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Isolation of Hyaluronidase Inhibitory Component from the Roots of *Astraglus membranaceus* Bunge (Astragali Radix)

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Abstract In order to isolate hyaluronidase (HAase) inhibitor from Astragali radix (AR), dried roots were extracted with ethanol, prior to sequential fractionations with n-hexane, chloroform, ethyl acetate, n-butanol, and aqueous fractions. The n-butanol soluble fraction was found to exhibit the most pronounced inhibitory effect (68%) on HAase, and the active components were separated using various chromatographic methods, including column chromatography and preparative HPLC. The active component was isolated from the n-butanol soluble fraction of AR and was structurally identified as calycosin-7-*O*-β-D-glucopyranoside by LC-MS, IR, ^{1}H NMR, and ^{13}C NMR analysis. The IC_{50} of calycosin-7-*O*-β-D-glucopyranoside's HAase activity was found to be 3.7 mg/mL.

Keywords: *Astragalus membranaceus* Bunge; calycosin-7-*O*-β-D-glucopyronoside; hyaluronidase inhibitor.

Introduction

Astragali radix (AR) is the root of *Astragalus membranaceus* Bunge, an herbaceous plant which belongs to the family, Leguminosae. AR has been used as an anti-perspirant, a diuretic, and a tonic in the traditional medicine protocols of many Asian countries. Studies regarding AR in pharmacology and clinical practice have demonstrated its immunostimulatory, cardiotonic, antioxidative, and anti-inflammatory properties (1, 2). This herbal drug is reported to contain triterpenoid glycosides and flavonoids, including astraisoflavans, formononetin, isorhamnetin, quercetin, and kaempferol. Flavonoids, in particular, have been established to be one of the more potent beneficial components present in AR, due to their various biological effects (3, 4).

Hyaluronidase (HAase) is a mucopolysaccharide-splitting enzyme, which hydrolyses the β-N-acetyl-D-glucosamine [1-4] glycosidic bonds in hyaluronic acid (HA), chondroitin and chondroitin sulfates (5). Depending on the mechanism of cleavage of HA, HAase is usually classified into three main types: hyaluronoglucosaminidase (E.C. 3.2.1.35, testicular, lysosomal and venom HAase), hyaluronoglucuronidase (E.C. 3.2.1.36, leech HAase), and HA lyase (E.C. 4.2.2.1, bacterial HAase) (6). HAase generally exists in an inactive form within sub-cellular lysosomes, and is released in an active form during certain experimental tissue injuries and human diseases, including rheumatoid arthritis. Therefore, HAase has also been reported to play an important role in mediating inflammatory response (7-9).

We have previously reported 5 herbal medicines, which, amongst 42 tested traditional herbal medicines, exhibited most profound HAase inhibitory activity (10). Thus, we have been attempting to identify the active components in such herbal medicines by analyzing the ethanol extracts from AR. In the present study, we describe the isolation and identification of the active compound with HAase inhibitory activity in the ethanol extract of AR, by using the Morgan-Elson method. The characterization of active components in AR can be useful in the development of natural anti-allergic and anti-inflammatory agents.

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Materials and Methods

Chemicals and apparatus Sodium HA (from human umbilical cord) and HAase (from bovine testes, Type I-S, 608 unit/mg), *p*-dimethyl aminobenzaldehyde, potassium tetraborate, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, USA). CD_3OD was obtained from CIL Co. (Andover, USA). Column chromatography was carried out using silica gel 60 (Merck Co., Darmstadt, Germany) and C_{18} (Alltech Co., Deerfield, USA). Other reagents, and all organic solvents used in this study, were of analytical grade. Recycling preparative HPLC was performed using a JAI LC-908 (JAI Co., Tokyo, Japan) with a JAIGEL GS 310 column (5 μm, 20 × 500 mm I.D), in-line degassor CASTORR-152 Double plunger type L-7100 pump, UV detector, RI detector, and Millennium 32 software. The analytical HPLC system used was Acme HPLC (Younglin Co., Seoul, Korea) with a Hypersil ODS column (reverse-phase type, 5 μ, 4.6 × 250 mm I.D, Thermo Co., San Jose, USA) and with a UV detector. IR spectra were recorded using a KBr disc with an FT-IR Spectrum 2000 instrument (Perkin-Elmer Co., Boston, USA). Mass spectra were obtained using LC-MS/MS (HP-1100 High performance Liquid Chromatography and a QUATTRO LC Triple Quadrupole Tandem Mass Spectrometer, Waters Ltd., Massachusetts, USA). NMR spectra were recorded on a Varian Unity INOVA400 NMR spectrometer (Varian, Inc., Palo Alto, USA) in $CDCl_3$.

Extraction and isolation of active component The dried AR was purchased from the Kyung-dong herbal drug store (Korea). Fifteen kg of dried AR was crushed and extracted

four times (24 h) with 95% ethanol at room temperature, using a shaking incubator. The filtered supernatant was evaporated in order to construct an ethanol AR extract under vacuum at 50°C. The crude ethanol extract (267 g) was suspended in water, and successively partitioned with organic solvents of different polarities, with the resultant production of *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and aqueous fractions. The fractionates were concentrated by evaporation or desiccation, yielding *n*-hexane residue (46.1 g), chloroform residue (50.2 g), ethyl acetate residue (15.8 g), and *n*-butanol residue (11.4 g), respectively.

The *n*-butanol soluble fraction (AR-Bu, 3.8 g) was subjected to column chromatography on silica gel, using a step-wise gradient solvent system of ethyl acetate, which contained increasing amounts of methanol (8:2, 6:4, 4:6, 2:8 and 0:1, 800 mL each), in order to yield six subfractions (AB-1, 2, 3, 4, 5 and 6, 240 mL each). Subfraction AB-4 (1.3 g) was subjected to re-column chromatography on C_{18} (Alltech Co., Deerfield, USA), and eluted with water which contained increasing amounts of methanol (10: 1, 5: 1, 1: 1 and 0: 1), in order to yield four subfractions (AB-4A, B, C and D). Subfraction AB-4C (148 mg) was further purified by preparative HPLC (JAI LC-908) with a JAIGEL GS 310 column (5 μm, 20 × 500 mm I.D), and eluted with 85% methanol to yield five compounds (AB-4C-I, CII, CIII, CIV and CV). The active component, AB-4C-IV, obtained from subfraction AB-4C (148 mg), was analyzed by HPLC with a Hypersil ODS column (5 μm, 4.6 × 250 mm I.D). The mobile phase contained 85% methanol, and was eluted at a flow rate of 0.5 mL/min. The effluent was monitored by a wavelength absorbance detector. Finally, active peak components were identified by their LC-MS/MS, IR, ^{1}H NMR and ^{13}C NMR spectra. Each sample was prepared by either evaporation or lyophilization in order to remove the residual solvents, and the final concentration of test samples was maintained as 10 mg/mL (in 5% DMSO) for the screening assay.

Determination of HAase inhibitory activity by Morgan-Elson assay HAase activity was determined by two different methods *in vitro*. First, the HAase inhibitory effect was determined by measuring the amount of *N*-acetylglucosamine formed from the sodium HA, according to the Morgan-Elson method (11). 50 μL of HAase (7,900 unit/mL) was mixed with 100 μL of the test samples, and then pre-incubated for 20 min at 37°C. The control group was treated with 100 μL of 0.1M acetate buffer (pH 3.5, 5 % DMSO) instead of with the test sample. The optical density at 585 nm was measured by a spectrophotometer. Percentage of inhibition was calculated as follows; Inhibition (%) = $[(OD_c\text{-}OD_s)/OD_c] \times 100$, where OD_c : the O.D of the control at 585 nm; OD_s : the O.D of the sample at 585 nm. The inhibitory ratio was plotted against the log concentration, and the dose response data were used to calculate the IC_{50}.

Determination of HAase inhibitory activity by microplate assay The HA activity was determined by microplate assay, according to the method previously described by Richman *et al.* (12). In brief, a plate consisting of 1 mg/mL HA dispersed in 1.5% (w/v) agarose gel was buffered with 0.05 M sodium citrate (pH 5.3, buffer A) containing 0.15 M NaCl and 0.02% (w/v) sodium azide. A stock solution of 3% agarose in buffer A was stored in 9.5 mL aliquots. A stock solution of 2 mg/mL HA in buffer A was prepared by stirring the powder into the buffer overnight at 4°C. A 9.5 mL aliquot of 3% agarose was melted in a boiling-water bath, cooled to 60°C, and added to 9.5 mL of 2 mg/mL HA in a 60°C bath with constant stirring. The solution was stirred for approximately 1 min in order to ensure complete mixing, and was then rapidly poured into a 9 × 9 cm plastic dish on a leveled surface. After the setting of the gel, cylindrical holes (2 mm in diameter) were punched into the plate and emptied by suction. 5 μL of bovine HAase (5,000 unit/mL) was mixed with 5 μL of the test samples (10 mg/mL), and then preincubated for 20 min at 37°C. The holes were filled with each treatment group, and then incubated for 20 h at 37°C. After incubation, the gel was covered with 10% (w/v in water) cetylpyridinium chloride as soon as the circles became distinct, and the diameters were measured using calipers and read against a dark background. The mean values for duplicate determinations of the zone diameter (in millimeters) were then plotted against the logarithm of the HAase activities.

Statistical analysis All results were expressed as the % mean ± standard deviation (S.D). IC_{50} values were calculated by linear regression analysis.

Results and Discussion

We have investigated the inhibitory effect of the solvent fractions isolated from AR ethanol extracts on the HAase activity (Table 1). Amongst the solvent fractions, the ethyl acetate and *n*-butanol soluble fractions exhibited significant activities, as 52.5 ± 0.6% (IC_{50} = 9.4 mg/mL) and 68.4 ± 0.1% (IC_{50} = 5.6 mg/mL) at 10 mg/mL of the concentration, respectively. It has been established that the inhibition of HAase is one of the possible mechanisms underlying anti-inflammatory and anti-allergy activities. Therefore, the *n*-butanol soluble fraction was further tested in order to isolate active compounds with HAase inhibitory activity.

The active compound was isolated using various chromatographic methods, as described in the Materials and Methods section. Our results suggested that the active fraction (AB-4C) harbored several compounds, including AB-4C-IV and some other unknown compounds also (Fig. 1). The strongest active component, AB-4C-IV, was isolated, and the purity of the compound was confirmed via analytical HPLC. We obtained an AB-4C-IV yield of 15 mg (0.0003% in root). The product was white in color, and had the most profound HAase inhibitory effect of all compounds tested (Table 2). The structural identification of AB-4C-IV was carried out by analysis of the MS, IR, ^{1}H NMR, and ^{13}C NMR spectra. The MS data are as follows; The ES-MS spectrum showed an M^++H at m/z 447 for the molecular formula $C_{22}H_{22}O_{10}$, and the MS/MS spectrum showed a 285 m/z (M^++H) (Fig. 2). The IR spectrum (Fig. 3) indicated the presence of a carbonyl group with strong absorption at 1730 cm^{-1} and a broad peak at 3422 cm^{-1} due to the presence of a hydroxyl group, and a strong peak at 1286 cm^{-1} was determined to be an

Table 1. HAase inhibitory effects of the fractions in AR

Sample fractions	Morgan-Elson assay	Microplate assay	IC_{50} (mg/mL)
	Inhibition (%)	Clear zone (mm)	
n-hexane fr.	3.5 ± 0.1	22	
Chloroform fr.	6.4 ± 0.1	22	
Ethyl acetate fr.	52.5 ± 0.6	7	9.4
n-butanol fr.*** [a]	68.4 ± 0.1	ND	5.6
Water fr.	0.5 ± 0.1	22	
AB-1	ND[b]	22	
AB-2	ND	22	
AB-3	ND	22	
AB-4 ***	67.1 ± 0.1	ND	6.2
AB-5	12.3 ± 0.1	18	
AB-6	7.4 ± 0.2	22	
AB-4A	ND	22	
AB-4B	ND	22	
AB-4C ***	70.2 ± 0.4	ND	3.7
AB-4D	16.8 ± 0.1	16	
HAase		22	

Data represents the means ± S.D.(N=3). The test concentration of each fraction was 10 mg/mL. HAase (from Bovine testicular) was treated with concentration of 7,900 unit/mL and 5,000 unit/mL, respectively.
[a]*** Active fractions
[b]ND : not detected

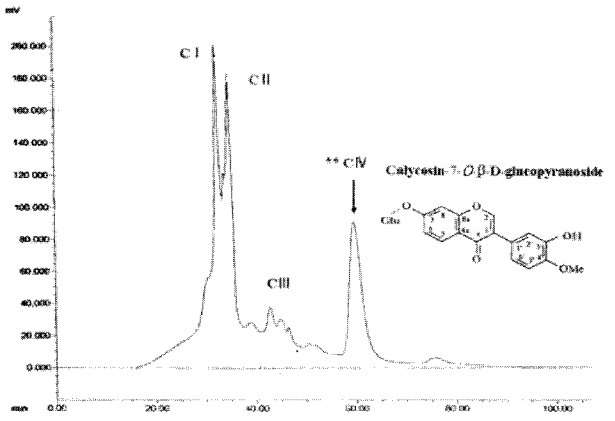

Fig. 1. HPLC chromatogram of AB-4C fraction in AR obtained using recycling preparative HPLC (JAIGEL GS column, 20 × 500 mm) by monitoring the eluent at 254 nm. Methanol (86%) was used as an eluent in isocratic mode with a flow rate of 3 mL/min. **Active component

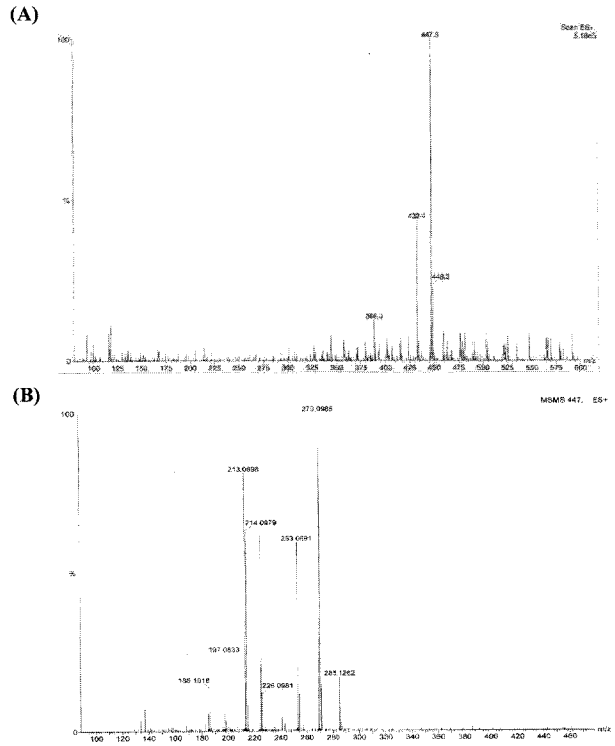

Fig. 2. ESI-MS (A) and ESI-MS/MS (B) spectrum of the active compound (AB-4C-IV) of AR. The spectrum was recorded on LC-MS/MS (HP-1100 High Performance Liquid Chromatography, QUATTRO LC Triple Quadrupole Tandem Mass Spectrometer). The ion source was ESI positive ion mode.

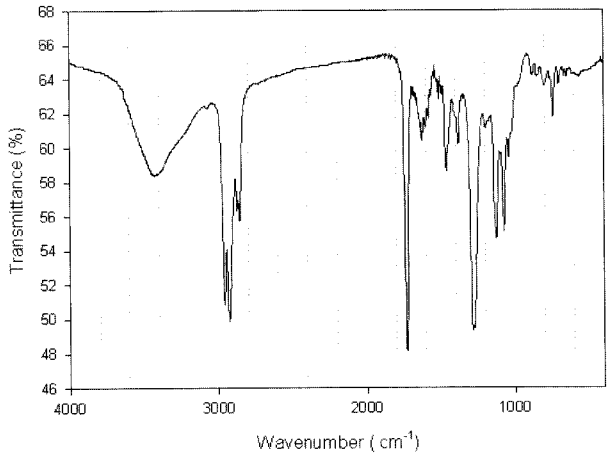

Fig. 3. FT-IR Chromatogram of the active compound (AB-4C-IV) of AR. The IR spectra was recorded on spectrum 2000 explore.

ester. The aromatic ring was shown by the presence of a signal in the 1380-1624 cm^{-1} spectrum. The ^{1}H NMR spectrum (Fig. 4) of the active compound manifested 7 proton signals at 7-8.5 ppm of the aromatic region. Two proton signals exhibited singlets at 8.232 ppm (H-2) and 7.011 ppm (H-2′). Anomeric proton signals were detected at 5.139 ppm, and the proton signal of methoxyl (OMe) resonated as a singlet, at 3.909 ppm. The ^{13}C NMR spectrum showed the signal of an oxygenated aromatic ring at 120-140 ppm. The active component, AB-4C-IV, was identified as calycosin-7-*O*-β-D-glucopyranoside by comparing its spectroscopic data with the literature (13, 14); however, this study is the first to assess the HAase inhibitory effect of this compound. This component exhibited high inhibitory activity towards HAase (Table 2). At a concentration of 10 mg/mL, the component was measured to have an inhibitory ability of 70.0 ± 0.8% (IC_{50} =3.7 mg/mL). However, further research should be carried out to increase the yield by optimal extraction process, because the yield of active component is too low.

Traditional herbal medicines have long been used in the

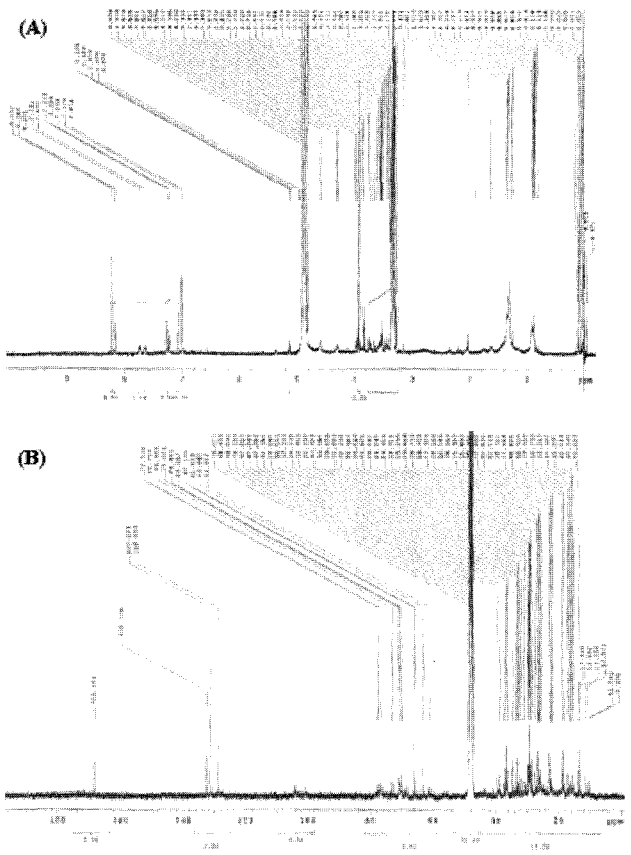

Fig. 4. ^{1}H NMR (A) and ^{13}C NMR (B) spectrum of active compound (AB-4C-IV) of AR. Sample was dissolved in MeOD (methy-*d3* alcohol-*d1*) and was recorded on high resolution NMR spectrometer (Varian Unity INOVA400 operating at 400 MHz and 25°C).

Table 2. HAase inhibitory effects of several peaks isolated from AB-4C by preparative HPLC

Sample	Compound Name	Inhibitory activity (%)	IC_{50} (mg/mL)
AB-4C-I	Unknown	26.7 ± 0.2	
AB-4C-II	Unknown	42.1 ± 0.5	
AB-4C-III	Unknown	23.3 ± 0.3	
AB-4C-IV*	Calycosin-7-*O*-β-D-glycoside	70.0 ± 0.8	3.7
AB-4C-V*	Unknown	50.1 ± 0.2	

Data represents the mean ± S.D.(N=3). The test concentration of each fraction was 10 mg/mL. Results are shown as mean ± S.D (n=3)
*Active fractions

prevention or cure of a host of diseases, and most of them contain compounds which exert various physiological effects. These compounds, in fact, have been demonstrated to possess antioxidative, anti-inflammatory, antiaging, and antimicrobial capacities. Some studies have also reported the isolation of two isoflavone glycosides, 7, 2'-dihydroxy-3', 4'-dimethoxyisoflavan-7-*O*-β-D-glucoside, and calycosin-7-*O*-β-D-glucopyranoside from AR. These components exhibited profound inhibitory effects on COX-2, an enzyme implicated in inflammatory reactions (2). *Moutan Radicis* Cortex and *Uncariae Ramulus et Uncus* also exhibited profound inhibitory effects against HAase activity, and their active components were identified to be ursolic acid and monoterpenoid glycosides, respectively (15). *Clematis mandshurica*, *Trichosanthes kirilowii* and *Prunella vulgaris* exhibit analgesic efficacy, antioxidant activity, and HAase inhibitory properties (16). Other plant-derived materials, including flavonoids, tannins, curcumines, and glycyrrhizin, also exhibited inhibitory effects against HAase activity, and some of these compounds and their synthetic derivatives have been used as anti-inflammatory drugs (17-19).

In conclusion, AR has been classically employed as a diuretic and tonic in Oriental medicine. It has been established that AR contains various active components, including sucrose, astragaloside, formononetin, calycosin, (3R) 7,2'-dihydroxy-5',6'-dimethoy-isofalvan-7-*O*-β-D-glucoside, β-sitosterol, palmatic acid, isorhamnetin, quercetin, kaempferol, polyamine, and daucosterol. Of these compounds, flavonoids are especially promising, exhibiting a variety of biological effects, including antioxidative effects, immunomodulatory activity, and even anticancer activity. Hence, not surprisingly, our findings indicated that the HAase inhibitory ability of AR was due to isoflavone glycoside compounds. These components, as HAase inhibitors, are expected to be useful in the development of multi-functional preventive agents against inflammation and /or allergic reactions. Also, our findings scientifically support the use and efficacy of herbal medicinal plants in treatment of inflammatory disorders.

Acknowledgements

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References

1. Zang YD, Wang YL, Shen JP, Li DX. Hypotensive and anti-inflammatory effects of *Astragalus saponin* 1. Acta Pharm. Sin. 19: 333-337 (1984)
2. Kim EJ, Oh OJ, Lee SK, Yang KS. Inhibitory effect of Astragali Radix on COX-2 activity. Korean J. Pharmacogn. 32: 311-315 (2001)
3. Kim JS, Kim CS. A study on the constituents from the roots of *Astragalus membranaceus* Bunge (β). Korean J. Pharmacogn. 28: 75-79 (1997)
4. Kim JS, Kim YT, Kim CS. A study on the constituents from the roots of *Astragalus membranaceus* Bunge (β). Korean J. Pharmacogn. 31: 109-111 (2002)
5. Feost GI, Csóka T, Stern R. The hyaluronidase: A chemical, biological and clinical overview. Trends Glycosci. Glycotech. 8: 419-434 (1996)
6. Hynes WL, Ferretti JJ. Assay for hyaluronidase activity. In: Methods in Enzymology. Academic Press, London, UK Vol. 235, pp.606-616 (1994)
7. Anderson AJ. The effect of anti-inflammatory drugs on the enzyme activity of a rat liver granular fraction high increases vascular permeability. Biochem. Pharmacol. 17: 2253-2264 (1968)
8. Cameron E, Pauling L, Leibovitz B. Ascorbic acid and cancer: a review. Cancer Res. 39: 663-681 (1979)
9. Golberg RL, Huff JP, Lenz ME, Glickman P, Katz R, Thonar EJMA. Elevated plasma levels of hyaluronic acid in patients with osteoarthritis and rheumatoid arthritis. Arth. Rheum. 34: 799-807 (1991)
10. Choi SI, Lee YM, Heo TR. Screening of hyaluronidase inhibitory and free radical scavenging activity *in vitro* of traditional herbal

medicine extracts. Korean J. Biotechnol. Bioeng. 18: 282-288 (2003)
11. Lee J, Lee SH, Min KR, Lee KS, Ro JS, Ryu JC, Kim Y. Inhibitory effects of hydrolysable tannins on Ca^{+2}-activated HAase. Planta Med. 59: 381-382 (1993)
12. Richman, P. G., Baer, H. A convenient plate assay for the quantitation of hyaluronidase in *Hymenoptera* venoms. Anal. Biochem. 109: 376-381 (1980)
13. Ma X, Tu P, Chen Y, Zhang T, Wei T, Ito Y. Preparative isolation and purification of two isoflavones from *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao by high-speed counter-current chromatography. J. Chromatogr. A 992: 193-197 (2003)
14. Lin LZ, He XG, Lindenmaier M, Nolan G, Yang J, Cleary M, Qiu SX, Cordell GA. Lipid chromatography-electrospray ionization mass spectrometry study of the flavonoids of the roots of *Astragalus mongholicus* and *A. membranaceus*. J. Chromatogr. A 876: 87-95 (2000)
15. Jeong SJ, Ko YS, Ahn NH, Kim YC. Hyaluronidase inhibitor from *Uncariae Ramulus et Uncus*. Korean J. Pharmacogn. 29: 169-172 (1998)
16. Choi JH, Choi JH, Kim DY, Yoon JH, Youn HY, Yi JB, Rhee HI, Ryu KH, Jung K, Han CK, Kwak WJ, Cho YB. Effects of SKI 306X, a new herbal agent, on proteoglycan degradation in cartilage explant culture and collagenase-induced rabbit osteoarthritis model. Osteoarthritis Cartilage 10: 471-478 (2002)
17. Kakegawa H, Matsumoto H, Satoh T. Inhibitory effects of some natural products on the activation of hyaluronidase and their anti-allergic actions. Chem. Pharm. Bull. 40: 1439-1442 (1992)
18. Kakegawa H, Matsumoto H, Satoh T. Inhibitory effects of hydrangenol derivatives on the activities. Planta Med. 54: 385-389 (1988)
19. Kim EJ, Yuh CS, Lee PJ, Choi MK, Kim SY. Mineral Contents and anti-inflammatory effect of ostrich extract combined with Korean herb medicine. Food Sci. Biotechnol. 12: 617-620 (2003)