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비자 유박 추출물이 최종당화산물과 콜라겐 교차결합에 미치는 효과

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Effect of Defatted *Torreya nucifera* Seed Extract on the Cross-linking of Advanced Glycation End Products to Collagen

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요 약: 최종당화산물은 환원당과 단백질, 지질 또는 핵산 사이의 당화반응에 의해서 생성되는 물질로 이 물질은 피부 노화 과정에 밀접하게 연관되어 있다고 보고되어 있다. 본 연구에서는 항산화 활성, 최종당화산물 생성 저해, 콜라겐과 최종당화산물의 교차결합 저해 및 억제, 엘라스타제 활성 저해 효능 시험을 통해서 비자나무 유박 추출물의 항노화 효과를 평가하였다. 시험 결과, 비자나무 유박 추출물은 폴리페놀과 플라보노이드를 함유하고 있으며, DPPH와 ABTS 자유라디칼을 50% 소거할 수 있는 농도는 각각 16.4 μg (Dried materials, DM)/mL과 16.7 μg DM/mL 로 관찰되었다. 또한, 비자나무 유박 추출물은 농도 의존적으로 최종당화산물 생성과 엘라스타제 활성을 저해하였고, 콜라겐과 최종당화산물의 교차결합을 저해 및 억제하였다. 따라서 본 연구 결과를 바탕으로 비자나무 유박 추출물은 항노화 효능이 있는 화장품 소재로서 활용 가치가 있음을 확인하였다.

Abstract: Advanced glycation end products (AGEs) are final products formed by glycation reaction between reducing sugars and proteins, lipids or nucleic acids. These AGEs are related to progress of skin aging. In this study, we evaluate anti-aging activity of Defatted *Torreya nucifera* seed extract (DTSE) through antioxidant, anti-glycation, anti-elastase and inhibitory and breaking activity on the cross-linking of AGEs to collagen assay. Results showed that DTSE contained polyphenols and flavonoids. The IC_{50} values of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity were 16.4 μg (Dried materials, DM)/mL and 16.7 μg DM/mL, respectively. DTSE also inhibited the formation of AGEs, elastase activity and cross-linking of AGEs to collagen as well as broke existing cross-linking of AGEs to collagen in a dose-dependent manner. Consequently, our findings suggest that DTSE could be useful as a cosmetic material with anti-aging activity.

Keywords: *Torreya nucifera, AGEs, anti-aging, antioxidant, anti-glycation*

1. Introduction

Advanced glycation end products (AGEs) are final products generated by glycation, which is a non-enzymatic browning process between reducing sugars and proteins, lipids or nucleic acids[1]. AGEs have been implicated in the pathogenesis of many diseases such as diabetes, arteriosclerosis, age-related cardiovascular diseases, Alzheimer's disease and aging[2,3].

Also, AGEs are known to trigger skin aging through various mechanisms. AGEs are cross-linked with extracellular matrix

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(ECM) such as collagen and elastic fibers during aging[3]. This non-enzymatic cross-linking contributes to skin stiffening and abnormal structural changes of ECM, causing decrease in skin elasticity[5,6]. Also, AGEs induce oxidative stress and cellular senescence by interacting with their cellular receptor (RAGE). In our previous study, we confirmed that AGEs are causative of the generation of reactive oxygen species (ROS) in human dermal fibroblasts (HDFs)[7]. Thus, it is a strategic approach to develop cosmetic material against AGEs-derived skin aging, which is able to scavenge oxidative stress and inhibit formation of AGEs and cross-linking of AGEs to collagen as well as break existing cross-linking of AGEs to collagen[8].

There are already identified AGEs inhibitor and breaker such as aminoguanidine (AG) and alagebrium (ALT-711). However, the development of these agents was stopped due to the problem of safety and cost[9].

Torreya nucifera is an evergreen coniferous tree, mainly found in areas of Korea and Japan. It has known to exhibit pharmacological effects such as antioxidant, antiproliferative, neuroprotective and anthelmintic activities[10-13]. While most studies have focused on various activities of its fruits, leaves and seeds, there are no studies about its defatted seed cakes. Therefore, the aim of this study was to investigate biological activities of defatted seed cakes of *T. nucifera* and to demonstrate its value of use in the cosmetic industry.

2. Materials and Methods

2.1. Materials

The seeds of *T. nucifera* were purchased from Jeju island (Korea). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, ethanol, bovine serum albumin (BSA), glycolaldehyde, tetramethylbenzidine (TMB) substrate and sulfuric acid (H_2SO_4) were purchased from Sigma (USA). Phosphate-buffered saline (PBS) was purchased form Welgene (Korea).

2.2. Preparation of Defatted *Torreya nucifera* seed cake extracts

The seeds of *T. nucifera* were pressed to remove oil and dried under the shade to pulverize into fine powders. 200 g of powders were extracted with 2 L of 70% ethanol by ultrasound sonication for 48 h. After filtering supernatants, the residues were evaporated to remove the ethanol by a rotary vacuum evaporator and freeze-dried to obtain final extracts of Defatted *Torreya nucifera* seed cakes (DTSE).

2.3. Determination of Antioxidant Activity

Total polyphenol contents

The content of total polyphenol was measured by the modified previous method[14]. The absorbance was read at 725 nm by a Gen5^{TM} UV-Vis spectrophotometer (BioTek, USA). The contents of total polyphenol were expressed as gallic acid equivalents (GAE). DM indicates dried materials.

Total flavonoid contents

The content of total flavonoid was measured by the modified previous method[15]. The absorbance was read at 420 nm by a spectrophotometer. The contents of flavonoid were expressed as rutin equivalents (RE).

DPPH radical scavenging activity

DPPH radical scavenging activity was measured by the modified previous method[16]. The scavenging activity was calculated by the following formula, % scavenging activity = $[(A_{control} - A_{sample})/A_{control} \times 100]$. $A_{control}$ and A_{sample} indicate the absorbance of only DPPH reagent and DTSE-treated groups, respectively. SC_{50} refer to the concentration of DTSE required to reduce 50% of DPPH and ABTS radicals. L-ascorbic acid was used as a positive control.

ABTS radical scavenging activity

ABTS radical scavenging activity was measured by the modified previous method[17]. It was calculated by the same method as the DPPH radical scavenging activity.

2.4. Anti-glycation assay

Anti-glycation activity was determined by the modified previous method[18]. Briefly, 10 mg/mL BSA was incubated with 10 mM glycolaldehyde at 37 ℃ with or without DTSE.

Table 1. Determination of total polyphenol, flavonoid contents and free radical scavenging activities of Defatted *Torreya nucifera* seed extract (DTSE). The contents of total polyphenol and flavonoid contents were expressed as gallic acid equivalents (GAE) and rutin equivalents (RE). DM indicates dried materials. The results are mean ± standard deviation (SD) (n=3)

	Total polyphenol contents (μg GAE/mg DM)	Total flavonoid contents (μg RE/mg DM)	DPPH · SC_{50} (μg DM/mL)	ABTS · SC_{50} (μg DM/mL)
DTSE	264.8 ± 0.5	5.6 ± 0.2	17.8 ± 1.8	16.7 ± 0.4
L-ascorbic acid	-	-	8.7 ± 0.8	10.1 ± 0.1

After incubation, fluorescence intensity was measured at 340 nm excitation and 430 nm emission by a Infinite F200 PRO (Tecan, Switzerland). Aminoguanidine (AG) was used as a positive control.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Inhibitory and breaking activity of DTSE on the cross-linking of AGEs to collagen were determined by the modified previous method[19]. Briefly, AGEs labeled with horseradish peroxidase (AGEs-HRP) were prepared using a peroxidase labeling kit-NH_2 Unit (Dojindo, Japan). To measure inhibitory activity on the cross-linking, AGEs-HRP were incubated in collagen-coated 96 well plate with or without DTSE. After washing with PBS containing 0.05% Tween 20 (PBST), AGEs cross-linked with collagen was detected using TMB substrate. This reaction was stopped by adding 1 N H_2SO_4. The absorbance was read at 450 nm by a spectrophotometer. In the case of breaking activity on the cross-linking, AGEs-HRP were incubated in collagen-coated 96 well plate. After washing with PBST, DTSE was treated. The subsequent procedures to detect AGEs cross-linked with collagen were equal as above.

2.6. Anti-elastase activity assay

Anti-elastase activity was measured by EnzChek Elastase Assay Kit (Thermo Scientific, USA) according to the manufacturer's instructions. N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (PC) was used as a positive control.

2.7. Statistical analysis

Statistical significance of data was determined by a Student's *t*-test. All results were expressed as the means ± standard deviation (n = 3). * $p < 0.05$ and ** $p < 0.01$ were considered to be significant.

3. Results and Discussion

3.1. Total polyphenol, total flavonoid contents and antioxidant activity of DTSE

AGEs are related to increased production of free radicals[20]. These free radicals such as reactive oxygen species (ROS) play a major role in the process of skin aging by causing deoxyribo nucleic acid (DNA) damage, the generation of matrix metalloproteinases (MMPs) and the expression of inflammatory genes[21]. Therefore, it is important to find agents with antioxidant activity because it could prevent skin aging by inhibiting formation of AGEs and oxidative stress. In a previous study, it was reported that green tea shows strong anti-glycation activity in addition to antioxidant activity[22]. We measured total polyphenol, flavonoid contents and free radical scavenging activity to confirm the antioxidant activity of DTSE. As shown in Table 1, total polyphenol and total flavonoid contents of DTSE was 264.8 ± 0.5 μg GAE/mg DM and 5.6 ± 0.2 μg RE/mg DM, respectively. Also, DTSE scavenged 50% of DPPH and ABTS radicals at the concentration of 16.4 ± 1.7 μg DM/mL and 16.7 ± 0.4 μg DM/mL, respectively. It was low antioxidant activity, compared with ascorbic acid used as positive control. However, DTSE could be considered an agent with strong antioxidant activity when it is considered a crude extract, which has not undergone the purified process.

3.2. Anti-glycation activity of DTSE

Glycation is a reaction between reducing sugar and proteins. This reaction lead to formation of AGEs that trigger pathogenic signalling pathways and cross-link extracellular

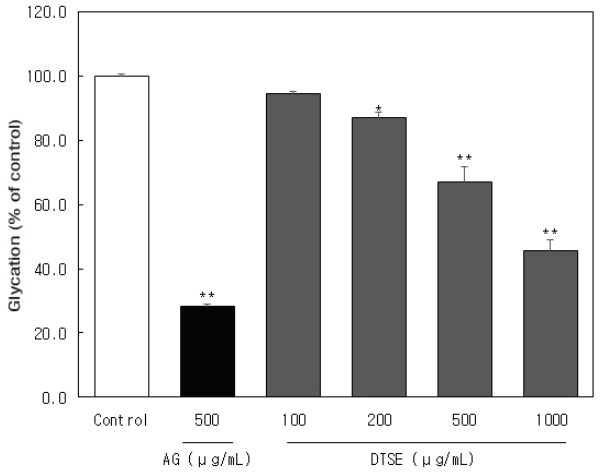

Figure 1. Anti-glycation activity of Defatted *Torreya nucifera* seed extract (DTSE). Aminoguanidin (AG) was used as a positive control. The results are mean ± standard deviation (SD) (n = 3). * $p < 0.05$ and ** $p < 0.01$ vs. Control.

matrix proteins[23]. Especially, accumulation of AGEs in dermal tissue not only lead to oxidative stress in the skin, but also turns skin colors yellow, which is not visually appealing[7]. In this study, we performed anti-glycation assay to confirm inhibitory activity of DTSE on formation of AGEs. As shown in Figure 1, DTSE inhibited formation of AGEs by 5.5, 12.9, 32.9 and 54.3% at concentrations of 100, 200, 500 and 1000 μg/mL, respectively. On the other hand, 500 μg/mL of AG inhibited formation of AGEs by 71.6%. AG is a representative positive control that could prevent formation of AGEs as well as cross-linking of AGEs to collagen. However, clinical application of AG is limited by reason of its side effects such as gastrointestinal problems, anaemia and hepatotoxicity[20,23]. There is a great demand for alternative candidate of AG, which can show less toxicity. DTSE can be considered as an alternative candidate of AG but need to be confirmed its safety in various condition.

3.3. Inhibitory and breaking activity of DTSE on the cross-linking of AGEs to collagen

Collagen, a major component of human skin, maintains skin elasticity in combination with elastin. However, accumulation of AGEs in the skin could induce loss of skin elasticity by cross-linking with collagen[24]. Previous study reported that non-enzymatic cross-linking of skin collagen via AGEs contributed to age-related skin stiffening[3]. Therefore, ability to inhibit or break the cross-linking of AGEs to collagen is important activity as an anti-aging agent. ALT-711 (4,5-dimethyl-3-phenacylthiazolium chloride), known as a drug candidate developed for AGE-breaker, has enzymatic characteristics and breaks the covalent bonds formed in cross-linked proteins. According to a previous study, application of ALT-711 increases skin elasticity of aged rats[3]. In this study, we confirmed inhibitory and breaking activity of DTSE on the cross-linking of AGEs to collagen by

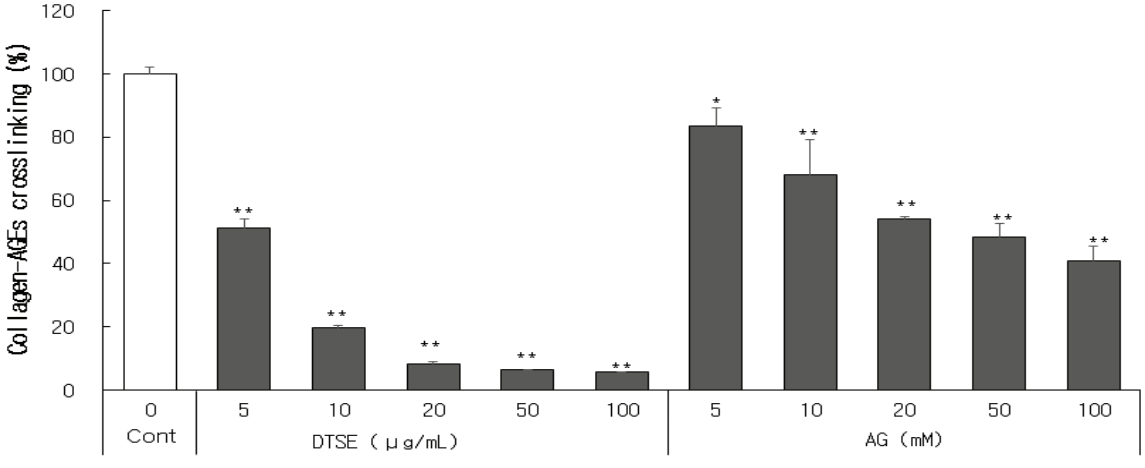

Figure 2. Inhibitory activity of Defatted *Torreya nucifera* seed extract (DTSE) on the collagen-AGEs cross-linking. Aminoguanidin (AG) was used as a positive control. The results are mean ± standard deviation (SD) (n = 3). * $p < 0.05$ and ** $p < 0.01$ vs. Control.

ELISA. Inhibitory activity of DTSE on the cross-linking was shown in Figure 2. As a result, DTSE prevented the cross-linking of AGEs to collagen by 48.6. 80.3, 91.6 and 93.7% at concentrations of 5, 10, 20 and 50 μg/mL, respectively. On the other hand, AG inhibited the cross-linking of AGEs to collagen by 16.7, 31.9, 45.7 and 51.6% at concentrations of 5, 10, 20 and 50 mM, respectively. Breaking activity of DTSE on the cross-linking of AGEs to collagen was shown in Figure 3. As a result, DTSE broke the cross-linking of AGEs to collagen by 22.1, 30.8, 48.5 and 85.9% at concentrations of 5, 10, 20 and 50 μg/mL, while ALT-711 reduced the cross-linking of AGEs to collagen by 27.2, 37.3, 56.0 and 67.4% at concentrations of 1, 2, 5 and 10 mM, respectively. It was reported that some extracts such as *Cornus officinalis* and *Trapa bispinosa* Roxb. have the activity as an inhibitor or breaker of cross-linking AGEs to collagen[25,26]. DTSE showed similar inhibitory effect on the cross-linking of AGEs to collagen. Taken together, it is expected that DTSE will be effective in skin aging as an inhibitor and breaker of collagen-AGEs cross-linking.

3.4. Inhibitory effect of DTSE on the elastase activity

Elastin is a major component of elastic fibers, which are responsible for elasticity of skin. However, elastin is degraded by elastase, which is degrading enzyme, leading to skin aging[27,28]. For this reason, inhibitor of elastase activity could prevent loss of skin elasticity. As shown in Figure 4, DTSE had an inhibitory effect on the elastase activity in a dose-dependent manner. DTSE inhibited elastase activity by 6.4, 20.3, 38.3 and 86.1% at concentrations of 5, 10, 20 and

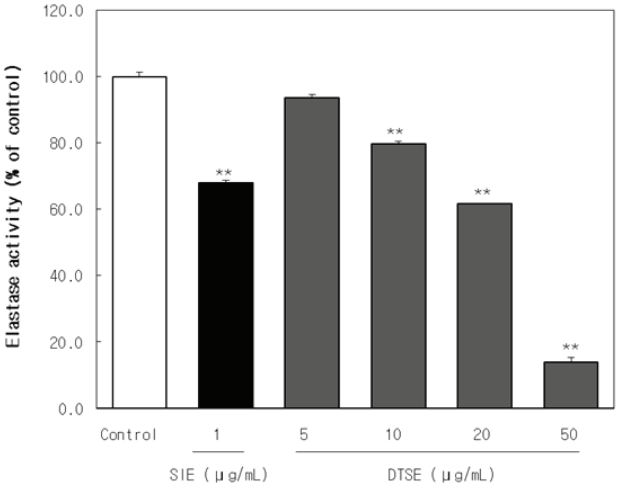

Figure 4. Inhibitory effect of Defatted *Torreya nucifera* seed extract (DTSE) on the elastase activity. Selective inhibitor of elastase (SIE), N-methoxysuccinyl-Ala-Ala-Pro-Val -chloromethyl ketone, was used as a positive control. The results are mean ± standard deviation (SD) (n = 3). ** $p < 0.01$ vs. Control.

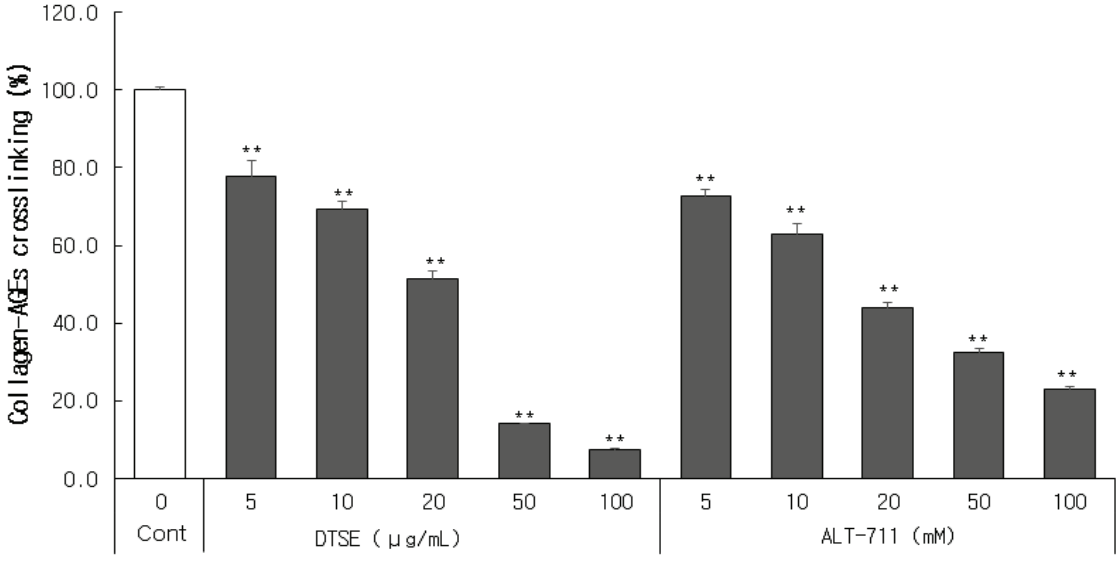

Figure 3. Breaking activity of Defatted *Torreya nucifera* seed extract (DTSE) on the collagen-AGEs cross-linking. ALT-711 was used as a positive control. The results are mean ± standard deviation (SD) (n = 3). ** $p < 0.01$ vs. Control.

50 μg/mL, while 1 μg/mL of PC used as a positive control had inhibited by 32.0%. These result s indicate that DTSE could be used as cosmetic material capable of inhibiting loss of skin elasticity.

4. Conclusion

In this study, we demonstrated that DTSE had anti-aging activity through various *in vitro* assay. DTSE had significant amount of polyphenol and flavonoid as well as strong free radical scavenging activity and showed anti-glycation activity, anti-elastase activity as well as inhibitory and breaking activity on the cross-linking of AGEs to collagen. In addition, DTSE is easy to acquire and affordable as the wastes of seed after removing oil. Consequently, these results suggest that DTSE could be used as an attractive cosmetic material for improving skin aging.

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