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Antioxidant activities of compounds isolated from *Dalbergia odorifera T. Chen* and their inhibition effects on the decrease of glutathione level of rat lens induced by UV irradiation

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Abstract

Four compounds were isolated from the root of *Dalbergia odorifera T. Chen* and identified by chemical and spectroscopic methods as 2′-*O*-methyl-isoliquiritigenin (**1**), odoriflavene (**2**), 5′-methoxy-vestitol (**3**) and formononetin (**4**). Their antioxidant activities were investigated in lard by the oxidative stability instrument; their inhibitory effects on the decrease of glutathione level of rat lens induced by UV irradiation were studied using a rat lens UV-damage model, and their *in vitro* cytotoxicity was measured on human SH-SY5Y neuroblastoma cells. Results indicated that all four compounds had obvious antioxidant effects, while none of them showed any synergistic effects on butylated hydroxytoluene or α-tocopherol. When $FeCl_3$ (4.0 μM) was added, the antioxidant activities of the four compounds markedly decreased. The inhibitory effects of compounds **1**, **2** and **4** on the decrease of glutathione level of rat lens induced by UV irradiation were comparable to that of α-tocopherol. All four compounds showed cytotoxic activity when evaluated against a SH-SY5Y cell line *in vitro*.

Keywords: *Dalbergia odorifera*; Antioxidant activity; Cataract; Glutathione; Lens; Cytotoxicity

1. Introduction

Lipids containing polyunsaturated fatty acids are readily oxidized by molecular oxygen and such oxidation proceeds by a free radical chain mechanism (Aruoma, 1998; De Groot & Noll, 1987).When oxygen is supplied in excess or its reduction is insufficient, reactive oxygen species would be generated and take part in lipid peroxidation, which causes food deterioration, aging of organisms and many pathological events (Ashok & Ali, 1999; Cerruti, 1994). Antioxidants act as radical-scavengers, inhibiting lipid peroxidation and other free radical-mediated process; thereby they play an important role in food preservation and human health (Takao, Kiatani, Watanabe, Yagi, & Sakata, 1994).

Active oxygen species deriving from oxygen, or from the pro-oxidant stimulation of oxygen metabolism, participate in the development of several diseases, such as cataract, ischemia-reperfusion disturbances in the brain and heart, arteriosclerosis, rheumatism, inflammatory disorders, gastric ulcer, and cancer (Nihro, Miyataka, Sudo, Matsumoto, & Satoh, 1991). Cataract is the leading cause of blindness, worldwide, accounting for over 50% of the world's blind population and affecting about 17 million people. UV radiation from sunlight and other ambient sources is considered the major causative factor for the onset of lens opacification and cataract processes (Mamdouh & Mohsen, 2001). Many investigators have reported that UV-induced cataractogenesis acts through increase of free radicals and the induction of photooxidation in the lens.

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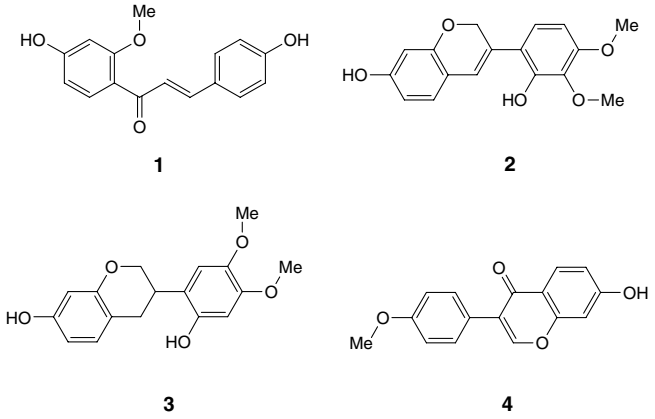

Fig. 1. Chemical structures of compounds **1**–**4**.

Numerous studies indicate that oxidative stress, mediated by active oxygen species in the lens, and lipid peroxides, produced in the crystalline lens, can initiate the process of cataractogenesis. It is of great importance to develop antioxidants in treatment of cataracts.

Glutathione (GSH) also exists in the lens, and is an essential antioxidant factor for maintenance of the tissue's transparency. Via an active GSH redox cycle located in the lens epithelium and superficial cortex, GSH detoxifies potentially damaging oxidants, such as H_2O_2 and dehydroascorbic acid, and indicates an important hydroxyl radical-scavenging function (Giblin, 2000).

Seven hundred species of plants of Chinese traditional medicines have been found to have obvious antioxidant activities and 24 plant species therein show strong antioxidant activities (Weng, Ren, Duan, & Jiang, 1998). One of the plants is *Dalbergia odorifera T. Chen*, a high tree. Its heart wood of stem and root is named as Jiangxiang in Chinese traditional medicine, which is indigenous in Guangdong, PR China and belongs to the *Leguminosae*. Its constituents of isoflavonoids and other compounds were reported in a previous study (Shoji et al., 1989; Tetsu, Shoji, Reiko, Ryoji, & Toshihiro, 1990; Yukihiro, Fumiyuki, Masaak, & Ushio, 1992).

In this paper, four compounds had been isolated from the root of *D. odorifera T. Chen* (Fig. 1). Their antioxidant activities were examined in lard by the oxidative stability instrument (OSI) at 100 °C. Their inhibitory effects on the decrease of GSH level of rat lens, induced by UV irradiation, were studied, using a rat's lens UV-damage model, and their cytotoxicity was also tested on SH-SY5Y neuroblastoma cells.

2. Materials and methods

2.1. Materials and equipment

The root of *D. odorifera* was purchased from Shandong Yantai Pharmaceutical Company in 1998 and stored at 4 °C before use. It was identified by Professor Runeng Zhao (Lanzhou Medical College, China). Butylated hydroxytoluene (BHT) and α-tocopherol were purchased from Beijing Chemical Company. Daily buffer (NaDPH), 5,5′-dithio bis(2-nitrobenzoic acid) and GSH were purchased from the Sino-American Biotechnology Company. $FeCl_3 \cdot 6H_2O$, C.P., was purchased from Jinshan Chemical Factory. Silica gel was obtained from Qingdao Ocean Chemical Factory. Lard was rendered in the laboratory from fresh pig fat tissue, purchased from Yantai Slaughter House. Wistar rats were obtained from the Experimental Animal Department of Peking University. Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin solution, fetal calf serum (FCS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich Chemical Company.

Melting points were determined on an X4-micro-melting point apparatus, uncorr. Optical rotations were measured with a polarimeter 241 (Perkin Elmer), using MeOH as solvent. IR-spectra were recorded on a Nicolet – 5DX IR spectrometer. ^1H and ^{13}C spectra were recorded with a Bruker AM-400 instrument, using solvents $CDCl_3$ or acetone-d_5, and using TMS as internal standard. EIMS data were determined at 70 eV (180 °C) on a ZAB-HS mass spectrometer. The absorbance of the MTT assay was measured on a Bio-Rad 550 microplate reader.

2.2. Extraction and isolation

The dried and powdered root of *D. odorifera* (3 kg) was extracted repeatedly with 95% ethanol for 2 weeks at room temperature. The extract was concentrated to dryness under reduced pressure, and the residue (318 g) was suspended in water (1 l). The water layer was extracted successively with petroleum ether, $CHCl_3$, EtoAc and *n*-BuOH, respectively. The EtoAc extract was evaporated *in vacuo* to give a residue of 112 g. The residue was subjected to column chromatography (CC) on silica gel, eluted with petroleum ether:EtoAc (10:1–0:10) to give nine fractions. Fraction 3 was rechromatographed on a silica gel column, using petroleum ether:EtoAc (15:1–6:1) to give compound **1** (24 mg). Fraction 6 was subjected to CC on a silica gel column eluted with benzene:acetone (6:1–4:1) to obtain compound **2** (28 mg). Fractions 7–9 were obtained by repeated CC on a silica gel column eluted with $CHCl_3$:acetone (10:1–4:1) to give compound **3** (25 mg), and compound **4** (24 mg), respectively.

2.3. Antioxidant activities

Antioxidant activities of the four compounds were studied in lard with an Omnion OSI (MA, USA) at 100 °C (Wang, Weng, & Cheng, 2000). The airflow rate was fixed at 20 l/h. Fe^{3+}, BHT and α-tocopherol were used as positive controls.

2.4. Inhibition effects on the decrease of GSH level of rat lens induced by UV irradiation

2.4.1. Lens solution preparation

The rat's eyes were freshly enucleated from Wistar rats (100–150 g) euthanized with overdoses of anesthetics. All animal procedures were in accordance with the ARVO resolution on animals and ophthalmic research. Then the lenses were carefully excised and homogenized with physiological saline solution. After centrifugation, the supernatant of the lens homogenate with tested compounds was exposed to UV irradiation (8 μW/cm^2) with wavelengths ranging from 365 to 254 nm for 15 min.

2.4.2. Quantification of total GSH

The GSH level was determined by a method previously described (Anderson, 1985). Daily buffer (NaDPH 0.248 mg/ml), 5,5′-dithio bis(2-nitrobenzoic acid) and water were pipetted in amounts into each cuvette. The cuvettes were placed at 30 °C in a water bath for 12–15 min. The tested compounds were added and mixed to initiate the assay. Absorbance was measured at 412 nm. The amount of GSH was determined as μmol/g tissue and a standard curve was made in which GSH equivalents were present.

2.5. Cytotoxicity assay

2.5.1. Cell culture

Human neuroblastoma SH-SY5Y cells were grown in DMEM supplemented with 10% FCS, 100 U/ml of penicillin and 100 μg/ml of streptomycin in a humidified atmosphere at 37 °C in 5% CO_2.

2.5.2. MTT assay

Cytotoxicity of the four compounds was determined on SH-SY5Y cells *in vitro* using a modified MTT assay previous described in detail (Plumb, Milroy, & Kaye, 1989). Briefly, SH-SY5Y cells were seeded in 96-well plates at 5×10^4 cells/well and allowed to attach. After incubation with medium containing a serial of doubling dilution of the tested compounds for 48 h, MTT was added to all wells to a final concentration of 0.5 mg/ml and incubated for 3 h at 37 °C. Then cells were briefly centrifuged and media aspirated off from the wells prior to adding DMSO (100 μl to each well) to dissolve the MTT formazan crystals. The absorbance was measured at 490 nm on a Bio-Rad microplate reader. Controls included untreated cells and medium alone, with all MTT assays performed in triplicate.

3. Results and discussion

3.1. Spectrometric identification of isolated compounds **1**–**4**

3.1.1. 2′-O-Methyl-isoliquiritigenin (**1**)

Pale yellow powder was obtained, m.p.: 186–188 °C. Its ^{1}H NMR spectrum showed the signals of nine protons consisting of a pair of ABX type signals [δ 6.51 (1H, d, $J = 2.0$ Hz), 6.43 (1H, dd, $J = 2.1$, 8.0 Hz), 7.53 (1H, d, $J = 8.2$ Hz)], a pair of A_2B_2 type signals [δ 6.80 (2H, d, $J = 8.0$ Hz), 7.53(2H, d, $J = 8.1$ Hz)] and two proton signals [δ 7.42(s)]. Besides the above signals, a methoxy signal [δ 3.85(3H, s)] and two hydroxyl signals (δ 10.10, brs) were observed, and compound **1** was identified as the known structure and showed a molecular ion at m/z 270 in EI-MS. The data and physical constants agreed completely with previous reports (Yukihiro et al., 1992).

3.1.2. Odoriflavene (**2**)

Colourless needles were obtained, m.p.: 177–179 °C. ^{1}H NMR spectral data agreed with odoriflavene isolated from this plant (Mamdouh & Mohsen, 2001). The IR spectrum indicated the presence of hydroxyl (ν_{max} 3245 cm^{-1}) and aromatic ring groups (ν_{max} 1610, 1495, 1460, 1438 cm^{-1}). Compound **2** showed a molecular ion at m/z 300 in EI-MS.

3.1.3. 5′-Methoxy-vestitol (**3**)

A pale brown powder was obtained, m.p.: 228–230 °C. Its ^{1}H NMR spectrum exhibited ABX-type signals [δ 6.30 (1H, d, $J = 2.1$ Hz), 6.36 (1H, dd, $J = 2.2$. 8.0 Hz), and 6.88 (1H, d, $J = 8.1$ Hz)] and two single signals at δ 6.57 and 6.76 due to phenolic protons. Moreover, signals due to two methoxyls at δ 3.70 and 3.73, one oxygenated methylene at δ 3.80–4.40 and one methylene at δ 2.90 were observed. Compound **3** showed a molecular ion at m/z 302 in EI-MS.

3.1.4. Formononetin (**4**)

A pale yellow powder was obtained, m.p.: 221–223 °C. It showed a molecular ion at m/z 263 in EI-MS. Compound **4** was determined to be formononetin by comparing MS, IR and ^{1}H NMR data with reported data (Mamdouh & Mohsen, 2001).

3.2. Examination of the antioxidant activities in lard

Antioxidant activities of the four compounds were studied in lard with an Omnion OSI (Wang et al., 2000). The OSI method is a standard method for the examination of antioxidant activities normalized by the American Lipid Chemists Association.

The antioxidant activities of the compounds were interpreted by the protection factor (Pf), which was calculated according to the expression:

$$\mathrm{Pf} = \frac{\text{Oxidant Induction Period for (lard + antioxidant)}}{\text{Oxidant Induction Period for lard}}$$

The Oxidant Induction Period for lard, with or without antioxidant, was managed and automatically reported by the OSI. A higher value of Pf means a greater antioxidant activity. Pf $\geqslant$ 2 means that the compound possesses obvious antioxidant activity.

As shown in Table 1, all of the four compounds had obvious antioxidant effects at 0.1 and 0.2 mM. Compounds

Table 1
The antioxidant protection factor (Pf[a]) of compounds at 100 °C ($n = 4$)

Compound	0.1 mM[b,c]	0.2 mM[b,c]
1	$2.32 \pm 0.05^{**,++}$	$3.50 \pm 0.08^{**,++}$
2	$3.31 \pm 0.17^{**,+}$	$4.81 \pm 0.21^{**,++}$
3	$2.70 \pm 0.18^{**,++}$	$3.33 \pm 0.12^{**,++}$
4	$2.79 \pm 0.17^{**,++}$	$4.67 \pm 0.25^{**,+}$
BHT	4.21 ± 0.03	5.82 ± 0.04
α-Tocopherol	3.72 ± 0.06	4.21 ± 0.07

[a] The antioxidant protection factor is used to interpret the antioxidant activity of the compounds. It is the ratio of the oxidant induction period for lard with antioxidant to that without antioxidant.
[b] ** $P < 0.01$, *vs* α-tocopherol.
[c] $^{+}P < 0.05$, $^{++}P < 0.01$, *vs* BHT.

2 and **4** produced significantly greater antioxidant activities than did α-tocopherol at 0.2 mM ($P < 0.01$), but smaller than did BHT ($P < 0.05$).

The synergistic effects (synergy%) of antioxidants A and B were calculated according to the expression:

$$\text{Synergy}\% = \frac{(\text{IP}_{\text{A+B combined}}) - \text{IP}_\text{A} - \text{IP}_\text{B}}{\text{IP}_\text{A} + \text{IP}_\text{B}} \times 100\%$$

where IP: Oxidant Induction Period.

Table 2 shows the synergistic antioxidant activities of the four compounds with equimolar BHT or α-tocopherol at 0.1 mM. When BHT or α-tocopherol was added, all of the four compounds produced greater antioxidant activities than did those alone. The antioxidant activities of compounds **2** and **4** with BHT or α-tocopherol were better than that of BHT (4.21 ± 0.03, in Table 1) or α-tocopherol (3.72 ± 0.06, in Table 1) alone. However, their synergistic effects were negative.

Table 2
The synergistic antioxidant activities of compounds (0.1 mM) with equimolar BHT or α-tocopherol at 100 °C ($n = 4$)

Compound	Pf with BHT[a]	S (%)[b]	Pf with α-tocopherol[c]	S (%)[b]
1	4.21 ± 0.04	−35.5	3.27 ± 0.05	−45.9
2	4.48 ± 0.11	−40.4	3.86 ± 0.21	−45.1
3	3.09 ± 0.17	−55.3	2.83 ± 0.15	−55.9
4	6.85 ± 0.22	−2.1	6.25 ± 0.26	−4.0

[a] The antioxidant protection factor of the compounds (0.1 mM) with equimolar BHT.
[b] Synergy%, the synergistic antioxidant effects of the compounds with BHT or α-tocopherol.
[c] The antioxidant protection factor of the compounds (0.1 mM) with equimolar α-tocopherol.

Table 3
The antioxidant protection factor (Pf) of compounds at 0.1 mM with Fe^{3+} at 100 °C ($n = 4$)

Compound	Pf with Fe^{3+}(4.0 μM)[a]
1	1.79 ± 0.03
2	1.96 ± 0.15
3	1.25 ± 0.21
4	0.90 ± 0.12

[a] The antioxidant protection factor of the compounds (0.1 mM) with Fe^{3+} (4.0 μM).

Fe^{3+} was one of the pro oxidant factors. The presence of Fe^{3+} resulted in a marked drop of antioxidant activities (Table 3). With reference to Table 1, when Fe^{3+} (4.0 μM) was added, the antioxidant activities of all four compounds clearly decreased.

3.3. Inhibitory effects on the decrease of GSH level of rat lens induced by UV irradiation

The lens is maintained in a very high reductive state, peroxidation of the lens constituents is known to be an early event in the development of cataracts. There is an antioxidant system known as the GSH redox cycle in the lens, and decrease of the activity of this system appears to be closely associated with lens oxidation.

From Table 4 we can see that, the GSH level of the model group treated with UV irradiation was obviously lower than that of the blank control group treated with from UV irradiation ($P < 0.05$), which indicated that UV irradiation could induce a decrease of the GSH level of rat lens. However, α-tocopherol could effectively inhibit the GSH level decrease ($P < 0.05$, as compared with the model control). Compounds **1**, **2** and **4**, at 0.43 and 0.86 mM, showed inhibitory effects on the GSH level decrease of rat lens induced by UV irradiation comparable with α-tocopherol ($P > 0.05$).

3.4. Cytotoxic effects on SH-SY5Y cells

The cytotoxic effects were assessed by MTT with SH-SY5Y cells. The IC_{50} values of compounds **1**–**4** were 32.5, 11.2, 28.5, and 13.4 μM, respectively. All four compounds showed cytotoxic activity on the SH-SY5Y cell line *in vitro*. Compound **2** exhibited the most potent cytotoxic effect compared to formononetin (**4**) which has been reported to show moderate cytotoxic activity on two human cancer cell lines (Ngamrojanavanich et al., 2007) (Table 5).

Previous phytochemistry studies have reported that *D. odorifera T. Chen* contained flavonoids and other compo-

Table 4
Effects of compounds on the GSH level in rats lens induced by UV irradiation (μg GSH/g tissue)

Compound	0.43 mM[c,d,e]	0.86 mM[c,d,e]
Blank control[a]	$43.3 \pm 9.66^{+}$	
Model control[b]	$13.6 \pm 3.02^{\#,*}$	
1	$29.7 \pm 8.25^{+}$	$39.4 \pm 11.3^{+}$
2	$27.1 \pm 4.8^{+}$	$38.2 \pm 10.9^{+}$
3	$22.8 \pm 7.21^{*}$	$32.1 \pm 9.36^{*}$
4	$27.1 \pm 7.04^{+}$	$38.8 \pm 11.7^{+}$
α-Tocopherol	$26.2 \pm 6.35^{+}$	$39.5 \pm 12.0^{++}$

[a] Lens solution was not dealt with UV irradiation.
[b] Lens solution was dealt with UV irradiation but without any tested compounds.
[c] $^{\#}$ $P < 0.05$, *vs* blank control.
[d] $^{+}$ $P < 0.05$, $^{++}$ $P < 0.01$, *vs* model control.
[e] * $P < 0.05$, *vs* α-tocopherol.

Table 5
Cytotoxic activities of compounds **1**–**4** on SH-SY5Y cell line

Compound	IC_{50} (μM)
1	32.5
2	11.2
3	28.5
4	13.4

nents (Shoji et al., 1989; Tetsu et al., 1990; Yukihiro et al., 1992). Flavonoids are a group of natural benzo-γ-pyran derivatives and are ubiquitous in photosynthesizing cells (Havsteen, 1983), which act as pharmacologically active constituents in many plant medicines and have multiple biological activities, including vasodilative, anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, antiallergic and antiviral effects (Middleton & Kandaswami, 1992). Compounds **1**–**4** and their homologues have been reported in many plant species and they exhibit various biological activities. Isoliquiritigenin and its homologues show antitumorigenic activities (Baba et al., 2000; Iwata et al., 1995; Yamazaki et al., 2002). Odoriflavene (**2**) can significantly inhibited prostaglandin biosynthesis, as well as platelet aggregation, induced by arachidonic acid (Goda, Kiuchi, Shibuva, & Sankawa, 1992). Vestitol is reported, not only to have potent CYP3A4 inhibitory activities (Tsukamoto et al., 2005), but also to exhibit anti-*Helicobacter pylori* activity against the CLAR- and AMOX-resistant strain as well as four CLAR, (AMOX)-sensitive strains, (Fukai et al., 2002), which may be a useful chemopreventive agent for peptic ulcer or gastric cancer in *H. pylori*-infected individuals. Formononetin (**4**) has been previously isolated from *Machaerium aristulatum* as an antigiardial constituent and also possess antitumor (Ngamrojanavanich et al., 2007), anti-fungal (Lopes, Kato, & Yoshida, 1999) and weak anti-*H. pylori* activity (El-Sohly, Joshi, & Nimrod, 1999; Fukai et al., 2002).

The antioxidants play a very important role in the food industry and are used to preserve food against oxidative deterioration of lipid. Synthetic antioxidants, such as BHT, are widely used in the food industry, but have been suspected to be responsible for liver damage and carcinogenesis in laboratory animals (Grice, 1986). From this point of view, governmental authorities and consumers are concerned about the safety of food and about the potential effects of synthetic additives on health (Reische, Lillard, & Eintenmiller, 1998). Therefore, the development and utilization of more effective antioxidants of natural origin, such as like the components of Chinese traditional medicines, are desired. Recently, the antioxidant activities of flavonoids have given rise to much attention. As polyphenolic compounds, flavonoid have the ability to act as antioxidants by a free radical-scavenging mechanism with the formation of reactive flavonoids phenoxyl radicals:

$$ROO^{\cdot} + FI - OH \rightarrow ROOH + FI - O^{\cdot}$$
$$HO^{\cdot} + FI - OH \rightarrow H_2O + FI - O^{\cdot}$$

D.odorifera T. Chen is valued, not only for its medicinal properties, but also for its antioxidant activities. In our work, all four compounds showed obvious antioxidant effects at 0.1 and 0.2 mM. Compounds **1**, **2** and **4** had inhibitory effects on the decrease of GSH level of rat lens induced by UV irradiation comparable to that of α-tocopherol, which could be useful for further investigation of the mechanism of the cataractogenesis process and the developing antioxidants in treatment of cataracts. In addition, all four compounds exhibited cytotoxic activity on the SH-SY5Y cell line *in vitro*. These experimental results may provide favourable evidence for further studies of these compounds.

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