Studies of Cuticle Drugs from Natural Sources. IV. Inhibitory Effects of Some *Arctostaphylos* Plants on Melanin Biosynthesis

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The inhibitory effect of 50% ethanol extracts obtained from the leaves of six *Arctostaphylos* plants (*A. patula*, *A. viscida*, *A. canescens*, *A. columbiana*, *A. nevadensis* and *A. uva-ursi*) (Ericaceae) was tested on melanin biosynthesis. Among them, the extracts of *A. patula* and *A. viscida* showed potent inhibition against tyrosinase. The two extracts not only inhibited the production of melanin from dopachrome by autoxidation, but exhibited SOD (superoxide dismutase)-like activity and had a moderate absorbance in the UV-B area. These results suggest that the leaves of *Arctostaphylos* plants, especially *A. patula* and *A. viscida* can be applied to a whitening agent for the skin.

Key words *Arctostaphylos patula*; *Arctostaphylos viscida*; melanin; tyrosinase inhibition; hyperpigmentation; whitening agent

Recently, much attention has been drawn to the application of naturally occurring crude drugs to cosmetics. From this point of view we reported that crude drugs originated from *Umbelliferous* plants, some *Prunus* plants and *Arctostaphylos uva-ursi*, all of which have been applied to cosmetic beauty preparations, have an inhibitory effect on tyrosinase activity closely related to hyperpigmentation.^{1–3)} In the present study, 50% ethanol extracts from the leaves of five *Arctostaphylos* plants (*A. canescens*, *A. columbiana*, *A. nevadensis*, *A. patula* and *A. viscida*) growing in the northeast part of North America were investigated *in vitro* as to their inhibitory effects on melanin biosynthesis and their SOD (superoxide dismutase)-like activity, in addition to their propensity for UV-B absorption in comparison with that of *A. uva-ursi*.

MATERIALS AND METHODS

Plants Materials Five *Arctostaphylos* species (*A. patula*, *A. viscida*, *A. canescens*, *A. columbiana* and *A. nevadensis*) used in this study were collected in North America. The voucher specimens are deposited in the Herbarium of Kinki University. Dried leaves (Uvae Ursi Folium) of *A. uva-ursi* collected in Spain were donated by Nippon Funmatsu Yakuhin Co., Ltd., Japan.

Preparation of Ethanol Extract and Measurement of Arbutin and Tannin Contents Respective dried and pulverized leaves were extracted with 50% ethanol at 85°C for 2 h × 2 times. After being combined, the extracts were concentrated under reduced pressure, and then freeze-dried. The lyophilized sample was supplied in the present study. The content of arbutin in the extract was determined by a HPLC method [conditions; column: YMC AG-303 (4.6 i.d. × 250 mm), detection: UV absorption at 280 nm, mobile phase: H₂O–MeOH (95:5), flow rate: 0.8 ml/min, column temperature: 30°C, injection volume: 10 µl, the calibration curve was prepared by an authentic arbutin purchased from Wako Pure

Chemicals (Japan)], and those of tannin were calculated according to the method of Japan Food Research Laboratories. Table 1 shows the contents of arbutin and total tannin in the extracts.

Tyrosinase Inhibitory Activity Anti-tyrosinase activity was measured according to the method of Mason and Peterson.⁴⁾ The test substance dissolved in 0.5 ml 1/15 M phosphate buffer (PBS, pH 6.8) and was incubated at 25°C for 10 min, and 0.5 ml mushroom tyrosinase (135 U/ml, Sigma Chemical Co., U.S.A.) and 0.5 ml 0.03% dopa (Nacalai Tesque, Japan) solution were there added. The reaction mixture was incubated for 5 min. The amount of dopachrome in the mixture was determined by the optical density (OD) at 475 nm using a Hitachi spectrophotometer, model 200-10. Kojic acid (Nacalai Tesque, Japan) and arbutin isolated from the leaves of *A. uva-ursi*³⁾ were used as standard agents. The inhibitory percentage of tyrosinase was calculated as follows: the activities of test substances were expressed as the 50% inhibitory concentration (IC₅₀; µg/ml or µM) by the method of Litchfield–Wilcoxon;

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

A: OD at 475 nm without test substance

B: OD at 475 nm without test substance and enzyme

Table 1. Arbutin and Tannin Content of Experimental Materials

Plants	Abbreviation	Yield (%) ^{a)}	Arbutin content (%) ^{b)}	Tannin content (%) ^{c)}
<i>A. patula</i>	APA-ext	35.3	3.95	12.2
<i>A. viscida</i>	AVI-ext	34.9	4.62	13.4
<i>A. nevadensis</i>	ANE-ext	33.8	3.19	12.9
<i>A. columbiana</i>	ACO-ext	30.3	5.75	11.6
<i>A. canescens</i>	ACA-ext	32.2	4.05	9.7
<i>A. uva-ursi</i>	AUV-ext	33.5	5.96	13.0

a) Yield % of 50% ethanolic extract from each leaves of *Arctostaphylos* plants. b) Arbutin content of 50% ethanolic extract. c) Tannin content of 50% ethanolic extract.

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C: OD at 475 nm with test substance

D: OD at 475 nm with test substance, but without enzyme

Production of Melanin from Dopachrome by Autoxidation The production of melanin from dopachrome by autoxidation was estimated by the modified method of Mason and Peterson.⁴⁾ The tyrosinase solution mentioned above was incubated at 25 °C for 10 min, to which a dopa solution was added, and the mixture incubated for 5 min. The test substance dissolved in PBS was then added to the solution and the reaction mixture was incubated for 60 min. The reaction was stopped by the addition of 0.2 ml 1 N HCl and the mixture was centrifuged at 1200 × g for 15 min. The precipitates obtained were washed with 1 ml 6 N HCl and distilled water (twice), and then dissolved in 2 ml solune 350 (Canberra Co., Netherlands).⁵⁾ The amounts of melanin were estimated by measurement of OD at 400 nm, and then the weight of standard melanin and the inhibitory percentage of the test substance were determined. Kojic acid and arbutin were used as standard agents.

SOD-like Activity SOD-like activity was measured according to the method of Oyanagui.⁶⁾ A 0.2 ml reagent A solution (0.5 mM hypoxanthine, 10 mM hydroxylamine hydrochloride) and a 0.8 ml EDTA-PBS (pH 8.2) solution containing the test substance were combined and preincubated at 37 °C for 10 min. Five mU/ml xanthine-oxidase solution, 0.2 ml, was added to the above solution, and the mixture was incubated for 30 min. Reagent B (2 ml coloring reagent to a final concentration of 20

μM *N*-1-naphthylethylenediamine, 2 mM sulfanilic acid and 16.7% acetic acid) was added to the reaction mixture. The resulting mixture was allowed to stand for 30 min at room temperature, and then OD was measured at 550 nm. SOD-like activity was expressed as an inhibitory percentage against the production of a superoxide anion from hypoxanthine-xanthine oxidase. A positive control agent, SOD, obtained from cow milk, was used.

Absorptions of UV-A and -B The UV spectrum of each extract (APA, AVI and AUV-ext), and of arbutin and kojic acid as a reference, was measured at the concentration of 50 ppm in methanol.

RESULTS

Tyrosinase Activity As shown in Table 2, six ethanol extracts from the leaves of *Arctostaphylos* plants showed anti-tyrosinase activity. Especially, the APA-ext (*A. patula* extract) and the AVI-ext (*A. vicida* extract) exhibited potent inhibition. Arbutin had weak inhibition. Kojic acid used as a standard agent inhibited the enzyme activity at IC₅₀ 0.0207 mM (2.9 μg/ml).

Production of Melanin from Dopachrome by Autoxidation Table 3 shows that the APA-ext, AVI-ext and ANE-ext (*A. nevadensis* extract) exhibited inhibitory effects against the production of melanin from dopachrome by autoxidation. The inhibitory effects of AUV-ext (*A. uva-ursi* extract) and ACA-ext (*A. canescens* extract) were small. The positive control of kojic acid also strongly inhibited the production, but arbutin did not.

Table 2. Inhibitory Effects on Tyrosinase Activity of 50% Ethanolic Extracts from the Leaves of Six *Arctostaphylos* Plants, Arbutin and Kojic Acid

Sample	Concentration	OD (×1000)	Inhibition (%)	IC ₅₀ ^{a)}
Control	—	1082 ± 7	—	—
APA-ext	50 (μg/ml)	863 ± 3	20.3	132.9 (μg/ml)
	200	460 ± 1	57.5	
	500	101 ± 3	90.6	
AVI-ext	50	853 ± 4	21.2	144.5
	200	473 ± 22	56.2	
	500	163 ± 5	85.0	
ANE-ext	50	877 ± 4	19.0	242.4
	200	628 ± 6	42.0	
	500	344 ± 5	68.2	
ACO-ext	50	1001 ± 8	7.4	246.3
	200	653 ± 12	39.7	
	500	254 ± 3	76.5	
ACA-ext	50	942 ± 7	12.9	225.7
	200	610 ± 10	43.7	
	500	273 ± 11	74.7	
AUV-ext	50	939 ± 8	13.2	190.9
	200	516 ± 9	52.3	
	500	227 ± 12	79.1	
Arbutin	0.1 (mM)	1063 ± 8	<5.0	>5.0 (mM)
	0.5	898 ± 3	17.0	
	1.0	886 ± 7	18.1	
	5.0	786 ± 11	27.4	
Kojic acid	0.005 (mM)	976 ± 42	9.7	0.0207 (mM)
	0.01	777 ± 4	28.2	
	0.05	297 ± 21	82.2	

Tyrosinase was preincubated with test substances at 25 °C for 10 min prior to incubation with dopa for 5 min, and the absorbance was read at 475 nm. Each value represents the mean ± S.E. of 3 experiments. a) 50% inhibitory concentration.

Table 3. Inhibitory Effects on Melanin Production from Dopachrome by Autoxidation of 50% Ethanolic Extracts

Sample	Concentration	OD (×1000)	Melanin product (μg/ml)	Inhibition (%)
Control	—	1218 ± 15	77.7	—
APA-ext	200 (μg/ml)	971 ± 14	62.0	20.3
	500	722 ± 20	46.1	40.7
	1000	684 ± 14	43.7	43.8
AVI-ext	200	987 ± 10	63.0	19.0
	500	715 ± 24	45.6	41.3
	1000	730 ± 6	46.6	40.1
ANE-ext	200	1243 ± 14	79.3	<5.0
	500	1048 ± 22	66.9	14.0
	1000	861 ± 26	54.9	29.3
ACO-ext	200	1093 ± 52	69.7	10.3
	500	896 ± 9	57.2	26.4
	1000	888 ± 7	56.7	27.1
ACA-ext	200	1031 ± 15	65.8	15.4
	500	842 ± 17	53.7	30.9
	1000	864 ± 18	55.1	29.1
AUV-ext	200	946 ± 17	60.3	22.4
	500	808 ± 14	51.6	33.7
	1000	791 ± 12	50.5	35.1
Arbutin	0.5 (mM)	1203 ± 7	77.1	<5.0
	1.0	1198 ± 4	76.4	<5.0
	5.0	1179 ± 2	74.3	<5.0
Kojic acid	0.05 (mM)	1365 ± 5	87.1	<5.0
	0.2	1103 ± 29	70.4	9.4
	0.5	632 ± 25	40.3	48.2

Tyrosinase was preincubated with dopa at 25 °C for 5 min prior to incubation with test substance for 60 min, and melanin produced was measured. Each value represents the mean ± S.E. of 3 experiments.

Table 4. SOD-like Activities of 50% Ethanolic Extracts and SOD

Sample	Concentration	OD ($\times 1000$)	Inhibition (%)	IC ₅₀ ^{a)}
Control	—	113.5 $\pm$ 1.5	—	—
APA-ext	2 (mg/ml)	92.7 $\pm$ 1.5	18.4	15.8 (mg/ml)
	10	67.3 $\pm$ 1.5	40.7	
	50	33.8 $\pm$ 0.9	70.2	
AVI-ext	2	93.3 $\pm$ 0.7	17.8	16.0
	10	67.8 $\pm$ 0.7	40.2	
	50	33.3 $\pm$ 0.3	70.6	
ANE-ext	2	88.3 $\pm$ 2.0	22.2	10.8
	10	58.3 $\pm$ 0.9	48.6	
	50	27.3 $\pm$ 0.3	75.9	
ACO-ext	2	88.8 $\pm$ 2.0	21.7	11.5
	10	62.2 $\pm$ 0.3	45.2	
	50	27.0 $\pm$ 0.0	76.2	
ACA-ext	2	92.0 $\pm$ 1.0	18.9	15.3
	10	67.7 $\pm$ 1.2	40.4	
	50	32.7 $\pm$ 0.3	71.2	
AUV-ext	2	98.2 $\pm$ 2.8	13.5	19.4
	10	71.8 $\pm$ 1.5	36.7	
	50	36.2 $\pm$ 0.3	68.1	
Arbutin	0.002 (mM)	112.7 $\pm$ 0.7	<5.0	>0.05 (mM)
	0.01	111.9 $\pm$ 0.3	<5.0	
	0.05	109.4 $\pm$ 0.2	<5.0	
SOD	0.01 (U/ml)	76.6 $\pm$ 0.8	32.5	0.02 (U/ml)
	0.02	58.2 $\pm$ 1.3	48.8	
	0.04	38.3 $\pm$ 0.3	66.2	

SOD-like activity was expressed as inhibitory percentage against the production of superoxide anion from hypoxanthine-xanthine-oxidase. Each value represents the mean $\pm$ S.E. of 3 experiments. a) 50% inhibitory concentration.

SOD-like Activity As shown in Table 4, these six extracts exhibited strong SOD-like activity. Kojic acid also showed the activity, but arbutin did not.

UV Absorption APA, AVI and AUV-extract at a concentration of 50 ppm showed a broad absorption band at 270–290 nm related to the UV-B area and another band, in spite of a weak absorption at 350–370 nm, corresponding to the UV-A region (Fig. 1). However, kojic acid and arbutin at a concentration of 25 ppm had an absorption band only at UV-B.

Correlation of the Arbutin and Tannin Contents and Anti-tyrosinase Activity As shown in Table 5, no correlation was observed between the contents of arbutin or tannins in the extracts and the anti-tyrosinase activity, which suggests that other chemical components in the extracts contribute to the activity.

Table 5. Correlation of Content of Arbutin or Tannin and Anti-tyrosinase Activity

Sample	Arbutin content (%) ^{a)}	Tannin content (%) ^{b)}	Inhibition of tyrosinase activity (IC ₅₀ ^{c)} mg/ml)
<i>A. patula</i>	3.95	12.2	132.9
<i>A. viscida</i>	4.62	13.4	144.5
<i>A. nevadensis</i>	3.19	12.9	242.4
<i>A. columbiana</i>	5.75	11.6	246.3
<i>A. canescens</i>	4.05	9.7	225.7
<i>A. uva-ursi</i>	5.96	13.0	190.9

a) Arbutin content in 50% ethanolic extract, b) tannin content in 50% ethanolic extract, and c) 50% inhibitory concentration.

Fig. 1. Absorption in the UV Spectrum

Sample concentration: *A. patula*, *A. viscida* and *A. uva-ursi*: 50 ppm; arbutin and kojic acid: 25 ppm.

DISCUSSION

In the present experiment, which sought to find a much stronger anti-tyrosinase activity than *A. uva-ursi*, the ethanol extracts of five species in the genus were investigated. The extracts of AVI, APA, ACA, ACO and ANE-ext showed more potent anti-tyrosinase activity and inhibition of melanin production from dopachrome by autoxidation than AUV-ext. Among them, the effects of APA-ext and AVI-ext are prominent. The SOD-like activity was related to the whitening effect and to the ability of UV absorption to prevent sunburn of APA-ext and AVI-ext, which are a little stronger than those of AUV-ext. Arbutin, which showed an anti-tyrosinase activity, and kojic acid, which also showed much stronger activity and inhibition of melanin production, showed neither SOD-like activity nor UV absorption at UV-A. Though the structure of the principle(s) which contributed

to the aforementioned effects must be made clear in the future, the *Arctostaphylos* ethanol extracts, in particular *A. patula* and *A. viscida*, are promising cosmetic agents which originate from a crude drug because of their potency of anti-tyrosinase activity, inhibition of melanin production, SOD-like activity and the ability to absorb UV-B and UV-A.

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