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Inhibitory Effects of Plant Extracts on Tyrosinase Activity and Melanin Synthesis

Hyen Joo Park[1], Kwang-Kyun Park[2], Jae-Kwan Hwang[3], Won-Yoon Chung[2], Seung-Eun Lee[4], and Sang Kook Lee[1,*]

[1]*College of Pharmacy, Seoul National University, Seoul 151-742, Korea*
[2]*College of Dentistry*
[3]*College of Engineering, Yonsei University*
[4]*Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science*

Abstract – In order to develop a new skin whitening agent, approximately 100 plant extracts were evaluated for their inhibitory activities against melanin biosynthesis in cultured mouse melanocyte melan-a cells. As a result, seven extracts exhibited over 50% inhibition of melanin synthesis compared to control at a concentration of 20 μg/ml. In particular, *Aster ageratoides* Turcz. var. *ageratoides* (branch, root, aerial, flower; IC_{50} = 17.3, 6.1, 13.6, 12.9 μg/ml, respectively) and *Physalis alkekengi* var. *francheti* (leaf, unripen fructus, aerial; IC_{50} =6 .5, 28.3, 23.9 μg/ml) markedly inhibited melanin synthesis. In addition, tyrosinase activity was monitored by the measurement of dopachrome formation from the oxidation of L-3,4-dihydroxyphenylalanine. Extracts of *A. ageratoides* Turcz. var. *ageratoides* (flower) and *P. alkekengi* var. *francheti* (leaf) showed the most potent tyrosinase inhibitory activity. These plants might be the potential candidate sources in the development of novel skin-whitening products.
Keywords – Melanin, Tyrosinase, Skin Whitening, *Physalis alkekengi* var. *francheti*, *Aster ageratoides* Turcz. var. *ageratoides*

Introduction

Melanin biosynthesis is initiated from hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosinase which continuously oxidizes DOPA to DOPA quinone formation (Shin *et al*., 1998). Tyrosinase (EC 1.14.18.1) is the key enzyme in the undesirable browning of fruits and vegetables, and the coloring of skin, hair and eyes in animals (Perez-Gilabert *et al*., 2001; Kubo *et al*., 2000). Since melanin has many functions this enzymatic oxidation of L-tyrosine to melanin is an important step. Indeed, the alterations of melanin biosynthesis occur in many disease states. Therefore, tyrosinase inhibitors have become increasingly significant in the food industry as well as in medicinal and cosmetic products (Mosher *et al*., 1983; Parvez *et al*., 2006). A number of tyrosinase inhibitors have been reported from natural or synthetic sources, but only a few are developed as skin-whitening agents. The major limitation is primarily due to various safety concerns. For example, linoleic acid, hinokitiol, kojic acid, naturally occurring hydroquinones, and catechols were reported to inhibit the enzyme activity, but these compounds also exhibited side effects (Seo *et al*., 2003). In addition, several phytochemicals have been reported as tyrosinase inhibitors including α-arbutin (Maeda *et al*., 1996; Curto *et al*., 1999; Virador *et al*., 1999), ellagic acid (Yoshimura *et al*., 2005), oxyresveratrol (Kim *et al*., 2002), chlorophorin, and norartocarpanone (Shimizu *et al*., 1998) for the development of better clinical relevant agents.

In our program to search for the inhibitors of melanin synthesis from natural products, we found the extracts of *A. ageratoides* Turcz. var. *ageratoides* and *P. alkekengi* var. *francheti* inhibited the tyrosinase activity and melanin formation in melan-a cell culture system. Although the chemical constituents of *A. ageratoides* Turcz. var. *ageratoides* (Clifford *et al*., 2006) and *P. alkekengi* var. *francheti* (Kawai *et al*., 2001; Asano *et al*., 1995) have been reported, the biological activities of *A. ageratoides* Turcz. var. *ageratoides* and *P. alkekengi* var. *francheti* have not been much studied yet.

We report herein for the first time the inhibitory effects of *A. ageratoides* Turcz. var. *ageratoides* and *P. alkekengi* var. *francheti* on melanin biosynthesis.

Experimental

Plant materials and preparation of extracts – Plant parts collected in the medicinal crop farm of Rural

*Author for correspondence
Tel: +82-2-880-2475; E-mail: sklee61@snu.ac.kr

Development Administration (RDA) in Korea, were dried, powdered, extracted with methanol. Solvent of extract was eliminated at 50°C in vacuum evaporator (JPSB1000, Eyela, Japan).

Enzyme assay – L-DOPA was used as a substrate, and tyrosinase (EC 1.14.18.1) activity was monitored by dopachrome formation at 475 nm for the appropriate time (usually not longer than 10 min). The assay was performed according to the procedure of Masatomo *et al* (1980) with minor modification. Briefly, the preincubation mixture consisted of 1.8 ml of 0.1 M phosphate buffer (pH 6.8), 0.6 ml of water, 0.1 ml of the sample solution, and 0.1 ml of the aqueous solution of mushroom tyrosinase (130 units). After preincubation for 5 min at room temperature, L-DOPA (0.4 ml of 6.3 mM) solution was added, and then the reaction was monitored at 475 nm for 3 min.

Cell line and culture – Melan-a cells (originally established by Dr. Bennett at the University of London) were kindly provided by Skin Research Institute, Amore-Pacific Co., Korea. Melan-a cells were grown in RPMI 1640 medium supplemented with antibiotics, 10% fetal bovine serum (FBS), and 20 nM TPA. The cells were incubated at 37 °C in a humidified atmosphere of 10% CO_2.

Inhibitory effect of melanin biosynthesis using cultured mouse melan-a cells – Confluent cultures of melan-a cells were rinsed with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) and lysed with 0.25% trypsin/EDTA. Cells were seeded into 6-well plastic culture plates at a density of 3×10^4 cells/well. At 72 h after plating, the media were replaced with or without test sample. After an additional 72 h incubation, the adherent cells exposed to the test samples were assayed.

Determination of melanin content in melan-a cells – The melanin content was determined as followings. After removing the media and washing the cells with Ca^{2+} and Mg^{2+}-free PBS, the cell pellet was dissolved in 0.3 ml of 1N NaOH, and incubated at 60°C for 10 min. The optical density at 475 nm was measured by an ELISA reader (Bio-Rad, U.S.A.). The melanin content was calculated by the standard curve of melanin.

Statistical analysis – Statistical analysis of data was performed using SigmaStat 2.03 (SPSS, Chicago, IL). One-way analysis of variance (ANOVA) was used to compare mean responses among the treatments. Statistical probability of $*P < 0.05$ was considered significant.

Results and discussion

In the present study, we screened for inhibitory activities of plant extracts against melanin biosynthesis in the mouse melanocytes melan-a cells. Of the extracts examined, nine extracts such as *Rubia akane* Nakai (Aerial), *Anthemis tinctoria* Kelwayi (Aerial), *Scabiosa tschiliensis* Gruning (Root), *Dioscorea batatus* Decne. (Root), *Isodon serra* (Maxim.) Kudo (Root), *Clematis heracleifolia* var. *davidiana* Hemsl. (Root), *Saponaria officinalis* L. (Flower, Root, Aerial) represented 20~40% inhibition of melanin synthesis compared to control at a concentration of 20 μg/ml. Especially, seven samples showed over 40% inhibition of melanin synthesis compared to control at a concentration of 20 μg/ml (Table 1). The extracts from *P. alkekengi* var. *francheti* (Leaf) inhibited melanin formation with 52%, 64%, and 72% in the concentration of 10, 20, and 30 μg/ml, respectively (IC_{50} = 6.5 μg/ml) as shown in Fig. 1 (A) and Fig. 1 (B). In addition, *A. ageratoides* Turcz. var. *ageratoides* (Root) showed a significant melanin formation inhibitory activity

Table 1. Effects of plant extracts on melanin levels in mouse melan-a cells

Plant	Part of Plant	Family Name	Activity*
Anthemis tinctoria Kelwayi	Root	Asteraceae	-
Anthemis tinctoria Kelwayi	Aerial	Asteraceae	+
Artemisia sieversiana Ehrh. ex Willd.	Aerial	Asteraceae	-
Artermisia absinthium L.	Aerial	Asteraceae	-
Aster ageratoides Turcz. var. *ageratoides*	Aerial	Asteraceae	+++
Aster ageratoides Turcz. var. *ageratoides*	Stem	Asteraceae	++
Aster ageratoides Turcz. var. *ageratoides*	Root	Asteraceae	+++
Aster ageratoides Turcz. var. *ageratoides*	Flower	Asteraceae	+++
Aster koraiensis Nakai	Aerial	Asteraceae	-
Benincasa cerifera Savi	Fructus	Cucurbitaceae	-
Benincasa cerifera Savi	Bark	Cucurbitaceae	-
Benincasa cerifera Savi	Flesh	Cucurbitaceae	-
Benincasa cerifera Savi	Seed	Cucurbitaceae	-

Table 1. Continued..

Plant	Part of Plant	Family Name	Activity*
Cannabis sativa L.	Root	Cannabaceae	-
Cannabis sativa L.	Leaf	Cannabaceae	-
Cannabis sativa L.	Stem	Cannabaceae	-
Carduus crispus L.	Root	Asteraceae	-
Carduus crispus L.	Aerial	Asteraceae	-
Carpesium macrocephalum Franch. &Sav.	Aerial	Asteraceae	-
Cassia occidentalis L.	Root	Fabaceae	-
Cassia occidentalis L.	Seed	Fabaceae	-
Cassia occidentalis L.	Aerial	Fabaceae	-
Celosia cristata L.	Root	Amaranthaceae	-
Celosia cristata L.	Aerial	Amaranthaceae	-
Clematis apiifolia DC.	Root	Ranunculaceae	-
Clematis apiifolia DC.	Aerial	Ranunculaceae	-
Clematis heracleifolia var. *davidiana* Hemsl.	Root	Ranunculaceae	+
Coix lacrymajobi var. *mayuen* (Rom.Caill.) Stapf	Root	Poaceae	-
Coix lacrymajobi var. *mayuen* (Rom.Caill.) Stapf	Arerial	Poaceae	-
Colocasia esculenta (L.) Schott	Root	Araceae	-
Cynanchum wilfordii (Maxim.) Hemsl.	Radix	Apocynaceae	-
Dendranthema boreale (Makino) Ling ex Kitam.	Root	Asteraceae	-
Dendranthema boreale (Makino) Ling ex Kitam.	Aerial	Asteraceae	-
Dendranthema indicum (L.) DesMoul.	Flower	Asteraceae	-
Dianthus japonicus Thunb. ex Murray	Root	Caryophyllaceae	-
Dianthus japonicus Thunb. ex Murray	Aerial	Caryophyllaceae	-
Dioscorea batatus Decne.	Root	Dioscoreaceae	+
Dioscorea batatus Decne.	Aerial	Dioscoreaceae	-
Echinacea purpurea (L.) Moench	Flower	Asteraceae	-
Echinacea purpurea (L.) Moench	Root	Asteraceae	-
Echinacea purpurea (L.) Moench	Aerial	Asteraceae	-
Erigeron annuus (L.) Pers.	Flower	Asteraceae	-
Erigeron annuus (L.) Pers.	Root	Asteraceae	-
Erigeron annuus (L.) Pers.	Aerial	Asteraceae	-
Gentiana scabra Bunge for. scabra	Root	Gentianaceae	-
Gentiana scabra Bunge for. scabra	Aerial	Gentianaceae	-
Helianthus annuus L.	Fructus	Asteraceae	-
Hibiscus trionum L.	Aerial	Asteraceae	-
Hypericum ascyron L.	Flower	Clusiaceae	-
Hypericum ascyron L.	Root	Clusiaceae	-
Hypericum ascyron L.	Aerial	Clusiaceae	-
Indigofera pseudotinctoria Matsum.	Root	Fabaceae	-
Indigofera pseudotinctoria Matsum.	Aerial	Fabaceae	-
Isatis tinctoria L.	Flower	Brassicaceae	-
Isodon inflexus (Thunb.) Kudo	Root	Labiatae	-
Isodon inflexus (Thunb.) Kudo	Aerial	Labiatae	-
Isodon japonicus (Burm.) Hara	Aerial	Labiatae	-
Isodon serra (Maxim.) Kudo	Root	Labiatae	+
Isodon serra (Maxim.) Kudo	Aerial	Labiatae	-
Ixeridium dentatum (Thunb. ex Mori) Tzvelev	Flower	Asteraceae	-

Table 1. Continued..

Plant	Part of Plant	Family Name	Activity*
Lagenaria leucantha Rusby	Unripen Fructus	Cucurbitaceae	-
Lagenaria leucantha Rusby	Seed	Cucurbitaceae	-
Lagenaria leucantha Rusby	Aerial	Cucurbitaceae	-
Lespedeza cuneata G.Don	Root	Fabaceae	-
Lespedeza cuneata G.Don	Aerial	Fabaceae	-
Lycium chinense Mill.	Dried Fructus	Solanaceae	-
Melilotus suaveolens Ledeb.	Root	Rosaceae	-
Melilotus suaveolens Ledeb.	Aerial	Rosaceae	-
Mosla dianthera (Buch.-Ham. ex Roxb.) ex Maxim.	Aerial	Compositae	-
Persicaria orientalis (L.) Spach	Flower	Polygonaceae	-
Persicaria orientalis (L.) Spach	Root	Polygonaceae	-
Persicaria orientalis (L.) Spach	Aerial	Polygonaceae	-
Physalis alkekengi var. *francheti* (Mast.) Hort	Unripen Fruit	Solanaceae	++
Physalis alkekengi var. *francheti* (Mast.) Hort	Rhizoma	Solanaceae	++
Physalis alkekengi var. *francheti* (Mast.) Hort	Aerial	Solanaceae	++
Physalis alkekengi var. francheti (Mast.) Hort	Leaf	Solanaceae	++
Physalis alkekengi var. francheti (Mast.) Hort	Stem	Solanaceae	++
Prunus mume Siebold &Zucc. for. *mume*	Stem	Rosaceae	-
Rehmannia glutinosa (Gaertn.) Libosch. ex Steud.	Flower	Scrophulariaceae	-
Rehmannia glutinosa (Gaertn.) Libosch. ex Steud.	Aerial	Scrophulariaceae	-
Ricinus communis L.	Root	Euphorbiaceae	-
Ricinus communis L.	Stem	Euphorbiaceae	-
Rubia akane Nakai	Root	Rubiaceae	-
Rubia akane Nakai	Aerial	Rubiaceae	+
Salvia miltiorrhiza Bunge	Flower	Labiatae	-
Saponaria officinalis L.	Flower	Caryophyllaceae	+
Saponaria officinalis L.	Root	Caryophyllaceae	++
Saponaria officinalis L.	Aerial	Caryophyllaceae	+
Scabiosa tschiliensis Gruning	Root	Dipsacaceae	+
Scabiosa tschiliensis Gruning	Aerial	Dipsacaceae	-
Sedum sarmentosum Bunge	Herba	Crassulaceae	-
Silybum marianum Gaertn.	Aerial	Asteraceae	-
Solanum nigrum L. var. nigrum	Root	Solanaceae	-
Taraxacum officinale Weber	Aerial	Compositae	-
Xanthium strumarium L.	Root	Compositae	-
Xanthium strumarium L.	Fructus	Compositae	-
Xanthium strumarium L.	Aerial	Compositae	-

*: +, weak inhibition (20~40%); ++, moderate inhibition (41~50%); +++, strong inhibition (51% above)

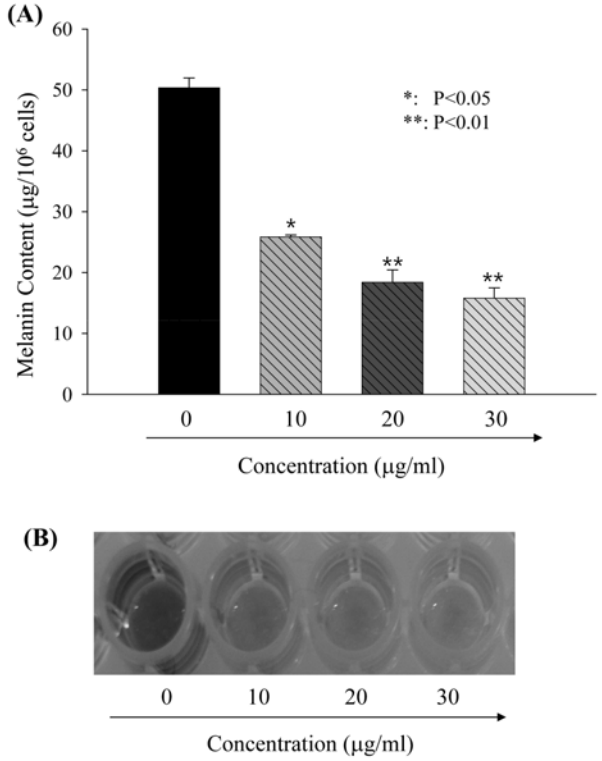

Fig. 1. (A) Effects of MeOH extracts of *P. alkekengi* var. *francheti* Mort (Leaf) on the melanin content. (B) Macroscopic views of the fraction of *P. alkekengi* var. *francheti* Mort (Leaf) on the melanin content. Data are representatives of three independent experiments. Statistical analyses were performed using the Student's t test and one-way ANOVA. The data shown are the means ± SD of four determinations. **P < 0.01, *P < 0.05 were considered statistically significant.

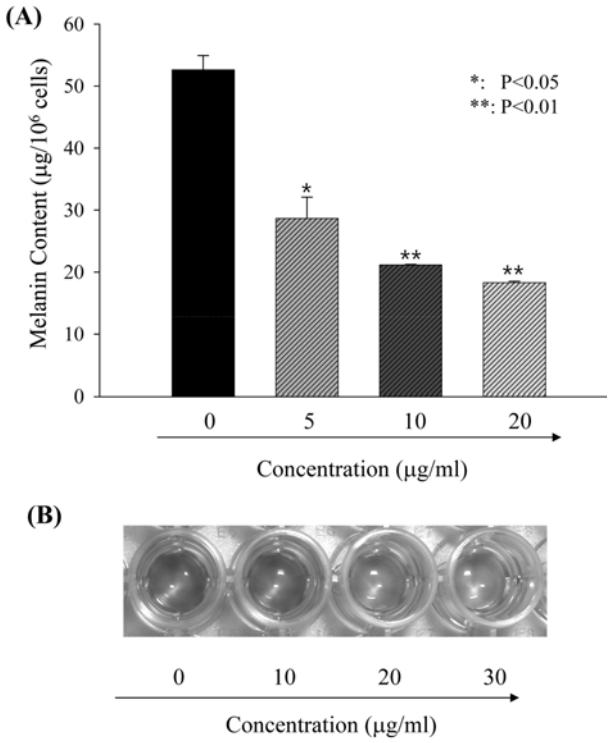

Fig. 2. (A) Effects of MeOH extracts of *A. ageratoides* Turcz. var. *ageratoides* (Root) on the melanin content. (B) Macroscopic views of the fraction of *A. ageratoides* Turcz. var. *ageratoides* (Root) on the melanin content. Data are representatives of three independent experiments. Statistical analyses were performed using the Student's t test and one-way ANOVA. The data shown are the means ± SD of four determinations. **P < 0.01, *P < 0.05 were considered statistically significant.

with 46.2%, 59.6%, and 65.4% inhibition in the level of 5, 10, and 20 μg/ml, respectively (IC_{50} = 6.1 μg/ml) (Fig. 2 (A), (B)).

In the development of skin-whitening agents the modulation of biosynthetic pathway of melanogenesis might be a primary target (Briganti *et al*., 2003). One of the well-known target biomolecules in this process is tyrosinase. Tyrosinase is a rate-limiting enzyme in melanin formation, and catalyzes the first step of oxidation of L-DOPA. On the line, many skin-whitening agents were screened by the inhibition of the enzyme activity. In our program to develop skin-whitening agents from natural products the assay for the inhibition of tyrosinase activity was also employed.

As a result, the extracts from *P. alkekengi* var. *francheti* exhibited the tyrosinase inhibitory activity of 62.7%, 53.0%, 57.7%, 67.6%, and 56.5% with unripe fruit, rhizoma, aerial, leaf, and stem part, respectively, at the test concentration of 250 μg/ml as shown in Fig. 3(A). In accordance with the result of melanin formation, the leaf extracts of *P. alkekengi* var. *francheti* showed the most potent inhibitory activity among the test extracts, and was dose-dependent manner (Fig. 3(B). In addition, the extracts of *A. ageratoides* Turcz. var. *ageratoides* also showed the inhibitory activity of tyrosinase of 71.8%, 34.8%, 19.6%, and 22.0 % with flower, stem, root, and aerial part, respectively, at the test concentration of 250 μg/ml as shown in Fig. 4(A). The flower extract of *A. ageratoides* Turcz. var. *ageratoides* was the most potent tyrosinase inhibitory activity among the extracts, and was dose-dependent as shown in Fig. 4(B).

These results indicate that the extracts from *P. alkekengi* var. *francheti* and *A. ageratoides* Turcz. var. *ageratoides* have potential tyrosinase inhibitory activities which might be useful in the application of anti-browning and skin-whitening agents. To our knowledge, this is the first report for the inhibitory activity of *P. alkekengi* var. *francheti* and *A. ageratoides* Turcz. var. *ageratoides* on the melanin biosynthesis and tyrosinase.

In conclusion, the present study revealed that *P.*

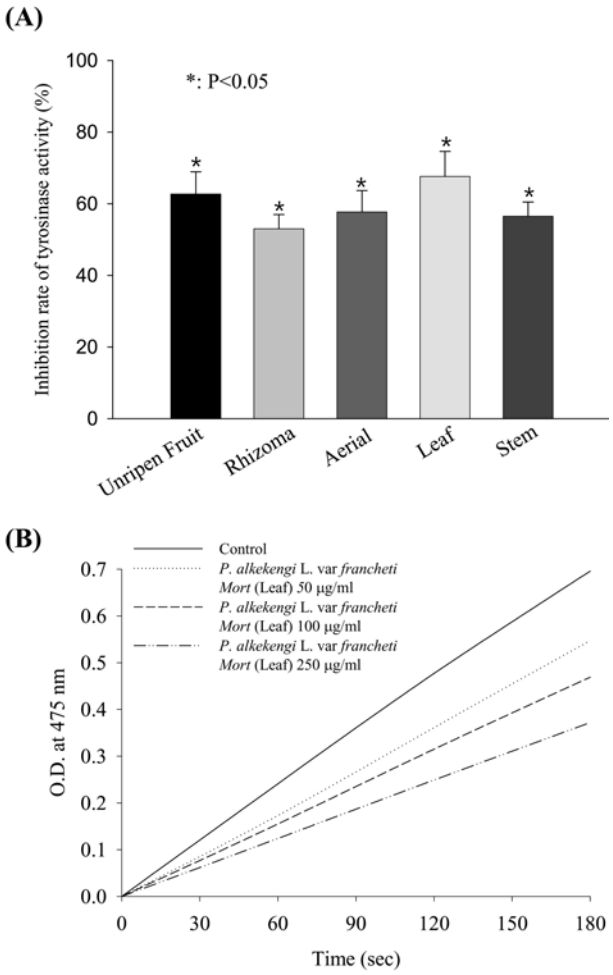

Fig. 3. (A) Inhibitory activity of dopachrome formation by *P. alkekengi* var. *francheti* Mort extract using mushroom tyrosinase (B) Time scanning of dopachrome formation by *P. alkekengi* var. *francheti* Mort (Leaf) extract. Statistical analyses were performed using the Student's t test and one-way ANOVA. The data shown are the means ± SD of four determinations. *P < 0.01 was considered statistically significant.

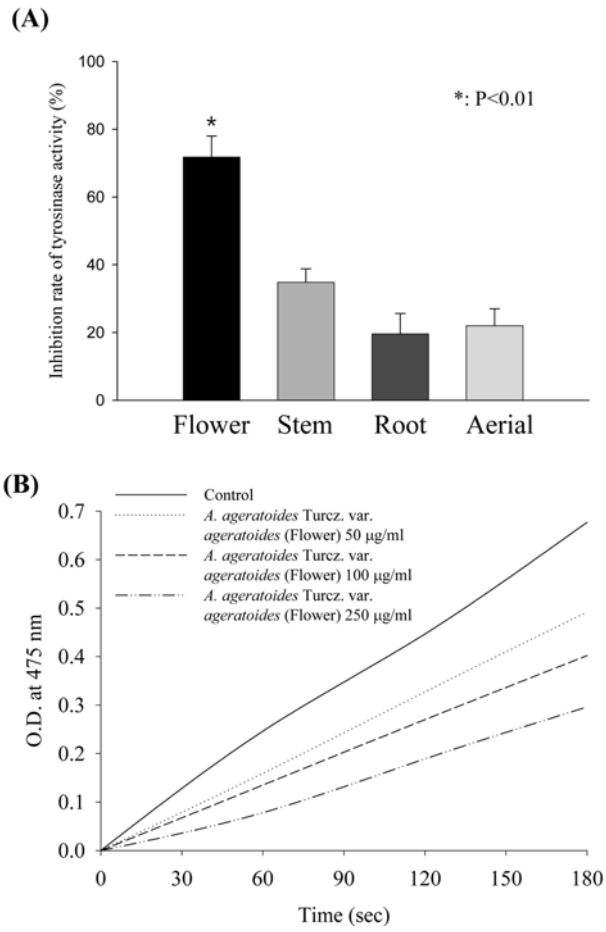

Fig. 4. (A) Tyrosinase inhibitory activity of MeOH extracts of *A. ageratoides* Turcz. var. *ageratoides* using mushroom tyrosinase (B) Time scanning of dopachrome formation by *A. ageratoides* Turcz. var. *ageratoides* (Flower) extract. Statistical analyses were performed using the Student's t test and one-way ANOVA. The data shown are the means ± SD of four determinations. *P < 0.01 was considered statistically significant.

alkekengi var. *francheti* and *A. ageratoides* Turcz. var. *ageratoides* have the potential inhibitory activity on the melanin synthesis which might be considered as promising candidates for preventing or treating hyperpigmentation, and also applying for a skin-whitening agent.

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