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Search for Skin-whitening Agent from *Prunus* Plants and the Molecular Targets in Melanogenesis Pathway of Active Compounds

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In our research program for discovering novel skin-whitening materials, screening of extracts from flowers of some *Prunus* species was performed using an anti-tyrosinase assay. Among the tested plants, the flowers of *P. persica* showed the most potent inhibitory activity. In addition, *P. persica* also showed suppression of melanogenesis in B16 rat melanoma cells. The active principles of tyrosinase inhibition and suppression of melanogenesis were revealed to be an afzelin (3-*O*-α-L-rhamnosylkaempferol) and a flavanone, naringenin. The mechanism of the anti-melanogenesis effect of these two compounds was disclosed, for the first time, as the suppression of the expression of tyrosinase protein, which was controlled by the inhibition of phosphorylation of p38 MAPK. These findings show that these compounds could be candidates for the novel molecular target for a skin-whitening agent.

Keywords: *Prunus genus*, Tyrosinase, Melanin, Afzelin, Naringenin, p38 MAPK.

Tyrosinase has been recognized as a key enzyme for the production of melanin, which is a colored protein that can cause melasma. Melasma can be considered as a self-defense reaction to avoid dermatitis and/or inflammation. However, this reaction would be an undesirable effect from a cosmetic viewpoint due to a bright complexion [1]. Skin-whitening has been paid much attention by women and has been the most important issue in the cosmetic area. Therefore, the development of skin-whitening agents has been urgently performed both in industrial and academic research areas. Various skin-whitening agents have been widely developed based on their inhibitory activity against tyrosinase and blended into many cosmetic products, i.e. sunscreens, creams and milky lotions. Some effective agents for skin-whitening have been discovered from natural product resources including hydroquinone, ascorbic acid, kojic acid and arbutin; these are recognized as safe and effective agents due to their natural origins. Among these agents, arbutin has been used as a strong skin-whitening agent. This compound was originally discovered from the leaves of *Arctostaphylos uva-ursi* [2]. This prompted us to screen another 5 *Arctostaphylos* species for anti-tyrosinase activity to discover *A. patula* to be the most effective agent, and which is stronger than *A. uva-ursi*; the others also possessed fairly strong activities [2]. Umbelliferae species also demonstrated anti-tyrosinase activities showing that they can be effective skin-whitening agents. Recently, we also reported that the fruit of *Citrus hassaku*, one of the most popular citrus fruits in Japan, possesses inhibitory activity on tyrosinase and suppressive activity against melanin production in B16 rat melanoma cells [3]. In another report, seeds from a tropical plant, "noni" (*Morinda citrifolia*), showed skin-whitening and prevention of wrinkles effects [4a-c]. Thus this plant was expected to be a promising skin-care agent. Moreover, a natural plant resource used as a food may be a rich resource for a novel, safe and effective skin-whitening agent.

Among the continuing research program for discovering novel skin-whitening agents, we focused on *Prunus* plants in our possession in the herbal garden of Kinki University. Screening of the extracts from leaves of 38 *Prunus* species was performed to reveal that the extracts of *P. yedoensis* and *P. zippeliana* possessed potent activities showing 80.6 and 79.3% inhibition at 500 μg/mL [4d], respectively. In addition, the cortex of *P. yedoensis* also showed 70.1% inhibition of tyrosinase. In these circumstances, we focused on the flowers of *Prunus* species. Flowers are known as a rich source of bioactive compounds [5a,b] and even as a multi-functional food. Although flowers of *Prunus* species have been extensively studied for their chemical constituents, no results on either their anti-tyrosinase activity or suppressive effect on melanin production have been reported. In this current work, screening of 5 *Prunus* species was performed for anti-tyrosinase and anti-melanin production activities. Among the tested plants, *P. persica* showed the most potent activity and its active constituents were identified. Furthermore, a preliminary investigation of the mechanism on the signaling pathway of tyrosinase expression was performed.

All the *Prunus* extracts tested showed significant inhibitory activity in the anti-tyrosinase assay (Table 1). Among them, *P. persica* (PP)-ext was the most potent (46.0 and 52.1% at 200 and 500 μg/mL, respectively). This result shows that flowers as well as leaves and barks of *Prunus* species might be promising agents for skin-whitening.

As a result of the tyrosinase inhibition, the effects of the extracts on melanin production in murine B16 cells were tested. Although tyrosinase has been known as the key enzyme in melanogenesis, auto-oxidation in cells has been recognized as another important factor in melanogenesis. Melanogenesis in B16 cells can be directly evaluated as a combination of various related biosyntheses using α-MSH (α-melanocyte stimulating hormone) as a stimulant of melanogenesis. α-MSH is a hormone which enhances melanogenesis when α-MSH binds to its receptor and activates phosphorylation of p38 while up-regulating phosphorylation of P70-S6K (ribosomal protein S6 kinase) and Erk (extracellular signal-regulated kinase). *P. yedoensis* (PY)-ext showed the most potent activity of the extracts tested (Table 2). It reduced the melanin content from 39.8±2.6 (vehicle control) to 24.2±2.0 and 11.7±0.7 at 100 and 500 μg/mL, respectively, without apparent

cytotoxicity. PP-ext and *P. mume* (PM)-ext showed moderate suppressive activity and *P. amygdalus (*PAY)-ext weak suppressive activity at 500 μg/mL. However, the activity might be attributed to weak cytotoxicity, as 14.3% of the cells were killed in the assay. Thus cytotoxicity of this extract to humans is a possibility and so might not be suitable for development as a skin-whitening agent. *P. ameniaca* (PAM)-ext showed no effect at the tested concentrations of 100 and 500 μg/mL.

Table 1: Inhibitory activities of extracts from *Prunus* species on tyrosinase.

Samples	Concentration (μg/mL)	O.D.	Inhibition (%)
Control	-	0.407 ± 0.007**	-
PP-ext	200	0.219 ± 0.003**	46.0
	500	0.195 ± 0.005**	52.1
PAY-ext	200	0.231 ± 0.005**	43.2
	500	0.201 ± 0.003**	50.6
PM-ext	200	0.245 ± 0.002**	39.7
	500	0.236 ± 0.001**	41.8
PY-ext	200	0.267 ± 0.008**	34.3
	500	0.233 ± 0.002**	42.6
PAM-ext	200	0.271 ± 0.009**	33.4
	500	0.241 ± 0.004**	40.7
Kojic acid	10 μM	0.296 ± 0.008**	27.2

PP-ext: *P. persica* extract; PAY-ext: *P. amygdalus* extract; PM-ext: *P. mume* extract; PY-ext: *P. yedoensis* extract; PAM-ext: *P. ameniaca* extract. Each value represents the mean ± S.D. of triplicates. ** Statistically significant in comparison with control group at p <0.01.

Table 2: Effects of extracts from *Prunus* species on α-MSH-stimulated melanogenesis in B16 cells.

Samples	Concentration (μg/mL)	Melanin content (μg/well)	Cell proliferation (%)
Control	-	8.6 ± 1.4	55.2 ± 0.3
Vehicle control	-	39.8 ± 2.6##	100.0 ± 0.6§§
PY-ext	100	24.2 ± 2.0**	97.6 ± 1.6
	500	11.7 ± 0.7**	107.3 ± 2.4
PP-ext	100	25.1 ± 3.0**	94.7 ± 5.7
	500	16.8 ± 0.4**	100.7 ± 2.3
PM-ext	100	29.7 ± 1.6**	101.6 ± 1.8
	500	20.7 ± 1.3**	100.9 ± 4.0
PAY-ext	100	38.0 ± 2.9	92.8 ± 2.0
	500	21.1 ± 1.0**	85.7 ± 0.6¶¶
PAM-ext	100	39.3 ± 3.6	99.6 ± 0.5
	500	38.2 ± 2.7	99.0 ± 0.3
Kojic acid	50 μM	19.6 ± 1.8**	95.8 ± 3.0

PY-ext: *P. yedoensis* extract; PP-ext: *P. persica* extract; PM-ext: *P. mume* extract; PAY-ext: *P. amygdalus* extract; PAM-ext: *P. ameniaca* extract. Each value represents the mean ± S.D. of triplicates. ## Statistically significant in comparison with control group at p <0.01. ** Statistically significant in comparison with vehicle control at p <0.01. Each value represents the mean ± S.D. of triplicates. §§ Statistically significant in comparison with control group at p <0.01.

Table 3: DPPH radical scavenging activities of extracts from *Prunus* plants.

Samples	Concentration (μg/mL)	O.D.	Inhibition (%)
Control	-	0.316 ± 0.003	-
PY-ext	10	0.269 ± 0.005**	14.9
	50	0.094 ± 0.019**	70.3
PAM-ext	10	0.287 ± 0.001*	9.2
	50	0.154 ± 0.017**	51.3
PM-ext	10	0.235 ± 0.004**	25.7
	50	0.051 ± 0.011**	41.8
PP-ext	10	0.287 ± 0.003	9.0
	50	0.199 ± 0.013**	37.1
PAY-ext	10	0.303 ± 0.003	4.0
	50	0.213 ± 0.009**	32.6
Ascorbic acid	50 μM	0.154 ± 0.007**	51.2

PY-ext: *P. yedoensis* extract; PAM-ext: *P. ameniaca* extract; PM-ext: *P. mume* extract; PP-ext: *P. persica* extract; PAY-ext: *P. amygdalus* extract. Each value represents the mean ± S.D. of triplicates. **; Statistically significant in comparison with control group at p <0.01.

As mentioned above, anti-oxidative activities are another important factor for melanogenesis. Therefore, as one indicator of anti-oxidative activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activities of the extracts of *Prunus* species were tested. PY-ext showed the most potent activity among the extracts tested with 14.9 and 70.3% inhibition at 10 and 50 μg/mL, respectively (Table 3). The result shows that the flowers of *Prunus* plants possess anti-oxidative activities.

We then focused on the extract with the most potent inhibitory activity on tyrosinase and suppression of melanogenesis. The most potent plant in melanogenesis suppression was *P. yedoensis*, but the raw flowers were not commercially available and we were unable to obtain enough material for investigation. Therefore we investigated the extract of *P. persica*, which possessed potent inhibitory activity on tyrosinase. Active principles involved in the inhibitory activity of tyrosinase and suppression of melanin production were isolated by fractionation of the MeOH extract. The tyrosinase inhibitory activities and suppression of melanogenesis produced by the different fractions are shown in Tables 4 and 5. The active principles were concentrated in the EtOAc soluble fraction in both assays. Thus this fraction was further purified to determine the active components using various liquid chromatography techniques. The isolated compounds were determined as afzelin (3-*O*-α-L-rhamnosylkaempferol, **1**) [5c] and naringenin (**2**); this is the first isolation of these from PP-ext. Compound **2** was found as the active principle responsible for the inhibitory activity on tyrosinase and suppression of melanogenesis, while **1** suppressed melanogenesis. A second tyrosinase inhibitory compound was isolated, along with compound **1**, but could not be characterized due to its unstable nature. Compound **1** showed weak suppression of melanogenesis (46.5% at 1 mM). In addition, **2** showed an IC_{50} value of 308 μM for suppression of melanogenesis, and 22.9% for tyrosinase inhibitory activity at 1 mM. These activities do not reflect the activity of the extract and other active compounds probably exist.

Table 4: Inhibitory effects on tyrosinase of fractions from partitioning.

Samples	Concentration (μg/mL)	O.D.	Inhibition (%)
Control	-	0.517 ± 0.002	-
n-Hexane fr.	10	0.527 ± 0.010	-1.9
	50	0.527 ± 0.003	-1.9
	100	0.506 ± 0.014**	2.3
EtOAc fr.	10	0.478 ± 0.006**	7.7
	50	0.327 ± 0.003**	36.7
	100	0.261 ± 0.002**	49.5
$CHCl_3$ fr.	10	0.526 ± 0.008	-1.6
	50	0.498 ± 0.006	3.7
	100	0.483 ± 0.006**	6.6
H_2O fr.	10	0.513 ± 0.013	0.9
	50	0.489 ± 0.003	5.5
	100	0.459 ± 0.010**	11.3
Kojic acid	10 μM	0.406 ± 0.003**	21.5

Each value represents the mean ± S.D. of triplicates. **; Statistically significant in comparison with control group at p <0.01.

The anti-melanogenesis effect of **1** has not been investigated so far. However, **2** has been reported to possess a melanogenesis activating effect promoting the protein producing cascade. In order to reveal the molecular target of **1** and to solve the disagreement with previous reports, the molecular targets for melanogenesis were investigated. As shown Figure 1, **1** and **2** suppressed α-MSH induced expression of tyrosinase in B16 cells at 250 μM.

In a previous report, **2** was shown to possess α-MSH like effects that increased the expression level of tyrosinase on untreated B16 cells at 100 μM [5d]. The reason for this discrepancy might be attributed to the difference of the experimental methods. The previous report investigated the effect of **2** on mature B16 cells, while we investigated the suppression effect on the high melanin content induced by α-MSH. Thus **2** might possess both suppression and promotion effects on melanogenesis depending on the concentrations of cellular melanin, namely **2** suppresses melanogenesis when melanin concentration is high, while it promotes melanogenesis when melanin concentration is low. Thus **2** could have a melanin controlling effect, which might be useful for protection from photo-aging in a moderate manner. This selectivity would be attractive for an anti-photoaging agent.

In a series of Western blot analyses, **1** and **2** suppressed the expression level of Mitf (microphthalmia-associated transcription factor, Figure 2). Mitf is widely recognized as the most critical transcription factor for the biosynthesis of tyrosinase protein. These data suggest that both compounds affect the expression of Mitf and

Table 5: Effects of fractions from partitioning on α-MSH-stimulated melanogenesis in cells.

Samples	Concentration (μg/mL)	Melanin content (μg/well)	Cell proliferation (%)
Control		8.6 ± 1.4	55.2 ± 0.3
Vehicle control		39.8 ± 2.6##	100.0 ± 0.6^{SS}
n-Hexane fr.	50	42.2 ± 2.5	102.9 ± 2.6
	100	41.7 ± 1.4	97.1 ± 2.7
	200	42.9 ± 0.8	95.3 ± 3.3
	500	31.2 ± 2.1**	87.1 ± 0.2¶¶
EtOAc fr.	50	24.7 ± 3.7**	97.4 ± 3.6
	100	22.7 ± 1.7**	101.6 ± 1.8
	200	17.2 ± 1.0**	104.9 ± 1.8
	500	9.3 ± 1.0**	104.6 ± 4.3
$CHCl_3$ fr.	50	34.3 ± 0.3	97.5 ± 3.7
	100	26.8 ± 0.7**	99.3 ± 0.3
	200	21.6 ± 0.7**	105.8 ± 3.0
	500	17.2 ± 1.0**	102.6 ± 4.5
H_2O fr.	50	45.3 ± 1.7	102.2 ± 3.7
	100	43.3 ± 2.9	100.7 ± 3.7
	200	42.6 ± 2.0	104.8 ± 1.9
	500	37.8 ± 1.9	104.4 ± 4.4
Kojic acid	50 μM	19.6 ± 1.8**	95.8 ± 3.0

Each value for melanin content represents the mean ± S.D. of triplicates. **; Statistically significant in comparison with control group at p <0.01. ¶¶; Statistically significant to the value of vehicle control group at p <0.01.

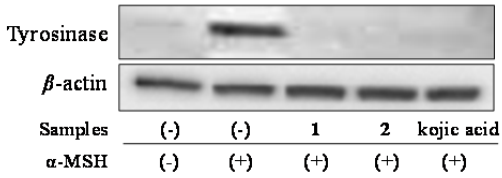

Figure 1: Suppression of tyrosinase expression
B16 cells were treated with 1 μM α-MSH, samples (250 μM) and kojic acid (250 μM) for 72 h. The cells were lysed and the soluble proteins were separated by SDS-PAGE. Proteins were transferred to PVDF membrane and treated with corresponding antibodies. After treating with HRP labeled secondary antibodies, the proteins were visualized by treating with chemiluminescent substrate. The images were obtained by image analyzer.

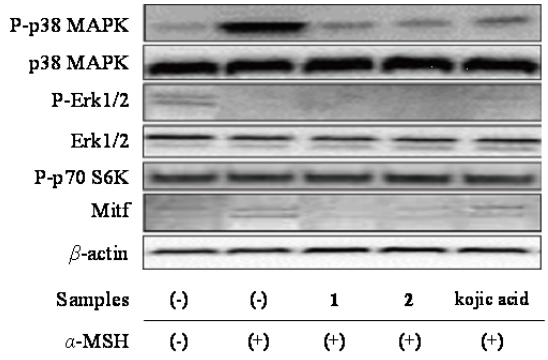

Figure 2: Suppression of tyrosinase expression
B16 cells were treated with 1 μM α-MSH, samples (250 μM) and kojic acid (250 μM) for 72 h. The cells were lysed and the soluble proteins were separated by SDS-PAGE. Proteins were transferred to PVDF membrane and treated with corresponding antibodies. After treating with HRP labeled secondary antibodies, the proteins were visualized by treating with chemiluminescent substrate. The images were obtained by image analyzer

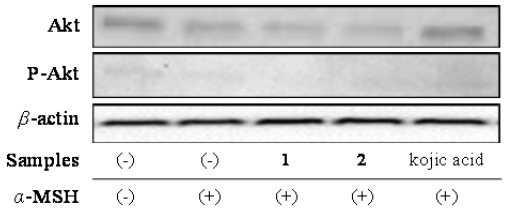

Figure 3: Effects of 1 and 2 on the expressions of Akt and P-Akt
B16 cells were treated with 1 μM of α-MSH, samples (250 μM) and kojic acid (250 μM) for 72 h. The cells were lysed and the soluble proteins were separated with SDS-PAGE. Proteins were transferred to PVDF membrane and treated with corresponding antibodies. After treating with HRP labeled secondary antibodies, the proteins were visualized by treating chemiluminescent substrate. The images were obtained by image analyzer.

control the expression of tyrosinase at its protein level. Mitf is regulated by the upstream proteins and then the levels of related proteins were investigated. Compounds **1** and **2** inhibited phosphorylation of p38 MAPK (mitogen-activated protein kinase), while other proteins remained unchanged compared with the property of α-MSH, which strongly promoted melanogenesis. From these results, **1** and **2** suppress melanogenesis by interfering with the p38 MAPK pathway, but not either the Erk or S6K pathways. In order to determine the protein in the upper stream, the expression levels of MKK (dual specificity mitogen-activated protein kinase kinase) 6 and phosphorylation of MKK3/MKK6 were investigated. However, the expression levels of these proteins were too low to be detected by Western blot (data not shown). In addition, **1** and **2** slightly suppressed the expression of Akt (protein kinase B, Figure 3). The expression of Akt is known to promote various protein bio-syntheses. These results suggest that **1** and **2** are new types of skin-whitening agents which inhibit melanogenesis by suppressing the tyrosinase level and also the melanin level itself. These findings are novel for these two compounds and provide important information for the further study on the melanogenesis signaling pathway. In addition, the target of the two compounds may be an ideal one for the inhibition of melanogenesis since they were of natural origin.

In conclusion, the flowers of *Prunus* species were found to be a novel source for a skin-whitening agent, along with leaves and barks. The active principles of the most potent material, the flowers of *P. persica*, were investigated. Afzelin (compound **1**) and naringenin (compound **2**) were identified as active principles and their preliminary modes of action were revealed. Both compounds showed inhibition of the p38 MAPK pathway, but not either Erk or S6K, to express the anti-tyrosinase production activities. Furthermore, both compounds inhibited the expression level of Akt, which might lead to the inhibition of melanin synthesis. These results suggest that *P. persica* flowers are a candidate for a promising and ideal skin-whitening agent. In spite of its potent skin-whitening effect, the plant has been used as a strong purgative agent. The appropriate application route must be considered in order to apply it to humans. Further investigation of the melanogenesis pathway is now underway in our laboratory.

Experimental

Prunus plants and preparation of extracts: The flowers of *P. persica*, *P. amygdalus* and *P. mume* were purchased from Tochimoto Tenkaido (Osaka, Japan). The flowers of *P. yedoensis* and *P. ameniaca* were collected in the Garden of Medicinal Plants at Kinki University. Voucher specimens were deposited at the Faculty of Pharmacy, Kinki University. Plant samples were pulverized with a blender and 100 g of powder was macerated with 1 L of 50% ethanol (EtOH). The suspension was refluxed for 1 h and then filtered. To the residue, the manipulation was repeated and the combined filtrate was evaporated under reduced pressure. The residue was lyophilized to obtain 50% EtOH extracts. The yields were as follows: *P. persica*; 25.0% (PP-ext), *P. yedoensis*; 32.8% (PY-ext), *P. amygdalus*; 29.0% (PAY-ext), *P. ameniaca*; 41.0% (PAM-ext) and *P. mume*; 33.0% (PM-ext).

Anti-tyrosinase activity: Anti-tyrosinase activity was evaluated by the method reported previously [6a] with optimization for the utility of a 96-well plate. To the sample solution of 50 μL in 5% DMSO in phosphate buffered saline (PBS), 0.03% L-DOPA solution (50 μL) in PBS was mixed and incubated for 10 min at 25ºC. After incubation, 50 μL tyrosinase solution in PBS was added and incubated for 5 min at 25ºC. After incubation, the optical density at 475 nm of the reaction mixture was measured with a microplate reader. The inhibition (%) was determined as the percentage compared with the control group. Kojic acid was used as a reference compound.

Cell culture: B16 melanoma cells (B16F1) were purchased from Dainippon Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10%, v/v, fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan) and 1% antibiotic–antimycotic solution (a mixture of 10000 U/mL penicillin, 10000 μg/mL streptomycin sulfate and 25 μg/mL amphotericin B, Invitrogen Corp., Carlsbad, CA) at 37°C in a humidified, CO_2-controlled (5%) incubator. Stimulation of melanogenesis was initiated by the addition of α-melanocyte stimulating hormone at 1 μM.

Melanogenesis assays: The amount of intracellular melanin in B16 cells was measured according to the method previously reported [6b]. Briefly, cells (2×10^4 cells) were seeded on 24-well plates with 800 μL of medium and treated with test samples (100 μL) and α-MSH (100 μL) at 24 h after seeding. Test samples were dissolved in dimethylsulfoxide (DMSO) and then diluted with DMEM to an appropriate concentration. The final concentration of DMSO was set to 0.1%, v/v. In the control and vehicle control groups, DMSO solution was used instead of the sample solution. α-MSH was added to the medium in the vehicle control and test groups. α-MSH was dissolved in aqueous acetic acid solution (5%, v/v) and then diluted with DMEM to a final concentration of 1 μM. The mixture was incubated at 37°C and the cells were washed twice with phosphate-buffered saline (PBS, pH 7.0) and dissolved in 2 N sodium hydroxide for 1 h at 65°C. The absorbance at 490 nm of each sample was measured by a microplate reader and melanin amount was determined by comparison of absorbance with that of the authentic melanin standard. Cell proliferation was assessed by WST-8 assay. Cell proliferation was shown as a percentage calculated by comparison with that of the vehicle control cells. Kojic acid was used as a reference compound.

DPPH free radical scavenging activity: Radical scavenging activity was measured according to the method previously reported [6c] with optimization for the usage of a 96-well plate. Test samples were dissolved in DMSO and diluted with 0.5 M acetate buffer (pH 5.5) to a final DMSO concentration of 1%, v/v. A mixture of test sample solution (100 μL), EtOH (64 μL), 0.5 M acetate buffer (pH 5.5) (16 μL) and 0.5 mM DPPH/EtOH solution (40 μL) was allowed to stand for 30 min at room temperature. The absorbance at 520 nm of the resulting mixture was measured with a microplate reader. L-Ascorbic acid was used as a reference compound. The scavenging activity of each sample was expressed as the percentage decrease of absorbance compared with that of the control DPPH solution.

Electrophoresis and Western blot analysis: The B16 cells were lysed in a lysis buffer containing protease inhibitors and centrifuged at 15,000 × *g*, at 4°C, for 20 min. The resulting supernatant (solubilized proteins) was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on either 7.5% or 10% polyacrylamide gel. Proteins were transferred electrophoretically onto a polyvinylidene-difluoride (PVDF) membrane. After blocking in 5% skimmed milk in PBS containing 0.05% Tween 20, PVDF membranes were incubated with an appropriate primary antibody and then further incubated with horseradish peroxidase-conjugated secondary antibody. Proteins were detected using the ECL Plus® Western blot detection kit (GE Healthcare Japan, Tokyo, Japan). Gel images were obtained with an image analyzer ChemiDoc™ XRS imaging system (Bio-Rad Laboratories, Inc., Hercules, CA).

Purification and identification of active constituents in PP-ext: Dried flowers of *P. persica* were extracted with methanol (MeOH) under reflux conditions. The aqueous solution extract was partitioned with *n*-hexane (yield; 14.7%), ethyl acetate (EtOAc, 18.8%) and chloroform ($CHCl_3$, 0.4%). The yield of theaqueous layer was 64.3%. The EtOAc layer was evaporated and the residue subjected to a repeated purification step with silica gel ($CHCl_3$:MeOH=4:1 as an eluent) and HPLC fractionation [column; SunFire ODS (Waters, 1 i.d. x 25 cm), mobile phase; 50% MeOH (0.1% acetic acid), flow rate; 5 mL/min]. These isolation procedures resulted in the isolation of **1** (4 mg) and **2** (10 mg).

Statistical analysis: Data were analyzed for significantly difference with Statcel3 software with multiple comparison test based on Bonferroni/Dunn algorithm at $p < 0.01$ and $p < 0.05$.

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