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LC-ESI-QTOF-MS/MS Characterization and Estimation of the Antioxidant Potential of Phenolic Compounds from Different Parts of the Lotus (*Nelumbo nucifera*) Seed and Rhizome

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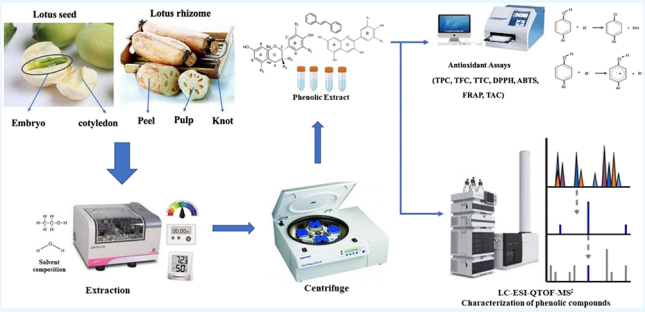
ABSTRACT: Edible lotus (*Nelumbo nucifera* G.) is widely consumed in Asian countries and treated as a functional food and traditional medicinal herb due to its abundant bioactive compounds. Lotus rhizome peels, rhizome knots, and seed embryos are important byproducts and processing waste of edible lotus (*Nelumbo nucifera* G.) with commercial significance. Nevertheless, the comprehensive phenolic profiling of different parts of lotus is still scarce. Thus, this study aimed to review the phenolic contents and antioxidant potential in lotus seeds (embryo and cotyledon) and rhizomes (peel, knot, and pulp) grown in Australia. In the phenolic content and antioxidant potential estimation assays by comparing to the corresponding reference standards, the lotus seed embryo exhibited the highest total phenolic content (10.77 ± 0.66 mg GAE/$g_{f.w.}$), total flavonoid content (1.61 ± 0.03 mg QE/$g_{f.w.}$), 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (9.66 ± 0.10 mg AAE/$g_{f.w.}$), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) scavenging activity (14.35 ± 0.20 mg AAE/$g_{f.w.}$), and total antioxidant capacity (6.46 ± 0.30 mg AAE/g), while the highest value of ferric ion reducing antioxidant power (FRAP) activity and total tannin content was present in the lotus rhizome knot (2.30 ± 0.13 mg AAE/$g_{f.w.}$). A total of 86 phenolic compounds were identified in five parts of lotus by liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS/MS), including phenolic acids (20), flavonoids (51), lignans (3), stilbenes (2), and other polyphenols (10). The most phenolic compounds, reaching up to 68%, were present in the lotus seed embryo (59). Furthermore, the lotus rhizome peel and lotus seed embryo exhibit significantly higher contents of selected polyphenols than other lotus parts according to high-performance liquid chromatography (HPLC) quantification analysis. The results highlighted that byproducts and processing waste of edible lotus are rich sources of phenolic compounds, which may be good candidates for further exploitation and utilization in food, animal feeding, and pharmaceutical industries.



1. INTRODUCTION

Lotus (*Nelumbo nucifera* G.) is an aquatic plant widely cultivated in China, Japan, India, Thailand, eastern Australia, and western Europe for more than 5000 years.[1−3] At present, the output of lotus-related traditional medicine in China has exceeded 80,000 tons/year.[4] Apart from its high ornamental value, almost all parts of lotus, including leaves, seeds, and rhizomes, have been used as functional foods and traditional medicine herbs due to their abundant bioactive compounds, including flavonols, procyanidins, alkaloids, and especially polyphenols.[4,5] Lotus seeds consist of the seed epicarp and seed kernel (white cotyledon), in between which lies a nonedible green embryo.[6] The knot and peel are nonedible parts of the lotus rhizome and are removed before consumption. Recently, many therapeutic effects of the lotus, including antiobesity, anticancer, anti-inflammatory, antioxidant, and antiaging, have been of great interest.[4,7]

Phenolic compounds are a group of compounds with polyhydroxy groups on the aromatic ring, which exhibit strong antioxidant properties via different mechanisms, including reactive oxygen species scavenger by donating electrons or transferring hydrogen atoms, metal chelators, oxidase inhibitors, and antioxidant enzyme cofactors.[8,9] According to the number of phenol units within the molecular structure, substituent groups, and the linkage type between phenol units, phenolic compounds can be classified into monomeric

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Table 1. Polyphenol Estimation and Antioxidant Activities of Lotus Samples

antioxidant assays	LR	LRP	LRK	LSE	LSC
TPC (mg GAE/$g_{f.w.}$)	0.34 ± 0.01^{c}	3.44 ± 0.07^{b}	3.49 ± 0.12^{b}	10.77 ± 0.66^{a}	1.11 ± 0.08^{c}
TFC (mg QE/$g_{f.w.}$)	0.01 ± 0.01^{e}	0.31 ± 0.02^{c}	0.24 ± 0.02^{d}	1.61 ± 0.03^{a}	0.61 ± 0.03^{b}
TTC (mg CE/$g_{f.w.}$)	0.02 ± 0.01^{d}	0.83 ± 0.01^{b}	1.77 ± 0.04^{a}	0.27 ± 0.02^{c}	0.32 ± 0.01^{c}
DPPH (mg AAE/$g_{f.w.}$)	0.60 ± 0.04^{e}	3.70 ± 0.17 ^{c}	4.36 ± 0.36^{b}	9.66 ± 0.10^{a}	1.82 ± 0.10^{d}
ABTS (mg AAE/$g_{f.w.}$)	0.58 ± 0.04^{e}	7.81 ± 0.15^{c}	8.85 ± 0.68^{b}	14.35 ± 0.20^{a}	2.09 ± 0.15^{d}
FRAP (mg AAE/$g_{f.w.}$)	0.11 ± 0.01^{c}	2.24 ± 0.13^{a}	2.30 ± 0.13^{a}	1.72 ± 0.02^{b}	0.22 ± 0.01^{c}
TAC (mg AAE/$g_{f.w.}$)	0.34 ± 0.01^{e}	1.83 ± 0.07^{c}	2.41 ± 0.09^{b}	6.46 ± 0.30^{a}	1.05 ± 0.04^{d}

^{a}The data are shown as mean ± standard deviation (n = 3); $^{a, b}$ indicate the means in a row with significant difference ($p < 0.05$) using a one-way analysis of variance (ANOVA) and Tukey's test. LR, lotus rhizome pulp; LRP, lotus rhizome peel; LRK, lotus rhizome knot; LSC, lotus seed cotyledon; LSE, lotus seed embryo; TPC, total phenolic content; TFC, total flavonoid content; TTC, total tannin content; DPPH, 2,2-diphenyl-1-picrylhydrazyl assay; FRAP, ferric reducing antioxidant power assay; ABTS, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid assay; TAC, total antioxidant capacity; GAE, gallic acid equivalents; QE, quercetin equivalents; CE, catechin equivalents; AAE, ascorbic acid equivalents.

polyphenols, including phenolic acids, flavonoids, stilbenes, and lignans, or polymeric polyphenols, such as tannins.[10,11] Modern research has demonstrated a significant positive correlation between phenolic compounds and antioxidant capacity, suggesting that the main contributor to antioxidant capacity might be the phenolic compounds in lotus.[12] The phenolic content can be estimated by various spectrometric assays, including the total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC), while various spectrophotometric-based *in vitro* antioxidant methods are used to estimate the overall antioxidant potential of plant materials, including the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay, ferric reducing antioxidant power (FRAP) assay, and total antioxidant capacity (TAC) assay.[13−18] However, TPC and other colorimetric methods neither separate nor quantify individual phenolic compounds. High-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS/MS) is an effective analytical tool for the characterization and quantification of individual phenolic compounds due to the high sensitivity and accuracy.[19−24] Previously, lotus seeds and rhizomes were reported to be rich in flavonoids, including flavonoid *C*-glycosides like schaftoside, quercetin derivatives, and catechin derivatives, by LC−MS and high-performance liquid chromatography equipped with a photodiode array (HPLC-PDA).[4,5]

The application of the lotus and its polyphenols in the food industry has gained much attention in recent years. Although previous studies have confirmed the antioxidant activity of lotus extracts effectively against lipid oxidation of the processed meat,[25,26] the comprehensive phenolic profiling of different parts of the lotus is still scarce. In this study, phenolic compounds in five parts of Australia-grown lotus including the lotus seed embryo (LSE), lotus seed cotyledon (LSC), lotus rhizome knot (LRK), lotus rhizome peel (LRP), and lotus rhizome pulp (LR) were extracted and subjected to various phenolic estimation methods (TPC, TFC, and TTC) as well as antioxidant assays (DPPH, ABTS, FRAP, and TAC). The further characterization and quantification of individual phenolic compounds in five parts of lotus were conducted by LC-ESI-QTOF-MS and HPLC-PDA, respectively. This study aimed to evaluate the antioxidant potential and provide a comprehensive phenolic profile of lotus to further explore and utilize the phenolic compounds in byproducts of lotus in the food, cosmetic, and pharmaceutical industries.

2. RESULTS AND DISCUSSION

2.1. Polyphenol Estimation (TPC, TFC, and TTC).

Previously, lotus seeds and rhizomes were reported to contain large amounts of phenolic compounds with strong antioxidant capacity, including flavonoids and phenolic acids. Thus, TPC, TFC, and TTC were conducted to estimate the phenolic content in ethanolic extracts of lotus (Table 1). The lotus seed embryo presented a significantly higher total phenolic content (10.77 ± 0.66 mg GAE/$g_{f.w.}$) at $p < 0.05$ than other tissues followed by the knot and peel of the lotus rhizome (3.49 ± 0.12 and 3.44 ± 0.07 mg GAE/$g_{f.w.}$, respectively). Our study is consistent with previous findings of Limwachiranon et al.[27] and Hu and Skibsted[12] that indicated that the phenolic contents of lotus knots are distinctive. The total phenolic content of our lotus rhizome peel is also comparable to that of 80% ethanolic extracts of lotus from Zhejiang, China (4.30 mg GAE/$g_{f.w.}$).[28] In the seeds of lotus, the pattern of the TPC results of Limwachiranon et al.[27] was contradictory to our research, as they found that the cotyledon had higher phenolic contents than embryos. This variation of TPC might be explained by several factors, including different growing regions, ripening stages, and drying processes and the choice of extraction reagent.[4,29−31] Yen et al.[31] compared the total phenolic contents in water extracts, acetone extracts, and ethyl acetate extracts of lotus seeds and found that water extracts of lotus seeds presented the highest TPC.

Flavonoids are the predominant class of phenolic compounds, which account for over 60% of the dietary phenols and exhibit health-promoting properties.[32−36] The highest flavonoid content was found in the lotus seed embryo (1.61 ± 0.03 mg QE/$g_{f.w.}$) followed by the lotus seed cotyledon (0.61 ± 0.03 mg QE/$g_{f.w.}$). Previously, Li et al.[33] found that the TFC value in the embryo of lotus seeds was higher than that in the cotyledon, which is in agreement with our study. The high content of flavonoid *C*-glycosides was reported in the embryo of lotus seeds, which consists of more than 70% of the total flavonoid content.[5,37]

Regarding the TTC, the content of tannins varied significantly among five parts of lotus in this study. The lotus rhizome knot presented the highest tannin content (1.77 ± 0.04 mg CE/$g_{f.w.}$) followed by the peel (0.83 ± 0.01 mg CE/$g_{f.w.}$), while the other parts of the lotus presented a relatively low tannin content. Only limited studies have been reported on the TTC of edible lotus. Huang et al.[26] found that the total tannin contents in water extracted lotus rhizome knot and lotus leaf were 13.0 ± 0.3 and 6.02 ± 0.2 (gallic acid equivalents g/100 g), respectively. Chen et al.[38] indicated that

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the content of tannins in lotus varied with different solvent extractions with the highest tannin content in 80% methanol extraction.

2.2. Antioxidant Activities (DPPH, FRAP, ABTS, and TAC). The phenolic contents are highly associated with their antioxidant properties.[39−41] Thus, several antioxidant assays were conducted to analyze the antioxidant capacity of the lotus samples. In our study, DPPH, ABTS, FRAP, and TAC were applied to estimate the antioxidant potential of different parts of the lotus.

DPPH is the most commonly used method to characterize the free radical scavenging capabilities of food extracts based on their hydrogen donating ability. The lotus seed embryo exhibited the highest DPPH free radical scavenging activity among five parts of lotus (9.66 ± 0.10 mg AAE/$g_{f.w.}$) followed by the knot and peel of the lotus rhizome (4.36 ± 0.36 and 3.70 ± 0.17 mg AAE/$g_{f.w.}$, respectively). In our study, the lotus rhizome knot showed a better scavenging capacity of DPPH• compared with the pulp, which agreed with the previous study conducted by Hu and Skibsted.[12]

The principle of the ABTS assay is similar to the DPPH method, which is based on the fact that the antioxidants in extracts reduce the preformed ABTS•$^+$ and form stable free radicals, resulting in decolorization.[42] As shown in Table 1, the scavenging activity of ABTS radicals ranged from 14.35 ± 0.20 to 0.58 ± 0.04 mg AAE/$g_{f.w.}$. The lotus seed embryo exhibited the highest scavenging activity (14.35 ± 0.20 mg AAE/$g_{f.w.}$) followed by the knot and peel of the lotus rhizome (8.85 ± 0.68 and 7.81 ± 0.15 AAE/$g_{f.w.}$, respectively). The knot exhibited a better scavenging capacity of ABTS radicals than the lotus rhizome pulp, which is in agreement with the study conducted by Yang et al.[28]

The FRAP assay measures the capacity of antioxidants to reduce the ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) to the ferrous complex (Fe^{2+}-TPTZ) at low pH. The reducing power of FRAP varied in five parts of lotus. The knot and peel of the lotus rhizome exhibited a significantly higher reducing power (2.30 ± 0.13 and 2.24 ± 0.13 mg AAE/$g_{f.w.}$, respectively) followed by the lotus seed embryo that presented 1.72 ± 0.02 mg AAE/$g_{f.w.}$ reducing power of FRAP. The FRAP value of the lotus rhizome peel was higher than that of the pulp, which agreed with the study of Yang et al.[28] Based on the previous study, the reducing power values vary with different maturity in both the seed and rhizome.[4,28]

In the TAC assay, which was based on the capacity of reducing phosphomolybdate ions, the lotus seed embryo exhibited a significantly higher total antioxidant capacity (TAC) among five parts of lotus (6.46 ± 0.30 mg AAE/$g_{f.w.}$) at $p < 0.05$ followed by the lotus rhizome knot obtaining a relatively high TAC value (2.41 ± 0.09 mg AAE/$g_{f.w.}$).

2.3. Correlation between Phenolic Compounds and Antioxidant Potential. Pearson's correlation between phenolic contents (TPC, TFC, and TTC) and four antioxidant assays (DPPH, ABTS, FRAP, and TAC) was performed to investigate the relationship between the phenolic contents and antioxidant capacities of lotus extracts. The correlation coefficients are summarized in Table 2.

A significantly positive correlation between the content of total phenolic compounds (TPC) and all antioxidant assays except FRAP (DPPH, $r = 0.993$, $p < 0.01$; ABTS, $r = 0.938$, $p < 0.01$; TAC; $r = 0.995$, $p < 0.01$) was observed. The positive correlation between TPC and antioxidant assays (DPPH, ABTS, and TAC) was also reported by previous studies,[12,28]

Table 2. Pearson's Correlation Coefficients for TPC, TFC, TTC, DPPH, FRAP, ABTS, and TAC

variables	TPC	TFC	TTC	DPPH	FRAP	ABTS
TFC	0.879[a]					
TTC	0.001	−0.271				
DPPH	0.993[b]	0.877[a]	0.109			
FRAP	0.523	0.180	0.746	0.590		
ABTS	0.938[a]	0.733	0.329	0.965[b]	0.781	
TAC	0.995[b]	0.911[a]	0.017	0.993[b]	0.494	0.929[a]

[a]The correlation between two assays is significant with $p < 0.05$. [b]Highly significant correlation with $p < 0.01$.

indicating that phenolic compounds were one of the contributors to the antioxidant activity of five lotus tissues. The low correlation between FRAP with other antioxidant activity measurements may be attributed to some slowly reacting polyphenolic compounds (quercetin, caffeic, ferulic, and tannic acids) having slower reactions, requiring a longer time until the complex reduction process was completed.[43]

The total flavonoid content was positively correlated with the TPC ($r = 0.879$, $p < 0.05$) as well as DPPH radical scavenging activity and total antioxidant capacitive ($r = 0.877$, $p < 0.05$ and $r = 0.911$, $p < 0.01$, respectively), suggesting that flavonoids are the predominant phenolic compounds in lotus, which significantly contributed to the antioxidant activities.

In general, the phenolic compounds are one of the contributors to the antioxidant activities of lotus seeds and rhizomes. Thus, screening of these phenolic compounds is essential. In this study, LC−MS/MS and HPLC-PDA were performed to further identify, characterize, and quantify phenolic compounds present in different lotus samples.

2.4. Characterization of Phenolic Compounds by LC-ESI-QTOF-MS/MS. Table 3 shows the phenolic compounds tentatively identified in five parts of lotus based on their m/z value and MS/MS spectral data using the Agilent LC-ESI-QTOF-MS/MS Mass Hunter workstation software (Qualitative Analysis, version B.03.01, Agilent) and Personal Compound Database and Library (PCDL) with an online database of Kansas State University, USA (Supporting Information, Figures S1 and S2). Compounds with PCDL scores higher than 80 and mess error <±5 ppm were further selected for m/z verification and MS/MS analysis.

Previously, more than 90 flavonoids and 12 phenolic acids have been reported in various parts of lotus, including leaves, seeds, rhizomes, and flowers.[27,38] In this study, a total of 86 phenolic compounds were characterized in lotus, including 20 phenolic acids (23%), 51 flavonoids (59%), 3 lignans (4%), 2 stilbenes (2%), and 10 other polyphenols (12%). Lignans, stilbene, other polyphenols, and some phenolic acids were first characterized in lotus.

2.4.1. Phenolic Acid. In our study, phenolic acids in five parts of lotus were tentatively characterized into four subclasses, including hydroxybenzoic acids, hydroxycinnamic acids, hydroxyphenylpentanoic acids, and hydroxyphenylacetic acids.

2.4.1.1. Hydroxybenzoic Acid Derivatives. Seventeen hydroxybenzoic acid derivatives were detected in five parts of lotus. Compound **1** with $[M - H]^-$ at m/z 331.0668 was tentatively identified as galloyl glucose in LSE. Upon fragmentation, it produced the product ions at m/z 169 and 125 due to the loss of the hexosyl moiety (162 Da) and a further loss of carbon dioxide (44 Da) from the precursor ion,

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Table 3. Characterization of Phenolic Compounds in Lotus by Using LC-ESI-QTOF-MS/MS[a]

no.	proposed compounds	molecular formula	RT (min)	ionization mode	molecular weight	theoretical (*m*/*z*)	observed (*m*/*z*)	mass error (ppm)	MS/MS product ions	samples
					Phenolic acid					
	hydroxybenzoic acids									
1	galloyl glucose	$C_{13}H_{16}O_{10}$	10.541	$[M-H]^-$	332.0743	331.0670	331.0668	−0.60	169, 125	LSE
2	2-hydroxybenzoic acid	$C_7H_6O_3$	11.038	[c]$[M-H]^-$	138.0317	137.0244	137.0248	2.92	93	LRP, LR, LSC,[b]LSE, LRK
3	4-hydroxybenzoic acid 4-*O*-glucoside	$C_{13}H_{16}O_8$	11.054	$[M-H]^-$	300.0845	299.0772	299.0770	−0.67	255, 137	LR,[b]LSE, LRK
4	gallic acid	$C_7H_6O_5$	12.893	$[M-H]^-$	170.0215	169.0142	169.0140	−1.18	125	LSC,[b]LSE
5	paeoniflorin	$C_{23}H_{28}O_{11}$	34.596	[c]$[M-H]^-$	480.1632	479.1559	479.1583	5.00	449, 357, 327	LSC,[b]LSE
	hydroxycinnamic acids									
6	cinnamic acid	$C_9H_8O_2$	12.479	[c]$[M-H]^-$	148.0524	147.0451	147.0453	1.36	103	LRP, LR, LSC,[b]LSE
7	3-*p*-coumaroylquinic acid	$C_{16}H_{18}O_8$	13.543	[c]$[M-H]^-$	338.1002	337.0929	337.0921	−2.37	265, 173, 162	[b]LRP, LR, LSE, LRK
8	*m*-coumaric acid	C9H8O3	39.486	[c]$[M-H]^-$	164.0473	163.0401	163.0405	3.03	119	LRP, LR,[b]LSC, LSE, LRK
9	caffeoyl glucose	$C_{15}H_{18}O_9$	19.603	$[M-H]^-$	342.0951	341.0878	341.0875	−0.88	179, 161	LSE
10	caffeic acid	$C_9H_8O_4$	19.619	$[M-H]^-$	180.0423	179.0350	179.0350	0.00	143, 133	LSE
11	3-feruloylquinic acid	$C_{17}H_{20}O_9$	20.847	[c]$[M-H]^-$	368.1110	367.1034	367.1025	−2.45	298, 288,192, 191	[b]LSE, LRK
12	ferulic acid 4-*O*-glucoside	$C_{16}H_{20}O_9$	23.330	$[M-H]^-$	356.1107	355.1034	355.1031	−0.84	193, 178, 149, 134	LR, LSC,[b]LSE
13	isoferulic acid	$C_{10}H_{10}O_4$	23.344	[c]$[M-H]^-$	194.0579	193.0506	193.0513	3.63	178, 149, 134	LRP, LR, LSC,[b]LSE, LRK
14	*p*-coumaric acid 4-*O*-glucoside	$C_{15}H_{18}O_8$	23.754	$[M-H]^-$	326.1002	325.0929	325.0940	3.38	163	[b]LR, LSE
15	sinapic acid	$C_{11}H_{12}O_5$	26.118	[c]$[M-H]^-$	224.0685	223.0612	223.0618	2.69	205, 163	LR,[b]LSC, LSE, LRK
16	verbascoside	$C_{29}H_{36}O_{15}$	31.531	$[M-H]^-$	624.2054	623.1981	623.1984	0.48	477, 461,315, 135	LSE
17	1-sinapoyl-2-feruloylgentiobiose	$C_{33}H_{40}O_{18}$	60.158	$[M-H]^-$	724.2215	723.2142	723.2122	−2.77	529, 499	LSE
	hydroxyphenylacetic acids									
18	3,4-dihydroxyphenylacetic acid	$C_8H_8O_4$	14.119	[c]$[M-H]^-$	168.0423	167.0350	167.0346	−2.39	149, 123	LRP, LR, LSC,[b]LSE, LRK
19	2-hydroxy-2-phenylacetic acid	$C_8H_8O_3$	14.616	[c]$[M-H]^-$	152.0473	151.0400	151.0394	−3.97	136, 92	LRP, LSC,[b]LSE, LRK
	hydroxyphenylpentanoic acids									
20	3-hydroxy-3-(3-hydroxyphenyl)propionic acid	$C_9H_{10}O_4$	18.327	$[M-H]^-$	182.0579	181.0506	181.0507	0.55	163, 135, 119	LSE
					Flavonoids					
	flavanols									
21	(+)-catechin 3-*O*-gallate	$C_{22}H_{18}O_{10}$	10.942	$[M-H]^-$	442.0900	441.0827	441.0842	3.40	289, 169, 125	LRK
22	(+)-gallocatechin 3-*O*-gallate	$C_{22}H_{18}O_{11}$	11.106	[c]$[M-H]^-$	458.0849	457.0776	457.0781	1.10	305, 169	LSE
23	(−)-epigallocatechin	$C_{15}H_{14}O_7$	21.832	[c]$[M-H]^-$	306.0740	305.0667	305.0675	2.62	261, 219	LRP, LSC,[b]LR
24	procyanidin trimer C1	$C_{45}H_{38}O_{18}$	22.246	[c]$[M-H]^-$	866.2058	865.1985	865.1989	0.46	739, 713, 695	LRP,[b] LR, LSC
25	cinnamtannin A2	$C_{60}H_{50}O_{24}$	24.081	[c]$[M-H]^-$	1154.2692	1153.2619	1153.2656	3.21	739	LR,[b]LSC
26	(−)-epicatechin	$C_{15}H_{14}O_6$	24.208	[c]$[M-H]^-$	290.0790	289.0717	289.0714	−1.04	245, 205, 179	LRP, LR, LRK, LSC,[b]LSE
27	4″-*O*-methylepigallocatechin 3-*O*-gallate	$C_{23}H_{20}O_{11}$	32.575	[c]$[M-H]^-$	472.1006	471.0933	471.0927	−1.27	169, 319	LRP, LSC,[b]LSE
28	procyanidin dimer B1	$C_{30}H_{26}O_{12}$	78.369	[c]$[M-H]^-$	578.1424	577.1351	577.1340	−1.90	451	[b]LRP, LR, LSC, LRK
	flavanones									
29	eriocitrin	$C_{27}H_{32}O_{15}$	34.931	[c]$[M-H]^-$	596.1741	595.1668	595.1650	−3.00	431, 287	[b]LSC, LSE
30	naringin	$C_{27}H_{32}O_{14}$	41.624	[c]$[M-H]^-$	580.1792	579.1719	579.1696	−4.00	271	[b]LRP, LSE

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Table 3. continued

no.	proposed compounds	molecular formula	RT (min)	ionization mode	molecular weight	theoretical (*m*/*z*)	observed (*m*/*z*)	mass error (ppm)	MS/MS product ions	samples
				Flavonoids						
31	8-prenylnaringenin	$C_{20}H_{20}O_5$	45.721	[M + H]+	340.1311	341.1384	341.1389	1.47	323, 137	[b]LRP, LR, LRK
32	hesperidin	$C_{28}H_{34}O_{15}$	52.573	[M + H]+	610.1898	611.1971	611.1962	−1.47	593, 465, 449, 303	LSE
33	hesperetin 3′-*O*-glucuronide	$C_{22}H_{22}O_{12}$	52.779	[c][M − H]$^-$	478.1111	477.1038	477.1048	2.10	301, 175, 113, 85	LRP,[b]LR, LSE, LRK
	flavones									
34	apigenin 7-*O*-apiosyl-glucoside	$C_{26}H_{28}O_{14}$	14.031	[c][M + H]+	564.1479	565.1552	565.1552	0.00	296	LR,[b]LSE
35	apigenin 7-*O*-glucuronide	$C_{21}H_{18}O_{11}$	22.201	[M + H]+	446.0849	447.0922	447.0933	2.46	271, 253	[b]LRP, LR
36	apigenin 6,8-di-C-glucoside	$C_{27}H_{30}O_{15}$	32.309	[c][M − H]$^-$	594.1585	593.1512	593.1532	3.37	503, 473	LSC,[b]LSE
37	chrysoeriol 7-*O*-glucoside	$C_{22}H_{22}O_{11}$	40.657	[c][M + H]+	462.1162	463.1235	463.1221	−3.00	445, 427, 409, 381	LSE
38	apigenin 6-C-glucoside	$C_{21}H_{20}O_{10}$	41.736	[c][M − H]$^-$	432.1056	431.0983	431.0984	0.23	413, 341, 311	LR, LRP, LSC,[b]LSE
39	neodiosmin	$C_{28}H_{32}O_{15}$	52.580	[c][M + H]+	608.1741	609.1814	609.1826	1.97	301, 286	[b]LSC, LSE
	flavonols									
40	patuletin 3-*O*-glucosyl-(1->6)-[apiosyl(1->2)]-glucoside	$C_{33}H_{40}O_{22}$	21.872	[M − H]$^-$	788.2011	787.1938	787.1907	−3.94	625, 463, 301, 271	LSE
41	quercetin 3-*O*-xylosyl-rutinoside	$C_{32}H_{38}O_{20}$	26.493	[M + H]+	742.1956	743.2029	743.2058	3.90	479, 317	LSC
42	myricetin 3-*O*-rutinoside	$C_{27}H_{30}O_{17}$	27.025	[c][M − H]$^-$	626.1483	625.1410	625.1393	−2.72	301	LSC,[b]LSE
43	quercetin 3-*O*-glucosyl-xyloside	$C_{26}H_{28}O_{16}$	27.754	[c][M − H]$^-$	596.1377	595.1304	595.1306	0.34	265, 138, 116	LSC,[b]LSE
44	kaempferol 3,7-*O*-diglucoside	$C_{27}H_{30}O_{16}$	28.897	[c][M − H]$^-$	610.1534	609.1461	609.1479	2.95	447, 285	LSC,[b]LSE
45	myricetin 3-*O*-glucoside	$C_{21}H_{20}O_{13}$	38.995	[c][M − H]$^-$	480.0904	479.0831	479.0834	0.63	317	LRP,[b]LR, LSC, LRK
46	kaempferol 3-*O*-glucosyl-rhamnosyl-galactoside	$C_{33}H_{40}O_{20}$	40.180	[c][M − H]$^-$	756.2113	755.2040	755.2025	−2.00	285	LSC
47	kaempferol 3-*O*-(2″-rhamnosyl-galactoside) 7-*O*-rhamnoside	$C_{33}H_{40}O_{19}$	41.143	[M − H]$^-$	740.2164	739.2091	739.2106	2.03	593, 447, 285	[b]LRP, LSE
48	quercetin 3′-*O*-glucuronide	$C_{21}H_{18}O_{13}$	45.016	[M − H]$^-$	478.0747	477.0674	477.0653	−4.40	301	LSC,[b]LSE
49	myricetin 3-*O*-rhamnoside	$C_{21}H_{20}O_{12}$	45.314	[c][M − H]$^-$	464.0955	463.0882	463.0871	−2.38	317	LRP, LR, LSC,[b]LSE, LRK
50	quercetin 3-*O*-arabinoside	$C_{20}H_{18}O_{11}$	45.598	[c][M − H]$^-$	434.0849	433.0776	433.0780	0.90	301	LRP,[b]LR, LSE
51	isorhamnetin	$C_{16}H_{12}O_7$	85.555	[c][M − H]$^-$	316.0583	315.0510	315.0510	0.00	300, 271	LSC,[b]LSE
	dihydrochalcones									
52	3-hydroxyphloretin 2′-*O*-xylosyl-glucoside	$C_{26}H_{32}O_{15}$	12.115	[M − H]$^-$	584.1741	583.1668	583.1688	3.43	289	LSE
53	3-hydroxyphloretin 2′-*O*-glucoside	$C_{21}H_{24}O_{11}$	38.973	[c][M − H]$^-$	452.1319	451.1246	451.1247	0.22	289, 273	LRP, LSC,[b]LRK
54	phloridzin	$C_{21}H_{24}O_{10}$	47.041	[c][M − H]$^-$	436.1369	435.1296	435.1303	1.61	273	LRP, LR,[b]LRK
	anthocyanins									
55	peonidin 3-*O*-diglucoside-5-*O*-glucoside	$C_{34}H_{43}O_{21}$	10.988	[c][M + H]$^+$	787.2297	788.2370	788.2399	3.68	625, 478, 317	[b]LRP, LR, LSE
56	cyanidin 3-*O*-(6″-*p*-coumaroyl-glucoside)	$C_{30}H_{27}O_{13}$	16.009	[c][M + H]$^+$	595.1452	596.1525	596.1515	−1.68	287	[b]LR, LRP
57	delphinidin 3-*O*-glucoside	$C_{21}H_{21}O_{12}$	22.187	[c][M + H]$^+$	465.1033	466.1106	466.1095	−2.36	303	LRP,[b]LR, LSC, LSK
58	delphinidin 3-*O*-glucosyl-glucoside	$C_{27}H_{31}O_{17}$	26.933	[M + H]$^+$	627.1561	628.1634	628.1664	4.78	465, 303	LSE
59	isopeonidin 3-*O*-arabinoside	$C_{21}H_{21}O_{10}$	41.565	[M + H]$^+$	433.1135	434.1208	434.1200	−1.84	271, 253, 243	[b]LSC, LSE
60	cyanidin 3,5-*O*-diglucoside	$C_{27}H_{31}O_{16}$	42.675	[c][M + H]$^+$	611.1612	612.1685	612.1672	−2.12	449, 287	[b]LSC, LSE
61	pelargonidin 3-*O*-rutinoside	$C_{27}H_{31}O_{14}$	50.950	[M + H]$^+$	579.1714	580.1787	580.1814	4.65	271, 433	LSE
	isoflavonoids									
62	6″-*O*-malonylglycitin	$C_{25}H_{24}O_{13}$	37.252	[M + H]$^+$	532.1217	533.1290	533.1274	−3.00	285, 270, 253	LSE
63	5,6,7,3′,4′-pentahydroxyisoflavone	$C_{15}H_{10}O_7$	37.837	[c][M + H]$^+$	302.0427	303.0500	303.0503	0.99	285, 257	[b]LRP, LR, LSC, LSE, LRK

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Table 3. continued

no.	proposed compounds	molecular formula	RT (min)	ionization mode	molecular weight	theoretical (m/z)	observed (m/z)	mass error (ppm)	MS/MS product ions	samples
				Flavonoids						
64	6″-*O*-acetyldaidzin	$C_{23}H_{22}O_{10}$	41.868	[c][M − H]$^-$	458.1213	457.1140	457.1129	−2.41	221	LRP, LR, LSC,[b]LSE
65	violanone	$C_{17}H_{16}O_6$	47.057	[c]M − H]$^-$	316.0947	315.0874	315.0881	2.22	300, 285, 135	LRP, LR, LSE,[b]LRK
66	3′-hydroxydaidzein	$C_{15}H_{10}O_5$	50.933	[M + H]$^+$	270.0528	271.0601	271.0613	4.43	253, 241, 225	LRP, LR, LSC,[b]LSE
67	6″-*O*-acetylglycitin	$C_{24}H_{24}O_{11}$	50.950	[c][M + H]$^+$	488.1319	489.1392	489.1410	3.68	285, 270	[b]LSE, LRP
68	3′-hydroxygenistein	$C_{15}H_{10}O_6$	51.305	[c][M + H]$^+$	286.0477	287.0550	287.0546	−1.39	269, 259	LSC, LSE,[b]LRK
69	dihydrobiochanin A	$C_{16}H_{14}O_5$	54.847	[M + H]$^+$	286.0841	287.0914	287.0922	2.79	269, 203,201, 175	LRP
70	2-dehydro-*O*-desmethylangolensin	$C_{15}H_{12}O_4$	75.685	[c][M − H]$^-$	256.0736	255.0663	255.0655	−3.14	135, 119	LRP,[b]LRK
71	3′,4′,7-trihydroxyisoflavanone	$C_{15}H_{12}O_5$	83.053	[c][M − H]$^-$	272.0685	271.0612	271.0608	−1.48	177, 151, 119, 107	LRP, LR, LSC,[b]LSE, LRK
				Other polyphenols						
	hydroxycoumarins									
72	coumarin	$C_9H_6O_2$	8.486	[M + H]$^+$	146.0368	147.0441	147.0442	0.68	103, 91	LRP
73	esculin	$C_{15}H_{16}O_9$	13.406	[M + H]$^+$	340.0794	341.0867	341.0862	−1.47	179, 151	[b]LRP, LR, LSC
74	salvianolic acid B	$C_{36}H_{30}O_{16}$	27.074	[M − H]$^-$	718.1534	717.1461	717.1485	3.35	519, 339, 321, 295	LSE
75	scopoletin	$C_{10}H_8O_4$	41.480	[c][M − H]$^-$	192.0423	191.0350	191.0357	3.66	176	LRP,[b]LR, LRK
	alkylmethoxyphenols									
76	4-vinylsyringol	$C_{15}H_{14}O_3$	21.803	[c][M + H]$^+$	242.0943	243.1016	243.1019	1.23	225, 211, 197	[b]LRP, LSE
	hydroxybenzoketones									
77	2,3-dihydroxy-1-guaiacylpropanone	$C_{10}H_{12}O_5$	9.879	[c][M − H]$^-$	212.0685	211.0612	211.0602	−4.70	167, 123, 105, 93	LRP
78	2-hydroxy-4-methoxyacetophenone 5-sulfate	$C_9H_{10}O_7S$	12.844	[M − H]$^-$	262.0147	261.0074	261.0069	−1.92	181, 97	LSE
	tyrosols									
79	hydroxytyrosol 4-*O*-glucoside	$C_{14}H_{20}O_8$	9.777	[c][M − H]$^-$	316.1158	315.1085	315.1076	−2.90	153, 123	[b]LSC, LR
80	demethyloleuropein	$C_{24}H_{30}O_{13}$	12.181	[M − H]$^-$	526.1686	525.1613	525.1633	3.81	495	LSE
81	3,4-DHPEA-AC	$C_{10}H_{12}O_4$	37.614	[c][M − H]$^-$	196.0736	195.0663	195.0659	−2.05	135	LRP, LR,[b]LRK
				Lignans						
82	todolactol A	$C_{20}H_{24}O_7$	39.718	[c][M − H]$^-$	376.1522	375.1449	375.1439	−2.67	313, 137	LRP,[b]LRK
83	7-hydroxymatairesinol	$C_{20}H_{22}O_7$	41.309	[c][M − H]$^-$	374.1366	373.1293	373.1298	1.30	343, 313, 298, 285	LR
84	matairesinol	$C_{20}H_{22}O_6$	45.898	[M − H]$^-$	358.1416	357.1343	357.1338	−1.40	342, 327, 313, 221	LRP, LR,[b]LRK
				Stilbenes						
85	resveratrol	$C_{14}H_{12}O_3$	31.317	[c][M − H]$^-$	228.0786	227.0713	227.0709	−1.80	212, 185, 157, 143	[b]LRP, LR, LSC, LRK
86	resveratrol 3-*O*-glucoside	$C_{20}H_{22}O_8$	42.667	[M − H]$^-$	390.1315	389.1242	389.1240	−0.51	227	LRK

[a]Lotus samples mentioned in abbreviations are lotus rhizome pulp (LR), lotus rhizome peel (LRP), lotus rhizome knot (LRK), lotus seed cotyledon (LSC), and lotus seed embryo (LSE). [b]Compound was detected in more than one lotus sample; data presented in this table are from the asterisk sample. [c]Compounds were detected in both negative [M − H]$^-$ and positive [M + H]$^+$ modes of ionization, while only single mode data were presented.

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respectively. The further confirmation of galloyl glucose was achieved by comparing the MS/MS spectra with a previous study, in which a similar MS/MS fragmentation behavior of galloyl glucose standard was observed.[44] Compound **2** detected in both modes with observed $[M - H]^-$ m/z at 137.0248 was discovered in all five parts of lotus and characterized as 2-hydroxybenzoic acid based on the product ion at m/z 93, corresponding to the loss of CO_2 (44 Da) from the precursor ion.[45] Previously, this compound was also characterized in lotus leaves and lotus seeds with the help of HPLC.[31,46] Compound **4** with $[M - H]^-$ m/z at 169.0140 was only detected from LSC and LSE and was characterized as gallic acid based on the product ion at m/z 125, corresponding to the loss of CO_2 from the precursor ion, which has already been reported in lotus leaves,[47] lotus seeds,[31] and lotus rhizomes.[7] Compound **5** with observed $[M - H]^-$ m/z at 479.1583 was tentatively characterized as paeoniflorin. The characteristic fragment ions at m/z 449 $[M - H - CH_2O]^-$, m/z 357 $[M - H - C_7H_6O_2]^-$, m/z 327 $[M - H - CH_2O - C_7H_6O_2]^-$, and m/z 121 $[M - H - C_{16}H_{22}O_9]^-$ confirmed the presence of paeoniflorin in the lotus seed. Previously, Tu et al.[48] isolated paeoniflorin from *Paeonia lactiflora*, a well-known traditional Chinese herb, and also reported the potent anti-inflammatory and immune regulatory effects of paeoniflorin.

2.4.1.2. Hydroxycinnamic Acids and Other Phenolic Acid Derivatives. In our study, 12 hydroxycinnamic acid derivatives, 2 hydroxyphenylacetic acids, and 1 hydroxyphenylpentanoic acid were identified in five parts of lotus. Compound **6** was tentatively characterized as cinnamic acid found in LRP, LR, LSC, and LSE based on m/z at 147.0453 in the negative mode. The identification was further supported by the MS^2 spectrum, which exhibited a typical product ion at m/z 103 formed by the neutral loss of a carboxylic acid moiety (45 Da).[49] Previously, cinnamic acid was found in bilberry fruit (*Vaccinium*) and reported to be a precursor for the synthesis of a vast number of plant substances, including lignin, tannins, flavonoids, and various alkaloids.[50]

m-Coumaric acid (compound **8**) were detected in both positive (ESI^+) and negative (ESI^-) modes in all five parts of lotus, with an observed $[M - H]^-$ m/z at 163.0405 and the primary product ion at m/z 119, corresponding to the loss of CO_2 (44 Da).[51] Compound **9** having a precursor ion $[M - H]^-$ m/z at 341.0875 was tentatively characterized as caffeoyl glucose and was present in LSE. The MS^2 fragmentation showed the product ions at m/z 179 $[M - H - 162]^-$ and m/z 161 $[M - H - 180]^-$, consistent with losses of the hexosyl moiety and further loss of H_2O.[52] Previously, derivatives of coumaric acids and caffeic acids have already been reported in different solvent extracts of lotus leaves and seeds by HPLC.[31,46]

Three other phenolic acid derivatives were also detected, including two hydroxyphenylacetic acid derivatives (compounds **18** and **19**) and one hydroxyphenylpentanoic acid derivative (compound **20**). To the best of our knowledge, this is the first time that hydroxyphenylacetic acids and hydroxyphenylpentanoic acids were identified in lotus.

2.4.2. Flavonoids. The study of flavonoids has always been the priority of research related to phenolic compounds in *N. nucifera*. In addition, flavonoids might be the predominant contributors to the antioxidant activity of lotus, as shown in the correlation section. In our study, a total of 51 flavonoids classified into 8 subclasses were characterized in 5 parts of lotus, including 8 flavanols, 5 flavanones, 6 flavones, 12 flavonols, 3 dihydrochalcones, 7 anthocyanins, and 10 isoflavonoids, as shown in Table 3.

2.4.2.1. Flavanol Derivatives. Compound **21** was characterized as (+)-catechin 3-*O*-gallate in LRK based on the precursor ion $[M - H]^-$ at m/z 441.0842, with product ions at m/z 289 ($[C_{15}H_{13}O_6]^-$), m/z 169 ($[C_7H_5O_5]^-$), and m/z 125 ($[C_6H_5O_3]^-$).[51] Previously, catechins and its derivatives have been detected in many tissues of lotus, including rhizomes,[53] leaves,[54] and seed epicarp,[4] which have been proved to be able to regulate the insulin secretion and blood glucose level in both *in vitro* and *in vivo* models.[55]

(−)-Epicatechin was proposed as compound **26**, detected from all five parts of lotus in both modes, with a precursor ion $[M - H]^-$ m/z of 289.0714. The MS^2 spectrum showed the product ions at m/z 245, 205, and 179, indicating the loss of CO_2 (44 Da), flavonid A ring (84 Da), and flavonid B ring (110 Da) from the precursor ion, respectively.[45] The procyanidin trimer C1 and procyanidin dimer B1 (compounds **24** and **28**) were also identified in LRP, LR, LSC, and LRK.[56,57] The presence of epicatechin and procyanidin in lotus has already been reported by Chen et al.[38] In addition, Xu et al.[58] extracted procyanidins in the seedpod of lotus and found that procyanidins improved age-related antioxidant deficits in an animal model and had antiaging effects.

2.4.2.2. Flavanone and Flavone Derivatives. Four apigenin derivatives (compounds **34**, **35**, **36**, and **38**) were tentatively identified from both seeds and rhizomes of lotus in our study. Compounds **36** and **38** were tentatively assigned as apigenin 6,8-di-*C*-glucoside and apigenin 6-*C*-glucoside, respectively, based on the $[M - H]^-$ ions at m/z 593.1532 and 431.0984, respectively.

The characteristic loss of 90 and 120 Da caused by the loss of cross-ring cleavages of the glycoside moiety was observed in these two compounds in the MS/MS fragmentation, confirming the identification of these two compounds.[59] Different from other apigenin derivatives, compounds **36** and **38** were only present in seeds of lotus, which are in agreement with the finding that the flavonoid *C*-glycosides were only present in lotus seeds[33] [Li, 2014 #40]. Previously, apigenin 6,8-di-*C*-glucoside (compound **36**) has been found in a 70% ethanolic extract of lotus seed embryo by HPLC−MS in the study of Zhu et al.[5] In contrast to the *O*-glucosyl bond, the *C*-glucosyl bond between the flavonoid carbon skeleton and the glycosyl group is more stable under acidity and enzymatic hydrolysis, resulting in significant differences in the bioactivity and pharmacokinetics.[60] Currently, the research of flavonoid *C*-glycosides is rare. Thus, the flavonoid *C*-glycosides need further study, which may have an application potential in the food industry.

2.4.2.3. Flavonol Derivatives. Based on the composition of the aglycone, a total of 12 flavonols identified in five parts of lotus were mainly classified into 4 different groups, including 3 kaempferol derivatives, 4 quercetin derivatives, 3 myricetin derivatives, and 1 isorhamnetin derivative.

Compound **47** with $[M - H]^-$ m/z at 739.2106 exhibiting characteristic fragment ions at m/z 593 $[M - H - C_6H_{10}O_4]^-$, m/z 447 $[M - H - 2C_6H_{10}O_4]^-$, and m/z 285 $[M - H - 2C_6H_{10}O_4 - C_6H_{10}O_5]^-$ was identified as kaempferol 3-*O*-(2″-rhamnosyl-galactoside) 7-*O*-rhamnoside.[61] Kaempferol derivatives were previously reported to spread in almost all lotus tissues.[62−67] Liao et al.[68] found that the kaempferol derivatives extracted from lotus leaves could prevent diabetes type 2 through the inhibition of α-amylase.

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2.4.2.4. Dihydrochalcone, Anthocyanin, and Isoflavonoid Derivatives. Compound **54** was only detected in lotus rhizomes in both positive (ESI^+) and negative (ESI^-) modes with an observed molecular ion peak $[M - H]^-$ m/z at 435.1303. This compound was assigned to phloridzin, a characteristic flavonoid found in apples based on its fragment at m/z 273 for the phloretin aglycon.[69] Compound **57** having a precursor ion $[M + H]^+$ m/z at 466.1095 was tentatively characterized as delphinidin 3-*O*-glucoside and was present in LRP, LR, LSC, and LRK. The MS^2 analysis showed the product ion at m/z 303 $[M + H - 162]^+$, consistent with losses of the hexosyl moiety.[70] Previously, delphinidin 3-*O*-glucoside has been identified in a methanolic extract of the lotus flower petal by HPLC−electrospray ionization−mass spectrometry in the study of Li et al.[33]

Compound **65** with $[M - H]^-$ m/z at 315.0881 exhibiting characteristic fragment ions at m/z 300 $[M - H - CH_3]^-$, m/z 285 $[M - H - 2CH_3]^-$, m/z 135 $[M - H - C_{10}H_{12}O_3]^-$, and m/z 91 $[M - H - C_{10}H_{12}O_3 - CO_2]^-$ was identified as violanone, which was previously found in the essential oil of traditional Chinese medicine *Dalbergia odorifera* by LC−MS.[71]

2.4.3. Other Polyphenols. Four hydroxycoumarins, one alkylmethoxyphenol, two hydroxybenzoketones, and three tyrosols were tentatively identified in our study. All of these compounds were first reported in lotus. Compound **81** from lotus rhizomes with $[M - H]^-$ ion at m/z 195.0659 was identified as 3,4-DHPEA-AC and showed the main fragment ion at m/z 135 was depicted on the cause of the $C_2H_4O_2$ deletion, which fit the fragment generated by the hydrolysis of the bound ester group from 3,4-DHPEA-AC. The hydroxytyrosol acetate was a critical antioxidant present in olive oil.[72]

2.4.4. Lignans and Stilbenes. Lignans were minor components present in the lotus. In the present study, a total of three lignans were shown to be only present in lotus rhizomes. Compound **84** was tentatively characterized as matairesinol and only found in lotus rhizomes based on $[M - H]^-$ m/z at 357.1338. The identification was further supported by the MS^2 spectrum that exhibited the product ions at m/z 342 $[M - H - 15]^-$, 327 $[M - H - 30]^-$, 313 $[M - H - 44]^-$, 221 $[M - H - 136]^-$, and 161 $[M - H - 196]^-$, corresponding to the loss of CH_3, two CH_3, CO_2, $C_8H_8O_2$, and $C_{10}H_{12}O_4$, respectively.[52] Matairesinol was previously found in the stem of traditional Chinese medicine *Acanthopanax senticosus*.[52]

Compounds **85** and **86** were aligned as resveratrol and resveratrol 3-*O*-glucoside by MS^2 spectrum[73] [Stella, 2008 #30]. According to previous research, resveratrol was identified primarily in fruit samples such as grape and was reported to be a new cancer chemopreventive agent that inhibits cellular events related to the initiation, promotion, and progression of tumors.[74] The lotus seed and rhizome are rich resources of phenolic compounds that might have a wide application prospect in pharmacy, feed, cosmetics, and food industries.

2.5. HPLC and Heat Map. Based on the characterization of phenolic compounds by LC-ESI-QTOF-MS (Table 3) and previous investigations involved in the phenolic composition of different lotus tissues,[5,31,33,46] a total of 10 polyphenols were selected for quantitative analysis in lotus by HPLC-PDA, including five phenolic acids (gallic acid, *p*-hydroxybenzoic acid, chlorogenic acid, syringic acid, and coumaric acid) and five flavonoids (catechin, epicatechin, quercetin-3-galactoside, quercetin, and kaempferol).

A heat map (Figure 1) shows the hierarchical clustering of targeted phenolic compounds in five parts of the lotus. The

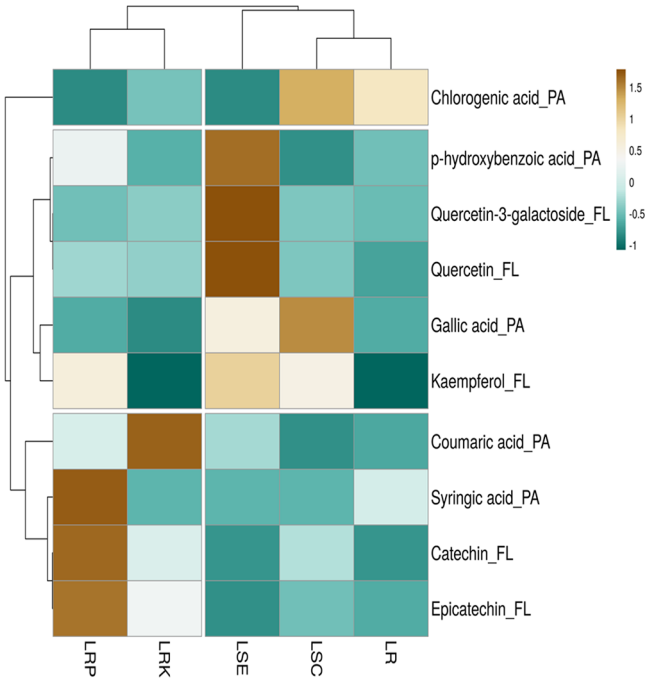

Figure 1. Heat map showing the distribution and concentration of phenolic compounds in five parts of lotus. Brown boxes show that constructions are higher among five samples. Blue boxes indicate lower concentrations. FL: flavonoids and PA: phenolic acids. Fruit peel samples are mentioned in abbreviations. LR: lotus rhizome pulp; LRP, lotus rhizome peel; LRK, lotus rhizome knot; LSC, lotus seed cotyledon; and LSE, lotus seed embryo.

axis of the map has samples and phenolic compounds, whereas the branching exhibits the similarity of the samples. The darker color (brown) represents a higher concentration, while the blue color has a lower content.

In general, quercetin-3-galactoside and quercetin were found in high concentrations in LSE (marked with dark brown color). Previously, Chen et al.[67] reported the concentration of quercetin in different tissues of lotus leaves ranging from 11.0 to 15.1 μg/g. In addition, gallic acid, chlorogenic acid, and kaempferol were also quantified in LSC. Previously, Yen et al.[31] already reported the concentration of caffeic acid, chlorogenic acid, *p*-hydroxybenzoic acid, and gallic acid in an aqueous extract of lotus seeds by HPLC and suggested that those phenolic acids may make contributions to the antioxidant activities of lotus seeds. Catechin and epicatechin were the most abundant flavanols quantified in the lotus rhizome. The LRP presents the highest syringic acid content, and the LRK exhibits the highest content of coumaric acid. Chlorogenic acid was the most abundant phenolic acid quantified in the LR.

3. MATERIALS AND METHODS

3.1. Chemicals and Reagents. The bioassay for the determination of phenolic compounds and antioxidant potential and the standards used including vanillin, catechin, gallic acid, and ascorbic acid were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia), and the chemicals including aluminum chloride hexahydrate, Folin−Ciocalteu reagent, ferric(III) chloride anhydrous, quercetin, 2,2-diphenyl-1-

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picrylhydrazyl (DPPH), sodium phosphate, 2,4,6-tripyridyl-*s*-triazine (TPTZ), potassium persulfate, ammonium molybdate, 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonate) (ABTS), and ammonium molybdate were obtained from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO, USA). Hydrated sodium acetate, anhydrous sodium acetate, hydrochloric acid, methanol, acetic acid, sodium carbonate (anhydrous), and sulfuric acid (98%) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA) and Chem-Supply Pty Ltd. (Adelaide, SA, Australia). Acetonitrile and acetic acid used in HPLC and LC−MS were analytical grade and purchased from Fisher Chemical Company (San Jose, CA, USA). Water used in this study was deionized by Millipore Milli-Q Gradient Water Purification System (Darmstadt, Germany). All standards used in HPLC analysis, including gallic acid, protocatechuic acid, caftaric acid, *p*-hydroxybenzoic acid, chlorogenic acid, caffeic acid, syringic acid, coumaric acid, ferulic acid, sinapic acid, catechin, epicatechin, epicatechin gallate, quercetin-3-glucuronide, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside, kaempferol-3-glucoside, diosmin, quercetin, kaempferol, polydatin, and resveratrol, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Sample Preparation. Seeds and rhizomes of ripened edible lotus were purchased from the Preston market in Melbourne, Victoria, Australia, in August 2019. Lotus rhizomes were cleaned before peeling and cutting into pulp (0.5 × 1 cm), peel (0.1 cm thick), and knot (0.5 × 1 cm). The peel, knot, and pulp were then blended into slurries using a 1.5 L blender (Russell Hobbs Classic, model DZ-1613, Melbourne, VIC, Australia). Lotus seeds were separated in half by a hammer to remove embryos from cotyledons followed by grinding in a grinder (Sunbeam Multi Grinder, EM0405, Melbourne, VIC, Australia). All materials were stored at −20 °C for further analysis.

3.3. Extraction of Phenolic Compounds. The polyphenols were extracted by modifying the method of Liu et al.[4] Each sample (5 g) was mixed with 15 mL of ethanol (80%, v/v) and homogenized by the Ultra-Turrax T25 Homogenizer (IKA, Staufen, Germany) at 10,000 rpm for 30 s followed by incubation in a ZWYR-240 incubator shaker (Labwit, Ashwood, VIC, Australia) at 120 rpm at 4 °C overnight. The extracts were centrifuged by a centrifugation incubator (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 10, 000 rpm for 10 min. The supernatants were transferred and filtered through a 0.45 μm syringe filter (Thermo Fisher Scientific Inc., Waltham, MA, USA) before being stored at −20 °C for further analysis.

3.4. Estimation of Polyphenols and Antioxidant Activities. All phytochemical and antioxidant assays were performed in triplicate and measured by a Multiskan Go microplate photometer (Thermo Fisher Scientific, Waltham, MA, USA). The standard curves were plotted with $R^2 > 0.995$. All results were based on fresh weight ($_{f.w.}$).

3.4.1. Determination of Total Phenolic Content (TPC). The total polyphenol content was measured using a modified Folin−Ciocalteu method of Gu et al.[13] A total of 25 μL of samples, 25 μL of 25% (v/v) Folin−Ciocalteu reagent, and 200 μL of deionized water were mixed in 96-well plates (Costar, Corning, NY, USA) followed by incubation for 5 min at 25 °C. After that, 25 μL of 10% (w/w) sodium carbonate was added followed by further incubation in the darkroom for 60 min at 25 °C. The absorbance was measured at 764 nm in a plate reader. The results were converted to total phenolic content expressed as mg of gallic acid equivalents per gram of the sample based on fresh weight (mg GAE/$g_{f.w.}$). Gallic acid ranging from 0 to 200 μg/mL was used to plot the calibration curve.

3.4.2. Determination of Total Flavonoid Content (TFC). A modified aluminum chloride coloration method was applied to evaluate the TFC values of lotus seeds and rhizomes.[13] Eighty milliliters of sample extracts, 80 μL of 2% (w/v) aluminum chloride ethanolic solution (analytical grade), and 120 μL 50 g/L sodium acetate were sequentially added into the 96-well plate. The mixture was incubated in the darkroom for 1 h at 25 °C before measuring the absorbance at 440 nm. The quercetin standard ranging from 0 to 50 μg/mL was used to plot the standard curve, and the result was expressed in quercetin equivalents (mg QE/$g_{f.w.}$).

3.4.3. Determination of Total Tannin Content (TTC). The TTC was determined by the colorimetric method of Gu et al.[13] with some modifications. The sample extract (25 μL) was mixed with 150 μL of 4% (w/v) methanolic vanillin solution and 25 μL of 32% (v/v) sulfuric acid (diluted with methanol) in the 96-well plates followed by incubation for 15 min at 25 °C. The absorbance was measured at 500 nm. Catechin (0−1000 μg/mL) was used as a calibration standard. The results were expressed as mg of catechin equivalents per gram of sample (mg CE/$g_{f.w.}$).

3.4.4. Determination of DPPH Free Radical Scavenging Activity. The ability to scavenge the DPPH radical was evaluated based on the method of Braca et al.[75] [Braca, 2001 #33] with some modifications. Forty microliters of lotus extracts was mixed with 260 μL of the DPPH radical methanol solution (0.1 mM). Absorbance was measured at 517 nm after 30 min incubation in the darkroom at 25 °C, and ascorbic acid (0−50 μg/mL) was used as the standard. The DPPH free radical scavenging activity was expressed as units of ascorbic acid equivalent (mg AAE/$g_{f.w.}$).

3.4.5. Determination of Ferric Reducing Antioxidant Power (FRAP). For the ferric reducing antioxidant power of lotus in this study, the method of Kim and Shin[76] was engaged with some modifications. In the reaction, the Fe^{3+}-TPTZ complex (ferric-2,4,6-tripyridyl-*s*-triazine) was reduced to a colored product (Fe^{2+}-TPTZ). The FRAP reagent was prepared freshly by mixing 10 mL of 20 mM $FeCl_3$, 10 mL of TPTZ solution (10 mM TPTZ and 40 mM HCl), and 100 mL of 300 mM sodium acetate solution. Then, 20 μL of extracts was mixed with 280 μL of the FRAP reagent in the 96-well plates. The absorbance was measured at 593 nm after incubation at 37 °C for 10 min, and ascorbic acid (0−50 μg/mL) was used as the control. The results were expressed as mg ascorbic acid equivalents per gram of sample weight (mg AAE/$g_{f.w.}$).

3.4.6. Determination of ABTS Free Radical Scavenging Activity. The determination of ABTS radical scavenging activity was based on a modified method of Sogi et al.[77] First, ABTS was dissolved in 140 mM potassium persulfate solution to a 7 mM concentration to produce the ABTS radical cation ($ABTS^{\bullet+}$) followed by incubation in the darkroom overnight before use. The stock solution was further diluted with ethanol (analytical grade) to give an absorbance of 0.70 ± 0.02 at 734 nm. After that, 10 μL of lotus extracts was added to 290 μL of the ABTS working solution in the 96-well plates. The absorbance was measured at 734 nm immediately after 6 min incubation at 25 °C, and ascorbic acid was used as control

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(0−2000 μg/mL). The results were expressed as mg ascorbic acid equivalent per gram of sample (mg AAE/$g_{f.w.}$).

3.4.7. Determination of Total Antioxidant Capacity (TAC). The total antioxidant capacity assay was carried out by modifying the method of Jan et al.,[78] which is based on reducing phosphomolybdate ions. Antioxidants in extracts reduce phosphomolybdate ion and form a green phosphate/MoV complex, which can be measured spectrophotometrically[78] [Jan, 2013 #13]. The sample (25 μL) was mixed with 250 μL of the prepared dye solution (0.6 M H_2SO_4 sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate, with the volume ratio of 1:1:1). The mixtures were incubated in the darkroom at 95 °C for 90 min. The absorbance was measured at 695 nm after cooling at room temperature for 10 min. Ascorbic acid (0−300 μg/mL) was used as standard, and the results were expressed as mg ascorbic acid equivalent per gram of sample (mg AAE/$g_{f.w.}$).

3.5. LC-ESI-QTOF-MS/MS Characterization. The characterization of phenolic compounds by LC-ESI-QTOF-MS/MS was carried out by the modified method of Ma et al.[79] An Agilent 1200 series HPLC (Agilent Technologies, CA, USA) equipped with an Agilent 6520 Accurate-Mass Q-TOF LC/MS (Agilent Technologies, CA, USA) was used in this work. The separation was achieved by a Synergi Hydro-RP (250 × 4.6 mm i.d.) reversed-phase column with a particle size of 4 μm (Phenomenex, Lane Cove, NSW, Australia) coupled with a Phenomenex 4.0 × 2.0 mm i.d. C18 ODS guard column. The column temperature was set at 25 °C, and the injection volume was 5 μL. The mobile phase consisted of two eluents. Eluent A is 0.5% acetic acid in water (0.5:99.5, v/v), and eluent B consists of acetonitrile/water/acetic acid (50:49.5:0.5, v/v/v). The gradient profile was 10−25% eluent B from 0 to 20 min, 25−35% eluent B from 20 to 30 min, 35−40% eluent B from 30 to 40 min, 40−55% eluent B from 40 to 70 min, 55−80% eluent B from 70 to 75 min, 80−90% B from 75 to 77 min, 90−100% B from 77 to 79 min, 100−10% B from 79 to 82 min, and isocratic 10% B from 82 to 85 min. The flow rate was 0.8 mL/min. The nitrogen gas pressure was set at 45 psi with a flow rate of 5 L/min at 300 °C, while the sheath gas was set at 11 L/min at 250 °C. The capillary was set at 3.5 kV. The nozzle voltage was set at 500 V. A complete mass scan ranging from m/z 50 to 1300 was used. MS/MS analyses were carried out in automatic mode with collision energy (10, 15, and 30 eV) for fragmentation. Peak identification was performed in both positive and negative modes, while the instrument control, data acquisition, and processing were performed using the MassHunter workstation software (Qualitative Analysis, version B.03.01) (Agilent Technologies, Santa Clara, CA, USA).

3.6. HPLC-PDA Quantification Analysis. The quantification of 10 targeted phenolic compounds present in five parts of the lotus was carried out by the modified method of Ma et al.[79] HPLC (chromatography separation module, Waters Alliance 2690) was equipped along with a photodiode array and a detector. The mention column and condition as stated in the LC-ESI-QTOF-MS/MS section were practical, excluding for a sample injection volume of 20 μL. The PDA detector noticed the phenolics of extracts under λ 280, 320, and 370 nm. The individual phenolic compound was quantified based on linear regression of the external standards' plotting peak area against concentration.

3.7. Statistical Analysis. Results from seven independent experiments (Sections 3.4.1 to 3.4.7: TPC, TFC, TTC, ABTS, DPPH, FRAP, and TAC) in spectrophotometric assays were expressed as mean ± standard deviation (SD). The analysis of variance was conducted using one-way analysis of variance (ANOVA), and the differences between the means of samples were carried out by Tukey's test using Minitab 18 Statistical Software (Minitab Inc., State College, PA, USA) at a significance level of $p < 0.05$. Pearson's correlation coefficient was used to analyze the correlation between antioxidant activities and total phenolic and flavonoid content in the extracts of lotus seeds and rhizomes.

4. CONCLUSIONS

Remarkable phenolic contents and antioxidant potentials were observed in all lotus samples, while among the five parts of lotus, the lotus seed embryo exhibits the highest total phenolic content, total flavonoid content, and total antioxidant capacity (DPPH, ABTS, and TAC). A total of 86 phenolic compounds were successfully separated and characterized in five parts of lotus seeds and rhizomes by the application of the LC-ESI-QTOF-MS/MS technique. Most compounds were discovered in the lotus seed embryo followed by the lotus seed cotyledon and lotus rhizome peel. In addition, the flavonoid *C*-glycosides identified in lotus seeds are not commonly found in most plants, which are valuable for further investigations on their effects on human health. Thus, the results of the present study revealed that byproducts of lotus seeds and rhizomes (lotus seed embryo, lotus rhizome knot, and lotus rhizome peel) have a prominent antioxidant effect and could be good sources of natural antioxidants. However, further investigations involving more detailed activity studies *in vitro* and *in vivo* are required to support further utilization in the food and pharmaceutical industries.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c07018.

LC-ESI-QTOF-MS/MS basic peak chromatograph (BPC) for the characterization of phenolic compounds of Australia-grown lotus (Figure S1) and LC-ESI-QTOF-MS/MS characterization of 2-hydroxybenzoic acid (Figure S2) (PDF)

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Notes

The authors declare no competing financial interest.

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