Anti-complement Activity of Constituents from the Stem-Bark of *Juglans mandshurica*

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Four known flavonoids and two galloyl glucoses isolated from the stem-bark of *Juglans mandshurica* (Juglandaceae), namely taxifolin (1), afzelin (2), quercitrin (3), myricitrin (4), 1,2,6-trigalloylglucose (5), and 1,2,3,6-tetragalloylglucose (6), were evaluated for their anti-complement activity against complement system. Afzelin (2) and quercitrin (3) showed inhibitory activity against complement system with 50% inhibitory concentrations (IC_{50}) values of 258 and 440 μM. 1,2,6-Trigalloylglucose (5) and 1,2,3,6-tetragalloylglucose (6) exhibited anti-complement activity with IC_{50} values of 136 and 34 μM. In terms of the evaluation of the structure–activity relationship of 3,5,7-trihydroxyflavone, compounds 2, 3, and 4 were hydrolyzed with naringinase to give kaempferol (2a), quercetin (3a), and myricetin (4a) as their aglycones, and these were also tested for their anti-complement activity. Of the three aglycones, kaempferol (2a) exhibited weak anti-complement activity with an IC_{50} value of 730 μM, while quercetin (3a) and myricetin (4a) were inactive in this assay system. Among the compounds tested, 1,2,3,6-tetragalloylglucose (6) showed the most potent anticomplement activity (IC_{50}, 34 μM).

Key words *Juglans mandshurica*; anti-complement activity; 1,2,3,6-tetragalloylglucose; 1,2,6-trigalloylglucose; 3,5,7-trihydroxyflavone

The invertebrate complement system can be activated by a cascade mechanism of the classical pathway (CP), an alternative pathway (AP), or the MBL/MASP (mannan binding lectin/MBL-associated serine protease) pathway.[1] The thirty odd complement fragments that make up the system include proteolytic pro-enzymes, non-enzymatic components that form functional complexes, co-factors, regulators, and receptors. The pro-enzymes are the complements, which become sequentially activated in the cascade of events leading to the activation of the complement system.[2] The proteolytic cascade allows for tremendous amplification, since each proteinase molecule activated at a single step can generate multiple copies of an activated enzyme later in the cascade, which in turn cleaves non-enzymatic components, such as C3, C4, and C5. The larger fragments derived from C3, C4, and C5 (*i.e.* C3b, C4b, and C5b) are involved in biologic effector functions, such as opsonization, phagocytosis, and immunomodulation. However, the smaller molecules, C3a, C4a, and C5a, called anaphylatoxins, induce the release of mediators from mast cells and lymphocytes, which cause a variety of inflammatory diseases, and may be fatal if they occur after organ transplantation.[2—4] Therefore, the modulation of complement activity should be useful in the therapy of inflammatory diseases.

During the course of our research on anti-inflammatory agents from natural sources, flavonoids and galloyl glucoses from the stem-bark of *Juglans mandshurica* MAXIMOWICZ (Juglandaceae) were tested for their anti-complement activity. *J. mandshurica* is used as a folk medicine for the treatment of cancer in Korea.[5] Several naphthoquinones, naphthalenyl glucoses, α-tetralonyl glucoses, diarylheptanoyl glucoses, and flavonoids have been isolated from this plant, and furthermore, these compounds have been shown to have, cytotoxic activity against human cancer cell lines, inhibitory effects on DNA polymerase and on the RNase H activities of HIV-1 reverse transcriptase, as well as anti-HIV-1 activity in MT-4 cells.[5—10] In this paper we describe the anti-complement activity of compounds from this plant.

MATERIALS AND METHODS

Plant Material The stem-bark of *J. mandshurica* was collected during September 1998 at a mountain area of Kimchun, Kyungbook, Korea, and dried at room temperature for 3 weeks. A voucher specimen is deposited at the herbarium of the College of Pharmacy, Chungnam National University, Korea.

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 (Man). Hemolysin, gelatin, $MgCl_2$, $CaCl_2$, sodium barbital, barbituric acid, and naringinase were purchased from Sigma Chemical (St. Louis, U.S.A.). Rosmarinic acid and tiliroside were isolated from *Agastache rugosa* and *Magnolia fargesii*, respectively. Silica gel was obtained from Merck Co. (Darmstadt, Germany). Sephadex LH-20 was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). ODS was obtained from Fuji Silysia Chemical (Aichi, Japan).

Extraction and Isolation Stem-bark of *J. mandshurica* (3.0 kg) was extracted with MeOH at room temperature for 24 h to give a dark-brown extract (390 g). The MeOH extract (300 g) was suspended in H_2O (2500 ml) and extracted with hexane (2500×3) to give a hexane-soluble fraction (48 g). The resulting H_2O layer was extracted with CH_2Cl_2 (3000 ml×3), EtOAc (3000 ml×3), and BuOH (3000 ml×3),

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successively. The EtOAc-soluble fraction (90 g) was chromatographed on a column of silica gel (1 kg). The column was eluted using a gradient of $CHCl_3$, MeOH, and H_2O to give six fractions (Fr. A—F: 2.7 g, 15.9 g, 23.9 g, 7.5 g, 5.4 g, and 10.2 g, respectively). Repeated column chromatography of Fr. B on silica gel ($CHCl_3$–MeOH, 9 : 1), Sephadex LH-20 ($CHCl_3$–MeOH, 1 : 9), and ODS column (50% aqueous MeOH), followed by MPLC on RP-18 (50% aq. MeOH and 70% aq. CH_3CN) afforded compounds **1** (165 mg) and **2** (189 mg). Repeated column chromatography of Fr. C on Sephadex LH-20 (MeOH and $CHCl_3$–MeOH, 1 : 9), silica gel ($CHCl_3$–MeOH, 8 : 2), and ODS column (40% aq. MeOH), followed by MPLC on RP-18 (40% aq. MeOH and 80% aq. CH_3CN) furnished compounds **3** (168 mg), **4** (345 mg), **5** (340 mg), and **6** (294 mg).[8]

Taxifolin (5,7,3′,4′-Tetrahyrdoxyflavanol, **1**): White amorphous powder, $[\alpha]_D$ +20° (c=0.1, MeOH). IR ν_{max} cm^{-1}: 3420, 1620, 1520, 1470, 1360, 1265, 1165. UV λ_{max} nm (log ε): 288 (4.2), 328 (sh).

Afzelin (Kaempferol 3-*O*-α-L-Rhamnopyranoside, **2**): Yellow amorphous powder, $[\alpha]_D$ −184° (c=0.1, MeOH). IR ν_{max} cm^{-1}: 3280, 1655, 1615, 1500, 1450, 1365. UV λ_{max} nm (log ε): 264 (4.3), 342 (4.1).

Quercitrin (Quercetin 3-*O*-α-L-Rhamnopyranoside, **3**): Yellow amorphous powder, $[\alpha]_D$ −178° (c=0.1, MeOH). IR ν_{max} cm^{-1}: 3320, 1660, 1610, 1500, 1450, 1360. UV λ_{max} nm (log ε): 254 (4.2), 314 (sh), 350 (4.1).

Myricitrin (Myricetin 3-*O*-α-L-Rhamnopyranoside, **4**): Yellow amorphous powder, $[\alpha]_D$ −141° (c=0.1, MeOH). IR ν_{max} cm^{-1}: 3270, 1655, 1610, 1500, 1455, 1340. UV λ_{max} nm (log ε): 254 (4.2), 312 (sh), 354 (4.1).

1,2,6-Trigalloylglucose (**5**): White amorphous powder, $[\alpha]_D$ −94° (c=0.1, MeOH). IR ν_{max} cm^{-1}: 3420, 1710, 1610, 1540, 1525, 1450, 1355. UV λ_{max} nm (log ε): 216 (4.6), 278 (4.4).

1,2,3,6-Tetragalloylglucose (**6**): White amorphous powder, $[\alpha]_D$ +39° (c=0.1, MeOH). IR ν_{max} cm^{-1}: 3400, 1700, 1610, 1540, 1455, 1355. UV λ_{max} nm (log ε): 216 (4.9), 278 (4.5).

Enzymatic Hydrolysis of 2, 3, and 4 Naringinase (100 mg) was added to a suspension of **2**, **3**, or **4** (each 10 mg) in 50 mM acetate buffer (pH, 5.5) and the mixture was stirred at room temperature for 3 h. The reaction mixture was extracted with EtOAc (20 ml) and evaporated to dryness. The each residue was chromatographed on silica gel eluting with $CHCl_3$–MeOH (1 : 0→8 : 2) to give kaempferol (**2a**), quercetin (**3a**), and myricetin (**4a**).

Kaempferol (**2a**): Yellow amorphous powder. $[\alpha]_D$: +5.0° (c=0.2, MeOH). ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 6.16 (1H, d, J=1.1 Hz, H-6), 6.40 (1H, d, J=1.1 Hz, H-8), 6.92 (2H, d, J=8.7 Hz, H-3′, 5′), 8.03 (2H, d, J=9.0 Hz, H-2′, 6′).

Quercetin (**3a**): Yellow amorphous powder. $[\alpha]_D$: +5.6° (c=0.2, MeOH). ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 6.14 (1H, d, J=1.8 Hz, H-6), 6.35 (1H, d, J=1.8 Hz, H-8), 6.87 (1H, d, J=8.4 Hz, H-5′), 7.53 (1H, dd, J=8.4, 2.4 Hz, H-6′), 7.65 (1H, d, J=1.8 Hz, H-2′).

Myricetin (**4a**): Yellow amorphous powder. $[\alpha]_D$: −4.0° (c=0.3, MeOH). ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 6.18 (1H, d, J=1.2 Hz, H-6), 6.37 (1H, d, J=1.2 Hz, H-8), 6.93 (2H, s, H-2′, 6′).

Anti-complement Assay A diluted solution of normal human serum (complement serum, 80 μl) was mixed with a gelatin veronal buffer (GVB^{2+}, 80 μl) with or without sample. Each sample was dissolved in DMSO, and it was used as negative control. The mixture was pre-incubated at 37° for 30 min, after which sensitized erythrocyte (sheep red blood cells, 40 μl) was added. After incubation under the same conditions, the mixture was centrifuged (4 °C, 1500 rpm) and the optical density of the supernatant (100 μl) was measured at 450 nm.[11] Tiliroside and rosmarinic acid were used as positive controls.[12] Anti-complement activity was determined as a mean of triplicate measurements and expressed as the 50% inhibitory concentrations (IC_{50}) values from complement-dependent hemolysis of the control.[13]

RESULTS AND DISCUSSION

The human complement system plays an important role in the host defense system against foreign invasive organisms, *i.e.* viruses, bacteria, and fungi, as well as an external wound. Its effects are normally beneficial to the host, but it can also cause adverse effects depending on the site, extent, and duration of complement activation.[14] Activation of the system may lead to pathologic reactions in a variety of inflammatory and degenerative diseases such as multiple sclerosis, systemic lupus erythematosus, Sjogren syndrome, dermatological disease, rheumatoid arthritis, and gout.[15]

As part of an ongoing program of research in our laboratory to detect natural products with anti-complement activity, the inhibitory properties of compounds from the stem-bark of *J. mandshurica* were investigated. Repeated column chromatography of the EtOAc-soluble fraction of the MeOH extract of *J. mandshurica* on silica gel, ODS, and Sephadex LH-20 led to the isolation of four flavonoids (**1**—**4**) and two galloyl glucoses (**5**, **6**). These compounds were identified as taxifolin (**1**), afzelin (**2**), quercitrin (**3**), myricitrin (**4**), 1,2,6-trigalloylglucose (**5**), and 1,2,3,6-trigalloylglucose (**6**) by comparing their spectral data to those previously reported (Fig. 1).[8] Furthermore, kaempferol (**2a**), quercetin (**3a**), and myricetin (**4a**) were obtained from afzelin (**2**), quercitrin (**3**), and myricitrin (**4**), respectively, by enzymatic hydrolysis.

The compounds **1**—**6** isolated were tested for their anti-complement activity and the results obtained are summarized in Table 1 (IC_{50} values). Afzelin (**2**), quercitrin (**3**), 1,2,6-tri-

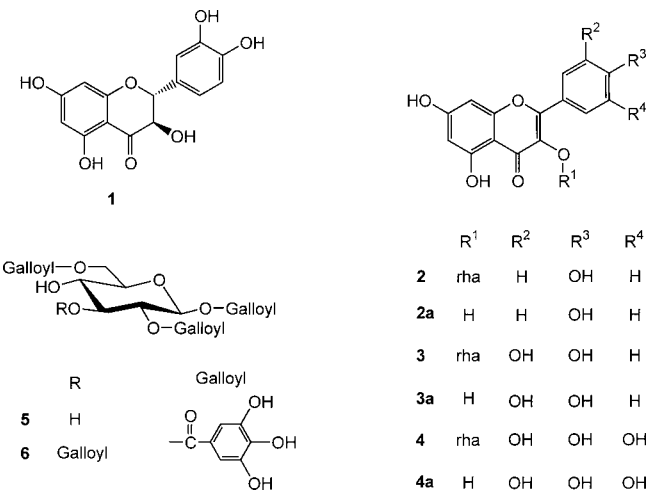

Fig. 1. Chemical Structures of Compounds from the Stem-Bark of *Juglans mandshurica*

Table 1. Inhibitory Effects of Compounds on the Complement System of Classical Pathway *in Vitro*

Compound	IC_{50} (μM)[a]
Taxifolin (**1**)	NE[c]
Afzelin (**2**)	258
Quercitrin (**3**)	440
Myricitrin (**4**)	NE
1,2,6-Trigalloylglucose (**5**)	136
1,2,3,6-Tetragalloylglucose (**6**)	34
Kaempferol (**2a**)	730
Quercetin (**3a**)	NE
Myricetin (**4a**)	NE
Rosmarinic acid[b]	180
Tiliroside[b]	101.2

a) Results are the mean (n=3). *b*) This compound was used as a positive control.[12] *c*) NE; no effect.

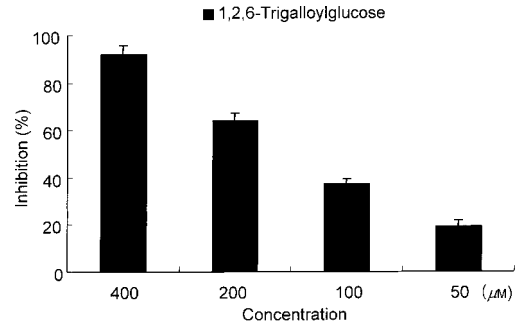

Fig. 2. Inhibitory Effects of 1,2,6-Trigalloylglucose (**5**) on Classical Pathway of Complement System (Mean±S.D., n=3)

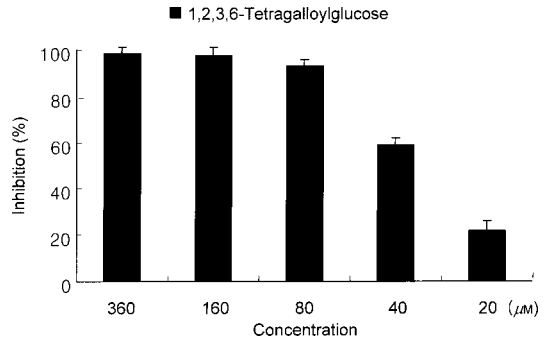

Fig. 3. Inhibitory Effects of 1,2,3,6-Tetragalloylglucose (**6**) on Classical Pathway of Complement System (Mean±S.D., n=3)

galloylglucose (**5**), and 1,2,3,6-tetragalloylglucose (**6**) showed anti-complement activity with IC_{50} values of 258, 440, 136, and 34 μM, respectively (Figs. 2, 3). On the other hand, taxifolin (**1**) and myricitrin (**4**) were completely incapable of inhibiting complement activity. Furthermore, kaempferol (**2a**), quercetin (**3a**), and myricetin (**4a**), which were obtained from compounds **2**, **3**, and **4**, were also evaluated for their anti-complement activity. Kaempferol (**2a**) exhibited weak anti-complement activity with an IC_{50} value of 730 μM, while quercetin (**3a**) and myricetin (**4a**) were inactive in this assay system. These results demonstrate the role which the number of hydroxyl groups on the B-ring of 3,5,7-trihydroxyflavone may play in structure–activity relationships: it seems that the inhibitory potencies of **2** (**2a**), **3** (**3a**), and **4** (**4a**) against anti-complement activity increased in inverse proportion to number of free hydroxyl groups on the B-ring of 3,5,7-trihydroxyflavone. These data agreed with the anti-complement properties of apigenin and its glycosides (apigenin 7-*O*-glucoside, apigenin 7-*O*-rutinoside), and luteolin and its glycosides (luteolin 7-*O*-glucoside, luteolin 4′-*O*-glycoside) isolated from *Ligustrum vulgare* and *Phillyrea latifolia*.[16]

Of the compounds tested, 1,2,3,6-tetragalloylglucose (**6**) was found to be the most active compound with an IC_{50} of 34 μM in this assay system. Comparing the galloylglucoses (**5**, **6**) with flavonoids (**1**—**4**) shown that the galloyl group might increase anti-complement properties. This result is supported by the anti-complement activity of 1,2,3,6-tetragalloylglucose (**6**; IC_{50}, 34 μM), which was found to be more potent than 1,2,6-trigalloylglucose (**5**; IC_{50}, 136 μM) against the complement system.

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