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Contributed Paper

Phytochemical Constituents of Thai Dark Purple Glutinous Rice Bran Extract [Cultivar Luem Pua (*Oryza sativa* L.)]

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ABSTRACT

The isolation and identification of phytochemical constituents in the bran of dark-purple glutinous rice cultivar Luem Pua, was conducted. Luem Pua rice bran was extracted with hexane and methanol. The investigation of phytochemicals in the hexane extract was carried out after the fats were eliminated by saponification reaction. The isolation of the unsaponified fraction by reverse-phase high-performance liquid chromatography resulted in four sub-fractions, from which seven sterols and two triterpenoids were identified. The structures of all identified compounds were confirmed by gas chromatography-mass spectrometry and proton nuclear magnetic resonance spectroscopy. The chemical constituents in the methanol extract were also investigated using column chromatography and semi-preparative HPLC. The chemical composition of each fractions were analyzed by liquid chromatography coupled with tandem mass spectrometry via electrospray ionization mode (LC-MS/MS), together with comparison with literature data. This resulted in the identification of cyanidin-3-*O*-glucoside, a major anthocyanin contributing to the dark-purple color and the highly oxidative property of the rice, along with six phenolic compounds and eight flavonoids. The findings from this work helps support the potentials of Luem Pua rice for further applications in either health or pharmaceutical products.

Keywords: Luem Pua rice bran, black rice bran, sterol, triterpenoid, phenolic compound, flavonoid, GC-MS, LC-MS, LC-MS/MS, *Oryza sativa* L.

1. INTRODUCTION

Luem Pua rice (*Oryza sativa* L.) is a glutinous upland rice cultivar having dark-purple pericarp and gaining high popularity in Thailand with a wonderful fragrant aroma and delicious chewy texture. It was selective breeding pure via Phitsanulok Rice Research Center and has been certified as a new rice variety by the Rice Department since 2012 [1]. Luem Pua rice has annual planted growing under the appropriate conditions with cool temperatures about 25 °C and loose soil especially in mountainous area located 400-800 meters above the sea level. There have been reports on nutritional value of Luem Pua rice that it contains various beneficial substances including omega-3, omega-6, omega-9, vitamin B1, vitamin B2, vitamin B6, vitamin B12, niacin, vitamin E, gamma-oryzanol, phytate, collagen, iron, calcium, folic acid, anthocyanins, protein, zinc, and manganese [2, 3]. It is also considered one of the pigmented rice that composes of high total phenolic contents [4] which has, therefore, made Luem Pua rice become even more attractive to consumer, not for only its delicious texture, but also its healthy anti-oxidative property.

One of four parts in a grain of rice is rice bran which can be obtained from the milling process. It is enriched with fiber, proteins and important antioxidant property. Generally, pigmented rice bran is well known that it contains bioactive compounds that have health and nutrition benefits for human. Black rice bran extract, in particular, is a potent source of natural antioxidants, which is important for the potential to increase immune system in the body and to protect and reduce the risk of cancer and heart diseases [5, 6]. Various studies reported the biological activities of pigmented rice bran such as inhibition of tyrosinase activity [7], anti-allergic and anti-inflammatory activities [8], anti-mutagenic, anti-carcinogenic, anti-amylase and anti-glycation activities [9, 10]. It has been confirmed that the colored rice bran, which has been proved exhibiting higher anti-oxidation property than the non-colored one [11], contained various types of phytochemicals such as phenolic compounds, flavonoids, alkaloids, triterpenoids, steroids and saponins [12-14].

In general, dark colored rice, black rice in particular, has outstanding bioactivity which makes it attractive for various application. Each variety composes of different chemical profile that contains different important bioactive compounds, both in quality and quantity [14-15]. Luem Pua rice is considered one of very few glutinous rice variety that have highly dark-purple color, which is expected to express some specific bioactivity. Indeed, the aqueous extract of Luem Pua rice has been recently found to be able to improve learning and memory in mice [16] and showed anti-oxidative and memory-enhancing effects in cell culture and mice [17]. It is therefore interesting to investigate for the chemical components of Luem Pua rice which may be relevant to its bioactivity. Furthermore, the understanding of its chemical profile provides fundamental knowledge of Luem Pua rice and reflects its potentials in product development for further applications including dietary supplement, cosmetic and pharmaceutical industries. We now report herein total analysis of the major bioactive phytochemicals of Luem Pua rice bran ranging from nonpolar phytosterols and triterpenoids to the polar phenolic compounds including flavonoids and anthocyanins.

2. MATERIALS AND METHODS

2.1 Plant Material

The bran of dark purple glutinous rice (*Oryza sativa* L.) cultivar Luem Pua used in this study was grown in Khao Kho, Phetchabun province in northern Thailand. The sample was collected from experimental field in Phitsanulok Rice Research center, Phitsanulok province, Thailand in October 2012.

2.2 Chemicals

All solvents used for TLC, CC, GFC including hexane, ethyl acetate, dichloromethane, and methanol were purchased in commercial grade and subjected for distillation to the satisfied purity prior to use for. Reagents for saponification reaction; pyrogallol (analytical grade) was purchased from Sigma-Aldrich Co. LLC (Steinheim Germany). Potassium hydroxide (analytical grade) was purchased from Carlo Erba Reagents (Val-de-Reuil, France). Sodium sulfate anhydrous (analytical grade) was purchased Merck (Darmstadt, Germany). Dichloromethane and ethanol (analytical grade) were purchased from Lab-scan (Bangkok, Thailand). Methanol for separation by HPLC technic was HPLC grade, purchased from Merck (Darmstadt, Germany). Deionized water was obtained from a MiliQ UV-Plus water purication system (Milipore Corp, Billerica, MA, USA). Anisaldehyde and sulfuric acid were purchased from Sigma-Aldrich Co. LLC (Steinheim Germany). Acetic acid for separation by HPLC technic was HPLC grade purchased from Fisher Scientific (Leicestershire, England). Chloroform-d for NMR spectroscopy purchased from Sigma-Aldrich Co. LLC (Steinheim Germany).

2.3 Extraction

One kilogram of the bran of Luem Pua rice (LPB) was successively macerated in *n*-hexane and methanol at room temperature (2×1000 mL for each solvent). The maceration period for each extraction was 7 days. After filtration, the combined extracts were separately evaporated to dryness under reduced pressure at temperature about 40 °C to give the hexane extract (LPBH, 236.14 g, 23.61%) and the methanol extract (LPBM, 73.47 g, 7.35 %).

2.4 Isolation and Identification of Hexane Extract of Luem Pua Rice Bran (LPBH)

2.4.1 Saponification and isolation of hexane extract (LPBH)

Hexane extract (LPBH) 20 g was saponified by refluxing with 0.5 M ethanolic potassium hydroxide solution (200 mL) in a presence of 0.2 g of pyrogallol, at 70 °C for 2 h. After evaporation to dryness the residue was subjected to extraction with dichloromethane (2×200 mL), washed with water, dried over anhydrous sodium sulfate. After filtration, the organic phase was then subjected to evaporation to remove the solvent using a rotary evaporator at 40 °C, yielding the unsaponified fraction, LPBHS (0.5154 g, 2.57 %). Separation of the LPBHS (200 mg) was performed with a high performance liquid chromatograph (Agilent Technologies, Santa Clara, CA, U.S.A.) equipped with a semi-preparative VertisepTM C-18 column (7.8 × 100 mm, 5 μm), injection volume: 20 μL, mobile phase: methanol 100 %, flow rate: 2 mL/min VWD: 254 nm at 25 °C. This resulted in four sub-fractions, LPBHS1 (2.7 mg, 1.35%), LPBHS2 (11.3 mg, 5.65%), LPBHS3 (20.6 mg, 10.30%), LPBHS4 (29.4 mg, 14.70%).

2.4.2 Identification of components in sub-fractions of unsaponified fraction of the hexane extract by GC-MS and NMR spectroscopy

Four sub-fractions of the unsaponified hexane extract (LPBHS1-4) were subjected for chemical components analysis by GC-MS system (Agilent 6890 and HP 5973 mass-selective detector, Agilent Technologies, USA) equipped with HP5-MS capillary column (30 m × 0.25 mm i.d. × 0.25 mm film thickness, Agilent Technologies). GC-MS was operated under a temperature program which was started at 60 °C and ramped to 280 °C at 3 °C/min. The injection temperature was 250 °C with an injection volume of 1 μL with a splitless mode. Helium gas at a flow rate of 1.2 mL/min was used as the carrier gas. The MS was operated in the electron impact mode with electron energy of 70 eV, ion source temperature at 230 °C, quadrupole temperature at 150 °C, and scan mass range of m/z 29-500. The identification of components in sub-fractions was performed by matching their mass spectra with reference spectra in the NIST08 and W8N08 mass spectral libraries, both purchased from Agilent Technologies. The structures of each compound identified were confirmed by ^{1}H-NMR spectroscopy which were recorded on a Bruker AVANCE 400 NMR spectrometer (400 MHz). The compounds were dissolved in chloroform-d ($CDCl_3$).

2.5 Isolation and Identification of Methanol Extract of Luem Pua Rice Bran (LPBM)

2.5.1 Isolation of chemical constituents of methanol extract (LPBM)

The methanol extract (LPBM; 40.0 g) was fractionated by column chromatography over silica gel (Merck cat. No. 9385, 40-63 μm, 250 g) and eluted under gradient condition: *n*-hexane, *n*-hexane-ethyl acetate, ethyl acetate-methanol and methanol with increasing amount of the more polar solvent, to afford three sub-fractions LPBM1 (8.40g, 21.00%), LPBM2 (3.96 g, 9.90%) and LPBM3 (14.59 g, 36.48%). LPBM2 (3.0 g) was separated into three sub-fractions including LPBM2.1 (0.9235 g, 30.78%), LPBM2.2 (0.3231 g, 10.77%), LPBM2.3 (0.2982 g, 9.94%) on a sephadex LH-20 and methanol was used as eluent. LPBM2.1 (500 mg) was then fractionated by column chromatography over Sephadex LH-20 and eluted under isocratic condition (CH_2Cl_2 : MeOH 1:1), affording 3 sub-fractions: LPBM2.1.1 (38.00 mg, 7.6%), LPBM2.1.2 (96.60 mg, 19.32%) and LPBM2.1.3 (10.00 mg, 2.00%). After that, LPBM2.1.1 (20 mg) was separated by reverse phase HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a semi-preparative VertisepTM C-18 column (7.8 × 100 mm, 5 μm), injection volume 20 μL, mobile phase: A: methanol B: 0.5 % acetic acid in water, with the gradient varied linearly from 20% to 100% (A) in 25 min, flow rate 0.3 mL/min, UV/Vis detector at 254 nm at 25 °C. The eluates were collected in 4 fractions: LPBM2.1.1.1 (1.00 mg, 5.0%), LPBM2.1.1.2 (2.30 mg, 11.5%), LPBM2.1.1.3 (2.70 mg, 13.5%) and LPBM2.1.1.4 (0.40 mg, 2.0%). LPBM2.2 (200 mg) was fractionated by column chromatography over Sephadex LH-20 and eluted under isocratic condition (CH_2Cl_2 : MeOH 1:1). The solvents were evaporated to dryness, affording 2 fractions: LPBM2.2.1 (61.5 mg, 30.75%) and LPBM2.2.2 (128.70 mg, 64.35%). Separation of LPBM2.2.2 (100 mg) was performed using HPLC with same conditions as LPBM2.1.1 to yield 7 sub-fractions: LPBM2.2.2.1 (1.10 mg, 1.10%), LPBM2.2.2.2 (1.00 mg, 1.00%), LPBM2.2.2.3 (1.00 mg, 1.00%), LPBM2.2.2.4

(5.50 mg, 5.50%), LPBM2.2.2.5 (0.30 mg, 0.30%), LPBM2.2.2.6 (0.60 mg, 0.60%) and LPBM2.2.2.7 (0.40 mg, 0.40%).

2.5.2 Identification of components in sub-fractions of methanol extract by MS/MS

Selected sub-fractions of methanol extract (LPBM2.1.1.1, LPBM2.1.1.2, LPBM2.1.1.3, LPBM2.1.1.4, LPBM2.2.2.4, LPBM2.2.2.5 and LPBM2.2.2.7) were subjected to structural identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using BRUKER Esquire 3000 Plus (Bruker Daltonies, Germany), direct infusion; micro syringe pump rate: 180 μL/hr, mass analyzer; octa quadrupole ion trap with an electrospray ionization (ESI) source operated in negative-ion mode. The mass spectra were recorded in the range of m/z 50-700 at a scan speed of 26,000 mass/sec. Nitrogen was used both as drying gas at flow rates of 5.0 L/min and as nebulizing gas with temperature set at 300 °C. All chemical components were identified by comparing with those of the literature data.

3. RESULTS AND DISCUSSION

3.1 Identification of Sterols and Triterpenoids in Sub-fractions from Unsaponified Hexane Extract (LPBHS)

The chemical components in all four sub-fractions (**LPBHS1-4**) obtained after the RP-HPLC isolation of the unsaponified fraction of the hexane extract have been identified by GC-MS from which their mass spectra were matched to those recorded in the MS library. The chemical structures were confirmed by ^{1}H-NMR spectroscopy which were found to be corresponded to literatures. It was found that LPBHS1 contained mainly two isomeric sterols, 24-methylene-ergosta-5-en-3β-ol (**1**) and 24-methylene-ergosta-7-en-3β-ol (**2**) with molecular ions of m/z 398. Sterols **1** and **2** showed characteristic fragment ions at m/z 383, 365, 314, 299, 281, 271 and at m/z 365, 314, 299, 271, with m/z 314 and 271 being base peaks, respectively. The ^{1}H-NMR spectrum of the mixture confirmed the structures of the isomers of which the signals of significant methine proton at C3 in two sterols were observed at the chemical shifts of 3.52 (*m*) and 3.62 (*m*) ppm and signals of the methylene protons at C28 of both compounds were observed at the chemical shifts of 4.65 (*s*) and 4.71 (*s*) ppm. The difference of the position of the double bonds between the two sterols, *i.e.* at C-5 in **1** and at C-7 in **2** were clearly confirmed by the observed olefinic H-6 in **1** and H-7 in **2** at the chemical shifts of 5.35 (*d*) and 5.15 (*d*) ppm, respectively. The H-6 signal was comparatively shifted slightly lower field than H-7 due to the closer position to the electron withdrawing hydroxyl group. Another two isomeric sterols, fucosterol (**3**) and gramisterol (**4**) have been identified as major components in LPBHS2. Both compounds have the same molecular ions of m/z 412 and showed fragment ions at m/z 397, 379, 314, 299, 281, 229, 213 and at m/z 397, 379, 328, 285, 269, respectively. The ^{1}H-NMR spectrum confirmed both structures by showing significant protons at C3 and C6 in **3** at the chemical shifts of 3.53 (*m*) and 5.35 (*d*) ppm, respectively, whereas **4** showed signals of protons at C3 and C7 at the chemical shifts of 3.11 (*dt*) and 5.12 (*d*) ppm, respectively. The observed quartet signal at 5.18 ppm was corresponded to the olefinic methine proton at C28 in **3**, while, in **4**, the olefinic methylene protons at C28 showed characteristic signals at 4.65 (*s*) and 4.71 (*s*) ppm. In addition, the presence of a methyl group at C4 in **4** was confirmed by the observed signal of the methine proton at 2.83 ppm. In LPBHS3, two sterols, campesterol (**5**) and stigmasterol (**6**) and a triterpenoid namely cycloartenol (**7**) have been

identified. Sterol **5** has a molecular ion at *m/z* 400 and showed fragment ions at *m/z* 385, 382, 367. The ^{1}H-NMR spectrum showed the methine proton at position 3 and the olefinic methine proton at C6 at chemical shifts 3.51(*m*) and 5.33 (*d*) ppm, respectively. **6** has a molecular ion at *m/z* 412 and showed fragment ions at *m/z* 397, 394, 369, 351, 314, 271, 255, 213. In addition to the ^{1}H-NMR signals of C3 and C6 which occurred at the chemical shifts 3.51 (*m*) and 5.33 (*d*) ppm, respectively, the signals of trans-olefinic protons at C22, 23 were observed as two doublets at 5.03 and 5.15 ppm. This clearly confirmed the additional side-chain double bonds found only in **6**. A triterpenoid **7** has a molecular ion at *m/z* 426 and showed fragment ions at *m/z* 411, 408, 393. ^{1}H-NMR spectrum showed significant methine protons at C3 and C24 at chemical shifts 3.28 (*m*) and 5.12 (*d*) ppm, respectively. The characteristic methylene protons of the cyclopropane ring were observed at d 0.33 (*d*) and 0.55 (*d*) ppm. β-Sitosterol (**8**) and 24-methylenecycloartanol (**9**) were found to be major components in LPBHS4. Sterol **8** has a molecular ion at *m/z* 414, while the fragment ions at *m/z* 399, 396, 381, 329, 303, 273, 255, 231, 213 were also observed. ^{1}H-NMR spectrum confirmed the structure by showing the signals of significant methine C3 and olefinic C6 protons which were observed at the chemical shift of 3.52 (*m*) and 5.35 (*d*) ppm. The triterpenoid **9** has a molecular ion at *m/z* 440 and showed fragment ions at *m/z* 425, 422, 407, 397, 379, 315, 300, 285, 203. The ^{1}H-NMR spectrum showed signals of methine C3 proton and methylene C19 protons at chemical shifts 3.28 (*dd*), 0.33 (*d*) and 0.55 (*d*) ppm, respectively. The olefinic methylene protons at C28 were observed at d 4.66 (*s*) and 4.71 (*s*) ppm. The MS and ^{1}H-NMR spectral data of each sterols and triterpenoids detected from the unsaponified fraction of the hexane extract were found to be agreeable with literatures [14]. The structures and data of these compounds are summarized in Figure 1 and Table 1.

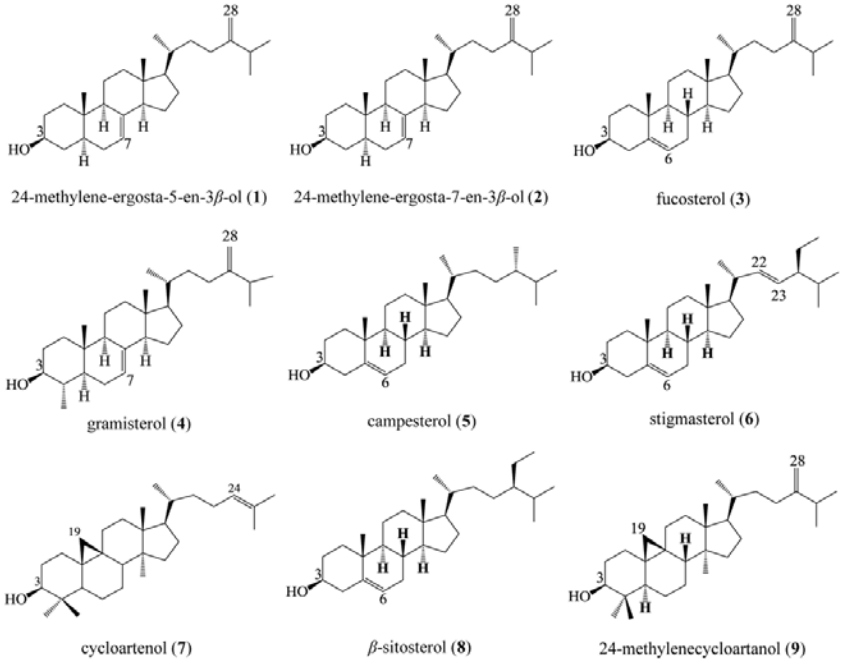

Figure 1. The structures of sterol and triterpenoid compounds obtained from the unsaponified fraction of the hexane extract of Luem Pua rice bran**.**

Table 1. Sterol and triterpenoid compounds found in sub-fractions of unsaponified fraction of the hexane extract.

Components identified	Molecular Formula	Molecular Weight	Mass fragments (*m/z*)	Selected ^{1}H NMR (δ, ppm)
LPBHS1				
24-Methylene-ergosta-5-en-3β-ol (**1**)	$C_{28}H_{46}O$	398	398, 383, 314, 299, 281, 271	3.52 (*m*, H_3), 4.65 (*s*, H_{28}), 4.71 (*s*, H_{28}), 5.35 (*d*, H_6)
24-Methylene-ergosta-7-en-3β-ol (**2**)	$C_{28}H_{46}O$	398	398, 365, 314, 271	3.62 (*m*, H_3), 4.65 (*s*, H_{28}), 4.71 (*s*, H_{28}), 5.15 (*d*, H_7)
LPBHS2				
Fucosterol (**3**)	$C_{29}H_{48}O$	412	412, 397, 379, 314, 299, 281, 229, 213	3.53 (*m*, H_3), 5.18 (*q*, H_{28}), 5.35 (*d*, H_6)
Gramisterol (**4**)	$C_{29}H_{48}O$	412	412, 397, 379, 328, 285	3.11 (*dt*, H_3), 4.65 (*s*, H_{28}), 4.71 (*s*, H_{28}), 5.12 (*d*, H_7)
LPBHS3				
Campesterol (**5**)	$C_{28}H_{48}O$	400	400, 385, 382, 367	3.51 (m, H3), 5.33 (d, H6)
Stigmasterol (**6**)	$C_{28}H_{48}O$	412	412, 397, 394, 369, 351, 314, 271, 255, 213	3.51(*m*, H_3), 5.00, 5.15 (*dd*, H_{22}, H_{23}), 5.33 (*d*, H_6)
Cycloartenol (**7**)	$C_{30}H_{50}O$	426	426, 411, 408, 393	0.33 (*d*, H_{19}), 0.55 (*d*, H_{19}), 3.28 (*m*, H_3), 5.12 (*d*, H_{24})
LPBHS4				
β-Sitosterol (**8**)	$C_{29}H_{50}O$	414	414, 399, 396, 381, 329, 303, 273, 255, 231, 213	3.52 (*m*, H_3), 5.35 (*d*, H_6),
24-Methylenecycloartanol (**9**)	$C_{31}H_{52}O$	440	440, 425, 422, 407, 397, 379, 315, 300, 285, 203	0.33(*d*, H_{19}), 0.55 (*d*, H_{19}), 3.28 (*dd*, H_3), 4.66 (*s*, H_{28}), 4.71 (*s*, H_{28})

3.2 Identification of Components in Sub-fractions of Methanol Extract (LPBM)

MS/MS analysis of selected fractions obtained after successive chromatographic separation of LPBM resulted in the identification of phenolic and flavonoid compounds, whose mass spectral data were all comparable to those reported in the literatures, as described below.

The ESI-MS/MS spectra of sub-fraction LPBM2.1.1.1 in negative ionization and full scan mode showed the deprotonated molecular ion $[M-H]^-$ of protocatechuic acid (**10**) at m/z 153 as a base peak together with the fragmented ion $[M-H-44]^-$ at m/z 109 which was resulted from the neutral loss of CO_2 from the carboxyl group [18-20].

Five components (**11-15**) of sub-fraction LPBM2.1.1.2 were identified from ESI-MS/MS spectra as follows; the first compound, identified as *p*-coumaric acid (**11**) [18-20], showed the deprotonated molecular ion at m/z 163 as a base peak. The fragment ion at m/z 119 was obtained via neutral loss of a CO_2 group from the carboxylic acid moiety $[M-H-44]^-$. The second compound showed deprotonated molecular ion at m/z 167 as a base peak with the MS^2 ion at m/z 123 $[M-H-44]^-$ which is a characteristic fragment ion obtained via neutral loss of CO_2 from the carboxylic acid moiety. A fragment ion at m/z 152 $[M-H-15]^-$ was obtained by losing a CH_3 group from the molecular ion. A characteristic MS^3 ion at m/z 108 $[M-H-15]^-$ which is a fragment ion resulted via loss of a CH_3 group from the m/z 123 precursor ion was also observed. Together with additional mass spectral data from published reports [19-21], this compound was identified as vanillic acid (**12**). The deprotonated molecular ion $[M-H]^-$ at m/z 517 was observed in the MS^2 spectra of the third component which was identified as feruloyl coniferin (**13**) [21, 22]. The fragment ion at m/z 337 resulting from the neutral loss of 180 Da was assumed to be associated with a coniferyl alcohol moiety $[M-H-180]^-$. The MS^2 base peak fragmented further to m/z 193 by losing 144 Da corresponding to a hexose moiety. The product ions at m/z 193 and 149 indicated that the hexose was attached to a ferulic acid moiety. The other fragment ions at m/z 175, 217 and 277 were also observed in the MS^2 spectrum. All data observed therefore strongly supported the structure of **13**. The deprotonated molecular ion $[M-H]^-$ at m/z 519 appeared as base peak and the product ions at m/z 314, 179 and 151 suggested that the fourth compound in this fraction was isorhamnetin-3-*O*-acetylglucoside (**14**) [23]. The dominant product ion at m/z 314 was present along with the minor ions of m/z 179 and 151. These two minor ions were specific to retro Diels-Alder reaction (RDA) in ring C of this flavonoid. Most of the product ions observed were similar to those reported for isorhamnetin-3-*O*-glucoside. The additional ion mass of 42 amu, corresponding to the acetyl group, confirmed that **14** was acetylglycoside derivative. The last compound, having the base peak molecular ion, $[M-H]^-$, at m/z 609, was identified as quercetin-3-*O*-rutinoside (**15**) [20, 23]. Low energy dissociation of this parent ion resulted in a daughter mass spectrum containing m/z 301 ion along with the C-ring fragmented ions at m/z 151 and 179.

LPBM2.1.1.3 provided a single compound identified as gallic acid (**16**) [18-21]. The first mass spectrum showed deprotonated molecular ion $[M-H]^-$ at m/z 169 while MS^2 fragmentation of this parent ion yielded a product ion at m/z 125 $[M-H-44]^-$ via neutral loss of a CO_2 group from the carboxylic acid moiety.

Two compounds (**17-18**) of LPBM

2.1.1.4 were identified from ESI-MS/MS as follows; the MS^2 spectra of **17** exhibited $[M-H]^-$ ion at m/z 193 and a fragment ion at m/z 149 $[M-H-44]^-$, which were corresponded to the characteristic neutral loss of CO_2 group from the carboxylic acid function in the negative mode. The observed product ions at m/z 178 and 134 were attributed to the neutral loss of the CH_3 group from the $[M-H]^-$ and $[M-H-44]^-$ ions, respectively. **17** was thus identified as ferulic acid [18-20]. The molecular ion $[M-H]^-$ at m/z 465 and the product ion of m/z 303 $[M-H-162]^-$ resulted from the loss of a glucose unit (162 Da) was observed for **18**, identified as 5, 3′, 4′, 5′-tetrahydroxyflavanone-7-*O*-glucoside [23]. Other product ions presented the existence of the characteristic ion of C-ring cleavage at m/z 151 and 179, with the ion of m/z 151 being observed as a base peak. The exceptionally high stability of this product ion was attributed to the C ring cleavage that led to the formation of product ions from ring B rather than ring A, both of which possessed the same m/z of 151. Therefore, the hydroxyl substitution pattern of **18** was likely to be matched with flavone type.

Cyanidin-3-*O*-glucoside (**19**) was found to be the only compound in sub-fraction LPBM2.2.2.4 [6]. The ESI-MS/MS spectra of **19** in positive ionization and full scan mode showed the molecular ion $[M^+]$ at m/z 449 and a fragment ion at m/z 287 which was corresponded to cyanidin aglycone obtained as a result from the loss of one glucose unit (162 Da). Its loss of H_2O followed by a series of CO losses, yielded some product ions of m/z 269, 241, 213, 185 and 157, which revealed cyanidin structure. Another cleavage of ring C resulted in the additional product ions at m/z 137, which was observed as a base peak, and at m/z 121.

Four components (**20-23**) have been identified from sub-fraction LPBM2.2.2.5. In the full scan mass spectra, $[M-H]^-$ at m/z 301, which was observed in negative mode for **20**, produced fragment ions at m/z 151 and 179, resulted from a cleavage of the heterocyclic C-ring by retro Diels-Alder reaction (RDA). Another fragmentation pathway concerning neutral losses of H_2O, CH_2O and CO_2 from the $[M-H]^-$ ion, m/z 301, leading to product ions at m/z 283, 271 and 257, respectively. These MS^2 data strongly suggested the identification of **20** as quercetin [18-20, 23]. Kaempferol-3-*O*-glucoside (**21**) [18, 20] was identified for the second compound detected in this fraction. The ESI-MS/MS spectra obtained in negative ion mode showed a deprotonated molecular ion $[M-H]^-$ at m/z 447. The daughter ion at m/z 285 was resulted from the loss of a glucose moiety (-162 Da). Other fragment ions at m/z 241 and 179 were also observed. The deprotonated molecular ion $[M-H]^-$ of **22**, identified as quercetin-3-*O*-glucoside [18, 20, 23], appeared at m/z 463. The major daughter ion $[M-H-162]^-$ at m/z 301 was a characteristic loss of glucose from the protonated aglycone ion of quercetin via homolytic cleavage at the *O*-glycosidic bond. This has been proposed as prominent indication of a quercetin glycoside derivative. The observed fragment ions of quercetin aglycone part at m/z 151 and m/z 179 were also supportive for the structure of **22**. The product ions of m/z 283 and 255 were consistent with the further fragmentation of $[M-H-162]^-$ by the successive losses of H_2O and CO molecules, respectively. The mass spectrum of the last compound exhibited the deprotonated molecular ion $[M-H]^-$ at m/z 465 and fragment ion at m/z 303, which was corresponded to characteristic loss of the glucose molecule $[M-H-162]^-$. The MS^2 base peaked was observed at m/z 285 for the product ion obtained from the loss of H_2O

[M-H-162-18]$^-$. Successive loss of CO_2 molecule resulted in the product ion at m/z 241. The characteristic ions of flavonoid fragmentation, m/z 151 was observed with the other characteristic ions of taxifolin fragmentation, m/z 179 and 125, which were corresponded to fragmented ions from ring B and ring A, respectively. This compound was thus identified as taxifolin-7-*O*-glucoside (**23**) [23]. The MS^2 spectra of LPBM2.2.2.7 presented a single compound identified as isorhamnetin (**24**) [20, 23]. The deprotonated molecular ion [M-H]$^-$ appeared at m/z 315, along with a fragment ion at m/z 300 [M-H-15]$^-$ corresponding to the loss of a CH_3 radical from the [M-H]$^-$ being observed as a base peak. Other daughter ion at m/z 283 was obtained due to further loss of a hydroxyl group. In addition, fragment ion at m/z 151 was produced by the cleavage of the C ring and was assigned to be RDA fragmented ion. The structures of all compounds identified from methanol extract is showed in Figure 2 and the details of MS/MS identification of **11**-**24** are summarized in Table 2.

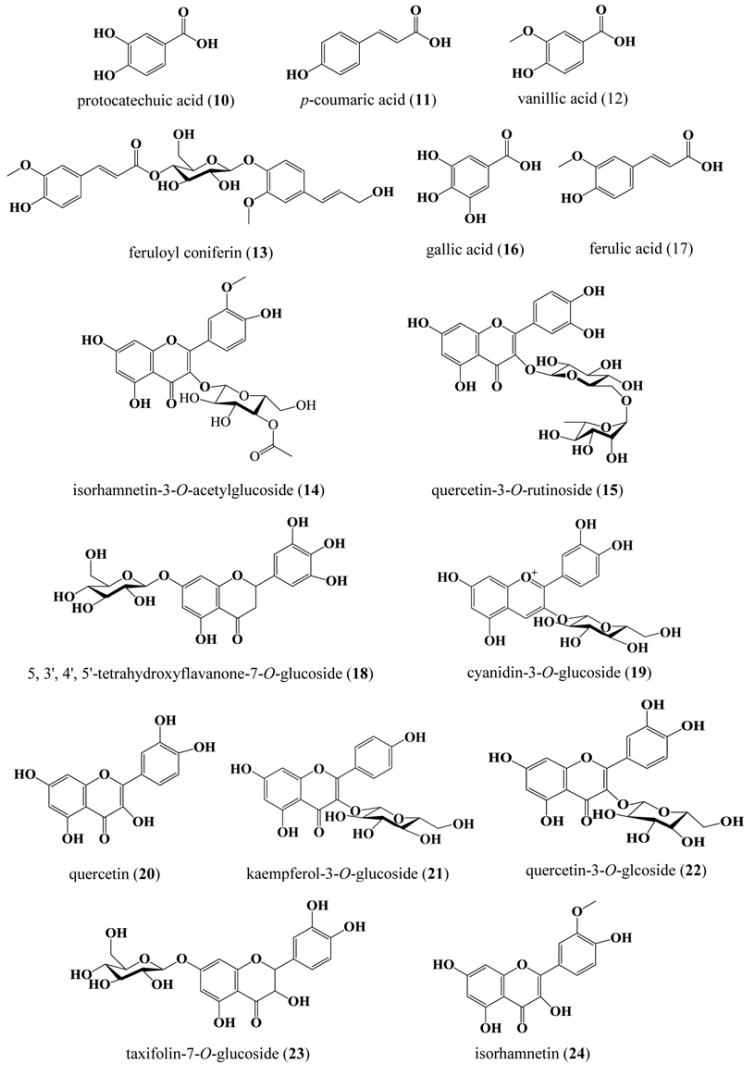

Figure 2. The structures of phenolic and flavonoid compounds obtained from the methanol extract of Luem Pua rice bran.

Table 2. Identification of phytochemical components in sub-fractions of LPBM by LC-MS/MS.

Components identified	Molecular Formula	$[M-H]^-$ ion (m/z)	$[M]^+$ ion (m/z)	MS^2 ion fragments (m/z)	MS^3 ion fragments (m/z)
LPBM2.1.1.1					
Protocatechuic acid (**10**)	$C_7H_6O_4$	153	-	109	-
LPBM2.1.1.2					
p-Coumaric acid (**11**)	$C_9H_7O_3$	163		119	-
Vanillic acid (**12**)	$C_8H_8O_4$	167		123, 152	108*
Feruloyl coniferin (**13**)	$C_{26}H_{30}O_{11}$	517		337, 193, 149, 175, 277, 217,	-
Isorhamnetin-3-*O*-acetylglucoside (**14**)	$C_{24}H_{24}O_{13}$	519		314, 151, 179	-
Quercetin-3-*O*-rutinoside (**15**)	$C_{27}H_{30}O_{16}$	609		301, 151, 179	-
LPBM2.1.1.3					
Gallic acid (**16**)	$C_7H_5O_5$	169		125	-
LPBM2.1.1.4					
Ferulic acid (**17**)	$C_{10}H_{10}O_4$	193		149, 134, 178	-
5,3′,4′,5′-tetrahydroxyflavanone-7-*O*-glucoside (**18**)	$C_{21}H_{22}O_{12}$	465		303, 151, 179	-
LPBM2.2.2.4					
cyanidin-3-O-glucoside (**19**)	$C_{21}H_{21}O_{11}$		449	287, 269, 241, 213, 185, 157,	
LPBM2.2.2.5				137, 121	
Quercetin (**20**)	$C_{15}H_{10}O_7$	301		151, 179, 283, 271, 257	-
Kaempferol-3-*O*-glucoside (**21**)	$C_{21}H_{20}O_{11}$	447		285, 241, 179	-
Quercetin-3-*O*-glucoside (**22**)	$C_{21}H_{19}O_{12}$	463		301, 151, 179, 283, 255	-
Taxifolin-7-*O*-glucoside (**23**)	$C_{21}H_{22}O_{12}$	465		303, 285, 241, 151, 179, 215	-
LPBM2.2.2.7					
Isorhamnetin (**24**)	$C_{16}H_{12}O_7$	315		300, 151, 283	-

* precursor ion = m/z 123

4. CONCLUSIONS

The isolation and identification of phytochemical constituents in the bran of Thai dark purple glutinous rice cultivar Luem Pua has been reported for the first time. Seven sterols (**1**-**6**, **8**) and two triterpenoids (**7**, **9**) have been identified from the unsaponified fraction of the hexane extract. Meanwhile, the investigation of the methanol extract, after successive chromatographic separation followed by MS/MS analysis of selected fractions, resulted in the identification of cyanidin-3-*O*-glucoside (**19**) together with six phenolic compounds (**10**-**13**, **16**, **17**) and eight flavonoids (**14**, **15**, **18, 20**-**24**). There have been various reports that **19** was a major anthocyanin found in various varieties of black rice and played predominant role in antioxidative activity of black rice bran extract [5, 6, 24]. It is, therefore, most plausible that **19** is also a major compound contributing to the dark-purple color of Luem Pua rice, and one of the compounds that plays important role in its anti-oxidative property. Moreover, many of the identified compounds have been reported for their biological activities. Phytosterols have been well approved for their ability in lowering blood cholesterol level [25] as well as having potentials in preventing cancer development [26]. Likewise, natural triterpenoids are known for their cytotoxicity to a wide range of cell lines [27]. In addition, phenolic compounds and flavonoids are potent sources of natural antioxidant for intake in the human diets [28, 29]. This study thus provide useful data to support the potential of Luem Pua rice to be further applied in either health or pharmaceutical products.

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