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Characterization of Antidiabetic Compounds from Extract of *Torreya nucifera*

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Natural products have gained increasing attention due to their advantage of long-term safety and low toxicity for a very long time. *Torreya nucifera* is widespread in southern Korea and Jeju Island and its seeds are commonly used as edible food. Oriental ingredients have often been reported for their insecticidal, antioxidant and antibacterial properties, but there have not yet been any studies on their antidiabetic effect. In this study, we investigated several biological activities of *T. nucifera* pericarp (TNP) and seeds (TNS) extracts and proceeded to characterize the antidiabetic compounds of TNS. The initial results suggested that TNS extract at 15 and 10 μg/ml concentration has inhibitory effects on α-glucosidase and protein tyrosine phosphatase 1B, that is 14.5 and 4.35 times higher than TNP, respectively. Thus, the stronger antidiabetic TNS was selected for the subsequent experiments to characterize its active compounds. Ultrafiltration was used to determine the apparent molecular weight of the active compounds, showing 300 kDa or more. Finally the mixture was then partially purified using Diaion HP-20 column chromatography by eluting with 50~100% methanol. Therefore we concluded that the active compounds of TNS have potential as therapeutic agents in functional food or supplemental treatment to improve diabetic diseases.

Key words : Diaion HP-20, α-glucosidase, protein tyrosine phosphatase 1B, *Torreya nucifera,* ultrafiltration

Introduction

Diabetes is a disease in which glucose levels in the blood are much higher than those in the general population. It has become a major public health problem around the world as a chronic metabolic disorder in which the body produces a small amount of insulin, stops its production, or gradually resists its action [12]. In particular, the prevalence and incidence of type 2 diabetes (T2DM) has been exploding worldwide over the past few decades [3]. T2DM is caused by a lack of pancreatic insulin, which accounts for more than 90% of all diabetes, and characterized by hyperglycemia, insulin resistance, and relative insulin secretion disorder. Liver, skeletal muscle and adipose tissue are major tissues that respond to insulin [2]. Liver insulin resistance (IR) contributes to hyperglycemia and T2DM, while long-term hyperglycemia leads to the development of insulin resistance [1, 10, 11]. However, these present oral hypoglycemia drugs used as a treatment for diabetes were often associated with adverse side-effects, including weight gain, bone loss and gastrointestinal side effects [7, 8, 16] In this regard, there was an urgent need to develop natural ingredients which improve hepatic insulin resistance with negligible side effects and have antidiabetic effects in the human body. These periodical demands for prevention of diabetes led to expectations for natural products and studies on their antidiabetic mechanism were increasing.

Torreya nucifera Siebold & Zucc. native to southern Korea, is an evergreen tree of the Taxaceae. There have been reported pharmacological effects such as appetite enhancement, digestion, constipation and hemorrhoid treatment. And its oil was used for food, hair product or fuel [4]. Previous studies have reported that several bioactive substances of its oil show insecticidal activity and antimicrobial activity [9]. However there have been few studies of seeds extract of *T. nucifera* on the separation and identification of active compounds concerning with antidiabetic activity.

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Therefore we investigated determination of α-glucosidase and protein tyrosine phosphatase 1B inhibitory activities to prevent diabetes and characterization of active compounds from seeds extract of *T. nucifera*.

Materials and Methods

Materials

Torreya nucifera seeds were purchased from a market of Namhae region in Korea. Methanol (MeOH), *n*-butanol (BuOH), and ethyl acetate (EtOAc) as solvents were purchased from SK Chemicals (Ulsan, Korea) and Diaion HP-20 was purchased from the Mitsubishi Kasei Co. (Tokyo, Japan). Enzymes and substrates for *in vitro* biological assays were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Extraction of *Torreya nucifera*

T. nucifera pericarps (TNP) and *T. nucifera* seeds (TNS) were washed three times in distilled water and used as an extraction sample. First, 3 volume of MeOH was added into each sample and standing overnight at room temperature. MeOH extracts by three times were filtered through a filter paper and concentrated under reduced pressure using a rotary vacuum evaporator (Changshin Science, Seoul, Korea). A concentrated extract was further dried in Modul 4080C speed vacuum (Hanil Science Industrial Co., Gimpo, Korea) and the extract was diluted to an appropriate concentration for future experiments of biological activity. Next, after selecting seed of *T. nucifera* as an active candidate, the optimal extraction condition was investigated as follows: hot water extraction at 100℃ for 10 minutes (HWE-1), MeOH extraction of remaining precipitates after hot water extraction at 100℃ for 10 minutes (HWME-1), hot water extraction at 121℃ for 15 minutes (HWE-2), MeOH extraction of remaining precipitates after hot water extraction at 121℃ for 15 minutes (HWME-2), and MeOH extraction at room temperature (ME). In addition, in order to carry out solvent extraction, water to solubilize the dried extract and equal volume of BuOH were mixed and vortexed. BuOH layer and water layer were harvested after centrifugation at 13,000 rpm for 5 minutes. The biological activity of each layer was evaluated for next experiments.

Biological activities of *Torreya nucifera* extracts

α-Glucosidase inhibitory activity

α-Glucosidase inhibitory activity was measured by spectroscopic method using enzyme-substrate reaction. Briefly, the sample was added with 2.5 mM *p*-nitrophenyl-α-D-glucopyranoside (pNPG, Sigma-Aldrich) which is a substrate in 100 mM sodium phosphate buffer (pH 6.8) and then incubated with 0.25 units α-glucosidase enzyme (Sigma-Aldrich) at 37℃ for 15 minutes. Its activity was determined by measuring the *p*-nitrophenol released from pNPG at 405 nm by SpectraMax M2e microplate reader (Molecular Devices, Toronto, Canada). MeOH was used as a negative control and acarbose (Sigma-Aldrich) at final concentrations of 15, 62.5, and 250 μg/ml as a positive control. The inhibitory activity was expressed as a percentage of the negative control.

Protein tyrosine phosphatase 1B (PTP1B) inhibitory activity

PTP1B inhibitory activity was performed by using *p*-nitrophenyl phosphate (pNPP, Sigma-Aldrich) as a substrate. PTP1B buffer solution was composed of 50 mM tris-base, 150 mM NaCl, 0.1 mg/ml BSA, and 3 mM dithiothreitol (DTT). PTP1B buffer solution was mixed with 10 mM pNPP and 1 μg/ml PTP1B enzyme (Cayman Chemical, Ann Arbor, USA) was added to the mixture and incubated at 37℃ for 30 minutes. Its activity was determined by measuring the pNPP at 405 nm by SpectraMax M2e microplate reader (Molecular Devices). MeOH was used as a negative control and ursolic acid (Sigma-Aldrich) at final concentrations of 0.1, 0.8, and 1.6 mM as a positive control. The inhibitory activity was expressed as a percentage of the negative control.

DPPH free radical scavenging activity

DPPH free radical scavenging inhibitory activity was measured by substrate 2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich) at a concentration of 1.5×10^{-4} M. Substrate and ascorbic acid (Sigma-Aldrich) were weak to light, so the experiment was conducted in dark room. After the sample was mixed with DPPH solution, it was incubated in the dark for 15 minutes. DPPH free radical scavenging inhibitory activity was determined by measuring absorbance at 510 nm by SpectraMax M2e microplate reader (Molecular Devices). The inhibitory activity was calculated as a percentage of the negative control.

Tyrosinase inhibitory activity

Tyrosinase inhibitory activity was measured by spectroscopic method using enzyme-substrate reaction. 100 mM L-tyrosine (Junsei Chemical Co., Tokyo, Japan) in 50 mM sodium phosphate buffer (pH 6.5) was prepared with 200 units of tyrosinase from mushroom (Sigma-Aldrich). Sample was added to the mixture and incubated at 37℃ for 30

minutes. MeOH was used as a negative control and kojic acid (Sigma-Aldrich) at final concentrations of 6, 12.5, and 25 μg/ml as a positive control. Its activity was determined by measuring absorbance at 475 nm by SpectraMax M2e microplate reader (Molecular Devices). The inhibitory activity was calculated as a percentage of the negative control.

Angiotensin-converting enzyme (ACE) inhibitory activity

ACE inhibitory activity was measured by spectroscopic method using enzyme-substrate reaction. The rabbit lung acetone powder (Sigma-Aldrich) was added to the 400 mM sodium borate buffer (pH 8.3) and mixed well with homogenizer, which was overnight with a mixer at 4℃ for 30 minutes. And after centrifugation at 3,000 rpm, then the ACE enzyme was prepared by selecting only the upper liquid (-20℃ storage). Briefly, the sample was mixed with 5 mg/ml hippuryl-His-Leu acetate salt in 400 mM sodium borate buffer (pH 8.3) and incubated with ACE enzyme at 37℃ for 15 minutes. After 1 N HCl was added to stop the reaction, the mixture was treated with EtOAc, gently vortexed, and left at room temperature for 30 minutes. Finally, the mixture was centrifuged at 13,000 rpm for 5 minutes. After transferring the supernatant to the new tube, it was vacuum-dried, dissolved in distilled water, and added to a 96-well microplate. And the absorbance was measured at 228 nm with a SpectraMax M2e microplate reader (Molecular Devices). The inhibitory activity was calculated as a percentage of the negative control.

Xanthine oxidase (XO) inhibitory activity

XO inhibitory activity was measured by fluorometrical method using pterine as substrate reactions. Briefly, sample was mixed with 0.4 mM pterin (Santa Cruz Biotechnology) dissolved in phosphate buffer (pH 8) and 0.5 unit of xanthine oxidase enzyme (Roche, Basel, Switzerland) at 37℃ for 30 minutes. And the reaction was stopped with 1 N HCl. The reaction mixtures were fluorometrically detected at 340 nm excitation and 410 nm emission by Victor3™ plate reader (PerkinElmer, Waltham, Massachusetts, USA). MeOH was used as a negative control and allopurinol (Sigma-Aldrich) at final concentrations of 0.25, 1, and 4 μg/ml as a standard inhibitor. The inhibitory activity was expressed as percentage of the negative control.

Characterization of active compounds from *Torreya nucifera* seeds (TNS)

Molecular weight cut-off (MWCO)

Molecular weight was measured using ultrafiltration by osmotic pressure. After dissolving the dried MeOH extract of TNS in water, its MWCO was performed using QuixStand BenchTop System Ultrafiltration (GE Healthcare, Chicago, IL, USA). Surface area of the membrane cartridge was 110 cm^2. The specimens were separated by membrane size and maintained at 150-115 rpm. The MWCO of sample was performed by using the 300 kDa membrane cartridge, yielding <300 kDa and ≥300 kDa fractions after ultrafiltration.

Solvent extraction and pH and heat stability

MeOH extract (ME) of TNS was solubilized in water and mixed well with the same volume of BuOH. Mixture was divided into BuOH layer and water layer after centrifugation for 5 minutes. Each layer was dried in speed vacuum apparatus and then tested for glucosidase inhibitory activity. On the other hand, TNS ME was checked for pH stability at pH 2, 7, and 10 after adjustment with 1 N HCl or 1 N NaOH. Also TNS ME was observed for heat stability at 100℃ for 10 minutes and 121℃ for 15 minutes. The remaining biological activities of TNS ME under different pH and temperature were examined by α-glucosidase assay.

Diaion HP-20 column chromatography

After filling 1 g of Diaion HP-20 resin into open column with an inner diameter of 14.5 mm, it was sequentially equilibrized with 100, 50, and 20% MeOH and finally replaced with distilled water. Molecular weight ≥300 kDa fraction of TNS ME was adjusted to pH 2 or pH 7 to observe its adsorption capability on the resin, depending on its pH. After loading each sample 5 mg/ml of TNS, fractions were sequentially eluted with 20, 50, and 100% MeOH. The elution volume of 2 ml per fraction was tested for α-glucosidase inhibition activity.

Statistical analysis

All results were confirmed in at least three times. Unless otherwise stated, data were expressed as the mean ± SD. Statistical comparisons were used by ANOVA. Results were statistically significant at $p<0.05$.

Results

Evaluation of biological activity of *Torreya nucifera* extract

To verify and compare the biological activities of *T. nucifera* pericarp (TNP) and seeds (TNS) extracts, many kinds

of *in vitro* assay were performed. α-Glucosidase is an enzyme that breaks down carbohydrates into monosaccharides to absorb them into the body. Its inhibitor can delay glucose absorption, reduce postprandial plasma glucose level, and inhibit postprandial hyperglycemia. Acarbose has been used as a treatment for diabetes as a-glucosidase inhibitor. When comparing with TNP and TNS at 15 μg/ml concentration, TNS showed 14.5 times of higher inhibitory activity than TNP (Fig. 1A). Protein tyrosine phosphatase 1B (PTP1B) is an enzyme that regulates insulin signaling and causes of tyrosine dephosphorylation in insulin receptor (IR) and insulin receptor substrate (IRS), leading to insulin-resistance and type 2 diabetes. At a concentration of 10 μg/ml, TNS showed 4.35 times higher PTP1B inhibitory activity than TNP (Fig.

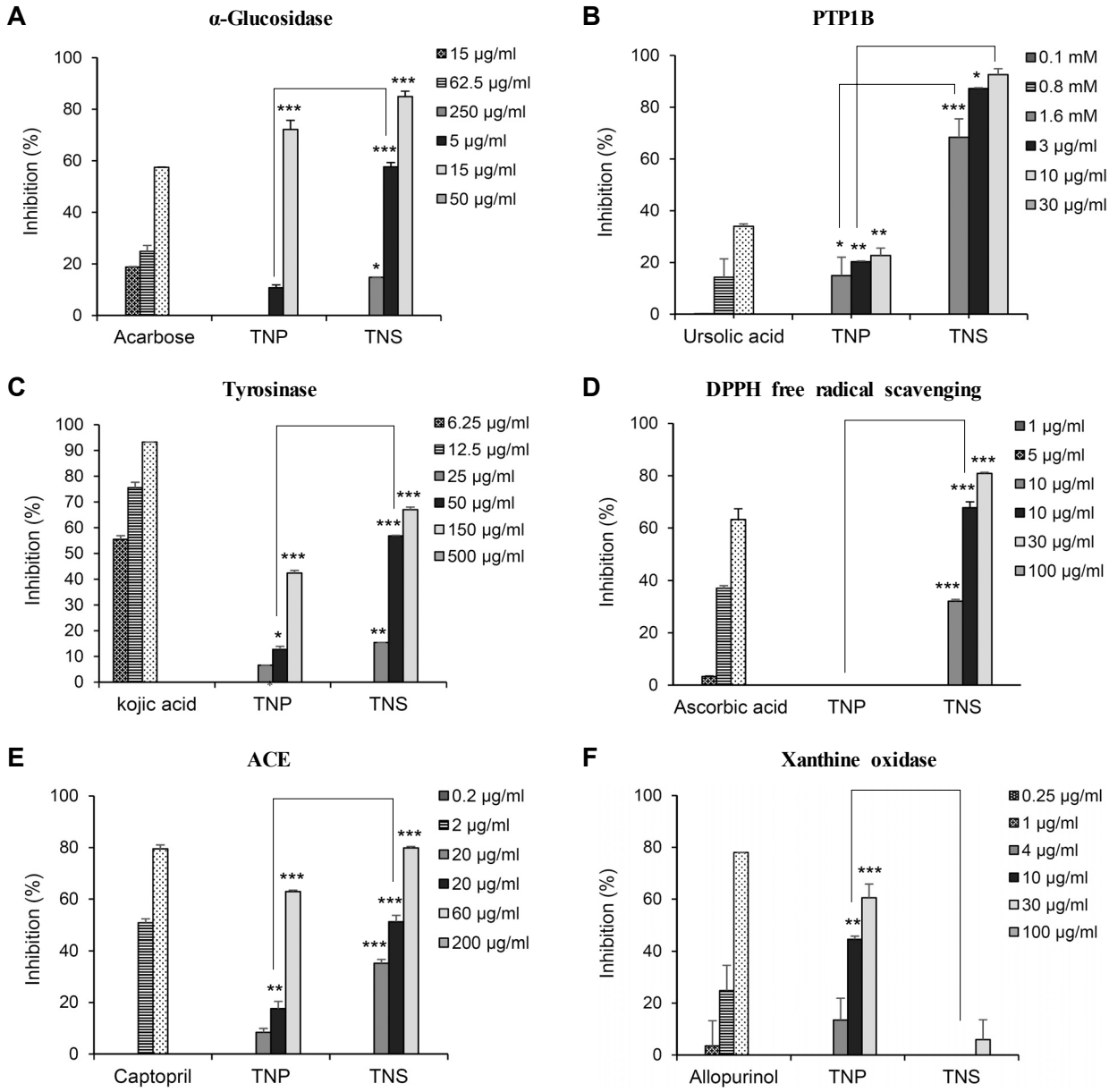

Fig. 1. Evaluation of biological activities of *Torreya nucifera* extract. (A) α-glucosidase inhibitory activity. The positive control was acarbose. (B) PTP1B inhibitory activity. The positive control was ursolic acid. (C) Tyrosinase inhibitory activity. The positive control was kojic acid. (D) DPPH free radical scavenging activity. The positive control was ascorbic acid. (E) ACE inhibitory activity. The positive control was captopril. (F) XO inhibitory activity. The positive control was allopurinol. Each experiment was performed in duplicate or triplicate. TNP, methanol extract of *Torreya nucifera* pericarp; TNS, methanol extract of *Torreya nucifera* seeds; PTP1B, Protein tyrosine phosphatase 1B; ACE, Angiotensin-converting enzyme; XO, xanthine oxidase. Data were presented as mean ± SD (n=3 in each group, *p<0.05, **p<0.01, ***p<0.001 compared to control).

1B). Tyrosinase inhibitory activity is to evaluate whitening ability by measuring dopachrome, a product by reacting with L-tyrosine and tyrosinase. At concentration of 150 μg/ml, TNS showed 3.6 times higher tyrosinase inhibitory activity than TNP (Fig. 1C). DPPH is a water-soluble material with chemically stabilized free radicals with maximum absorbance at 515 to 520 nm. When the DPPH free radical scavenging activity was compared, at concentration of 150 μg/ml, TNS was 67.8 times higher than TNP (Fig. 1D). These results suggested that TNS contains more antioxidant substances than TNP. Captopril is a synthetic angiotensin-converting enzyme (ACE) inhibitor, which is associated with oxidative stress and hypertension. At concentration of 20 μg/ml, TNS was 2.9 times higher inhibitory activity than TNP (Fig. 1E). The pterine (2-amino-4-hydroxypteridine) was used as a substrate of xanthine oxidase (XO), which is converted to phosphorylation of isoxanthopterin (2-amino-4,7-pterinediol). When comparing with TNS and TNP, XO inhibitory activity of TNP was 44.5 times higher than TNS (Fig. 1F), which was different from others results that TNS was higher than TNP. Taken together, it seemed that TNS has stronger antidiabetic activity on the base of the results from α-glucosidase and PTP1B inhibitory activities. Therefore TNS was selected as a candidate for the subsequent experiments to characterize its active compounds.

Extraction of active compounds from *Torreya nucifera* seeds (TNS)

To determine the optimal physiological extraction from *T. nucifera*, showing higher α-glucosidase inhibitory activity, extraction experiments were carried out under five conditions as described in section of materials and methods. As a result, methanol (MeOH) was more efficient to extract active compounds than hot water at high temperature, based on the observation of higher α-glucosidase activity in the MeOH extraction than HWE of *T. nucifera* seeds. HWME-1 showed as much inhibitory activity as ME without heating at room temperature (Fig. 2A). It was supposed that active compounds were extractable with MeOH not hot water and higher stable without heating and at lower-temperature if heated. Therefore MeOH extraction from TNS at room temperature was chosen as a best extraction process to yield active compounds with α-glucosidase inhibitory activity. On the other hand, organic solvent extraction process was performed to divide various components of TNS into groups of substances with similar polarities. After *n*-butanol (BuOH) extraction with 25 mg of TNS, the total solid of BuOH layer was 6 mg and water layer was 19 mg. As a result of examination of α-glucosidase activity with each layer, the water layer showed higher inhibitory ability than the BuOH layer (Fig. 2B).

Molecular weight fractionation of active compounds from *Torreya nucifera* seeds (TNS) extract

At first of preliminary experiment, the apparent molecular weight of active compound from the TNS extract was determined using centricon tubes, Amicon Ultra-0.5 Centrifugal Filter Unit with molecular weight cut-off of 30 kDa (Sigma-

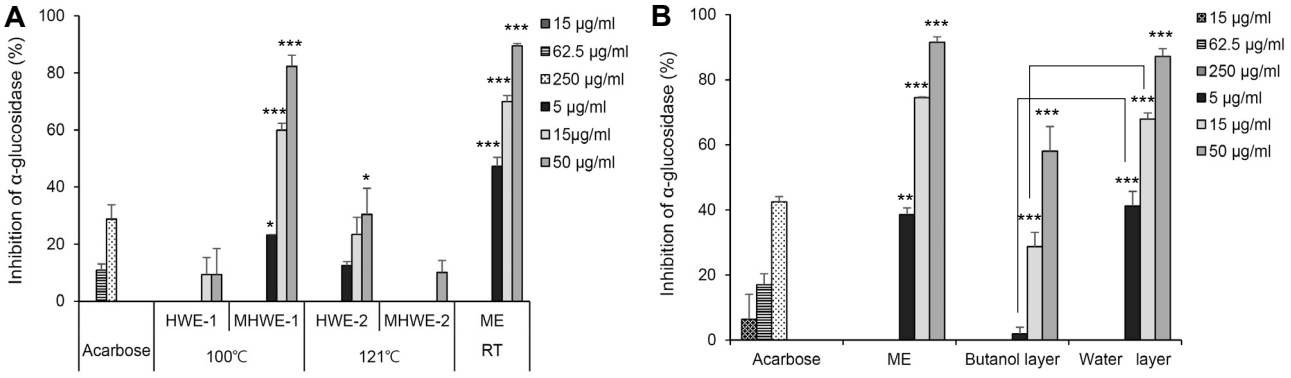

Fig. 2. Extraction conditions of active compounds from *Torreya nucifera* seeds (TNS). (A) Extraction with hot water or methanol. To confirm the maximum extraction conditions for the active substance of TNS, methanol extract of remaining precipitates (HWME-1) after heating at 100℃ for 10 minutes (HWE-1), methanol extract of remaining precipitates (HWME-2) after heating at 121℃ for 15 minutes (HWE-2), and methanol extract (ME) at room temperature (RT) were prepared and investigated to observe α-glucosidase inhibition activity. (B) Organic solvent extraction. Each sample was fractionated into butanol layer and water layer and their inhibitory activities were determined on α-glucosidase. Data were presented as mean ± SD (n=3 in each group, *p<0.05, **p<0.01, ***p<0.001 compared to control).

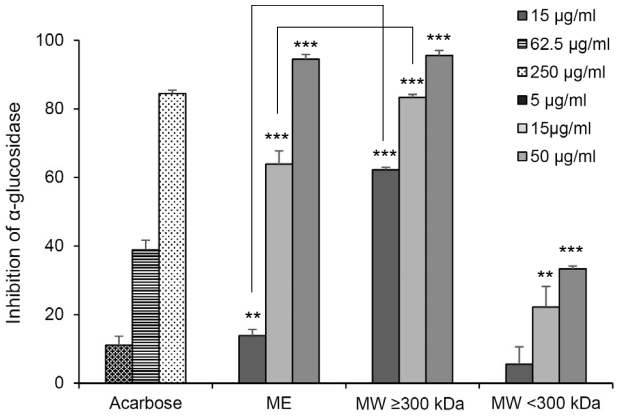

Fig. 3. Molecular weight fractionation of active compounds from *Torreya nucifera* seeds (TNS) extract. After ultrafiltration of TNS extract using 300 kDa molecular weight cut-off membrane, their fractions were assayed to observe α-glucosidase inhibition activity. Data were presented as mean ± SD (n=3 in each group, ***p*<0.01, ****p*<0.001 compared to control).

Aldrich). TNS extract was loaded and centrifuged for 45 minutes at 3,000 rpm using a centrifugal force. Filtrate and concentrate were assayed on α-glucosidase inhibitory activity. A large of inhibitory activity was detected in concentrated fraction, showing existing of active compounds. It seemed that active compounds from TNS extract belong to molecular weight of 30 kDa or more (data not shown). After a pretest using centricon tubes, larger volume of TNS extract was fractionated using the principle of osmotic pressure for ultrafiltration, depending on its molecular weight. Then 11 g of ME of TNS was loaded and finally 6.38 g of MW≥300 kDa fraction and 4.5 g of MW<300 kDa were separated to yield the products. As a result, fraction of molecular weight of 300 kDa or higher from TNS ME was most on α-glucosidase inhibitory activity (Fig. 3). And its specific activity was increased by 2~3 times, compared to original ME as loading sample. Finally the most active MW≥300 kDa fraction of TNS extract was used for further experiments to characterize its active compounds.

Heat and pH stability of active compounds from *Torreya nucifera* seeds (TNS) extract

Thermal stability and pH stability of MW≥300 kDa fraction from TNS extract were performed to determine whether active compounds were stable or unstable at those conditions. The fraction was adjusted to pH 2, 7, and 10, respectively, and heated at 100℃ for 10 minutes and 121℃ for 15 minutes, respectively. In the pH stability, there was no significant change of the α-glucosidase inhibitory activity, despite of pH variation (Fig. 4A). In the thermal stability, heating at 121℃ caused to more slight decrease of inhibitory activity than 100℃ (Fig. 4B). Taken together, it suggested that the active compounds of TNS were stable even under pH change but a little bit relatively unstable at higher temperature of 121℃.

Diaion HP-20 column chromatography of active compounds from *Torreya nucifera* seeds (TNS) extract

To determine chromatographic adsorption, 2 ml of TNS extract at 5 mg/ml was adjusted to pH 2 and pH 7 and loaded on Diaion HP-20 resin, respectively. Each adsorption resin was sequentially eluted with 2 ml of passing (fr.#1 ~fr.#2), 20% MeOH (fr.#1~fr.#4), 50% MeOH (fr.#1~fr.#4), and 100% MeOH (fr.#1~fr.#4). In the case of pH 2, active

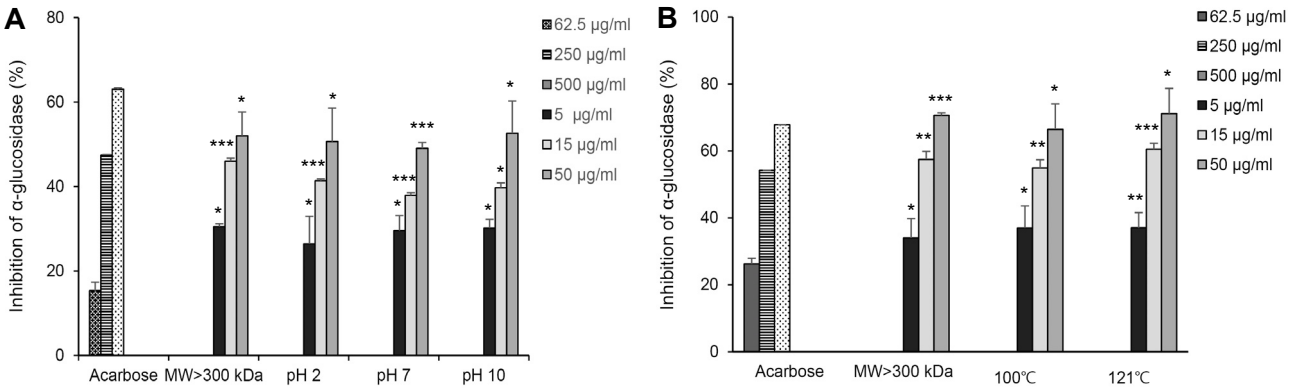

Fig. 4. Heat and pH stability of active compounds from *Torreya nucifera* seeds. (A) pH stability. (B) Heat stability. TNS extracts were adjusted to pH 2, 7, and 10 and heated at 100℃ for 10 minutes and 121℃ for 15 minutes, respectively. Each extract was assayed to observe remaining α-glucosidase inhibition activity. Data were presented as mean ± SD (n=3 in each group, ***p*<0.01, ****p*<0.001 compared to control).

fractions were eluted with 50~100% MeOH from Diaion HP-20 resin, appearing concentrated active compounds in a few fractions (Fig. 5A). However, in the case of pH 7, TNS extract was not well adsorbed on Diaion HP-20, resulting from broad dispersed distribution of active fractions (Fig. 5B).

Partial purification of active compounds from *Torreya nucifera* seeds (TNS) extract

According to previous adsorptional property of Diaion HP-20 chromatography, a larger of TNS extract at pH 2 was applied on Diaion HP-20 column to further separate active compounds. The concentrate of TNS ME was dissolved in water and ultrafiltrated to obtain active fraction having a molecular weight of 300 kDa or more. Then active fractions of higher molecular weight were subjected to Diaion HP-20 column chromatography at pH 2. The fractions eluted with 50~100% MeOH from Diaion HP-20 were determined their α-glucosidase inhibitory activity, yielding active compounds. Finally active fractions were collected to dry and named as HP-20 eluent (HPE) (Fig. 6A). As shown in Fig. 6B, HPE was increasing on α-glucosidase inhibitory activity at same concentration than previous original ME and ultrafiltration fractions, displaying increased specific activity (increased purity fold) of HPE to 9~10 times, based from starting ME. In *in vitro* PTP1B assay, HPE showed the same pattern of inhibitory activity with α-glucosidase at same concentrations (Fig. 6C), consistent with the result of PTP1B inhibitory activity of TNS in Fig. 1B.

Discussion

Diabetes has emerged as a major health threat worldwide and more than 90% of diabetic patients have type 2 diabetes (T2DM) [17]. Insulin is the most important biohormone that controls energy metabolism utilizing sugars, lipids, and proteins. It is also involved in growth and regulation of electrolytes. Insulin resistance (IR) can be defined as a metabolic state in which the insulin action (insulin sensitivity) is lower at physiological insulin concentration [5, 6, 14, 15]. Improving insulin resistance in the liver is believed to be an important strategy for T2DM treatment [13]. Due to the

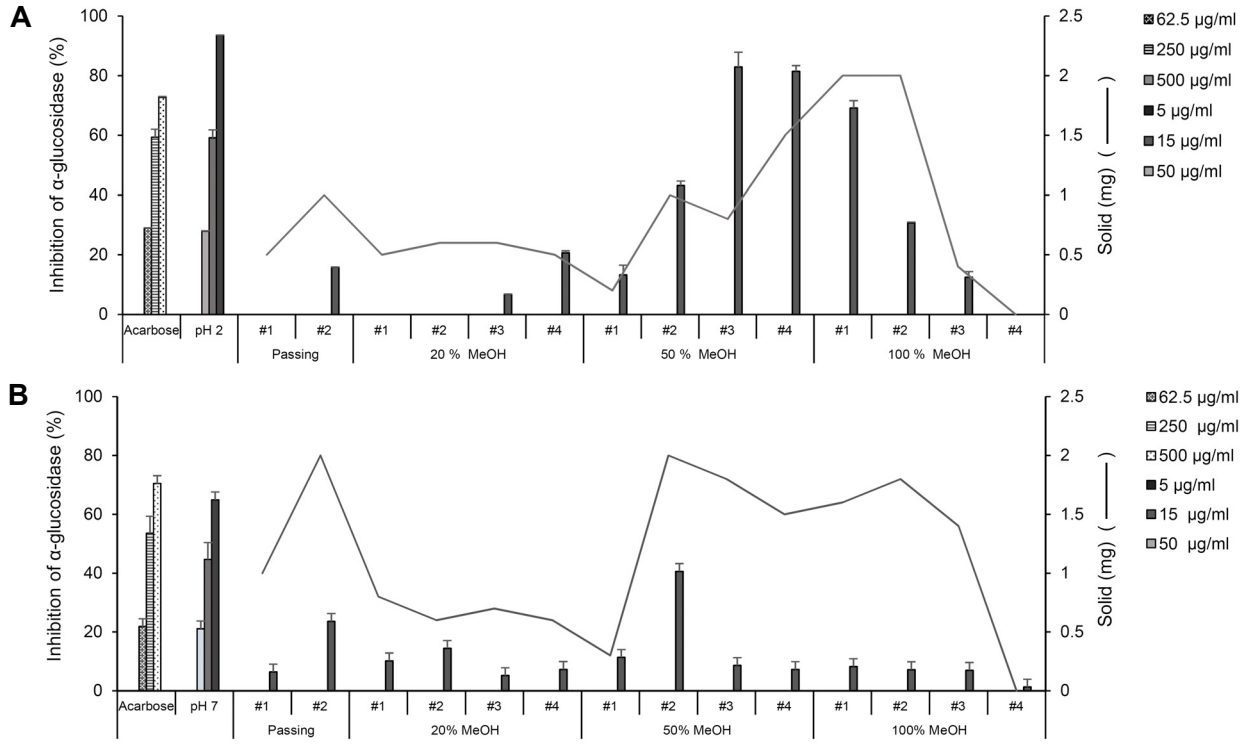

Fig. 5. Preliminary adsorption experiment on Diaion HP-20 resin of the *Torreya nucifera* seeds (TNS) extract. (A) Adsorption of TNS extract at pH 2. (B) Adsorption of TNS extract at pH 7. Passing fractions from Diaion HP-20 were collected in 2 ml of each fraction (fr.#1~fr.#2). In 20% methanol (MeOH), 50% MeOH, and 100% MeOH elution, each 2 ml was collected into 4 fractions (fr.#1~fr.#4). The main bar showed the α-glucosidase inhibitory ability and the line showed the total solid (mg) of each fraction.

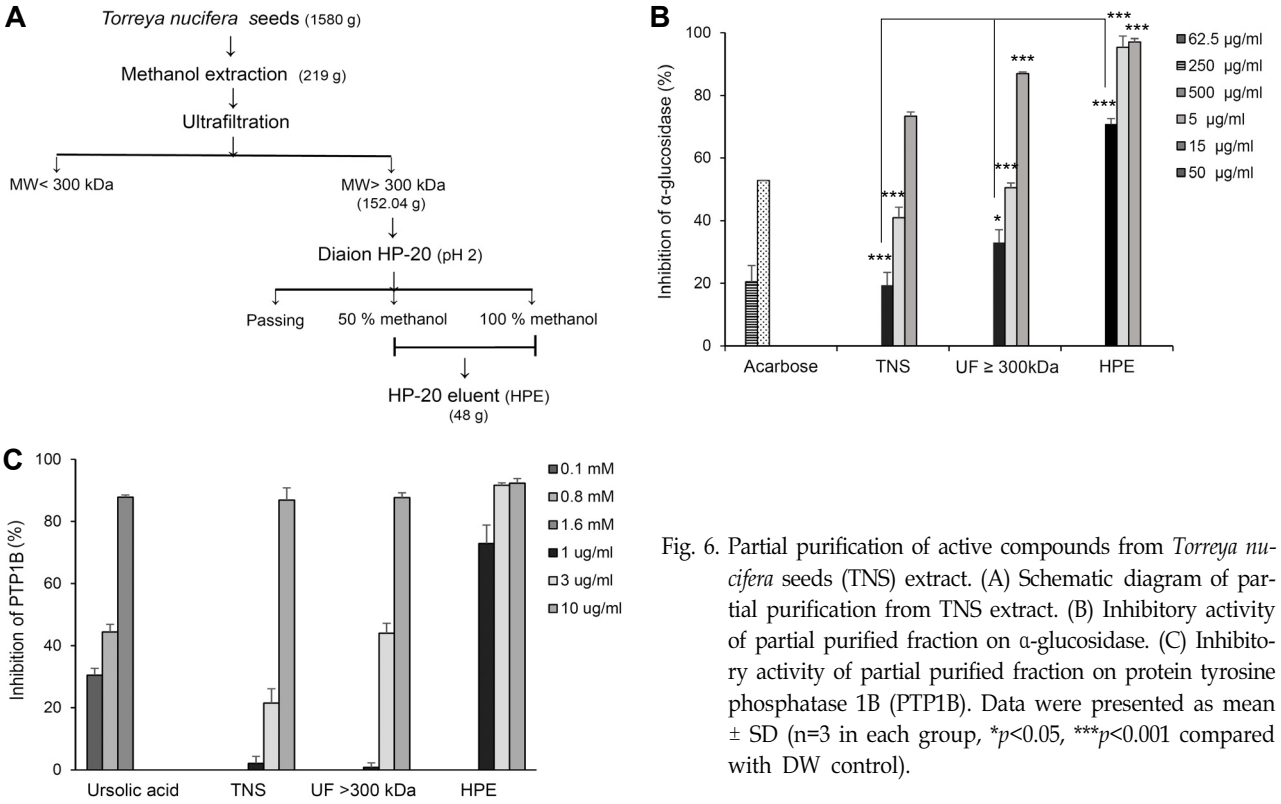

Fig. 6. Partial purification of active compounds from *Torreya nucifera* seeds (TNS) extract. (A) Schematic diagram of partial purification from TNS extract. (B) Inhibitory activity of partial purified fraction on α-glucosidase. (C) Inhibitory activity of partial purified fraction on protein tyrosine phosphatase 1B (PTP1B). Data were presented as mean ± SD (n=3 in each group, *p<0.05, ***p<0.001 compared with DW control).

limitations of current treatments, researchers have been finding new safer and more powerful antidiabetic drugs. Natural products and plants provide new insights into more efficient and safe drug development with low toxicity, because those have been used for a very long time.

Torreya nucifera seeds (TNS) have been used as edible and oriental ingredients but there have not been reported on antidiabetics of TNS extracts. In this study, active substances included in TNS were likely to be therapeutic agents that can improve diabetes, because its seeds showed significant α-glucosidase inhibitory activity (Fig. 1A) and protein tyrosine phosphatase 1B (PTP1B) inhibitory activity (Fig. 1B). TNS showed higher inhibitory activities than *T. nucifera* pericarps (TNP) and was selected to characterize its active compounds. For the optimal physiological extraction from *T. nucifera*, methanol (MeOH) was more efficient to extract active compounds (Fig. 2A). It was supposed that active compounds were significantly stable under conditions without heating and at lower-temperature if heated. In *n*-butanol (BuOH) extraction process, the water layer showed higher inhibitory ability than the BuOH layer (Fig. 2B), suggesting high polarity of active compounds. Also the apparent molecular weight of active compounds was determined to be 300 kDa or more by using the principle of osmotic pressure for ultrafiltration (Fig. 3). Stability of MW≥300 kDa fraction was performed at those thermal and pH conditions to determine whether active compounds were stable or unstable. There was no significant change of the α-glucosidase inhibitory activity, despite of pH variation (Fig. 4A) and heating at 121℃ caused to slight decrease of inhibitory activity (Fig. 4B). It seemed that the active compounds of TNS were stable even under pH change and higher temperature. Finally a larger concentrate of TNS methanol extract (ME) was ultrafiltrated to obtain a molecular weight of 300 kDa or more and the fractions were subjected to Diaion HP-20 column chromatography at pH 2, which was eluted with 50~100% MeOH. Active fractions were collected and named as HP-20 eluent (HPE) (Fig. 6A). HPE displayed increased specific inhibitory activity (increased purity fold) to 9~10 times compared to starting ME, based on its α-glucosidase inhibitory activity (Fig. 6B) and PTP1B inhibitory activity (Fig. 6C).

This suggested the possibility that Diaion HP-20 eluent (HPE) from *T. nucifera* seeds could treat type 2 diabetes by improving the insulin resistance signaling by regulating α-glucosidase and PTP1B activities. Therefore, by analyzing their antidiabetic activity in additional *in vivo* experiments using animal models in the future, active substances of TNS can be an appropriate functional food and supplemental

treatment in T2DM in various experimental aspects.

Acknowledgement

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 비자나무 추출물의 항당뇨 활성물질의 특성 연구

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비자나무는 한반도 남부지역과 제주도에 자생하고 있으며 식용으로 이용이 가능하며, 전통적으로 구충 빛 변비 예방의 목적으로 사용되어 왔었다. 그러나 항산화 활성에 대한 연구는 보고되어 왔지만 항당뇨 활성에 대한 연구는 제대로 이루어지지 않고 있다. 새로운 당뇨 치료제로서 천연물은 독성의 염려가 낮고 매우 오랫동안 사용되어 왔기 때문에 약물에 대한 안전성의 장점이 있다. 식용 및 한방 재료로 사용되고 있는 비자나무는 지금까지 살충 및 항균성에 대해 보고되어 왔으나 항당뇨에 관한 연구는 이루어진 바가 없다. 따라서 본 연구에서는 비자나무 추출물의 항당뇨 효과를 조사하고 그 특성을 조사하였다. 먼저, 비자나무의 과육과 종자를 메탄올로 각각 추출한 후, 항당뇨 활성과 관련된 α-glucosidase와 protein tyrosine phosphatase 1B (PTP1B)에 대한 저해활성을 포함한 다양한 생리활성을 조사하였다. 그 결과, 비자나무의 종자 추출물에서 항당뇨활성을 나타내는 α-glucosidase와 PTP1B 효소에 대한 억제 효능이 높게 나타났으며, 특히 과육에 비해 종자 추출물이 각각 14.5배, 4.3배 높은 저해활성을 보였다. 또한 비자나무의 과육 추출물의 pH와 열 안정성 테스트를 수행한 결과로, 활성 물질은 산, 알카리 조건과 고온의 조건에서 안정한 저해활성을 보였다. 비자 과육의 메탄올 추출물을 한외 여과(ultrafiltration)한 결과, 항당뇨 활성물질은 분자량이 300 kDa 이상에 해당하였고 Diaion HP-20 수지에 대한 흡착능을 조사한 결과, 50-100% 메탄올 조건에서 항당뇨 활성물질이 용출되었다. 따라서 비자나무 종자의 메탄올 추출물로부터 buthanol 추출, 한외 여과, Diaion HP-20 컬럼 크로마토그래피를 통해 비자나무의 항당뇨 활성 물질의 분리, 정제를 시도하였다. 따라서 비자나무 종자 추출물의 인슐린 저항성에 대한 개선 효과에 대한 추가 연구를 통해 천연물 유래 당뇨병 예방 및 치료용 조성물로서의 가능성을 보여주었다.