In Vitro Evaluation of Secoiridoid Glucosides from the Fruits of *Ligustrum lucidum* as Antiviral Agents

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Six secoiridoid glucosides, lucidumoside C (1), oleoside dimethylester (2), neonuezhenide (3), oleuropein (4), ligustroside (5) and lucidumoside A (6), isolated from the fruits of *Ligustrum lucidum* (Oleaceae), were examined *in vitro* for their activities against four strains of pathogenic viruses, namely herpes simplex type 1 virus (HSV-1), influenza type A virus (Flu A), respiratory syncytial virus (RSV) and parainfluenza type 3 virus (Para 3). Antiviral activities were evaluated by the cytopathic effect (CPE) inhibitory assay. The purpose was to check if the anti-oxidative potency of these glucosides correlated with their antiviral potency. Results showed that none of the glucosides had any significant activity against HSV-1 and Flu A. Oleuropein, however, showed significant antiviral activities against RSV and Para 3 with IC_{50} value of 23.4 and 11.7 μg/ml, respectively. Lucidumoside C, oleoside dimethylester and ligustroside showed potent or moderate antiviral activities against Para 3 with IC_{50} values of 15.6–20.8 μg/ml. These results also documented that the anti-oxidative potency of these secoiriodoid glucosides was not directly related to their antiviral effects.

Key words secoiridoid glucoside; antiviral activity; oleuropein; lucidumoside C

Oleuropein is a phenolic secoiridoid glucoside widespread in members of the family Oleaceae. It has been shown to possess a wide range of biological activities. It increased coronary blood flow and showed antiarrhythmic and spasmolytic effects.[1,2] Oleuropein exhibited hypoglycemic effect and increased tolerance of orally administered glucose. It also showed anti-oxidative and anti-inflammatory properties.[3—7] Other reported effects of oleuropein included the potentiation of cellular and organismal protection through the macrophage-mediated response,[4] the inhibition of platelet aggregation and eicosanoid production,[8] reduction of the low density lipoproteins (LDL) level,[9] the potent and protective antioxidant action on LDL,[10] the inhibitory effects on cytochrome P450 and 17β-hydroxysteroid dehydrogenase activity,[11] potent cytotoxic effects on tumor cell lines including P-388, L-1210. SNU-5 and HL-60,[12] inhibitory effects on of lipoxygenase activity,[13] and antibacterial functions.[14—18] Recently, oleuropein was also claimed in a U.S. patent to have potent antiviral activities against herpes mononucleosis, hepatitis virus, rotovirus, bovine rhinovirus, canine parvovirus and feline leukaemia virus.[19] On the other hand, But and his research team recently reported the isolation of oleuropein (**4**), lucidumoside C (**1**) and other secoiridoid glucosides from the fruits of *Ligustrum lucidum*, another member of Oleaceae.[3] They demonstrated strong anti-oxidative effects in these secoiridoid glucosides (Table 1). Oleuropein and lucidumoside C were found to have stronger anti-oxidative potency than trolox, their IC_{50} being 25.0, 9.3, and 55.0 μM, respectively. Based on such anti-oxidative capabilities and also the antiviral effects claimed in the U.S. patent, the authors suggested that "it would be interesting to check if oleuropein's antiviral effect is related to its anti-oxidant property and also if lucidumoside C has stronger antiviral function".[3] This suggestion actually posed two important questions: 1) is anti-oxidative potency of a compound correlated with its antiviral potency? and 2) is anti-oxidation a mechanism against viruses?

In the present paper, we present the *in vitro* evaluation of six secoiridoid glucosides from the fruits of *Ligustrum lucidum* against herpes simplex type 1 virus (HSV-1), influenza type A virus (Flu A), respiratory syncytial virus (RSV) and parainfluenza type 3 virus (Para 3). The relationship of antiviral activities of secoiridoid glucosides with their anti-oxidative effects were analysed.

Results and Discussion

The six secoiridoid glucosides isolated from *L. lucidum* did not show any antiviral activities against Flu A and HSV-1, except oleoside dimethylester (**2**) which showed a little antiviral activity against HSV-1 (Tables 2, 3).

The *in vitro* antiviral activities of the six isolated compounds against RSV are summarized in Table 4. Oleuropein showed potent anti-RSV activity with an IC_{50} value of 23.4 μg/ml and a large therapeutic index comparable to that of ribavirin, an approved drug for the treatment of RSV infections in human.

Oleuropein also showed a potent antiviral activity against Para 3 with an IC_{50} value of 11.7 μg/ml and a larger therapeutic index than ribavirin. Ligustroside (**5**) also showed potent antiviral activity against Para 3 with an IC_{50} value of 15.6 μg/ml. Lucidumoside C and oleoside dimethylester ex-

Table 1. Inhibitory Effects of Six Secoiridoid Glucosides Isolated from *Ligustrum lucidum* on 2,2′-Azo-bis-(2-amidinopropane)dihydrochloride-Induced Hemolysis of Rat Red Blood Cell *in Vitro*[3]

Compound	IC_{50} (μM)[a]
Lucidumoside C	9.3
Oleoside dimethylester	65.0
Neonuezhenide	35.0
Oleuropein	25.0
Ligustroside	>200.0
Lucidumoside A	>200.0
Trolox[b]	55.0

a) IC_{50}, 50% inhibitory concentration (μM). *b*) Positive control drug.

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Table 2. Inhibitory Effects of Six Secoiridoid Glucosides Isolated from *Ligustrum lucidum* on Flu A-Induced Cytopathogenicity in MDCK Cells

Compound	IC_{50} (μg/ml)[a]	TC_{50} (μg/ml)[b]	TI[c]
Lucidumoside C	>100.0	ND	—
Oleoside dimethylester	>100.0	ND	—
Neonuezhenide	>100.0	ND	—
Oleuropein	>100.0	ND	—
Ligustroside	>100.0	ND	—
Lucidumoside A	>100.0	ND	—
Ribavirin[d]	31.3	>166.7	>5.0

a) IC_{50} is the concentration of the sample required to inhibit virus-induced CPE 50%. *b*) TC_{50} is the concentration of the 50% cytotoxic effect. *c*) $TI=TC_{50}/IC_{50}$. *d*) Ribavirin, an approved drug for the treatment of RSV infections. ND, not done.

Table 3. Inhibitory Effects of Six Secoiridoid Glucosides Isolated from *Ligustrum lucidum* on HSV-1-Induced Cytopathogenicity in Vero Cells

Compound	IC_{50} (μg/ml)[a]	TC_{50} (μg/ml)[b]	TI[c]
Lucidumoside C	>200.0	166.7	<0.8
Oleoside dimethylester	83.3	166.7	2.0
Neonuezhenide	291.7	291.7	1.0
Oleuropein	214.3	214.3	1.0
Ligustroside	>250.0	250.0	<1.0
Lucidumoside A	>250.0	250.0	<1.0
ACV[d]	1.3	>166.7	>128.0

a—*c*): See footnotes of Table 2. *d*) ACV, acyclovir, an approved drug for the treatment of HSV infections.

Table 4. Inhibitory Effects of Six Secoiridoid Glucosides Isolated from *Ligustrum lucidum* on RSV-Induced Cytopathogenicity in Hep 2 Cells

Compound	IC_{50} (μg/ml)[a]	TC_{50} (μg/ml)[b]	TI[c]
Lucidumoside C	>150.0	250.0	<1.7
Oleoside dimethylester	83.3	125.0	1.5
Neonuezhenide	145.8	145.8	1.0
Oleuropein	23.4	562.5	24.0
Ligustroside	>150.0	375.0	<1.7
Lucidumoside A	>150.0	250.0	<1.7
Ribavirin[d]	2.6	62.5	24.0

a—*c*): See footnotes of Table 2. *d*) Ribavirin, an approved drug for the treatment of RSV infections.

hibited moderate antiviral activity against Para 3 with the same IC_{50} value of 20.8 μg/ml. Neonuezhenide (**3**) and lucidumoside A (**6**) showed weak activity against Para 3 (Table 5).

Our results confirmed that oleuropein isolated from *L. lucidum* showed significant antiviral activities against respiratory viruses including RSV and Para 3 *in vitro*. It should be noted that elenolic acid is a main fragment in oleuropein. Calcium elenolate, a calcium salt of elenolic acid, was shown to have potent antiviral activities against herpes, influenza A and B, parainfluenza 1, 2 and 3 viruses.[20,21]

Lucidumoside C, neonuezhenide and oleuropein were shown to have strong antioxidant effects with IC_{50} values of 9.3, 35.0, and 25.0 μM, respectively.[3] Lucidumoside C exhibited the most potent activity. However in our *in vitro* evaluation against respiratory viruses, lucidumoside C and neonuezhenide did not show any antiviral activities against Flu A, HSV-1 and RSV, except moderate or weak antiviral activity against Para 3. Oleuropein demonstrated potent antiviral activities against RSV and Para 3. Results of our evaluation confirmed that oleuropein has potent antiviral effects, and also suggested that the anti-oxidative potency of secoiridoid glucosides is not directly related to their antiviral properties against viruses.

Table 5. Inhibitory Effects of Six Secoiridoid Glucosides Isolated from *Ligustrum lucidum* on Para 3-Induced Cytopathogenicity in Hep 2 Cells

Compound	IC_{50} (μg/ml)[a]	TC_{50} (μg/ml)[b]	TI[c]
Lucidumoside C	20.8	250.0	12.0
Oleoside dimethylester	20.8	125.0	6.0
Neonuezhenide	72.9	145.8	2.0
Oleuropein	11.7	562.5	48.0
Ligustroside	15.6	375.0	24.0
Lucidumoside A	41.7	250.0	6.0
Ribavirin[d]	2.6	62.5	24.0

a—*c*): See footnotes of Table 2. *d*) Ribavirin, an approved drug for the treatment of RSV infections.

Experimental

Materials The secoiridoid glucosides used in this investigation were isolated from the dried fruits of *Ligustrum lucidum* in our laboratory.[3] Dulbecco's modified Eagle's medium (DMEM) and phosphate buffered saline (PBS) were purchased from Sigma Co. (U.S.A.). Trypsin–EDTA (×10) and trypsin (1 : 250) were from Gibco Co. (U.S.A.). Fetal bovine serum (FBS) was from Biofluids Inc. (U.S.A.).

Viruses and Cells RSV strain Long, HSV-1 (15577) strain, Para 3, Madin Darby canine kidney (MDCK) cells, Vero cells and Hep 2 cells were obtained from American Type Culture Collection. Flu A (H3N2) strain was obtained from Guangzhou province, P. R. China.

Cytotoxicity Assay Cell toxicity was monitored by determining the effect of the natural products on cell morphology and cell viability. Serial twofold dilutions of the natural products were added to confluent cell monolayers and the cells were cultivated at 37 °C for 2—5 d. The morphology of the cells was inspected daily and observed for microscopically detectable alterations, including the loss of monolayer, rounding, shrinking of the cells, granulation, and vacuolisation in the cytoplasm. The cytopathic effect (CPE) was scored (scores: 0=0% CPE, 1=0—25% CPE, 2=25—50% CPE, 3=50—75% CPE, 4=75—100% CPE). The 50% toxic concentration (TC_{50}), the concentration required to cause visible changes in 50% of intact cells, was estimated from graphic plots. The maximal non-cytotoxic concentration (MNCC) was determined as the maximal concentration of the natural products that did not exert toxic effect detected by microscopic monitoring.

Cytopathic Effect Reduction Assay The antiviral activity of test samples against viruses was measured by the CPE inhibition assay.[22,23] Twofold serial dilutions of natural products were seeded into cells monolayers cultivated in 96-well culture plates, using the MNCC as the higher concentration. An infection control was made in the absence of natural products. An equal volume of virus suspension (100 $TCID_{50}$/ml) was added to the cells monolayers. The plates were incubated at 37 °C in a humidified CO_2 atmosphere (5% CO_2) for 2—5 d (2 d for HSV-1, 2—3 d for Flu A, 3—4 d for Para 3, and 4—5 d for RSV). After that, CPE was observed. The virus induced CPE was scored as described above in cytotoxicity assay. The reduction of virus multiplication was calculated as % of virus control (%virus control=$CPE_{exp}/CPE_{virus\ control}\times100$). The concentration reducing CPE by 50% with respect to virus control was estimated from graphic plots and was defined as 50% inhibited concentration (IC_{50}) expressed in μg/ml. The therapeutic index (TI) was calculated from the ratio TC_{50}/IC_{50}.

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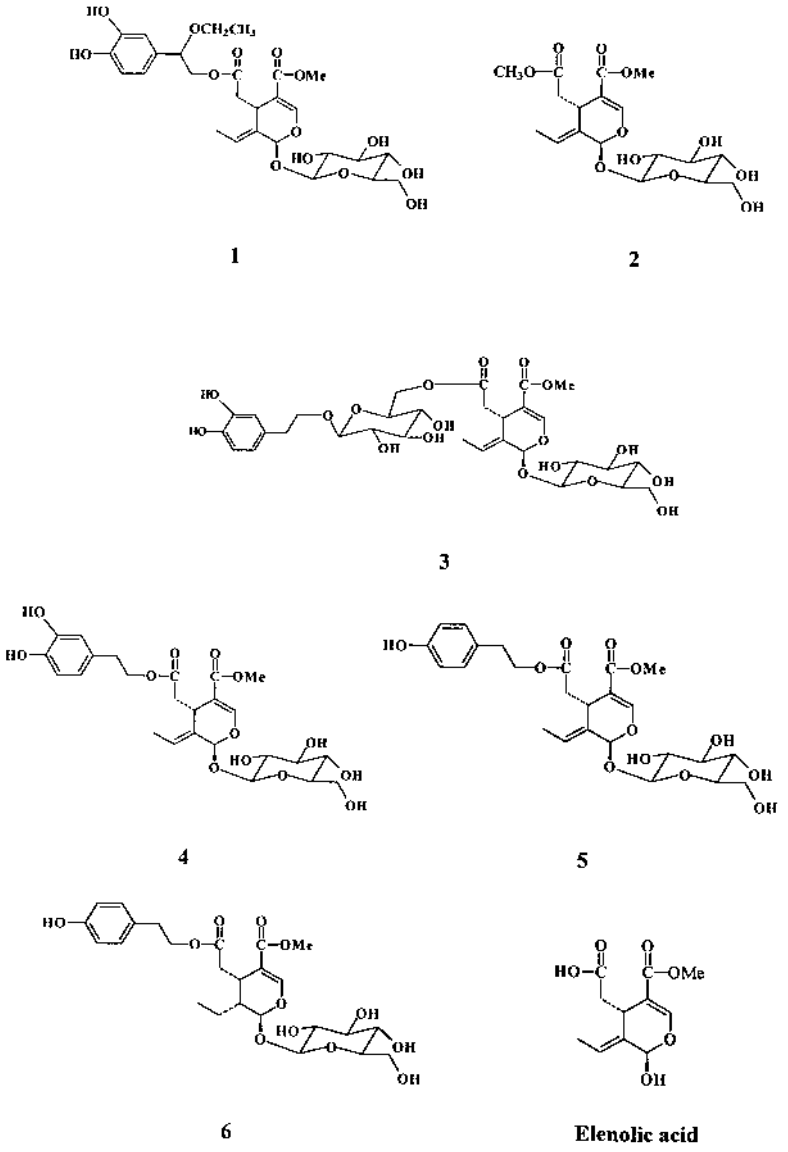

Fig. 1. Chemical Structures of Compounds from *Ligustrum lucidum* and Elenolic Acid

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