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Original Article

Secondary Metabolites with Anti-complementary Activity from the Stem Barks of *Juglans mandshurica* Maxim[1]

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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 comparation with published literatures. The epimeric ellagitannins I and II exhibited inhibitory properties against a classical pathway of complementary system with 50 % inhibitory concentrations (IC_{50}) values of 65.3 and 47.7 μM, respectively, comparing with riliroside (IC_{50}=104 μM) and rosmarinic acid (IC_{50}=182 μM), 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

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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}H-NMR (400 MHz) and ^{13}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-H_2O (3 : 1 : 1, v/v/v, solvent A) and HOAc-H_2O (3 : 47, v/v, solvent B). TLC spots were detected by UV-light (254 and 365 nm) and by spraying with 1% $FeCl_3$ (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 (GVB^{2+}, 80 μL) without or with samples. The mixture was pre-incubated at 37ºC for 30 min, followed by adding sensitized erythrocyte (sheep red blood cells, 40 μL). After incubation under the same conditions, the mixture was centrifuged (4ºC, 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 (IC_{50}) 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 (CH_2Cl_2), 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%), CH_2Cl_2 (20.6 g, yield 0.6%), EtOAc (130 g, yield 4.1%) and H_2O (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 H_2O soluble Fr. (40.0 g) was applied to a Sephadex LH-20 column eluting with MeOH-H_2O(1:1, v/v) to give four fractions: H_1 (12.4 g), H_2 (8.5 g), H_3 (16.2 g) and H_4 (2.8 g), which were sampled and their composition were monitored by Paper TLC. Fraction H_3 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 H_{33} (8.9 g) was stepwisely rechromatographed with MeOH-H_2O (1 : 3, v/v) to give subfractions H_{331} (1.8 g) and H_{332} (6.2 g) and compound

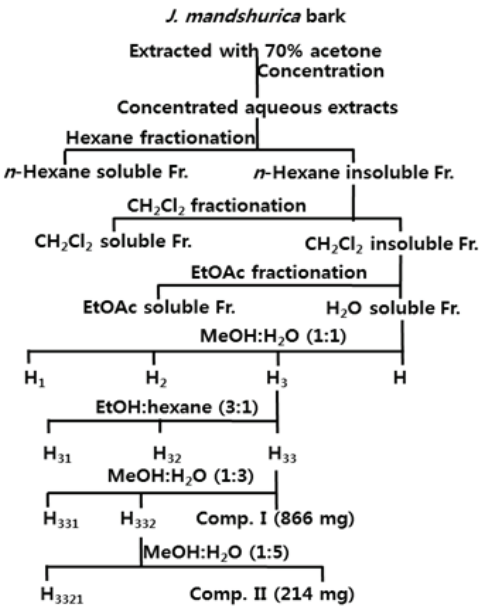


Fig. 1. Extraction, fractionation and isolation procedures of tannins from *J. mandshurica* stem bark.

I (866 mg). Subfraction H_{332} was resubjected to a Sephadex LH-20 column and eluted with MeOH-H_2O (1 : 5, v/v) for further separation to give subfraction H_{3321} (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 ºC and optical rotation of $[\alpha]_D^{20}$ + 18.5º (*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 $C_{20}H_{18}O_{14}$ 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 $FeCl_3$

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 ^{1}H- 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.

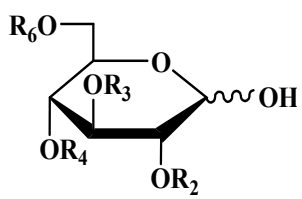
Compound **II** was obtained as a pale brown amorphous powder for the first time from this species. The melting point was 135–137ºC. We found its optical rotation $[\alpha]_D^{20}$ of +106° (*c* 0.1 in MeOH). Its IR and UV absorption bands were similar with those of compound I described above. R_f of 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 $[M+Na]^+$at 807, $[2M+K]^+$ at 823, $[2M+Na]^+$ at 1591, $[2M+K]^+$ at 1607, respectively, corresponding to the molecular formula $C_{20}H_{18}O_{14}$. Similar to that of compound **I**, compound **II** was also an epimeric compound, which was existing in equilibrium as anomeic mixture of α- and β-D-glucoses as described in previously published literatures (Tsujita *et al*., 2017; Seikel *et al*., 1970). In the ^{1}H-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 ^{1}H- 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)

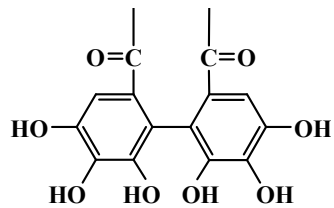
Colorless amorphous powder; mp 112–114 ºC; $[\alpha]_D^{20}$ +18.5º (c 0.1 in MeOH); IR (KBr) νmax cm^{-1} 3380, 1738, 1615, 1511, 1435, 1230, 1185, 875, 830, 735; UV λ max (MeOH) nm 262; R_f : 0.68 (solvent A) and 0.77 (solvent B); MALDI-TOF-MS : m/z $[M+Na]^+$ at 505, $[M+K]^+$ at 521, $[2M+Na]^+$ at 987 and $[2M+K]^+$ at 1003; ^{1}H- 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 135–137ºC; $[\alpha]_D^{20}$ + 106° (*c* 0.1 in MeOH); IR (KBr) ν_{max} cm^{-1} 3380,



	R_2	R_3	R_4	R_6
I	HHDP		H	H
II	HHDP		HHDP	



HHDP

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	$IC_{50}(\mu M)^a$
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; ^{1}H- and ^{13}C-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 IC_{50} values were 65.3 and 47.7 μM, respectively, comparing tiliroside and rosmarinic acid 104 and 182 μM, 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.

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