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Research article

Quantification of polyphenol content, antioxidant properties and LC-MS/MS analysis in Malaysian indigenous rice cultivars (*Oryza sativa* L.)

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Abstract

The study was carried out to evaluate the differences in the total phenolic content (TPC), total flavonoid content (TFC), antioxidant activities via 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic) and 2,2-diphenyl-1-picrylhydrazyl assays and quantification of targeted polyphenol compounds in Malaysian upland and wetland rice (*Oryza sativa* L. *indica*) varieties. Of note, the Bario cultivar of upland rice-seed grain extracts had the highest TPC (47.84 mg gallic acid equivalents/g), TFC (7.14 mg rutin equivalents/g) and antioxidant activities 25% higher than for the other cultivars. Ferulic acid, salicylic acid and *p*-coumaric acid, (the most abundant polyphenol compounds and known as a group of flavonoids responsible for high antioxidant properties) were detected in all extracts tested; upland rice of the Bario cultivar had a higher portion of ferulic acid (10.31 mg/100g), salicylic acid (3.25 mg/100g) and *p*-coumaric acid (1.10 mg/100g). This finding of antioxidant compounds was further confirmed using liquid chromatography combined with tandem mass spectrometry analysis in which ferulic acid produced the most substantial peak at $[M-H]^-$ m/z 193 to form the daughter ion of 134 at a retention time of 5.79 min, while p-coumaric acid was detected at m/z 163 to form the daughter ion of m/z 119 at a retention time of 4.95 min. The results indicated that the increased antioxidant quantity in upland rice could support the available upland rice grain database for the development of enriched health-promoting foods.

Introduction

Rice (*Oryza sativa* L. *indica*) is considered an important grain crop for human consumption and has the second highest global production among world staple foods after maize in the world (Zuraida et al., 2011). In Malaysia, most of the rice-growing area is located in the irrigated lowlands and approximately 660,000 ha of arable land is used to grow the most favorable variety, MR219 (Abdul Fatah, 2017). Upland rice, one of the preferable types eaten by rural communities and only at low scale production is cultivated under limited irrigation and contributes 11% of total global rice production (Mohd Din et al., 2016). The Malaysian upland rice yield is categorized low

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and variable in production in the range 0.46–1.1 t/ha (Sohrabi et al., 2013). Worldwide, researchers have put considerable effort scientifically into upland rice cultivars in order to improve the grain yield and crop morphological traits. In addition, the poor nutritional value of upland rice is commonly because of the lack of good nutrient management in upland rice cultivation. Notwithstanding ongoing improvement efforts, these upland rice cultivars can withstand harsh environmental changes that contribute to their advantages over other rice types (Hanafi et al., 2009). Malaysian agriculture depends greatly on wetland rice varieties such as MR219 and MR220 for national food security and there is a need for diversification to other potential candidates of upland rice with similar yield characteristics. Alternatively, there must be effort to intensify cultivation and improve the functional healthy characteristics that may appeal to the consumer preference. Because rice is a staple food globally, rice with higher nutritional as well as antioxidant capacity is being considered a requirement for having a healthy society. The strategy should be to develop good quality rice varieties looking not only at special genotypic characteristics but also considering those rich in functional bioactive compounds showing a high natural antioxidant trait. Furthermore, malnutrition and increased incidence of chronic disease phenomena have become a major problem in developed countries increasing the need to urgently provide highly nutritious rice grain (Hefferon, 2015). Aune et al. (2016) clarified the comprehensive meta-analysis on whole-grain intake in relation to cardiovascular diseases incidence risk. They mentioned the importance of consuming whole-grain rice packed with nutrition instead of refined rice as the former food has been associated with a lower prevalence of chronic diseases. Thus, whole rice grain with its functional health benefit is part and parcel of the whole suggested solution for the improvement of living standards around the world.

Therefore, more attention should be paid to consuming rice varieties with high nutritional components in the rice grain resulting in both nutritional (calorific) and health benefits. These ubiquitous compounds are believed to exhibit important therapeutic roles against degenerative and chronic diseases, thanks to their antioxidant properties (Burlando and Cornara, 2014). Antioxidant properties, particularly in phenolic acid, exhibit a wide range of physiological properties such as anti-allergenic, anti-artherogenic, anti-microbial, cardioprotective and vasodilatory effects (Vichapong et al., 2010). It has been shown that consumption of high-value rice grain can lead to decreased oxidative stress and a simultaneous increase in antioxidant capacity in the tested cells (Chi et al., 2007; Lin and Tang, 2007). Phytochemical investigation on phenolic compounds and their antioxidant properties from different rice varieties has gradually increased over recent decades. The predominant phenolic compounds are considered to be responsible for the antioxidant activity in rice and include: flavonoids, anthocyanins, isovitexin, proanthocyanidins, oryzanol, α-tocopherol, tocotrienol (also called tocols or vitamin E) and phytic acid (Shao et al., 2014). To date, at least 29 phenolic acids have been identified being predominantly classified as ferulic, ρ-coumaric and sinapic acids according to Goufo and Trindade (2014). The phenolic concentration (polyphenols) in the whole rice grain is normally dependent on the cultivar, processing conditions, growth stage of the pericarp color and abiotic factors (Walter et al., 2013). Until now, research on phytochemicals and antioxidant properties has been widely reported on brown rice, black rice, purple rice, red rice, white rice and wild brown rice (Chi et al., 2007; Moongngarm and Saetung, 2010; Chen et al., 2012; Shao and Bao 2015; Zhang et al., 2015; Das et al., 2017; Gong et al., 2017). Phenolic compounds analysis is normally determined in three steps. In the first stage, extraction of phenolic compounds from samples using ultrasonic-assisted extraction, Soxhlet extraction, microwave assisted extraction, supercritical fluid extraction or pressurized liquid extraction. Then, the extracts are cleaned up to eliminate interference and finally the precise amount of phenolic compounds can be analyzed using several methodologies based on the objectives of the particular study. In the current study, liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) analysis was selected due to its high selectivity and sensitivity. It is worthwhile utilizing this analytical method as it is possible to separate the isomeric structures of phenols within one chromatographic run. Jaitz et al. (2010) highlighted 11 analytes belonging to phenol classes of red wine using LC-MS/MS detection. However, the current study only focused on rice varieties planted in wetland areas. To the best of the current authors' knowledge, there is still scarce data available on the phytochemical contents and their antioxidant properties for Malaysian upland rice cultivars; hence the novelty of this study. In addition, the current study focused on the identification of polyphenols using the combination of the separation capabilities of LC and the power of MS as an authentic confirmation method. The significance of the existence of some phenolic compound in upland rice is crucial for rice breeders to screen for local varieties with functional properties through comparative evaluation of phytochemical profiling. Thus, some rice varieties with low yield may have a high content in terms of the functional quality of bioactive compounds. Therefore, the purpose of this study was to investigate the phytochemical constituents including the phenolic and flavonoids contents and obtain further confirmation of the specific bioactive compounds to ascertain antioxidant properties and LC-MS/MS analysis based on theoretical mass fragmentation patterns. These characterization data will support the critical estimation of available upland rice grain data for the potential development of health-promoting foods.

Material and Methods

Chemicals and reagents

Ethanol (reagent grade), sodium carbonate and aluminum chloride were obtained from Fisher Scientific (Pittsburgh, PA, USA). Distilled water and deionized water were produced using a Barnstead E-pure water system (Thermo; Waltham, MA, USA). The high performance liquid chromatography (HPLC) grade of acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic) or ABTS, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu's phenol reagent, *p*-coumaric

acid, salicylic acid and ferulic acid standards were obtained from Sigma-Aldrich (St Louis, MO, USA). The reference standards consisting of gallic acid and rutin were obtained from Acros Organics (Pittsburgh, PA, USA). All chemicals used were of analytical grade.

Rice material

Manually dehusked seed samples of Malaysian upland rice (*Oryza sativa* L.) Bario and Bukit Pulut cultivars were collected from Sabah and Sarawak provinces, West Malaysia. Two wetland rice varieties named as Mahsuri and Sri Malaysia 1 cultivars were identified by and released from the Malaysian Agricultural Research and Development Institute, Malaysia. The samples were stored at 4°C prior to analysis.

Sample extraction

Samples of 2 g of ground rice were extracted using 10 mL of 80% ethanol. Samples were continuously shaken (SP 200 Digital Orbital Shaker; Daihan Scientific; Gang-Won-Do, Korea) for 3 hr at ambient temperature. The extracted samples were centrifuged (Mikro 200; Hettich Centrifuge; Tuttlingen, Germany) at 2,500 revolutions per minute for 10 min and the supernatants were collected. The extracted samples (a combination of both extract and supernatant) were further concentrated and kept continuously shaken for 1 hr before solvent drying using a rotary evaporator (Heidolph; Schwabach, Germany). The dried ethanol extract was resuspended in 5 mL of 80% ethanol. Evaporation of the extracts was done under pressure using a rotary evaporator at 40° C for 30 min. The rice-grain extract solutions were stored in the refrigerator at -25°C until assays for antioxidant activity and polyphenol determination were carried out.

Determination of total phenolic content

The total phenolic content (TPC) was measured using the Folin-Ciocalteu method described by Bonoli et al. (2004) with minor modifications. Rice-grain extract (200 µL) was mixed with 1 mL of Folin-Ciocalteu reagent (2N) and left for 5 min before adding with 800 µL 10% sodium carbonate (Na_2CO_3). Then, the mixture was diluted with 5 mL of distilled water. The TPC of the rice-grain extract was measured by reading the absorbance of the mixture at 760 nm using an ultraviolet-visible spectrum (UV-VIS) spectrophotometer (UV-1800; Shimadzu Corp.; Kyoto, Japan) after incubation for 2 hr. As a comparison, gallic acid was used as a chemical standard of calibration. The TPC was expressed as milligrams of gallic acid equivalents (GAE) per gram of rice grain extract (mg GAE/100 g). Additional dilution was performed if the absorbance value exceeded the linear range of the standard curve.

Determination of total flavonoid content

The total flavonoid content (TFC) was determined using the formation of a complex flavonoid-aluminum method (Djeridane et al., 2006). About 1 mL of rice-grain extract and standard solutions were added with 1mL of 2% aluminum chloride ethanolic solution. The absorbance of the mixture was measured at 430 nm using the UV-VIS spectrophotometer after incubation at room temperature for 15 min. Rutin was used as a chemical reference and calibration curve. TFC measurement was calibrated to a standard curve of prepared rutin solution and was expressed as milligrams of rutin equivalents (RE) per 1 g of rice-grain extract (mg RE/100g).

Radical scavenging activities of rice grain extracts

For both DPPH and ABTS assays, samples were diluted appropriately with ethanol. Ascorbic acid was used as an antioxidant standard. DPPH assay was measured according to the method described by Fasahat et al. (2012). For the DPPH reaction, 100 µL of rice-grain extract was mixed with 1.9 mL DPPH solution (0.1 mM in 80% ethanol) and incubated for 30 min at room temperature in the dark. The reaction mixture was measured at 517 nm using the UV-VIS spectrophotometer. The reactions were performed in triplicate and mean values were obtained for both the samples and standard. Ascorbic acid was used as a control. The percentage of scavenging activity was calculated as caused by the hydrogen donor activity of each sample using the formula: scavenging activity (%) = 1 - absorbance of sample / absorbance of control, ascorbic acid) × 100. The antioxidant activity was described using the amount of antioxidant required to scavenge the initial DPPH concentration by 50% (IC_{50}) by comparison to a standard curve and was expressed as a concentration in micrograms per milliliter.

Slight modification was made to the 2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay procedure of van den Berg et al. (1999). ABTS reagent was dissolved in water to a 7 mM concentration. Briefly, to produce the radical cation ($ABTS^{\cdot+}$), 2.5 mM potassium persulfate was mixed with ABTS reagent at ratio of 1:1 and the solution was incubated at room temperature under minimal light for 6 hr to complete the chemical reaction. To perform the assays, the ABTS solution was diluted with ethanol and spectrophotometrically measured until it reached an absorbance value of 0.70 ± 0.02 at 734 nm with phosphate buffered saline at pH 7.4. Freshly prepared samples of rice-grain extract and standard (50 µL) at different concentrations were mixed with 1.5ml $ABTS^{\cdot+}$ solution. After 5 min, these mixture solutions were measured at 734 nm using the UV-VIS spectrophotometer. $ABTS^{\cdot+}$ solution (ascorbic acid, vitamin C standard) was used as the control. The percentage of inhibition of the samples and standard were calculated using the concentration-response curve. The affinity of the test material to quench ABTS free radicals was evaluated according to the formula: scavenging activity (%) = 1 - absorbance of sample / absorbance of control, without sample) × 100. The antioxidant activity was mentioned as the IC_{50} by comparison with the standard curve and results were expressed in concentration units of micrograms per milliliter.

Quantification of polyphenolic compounds in rice grain extract using liquid chromatography mass spectrometry

Quantification of three of the selected phenol compounds (*ρ*-coumaric, salicylic acid and ferulic acid) was performed using LC-MS/MS in the negative ionization mode. The chromatographic system consisted of an Acquity HPLC unit equipped with a binary pump, autosampler, a thermostatted column compartment, a degasser (Waters Corporation; Milford, MA, USA) connected to an ESI-MS interface 4000 Q TRAP system (Applied Biosystems AB Sciex; Carlsbad, CA, USA). The C-18 reversed phase separation was operated on a 150 mm × 4.6 mm column with a 1.7 µm particle diameter. The Analyst software (version 1.4.2; Applied Biosystems AB Sciex; Carlsbad, CA, USA) was used for instrument control and data analysis. Solvent A was 99.9% water with 0.1% formic acid while 99.9% acetonitrile with 0.1% formic acid was used as Solvent B. The LC system was operated for 30 min with the designated gradient 0–10 min, 10% B; 10–12 min, 10–90% B; 12–14 min, 90% A; 14–15 min, 90–10% B; for the final washing and equilibration of the column before the next injection. The injection volume was set at 5 µL per sample. All samples and the mobile phase were filtered using 0.2 µm nylon prior to injection.

Operational mass spectrum analysis was set in the range 50–300 m/z with 250 ms ion accumulation time. The voltage and capillary were set and maintained at 400° C and -4.5kV, respectively. The mass spectrometer parameters were: nitrogen gas for nebulization, 40 pounds per square inch (psi); for drying solvent, 40 psi; nitrogen gas, 10 psi; collision gas set at high; de-clustering potential at 30–50 V and collision exit energy at 10–20 V. Three of the selected phenol compounds were separately determined using multiple reaction ions monitoring (MRM) in the infusion mode. The identified analytes were quantified based on their peak areas and comparison with a calibration curve obtained with the corresponding standards.

Statistical analysis

The experimental data of TPC and TFC and antioxidant properties of the rice samples were analyzed using analysis of variance. Then means were compared using Duncan's multiple range test in the SPSS 16.0 statistical software (SPSS Inc.; Chicago, IL, USA). Significance was tested at $p < 0.05$.

Results and Discussion

Bleaching of DPPH was determined to verify their availability of free radical scavenging activity. The DPPH and ABTS assays are frequently preferable techniques to measure antioxidant properties in different ranges of plant samples including rice grain by providing an index of the free radical quenching capacity. The results showed that the antioxidant capacity (DPPH & ABTS) of the tested rice grain samples followed the rank order: Bario cultivar > Bukit Pulut cultivar > Mahsuri cultivar > Sri Malaysia cultivar. DPPH scavenging assay is considered a suitable and easy colorimetric technique for evaluation of antioxidant effects where there is a known lipophilic character. The chemically generated ABTS was used to examine both hydrophobic and lipophilic antioxidants. The ABTS method is normally based on the ability of antioxidant molecules to quench the long-lived $ABTS^{\cdot+}$ radical (Kim et al., 2002). A lower concentration value of IC_{50} indicates a higher antioxidant activity. In this study, ascorbic acid was used as a scavenger indicator to measure the scavenging response within the rice-grain extracts. The DPPH assay results showed that ascorbic acid was capable of scavenging about 93.84 ± 0.1% at a concentration below 1,000 µg/mL (result not shown), with an IC_{50} of 262.5 ± 0.4 µg/mL (Table 1). In general, the rice varieties were significantly different using both assays. The antioxidant activity results revealed that two upland rice cultivars had a better scavenging effect compared to both wetland rice cultivars. For the upland rice variety, the IC_{50} values obtained in ascending order were from Bukit Pulut to Bario. However, the lower antioxidant activity in the wetland rice was probably due to less active antioxidant compound existing in polished rice cultivars. Some of the nutritional value was probably removed through the milling and bleaching processes. The study done by Faiz et al. (2015) confirmed that the percentage of scavenging activity of selected upland rice genotypes from Malaysia was about 31.85–98.45% which was higher than for non-pigmented white rice. The current results showed the percentage of scavenging activity measured at a concentration of 1,000 µg/mL was within their acceptable ranges of 67.47% (upland rice Bario, result not shown) and 43.83% (upland rice Bukit Pulut, result not shown). However, Faiz et al. (2015) did not mention the effective concentration (IC_{50}) for their tested upland rice cultivars. In the current study, the IC_{50} value for DPPH for Bario was 810.47±7.46 µg/mL and 2250.34±30.03 µg/ml for the Bukit Pulut cultivar. These results could be explained by upland rice being a type of pigmented brown rice reported to have

Table 1 IC_{50} values (mean±SD) for ABTS and DPPH scavenging activities of ethanolic extracts of different rice grain varieties

Sample	DPPH scavenging activity IC_{50} (µg/mL)	$ABTS^{\cdot+}$ scavenging activities IC_{50} (µg/mL)
Control	262.48 ± 3.45^{a}	42.88 ± 4.33^{a}
UR Bario	810.47 ± 7.46^{b}	3480.93 ± 56.25^{b}
UR Bukit Pulut	2250.34 ± 30.03^{bc}	3820.59 ± 20.62^{b}
WR Mahsuri	7835.05 ± 45.53^{cd}	14910.69 ± 633.28^{c}
WR Sri Malaysia	8709.82 ± 6466.28^{e}	27038.82 ± 69.77^{d}

ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic); DPPH = 2,2-diphenyl-1-picrylhydrazyl; UR = upland rice; WR = wetland rice; IC_{50} = amount of antioxidant required to scavenge the initial DPPH concentration by 50%.

Means in the same column with different lowercase superscripts are significantly different ($p < 0.05$).

high nutritional value content and antioxidant properties (Vichapong et al., 2010). Furthermore, Faiz et al. (2015) discovered that upland rice cultivars contained high micronutrient contents and antioxidant properties compared with commercialized white rice. Goufo and Trindade (2014) explained in detail the factors contributing to varying amounts of phenolic compounds in rice that included: soil type, chemical inputs, different extraction solvent and techniques, degree of ripening and different processing methods.

The antioxidant capacity using ABTS assay was significantly different and consistently higher than the antioxidant capacity determined using DPPH. ABTS requires a higher concentration to scavenge the oxidized molecules at their effective concentration. For example, the upland rice Bario cultivar was about four times lower in responding to ABTS compared to DPPH assay. The extraction efficiency of organic compounds is greatly dependent on the extraction method used. In the current study, Soxhlet extraction was used and the ultrasound assisted extraction (UAE) technique was suggested as this approach could potentially penetrate the matrix material, rupturing the cell walls to easily release the extracted compounds from the matrix (Oniszczuk and Olech., 2016). In addition, 80% ethanol was used as an extractant and appeared to be the superior solvent. However, the molecular affinity and polarity of the analyzed compounds may be attributed to the reduced extraction efficiency thus generating a low ABTS reading. The difference in the ABTS and DPPH determination was probably due to the different test methods which are based on hydrophilic and hydrophobic antioxidant systems. This needs further evaluation. The two ABTS highest IC_{50} values were found in the upland rice Bario and Bukit Pulut cultivars, (3,480.93 µg/mL and 3,820.59 µg/mL respectively). It can be clearly seen from the data that a higher concentration is required to achieve 50% of scavenging effect for non-pigmented wetland rice. There was a similar trend in the antioxidant activity observed using both DPPH and ABTS methods. However, the difference between these antioxidant capacities was greatly dependent on the highly colored pigmented food as discussed by Floegel et al. (2011).

Different rice varieties produced different concentrations of phenolic compounds. Therefore, TPC and TFC may directly predict the antioxidant activities. The TPC was expressed as milligrams of GAE per gram of rice whereas the TFC was expressed as milligrams of RE per gram of sample. Overall, the ratio of TFC:TPC for all rice-grain extract samples is presented in Table 2. The TPC was in the range 20.00–47.84 mg GAE/g of dry grain weight for both rice grains, while the TFC was in the range 3.35–7.14 mg RE/g of sample. Indeed, the upland rice cultivars had the highest phenolic content (TPC and TFC) with the exception of the TFC value in the Bukit Pulut sample (Table 2). This phenolic compound can be predicted to contribute to the higher antioxidant activities than had been reported previously. These findings prove the phenolic compounds are the major contributor toward antioxidant activity in the rice grain. Presumably, this is provided by the flavonoids and also non flavonoid phenolics. The TPC values of the two upland cultivars were significantly different. The Bario cultivar produced a TPC value of 47.84 mg GAE/g followed by the Bukit Pulut cultivar with 28.78 mg GAE/g. Furthermore, the Bario cultivar had a good TPC among the rice grain extracts tested. The TFC values of the Mahsuri and Sri Malaysia cultivars were 27.19 mg GAE/g and 20.00 mg GAE/g, respectively. There was a significant difference between the wetland rice samples. The maximum concentration of TPC in wetland rice was observed for the Mahsuri cultivar (27.11 mg GAE/g). In addition, there was a significant difference in the TFC among the wetland rice cultivars with similar trends showing that Mahsuri had a higher TFC reading compared to Sri Malaysia. Data from the present study were in agreement with the study reported by Tian et al. (2005) who reported the presence of a higher phenolic content in pigmented brown rice. However, many studies have recorded differences among rice varieties due to the solvent extraction used, storage conditions and the rice color. There are other phytochemicals such as carotenoids, tocols and γ-oryzanols that contribute to the antioxidant capacity rather than just phenolic compounds (Choi et al., 2007; Shen et al., 2009). Previous reports have mentioned that the antioxidant content of colored rice species was five times higher than for commercial white rice because the rice bran-linked nutritional components generally consumed in such rice had been polished and milled (Zhang et al., 2015; Walter et al., 2013). Moreover, these reports identified phenolic acids (including ferulic acid, caffeic acid and syringic acid) in the rice grains at similar levels to in other grains (Semsang et al., 2016). However, there has been no reported data on the phenolic content in upland rice except for pigmented rice types.

The LC-MS/MS analysis elucidated three phenol compounds in the rice varieties based on the primary fragmentation pattern of the parent ion to the product ion. High accuracy and sensitive mass detection MRM produced intense peaks for the corresponding compound at $[M-H]^-$. Thus, separation was achieved within 6 min retention time (Table 3). Table 3 indicate the detection of ferulic acid, *ρ*-coumaric acid and salicylic acid based on the linear calibration curve with ferulic acid having the most prominent peak at $[M-H]^-$ m/z 193 to form the daughter ion of 134 at a retention time of 5.79 min, while *ρ*-coumaric acid was detected at m/z 163 to form the daughter ion of m/z 119 at a retention time of 4.95 min (Fig. 1). Figure 1 also shows the separation chromatograms for ferulic and *ρ*-coumaric acids

Table 2 Total phenolic content (TPC) and total flavonoid content (TFC) (mean±SD) of ethanolic extracts of different rice grain varieties

Sample	TPC (mg GAE/g)	TFC (mg RE/g)	TFC:TPC ratio (mg RE/mg GAE)
UR Bario	47.84 ± 0.31^{a}	7.14 ± 0.23^{a}	0.14
UR Bukit Pulut	28.78 ± 0.28^{c}	3.52 ± 0.18^{b}	0.12
WR Mahsuri	27.19 ± 0.11^{c}	6.36 ± 0.01^{a}	0.23
WR Sri Malaysia	20.00 ± 0.35^{b}	3.35 ± 0.60^{b}	0.17

UR = upland rice; WR = wetland rice; GAE = gallic acid equivalents; RE = rutic acid equivalents.

Means in the same column with different lowercase superscripts are significantly different ($p < 0.05$).

Values expressed in milligrams per gram of dry weight.

obtained from both upland rice cultivars (Bario and Bukit Pulut). Another compound of salicylic acid showed an intense peak from m/z 137 as a fragment of the daughter ion of m/z 92 at a retention time of 5.95 min. Interestingly, significantly higher amounts of ferulic acid and *ρ*-coumaric acid were detected in the upland rice cultivars compared to the wetland rice. However, the amount of salicylic acid was significantly different among the rice-seed grain extracts tested with the exception of the two upland rice samples. It seemed to show that the wetland rice Mahsuri cultivar was rich in salicylic acid. This may partly be explained by compound variations and the uniqueness of wetland rice compared to upland rice.

This study demonstrated that ferulic acid and *ρ*-coumaric acid are rich in the upland rice. This was in agreement with the studies conducted by Vichapong et al. (2010) which identified these two phenolic acids in pigmented and non-pigmented rice samples using HPLC detection. Nakornriab et al. (2008) identified two flavonoids (quercetin and isohamnetin) from black rice using LC-MS/MS. Many reports have used the LC-MS/MS analytical method to quantify phenolic compounds mediated with chromatographic separation in many plants such as leafy vegetables (Oniszczuk and Olech, 2016), medicinal plants (Oniszczuk and Podgorski et al., 2015; Kuźniewski et al., 2018), lentils seeds (Mirali et al., 2014), fruit juice and beverages (Sapozhnikova, 2014) and puffed cereal (Blicharski et al., 2017). Nevertheless, no such information on polyphenol quantification via LC-MS/MS has been previously reported for upland rice varieties. Based on the above-mentioned rice studies, the range of ferulic acid in pigmented rice is 1.13–25.90 mg/100g and for *ρ*-coumaric is in the range 0.62–7.10mg/100g. The current results were consistent with these earlier findings due to the similarity of the plant genotype of the pigmented rice. Reports have commented on the medicinal effects of ferulic acid, *ρ*-coumaric acid and salicylic acid including antioxidant, anti-inflammatory, anti-allergic and antimicrobial properties (Chua et al. 2011; Kumar and Pruthi, 2014). Those authors believed that there were many phenol compounds that could be found in the whole rice, but the current study identified only three particular compounds (ferulic acid, *ρ*-coumaric acid and salicylic acid) based on the leading preference of phenol metabolites found in rice.

Table 3 Phenolic compounds (mean±SD, mg/100g) of rice seed extracts and mass spectral data

Compound	UR Bario	UR Bukit Pulut	WR Mahsuri	WR Sri Malaysia	$[M-H]^-$	Retention time (min)
Ferulic acid	10.31[a]	8.64[a]	5.10[b]	4.69[b]	193>134	5.79
p-coumaric acid	1.11[a]	1.42[a]	0.40[b]	0.60[b]	163>119	4.95
Salicyclic acid	3.24[b]	3.47[b]	5.86[c]	1.37[a]	193>95	5.95

UR = upland rice; WR = wetland rice

Means in the same row with different lowercase superscripts are significantly different ($p < 0.05$).

Values are expressed in milligrams per gram of dry weight of samples

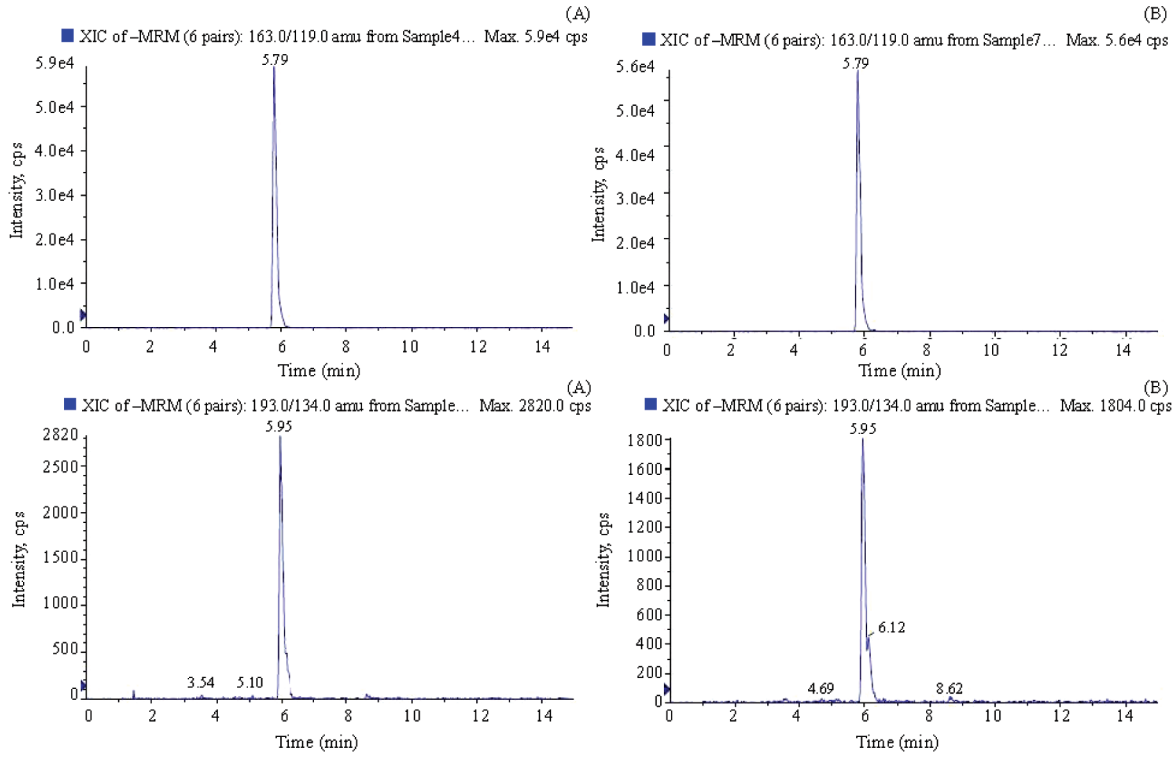

Fig. 1 Mass chromatograms obtained from upland rice Bukit Pulut and Bario. Upper figures designated by (A) Bukit Pulut, *p*-coumaric acid; (C) Bukit Pulut, ferulic acid; (B) Bario, *p*-coumaric acid; (D) Bario, ferulic acid.

Thus, the current study succeeded in developing a fast and accurate detection method of phenolic acid via a mass spectrometry technique at lower concentrations from 0.01 μg/mL. The quantitative technique using LC-MS/MS and MRM methods provided sensitive detection to differentiate among phenolic compounds within the rice samples.

In conclusion, this study chemically identified an improved phytochemical profile and antioxidant properties as well as the specific polyphenol compounds (ferulic acid, *ρ*-coumaric acid and salicylic acid) in upland rice compared to the wetland rice samples. Based on the current results, upland rice could be further highlighted as having a considerable amounts of polyphenols compounds and phenolic acids, reflecting that they are responsible for antioxidant properties. Future studies profiling all phenolic metabolites in selected, prominent, upland rice cultivars need to be performed to assist nutritionists in estimating dietary intake. The results provide opportunities to further increase the content of polyphenols in the whole-rice grain using fertilizer strategy and breeding approaches, especially in white rice. These data could inform functionalities and economic potential with new hope to promote the production of new upland rice breeds with enhanced levels of antioxidant ratios for incorporation into functional foods.

Conflict of Interest

The authors declare that there are no conflicts of interest

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