

Secondary Metabolites with Anti-complementary Activity from the Stem Barks of *Juglans mandshurica* Maxim¹

Zi-Jiang Li^{2,3} · Shilin Chen² · Xiang-Hao Yang² · Rui Wang² · Hee-Jeong Min⁴ ·
Lei Wu⁵ · Chuan-Ling Si^{2,4,5,†} · Young-Soo Bae^{4,5,†}

ABSTRACT

Juglans mandshurica is a fast growing hard species, which is a tree in family of Juglandaceae and has a wide distribution in China, Korea and eastern Russia. Plant materials from *J. mandshurica* have extensively been used in folk medicines to prevent or cure gastric, esophageal, lung and cardiac cancer. As one chain of our searching for anticomplementary agents from natural sources, two epimeric ellagitannins, [2,3-*O*-4,4',5,5',6,6'-hexahydroxydiphenoyl (HHDP))-(α,β)-D-glucose] (I) and pedunculagin (II) were purified from 70% acetone extracts of the stem barks of *J. mandshurica* by Thin Layer Chromatography and Sephadex LH-20 column chromatography approaches. The chemical structures of the isolated compounds were characterized by MS, NMR, and a careful comparison with published literatures. The epimeric ellagitannins I and II exhibited inhibitory properties against a classical pathway of complementary system with 50 % inhibitory concentrations (IC₅₀) values of 65.3 and 47.7 μ M, respectively, comparing with riliroside (IC₅₀=104 μ M) and rosmarinic acid (IC₅₀=182 μ M), which were used as positive controls. Thus, the work indicated both the two secondary metabolites possess excellent inhibitory activity and might be developed as potential anticomplementary chemicals.

Keywords: anticomplementary activity, epimeric ellagitannins, *Juglans mandshurica* Maxim., spectroscopic technique, secondary metabolites

1. INTRODUCTION

The human complement system plays an important role in the host defense system against foreign invasive organisms such as 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 (Cimanga *et al.*, 1995). 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

¹ Date Received November 8, 2017, Date Accepted December 22, 2017

² Tianjin Key Laboratory of Pulp & Paper, College of Papermaking Science & Technology, Tianjin University of Science & Technology, Tianjin 300457, China

³ Shandong Institute of Commerce and Technology, Jinan 250103, China

⁴ Department of Forest Biomaterials Engineering, College of Forest & Environment Sciences, Kangwon National University, Chuncheon 24341, Korea

⁵ Institute of Applied Chemistry, Jiangxi Academy of Sciences, Nanchang 330096, China

† Corresponding author: Young-Soo Bae (e-mail: bae@kangwon.ac.kr, ORCID: 0000-0003-1108-9269)

† Corresponding author: Chuan-Ling Si (e-mail: sichli@tust.edu.cn, ORCID: 0000-0003-1630-7800)

syndrome, dermatological disease, rheumatoid arthritis, and gout. Therefore, the modulation of complement activity is important and there is a need to develop anti-complementary agents from various sources such as plants (Park *et al.*, 1999; Min *et al.*, 2003).

Juglans mandshurica Maxim (Juglandaceae), a fast growing deciduous tree, is widely distributed in China, Siberia and Korean peninsula. The tree has been used as a folk medicinal plant for the treatment of esophageal, gastric, cardiac and lung cancer. It was reported that volatile constituents from the species inhibited the growth of the neighboring plants and can be developed for chemurgy (Min *et al.*, 2003; Kim, 1994). Some of the valuable constituents such as α -tertalonyl glucopyranosides, naphthoquinones, naphthalenyl glycosides, flavonoids, galloyl glucopyranosides and diarylheptanoyl glucopyranosides have already been isolated from this species (Park *et al.*, 2017; Wang *et al.*, 2017; Min *et al.*, 2003; Li *et al.*, 2003). In addition, several studies have reported the anti-complement activity, inhibition of human immunodeficiency virus type 1 reverse transcriptase and ribonuclease H activities of *J. mandshurica* extract (Bi *et al.*, 2016; Min *et al.*, 2003; Min *et al.*, 2000).

Thus, and as a part of a project aimed at discovering bioactive and structurally novel compounds from plant sources, the chemical constituents of *J. mandshurica* bark and their anti-complement activity were investigated.

2. MATERIALS and METHODS

2.1. Instruments

Melting points (uncorrected) were determined with an Electro Thermal 9100 apparatus. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter in MeOH. IR spectra were obtained on a Perkin-Elmer BX FT-IR spectrometer in a KBr disk. UV spectra were

recorded in MeOH (Jenway 6405 spectrophotometer). $^1\text{H-NMR}$ (400 MHz) and $^{13}\text{C-NMR}$ (100 MHz) spectra were recorded in MeOH- d_4 with TMS (Tetramethylsilane) as an internal standard using a Bruker Avance DPX 400 spectrometer. MALDI-TOF-MS spectroscopy was measured on a Model Voyager-DE STR spectrometer.

Paper TLC analysis were carried out on DC-Plastikfolien Cellulose F (Merck Co.) plates and developed with *t*-BuOH-HOAc-H₂O (3 : 1 : 1, v/v/v, solvent A) and HOAc-H₂O (3 : 47, v/v, solvent B). TLC spots were detected by UV-light (254 and 365 nm) and by spraying with 1% FeCl₃ (in EtOH) solution followed by heating.

2.2. Anti-complement Assay

Anti-complement properties of the isolated epimeric ellagitannins were evaluated by a method adopted from Yamada *et al.*(1985). A diluted solution of normal human serum (complement serum, 80 μL) was mixed with a gelatin veronal buffer (GVB²⁺, 80 μL) without or with samples. The mixture was pre-incubated at 37°C for 30 min, followed by adding sensitized erythrocyte (sheep red blood cells, 40 μL). After incubation under the same conditions, the mixture was centrifuged (4°C, 1500 rpm) and the absorbance of the supernatant (100 μL) was measured at 450 nm by a UV spectrometer (Libra S32, Biochrom). Each sample was dissolved in DMSO as negative control, while tiliroside and rosmarinic acid were used as positive controls. Anti-complement activity was determined as a mean of three independent trials and expressed as the 50% inhibitory concentration (IC₅₀) values from complement-dependent hemolysis of the control (Jung *et al.*, 1998; Oh *et al.*, 2000).

2.3. Plant Material

The stem bark was stripped from a 10-year-old *J. mandshurica* tree grown in the experimental forest of

Kangwon National University, Korea in April, 2005 and was identified by Prof. Wan-Keun Park, School of Forest, Kangwon National University, Korea. A voucher specimen has been deposited at the herbarium of Laboratory of Natural Products, Kangwon National University, Korea.

2.4. Extraction and Fractionation

The bark of *J. mandshurica* was air-dried and ground with a Wiley mill. A precisely weighted amount (3.2 kg) was extracted in 70 % acetone aqueous solution (each 20 L for 72 h×3 times) at room temperature. The extracting solutions were filtered, combined, and concentrated with an evaporator under reduced pressure to afford the crude extract, which was suspended in water and then successively submitted to liquid-liquid fractions with in separation funnels using *n*-hexane, methylene chloride (CH₂Cl₂), ethylacetate (EtOAc) and *n*-buthanol (*n*-BuOH) (Si *et al.*, 2017). For the detailed extraction and fractionation procedures see Fig. 1. The soluble fractions in *n*-hexane (15.6 g, yield 0.5%), CH₂Cl₂ (20.6 g, yield 0.6%), EtOAc (130 g, yield 4.1%) and H₂O (337.6 g, yield 10.6%) were obtained after freezing-drying as powders.

2.5. Isolation of Ellagitannins

As shown in Fig. 1, a portion of the resulting H₂O soluble Fr. (40.0 g) was applied to a Sephadex LH-20 column eluting with MeOH-H₂O(1:1, v/v) to give four fractions: H₁ (12.4 g), H₂ (8.5 g), H₃ (16.2 g) and H₄ (2.8 g), which were sampled and their composition were monitored by Paper TLC. Fraction H₃ was reapplied to a Sephadex LH-20 column using EtOH-hexane (3 : 1, v/v) as eluent for further purification to yield three subfractions and the third subfraction H₃₃ (8.9 g) was stepwisely rechromatographed with MeOH-H₂O (1 : 3, v/v) to give subfractions H₃₃₁ (1.8 g) and H₃₃₂ (6.2 g) and compound

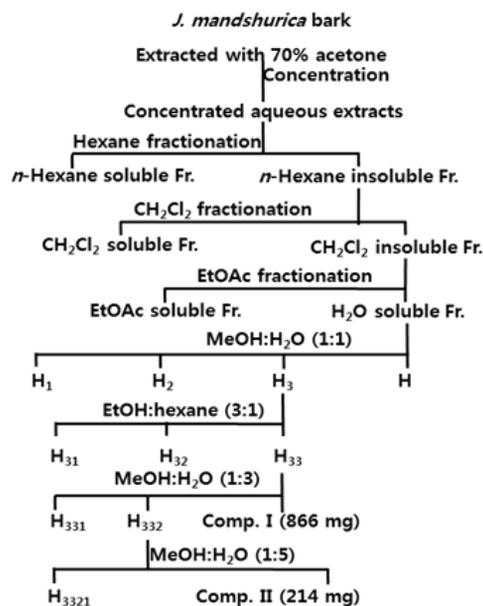


Fig. 1. Extraction, fractionation and isolation procedures of tannins from *J. mandshurica* stem bark.

I (866 mg). Subfraction H₃₃₂ was resubjected to a Sephadex LH-20 column and eluted with MeOH-H₂O (1 : 5, v/v) for further separation to give subfraction H₃₃₂₁ (5.9 g) and compounds **II** (214 mg).

3. RESULTS and DISCUSSION

Compound I, which has never been reported from *J. mandshurica* previously, was isolated as a colorless amorphous powder with melting point of 112–114 °C and optical rotation of $[\alpha]_D^{20} + 18.5^\circ$ (*c* 0.1 in MeOH). On paper TLC, its *R_f* (Retention Factor) values are 0.68 (solvent A) and 0.77 (solvent B). Its molecular weight 482 and molecular formula C₂₀H₁₈O₁₄ was determined based on the quasi-molecular ion peaks *m/z* [M+Na]⁺ at 505, [M+K]⁺ at 521, [2M+Na]⁺ at 987 and [2M+K]⁺ at 1003 in the MALDI-TOF-MS spectrum. The presence of phenolic hydroxyl group in the molecule was recognized from grey-green color with ethanolic FeCl₃

solution on TLC (Imakura *et al.*, 1985). In its IR spectrum, absorption bands at 3380, 1738, 1615, 1511, 1435, 1230, 1185, 875, 830, 735 (cm^{-1} in KBr) were observed. Its UV spectrum showed maxima at 262 nm (in MeOH). In ^1H - and ^{13}C -NMR spectra, A characteristic anomeric mixture of α - and β -D-glucose and connecting 4,4',5,5',6,6',-hexahydroxydiphenoyl (HHDP) residues were observed. However, compound **I**, a pair of epimers, could not be completely separated by column chromatography though it appeared as only one spot on two-dimensional paper TLC. Its spectroscopic data were identical with those reported by Seikel *et al.* (1970), thus compound **I** was identified as 2,3-O-4,4',5,5',6,6'-HHDP-(α/β)-D-glucose.

Compound **II** was obtained as a pale brown amorphous powder for the first time from this species. The melting point was 135–137°C. We found its optical rotation $[\alpha]_D^{20}$ of $+106^\circ$ (c 0.1 in MeOH). Its IR and UV absorption bands were similar with those of compound **I** described above. R_f of this compound are 0.29 (solvent A) and 0.55 (solvent B). The MALDI-TOF-MS spectrum of compound **II** gave peaks of m/z $[\text{M}+\text{Na}]^+$ at 807, $[\text{2M}+\text{K}]^+$ at 823, $[\text{2M}+\text{Na}]^+$ at 1591, $[\text{2M}+\text{K}]^+$ at 1607, respectively, corresponding to the molecular formula $\text{C}_{20}\text{H}_{18}\text{O}_{14}$. Similar to that of compound **I**, compound **II** was also an epimeric compound, which was existing in equilibrium as anomeric mixture of α - and β -D-glucoses as described

in previously published literatures (Tsujita *et al.*, 2017; Seikel *et al.*, 1970). In the ^1H -NMR spectrum of compound **II**, two sets of partially overlapped α - and β -D-glucose were confirmed. In addition, four sets of 4,4',5,5',6,6',-hexahydroxydiphenoyl (HHDP) moieties were observed. The ^{13}C -NMR of compound **II** also presented duplicated signals due to the presence of a pair of epimers. These ^1H - and ^{13}C -NMR data were coincided with those reported in literature (Seikel *et al.*, 1970) and compound **II** was elucidated as pedunculagin consequently.

3.1 2,3-O-4,4',5,5',6,6'-HHDP-(α/β)-D-Glucose (**I**)

Colorless amorphous powder; mp 112–114 °C; $[\alpha]_D^{20} +18.5^\circ$ (c 0.1 in MeOH); IR (KBr) ν_{max} cm^{-1} 3380, 1738, 1615, 1511, 1435, 1230, 1185, 875, 830, 735; UV λ max (MeOH) nm 262; R_f : 0.68 (solvent A) and 0.77 (solvent B); MALDI-TOF-MS : m/z $[\text{M}+\text{Na}]^+$ at 505, $[\text{M}+\text{K}]^+$ at 521, $[\text{2M}+\text{Na}]^+$ at 987 and $[\text{2M}+\text{K}]^+$ at 1003; ^1H - and ^{13}C -NMR data were identical with those in the literature (Seikel *et al.*, 1970).

3.2 Pedunculagin (**II**)

Pale brown amorphous powder; mp 135–137°C; $[\alpha]_D^{20} + 106^\circ$ (c 0.1 in MeOH); IR (KBr) ν_{max} cm^{-1} 3380,

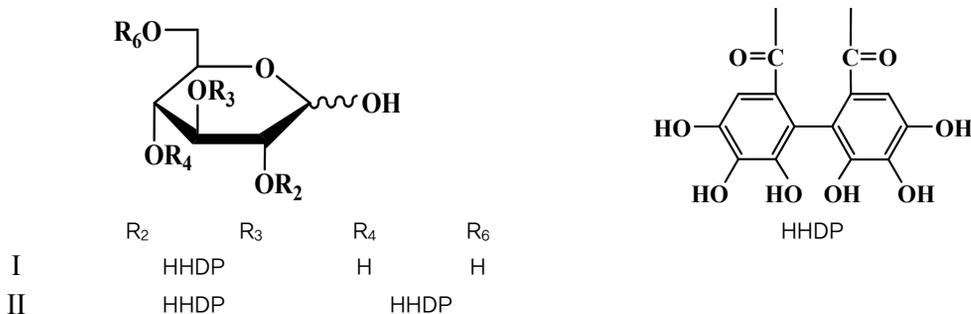


Fig. 2. Structures of isolated epimeric ellagitannins from *J. mandshurica* stem bark.

Table 1. Anti-complementary effects of isolated epimeric ellagitannins by complementary system of classical pathway *in vitro*.

| | Sample | IC ₅₀ (μ M) ^a |
|-------------------|--|--|
| Ellagi-tannins | 2,3-O-4,4',5,5',6,6'-HHDP-(α/β)-D-glucose (I) | 65.3 |
| | pedunculagin (II) | 47.7 |
| Positive controls | Tiliroside | 104 |
| | Rosmarinic acid | 182 |

^a Data were expressed as the mean of three independent replicates.

1747, 1615, 1511, 1435, 1230, 1185, 875, 830, 735; UV λ_{\max} (MeOH) nm 260; R_f : 0.29 (solvent A) and 0.55 (solvent B); MALDI-TOF-MS: m/z [M+Na]⁺ 807, [2M+K]⁺ 823, [2M+Na]⁺ 1591, [2M+K]⁺ 1607; ¹H- and ¹³C-NMR data were in agreement with those of the literature (Tanaka *et al.*, 1993).

3.3 Anti-complementary Activity

Compounds **I** and **II** isolated from *J. mandshurica* stem bark were assayed for their anti-complement activity on the complement system of classical pathway (CP) *in vitro* and the results were summarized in Table 1. Both the two epimeric ellagitannins exhibited strong anti-complement activity with IC₅₀ values were 65.3 and 47.7 μ M, respectively, comparing tiliroside and rosmarinic acid 104 and 182 μ M, respectively, which were used as positive controls. These facts suggested that the two epimeric ellagitannins could be used as anti-complement agents.

4. CONCLUSION

By successive liquid-liquid fractionation and repeated TLC-monitored purification of 70% aqueous acetone extraction of the *J. mandshurica* barks, two ellagitannins were isolated and their chemical structures (Fig. 2) were elucidated as 2,3-O-4,4',5,5',6,6'-HHDP-(α/β)-D-glucose (I) and pedunculagin (II) based on their chemical and

spectroscopic evidences, and a careful comparison with previously published data. Compounds I and II were epimeric ellagitannins and they were isolated as anomeric mixtures of α - and β -D-glucoses in the current work as described in literatures. It is noteworthy that this is the first report of compounds I and II from *J. mandshurica*.

The activity of the two epimeric ellagitannins (I and II) were evaluated by a classical pathway of complementary system assay, and our investigation results indicated that both the two compounds inhibited excellent effects and could be used as potential anti-complementary agents.

ACKNOWLEDGMENT

This work was kindly supported by National Key Research and Development Program of China (Grant No.2017YFB0307903), Foundation of Key Project of Research and Development Program of Jiangxi Province (No. 20171BBH80017 and 20171ACF60009), the Science and Technology Major Project Foundation of Jiangxi Academy of Sciences (2018-YZD1-05 and 2018-YZD2-18), Science Foundation for Young Doctors of Jiangxi Academy of Science (2016-YYB-07), and Introduction of Overseas Technical and Managerial Personnel Program of State Administration of Foreign Experts Affairs (20173600003), P.R. China.

REFERENCES

- Bi, D.D., Zhao, Y.C., Jiang, R., Wang, Y., Tian, Y.X., Chen, X.Y., Bai, S.J., She, G.M. 2016. Phytochemistry, bioactivity and potential impact on health of *Juglans*: the original plant of walnut. *Natural Product Communications* 11(6): 869-880.
- Cimanga, K., Bruyne, T.D., Lasure, A., Poel, B.V., Pieters, L., Berghe, D.V., Vlietinck, A., Kambu, K., Tona, L. 1995. In vitro anticomplementary activity of constituents from *Morinda morindoides*. *Journal of Natural Products* 8(3): 372-378.
- Imakura, Y., Kobayashi, S., Mima, A. 1985. Bitter phenyl propanoid glycosides from *Campsia chinensis*. *Phytochemistry* 24(1): 139-146.
- Jung, K.Y., Oh, S.R., Park, S.H., Lee, I.S., Ahn, K.S., Lee, J.J., Lee, H.K. 1998. Anti-complement activity of tiliroside from the flower buds of *Magnolia fargesii*. *Biological & Pharmaceutical Bulletin* 21(10): 1077-1078.
- Kim, T.W. 1994. *The Woody Plants of Korea*. Kyohak Press: Seoul, Korea. pp.58-59.
- Li, G., Xu, M.L., Choi, H.G., L, S.H., Jahng, Y.D., Lee, C.S., Moon, D.C., Woo, M.H., Son, J.K. 2003. Four new diarylheptanoids from the roots of *Juglans mandshurica*. *Chemical & Pharmaceutical Bulletin* 51(3): 262-264.
- Min, B.S., Nakamura, N., Miyashiro, H., Kim, Y.H., Hattori, M. 2000. Inhibition of human immunodeficiency virus type 1 reverse transcriptase and ribonuclease H activities by constituents of *Juglans mandshurica*. *Chemical & Pharmaceutical Bulletin* 48(2): 194-200.
- Min, B.S., Lee, S.Y., Kim, J.H., Lee, J.K., Kim, T.J., Kim, D.H., Kim, Y.H., Joung, H., Lee, H.K., Nakamura, N., Miyashiro, H., Hattori, M. 2003. Anti-complement activity of constituents from the stem-bark of *Juglans mandshurica*. *Biological & Pharmaceutical Bulletin* 26(7): 1042-1044.
- Oh, S.R., Kinjo, J., Shii, Y., Ikeda, T., Ahn, K.S., Kim, J.H., Lee, H.K. 2000. Effects of triterpenoids from *Pueraria lobata* on immuno-hemolysis: β -D-glucuronic acid plays an active role in anticomplementary activity in vitro. *Planta Medica* 66(6): 506-510.
- Park, S.H., Oh, S.R., Jung, K.Y., Lee, I.S., Ahn, K.S., Kim, J.H., Kim, Y.S., Lee, J.J., Lee, H.K. 1999. Acylated flavonol glycosides with anti-complement activity from *Persicaria lapathifolia*. *Chemical & Pharmaceutical Bulletin* 47(10): 1484-14686.
- Park, S., Kim, N., Yoo, G.J., Kim, S.N., Kwon, H.J., Jung, K.W., Oh, D.C., Lee, Y.H., Kim, S.H. 2017. Phenolics and neolignans isolated from the fruits of *Juglans mandshurica* Maxim. and their effects on lipolysis in adipocytes. *Phytochemistry* 137(8): 87-93.
- Seikel, M.K., Hillis, W.E. 1970. Hydrolysable tannins of *Eucalyptus delegatensis* wood. *Phytochemistry* 9(5): 1115-1128.
- Si, C.L., Gao, Y., Wu, L., Liu, R., Wang, G.H., Dai, L., Li, X.H., Hong, Y.M. 2017. Isolation and characterization of triterpenoids from the stem barks of *Pinus massoniana*. *Holzforchung* 71(9): 697-703.
- Tanaka, T., Tachibana, H., Nonaka, G., Nishioka, I., Hsu, F.L., Kohda, H., Tanaka, O. 1993. Tannins and related compounds. CXXII. New dimeric, trimeric and tetrameric ellagitannins, lambertianins A-D, from *Rubus lambertianus* Seringe. *Chemical & Pharmaceutical Bulletin* 41(7): 1214-1220.
- Tsujita, T., Matsuo, Y., Saito, Y., Tanaka, T. 2017. Enzymatic oxidation of ellagitannin and a new ellagitannin metabolite from *Camellia japonica* leaves. *Tetrahedron* 73(5): 500-507.
- Wang, T.M., Liu, J., Yi, T., Zhai, Y.J., Zhang, H., Chen, H.B., Cai, S.Q., Kang, T.G., Zhao, Z.Z. 2017.

Multiconstituent identification in root, branch, and leaf extracts of *Juglans mandshurica* using ultra high performance liquid chromatography with quadrupole time-of-flight mass spectrometry. *Journal of Separation Science* 40(17): 3340-3452.

Yamada, H., Ohtani, K., Kiyohara, H., Cyong, J.C., Otsuka, Y., Ueno, Y., Omura, S. 1985. Purification and chemical properties of anti-complementary polysaccharide from the leaves of *Artemisia princeps*. *Planta Medica* 51(2): 121-125.