Antioxidant capacity and phenolic contents of three *Quercus* species

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ABSTRACT. The antioxidant capability and phenolic contents of ethanol extracts (free phenolics) and ethyl acetate extracts (bound phenolics) of three *Quercus* species were estimated in this work. The antioxidant activities were examined by 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) free radical, reducing power and *β*-carotene bleaching methods. HPLC was employed to detect major phenolic acids. The leaf extract of *Q. salicina* contained maximum total phenolics while the highest total flavonoid content was found in the leaf extract of *Q. serrata*. The antioxidant activities varied among three species. Bark extract of *Q. salicina* was the most potential and it was closed to levels of the standard antioxidative dibutyl hydroxytoluene (BHT). The bark extract of *Q. serrata* also showed promising antioxidant activities despite their eminence was negligibly lower than *Q. salicina*. Stronger antioxidant activities of free phenolics than those of the bound phenolics may be attributed to higher quantities of free phenolics in the barks of *Quercus* species, however total flavonoids may not contribute a critical role. By HPLC analysis, thirteen phenolic acids were detected in the leaf and bark extracts. Of them, *Q. salicina* showed maximum in number (ten compounds) and quantities of detected phenolic acids. Ellagic, chlorogenic and benzoic acids were dominant in *Quercus* species. Findings of this study revealed that leaves and barks of three *Quercus* species are rich source of antioxidants, and *Q. salicina* is the most promising and should be elaborated to exploit its pharmaceutical properties.

1. INTRODUCTION

In recent years, plant phenolic compounds have a great deal of attention because of multifunctional properties and beneficial effects on human health [1,2]. Phenolic including phenolic acids and flavonoids, which are characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups [3-6], are naturally synthesized via the shikimate pathway [7]. It has been estimated that there are over six thousand phenolic compositions identified and still a large percentage remains unexplored [4,8]. Phenolics are powerful antioxidants due to their beneficial properties to neutralize free radicals effectively by providing a hydrogen or an electron [2]. Antioxidants act as an inhibitor in the initiation of deleterious free radical reactions because they can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction [8]. Antioxidant compounds are widely distributed in vegetables, fruits, herbs and many food sources [9-11]. Thus, natural antioxidants need to be explored in supporting human health.

Quercus (Oak) trees belong to *Fagaceae* family and they are dominant species in forest ecosystem with 450 species distributed worldwide [12]. Oaks are a major source of hardwood for use in construction, furniture, veneer barrels and other purposes because the wood of *Quercus* is durable and tough, and wood grain is really attractive [13]. It was reported that there were 20

bioactive compounds isolated in barks and leaves of oaks such as oligomeric, (+)-catechin, (-)-epicatechin, tiliroside, (-)-epigallocatechin, syringic acid, *p*-coumaric acid, sinapic acid and naringenin [11,14,15]. Moreover, thirteen species in the *Quercus* genus are herbal medicines. They have been extensively used in folk and scientific medicines in China and Korea for treatment of diarrhea, dysentery, dermatitis and diabetes [16,17].

Quercus crispula Blume, *Quercus salicina* Blume and *Quercus serrata* Thunb. distributed widely in Japan, Taiwan, Korea, China, Russia and the East Himalaya [18]. *Q. crispula* is the most dominant tree species in cool temperate, mixed broadleaf/conifer forests in northern Japan [19]. This species is cultivated for timber, charcoal, ornament in Japan [18]. *Q. serrata* is a very important species in Japan. This species is not only used for timber and charcoal, but it also is used as bed logs for Shiitake mushroom (*Lentinus edodis*) production [18,20]. *Q. salicina* has been used as herbal medicine in Japan for nearly 50 years in treatment of urolithiasis because its extract might prevent stone formation and recurrence in urolithiasis patients [21].

To the best of our knowledge, the biological activities and phenolic compositions of *Q. crispula*, *Q. serrata* and *Q. salicina* have not yet been well examined. Hence, in this study the total phenolics, flavonoids, and antioxidant activities in leaves and barks of three *Quercus* species were estimated and major phenolic acids of extracts were identified and quantified. The correlations of these compounds to the antioxidant activities were also discussed.

2. MATERIALS AND METHODS

Standards and reagents

Standard phenolic compounds including benzoic acid, caffeic acid, catechol, cinnamic acid, chlorogenic acid, ellagic acid, ferulic acid, gallic acid, protocatechuic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, sinapic acid, syringic acid, vanillic acid, vanillin, gallic acid and rutin, were purchased from KANTO chemical, Tokyo, Japan.

Reagents: Folin-Ciocalteu's reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), dibutyl hydroxytoluene (BHT), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate ($K_2S_2O_8$) aluminium (III) chloride hexahydrate ($AlCl_3.6H_2O$), β-carotene, linoleic acid, polyoxyethylen esorbitan monopalmitate (Tween-40), trichloroacetic acid (CCl_3COOH), iron (III) chloride ($FeCl_3$), sodium carbonate (Na_2CO_3), disodium hydrogenphosphate (Na_2HPO_4), sodium dihydrogenphosphate (NaH_2PO_4), sodium hydroxide (NaOH), hydrochloric acid (HCl) were obtained from Kanto Chemical Co., Inc. All other solvents used were of analytical grade.

Plant materials

Three species, namely, *Quercus crispula* Blume, *Quercus salicina* Blume and *Quercus serrata* Thunb. were sampled from Ashiu Forest Research Station, Kyoto University, Japan in October, 2014. Voucher specimens have kept in laboratory of Department of Development Technology, Graduate School for International Development and Cooperation, Hiroshima University, Japan. For each species, leaves were collected at lower canopy from three individual trees in sunny places whereas barks were collected at 1 - 1.5 m in height by shaving with 3 mm thickness of the outer part. All samples were cut into small pieces and separately dried in an oven at 30 °C to obtain 14 - 15% moisture content. Afterward, leaves and barks were pulverized into fine powder in a grinding machine. An amount of ten grams (leaf or bark) powder of three individual trees of one species were combined and considered one sample.

Extraction of free phenolics

Dried powdered leaves and barks (3 g) of the three species were extracted with 100 mL ethanol (99.5%), stirred for 12 h at room temperature (25 °C). After extraction, the mixtures were centrifuged at 5000 rpm for 10 min followed by filtration. The residues were re-extracted using the same procedure once more. The two extracts were combined and then the solvent was removed in a

rotary evaporator at 30 °C. The precipitates were weighted, dissolved in methanol and kept in the dark at 4 °C for further analysis.

Extraction of bound phenolics
Bound phenolics were extracted following the method described previously by Xuan et al. [22] with some modifications. The residue from above free phenolic extraction was hydrolyzed with 50 mL of 4 M NaOH and stirred at 50 °C for 4 h. This suspension was centrifuged at 5000 rpm for 10 min followed by filtrating through the Advantec No. 1 filter paper (Toyo Roshi Kaisha, Ltd., Japan) and then neutralized to pH 1.5 with 37% hydrochloric acid. The filtrate was extracted four times with ethyl acetate. Subsequently, the extracts were combined and evaporated to dryness on a rotary evaporator at 30 °C. The dried extract was dissolved in methanol and then stored in the dark at 4 °C to further use.

Determination of total phenolic content
The total phenolic contents of the extracts were determined using the Folin-Cicalteau method described by Medini et al. [23]. Briefly, an amount of 0.125 mL of the extracts (free or bound) was mixed with 0.5 mL of distilled water and 0.125 mL of Folin-Cicalteau's reagent was added. After 6 min, 1.25 mL of 7.5% aqueous Na_2CO_3 solution was added. The solution was then adjusted to a final volume of 3 mL with distilled water and mixed vigorously. The mixture was incubated for 90 min at room temperature. The absorbance at 760 nm was recorded using a spectrophotometer (HACH DR/4000U-USA). The total phenolic content was expressed as mg of gallic acid equivalents (GAE)/g dry weight (DW).

Determination of total flavonoid content
The flavonoid content in extracts was determined using the method described by Djeridane et al. [24]. Sample extract (0.5 mL) was mixed with 0.5 mL of 2% aluminum chloride methanol solution. After 15 min at room temperature, the absorption was measured at 430 nm using a spectrophotometer (HACH DR/4000U-USA). The total flavonoid content was expressed as mg of rutin equivalents (RE)/g DW.

Antioxidant properties
DPPH radical scavenging activity
DPPH scavenging activity was evaluated according to the method described by Elzaawely et al. [10]. The various concentrations of 0.5 mL sample extracts were mixed with 0.25 mL of 0.5 mM DPPH and 0.5 mL of 0.1 M acetate buffer (pH 5.5). The mixtures were shaken vigorously and left at room temperature in the dark for 30 min. The reduction of the DPPH radical was determined by reading absorbance at 517 nm using a spectrophotometer (HACH DR/4000U-USA). BHT concentrations of 0.01 mg/mL to 0.05 mg/mL were used as a positive control. The percentage of DPPH radical scavenging was calculated as follows:

$$\text{Radical scavenging (\%)} = [(\text{abs}_{control} - \text{abs}_{sample})/\text{abs}_{control}] \times 100$$

The $\text{abs}_{control}$ is the absorbance of reaction without sample and abs_{sample} is the absorbance of reaction with sample. IC_{50} (inhibitory concentration) values were determined as inhibitory concentration of the extract necessary to decrease the initial DPPH radical concentration by 50% and were expressed in mg/mL. Lower IC_{50} value indicates higher DPPH radical scavenging activity. All measurements were performed in triplicate.

ABTS radical scavenging activity
The ABTS radical cation decolorization assay was used according to method of Re et al. [25]. Briefly, ABTS radical solution was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulfate in water and kept at room temperature in the dark for 16 h. Prior to use in the assay, ABTS radical solution was diluted with 80% methanol to give an absorbance of 0.70 ± 0.03 at 734 nm using a spectrophotometer (HACH DR/4000U-USA). Free radical scavenging activity was

assessed by mixing 30 µL of test sample or standard with 1 mL of methanolic ABTS radical solution and mixed thoroughly. The mixture was left in the dark at room temperature for 30 min and the absorbance was recorded at 734 nm. BHT standard solutions (concentrations 0.1 to 0.5 mg/mL) were prepared and assayed at the same conditions. All measurements were performed in triplicate. The percentage inhibition was calculated according to the formula:

$$\text{Radical scavenging (\%)} = [(\text{abs}_{control} - \text{abs}_{sample})/\text{abs}_{control}] \times 100$$

The $\text{abs}_{control}$ is the absorbance of reaction without sample and abs_{sample} is the absorbance of reaction with sample. The antioxidant property of test sample was defined by IC_{50} as the inhibitory concentration of the extract necessary for 50% reduction of ABTS and was expressed in mg/mL.

Reducing power

The reducing power was assayed by using the method described previously [2] with some slight modifications. Two hundred microliters of each extract or BHT was mixed with 0.5 mL phosphate buffer 0.2 M (pH 6.6) and 0.5 mL potassium ferricyanide $[K_3Fe(CN)_6]$ (10 g/L) in a test tube. The mixture was then incubated at 50 °C for 30 min. Afterwards, 0.5 mL of trichloroacetic acid (100 g/L) was added to the mixture, then centrifuged at 4000 rpm for 10 min. Finally, 0.5 mL of the supernatant was diluted with 0.5 mL of distilled water and 0.1 mL $FeCl_3$ solution (1 g/L) was added. The mixture was mixed thoroughly and the absorbance was measured at 700 nm using a spectrophotometer (HACH DR/4000U-USA). The assays were carried out in triplicate. The IC_{50} values were calculated at which the absorbance was 0.5. Lower IC_{50} indicates higher reducing power.

Antioxidant assay using the *β*-carotene bleaching system

The antioxidant activity of sample extracts was evaluated by the *β*-carotene linoleate bleaching system described by Elzaawely et al. [26]. *β*-carotene (2 mg) was dissolved in 10 mL of chloroform and 1 mL of the chloroform solution was mixed with 20 µL linoleic acid and 200 mg Tween-40. The chloroform was evaporated under vacuum at 45 °C. Afterward, 50 mL oxygenated water was added, the emulsion was vigorously shaken until complete homogenization was achieved. The obtained emulsion was freshly prepared before each experiment. A methanolic solution 0.12 mL of sample extract or BHT (1 mg/mL) were mixed with 1 mL of the emulsion. An equal amount of methanol was used for negative control. The solutions were incubated at 50 °C and recorded at 492 nm using a spectrophotometer (HACH DR/4000U-USA). All extracts were measured at zero time and every 15 min up to 180 min. The assays were carried out in triplicate for each extract. Lipid peroxidation inhibition (LPI) was calculated using the following equation: LPI (%) = $A_1/A_0 \times 100$. Where A_0 is the absorbance value measured at zero time for the test sample, while A_1 is the corresponding absorbance value measured after incubation for 180 min. Higher LPI value shows the higher antioxidant activity.

Identification and quantification by HPLC

Phenolic acid identification was performed using liquid chromatography with UV detection. The phenolic compositions of different samples were determined at 254 nm by using HPLC system, Japan (LC-Net II/ADC, UV-2075 Plus and PU-2089 Plus), the column Jasco RPC18 (250 mm x 4.6 mm x 5 µm). The mobile phase was methanol 99.8% (solvent A) and 0.1% acid acetic (v/v) (solvent B). A gradient elution was run with 1 mL/min flow-rate using the following time gradients: 5% A (0 - 5 min), 20% A (5 - 10 min), 50% A (10 - 20 min), 80% A (20-30 min), 100% A (30 - 50 min), 5% A (50-60 min). Phenolic standards (0.01 - 0.1 mg/mL) and extracts (1 mg/mL) were injected to HPLC with an amount of 5 µL.

Statistical analysis

The data were analyzed by one – way ANOVA using the Minitab 16.0 software for Window. Upon significant differences, means were separated using Tukey's test at $p < 0.05$ with three replications and expressed as the mean ± standard errors (SE).

3. RESULTS

Total phenolics and flavonoids

The phenolic and flavonoid contents in both free and bound forms from leaves and barks varied among *Q. crispula, Q. salicina and Q. serrata* (Table 1). In general, amounts of free phenolics and flavonoids were much higher than the bound forms. The leaf and bark extracts of *Q. salicina* contained significant higher amount of total phenolics than other extracts. Meanwhile, the leaf extract of *Q. serrata* showed the highest value of total flavonoids.
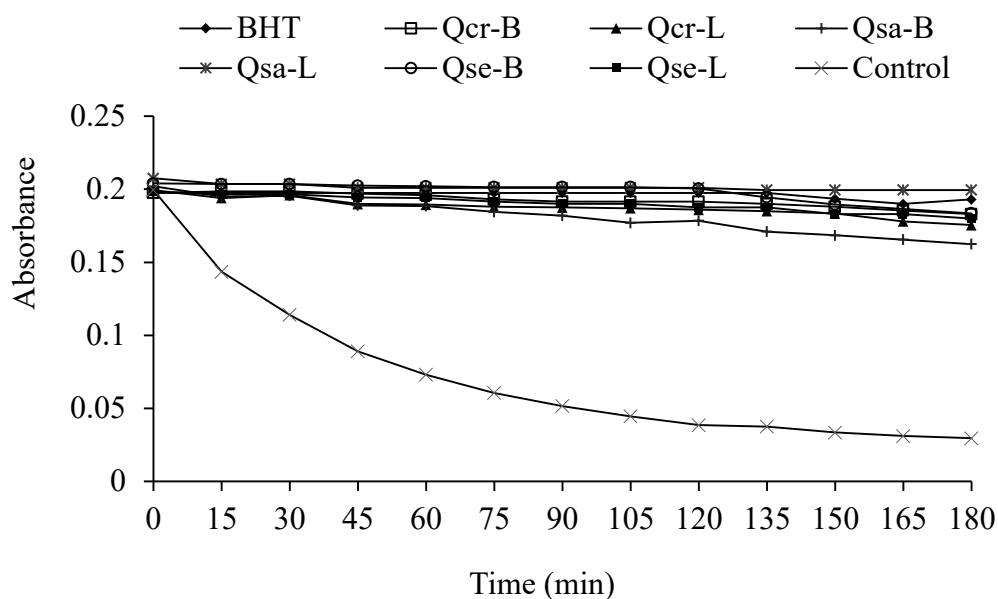
Antioxidant activities

In DPPH scavenging activity, the free phenolics in barks of *Q. salicina* and *Q. serrata* showed similar strength (IC_{50} = 0.031 and 0.026 mg/mL, respectively) to that of BHT ($p > 0.05$), which was used as a positive control in all assays of antioxidant activity trials. Both free and bound extracts in leaves of *Q. salicina* were potential (IC_{50} = 0.067 and 0.079 mg/mL, respectively) (Table 2). In the ABTS assay, none of the extracts showed similar levels as the BHT did, but the maximum activities were the free extracts of barks of *Q. salicina* and *Q. serrata* (IC_{50} = 0.287 and 0.286 mg/mL, respectively (Table 2).

In the reducing power assay, the antioxidant activity of free phenolic extracts in bark of *Q. serrata* was the most potential (IC_{50} = 0.227 mg/mL) and it was not significantly different from that of BHT ($p > 0.05$), followed by free phenolic extracts in bark of *Q. salicina* (IC_{50} = 0.274 mg/mL). The other free phenolic extracts were much lower magnitudes of antioxidant activities, as compared to that of BHT (Table 3). Regarding bound phenolics, the leaf extract of *Q. salicina* showed the strongest antioxidant activity, which was not significantly different from that of BHT ($p > 0.05$); other bound phenolic extracts did not show any potential reducing power activity (Table 3).

In the *β*-carotene bleaching method, all the extracts inhibited *β*-carotene oxidation. The free phenolic extracts exhibited a superior inhibition compared to BHT (Figure 1A) whereas bound phenolic extracts showed a lower inhibition than BHT (Figure 1B). Additionally, as showed in Table 3, the LPI% of the free phenolics in leaves of *Q. salicina* and *Q. serrata*, and barks of *Q. crispula* indicated the strongest activity comparable to that of BHT ($p > 0.05$). Beside, the free extract in bark of *Q. salicina* was also potential. In contrast to the free phenolic extracts, none of the bound phenolic extracts showed similar antioxidant activity as BHT did (Table 3).
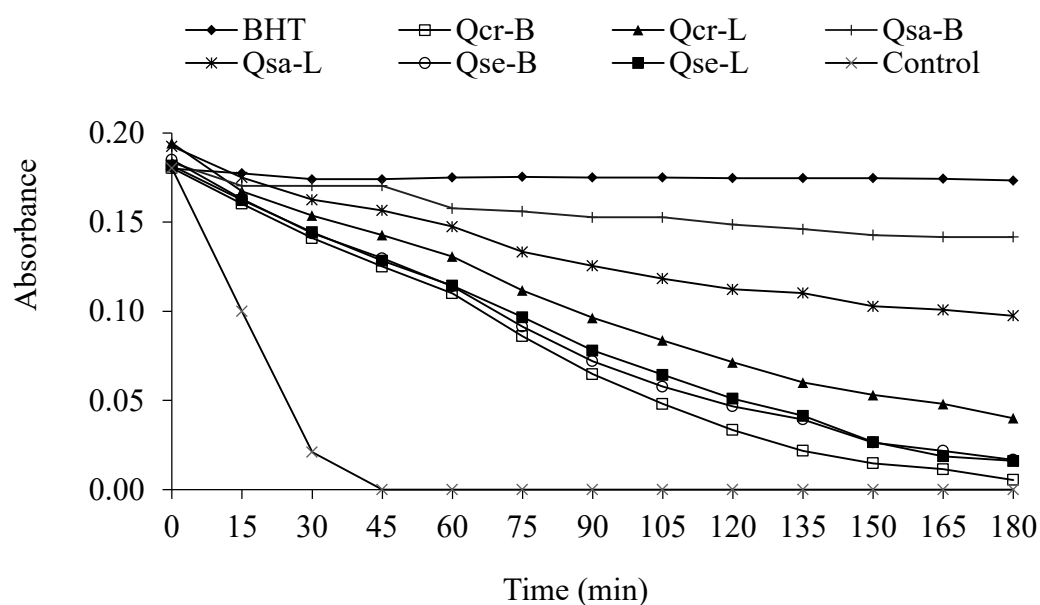
(A)

(B)

Figure 1. Antioxidant activity of (A) free phenolics and (B) bound phenolics of *Quercus* extracts measured by *β*-carotene bleaching method.

Qcr: *Q. crispula*, Qsa: *Q. salicina*, Qse: *Q. serrata*

B: bark; L: leaf

BHT was used as positive control

Table 1. Free, bound and total phenolics and flavonoids of *Quercus* extracts

Sample	Phenolics (mg GAE/g DW)			Flavonoids (mg RE/g DW)		
	Free	Bound	Total phenolics	Free	Bound	Total flavonoids
Qcr-B	9.67 ± 0.04e	6.79 ± 0.09c	16.45 ± 0.12d	1.33 ± 0.04c	1.01 ± 0.01c	2.33 ± 0.04d
Qcr-L	4.22 ± 0.05f	7.95 ± 0.16b	12.25 ± 0.12e	1.22 ± 0.02c	2.97 ± 0.11b	4.18 ± 0.13c
Qsa-B	32.26 ± 0.13a	3.64 ± 0.11e	35.89 ± 0.24b	0.74 ± 0.01c	0.68 ± 0.00c	1.41 ± 0.00d
Qsa-L	27.93 ± 0.28b	18.37 ± 0.43a	46.30 ± 0.18a	7.88 ± 0.15b	16.84 ± 0.52a	24.72 ± 0.66b
Qse-B	11.49 ± 0.05d	4.66 ± 0.12d	16.15 ± 0.13d	1.26 ± 0.03c	1.23 ± 0.03c	2.49 ± 0.00d
Qse-L	22.59 ± 0.08	3.38 ± 0.09e	25.97± 0.17c	27.10 ± 0.32a	1.08 ± 0.01c	28.18 ± 0.33a

Values represent means ± SE (n = 3). Different letters in the same column indicate significant differences ($p < 0.05$)
Qcr: *Q. crispila*; Qsa: *Q. salicina*; Qse: *Q. serrata*
B: bark; L: leaf

Table 2. DPPH and ABTS radical scavenging assay of *Quercus* extracts

	DPPH (IC$_{50}$ mg/mL)		ABTS (IC$_{50}$ mg/mL)	
	Free	Bound	Free	Bound
Qcr-B	0.158 ± 0.000a	0.446 ± 0.009c	1.047 ± 0.008a	2.074 ± 0.054c
Qcr-L	0.100 ± 0.000c	0.333 ± 0.003d	1.008 ± 0.007a	1.803 ± 0.015c
Qsa-B	0.031 ± 0.000e	0.302 ± 0.004e	0.287 ± 0.002d	1.565 ± 0.052d
Qsa-L	0.067 ± 0.001d	0.079 ± 0.001f	0.523 ± 0.011c	0.559 ± 0.006e
Qse-B	0.026 ± 0.002e	565 ± 0.006b	0.296 ± 0.016d	2.796 ± 0.154b
Qse-L	0.118 ± 0.003b	0.662 ± 0.012a	0.619 ± 0.003b	3.158 ± 0.064a
BHT	0.027 ± 0.001e	0.027 ± 0.001g	0.184 ± 0.003e	0.184 ± 0.003f

Values represent means ± SE (n = 3). Different letters in the same column indicate significant differences ($p < 0.05$)
BHT (dibutyl hydroxytoluene) was used as positive control
Qcr: *Q. crispila*; Qsa: *Q. salicina*; Qse: *Q. serrata*
B: bark; L: leaf

Table 3. Reducing power and β-carotene/linoleic acid bleaching assay of *Quercus* extracts

Sample	Reducing power (IC$_{50}$ mg/mL)		β-carotene/linoleic acid inhibition (LPI %)	
	Free	Bound	Free	Bound
Qcr-B	0.940 ± 0.017a	1.828 ± 0.040b	92.66 ± 1.25abc	2.95 ± 1.20e
Qcr-L	0.578 ± 0.002b	1.549 ± 0.107b	87.97 ± 1.47c	20.61 ± 1.01d
Qsa-B	0.274 ± 0.001e	1.223 ± 0.019bc	80.43 ± 1.03d	77.26 ± 3.41b
Qsa-L	0.371 ± 0.004d	0.557 ± 0.025cd	96.15 ± 0.01ab	64.31 ± 1.19c
Qse-B	0.227 ± 0.001ef	3.316 ± 0.373a	89.92 ± 1.42bc	9.01 ± 0.83e
Qse-L	0.490 ± 0.018c	3.256 ± 0.097a	90.92 ± 1.61abc	8.81 ± 0.64e
BHT	0.186 ± 0.002f	0.186 ± 0.002d	97.47 ± 1.01a	96.49 ± 1.01a

Values represent means $\pm$ SE (n = 3). Different letters in the same column indicate significant differences ($p < 0.05$)
BHT was used as positive control
Qcr: *Q. crispila*; Qsa: *Q. salicina*; Qse: *Q. serrata*
B: bark; L: leaf

Correlation between phenolic, flavonoid contents and antioxidant activities

As shown in Table 4, highly significant and positive correlation ($R^2 = 0.532$ - 0.730) were observed between phenolic contents and DPPH, ABTS, reducing power and β-carotene/linoleic acid inhibition, while non significant correlation was found between flavonoid contents and antioxidant activities. These results may confirm that phenolic contents are responsible for the antioxidant activities of extracts.

Table 4. Correlation coefficients between antioxidant activities and phenolic, flavonoid contents of *Quercus* extracts

|	Correlation R^2	
	Phenolic contents	Flavonoid contents
1/IC$_{50}$ of DPPH radical scavenging ability	0.557^{**}	-0.041
1/IC$_{50}$ of ABTS radical scavenging ability	0.730^{**}	0.143
1/IC$_{50}$ of Reducing power	0.701^{**}	0.199
β-carotene/linoleic acid inhibition (LPI%)	0.532^{**}	0.326

** Correlation is significant at the 0.01 level

Identification and quantification of phenolic compounds

Phenolic compounds of extracts were identified by comparing retention times of fifteen standard phenolic acids separated by HPLC (Figure 2A). The results showed that thirteen phenolic acids including gallic, protocatechuic, chlorogenic, *p*-hydroxybezoic, vanillic, syringic, ferulic, sinapic, *p*-coumaric, benzoic, ellagic, cinnamic acids and vanillin in free and bound phenolic extracts were identified (Table 4). Ellagic, chlorogenic and benzoic acids were the major phenolic compounds and their amounts varied among *Quercus* species, plant organs and extracting solvents. Gallic and vanillic acids were solely detected in *Q. salicina* whereas syringic and cinnamic acids were only found in *Q. serrata*. In general, the free phenolic extracts obtained higher quantities as well as greater numbers of phenolic acids as compared to that of bound phenolic extracts, with exception of *Q. salicina* leaves (Table 4). In the free phenolics in leaf extracts of *Q. serrata* had the highest amount of phenolic acids (3.99 mg/g DW), followed by bark and leaf extracts of *Q. salicina* (3.63 mg/g 3.24 DW, respectively) (Table 4). The bound phenolics in leaf extract of *Q. salicina* was detected eight phenolic acids (Figure 2B) followed by the free phenolics in bark extract of *Q.*

serrata (six phenolic acids), whereas other extracts detected either three or four phenolic acids among the fifteen standard constituents (Table 4).
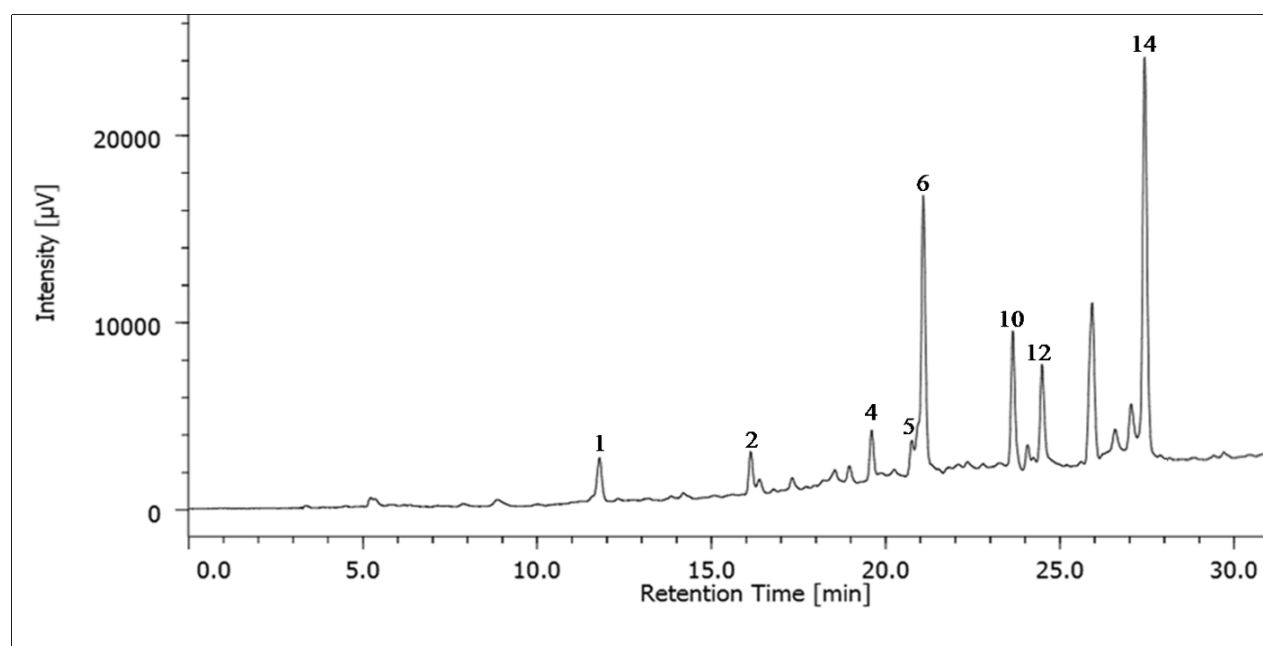
(A)
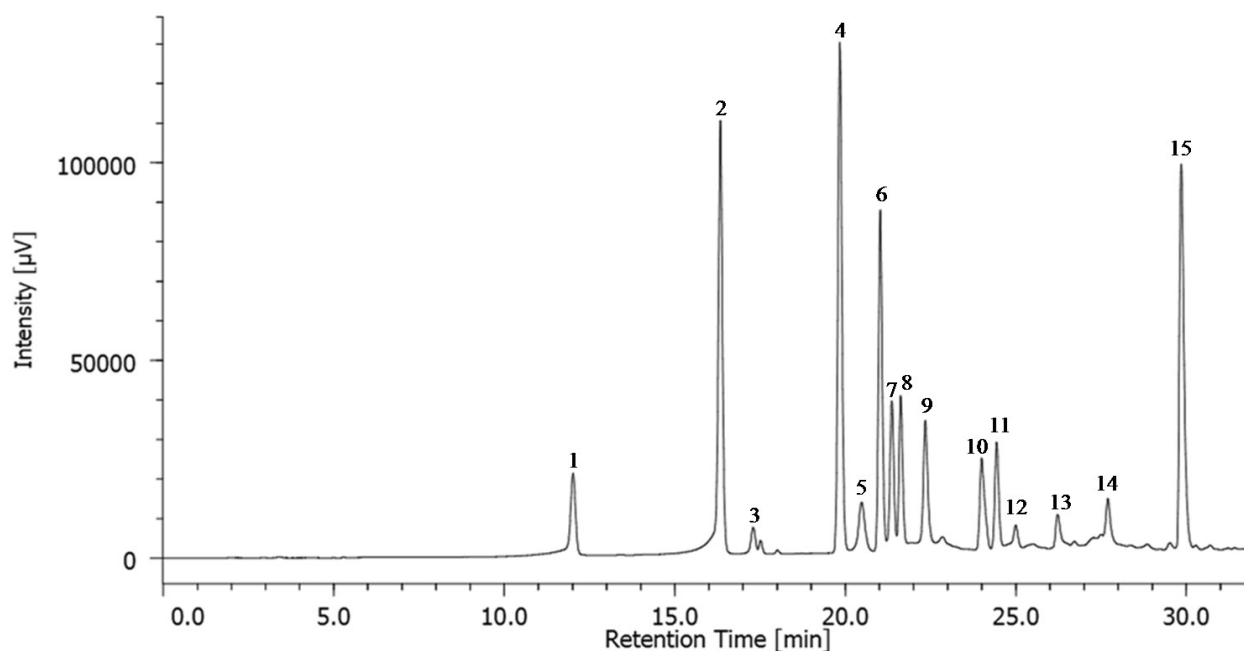
(B)

Figure 2. Chromatogram of phenolic standard mixture at 254 nm (A) and chromatogram of bound phenolic extract of *Q. salicina* leaves (B)
Peak 1: gallic acid; 2: protocatechuic acid; 3: catechol; 4: chlorogenic acid; 5: *p*-hydroxybenzoic acid; 6: vanillic acid; 7: caffeic acid; 8: syringic acid; 9: vanillin; 10: ferulic acid; 11: sinapic acid; 12: *p*-coumaric acid; 13: benzoic acid; 14: ellagic acid; 15: cinnamic acid.

Table 5. Quantification of major phenolic acids (mg/g DW) in *Quercus* extracts

Sample	GA	PA	ChA	p-HY	VA	SyA	V	FA	SiA	p-CA	BA	EA	CA	Total
Qcr-B[f]	-	-	-	-	-	-	0.47 ± 0.00a	-	-	-	-	2.33 ± 0.02a	-	2.8
Qcr-B[b]	-	-	-	-	-	-	-	0.63 ± 0.00a	-	-	-	-	-	0.63
Qcr-L[f]	-	-	-	-	-	-	-	-	0.31 ± 0.01b	0.06 ± 0.00b	0.47 ± 0.10bc	0.08 ± 0.01d	-	0.92
Qcr-L[b]	-	-	1. 1.01 ± 0.05ab	-	-	-	-	-	-	-	-	0.83 ± 0.16bcd	-	1.84
Qsa-B[f]	-	-	1.55 ± 0.01a	0.25 ± 0.06a	-	-	-	-	-	-	-	1.83 ± 0.17ab	-	3.63
Qsa-B[b]	0.03 ± 0.00b	-	0.32 ± 0.00c	-	-	-	0.16 ± 0.01c	-	-	0.11 ± 0.01b	-	-	-	0.62
Qsa-L[f]	-	-	-	-	-	-	-	-	-	-	2.87 ± 0.00a	0.37 ± 0.00cd	-	3.24
Qsa-L[b]	0.12 ± 0.01a	0.15 ± 0.00a	0.93 ± 0.01ab	0.11 ± 0.00ab	0.72 ± 0.04	-	-	0.59 ± 0.01b	-	0.54 ± 0.03a	-	1.13 ± 0.03bc	-	4.29
Qse-B[f]	-	0.06 ± 0.00b	0.35 ± 0.00c	-	-	0.39 ± 0.01	0.22 ± 0.00b	-	-	-	-	1.29 ± 0.05b	0.30 ± 0.01	2.61
Qse-B[b]	-	-	0.68 ± 0.00bc	-	-	-	-	0.35 ± 0.00c	-	-	0.36 ± 0.08c	-	-	1.39
Qse-L[f]	-	-	1.13 ± 0.04ab	-	-	-	-	-	1.81 ± 0.18a	-	1.05 ± 0.20 b	-	-	3.99
Qse-L[b]	-	-	-	0.02 ± 0.00b	-	-	-	-	0.59 ± 0.02b	-	-	0.33 ± 0.01d	-	0.94

Values represent means ± SE (n = 3). Different letters in the same column indicate significant differences ($p < 0.05$)

(-) not detected

GA: gallic acid; PA: protocatechuic acid; ChA: chlorogenic acid; p-HY: *p*-hydroxybenzoic acid; VA: vanillic acid; SyA: syringic acid; V: vanillin; FA: ferulic acid; SiA: sinapic acid; p-CA: *p*-coumaric acid; BA: benzoic acid; EA: ellagic acid; CA: cinnamic acid

Qcr: *Q. crispila*; Qsa: *Q. salicina*; Qse: *Q. serrata*

B: bark; L: leaf

[b]: bound phenolic extract (ethyl acetate extract)

[f]: free phenolic extract (ethanol extract)

4. DISCUSSION

Plants are rich in phenolics and flavonoids that produce beneficial effects by protecting cells against the oxidative damage caused by free radicals [27]. They are considered as one of the most potent and therapeutically useful natural components because of the antioxidant, anticancer, antimicrobial, antiviral and anti-inflammatory activities [28]. Total phenolic contents of plants depend on species and different organs [23]. Comparing with previous results reported in some other *Quercus* species, total phenolic contents of all extracts in this study showed higher quantities. Santos et al. [29] noted that cork of *Quercus super* obtained 2.4 - 10.6 mg GAE/g DW while twigs and acorns extracts of *Quercus robur* and *Quercus petraea* showed 7.44 and 16.25 mg catechin/g DW respectively. Other reports included the total phenolic contents in leaf extracts of *Q. robur* and *Q. petraea* ranged from 32 - 35 mg catechin/g DW [30] and *Quercus resinosa*, *Quercus laeta,* and *Quercus obtusata* (5.7 - 7.1 mg GAE/g DW) [31]. In addition, total phenolic contents in leaf and bark extracts of *Q. salicina* were higher than some medicinal plants in previous studies such as *Aristolochia mollissima, Lobelia chinensis*, *Dianthus superbus* and *Centipeda minima* (3.9 - 5.9 mg/g DW) [32]. Flavonoids are the chief of phenolic compounds and they also have potent antioxidant activities with positive effects on human health [33]. The total flavonoid content in leaves of *Q. salicina* and *Q. serrata* were much higher than that of *Launaea procumbens* which as a food supplement in Pakistan (4 - 13 mg RE/g DW) [34]. From the results, this study concluded that leaves of *Q. salicina* and bark of *Q. serrata* may obtain rich phenolics and should be further elaborated to exploit their biological properties.

Antioxidant capacity is widely used as a parameter for checking the activity of plant extracts [2]. Natural bioactive compounds are considered as an important alternative to the synthetic antioxidants, which are used as functional food to prevent human diseases [35]. Many previous studies showed a direct correlation between the phenolic contents and the antioxidant activity [36,37]. This study showed a highly significant and positive correlation between phenolic contents and DPPH, ABTS. Therefore, free extracts contained a higher amounts of phenolic contents which may be the reason for stronger radical scavenging activities. However, the antioxidant activity of plant extracts may relate to the presence of some individual phenolic compounds and their structures [24]. In addition, the position and quantitation of hydroxyl groups are possibly one of the main factors [38]. If an additional hydroxyl group is added to *ortho* or *para* position of the benzen ring, the antioxidant activity will be significantly increased, principally the *ortho* position can create the intramolecular hydrogen bond, so such position is proposed more effective than *para* and *meta* one [39,40]. As can be seen in the free phenolic extract of *Q. serrata* bark (Table 1), this species showed moderate phenolic contents but it had the highest antioxidant activity in DPPH assay. Moreover, syringic and cinnamic acids were only found in bark extract of *Q. serrata.* These compounds may have an important contribution to antioxidant activity. Comparing to previous studies, results of the DPPH assay revealed that the free phenolic extracts from *Q. salicina* and *Q. serrata* were superior to stem, flower and leaf extracts of *Alpinia zerumbet* [41]. Moreover, the antioxidant activity in term of DPPH scavenging in bark extracts of *Q. salicina* and *Q. serrata* were higher than all *Limnophila aromatic* extracts which were obtained by vigorously stirring with different aqueous solvents including methanol, ethanol and acetone [42]. Similarly, in ABTS assay we found that both bark and leaf of *Q. salicina* extracts contained higher concentration of total identified phenolics. From the results we stated that *Q. salicina* extracts are the most potential sources of natural antioxidant. Reducing power represents a crucial mechanism of phenolic antioxidant action because the total phenolic content and ferric reducing power have high relation [43]. Furthermore, an amino group and different substituents on the phenyl of chalcone moiety play an important role in the reducing power compounds [44]. The phenolic compounds obtained from three *Quercus* species might contain a number of reductants which may react with the free radicals to stabilize and terminate from free radical chain reaction. The reducing power of free phenolic compounds were more pronounced than those of bound phenolics probably because the free phenolics act as strong reducing agents by donating electrons and converted free radicals to more stable molecules [2,45]. The *β*-carotene bleaching method is based on loss of yellow color of *β*-carotene because the free radical was generated from linoleic acid can highly attack unsaturated *β*-carotene. The rate inhibition of linoleic acid oxidation by free phenolics was high compared to standard BHT and the values from previous research [46]. In this study, leaves of *Q. salicina* and *Q. serrata*, and barks of *Q. crispula* were the most potent in *β*-carotene assays (Table 3). By comparing the antioxidant activities using DPPH radical scavenging, ABTS radical scavenging, reducing power, and *β*-carotene-linoleate bleaching methods, it is observed that their activities varied among solvents and plant parts (leaves and barks). In general, the free phenolic extracts of all samples showed stronger antioxidative levels than the bound phenolic extracts (Tables 2,3).

Results of HPLC analysis demonstrated that amounts and types of phenolic compounds varied among oak species, leaves and barks (Table 4). In previous reports, several phenolic acids were identified in other *Quercus* species such as chlorogenic, cinnamic, gallic, gentisic acids and (+) catechin [47]; gallic, protocatechuic, ellagic acids and esculetin were the major compounds in corks of *Q. super* [29] or quercetin in stems of *Q. salicina* [16]. Ellagic acid also was identified as the major constituent of *Q. suber* from Spain and Portugal [35]. In this study, ellagic acid was the predominant composition, followed by chlorogenic and benzoic acids. Ellagic acid was a compound has four hydroxyl groups with multi-functions for anti-mutagenic, antimicrobial and antioxidant activities. Moreover, this compound was an inhibitor of human immune deficiency virus (HIV) [48,49]. As a result of inhibiting cell cycle progression and inducing apoptosis, ellagic acid may be considered as a product of cytotoxicity in tumor cells [50]. Chlorogenic acid is an important antioxidant in plants to ameliorate the damaging effects of oxidative stress in plants and hinder

degenerative diseases in animals when supplied in their diet [51]. Benzoic acid is extensively used as preservatives and flavor enhancers, analgesics, antiseptics, chemotherapeutics for chemical syntheses [52,53].

5. CONCLUSIONS

This study proposed that *Q. crispula, Q. salicina, Q. serrata* possessed high total phenolic contents and appeared to be strong radical scavengers. Thirteen phenolic compounds were identified from bark and leaf extracts. Ellagic, chlorogenic and benzoic acids were the dominant phenolic compounds in *Quercus* species. Evidences obtained in this study also suggested that among three species of oak, *Q. salicina* was the most potential and should be considered as a rich source of natural antioxidants. Further searches for potent and novel antioxidants in these *Quercus* species should be needed.

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