Antioxidative Activity and Anti-melanogenic Effect of the Extract from the Leaves of Robinia Pseudo-acacia L.

Jin-Su Chung, Ghee-Hong Jin and Yoon-Kee Park, M.D.*

R&D Center of Household Products & Personal Care, Cheiljedang Co., Inchon, and Department of Dermatology, Yonsei University College of Medicine, Seoul, Korea*

***Background*: Plant extracts have been widely used as important therapeutic drugs for many centuries all over the world. There have been many reports that natural products have various kinds of biological activities such as anti-allergy, anti-inflammatory and anti-microbial activities. Recently, the screening for the efficacy and safety of natural products has been extensively performed.**

***Objective*: This study was carried out to find a beneficial plant extract possessing excellent antioxidative and anti-melanogenic activities. We have found that the leaf of Robinia pseudo-acacia L. has active substances which are involved in those activities.**

***Methods*: To confirm the antioxidative activity of the extract obtained from the leaves of Robinia pseudo-acacia L., scavenging ability of the extract on DPPH free radicals and its inhibitory effects on lipid autoxidation and peroxidation were investigated.**

In addition, inhibitory effects of the extract on mushroom tyrosinase as well as melanin biosynthesis in cultured B16 melanoma cells were evaluated.

***Results*: The acacia extract showed not only powerful antioxidative activity but also anti-melanogenic acitivity as strong as that of arbutin which is a well known inhibitor of melanogenesis.**

***Conclusion*: These results suggest that the extract from the leaves of Robinia pseudo-acacia L. could be used as a lightening and antioxidative agent for the skin.**

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Key Words : Acacia extract, B16 melanoma, Anti-melanogenic, Antioxidative

Recently, plant extracts which are known to have various biological activities such as anti-inflammatory, anti-microbial, anti-aging and anti-melanogenic activities, have been reported[1]. These natural products have been used as main additives in a wide range of personal-care products. Especially, the antioxidative and anti-melanogenic substances present in natural plants have recently received much attention in the cosmetic field, because many synthetic antioxidative and anti-melanogenic substances are being questioned regarding their safety.

In this study, we examined antioxidative and anti-melanogenic activities of the extract obtained from the leaves of Robinia pseudo-acacia L.

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Reprint request to : Jin-Su Chung, R&D Center of Household Products & Personal Care, Cheiljedang Co., Inchon, 400-103, and Department of Dermatology*, Yonsei University College of Medicine, Seoul, Korea

MATERIALS AND METHODS

Preparation of extract from the leaves of Robinia pseudo-acacia L.

Fresh leaves of Robinia pseudo-acacia L. were collected in Inchon, Korea.

The air-dried leaves were frozen in liquid nitrogen

and powdered in porcelain mortar. Ten gram mass of the powdered leaves was extracted with 200 ml of 90% ethanol, stirred overnight at room temperature. The extract solution was centrifuged at 3,000 rpm for 10 min. The supernatant was filtered (0.45μm filter, Gelman Sciences, Michigan, USA) and concentrated in a vacuum evaporator.

Free-radical scavenging activity

The modified method of Fujita et al[2] using a moderately stable free-radical, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) (Sigma, St. Louis, Mo., USA) was used to determine the free-radical scavenging activities of the acacia extract and reference compounds including L-ascorbic acid (Sigma, St. Louis, Mo., USA), butylated hydroxytoluene (BHT) (Ueno, Osaka, Japan) and rutin (Sigma, St. Louis, Mo., USA). The test sample was dissolved in absolute ethanol, and 1.0 ml of the ethanolic sample solution was added to 1.0 ml of 0.1 mM DPPH methanolic solution. The mixture was incubated at 37℃ for 30 min, and the amount of free-radical to be left was measured at 516 nm by a spectrophotometer. The scavenging activity of the sample was expressed as its concentration that scavenges 50% of the free radicals generated.

Antioxidative activity against lipid autoxidation

The antioxidative activity of test sample toward linolenic acid (Sigma, St. Louis, Mo., USA) was determined by measuring the peroxide value as follows. One milliliter of the reaction mixture containing 2 mg of linolenic acid, 10 mg of Tween 20 and 0.1 mg of test sample per ml of 0.2 M potassium phosphate buffer (pH 7.4) was incubated for 24 hours at 37℃. After incubation, 0.1 ml of the mixture was mixed with 4.7 ml of 80% ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 20 mM ferrous ammonium sulfate in 3.5% hydrochloric acid. After 3 min, the absorbance of the mixture was measured at 500 nm[3].

Soybean lipoxygenase inhibition assay

The inhibitory effects of test samples on soybean lipoxygenase (Type I) (Sigma, St. Louis, Mo., USA) were assayed by the method of Baumann et al[4]. All samples were tested in duplicate.

Mushroom tyrosinase inhibition assay

For this assay, the modified method of Tomita et al was used[5], and arbutin (Kaden, Hamburg, Germany) was used as a reference compound. The reaction mixture consisted of 1.15 ml of 0.1 M potassium phosphate buffer (pH 6.5), 0.1 ml of 3 mM L-tyrosine solution, 0.05 ml of 2000 U/ml mushroom tyrosinase (Sigma, St. Louis, Mo., USA), in 0.05 M potassium phosphate buffer (pH 6.5) and 0.2 ml of test sample. After incubation at 37 ℃ for 10 min, the optical density of the reaction mixture was measured at 475 nm, and the percent inhibition of mushroom tyrosinase activity was calculated as follows.

$$\% \text{ inhibition} = \{(A-(B-C))/A\} \times 100$$

A = Optical density at 475 nm after incubation without test sample.

B = Optical density at 475 nm after incubation with test sample.

C = Optical density at 475 nm after incubation with test sample, without mushroom tyrosinase.

Inhibition of melanin biosynthesis in cultured B16 melanoma cells

B16 melanoma cells provided from KCLB (Korea Cell Line Bank, Seoul, Korea) were grown in DMEM (Gibco BRL, Grand Island, N.Y., USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, N.Y., USA) in a 5% CO2 humidified incubator. The B16 melanoma cells were suspended in fresh DMEM containing 10% FBS at 5×10^4 cells/ml. Four point eight milliliter of the cell suspension was poured into a T25 tissue culture flask (Nunc, Roskilde, Denmark) and allowed overnight to completely adhere to the flask. Test sample (0.2 ml) dissolved in sterile water was added to the flask and incubated at 37℃ for 3 days. After 3 days, the adherent cells were washed twice with phosphate-buffered saline (PBS) (Gibco BRL, Grand Island, N.Y., USA) and detached from the flask by trypsin treatment. The detached cells were collected by centrifugation and counted by hemocytometer. After centrifugation of the counted cells, the cell pellets were dissolved in 1.0 ml of 1 N NaOH, and the absorbance of the solution was measured at 475 nm[6]. The effects of the test samples on melanin content per 1×10^6 cells were expressed as the percent inhibition of the value obtained in B16 melanoma cells cultured with sterile water alone (control).

RESULT

Effect of the extract of Robinia pseudo-acacia L. on free-radical scavenging activity

To evaluate free-radical scavenging activity, SC_{50} value defined as the sample concentration (μg/ml) required for scavenging 50% of the free radicals which are produced in DPPH free radical generating system, was used. The free radical scavenging activity of the extract of Robinia pseudo-acacia L. was shown in Table 1. Compared with other pure antioxidants (L-ascorbic acid, BHT and rutin), the extract showed somewhat low radical-scavenging activity.

Inhibitory effect on the autoxidation of linolenic acid

For this assay, the final concentrations of all samples tested were adjusted to 0.1 mg of test sample in 1.0 ml of reaction mixture. The extract of Robinia pseudo-acacia L. remarkably suppressed the autoxidation of linolenic acid. The antioxidative activity was strong or similar in comparison with those of other antioxidants (L-ascorbic acid, BHT and maltol) used in this assay (Table 2).

Table 1. Scavenging activity of various antioxidants against DPPH free radicals

Sample	SC_{50} (μg/ml)
acacia extract	12.9
L-ascorbic acid	2.3
BHT	6.1
rutin	5.1

Data on L-ascorbic acid, BHT and rutin are given as reference values and expressed as SC_{50} defined as the sample concentration required to scavenge 50% of the free radicals generated.

Table 2. Antioxidative activity against the autoxidation of linolenic acid

Sample	Inhibition (%)
acacia extract	51.8 ± 0.2
L-ascorbic acid	34.9 ± 0.5
BHT	50.7 ± 1.0
maltol	50.8 ± 1.3

The final concentration of sample tested was adjusted to 0.1 mg of test sample in 1.0 ml of reaction mixture. Data is expressed as the mean ±difference of duplicate.

Effect of the extract of Robinia pseudo-acacia L. on soybean lipoxygenase

The extract of Robinia pseudo-acacia L. was also tested against soybean lipoxygenase to ascertain whether the extract affects the enzymatic peroxidation of lipid or not. Of all samples tested, the extract of Robinia pseudo-acacia L. showed the strongest antioxidative activity against lipid peroxidation (Table 3). However, L-ascorbic acid did not prevent the lipid peroxidation by soybean lipoxygenase, although it is a potent free radical scavenger at very low concentration as shown in Table 1. Thus, it was reconfirmed that there is a poor correlationship between the free radical scavenging activity and the antioxidative activity[7].

Effect of the extract of Robinia pseudo-acacia L. on mushroom tyrosinase

To confirm the anti-melanogenic ability of the extract of Robinia pseudo-acacia L., inhibitory effect of the extract on mushroom tyrosinase was investigated by in vitro enzyme assay. The extract inhibited tyrosinase in a dose-dependent manner (Fig. 1). The IC_{50} value defined as the sample concentration (μg/ml) required to inhibit 50% of mushroom tyrosinase activity was calculated. Compared with arbutin known well as a tyrosinase inhibitor, the IC_{50} values of the extract and arbutin were 234.5 μg/ml and 132.1 μg/ml, respectively.

Table 3. Inhibitory effect on soybean lipoxygenase

Sample	Inhibition (%)
acacia extract	82.1 ± 5.1
L-ascorbic acid	0.4 ± 2.9
BHT	66.6 ± 0.8
rutin	49.0 ± 6.5

The final concentration of sample tested was adjusted to 0.1 mg of test sample in 1.0 ml of reaction mixture. Data is expressed as the mean ±difference of duplicate.

Effect of the extract of Robinia pseudo-acacia L. on cultured B16 melanoma cells

To confirm whether the acacia extract affects

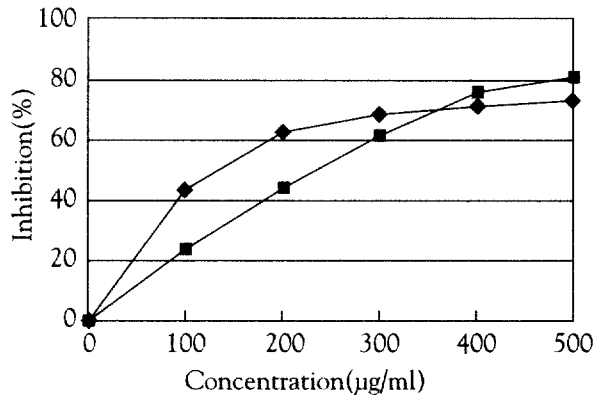

Fig. 1. Inhibitory effect on mushroom tyrosinase (◆ ; arbutin, ■ ; acacia extract)

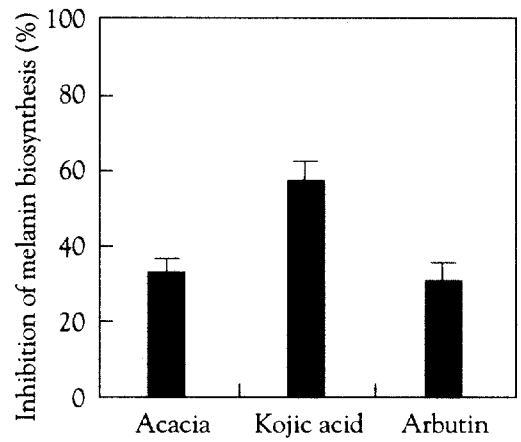

Fig. 2. Inhibitory effect of the acacia extract on melanogenesis in cultured B16 melanoma cells.
The effects of the acacia extract, kojic acid (Sigma, St. Louis, Mo., USA) and arbutin on melanogenesis in cultured B16 melanoma cells were investigated. B16 melanoma cells were cultured with 200 ug/ml of the test sample. The anti-melanogenic activity of the test sample was expressed as the percent inhibition of the value obtained in the control cells. The percent inhibition value is the average of the duplicate cultures, and the error bar represents the difference between duplicates.

melanogenesis in B16 melanoma cells, B16 melanoma cells were cultured with 200 μg/ml of the extract. Melanin biosynthesis was significantly decreased by the extract as shown in Fig. 2. These results indicate that the extract of Robinia pseudo-acacia L. has inhibitory effects on tyrosinase in cell-free system and melanin biosynthesis in cell culture system.

DISCUSSION

It has been reported that plants used in Chinese medicines have various radical-scavenging active components such as flavonoids, carotenoids, tannins and ascorbic acid[8]. Furthermore, polyphenols from plants have been reported to have generally various biological functionalities of anti-melanogenic activity, anti-allergy, anti-mutagenicity and so on. For example, polyphenols such as arbutin[9] and kojic acid[10] have been known to inhibit melanin biosynthesis. Melanin, which is the pigment of the hair and skin color, is synthesized in the melanosomes of melanocytes in the epidermal basal layer and transferred via dendrites to the surrounding keratinocytes. Biosynthesis of melanin starts from the amino acid tyrosine, which is converted to L-dopa and then to dopaquinone by the enzyme tyrosinase known to be a key enzyme for melanin biosynthesis in plants, microorganisms and mammalian cells[5]. One of the major functions of melanin is believed to be as a photoprotector which absorbs the radiation energy of UV, thus protecting the skin from inflammatory damage. Melanin also possesses SOD-like activity to scavenge superoxide anion radicals.

The involvement of free radicals and reactive oxygen species (ROS) in skin aging has been investigated in detail. Reactive oxygen species damage the skin directly and form lipidperoxide which results in the formation of insoluble pigments such as eumelanin (black) and phaeomelanin (yellowish, reddish). Of ROS, hydroxyl radical causes much physiological damage in skin, because it is the highest reactive radical which can react with fatty acids (particularly with polyunsaturated acids) of membrane, which leads to membrane disorganization and denatures the proteins of the connective tissue such as collagens, elastins and other membrane proteins. Hydroxyl radical also causes DNA damage. Consequently, in connective tissue, the loss of its elasticity and the decrease of cell proliferation are closely linked to skin aging[11].

In this study, we have focused our research on antioxidative activity and anti-melanogenic activity of the extract of Robinia pseudo-acacia L.. The leaf of Robinia pseudo-acacia L. has various abundant flavonoids such as acacetin, acaciin, quercetin and so on. These flavonoid components were expected to show strong antioxidative and anti-melanogenic activities. The extract obtained from the leaves of

Robinia pseudo-acacia L. showed somewhat low radical-scavenging activity when compared with other antioxidants including synthetic antioxidant BHT and rutin which has been demonstrated to be an effective inhibitor of iron-dependent lipid peroxidation[12]. However, if the pure material which has an effect on scavenging free radicals were purified from the extract, it could be expected relatively as a good radical scavenger. In particular, the extract inhibited more effectively soybean lipoxygenase than other antioxidants used in this assay. It also inhibited effectively tyrosinase, a key enzyme for melanin biosynthesis, like melanogenesis inhibitors such as arbutin and kojic acid used as cosmetic additives.

In this report, we have shown that the extract obtained from the leaves of Robinia pseudo-acacia L. has not only powerful antioxidative activity but also strong anti-melanogenic activity in cell-free system as well as melanin biosynthesis in cultured B16 melanoma cells. These results suggest that the extract may contribute to prevent skin aging and hyperpigmentation.

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