Antioxidant Activity of *Cercis chinensis* and Its Protective Effect on Skin Aging

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Summary

Reactive oxygen species are capable of damaging biomolecules such as lipids, proteins, and DNA, which can not only lead to various diseases, but also oxidative damage resulting aging. In our previous study, *Cercis chinensis* (Leguminosae) showed a potent antioxidant activity. Nineteen compounds were isolated through antioxidant activity-guided fractionation. The *C. chinensis* extract and some of the constituents exhibited a potent antioxidant activity on the free radicals and lipid peroxidation and a notable protective effect on the *t*-BuOOH induced oxidative damage. *In vivo* test of skin damage induced by UVB irradiation, the extract of *C. chinensis* and a constituent, piceatannol, exhibited a significant protective effect. The life-span of the HEK-N/F cells were extended by 1.21—2.12 fold as a result of the continuous administration of 3 μg/ml of the *C. chinensis* extract and the active constituents compared to that of the control. These observations were attributed to the inhibitory effect of the *C. chinensis* extract and its constituents on the age-dependent shortening of the telomere. Thus, *C. chinensis* was demonstrated to protect the skin cells against oxidative stress and inhibit thereby the cellular aging, followed by expectation as anti-aging cosmetic ingredient.

Introduction

It is well known that reactive oxygen species (ROS) generated during the metabolic pathway damage biomolecules such as lipids, proteins, sugars and DNA, which can not only lead to various diseases [1], but also oxidative damage resulting aging [2],[3]. Indeed, oxidized biomolecules such as the 8-oxo-2'-deoxyguanosine residues in DNA, carbonyls and dityrosines in proteins and hydroperoxides in lipids have been reported to accumulate in the tissues of aged animals [4—6]. This oxidative stress theory of aging is supported by many studies, and it is now accepted one of the most important theories of aging. Accordingly, antioxidants, which can prevent oxidative damage from ROS, are expected to inhibit the aging process. Recently, another theory, the telomere hypothesis of aging, has also been accepted together with the oxidative stress theory. Telomeres located at the end of linear chromosome shorten with each cell division, and DNA damage if the telomeric DNA reaches a critical length, which can ultimately, lead to cell cycle arrest in senescent cells [7],[8]. Shortening of the telomeric DNA has been observed in human cells during the aging processes [9]. A life span extension has been observed after introducing telomerase, which can maintain the telomere length, into normal human cells [10].

In our previous study, the *Cersis chinensis* extract exhibited potent antioxidant activity on DPPH radical and lipid peroxidation. *C. chinensis* which belongs to the Leguminosae is widely cultivated as an ornamental plant. The stem bark, root bark and stem of *C. chinensis* have been used for a

promotion of blood circulation, dysmenorrhea, edema, a bruise, and an injury [11].

Based on the two aging theories, this study investigated the antioxidant activity of the *C. chinensis* extract and its constituents, and the protective effect on the oxidative stress induced by tertiary butyl hydroperoxide (*t*-BuOOH) and ultraviolet (UV)-B. Furthermore, the inhibitory effect of the medicinal plant on the cellular aging of human epidermal kerainocytes was examined by measuring the telomere length.

Materials and Methods

Plant Material - The leaves and stem of *C. chinensis* were collected at Daejeon, Korea in September 2001 and was identified by Prof. KiHwan Bae, College of Pharmacy, Chungnam National University. A voucher specimen (HK 1122) was deposited in the Jakwang Research Institute of the Hansaeng Cosmetics Co., Ltd.

Extraction and Isolation - The dried leaves and stem from *C. chinensis* (1 kg) were extracted with 60% EtOH at room temperature for 2 weeks. The EtOH extract (75 g) was suspended in water and then partitioned with hexane, EtOAc and BuOH, sequentially. The EtOAc and BuOH fraction (Fr.) exhibited a potent activity with an IC_{50} value of 24.0 and 27.0 μg/ml, respectively, in the DPPH assay. YMC gel and silica gel column chromatography, and preparative HPLC of the active EtOAc-

and BuOH-soluble fraction led to the isolation of nineteen compounds (**1**—**19**). They were identified as isoliquiritigenin (**1**), liquiritigenin (**2**), 2′,4′-dihydroxy-4-methoxychalcone (**3**), resveratrol (**4**), piceatannol (**5**), gallic acid (**6**), methyl gallate (**7**), ethyl gallate (**8**), myricetin (**9**), afzelin (**10**), quercitrin (**11**), myricitrin (**12**), myricetin-3-*O*-(2″-*O*-galloyl)-α-L-rhamnopyranoside (**13**), syringetin-3-*O*-rutinoside (**14**), (+)-catechin (**15**), (-)-epicatechin-3-*O*-gallate (**16**), (-)-epigallocatechin-3-*O*-gallate (**17**), (-)-lyoniresinol-3a-*O*-β-D-xylopyranoside (**18**) and (+)-lyoniresinol-3a-*O*-β-D-glucopyranoside (**19**) by comparing their spectral data with those previously reported [12—18].

Antioxidant activity on the free radicals and lipid peroxidation - The DPPH radical, superoxide radical scavenging activity was measured using a method described previously [19]. The lipid peroxidation inhibitory activity in a rat brain homogenate was evaluated using the thiobarbituric acid (TBA) method described previously [19].

Oxidative stress induced cell damage

Cell Culture - The human epidermal keratinocytes-Neonatal/Foreskin (HEK-N/F) cells were purchased from Modern Tissue Technologies, Inc. (MC1312, Korea). The HEK-N/F cells were cultured in a type IV collagen coated plate with KGM® Bulletkit medium (CC-3111, Clonetics) in humidified atmosphere of 5% CO_2/95% air at 37°C, and cultured to 90% confluence.

***t*-BuOOH induced oxidative stress** - The HEK-N/F cells (1×10^4 cells/100 μl) were seeded on a 96

well microplate and precultured for 24 h. The cells were then treated with 1 μl of the sample and 10 μl of t-BuOOH (1.5 mM) dissolved in Hank's balanced salt solution (HBSS, Gibco. BRL) for 3 h in order to induce cellular peroxidation. The cell viability was measured using the MTT method. The inhibitory activity of lipid peroxidation was also determined using the thiobarbituric acid (TBA) method, as previously described [20].

***In vivo* study**

Female SKH-1 hairless mice (5 weeks old) were housed under standard conditions (temperature 24 ± 2°C; relative humidity 50 ± 10%; 12 hr/day light/dark cycle) and given a commercial diet and water *ad libitum*. The mice were divided into four groups containing five mice each. Samples were administered topically at dose of 50, 30 and 10 mg/kg on their dorsal skin, and the dorsal skin of the mice was irradiated with UVB within 30 min of administration as described previously [21]. After 24 hr, the dorsal skin was collected from each mouse and frozen at -70°C until used.

Assay of lipid peroxidation - The dorsal skin was homogenized in 10 volumes of a 50 mM phosphate buffer (pH 7.8) under 4°C. The lipid peroxidation level was measured using the TBA method. The TBARS concentration was presented as nmol malondialdehyde/mg protein. The proteins were determined using the dye binding method using a Bio-Rad protein assay kit (Bio-Rad Lab., USA).

Inhibitory activity of telomere length shortening

Cell culture - The HEK-N/F cells were successively subcultured at a dilution rate of 1:8. The cells were grown to the population doubling level (PDL) of approximately 3 in the absence of the sample, and fed with or without the sample at a dose of 3 μg/ml successively upon each culture passage. The PDL was regarded to be zero for the culture starting immediately after the primary culture of the HEK-N/F cells, and was calculated to increase according to the following equation: $\log_2$ [(the number of collected cells)/(the number of seeded cells)]

Determination of telomere length by Southern blots - The genomic DNA was extracted using a nucleic acid extraction kit IsoQuick (ORCA Research Inc.) from 10^6 cells at each passage. The extracted DNA was cleaved with the restriction enzyme, *Hinf* I (TaKaRa), to produce terminal restriction fragments (TRFs). The telomere length was determined by Southern blots as previously described [22].

Statistical analysis

The Data is expressed as a mean ± SD, and the differences between the control and test groups were analyzed using a two-tailed Student's t-test. Differences $P < 0.05$ were considered to be statistically significant.

Results and Discussion

Antioxidant activity on the free radicals and lipid peroxidation

The antioxidant activity of the *C. chinensis* extract and its constituents (**1**—**19**) on the free radicals and lipid peroxidation is shown in Table I. Piceatannol (**5**) and myricetin (**9**) exhibited the potent radical scavenging activity and the lipid peroxidation inhibitory activity.

Cytoprotective effect on the *t*-BuOOH induced oxidative stress

t-BuOOH is an hydroperoxidant that can be metabolized to free radical intermediates, which can subsequently initiate lipid peroxidation resulting in cell damage. The cytoprotective effect of the *C. chinensis* extract and its constituents induced by *t*-BuOOH is shown in Table II. The cell viability of the HEK-N/F cells was significantly decreased by 11.2 ± 1.2% when they were treated with 1.5 mM *t*-BuOOH for 3 h, whereas the cell viability was increased by 53.5 ± 3.8% when they were treated with the *C. chinensis* extract at a concentration of 50 μg/ml. Some of the constituents exhibited potent cytoprotective activity (Table II). The cytoprotective activity was increased according to the number of hydroxyl groups on the aromatic ring, which suggests that their cytoprotective activity results in their electron donating activity, and in accordance with the radical scavenging activity [23]. Piceatannol (**5**) exhibited the most potent cytoprotective activity (84.7 ± 6.9%) on the tested compounds. Glycosylation of a hydroxyl group is completely inactive on the cytoprotective activity.

The aglycone, myricetin (**9**), exhibited higher activity than the glycoside, myricitrin (**12**). These results are in accordance with those reported in other assay systems [23],[24]. Although, Myricetin-3-*O*-(2″-*O*-galloyl)-α-L-rhamnopyranoside (**13**), (-)-Epicatechin-3-*O*-gallate (**16**) and (-)-Epigallocatechin-3-*O*-gallate (**17**) exhibited strong activity on radical scavenging and lipid peroxidation due to the residual galloyl group, they did not show a high activity in this assay system. The cytoprotective effect of the *C. chinensis* extract and its constituents (**1**—**19**) against the *t*-BuOOH induced oxidative stress was also confirmed by determining the level of lipid peroxidation (Table II). The TBA reactive substance (TBARS) of the cells treated with *t*-BuOOH (1.5 mM) for 3 h was increased 23.6-fold compared to the untreated control. As shown in Table II, the TBARS of the cells treated with the *C. chinensis* extract and some of the constituents, in particular, **5** were also significantly decreased, which means that the *C. chinensis* extract and its constituents could protect the cells from oxidative stress.

Protective effect on UVB-induced skin lipid peroxidation (*In vivo*)

The *C. chinensis* extract and piceatannol (**5**), which showed the most potent cytoprotective activity on oxidative stress, were further investigated to determine the protective effect against the UVB-induced skin lipid peroxidation *in vivo*. UVB radiation (in the range of 290-320 nm) is believed to be the major cause of skin cancer [25]. UVB is also known to induce excessive ROS generation, and lipid peroxidation is a marker of oxidative stress [25],[26]. As shown in Table III, the UVB exposed

mice skin had a significantly higher amount of TBARS (0.68 ± 0.10 nmol/mg protein) in 24 h after UVB irradiation. In contrast, UVB-induced skin lipid peroxidation was inhibited when the mice were topically administered with the *C. chinensis* extract at a dose of 50 and 30 mg/kg (Table III). The TBARS concentration was significantly reduced by 0.07 ± 0.03 nmol/mg protein ($P < 0.001$) when it was topically administered with **5** at a dose of 30 mg/kg. The amount of TBARS (0.16 ± 0.03 nmol/mg protein) was also significantly decreased even at dose as low as 10 mg/kg. As a result, it is demonstrated that the *C. chinensis* extract and **5** affords protection against the oxidative damage induced by UVB irradiation due to its potent antioxidant properties that efficiently reduce oxidative stress.

Elongating effect on cellular life-span

Most mammalian cells cultivated *in vitro* undergo a limited number of cell divisions and then arrest in a stage known as replicative senescence. In a report [22], it was found that cultivating the cells under enhanced oxidative stress accelerates the replicative senescence, and antioxidants could elongate the life span. To investigate the effect of the *C. chinensis* extract and its active constituents, **5** and **9**, on the cellular doubling potential, the HEK-N/F cells were serially subcultured until the spontaneous stoppage of cell division in the presence or absence of the samples. The untreated cells rapidly grew to a PDL of 14.1, whereas 3 μg/ml of the *C. chinensis* extract, **5** and **9** added cells enhanced the maximum PDL to 17.0, 29.9 and 23.2, respectively. The cellular life was

extended 1.21-, 2.12- and 1.65-fold, respectively, in comparison to the control group (Fig. 1).

Inhibitory effect of age-dependent telomere shortening

Based on the telomere theory of aging, the elongating effect of the cellular life span was evaluated by measuring the telomere length in each culture passage. The DNA extracted from the HEK-N/F cells of each culture passage was restricted by *Hinf* I to produce the TRFs, which were analyzed by Southern blot analysis. As shown in Fig. 2, the telomeres in the control cells gradually shortened from 12.42 kbp (PDL 2.8) to 7.90 kbp (PDL 14.1). In comparison to this, when the *C. chinensis* extract, **5** and **9** were continuously administered to the cells, the telomere length shortening was inhibited. It was observed that in the case of the cells administered to **5**, the telomere length had shortened by an average of 169 bp/PDL, which was slower than the 368 bp/PDL observed in the control group (Fig. 2). Cell division can be continuously promoted as far as the telomeric DNA is kept at more than a critical value (in this report, it is assumed to be approximately 7.9 kbp). From this viewpoint, it was found that the *C. chinensis* extract, **5** and **9** could be delay the shortening rate of the telomeric DNA and thereby, the rate to reach the critical value to be delayed and therefore it could extend a period for the termination of cell division.

Anti-aging effect of *T. chebula* based on the two aging theories

The two aging theories, oxidative stress theory and telomere theory, have been established well by

many related evidences. Recently, it was recognized that oxidative damage is repaired less well in the telomeric DNA than elsewhere in the chromosome, and oxidative stress accelerates telomere loss [22],[27—29]. Measuring both antioxidant activity on oxidative stress and telomere shortening rate, we found that the *C. chinensis* extract and its constituents could slowdown the telomere shortening rate by inhibition of oxidative stress. Therefore, it is suggested that the *C. chinensis* and its constituents protect the skin cells against oxidative stress and prevent thereby the cellular aging, followed by expectation as anti-aging cosmetic ingredient.

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Legend of Figures

Fig. 1. Elongating effect of the *C. chinensis* extract and its active constituents, piceatannol (**5**) and myricetin (**9**), on the life-span in the HEK-N/F cells. Cellular life-spans were elongated from PDL 14.1 for control to PDL 17.0, 29.9 and 23.2, respectively, for the *C. chinensis* extract, **5** and **9**-added cells.

Fig. 2. Dependence of the telomeric DNA length on the cellular ages (PDL) of the HEK-N/F cells administered with or without the *C. chinensis* extract, piceatannol (**5**) and myricetin (**9**). Mean TRF length is estimated as a center of mass and expressed in kbp based on the following equation: $\Sigma(MWi \times ODi)/\Sigma(ODi)$, where ODi is densitometric output and MWi is the length of the DNA at position i.

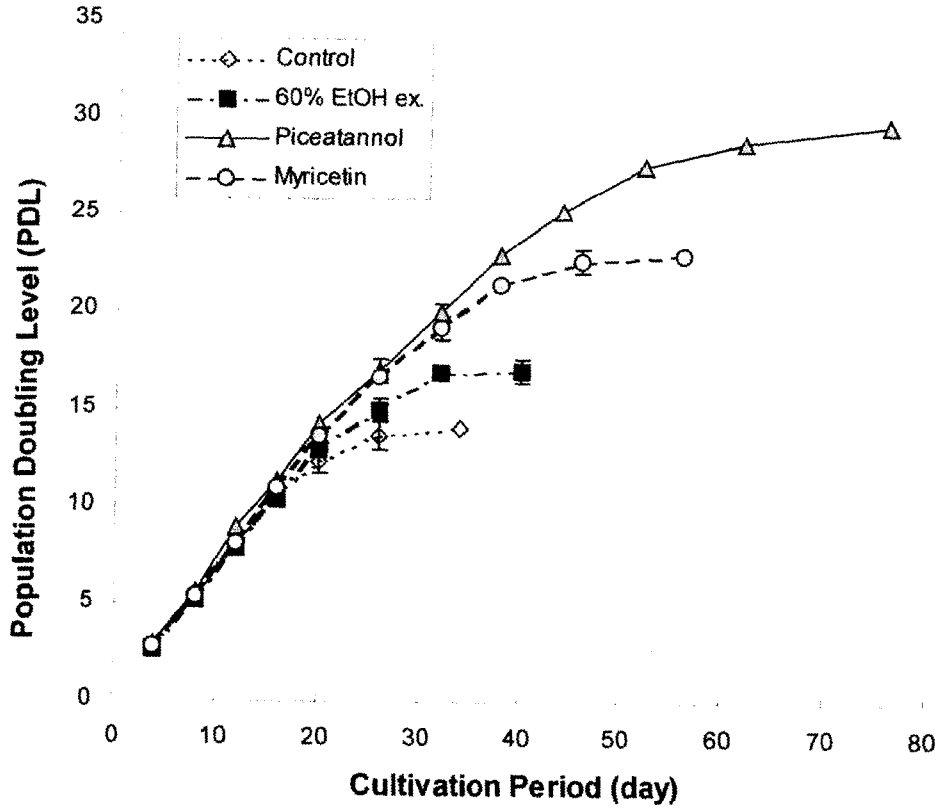
Control
60% EtOH ex.
Piceatannol
Myricetin
Population Doubling Level (PDL)
Cultivation Period (day)
0
5
10
15
20
25
30
35
0
10
20
30
40
50
60
70
80

Fig. 1

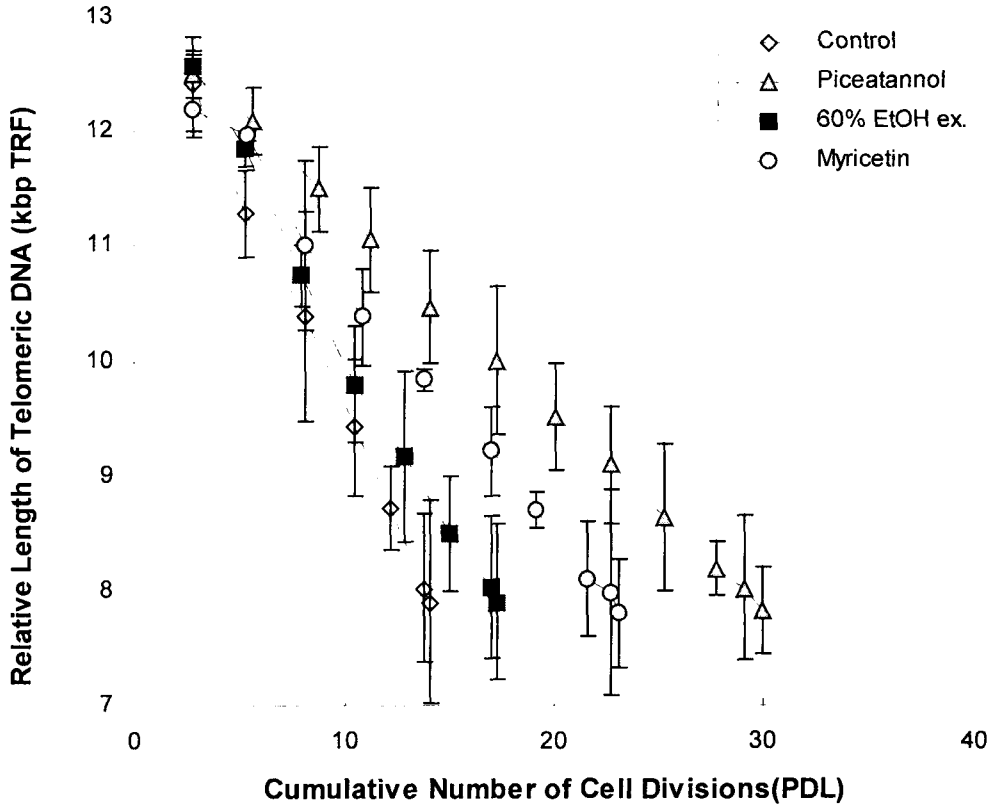
Relative Length of Telomeric DNA (kbp TRF)
13
12
11
10
9
8
7
0
10
20
30
40
Cumulative Number of Cell Divisions(PDL)
Control
Piceatannol
60% EtOH ex.
Myricetin

Fig. 2

Table I. Antioxidant activity of the *C. chinensis* extract and its constituents on the free radicals and lipid peroxidation

Compounds	DPPH radical scavenging activity IC_{50} (μg/ml)	Superoxide radical scavenging activity IC_{50} (μg/ml)	Lipid peroxidation inhibitory activity IC_{50} (μg/ml)
EtOH extract	35.6 ± 2.1	39.1 ± 4.0	12.5 ± 1.6
Chalcones			
Isoliquiritigenin (**1**)	> 50	47.6 ± 4.0	3.33 ± 0.50
Liquiritigenin (**2**)	> 50	49.8 ± 3.1	12.81 ± 1.86
2′,4′-Dihydroxy-4-methoxychalcone (**3**)	> 50	59.2 ± 3.8	6.05 ± 0.99
Stilbenes			
Resveratrol (**4**)	39.5 ± 2.8	45.3 ± 1.9	0.89 ± 0.10
Piceatannol (**5**)	20.9 ± 1.3	12.1 ± 1.7	0.09 ± 0.01
Phenolics			
Gallic acid (**6**)	5.1 ± 0.4	34.1 ± 2.4	6.80 ± 0.63
Methyl gallate (**7**)	5.3 ± 0.3	16.5 ± 1.4	7.05 ± 0.87
Ethyl gallate (**8**)	7.0 ± 1.0	15.8 ± 1.6	7.01 ± 0.62
Flavonols			
Myricetin (**9**)	7.3 ± 0.3	12.1 ± 1.1	0.95 ± 0.06
Afzelin (**10**)	33.4 ± 1.6	50.2 ± 3.4	10.25 ± 0.91
Quercitrin (**11**)	12.4 ± 0.6	32.1 ± 2.0	6.21 ± 0.40
Myricitrin (**12**)	11.6 ± 0.4	29.7 ± 1.8	5.27 ± 0.32
Myricetin-3-*O*-(2″-*O*-galloyl)-α-L-rhamnopyranoside (**13**)	8.6 ± 0.7	13.2 ± 2.5	4.73 ± 0.41
Syringetin-3-*O*-rutinoside (**14**)	35.1 ± 1.5	45.2 ± 2.2	11.0 ± 1.2
Flavanols			
(+)-Catechin (**15**)	15.6 ± 0.8	16.5 ± 2.0	4.71 ± 0.26
(-)-Epicatechin-3-*O*-gallate (**16**)	6.8 ± 0.5	11.9 ± 2.1	2.90 ± 0.06
(-)-Epigallocatechin-3-*O*-gallate (**17**)	6.7 ± 0.4	24.0 ± 3.5	1.00 ± 0.08
Lignans			
(-)-Lyoniresinol 3a-*O*-β-D-xylopyranoside (**18**)	45.7 ± 4.0	>100	37.42 ± 2.06
(+)-Lyoniresiol 3a-*O*-β-D-glucopyranoside (**19**)	42.6 ± 3.1	>100	39.10 ± 3.11
α-tocopherol	25.4 ± 0.9		6.61 ± 0.95
Caffeic acid		11.0 ± 1.8	
BHA	15.3 ± 0.6	48.8 ± 2.5	0.11 ± 0.02

Values are expressed as mean ± SD of three replicates.

Table II. Cytoprotective effect of the *C. Chinensis* extract and its constituents on *t*-BuOOH-induced oxidative damage

Compounds	Cell viability (%)	TBARS (pmol/mg protein)
Blank	100	370.2 ± 51.2**
Control	11.2 ± 1.2	8721.5 ± 809.5
EtOH extract	53.5 ± 3.8**	984.0 ± 95.2**
Chalcones		
Isoliquiritigenin (**1**)	13.9 ± 2.6	5118.1 ± 410.5*
Liquiritigenin (**2**)	16.2 ± 3.2*	3449.7 ± 305.8**
2′,4′-Dihydroxy-4-methoxychalcone (**3**)	12.1 ± 1.0	8255.1 ± 576.4
Stilbenes		
Resveratrol (**4**)	18.1 ± 1.6*	2792.4 ± 259.1**
Piceatannol (**5**)	84.7 ± 6.9**	520.2 ± 60.7**
Phenolics		
Gallic acid (**6**)	41.5 ± 3.1**	1245.7 ± 112.4**
Methyl gallate (**7**)	43.0 ± 5.6**	1024.4 ± 91.9**
Ethyl gallate (**8**)	43.1 ± 4.1**	1115.6 ± 96.2**
Flavonols		
Myricetin (**9**)	61.0 ± 4.5**	810.7 ± 77.0**
Afzelin (**10**)	16.8 ± 1.3*	3295.1 ± 304.5**
Quercitrin (**11**)	21.4 ± 1.9*	2217.2 ± 200.3**
Myricitrin (**12**)	25.4 ± 1.7*	1852.6 ± 126.8**
Myricetin-3-*O*-(2″-*O*-galloyl)-α-L-rhamnopyranoside (**13**)	45.1 ± 3.6**	1019.8 ± 154.0**
Syringetin-3-*O*-rutinoside (**14**)	20.0 ± 2.6*	2928.1 ± 209.9*
Flavanols		
(+)-Catechin (**15**)	24.5 ± 1.9*	1912.4 ± 112.0**
(-)-Epicatechin-3-*O*-gallate (**16**)	49.4 ± 6.4**	1007.3 ± 95.2**
(-)-Epigallocatechin-3-*O*-gallate (**17**)	46.6 ± 5.9**	1032.6 ± 103.5**
Lignans		
(-)-Lyoniresinol 3a-*O*-β-D-xylopyranoside (**18**)	13.5 ± 2.0	5981.1 ± 412.6*
(+)-Lyoniresiol 3a-*O*-β-D-glucopyranoside (**19**)	12.9 ± 2.1	7635.7 ± 631.2

Values are expressed as mean ± SD of three replicates.

* $P < 0.05$, ** $P < 0.001$, were significantly different from the group treated *t*-BuOOH.

Table III. Effect of the *C. chinensis extract* and piceatannol (**5**) on UVB-induced lipid peroxidation in SKH-1 mice

Sample treatment (mg/kg)		Amount of TBARS (nmol/mg protein)
Control		0.32 ± 0.05*
UVB irradiated		0.68 ± 0.10
C. chinensis extract	50	0.21 ± 0.10*
+ UVB irradiated	30	0.34 ± 0.09*
	10	0.64 ± 0.10
Piceatannol (**5**)	30	0.07 ± 0.03**
+ UVB irradiated	10	0.16 ± 0.05*

Values are expressed as mean ± SD.

* $P < 0.05$, ** $P < 0.001$, were significantly different from the UVB irradiated group.