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Flavonoids from the leaves and twigs of *Lindera sericea* (Seibold et Zucc.) Blume var. *sericea* (Lauraceae) from Japan and their bioactivities

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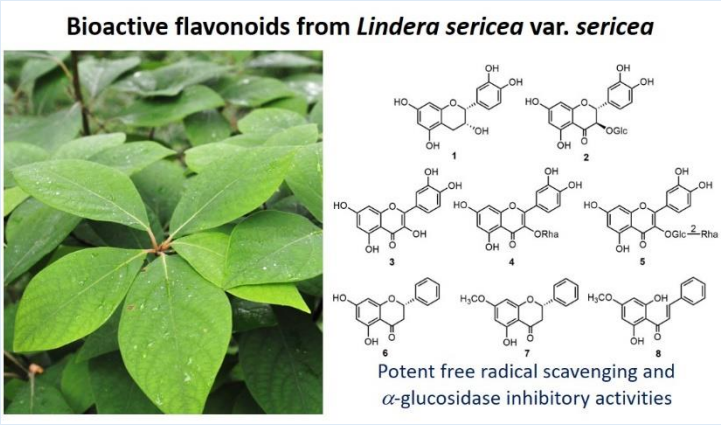
ABSTRACT



Background: The leaves and twigs of *Lindera sericea* (Seibold et Zucc.) Blume var. *sericea* (Lauraceae) are used as traditional medicines for treating indigestion, stomachache, anxiety, etc. In recent years, there has been a growing interest in these plant materials as a source of healthy drinks and functional foods. The main aim of this study was to characterize the major phenolic compounds from the leaves and twigs and to evaluate their free radical scavenging and α-glucosidase inhibitory activities.

Methods: The dried leaves and twigs were extracted with 70% methanol. The dried extract was then subjected to repeated column chromatography to isolate eight flavonoids. The compounds were then evaluated for their 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity and α-glucosidase inhibitory activity.

Results: The isolated compounds were identified as (-)-epicatechin (**1**), taxifolin 3-*O*-glucoside (**2**), quercetin (**3**), quercitrin (**4**), quercetin 3-*O*-neohesperidoside (**5**), pinocembrin (**6**), pinostrobin (**7**) and pinostrobin chalcone (**8**) based on their nuclear magnetic resonance (NMR), spectroscopic data and comparison with literature values. All these compounds were isolated for the first time from this plant. All flavonoids except pinocembrin (**6**), pinostrobin (**7**) and pinostrobin chalcone (**8**) showed potent free radical scavenging activity. In α-glucosidase inhibitory activity assay, quercetin (**3**), quercitrin (**4**) and taxifolin 3-*O*-glucoside (**2**) showed potent activity.

Conclusions: Eight flavonoids were reported for the first time from the leaves and twigs of the title plant. Some of these compounds showed potent free radical scavenging and α-glucosidase inhibitory activities.

Keywords: *Lindera sericea* var. *sericea*; Lauraceae; Kekuromoji; free radical; α-glucosidase

INTRODUCTION

Medicinal plants are an integral part of primary healthcare for more than 70% of the world's population and plant-derived natural products have also played an important role in the discovery and development of modern drugs [1–4]. Besides that, various medicinal plants are also used as foods, spices, food supplements, food colorants, food preservatives, sweeteners, etc. [5–8]. In recent years, there has been a growing interest in plant-based functional foods, food supplements and nutraceuticals as potential agents in the prevention and treatment of diseases [9–11]. Plant polyphenols, including flavonoids and phenolic acids, are widely studied for their health beneficial properties [11–14]. For the development of safe and effective functional foods, the bioactive constituents in plant materials, extracts and final formulations should be adequately characterized. Similarly, their pharmacological activities should be evaluated using *in vitro* and *in vivo* activity evaluation methods. Their effectiveness on humans should be evaluated in clinical studies; on the other hand, the toxicity profiles of these extracts/products should also be assessed.

The Lauraceae family is comprised of about 50 genera and about 3,000 plant species having pantropical distribution, and many of these species are widely used as nutritious foods, medicines and spices [15,16]. The genus *Lindera* consists of about 100 species distributed in the temperate to tropical regions of Asia and North America [16–18]. Many species of *Lindera* genus are used for the treatment of cholera, diarrhea, nausea, rheumatoid arthritis, stroke, toothache, etc. in various traditional medicines [17,19,20]. Fatty and essential oils obtained from *Lindera* plants have a pleasant smell and are used to make fragrances, lubricants, soaps and spices [17,21]. Twigs are also used to make toothpicks [21]. In Japan, nine species of *Lindera* are reported along with some varieties [18]. *Lindera*

sericea (Seibold et Zucc.) Blume var. *sericea* ("Kekuromoji" in Japanese) is a deciduous shrub distributed in the Honshu, Shikoku and Kyushu islands of Japan [18]. Various plant parts of *Lindera sericea* var. *sericea*, *Lindela umbellata* Thunb. var. *umbellata* ("Kuromoji" in Japanese) and related species are widely used as traditional medicines for treating indigestion, stomachache, anxiety, etc. [22]. These plants have been used as the source of healthy drinks and functional foods in recent years. There are few studies reported about the constituents for essential oils obtained from the leaves [21,23,24] and alkaloids from the roots of *L. sericea* var. *sericea* [25]. However, there is only one report about the flavonoid of the leaves, where kaempferol-3,7-*O*-di-rhamnopyranoside was isolated and identified [26]. During the preliminary screening of different plant extracts for their free radical scavenging and enzyme inhibitory activities, the 70% methanol (MeOH) extract of the leaves and twigs of *Lindera sericea* var. *sericea* showed potent α-glucosidase inhibitory activity (IC_{50} = 9.51 μg/ml) and moderate antioxidant activity on DPPH free radical assay (IC_{50} = 25.82 μg/ml) [27]. As there are no detailed studies reported about the bioactive constituents of *L. sericea* var. *sericea*, the main aim of this study was to isolate and identify the major phenolic compounds from the leaves and twigs and to evaluate their free radical scavenging and α-glucosidase inhibitory activities.

Figure 1. Photographs of *Lindera sericea* var. *sericea*

METHODS

General Experimental Procedure: ^{1}H- and ^{13}C-NMR spectra were measured on BRUKER AVANCE 600 NMR Spectrometer (Bruker, Billerica, MA, USA) (^{1}H-NMR: 600 MHz and ^{13}C-NMR: 150 MHz). Chemical shift values (δ_H and δ_C) are given in ppm with reference to tetramethylsilane (TMS). Absorbance was recorded on ARVO MX 1420 Multilabel Counter Microplate Reader (Perkin-Elmer, Yokohama, Japan) at room temperature. Column chromatography (CC) was carried out with MCI gel CHP20P (75~ 150 µm, Mitsubishi Chemical Industries Co. Ltd., Tokyo, Japan), Sephadex LH-20 (Amersham Pharmacia Biotech, Tokyo, Japan) and silica gel 60 (0.040-0.063 mm, Merck KGaA, Darmstadt, Germany). Thin-layer chromatography (TLC) was performed on a pre-coated silica gel 60 F_{254} (Aluminum sheet, Merck KGaA, Darmstadt, Germany).

Plant Material: The fresh leaves and twigs of *L. sericea* var. *sericea* were collected from Yasuhara Town, Kochi Prefecture, Japan in October 2015 and identified by one of the authors (Dr. T. Watanabe). They were dried under shade for two weeks. A voucher specimen (Voucher No.: KUT(Exp)320) was deposited at the Museum of Traditional Medicines, School of Pharmacy, Kumamoto University, Kumamoto, Japan.

Chemicals: 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and α-glucosidase (from *Saccharomyces cerevisiae*) were purchased from Sigma Aldrich, Co. (Tokyo, Japan). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and Acarbose were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). 2-Morpholinoethanesulfonic acid monohydrate (MES) was purchased from Dojindo Chemical Research (Kumamoto, Japan). All other chemicals used were of analytical grade.

Extraction and Isolation: The dried leaves and twigs of *L. sericea* var. *sericea* (2.6 kg) were extracted twice with 70% MeOH (18 L) at room temperature for two weeks. The filtered extracts were combined and evaporated under reduced pressure to give 255.0 g extract. The extract was then subjected to MCI gel CHP20P column chromatography (CC) and eluted successively with water, 40%, 60%, 80% and 100% MeOH to give ten fractions (Fr. 1~Fr. 10). Fraction 2 (20.5 g, water eluate) was subjected to Sephadex LH-20 CC (MeOH) to obtain six subfractions (SubFr. 2-1~SubFr. 2-6). Among them, subfraction 2-2 (651.4 mg) was subjected on ODS CC (30%, 35%, 40% MeOH) to obtain compounds **2** (42.7 mg) and **5** (11.2 mg). Subfraction 2-4 (2.8 g) was subjected on ODS CC (30% MeOH) to obtain compound **1** (157.1 mg). Fraction 3 (23.6 g, 40% MeOH eluate) and fraction 4 (4.9 g, 40% MeOH eluate) were combined and subjected to Sephadex LH-20 CC (MeOH) to obtain four subfractions (SubFr. 3-1~ SubFr. 3-4). Subfraction 3-2 (3.6 g) was subjected on ODS CC (10%, 20%, 30%, 40%, 50% MeOH) and silica gel CC (CH_2Cl_2:MeOH:H2O = 7:3:0.5) to obtain compound **1** (88.8 mg), **4** (225.8 mg) and **3** (228.0 mg). Fraction 7 (19.9 g, 80% MeOH eluate) was subjected to Sephadex LH-20 CC (MeOH) to obtain compound **6** (6015 mg). Fraction 8 (3.2 g, 100% MeOH) was subjected to Sephadex LH-20 CC (MeOH) to obtain three subfractions (SubFr. 8-1~8-3). Subfraction 8-2 (1.2 g) was subjected to silica gel CC (CH_2Cl_2:MeOH = 10:1) to obtain nine subfractions (8-2-1 ~ 8-2-9). Subfraction 8-2-2 (782.2 mg) was repeatedly applied to silica gel CC (CH_2Cl_2:MeOH:H_2O = 7:3:0.5) to obtain compounds **7** (65.2 mg) and **8** (11.5 mg). Fraction 9 (2.2 g, 100% MeOH eluate) was subjected to silica gel CC (CH_2Cl_2:MeOH = 10:1) to obtain eight subfractions (SubFr. 9-1~ SubFr.9-8). Subfraction 9-3 (14.1 g) was subjected to silica gel CC (CH_2Cl_2:MeOH = 50:1) to obtain compound **7** (7400 mg).

Evaluation of Biological Activities: The evaluations of DPPH free radical scavenging activity and α-glucosidase inhibitory activity were performed using the methods reported previously [28].

RESULTS

The dried leaves and twigs of *L. sericea* var. *sericea* (2.6 kg) were extracted with 70% MeOH to obtain 255.0 g of extract. The extract was then subjected to repeated column chromatography on MCI gel CHP20P, Sephadex LH20, ODS and silica gel to isolate eight pure compounds (**1**–**8**). The structures of these compounds were elucidated on the basis of NMR spectral data and by comparison with literature as (-)-epicatechin (**1**) [29], taxifolin 3-*O*-glucoside (**2**) [30], quercetin (**3**) [31], quercitrin (**4**) [31], quercetin 3-*O*-neohesperidoside (**5**), pinocembrin (**6**), pinostrobin (**7**) [32,33] and pinostrobin chalcone (**8**) [34] (Figure

2). The ^{1}H- and ^{13}C- NMR spectral data of these compounds are given in Table 1 and 2, respectively. All of these compounds were isolated for the first time from this plant. Pinocembrin (**6**, 6015 mg) and pinostrobin (**7**, 7465 mg) were isolated in large quantities, which can be considered as marker compounds in the leaves and twigs of *L. sericea* var. *sericea*.

Table 1. ^{1}H-NMR spectroscopic data of isolated compounds

Position	1[a]	2[a]	3[b]	4[b]	5[a]	6[a]	7[a]	8[a]
2	4.84, brs	5.23, d (9.7)				5.44, dd (3.1, 12.8)	5.48, dd (2.9, 12.8)	7.62, dd (1.7, 7.7)
3	4.21, brs	4.93, d (9.6)				2.76, dd (12.8, 17.1) 3.07, dd (3.1, 17.1)	2.80, dd (2.9, 17.1) 3.11, dd (12.8, 17.1)	7.41, m
4	2.88, dd (16.7, 4.6) 2.79, dd (16.7, 2.8)							7.41, m
6	5.98, brs	5.89, d (1.9)	6.21, d (1.8)	6.19, d (2.1)	6.19, d (2.1)	5.89, d (2.2)	6.05, d (2.2)	7.62, dd (1.7, 7.7)
8	5.98, brs	5.91, d (1.9)	6.44, d (1.8)	6.34, d (2.1)	6.38, d (2.1)	5.93, d (2.2)	6.08, d (2.2)	7.74, d (15.7)
2'	6.99, d (1.8)	6.96, d (1.9)	7.70, d (1.8)	7.34, d (2.1)	7.34, d (2.1)	7.37, m	7.40, m	
3'						7.37, m	7.40, m	6.05, d (2.3)
4'						7.37, m	7.40, m	
5'	6.80, d (8.2)	6.78, d (8.1)	6.92, d (8.5)	6.91, d (8.2)	6.91, d (8.3)	7.37, m	7.40, m	6.09, d (2.3)
6'	6.82, dd (1.8, 8.2)	6.83, dd (1.9, 8.1)	7.57, dd (1.8, 8.5)	7.32, dd (2.1, 8.2)	7.30, dd (2.1, 8.3)	7.37, m	7.40, m	
OCH_3							3.81, s	3.79, s
Glc-1		3.87, d (7.7)			5.15, d (7.8)			
Rha-1				5.36, d (1.5)	5.35, d (1.6)			
Rha-6				0.96, d (6.1)	0.95, d (6.2)			

[a]in CD_3OD, [b]in DMSO-d_6,

These isolated compounds were evaluated for their DPPH free radical scavenging activity and *α*-glucosidase inhibitory activity. IC_{50} values of active compounds for both assays are given in Table 3. All flavonoids except pinocembrin (**6**), pinostrobin (**7**) and pinostrobin chalcone (**8**) showed potent free radical scavenging activity (Table 3), and among them, (-)-epicatechin (**1**) and quercetin (**3**) were most potent. In *α*-glucosidase inhibitory activity assay, quercetin (**3**), quercitrin (**4**) and taxifolin 3-*O*-glucoside (**2**) showed potent activities (Table 3). Pinocembrin (**6**) also showed potent inhibitory activity, however, pinostrobin (**7**) and pinostrobin chalcone (**8**) were inactive. As pinocembrin (**6**) and pinostrobin (**7**) were isolated in high quantities, these compounds may have different biological activities which should be studied in detail in the future

Table 2. ^{13}C-NMR spectroscopic data of isolated compounds

Position	1[a]	2[a]	3[b]	4[b]	5[a]	6[a]	7[a]	8[a]
1								137.0
2	79.4	83.5	146.8	158.4	159.3	80.5	80.6	129.4
3	67.0	77.2	135.7	136.2	136.2	44.2	44.3	130.0
4	28.8	195.9	175.8	176.5	179.6	197.3	197.8	130.0
5	157.3	165.5	160.7	163.0	163.1	165.5	165.3	130.0
6	96.2	97.4	98.1	99.7	99.9	96.3	95.9	129.4
7	157.0	169.0	163.8	165.7	166.1	168.5	169.6	143.2
8	95.6	96.3	93.3	94.7	94.8	97.2	95.1	128.8
9	156.7	164.1	156.1	159.2	158.4	164.7	164.5	194.4
10	99.7	102.6	102.9	105.8	105.9	103.4	104.1	
1'	131.6	129.0	121.9	122.9	123.1	140.5	140.3	106.7
2'	114.8	115.9	115.4	116.3	116.9	127.4	127.4	169.6
3'	145.3	147.3	145.0	146.3	146.4	129.6	129.7	95.0
4'	145.4	146.4	147.6	149.6	149.9	129.7	129.8	167.9
5'	115.7	116.3	115.5	116.9	116.4	129.6	129.7	94.6
6'	119.1	121.1	119.9	122.8	123.0	127.4	127.4	165.8
OCH_3							56.3	55.8
Glc-1		102.5			99.9			
Glc-2		74.6			78.7			
Glc-3		78.2			78.3			
Glc-4		71.3			71.9			
Glc-5		77.8			77.2			
Glc-6		62.6			62.6			
Rha-1				103.4	103.6			
Rha-2				71.9	73.3			
Rha-3				71.8	73.2			
Rha-4				72.1	75.1			
Rha-5				73.2	70.1			
Rha-6				17.6	17.7			

[a]in CD_3OD, [b]in DMSO-d_6

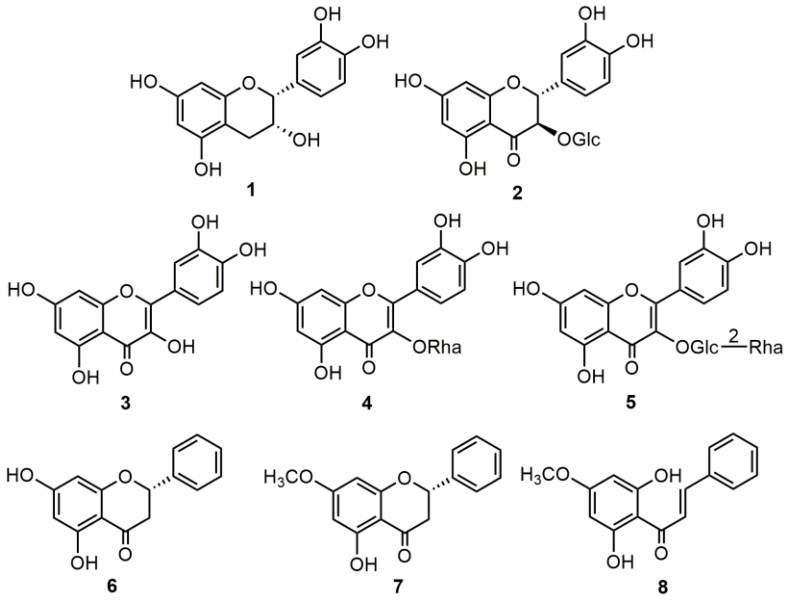


Figure 2. Chemical structures of compounds isolated from *L. sericea* var. *sericea*

Table 3. IC_{50} (μM) values[a] of compounds isolated from *L. sericea* var. *sericea* for their bioactivities

Compounds	DPPH free radical scavenging activity (μM)	α-glucosidase inhibitory activity (μM)
1	20.1 ± 0.09	25.4 ± 1.28
2	29.6 ± 0.72	8.6 ± 0.52
3	21.8 ± 0.15	3.0 ± 0.26
4	31.0 ± 1.31	5.1 ± 0.27
5	27.2 ± 1.40	-
6	-	59.3 ± 3.95
Trolox	38.9 ± 1.09	-
Acarbose	-	331.6 ± 6.49

[a]Values are represented as mean±SD (n=3).

DISCUSSION

Some of the compounds isolated in this study were previously isolated from other species of *Lindera* genus. Takizawa et al. [22] has reported the presence of eight flavonoids i.e. kaempferol, afzelin, quercitrin, isoquercitrin, quercetin, avicurarin, hyperin and rutin from the leaves of related species, i.e. *L. umbellata* var. *umbellata, L. umbellata* var. *membranacea* (Maxim.) Momiy. ex H. Hara et M. Mizush. and *L. sericea* var. *glabrata* Blume. In the present study, quercetin (**3**) and its two derivatives (**4** and **5**) were isolated from *L. sericea* var. *sericea* which showed close similarity to these species. Park et al. [26] had reported kaempferol-3,7- *O*-di-rhamnopyranoside from the leaves of this plant, however, neither kaempferol nor its derivatives were

isolated in present study. We had previously reported various kaempferol derivatives from the leaves of *L. neesiana* (Wall. ex Nees) Kurz from Nepal. However, the chemical composition of these two species seems to be different from the current analysis [35]. Further comparative studies among different *Lindera* species may help in understanding their chemotaxonomic relationships.

Flavonoids are reported as common chemical constituents in *Lindera* plants such as *L. aggregata*, *L. erythrocarpa*, *L. lucida*, *L. neesisana, L. obtusiloba, L. umbellata*, etc. [35–37]. Various compounds of other chemical classes such as alkaloids, butanolides, isocoumarins, lignans, lucidones, phenylpropanoids, sesquiterpenoids, etc. are reported from this genus [17,38–40]. Extracts and isolated compounds of *Lindera* species have shown potent pharmacological activities such as antioxidant [41,42], anti-inflammatory [43,44], antihypertensive [45], hepatoprotective [46] and cytotoxic [47,48] activities among others.

(-)-Epicatechin (**1**) is among the most studied natural products and has been isolated from various plant sources having multiple pharmacological activities [14,49–51]. Quercetin (**3**) and its derivatives (**4** and **5**) are abundant in many vegetables, fruits and other plant parts and have shown multiple biological activities such as antioxidant, anticancer and anti-inflammatory activities [52–54]. Pinocembrin (**6**) has been isolated from various plant species and various biological activities such as anti-inflammatory activity, antimicrobial activity and neuroprotective activities were reported [55]. Further studies are necessary to evaluate the other biological activities of these compounds.

CONCLUSIONS

In this study, a total of eight flavonoids, (-)-epicatechin (**1**), taxifolin 3-*O*-glucoside (**2**), quercetin (**3**), quercitrin (**4**), quercetin 3-*O*-neohesperidoside (**5**), pinocembrin (**6**), pinostrobin (**7**) and pinostrobin chalcone (**8**), were isolated and identified from the leaves and twigs of *L. sericea* var. *sericea* for the first time. In previous reports, these flavonoids were reported to possess various pharmacological activities. In this study, some of the isolated compounds showed potent free radical scavenging and α-glucosidase inhibitory activities, which may support their traditional uses as medicines and as functional foods;however, further studies are necessary to evaluate their activities in animal models and human subjects to provide necessary scientific evidence.

***List of Abbreviations*:** CC: column chromatography, DPPH: 1,1-diphenyl-2-picrylhydrazyl, MES: 2-morpholinoethanesulfonic acid monohydrate, TLC: thin-layer chromatography.

Competing Interests: There are no conflicts of interest to declare.

Author's Contributions: Hari Prasad Devkota conceived and designed the experiments. Hari Prasad Devkota, Ayumi Kurizaki, Kazuki Tsushiro, Anjana Adhikari-Devkota and Kengo Hori performed the experiments, analyzed the data and wrote the paper. Mikiyo Wada and Takashi Watanabe provided the resources and contributed to data analysis. All authors checked and approved the final version of the manuscript.

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