Tropical Journal of Pharmaceutical Research August 2011; 10 (4): 431-436

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v10i4.8

Research Article

High Levels of Phytophenolics and Antioxidant Activities in *Oryza Sativa* – Unpolished Thai Rice Strain of Leum Phua

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Abstract

Purpose: *To investigate phenolic levels, phytophenolic profiles and total antioxidant activities of Oryza sativa, unpolished Thai rice.*
Methods: *Unpolished Thai rice strains of Leum Phua, Klam, Hawm Nil and Black Rose were measured for antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) methods. Phytophenolic chromatograms were obtained by a high performance liquid chromatography (HPLC) technique. Additionally, phenolic content and anthocyanin pigment were also assayed.*
Results: *Oryza sativa, unpolished Thai rice strain of Leum Phua showed the highest antioxidant activity. It was also highest in anthocyanin pigment. The strains of Leum Phua, Klam, Hawm Nil and Black Rose showed high levels of phenolic content: 1.36±0.03, 0.78±0.02, 0.61±0.01 and 0.57±0.02 mg gallic acid/ g sample, respectively. Interestingly, phytophenolic chromatogram and anthocyanin pigment levels showed a strong correlation.*
Conclusion: *Oryza sativa, unpolished Thai rice strain of Leum Phua, has high levels of anthocyanin pigment, phenolic content and antioxidant activity. Its pure phytochemical contents should be further studied for their pharmaceutical benefits.*

Keywords: *Antioxidant, Oryza sativa, Phenolics, Anthocyanin, Phytochemicals, Thai rice*

Received: 14 February 2011 *Revised accepted: 30 June 2011*

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INTRODUCTION

Cellular oxidative stress is defined by the overproduction of radicals in biological systems leading to cellular oxidative injury [1]. Free radicals, which are unstable molecules, are highly reactive with macromolecules including lipid, protein and nucleic acids which progress to oxidative injury [2]. Clinically chronic diseases such as cancer, neurodegenerative disorders, cardiovascular disease and aging have been related to cellular oxidative damage [3,4]. Phytophenolic compounds of plants are claimed to have a role in the effective prevention of cellular oxidative injury [5].

The essential groups of plant phytophenolics are forms of flavonoids, phenolic acids and polyphenols [6]. Anthocyanin pigments, a member of flavonoid group, are presented as the blue, purple, black and red color pigments of fruits and vegetables [7,8]. Phenolics in phytochemical plants are highly efficient in radical scavenging activities [8,9]. In addition, the phytophenolics of dietary fruits and vegetables are also related to cancer prevention [9,10]. High consumption of the red color in unpolished Thai rice in rats has been associated with low levels of oxidative stress marker [11]. *Oryza sativa*, unpolished Thai rice, presents black color pigments. They might be indicative of high phytophenolic compounds.

The objective of this study was to evaluate the distribution of phytophenolic compounds in *Oryza sativa*, Thai rice strains of Leum Phua, Klam, Hawm Nil and Black Rose, as well as determine their total antioxidant activity.

EXPERIMENTAL

Oryza sativa, unpolished Thai rice samples Black-colored strains of unpolished Thai rice, including Leum Phua, Klam, Hawm Nil and Black Rose, were purchased from local markets in Thailand. The rice extract of each strain was homogenized in an electric blender. The homogenized samples were extracted with 1:4 w/v of 95 % ethanol. The extract was mixed using Multi RS-60 Rotatormixer for 90 min, centrifuged at 3000 rpm for 10 min and filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter prior to further use.

Assessment of total antioxidant activity by DPPH method

Total antioxidant activity was obtained by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method [12]. The working solution of DPPH was freshly prepared with 95 % ethanol with an absorbance of 540 nm at 0.95 ± 0.01 unit. The extract (20 µl) was mixed with 180 µl of working DPPH and the absorbance of the mixture immediately measured spectrophotometrically (Shimadzu UV-2550, Japan) at a wavelength of 540 nm. Vitamin C equivalent antioxidant capacity (VCEAC) was used as a reference. Total antioxidant activity of the extracted Thai rice was expressed as mg Vit C/ g sample equivalent, obtained from the calibration curve ($y = 0.7748x$, $r^2 = 0.9988$) of standard vitamin C at concentrations ranging from 10 to 100 mg/ml.

Assessment of total antioxidant activity by ABTS method

Total antioxidant activity of the extracts was assayed by 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method [12]. A working ABTS solution was freshly prepared by mixing equal volumes of 7mM ABTS and 2.4mM potassium persulfate. The mixture was incubated in the dark for 12 h at room temperature. The mixed reaction was diluted with 95 % ethanol to obtain a working solution with an absorbance of 734 nm at 0.95±0.01 unit,. The extract (70 µl) was added to 630 µl of the working solution and incubated for 30 min. The absorbance of the mixture was measured spectrophotometrically at 734 nm. Trolox equivalent antioxidant capacity (TEAC) was used as a standard. Total scavenging activity of the extracts was expressed as mg trolox/ g sample equivalent, obtained from the calibration curve (y = 1.4103x, r^2 = 0.999) of trolox standard at concentrations ranging from 10 to 50 mg/ml.

Evaluation of total antioxidant activity by FRAP method

Ferric reducing antioxidant power (FRAP) method, based on the reduction of a ferric complex (Fe^{3+}-TPTZ) to the ferrous complex (Fe^{2+}-TPTZ) [11], was also to evaluate total antioxidant activity of the rice extracts. A working FRAP reagent was freshly prepared by mixing together 2.5 ml of 10mM 2,4,6-tripyridyl-s-triazine (TPTZ solution), 2.5 ml of 20mM ferric chloride hexahydrate ($FeCl_3.6H_2O$) and 25 ml of acetate buffer at pH 3.6. The extract was mixed with 180 µl of the working FRAP reagent in 96 well plates and then incubated in dark conditions at room temperature for 15 min. The blue color of the antioxidant was investigated using a multimode detector (Beckman, DTX 880, Australia) at 595 nm. Ferrous sulfate (Fe_2SO_4) was used as a reference. Total antioxidant activity of the extracts was expressed as µmol Fe^{2+}/g sample equivalent, obtained from the calibration curve (y = 0.0201x, r^2 = 0.9937) of ferrous sulfate at concentrations ranging from 100 to 1000 µmol/ml.

Determination of total phenolic content

Total phenolic content of the extracts of the black-colored unpolished rice was measured using a Folin-Denis reagent [12]. Ten microliters of the extract was diluted with 790 µl of distilled water and then mixed meticulously with 50 µl of fresh reagent of Folin-Denis. An aliquot (150 µl) of 7.5 %w/v of sodium carbonate was added and incubated in dark conditions for 30 min. The spectrophotometric absorbance of the phenolic content was measured at 765 nm. Gallic acid equivalent (GAE) was used as a reference. Total phenolic content of the samples was expressed as mg gallic acid/ g sample equivalent, obtained from the calibration curve (y = 1.085x, r^2 = 0.9997) of gallic acid at concentrations ranging from 0.2 to 1.0 mg/ml.

Determination of total anthocyanin pigment

The rice extracts were investigated for monomeric anthocyanins by pH-differential method [13]. The method used consists of assaying total monomeric anthocyanin content, based on the structural change of the anthocyanin chromophore between pH 1.0 and 4.5 in two dilutions of the sample, one with a buffer of 0.025 M potassium chloride (pH 1.0) and the other 0.4 M sodium acetate (pH 4.5). Monomeric anthocyanins undergo a reversible structural transformation as a function of pH (colored oxonium form at pH 1.0 and colorless hemiketal form at pH 4.5). The pigment content was calculated based on cyanidin-3-glucoside, with a molecular weight of 445.2 g/ L and molar absorbance (ε) of 29600 cm^{-1} mg^{-1}. The computed anthocyanin pigment was expressed as cyanidin-3-glucoside equivalents. Anthocyanin pigment (cyanidin-3-glucoside equivalents, mg/L) content (cC) was calculated as in Eq 1.

$$C = (A \times MW \times DF \times 10^3)/ (\varepsilon \times 1) \quad \ldots\ldots\ldots (1)$$

where A = (*A*520nm – *A*700nm) pH 1.0 – (*A*520nm – *A*700nm) pH 4.5; MW = molecular weight; DF = dilution factor established in D; 1 = path length in cm; and ε = molar extinction coefficient

Assessment of phytophenolic profiles

A scan of the extract of the black-colored strains of unpolished Thai rice were obtained spectrophotometrically in the wavelength range of 250 - 700 nm. An absorbance that showed the highest peak in the scan wavelength range was used for UV-VIS detection in high performance liquid chromatography (HPLC). Chromatogram profiles for the phytophenolic compounds of the rice strains were obtained. In this method, the extract (100 µl) was evaporated under nitrogen gas at 40 °C. The residue was reconstituted with 100 µl of freshly prepared mobile phase (methanol and 3 % acetic acid, 1: 1). Butylated hydroxytoluene (BHT, 0.01 g) was added to the mobile phase and the phenolics measured by a reverse-phase HPLC system (HP1100, Agilent) with a UV-VIS detector at 217 nm. The gradient mobile phase was delivered to a 250 x 4.6 mm Column Luna C18, 5 μm (P/N 00G-4252-E0) at a flow rate of 1 ml/min, maintained at 40 ℃. The gradient of the mobile phase is shown in Table 1.

Table 1: The gradient of the HPLC mobile phase

Time (min)	Ratio (%) of mobile phase	
	3% acetic acid	***Methanol***
0	100	0
10	90	10
20	70	30
30	50	50
40	40	60

Statistical analysis

All the data were expressed as mean ± standard error (SE). Data correlations were obtained by Pearson correlation using SPSS program, version 13.0. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Antioxidant activity of unpolished Thai rice

As Table 2 shows, the extract of Leum Phua strain had a higher antioxidant activity than other strain extracts.

Phenolic compounds of unpolished Thai rice

The rice strain extracts of Leum Phua, Klam, Hawm Nil and Black Rose showed high levels of phenolic content: 1.36 ± 0.03, 0.78 ± 0.02, 0.61 ± 0.01 and 0.57 ± 0.02 mg gallic acid/ g sample, respectively. In addition, the strain of Leum Phua presented the highest level of anthocyanin pigment at 36.94 ± 0.97 μg/ g sample (Table 2). Total phenolic content showed a high correlation (r) with r = 0.991 (p = 0.009), r = 0.994 (p = 0.006) and r = 0.981, (p = 0.019) for DPPH, ABTS and FRAP antioxidant methods, respectively.

The UV-Vis spectra of the rice strain extracts showed similar peaks (Fig 1) with the highest peak absorbance for all the strains occurring at a wavelength of 217 nm.

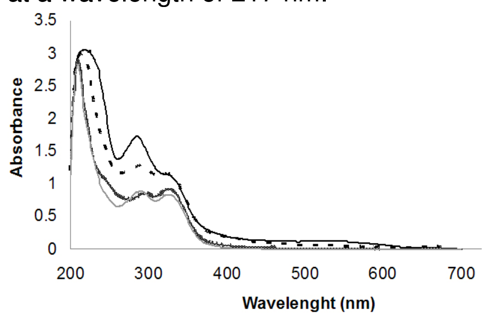

Fig 1: UV-Vis spectra of *Oryza sativa*, Thai rice strains: Leum Phua (black line), Klam (dash line), Hawm Nil (dotted line) and Black Rose (gray line).

Although, the rice strains exhibited similar UV-Vis spectra, as Fig 2 shows, their high performance liquid chromatography (HPLC) chromatograms differed substantially (Fig 2).

Table 2: Phenolic levels and antioxidant activities in unpolished Thai rice

Thai rice strain	Total phenolic content (mg gallic acid/ g sample)	Monomeric anthocyanin pigment (mg/ L equivalent)	DPPH (mg Vit C/ g sample)	ABTS (mg trolox/g sample)	FRAP (μmol Fe^{2+}/g sample)
Leum Phua	1.36±0.03	36.94±0.97	5.54±0.01	3.21±0.04	18.20±0.04
Klam	0.78±0.02	7.36±0.26	2.46±0.02	1.38±0.02	12.06±0.02
Hawm Nil	0.61±0.01	1.08±0.06	2.17±0.02	0.46±0.03	8.78±0.01
Black Rose	0.57±0.02	0.06±0.01	1.93±0.03	0.31±0.03	6.97±0.03

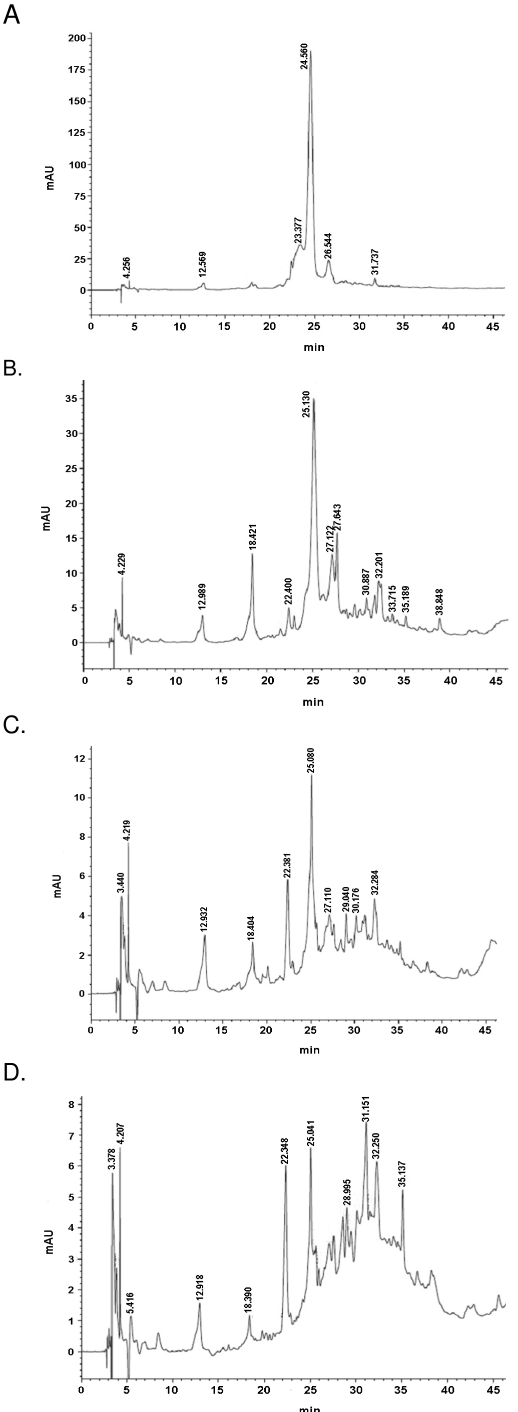

Fig 2: Chromatograms of the extract of (A) Leum Phua, (B) Klam, (C) Hawm Nil and (D) Black Rose strains of Thai rice (*Oryza sativa).*

When the same concentration of the samples were injected, the magnitude of the peak was greatest for the extracts of Leum Phua (Fig 2A) and Klam (Fig 2B) strains, 191.34 and 35.11 mAU, respectively, while those of Hawm Nil and Black Rose much lower at 11.2 mAU (Fig 2C) and 7.42 mAU (Fig 2D), respectively.

The peak level of the phytophenolic profiles of the extracts showed a high correlation with anthocyanin pigment levels (r = 0.999, *p* = 0.001), as illustrated in Fig 3.

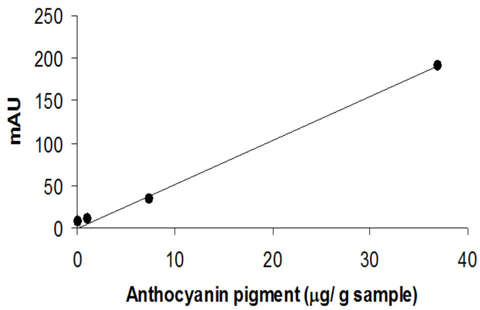

Fig 3: Correlation between phytophenolic peaks and anthocyanin pigment levels (r=0.999, *p*=0.001)

DISCUSSION

A high correlation between phenolic content and antioxidant activity was found in the unpolished Thai strains of Leum Phua, Klam, Hawm Nil and Black Rose. This finding supports previous studies that found a direct relationship between phenolic compounds and the levels of color pigments in fruits and vegetables [9,13]. In other studies, the level of color pigments correlated with antioxidant activity [14,15]. High phytophenolic content was found to correlate with the color pigments of unpolished Thai rice in previous study [12]. Although, unpolished Thai rice strains exhibited similar UV-Vis spectra at a spectrophotometric wavelength of 217 nm, their chromatographic profiles differed substantially. This finding may be due to the presence of several phytophenolics in the black pigment of the rice strain.

Phytophenolics are said to be the largest category of phytochemicals in the plant kingdom.

Anthocyanin pigments are flavonoids that present as the color pigments of fruits and vegetables [7]. Furthermore, phytophenolic substances have been associated with clinically oxidative disease prevention [3,16]. In this work, anthocyanin pigment showed strong correlation with phytophenolic content. Therefore, the high anthocyanin pigment content of unpolished Thai rice strain, Leum Phua, may make it a clinically beneficial in preventing oxidative stress-related diseases such as coronary heart disease and cancer [17,18]. There is need, therefore, to investigate the pure phytophenolic compounds of unpolished Thai strain of Leum Phua for possible health benefits.

CONCLUSION

Of all the four strains of unpolished Thai rice studied, Leum Phua strain contained the highest levels of phytophenolics, anthocyanin pigment and antioxidants. The pure compounds of this strain should be further studied for its beneficial health effects, including prevention of cellular oxidative damage.

ACKNOWLEDGMENT

This research was supported by the Faculty of Science, Mahidol University, Thailand.

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