Inhibitory effects of medicinal plants on elastase activity and biological activities in the active plant extracts

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Abstract

Inhibitory effects of 200 medicinal plants on elastase activity were screened. Among the 200 plants, six plant extracts exhibited more than 65% of inhibition on elastase activity by their total methanol extracts at 1,000 ㎍/㎖ as a final concentration. For six active plants including *Areca catechu*(IC_{50}:42.4 ㎍/㎖), *Cinnamonum cassia* (IC_{50}:208.7 ㎍/㎖), *Myristica fragrans*(IC_{50}:284.1 ㎍/㎖), *Curcumma longa*(IC_{50}:398.4 ㎍/㎖), *Alponia katsumadai*(IC_{50}:465.7 ㎍/㎖), and *Dryopteris crassirrhizoma*(IC_{50}:714.4 ㎍/㎖), the inhibitory effects of their constituents were examined on the activity of human leukocyte elastase, hyaluronidase and lipid peroxidation. In lipid peroxidation assay using TBA method, three of the 6 plants including *Curcumma longa*(IC_{50}:45.5 ㎍/㎖), *Areca Catechu*(IC_{50}:51.0 ㎍/㎖) and *Alponia katsumadai*(IC_{50}:116.3 ㎍/㎖) exhibited more than 70% of inhibition at the concentration of 1,000 ㎍/㎖, but only one plant, *Areca Catechu*(IC_{50}:563 ㎍/㎖) showed high inhibitory effect on

hyaluronidase activity. The results suggest that medicinal plants showing several biological activities may be potent inhibitors of anti-aging process on skin and that might be useful for application in cosmetics.

Introduction

Elastase[EC3.4.4.7] is the only enzyme capable of degrading elastin, an insoluble elastic fibrous protein of animal connective tissues. It hydrolyzes practically all proteins, including the supporting and structural proteins of the connective tissue such as collagen and elastin[1]. Elastin is the main component of the elastic fibers of the connective tissue and tendons. In the skin, the elastic fiber together with the collagenous fibers form a network developing under the epiderm[2]. Elastase is the sole proteinase which is able to spilit elastin, and plays a critical role in inflammation processes[3]. Elastase has received a great attention, primarily because of its reactivity and unspecificity. It is able to attack all major connective tissue matrix proteins, e.g. elastin, collagen, proteoglycans and keratins. In contrast to elastase, collagenase is rather a specific proteinase with a limited number of substrates[4]. In the UVA-irradiated skin, mild inflammation occurs repeatedly in the dermis, and it is assumed that connective tissue proteins may be attacked by elastase released from polymorphonuclear leukocytes(PMNs), resulting in damages of elastin and collagen fibers and finally causing sagging[5]. With age, specially those over 40, the elasticity of skin is significantly decreased by elastase and also results in sagging, Histological examination revealed thickened epidermis and dermis, increased numbers of mast cells, hypertrophic cysts, infiltration of inflammatory cells, partial absence and aggregation of elastin fibers and a decrease in collagen fibers. Biochemically, elastase acitivity was significantly increased with age[6].

Hyaluronic acid with high molecular weight inhibits the phagocytic ability of macrophages, which is one of the important reaction in inflammation[7]. Hyaluronic

acid with high molecular weight has an important sole in regulation of scarless repair in fetal wound healing by markedly diminishing in inflammatory response[8]. However, degradation products of hyaluronic acid lead to increase inflammation, angiogenesis, fibrosis, and collagen deposition in wound healing. High level of hyaluronic acid with low molecular weight has been detected in patients with inflammatory diseases such as rheumatoid arthritis[9]. Hyaluronidase is an endohexosaminidase that initiates the degradation of hyaluronic acid with high molecular weight. Recently, a number of studies have been interested in interactions between elastase and its inhibitors[10,11,12]. Until now, it has been proposed but not yet fully suggested that the topical application of specific inhibitors on human skin surface may show beneficial effects on UV-irritated and dry skin. Furthermore, plant sources have been evaluated for developing natural antioxidants that may be involved in antiaging and antiwrinkle care[13,14]. In this study, we have investigated the inhibitory effects of 200 medicinal plants on elastase activity and examined the other biological activities of the active extracts which can be developed as possible antiinflammatory and antiaging agents on human skin.

Experimental methods

Chemicals

All solvents were of analytical grade. Human leukocyte and porcine pancreatic elastase, hyaluronidase, hyaluronic acid, ethyl linolate, BHT and TBA were purchased from Sigma chemical Co.,(USA, St. Louis). [N-Succ-(Ala)3-p-nitroanilide] and Meo-Succ-(Ala)2-Pro-Val-p-nitroanilide were purchased from Calbiochem(USA, MA). Elhibin and Sophorine were used commerical grade from Pentapharm Ltd. (Switzerland, Basel) and Secma Co(France, Pontrieux).

Apparatus

Spectrophotometric measurements were performed with a UV-Visible spectrophotometer (Beckman).

Plant extract

Medicinal plants were purchased from the oriental medicinal market located in Seoul, South Korea. Each of the plants was sliced and weighted. Each plant powder (100g) was extracted with 300㎖ to 500㎖ of methanol : water(80 : 20, v/v) in a boiling water bath for 7days. After filtration, this extract solution was evaporated to dryness under vacuum, and then completely dried by lyophilization. The dried extract was called as "total MeOH extract". The dried total MeOH extracts were used as sample in this study.

Elastase activity assay

Porcine pancreatic elastase(PPE ; Sigma ; type IV) was assayed spectrophotometrically by the modified method of James *et al.*,[15] using [N-Succ-$(Ala)_3$-p-nitroanilide] as the substrate, and monitoring the release of p-nitroaniline for 20 min at 25℃. The amount of p-nitroaniline was determined by measuring the absorbance at 410 ㎚. The reaction mixture contained: 0.2M Tris-HCl buffer(pH 8.0), 1 ㎍/㎖ elastase, 0.8 mM succnyl-Ala-Ala-Pro-p-nitroanilide(ESIV;elastase substrate IV; Calbiochem) as substrate and different inhibitors(dissolved in methanol). Each inhibitor was preincubated for 20 min. at 25℃ and the reaction was started by adding substrate. Blanks contained all components except enzyme. Human leukocyte elastase(HLE; Sigma) activity was spectrophotometically determined by measuring the amount of p-nitroaniline at 410 nm for 20 min. at 25℃. The reaction mixture contained ; 0.1M HEPES buffer(pH 7.5), 1 ㎍/㎖ elastase, 0.5M NaCl, 9.8% DMSO, 1% v/v 10 ㎎/㎖ BSA(Sigma ; fraction V), 1.12 mM Meo-Succinyl-Ala-Ala-Pro-Val-p-nitroanilide(ESI ; elastase substrate I ; Calbiochem) as a substrate and different inhibitors. Inhibitor(total MeOH extract) dissolved in methanol was preincubated for 20 min. at 25℃ and the reaction was started by adding substrate. The rate of the reaction is the slope of the line recorded and is proportional to elastase activity. A control curve was prepared with elastase in the absence of

inhibitor. One unit of elastolytic activity is defined as the activity releasing 1 μM of p-nitroaniline/min. For p-nitroaniline an ε of 8800 at 410 nm was used[16]. The percentage of inhibition was calculated as follow;

$$\text{Inhibition}(\%) = (1 - B/A) \times 100$$

where, A is the enzyme activity without inhibitor and B is the activity in the presence of inhibitor.

Antioxidative activity

A lipid peroxidation system was induced by Fenton's reagent. Each test sample(0.1 mℓ) and ethyl linoleate (10 μℓ) were added to incubation medium(4.89 mℓ) containing 2% sodium dodecyl sulfate, 1 μM ferrous chloride and 0.5 mM hydrogen peroxide. The known synthetic antioxidant, butylated hydroxyl toluene(BHT) was used as a reference compound. The incubation medium was keep at 55℃ for 16 hrs. Each reaction mixture(0.2 mℓ) was transferred into a test tube, followed by addition of 4% BHT (50 ul) to prevent further oxidation. Antioxidative activity of the sample was measured using thiobarbituric acid(TBA) assay according to the method of Ohkawa *et al.*, [17]. The absorbance was measured at 535 nm.

Hyaluronidase activity assay

Hyaluronidase activity was spectrophotometically determined by measuring the amount of N-acetylglucosamine formed from sodium hyaluronate[18]. 50 μℓ of bovine hyaluronidase(7,900 units/mℓ) dissolved in 0.1 M acetate buffer(pH 3.5) was mixed with 100 μℓ of a designated concentration of sample(total MeOH extract) dissolved in 5% dimethyl sulfoxide, and then incubated in a water bath at 37℃ for 20 min. The control group was treated with 100 μℓ of 5% dimethyl sulfoxide instead of the sample. 100 μℓ of 12.5 mM calcium chloride was added to the reaction mixture, and then the mixture was incubated in a water bath at 37℃ for 20 min. This Ca^{2+} -activated hyaluronidase was treated with 250 μℓ of sodium hyaluronate(1.2 mg/mℓ) dissolved in 0.1 M acetate buffer(pH 3.5), and then incubated in a water bath at 37

℃ for 40 min. 100 μℓ 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 dimethyl-aminobenzaldehyde solution(4 g of p-dimethylamino- benzaldehyde dissolved in 350mℓ 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 at 37℃ for 20 min. Optical density at 585 nm of the reaction mixture was measured by using a spectrophotometer. The percentage of inhibition was calculated as follows;

$$\text{Inhibition}(\%) = [(\text{control } OD_{585nm} - \text{sample } OD_{585nm})/\text{control } OD_{585nm}] \times 100$$

Results and discussions

Inhibitory effects of 200 medicinal plants on porcine pancreatic elastase(PPE) were screened(published in Inter. J. cosm. sci.). In the inhibition of PPE assay, several plant extracts including *Areca catechu, Cinnamomum cassia, Myristica fragrans* and *Curcumma longa* showed more than 30% inhibition at the concentration of 100 μg/mℓ or 80% inhibition at the concentration of 1,000 μg/mℓ. Nine of the 200 medicinal plants exhibited more than 65% of inhibition on elastase activity by their total methanol extracts at 1,000 μg/mℓ as a final concentration. Six of these active extracts were prepared from semen of *Areca Catechu,* cortex of *Cinnamomum cassia,* semen of *Myristica fragrans,* radix of *Curcumma longa,* radix of *Dryopteris crassirrhi-zoma,* and semen of *Alponia katsumadai.* Among the 200 medicinal plants 120 plants did not exhibit significant inhibition on PPE activity. For six active extracts showing high inhibitory activities against PPE in the initial screening, were examined that several biological activities such as human leukocyte elastase activity, antioxidative and/or hyaluronidase inhibition activity were examined. In order to determine IC_{50} values of 6 plant extracts showing high biological activities, the experiments for dose-response relationship were performed.

Fig. 1 shows the concentration-dependent inhibition of porcine pancreatic elastase (PPE) and Human leukocyte elastase(HLE) by six plant extracts. *Areca Catechu* had far greater inhibitory effect of PPE activity with minimal effective concentration of 10 ㎍/㎖(33.2 % inhibition), at 50 ㎍/㎖ PPE was inhibited by 59% and plateau at 500 ㎍/㎖(95 % inhibition). Six selected plant extracts showed a similar pattern of inhibition on the hydrolytic activity of PPE and HLE. For the inhibition of PPE and HLE IC_{50} values of these 6 plant extracts including *Areca Catechu*(42.4 ㎍/㎖, 51.3 ㎍/㎖), *Cinnamomum cassia*(208.7 ㎍/㎖, 190.8 ㎍/㎖), *Myristica fragrans*(284.1 ㎍/㎖, 289.4 ㎍/㎖), *Curcumma longa*(398.4 ㎍/㎖, 416.7 ㎍/㎖), *Alponia katsumadai*(465.7 ㎍/㎖, 481.9 ㎍/㎖) and *Dryopteris crassirrhizoma*(714.4 ㎍/㎖, 649.3 ㎍/㎖) respectively, are comparable to the reference compounds, ELHIBIN(980 ㎍/㎖) and Sophorine(13.3 ㎎/㎖)[19, 20]. Among the plant extract tested, *Areca Catechu* showed the highest inhibitory activity against PPE and HLE, which showed the higher potent activity than Sophora flavescens extracts developed in Shiseido(Japan) ; (30 ㎍/㎖ : 20% inhibition, 60 ㎍/㎖ : 40% inhibition, 300 ㎍/㎖ : 85% inhibition)[21].

These active plant extracts showing high inhibitory activity against elastase in the initial screening were tested for antioxidative activity using Fenton's reagent/ethyl linolate system and for inhibitory effect on hyaluronidase activity. Table 1 represent the inhibition of human leukocyte elastase, antioxidative activity and anti-hyaluronidase activity of plant extracts. In lipid peroxidation assay using TBA method, three out of the plant extracts including *Curcumma longa, Areca Catechu,* and *Alponia katsumadai* exhibited more than 70% and 20% inhibition at the concentration of 1,000 ㎍/㎖ and 10 ㎍/㎖, respectively. In anti-hyaluronidase activity assay, *Areca Catechu* out of the 6 plant extracts exhibited more than 60% and 20% of inhibition at the concentration of 1 ㎎/㎖ and 2 ㎎/㎖, respectively. The other extracts including *Cinnamomum cassia, Dryopteris crassirrhizoma* and *Alponia katsumadai* at 2 ㎎/㎖ as the final concentration exhibited more than 50% of inhibition.

Fig. 2 shows the antioxidative activity of three active plant extracts. IC_{50} values of

plant extracts selected were 45.5 μg/mℓ for *Curcumma longa,* 51.0 μg/mℓ for *Areca Catechu* and 116.3 μg/mℓ for *Alponia katsumadai*, which showed similar potency to dl-α-tocopherol or more potent activity than L-ascorbic acid. BHT was the potent inhibitor of TBA-reactive material formation. IC_{50} value of BHT was 1.5 μg/mℓ, while other reference compounds, dl-α-tocopherol and L-ascorbic acid showed 33.6 μg/mℓ and 219 μg/mℓ, respectively(not shown)[22]. Natural antioxidants are usually phenolic or polyphenolic compounds and these antioxidants include tocopherol, flavonoid and cinnamic acid derevatives[23]. It is known that there are two types of antioxidant[24]. The first type of antioxidant inhibits the formation of free radicals which may initiate oxidation. In most case, they are chelators of metal ions such as flavonoids. The second type of antioxidant inhibits the free radical chain propagation reaction. Therefore, some of plant extracts may be act at the initiation stage of peroxidation interfering with Fenton's reaction, thus breaking the chain reaction.

As shown in Fig. 3 the *Areca Catechu* extract at 1 mg/mℓ to 5 mg/mℓ as the final concentration exhibited 65% to 88% of inhibition and at 0.5 mg/mℓ did 45% of inhibibion on hyaluronidase activity. The plant extract of *Glycyrrbiza uralensis* at 0.5 mg/mℓ to 5 mg/mℓ as the final concentration exhibited 76% to 95% of inhibition on the enzyme activity and at 0.1 mg/mℓ did 10% of inhibition. IC_{50} values of *Glycyrrbiza uralensis* and *Areca Catechu* were found to be 330 μg/mℓ and 563 μg/mℓ, respectively. On the other hand, IC_{50} values of the other plant extracts tested showed much lower activity than the well-known reference compounds. Most of them showed IC_{50} values of more than 1.5 mg/mℓ except *Areca Catechu* showing relative higher activiy(563 μg/mℓ). Major constituents of *Areca Catechu* are phenolic compounds such as flavonoids and tannins[27], thus active constituents of *Areca Catechu* and *Glycyrrbiza uralensis* with inhibitory effects on hyaluronidase activity would be speculated as the phenolic compounds. *Glycyrrbiza uralensis* is triterpenoid including Glycyrrbizin and flavonoids including liquiritin phenolic compounds such as flavonoids and tannins are known as potent inhibitors on hyaluronidase activity[25,26]. From all of these results, it is sure that six plant extracts possessed the inhibitory

effect of elastase activity, at least *in vitro*. Therefore, it suggested that the topical application of plant-based inhibitors of unspecific elastase to cosmetics may provide beneficial effects on UV-irradiated and dry skin.

In this study, we have also investigated the other inhibitory effects of 6 plant extracts showing anti-elastase activity to develop antiinflammatory and antioxidative cosmetics. We demonstrated that several palnt extracts have both anti-elastase activity and anti-hyaluronidase activity. These plant extracts can be applied to cosmetics as plant-based materials having total anti-aging effects on human skin. However, we have not determined the components in the plant extracts showing these biological activities. Isolation and purification of the active components from plant extracts with biological activity is under the investigation.

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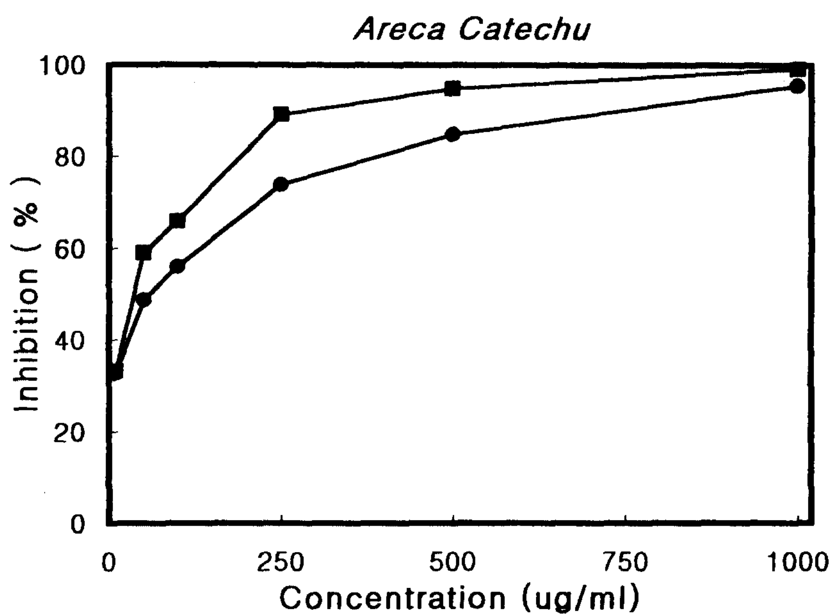


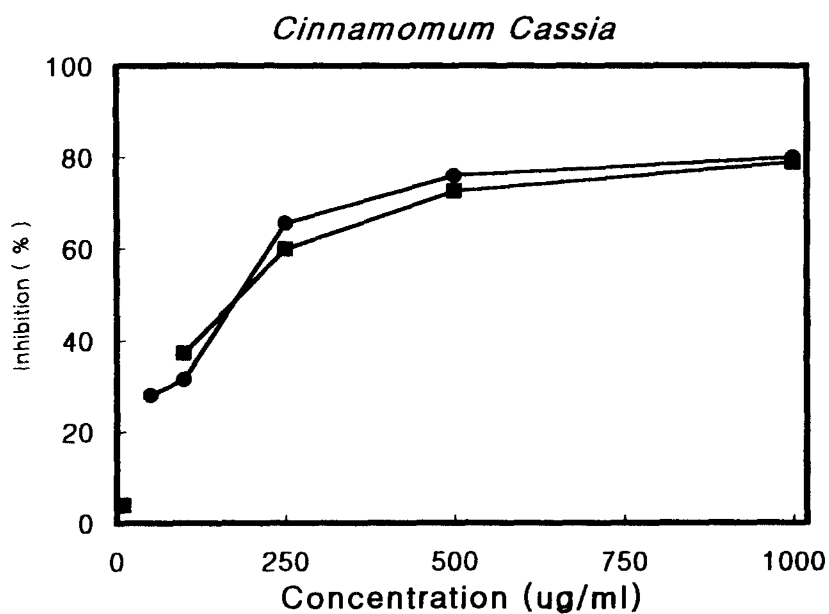
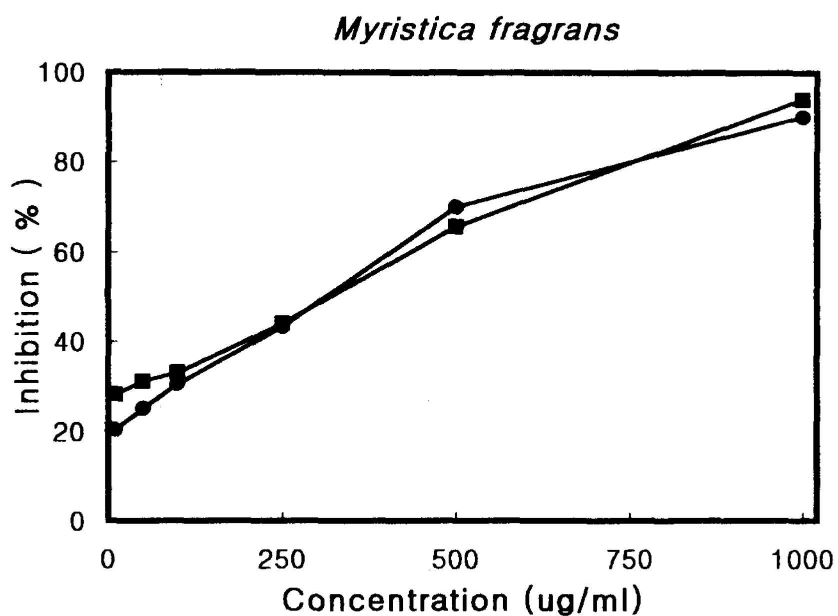
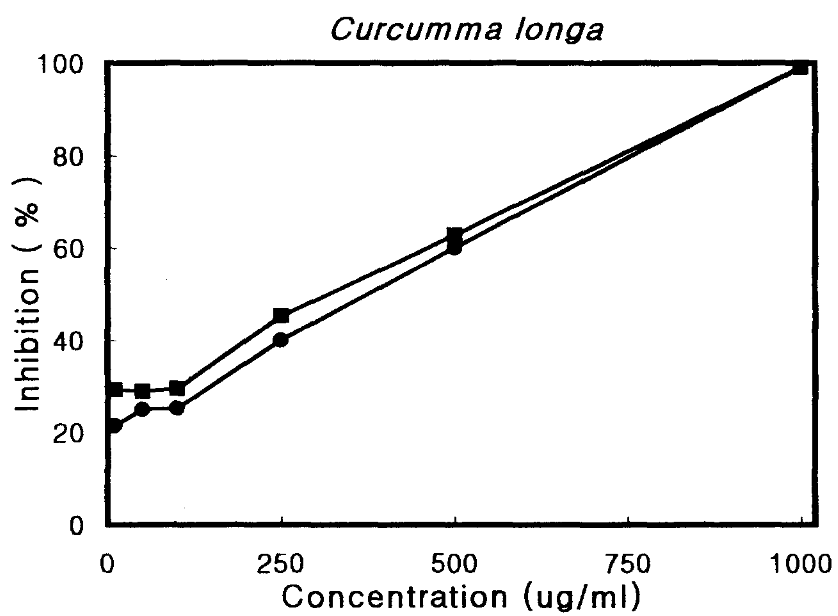


Fig 1. Anti-elastase activity of several plant extracts : dose-response curve Inhibitory effects on the PPE(■) and HLE(●) activities by plant extracts





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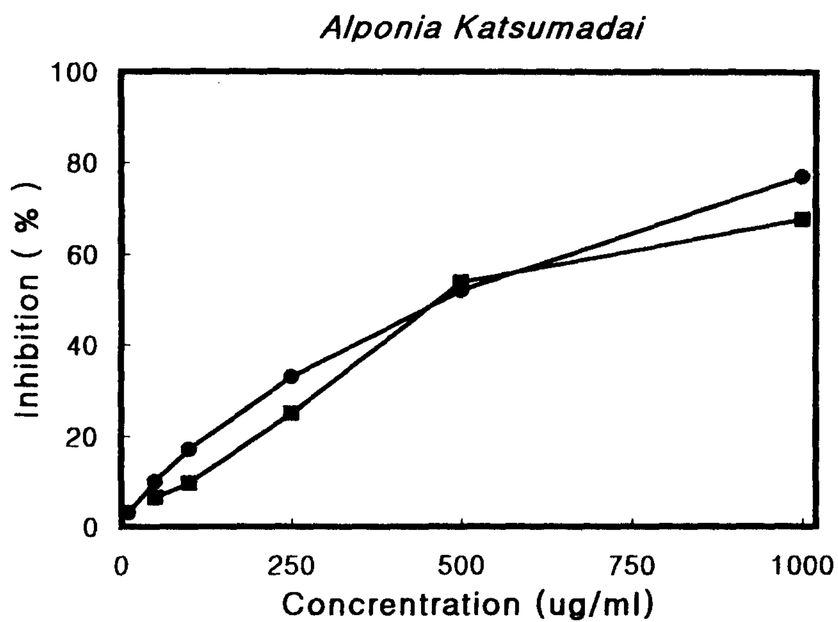

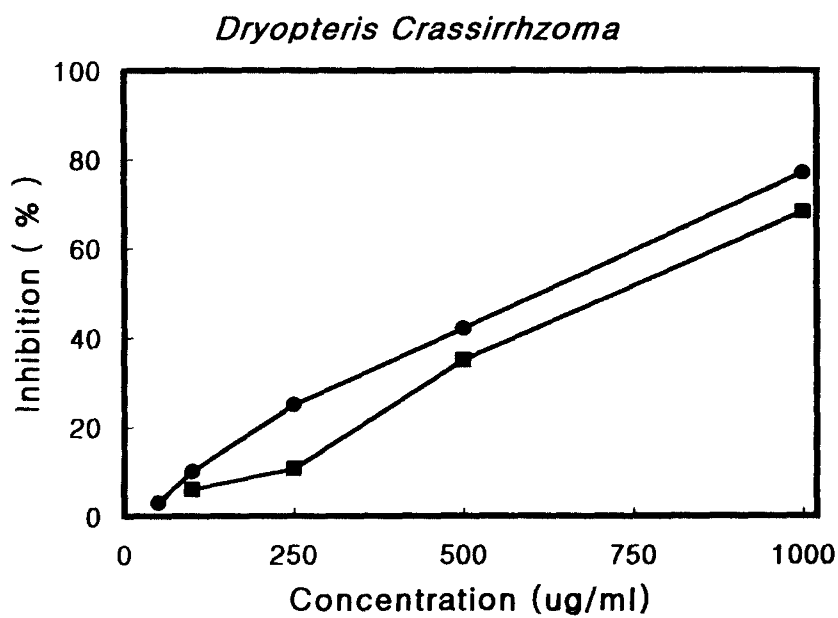

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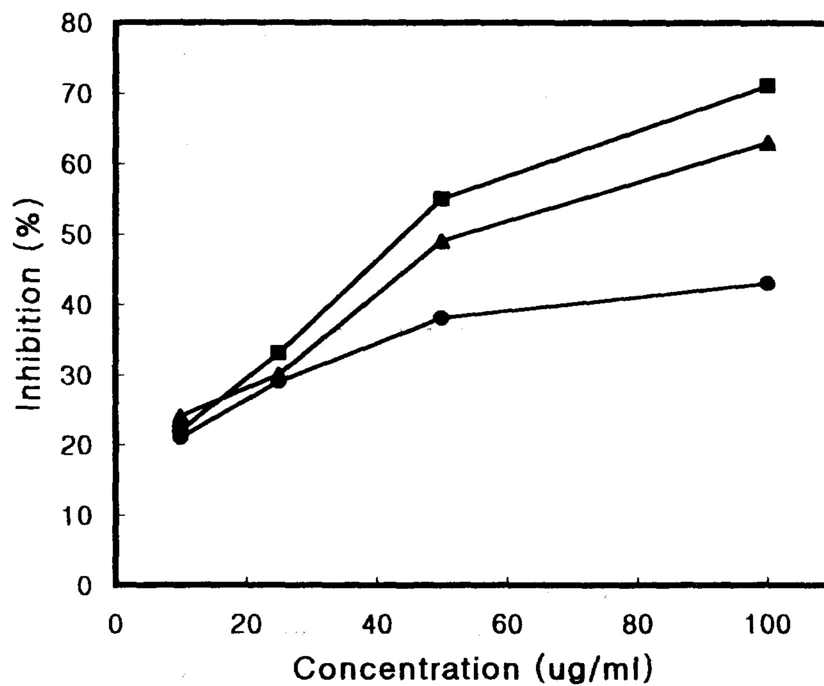

Fig 2. Dose-dependent inhibition on antioxidative activity

—●— *Alponia Katsumada* —■— *Curcumma longa*
—▲— *Areca Catechu*

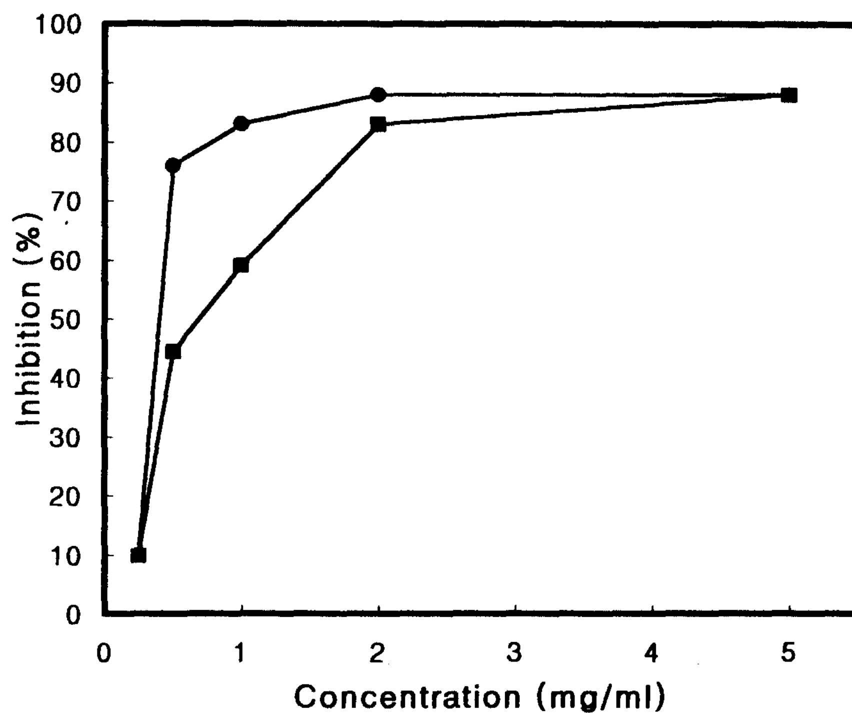

Fig 3. Dose−dependent inhibition on hyaluronidase activity
Effects on the enzyme activity of *Areca Catechu* (■) *and Glycyrrbiza uralensis* (●) are indicated as % of inhibition compared with control

Table 1. Inhibition of biological activities in 7 plant extracts

Medicinal plants	Inhibition of HLE(%)		Ihibition of Hyaruronidase(%)		Antioxidative effect(%)	
	100ug/ml	1000ug/ml	1mg/ml	2mg/ml	10ug/ml	1000ug/ml
Alponia Katsumadai	17	77	19	66	21	73
Areca catechu	56	95	65	83	24	75
Cinnamomum cassia	30	90	5	50	15	44
Curcumma longa	25	100	4	7	22	81
Dryopteis crassirrhzona	10	77	9	68	11	37
Myristica fragrans	31	80	<0	<0	15	53
Glycyrrbiza uralensis	18	35	81	95	1	71

Glycyrrbiza uralensis are indicated as % of inhibition compared with the control.
* Data are indicated as mean (n=3), and their signigicances are P < 0.01