Indones. J. Chem., 2022, 22 (5), 1304 - 1320

Profiling of Phytochemical Compounds of East Java Red Rice Bran Has the High-Value Biological Activities as Antioxidant and Antidiabetic

Yoravika Dwiwibangga[1,2], Anna Safitri[1,2], and Fatchiyah Fatchiyah[1,3*]

[1]*Research Center of Smart Molecule of Natural Genetics Resource, Brawijaya University, Malang 65145, East Java, Indonesia*

[2]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Brawijaya University, Jl. Veteran, Malang 65145, East Java, Indonesia*

[3]*Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Jl. Veteran, Malang 65145, East Java, Indonesia*

* ***Corresponding author:***

tel: +62-341-575-841
email: fatchiya@ub.ac.id

Received: March 7, 2022
Accepted: June 20, 2022

DOI: 10.22146/ijc.73432

Abstract: *The phytochemicals contained in rice bran, mainly flavonoid compounds, are predicted to have biological activity. Flavonoids are able to counteract the free radicals and degrade insulin resistance. The East Java Red Rice Bran samples, e.g., Mentik Wangi, Aek Sibundong, and Blambangan, were used in the study. Their phytochemical profiles, functional groups, antioxidant, and antidiabetic activities were investigated. The phytochemical analysis showed that the bran of Mentik Wangi, Aek Sibundong, and Blambangan contained flavonoid, triterpenoid, phenolic, tannin, and glycoside. Based on the FTIR, some functional groups were identified in three rice bran varieties, namely, the O-H stretching, C-H aliphatic, C-H sp^3 stretching, C=C stretching aromatics, C=C stretching alkenes, CH_2 and CH_3 bonds rocking, C-H aromatic, CH-OH stretching alcohols, and C-O stretching ether or ester suggesting that rice brans are rich in phytochemical compounds. Through LC-HRMS analysis in positive ion mode, several types of flavonoids were confirmed. Pinocembrin was found in the three brands. The highest antioxidant and antidiabetic activity were observed in Blambangan rice bran with an IC_{50} value of 1.09 and 75.76 µg/mL, respectively. To conclude, the red rice bran phytochemical compounds exhibit potential biological activities as antioxidant and antidiabetic agents.*

Keywords: *antidiabetic; antioxidant; phytochemical; rice bran*

■ INTRODUCTION

Rice (*Oryza* spp.) is the most popular member of the Poaceae family [1]. Rice consists of more than 40,000 varieties worldwide and it is commonly divided into two widely cultivated types, *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice) [2]. In developing countries, rice is an important cereal crop consumed both as a staple food as well as processed products [3]. In general, white rice dominates the rice market. Nonetheless, pigmented rice cultivars nowadays attract great interest from researchers, nutritionists, and clinicians. Attention is currently being given to the antioxidative and radical-scavenging properties of pigmented rice cultivars because of their potential to provide and promote human health by reducing the concentration of reactive oxygen species and free radicals [1,4-6].

The pigmented rice cultivars include black rice, red rice, purple rice, and brown rice [6-7]. Red rice is suggested to contain various beneficial compounds for human health [5,8-9]. The major phytochemicals of red rice include phenolics, flavonoids, pro-anthocyanidins, and anthocyanins; which are believed to have several biological functions, such as antioxidants, antidiabetics, and anticholesterol [3,9-10].

Even though red rice and its biological capacities have been widely studied, research on red rice bran biological activities is still limited. The rice bran has been

generally used as livestock feed, although, it is potential to be explored for its contents and biological activities. The phytochemical investigations of red rice bran have shown that it contains flavonoids, phenolics, anthocyanins, proanthocyanidins, tannins, alkaloids, and some essential oils [9,11-12]. The flavonoid compounds detected in red rice bran include quercetin, apigenin, catechin, luteolin, and myricetin [9,13-14]. Moreover, several lipophilic components were identified, including γ-oryzanol, tocotrienols, and tocopherols [15-16]. These compounds have been suggested to possess high antioxidant activity [17-19].

Antioxidants are molecules that neutralize free radicals and prevent the damages that lead to degenerative diseases, such as cardiovascular, diabetes, hyperlipidemia, and other diseases. Antioxidants are substances that can protect cells from damage caused by unstable molecules known as free radicals [20-23]. Flavonoid compounds in pigmented rice are known to have a biological activity on metabolic disorders, such as cardiovascular disease, obesity, cancer, and diabetes mellitus. Flavonoid compounds act as antidiabetic agents due to their ability to regulate carbohydrate digestion, insulin signaling, insulin secretion, glucose uptake, and adipose deposition [24]. Flavonoids have been shown to decrease the pathogenesis of diabetes and its complications. Flavonoid compounds can reduce apoptosis and insulin resistance. Furthermore, flavonoids can increase insulin secretion and GLUT 4 translocation [25]. Flavonoid compounds can interact with α-amylase and α-glucosidase enzymes to form complex structures through hydrogen bonds and hydrophobic interactions. The interaction of flavonoids and enzymes causes inhibition of substrate binding to enzymes so that enzyme activity decreases [26-27].

To the best of our knowledge, exploration of biological activities from red rice bran from East Java has not been conducted. In the current study, three varieties of red rice, i.e., Aek Sibundong, Mentik Wangi, and Blambangan were studied. The study aims to identify compounds and investigate the antioxidant and antidiabetic activity of the extracts from red rice bran from East Java through phytochemical analysis, FTIR, LC-HRMS, antioxidant, and antidiabetic assays.

■ EXPERIMENTAL SECTION

Materials

The materials used were purchased from Sigma-Aldrich (Darmstadt, Germany): methanol (99.9%), sodium hydroxide (98%), sulfuric acid (99.9%), chloroform (99.5%), iron(III) chloride (97%), glacial acetic acid (99%), hydrogen chloride (37%), sodium carbonate (99%), trichloroacetic acid (99%), potassium ferricyanide (99%), and aluminium chloride (99.9%). The following materials were obtained from Merch: ascorbic acid (99%), gallic acid (97.5%), quercetin (95%), dinitrosalicylic acid reagent (98%), acarbose (95%), Wagner reagent, Folin-Ciocalteu reagent (1:1), phosphate buffer, starch, and α-amylase enzyme (from *Aspergillus oryzae*). Three varieties of rice were obtained from three regencies in East Java, Indonesia. They were Mentik Wangi from Ngawi, Aek Sibundong from South Malang, and Blambangan from Banyuwangi.

Instrumentation

The instruments used in this study were a Genesys 150 Thermo Scientific UV-Vis spectrophotometer and a Fourier Transform Infrared (FTIR) spectrophotometer (Shimadzu) IR Type Prestige 21. Identification and separation of components were performed using Liquid Chromatography-High-Resolution Mass Spectrometry (LC-HRMS) Thermo Scientific Dionex UltiMate 3000 RSLCnano in the Laboratorium Sentral Ilmu Hayati (LSIH), Brawijaya University.

Procedure

Maceration of red rice bran

The powder of three varieties of red rice bran samples, each 200 g, was macerated with 99.9% methanol for 3 × 24 h at room temperature [28]. Each extract was filtered with Whatman paper no. 42 and then evaporated by rotary evaporator vacuum with a slow speed at 95 rpm, at 45 °C. The extracts were then stored under freezing temperature (4 °C) for further analysis [8,28].

Phytochemical analysis of red rice bran

The phytochemical analysis of flavonoid, triterpenoid, phenolic, tannin, alkaloid, glycoside, and saponin was conducted based on previous studies [29-

30]. Different wavelengths were used in each test to measure the absorbance of the samples using a spectrophotometer UV-Vis, i.e., 430 nm for the flavonoid test, 544 nm for the triterpenoid test, 276 nm for the steroid test, 276 nm for the phenolic test, 725 nm for tannin test, 470 nm for alkaloid test, 600 nm for glycoside test, and 435 nm for saponin test [8].

Determination of total phenolic content

The total phenolic content in extracted red rice bran was determined by a spectrophotometric method using the Follin-Ciocalteu's reagent [31]. The 0.2 mL sample (4 mg/mL) was mixed with 0.6 mL of distilled water and 0.2 mL of Folin-Ciocalteu's reagent. After 5 min, 1 mL of saturated sodium carbonate solution (8% w/v in distilled water) was added to the mixture, and the volume was made up to 3 mL with distilled water. The final mixture was kept in the dark at ambient conditions for 30 min to complete the reaction. The absorbance was measured by a UV-Vis spectrophotometer at 745 nm. All measurements were determined in triplicate, and the data were expressed as mg Gallic Acid Equivalent (GAE)/100 g of crude extract of rice bran. The phenolic content was calculated as GAE/g of dry plant material based on a standard curve of gallic acid (5–100 mg/mL, $Y = 0.0104x - 0.0314$, $R^2 = 0.9644$). All determinations were carried out in triplicates.

Determination of total flavonoids content

The aluminium chloride colorimetric method was used for the determination of the total flavonoid content of the sample [31]. An amount of 0.6 mL extract was mixed with 0.6 mL of 2% aluminium chloride. After mixing, the solution was incubated for 60 min at room temperature. The absorbance of the reaction mixtures was measured against blank at 418 nm wavelength with a UV-Vis spectrophotometer. The concentration of total flavonoid content in the test samples was calculated from the calibration plot (5–20 mg/mL, $Y = 0.0506x + 0.0019$, $R^2 = 0.9856$) and expressed as mg quercetin equivalent (QE)/g of dried plant material. All the determinations were carried out in triplicates.

Identification of red rice bran extract using FTIR

Identification of functional groups contained in the extract of red rice bran was observed using FTIR spectrophotometer. A small amount of sample was dropped on one part of the KBr window. Then, another part of the KBr window was attached. Thus, the sample was evenly distributed on the window surface. The KBr window is placed in the holder, and the FTIR instrument is switched on. The IR spectra were recorded in the wavenumber range from 4500 to 400 cm^{-1}.

Identification of red rice bran extract using LC-HRMS

LC-HRMS was conducted in LSIH (Laboratorium Sentral Ilmu Hayati), Brawijaya University. The column used was a Hypersil GOLD aQ 50 mm × 1 mm × 1.9 µm particle size with an injection volume of 100 µL. Solvents used were solvent A = 0.1% formic acid in water and solvent B = 0.1% formic acid in acetonitrile. The elution gradient was 30 min with an analytical flow rate of 40 µL/min and the solvent ratio was set according to Table 1. Liquid chromatography was followed by mass spectrometer analysis (Thermo Scientific Q Exactive mass spectrometer) in the Electrospray ionization (ESI) method with positive ion mode detection. Experiments were set as follows: sheath gas (N_2) pressure = 50 psi, spray voltage = 4.5 kV, capillary temperature = 300 K, and *m/z* range = 50–750. Spectra were recorded in full mass scan condition with resolution = 70000 followed by data-dependent MS^2 scan with resolution = 17500. The compounds were determined using Compound Discoverer software version 3.2 with mzCloud MS/MS library.

Ferric reducing/antioxidant power (FRAP) assay

Various concentrations of each red rice bran extract were made with a range of 0–200 µg/mL for Mentik Wangi and Aek Sibundong; and 0–10 µg/mL for Blambangan. The solution was mixed with 2.5 mL of 0.2 M phosphate

Table 1. Gradient elution

Time (min)	%A : %B
0	95:5
2	95:5
15	40:60
22	5:95
25	5:95
25.1	95:5
30	95:5

buffer pH 6.6 and 2.5 mL of 1% potassium ferricyanide. The solution mixture was incubated at 50 °C for 20 min, and then 2.5 mL of 10% TCA was added and homogenized. An aliquot of the solution was put in a new test tube, mixed with distilled water and 0.1% ferric chloride solution. The absorbance was measured at 700 nm using a UV-Vis spectrophotometer [32]. The antioxidant activity of ascorbic acid with various concentrations of 0, 2, 4, 6, 8, and 10 µg/mL was also measured for a positive reference. The antioxidant activity was then calculated using Eq. (1).

$$\%\ \text{antioxidant} = \left[\frac{\text{sample absorbance} - \text{control absorbance}}{\text{sample absorbance}}\right] \times 100\% \quad (1)$$

The IC_{50} value was determined by a linear regression equation between the sample concentration and its antioxidant activity as the X and Y-axis. The IC_{50} value of each sample was expressed as a value of Y = 50, and the value of X was obtained as IC_{50}.

α-Amylase inhibitory activity

Briefly, 250 µL of bran extracts (Mentik Wangi and Aek Sibundong (0–200 µg/mL), Blambangan (0–100 µg/mL)) and acarbose (0–10 g/mL) were put into a test tube and added 250 µL of the α-amylase enzyme (50 µg/mL). Each solution was homogenized and incubated at 37 °C for 30 min. Then, 250 µL of 1% (w/v) starch was added and incubated again at 25 °C for 10 min. After that, 500 µL of DNS reagent was added and heated in boiling water for 5 min. The solution was cooled, and 5 mL of distilled water was added. Absorbance measurements at 480 nm and the IC_{50} value were calculated [28]. The antidiabetic activity was then calculated using Eq. (2).

$$\%\ \text{antioxidant} = \left[\frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}}\right] \times 100\% \quad (2)$$

Statistical analysis

All values were written as means ± standard deviation (SD) of three replicates. Statistical analysis of total phenolic content, total flavonoid content, antioxidant, and antidiabetic activity was performed using one-way analysis of variance (ANOVA), followed by the Tukey test for homogeneous data. Inhomogeneous data were analyzed by Brown-Forsythe and Games-Howell in IBM Statistical Product and Service Solutions software version 23 with a 95% confidence level ($p < 0.05$).

■ RESULTS AND DISCUSSION

Phytochemical Analysis

The phytochemical constituents contained in the extracts are important for predicting the biological and pharmacological activities. The phytochemical analysis was conducted based on the color changes after extracts reacted with the standard reagents for secondary metabolite detection. Three varieties of red rice bran were subjected to phytochemical identification. Results are presented in Table 2. The phytochemical analysis revealed that the red rice bran of Mentik Wangi, Aek Sibundong, and Blambangan contained flavonoids, triterpenoid, phenolic, tannin, and glycoside. Interestingly, steroids were detected only on Aek Sibundong bran. From the measurement results of the absorbance value, the higher absorbance indicates the higher concentration of secondary metabolites contained in the bran extract. These results suggest that varieties of red rice bran are rich in secondary metabolite compounds, thus, they have high nutrition contents. Triterpenoids and flavonoids were considered as the main

Table 2. The phytochemical screening in pigmented rice

Sample	Flavonoid	Triterpenoid	Steroid	Phenolic	Tannin	Alkaloid	Glycoside	Saponin
Menthik Wangi	+++	++++	-	++	++	-	+	-
Aek Sibundong	++	++	+	++	++	-	+	-
Blambangan	++	++	-	+	+	-	+	-

\- = not detected
\+ = detected phytochemical in low intensity of color
++ = detected phytochemical compound with medium color intensity
+++ = detected phytochemical compound with high color intensity
++++ = detected phytochemical compound with very high color intensity

class of secondary metabolites identified in three red rice bran based on absorbance values.

FTIR Analysis

FTIR spectra (Fig. 1) described absorbances of various chemical components in the three red rice bran extracts. The FTIR spectra of the samples visually do not show major differences demonstrating that the chemical components contained are generally similar (Table 3). Ten specific regions appeared in the three FTIR spectra. They were from O-H stretching, C-H aliphatic, C-H sp^3 stretching, C=C stretching aromatic, C=C stretching alkenes, CH_2 and CH_3 rocking, C-H aromatic, CH-OH stretching alcohols, C-O stretching ether or ester, and fingerprint region. However, some specific wavenumbers shifted. The O-H stretching absorbance was found in around 3433–3217 cm^{-1}. The O-H stretching region commonly comes from the flavonoid, phenolic, glycoside, and tannin compounds in the extracts.

FTIR results also supported the results of the phytochemical analysis. The C-H aliphatic and C-H sp^3 stretching vibrations at 3000–2856 cm^{-1} with the presence of C-H bending vibrations on CH_2 and CH_3 at 1461–1459 cm^{-1} region indicated a dimethyl group as triterpenoid compounds [33]. The band detected in 1744–1711 cm^{-1} indicated the presence of ester, which showed a hydrolyzed tannin compound formed from a hydroxyl group and a carboxyl group from phenolic acid. Also, the form of O-H stretching, C–H aliphatic, C=C alkenes, C=C aromatics, C-O-H, and C-O-C ethers indicated tannin groups [34]. The bands at 3433–3417, 3010–3007, 1744–1711, 1377–1370, 1268–1242, and 1169–1073 cm^{-1} indicated the presence of phenolic compounds, glycosides, and flavonoids [35]. The absorption band in

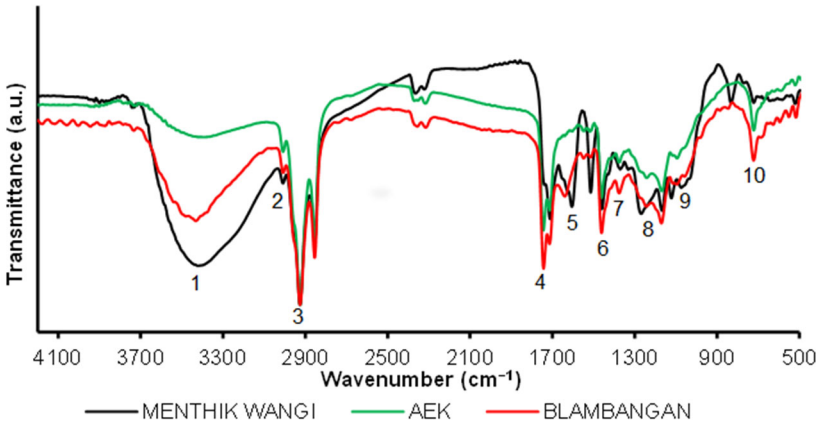

Fig 1. FTIR spectrum of red rice bran

Table 3. The wavenumber values at the FTIR absorption peaks of red rice bran and their probable functional groups

No.	Wavenumber (cm^{-1})			Probable functional group
	Mentik Wangi	Aek Sibundong	Blambangan	
1	3417.20	3421.48	3432.89	O-H stretching
2	3009.31	3007.88	3007.88	C-H aliphatic
3	2926.59–2855.28	2925.16–2855.28	2925.16–2855.28	C-H sp^3 stretching
4	1739.98–1711.45	1744.26–1712.88	1742.83–1714.31	C=C stretching aromatic
5	1605.91–1514.64	1526.05	1638.72–1548.87	C=C stretching alkenes
6	1459.01	1460.44	1460.44	CH_2 and CH_3 (the vibrational rocking of the C-H)
7	1370.59	1374.87	1376.29	C-H aromatic and carbonyl-carbonate
8	1267.90	1242.23	1243.66	CH-OH stretching alcohols
9	1168.07–1073.94	1168.07–1095.33	1170.92–1095.33	C-O stretching ether or ester
10	1000–400	1000–400	1000–400	Fingerprint region

the region 1000–400 cm^{-1} was evidenced as fingerprinting region. This region provides some information related to organic compounds that are probably present in the brans, such as carbohydrates, proteins, and organic acids.

LC-HRMS Analysis

LC-HRMS analysis aims to identify the flavonoid compounds of bran extracts. LC-HRMS analysis led to the interim identification of 20 compounds for each East Java red rice bran extract (Table 4). The results of the LC-HRMS identified many components, including flavonoids, phenolic acids, amino acids, saponin, alkaloids, vitamins, and fatty acids. The compounds that were tentatively identified in the three extracts of East Java red rice bran were pinocembrin, 4-coumaric acid, ferulic acid, hexadecanamide, 2-(methylthio)benzothiazole, 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde, monoolein, methyl 9*Z*,11*E*,13*E*-octadecatrienoate, 4-methoxycinnamic acid, bis(2-ethylhexyl) phthalate, and stearamide.

Table 4. LC-HRMS results of the East Java red rice bran

Samples	Proposed Compound	RT (min)	mzCloud best match	Dm (error mass)
Mentik Wangi	4-Coumaric acid	6.74	99.3	0.66
	Ferulic acid	7.25	98.0	0.59
	Dibenzylamine	7.71	98.2	0.21
	Tricin 5-O-β-D-glucoside	7.81	98.3	0.35
	Pinocembrin	13.72	98.3	0.81
	2-(Methylthio)benzothiazole	14.37	97.9	1.88
	Bis(4-ethylbenzylidene)sorbitol	15.10	99.6	0.56
	Tributyl phosphate	17.14	99.0	0.94
	2,4-dihydroxyheptadec-16-en-1-yl acetate	17.27	98.7	0.79
	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	17.37	97.9	1.07
	Dibutyl phthalate	18.43	98.6	2.05
	Linoleoyl Ethanolamide	19.34	97.2	1.52
	Methyl 9*Z*,11*E*,13*E*-Octadecatrienoate	19.72	98.3	1.70
	4-Methoxycinnamic acid	20.95	98.7	1.02
	Monoolein	21.31	98.7	2.52
	Hexadecanamide	21.95	99.0	1.92
	Bis(2-ethylhexyl) phthalate	23.18	99.8	2.70
	Bis(2-ethylhexyl) adipate	23.29	98.7	1.66
	Di(2-ethylhexyl) phthalate	23.32	99.7	2.70
	Stearamide	23.96	98.2	2.02
Aek Sibundong	Diisodecyl phthalate	5.31	98.6	0.20
	Bis(3,5,5-trimethylhexyl) phthalate	5.30	98.6	0.52
	4-Coumaric acid	6.75	99.3	0.95
	Ferulic acid	7.27	96.3	0.60
	Dibenzylamine	7.72	98.7	0.36
	12*Z*-9,10,11-trihydroxyoctadec-12-enoic acid	11.68	99.1	1.45
	Pinocembrin	13.74	97.4	0.93
	2-(Methylthio)benzothiazole	14.39	98.6	1.04
	Triisobutyl phosphate	17.15	98.1	1.40
	3,5-di-*tert*-Butyl-4-hydroxybenzaldehyde	17.37	98.3	0.87
	9-Oxo-10*E*,12*E*-octadecadienoic acid	18.32	98.2	1.45

Table 4. LC-HRMS results of the East Java red rice bran (*Continued*)

Samples	Proposed Compound	RT (min)	mzCloud best match	Dm (error mass)
Aek Sibundong	Dibutyl phthalate	18.36	96.9	1.09
	Monoolein	19.14	96.8	0.80
	Methyl 9*Z*,11*E*,13*E*-Octadecatrienoate	19.73	98.7	2.12
	1-Linoleoyl glycerol	20.03	98.3	1.27
	Palmitoyl ethanolamide	20.65	99.2	1.13
	4-Methoxycinnamic acid	20.75	98.9	0.56
	Hexadecanamide	21.92	98.9	1.09
	Bis(2-ethylhexyl) phthalate	23.17	99.7	1.37
	Stearamide	23.99	98.3	1.37
Blambangan	4-Coumaric acid	6.76	99.0	0.59
	Ferulic acid	7.28	97.5	0.65
	7-hydroxy-5-methoxy-2-phenyl-3,4-dihydro-2*H*-1-benzopyran-4-one	11.40	98.5	0.98
	Pinocembrin	13.75	97.8	0.93
	2-(Methylthio)benzothiazole	14.41	98.2	1.63
	Bis(4-ethylbenzylidene)sorbitol	15.11	99.5	1.10
	2,4-dihydroxyheptadec-16-en-1-yl acetate	17.27	98.9	0.79
	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	17.38	99.2	1.26
	Diisobutyl phthalate	18.43	98.7	2.16
	Methyl 9*Z*,11*E*,13*E*-Octadecatrienoate	19.73	99.3	1.18
	1-Linoleoyl glycerol	19.86	97.9	1.08
	Palmitoyl ethanolamide	20.68	98.9	1.13
	4-Methoxycinnamic acid	20.94	98.2	0.97
	Octadec-9-ynoic acid	21.09	97.5	0.11
	Monoolein	21.54	98.4	0.88
	Oleamide	21.86	97.9	1.94
	Hexadecanamide	21.96	99.1	1.68
	Bis(2-ethylhexyl) phthalate	23.33	99.6	1.91
	Methyl palmitate	24.40	98.3	0.58
	Stearamide	24.52	98.7	2.12

Table 5. Flavonoid and phenolic components of the three bran samples

Flavonoids	Phenolics
Pinocembrin	4-Coumaric acid
Monoolein	Ferulic acid
	4-Methoxycinnamic acid

The total ion chromatogram of each bran variety is shown in Fig. 2. In Mentik Wangi bran, LC-HRMS chromatograms and mass spectra show the compound 4-coumaric acid with the molecular formula $C_9H_8O_3$, established by *m/z* 165.05453 $[M+H]^+$. Tricin 5-O-β-D-glucoside has a molecular formula $C_{23}H_{24}O_{12}$, which appeared at *m/z* 493.13397 $[M+H]^+$. The molecular formula of pinocembrin, 4-methoxycinnamic acid, and bis(2-ethylhexyl) phthalate is $C_{15}H_{12}O_4$, $C_{10}H_{10}O_3$, and $C_{24}H_{38}O_4$, respectively, with the *m/z*, emerged at 257.08063 $[M+H]^+$, 179.06999 $[M+H]^+$, and 391. 2834 $[M+H]^+$ (Fig. 3). In the LC-HRMS chromatograms and mass spectra of Aek Sibundong's bran, ferulic acid, 3,5-di-tert-butyl-4-hydroxybenzaldehyde, and monoolein with the molecular formula $C_{10}H_{10}O_4$, $C_{15}H_{22}O_2$, and $C_{21}H_{40}O_4$, respectively, with the *m/z* appeared at 195.06525 $[M+H]^+$,

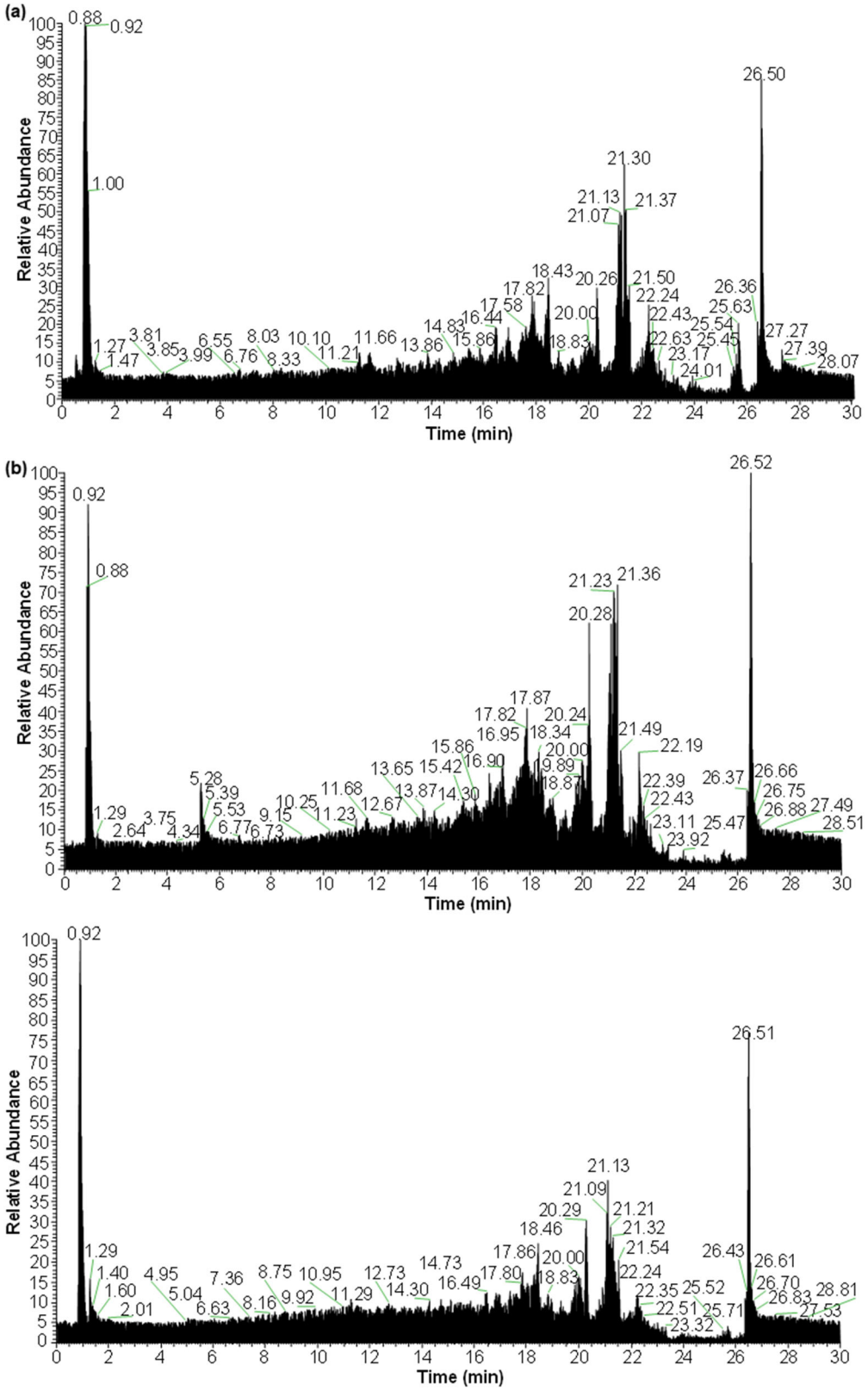

Fig 2. Total ion chromatograms (a) Mentik Wangi, (b) Aek Sibundong, (c) Blambangan

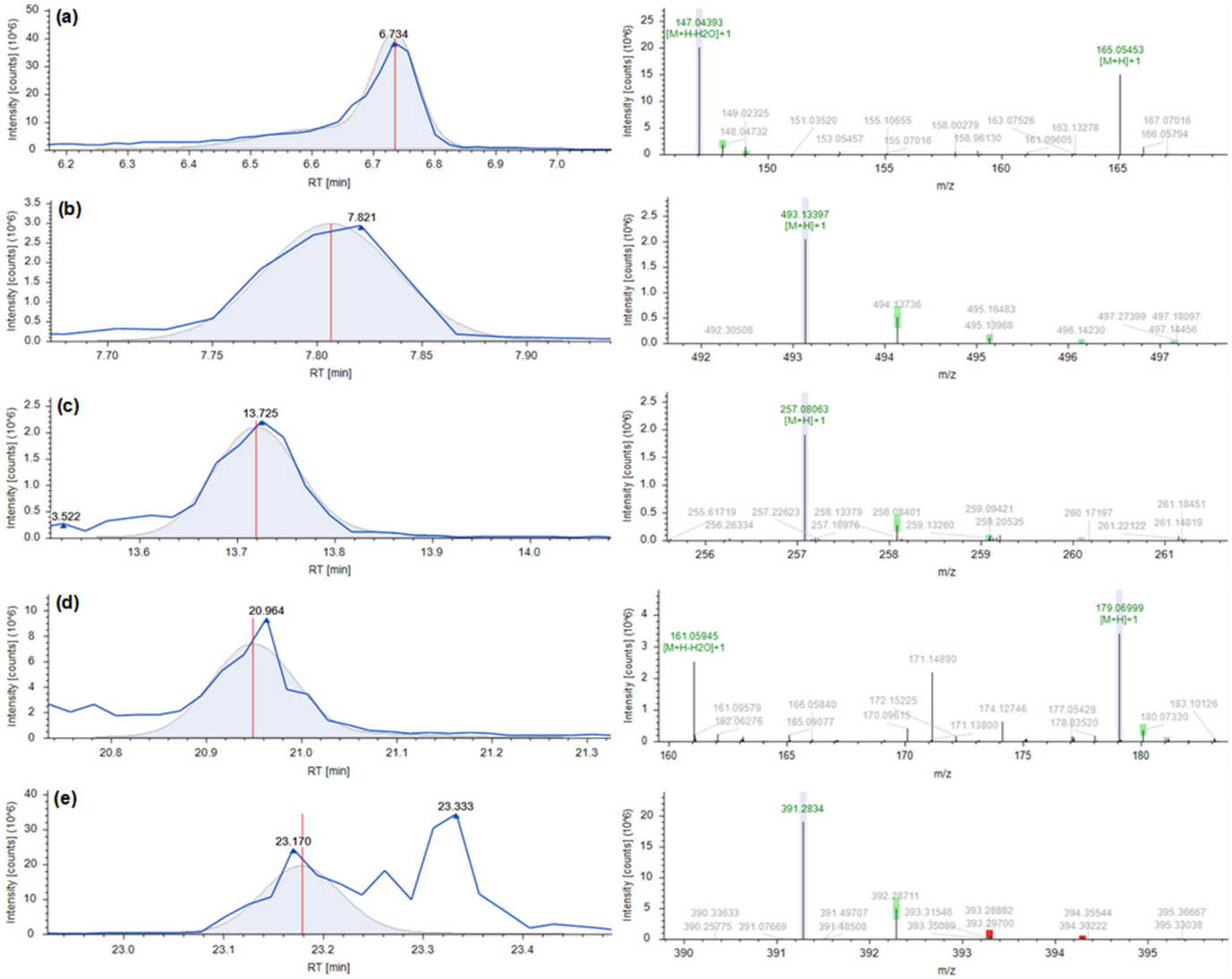

Fig 3. LC-HRMS chromatograms and mass spectra of (a) 4-Coumaric acid, (b) Tricin 5-O-β-D-glucoside, (c) Pinocembrin, (d) 4-Methoxycinnamic acid, and (e) Bis(2-ethylhexyl) phthalate from the East Java Mentik Wangi bran extract

235.16925 $[M+H]^+$, and 357.29965 $[M+H]^+$. The 1-Linoleoyl glycerol and palmitoyl ethanolamide compounds have a molecular formula $C_{21}H_{38}O_4$, and $C_{18}H_{37}NO_2$, established by *m/z* 355.28387 $[M+H]^+$ and 300.28937 $[M+H]^+$ were identified (Fig. 4). Furthermore, Blambangan bran identified the molecular formula for the compound 7-hydroxy-5-methoxy-2-phenyl-3,4-dihydro-2*H*-1-benzopyran-4-one, bis(4-ethylbenzylidene)sorbitol, and 2,4-dihydroxyheptadec-16-en-1-yl acetate are $C_{16}H_{14}O_4$, $C_{24}H_{30}O_6$, and $C_{19}H_{36}O_4$ with the *m/z* value at 271.09631 $[M+H]^+$, 415.21106 $[M+H]^+$, and 351.25052 $[M+H]^+$. Diisobutyl phthalate and methyl palmitate with the molecular formula $C_{16}H_{22}O_4$ and $C_{17}H_{34}O_2$, recognized by *m/z* 279.15848 $[M+H]^+$ and 271.26312 $[M+H]^+$ (Fig. 5).

From the results of the LC-HRMS analysis, it is known that pinocembrin, tricin 5-O-β-D-glucoside, monoolein, and 7-hydroxy-5-methoxy-2-phenyl-3,4-dihydro-2*H*-1-benzopyran-4-one are part of the flavonoid compound. In addition, it's also known that 4-coumaric acid, ferulic acid, and 4-methoxycinnamic acid are part of the phenolic. In the results of the LC-HRMS, pinocembrin, monoolein, tricin 5-O-β-D-glucoside, 4-coumaric acid, 4-methoxycinnamic acid, and ferulic acid

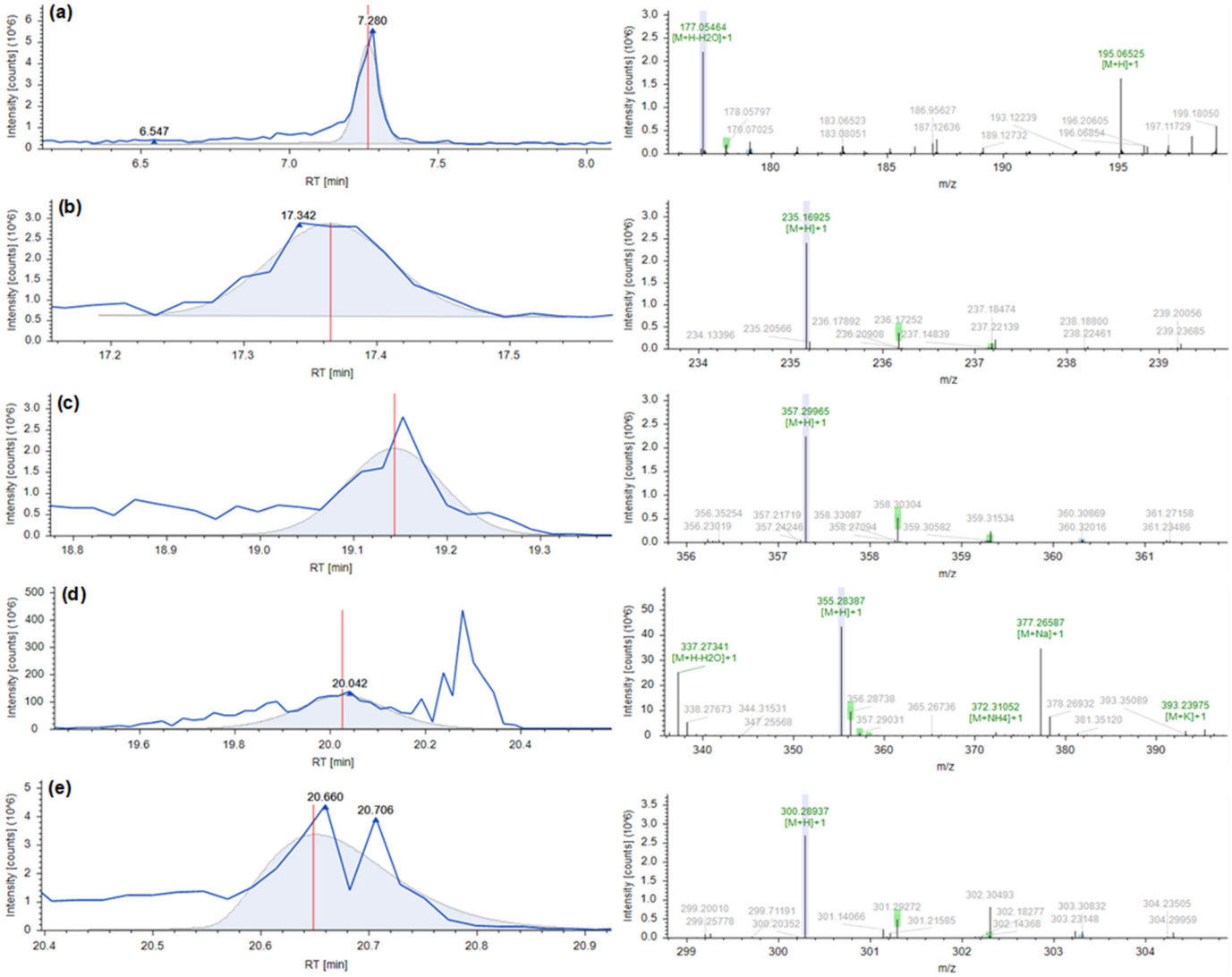

Fig 4. LC-HRMS chromatograms and mass spectra of (a) Ferulic acid, (b) 3,5-di-tert-butyl-4-hydroxybenzaldehyde, (c) Monoolein, (d) 1-Linoleoyl glycerol, and (e) Palmitoyl ethanolamide from the East Java Aek Sibundong bran extract

were found in Mentik Wangi rice were identified. In Aek Sibundong's bran, pinocembrin and monoolein were identified as flavonoid compounds. In addition, 4-coumaric acid, 4-methoxycinnamic acid, and ferulic acid were identified as phenolic compounds. In Blambangan's bran, pinocembrin, monoolein, 7-hydroxy-5-methoxy-2-phenyl-3,4-dihydro-2*H*-1-benzopyran-4-one, 4-coumaric acid, 4-methoxycinnamic acid, and ferulic acid were identified as flavonoid and phenolic compounds. The flavonoid and phenolic components in the three bran samples are shown in Table 5. Antidiabetic and antioxidant activity of each bran variety was influenced by flavonoid and phenolic compounds. Flavonoid compounds have an important role in the health segment, such as antidiabetic, antioxidant, antiviral, anti-inflammatory, and antibacterial. Flavonoids show beneficial antioxidant activity in managing diabetes mellitus [36].

Antioxidant Activity

Investigation of flavonoid and phenolic in red rice bran continued using UV-Vis spectrophotometer to analyze total flavonoid and phenolic content. Table 6 displays the calculation of total phenolic and flavonoid content. The highest total phenolic and flavonoid content is in Mentik Wangi (2200.97 ± 0.056 mg GAE/g sample

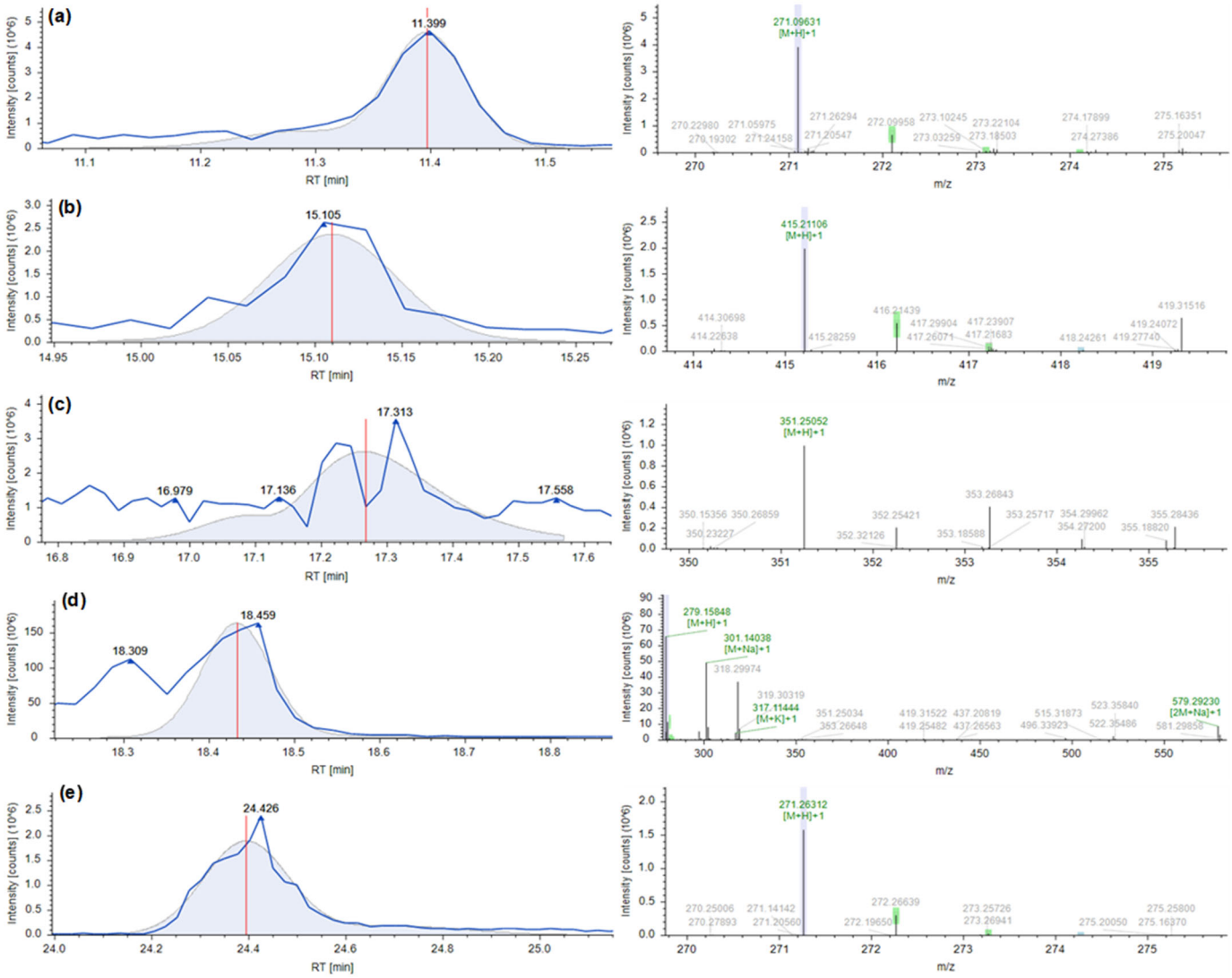

Fig 5. LC-HRMS chromatograms and mass spectra of (a) 7-hydroxy-5-methoxy-2-phenyl-3,4-dihydro-2*H*-1-benzopyran-4-one, (b) Bis(4-ethylbenzylidene)sorbitol, (c) 2,4-dihydroxyheptadec-16-en-1-yl acetate, (d) Diisobutylphthalate, and (e) Methyl palmitate from the East Java Blambangan bran extract

and 1467.96 ± 0.011 mg QE/g sample). Based on the One-Way ANOVA test, it was known that the significance value was $p > 0.05$. Therefore, it was assumed that the value of total flavonoid and phenolic content in the three varieties of red rice bran were not significantly different. Furthermore, the total flavonoid and phenolic content values also supported the results of phytochemical analysis, FTIR spectra, and LC-HRMS results. The highest total flavonoid and phenolic content were found in Mentik Wangi bran. These results indicated that the concentration of flavonoid and phenolic compounds in Mentik Wangi bran was higher than in the other two rice brans.

Ascorbic acid as the positive reference resulted in the highest antioxidant activity with the IC_{50} value of 0.28 ± 0.93 µg/mL. The reducing power activity was found in the order of ascorbic acid > Blambangan > Aek Sibundong > Mentik Wangi. The antioxidant activities of all red rice bran were lower than that of ascorbic acid. Based on the Games-Howell test, the antioxidant activity in the three varieties of red rice bran was significantly different. The lowest antioxidant activity was shown in Mentik Wangi bran with the IC_{50} value of 124.30 ± 1.27µg/mL. Both Mentik Wangi and Aek Sibundong brans resulted in an IC_{50} value of less than 250 µg/mL,

which is categorized as weak antioxidant activity; whereas Blambangan bran and ascorbic acid had an IC_{50} value of less than 10 μg/mL, which is categorized as very strong antioxidant activity (Table 6) [9]. A lower IC_{50} value represents a stronger free radical inhibitor (strong free radical inhibitors are active at low concentrations) [37].

Unpaired electrons or free radicals tend to attract electrons from other compounds to achieve atomic or molecular stability. If these free radicals are not inactivated, they can damage cell-forming macromolecules, such as proteins, carbohydrates, fats, and nucleic acids. The reducing power in the FRAP method is an indicator of the potential of antioxidant compounds [38]. Compounds that have reducing power may be able to act as antioxidants because compounds can stabilize radicals by donating electrons or hydrogen atoms. This interaction will stabilize the radical compound and stop the chain reaction of the formation of other free radicals [38-39].

Antidiabetic Activity

Inhibition of α-amylase and α-glucosidase is considered effective for controlling type 2 diabetes mellitus. The α-amylase secreted by the salivary glands and pancreas functions to break the α-1,4-glycoside bond to produce maltose and glucose. When the α-amylase enzyme is inhibited, the digestion of carbohydrates will also be blocked so as the level of glucose absorption into the blood will decrease [40]. Acarbose was used as a comparison for the α-amylase enzyme inhibition assay. The maximum potential of the sample in inhibiting α-amylase was indicated by the smallest amount of IC_{50}. Assays for the inhibitory activity of the α-amylase enzyme are shown in Table 7. Acarbose as a reference resulted in the highest antidiabetic activity with the IC_{50} value of 6.12 ± 1.68 μg/mL. The highest antidiabetic activity was shown in Blambangan bran with the IC_{50} value of 75.76 ± 0.36 μg/mL. The antidiabetic activity was found in the order of acarbose > Blambangan > Aek Sibundong > Mentik Wangi. Based on the Games-Howell test, the antidiabetic activity in the three varieties of red rice bran was significantly different.

Flavonoids have been extensively studied as α-glucosidase and α-amylase inhibitors [41-42]. The hydroxyl group of flavonoid compounds can effectively be conjugated to the residue of the active site of α-amylase and α-glucosidase enzymes. The 4-oxo group on the C ring of flavonoids contributes to the distribution of the electron cloud by donating a hydrogen atom to form hydrogen bonds with the active site residue. This interaction will inhibit the enzyme activity [42-43].

The phytochemical profiles, total flavonoid and phenolic content values, FTIR spectra, and LC-HRMS results contained in the extracts are important for predicting the biological and pharmacological activity of plants. All phytochemical profiles detected in red rice bran have many biological activities. Flavonoid and phenolic compounds are commonly known to have high antioxidant activity [44-45]. The potential of phenolic and flavonoid compounds as antioxidants are caused by the hydroxyl groups in phenolic compounds. The hydroxyl group acts as a hydrogen atom donor when it

Table 7. The antidiabetic activity of red rice bran in inhibiting α-amylase

Sample	IC_{50} (μg/mL)
Mentik Wangi	171.82^{a} ± 0.49
Aek Sibundong	157.29^{b} ± 1.28
Blambangan	75.76^{c} ± 0.36

*Different notations show significant differences at the α = 0.05 level

Table 6. Total phenolic, total flavonoid, and the antioxidant activity of red rice bran

Sample	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)	IC_{50} (μg/mL)
Mentik wangi	2200.97 ± 0.056	1467.96 ± 0.011	124.30^{a} ± 1.27
Aek sibundong	1578.00 ± 0.110	1197.00 ± 0.011	116.83^{b} ± 1.42
Blambangan	949.48 ± 0.056	1122.77 ± 0.011	1.09^{c} ± 0.82

*GAE = Gallic acid equivalent, QE = Quercetin equivalent.

*Different notations show significant differences at the α = 0.05 level

reacts with free radicals through an electron transfer mechanism so that the oxidation process can be inhibited [46-47]. Flavonoid and phenolic compounds are most effective at stabilizing free radicals (hydroxyl, superoxide, and peroxyl radicals) and can inhibit oxidation reactions because they can produce phenolic radicals that are stabilized by the resonance effect of aromatic rings [48]. Flavonoids and phenolic compounds are useful to stop free radicals in the body and to prevent aging factors. Flavonoids with antioxidant activity are beneficial in the management of diabetes mellitus [49-50]. The number of hydroxyl groups, hydroxyl configuration, C2-C3 double bonds, and C-4 ketonic functional groups is essential in the manifestation of flavonoid bioactivity, especially for antidiabetic effects [51-52].

The FRAP method was applied to study the antioxidant activity. The FRAP method is often used to evaluate the ability of an antioxidant to donate an electron. In this examination, the ability of extracts to reduce the ferric cyanide complex to the ferrous cyanide complex was determined [53]. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates so they can act as antioxidants. Results showed that red rice bran had various antioxidant activities. Mentik Wangi and Aek Sibundong were categorized as having weak antioxidant activity, whereas Blambangan was very strong antioxidant activity. Based on previous research, Aek Sibundong bran with the IC_{50} value of 116.83 ± 1.42 g/mL had weaker antioxidant activity than Aek Sibundong red rice with the IC_{50} value of 6.65 g/mL, whereas Blambangan bran with the IC_{50} value of 1.09 ± 0.82 g/mL had stronger antioxidant activity than Blambangan red rice with the IC_{50} value of 34 g/mL [54].

The α-amylase inhibition test was used to evaluate the ability of flavonoid and phenolic compounds to inhibit enzyme activity. The results showed that red rice bran has various antidiabetic activities. The bran of Mentik Wangi, Aek Sibundong, and Blambangan had antidiabetic activity, respectively, with IC_{50} values of 171.82 ± 0.49, 157.29 ± 1.28, and 75.76 ± 0.36 μg/mL. This inhibition was influenced by the interaction of hydrogen bonds between the hydroxyl groups of flavonoids and the catalytic residues of the enzyme. The interaction between enzymes and flavonoids can reduce starch digestion and postprandial glycemia. Flavonoids can also prevent the glucose absorption by inhibiting glucose transporters [55-56].

The results of antioxidant and antidiabetic activity were not positively correlated with the results of phytochemical analysis, total phenolic, and flavonoid content. Flavonoid and phenolic compounds were indeed responsible for the antioxidant and antidiabetic activity. However, the number and position of the –OH group in the compound were more influential. Compounds having hydroxyl groups in the 5,7-*meta* position have higher antioxidant activity. Substitution of the alkyl group at the -*ortho* or -*para* position in ring B will increase the electron density of the hydroxyl group with an inductive effect. However, long or branched alkyl chains will decrease antioxidant activity due to the influence of steric effects. In addition, the lower the dissociation energy of the –OH bond, the greater the ability of antioxidant activity because it is easier to react with free radicals [57-58].

Flavonoids have a stronger free radical scavenging ability than phenolic acids because they have a higher number of hydroxyl groups. The high antioxidant and antidiabetic activity of Blambangan bran are influenced by the flavonoid content. The highest peak area of pinocembrin and monoolein compared to other bran varieties indicated the high concentration of these compounds. Pinocembrin and monoolein are known to be effective in inhibiting the activity of the α-amylase enzyme. The conjugation of 4-oxo group on C ring plays an important role in the antioxidant and antidiabetic activities. The hydroxyl group of flavonoids enhances the interaction with enzymes, especially by forming hydrogen bonds. The hydroxyl group at the C-3 position in ring B, the C-5 position in ring A, and the carbonyl compound at the C-4 position in ring C have the potential to inhibit the activity of α-amylase enzymes. In addition, the 5,7-*meta*-dihydroxylation arrangement on the A ring also increases the antioxidant and antidiabetic capabilities of flavonoids [50,59-60]. Therefore, further *in vitro* and *in vivo* analyses are needed to elucidate the biological function of East Java red rice brans.

■ CONCLUSION

This study shows that each red rice bran has antioxidant activity and potential as an inhibitor α-amylase enzyme. The highest antioxidant and antidiabetic activity were found in Blambangan red rice bran with an IC_{50} value of 1.09 ± 0.82 and 75.76 ± 0.36 μg/mL, respectively. The use of phytochemical analysis, FTIR, and LC-HRMS tentatively identified many compounds contained in the rice bran extracts. The phytochemical compounds discovered in red rice bran included flavonoids, triterpenoid, phenolic, tannin, and glycoside. FTIR analysis supported the results of the phytochemical analysis. Various flavonoid and phenolic compounds found in the results of the LC-HRMS analysis affect the biological activity of the rice brans. East Java red rice bran is prospective to be used as an antioxidant and antidiabetic. Alpha-amylase inhibition has led to the discovery of new plant-based therapeutic products, specifically for diabetes. Further studies with an *in vivo* and *in silico* approach are needed to confirm the results of the current work.

■ ACKNOWLEDGMENTS

This work was a part of the Professor and Doctoral Research Grant Program for Fiscal Year 2021 number 1568/UN10.F09/PN/2021 from the Brawijaya University, Malang.

■ AUTHOR CONTRIBUTIONS

Yoravika Dwiwibangga carried out the experiment, interpretation of data, and wrote the manuscript. Fatchiyah supervised the experiment, analyzed data, and revised the manuscript. Anna Safitri analyzed data and wrote the manuscript.

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