FINAL REPORT

Set for Examining Skin Whitening/Dullness

324eco MgH2

Evaluation on

Inhibition of Melanin Production While B16 Melanoma Cells Were Used,

Inhibition of Darkening Melanin Precursor (DHICA),

Inhibition of Advanced Glycation End Products (AGEs) Production While Collagen Was Used

TEST FACILITY

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IDENTIFICATION	WS20052801
TEST SUBSTANCE	324eco MgH2
TEST TITLE	Set for Examining Skin Whitening/Dullness ・Inhibition of melanin production while B16 melanoma cells were used ・Inhibition of darkening a melanin precursor (DHICA) ・Inhibition of advanced glycation end products (AGEs) production while collagen was used
DOCUMENT STORAGE LOCATION	Kirei Testing Labo Co., Ltd.
TEST DATES	July 31, 2020
DOCUMENT STORAGE PERIOD	5 years after the test

Report Content

1. Summary

After 324eco MgH2 was applied,

- The rate (%) of darkening DHICA was significantly lower than that of control.
- The rate (%) of AGEs production was marginally lower than that of control.

From the above, 324eco MgH2 can exert advantageous effects on skin whitening/dullness.

2 Test objective

The test substance (investigational product) has been used to investigate the effects of the test substance on skin whitening and dullness by evaluating the inhibition of melanin production, the inhibition of darkening a melanin precursor (DHICA), and the inhibition of advanced glycation end products (AGEs) production.

3 Experimental design

The “skin spots and dullness”, which are top-ranked skin troubles, are affected by various factors. Melanin in epidermal pigmented cells (melanocytes) may be over-produced over the whole skin or like spots. This is a cause for the skin spots and dullness. In addition, oxidative polymerization of a melanin precursor DHICA (5,6-dihydroxyindole-2-carboxylic acid) is an essential step of producing dark melanin. Meanwhile, collagen glycation in the dermis is a cause for yellowing, which is not due to melanin.

Here, this assay set was used to evaluate how the test substance (investigational product) affected the level of production of melanin in pigmented cells, darkening of a colorless melanin precursor DHICA, oxidation of collagen. These assays were used to examine utility of the test substance (investigational product) on “skin whitening/dullness” including yellowing.

4 Materials and methods

4-1. Inhibition of melanin production while B16 melanoma cells were used [1, 2)]

4-1-1. Cells

Mouse B16 melanoma cells were cultured in a CO_2 incubator (at a CO_2 level of 5% and at 37°C). Then, this assay was performed.

4-1-2. Medium

Dulbecco's Modified Eagle Medium (DMEM, Cat No. 10566-016, Gibco, USA) containing 10.0% (v/v) Fetal Bovine Serum (FBS, Cat No. SH30071.03, Hyclone, UK) and 1.0% (v/v) Antibiotic-Antimycotic 100x (Cat No. 15240-062, Invitrogen, USA) was used.

4-1-3. How to prepare the test substance

DMEM containing 100 nM α-Melanocyte stimulating hormone (α-MSH, Cat No. M4135, Sigma-Aldrich, USA), 100 μM theophyline (Cat No. T1633, Sigma-Aldrich, USA), and 10% FBS was prepared and used as an assay culture medium.

An assay culture medium was used to dilute the test substance in a 10-fold dilution series to prepare, when used, the test substance at 3 concentrations (0.001%, 0.01%, 0.1%). An assay culture medium was used for control.

4-1-4. Assay protocol

1) First, 100 μL of B16 cells were seeded at 5.0×10^4 cells/mL per well of a 96-well plate (Cat No. 3595, Corning, USA), and were cultured in a CO_2 incubator for 24 h. In addition, 200 μL of phosphate buffer saline (PBS (-), Lot. 198601, Nissui, Japan) was added to wells not used for the assay so as to prevent drying during culture.
2) Next, 100 μL of the test substance or control was added to the 96-well plate, from which the culture supernatant had been removed, and the cells were then cultured in the CO_2 incubator for 72 h
3) The culture supernatant was removed and each well of the 96-well plate was washed gently once with 200 μL of PBS(-) warmed to 37°C.
4) Next, 120 μL of AlamarBlue (Cat No. DAL1025, Thermo Fisher Scientific, USA) solution prepared by 10-fold dilution with serum-free DMEM was added, and the mixture was incubated in the CO_2 incubator for 1 h.
5) Then, 100 μL of the culture supernatant was pipetted onto a 96-well plate (Lot. 9017, Corning, USA). After that, a microplate reader (SPARK® 10M, TECAN, Switzerland) was used to measure absorbance at 570 nm (OD_{570}) and absorbance at 650 nm (OD_{650}).
6) Each well of the 96-well plate, on which the cells were cultured, was washed gently once with 200 μL of PBS(-).
7) Next, 100 μL of aqueous solution containing 1 M sodium hydroxide (CAS No. 1310-73-2, Kanto Chemical, Japan) and 10% DMSO (Cat No. 043-07216, Wako, Japan) was added to each well of the 96-well plate. The plate was sealed and then incubated at 85°C for 10 min.
8) The 96-well plate was shaken using a plate mixer (NS-4P, AS ONE, Japan) at 270 rpm for 60 s. Then, a microplate reader was used to measure absorbance at 405 nm (OD_{405}).

9) OD_{570}, OD_{600}, and OD_{405} values of the test substance and the control were used to calculate, using the following formula, the rate of melanin production per cell count by the test substance.

Rate (%) of melanin production per cell count = [SM / (S - SR)] / [CM / (C - CR)] × 100.

CM: Control OD_{405} C: Control OD_{570} CR: Control blank OD_{650}

SM: Test substance OD_{405} S: Test substance OD_{570} SR: Test substance OD_{650}

4-2. Inhibition of darkening a melanin precursor (DHICA)[3)]

4-2-1. How to prepare the test substance

Ultra pure water (CAS No.7732-18-5, Wako, Japan) was used to dilute the test substance in a 10-fold dilution series to prepare, when used, the test substance at 3 concentrations (0.01%, 0.1%, 1%). In the control, PBS(-) was used.

4-2-2. Assay protocol

1) After 100 μL of 20 μg/mL DHICA (D452825, Toronto Research Chemicals, USA) solution was added to a 96-well plate (Lot. 9107, Corning, USA), 100 μL of the test substance (investigational product) or control was added. In the control, PBS(-) was used.

2) The 96-well plate was shaken using a plate mixer at 270 rpm for 30 s. Then, a microplate reader was used to measure absorbance at 405 nm (OD_{405}).

3) The 96-well micro plate of interest was set in a quartz reaction container (Ozawa science, Japan), and was irradiated using a solar simulator (Suntest CPS+, Atlas, USA) for 30 min.

4) The 96-well plate was shaken using a plate mixer at 270 rpm for 30 s. Then, a microplate reader was used to measure absorbance at 405 nm (OD_{405}).

5) The OD_{405} values of the test substance (investigational product) and the control before and after the irradiation were used to calculate, using the following formula, the rate of darkening DHICA by the test substance (investigational product).

Rate (%) of darkening DHICA = $(S_{30} - S_0) / (C_{30} - C_0) \times 100$.

C_{30}: Control OD_{405} after irradiation C_0: Control OD_{405} before irradiation

S_{30}: Test substance OD_{405} after irradiation S_0: Test substance OD_{405} before irradiation

4-3. Inhibition of advanced glycation end products (AGEs) production while collagen was used [4)]

4-3-1. How to prepare the test substance

Ultra pure water (CAS No.7732-18-5, Wako, Japan) was used to dilute the test substance in a 10-fold dilution series to prepare, when used, the test substance at 3 concentrations (0.01%, 0.1%, 1%). Ultrapure water was used for control.

4-3-2. Assay protocol

1) Here, 10% collagen solution, 1 M glucose solution, and 100 μL of the test substance (investigational product) or control were added to a 1.5-mL tube (Cat No. 0030125150, Eppendorf, Germany). The mixture was mixed by vortex.

2) The mixture was then centrifuged (ROTER: 41, CF15RN, himac, Japan) at 12,000 × g for 3 min. Then, 100 μL of each supernatant was pipetted onto a 96-well plate (Lot. 9107, Corning, USA).

3) The supernatant was excited at 360 nm and the fluorescence intensity (a.u.) at 465 nm was measured using a microplate reader.

4) The supernatant pipetted onto the 96-well plate was returned to a 1.5-mL tube, and the 1.5-mL tube was then heated at 100°C for 30 min.

3) Subsequently, the supernatant was centrifuged at 12,000 × g for 3 min, and 100 μL of the supernatant was added to a 96-well plate.Then, a microplate reader was used to measure the fluorescence intensity (a.u.).

4) The fluorescence intensities of the test substance (investigational product) and the control before and after the irradiation were used to calculate, using the following formula, the rate of AGEs production by the test substance (investigational product).

Rate (%) of AGEs production = $(S_{30} - S_0) / (C_{30} - C_0) \times 100$.

C_{30}: Control fluorescence intensity after heating C_0: Control fluorescence intensity before heating

S_{30}: Test substance fluorescence intensity after heating S_0: Test substance fluorescence intensity before heating

5 Significance test

For each assay, a significance test, which was an unpaired t-test between the test substance treatment group and control, was conducted. Each test was two-sided, and the significance level was less than 5%.

6 Results and conclusions

6-1. Inhibition of melanin production while B16 melanoma cells were used

While the melanin production rate of the control is set to 100%, Table 1 and Fig. 1 show the melanin production rate of the test substance. In addition, Table 1 shows the inhibition of melanin production by the test substance (investigational product).

6-2. Inhibition of darkening a melanin precursor (DHICA)

While the rate of darkening DHICA by the control is set to 100%, Table 2 and Fig. 2 show the rate of darkening DHICA by the test substance (investigational product). In addition, Table 2 shows the inhibition of darkening DHICA by the test substance.

6-3. Inhibition of advanced glycation end products (AGEs) production while collagen was used

While the AGEs production rate of the control is set to 100%, Table 3 and Fig. 3 show the AGEs production rate of the test substance. In addition, Table 3 shows the inhibition of AGEs production by the test substance (investigational product).

7 References

1) S. Akiu, *et al.* , Nihon Hifuka Gakkai Zasshi., 101(6), 609-613, 1991.

2) K. Ohguchi, *et al.* , Biosci. Biotechnol. Biochem., 72, 1107-1110, 2008.

3) K. Maeda and M. Hatao, J. Invest. Dermatol., 122, 503-509, 2004.

4) H. Masaki, *et al.* , Biochim. Biophys. Acta., 1428, 45-56, 1999.

Table 1. Absorbance (OD_{570}, OD_{600}, OD_{405}) and the rate of melanin production per cell.

	Concentration	OD_{570}			OD_{600}			OD_{405}			OD_{405} / (OD_{570} - OD_{600})						Rate (%) of melanin production per cell count						
	(%)	1	2	3	1	2	3	1	2	3	1	2	3	mean	±	s.d.	1	2	3	mean	±	s.d.	*t*-test vs. Control (+)
Control (+)	-	0.600	0.592	0.601	0.397	0.394	0.388	0.097	0.099	0.095	0.478	0.500	0.446	0.475	±	0.027	100.7	105.3	94.0	100.0	±	5.7	-
Test substance	0.001	0.628	0.612	0.613	0.405	0.392	0.393	0.106	0.107	0.110	0.475	0.486	0.500	0.487	±	0.013	100.1	102.4	105.3	102.6	±	2.6	-
	0.01	0.609	0.609	0.619	0.394	0.396	0.406	0.114	0.114	0.111	0.530	0.535	0.521	0.529	±	0.007	111.7	112.7	109.8	111.4	±	1.5	-
	0.1	0.596	0.606	0.602	0.407	0.415	0.410	0.103	0.104	0.104	0.545	0.545	0.542	0.544	±	0.002	114.8	114.8	114.2	114.6	±	0.3	-

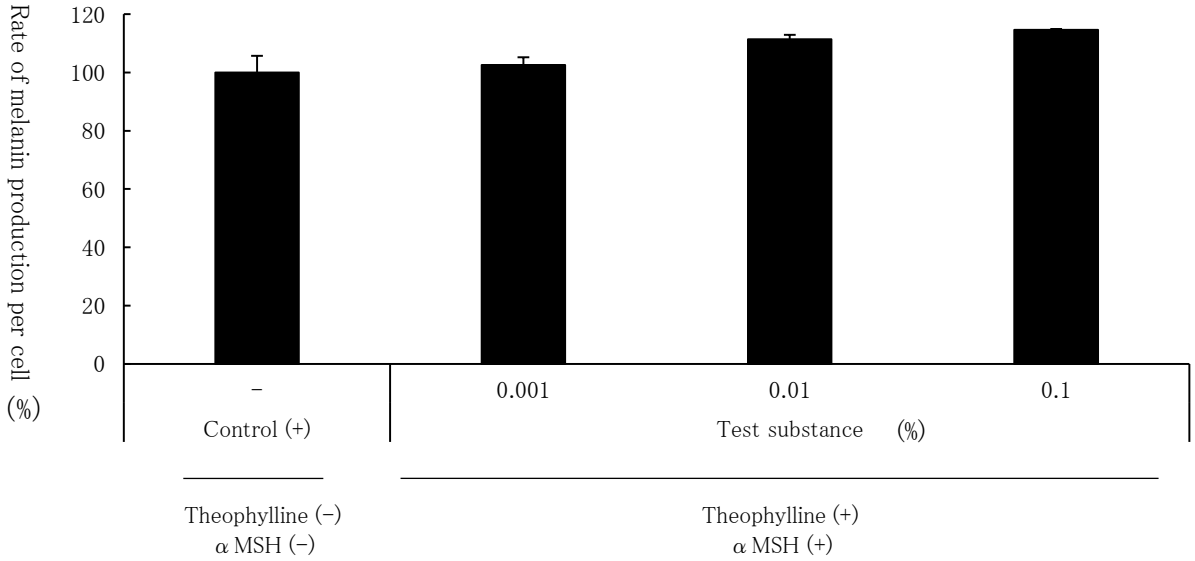

Fig. 1. How the test substance (investigational product) affected the rate of melanin production.

n = 3, mean ± s.d., unpaired t-test, $^{\dagger}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

s.d.: standard deviation

Table 2. Absorbance (OD_{405}), the rate of darkening DHICA, and the inhibition of darkening DHICA.

	Concentration	OD_{405}									Rate of darkening DHICA						Inhibition (%) of darkening DHICA						
		0min			30 min			30 min - 0 min															
	(%)	1	2	3	1	2	3	1	2	3	1	2	3	mean	±	s.d.	1	2	3	mean	±	s.d.	*t*-test
Control	-	0.047	0.048	0.047	0.119	0.120	0.120	0.072	0.072	0.073	99.5	99.5	100.9	100.0	±	0.8	0.5	0.5	-0.9	0.0	±	0.8	-
Test substance	0.01	0.048	0.048	0.049	0.129	0.130	0.129	0.081	0.082	0.080	112.0	113.4	110.6	112.0	±	1.4	-12.0	-13.4	-10.6	-12.0	±	1.4	
	0.1	0.055	0.052	0.053	0.128	0.127	0.126	0.073	0.075	0.073	100.9	103.7	100.9	101.8	±	1.6	-0.9	-3.7	-0.9	-1.8	±	1.6	
	1	0.055	0.057	0.064	0.115	0.115	0.118	0.060	0.058	0.054	82.9	80.2	74.7	79.3	±	4.2	17.1	19.8	25.3	20.7	±	4.2	**

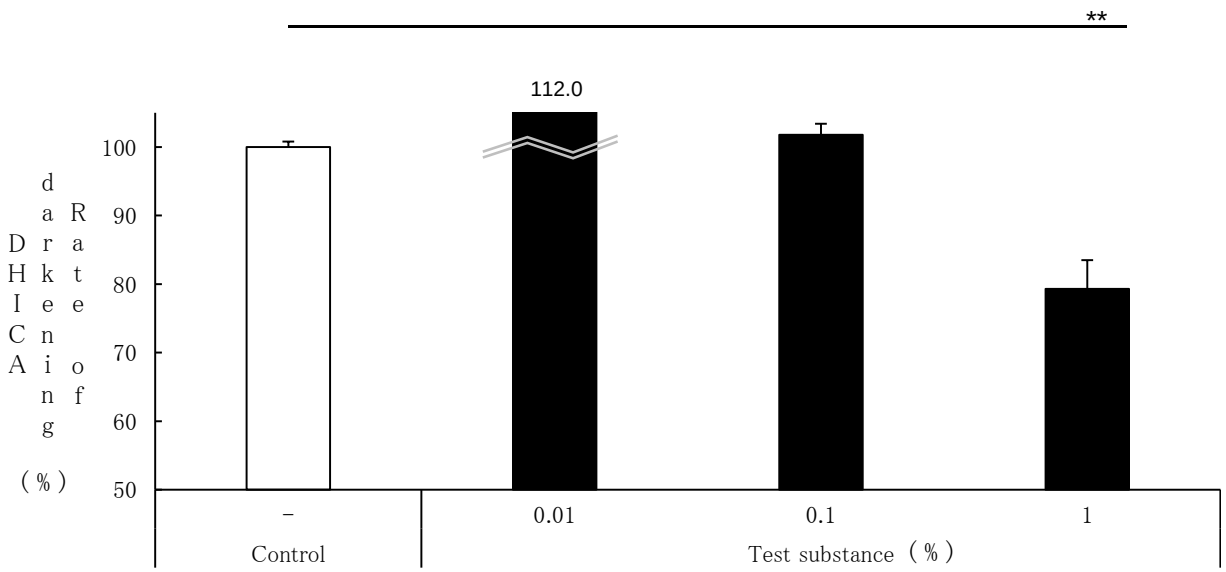

Fig. 2. How the test substance (investigational product) affected the darkening of DHICA.

n = 3, mean ± s.d., unpaired *t*-test, $^{\dagger}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

s.d.: standard deviation

Table 3. Fluorescence intensity, the rate of AGEs production, and the inhibition of AGEs production.

	Concentration	Fluorescence intensity (a.u.)									Rate (%) of AGEs production						Inhibition (%) of AGEs production						
		0min			30 min			30 min - Ave. 0 min															
	(%)	1	2	3	1	2	3	1	2	3	1	2	3	mean	±	s.d.	1	2	3	mean	±	s.d.	*t*-test
Control	-	10072	10159	10062	12747	12711	12697	2649	2613	2599	101.1	99.7	99.2	100.0	±	1.0	-1.1	0.3	0.8	0.0	±	1.0	-
Test substance	0.01	9815	9788	9682	12112	12171	12119	2350	2409	2357	89.7	91.9	90.0	90.5	±	1.2	10.3	8.1	10.0	9.5	±	1.2	***
	0.1	9851	9944	9728	12087	12095	12235	2246	2254	2394	85.7	86.0	91.4	87.7	±	3.2	14.3	14.0	8.6	12.3	±	3.2	**
	1	9843	9621	9747	11997	11799	12039	2260	2062	2302	86.3	78.7	87.9	84.3	±	4.9	13.7	21.3	12.1	15.7	±	4.9	**

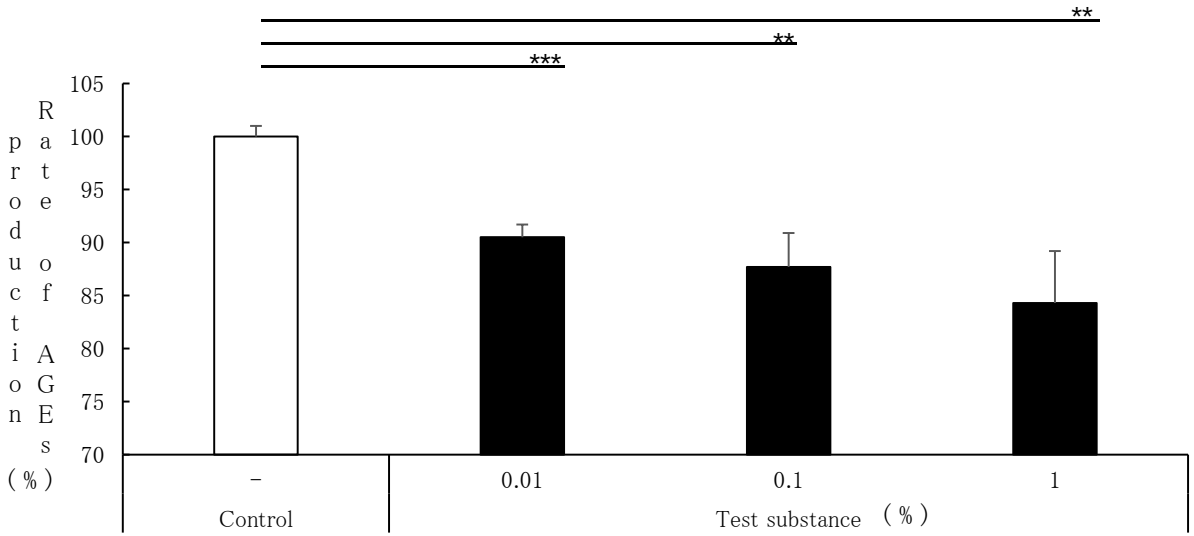

Fig. 3. How the test substance (investigational product) affected the AGEs production.

n = 3, mean ± s.d., unpaired *t*-test, $^{\dagger}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

a.u.: arbitrary unit, s.d.: standard deviation