



Anti-oxidant activity of avicularin and isovitexin from *Lespedeza cuneata*

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Abstract This study evaluated the anti-oxidant activity of avicularin and isovitexin from *Lespedeza cuneata* using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl (OH) scavenging assays. The results showed that among the four fractions, the highest OH radical scavenging activity was exhibited by the *n*-butanol fraction. The DPPH radical scavenging activities of avicularin and isovitexin were higher than 50% at a concentration of 100 µg/mL. Moreover, one hundred micrograms per liter of avicularin and isovitexin exhibited effective anti-oxidant activity, with the OH radical scavenging rates being 87.54 and 91.48%, respectively. These results suggest that *Lespedeza cuneata* would be useful anti-oxidant agents from natural sources.

Keywords Avicularin · High performance liquid chromatography · Isovitexin · *Lespedeza cuneata* · Radical scavenging activity

Introduction

The production of reactive oxygen species during metabolism and the downregulation of anti-oxidant defense systems in the human

body lead to oxidative stress and cellular dysfunction, resulting in chronic disorders such as cancer, cardiovascular diseases, and diabetes, among others [1,2]. Therefore, reducing the oxidative stress is important for the anti-oxidant defense system. Plants have been known to contain natural anti-oxidants, such as flavonoids, which have well-known anti-oxidant properties. Flavonoids are polyphenolic compounds comprised of two benzene rings that are linked together with a heterocyclic pyran or pyrone ring [3]. This particular structure of flavonoids, combined with the position of its hydroxyl groups and the polarity of its glycoside derivatives, contributes to its anti-oxidant properties [4-6]. Flavonoids can also act as modulators of signaling pathways that are directly involved in cellular function and survival, such as the phosphoinositide 3-kinase (PI3K), Akt/PKB, and MAPK pathways [7].

Lespedeza cuneata, commonly known as the Chinese bush clover, is a perennial legume native to Asian countries including Korea, Japan, China, and Taiwan. It is used in traditional medicine for the treatment of asthma, chronic cough, abscess, and for eyesight strengthening. In addition, the aerial parts of this plant protect against inflammation, diabetes, and glucose toxicity [8-10]. Previous studies reported that *L. cuneata* is rich in pinitol, tannins, β-sitosterol, and flavonoids such as quercetin, kaempferol, and vitexin [11,12]. It is also rich in *C*-glycosyl flavones such as desmodin, vicenin II, and isovitexin, and *O*-glycosyl flavonols such as avicularin and trifolin [3]. Among these, avicularin and isovitexin in particular, have various pharmacological activities. Avicularin has hepatoprotective abilities mediated through its radical scavenging properties [13]. It also protects cardiac cells by reducing the release of lactate dehydrogenase from H9c2 cardiomyocytes exposed to oxidative stress [14]. Isovitexin, on the other hand, possesses effective anti-oxidant, anti-inflammatory, and anti-neoplastic properties [15] and accumulates in the tissues of seedlings exposed to blue light [16,17].

The present study was conducted in an effort to closely investigate the anti-oxidant properties of *L. cuneata* by analyzing four solvent fractions of the plant. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl (OH) radical scavenging activities were measured to

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evaluate the anti-oxidant ability of these fractions. The average contents of avicularin and isovitexin in the samples collected from different geographical regions in Korea were simultaneously detected by using high-performance liquid chromatography (HPLC).

Materials and Methods

Collection of plant materials and preparation of solvent fractions

The aerial parts of *L. cuneata* were collected from Gokseong, Inje, and Boeun, in Korea in the month of October in 2016 and in the month of November in 2017. The voucher specimens were identified by Prof. S. S. Roh, Daegu Haany University and deposited at the herbarium of the Department of Plant Science and Technology, Chung-Ang University, Anseong, Korea. Dried samples of *L. cuneata* were refluxed with methanol (MeOH), and the resulting extract was filtered and evaporated to dryness *in vacuo*. The extract was solvent partitioned using *n*-hexane, chloroform (CHCl₃), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH).

Chemicals and reagents

HPLC grade acetonitrile and water were obtained from J. T. Baker (Parkway Center, PA, USA). The 1290 Infinity II LC system with INNO C18 column (250 mm×4.6 mm, 5 μm) from Agilent Technology was used for the chemical analyses. The dried aerial portions of *L. cuneata* were extracted three times with 80% MeOH. The resultant filtrate was evaporated under reduced pressure using a rotavap to obtain the MeOH extract, which was suspended in distilled H₂O and successively solvent-partitioned with *n*-hexane, CHCl₃, EtOAc, and *n*-BuOH. The compounds from the polar fractions were isolated and identified as avicularin and isovitexin (Fig. 1) [18].

DPPH and ·OH radical scavenging activity

In a 96-well microtiter plate, 100 μL of each sample was added to 60 μM of an ethanolic solution of DPPH [19]. After mixing with a vortex mixer, the mixture was incubated for 30 min at room temperature and the absorbance was measured at 540 nm. The DPPH radical scavenging activity was recorded as a percentage in comparison to the control. For the quantification of OH radical scavenging activity, separate reaction mixtures were prepared,

containing 10 mM FeSO₄·7H₂O-EDTA, 10 mM 2-deoxyribose, and the sample solutions [20]. After incubation at 37 °C for 4 h, the reaction was terminated by adding 2.8% trichloroacetic acid and 1.0% solution of thiobarbituric acid. The solution was boiled for 20 min in a water bath and then cooled on ice. The OH radical scavenging activity was measured at 490 nm.

Preparation of standards and samples for HPLC

Standard stock solutions of avicularin and isovitexin were separately dissolved in 1 mg/mL MeOH. The working solutions used to construct the calibration curve were prepared by serially diluting the stock solution to the desired concentrations. Fifteen grams of each sample was extracted with 210 mL of distilled water for 8 h. All the samples were concentrated *in vacuo* using an Eyela rotary evaporator. The concentrated samples were then oven-dried for 48 h. The samples were subsequently powdered by freeze-drying. Each of the samples was dissolved in 20 mg/mL MeOH. Both the standard and the sample solutions were filtered by using a PVDF filter (Cat No. 6779, Piscataway, NJ, USA) having a pore size of 0.45 μm.

HPLC analytical conditions

Quantitative analyses of avicularin and isovitexin were performed using a reverse-phase HPLC system and chromatographic separation was achieved using an INNO C₁₈ column (250 mm×4.6 mm, 5 μm). A gradient elution system, with a mobile phase comprising of 0.5% acetic acid in water (A) and acetonitrile (B), was utilized. The ratio of the solvents (A:B) in the gradient solvent system was initially 90:10, which was increased in linear gradients to 83:17 for 5 min, then to 81:19 for 15 min, and finally the mobile phase was maintained for up to 30 min. The temperature of the column was maintained at 35 °C and the flow rate was set at 1 mL/min. The injection volume was 10 μL and peaks were monitored at 278 nm.

Calibration curve

Avicularin and isovitexin were dissolved in HPLC grade methanol to prepare solutions having concentrations ranging between 0.25 and 0.015625 μg/g. The standards were calculated by using the peak area (Y), concentration (X, μg/g), and the values of the mean (n =5) ± standard deviation (SD). Linear regression was used to determine the linearity of the calibration curve. The contents of

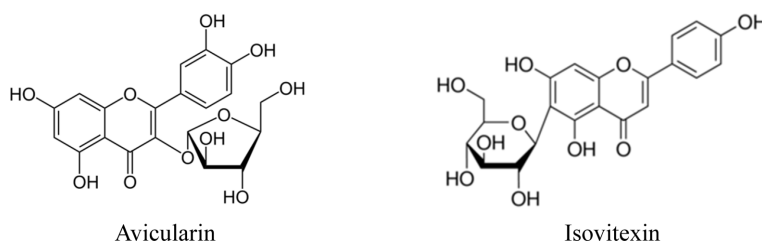


Fig. 1 Structures of avicularin and isovitexin

avicularin and isovitexin were determined from the corresponding calibration curves.

Statistical analysis

Significant differences in the values measured during the experiments were determined using Duncan’s Multiple Range Test using the SAS software (version 6.0, SAS Institute, Cary, NC, USA).

Results and Discussion

The anti-oxidant activities of the solvent fractions of *L. cuneata* and the two compounds, avicularin and isovitexin, were evaluated by using the DPPH and OH radical scavenging assays. The OH radical is the most reactive radical and induces oxidative damage in cells [21]. As shown in Table 1, among the four fractions (*n*-hexane, CHCl₃, EtOAc, and *n*-BuOH), the OH radical scavenging activities of the CHCl₃ and *n*-BuOH fractions were higher than 50% at a concentration of 250 µg/mL. The *n*-BuOH fraction (50-250 µg/mL) had the highest anti-oxidant activity among the four fractions. Our results support the observations of a previous study on the anti-oxidant activity of *L. cuneata* extracts [22] and demonstrate that the EtOAc and *n*-BuOH fractions have the highest anti-oxidant activities compared to those of the other fractions [4].

The DPPH radical scavenging activities of avicularin and

Table 1 The hydroxyl radical scavenging activity of the four solvent fractions of *L. cuneata*

Concentration (µg/mL)	Scavenging activity (%)			
	<i>n</i> -Hexane	CHCl ₃	EtOAc	<i>n</i> -BuOH
10	45.12±0.28 ^b	47.59±1.22 ^b	40.28±1.80 ^a	49.97±0.05 ^c
50	49.07±0.61 ^a	47.03±0.83 ^b	43.37±0.05 ^a	53.02±0.19 ^b
100	40.55±1.04 ^c	44.04±0.49 ^c	41.65±0.58 ^a	52.21±0.23 ^b
250	37.67±1.24 ^c	56.26±0.47 ^a	41.60±0.29 ^a	55.66±0.43 ^a

The values represent the mean percent scavenging activity ± SD
^{a-c}The means denoted by different superscript letters differ significantly (*p* < 0.05) by Duncan's Multiple Range Test

Table 2 The DPPH scavenging activity of avicularin and isovitexin

Concentration (µg/mL)	Scavenging activity (%)	
	Avicularin	Isovitexin
100	71.28±0.43	58.20±3.2

The values represent the mean percent scavenging activity ± SD

Table 3 The hydroxyl radical scavenging activity of avicularin and isovitexin

Concentration (µg/mL)	Scavenging activity (%)	
	Avicularin	Isovitexin
100	87.54±0.16	91.48±0.11

The values represent the mean percent scavenging activity ± SD

isovitexin are shown in Table 2. The DPPH radical scavenging effect of avicularin at a concentration of 100 µg/mL was significant, being 71.28%. At a value of 58.20%, the DPPH radical scavenging effect of isovitexin was relatively lower than that of avicularin. However, both avicularin and isovitexin had effective OH radical scavenging capacities, with values of 87.54 and 91.48%, respectively (Table 3). A number of studies have demonstrated a correlation between the anti-oxidant effect and the structures of flavonoids and phenolic compounds [23,24]. According to Kim and coworkers [13], avicularin, which contains the quercetin aglycone in its structure, exhibits radical scavenging effects, whereas isovitexin, which contains the apigenin aglycone, does not have any 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging effects. However, our results indicated that both avicularin and isovitexin have anti-oxidant properties possibly because they can both act as hydrogen donors and/or metal ion chelators [3]. This study further supports this claim and highlights the use of the *n*-BuOH fraction for future studies on the anti-oxidant properties of *L. cuneata*.

For quantitative analyses, avicularin and isovitexin were simultaneously analyzed. The linear calibration results (*r*² = 0.9999) for both compounds are shown in Table 4 and the quantities of avicularin and isovitexin in *L. cuneata* are reported in Table 5. The contents of avicularin and isovitexin in the samples of *L. cuneata* collected from the different geographical locations were found to vary (Fig. 2). The contents of avicularin and isovitexin were highest in the samples collected from Gokseong in October 2016, being 0.66 and 1.18 mg/g, respectively. On the other hand, the contents of avicularin and isovitexin were lowest in the samples collected from Inje in November 2016 and from

Table 4 Calibration curves for avicularin and isovitexin

Compound	t _R	Calibration equation ^a	Correlation factor, <i>r</i> ² ^b
Avicularin	23.7	Y=951.79X-0.1937	0.9999
Isovitexin	17.7	Y=1867X+2.7532	0.9999

^aY = peak area, X = concentration of the standard in mg/mL
^b*r*² = correlation coefficient for three data points in the calibration curve.
 t_R = retention time

Table 5 Contents of avicularin and isovitexin of *L. cuneata* collected from different regions

Region	Harvest Time	Content (mg/g)		
		Avicularin	Isovitexin	Total
Gokseong	2016.10	0.660±0.011	1.182±0.008	2.214
	2017.11	0.323±0.042	0.049±0.012	
Inje	2016.10	0.156±0.047	0.559±0.044	1.177
	2017.11	0.128±0.026	0.334±0.010	
Boeun	2016.10	0.296±0.021	1.140±0.027	1.677
	2017.11	-	0.241±0.009	
Average		0.052	0.097	

-: not detected

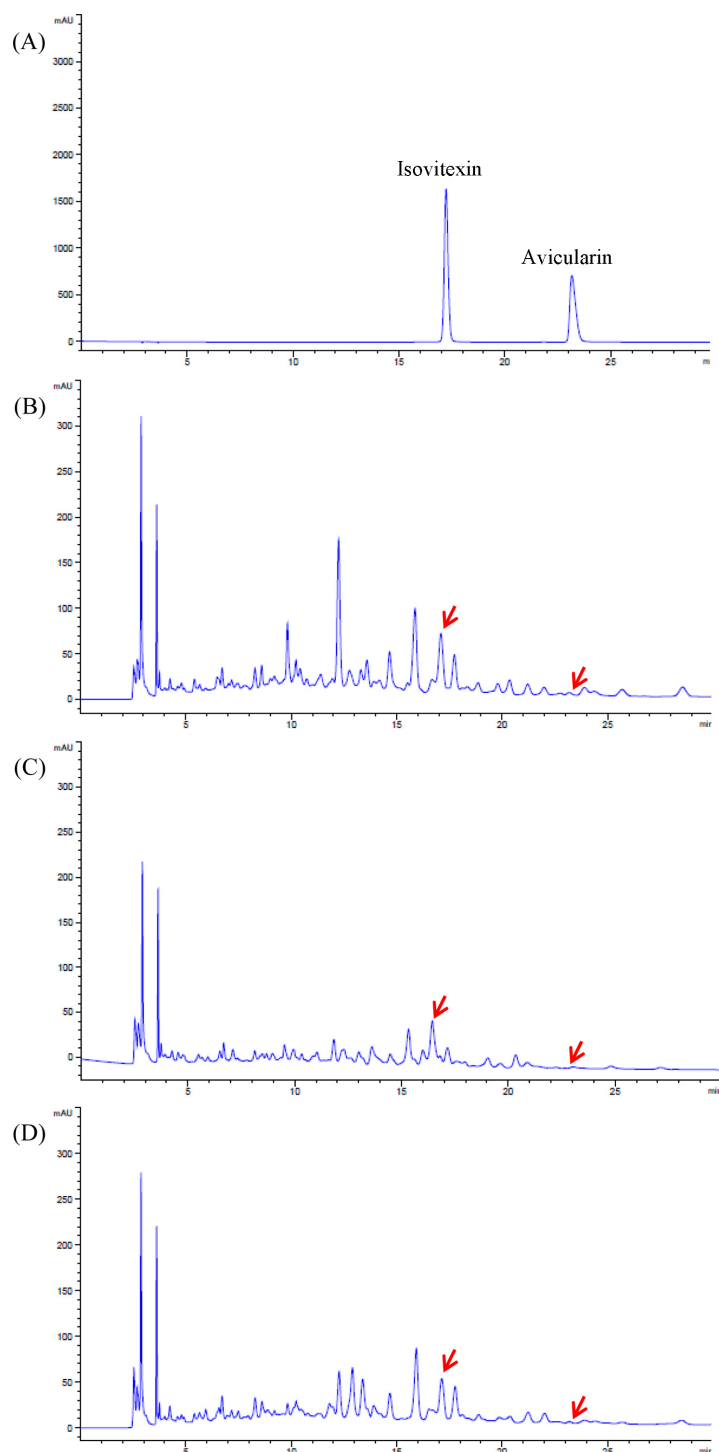


Fig. 2 HPLC chromatograms of the standards (A) and the distilled water extracts of the samples collected from Gokseong (B), Inje (C), and Boeun (D) in 2016

Gokseong in November 2017, respectively. Avicularin was not detected in the samples collected from Boeun in 2017. The difference in the contents could be attributed to seasonal differences, geographic variations, differences in the plant part processed, and the time of harvest. These variations indicated that

the use of samples of *L. cuneata* collected from Gokseong for the isolation of avicularin and isovitexin and other related studies would produce better yields of avicularin and isovitexin if collected the in the month of October than in November. The higher average temperature and monthly total precipitation could

Table 6 Temperature and precipitation of collection regions

Region	Time	Average temperature (°C)	Monthly total precipitation (mm)
Gokseong	2016.10	15.5	255.0
	2017.11	5.7	4.5
Inje	2016.10	13.1	107.1
	2017.11	3.8	28.2
Boeun	2016.10	14.1	135.7
	2017.11	4.3	13.4

Source: Korea Meteorological Administration

be attributed the higher content of avicularin and isovitexin (Tables 5 and 6).

Based on our results, the *n*-BuOH fraction of *L. cuneata*, which contains higher amounts of flavonoids than the other fractions, exhibited the most effective free radical scavenging activity. The anti-oxidant activity of the *n*-BuOH fraction can be attributed to the compounds avicularin and isovitexin. This study also established that the month of October is a better time for harvesting *L. cuneata* in Gokseong for obtaining maximum yields of avicularin and isovitexin. This study can further improve the development of functional foods with high anti-oxidant effects.

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