

**Research Article**
**Open Access**

## Changes in Contents of Simple Phenols and Volatile Compounds in Leaves of Black Rice Varieties Following Growth Stages

 Pasin Thitisut<sup>1</sup>, Wichwara Nawara<sup>1</sup>, Chonlada Bennett<sup>1</sup>, Phumon Sookwong<sup>1,2</sup> and Sugunya Mahatheeranont<sup>1,2,3\*</sup>
<sup>1</sup>Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand

<sup>2</sup>Research Center on Chemistry for Development of Health Promoting Products from Northern Resources, Chiang Mai University, Chiang Mai, Thailand

<sup>3</sup>Center of Excellence for Innovation in Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand

**ABSTRACT**

In this study, we aimed to explore bioactive volatile compounds in leaves of black rice which emphasized on phenolic compounds. Leaves of four black rice varieties including IR1552 and three Thai black glutinous rice varieties, Kam Huai Hong Khrai (KHHK), Kam Chiang Mai (KCM), and Kam Muang Nan (KMN) were extracted by acidic and basic aqueous solution. Gas chromatography–mass spectrometry analysis of the volatile components in these black rice extracts revealed twelve simple phenols among fifty-five volatile compounds identified. 2-Methoxy-4-vinylphenol was detected as the most abundant volatile, especially, in the booting stage for KHHK followed by the tillering stage of the same variety. Its highest content was approximately 10-folds greater than that of vanillin at the booting stage. To the best of our knowledge, seven simple phenols were detected in black rice leaves for the first time, which included 4-vinylphenol, 2,6-dimethoxyphenol, 4-hydroxybenzaldehyde, 2-methoxy-4-(1-propenyl)phenol, 2,4-bis(1,1-dimethylethyl)phenol, 4-hydroxy-3,5-dimethoxy-benzaldehyde and 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol. 3-Hexen-1-ol was detected at the highest amount and was only identified in the dough-grain stage for KHHK, KCM and KMN. Two major aldehydes (2E)-2-hexenal and nonanal were detected in high quantities in KCM black rice variety in the dough-grain and mature-grain stage. Among the twelve fatty acids identified, (Z,Z,Z)-9,12,15-octadecatrienoic acid was detected at the highest amount, on average, followed by hexadecadienoic acid and octadecanoic acid. Therefore, it could be suggested that the leaves of black rice are rich in phenolic compounds and could be used to promote natural sources of food ingredients that are of great interest to the food industry.

**\*Corresponding author**

Sugunya Mahatheeranont, Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand. E-mail: sugunya.m@cmu.ac.th

**Received:** September 25, 2020; **Accepted:** October 03, 2020; **Published:** October 10, 2020

**Keywords:** Black rice, leaves, simple phenols, GC-MS, 2-methoxy-4-vinylphenol

**Introduction**

Rice (*Oryza sativa* L.) is a major crop that is consumed as a staple food by two-thirds of the world's population [1]. More than forty thousand rice varieties have been developed thus far and are cultivated in different regions around the world. All rice plants share common features such as round hollow stems, flat leaves, and flower clusters. The stems supporting the leaves transport essential nutrients between the roots, leaves and reproductive structures of the rice plants. The leaves are the main organs for photosynthesis and respiratory functions. They contain a huge amount of phytochemicals, some of which are essential defensive chemicals. The defensive compounds are secondary plant metabolites, including alkaloids, phenols, and terpenes [2]. In terms of human benefit, these three classes of plant metabolites are of great interest for researchers to identify and develop new drugs to treat various kinds of diseases [3].

Volatile organic compounds are the major metabolites in plants that are produced to protect themselves from the surrounding organisms. This is commonly done by releasing signals and messages [4]. A great number of volatile organic compounds

have been widely investigated throughout the plant kingdom and are exploited in different sectors, including sustainable agriculture, foods, cosmetics, and medicinal application due to their therapeutic benefits [5]. Several studies have investigated the volatile compounds of rice seeds and have found a broad spectrum of more than 200 volatile compounds, which are classified in groups of aliphatic alcohols, aromatics, aliphatic aldehydes, N-containing compounds, terpenoids, aliphatic ketones, fatty acids and esters. Our previous report has revealed more than 146 black rice volatiles, analysed by solid-phase micro-extraction (SPME) and comprehensive two-dimensional gas chromatography-mass spectrometry (GC×GC-MS). Among these headspace volatiles, 28 terpenoids were confirmed as the flavour or odorant compounds of black rice [6]. In contrast to rice seed volatiles, some defensive chemicals of rice plants have been identified more specifically in the rice leaves, as they function against pathogenic diseases, biotic and abiotic stresses. For instance, a study on allelopathic potential of some Vietnamese rice cultivars on barnyardgrass (*Echinochola crus-galli* (L.) Beauv.) found five allelochemicals that possessed the greatest phytotoxicity on barnyardgrass shoot which were salicylic acid, vanillic acid, p-coumaric acid, 2,4-dimethoxybenzoic acid, and cinnamic acid [7]. A few studies have reported the analysis of total volatile compounds in rice leaves. The recent one comparatively investigated aroma volatiles

between two scented and non-scented rice (*Oryza sativa* L.) cultivars at various developmental stages [8]. It revealed that N-heterocyclic class was the major distinguishing class between scented and non-scented rice. To date, there have been no reports on the volatile compounds in rice having black-coloured leaves.

With the expectation of some added bioactive compounds which are involved in the biosynthesis or biodegradation pathways of anthocyanin pigments, this study investigated the volatile compounds in three Thai black rice varieties. A fourth black rice variety, IR1552, obtained from the International Rice Research Institute (IRRI) in the Philippines through the Rice Department of Thailand, was also used for comparative studies. The changes in the contents of some potential volatile constituents, especially simple phenols which are reported to have high antioxidant activity were monitored. The investigation was performed following the development of black rice plants at five growth stages, including tillering, booting, heading, dough-grain and mature-grain stage.

## Materials and Methods

### Plant Material

Black rice variety IR1552 and three Thai black glutinous rice varieties, Kam Huai Hong Khrai (KHHK), Kam Chiang Mai (KCM), and Kam Muang Nan (KMN) were obtained from Rice Department Ministry of Agriculture and Cooperatives, Thailand. They were grown in the experimental field of the Chiang Mai Rice Research Center, Sanpatong, located in Chiang Mai province, northern Thailand. Black rice leaves were collected at 5 developmental stage, namely; tillering (45 days after sowing), booting (68 days), heading (82 days), dough-grain (119 days) and mature-grain (134 days). The leaves were air dried to reduce moisture (10%), vacuum sealed in aluminum bags, and stored in refrigerator at 4 °C until analysis.

### Chemicals

Analytical grade solvents and reagents used in this study, including methanol, dichloromethane were purchased from RCI Labscan (Thailand). Deionized water was obtained from a Milli-Q UV-Plus water purification system (Millipore Corp., Billerica, MA). 2,4,6-trimethylpyridine (TMP) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

### GC-MS analysis

#### Extraction of black rice leaf volatiles

Rice leaf samples were chopped and grounded with a blender. The grounded rice leaves were weighed (3.00 g) and placed into a 250 mL flask containing 100 mL of 0.25 ppm TMP internal standard in 0.01 M HCl solution for the acid extraction. The mixture was stirred for 30 min before filtration. The filtrate was transferred to a 500 mL pear-shaped separatory funnel, followed by the addition of approximately 1.5 mL of 5.0 M NaOH to make the solution slightly basic. Dichloromethane (200 mL) was immediately added as an organic solvent. After agitation, the dichloromethane portion in the organic layer was transferred to a 500 mL flask.

Thereafter, grounded rice leaves were weighed (3.00 g) again and placed into a 250 mL flask for the basic extraction. The rice leaf volatiles were extracted with 100 mL of 0.25 ppm TMP internal standard in 0.01 M NaOH solution and approximately 1.5 mL of 5.0 M HCl was added to make the solution slightly acidic. The extraction by dichloromethane was performed using the same procedure as the above. The dichloromethane portion was transferred to the same flask as above. The solution was dried with sodium sulfate anhydrous and concentrated to 1.00 mL using a rotary evaporator under reduced pressure at a temperature of 26

°C. The supernatant was transferred to a V-shaped vial prior to analysis by GC-MS.

### GC-MS condition

The separation and identification of volatile constituents in black rice leaf extracts were analyzed by a HP model 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled with a HP model 5973 mass-selective detector with a capillary column HP-5MS (30 m × 0.25 mm and 0.25 μm). Purified helium (99.99%) was used as the GC carrier gas under a constant flow rate of 28.6 cm s<sup>-1</sup> (1 mL min<sup>-1</sup>). The oven temperature was initially held at 40 °C and was increased at a rate of 4 °C min<sup>-1</sup> to a final temperature of 250 °C. The injector temperature was 250 °C. The sample was injected with a split ratio of 10:1. The mass spectrometer was operated in electron impact (EI) mode with an electron energy of 70 eV. The ion source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. The mass range (m/z) was 29-550 with a scan rate of 0.68 s/scan. The electron multiplier voltage was 1150 V and the GC-MS transfer line was set to 280 °C.

Identification of volatile components in rice leaf extracts were performed by matching their mass spectra with reference spectra in the Wiley7n Mass Spectral Library (Revision C.00.00) and the NIST98 Mass Spectral Library (Revision D.01.00/1.6d.). Quantitative analysis of each volatile component was performed by peak area normalization measurements.

### Statistical analysis

The data was analyzed using Statistica Version 8.0. The quantitative data were expressed as the mean ± standard deviation. Analysis of standard deviation was used to find the level of significant differences at P<0.05 performed using one-way ANOVA, SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA).

## Results and Discussion

Of all the volatile compounds detected in the four black rice varieties (Table 1), fifty-five compounds were identified and were categorized mainly as ketones (15) followed by simple phenols (12), fatty acids (6), alcohols (4), and aldehydes (3), respectively. Overall, 2-methoxy-4-vinylphenol was detected as the most abundant volatile, especially, in the booting stage for KHHK black rice variety followed by the tillering stage of the same rice variety. It was also detected roughly 10-folds greater than vanillin at the booting stage. It should be noted that vanillin was detected in every growth stage for every rice variety but was the highest at the mature-grain stage for all black rice varieties. To the best of our knowledge, this is the first study that shows the highest number of simple phenols accumulated in rice leaves. Moreover, seven simple phenols were detected in black rice leaves for the first time. These include 4-vinylphenol, 2,6-dimethoxyphenol, 4-hydroxybenzaldehyde, 2-methoxy-4-(1-propenyl)phenol, 2,4-bis(1,1-dimethylethyl)phenol, 4-hydroxy-3,5-dimethoxy-benzaldehyde and 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol. These simple phenols possess a wide range of therapeutic properties, some of which are listed in Table 2. Other important simple phenolic compounds include vanillin, 4-vinylphenol, and 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol, which were detected in every black rice variety and in every growth stage. 2,4-Bis(1,1-dimethylethyl)phenol is a common toxic metabolite produced by various groups of organisms. It exhibits potent toxicity against almost all testing organisms, including the producers, however, it is not clear why organisms produce phenolic compound and its analogues [9].

**Table 1.** The identified volatile components in leaves of IR1552, Kam Huai Hong Khrai (KHHK), Kam Chiang Mai (KCM), and Kam Muang Nan (KMN) at different growth stages.

Structural Assignment	Area ratio of volatile compound per internal standard																			
	Retention Index (RI)			Tillering stage			Booting stage			Heading stage			Dough-grain stage			Mature-grain stage				
	Exp	Ref	IR1552	KHHK	KCM	KMN	IR1552	KHHK	KCM	KMN	IR1552	KHHK	KCM	KMN	IR1552	KHHK	KCM	KMN		
<b>1. Alkene</b>																				
2,6,10-Trimethyl-1,4-ethylene-14-pentadecene	1839	1839	0.39	0.50	0.32	0.36	-	-	-	-	-	-	-	0.11	-	-	0.70	-	-	
<b>2. Ketone</b>																				
4-Methyl-2-hexanone	850	850	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5-Ethylidihydro-2(3H)-furanone	1063	1064	0.33	-	-	0.23	0.22	0.20	0.22	-	-	-	-	0.13	-	-	-	-	0.59	-
2,6,6-Trimethylcyclohex-2-ene-1,4-dione	1154	1152	-	-	-	0.04	-	-	-	-	0.06	0.16	0.16	-	-	-	-	0.05	0.06	0.10
4-Methyl-2,3-dihydropyran-6-one	1174	1169	-	-	-	-	-	-	-	-	-	0.17	-	-	-	-	-	-	-	-
2,2,6-Trimethylcyclohexane-1,4-dione	1179	1169	-	-	-	-	-	-	-	-	-	0.19	0.26	-	-	-	-	-	-	-
1-(Methylphenyl)-ethanone	1195	1181	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.10	0.14	0.08
3-Ethyl-4-methyl-1H-pyrrole-2,5-dione	1251	1265	0.94	-	-	-	0.32	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-1-(2,6,6-Trimethylcyclohexa-1,3-dien-1-yl)but-2-en-1-one	1396	1388	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.06	0.07	-	-
(3E)-4-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-3-buten-2-one	1497	1498	-	-	0.21	0.26	0.12	0.10	0.08	0.11	0.09	0.08	-	0.19	-	0.08	-	0.48	-	-
1-(4-Hydroxy-3-methoxyphenyl)ethanone	1507	1499	-	-	-	-	-	-	-	-	0.16	0.12	0.37	0.26	0.16	0.18	0.37	0.32	-	0.27
4,4,7a-Trimethyl-6,7-dihydro-5H-1-benzofuran-2-one	1543	1538	1.83	-	1.22	1.46	1.00	0.68	0.49	0.56	0.85	0.57	1.49	1.22	0.47	0.40	0.05	1.55	0.38	0.68
2-(Methoxymethyl)-3,5-dimethylcyclohexa-2,5-diene-1,4-dione	1583	1573	3.35	0.33	3.54	2.74	4.57	9.07	4.22	7.56	7.72	0.60	5.95	0.84	8.27	12.26	13.36	4.41	-	2.10
6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(1H)-one	1795	1800	2.56	3.68	2.98	4.14	2.31	2.74	1.99	2.52	3.12	0.60	1.33	2.14	2.59	1.11	1.55	1.85	-	-
4-Hydroxy-3,5,5-trimethyl-4[(E)-3-oxobut-1-enyl]cyclohex-2-en-1-one	1808	1802	0.35	0.15	-	-	-	-	-	-	-	0.17	0.39	0.47	-	-	-	-	-	-
6,10,14-Trimethylpentadecan-2-one	1865	1869	-	-	-	-	-	-	-	-	-	-	-	0.63	-	-	-	-	-	1.05

**Table 1. (Continued)**

Structural Assignment		Area ratio of volatile compound per internal standard																											
		Retention Index (RI)						Tillering stage				Booting stage				Heading stage				Dough-grain stage				Mature-grain stage					
		Exp	Ref	IR1552	KHHK	KCM	KMN	IR1552	KHHK	KCM	KMN	IR1552	KHHK	KCM	KMN	IR1552	KHHK	KCM	KMN	IR1552	KHHK	KCM	KMN	IR1552	KHHK	KCM	KMN		
<b>3. Alcohol</b>																													
	3-Hexen-1-ol	862	861	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	2-Ethyl-1-hexanol	1040	1045	-	-	-	-	-	-	-	-	0.10	0.33	0.18	-	-	-	-	-	-	-	-	-	-	-	0.13	0.20		
	2-Phenoxyethanol	1236	1229	-	-	-	-	-	-	-	-	-	-	0.09	0.15	-	-	-	-	-	-	-	-	-	-	-	-		
	Benzyl alcohol	1056	1057	0.54	0.68	0.48	0.82	0.25	0.23	0.17	0.20	0.19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.29		
<b>4. Aldehyde</b>																													
	(2E)-2-Hexenal	857	857	0.04	0.07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	Nonanal	1111	1111	0.05	-	0.15	-	-	0.09	-	0.09	0.10	0.11	0.07	0.09	-	-	-	-	-	-	-	-	-	-	-	0.17		
	2,6,6-Trimethylcyclohexa-1,3-diene-1-carbaldehyde	1208	1207	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.05		
<b>5. Ester</b>																													
	Dimethyl benzene-1,2-dicarboxylate	1468	1469	-	-	-	-	-	-	-	-	-	-	0.05	-	-	-	-	-	-	-	-	-	-	-	-	-		
	Diethyl benzene-1,2-dicarboxylate	1609	1605	-	-	-	-	-	-	-	-	-	-	0.09	-	-	-	-	-	-	-	-	-	-	-	-	-		
	2-Ethylhexyl salicylate	1822	1817	-	-	-	-	-	-	-	-	-	-	1.10	0.11	0.08	0.13	0.30	-	-	-	-	-	-	-	-	-	9.00	0.47
	(3,3,5-Trimethylcyclohexyl) 2-hydroxybenzoate	1901	1904	0.05	0.20	0.05	0.15	0.16	0.09	3.49	0.30	0.18	-	0.47	0.89	0.21	0.11	0.12	-	-	-	-	-	-	-	-	-	1.12	
	Methyl 11-octadecanoate	2124	2115	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	Tetradecyl benzoate	2438	2447	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
<b>6. Fatty acid</b>																													
	Hexanoic acid	1013	1013	0.59	0.96	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	(3E)-3-Hexenoic acid	1032	1023	1.30	1.33	0.82	0.97	0.39	1.63	0.79	1.38	0.25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	(2E)-2-Hexenoic acid	1058	1047	-	-	-	-	-	-	-	-	-	-	0.32	-	0.28	0.16	0.10	0.11	-	-	-	-	-	-	-	-	-	-
	Benzoic acid	1201	1210	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	Octanoic acid	1203	1203	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	Nonanoic acid	1299	1293	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	Dodecanoic acid	1593	1592	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	Tetradecanoic acid	1788	1787	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	Hexadecadienoic acid	1992	1991	6.56	8.49	5.57	6.85	2.53	3.86	0.65	3.09	2.94	7.23	8.33	20.61	2.50	2.66	3.03	4.57	2.61	4.12	4.63	5.73	5.73	5.73	5.73	5.73		

**Table 1. (Continued)**

Structural Assignment	Area ratio of volatile compound per internal standard																					
	Retention Index (RI)			Tillering stage			Booting stage			Heading stage			Dough-grain stage			Mature-grain stage						
	Exp	Ref	IRI552	KHHK	KCM	KMN	IRI552	KHHK	KCM	KMN	IRI552	KHHK	KCM	KMN	IRI552	KHHK	KCM	KMN	IRI552	KHHK	KCM	KMN
(Z,Z)-9,12-Octadecadienoic acid	2134	2134	0.48	-	-	-	0.40	0.98	2.61	0.78	-	1.94	2.97	15.28	0.41	0.30	0.52	0.62	0.64	1.14	2.56	2.11
(Z,Z,Z)-9,12,15-Octadecatrienoic acid	2141	2143	4.34	14.74	12.45	11.32	3.66	7.83	0.79	6.06	5.82	12.71	12.17	15.96	2.54	3.81	4.40	5.60	0.00	0.00	2.97	2.92
Octadecanoic acid	2194	2188	0.45	0.61	0.94	0.89	0.32	0.27	5.69	0.34	0.41	1.89	2.36	4.77	0.24	0.20	0.22	0.31	-	-	0.97	0.92
<b>7. Simple Phenols</b>																						
Phenol	1005	1004	-	-	-	-	1.07	1.52	1.57	1.30	0.37	-	-	-	0.39	-	-	-	-	-	-	-
2-Methoxyphenol	1098	1097	0.15	0.23	0.40	0.51	0.48	1.05	0.74	0.68	0.48	0.21	0.74	0.61	0.79	0.88	0.83	0.86	0.60	0.31	1.08	1.20
4-Vinylphenol	1246	1247	0.73	1.74	1.27	2.16	0.89	1.97	1.38	1.83	0.56	0.49	1.35	0.80	0.75	1.00	2.23	1.46	0.33	0.25	2.06	1.74
2-Methoxy-4-vinylphenol	1328	1328	25.09	48.78	34.77	32.07	24.04	52.34	36.31	32.54	31.44	10.73	28.35	16.97	34.55	41.71	41.64	26.14	14.51	1.24	29.68	33.35
2,6-Dimethoxyphenol	1367	1367	0.14	0.17	0.14	0.26	0.35	0.63	0.39	0.48	0.17	0.10	0.60	0.45	0.35	0.34	0.49	0.45	0.67	-	1.34	1.52
4-Hydroxybenzaldehyde	1406	1409	0.98	-	-	1.09	0.61	0.48	0.44	0.92	0.44	-	1.50	0.86	0.61	1.72	0.41	2.01	0.66	0.64	1.62	2.38
Vanillin	1415	1415	5.26	4.76	4.06	5.07	3.09	5.91	4.01	4.61	3.16	4.32	8.32	5.62	3.16	4.96	5.49	4.40	6.77	6.34	9.74	7.08
2-Methoxy-4-(1-propenyl)phenol	1468	1463	-	-	-	-	-	-	-	-	-	-	0.13	0.18	-	-	-	-	-	-	-	-
2,4-Bis(1,1-dimethylethyl)phenol	1531	1525	0.20	0.15	0.16	0.17	0.11	0.10	0.14	0.13	0.10	0.12	0.42	0.29	0.08	0.17	0.12	0.13	0.14	0.21	0.30	0.22
4-Hydroxy-3,5-dimethoxybenzaldehyde	1682	1670	0.50	1.06	1.15	1.14	0.50	2.50	1.70	1.78	0.56	0.98	1.86	1.56	0.42	1.59	1.84	0.79	-	-	-	-
4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	1746	1744	0.76	2.52	1.89	2.20	0.87	3.61	2.49	2.59	0.84	2.96	7.67	5.81	0.80	1.08	2.15	1.40	1.58	3.09	2.25	2.37
4,4'-(1-Methylethylidene)bisphenol	2224	2022	-	-	-	-	-	-	-	-	-	-	0.40	-	-	-	-	-	-	-	-	-
<b>8. Nitrogen-containing</b>																						
N,N-Dibutyl-formamide	1318	1319	-	-	-	-	-	-	-	-	-	-	0.17	-	-	-	0.09	-	-	-	-	-
3,3-Diphenylprop-2-enenitrile	1889	1896	0.20	0.39	0.28	0.35	-	-	-	-	-	-	-	-	0.12	-	0.13	0.11	-	-	0.23	0.15

**Table 2.** Phenolic compounds found in black rice leaves and their bioactivities.

Compound	Bioactivities	Literature
2,4-Bis(1,1-dimethylethyl)phenol	Antioxidant, anticancer, antifungal, anti-inflammatory and antibacterial activities	[11-15]
2,6-Dimethoxyphenol	Inhibiting the growth of vaginitis pathogens	[16]
4-Hydroxy-3,5-dimethoxy-benzaldehyde	Radioprotection	[17]
4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	Antioxidant, anti-microbial, anti-inflammatory	[18]
2-Methoxy-4-vinylphenol	Antioxidant, antimicrobial and anti-inflammatory	[18,19]
Vanillin	Antioxidant, antimicrobial, antimutant, antifungal and antibacterial activities	[20-22]
4-Vinylphenol	Inhibition of hepatotoxicity and pneumotoxicity in rats and mice, inhibition of metastasis and cancer stemness in breast cancer cells	[23,24]

Among the fifteen ketones identified, 2-(methoxymethyl)-3,5-dimethylcyclohexa-2,5-diene-1,4-dione, was detected at the highest amount, especially in the dough-grain stage. The compounds 2-(methoxymethyl)-3,5-dimethylcyclohexa-2,5-diene-1,4-dione and 4,4,7a-trimethyl-6,7-dihydro-5H-1-benzofuran-2-one were the only ketones that were detected in almost every black rice variety in nearly every growth stage. The main alcohol compound, 3-hexen-1-ol, was detected at the highest amount and was only identified in the dough-grain stage for KHHK, KCM, and KMN black rice varieties. It is an aroma-active compound produced in small amounts by most plants and it acts as an attractant to many predatory insects [10].

The main aldehydes (2E)-2-hexenal and nonanal were detected the most in KCM black rice variety in the dough-grain stage and mature-grain stage. Among the twelve fatty acids identified, it was found that (Z,Z,Z)-9,12,15-octadecatrienoic acid was detected at the highest amount, on average, followed by hexadecadienoic acid and octadecanoic acid. These compounds were identified predominantly in KMN black rice variety at the heading stage.

Noteworthy, the absence of terpenoids and other relatively nonpolar components was due to the acidic and basic solution extraction, which accommodates the partition of polar substances more efficiently.

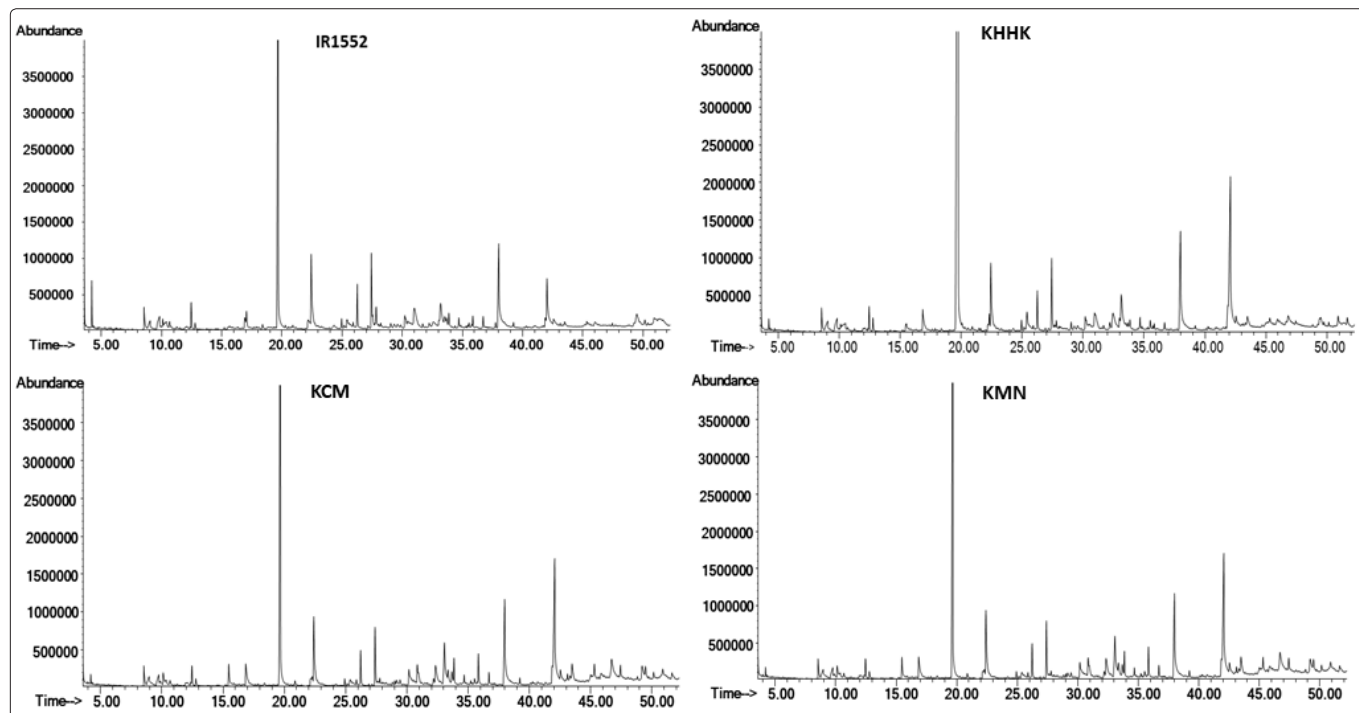
Figure 1 depicts the important antioxidant compounds found in the leaf of the four black rice varieties at the different growth stages. The line graph for 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol and 2,4-bis(1,1-dimethylethyl)phenol showed a similar trend where an increase in antioxidant compounds at the heading stage was observed, mainly for the rice varieties KCM and KMN. On the contrary, 2-methoxy-4-vinylphenol, 2-methoxyphenol, 4-vinylphenol, and especially benzyl alcohol was detected the least at the heading stage. Furthermore, 2-methoxyphenol, vanillin, 4-hydroxy-3,5-dimethoxy-benzaldehyde had the highest detectable amount at the tillering stage and decreased in content toward the mature-grain stage.

Simple phenols are bioactive natural occurring compounds found commonly in food plants and are well-recognized natural antioxidants. The biological activities of phenolic compounds, especially antioxidant activity, have been acknowledged for decades. Recently, their many observed inhibitory effects on mutagenesis and carcinogenesis have driven the search for new antioxidants of natural origins. Leaves of black rice are rich in phenolic compounds, not only the simple phenols but also their natural pigments, anthocyanins, and many other types of polyphenols, which could be used as novel natural sources for food ingredients that are of great interest to the food industry.



**Figure 1:** Relative contents of 8 bioactive compounds at 5 developmental stages (tillering, booting, heading, dough-grain and mature-grain) in leaves of 4 black rice varieties; IR1552, Kam Huai Hong Khrai (KHHK), Kam Chiang Mai (KCM), and Kam Muang Nan (KMN).

## Supplementary document



## Conclusions

This study revealed that black rice leaves are the potential source of bioactive phytochemicals, especially antioxidants in the group of simple phenols, seven of which were detected for the first time. The three Thai black rice varieties and a variety from the IRRI showed similar GC-MS profiles of volatile components extracted by acidic and basic aqueous solutions. However, the contents of these volatile compounds in leaves of each black rice variety changed variously following growth stages.

## Acknowledgments

The Research Center on Chemistry for the Development of Health-Promoting Products from Northern Resources, Chiang Mai University (CMU) and the Centre of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, Science, Research and Innovation, Thailand are gratefully acknowledged.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Cha HM, Han G, Chung HJ (2012) A study on the trend analysis regarding the rice consumption of Korean adults using Korean National Health and Nutrition Examination Survey data from 1998, 2001 and 2005. *Nutrition Research and Practice* 6: 254-262.
2. Levin DA (1976) The chemical defenses of plants to pathogens and herbivores. *Annual Review of Ecology and Systematics* 7: 121-159.
3. Mithofer A, Boland, W (2012) Plant defense against herbivores: chemical aspects. *Annual Review of Plant Biology* 63: 431-50.
4. Effah E, Holopainen JK, McCormick AC (2019) Potential roles of volatile organic compounds in plant competition. *Perspectives in Plant Ecology, Evolution and Systematics* 38: 58-63.
5. Raut JS, Karuppayil SM (2014) A status review on the medicinal properties of essential oils. *Industrial Crops and Products* 62: 250-264.
6. Chumpolsri W, Wijit N, Boontakham P, Nimmanpipug P, Sookwong P, et al. (2015) Variation of Terpenoid Flavor odorants in bran of some black and white rice varieties Analyzed by GC×GC-MS. *Journal of Food and Nutrition Research* 3: 114-120.
7. Ho TL, Nguyen TTC, Vu DC, Nguyen NY, Nguyen T, et al. (2020) Allelopathic potential of rice and identification of published allelochemicals by cloud-based metabolomics platform. *Metabolites* 10: 244.
8. Hinge VR, Patil HB, Nadaf AB (2016) Aroma volatile analyses and 2AP characterization at various developmental stages in basmati and Non-Basmati scented rice (*Oryza sativa* L.) cultivars Rice 9: 38.
9. Zhao F, Wang P, Lucardi RD, Su Z, Li S (2010) Natural sources and Bioactivities of 2,4-di-tert-butylphenol and its analogs. *Toxins* 12: 35-60.
10. Ruther J, Hilker M, (2003) Attraction of forest cockchafer *Melolontha hippocastani* to (Z)-3-hexen-1-ol and 1,4-benzoquinone: Application aspects. *Entomologia Experimentalis et Applicata* 107: 141-147.
11. Fitzgerald DJ, Stratford G, Jasson M, Ueckert J, Bos A, et al. (2004) Mode of antimicrobial action of vanillin against *Escherichia coli*, *Lactobacillus plantarum* and *Listeria innocua*. *Journal of Applied Microbiology* 97: 104-113.
12. Rana VS, Blazquez MA (2007) Chemical constituents of *Gynura cusimbua* aerial parts. *Journal of Essential Oil Research* 19: 21-22.
13. Malek SN, Shin SK, Wahab NA, Yaacob H (2009) Cytotoxic components of *Pereskia bleo* (Kunth) DC. (Cactaceae) leaves. *Molecules* 14: 1713-1724.
14. Zhou BL, Chen ZX, Du L, Xie YH, Zhang Q, et al. (2011) Allelopathy of root exudates from different resistant eggplants to *Verticillium dahliae* and the identification of allelochemicals. *African Journal of Biotechnology* 10: 8284-8290.
15. Abdullah A-SH, Mirghani MES, Jamal P (2011) Antibacterial activity of Malaysian mango kernel. *African Journal of Biotechnology* 10: 18739-18748.
16. Rao H, Li P, Wu H, Liu C, Peng W, et al. (2019) Simultaneous determination of six compounds in destructive distillation

- extracts of hawthorn seed by GC-MS and evaluation of their antimicrobial activity. *Molecules* 24: 4328.
17. Zheng H, Chen ZW, Wang L, Wang SY, Yan YQ, et al. (2008) Radioprotection of 4-hydroxy-3,5-dimethoxybenzaldehyde (VND3207) in culture cells is associated with minimizing DNA damage and activating Akt. *European Journal of Pharmaceutical Sciences* 33: 52-59.
  18. Ravikumar V, Gopal V, Sudha T (2012) Analysis of phytochemical constituents of stem bark extracts of *Zanthoxylum Tetraspermum* Wight & Arn. *Research Journal of Pharmaceutical, Biological and Chemical Sciences* 3: 391-402.
  19. Al-Marzoqi AH, Hadi MY, Hameed IH (2016) Determination of metabolites products by *Cassia angustifolia* and evaluate antimicrobial activity. *Journal of Pharmacognosy and Phytotherapy* 8: 25-48.
  20. Burri J, Graf M, Lambelet P, Lo LJ (1989) Vanillin: More than a flavouring agent a potent antioxidant. *Journal of the Science of Food and Agriculture* 48: 49-56.
  21. Shyamala BN, Naidu M, Sulochanamma G, Srinivas P (2007) Studies on the antioxidant activities of natural vanilla extract and its constituent compounds through in vitro models. *Journal of Agriculture and Food Chemistry* 55: 7738-77435.
  22. Mourtzinou I, Konteles S, Kalogeropoulos N, Karathanos VT (2009) Thermal oxidation of vanillin affects its antioxidant and antimicrobial properties. *Food Chemistry* 114: 791-797.
  23. Carlson GP (2002) Effect of the inhibition of the metabolism of 4-vinylphenol on its hepatotoxicity and pneumotoxicity in rats and mice. *Toxicology* 179: 129-136.
  24. Leung H, Ko C, Yue GG, Herr I, Lau C (2018) The natural agent 4-vinylphenol targets metastasis and stemness features in breast cancer stem-like cells. *Cancer Chemotherapy and Pharmacology* 82: 185-197.