Fractionation and Identification of Antioxidant Compounds from Bran of Thai Black Rice *cv.* Riceberry

Panawan Suttiarporn, Phumon Sookwong, and Sugunya Mahatheeranont

***Abstract*—Bran of Thai Black Rice *cv.* Riceberry was extracted using solvent extraction (hexane, dichloromethane and methanol). The crude extracts obtained were investigated for their antioxidant property and total phenolic contents which were assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay and Folin-Ciocalteu's method, respectively. Results showed that the methanolic extract had the strongest total antioxidant activity and phenolic content at 97.83 % and 9.87 mg GAE/ml of extract, respectively. The methanolic extract was further fractionated by column chromatography to obtain ten fractions. The chemical components of the crude extracts and the fractions were analyzed by gas chromatography-mass spectrometry (GC-MS). Seventy-nine constituents were identified in all extracts of Riceberry bran. Among them, 9 simple phenolics and 4 flavonoids, which were previously reported to have antioxidant activities, were quantified. The major simple phenolic was 4-vinylguaiacol and the major flavonoid was apigenin.**

***Index Terms*—Riceberry, antioxidants, fractionation, gas chromatography mass-spectrometry.**

I. Introduction

Black rice brans**,** the black outer layers removed during the milling of black rice, have potential to promote human health due to the great antioxidant potency of their phytochemicals. These compounds include anthocyanidins (cyanidin-3-O-glucoside and peonidin-3-O-glucoside [1]), flavonoids glycosides (quercetin-3-O-glucoside, isorhamnetin-3-O-glucoside and myricetin-7-O-glucoside [2]), simple phenolics (2-methoxy-4-vinylphenol (4-vinylguaiacol), 2-methoxy-phenol (guaiacol), and 1,2-benzendiol (catechol) [3]), vitamin E, (tocopherols and tocotrienols [4]), and γ-oryzanols (ferulate derivatives of 24-methylenecycloartenol, cycloartenol, campesterol and β-sitosterol [5]).

Riceberry rice, recently developed for nutritional benefit to consumers, a combination of Hom Nin rice, with its well-known antioxidant properties, and Thai Hom Mali rice, which is also called Thai Jasmine or Khao Dawk Mali 105. The extracts of the bran had been reported to have potential anticancer activity [6], hypoglycemic, hypolipidemic, antioxidant, and anti-inflammation properties [7].

There are a few reports about the identification of chemical components in pigmented rice by GC-MS. However, GC-MS analysis of some specific chemical components from fractions isolated by column chromatography have not been reported. Therefore, the objective of this research was to isolate complex crude extracts of Riceberry bran, to subfractionate the extract using column chromatography, and identify of antioxidant components by GC-MS.

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The authors are with Center of Excellence for Innovation in Chemistry and Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand (e-mail: Panawansut@gmail.com, phumon@gmail.com, sugunya.m@cmu.ac.th).

II. Materials and Methods

A. Plant Material

Thai black rice cv. Riceberry was collected from the experimental field in Kasetsart University, Kamphaengsaen Campus, Nakorn Pathom province in the central Thailand.

B. Extraction and Fractionation

Bran of Riceberry (1.00 kg) was extracted with 2.5 L of hexane, 2.5 L of dichrolomethane, and 2.5 L of methanol, respectively. Then, each extract was evaporated to dryness yielding hexane extract (CH, 141.19 g), dichloromethane extract (CDCM, 44.21 g) and methanol extracts (CM, 58.66 g). Part of the methanol extract (30.00 g) was fractionated with siliga gel and eluted by a series of solvents with increasing polarity; hexane, hexane:ethyl acetate, ethyl acetate:methanol and methanol yielding 10 fractions which were in agreement of those on TLC patterns; CM1 (6.01 g), CM2 (1.18 g), CM3 (6.49 g), CM4 (1.42 g), CM5 (0.58 g), CM6 (0.73 g), CM7 (0.36 g), CM8 (6.35 g), CM9 (3.63 g) and CM10 (2.55 g).

C. 1,1-Diphenyl-2-Picrylhydrazyl Free Radical Scavenging Assay

An aliquot 3 mL of the DPPH radical solution (0.3 mM, in methanol) was added to 1 mg/mL of the extract. The mixture was shaken and left to stand for 30 min at room temperature in the dark. The absorbance was measured at 517 nm. The radical scavenging effect was calculated by the following equation: scavenging activity (%) = 100 × [(Abs of control – Abs of Sample)/ Abs of control].The experiment was performed in triplicate.

D. Determination of Total Phenolic Contents

Total phenolics in the extracts were investigated using Folin-Ciocalteu's method. The reaction mixture was prepared by mixing 0.2 ml of methanolic solution of extract, 1 ml of Folin-Ciocalteu's reagent, 1 ml 7% $NaHCO_3$ and 5 ml of distilled water. The samples were stand for 1 hour at room temperature. The absorbance was determined at 765 nm. The content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GAE/ml of extract).

E. GC-MS Analysis of Chemical Components

The chemical compositions of crude extracts and all

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fractions were analyzed by gas chromatograph (GC-Agilent 6890) equipped with HP5-MS (30 m × 0.25 mm × 0.25 µm film thinkness) interfaced to a mass selective detector (HP 5973). The oven temperature was started from 60 ℃ to 280 ℃ at a rate 3 ℃/min and held for 16.67 min. Injector temperature was 250 ℃. A split ratio of injection was 10:1. Helium was used as a carrier gas with the flow of 1 ml/min. The mass spectrometer was operated in electron impact (EI) mode with an electron energy at 70 eV. All chemical compounds were identified by matching their mass spectra with reference spectra in the W8N08 and Wiley7n mass spectral libraries (Agilent Technologies, U.S.A.) and compared their retention indices with the published Kovάts retention indices, relative to C8–C22 *n*-alkanes.

F. Statistical Data Analysis

Significant differences were employed at for $P < 0.05$. All analyzes were conducted using variance (ANOVA) test of CropStat version 7.2.3 (CropStat, IRRI).

III. Result and Discussion

The Riceberry bran was extracted using solvents (hexane, dichloromethane and methanol) yielding three crude extracts; crude hexane as viscous yellow oil (14.11%), crude dichloromethane as viscous dark green oil (4.42%) and crude methanol as viscous dark purple oil (5.87%). These extract were tested for radical scavenging and total phenolic contents and were then subjected to separation by GC–MS in order to identify the chemical constituents. GC-MS chromatogram of fraction CM6 is shown in Fig. 1.

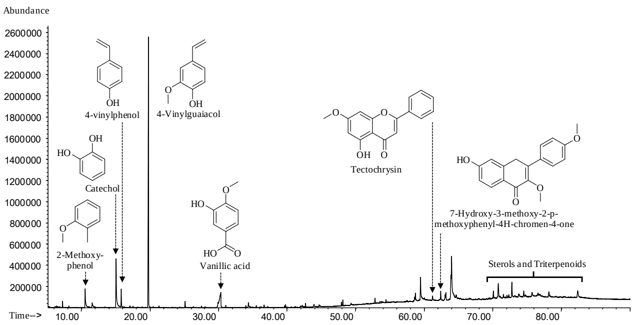

Fig. 1. GC-MS chromatogram of the Fraction CM6.

The percentage of radical scavenger (DPPH assay) and total phenolic contents of the extracts were presented in Table I. Among all the extracts, the crude methanol extract (% radical scavenger = 94.83 %) exhibited the highest scavenging ability for DPPH radicals, the crude dichloromethane extract performed rather moderate activity (49.61%), but the crude hexane did not have an antioxidant activity.

In Folin-Ciocalteu's assay, the standard curve equation for total phenolic content (mg GAE/mL) was $y = 0.0051x - 0.004$ ($R^2 = 0.9993$). Among all, the methanol extract contained the highest amounts of total phenolic (9.87 mg GAE/ml of extract) follow by the dichloromethane extract (3.52 mg GAE/ml of extract). Whereas, crude hexane extract did not show total phenolic content.

TABLE I: Percentage of Radical Scavenging and Total Phenolic Contents of the Extracts

Extract	% Radical Scavenger	Total Phenolic Content (mg/ml)
Crude Hexane	0.00 ± 0.00^{c}	0.72 ± 0.41^{c}
Crude Dichloromethane	49.61 ± 0.47^{b}	3.52 ± 0.00^{b}
Crude Methanol	94.83 ± 0.08^{a}	9.87 ± 0.93^{a}

Because the crude methanol extract showed the highest value both of radical scavenging assay and total phenolic content, the extract was considered to be further isolate by column chromatography into 10 fractions with a percentage yield of 20.03, 3.93, 21.63, 4.73, 1.93, 2.43, 1.20, 21.17, 12.10 and 8.49 (w/w), respectively. These fractions also were analyzed by GC–MS in order to identify the chemical constituents. Their relative area percentages and their retention indices (RI) are summarized in Table II. Overall, 38 constituents were identified among three crude extracts. However, after the fractionation process, the identified constituents were raised up to 79 compounds. These compounds could be categorized as 3 aliphatic hydrocarbons, 5 aliphatic aldehydes, an aliphatic alcohols, 2 cyclic ketones, 6 aromatics, 8 heterocyclics, 9 simple phenolics, 29 fatty acids and fatty acid esters, 2 diterpenes, 4 flavonoids, 6 sterols, 1 triterpene and 3 cycloartane triterpenoids. The fractionation by column chromatography could enhance the ability to identify chemical components in such complex rice bran extracts.

TABLE II: Structural Assignment and Relative Peak Area Percent of the Chemical Components in the Extracts of Riceberry Bran (Part I)

Structural Assignment[a]	RI	Relative Abundance (%)					
		CH	CDCM	CM	CM1	CM2	CM3
Aliphatic Hydrocarbons							
1-Decene[1,2]	1100					0.06	0.01
Neophytadiene[1,2]	1841						
3,7,11,15-Tetramethyl-2-hexadecene[1,2]	1848						
Aliphatic Aldehydes							
Heptenal[1,2,3]	965	0.20					0.07
(E)-2-Decenal[1,2]	1269	0.39					0.11
(E,Z)-2,4-Decadienal[1,2]	1301						0.05
(E,E)-2,4-Decadienal[1,2]	1323						0.06
(Z)-9-Octadecenal[1]							
Aliphatic Alcohols							
3,7,11,15-Tetramethyl-2-hexadecen-1-ol[1,2]	1883						
Cyclic Ketones							
1,2-Cyclopentanedione[1,2]	934			1.53			
4,7,7-Trimethylbicyclo[3,3,0]octan-2-one[1,2]	1125						

Aromatics							
1-Ethyl-3-methyl-benzene[1,2]	964						
1,2,4-Trimethyl-benzene[1,2]	972						
1-Ethyl-4-methyl- benzene[1,2]	992						
1,2,3-Trimethyl-benzene[1,2]	1006						
Benzene acitic acid[1,2]	1258						
1,4-Dihydrophenanthrene[1,2]	1572						
Heteroacyclics							
2,2-Dimethyl-1,3-dioxolane-4-methanol[1,2]	947						
2H-Pyran-2,6(3H)-dione [1,2]	1005			0.12			
Thymine[1,2]	1086						
Methyl nicotinate[1,2]	1146						
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one[1,2]	1149			0.26			
5-Hydroxymethyl-2-furancarboxaldehyde[1,2]	1237			1.05			
Niacin[1,2]	1242						
4-(1-Pyrrolyl)butanoic acid[1,2]	1374						
Simple Phenolics							
2-Methoxy-phenol (Guaiacol) [1,2]	1100						
1,2-Benzenediol (Catechol) [1,2]	1212			0.68			
4-Vinylphenol[1,2]	1229						
2-Methoxy-vinylphenol (4-vinylguaiacol) [1,2]	1322		0.29	0.46			
2,6-Dimethoxy-phenol (Syringol) [1,2]	1358						
4-Hydroxy-benzeneethanol (Tyrosol) [1,2]	1431						
Methyl 4-hydroxy-3-methoxybenzoate (Methyl vanillate) [1,2]	1522			0.51			0.03
4-Hydroxy-3-methoxybenzoic acid(Vanillic acid) [1,2]	1571			0.69			
Methyl 3,5-Dihydroxybenzoate[1,2]	1688			0.34			
Fatty Acids and Fatty acid Esters							
Octanoic acid[1,2]	1184						0.05
Dodecyl acrylate [1,2]	1697						
2-(Dodecyloxy)-ethanol[1,2]	1719					0.10	
Methyl myristate[1,2]	1729		0.03	0.27		0.27	0.03
Myristic acid[1,2]	1768	0.18	0.26	0.17			0.01
Methyl 9-methyltetradecanoate[1,2]	1828					0.03	
(Z)-Methyl 9-hexadecenoate[1,2]	1905					0.13	
Methyl palmitate[1,2]	1929		1.49	7.35		13.58	1.84
Palmitic Acid[1,2,3]	1974	12.95	13.6	9.72		1.83	9.31
Ethyl palmitate[1,2]	1995		0.03			0.14	
Methyl 8,11-octadecadienoate[1,2]	2094		3.12	8.66		17.48	2.47
Methyl oleate[1,2]	2101		4.12	10.65		27.59	3.45
Methyl stearate[1,2]	2127			0.07		1.93	
Linoleic acid[1,2]	2131	76.91	62.3	18.83			
Oleic acid[1,2,3]	2156			14.55		9.93	64.66
Ethyl linoleate[1,2]	2160					0.42	
Ethyl Oleate[1,2]	2167					0.78	
Stearic acid[1,2]	2172			0.70			
Methyl 11-eicosenoate[1,2]	2294			0.13		0.49	
Methyl arachidate[1]				0.04		0.80	0.09
Arachidic acid[1]				0.16			
Butyl 9,12-octadecadienoate [1]							
2-Hydroxy-1-(hydroxypropyl) ethyl hexadecanoate[1]				0.90			
Methyl behenate[1]				0.90		0.25	
3-Hydroxypropyl oleate[1]							
β-Monolinolein[1]				0.91			
3-Hydroxypropyl oleate[1]				1.98			
Methyl tetracosanoate[1]				0.14			
Methyl hexacosanoate[1]						0.11	0.03
Diterpenes							
Phytol[1]	2111						
2-Methyl hydrogen 8,13-epoxy-3-oxo-1,2-secolabdane-1,2-dioat[1]					17.51	1.80	0.22
Flavonoids							
Tectochrysin[1]							0.14
7-Hydroxy-3-methoxy-2-p-methoxyphenyl-4H-chromen-4-one[1]							0.14
Apigenin[1]							
2-(3-Hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4 -one[1]							0.12
Sterols							
Stigmasta-3,5,22-trien[1]					3.26	0.34	0.03
Ergosta-4,6,22-trien-3β-ol[1]			0.4	0.4		0.27	
Stigmastan-3,5-diene[1]					12.43	1.46	0.17

Campestrol[1]	0.73	0.58	0.38	3.10		0.12
Stigmasterol[1,3]	0.50	0.56	0.58			0.11
β-Sitosterol[1,3]	1.58	1.61	1.24	2.49		0.22
Triterpene						
Squalene[1]		0.29	0.23	23.50		
Cycloartane Triterpenoids						
Cycloartenol[1]						0.17
24-Methylenecycloartan-3-one[1]		1.37			1.64	0.35
24-Methylene-cycloartanol[1]	0.64		0.64	7.02	0.29	1.26

TABLE II: STRUCTURAL ASSIGNMENT AND RELATIVE PEAK AREA PERCENT OF THE CHEMICAL COMPONENTS IN THE EXTRACTS OF RICEBERRY BRAN (PART II)

Structural Assignment[a]	**Relative Abundance (%)**						
	CM4	**CM5**	**CM6**	**CM7**	**CM8**	**CM9**	**CM10**
Aliphatic Hydrocarbons							
1-Decene[1,2]	0.52	0.69			0.50		
Neophytadiene[1,2]	0.80						
3,7,11,15-Tetramethyl-2-hexadecene[1,2]	0.17						
Aliphatic Aldehydes							
Heptenal[1,2,3]	0.71		0.09				
(E)-2-Decenal[1,2]	0.66						
(E,Z)-2,4-Decadienal[1,2]	0.35						
(E,E)-2,4-Decadienal[1,2]	0.46						
(Z)-9-Octadecenal[1]	2.45						
Aliphatic Alcohols							
3,7,11,15-Tetramethyl-2-hexadecen-1-ol[1,2]	0.17						
Cyclic Ketones							
1,2-Cyclopentanedione[1,2]				4.22	3.27	0.58	10.06
4,7,7-Trimethylbicyclo[3,3,0]octan-2-one[1,2]			0.90				
Aromatics							
1-Ethyl-3-methyl-benzene[1,2]			0.19				
1,2,4-Trimethyl-benzene[1,2]			0.10	0.14			
1-Ethyl-4-methyl- benzene[1,2]			0.06				
1,2,3-Trimethyl-benzene[1,2]				1.49			
Benzene acitic acid[1,2]			0.90				
1,4-Dihydrophenanthrene[1,2]						0.65	
Heteroacyclics							
2,2-Dimethyl-1,3-dioxolane-4-methanol[1,2]				1.41			
2H-Pyran-2,6(3H)-dione [1,2]							1.58
Thymine[1,2]						2.44	0.52
Methyl nicotinate[1,2]						0.35	1.17
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one[1,2]							0.34
5-Hydroxymethyl-2-furancarboxaldehyde[1,2]						0.34	
Niacin[1,2]						0.60	0.34
4-(1-Pyrrolyl)butanoic acid[1,2]							0.53
Simple Phenolics							
2-Methoxy-phenol (Guaiacol) [1,2]			3.37			0.68	0.67
1,2-Benzenediol (Catechol) [1,2]			8.71	11.62	1.77	1.88	0.95
4-Vinylphenol[1,2]			1.96	0.51			
2-Methoxy-vinylphenol (4-vinylguaiacol) [1,2]			35.00	10.44	1.30	0.72	0.57
2,6-Dimethoxy-phenol (Syringol) [1,2]				0.52			
4-Hydroxy-benzeneethanol (Tyrosol) [1,2]			0.09				
Methyl 4-hydroxy-3-methoxybenzoate (Methyl vanillate) [1,2]	0.31			0.22	0.40	0.35	0.50
4-Hydroxy-3-methoxybenzoic acid(Vanillic acid) [1,2]			0.99	2.52	1.93	0.85	0.39
Methyl 3,5-Dihydroxybenzoate[1,2]			0.73				
Fatty Acids and Fatty acid Esters							
Octanoic acid[1,2]	0.23						
Dodecyl acrylate [1,2]	2.18						
2-(Dodecyloxy)-ethanol[1,2]		1.00			0.88		
Methyl myristate[1,2]							
Myristic acid[1,2]							
Methyl 9-methyltetradecanoate[1,2]							
(Z)-Methyl 9-hexadecenoate[1,2]							
Methyl palmitate[1,2]	2.26	1.02	0.42		0.14		
Palmitic Acid[1,2,3]		0.68	0.40	1.23	8.29		6.44
Ethyl palmitate[1,2]							
Methyl 8,11-octadecadienoate[1,2]	4.44	1.32	0.42			0.35	12.84
Methyl oleate[1,2]	4.51	1.87					
Methyl stearate[1,2]	0.31						

Linoleic acid[1,2]					2.21	2.00	
Oleic acid[1,2,3]					16.36	1.16	
Ethyl linoleate[1,2]							
Ethyl Oleate[1,2]							
Stearic acid[1,2]							
Methyl 11-eicosenoate[1,2]							
Methyl arachidate[1]							
Arachidic acid[1]							
Butyl 9,12-octadecadienoate [1]	1.02						
2-Hydroxy-1-(hydroxypropyl) ethyl hexadecanoate[1]			3.78			1.07	
Methyl behenate[1]							
3-Hydroxypropyl oleate[1]					3.94	4.52	
β-Monolinolein[1]							
3-Hydroxypropyl oleate[1]							
Methyl tetracosanoate[1]							
Methyl hexacosanoate[1]							
Diterpenes							
Phytol[1]	0.26						
2-Methyl hydrogen 8,13-epoxy-3-oxo-1,2-secolabdane-1,2-dioat[1]	4.38			11.35	7.31	5.02	4.09
Flavonoids							
Tectochrysin[1]			0.60	1.33			
7-Hydroxy-3-methoxy-2-p-methoxyphenyl-4H-chromen-4-one[1]			1.00	2.14			
Apigenin[1]						5.97	
2-(3-Hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one[1]				4.02			
Sterols							
Stigmasta-3,5,22-trien[1]	0.90	3.37	0.54	2.08	0.73	1.20	
Ergosta-4,6,22-trien-3β-ol[1]							
Stigmastan-3,5-diene[1]	3.42	13.63	2.04		4.84	3.63	3.62
Campestrol[1]	10.85	2.13	1.13	2.00	1.65	1.18	0.87
Stigmasterol[1,3]	10.27	1.20					
β-Sitosterol[1,3]	21.25	7.15	0.95	1.97	1.25	0.77	1.18
Triterpene							
Squalene[1]							
Cycloartane Triterpenoids							
Cycloartenol[1]							
24-Methylenecycloartan-3-one[1]							
24-Methylene-cycloartanol[1]	8.77	4.79	1.47	3.91	2.60	2.67	1.58

[a] Identification: 1, mass spectrum (tentative); 2, retention indices (RI); and 3, standard compound.

The major component in all of the three crude extracts was linoleic acid (76.91%, 62.3% and 18.83%) and the major compounds of the methanol fractionates were squalene (23.50%), methyl oleate (27.59%), oleic acid (64.66%), *β*-sitosterol (21.25%), stigmastan-3,5-diene(13.63%), 4-vinylguaiacol (35.00%), catechol (11.62%), oleic acid (16.36%), apigenin (5.97%) and methyl 8,11-octadecadienoate (12.84%), respectively.

Among these identified compounds, simple phenolics and flavonoids are responsible for antioxidantion, anti-inflammation and anticancer, for instance, 4-vinylguaiacol is a flavoring substance with an antioxidant property [8], apigenin has antioxidant, anti-inflammatory and anticancer activities [9].

Simple phenolics were reported to possess important biological activities and to be especially involved in the reduction of oxidative stress as a major cause of age-related diseases and cancers [10].The identified simple phenolics in the rice bran extracts were guaiacol, catechol, 4-vinylphenol, 4-vinylguaiacol, syringol, tyrosol, methyl vanillate, vanillic acid and methyl 3,5-dihydroxybenzoate.

Flavonoids are typical phenolic compounds and powerful chain-breaking antioxidants. The identified flavonoids in the extracts were tectochrysin, 7-hydroxy-3-methoxy-2-p-methoxyphenyl-4H-chromen-4-one, apigenin and 2-(3-hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one.

It is implied that simple phenolics and flavonoids present in the methanol extract of Riceberry bran play an important role in antioxidant activity.

IV. Conclusion

Identification of some antioxidants in bran of the Thai black rice cultivar Riceberry was accomplished using solvent extraction, fractionation by column chromatography, and then analysis by GC-MS. All crude extracts were also investigated for antioxidant capacity and total phenolic content. The study revealed that the crude methanolic extract of Riceberry bran had the strongest antioxidant capacity. Among overall identified compounds, simple phenolic compounds and flavonoids were antioxidant compounds. The major phenolic compounds in this rice bran was 4-vinylguaiacol and the major flavonoids in which was apigenin. The result showed that Riceberry rice had great potential for use as a functional food.

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Panawan Suttiarporn is a Ph.D. student in organic chemistry, the Department of Chemistry, Faculty of Science, Chiang Mai University, Thailand. She got her B.Sc. degree in chemistry in 2006 from Chiang Mai University, Thailand. Her current research interests include chromatographic-mass spectrometric technique for analysis of phytochemicals in rice and natural products.

Phumon Sookwong received his doctoral degree in agricultural science from Tohoku University, Japan, in 2009. He is specialized in researching the analysis and development of food and nutraceuticals.

Sugunya Mahatheeranont received her doctoral degree from University of Wales Cardiff, in United Kingdom. She is specialized in researching the rice phytochemicals by combined chromatographic-mass spectrometric, and hyphenated techniquese; GC-MS, GCxGC-MS, HPLC-MS/MS.