Aldose Reductase Inhibitors from the Fruiting Bodies of *Ganoderma applanatum*

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The isolation and characterization of rat lens aldose reductase (RLAR) inhibitors from the fruiting bodies of *Ganoderma applanatum* were conducted. Among the extracts and fractions from *G. applanatum* tested, the MeOH extract and EtOAc fraction were found to exhibit potent RLAR inhibition *in vitro*, their IC_{50} being 1.7 and 0.8 $\mu\text{g/ml}$, respectively. From the active EtOAc fraction, seven compounds with diverse structural moieties were isolated and identified as D-mannitol (**1**), 2-methoxyfatty acids (**2**), cerebrosides (**3**), daucosterol (**4**), 2,5-dihydroxyacetophenone (**5**), 2,5-dihydroxybenzoic acid (**6**), and protocatechualdehyde (**7**). Among them, protocatechualdehyde (**7**) was found to be the most potent RLAR inhibitor ($IC_{50}=0.7 \mu\text{g/ml}$), and may be useful for the prevention and/or treatment of diabetic complications.

Key words *Ganoderma applanatum*; Polyporaceae; protocatechualdehyde; aldose reductase activity; diabetic complication

The fruiting bodies of *Ganoderma lucidum* are a well-known Chinese medicine that has been used clinically in China, Japan and Korea, for a long time. More than 130 highly oxygenated lanostane-type triterpenoids have been isolated from the fruiting bodies, mycelia and spores of *G. lucidum*, including common fungal steroids derived from ergosterol, some of them exhibiting a bitter taste or useful biological activities.¹⁾ Other *Ganoderma* spp. have also been used in traditional medicine for the treatment of cancer, hypertension and chronic bronchitis, and as a tonic or sedative in China, Japan and Korea.^{2,3)} In our continuing search for the bioactive principles from crude drugs, the extracts and fractions of *G. applanatum* were evaluated, particularly in relation to their inhibitory effects on rat lens aldose reductase (RLAR). Here, the effects of compounds isolated from *G. applanatum* on RLAR activity *in vitro* are described.

MATERIALS AND METHODS

Plant Material The fruiting bodies of *Ganoderma applanatum* (LEYSS. ex FR.) KARST. (Polyporaceae) were provided by St. Clair Milk and Grocery (Niagara Falls, Canada) in March 2002. The botanical identification was made by Mr. Gregory J. Belmore (Ministry of Natural Resources, Ontario, Canada). A voucher specimen (No. 2002-02) is deposited in the Herbarium of our Institute.

Extraction and Isolation The ground fruiting bodies (2.1 kg) of *G. applanatum* were extracted with MeOH (3 l $\times$ 5) under reflux to give an extract (85 g). The MeOH extract was suspended in water and successively partitioned with *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol, to yield 15, 25, 30 and 15 g fractions, respectively. The precipitate from the EtOAc fraction was crystallized from MeOH to yield mannitol (**1**, 35 mg). The filtrate from the EtOAc extract (30 g) underwent silica gel chromatography with hexane–EtOAc–MeOH (10:3:1) as the eluent, to afford 15 fractions (EA01–EA15). Fraction EA01 was crystallized from MeOH to yield 2-methoxyfatty acid (**2**, 10 mg). Fraction EA03 was separated by column chromatography with CHCl_3 –MeOH– H_2O (7:1:0.5) as the eluent, to afford a cerebroside (**3**, 5 mg) and daucosterol (**4**, 6 mg). Fraction EA04 was treated in the same way as EA03, yielding a cere-

broside (**3**, 12 mg). Fraction EA05 was applied to silica gel column, with hexane–EtOAc (5:8) as the eluent, to afford 2,5-dihydroxyacetophenone (**5**, 5 mg). Fraction EA06 was further purified on the same silica gel column, with toluene–EtOAc–HOAc (5:3:1) as the eluent, yielding 2,5-dihydroxybenzoic acid (**6**, 7 mg). Fraction EA07 was similarly subjected to a silica gel column, with toluene–EtOAc–HOAc (2.5:1:0.5) as the eluent, to yield protocatechualdehyde (**7**, 2 mg). The purity of each isolate was >95%. GC analysis of **2** revealed it was composed of C_{22} – C_{25} fatty acids (major C_{24} : 59%). However, the fatty acid moiety of **3** was composed of C_{16} , C_{17} (major), and C_{18} .

Fig. 1. Structures of Compounds 1–7

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Chemicals DL-Glyceraldehyde, sodium phosphate and adenine dinucleotide phosphate reduced form (NADPH) were purchased from Sigma Chem. Co. (St. Louis, MO, U.S.A.). All other chemicals and reagents were analytical grade and commercially available.

AR Activity *in Vitro* Rat lenses were removed from Sprague-Dawley rats weighing 250–280 g and frozen until use. The supernatant fraction of the rat lens homogenate was prepared according to the procedures of Hayman and Kinoshita.⁴⁾ Partially purified enzyme was routinely used to test for enzyme inhibition. The partially purified material was separated into 1.0 ml aliquots and stored at -40°C . Rat lenses were homogenized and centrifuged at 12000 *g* and the supernatant was used as an enzyme source. AR activity was assayed spectrophotometrically by measuring the decrease in the absorption of NADPH (16 mm) at 340 nm over a 5 min period, with DL-glyceraldehyde as the substrate.⁵⁾ The concentrations of the inhibitors producing 50% inhibition of the enzyme activity (IC_{50}) were calculated from the least-squares regression line of the logarithmic concentrations plotted against the remaining activity.

RESULTS AND DISCUSSION

The effects of the MeOH extract, the water extract, and the various fractions from *G. applanatum* were estimated with the RLAR enzyme, using DL-glyceraldehyde as a substrate. The inhibitory potencies and IC_{50} values on the RLAR enzyme were estimated. As shown in Tables 1 and 2, the IC_{50} values of the MeOH and the water extracts were determined to be 1.7 and 8.7 $\mu\text{g/ml}$, respectively, while that of TMG, a known AR inhibitor,⁶⁾ was 0.5 $\mu\text{g/ml}$. To evaluate the AR inhibitory compounds from *G. applanatum*, activity-guided fractionation was carried out and their effects on RLAR were tested. The EtOAc fraction from the MeOH extract was found to exhibit the strongest RLAR inhibitory activity, its IC_{50} being 0.8 $\mu\text{g/ml}$.

The active EtOAc fraction was subjected to repeated silica gel chromatography yielding seven compounds. They were identified as D-mannitol (**1**),⁷⁾ 2-methoxyfatty acids (**2**),^{8,9)} cerebrosides (**3**),^{10–13)} daucosterol (**4**),¹⁴⁾ 2,5-dihydroxyacetophenone (**5**),¹⁵⁾ 2,5-dihydroxybenzoic acid (**6**)¹⁶⁾ and protocatechualdehyde (**7**)^{17,18)} by spectral analysis and comparison of their spectroscopic data with those reported. All of these compounds were isolated for the first time from the fruiting bodies of *G. applanatum*. All compounds were tested for their effects on RLAR, and the results are summarized in Table 3. The results show that only protocatechualdehyde (**7**) showed significant inhibitory activity towards AR, with an IC_{50} of 0.7 $\mu\text{g/ml}$, which is equivalent to that of the positive control TMG ($\text{IC}_{50}=0.6 \mu\text{g/ml}$). The results suggest that two simple phenolic compounds (**5**, **6**) having 2,5-dihydroxy benzene moieties exhibit somewhat lower potencies than that carrying a catechol moiety as suggested in flavonoids and related compounds for AR inhibitory activity.¹⁹⁾ We concluded that the observed activity in the crude EtOAc extract ($\text{IC}_{50}=0.8 \mu\text{g/ml}$) was probably attributable to the contribution of the inhibitory effects of unknown polyphenols or other compounds present and/or the synergistic effect of individual AR inhibitory constituents when tested in the form of a complex mixture. It has been acknowledged that plant-de-

Table 1. Inhibitory Effects of the MeOH and Water Extracts on the RLAR

Extract	Concentration ($\mu\text{g/ml}$)	Inhibition (%)	IC_{50} ($\mu\text{g/ml}$)
TMG	10	94.7	0.5
	1	81.7	
	0.1	10.8	
MeOH extract	100	99.9	1.7
	10	80.3	
	1	39.8	
Water extract	100	98.5	8.7
	10	56.5	
	1	3.6	

Inhibition rates were calculated as percentages with respect to the control value. TMG: tetramethylene glutaric acid.

Table 2. Inhibitory Effects of the Various Fractions on the RLAR

Fraction	Concentration ($\mu\text{g/ml}$)	Inhibition (%)	IC_{50} ($\mu\text{g/ml}$)
TMG	10	92.7	0.5
	1	68.7	
	0.1	19.8	
<i>n</i> -Hexane	100	91.1	5.0
	10	52.7	
	1	32.7	
CH_2Cl_2	10	87.2	1.7
	5	68.2	
	1	41.2	
EtOAc	10	90.5	0.8
	1	65.8	
	0.5	34.7	
<i>n</i> -BuOH	10	94.4	1.3
	1	37.7	
	0.5	35.4	

Inhibition rates were calculated as percentages with respect to the control value. TMG: tetramethylene glutaric acid.

Table 3. Inhibitory Effects of Compounds **1**–**7** on the RLAR

Compound	Concentration ($\mu\text{g/ml}$)	Inhibition (%)	IC_{50} ($\mu\text{g/ml}$)
TMG	10	91.5	0.6
	1	62.9	
	0.1	18.4	
1	100	2.6	>100
2	100	0.73	>100
3	100	94.3	30.0
	50	66.3	
	10	11.4	
4	100	1.0	>100
5	100	27.5	>100
6	100	83.2	39.4
	50	53.5	
	10	6.9	
7	10	89.7	0.7
	5	77.4	
	0.5	46.7	

Inhibition rates were calculated as percentages with respect to the control value. TMG: tetramethylene glutaric acid.

rived extracts and phytochemicals are potential alternatives to synthetic inhibitors against AR.^{19,23,27)} Currently, the compounds isolated from plants as AR inhibitors are classified as flavonoids,^{19–26)} stilbenes,¹⁹⁾ terpenoids,²¹⁾ ellagic acid and its derivatives,²⁸⁾ and alkaloids.²⁹⁾

The present study was carried out in a search for new potential AR inhibitors from the fruiting bodies of *G. applanatum*, and cerebroside (3), 2,5-dihydroxybenzoic acid (6) and protocatechualdehyde (7) were isolated as active principles which may be useful for the treatment of diabetic complications.

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