Antioxidative Glucosides from the Fruits of *Ligustrum lucidum*

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The ethanol extract of the fruits of *Ligustrum lucidum* was shown to have inhibitory effects on the hemolysis of red blood cells induced by 2,2'-azo-bis-(2-aminopropane) dihydrochloride. Bioassay-guided analysis led to the isolation of ten secoiridoid glucosides. Two of them were new, lucidumosides C and D. Their structures were elucidated by spectroscopic methods. The other eight compounds were identified as oleoside dimethyl ester, ligustroside, oleuropein, nuzhenide, isonuezhenide, neonuezhenide, lucidumoside A and lucidumoside B. Five compounds, oleoside dimethyl ester, oleuropein, neonuezhenide, lucidumoside B and lucidumoside C, exhibited strong antioxidant effect against hemolysis of red blood cells induced by free radicals.

Key words  *Ligustrum lucidum*; Oleaceae; antioxidant; lucidumoside C; lucidumoside D

The metabolic processes in the human body, inevitably, often produce harmful free radicals. These free radicals have been shown to possibly contribute to aging and various diseases, such as atherosclerosis and cancer.1—2) Therefore, it is important to identify effective radical scavengers to help relieve the damaging effects of free radicals. Our screening program for potent antioxidants from natural products has confirmed that polyphenolic compounds present in foods of plant origin do have strong radical scavenging and oxidation-inhibiting effects. For example, the phenylethanoids glycosides from *Brandisia hancei* were found to have potent antioxidative properties.3)

Recently, our attention on the fruit of *Ligustrum lucidum* Arr. (Oleaceae), which is known as “Nuzhenzi” in China. It is commonly used in Chinese medicine to supplement “kidneys”, nourish “yin”, strengthen liver and clear vision.4) Previous studies reported that the fruit of *L. lucidum* has immunomodulatory,5) hypolipemic,6) antiinflammatory,7) hepatoprotective,8) antitumor9) and antiaging effects.10) Relevant species of the same genus showed inhibitory effect on acyl CoA cholesterol acyltransferase,11) and immunomodulatory,12) and anticomplementary activities.13) These biological effects appear to support some of the ethnomedical claims. Chemical studies have found volatile components, triterpenes, flavonoids, secoiridoid glucosides, and phenolic compounds from this plant, and constituents such as phenylethanoids, monoterpenes, and secoiridoid glucosides from other *Ligustrum* species.11,14—19)

In our screening program, ethanol extract of the fruits of *L. lucidum* showed antioxidative effect. Through bioassay-guided analysis, ten secoiridoid glucosides were isolated from the active fractions. Two of the secoiridoid glucosides, lucidumosides C (9) and D (10), were new. Here we report the antioxidative activity of these constituents of *Nuzhenzi* against hemolysis of red blood cells (RBC) induced by free radicals, and also the structure elucidation of compounds 9 and 10.

The fruits of *L. lucidum* were extracted with ethanol and the ethanol extract showed significant inhibitory effects (IC₅₀ = 125 µg/ml) on the hemolysis of RBC induced by free radicals (Fig. 1). The ethanol extract was partitioned with water into an aqueous fraction (A) and an insoluble fraction (B). The bioactivity of the ethanol extract against hemolysis of RBC test system was found concentrated in fraction A (IC₅₀ = 40 µg/ml). This fraction A was chromatographed on a porous polymer gel D-101 column with water, 60% aqueous ethanol and ethanol, to give fractions A₁, A₂ and A₃ (Fig. 2). The biological activity was found concentrated in fraction A₂, IC₅₀ being 31 µg/ml. From the active fraction A₂, two new secoiridoid glucosides, lucidumosides C (9) and D (10), were isolated together with eight known secoiridoid glucosides, oleoside dimethyl ester (1), ligustroside (2), oleuropein (3), nuzhenide (4), isonuezhenide (5), neonuezhenide (6), lucidumoside A (7) and lucidumoside B (8). Compounds 1—8 were identified by comparison of spectral data with reported data.18)

Compound 9 was obtained as an amorphous powder, [α]D ~112° (MeOH). Its molecular formula was identified as C₂₅H₃₆O₁₄ from its ESI-MS spectrum (m/z 583 [M−H]− and HR-FAB mass spectrum (m/z 585.5817 [M+H]+). The presence of an aromatic moiety and a conjugated ester group was indicated by the absorptions in the UV (235, 282 nm) and IR (1640, 1450 cm⁻¹) spectra of 9. The ¹H-NMR spectrum showed the following signals due to a feature oleosidic aglycone moiety: an olefinic proton [δ 7.52 (1H, s, H-3)], an acetalcarbinal proton [δ 5.92 (1H, s, H-1)], three olefinic methyl protons [δ 1.70 (3H, d, J = 6.9 Hz, H₁-10)] and an olefinic proton [δ 6.10 (1H, q, J = 6.9 Hz, H-8)]. Its ¹H-NMR spectrum (Table 1) indicated an ABX system which belongs to the phenyl. ¹H-NMR signals of an anomic proton at δ 4.81 (1H, d, J = 7.8 Hz) is consistent with the configuration for β-α-glucose. When comparing the ¹H signals of 9 with those of 3, their ¹H-NMR signals were similar, but the signals of H-α and β in the phenethyl moiety were different. Also, there was one more set of ethoxy which was shown at δ 4.59 (1H, t, J = 5.4 Hz) and δ 3.44 (2H, m) in 9.

As the signal of CD₃OD solvent overlapped with other signals, the 2D NMR spectrum of glucoside 9 was measured in pyridine-d₅. The signals of proton and carbon of 9 were excited by ¹H−¹H and ¹H−¹³C COSY experiment. The chemical shift of H-2β in phenethyl moiety of 9 shifted downfield from δ 2.85 (2H, t, J = 6.7 Hz) to δ 4.59 (1H, t, J = 7.1 Hz) as compared with those of 3. Furthermore, in the HMBC experiment, the correlation between H′-1′ (δ 5.47) of the glucose

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unit and the C-1 atom ($\delta$ 94.9) of the aglycone moiety, three methoxy protons ($\delta$ 3.65) and the C-11 of the aglycone moiety, H-\(\alpha\) ($\delta$ 4.33) of the phenylethyl moiety and the C-7 ($\delta$ 171.5) of the aglycone unit, the H-\(\beta\) ($\delta$ 4.59) of the phenylethyl moiety and the –OCH\(_2\)CH\(_3\) ($\delta$ 64.4) were observed. Additional significant long-range correlation confirming the proposed structure were shown in Fig. 4. The structure of this glucoside was concluded to be as shown in 9. This new secoiridoidic glucoside is designated as lucidumoside C.

Compound 10 was obtained as an amorphous powder. The ESI-MS of compound 10 exhibited a pseudomolecular ion [M–H]\(^{-}\) at \(m/z\) 567 and its HR-FAB mass spectrum \(m/z\) 569.5721 [M+H]\(^{+}\), compatible with the molecular formula C\(_{27}\)H\(_{36}\)O\(_{13}\). The UV (278 nm) and IR (1520 cm\(^{-1}\)) spectra of 10 displayed the presence of an aromatic moiety. Its \(^1\)H spectrum and \(^{13}\)C DEPT experiment exhibited 3,4-dihy-
doxyphenylethyl group by the observation of typical ABX spin system and six aromatic carbon [δ 130.5 (C), 112.1 (CH), 149.6 (C), 147.6 (C), 113.1 (CH), 121.8 (CH)], in addition to the occurrence of a CH$_2$CH$_2$O group [δ 4.23 (t, J = 6.9 Hz) and 2.90 (t, J = 6.9 Hz)]. The $^1$H-NMR spectrum in Table 1 exhibited one set of signals for a methoxy group,
two olefinic protons (H-3, H-8), an acetalcalbinol proton (H-1), and an olefinic methyl group (H-10) signals. The 1H-NMR signals of an anemic proton at \( \delta 4.83 \) (1H, \( d, J= 7.7 \) Hz) is consistent with configuration for \( \beta \)-d-glucose. These spectral features proved the presence of an identical moiety of oleo-side dimethyl ester (1) in compound 10. The 1H- and 13C-NMR spectra of glucoside 10 were very similar to those of 3 except for the appearance of signals of two methoxy group in 3 (Tables 1 and 2). By comparison of 13C-NMR signals of 10 with those of 3, the chemical shift assignable to C-3" and 4" of 3 shifted downfield 2.9 and 3.0 ppm, respectively. The two methoxy group should be located at the C-3" and 4" position of the phenylethyl moiety in 10. Thus, the structure of this glucoside was demonstrated as 10; it is named lucidumoside D.

Compounds 1—10 were tested for their effect on free radical induced hemolysis of RBC. Our results demonstrated that compounds 3, 6 and 9 showed strong antioxidant effects (IC50 = 9.3—37.5 \( \mu \)M). Compound 9 exhibited the most potent activity (IC50 = 9.3 \( \mu \)M), which was 4 times stronger than that of trolox. The activity of compounds 3 and 6 was also stronger than that of trolox, but slightly weaker than that of compound 9. Compounds 1 and 8 showed weaker activity than trolox. The IC50 of compounds 2, 4, 5, 7 and 10 exceeded 200 \( \mu \)M. These experimental results suggested that the hemolysis inhibitory effect of these compounds might be related to the number of their phenolic hydroxyl groups.

Additionally, 3 was recently reported to have antiviral effects. It would be interesting to check if its antiviral effect is related to its antioxidant property and also if 9 has stronger antiviral function.

Experimental
Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were recorded using a Shimadzu UV-1300PC spectrophotometer. IR absorption spectra were obtained with an IR-450 instrument as a film on KBr disk. FAB-MS were recorded on VG Autospec 3000 system, and ESI-MS on Finnigan TSQ 7000. 1H and 13C spectra were obtained with Bruker 400 instrument operating at 400 MHz for 1H, 100 MHz for 13C, respectively. Chemical shifts are reported in parts in million on the \( \delta \) scale as referenced to TMS as the internal standard, and coupling constants are in Hertz. Column chromatographies were performed with silica gel (Qingdao Haiyang Chemical Group Co. Ltd., China), Chromatorex ODS (Fuji Silysia Chemical Ltd., Japan), D-101 (Tianjin Agricultural Chemical Co. Ltd., China), and Sephadex LH-20 (Pharmacia Fine Chemical Co. Ltd.). TLC were performed on Silica gel [CHCl3–MeOH (9 : 1) as eluent] and then by ODS [MeOH–H2O (6 : 5)] to yield Lucidumoside D (9.1 mg) and isonuezhenide (4.8 mg) (9.1 mg) and isonuezhenide (5.1 mg). The Fr. 6 (1.5 g) was chromatographed on Sephadex LH-20 [MeOH–H2O (6 : 4)] and purified by silical gela (CHCl3–MeOH (8 : 2)) and Rp-18 [MeOH–H2O (6 : 5)] to yield neounezhene (4) (20.1 mg).

Lucidumoside C (9): Powder, \([\alpha]_{D}^{20} = -122^\circ \) (c 0.22, MeOH); UV (EtOH) \( \lambda_{\text{max}} \) (log \( \epsilon \)) 235 (4.10), 282 (3.43); IR (KBr) \( \nu_{\text{max}} \) 1725, 1644, 1450, 1390, 1075 cm\(^{-1}\); The 1H- and 13C-NMR spectral data (see Tables 1 and 2) and ESI-MS \( m/z \) 583 [M—OH]−, 327 [M—129H]−, 283 [M—155H]−; HR–FAB–MS \( n/z \) found 585.5817 [M+H]+ \( (\text{C}_{27}\text{H}_{37}\text{O}_{13}) \), requires 585.5824.

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References