FINAL REPORT

Antioxidant capacity-measuring set

In vitro evaluation of
SOD-like activity,
OH radical scavenging capacity,
Lipid peroxide production inhibition
for SHAMPOO BAR H2 ingredient MgH2

TEST FACILITY

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1. Summary

This study showed that

- Compared with control, the test substance significantly increased the SOD-like activity rate.
- Compared with control, the test substance significantly increased the OH radical scavenging rate.
- Compared with control, the test substance significantly increased the lipid peroxide production inhibition rate.

, suggesting that SHAMPOO BAR H2 ingredient MgH2 is useful as an antioxidant.

2. Test objective

The test substance is evaluated for the SOD-like activity, OH radical scavenging capacity, and lipid peroxide production inhibition. Thus, the efficacy of the test substance as an antioxidant is examined.

3. Experimental design

Reactive oxygen species are known to mediate, for instance, skin aging or dryness, spots, wrinkles, resilience/elasticity reduction, dullness, and rough skin. As a counter-measure, it is thus important to scavenge reactive oxygen species.
In this test, evaluated were: whether the test substance scavenged super oxide anions（O_2-）, which are known to be produced in an early stage of inflammation and metabolism after UV irradiation and hydroxy radicals（•OH）, which are called reactive oxygen having very high reactivity; whether the test substance inhibited production of lipid peroxide (LOOH), which can be generated after skin lipid oxidation. In this way, the antioxidant capacity of the test substance was evaluated extensively.

4. Materials and methods

4-1. How to prepare the test substance

Ultrapure water (CAS No.7732-18-5, Wako, Japan) was used to prepare 10% MgH_2, and the total of 7 solutions at concentrations of (0.00001%, 0.0001%, 0.001%, 0.01%, 0.1%, 1%, 10%) were prepared by 10-fold serial dilution using ultrapure water. Then, It was left to stand at room temperature for 1 hour to generate hydrogen. After that, they were centrifuged at 12,000 × g for 3 min, and 300 μL of the supernatant was collected in a 1.5-mL tube (Cat No. 0030125150, Eppendorf, Germany) for the test. Ultrapure water was also used for control.

4-2. Test protocol

4-2-1. SOD-like activity (in vitro)[1-3)]

The super oxide anion (O_2^-) scavenging capacity is measured as superoxide dismutase (SOD)-like activity. O_2^- scavenging capacity was measured with a SOD Assay Kit-WST (Cat No. S311, Dojindo, Japan).

1) Here, 90 µL of the sample or control, 900 µL of WST working solution, and 90 µL of enzyme working solution were added to a 1.5-mL tube. For a blank, ultrapure water was used instead of the enzyme working solution.
2) The mixed solution was uniformly mixed using vortex mixer, which was then incubated at 37ºC for 20 min.
3) After 20 min, they were centrifuged at 3,000 rpm for 3 min, and 240 µL of the supernatant was dispensed into each well of a 96-well plate.
4) Then, the absorbance at 450 nm was measured using a microplate reader (SPARK® 10M, TECAN, Switzerland).
5) The sample and control OD_{450} values were used to calculate, using the following equations, the O_2^- residual percentage caused by and the O_2^- scavenging rate of the test substance. This O_2^- scavenging rate was defined as the SOD-like activity rate.

O_2^- residual percentage (%) = $(S - SB)/(C - CB) \times 100$

O_2^- scavenging rate (%) = $\{(C - CB) - (S - SB)\}/(C - CB) \times 100$ = SOD-like activity rate (%)

C : Control OD_{450}

CB : Control blank OD_{450}

S : Test substance OD_{450}

SB : Test substance blank OD_{450}

4-2-2. OH radical scavenging capacity (in vitro)[4, 5)]

The test substance is measured for effects on scavenging radicals ・OH generated from hydrogen peroxide.

1) Here, 100 µL of the sample or control and 100 µL of 100 µM hydrogen peroxide (H_2O_2, CAS No. 7722-84-1, Wako, Japan) were added to a 1.5-mL tube. For a blank, purified water was used instead of 100 µM H_2O_2.
2) Uniformly mixed using vortex mixer and was incubated at 37ºC for 20 min.
3) After 20 min, they were centrifuged at 3,000 rpm for 3 min, and 50 µL of the supernatant was dispensed into each well of a 96-well plate.
4) Then, 150 µL of 100 mM PIPES (CAS No. 5625-37-6, Dojindo, Japan) buffer containing 0.5% TritonX-100 (CAS No. 9002-93-1, Sigma, USA), 100 µM DA-64 (CAS No. 115871-19-7, Wako, Japan) and 2000 mU/mL Peroxidase (ACS No. 9003-99-0, Wako, Japan) was added into each well of a 96-well plate and incubated at 37°C for 5 min.
5) After that, the absorbance at 727 nm was measured using microplate reader.

6) The sample and control OD_{727} values were used to calculate, using the following equations, the OH radical residual percentage caused by and the OH radical scavenging rate of the test substance.

OH radical residual percentage (%) = $(S - SB)/(C - CB)\times 100$

OH radical scavenging rate (%) = $\{(C - CB) - (S - SB)\}/(C - CB)\times 100$

C : Control OD_{727}

CB : Control blank OD_{727}

S : Test substance OD_{727}

SB : Test substance blank OD_{727}

4-2-3. Lipid peroxide production inhibition (in vitro)[6, 7]

Conjugated diene is measured that is produced by oxidation of linoleic acid, an unsaturated fatty acid.

1) Here, 1400 µL of 10 mM sodium dodecyl sulfate (SDS, CAS No. 151-21-3, Sigma, USA) solution containing 0.26 mM linoleic acid (CAS No. 60-33-3, Wako, Japan), and 20 µL of the sample or control were added to a 1.5-mL tube. The resulting solution was mixed by sonication. For a blank, ultrapure water was used.
2) Next, 10 µL of 0.2% 2,2'-Azobis(2-methylpropionamidine) Dihydrochloride (AAPH, CAS No. 2997-92-4, Wako, Japan) solution was added mixed solution, and uniformly mixed by sonication and vortex. Then, they were centrifuged at 3,000 rpm for 3 min.
3) After centrifugation, 100 µL of the supernatant was dispensed into each well of a UV-permeable 96-well plate (Cat No. 8404, Thermo Fisher, USA). The conjugated dienes were measured absorbance at 233 nm using a microplate reader.
4) After that, 1.5-mL tubes were incubated at 50℃ for 1 hour.
5) After 1 hour, they were centrifuged at 3,000 rpm for 3 min, and 100 µL of the supernatant was dispensed into each well of a UV-permeable 96-well plate.
6) The sample and control OD_{233} values were used to calculate, using the following equations, the lipid peroxide production rate and the lipid peroxide production inhibition rate.

Lipid peroxide production rate (%) = $(S - SB)/(C - CB)\times 100$

Lipid peroxide production inhibition rate (%) = $\{(C - CB) - (S - SB)\}/(C - CB)\times 100$

C : Control OD_{233}

CB : Control blank OD_{233}

S : Test substance OD_{233}

SB : Test substance blank OD_{233}

4-3. Significance test

A paired t-test, as a significance test, was conducted per assay with respect to a control group and a test substance addition group. Each test was two-sided, and the significance level was less than 5%.

5 Results and conclusions

The mean ± SD of the measured results and the bar graphs of the results are provided in Annex.

5-1. SOD-like activity

While the O_2^- residual percentage caused by the control is set to 100%, the O_2^- residual percentage caused by the test substance is shown in Table 1 and Fig. 1. In addition, the O_2^- scavenging rate, namely the SOD-like activity of the test substance is shown in Table 1.

5-2. OH radical scavenging capacity

While the OH radical residual percentage caused by the control is set to 100%, the OH radical residual percentage caused by the test substance is shown in Table 2 and Fig. 2. In addition, the OH radical scavenging rate of the test substance is shown in Table 2.

5-3. Lipid peroxide production inhibition

While the lipid peroxide production rate of the control is set to 100%, the lipid peroxide production rate of the test substance is shown in Table 3 and Fig. 3. In addition, Table 3 shows the lipid peroxide production inhibition rate of the test substance.

6 References

1) H. Ukeda., et al., *Anal. Sci.* , 15, 353, 1999.

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4) K. Miyata., et al., *Biosci. Biotechnol. Biochem.* , 70(9), 2138-2144, 2006.

5) M. Miyashita., et al., *J. Agric. Food Chem.* , 55(3), 806-811, 2007.

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Table 1. The O_2^- residual percentage caused by and the O_2^- scavenging rate（SOD-like activity）of the test substance

	Concentration (%)	O_2^- residual percentage (%) 1	2	3	mean	±	s.d.	O_2^- scavenging rate（SOD-like activity rate）(%) 1	2	3	mean	±	s.d.	*P* value
Control	-	98.9	100.9	100.2	100.0	±	1.0	1.1	-0.9	-0.2	0.0	±	1.0	-
Test substance	0.00001	98.8	98.2	98.9	98.6	±	0.4	1.2	1.8	1.1	1.4	±	0.4	$P < 0.1$
	0.0001	99.0	99.0	98.4	98.8	±	0.4	1.0	1.0	1.6	1.2	±	0.4	-
	0.001	97.8	97.3	97.5	97.5	±	0.3	2.2	2.7	2.5	2.5	±	0.3	$P < 0.05$
	0.01	98.6	98.0	98.6	98.4	±	0.4	1.4	2.0	1.4	1.6	±	0.4	$P < 0.1$
	0.1	97.7	99.2	99.0	98.6	±	0.8	2.3	0.8	1.0	1.4	±	0.8	-
	1	98.3	99.8	100.3	99.5	±	1.0	1.7	0.2	-0.3	0.5	±	1.0	-
	10	85.3	84.8	84.5	84.9	±	0.4	14.7	15.2	15.5	15.1	±	0.4	$P < 0.001$

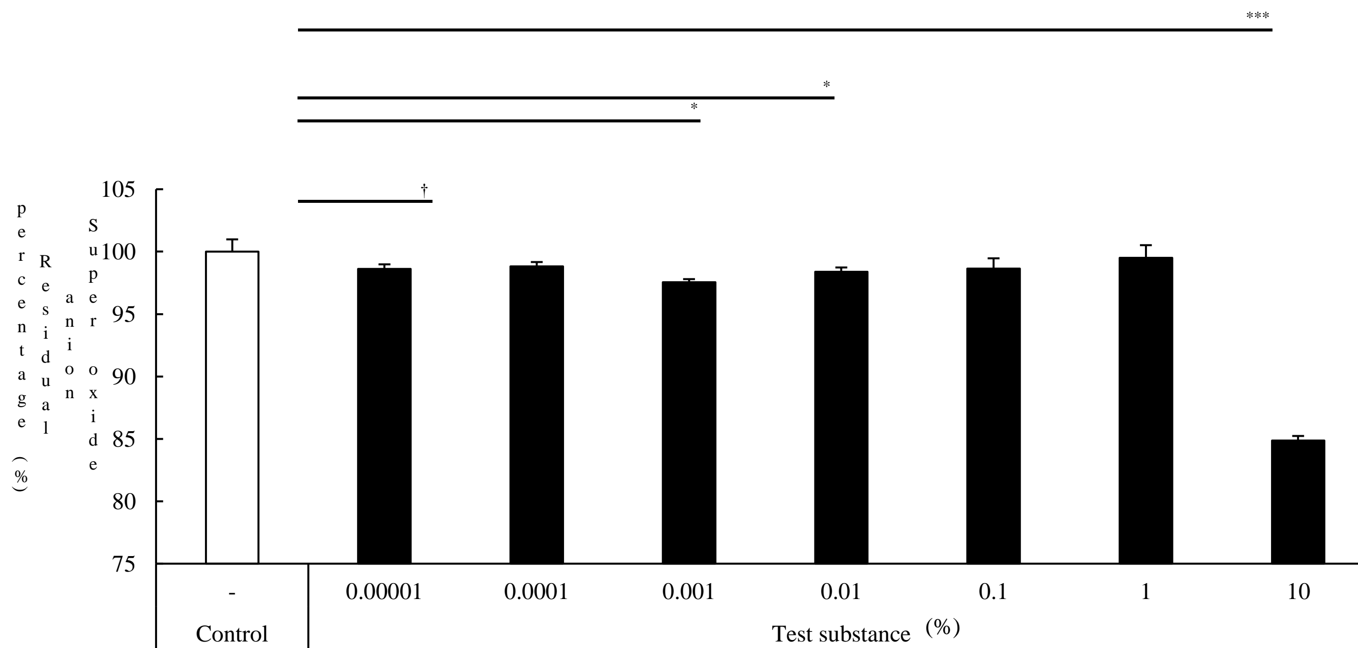

Fig. 1. The O_2^- residual percentage caused by the test substance
$n = 3$, unpaired t-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

Table 2. The OH radical residual percentage caused by and the OH radical scavenging rate of the test substance.

	Concentration (%)	・OH residual percentage (%)						・OH scavenging rate (%)						P value
		1	2	3	mean	±	s.d.	1	2	3	mean	±	s.d.	
Control	-	97.5	100.9	101.6	100.0	±	2.2	2.5	-0.9	-1.6	0.0	±	2.2	-
Test substance	0.00001	99.4	101.6	102.3	101.1	±	1.5	0.6	-1.6	-2.3	-1.1	±	1.5	-
	0.0001	100.9	101.0	100.8	100.9	±	0.1	-0.9	-1.0	-0.8	-0.9	±	0.1	-
	0.001	98.8	101.1	100.7	100.2	±	1.3	1.2	-1.1	-0.7	-0.2	±	1.3	-
	0.01	99.2	101.0	100.9	100.3	±	1.0	0.8	-1.0	-0.9	-0.3	±	1.0	-
	0.1	98.7	100.4	101.3	100.1	±	1.3	1.3	-0.4	-1.3	-0.1	±	1.3	-
	1	98.9	100.6	101.5	100.4	±	1.3	1.1	-0.6	-1.5	-0.4	±	1.3	-
	10	91.4	92.5	92.2	92.0	±	0.6	8.6	7.5	7.8	8.0	±	0.6	*P < 0.01*

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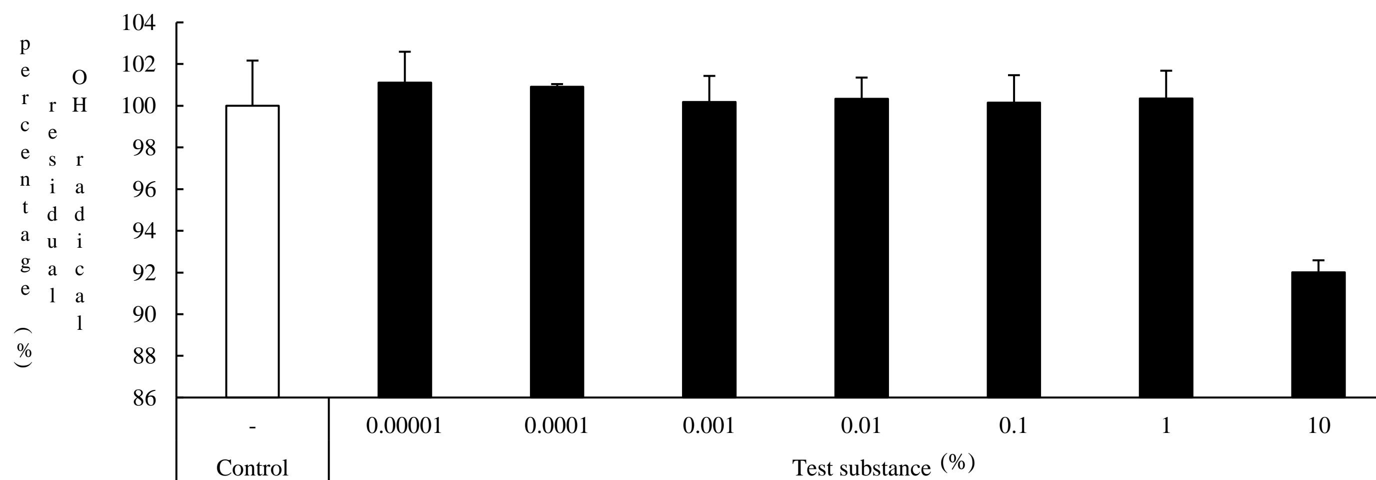

Fig. 2. The OH radical residual percentage caused by the test substance.
$n = 3$, unpaired t-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

Table 3. The lipid peroxide production rate and production inhibition rate of the test substance.

	Concentration (%)	Lipid peroxide production rate (%) 1	2	3	mean	±	s.d.	Lipid peroxide production inhibition rate (%) 1	2	3	mean	±	s.d.	P value
Control	-	103.5	99.8	96.7	100.0	±	3.4	-3.5	0.2	3.3	0.0	±	3.4	-
Test substance	0.00001	72.9	80.8	80.3	78.0	±	4.4	27.1	19.2	19.7	22.0	±	4.4	*P < 0.01*
	0.0001	82.4	83.5	84.5	83.5	±	1.1	17.6	16.5	15.5	16.5	±	1.1	*P < 0.01*
	0.001	86.6	79.8	79.8	82.0	±	4.0	13.4	20.2	20.2	18.0	±	4.0	*P < 0.01*
	0.01	76.6	86.6	79.8	81.0	±	5.1	23.4	13.4	20.2	19.0	±	5.1	*P < 0.01*
	0.1	84.0	78.7	83.5	82.0	±	2.9	16.0	21.3	16.5	18.0	±	2.9	*P < 0.01*
	1	51.8	55.5	55.5	54.2	±	2.1	48.2	44.5	44.5	45.8	±	2.1	*P < 0.001*
	10	11.6	2.6	5.8	6.7	±	4.6	88.4	97.4	94.2	93.3	±	4.6	*P < 0.001*

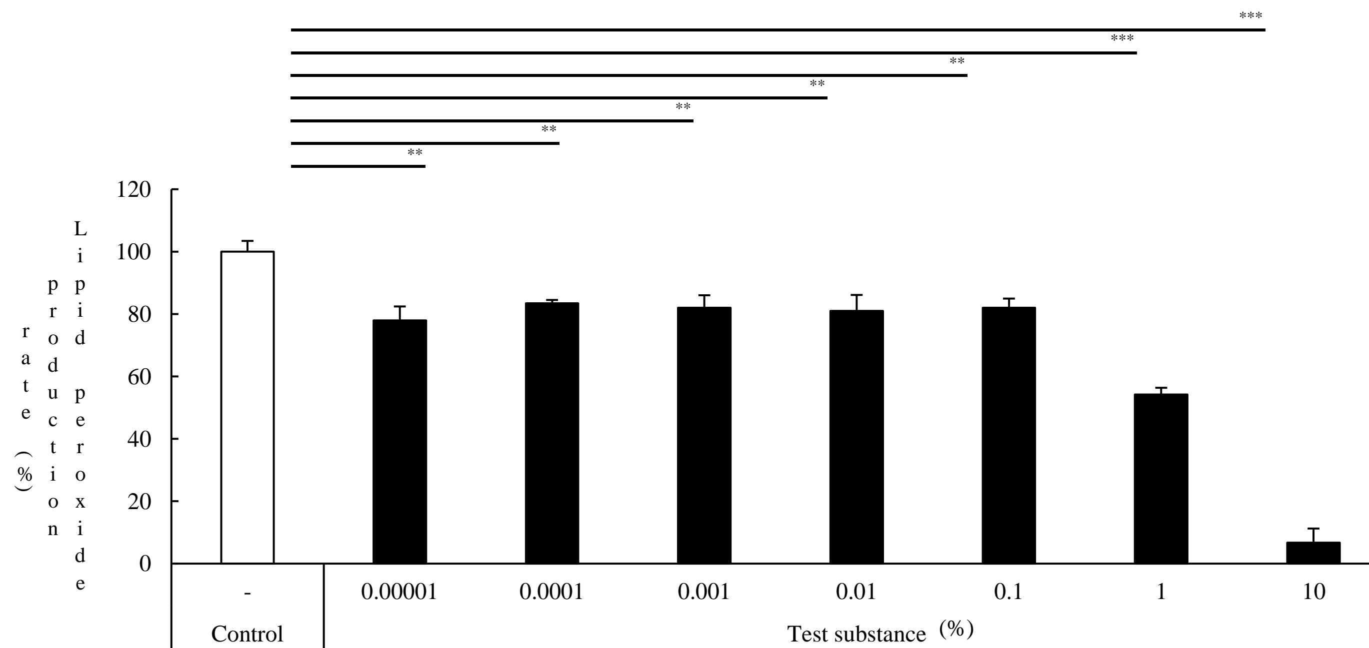

Fig. 3. The lipid peroxide production rate of the test substance.
n = 3, unpaired t-test, *P < 0.05, **P < 0.01, ***P < 0.001