The Effects of *Alpinia katsumadai* Extract on Anti-inflammation and Melanogenesis

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Abstract

Several solvent extracts from *Alpinia katsumadai* were prepared and their various anti-inflammatory activities were evaluated. *Alpinia katsumadai* extract showed high various anti-inflammatory effects among the 8 medicinal plant extracts. Butanol extract from *Alpinia katsumadai* showed a potent anti-oxidative and free radical scavenging activities. Free radical scavenging effect of butanol fraction of *Alpinia katsumadai*(IC_{50} : 5㎍/㎖) was higher than butylated hydroxytoluene(IC_{50} : 50㎍/㎖) and ascorbic acid(IC_{50} : 22㎍/㎖). *Alpinia katsumadai* butanol fraction exhibited relatively high antioxidative activity(IC_{50} : 80㎍/㎖) compared to ascorbic acid. The inhibitory effect of *Alpinia katsumadai* ethanol extract on elastase exhibited 10 to 78% at 100 to 1000㎍/㎖ concentration ; the IC_{50} values with 465.7㎍/㎖ for porcine pancreatic elastase(PPE) and 481.9㎍/㎖ for human leukocyte elastase(HLE), respectively. The *Alpinia katsumadai* extract inhibited effectively hyaluronidase activity(IC_{50}

: 335㎍/㎖), and showed inhibition *in vitro* on delayed hypersensitivity when it was topically applied. These results suggest that *Alpinia katsumadai* extract may reduce inflammatory skin trouble. The *Alpinia katsumadai* extract also showed higher inhibitory effect of melanin biosynthesis on cultured melanoma cell compared to arbutin and kojic acid.

Introduction

Plant extracts have been evaluated for developing natural antioxidants and melanogenesis inhibitors that are be involved in anti-aging(1). Many endogeneous plant compounds have been reported to retard the oxidation process in their natural environment and in products to which they have been added(2). Natural antioxidants occur in all higher plants and in all parts of the plant. Typical compounds that possess antioxidative activities are including tocopherols, flavonoids, cinnamic acid derivatives, and polyfunctional organic acids. Recent studies indicated that the compounds with antioxidative and free radical scavenging activities could inhibit mutagenesis and carcinogenesis in addition to retardation of aging(1-3). High molecular weight hyaluronic acid has an important role in the regulation of scarless repair in fetal wound healing by markedly diminishing the inflammatory response(4). However, degradation products of hyaluronic acid lead to increase inflammation, angiogenesis, fibrosis, and collagen deposition in wound healing. Recently, the harmfulness of ultraviolet(UV) radiation is increasing due to destruction of ozone layer. Excessive exposure to UV radiation causes post-inflammatory pigmentation(4-5). Pigmentary disorders are caused by various factors, including inflammation, imbalance of hormones, and genetic disorder(6). Melanins play a critical role in the absorption of free radicals and melanogenesis in the skin in a kind of process that produces photoprotective

agents against damaging effect of UV. Aging of the skin results from two main factors : genetic programming and permanent actinic damage due to environmental stress(UV, detergents, mechanical shocks)(7). In UV-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 damage to elastin and collagen fibers and finally causing sagging(8). Recently, a number of studies have been interested in interactions between elastase and its inhibitors including unsaturated fatty acids, peptides, flavonoids, and terpenoids(9-15). It has proposed but not yet fully demonstrated(16) that the benefical effects of these inhibitors on the typical signs of cellulitis are obtained by inhibition of elastase, the lysosomal enzyme which regulate the turnover of the structural constituents of the extravascular matrix that surround the capillary walls(17-20). Therefore, with the aim of finding alternative anti-inflammatory agents that can safely be used in cosmetics, we have screened various medicinal plants extracts for their anti-inflammatory effects. Plant extracts having such biological activities may be a good choice for cosmetic purpose because of their relatively lower side effects.

We have screened the inhibitory effects of over 100 medicinal plants on elastase activity, and examined their anti-inflammatory effects. We have selected the *Alpinia katsumadai* extract as anti-inflammatory agents.

Alpinia katsumadai is native to Hainan Island in Soutern China, but is widely distributed in shaded woodland in Hong Kong. It is used in traditional Chinese medicine as an antiemetic and for treatment of stomach disorders. Previous investigations of *Alpinia katsumadai* have reported a variety of diaryheptanoids, chalcones and flavonoids, monoterpenes and sesqiterpenoids (21). To clarify anti-inflammatory effects of *Alpinia katsumadai* extract, we have studied the anti-oxidative activity, free radical scavenging effect,

inhibition of elastase and inhibition of hyaluronidase *in vitro*. Inhibitory effect of *Alpinia katsumadai* extract on melanogenesis was evaluated using B16 melanoma cell culture. The safety of *Alpinia katsumadai* was also investigated by cytotoxicity on human fibroblasts and skin irritation testing.

Materials and 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.(St. Louis, USA). [N-Succ-$(Ala)_3$-p-nitroanilide] and Meo-Succ-$(Ala)_2$-Pro-Val-p-nitroanilide were purchased from Calbiochem(MA, USA). Other reagents were analytical grade from commercial sources.

Preparation of plant extracts

Medicinal plants were purchased from the oriental medicine market located in Seoul, South Korea. Each of the plants was sliced and weighed. Powder(100g) from each plant was extracted with 500㎖ of ethanol : water(80 : 20, v/v) at room temperature for 7 days. After filtration, this extract was evaporated to dryness under vacuum, and then completely dried by lyophilization. *Alpinia katsumadai* extract was fractionated with different solvents(ether, hexane, chloroform, ethylacetate, butanol, and water), and was used as the sample in this study.

Antioxidative activity

A lipid peroxidation system was induced by Fenton's reagent. Each test sample(0.1㎖) and ethyl linoleate(10㎕) were added to incubation medium(4.89

㎖) containing 2% sodium dodecyl sulfate, 1㎛M ferrous chloride and 0.5㎛M hydrogen peroxide. The known synthetic antioxidant, butylated hydroxytoluene (BHT) was used as a reference compound. The incubation medium was kept at 55℃ for 16hrs. Each reaction mixture(0.2㎖) was transferred into a test tube, followed by addition of 4% BHT(50㎕) to prevent further oxidation. Antioxidative activity of the sample was measured using thiobarbituric acid(TBA) assay according to the method of Ohkawa *et al.*(22).

Free radical scavenging activity

Scavenging effect against free radical generation was measured by following the procedure of Fugita *et al.*(23). The sample solution(2㎖) was added to 2㎖ of 60㎛M 1,1-diphenyl-2-picryl hydrazine(DPPH) ethanolic solution and kept at room temperature for 30min. The absorbance was measured at 520㎚.

Assay for elastase and tyrosinase activity

Porcine pancreatic elastase(PPE : Sigma) and human leukocyte elastase (HLE, Sigma) was assayed spectrophotometrically by the modified method of James *et al.*(24), a using N-Succ-$(Ala)_3$-nitroanilide(S.A.N.A.) as a substrate, and monitoring the releasé of p-nitroaniline for 20min at 25℃ by measuring the absorbance at 410㎚. Tyrosinase activity is generally determined by spectrophotometry. The procedure followed that described by Vanni *et al.*(25). The reaction mixture consisted of 0.05M phosphate buffer(pH 6.8, 2.3㎖), 1.5mM L-tyrosine solution(0.4㎖) and 2000U/㎖ mushroom tyrosinase(Sigma), in 0.05M phosphate buffer(pH 6.8, 0.1㎖). A sample solution(0.2㎖) was added to reaction mixture and incubated at 37℃ for 10min. The optical density at 475㎚ was measured by a spectrophotometer(Beckman). The percentage of inhibition was calculated as :

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

where, A is the enzyme activity without *Alpinia katsumadai* extract, and B is the activity in the presence of *Alpinia katsumadai* extract.

Anti-inflammatory effect by assay of hyaluronidase activity

Hyaluronidase activity was determined spectrophotometrically by measuring the amount of N-acetylglucosamine formed from sodium hyaluronate(26). 50㎕ of bovine hyaluronidase(7,900units/㎖) dissolved in 0.1M acetate buffer(pH 3.5) was mixed with 100㎕ of a designated concentration of *Alpinia katsumadai* extract dissolved in 5% DMSO, and then incubated in a water bath at 37℃ for 20min. The control group was treated with 100㎕ of 5% DMSO instead of the *Alpinia katsumadai* extract incubated in a water bath at 37℃ for 20min. This Ca^{2+} activated hyaluronidase was treated with 250㎕ of sodium hyaluronate(1.2㎎/㎖) dissolved in 0.1M acetate buffer(pH 3.5), and then incubated in a water bath at 37℃ for 40min. 100㎕ of 0.4N sodium hydroxide and 100㎕ of 0.4M potassium borate were added to reaction mixture, and then incubated in a boiling water bath for 3min. After cooling to room temperature, 3㎖ of dimethyaminobenzaldehyde solution(4g of p-dimethyamino-benzaldehyde dissolved in 350㎖ of 100% acetic acid and 50㎖ of 10N hydrochloric acid) was added to the reaction mixture, and then incubated in a water bath at 37℃ for 20min. Optical density at 585㎚ of the reaction mixture was measured by using a spectrophotometer. The percentage of inhibition was calculated as :

$$\text{Inhibition}(\%) = [(OD_c - OD_s)/OD_c] \times 100$$

where OD_c is the OD at 585㎚ of the control, and OD_s is the OD at 585㎚ of the sample.

Anti-inflammatory activity *in vivo*

Inhibitory activity against delayed hypersensitivity was measured according

to the method of Tarayre *et al.*(27). Briefly, 3% picryl chloride in acetone was applied to abdomen of mice(18-22g). One week later, 3% picryl chloride was applied to ears of mice and ear thickness was measured 24hrs after the treatment of picryl chloride solution. Preparations of the test compounds were applied to ears of mice daily for 7 days. The differences between ear thickness of the extract-treated group and the control group treated with picryl chloride and vehicle only were regarded as an inhibitory activity.

Inhibition of Melanogenesis

We examined the inhibition of melanogenesis in B-16 melanoma cell. B-16 melanoma cells were placed in 35㎜ dish at a density of 2.0×10^4cells/dish and cultured at 37℃ and 6.5% CO_2 in Dulbecco's modified eagle's medium (DMEM) containing 10% Fetal Bovine Serum(FBS) and 1% antibiotics. After 48hrs of cultivation, we replaced the medium containing extracts of various concentration. After 2 days, we replaced the cells with phosphate buffer saline(PBS) and collected the cells by trypsinization and centrifugation. We separated melanin from the pellet of the cells using 5% trichloroacetic acid and dissolved the melanin in 1N NaOH solution. We determined the melanin contents with an absorbance at 475㎚. A standard curve for melanin determination was prepared using synthetic melanin(Sigma). The cell number was determined with the coulter counter.

Cytotoxicity test

Human fibroblasts were seeded in 96 well plate at a density of 10^4cell/well, supplemented with 0.2㎖ of Eagle's minimal essential medium(EMEM) containing 2% FBS, and incubated for 24 hours. After adding sample, the cells were incubated for another 24 hours, the survival and proliferation of cells were evaluated by MTT assay(28). MTT solution(0.1㎖) was added to each

wells and incubated for 3 hours. After removing the media, 0.5㎖ of DMSO was added and formed formazan was measured by reading the absorbance at 570㎚ using ELISA reader.

Human skin irritation test

We studied the potential of the *Alpinia katsumadai* to irritate human skin in 50 healthy female volunteers using 48hrs closed patch. No skin irritation occurred after application in 50 volunteers.

Results and Discussion

The anti-inflammatory effects of 8 plant extracts in the initial screening are shown in Table I. In this assay, the extract of *Alpinia katsumadai* among 8 plant extracts including *Alpinia katsumadai, Cinnamomum cassia, Miristica fragrans, Dryopteris crassirrhizoma, Scutellaria baicalensis, Lonicera japonica, Thea sinensis*(Green tea), *and Thea sinensis*(Oolong tea) shows high anti-inflammatory activities. Table II shows the antioxidative and free radical scavenging activities against several solvent fractions of *Alpinia katsumadai.* The butanol fraction exhibited higher activities than other solvent fractions.

The antioxidative activity of the *Alpinia katsumadai* butanol fraction as well as reference compounds such as L-ascorbic acid and BHT, gave good dose response relationships(data not shown). BHT is one of the most potent inhibitor of TBA-reactive material formation. IC_{50} value of BHT was reported to be 1.5㎍/㎖, while other reference compound, L-ascorbic acid showed IC_{50} values of 219㎍/㎖. IC_{50} value of *Alpinia katsumadai* butanol fraction was found to be 80㎍/㎖(Table II), which showed much higher activity than the ascorbic acid. Natural anti-oxidants are usually phenolic or polyphenolic compounds and these compounds include tocopherol, flavonoid, and cinnamic

acid derivatives(29). The first type of antioxidant inhibits the formation of free radicals which may initiate oxidation. The second type of antioxidant inhibits the free radical chain propagation reactions. Therefore, some of plant extracts may act at the initiation stage of peroxidation interfering with Fenton's reaction. Figure 1 shows the free radical scavenging activity of *Alpinia katsumadai* ethanol extract and reference compounds. IC_{50} values of ascorbic acid and BHT were found to be 22㎍/㎖ and 50㎍/㎖, respectively. L-Ascorbic acid is known to be one of the most potent free radical scavenger. On the other hand, IC_{50} value of *Alpinia katsumadai*(IC_{50} : 18.2㎍/㎖) showed much higher activity than the well-known reference compounds. Furthermore, butanol fraction exhibited much higher activity for free radical scavenging (IC_{50} : 5㎍/㎖).

Free radical damage to biosystem is one of the major processes that contribute to the degenerative diseases like cancer and aging(30). Detailed free radical mechanisms and their quantitiative contributions are still not clear. Despite these uncertainties, it is clear that free radical scavengers may inhibit endogeneous, metabolically driven, oxidative DNA damage, as well as mutations and aging caused by exogeneous agents(31-33).

As shown in Figure 2, the *Alpinia katsumadai* with 0.1~1㎎/㎖ as the final concentration exhibited 15% to 84% inhibition. Anti-hyaluronidase activity of *Glycyrrhiza uralensis* as a control exhibited 10~84% of inhibition at the concentration of 0.1~1㎎/㎖. IC_{50} values of *Glycyrrhiza uralensis* and *Alpinia katsumadai* were found to be 330㎍/㎖ and 335㎍/㎖, respectively. Figure 3 shows the dose-dependent inhibition of PPE and HLE by *Alpinia katsumadai* extract. The *Alpinia katsumadai* extract at 100 to 1000㎍/㎖ as the final concentration exhibited 10% to 78% inhibition, IC_{50} values are 465.7㎍/㎖(PPE) and 481.9㎍/㎖(HLE), respectively. The 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 and collagen(34). One major target of cosmetologic research is to find effective elastase inhibitors. Table III presented the topical anti-inflammatory activity of the *Alpinia katsumadai* extract. Although the activity was found to be weak compared to the potent activity of the reference compounds, prednisolone, but *Alpinia katsumadai* showed anti-hypersensitivity.

Mouse ear edema assay is frequently used animal model for topical application of the various compounds(35). In croton oil induced ear edema, 12-o-tetradecanoylphobol-13-acetate(TPA) is suggested to produce edema and leukotriene B_4 is found in ear area. In arachidonic acid induced ear edema, ecosanoids such as prostaglandins and leukotriens are involved in the inflammation. The *Alpinia katsumadai* extract showed the anti-inflammatory activity against both inflammator and mediator, which suggested that the *Alpinia katsumadai* extract might reduce the inflammation in the skin induced by various inducers of inflammation. Because the *Alpinia katsumadai* extract also showed the inhibitory activity against delayed hypersensitivity, the *Alpinia katsumadai* extract may be an useful agent to treat various skin problems.

The active ingredient in *Alpinia katsumadai* may be a xanthine derivative. Plants and plant alkaloid derivatives, such as caffeine in coffee and tea, have been used for centuries as medicinals and as poultices on wounds. Theophyllin is a phosphodiesterase inhibitor and a potent antagonist to histamine. Aminophylline is a potent vasodilator. Xanthine have even been shown to inhibit histamine release from human leukocytes(36). Similar effects would be anticipated from *Alpinia katsumadai* extract. These reports indicate that *Alpinia katsumadai* extract may contain the compounds analogeous to alkaloids, contributing the anti-inflammatory activities *in vivo* and *in vitro*. We are undergoing the seperation of active compound of *Alpinia katsumadai* and will report the results later. The safety of *Alpinia katsumadai* extract

was evaluated by cytotoxicity on human fibroblasts and the skin irritation testing. In cytotoxicity testing on human fibroblasts, LD_{50} of *Alpinia katsumadai* extract was about 50㎎/㎖, not change cell proliferation at a concentration of 50㎎/㎖. According to the test, noticeable primary irritation did not appear, and minimal erythema reaction was detected in 4 out of 50 volunteers at 24hrs observation, but, all disappeared at 48hrs. For the patch base, 2 from 50 volunteers showed minimal erythema at 24hrs, but the reaction disappeared at 48hrs.

In this study, the effect of *Alpinia katsumadai* on melanogenesis was examined using tyrosinase assay(IC_{50} : 250㎍/㎖) and B-16 melanoma cells *in vitro*. *Alpinia katsumadai* extract showed higher inhibitory effect on cultured melanoma cell(IC_{50} : 35㎍/㎖) but exhibited lower effect on inhibition of tyrosinase. *Alpinia katsumadai* extract inhibited cellular pigmentation by reducing oxidative stress and inhibiting of tyrosinase, and it is expected to have whitening effect on human skin. In conclusion, we suggests that *Alpinia katsumadai* extract as an active ingredients for anti-aging may be sufficient to satisfy consumers and cosmetic scientist.

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Captions of Figure

Figure 1. Free radical scavenging activities of ethanol extract of *Alpinia katsumadai* and reference compound. IC_{50} Values are 22㎍/㎖ (◆), 18.2㎍/㎖(■), and 50㎍/㎖(▲)

Figure 2. Dose-dependent inhibition of hyaluronidase activity. The effects of *Alpinia katsumadai* 80% ethanol extract(■) and *Glycyrrhiza uralensis*(●) on enzyme activity are indicated as % inhibition compared to the control. IC_{50} values are 335㎍/㎖(■) and 330㎍/㎖(●)

Figure 3. Anti-elastase activity of *Alpinia katsumadai* 80% ethanol extract. Dose-response curse for the inhibitory effects on porcine pancreatic elastase(PPE)(IC_{50} : 465.7㎍/㎖), human leukocyte elastase(HLE) (IC_{50} : 481.9㎍/㎖), and ELHIBIN(IC_{50} : 980㎍/㎖, Pentapham, Switzerland) as a control.

Table I. Screening of several anti-inflammatory effects in ethanol extracts of the 8 medicinal plants. Data shown in table are the IC_{50} values compared to standard samples.

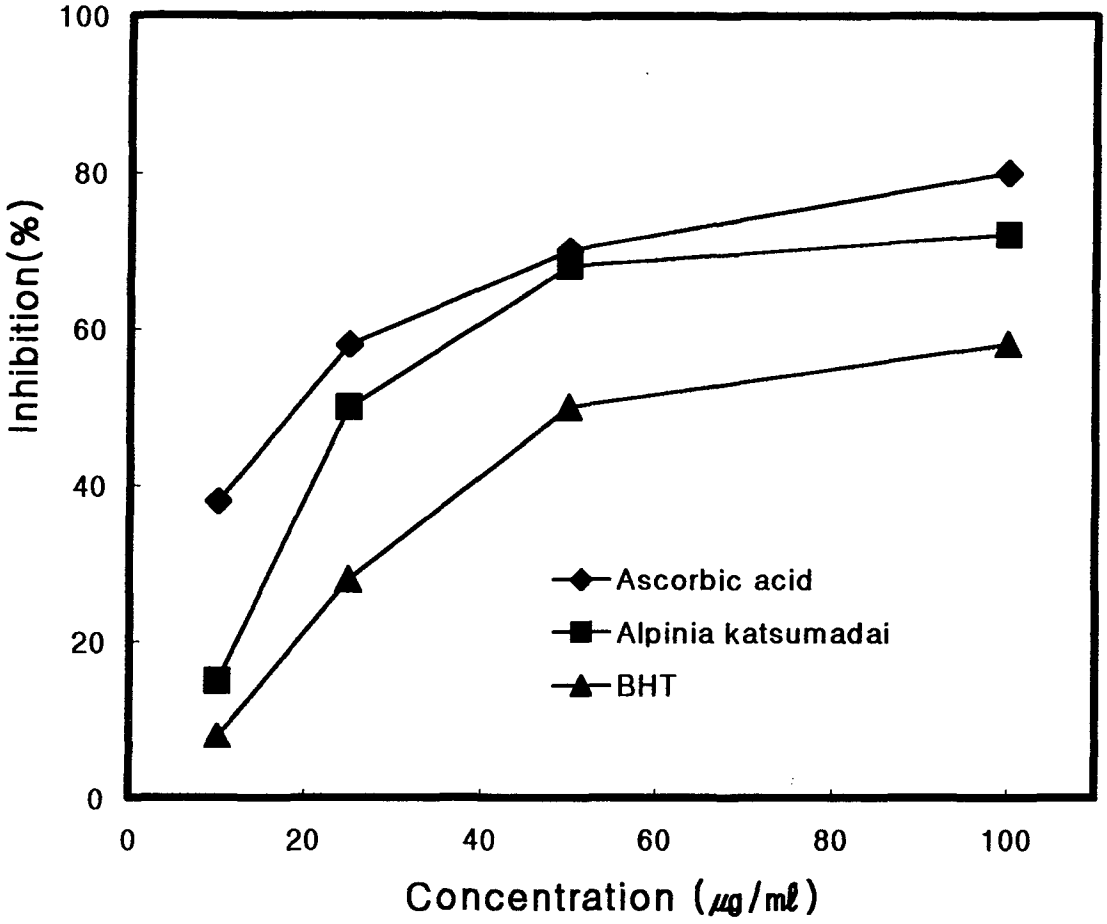
Plants	Anti-oxidative activity (IC_{50}, μg/mℓ)	Free radical scavenging (IC_{50}, μg/mℓ)	Tyrosinase inhibition (IC_{50}, μg/mℓ)	Anti-elastase activity (IC_{50}, μg/mℓ)
Alpinia katsumadai	190	18.2	250	465.7
Cinnamomun cassia	340	9.2	450	208.7
Thea sinensis (green)	260	15.5	950	-
Myristica fragrans	>1000	72.5	-	>1000
Dryopteris crassirrhizoma	750	21.5	-	714.4
Scutellaria baicalensis	380	42.5	-	-
Thea sinensis (oolong)	670	35.5	1000	-
Lonicera japonica	>1000	40	>1000	-
Ascorbic acid	217	22	-	-
Kojic acid	-	-	37	-
ELHIBIN	-	-	-	980

Table II. Anti-oxidative and free radical scavenging activities of several solvent fractions from *Alpinia katsumadai*.

Solvent fractions	Anti-oxidative activity (IC_{50}, μg/mℓ)	Free radical scavenging (IC_{50}, μg/mℓ)
Ethanol	190	18.2
Hexane	>1000	>1000
Ethyl acetate	180	15
Buthanol	80	5
Water	260	12
Ascorbic acid	217	22

Table III. Inhibition of delayed hypersensitivity by the *Alpinia katsumadai* 80% ethanol extract. P > 0.001, significantly different from control (n= 10)

Groups	Dose (μg/ear)	Thickness increased mm, (% inhibition)
Control	-	0.25 ± 0.05
Prednisolone	7 x 0.1	0.05 ± 0.005 (77.0)
5% *Alpinia katsumadai*	7 x 50	0.16 ± 0.04 (27.0)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Inhibition(%)
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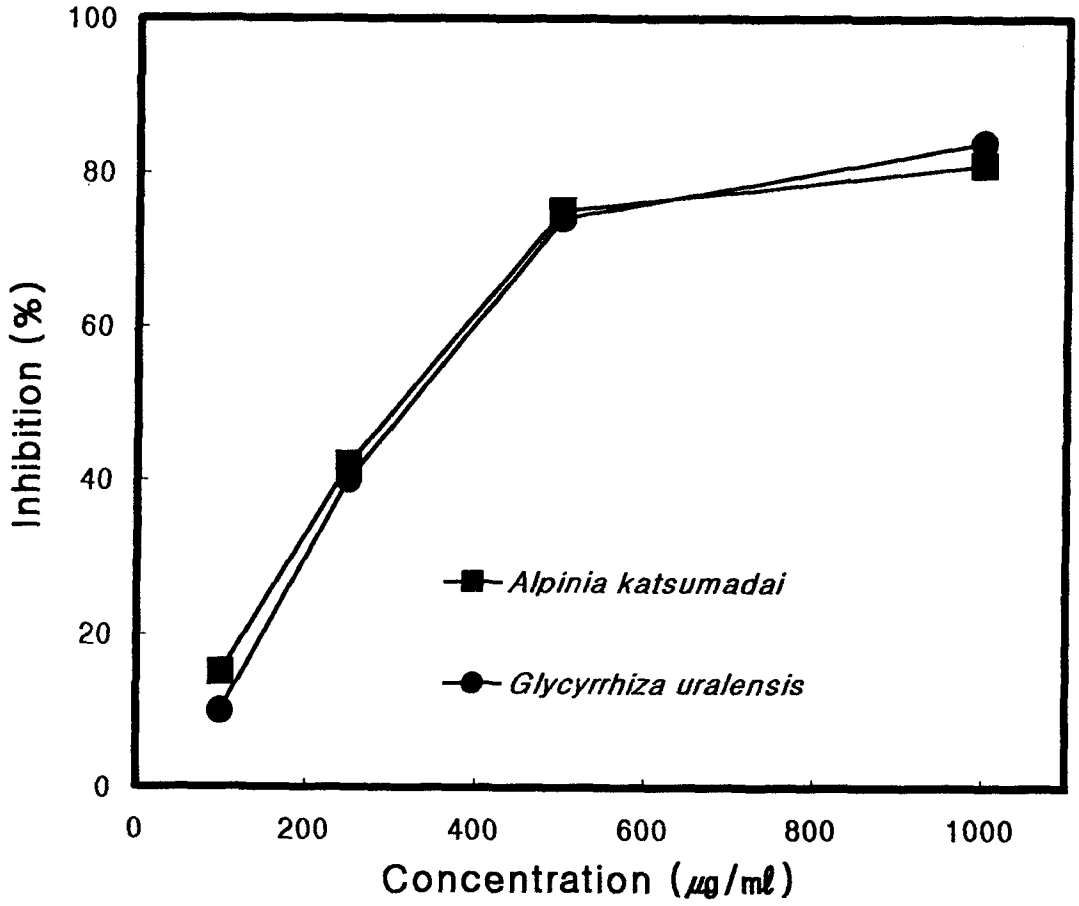
Concentration (㎍/㎖)
Ascorbic acid
Alpinia katsumadai
BHT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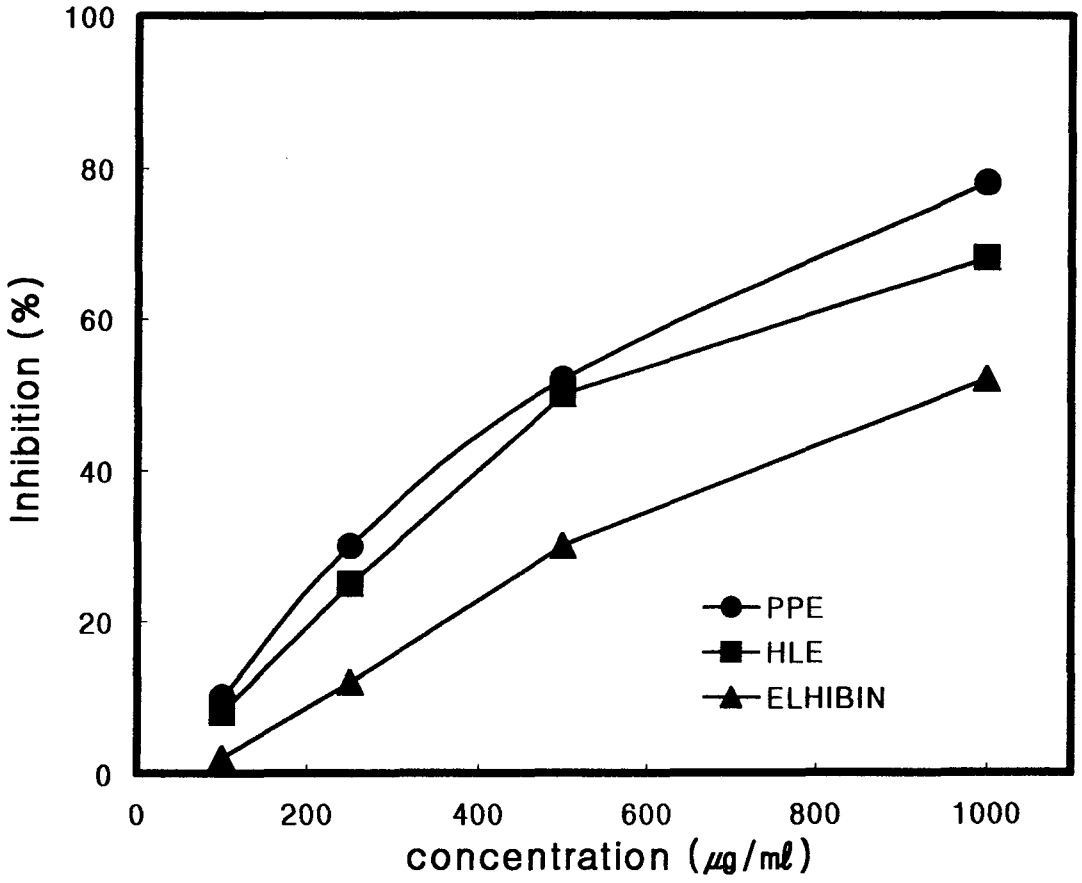
Glycyrrhiza uralensis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