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Antioxidant Activity from the Stem Bark of *Albizzia julibrissin*

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The antioxidant activity of the stem bark from *Albizzia julibrissin* was evaluated for its potential to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, to inhibit the generation of the hydroxyl radical (·OH), total reactive oxygen species (ROS) and to scavenge authentic peroxynitrites ($ONOO^-$). The methanol extract of *A. julibrissin* exhibited strong antioxidant activity in the tested model systems. Therefore, it was further fractionated using several solvents. The antioxidant activity of the individual fractions were in the order of ethyl acetate (EtOAc) > *n*-butanol (*n*-BuOH) > dichloromethane (CH_2Cl_2) > and water (H_2O). The ethyl acetate soluble fraction, which exhibited strong antioxidant activity, was further purified by repeated silicagel, Sephadex LH-20 and RP-18 gel column chromatography. Sulfuretin (**1**) and 3',4',7-trihydroxyflavone (**2**) were isolated as the active principles. Compounds **1** and **2** exhibited good activity in all tested model systems. Compound **1** exhibited five times more inhibitory activity on the total ROS than Trolox. Compound **2** showed six times stronger DPPH radical scavenging activity than L-ascorbic acid. These results show the possible antioxidant activity of the *A. julibrissin* crude extract and its major constituents.

Key words: *Albizzia julibrissin*, Leguminosae, Sulfuretin, 3',4',7-Trihydroxyflavone, Antioxidant

INTRODUCTION

Reactive oxygen or nitrogen species including ·OH, hydrogen peroxide (H_2O_2), superoxide anion ($\cdot O_2^-$), nitric oxide (NO·), and $ONOO^-$ are associated with a wide variety of human diseases such as *Alzheimers disease*, rheumatoid arthritis, cancer, and atherosclerosis (Squadrito and Pryor, 1998; Podrez *et al.*, 1999). Many researchers have long sought powerful, nontoxic natural antioxidants from edible or medicinal plants in order to prevent these reactive species related disorders in humans as well as to replace synthetic compounds, which may be carcinogenic and harmful to the lungs and liver (Branen, 1975). In an ongoing study aimed at identifying antioxidants from natural products, this particular study focused on *Albizzia julibrissin*.

The dried stem bark of *Albizzia julibrissin* Durazz (Leguminosae) has been used to treat insomnia, diuresis, sthenia, ascaricide and contusions in China, Japan and Korea (Kim, 1996). Previously, we reported the isolation of 3',4',7-trihydroxyflavone, and α-spinasterol glucoside (Chamsuksai *et al.*, 1981), triterpenes (Kang and Woo, 1983; Woo and Kang, 1984) and a new acid (Jung *et al.*, 2003) from *Albizzia julibrissin*.

This paper, reports an evaluation of the antioxidant activity of all the extracts and compounds isolated from the *A. julibrissin* for their potential to scavenge the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, to inhibit the generation of total reactive oxygen species (ROS), and hydroxyl radicals (·OH), and to scavenge authentic peroxynitrite ($ONOO^-$).

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MATERIALS AND METHODS

General experimental procedures

The EI mass data were recorded using a JEOL JMS-700 spectrometer. The ^1H- and ^{13}C-NMR spectra were measured using a JEOL JNM-ECP 400 (400 MHz for ^1H, 100 MHz for ^{13}C) spectrometer. The chemical shifts were referenced to the respective residual solvent peaks (δ_H

3.30 and δ_C 49.0 for CD_3OD and δ_H 2.50 and δ_C 39.5 for DMSO-d_6). The DEPT, HMQC, and HMBC spectra were recorded on a JEOL JNM-ECP 400 using pulsed field gradients. Column chromatography was carried out using Si gel (Merck, 70-230 mesh), Sephadex LH-20 (Sigma, 25-100 μ) and RP-18 gel (Merck, 40-63 mm). The TLC was performed on a precoated Merck Kieselgel 60 F_{254} plate (0.25 μm) and a Merck 25 RP-18 F_{254s} plate (5×10 cm). 50% H_2SO_4 was used as the spray reagent.

Chemicals

The 1,1-diphenyl-2-picrylhydrazyl, L-ascorbic acid, and penicillamine were purchased from the Sigma Chemical Company (St. Louis, MO, USA). The 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA), dihydrorhodamine 123 (DHR 123), and peroxynitrite were obtained from Molecular Probes (Eugene, Oregon, USA), and Cayman (Ann Arbor, MI, USA), respectively.

Plant material

The stem bark from *Albizzia julibrissin* Durazz (Leguminosae) was purchased from the herbal medicine co-operative association in Busan Province, in August 2001. A voucher specimen was deposited at the Faculty of Food Science and Biotechnology, Pukyong National University.

Isolation of compounds 1 and 2

The stem bark (18.2 kg) of *A. julibrissin* was refluxed with MeOH for three hours (9 L×3). The total filtrate was concentrated and dried in vacuo at 40°C to render the MeOH extract (2.97 kg). The extract was then suspended in distilled water and sequentially partitioned with CH_2Cl_2 (931.8 g), EtOAc (86.2 g), *n*-BuOH (649.5 g), and H_2O (1181.8 g). Each extract was tested for its antioxidant activity in the tested model systems, and the EtOAc fraction exhibited strong activity. Therefore, the EtOAc (86.2 g) fraction was chromatographed on a Si gel column using CH_2Cl_2-MeOH (gradient) to yield 29 subfractions. Fraction 19 (14.6 g) was further chromatographed on a Sephadex LH-20 and RP-18 gel column using a H_2O-MeOH gradient to give compound **1** (22 mg). Fraction 20 (17.5 g) was chromatographed on a Sephadex LH-20 column with MeOH, which yielded compound **2** (300 mg).

Sulfuretin (1)

Amorphous orange powder; EIMS *m/z* (%) : 270 (100), 253 (20), 242 (12), 213 (18), and 92 (27); ^{1}H-NMR (400 MHz, CD_3OD) : δ 7.59 (1H, d, *J* = 8.4 Hz, H-4), 7.50 (1H, brs, H-2'), 7.21 (1H, d, *J* = 7.9 Hz, H-6'), 6.83 (1H, d, *J* = 8.2 Hz, H-5'), 6.69 (1H, d, *J* = 8.4 Hz, H-5), 6.68 (1H, brs, H-7), 6.67 (1H, brs, H-10) ; ^{13}C-NMR (100 MHz, CD_3OD) : δ 185.3 (C-3), 170.6 (C-6), 169.2 (C-8), 150.2 (C-4'), 148.5 (C-2), 147.5 (C-3'), 127.6 (C-4), 127.2 (C-6') , 126.3 (C-1'), 119.7 (C-2'), 117.5 (C-5'), 115.7 (C-9), 115.5 (C-10), 114.9 (C-7), 100.2 (C-5)

3',4',7-Trihydroxyflavone (2)

Amorphous yellowish powder; EIMS *m/z* (%) : 270 (100), 242 (35), 137 (81), and 121 (30); ^{1}H-NMR (400 MHz, DMSO-d_6) : δ 10.75 (1H, brs, OH), 9.80 (1H, brs, OH), 9.37 (1H, brs, OH), 7.86 (1H, d, *J* = 8.6 Hz, H-5), 7.39 (1H, d, *J* = 2.0 Hz, H-2'), 7.38 (1H, dd, *J* = 2.0 and 8.6 Hz, H-6'), 6.60 (1H, s, H-3), 6.93 (1H, d, *J* = 2.0 Hz, H-8) 6.89 (1H, d, *J* = 8.6 Hz, H-5'), 6.88 (1H, d, *J* = 2.0 and 8.6 Hz, H-6); ^{13}C-NMR (100 MHz, DMSO-d_6) : δ 176.2 (C-4), 162.6 (C-2), 157.3 (C-9), 149.1 (C-4), 145.7 (C-3'), 126.5 (C-5), 122.1 (C-1'), 118.5 (C-6'), 116.1 (C-5'), 115.9 (C-10), 114.7 (C-6), 113.1 (C-2), 104.5 (C-3), 102.3 (C-5)

Measurement of DPPH radical scavenging activity

The DPPH radical scavenging effect was evaluated using the slight modification of the method reported by Blois (Blois, 1958). One hundred sixty microliters (μL) of a MeOH solution with various concentrations were added to a 40 μL DPPH methanol solution (1.5×10^{-4} M). After mixing gently and standing at room temperature for 30 min, the optical density was measured at 520 nm using a microplate reader spectrophotometer VERSAmax (Molecular Devices, CA, USA). The antioxidant activity of each sample was expressed in terms of the IC_{50} (μg/mL or μM required to inhibit DPPH radical formation by 50%), which was calculated from the log-dose inhibition curve.

Measurement of the inhibition of the ·OH generation

The extracts (final 0.5 mg/mL) were added to 1 mM H_2O_2 and 0.2 mM $FeSO_4$, and incubated at 37°C for 5 min. The esterase-treated 2 μM DCHF-DA was then added and the changes in the fluorescence were monitored at an excitation and emission wavelength of 485 nm and 530 nm for 30 min, respectively.

Measurement of the inhibition of the total ROS generation

Rat kidney homogenates, which were prepared from the kidneys of freshly killed male Wistar rats weighing 150-200 g, were mixed with or without the suspension of the extracts/or compounds. The mixtures were, then incubated with 12.5 μM DCHF-DA at 37°C for 30 min. A 50 mM phosphate buffer solution at pH 7.4 was also used. The fluorescence intensity of the oxidized 2',7'-dichlorodihydrofluorescein (DCF) was monitored using a microplate fluorescence spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT), with an excitation and emission wavelength of 460 and 530 nm (Label and Bondy, 1990), respectively.

Measurement of the $ONOO^-$ scavenging activity

The peroxynitrite scavenging was measured by monitoring the oxidation of DHR 123 using a slight modification of the method reported by Kooy *et al* (Kooy *et al.*, 1994). DHR 123 (5 mM) in dimethylformamide, which was purged with nitrogen, was stored as a stock solution at -80°C. This solution was then placed in ice and kept in the dark prior to the study. The buffer used consisted of 90 mM sodium chloride, 50 mM sodium phosphate, 5 mM potassium chloride at pH 7.4, and 100 μM diethylenetriaminepentaacetic acid (DTPA), each of which was prepared using high quality deionized water and purged with nitrogen. The final concentration of the DHR 123 was 5 μM. The background and final fluorescent intensities were measured 5 min after the treatment with and without the authentic peroxynitrite. DHR 123 was oxidized rapidly by the authentic peroxynitrite, and its final fluorescent intensity remained unchanged over time. The fluorescence intensity of the oxidized DHR 123 was measured using a microplate fluorescence reader (FL 500, Bio-Tek Instruments) at excitation and emission wavelengths of 480 nm and 530 nm, respectively. The results are expressed as a means±S.E. (n = 3) for the final fluorescence intensity minus the background fluorescence. The effects are expressed as the percentage inhibition of DHR 123 oxidation.

Statistical analysis

The data is expressed as a mean standard error of three experiments.

RESULTS AND DISCUSSION

DPPH is a stable free radical compound, which has been widely used as a substrate to evaluate the antioxidative action of the antioxidants. ROS are formed in the body as a consequence of the aerobic metabolism, and damages all intracellular components (Sagar *et al.*, 1992). The $ONOO^-$, which is formed from a reaction between superoxide and nitric oxide, is a cytotoxic species that can oxidize several cellular components such as proteins, lipids, and DNA (Squadrito and Pryor, 1998).

This study investigated the general antioxidant effects of the *Albizzia julibrissin* extract for its potential to scavenge the stable DPPH free radicals, to inhibit the generation of hydroxyl radicals and the total ROS in kidney homogenates using DCFH-DA, and to scavenge the authentic peroxynitrites of the MeOH extract along with its solvent partitioned fractions such as CH_2Cl_2-, EtOAc-, n-BuOH-, and H_2O-soluble fractions. Further studies aimed at identifying the active principles from the active fractions are planned. As summarized in Table I, the scavenging activity of the MeOH extract and its fractions on DPPH increased in the order of EtOAc > n-BuOH > MeOH > CH_2Cl_2 > H_2O, which were 19.09, 47.85, 86.56, 156.09 and 194.86 μg/mL in their IC_{50} (50% inhibition concentration), respectively. The CH_2Cl_2-, EtOAc-, n-BuOH and the H_2O-soluble fractions on the total ROS exhibited strong activity at a concentration of 20 μg/mL with 43.91±2.03%, 43.79±1.16%, 38.25± 4.93% and 50.32±6.25% inhibition, respectively. This, is comparable to that (45.36±14.64%) of Trolox at a concentration of 40 μg/mL, which was used as the positive control. In contrast, the inhibition activity of the MeOH extract on the total ROS was weaker than that of Trolox at a concentration of 40 μg/mL. The inhibitory activity of the EtOAc fraction on inhibiting ·OH was stronger than that of the other fractions. In the $ONOO^-$ system, the MeOH extract and its fractions tested exhibited moderate antioxidant activity with an IC_{50} < 15 μg/mL, which is comparable to penicillamine (IC_{50} 2.55±0.14 μg/mL), specially like the n-BuOH soluble fraction with IC_{50} 2.33±0.12 μg/mL. These results suggest that the EtOAc fraction of the MeOH extract as well as the MeOH extract of *A. julibrissin* are scavengers of free radicals, $ONOO^-$ and ROS including hydroxyl radicals, and their capacity to scavenge free radicals, $ONOO^-$ and ROS may contribute to their antioxidant activity. It is interesting to note that the EtOAc fraction had a high scavenging activity, whereas both the CH_2Cl_2 and H_2O fractions exhibited weak activity in the model systems tested. The comparative scavenging effects of the fractions derived from the MeOH extract of *A. julibrissin* on a variety of *in vitro* model systems can be explained in terms of their compositional differences. It was expected that flavonoids and phenolic acids, which are of great interest for their radical-scavenging activity, would be present in the EtOAc fraction.

Therefore, the active EtOAc-soluble fraction was subjected

Table I. Antioxidant activities of the *Albizzia julibrissin* extracts on DPPH, ·OH, $ONOO^-$, and ROS

Samples	DPPH[a]	$ONOO^-$[b]	·OH[c]	ROS[d]
Methanol	86.56	11.76 ± 0.76	-165.95 ± 20.64[#]	31.95 ± 5.90
Dichloromethane	156.09	17.42 ± 0.79	-428.17 ± 16.29	43.91 ± 2.03*
Ethyl acetate	19.09	11.62 ± 0.76	58.71 ± 5.49	43.79 ± 1.61*
n-Butanol	47.85	2.33 ± 0.12	29.46 ± 3.36	38.25 ± 4.93*
Water	194.86	16.83 ± 1.40	20.57 ± 8.88	50.32 ± 6.25*
L-Ascorbic acid	1.63		55.20 ± 5.50	
Trolox				45.36 ± 14.64
Penicillamine		2.55 ± 0.14		

[a]DPPH is the free radical scavenging activity (IC_{50} : μg/mL). [b]$ONOO^-$ is the inhibitory activity of peroxynitrite (IC_{50} : μg/mL). [c]OH is the inhibition percent of hydroxyl radical generation in 1.0 mM H_2O_2 and 0.2 mM $FeSO_4$ at the test concentration of 40 μg/mL. [d]ROS is the inhibition percent of total free radical generation in kidney postmicrosomal fraction at the concentration of 40 μg/mL. (*ROS inhibitory concentration at 20 μg/mL). [#]· indicate free radical generation.

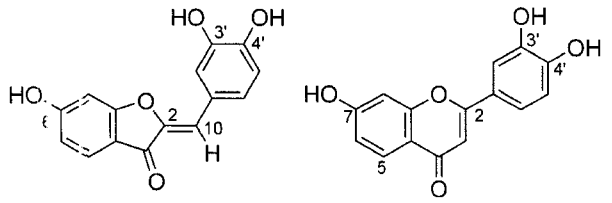

Fig. 1. Structures of compounds **1** and **2**

to further chemical analysis, and after successive column chromatography, two known flavonoids (**1** and **2**) were isolated (Fig. 1). These compounds were readily identified by comparison of reported spectroscopic data as sulfuretin (**1**) and 3',4',7-trihydroxyflavone (**2**) (Chamsuksai *et al.*, 1981; Farag *et al.*, 1997; Park *et al.*, 2000). The complete assignments of the ^{1}H- and ^{13}C-NMR spectra were made using the HMQC and HMBC spectra. The isolation of compound **1** is the first report of its occurrence in the *Albizzia* species.

The antioxidant activities of the two isolated compounds, **1** and **2**, are shown in Table II. Compounds **1** and **2** exhibited strong antioxidant activity on the DPPH radical with an IC_{50} of 8.52 μM and 2.20 μM, respectively. Their IC_{50} values were two times and six times lower than the IC_{50} of 12.78 μM for L-ascorbic acid, respectively. Compounds **1** and **2** also exhibited strong inhibition activity with an C_{50} of 0.73±0.07 μM and 3.90±0.15 μM on the total ROS, respectively. Compound **1** in particular had five times stronger activity than that (IC_{50} 3.66±0.17 μM) of Trolox. In the $ONOO^-$ system, compounds **1** and **2** exhibited good inhibitory activity with an IC_{50} 4.21±0.12 μM and 5.78±0.21 μM, which is comparable to that (IC_{50} 4.46± 0.11 μM) of penicillamine, respectively. Compounds **1** and **2** had moderate activity on ·OH radicals with an IC_{50} of 4.59±0.21 μM and 8.58±0.66 μM, respectively. Although the DPPH scavenging, anti-rheumatoidal and cytotoxic activities of compound **1**, and the anti-inflammatory activity of compound **2** have been reported (Lee *et al.*, 1993; Westenburg *et al.*, 2000; Park *et al.*, 2000; Choi *et al.*, 2002), this study reports the antioxidant activities of **1** and **2** for the first time. As for the structure-activity relationship, compounds **1** and **2** exhibited good activity in all the tested model systems. The above results suggest that the 3',4'-*ortho*-functional group on the B-ring are the most important for the antioxidant activity. Flavonoids, hydroxycinnamates and its related phenolic acids have been reported to function as potent antioxidants by virtue of their hydrogen-donating properties (Rice-Evans *et al.*, 1996) and their metal-chelating properties (Morel *et al.*, 1993; Salah *et al.*, 1995). Several studies have reported that these compounds can prevent the $ONOO^-$-mediated nitration of the protein-bound and free tyrosine, and can inhibit the $ONOO^-$-mediated oxidation of dihydrorhodamine 123 and DNA (Oshima *et al.*, 1998). These results show that an investigation into the structural requirements for the scavenging activities of the different classes of compounds on the free radicals and reactive oxygen or nitrogen species including hydroxyl radicals and peroxynitrites is needed.

Table I. Antioxidant activities of compounds derived from *Albizzia julibrissin* on DPPH, ·OH, $ONOO^-$, and total ROS

Compounds	IC_{50} (μM)			
	DPPH[a]	$ONOO^-$[b]	·OH[c]	Total ROS[d]
Sulfuretin (1)	8.52	4.21 ± 0.12	4.59 ± 0.21	0.73 ± 0.07
3',4',7-Trihydroxyflavone (2)	2.20	5.78 ± 0.21	8.58 ± 0.66	3.90 ± 0.15
L-Ascorbic acid	12.78			
Trolox			3.98 ± 0.07	3.66 ± 0.17
Penicillamine		4.46 ± 0.11		

[a]DPPH is the free radical scavenging activity. [b]$ONOO^-$ is the inhibitory activity of peroxynitrite. [c]OH is the inhibitory activity of hydroxyl radical. [d]ROS is the inhibitory activity of total free radical.

The results in this study indicate that the methanol extract of *A. julibrissin* and its various fractions, as well as its components, may be useful for treating oxidative damage. A further investigation into the antioxidant activity of these natural compounds in view of preventing various radical-mediated injuries in pathological situations *in vivo* is currently underway.

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