Bioactive Compounds, Free Radical Scavenging Activity and Xanthine Oxidase Inhibitory Activity from Local Pigmented Rice Extracts of Loei Province

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Abstract

Pigments in pigmented rice are usually in the pericarp or the seed coat. The color of the seed coat varies from black, purple, red or brown. Pigmented rice contains a range of bioactive compounds including phenolic acids, flavonoids and anthocyanins. This study investigates the free radical scavenging activity and evaluates xanthine oxidase inhibitory activity of the phenolic compound, flavonoid compound and anthocyanin from local pigmented rice extract of Loei province. Khao Pae Daeng, Khao Daeng Mueang Loei, Khao Jao Dam, Khao Niaw Kam, Khao Kam Poon and Khao Dam Leum Pua were extracted with absolute ethanol for phenolic compound and flavonoid compound analysis. Whereas, 1% of hydrochloric acid diluted in methanol is generally used to extract anthocyanin. Total phenolic, total flavonoid and total anthocyanin were analyzed by spectrophotometric method. High-performance liquid chromatography technique was used to identify and quantify phenolic acid, flavonoid and anthocyanin in local pigmented rice extract. Furthermore, the extracts were evaluated for their free radical scavenging activity using DPPH radical and ABTS radical as a substrate and xanthine oxidase inhibitory activity. The extract from Khao Dam Leum Pua showed both maxima of total phenolic contents (88.32± 6.03 mgGAE/ 100g) and total flavonoid contents (364.19± 7.12 mgQE/100g). Five phenolic acids were detected in all extract but found in different quantities. Khao Dam Leum Pua, Khao Niaw Kam and Khao Kam Poon was containing greater phenolic acid contents when compared with the other local pigmented rice ($p<0.05$). Likewise, the extract from Khao Dam Leum Pua and Khao Kam Poon showed the maximum of quercetin contents (0.44-0.45 mg/100g). The most of total anthocyanin contents were found in Khao Dam Leum Pua and Khao Kam Poon ($p<0.05$). Khao Dam Leum Pua contained greater cyanidin-3-glucoside (60.03± 3.88 mg/100g) and cyanidin contents (16.43± 0.93 mg/100g) when compared with the other extract ($p<0.05$). Finally, the extract from Khao Dam Leum Pua displayed the strongest DPPH radical anion scavenging activity (IC_{50}= 49.78 ± 3.57 μg/ml). Also, the strongest ABTS radical cation scavenging activity was found in Khao Dam Leum Pua (IC_{50}=16.54± 1.06 μg/ml). The extract from Khao Dam Leum Pua exhibited the strongest xanthine oxidase inhibitory activity with IC_{50} values of 64.16± 4.42 μg/ml. Significant positive correlations (r^2) demonstrate that the free radical scavenging effects of local pigmented rice extract might generally be considered a result of the presence of the phenolic compounds (0.7278) flavonoid compounds (0.6591) and anthocyanin (0.8459). Similarly, correlation analysis showed that anthocyanin such as cyanidin was positively correlated to xanthine oxidase inhibitory activity (0.9192). This study provides new insights into the potential use of local pigmented rice extracts, especially black rice such as Khao Dam Leum Pua and Khao Kam Poon as a source of antioxidants and xanthine oxidase inhibitor.

Keywords: bioactive compounds, free radical scavenging activity, xanthine oxidase inhibitory activity, local pigmented rice, *Oryza sativa* L.

Introduction

In normal conditions, the human body has a redox system trying to keep human life at a healthy balance. The presence of free radicals will adversely affect cells or the human body. Some free radicals such as nitric oxide, superoxide radical anion and related reactive oxygen species (ROS) and or reactive nitrogen species

(RNS) mediate cells in signaling processes (Drö ge, 2002). Oxidative stress is a state in which there are excessive amounts of free radicals. The imbalance of antioxidant capacity causes the damage of biomolecules such as enzymes, proteins, lipids and DNA. Oxidative stress is essential in developing chronic degenerative diseases, including cancer, coronary heart disease and aging (Dai & Mumper, 2010).

Uric acid is the final oxidation product of purine catabolism in humans catalyzed by xanthine oxidoreductase (XOR) enzymes. Overall production or underexcretion of uric acid results in elevated serum uric acid levels, term hyperuricemia, which has long been established as the primary etiologic factor in gout (Galassi & Borghi, 2015). XOR is a rate-limiting enzyme in uric acid production. XOR catalyzes the last two steps of purine catabolism, the oxidation of hypoxanthine to xanthine and the oxidation of xanthine to uric acid, by utilizing either NAD^+ or O_2. As a result of the reaction, two reactive oxygen species, superoxide anion and hydrogen peroxide are produced (Changyi, Jian-Ming, & Qizhi, 2016). Superoxide anion and hydrogen peroxide directly cause oxidative damage to various biomolecules. Increased XOR activity and oxidative stress have been observed in multiple diseases in humans and experimental animals (Minhas et al., 2006). For example, the overall risk of cardiovascular disease mortality increased by 15% for each increase of 1 mg/dl of uric acid (Borghi & Desideri, 2016).

Antioxidants can inhibit free radicals and prevent oxidation of biomolecules by inhibiting free radicals and reducing oxidative stress. Antioxidants are found in many sources such as plants, fruits, beverages and food can be evaluated using various *in vitro* assays (Miguel, 2010). Bioactive compounds such as phenolic compounds, flavonoid compounds and anthocyanin are a source of natural antioxidants. Bioactive compounds are found in algae, marine algae and various plants (Ambrozova et al., 2014). Phenolic acids are one type of phenolic compound and classified into two groups (hydroxycinnamic acids and hydroxybenzoic acids). These compounds exhibit a wide range of physiological properties such as antioxidant, anti-allergenic, anti-inflammatory, cardioprotective and vasodilatory effects (Manach, Mazur, & Scalbert, 2005). In previous research, sinapinic acid in the cinnamic acid group shown an antiproliferative activity in cervical cancer cell lines and have a novel histone deacetylase inhibitor (Senawong et al., 2013). A flavonoid is a large group of polyphenol compounds in plants. Flavonoids are a diverse group of phytochemical compounds found in plants, fruits, vegetables and beverages like tea and wine. These are classified into five subclasses, flavonols, flavones, flavanones, flavan-3-ols and anthocyanins depend on the chemical structure. Some flavonoids such as quercetin show biological properties include inhibiting free radical and antioxidant activity, anti-inflammatory, anti-cancer and anti-allergic (Ling-Shan, Jin-Ping, Xiao-Qing, & Xue-Rong, 2009).

Anthocyanins are members of the flavonoid group of phytochemicals and generally accepted as the largest and most important group of water-soluble pigments in nature. Anthocyanins are responsible for the purple, blue, red and orange colors of many vegetables, fruits and some cereal. The variety of anthocyanins are due to the number and position of hydroxyl (-OH) and methoxy ($-OCH_3$) groups on the basic anthocyanin skeleton (C6-C3-C6 skeleton), the identity, number, and positions at which sugars are attached, and the extent of sugar acylation and the identity of the acylating agent (Prior & Wu, 2006). Epidemiologic studies suggest that anthocyanins consumption lowers the risk of antioxidant properties cardiovascular disease, diabetes, arthritis and cancer (Miguel, 2011). Foods that contain anthocyanin are becoming more popular (Miguel, 2011). As mentioned previously, food containing plant phytochemicals, phenolic compound flavonoid compound or

anthocyanin, is advantageous for our health since it can protect the human body from free radicals and retard the progress of many chronic diseases (Lobo, Patil, Phatak, & Chandra, 2010).

Pigmented rice is a type of rice species *Oryza sativa* L. which is glutinous, packed with a high level of nutrients and mainly cultivated in Northeast Thailand. The pericarp or seed coat (outer part) of this rice color's kernel is black, purple, red, black-red and red-black due to a pigment known as anthocyanin. A commonly found anthocyanin in pigmented rice is acetylated procyanidins, which are reported to possess antioxidant properties (Oki et al., 2002). Six varieties of local pigmented rice, Khao Pae Daeng (KPD), Khao Daeng Mueang Loei (KDML), Khao Jao Dam (KJD), Khao Niaw Kam (KNK), Khao Kam Poon (KKP) and Khao Dam Leum Pua (KDLP) were cultivated in Loei province of Northeast Thailand. Six varieties of local pigmented rice are different colors such as brown-red, red-black or black-red. Several studies have been conducted to explore the quantitation of bioactive compounds such as phenolic compound, flavonoid compound and anthocyanin from this rice species (Sompong, Siebenhandl, Linsberger & Berghofer, 2011; Pengkumsri et al., 2015), but not included xanthine oxidase inhibitory activity. In this work, we investigate antioxidant activity and evaluate XOR inhibitors to explore the potential inhibitory activity of six varieties of local pigmented rice extracts. These extract exhibit promising XOR inhibition and may warrant further studies at the cellular level.

Methods and Materials

Sample and chemical preparation

- Plants material

Six varieties of local pigmented rice, as shown in Figure 1. KPD, KDML, KJD, KNK, KKP and KDLP were collected during November-December 2016 from Loei Province, Thailand. All samples were cleaned and dried in a hot air oven at 60°C to 65°C for 24 h. The dried sample was powered using a blender and stored at -20°C

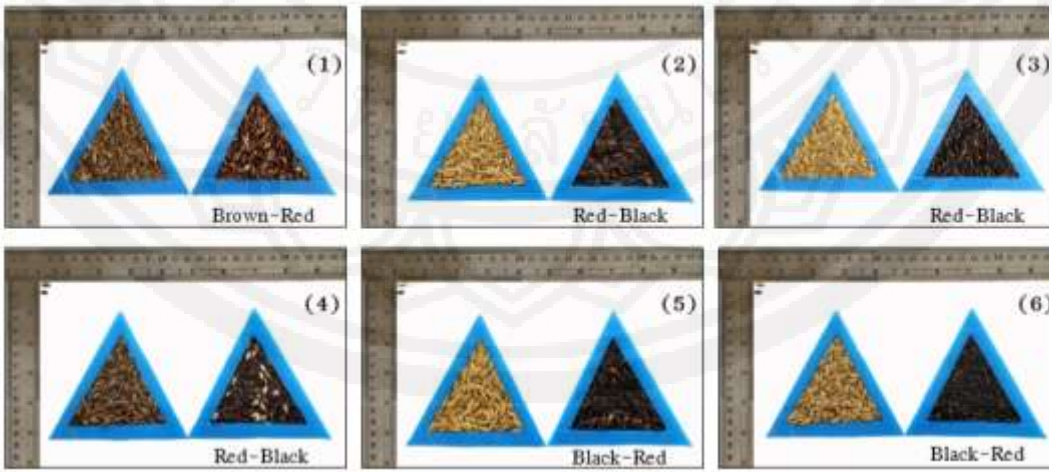

Figure 1 The morphological of local pigmented rice in experiment. (1) KPD (2) KDML (3) KJD (4) KNK (5) KKP (6) KDLP

- Chemicals

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) was obtained from TCI America Corporation. 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) (ABTS), folin-ciocalteu's phenol reagent, aluminium chloride, sodium nitrite, gallic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, quercetin, genistein, cyanidin-3-glucoside, cyanidin, allopurinol, xanthine and xanthine oxidase were purchased from Sigma-Aldrich Corporation. Sodium hydroxide and sodium carbonate were purchased from Ajax Finechem Corporation. Acetonitrile and methanol were of HPLC grade. All the other reagents were of analytical grade.

Samples extraction

- Ethanolic extraction

The dried samples were ground into fine powder and 10 gram of each sample was extracted by using 100 ml of absolute ethanol for 48 h. at room temperature. The extract obtained was filtered through filter paper (Whatman No. 4) and the solvent was removed by using a rotary evaporator to dryness the ethanolic extracts for total phenolic and total flavonoid content analyses.

- Anthocyanin extraction

Ten grams of dried samples were macerated and periodically stirred in 100 ml of 1% hydrochloric acid in methanol for 6 h. at room temperature. The suspension was filtered through Whatman No. 4 filter paper and centrifuged at 5,000 rpm for 15 min. The supernatant was air-dried to yield an anthocyanin extracts for total anthocyanin content analyses. The residue was reconstituted in 1% hydrochloric acid in methanol before testing and the solvent was used as a negative control.

- Phenolic acid extraction

The phenolic acid extraction was performed using the acidic hydrolysis method with some modification (Sani, Iqbal, Chan & Ismail, 2012). In short, the dried sample of each local pigmented rice (10 g) was suspended in 70% methanol (200 ml). The mixture was stirred at room temperature for 2 h. and then filtered through the filter paper (Whatman No. 4). The filtrate was concentrated until the final volume was 60 ml by rotary evaporator. 2 M of NaOH (50 ml) was added into the remaining filtrate and stirred continuously for 12 h. at room temperature. The mixture was centrifuged at 3,000 rpm about 10 min and then filtered through filter paper. The supernatant was repeatedly extracted three times with 80 ml of diethyl ether. The combined aqueous phases were acidified with 10 M of HCl until pH 1.5 and filtered through filter paper. The filtrate was further extracted with diethyl ether (80 ml) for three times. The combined diethyl ether layers were concentrated by a rotary evaporator to yield a phenolic acid extracts.

Determination of total phenolic content

Total phenolic content (TPC) in ethanolic extract of each local pigmented rice was determined by a modification of the Folin-Ciocalteu assay (Oskoueian, Abdullah, Hendra & Karimi, 2011). Briefly, 0.5 ml of ethanolic extracts (10-200 μg/ml) solution was mixed with 1.5 ml of 10% (v/v) folin-ciocalteu reagent. After 5 min, 2.0 ml of Na_2CO_3 (75%) was subsequently added to the mixture and incubated at 50 °C for 10 min. The absorbance was measured by Shimadzu UV-160A spectrophotometric at 750 nm. Gallic acid was used as a standard and the result was calculated as mgGallic Acid Equivalent (GAE) per one hundred gram of dry weight. The calibration curve of gallic acid was $Y=0.0933X-0.0566$, $R^2=0.9996$

Determination of total flavonoid content

Total flavonoid content (TFC) in ethanolic extract of each local pigmented rice was determined by a modification of the aluminium chloride colorimetric assay (Oskoueian et al., 2011). An aliquot of 0.1 ml of ethanolic extracts (10-100 μg/ml) solution were mixed with 0.2 ml of 10% (w/v) $AlCl_3$ solution in methanol, 0.2 ml (1 M) potassium acetate and 4.5 ml distilled water. The mixture was incubated for 30 min at room temperature. The absorbance was measured by Shimadzu UV-160A spectrophotometric at 420 nm. Quercetin was used as a standard and the result was calculated as mgQuercetin Equivalent (QE) per one hundred grams of dry weight. The calibration curve of quercetin was $Y=1.1561X+0.0249$, $R^2=0.9953$

Determination of total anthocyanin content

The assessment of total anthocyanin content (TAC) was carried out by the pH differential method according to AOAC as described by (Lee, Durst, & Wrolstad, 2005). Briefly, 2.5 ml of buffer solution pH of 1.0 or 4.5 and 0.5 ml of anthocyanin extracts with different concentrations (10-100 μg/ml) were mixed and incubated at room temperature for 20 min. Absorbance was measured at 510 and 700 nm in buffers at pH 1.0 and 4.5. Anthocyanin concentration is expressed as mgCyanidin Equivalents (CE) per one hundred gram of dry weight and calculated using the formula (1) below:

$$TAC = \frac{A \times M.W. \times DF \times 10^3}{\varepsilon \times 1} \quad (1)$$

A = (A510 nm-A700 nm) at pH 1.0-(A510 nm-A700 nm) at pH 4.5, M.W. (Molecular weight) = 322.70 g/mol, DF = Dilution factor, 1 = Cuvette path length in centimeter, ε (Molar extinction coefficient for cyanidin) = 35,000 L/ mol.cm^{-1}, 10^3 = Factor to convert gram to milligram. All spectrometric measurements were performed using a Shimadzu UV-160A spectrophotometer.

Liquid chromatography analysis

- HPLC analysis of phenolic acid, flavonoid and anthocyanin

Chromatographic separation of individual phenolic acids was carried out using a SHIMADZU HPLC-UV-VIS system, based on matching spectrum and retention times of phenolic acid standard. The HPLC system consisted of LC-10AD separation module unit, SCL-10A UV-VIS detector and a 250 x 4.6 mm, 5 μm, 10 A°, ODS C-18 column (GL Science, USA), with 100% acetonitrile (solvent A) and a gradient system of acetic acid in deionized water (solvent B) by increased from 1% to 8% in 5 min and maintaining at 8% for 10 min, then increasing to 20% within 5 min and maintaining at 20% for 10 min, followed by returning to the initial condition (1%) in 10 min and keeping for 5 min before next injection. The UV-VIS detector monitored the phenolic acids at a wavelength of 280 nm. The flow rate and the injection volume were 1.0 ml/min and 20 μl, respectively.

A portion of ethanolic extract (0.1 g) was dissolved in 5 ml of 80 % methanol and filtered through a 0.45 μm membrane before use and was injected into the HPLC equipment. Quercetin content was analyzed using an HPLC system, with 100% methanol (solvent A) and a linear gradient solvent system of acetic acid in deionized water (solvent B) by increased from 50% to 75% in 5 min, then increasing to 90% within 5 min and increasing to 100% within 5 min followed by returning to the initial condition (50%) in 10 min and

keeping for 5 min before next injection. The UV-VIS detector monitored the quercetin at a wavelength of 510 nm. The flow rate and the injection volume were 1.0 ml/min and 20 µl, respectively.

A portion of anthocyanin crude extract obtained as explained above was dissolved in 5 ml of 1% HCl in MeOH and filtered through a 0.45 µm membrane before use and was injected into the HPLC equipment. Qualitative HPLC analysis with a linear gradient solvent system of 4% aqueous formic acid (solvent A) and 100% acetonitrile (solvent B) as follows: 90%-80% solvent A until 5 min, followed by 80%-70% solvent A over 15 min, then going back to 90% solvent A until 30 min and finally reconditioning the column with 90% solvent A isocratic for 15 min. The flow rate and the injection volume were 1.5 ml/min and 20 µl, respectively. The UV-VIS detector monitored the anthocyanin at a wavelength of 520 nm.

The amount of phenolic acid, flavonoid and anthocyanin was performed using the standard addition method and consequent calculation.

- Quantification of the phenolic acid, flavonoid and anthocyanin

External standardization of phenolic acids (gallic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid), flavonoid (quercetin, genistein) and anthocyanins (cyanidin, cyanidin-3-glucoside) was used for quantification of all bioactive compounds. Calibration curves were prepared in the concentration range between 1 to 80 µg/ml for phenolic acid and flavonoid. The concentration of calibration curves between 1 to 60 µg/ml was used for the determination of anthocyanin. The linearity was estimated by linear regression analysis applying the least square method. Limit of detection (LOD) and limit of quantification (LOQ) were calculated by linear regression equations.

In vitro antioxidant activity

- DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described (Li, Wu, & Huang, 2009) with some modification. 100 µM of DPPH solution was prepared by dissolving 4 mg of DPPH with 100 ml ethanol. A 200 µl of DPPH solution was mixed with 10 µl of ethanolic extract at various concentrations (1-100 µg/ml) in 96-well plate. The solution was mixed and incubated in the dark for 10 min at room temperature. Then, the absorbance was taken by a microplate reader (SPECTROstar^{nano}, BMG Labtech, Germany) at 515 nm. The control was prepared using the same procedure without any sample. Gallic acid, quercetin and cyanidin was used as standard controls. The scavenging activity was represented as 50 percent inhibition concentration (IC_{50}) and the percentage DPPH inhibition of the test samples was calculated using the formula (2) below:

$$\text{DPPH inhibition } \% = \frac{(A_0 - A) \times 100}{A_0} \qquad (2)$$

Where A_0 is the absorbance at 515 nm of DPPH without sample, and A is the absorbance at 515 nm of the reaction mixture containing DPPH and sample.

- ABTS radical scavenging activity

ABTS radical scavenging activity was determined as described (Li et al., 2009) with some modification. Briefly, a mixture of 7.4 mM of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) solution and 2.6 mM of potassium persulfate solution was kept overnight in the dark. Prior to use in the assay,

the ABTS radical cation was diluted with 50 % ethanol for an initial absorbance of about 0.70± 0.02 at 745 nm. Free radical scavenging activity was assessed by mixing 10 µl of ethanolic extract at various concentrations (0.5–100 µg/ml) with 200 µl of ABTS working solution. The solution was incubated in the dark for 30 min at room temperature. Then the absorbance was taken by microplate reader (SPECTROstar^{nano}, BMG Labtech, Germany) at 745 nm. Gallic acid, quercetin and cyanidin was used as standard controls. The scavenging activity was represented as 50 percent inhibition concentration (IC_{50}) and the percentage ABTS inhibition of the test samples was calculated using the formula (3) below:

$$\text{ABTS inhibition \%} = \frac{(A_0 - A) \times 100}{A_0} \quad (3)$$

Where A_0 is the absorbance at 745 nm of ABTS without sample, and A is the absorbance at 745 nm of the reaction mixture containing ABTS and sample.

In vitro xanthine oxidase (XO) inhibitory activity

The xanthine oxidase activity was measured by spectrophotometer using xanthine as the substrate (Owen & Johns, 1999) with the following modifications. Total volume of the assay mixture is 1.5 ml and consists of ethanolic extract at various concentrations (0.3–96 µg/ml) under study, 0.15 M phosphate buffer (pH 7.5) and 50 µl of 0.2 U/ml xanthine oxidase enzyme solution. After preincubation at 37° C for 30 min, the reaction was initiated by addition of 500 µl of 0.06 mM xanthine and incubation at 37° C for 15 min. The reaction was stopped by adding 0.5 M HCl 50 µl and monitored absorption at 290 nm using a Shimadzu UV-160A spectrophotometer. Allopurinol was used as a positive control. A blank was also prepared in the same way, but the enzyme solution added to the assay mixture after adding 0.5 M HCl. The percent xanthine oxidase inhibitory activity of the assayed samples was calculated using the formula (4) below:

$$\text{Xanthine oxidase inhibition \%} = \frac{(A-B)-(C-D) \times 100}{A-B} \quad (3)$$

Where A is the activity of the enzyme without test extract, B the control of A without test extract and enzyme, C and D are the activities of the test extract with and without xanthine oxidase enzyme.

IC_{50} values were obtained through linear regression analysis the plot of concentration (X axis) against percent inhibition (Y axis).

Statistical and data analysis

Data are given as the mean ± SD of three measurements. All linear regression in this paper was analyzed by IBM SPSS statistics software. The data were analyzed by one-way analysis of variance (ANOVA). A difference was considered statistically significant if $p \leq 0.05$.

Results

Yield of the local pigmented rice extract

The ethanolic extract content of six local pigmented rice is between 10.97–13.52 g/100g dry weight (DW) (Table 1). Whereas, the number of anthocyanin extracts is between 9.63–21.68 g/100g DW (Table

1). While the content of phenolic acid extracts using acidic hydrolysis ranged from 75.83 to 84.32 mg/100g DW (Table 1). KDLP contained the highest of all extracts (Table 1).

Total phenolic (TPC), total flavonoid (TFC) and total anthocyanin (TAC) contents of local pigmented rice extracts

TPC, TFC and TAC of local pigmented rice extract were determined and are presented in Figure 2. KDLP

Table 1 Extraction yields of crude extract from local pigmented rice

Local pigmented rice sample	Sample code	Extraction yields		
		Ethanolic extract (g/100g)	Anthocyanin extract (g/100g)	Phenolic acid extract (mg/100g)
Khao Pae Daeng	KPD	11.62± 0.08[a]	9.63± 0.02[a]	75.83± 1.62[a]
Khao Daeng Mueang Loei	KDML	12.43± 0.04[a]	12.82± 0.02[b]	76.47± 2.74[a]
Khao Jao Dam	KJD	10.97± 0.17[a]	13.18± 0.07[b]	78.53± 2.07[a]
Khao Niaw Kam	KNK	13.15± 0.03[a]	14.04± 0.01[b]	76.48± 1.18[a]
Khao Kam Poon	KKP	11.87± 0.05[a]	20.03± 0.03[c]	80.11± 3.21[b]
Khao Dam Leum Pua	KDLP	13.52± 0.06[a]	21.68± 0.04[c]	84.32± 2.23[b]

Values in the same column followed by a different letter are significantly different ($p<0.05$)

contained the maximum TPC (88.32± 6.03 mgGAE/100g) in terms of gallic acid equivalents followed by KKP, KNK, KJD, KPD and KDML, respectively (Figure 2A). TFC of 364.19± 7.12 mgQE/100g DW of KDLP were scientifically ($p<0.05$) higher than that of other local pigmented rice (Figure 2B). Different rice varieties had significantly different average levels of TAC; the highest level was found in the KDLP-KKP (26.45-28.79 mgCE/100g DW) followed by KJD-KDML-KNK and KPD group, respectively ($p<0.05$) (Figure 2C). KDLP exhibited the highest of all bioactive compounds (Figure 2A to 2C).

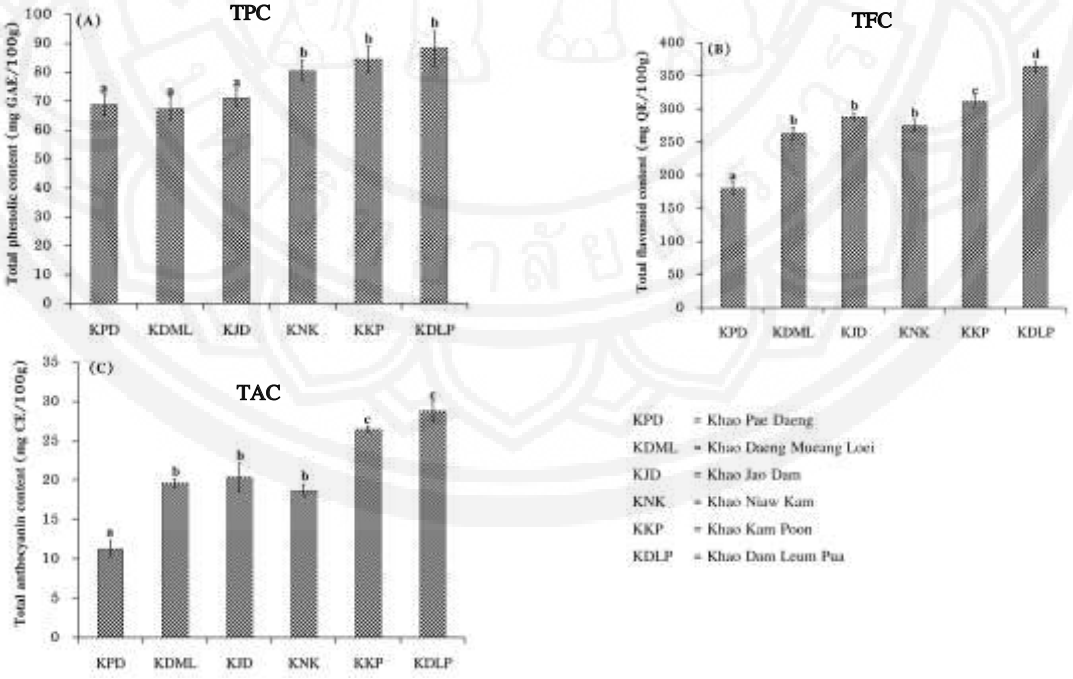

Figure 2 Bioactive compounds in six local pigmented rice. (A) TPC, (B) TFC and (C) TAC from local pigmented rice. Results represented as means ± standard deviation of three independent determinations. Different letters indicate significant differences ($p<0.05$)

Determination of phenolic acids by HPLC

From this study, the phenolic acid extracts were assayed by chromatographic analysis, using the HPLC-UV, to determine the content of phenolic acids. Gallic acid, vanillic acid, syringic acid, *p*-coumaric acid and ferulic acid were found in all local pigmented rice in different quantities (Figure 3A-3C and Table 2). The major component of phenolic acid was ferulic acid followed by vanillic acid, *p*-coumaric acid, syringic acid and gallic acid, respectively (Table 2). KDLP, KKP and KNK indicated the maximum of total phenolic acid contents (45.05-48.24 mg/100g DW) when compared to the other local pigmented rice (Table 2).

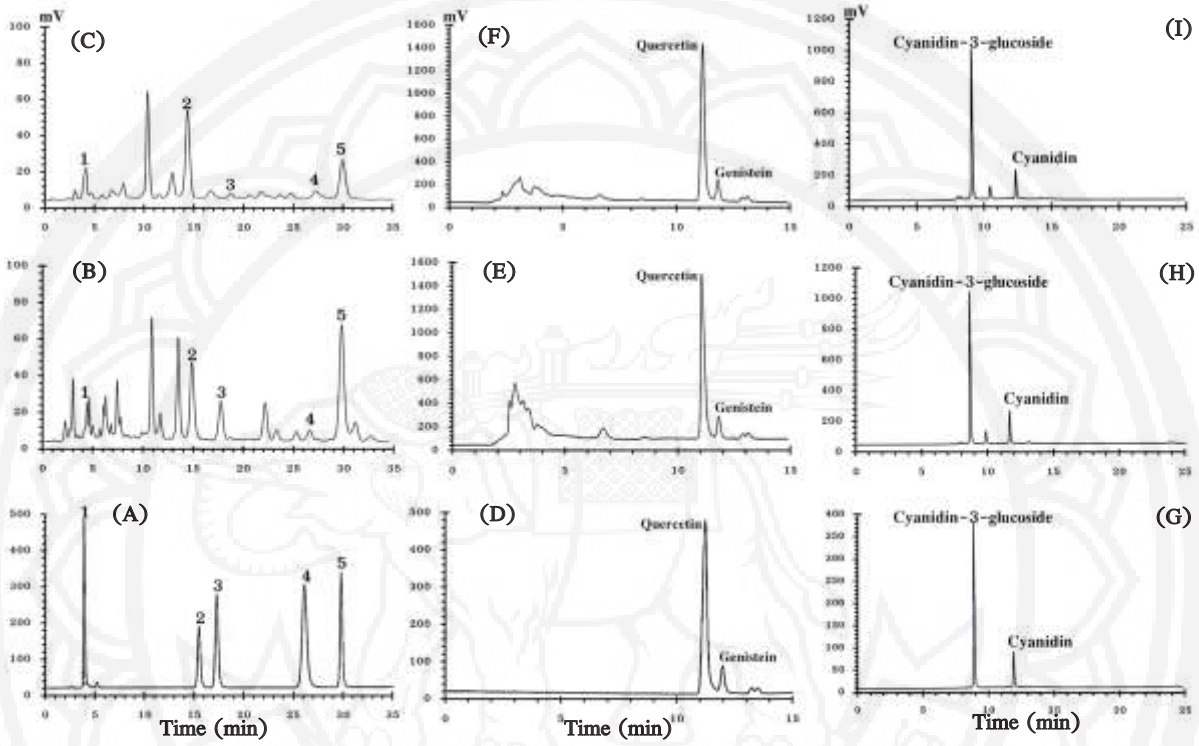

Figure 3 HPLC-UV-VIS chromatograms of bioactive compounds from local pigmented rice
(A) Chromatograms of a phenolic acid standards as 1=gallic acid, 2=vanillic acid, 3=syringic acid, 4=*p*-coumaric acid, 5=ferulic acid. (B-C) Chromatograms of phenolic acids from KDLP and KKP, respectively
(D) Chromatograms of a flavonoid standards. (E-F) Chromatograms of flavonoids from KDLP and KKP, respectively
(G) Chromatograms of an anthocyanin standards. (H-I) Chromatograms of anthocyanins from KDLP and KKP, respectively

Determination of flavonoids by HPLC

The major flavonoids components identified based on the standards using high performance liquid chromatography are shown in Figure 3 (D-F). Of the two flavonoids (quercetin and genistein) that are usually present in this experiment. Quercetin appears to be the major flavonoid in the local pigmented rice (Figure 3E-F and Table 2). KDLP and KKP presented the highest of flavonoid content is 0.47-0.48 mg/100g DW (Table 2). The calibration curves of quercetin and genistein also showed good linearity (r= 0.9990 and 0.9973, respectively) (Table 3).

Determination of anthocyanins by HPLC

About two anthocyanins of local pigmented rice have been quantified (cyanidin-3-glucoside and cyanidin) (Figure 3 G-I). The mean value of the sum of the two anthocyanins in local pigmented rice ranges from

22.31 to 76.46 mg/100g DW (Table 2). The result of this present study suggested that the local pigmented rice contains a higher amount of anthocyanin content with the following richness order: cyanidin-3-glucoside > cyanidin (Table 2). KDLP indicated the maximum of anthocyanin contents (76.46± 4.81 mg/100g DW) (Table 2). The calibration curves of cyanidin-3-glucoside and cyanidin also showed good linearity (r=0.9991 and 0.9999, respectively) (Table 3).

Linear regression equation, correlation coefficient (r), limit of detection (LOD) and limit of quantitation (LOQ) of phenolic acid, flavonoid and anthocyanin are shown in Table 3.

Table 2 Contents of phenolic acid, flavonoid and anthocyanin from six local pigmented rice (mg/100g DW)

Bioactive compounds	Local pigmented rice sample					
	KPD	KDML	KJD	KNK	KKP	KDLP
Phenolic acid						
Gallic acid	0.89± 0.01^{a}	0.76± 0.02^{a}	0.91± 0.02^{a}	1.02± 0.01^{a}	1.14± 0.02^{a}	1.03± 0.01^{a}
Vanillic acid	5.61± 0.22^{a}	5.82± 0.17^{a}	5.42± 0.23^{a}	5.54± 0.18^{a}	5.96± 0.31^{a}	6.33± 0.24^{a}
Syringic acid	2.62± 0.17^{a}	3.11± 0.25^{a}	3.13± 0.47^{a}	3.96± 0.56^{a}	3.81± 0.24^{a}	3.78± 0.17^{a}
p-Coumaric acid	3.53± 0.11^{a}	3.68± 0.41^{a}	3.72± 0.18^{a}	4.77± 0.51^{a}	6.02± 0.46^{a}	5.47± 0.35^{a}
Ferulic acid	26.88± 4.46^{a}	25.57± 3.41^{a}	28.43± 2.19^{a}	29.76± 2.82^{a}	30.14± 3.51^{a}	31.63± 3.07^{a}
Total	39.53± 4.97^{a}	38.94± 4.62^{a}	41.61± 3.09^{a}	45.05± 4.08^{b}	47.07± 4.54^{b}	48.24± 3.84^{b}
Flavonoid						
Quercetin	0.14± 0.01^{a}	0.11± 0.01^{a}	0.24± 0.01^{b}	0.28± 0.01^{b}	0.44± 0.04^{c}	0.45± 0.09^{c}
Genistein	0.02± 0.01^{a}	0.03± 0.01^{a}	0.02± 0.01^{a}	0.02± 0.01^{a}	0.03± 0.01^{a}	0.03± 0.01^{a}
Total	0.16± 0.02^{a}	0.14± 0.02^{a}	0.26± 0.02^{b}	0.30± 0.02^{b}	0.47± 0.05^{c}	0.48± 0.20^{c}
Anthocyanin						
Cyanidin-3-glucoside	21.17± 1.68^{a}	23.81± 1.46^{a}	25.32± 2.07^{a}	41.53± 2.60^{b}	57.86± 3.42^{c}	60.03± 3.88^{c}
Cyanidin	1.14± 0.07^{a}	2.42± 0.17^{a}	9.45± 0.43^{b}	10.94± 0.06^{b}	11.35± 0.23^{b}	16.43± 0.93^{c}
Total	22.31± 1.75^{a}	26.23± 1.63^{a}	34.77± 2.50^{b}	52.47± 2.66^{c}	69.12± 3.65^{d}	76.46± 4.81^{e}

Results represented as means ± standard deviation of three independent determinations. Different letters in the same row indicate significant differences ($p<0.05$)

Table 3 Limit of detection (LOD), limit of quantitation (LOQ) and linear regression equation of phenolic standards, flavonoid standards and anthocyanin standards analyzed by HPLC method

Standard	Linear regression equation				
	LOD* (μg/ml)	LOQ* (μg/ml)	Regression equation	Correlation coefficient (r)	Retention time (min)
Phenolic acid					
Gallic acid	0.3593	0.7374	Y=39355X-3881	0.9979	3.934
Vanillic acid	0.1994	0.5512	Y=26908X-654	0.9985	15.142
Syringic acid	0.1852	0.5560	Y=42062X-537	0.9984	17.307
p-Coumaric acid	0.4214	0.8523	Y=76971X-9075	0.9979	25.839
Ferulic acid	0.2041	0.6811	Y=37231X+25398	0.9957	29.812
Flavonoid					
Quercetin	0.2105	0.7013	Y=49287X+5565	0.9990	11.015
Genistein	0.2974	0.5892	Y=8727X+2558	0.9973	11.977
Anthocyanin					
Cyanidin-3-glucoside	0.0432	0.1804	Y=35763X+10601	0.9991	8.937
Cyanidin	0.0641	0.2034	Y=48943X+39001	0.9999	12.081

* Detection limits were calculated by the linear regression equation

DPPH and ABTS free radical scavenging activity

The free radical scavenging activities of local pigmented rice extracts were determined using two different methods (DPPH radical scavenging and ABTS radical scavenging assays). In the DPPH radical scavenging assay, Figures 4(A) show the free radical scavenging activities of extracts obtained from KPD, KDML, KJD, KNK, KKP and KDLP in the reactions with 1,1-diphenyl-2-picrylhydrazil. The extracts inhibited the DPPH in a dose dependent manner. The IC_{50} concentrations (Table 4) show significant ($p<0.05$) differences in DPPH scavenging activity among samples, where KDLP extracts show the lowest IC_{50} value of 49.78± 3.57 μg/ml (Table 4).

In the ABTS assay as presented in Figures 4(B), the ABTS inhibition percentage values were dose dependent, whereby they increased in the range of the tested concentrations, for the local pigmented rice extracts and the positive control (gallic acid, quercetin and cyanidin). The IC_{50} values of the local pigmented rice extracts and positive control were calculated and are listed in Table 4. The highest average scavenging activity was found in KDLP extracts (IC_{50}=16.54± 1.06 μg/ml) and KKP extracts (IC_{50}=19.60± 1.36 μg/ml) (Table 4).

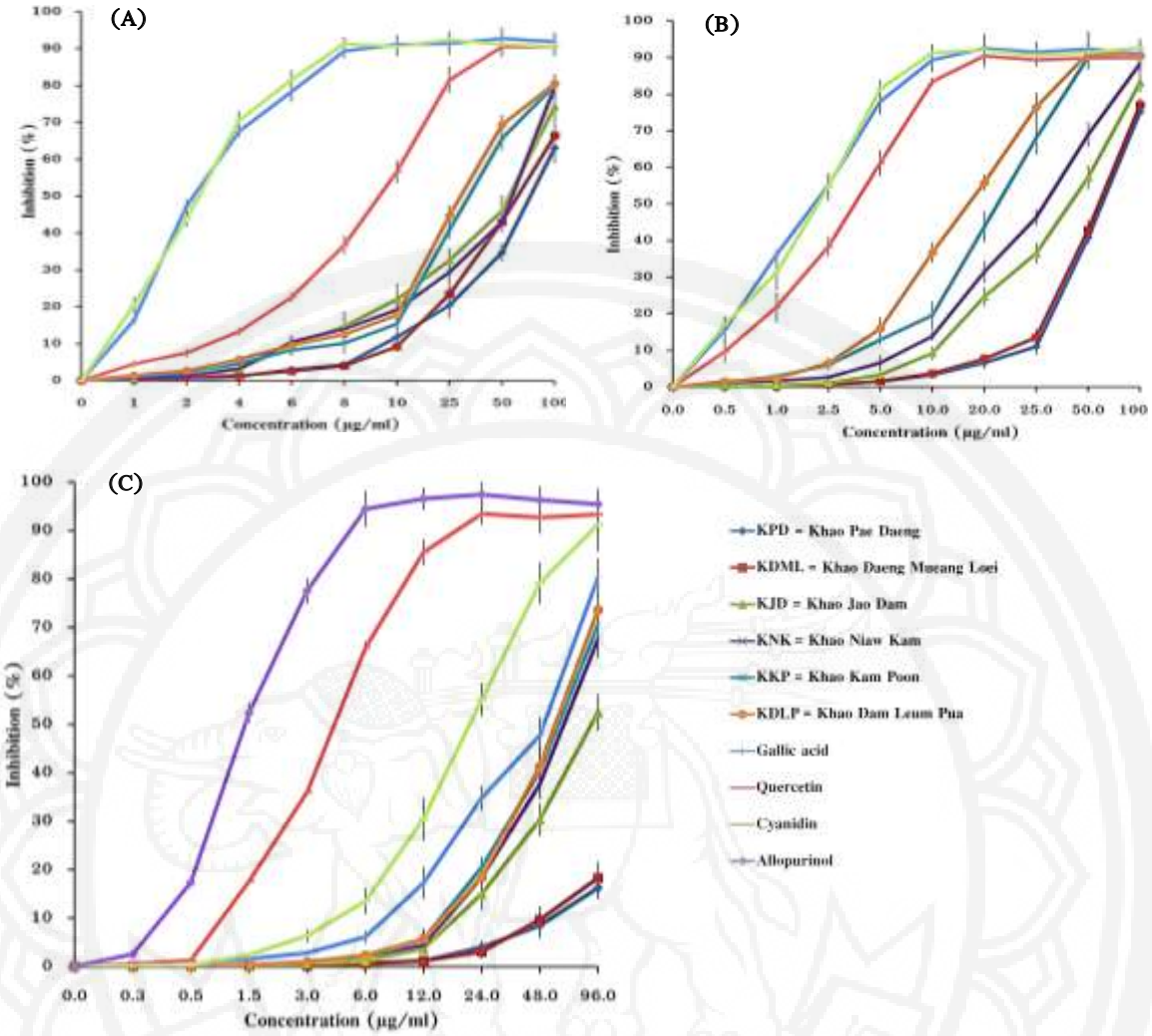

Figure 4 Effect of different concentrations from local pigmented rice extract in free radical scavenging activity and xanthine oxidase inhibitory effect. (A) = DPPH assay, (B) = ABTS assay, (C) = Xanthine oxidase inhibition test

Xanthine oxidase inhibitor activity

The extracts XO inhibitory activities are presented in Figure 4(C). All the extracts inhibited the XO activities in a dose dependent manner (Figure 4 C). The IC_{50} concentrations of KDLP, KKP and KNK extracts were significantly ($p<0.05$) lower than that of the KPD, KDML and KJD (Table 4). The results indicated that those extracts could be potential sources of bioactive compounds to inhibit XO activity.

Table 4 The value of IC_{50} and $1/IC_{50}$ of the local pigmented rice extracts

Extracts	Pigmented rice	Free radical scavenging activity				Xanthine oxidase inhibitory activity		Category*
		DPPH		ABTS				
		IC_{50}(μg/ml)	$1/IC_{50}$	IC_{50}(μg/ml)	$1/IC_{50}$	IC_{50}(μg/ml)	$1/IC_{50}$	
Ethanolic extract	KPD	>50	0.013	>50	0.015	>100	0.003	E
	KDML	>50	0.014	>50	0.015	>100	0.004	E
	KJD	>50	0.017	>50	0.017	88.56± 5.25[a]	0.011	D
	KNK	>50	0.017	34.62± 1.71[b]	0.029	69.65± 4.13[b]	0.014	C

Table 4 (Cont.)

Extracts	Pigmented rice	Free radical scavenging activity				Xanthine oxidase inhibitory activity		Category*
		DPPH		ABTS				
		IC_{50}(μg/ml)	1/ IC_{50}	IC_{50}(μg/ml)	1/ IC_{50}	IC_{50}(μg/ml)	1/ IC_{50}	
	KKP	>50	0.019	19.60± 1.36^{c}	0.051	66.15± 3.76^{b}	0.015	C
	KDLP	49.78± 3.57^{c}	0.020	16.54± 1.06^{c}	0.060	64.16± 4.42^{b}	0.016	A
Positive control	Gallic acid	3.92± 0.55^{d}	0.255	2.68± 0.14^{d}	0.373	54.99± 3.41^{c}	0.018	A
	Quercetin	13.04± 1.54^{e}	0.077	4.93± 0.13^{d}	0.203	5.83± 0.89^{d}	0.172	A
	Cyanidin	3.10± 0.66^{d}	0.323	2.46± 0.08^{d}	0.407	27.28± 2.50^{e}	0.037	A
	Allopurinol	N.D.	N.D.	N.D.	N.D.	1.90± 0.04^{f}	0.513	B

Results represented as means ± standard deviation of three independent determinations. Different letters in the same column indicate significant differences ($p<0.05$). * According to their effect on free radical scavenging activity and xanthine oxidase inhibitory activity of local pigmented rice extract were classified into specific categories (see Table 5). N.D. = Not detected

Table 5 Summary of the classification of local pigmented rice extract into five categories according to their free radical scavenging activity and inhibition of xanthine oxidase

Category	Free radical scavenging activity[a]	Xanthine oxidase inhibitory activity[a]	Example
A	+	+	Quercetin, Cyanidin, Gallic acid, KDLP
B	N.D.	+	Allopurinol
C	−	+	KNK, KKP
D	0	+	KJD
E	0	0	KPD, KDML

[a]Key: 0 = no effect; + = effect; − = pro-oxidant effect; N.D. = Not detected

Correlation between antioxidant activity and the local pigmented rice extracts

Table 6 shows the total phenolic (TPC), total flavonoid (TFC) and total anthocyanin (TAC) contents were highly correlated to free radical scavenging activities with a high correlation coefficient (r^2) values ranging from 0.7562 to 0.8962. Similar correlation coefficients were observed when *p*-coumaric acid, ferulic acid, quercetin, cyanidin and cyanidin-3-glucoside were compared to the free radical scavenging activities, r^2= 8408, 0.8122, 0.9255, 0.8153 and 0.9207, respectively (Table 6). When the correlations between anthocyanin contents and the free radical scavenging activities are compared for the phenolic contents and flavonoid contents separately, it becomes clear that the anthocyanin contents values are better correlated with the free radical scavenging activities (r^2=0.8459) as opposed to phenolic contents (r^2=0.7278) and flavonoid contents (r^2=0.6591) (Table 6).

Correlation between XO inhibition and the local pigmented rice extracts

The correlation coefficient value of local pigmented rice extracts as XO inhibitors is presented in Table 6. Low positive correlations (r^2<0.8000) were observed between phenolics, flavonoids and anthocyanins with xanthine inhibitory activities in this study. For the anthocyanins, we found a higher significant correlation between XO inhibition (r^2=0.7470) than phenolic contents (r^2=0.6796) and flavonoid contents (r^2=0.5071) (Table 6). Cyanidin, a member of anthocyanin, in local pigmented rice shows the highest positive correlations (r^2=0.9192) with xanthine inhibitory activity (Table 6).

Table 6 The correlation coefficients values (r^2) between free radical scavenging activities ($1/IC_{50}$) or xanthine oxidase inhibitory activity ($1/IC_{50}$) and bioactive compounds

Bioactive compounds	DPPH	ABTS	Average	XO inhibition
Total phenolic contents (TPC)	0.8720	0.9204	0.8962	0.8131
Gallic acid	0.6510	0.6336	0.6423	0.7418
Vanillic acid	0.3915	0.6907	0.5411	0.1298
Syringic acid	0.7090	0.5591	0.6341	0.8003
p-Coumaric acid	0.8063	0.8753	0.8408	0.7004
Ferulic acid	0.8518	0.7725	0.8122	0.8919
Average	-	-	**0.7278**	**0.6796**
Total flavonoid contents (TFC)	0.8568	0.6555	0.7562	0.6571
Quercetin	0.9413	0.9096	0.9255	0.8324
Genistein	0.2232	0.3681	0.2957	0.0319
Average	-	-	**0.6591**	**0.5071**
Total anthocyanidin contents (TAC)	0.8605	0.7426	0.8016	0.5883
Cyanidin-3-glucoside	0.8771	0.9642	0.9207	0.7335
Cyanidin	0.9212	0.7093	0.8153	0.9192
Average	-	-	**0.8459**	**0.7470**

Discussion

The antioxidant compounds in rice were classified into six groups: phenolic acids, flavonoids, anthocyanins, tocopherols, γ-oryzanol and phytic acid (Goufo & Trindade, 2014). In this research, phenolic acids, flavonoids and anthocyanins related to quantifying antioxidant compounds in local pigmented rice are a highlight. Six local pigmented rice are composed of all compounds but different quantities (Table 1, Table 2, Figure 2 and Figure 3).

Phenolic acids are molecules containing a phenolic ring and an organic carboxylic acid function. The C6-C1 skeleton of the phenolic ring is hydroxybenzoic acid derivative (gallic acid, vanillic acid, syringic acid). While, C6-C3 skeleton of the phenolic ring is a hydroxycinnamic acid derivative such as *p*-coumaric acid, ferulic acid (Goufo et al., 2014). In this research, five phenolic acids are usually identified in local pigmented rice, with their sum ranging from 38.94 to 48.24 mg/100g DW (Table 2). The data indicate the portion of hydroxycinnamic acid derivative higher than a hydroxybenzoic acid derivative in local pigmented rice (Figure 3A to 3C, Table 2). Our results are similar to those of previous studies reporting that the phenolic acids in rice (Goufo & Trindade, 2014; Moongngarma, Daomukda, & Khumpika, 2012; Sompong et al., 2011).

In general, the phenolic acids have an aromatic ring with one or more hydroxyl groups. The number and position of a hydroxyl group on a phenolic ring leads to the variation in their antioxidant potential (Goufo & Trindade, 2014). Both DPPH and ABTS are stable free radicals that dissolve in ethanol (Li et al., 2009). When a free radical scavenger by hydrogen donation, the colors in the DPPH and ABTS assay solutions become lighter. As presented in Figures 4A and 4B, both the DPPH and ABTS inhibition percentage values were dose dependent for the local pigmented rice extracts and the positive control (gallic acid). The free radical scavenging activity of the DPPH and ABTS in the local pigmented rice correlated with that observed with the TPC (r^2=0.8962) and phenolic acids (r^2=7278), especially *p*-coumaric acid and ferulic acid (Table

6). This result was similar to the study by Kitisin, Saewan, and Luplertlop (2015), which reported the antioxidant properties of Thai pigmented rice extracts. This research shows the pigmented rice extract from KDLP may act as an electron donating substance, showing the highest free radical scavenging activity (Table 4 and Table 5).

The structure of flavonoids consists of a 15-carbon skeleton that is organized in two A and B of aromatic rings interlinked by a three-carbon chain (C6-C3-C6) (Prior & Wu, 2006). Flavonoids can be classified into six groups (flavones, flavonols, flavanols, flavanonols, isoflavones and flavanones). In this study, total flavonoid content (TFC) was highest in KDLP followed by KKP, KJD, KNK, KDML and KJD (Figure 2B). All local pigmented rice showed the different composition of flavonoids (Figure 3 and Table 2). Quercetin, a type of flavonols group, is a major component found in all local pigmented rice (Figure 3 and Table 2). This result was similar to the study by Prior and Wu (2006), which reported the quercetin content in pigmented rice (whole grain) range from 0.42 to 3.68 mg/100g DW.

Flavonoids are recognized for both their ability to donate electrons and to stop the chain reaction. These activities are attributed to the phenolic hydroxyls, particularly in the 3′OH and 4′OH of the three-carbon chain (Goufo & Trindade, 2014). In this research, the ethanolic extract of all local pigmented rice showed a similar percentage inhibition value to that of quercetin standard in DPPH assay and ABTS assay (Figure 4A and 4B). The free radical scavenging activity of the DPPH and ABTS in the local pigmented rice correlated with that observed with the TFC (r^2=0.7562), especially quercetin (r^2=0.9255) (Table 6).

Anthocyanins, another class of flavonoids, are intermediates in the synthesis of proanthocyanidins. Anthocyanins are colored water-soluble pigments representing one of the major subclasses of compounds. Goufo and Trindade in the 2014 year showed the 18 anthocyanins have been identified in rice, especially four anthocyanins have been quantified (cyanidin-3-glucoside, peonidin-3-glucoside, cyanidin-3-rutinoside and cyanidin-3-galactoside). This study revealed that black-red pigmented rice (KDLP, KKP) accumulated significant amounts of anthocyanins as total anthocyanin content (TAC), compared with other local pigmented rice varieties (Figure 2 and Table 2). The results were similar to Zhang, Zhang, Zhang, and Liu (2010), who found that high amounts of anthocyanins in black rice. In this study, cyanidin-3-glucoside (21.17-60.03 mg/100g DW) and cyanidin (1.14-16.43 mg/100g DW) were identified and measured in local pigmented rice (Figure 3G-3I and Table 2). This result was similar to the study by Sompong et al. (2011), which reported the cyanidin-3-glucoside is present at the highest level among the red and black rice.

The free radical scavenging activity of red rice of Thailand and Srilanka were previously assessed and the report suggested that Thai red rice is superior to Srilankan rice, but black rice is more effective than red rice varieties (Pengkumsri et al., 2015). Whereas, the current investigation employed the free radical scavenging assay for the comprehensive analysis of free radical scavenging property of local pigmented rice extract. Collectively, the data obtained from the free radical scavenging property evaluation studies, resulted in a KDLP of local pigmented rice extract (black-red) rich in free radical scavenging compounds than other tested rice varieties (Table 2, Table 5 and Table 6).

XO inhibitory activity of local pigmented rice extracts was determined by using xanthine as a substrate with a different concentration of allopurinol standard and extracts ranging from 0.3 to 96 μg/ml (Figure 4C). The local pigmented rice extract showed a similar percentage inhibition value to that of the allopurinol standard (Figure 4C). Likewise, ethanolic extracts from KDLP and KKP showed the lowest IC_{50} value when compared

with other rice (Table 4). These indicate that, ethanolic extract from KDLP and KKP unable to inhibit the xanthine oxidase enzyme. Besides, KDLP and KKP exhibit the highest of TPC, TFC and TAC (Table 2 and Figure 2). Moreover, The XO inhibitory activity of local pigmented rice correlated with that observed with the TPC, TFC and TAC, especially cyanidin (r^2=0.9192) (Table 6). Therefore, the xanthine oxidase inhibitory activity of KDLP and KKP might be due to the presence of anthocyanin compounds in the local pigmented rice extract.

Phenolic compounds are reported to possess XO inhibitory activities. XO inhibitory activities of some plants (*Vicia faba* and *Lotus edulis*) showed the potent inhibition of XO enzyme with IC_{50} values range from 40-135 µg/ml and 55-260 µg/ml, respectively (Metha & Nayeem, 2014). The structure-activity of flavonoids influences the XO inhibition via interaction with the molecular target of flavonoids (Wong, Ng, Chuah, Koh, & Ling, 2014). Previous studies showed that the structure-activity relationship of flavonoids will determine the XO inhibitory activity and radical scavenging activity (Cos et al., 1998). Flavonoids that are selected as potential XO inhibitors should consist of the either hydroxyl group at C-5 and C-7, double bond between C-2 and C-3 or planar structure in flavones are essential. For example, quercetin exhibit a higher inhibition rate of XO enzyme compared to the glycosides of these flavonoids (Cos et al., 1998).

Anthocyanins, phenolic phytochemical compounds, are widespread in plants. Previous reports showed cyanidin and cyanidin-3-glucoside have significant inhibition of XO activity (Acquaviva et al., 2003). In this assay, the cyanidin standard showed a more potent XO inhibitory activity (Table 4 to 6). This result was similar to the study by Yamasaki, Uefuji, and Sakihama (1996), which reported the anthocyanin could be bleached by the non-enzymatic reaction with the superoxide radical. The XO inhibitory action of local pigmented rice extract in the xanthine-xanthine oxidase system may be due either to the scavenger activity of anthocyanin compounds or to their action on the primary function of the enzyme. To the best of our knowledge, this is the first report on the potential ability of local pigmented rice extracts (KDLP and KKP) to inhibit xanthine oxidase enzyme.

Conclusion and Suggestions

This study had established the bioactive compounds, antioxidant activity and xanthine inhibitory action from six local pigmented rice of Loei province. Six local pigmented rice contained antioxidant compounds, including phenolic acids, flavonoids and anthocyanins. These compounds may play a role in the antioxidant activities observed in six pigmented rice extract. Pigmented rice (KDLP) is found superior in bioactive compounds and bioactive properties than other pigmented rice varieties. Anthocyanins, especially cyanidin, exhibit a high potential ability to inhibit xanthine oxidase. Correlation analysis showed that anthocyanin was positively correlated to xanthine oxidase inhibitory activity. It may be useful for the treatment of hyperuricemia and gout.

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