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# A Potent Anti-Complementary Acylated Sterol Glucoside from *Orostachys japonicus*

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In order to isolate substances that inhibit the hemolytic activity of human serum against erythrocytes, we have evaluated whole plants of the *Orostachys japonicus* species with regard to its anti-complement activity, and have identified its active principles following activity-guided isolation. A methanol extract of the *O. japonicus*, as well as its *n*-hexane soluble fraction, exhibited significant anti-complement activity on the complement system, which was expressed as total hemolytic activity. A bioassay-guided chromatographic separation of the constituents resulted in the isolation of three known compounds **1-3** from the active *n*-hexane fraction. The structure of these compounds were analyzed, and they were identified as hydroxyhopanone (**1**), β-sitosteryl-3-*O*-β-D-glucopyranosyl-6'-*O*-palmitate (**2**), and β-sitosteryl-3-*O*-β-D-glucopyranoside (**3**), respectively. Of these compounds, compound **2** exhibited potent anti-complement activity ($IC_{50}$ = 1.0 ± 0.1 μM) on the classical pathway of the complement, as compared to tiliroside ($IC_{50}$ = 76.5 ± 1.1 μM), which was used as a positive control. However, compounds **1** and **3** exhibited no activity in this system.

**Key words:** *Orostachys japonicus* A. Berger, Crassulaceae, Anti-complement activity, β-Sitosteryl-3-*O*-β-D-glucopyranosyl-6'-*O*-palmitate

## INTRODUCTION

The complement system is a major effector of humoral immunity, and can be activated either by a cascade mechanism in the classical pathway (CP), an alternative pathway (AP), or by the mannan-binding lectin (MBL)-associated serine protease (MBL/MASP) pathway (Kirschfink, 1997). The thirty-odd complement fragments comprising the complement system include proteolytic pro-enzymes, non-enzymatic components that form functional complexes, co-factors, regulators, and receptors (Ember & Hugli, 1997). The proteolytic cascade makes significant amplification possible, as each protease molecule activated in one step can subsequently generate multiple copies of activated enzyme later in the cascade. These enzymes then cleave non-enzymatic components, including C3, C4, and C5. The larger fragments derived from C3, C4, and C5 (*i.e.* C3b, C4b, and C5b) appear to be involved in biologic effector functions, including opsonization, phagocytosis, and immunomodulation. However, the smaller molecules, C3a, C4a, and C5a, all of which have been designated anaphylatoxins, induce the release of mediators from mast cells and lymphocytes. These mediators, in turn, cause a variety of inflammatory diseases, and may ultimately prove fatal if their release occurs after organ transplantation (Ember & Hugli, 1997; Min *et al.*, 2001). Therefore, the ability to modulate complement activity would clearly be beneficial in the therapy of inflammatory diseases.

In the course of research into the isolation from natural products of biologically active substances that exert modulatory activity against inflammatory diseases, we discovered that the methanol extract of *Orostachys japonicus* exhibited potent anti-complement activity. The *O. japonicus* A. Berger (Crassulaceae) is a perennial herb, which is found fairly ubiquitously in Korea, China, and Japan. The dried whole plants of this species, in particular, have been used as a Chinese crude drug for the treatment of fever, hemostasis, hepatitis, arthritis,

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eczema, and intoxication, and have also been used in folk medicine as an anti-cancer agent (Kim, 1984). Earlier phytochemical investigations into the properties of this species have resulted in the isolation of such diverse compounds as flavonoids (Park *et al.*, 1991a; Sung *et al.*, 2002), phenolic acids (Park *et al.*, 2000), triterpenoids (Park *et al.*, 1994), and sterols (Park *et al.*, 1991b; Lee *et al.*, 2004). The biological activities of *O. japonicus,* which include anti-mutagenic (Park *et al.*, 1991b), and anti-HIV-1 protease activity (Park *et al.*, 2000), as well as a protective effect against $H_2O_2$-induced apoptosis (Yoon *et al.*, 2000), have been reported in previous studies. Although the biological activities exhibited by this plant have already been intensively studied, no documentation regarding its modulatory properties with respect to complement activity currently exists. Thus, we embarked upon the current investigation in order to determine the degree to which *O. japonicus* might exert anti-complement effects, and to further identify the active compounds of the plants.

## MATERIALS AND METHODS

### General experimental procedures

$^1$H-NMR and $^{13}$C-NMR spectra were recorded on a JEOL JNM ECP-400 spectrometer (400 MHz for $^1$H and 100 MHz for $^{13}$C). The HMQC and HMBC experiments were recorded using pulsed field gradients. EI-MS data were recorded on a JEOL JMS-700 spectrometer. Positive-ion LR FAB-MS data was collected on a JEOL JMS-HX110/110A Tandem mass spectrometer (JEOL). Column chromatography was done with Si gel (Merck, 70~230 mesh) and Sephadex LH-20 (Sigma, 25~100 μm). Thin layer chromatography (TLC) was carried out on pre-coated Merck Kieselgel 60 $F_{254}$ plate (0.25 mm) and 50% $H_2SO_4$ was used as spray reagent.

### Chemicals

Sheep red blood cell (SRBC) was obtained from college of agriculture, Chungnam National University (Daejeon, Korea). Normal human serum was collected from a healthy volunteer (male). Hemolysin, gelatin, $MgCl_2$, $CaCl_2$, sodium barbital, and barbituric acid were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Tiliroside was isolated from *Magnolia fargesii* (Min *et al.*, 2003b).

### Plant materials

The whole plant of *Orostachys japonicus* A. Berger was collected on August 2003 from Hapchon, Kyongnam province, Korea. A voucher specimen is deposited at herbarium of Suncheon National University.

### Extraction and isolation

The dried whole plant of *O. japonicus* (4.1 kg) was refluxed with MeOH for three hours. The total filtrate was concentrated to dryness *in vacuo* at 40°C to render the MeOH extract (853 g). This extract was suspended in $H_2O$ and then partitioned with *n*-hexane, $CH_2Cl_2$, EtOAc, and *n*-BuOH, successively, to afford the *n*-hexane extract (114 g), $CH_2Cl_2$ extract (11 g), EtOAc extract (66 g), *n*-BuOH extract (366 g), and the $H_2O$ residue (294 g). The *n*-hexane extract (114 g) was chromatographed over Si gel column (12 × 60, Si gel 60, Merck, 2 kg) and eluted with *n*-hexane-EtOAc (100:1 to 1:1) to obtain 24 fractions (Fr. 1-Fr. 24). The fraction 11 (4.54 g) was subjected to column chromatography on the Si gel (*n*-hexane:EtOAc = 7:1) gave compound **1** (21 mg). The fraction 20 (5.77 g) was chromatographed over the Si gel with *n*-hexane: EtOAc = 1:1 to obtained 6 subfractions (Fr. 20-1 to 20-6). Fraction 20-4 was further purified by Sephadex LH-20 with MeOH and Si gel with $CH_2Cl_2$:EtOAC:MeOH = 35:1:1 to yield compound **2** (17.6 mg). The fraction 22 (9.33 g) was subjected to column chromatography on the Si gel with *n*-hexane:EtOAc = 1:1 to obtained 5 subfractions (Fr. 22-1 to 22-5). Fraction 22-5 was dissolved in MeOH kept overnight at room temperature to yield compound **3** (1.8 g) as white amorphous powder.

### Hydroxyhopanone (1)

Amorphous white powder. EI-MS *m/z* 442 $[M]^+$, IR $\nu_{max}$ (KBr) $cm^{-1}$: 3467, 1708. $^1$H-NMR (400 MHz, pyridine-$d_5$) δ: 1.41 (3H, s, H-30), 1.36 (3H, s, H-29), 1.13 (3H, s, H-23), 1.03 (3H, s, H-24), 0.95 (3H, s, H-26), 0.94 (3H, s, H-27), 0.93 (3H, s, H-25), 0.84 (3H, s, H-28); $^{13}$C-NMR (100 MHz, pyridine-$d_5$) δ: 216.4 (C-3), 72.4 (C-22), 54.8 (C-5), 51.5 (C-21), 50.2 (C-13), 49.7 (C-9), 47.3 (C-4), 44.3 (C-18), 42.1 (C-14), 41.7 (C-8), 41.7 (C-19), 39.5 (C-1), 36.8 (C-10), 34.7 (C-15), 34.3 (C-2), 32.8 (C-7), 31.4 (C-30), 29.8 (C-29), 26.9 (C-23), 26.6 (C-20), 24.3 (C-12), 22.2 (C-16), 21.7 (C-11), 21.2 (C-24), 19.9 (C-6), 17.0 (C-27), 16.5 (C-26, 28), 15.7 (C-25).

### β-Sitosteryl-3-*O*-β-D-glucopyranosyl-6'-*O*-palmitate (2)

Amorphous powder, Positive LR-FABMS *m/z* 838 $[C_{51}H_{90}O_7 + Na + H]^+$; $^1$H-NMR (400 MHz, $CDCl_3$) δ: 5.36 (1H, m, H-6), 4.42 (1H, d, *J*=5.3 Hz, H-6a'), 4.38 (1H, d, *J*=7.4 Hz, H-1'), 4.30 (1H, d, *J*=11.0 Hz, H-6b'), 3.58 (1H, s, H-3'), 3.55 (1H, d, *J*=7.8 Hz, H-3), 3.46 (1H, d, *J*=3.5 Hz, H-1), 3.40 (1H, s, H-4'), 3.37 (1H, d, *J*=6.5 Hz, H-5'), 2.33 (2H, t, *J*=7.5 Hz, H-2"), 2.27 (2H, d, H-4), 2.03 (2H, d, *J*=6.5 Hz, H-11, H-12), 1.95 (1H, s, H-2), 1.85 (2H, d, *J*=12.9 Hz, H-1), 1.61 (2H, m, H-3"), 1.49 (2H, s, H-8, H-11), 1.26 (2H, br s, H-28, 4", 5", 6", 7"~12", 13", 14", 15"), 1.00 (3H, s, H-19), 0.93 (1H, s, H-24), 0.91 (1H, s, H-4'), 0.88 (3H, s, H-16"), 0.85 (1H, s, H-26), 0.68 (3H, s, H-18); $^{13}$C-NMR (100 MHz, $CDCl_3$) δ: 174.5 (C-1"), 140.3 (C-5), 122.1 (C-6), 101.2 (C-1'), 79.7 (C-3), 76.1 (C-3'), 73.9 (C-5'), 73.5 (C-

2'), 70.2 (C-4'), 63.4 (C-6'), 56.8 (C-14), 56.1 (C-17), 50.2 (C-9), 45.8 (C-24), 42.3 (C-13), 39.8 (C-12), 38.9 (C-4), 37.3 (C-1), 36.7 (C-10), 36.2 (C-20), 34.3 (C-2"), 33.9 (C-22), 31.9 (C-7), 31.9 (C-8), 29.8 (C-7"~12"), 29.7 (C-6"), 29.6 (C-5"), 29.4 (C-2), 29.1 (C-25), 28.2 (C-16), 25.0 (C-23), 24.3 (C-15), 23.1 (C-28), 22.7 (C-15"), 21.1 (C-11), 19.8 (C-26), 19.4 (C-19), 19.0 (C-27), 18.8 (C-21), 14.1 (C-16").

### Anti-complement assay

A diluted solution of normal human serum (complement serum, 80 μL) collected from healthy volunteer (male) was mixed with gelatin veronal buffer ($GVB^{2+}$, 80 μL) with or without sample. Each sample was dissolved in DMSO, which was used as a negative control. The mixture was pre-incubated at 37°C for 30 min, and sensitized erythrocytes (sheep red blood cell, 40 μL) were added. After incubation under the same conditions, the mixture was centrifuged (4°C, 1500 × g) and the optical density of the supernatant (100 μL) measured at 405 nm (Yamada *et al.*, 1985). Tiliroside was employed as positive controls (Kim *et al.*, 1998; Jung *et al.*, 1998). The purity of the compounds used for the assay was above 95% (determined by HPLC). Anti-complement activity was determined by means of triplicate measurements and expressed as the 50% inhibitiory concentration ($IC_{50}$ value) from complement-dependent hemolysis of the control.

### Statistical analysis

All results were expressed as mean values ± standard error of triplicate experiment.

## RESULTS AND DISCUSSION

The human complement system performs an important function in the host's defense system against foreign invasive organisms, *i.e.* viruses, bacteria, and fungi. It also plays a role in the defense system associated with external wounds. Its effects are normally beneficial to the host, but can sometimes exert adverse effects, depending on the site, extent, and duration of complement activation. Activation of the complement system may result in pathologic reactions in a variety of inflammatory and degenerative diseases, including multiple sclerosis, systemic lupus, erythematosus, Sjogren syndrome, dermatological disease, rheumatoid arthritis, and gout (Vogt, 1985; Walport, 1993).

We attempted to characterize the effects of various extracts *O. japonicus* on the complement system. As shown in Table I, the methanol extract of *O. japonicus* clearly exhibited anti-complement activity, with an $IC_{50}$ value of 41.3 ± 1.6 μg/mL, as compared to tiliroside ($IC_{50}$ = 76.5 ± 1.1 μg/mL), which was used as a positive control.

**Table I.** Anti-complement activity of MeOH extract and its various fractions from *O. japonicus*

| Fractions | $IC_{50}$ (μg/mL) |
|---|---|
| MeOH extract | 41.3 ± 1.6 |
| *n*-Hexane | 15.9 ± 0.3 |
| $CH_2Cl_2$ | 16.3 ± 0.5 |
| EtOAc | 130.7 ± 2.1 |
| *n*-BuOH | >200 |
| $H_2O$ | >200 |
| Tiliroside[a] | 76.5 ± 1.1 |

[a]Tiliroside used as a positive control.

We also attempted to determine the levels of activity of the organic-soluble fractions, including the *n*-hexane, dichloromethane ($CH_2Cl_2$), and ethyl acetate (EtOAc), *n*-BuOH fractions, as well as the water ($H_2O$) layer, obtained from the MeOH extract of the *O. japonicus* whole plants. Among the five fractions tested, the *n*-hexane fraction ($IC_{50}$ = 15.9 ± 0.3 μg/mL) was found to exhibit a more profound anti-complement activity than did any of the other fractions. Although the $CH_2Cl_2$ fraction exhibited activity similar to that of the *n*-hexane fraction, it generated a much lower yield than did the latter (yield of $CH_2Cl_2$ fraction = 1.3%, yield of *n*-hexane fraction = 13.4%). Therefore, we attempted to isolate the active compounds, using the *n*-hexane fraction. A combination of Si gel and Sephadex LH-20 column chromatography of the *n*-hexane fraction of the MeOH extract of *O. japonicus* resulted in the isolation of three compounds **1-3**. These compounds were ultimately determined to be hydroxyhopanone (**1**, Poehland

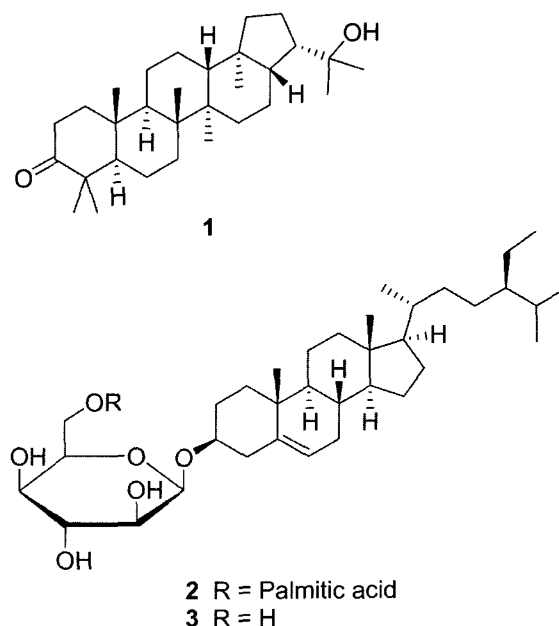


**Fig. 1.** Chemical structures of compounds **1-3**

*et al.*, 1987), β-sitosterol-3-*O*-β-D-glucopyranosyl-6'-*O*-palmitate (**2**, Nguyen *et al.*, 2004), and β-sitosterol-3-*O*-β-D-glucopyranoside (**3**), respectively (Fig. 1), according to a comparison of their spectral data with published data. Compounds **1** and **2** had never before been reported to exist in this plant.

All of the compounds (**1**-**3**) were subjected to *in vitro* bioassays for their classical pathway complement inhibitory activity. The results are summarized in Table II. Compound **2** exerted a clear inhibitory effect on the CP of the complement system, with an $IC_{50}$ value of 1.0 ± 0.1 μM (Fig. 2). Tiliroside, used as a positive control, exhibited an $IC_{50}$ value of 76.5 ± 1.1 μM (Table II). Compounds **1** and **3**, however, exhibited no significant relevant effects in this assay system.

Our results indicate that, compared with compounds **2** and **3**, compound **2** contained a palmitic acid at C-6' on the compound **3**. This result suggests that the palmitic acid at C-6' enhanced the activity of compound **2**, as compared to the activity of compound **3**.

It has been previously reported that flavonoids (Cimanga *et al.*, 1995; Park *et al.*, 1999a; Min *et al.*, 2003b), triterpenoids (Min *et al.*, 2001; Lee *et al.*, 2003, 2004), polyacetylene (Park *et al.*, 2004), lactones (Min *et al.*, 2003a), and saponins (Park *et al.*, 1999b) all exhibit significant anti-complement activity. To the best of our knowledge, this constitutes the first report of anti-complement activity against the CP of the complement system exerted by the acylated sterol glucoside from *O. japonicus*.

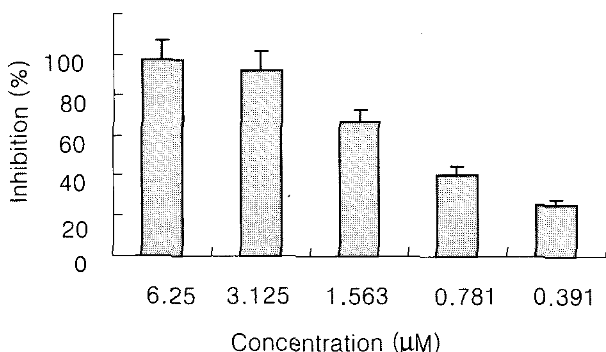


**Fig. 2.** Inhibitory effects of β-sitosteryl-3-*O*-β-D-glucopyranosyl-6'-*O*-palmitate (**2**) on classical pathway of complement system

**Table II.** Inhibitory effects of compounds isolated from *O. japonicus* on the classical pathway of the complement system *in vitro*

| Compounds | $IC_{50}$ (μM)[a] |
|---|---|
| Hydroxyhopanone (**1**) | >100 |
| β-Sitosteryl-3-*O*-β-D-glucopyranosyl-6'-*O*-palmitate (**2**) | 1.0 ± 0.1 |
| β-Sitosteryl-3-*O*-β-D-glucopyranoside (**3**) | >100 |
| Tiliroside[b] | 76.5 ± 1.1 |

[a]*$IC_{50}$ value obtained from three separate experiments are shown.* [b]Tiliroside used as a positive control.

A further investigation of the anti-complement active constituents of *O. japonicus* will surely prove to be both informative and useful.

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