing a common single letter for each measurement were significantly different at $p < 0.05$.


Figure 1. Free radical scavenging activity (DPPH assay) of the different extract of Hashemi rice bran. Error bars represent standard errors of the mean ($n = 3$).

2.3.2. β-Carotene Bleaching Assay

In this method, peroxyl radicals are made by oxidation of linoleic acid and these radicals oxidize unsaturated β-carotene. Thus, if antioxidants are present in the evaluated sample, β-carotene degradation is reduced. Therefore, the amount of decomposed β-carotene is related to the antioxidant activity of the extract [45,46]. The effects on β-carotene oxidation of Hashemi rice bran extracts obtained using different extraction methods are shown in Table 2. It is obvious that extracts can scavenge free radicals from the heterogeneous medium. As shown in Table 2, the ethanol-water (50:50 *v/v*) ultrasonic method was the most effective extraction method in preserving the antioxidant activity of rice bran extracts (65.27%) followed by the ethanol ultrasonic (54.76%), ethanol-water (50:50 *v/v*) maceration (53.67%), and ethanol maceration (47.23%) methods. Ascorbic acid showed the highest oxidation inhibition (89.16%). Therefore, we focused on the use of the ethanol-water (50:50 *v/v*) ultrasonic method to obtain Hashemi rice bran extracts due to the high amounts of total phenolic and tocopherol contents, high radical scavenging activity, and high β-carotene bleaching inhibition. A linear correlation between the antioxidant activity and polyphenolic contents has been reported as variable ranges in different plants [47–49]. Arab *et al.* [50] reported that Fajr rice bran with high total phenolic content showed high antioxidant activity measured using the DPPH radical scavenging assay.

2.3.3. Nitric Oxide Scavenging Activity

As shown in Table 2 and Figure 2, the highest nitric oxide scavenging activity (82.20 μg/mL) with an IC_{50} value of 58.25 μg/mL was observed when the ethanol-water (50:50 *v/v*) ultrasonic extraction technique was used. The IC_{50} values of the ethanol ultrasonic, ethanol-water (50:50 *v/v*) maceration, and ethanol maceration extraction methods were 70.15, 93.20, and 112.85 μg/mL, respectively (Figure 2). Gallic acid and ascorbic acid showed an IC_{50} value of 27.5 and 15.90 μg/mL, respectively. Excess nitric oxide, which is known to accumulate in the acidic environment of the stomach, reacts with oxygen to form nitrite ions and induce mutagenic reactions [51]. Thus, the nitric oxide produced must be scavenged from the human body and previous studies have shown that phenolic compounds have a great nitrite scavenging activity [52]. In the present study, we used three different antioxidant assays and the results of all three assays showed that the ethanol-water (50:50 *v/v*) ultrasonic extraction method resulted in the highest antioxidant activity. In addition, secondary metabolites analysis showed that the ethanol-water (50:50 *v/v*) ultrasonic extraction method produced the highest TP, TF, and total tocotrienols content values. Phenolic acids and flavonoids are phytochemicals and act as potent antioxidant agents. Consequently, the highest antioxidant activity recorded in extracts obtained using the ethanol: water (50:50 *v/v*) ultrasonic extraction method might be related to high secondary metabolite contents. A correlation analysis was carried out in order to test this hypothesis.
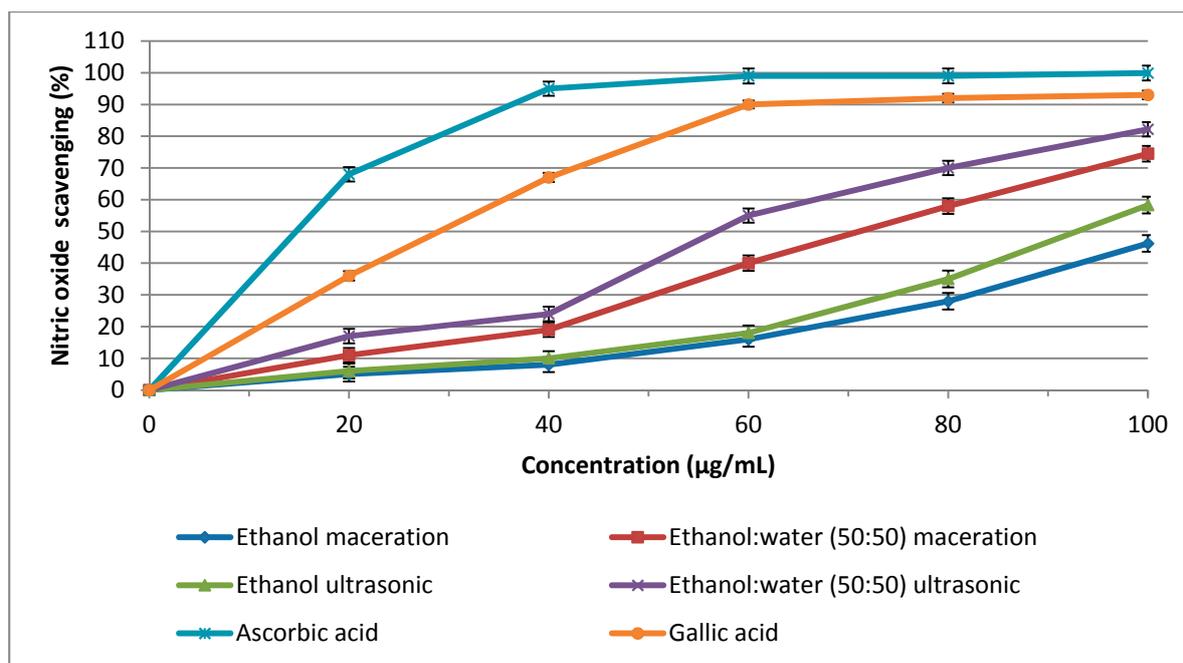
Figure 2. Nitric oxide scavenging activity of Hashemi rice bran extracts from different extraction methods. Error bars represent standard errors of the mean (*n* = 3).

2.4. Correlation Analysis

Table 3 shows the intercorrelations among the different measurements carried out in Hashemi rice bran extracts. A significant (*p* < 0.01) positive correlation was observed between TP, TF, DPPH, and nitric oxide scavenging activities. In addition, a positive and significant (*p* < 0.01) correlation was found between total tocotrienols, DPPH, and nitric oxide scavenging activities.

Table 3. Correlation analysis between secondary metabolites and antioxidant activity of Hashemi rice bran.

		1	2	3	4	5	6	7
1	TP	1						
2	TF	0.825 **	1					
3	Total tocopherol	0.526	0.663	1				
4	Total tocotrienols	0.792 *	0.677	0.546	1			
5	DPPH	0.925 **	0.911 **	0.946 **	0.988 **	1		
6	β-Carotene-linoleic acid bleaching	0.668	0.871 *	0.719	0.844 *	0.612	1	
7	Nitric oxide scavenging activity	0.950 **	0.936 **	0.812 *	0.969 **	0.905 **	0.746	1

* = significant at *p* < 0.05; ** = significant at *p* < 0.01.

In the present study, no significant correlation (*p* > 0.05) was found between β-carotene bleaching assay, TP, and total tocopherol content. The β-carotene bleaching assay only showed a significant correlation (*p* < 0.05) with TF and total tocotrienols. Correlation coefficients and regression analyses showed that phenolic compounds, tocopherols, and tocotrienols were responsible for the antioxidant activity in the Hashemi rice bran extracts. A positive and significant correlation, between polyphenols and antioxidant activity has already been reported in several previous studies [9,28,53,54].

2.5. Phenolic Acid and Flavonoid Composition of Hashemi Rice Bran Extracts

Extracts obtained using the ethanol-water (50:50, *v/v*) ultrasonic method were chosen for phenolic and flavonoid profiling because they showed the highest phenolic and flavonoid contents when compared with other extraction methods. As shown in Table 4, Hashemi rice bran is a good, source of phenolic compounds.

Table 4. Concentration of identified phenolic acids and flavonoids and their recovery test in Hashemi rice bran extract.

Phenolic Acids	Concentration in Sample (mg/100 g DM)	Standard Added (mg)	Recovery		Recovery (%)	RSD (%)
			Expected	Actual		
Gallic acid	11.56 ± 0.88	1.00	12.56	12.16 ± 0.26	96.81	2.13
	11.56 ± 0.88	2.50	14.06	13.68 ± 0.37	97.30	2.70
	11.56 ± 0.88	5.00	16.56	16.24 ± 0.24	98.07	1.47
Protocatechuic acid	6.72 ± 0.16	1.00	7.72	8.80 ± 0.10	113.99	1.13
	6.72 ± 0.16	2.50	9.22	10.12 ± 0.15	109.76	1.48
	6.72 ± 0.16	5.00	11.72	11.95 ± 0.13	101.96	1.08
Syringic acid	10.39 ± 0.11	1.00	11.39	10.55 ± 0.075	92.63	0.71
	10.39 ± 0.11	2.50	12.89	11.47 ± 0.03	88.98	0.26
	10.39 ± 0.11	5.00	15.39	16.42 ± 0.09	106.69	0.54
Chlorogenic acid	11.12 ± 0.28	1.00	12.12	11.80 ± 0.21	97.36	1.77
	11.12 ± 0.28	2.50	13.52	14.19 ± 0.18	104.96	1.26
	11.12 ± 0.28	5.00	16.12	15.33 ± 0.17	95.10	1.10
Caffeic acid	10.59 ± 0.16	1.00	11.59	10.64 ± 0.09	91.80	0.84
	10.59 ± 0.16	2.50	13.09	12.77 ± 0.11	97.56	0.86
	10.59 ± 0.16	5.00	15.59	16.36 ± 0.07	104.94	0.42
Ferulic acid	12.28 ± 0.69	1.00	13.28	12.91 ± 0.16	97.21	1.23
	12.28 ± 0.69	2.50	14.78	15.10 ± 0.19	102.17	1.25
	12.28 ± 0.69	5.00	17.28	16.52 ± 0.23	95.60	1.39
Cinnamic acid	8.23 ± 0.86	1.00	9.23	10.66 ± 0.14	115.49	1.31
	8.23 ± 0.86	2.50	10.73	11.91 ± 0.17	111.00	1.42
	8.23 ± 0.86	5.00	13.23	12.46 ± 0.18	94.18	1.44
Apigenin	2.65 ± 0.52	0.25	2.90	3.35 ± 0.12	115.51	3.58
	2.65 ± 0.52	0.50	3.15	3.60 ± 0.09	114.28	2.50
	2.65 ± 0.52	1.00	3.65	3.40 ± 0.06	93.15	1.76
Catechin	4.28 ± 0.88	0.50	4.78	4.20 ± 0.10	87.86	2.38
	4.28 ± 0.88	1.00	5.28	5.00 ± 0.11	95.23	2.20
	4.28 ± 0.88	2.00	6.28	5.85 ± 0.13	93.15	2.22
Quercetin	1.36 ± 0.22	0.25	1.61	1.50 ± 0.01	93.16	0.66
	1.36 ± 0.29	0.50	1.86	1.62 ± 0.03	87.09	1.85
	1.36 ± 0.18	1.00	2.36	2.46 ± 0.04	104.23	1.62

In the present study, seven phenolic acids (gallic, protocatechuic, syringic, chlorogenic, caffeic, ferulic, and cinnamic acids) and three flavonoids (apigenin, catechin, and quercetin) were detected in the extracts. The results showed that ferulic acid (12.28 mg/100 g D.W), gallic acid (11.56 mg/100 g DM), and chlorogenic acid (11.12 mg/100 g DM) were the most abundant phenolic acids within the identified

compounds. Three flavonoid compounds, namely apigenin, catechin, and quercetin, were identified in rice bran extracts at concentrations of 2.65, 4.28, and 1.36 mg/100 g DM, respectively. The rest of the phenolic compounds were present in the following order of decreasing concentration: ferulic, gallic, chlorogenic, caffeic, syringic, and cinnamic acids. Recently other flavonoids have been identified in other rice varieties, including naringenin [55], luteolin, apigenin [56], rutin [57], and myricetin [58].

2.6. Validation Method

A recovery study, of extracts from rice bran was carried out, by adding different spike levels (low, medium, and high) of phenolic and flavonoid standards in order to evaluate, the accuracy of the analytical methods. The mean recovery was 97.30%–105.19%, 101.96%–113.99%, 88.98%–106.69%, 95.10%–104.96%, 91.80%–104.94%, 95.60%–102.17%, 94.18%–115.49%, 93.15%–115.51%, and 87.86%%–95.23% for gallic, protocatechuic, syringic, chlorogenic, caffeic, ferulic, cinnamic acids, and for apigenin, and catechin, respectively. The %RSD of the average recovery was 2.11%, 1.24%, 0.51%, 1.39%, 0.90%, 1.30%, 1.40%, 2.61%, and 2.26% for gallic, protocatechuic, syringic, chlorogenic, caffeic, ferulic, cinnamic acids, and for apigenin, and catechin, respectively. The low RSD values indicated that UHPLC system was suitable. The observed variation confirmed the robustness of the analysis system. A calibration curve was constructed to assess the linearity between the six concentrations of each phenolic compound and the corresponding peak area of the UHPLC methods. The limit of detection (LOD) is usually defined as the lowest quantity or concentration of a component that can be reliably detected with a given analytical method, but do not have to quantitate as an appropriate value. The limit of quantification (LOQ) is usually defined as the lowest sample concentration which can still be quantitatively detected with accuracy and an acceptable precision. In current study, LOQ, and LOD ranged from 0.2–0.4 (µg/mL) and 0.01–0.5, respectively.

Table 5 Analytical characteristics for determination of phenolic compounds.

Compounds	Regression Equation (y = ax ± b)	R^2	LOD (µg/mL)	LOQ (µg/mL)
Gallic acid	y = 1846.5x − 5.2530	0.9954	0.50	0.25
Protocatechuic acid	y = 2348.1x + 14.863	0.9990	0.10	0.30
Syringic acid	y = 2245.2x − 3.5633	0.9992	0.10	0.30
Chlorogenic acid	y = 2988.4x − 29.643	0.9984	0.01	0.20
Caffeic acid	y = 3357.1x + 8.7350	0.9990	0.13	0.40
Ferulic acid	y = 1969.5x − 14.299	0.9980	0.01	0.20
Cinnamic acid	y = 3144.8x + 21.206	0.9982	0.03	0.20
Apigenin	y = 1654.7x − 22.30	0.9975	1.07	3.25
Catechin	y = 2459.4x + 71.083	0.9990	0.85	2.58
Quercetin	y = 14692x + 2972.80	0.9981	0.19	0.49

y = peak area; R^2 = coefficient of determination; LOD: Limit of detection; LOQ: Limit of quantification.

3. Experimental Section

3.1. Preparation of Hashemi Rice Bran Extracts

Hashemi rice variety was donated by the Rice Research Institute of Iran (RRII). All paddy rice samples were dehulled and polished using rice dehusker and rice milling machine, set at 8% degree of milling, to obtain the milled rice bran and then, in order to separate the grains from the rice bran, they were sieved through 180 μm sieve (80 mesh). In order to inactivate endogenous lipases, rice bran was heated at 100 °C for 15 min. Rice bran powder (100 gr) was extracted, with 1 L ethanol and ethanol-water (50:50 *v/v*) for 1 h in a shaking water bath set at 160 rpm and 45 °C. The solvents were evaporated after filtering through a Whatman No. 1 filter paper. The remaining solid residues of rice bran were extracted twice more using a similar procedure and the extracts were mixed, before removing the solvent using a vacuum oven. For the ultrasonic extraction, the temperature was set at 45 °C and the ultrasonic power was adjusted to 150 W. Samples were extracted for 1 h. All extracts were, stored at −20 °C for future analysis.

3.2. Total Phenolic Content

Hashemi rice bran extracts (100 μL) were diluted in 10 mL of distilled, water. Folin-Ciocalteu reagent (500 μL) was added to this solution and incubated in total darkness for 10 min at room temperature. Later, sodium carbonate 20% (1 mL) was added and solutions were incubated again for 20 min. After incubation, the absorbance of the solutions was read at 760 nm using a spectrophotometer (UV2550, Shimadzu, Kyoto, Japan). Gallic acid, standard at different concentrations was used for the calibration curve calculation [59].

3.3. Total Flavonoid Content

Hashemi rice bran extracts (1 mL) were mixed with $NaNO_2$ in a methanolic solution (4 mL, 1:5, *w/v*) and kept at room temperature for 6 min. Thereafter, $AlCl_3$ solution, (0.3 mL, 1:10, *w/v*) was added, mixed well, and allowed to stand for another 6 min. Immediately thereafter, 1 M NaOH solution (2.0 mL) was added to each extract and incubated for 10 min at room temperature. The absorbance of the solutions was read at 510 nm using a spectrophotometer, (UV2550, Shimadzu, Kyoto, Japan). Quercetin standards of different concentrations was used for the calibration curve calculation [60].

3.4. Total Tocopherol Content

Extracts (1 mL) were transferred, to a flask, and toluene (5 mL) and 2.2′-bipyridine (3.5 mL, 0.07% *w/v* in 95% ethanol) were added to the mixture. Next, $FeCl_3.6H_2O$ (0.5 mL, 0.2% *w/v* in 95% ethanol) was added and solutions were kept for 1 min. The absorbance of the solutions was read at 520 nm using a spectrophotometer (UV2550, Shimadzu, Kyoto, Japan). During their preparation, all solutions were kept away from the light. α-tocopherol standard was dissolved in toluene in a concentration, range of 0–320 μg/mL and the calibration curve was calculated. The total tocopherol amount was calculated based on μg of α-tocopherol per mL of extract [61].

3.5. In Vitro Evaluation of Antioxidant Activity

3.5.1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Assay

The DPPH assay was used in order to evaluate the free radical scavenging activity of Hashemi rice bran extracts [62]. DPPH was dissolved in methanol at a concentration of 200 μM. The DPPH solution (2 mL) was mixed with Hashemi rice bran extract (2 mL) and incubated in a dark room for 30 min at 28 °C. After incubation, the absorbance of the samples was read at 517 nm using a spectrophotometer (UV.2550, Shimadzu, Kyoto, Japan). Gallic acid and ascorbic ac.id were used as a positive control. The scavenging activity was calculated using the following formula:

$$\% \text{ inhibition} = [(\text{absorbance}_{control} - \text{absorbance}_{sample})/\text{absorbance}_{control})] \times 100 \qquad (1)$$

3.5.2. β-Carotene Bleaching Assay

Lipid peroxidation, inhibition activity of Hashemi rice bran extracts was determined using the β-carotene bleaching method [63]. β-Carotene (5 mg) was dissolved in HPLC-grade chloroform (10 mL). Tween 40 (400 mg) was mixed with linoleic acid (40 mg); then the β-carotene solution (600 μL) was added and mixed gently. Chloroform was evaporated from the solution using a rotary evaporator (Cole-Parmer Diagonal Rotary, Evaporator System, Chicago, IL, USA). The residue was dissolved in distilled water (100 mL) and an aliquot of this solution (2.5 mL) was transferred to a test tube. Hashemi rice bran extract (350 μL, 2 g/L) was added. After this, samples were heated in a water bath for 120 min at 50 °C. The absorbance values of the samples were read spectrophotometrically at 470 nm. The antioxidant capacity, of the extracts was expressed as percentage inhibition:

$$\% \text{ inhibition} = [(\text{absorbance}_{control} - \text{absorbance}_{sample})/\text{absorbance}_{control})] \times 100 \qquad (2)$$

3.5.3. Nitric Oxide Scavenging Activity

Hashemi rice bran extract (3 mL) at different concentrations (50–250 μg/mL) was transferred to the test tubes. Thereafter, 2 mL of the reaction mixture [10 mM sodium nitroprusside (SNP) in 0.5 M phosphate buffer, pH 7.4] were added and mixed well. The mixture was incubated for 60 min at 37 °C. After incubation, Griess reagent (0.1% α-naphthyl-ethylenediamine in water and 1% H_2SO_4 in 5% H_3PO_4) was added to the mixtures. The absorbance of the samples was measured spectrophotometrically, at 540 nm (UV2550, Shimadzu, Kyoto, Japan). Gallic acid and ascorbic acid were used as a positive control. Nitric oxide (NO) scavenging activity (%) was calculated by, using the formula:

$$\% \text{ NO scavenging activity} = [(\text{absorbance}_{control} - \text{absorbance}_{sample})/\text{absorbance}_{control})] \times 100 \qquad (3)$$

3.6. Separation and Analysis of Flavonoids and Phenolic Acids

Ultra-high performance liquid chromatography (UHPLC, 1290 Infinity Quaternary, LC System, Agilent, Santa Clara, CA, USA) was used for flavonoid separation and identification. The chromatographic system conditions were set as follows: mobile phase, 0.03 M orthophosphoric acid (A) and HPLC grade methanol (B); detector, UV 360 nm; column, C18 column (5.0 μm, 4.6 mm inner diameter [ID] × 250 mm); column oven temperature, 35 °C; and flow rate, 1.0 mL/min. Gradient elution was performed as follows:

0 min 40% B, 10 min, 100% B, 15 min 100% B, and 20 min 40% B. To prepare the standard solution, gallic acid monohydrate (CAS Number 5995-86-8), chlorogenic acid (CAS Number 327-97-9), protocatechuic acid (CAS Number 99-50-3), syringic acid (CAS Number 530-57-4), caffeic acid (CAS Number 331-39-5), ferulic acid (CAS Number 537-98-4), *trance*-cinnamic acid (CAS Number 140-10-3), (+)-catechin hydrate (CAS Number 225937-10-0), apigenin (CAS Number 520-36-5) and quercetin dihydrate (CAS Number 6151-25-3) were dissolved in methanol (HPLC grade) at various concentrations. Linear, regression equations were calculated using the expression $Y = aX \pm b$, where X was the concentration of the related compound and Y the peak area of the compound obtained from UHPLC. The linearity was established by the coefficient, of determination (R^2) [64].

3.7. Recovery Test

A recovery study was carried out in order to test the accuracy of the method. Known amounts of phenolic acid and flavonoid standards at three different concentrations were added to rice bran extracts. The mixtures were injected into UHPLC and the percentage recovery of each phenolic compound from the spiked samples was calculated as follow:

$$\% \text{ Recovery} = (\text{amount of phenolic acids or flavonoids after spiking} \times 100)/(\text{original concentration of phenolic acid or flavonoids} + \text{spiked amount}) \tag{4}$$

$$\text{Percent relative standard deviation (\%RSD)} = (\text{standard deviation of phenolic acid or flavonoids} \times 100)/(\text{average content of phenolic acid or flavonoids}) \tag{5}$$

3.8. Statistical Analysis

All data from the study were shown as mean $\pm$ SD of three replicates, and means were compared using analysis of variance, (ANOVA) using the Statistical Analysis System software (SAS 9.0, SAS Institute, Cary, NC, USA). The data obtained were manipulated, to calculate statistical values such as means and standard deviations (SD) using Microsoft Excel (Microsoft Inc., Redmond, WA, USA). Group means were compared using Duncan's tests. The differences were considered significant at $p < 0.05$.

4. Conclusions

In this study, a simple and reliable extraction technique, and a validated UHPLC method were developed for the simultaneous separation and quantification of phenolic acids and flavonoids in Hashemi rice bran. In general, the aqueous organic solvent ethanol-water (50:50 *v/v*) ultrasonic extraction technique gave the greatest secondary metabolites content and also showed valuable antioxidant activity which was assessed using three different methods. Ferulic, gallic and chlorogenic acids were the most abundant phenolic compounds in Hashemi rice bran. Hashemi rice bran with its large amounts of potent phenolic compounds has good levels of antioxidant activity and the correlation analysis showed that the antioxidant activity in Hashemi rice bran extracts depended on its secondary metabolite contents, especially of phenolic and tocotrienol compounds. Characterization of the phytochemical profile and antioxidant activity of Hashemi rice bran provides insights into its potential application to promote health. Further investigation and experimentation into optimization of the ultrasonic extraction technique with ethanol-water

(50:50 *v/v*) in order to find best range of variables (liquid to solid ratio, temperature, extraction time) is strongly recommended.

Author Contributions

Study design, experimental work and writing, of first draft were performed by A. Ghasemzadeh, under supervision of H.Z.E. Jaafar. The first draft of paper was reviewed by H.Z.E. Jaafar and A.S. Juraimi. A. Tayebi-Meigooni participated, in the extractions and interpreted the experimental data. All authors reviewed and approved the final version.

Conflicts of Interest

The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds flavonoids and phenolic acids standards are available from the authors.