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Inhibitory Effects of Herbal Medicines on Hyaluronidase Activity

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Abstract—Inhibitory effects of 130 medicinal plants on hyaluronidase activity were analyzed. The medicinal plants are clinically used as herbal medicines for korean traditional prescriptions. Six out of the 130 herbal medicines exhibited more than 50% of inhibition on hyaluronidase activity by their total methanol extracts with 5 mg/ml as a final concentration. The active total methanol extracts were prepared from cortex of *Acanthopanax gracilistylus*, lignum of *Caesalpinia sappan*, radix of *Glycyrrhiza uralensis*, radicis cortex of *Morus alba*, herba of *Prunella vulgaris*, and radix of *Sanguisorba officinalis*. These active total methanol extracts were sequentially fractionated with dichloromethane, ethyl acetate, *n*-butanol, and then water. Among the solvent-fractionated extracts, the butanol fractions of *Acanthopanax gracilistylus* and *Glycyrrhiza uralensis* with 1 mg/ml as the final concentration exhibited more than 50% of inhibition on hyaluronidase activity, and the other fractions with the same concentration did less than 20% of inhibition.

Keywords—Hyaluronidase activity · *Acanthopanax gracilistylus* · *Caesalpinia sappan* · *Glycyrrhiza uralensis* · *Morus alba* · *Prunella vulgaris* · *Sanguisorba officinalis*

Hyaluronic acid, a mucopolysaccharide composed of alternating glucuronic acid and N-acetylglucosamine residues, is the major component of extracellular matrix, and biologic fluids[1]. The mucopolysaccharide has a variety of biological activities to provide the stability and elasticity to the extracellular matrix, and regulate the cell-cell and cell-matrix interactions, and the movement of interstitial fluids and biomolecules[2-5]. Hyaluronic acid can be bound with a variety of proteins such as hyaluronectin, link proteins, and fibrinogen[6-8]. The interaction of hyaluronic acid with its binding proteins strongly depends on the chain length of hyaluronic acid[9]. Hyaluronic acid with high molecular weight inhibits the phagocytic ability of macrophages, which is one of the important reactions in inflammation[10]. Hyaluronic acid with high molecular weight is an important regulator of scarless repair in fetal wound healing by markedly diminishing the inflammatory response[11,12]. However, degradation products of hyaluronic acid lead to increased inflammation, angiogenesis, fibrosis, and collagen deposition in wound healing[11,12]. High level of hyaluronic acid with decreased molecular weight has been detected in patients with inflammatory diseases including rheumatoid arthritis[13].

Hyaluronidase is an endohexosaminidase that initiates the degradation of hyaluronic acid with high molecular weight. The hyaluronidase activity is strongly inhibited by antiinflammatory drugs such as indomethacin and aspirin, and

antiallergic drugs such as disodium cromoglycerate and *N*-3′,4′-dimethoxycinnamoylanthranilic acid (tranilast)[14,15]. In this study, we have investigated the inhibitory effects of 130 herbal medicines on hyaluronidase activity in order to screen the bioactive substances which can be developed as possible antiinflammatory and/or antiallergic agents.

Experimental Methods

Chemicals - *p*-Dimethylaminobenzaldehyde, sodium hyaluronate, and bovine hyaluronidase were purchased from Sigma Chemical Co., USA. Other chemicals used in this study were the good grade for enzyme assay.

Medicinal plants and their extracts - Medicinal plants listed in Table I were purchased from a drug store (Dongyang Yakup Co., Korea). The plants are herbal medicines which are clinically used for korean traditional prescriptions. The purchased herbal medicines were taxonomically identified with respect to plant morphology, and voucher specimens were deposited at the herbarium of our department. Each of the herbal medicines was sliced, and weighted. One hundred gram of the sliced herbal medicine was extracted twice with 300 ml to 500 ml of methanol:water (80:20 v/v) in a boiling water bath under reflux for 3 h. This extract solution was evaporated under reduced pressure at 50°C, and then completely dried by lyophilization. The dried extract was called as "total MeOH extract".

Some of the total MeOH extracts were subjected to sequential fractionations with dichloromethane, ethyl acetate, and then *n*-butanol. Five gram of the total MeOH extract was suspended in 300 ml of distilled water, and then extracted several times with 100% dichloromethane until colored constituents were not transferred to the dichloromethane layer. The remaining aqueous layer was subjected to extraction with 100% ethyl acetate. This extraction was continued until no colored constituents were transferred to the ethyl acetate layer. The remaining aqueous layer after the ethyl acetate extraction was further extracted with 100% *n*-butanol until colored constituents were not transferred to the butanol layer. The fractions extracted with each of dichloromethane, ethyl acetate, and *n*-butanol, and the aqueous layer remained after the *n*-butanol extraction were evaporated under reduced pressure with 50°C, and then completely dried by lyophilization. The dried total MeOH extracts and solvent fractions were used as samples in this study.

Hyaluronidase activity assay - Hyaluronidase activity was spectrophotometrically determined by measuring the amount of *N*-acetylglucosamine formed from sodium hyaluronate. Fifty ul of bovine hyaluronidase (7,900 units/ml) dissolved in 0.1 M acetate buffer (pH 3.5) was mixed with 100 ul of a designated concentration of sample (total MeOH extract or the solvent fraction) dissolved in 5% dimethyl sulfoxide, and then incubated in a water bath with 37°C for 20 min. The control group was treated with 100 ul of 5% dimethyl sulfoxide instead of the sample. The reaction mixture was added with 100 ul of 12.5 mM calcium chloride, and then incubated in a water bath with 37°C for 20 min. This Ca^{++}-activated hyaluronidase was treated with 250 ul of sodium hyaluronate (1.2 mg/ml) dissolved in 0.1 M acetate buffer (pH 3.5), and then incubated in a water bath with 37°C for 40 min. One hundred ul of 0.4 N sodium hydroxide and 100 ul of 0.4 M potassium borate were added to the reaction mixture, and then incubated in a boiling water bath for 3 min. After cooling to room temperature, 3 ml of dimethylaminobenzaldehyde solution (4 g of *p*-dimethylaminobenzaldehyde dis-

Table I. Inhibition on the hyaluronidase activity by total MeOH extracts.

Medicinal plants (part of use)	Family name	% of Inhibition[a]
Acanthopanax gracilistylus (cortex)	Araliaceae	85±1
Acorus gramineus (rhizoma)	Araceae	NE
Adenophora trachelioides (radix)	Campanulaceae	13±1
Agastache rugosa (herba)	Labiatae	NE
Agrimonia pilosa var. *japonica* (herba)	Rosaceae	9±2
Akebia quinata (caulis)	Lardizabalaceae	41±3
Albizzia julibrissin (cortex)	Leguminosae	NE
Alisma orientale (rhizoma)	Alismataceae	NE
Alpinia oxyphylla (fruit)	Zingiberaceae	NE
Amomum cardamomum (fruit)	Zingiberaceae	NE
Amomum tsao-ko (fruit)	Zingiberaceae	23±1
Amomum villosum (semen)	Zingiberaceae	NE
Anemarrhena asphodeloides (rhizoma)	Liliaceae	NE
Angelica dahurica (radix)	Umbelliferae	NE
Angelica gigas (radix)	Umbelliferae	NE
Angelica koreana (radix)	Umbelliferae	NE
Aquilaria agallocha (lignum)	Thymelaceae	NE
Aralia continentalis (radix)	Araliaceae	NE
Arctium lappa (semen)	Compositae	NE
Areca catechu (pericarpium)	Palmae	9±1
Areca catechu (semen)	Palmae	36±3
Arisaema consanguineum (rhizoma)	Araceae	7±1
Artemisia argyi (folium)	Compositae	NE
Asarum heterotropoides var. *mandshuricum* (radix)	Aristolochiaceae	NE
Asparagus cochinchinensis (radix)	Liliaceae	NE
Astragalus membranaceus (radix)	Leguminosae	NE
Atractylodes japonica (rhizoma)	Compositae	NE
Benincasa hispida (semen)	Cucurbitaceae	NE
Biota orientalis (semen)	Cupressaceae	NE
Boswellia carterii (resin)	Burseraceae	NE
Bupleurum falcatum (radix)	Umbelliferae	NE
Caesalpinia sappan (lignum)	Leguminosae	71±2
Carthamus tinctorius (flower)	Compositae	8±2
Chrysanthemum morifolium (flower)	Compositae	NE
Cimicifuga heracleifolia (rhizoma)	Ranunculaceae	3±1
Cinnamomum cassia (cortex)	Lauraceae	NE
Cistanche salsa (herba)	Orobanchaceae	NE
Citrus aurantus var. *tachibana* (pericarpium)	Rutaceae	NE
Clematis chinensis (radix)	Ranunculaceae	NE
Cnidium monnieri (fruit)	Umbelliferae	NE
Cnidium officinale (rhizoma)	Umbelliferae	7±1
Coix lachryma-jobi var. *ma-yuen* (semen)	Graminae	NE
Commiphora molmol (resin)	Burseraceae	NE
Coptis chinensis (rhizoma)	Ranunculaceae	NE
Cornus officinalis (fruit)	Cornaceae	NE

Table I. Continued

Medicinal plants (part of use)	Family name	% of Inhibition[a]
Corydalis yanbusuo (tuber)	Fumariaceae	NE
Crataegus pinnatifida (fruit)	Rosaceae	NE
Curcuma longa (rhizoma)	Zingiberaceae	NE
Curcuma zedoaria (rhizoma)	Zingiberaceae	NE
Cynomorium songaricum (herba)	Cynomoriaceae	NE
Cyperus rotundus (rhizoma)	Cyperaceae	NE
Dioscorea batatas (radix)	Dioscoreaceae	NE
Dipsacus asper (radix)	Dipsacaceae	NE
Dolichos lablab (semen)	Leguminosae	NE
Ephedra sinica (herba)	Ephedraceae	NE
Epimedium grandiflorum (herba)	Berberidaceae	NE
Eucommia ulmoides (cortex)	Eucommiaceae	NE
Eugenia caryophyllata (flower)	Myrtaceae	17±2
Euphorbia kansui (radix)	Euphorbiaceae	NE
Euphorbia pekinensis (radix)	Euphorbiaceae	NE
Euphoria longan (fruit)	Sapindaceae	NE
Evodia officinalis (fruit)	Rutaceae	NE
Foeniculum vulgare (fruit)	Umbelliferae	NE
Forsythia viridissima (fruit)	Oleaceae	NE
Fritillaria verticillata (tuber)	Liliaceae	NE
Gardenia jasminoides (fruit)	Rubiaceae	NE
Gastrodia elata (rhizoma)	Orchidaceae	NE
Gentiana scabra var. *buergeri* (radix)	Gentianaceae	NE
Gleditsia sinensis (spina)	Leguminosae	NE
Glycyrrhiza uralensis (radix)	Leguminosae	83±2
Hordeum vulgare (semen)	Graminae	NE
Kalopanax septemlobus (cortex)	Araliaceae	NE
Ligusticum tenuissimum (radix)	Umbelliferae	NE
Lindera strychnifolia (radix)	Lauraceae	NE
Liriope graminifolia (tuber)	Liliaceae	NE
Lonicera japonica (flower)	Caprifoliaceae	NE
Loranthus parasiticus (herba)	Loranthaceae	NE
Lycium chinense (fruit)	Solanaceae	11±3
Lycium chinense (radicis cortex)	Solanaceae	NE
Machilus thunbergii (cortex)	Lauraceae	31±5
Magnolia liliflora (flower)	Magnoliaceae	NE
Mentha arvensis (herba)	Labiatae	NE
Morus alba (radicis cortex)	Moraceae	55±1
Nelumbo nucifera (semen)	Nymphaeaceae	NE
Pachyma hoelen (sclerotia)	Polyporaceae	NE
Paeonia albiflora (radix)	Ranunculaceae	8±1
Paeonia suffruticosa (cortex)	Paeoniaceae	NE
Panax ginseng (radix)	Araliaceae	NE
Perilla frutescens (herba)	Labiatae	NE
Peucedanum japonicum (radix)	Umbelliferae	8±1

Table I. Continued

Medicinal plants (part of use)	Family name	% of Inhibition[a]
Peucedanum praeruporum (radix)	Umbelliferae	3±1
Phellodendron amurense (cortex)	Rutaceae	NE
Phyllostachys nigra var. *henonis* (caulis)	Graminae	NE
Pinellia ternata (tuber)	Araceae	NE
Plantago asiatica (semen)	Plantaginaceae	NE
Platycodon grandiflorum (radix)	Campanulaceae	NE
Polygala tenuifolia (radix)	Polygalaceae	NE
Polygonatum sibiricum (rhizoma)	Liliaceae	NE
Polygonum multiflorum (radix)	Polygonaceae	NE
Poncirus trifoliata (fruit)	Rutaceae	3±1
Potentilla chinensis (herba)	Rosaceae	NE
Prunella vulgaris (herba)	Labiatae	79±1
Prunus persica (semen)	Rosaceae	NE
Psoralea corylifolia (semen)	Leguminosae	NE
Pueraria thunbergiana (radix)	Leguminosae	5±2
Raphanus sativus (semen)	Cruciferae	NE
Rehmannia glutinosa (rhizoma)	Scrophulariaceae	NE
Rheum undulatum (rhizoma)	Polygonaceae	24±1
Rosa laevigata (fruit)	Rosaceae	NE
Rubus coreanus (fruit)	Rosaceae	12±6
Sanguisorba officinalis (radix)	Rosaceae	51±1
Saussurea lappa (radix)	Compositae	NE
Schizandra chinensis (fruit)	Magnoliaceae	42±10
Schizonepeta tenuifolia (herba)	Labiatae	NE
Scrophularia ningpoensis (radix)	Scrophulariaceae	NE
Scutellaria baicalensis (radix)	Labiatae	32±6
Solanum nigrum (herba)	Solanaceae	NE
Sparganium stoloniferum (rhizoma)	Sparganiaceae	NE
Stephania tetrandra (radix)	Menispermaceae	18±2
Taraxacum mongolicum (herba)	Compositae	NE
Thuja orientalis (folium)	Cupressaceae	NE
Trichosanthes kirilowii (radix)	Cucurbitaceae	NE
Trichosanthes kirilowii (semen)	Cucurbitaceae	NE
Tripterygium regelii (herba)	Celastraceae	NE
Uncaria rhynchophylla (ramulus et uncus)	Rubiaceae	10±6
Vitex rotundifolia (fruit)	Verbenaceae	NE
Zanthoxylum bungeanum (pericarpium)	Rutaceae	NE
Zingiber officinale (rhizoma)	Zingiberaceae	NE
Zizyphus vulgaris var. inermis (fruit)	Rhamnaceae	NE
Zizyphus vulgaris var. spinosus (fruit)	Rhamnaceae	NE

[a]Data are indicated as mean ± standard error (n=5). NE means "not effective".

solved in 350 ml of 100% acetic acid and 50 ml of 10 N hydrochloric acid) was added to the reaction mixture, and then incubated in a water bath with 37°C for 20 min. Optical density at

Table II. Inhibition on the hyaluronidase activity by solvent-fractionated extracts.

Medicinal plants	% of Inhibition[a]			
	CH_2Cl_2 fr.	EtOAc fr.	BuOH fr.	H_2O fr.
Acanthopanax gracilistylus	5 ± 1	88 ± 1*	88 ± 1*	17 ± 8
Caesalpinia sappan	23 ± 1	78 ± 1*	67 ± 2*	35 ± 2**
Glycyrrhiza uralensis	44 ± 1*	68 ± 1*	95 ± 2*	5 ± 2
Morus alba	< 0	56 ± 2*	17 ± 2	8 ± 1
Prunella vulgaris	8 ± 4	78 ± 1*	87 ± 1*	73 ± 1*
Sanguisorba officinalis	< 0	28 ± 1	9 ± 1	34 ± 2**

[a]Data are indicated as mean ± standard error (n=5), and their significances are $p < 0.001$ (*) and $p < 0.01$ (**). Each of the total methanol extracts were sequentially fractionated with dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and then *n*-butanol (BuOH), where H_2O fraction (fr.) was the aqueous layer after the *n*-butanol extraction. Each of the fractions was treated with 5 mg/ml as a final concentration.

585 nm of the reaction mixture was measured by using a spectrophotometer (JASCO, Japan).

Statistics - Inhibitory effect of sample on hyaluronidase activity was expressed as follows: % of inhibition = [(control OD_{585}-sample OD_{585}) / control OD_{585}] x 100, where OD_{585} is the optical density at wavelength 585 nm. Data were collected as mean ± standard error by 5 independent tests (n=5), and significance of the data was analyzed by the Student's t-test.

Results and Discussion

Inhibitory effects on hyaluronidase activity by 130 medicinal plants were analyzed (Table I). The medicinal plants are clinically used as herbal medicines for korean traditional prescriptions. Six out of the 130 herbal medicines exhibited more than 50% of inhibition on hyaluronidase activity by their total MeOH extracts with 5 mg/ml as a final concentration. These active extracts were prepared from cortex of *Acanthopanax gracilistylus*, lignum of *Caesalpinia sappan*, radix of *Glycyrrhiza uralensis*, radicis cortex of *Morus alba*, herba of *Prunella vulgaris*, and radix of *Sanguisorba officinalis*. Significant inhibition but less than 50% of inhibition on the enzyme activity was exhibited by total MeOH extracts prepared from caulis of *Akebia quinata*, fruit of *Amomum tsao-ko*, semen of *Areca catechu*, cortex of *Machilus thunbergii*, rhizoma of *Rheum undulatum*, fruit of *Schizandra chinensis*, and radix of *Scutellaria baicalensis*. The other 117 herbal medicines did not exhibit significant inhibition on hyaluronidase activity.

The total MeOH extracts exhibited more than 50% of inhibition at 5 mg/ml of final concentration were independently subjected to sequential fractionations with dichloromethane, ethyl acetate, and then *n*-butanol. Inhibitory effects on hyaluronidase activity by each of the solvent fractions with 5 mg/ml as a final concentration were analyzed (Table II). Both ethyl acetate and butanol fractions of *Acanthopanax gracilistylus* exhibited strong inhibitions on hyaluronidase activity but other fractions of this herbal medicine did not. All solvent fractions of *Caesalpinia sappan* inhibited the enzyme activity, where ethyl acetate and butanol fractions exhibited more than 50% of inhibition. Among the solvent-fractionated extracts, the butanol fraction of *Glycyrrhiza uralensis* exhibited the highest inhibition on hyaluronidase activity,

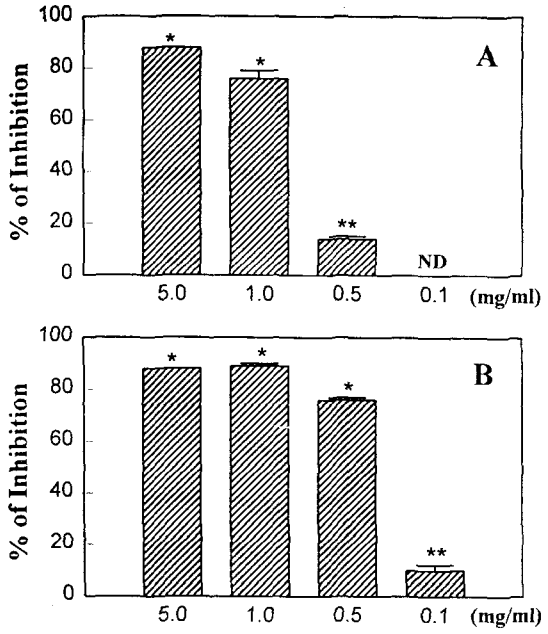

Fig. 1. Dose-dependent inhibition on hyaluronidase activity by the butanol fractions of *Acanthopanax gracilistylus* and *Glycyrrhiza uralensis*. Effects on the enzyme activity by the butanol fractions of *Acanthopa-nax gracilistylus* (A), and *Glycyrrhiza uralensis* (B) are indicated as % of inhibition compared with the control. Inhibitory effect of the butanol fraction of *Acantho-panax gracilistylus* with 0.1 mg/ml as a final concentration was not determined (ND). Significances of the data are $p < 0.001$ (*) and $p < 0.01$ (**).

and ethyl acetate and dichloromethane fractions of this herbal medicine exhibited significant inhibitions. The ethyl acetate fraction of *Morus alba* inhibited the hyaluronidase activity but other fractions of this herbal medicine did not. All of the fractions except dichloromethane fraction of *Prunella vulgaris* exhibited strong inhibition on the enzyme activity. The ethyl acetate and aqueous fractions of *Sanguisorba officinalis* tended to inhibit the hyaluronidase activity but other fractions of this herbal medicine did not at all. Thus, more than 50% of inhibition on hyaluronidase activity was exhibited by ethyl acetate and butanol fractions of *Acanthopanax gracilistylus*, *Caesalpinia sappan*, and *Glycyrrhiza uralensis*, ethyl acetate fraction of *Morus alba*, and ethyl acetate, butanol and water fractions of *Prunella vulgaris*.

Among the active fractions, the butanol fractions of *Acanthopanax gracilistylus* and *Glycyrrhiza uralensis* with 1 mg/ml as a final concentration exhibited more than 50% inhibition, but the other fractions with the same concentration did less than 20% of inhibition on hyaluronidase activity. As shown in Fig. 1, the butanol fraction of *Acanthopanax gracilistylus* with 1 mg/ml to 5 mg/ml as the final concentration exhibited 83% to 88% of inhibition, and the same fraction with 0.5 mg/ml did 10% of inhibition on hyaluronidase activity. The butanol fraction of *Glycyrrhiza uralensis* with 0.5 mg/ml to 5 mg/ml as the final concentration exhibited 76% to 95% of inhibition on the enzyme activity and the same fraction with 0.1 mg/ml did 10% of inhibition.

Major constituents of *Acanthopanax gracilistylus* are lignans including acanthoside, triterpenoids including chisanoside, and flavonoids including isoquercitrin, and those of *Glycyrrhiza uralensis* are triterpenoids including glycyrrhizin and flavonoids including liquiritin. Phenolic compounds such as flavonoids and tannins are known as potent inhibitors on hyaluronidase activity[14,16]. Thus, active constituents of *Acanthopanax gracilistylus* and *Glycyrrhiza uralensis* with inhibitory effects on hyaluronidase activity would be speculated as the phenolic compounds.

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