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Original article

Effect of mangosteen pericarp extract-containing black vinegar drink on skin quality through anti-glycative actions.

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Abstract

***Purpose*:** The accumulation of advanced glycation end products (AGEs) in a living body caused by glycative stress contributes to the progress of aging of various biological tissues including skin. It is reported that mangosteen pericarp extract has the effects on the inhibition of the generation of AGEs and the improvement of skin elasticity. It is also reported that acetic acid included in black vinegar has an inhibitory effect on the rising of postprandial blood glucose levels. The evaluations of anti-glycative effect, skin-quality improving effect and the safety of long-term intake of black vinegar drink blended with mangosteen pericarp extract (hereinafter referred to as "test product") were conducted in this research.

***Method*:** 24 subjects were selected from 73 post-menopausal females 45 years or older and younger than 65 whose skin AGE fluorescence intensity was high, and they were divided into a test group and control, consisting of 12 subjects in each group. The subjects of the test group and control were asked to take the test product and placebo, respectively, for 12 weeks consecutively. The double-blind placebo-controlled parallel-group comparison tests including blood test, skin AGEs fluorescence intensity test and skin function test were conducted before the intake and 4, 8 and 12 weeks after the start of intake.

***Results*:** The AGEs fluorescence intensity on the skin on the inside of the upper arm of the subjects of the test group (n = 10) who had completed the test was significantly lower than those of control (n = 11) 4, 8 and 12 weeks after the start of intake ($p < 0.05$). The skin elasticity (R6) of the test group measured using a cutometer 12 weeks after the start of intake was significantly lower than the control ($p = 0.004$). Although no significant change in skin moisture was observed in the control, it significantly increased in the test group 8 and 12 weeks after the start of intake. (Before intake: 32.0%, 8 weeks after the start of intake: 37.4% ($p = 0.006$), 12 weeks after the start of intake 37.7% ($p = 0.004$). No adverse event was recognized during the test period and after the completion of test.

***Conclusion*:** It was expected from the result of the test of long-term intake that there is no problem concerning the safety of black vinegar drink blended with mangosteen pericarp extract, that it inhibits the accumulation of AGEs in skin while it does not have an affect on glycolipid metabolic indicator and that it improves skin elasticity.

***KEY WORDS*:** mangosteen (*Garcinia mangostana*), black vinegar, skin AGE fluorescence, skin elasticity, glycative stress.

Introduction

The accumulation of the advance glycation end products (AGEs) in living body caused by glycative stress contributes to the progress of aging of various living tissues including skin [1, 2]. It is reported that mangosteen pericarp extract has the effect of inhibiting the generation of AGEs and improving skin elasticity [3-6]. It is also reported that the acetic acid included in black vinegar has the effect of inhibiting the rise of postprandial blood glucose levels [7-10]. The purpose of this research was to conduct the evaluations of the anti-glycative effects and skin quality improving effect by the intake of a black vinegar drink blended with mangosteen pericarp extract. Targeting post-menopausal healthy females 45 years or older and younger than 75, various skin tests and clinical tests concerning the improvements of glycative actions and skin quality by the intake of the black vinegar drink blended

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with mangosteen pericarp extract for 12 consecutive weeks and the safety of its long-term intake were conducted and the double-blind placebo-controlled parallel-group comparison tests were conducted and discussed.

Method

Subjects

The subjects were healthy Japanese females 45 years or older and younger than 75, who were worried about their skin wrinkles and sagging. Recruitment of the subjects was implemented through hearing investigation to the members registered in a clinical test volunteers' association through telephone, and a preliminary survey was conducted for 73 females eligible for the test (55.6 ± 4.1 years, body mass index [BMI] 21.1 ± 3.1) and among them, 24 candidates (55.1 ± 4.3 years, BMI 19.6 ± 2.3) were selected as subjects, whose skin AGEs fluorescence intensity tested by a skin AGE sensor (Sharp Life Science Corporation, Kobe, Hyogo, Japan) [11] was high and R2 value by a cutometer (CT580; Courage-Khazaka, Cologne, Germany) was low.

Those who fell in the following exclusion criteria were excluded: 1) other supplement taker, 2) patient under the treatment of liver disease, kidney disease, or type-2 diabetes, 3) patient with atopic dermatitis, 4) person having a history of serious disease or under treatment 5) person who will be participating in other clinical tests when the present test starts, 6) person who has difficulties in maintaining lifestyles (taking meals, exercise, sleep, skin care etc.), 7) person who has difficulties in keeping a subject's diary, and 8) person who was judged as inappropriate as a subject by a doctor responsible for the test.

The subjects were asked to comply with the following notes during the test period. 1) Avoid overeating, excessive exercise and lack of sleep, 2) eat nothing from 4 hours before blood collection until the completion of the test (drinking water is permitted), 3) abstain from alcohol from the day before the test until the completion of the test, 4) have enough sleep the night before the test, 5) don't change lifestyle, 6) avoid excessive sunburn and traveling to areas where ultraviolet (UV) rays are strong during the test period, 7) avoid eating new healthy foods, 8) don't change skin care products and method, 9) avoid using new bathwater additive and body care products, 10) if you want to use a moisturizing cream because of extreme dryness of hands and feet, use it except on the measurement parts (face and arm), however, in that case, log it in diary, 11) during the test period, avoid actions that affect the quality of skin, such as skin scrubbing, and 12) don't shave the measurement parts in the face and don't remove excess hair in measurement pats from a week before the test.

Test product

The test product was black vinegar drink with a dilutable type developed as one that can be expected of anti-glycative effect and skin-quality improving effect from the research achievements so far by Yomeishu Seizo Co., Ltd. (Shibuya-ku, Tokyo, Japan) and the research achievements by "Agriculture, Forestry and Fisheries Creative Technology in Next Generation," SIP (Strategic Innovation Creative Program, Research No.1433567), that has been implemented by Doshisha University.

The subjects ingested a black vinegar drink blended with mangosteen pericarp extract (test product) or placebo drink (control product), both 25 mL diluted with water to 4 times (100 mL in total).

The intake amounts of the functional ingredients of the product for one time were 16.7 mL of black vinegar (750 mg as acetic acid), 100 mg of mangosteen pericarp extract and 4.5 g of indigestible dextrin. Those of control products were 0.167 mL of black vinegar, 0 mg of mangosteen pericarp extract and 0 g of indigestible dextrin. The composition and nutrient composition of each product are shown in ***Table 1*** and ***2***. The test product and control product were offered free for the test by Yomeishu Seizo Co., Ltd.

Table 1. Composition (single dose).

Test group	Control
Black vinegar (16.7 mL containing 750 mg acetic acid)	Black vinegar (0.167 mL)
Citric acid	Citric acid
Apple juice	–
Mangosteen pericarp extract (100 mg)	–
Kinkotsu-so (*Ajuga decumbens*) extract	–
Tian cha (Chinese blackberry tea) extract	–
Stevia	Stevia
Erythritol	Erythritol
Digestion resistant dextrin (4.5 mg)	–
–	Caramel

Table 2. Nutrient composition (per 25 mL).

	Test group	Control
Energy (kcal)	31.1	10.7
Protein (g)	0.0	0.0
Fat (g)	0.0	0.0
Carbohydrate (g) (DF + others)	13.0 (4.5 + 8.5)	2.6
Na (mg)	3.1	0.0

DF, dietary fiber. Others contain sugar and starch.

Test design

Stratified randomization double-blind parallel group comparison placebo controlled tests were conducted, in which AGE sensor score and skin elasticity score (R2 and R7) were used as stratification factors. The responsible person for the allocation randomly divided the 24 subjects selected through screening into 2 groups, one for test group and another for control. The allocation table was sealed and kept until opened by key. The period of the intake of test products was 12 weeks, and before the period (the 0th week), in the 4th and the 8th week and at the completion of the period of intake (the twelfth week), investigations were conducted, which consisted of skin function test, evaluations of oxidative stress markers and glycative stress markers, blood and urine tests (safety), basic measurements (body composition and blood pressure measurements), medical interview by doctor and a survey by anti-aging QOL (quality of life) questionnaire. The subjects were asked to keep a diary every day during the test period. The test period was from March to September 2016.

Evaluation items

Body measurements

Body height, body weight, blood pressure (systolic phase and diastolic phase) and pulse rate were measured. Body composition test was conducted using body composition meter BC118D (Tanita Co., Tokyo, Japan).

Subjective symptom

Anti-aging QOL common questionnaire (AAQOL) was used for the evaluation of subjective symptom [12].

Skin function test

Facial skin image analysis and the tests of skin AGEs fluorescence intensity, skin elasticity, skin moisture, transepidermal water loss amount and color difference were conducted for skin conditions. The tests were conducted after cleaning in a constant temperature and humidity room (21 ± 1°C, 50 ± 5%) after the subjects were acclimatized to this condition for 20 minutes.

VISIA Evolution (Canfield Imaging Systems, Inc., NJ. USA) was used for the facial skin image analysis.

TruAge scanner (TAS00100; DiagnOptics, Groningen, Netherlands) was used for the measurement of skin AGEs fluorescence intensity on the insides of upper arm and lower arm, and AGE sensor was used for the measurement of the tip of finger.

Skin elasticity was measured by single extraction method using a cutomter (MPA580: Courage-Khazaka, Cologne, Germany) and R2, R5. R6 and R7 were used as evaluation indices. A corneometer (CM825; Courage-Khazaka) was used for the measurement of skin moisture, and Tewameter (TM300; Courage-Khazaka) was used for the measurement of transepidermal water loss. A spectrophotometer (CM-2600d; Konika-Minoruta Co. Ltd., Tokyo, Japan) was used for the measurement of color difference. Skin elasticity, skin moisture, transepidermal water loss and color difference were measured 5 times centering on the inside of upper arm and the average values of three – time measurements excluding the maximum and minimum values were used.

Glycative stress markers

3-deoxyglucosone in plasma (3DG), N^{ε}-carboxymethyllysine (CML), pentosidine in plasma and urine, and immuno reactive insulin (IRI) were measured as glycative stress markers. Out of safety evaluation indices, fasting plasma glucose (FPG), HbA1c [NGSP], low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), triglyceride (TG) were used. 3DG (high performance liquid chromatography [HPLC] method), CML (enzyme-linked immunosorbent assay [ELISA] method) and pentosidine (ELISA method) were measured at the Japan Institute for the Control of Aging (JaICA), Nikken Seil Co., Ltd. (Fukuroi, Shizuoka, Japan).

Oxidative stress markers

A biological anti-oxidant potential (BAP) test and a Diacron-reactive oxygen metabolites (d-ROM) measurement

were conducted for the anti-oxidative potential and oxidative stress in the body [13]. Serum oxidized LDL, 8-hydroxy-2'-deoxyguanosine (8-OHdG) in urine and isoprostane in urine were measured. BAP test and d-ROM measurement were conducted in Olive Takamatsu Medical Clinic (Takamatsu, Kagawa, Japan) and the measurements of oxidized LDL, 8-OHdG and isoprostane were conducted in the Japan Institute for the Control of Aging, Nikken Seil Col. Ltd. The measurement of insulin was conducted in the Health and Science Research Institute (Yokohama, Kanagawa, Japan). Malondialdehyde-modified LDL (MDA-LDL) was measured using the ELISA method for the measurement of oxidized LDL.

Safety indices

The measurements of blood pressure, heart rate, body weight, body fat percentage and BMI, hematologic test, biochemical test and urine test were performed for the evaluation of safety. The items of hematologic test were as follows: white cell count, red cell count, hemoglobin count, hematocrit count, erythrocyte indices (MCV, MCH and MCHC), platelet count, eosinophil, basophil, lymphocyte, monocyte, neutrophil, erythroblast, anisocylosis, polychromasia and deformation. Biochemical test items are shown as follows: Total protein (TP), albumin (ALB), A/G ratio, blood urea nitrogen (BUN), creatinine (CRE), uric acid (UA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ–GTP), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine kinase (CK), total cholesterol (TC), TG, LDL-C, HDL-C, total bilirubin (T-BIL), sodium (Na), potassium (K), chlorine (Cl), calcium (Ca), magnesium (Mg), iron (serum), FPG and HbA1c [NGSP].

Statistical analysis

Subjects' backgrounds were shown as average value ± standard deviation and evaluation items as average value ± standard error. "The subjects who were incorporated into the test and to whom the use of test products started" were regarded as "full analysis set (FAS)" and those who had no serious protocol deviation that the "full analysis set" has were regarded as "per-protocol set (PPS)," as the most appropriate group for the implementation of test plan. FAS analysis was applied for the background and safety of subjects and PPS analysis was applied for the analysis of the effectiveness. As test methods, a Dunnett's test or a Steel's test was used for the comparison between before and after the intake, and an unpaired t-test or a Wilcoxon rank sum test was used for the comparison between the groups. However, a paired t-test was used for the comparison of only the data before and 12 weeks after the start of intake, such as the facial skin image analysis by VISIA, and an unpaired t-test was used for the comparison between the groups. For the comparison analysis between the groups, the change ratios based before the intake were compared between the test group and control. SAS (SAS 9.4) or SPSS (Statistics 19) was used for the statistical analysis and significance level of two-sided test was less than 5%.

Ethical guideline

This test was implemented in compliance with Helsinki Declaration (revised in the WMA General Assembly in 2013, Fortaleza) and the ethical guidance for Medical and Health Research Involving Human Subjects (announced by the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Heath, Labour and Welfare). In order to protect the human rights and safety of the subjects and assure the reliability of the test data, this test was conducted through the deliberation and approval by the Ethical Review Committee concerning the "research involving human subjects," of the Society for Glycative Stress Research (Approval No.: GSE 2016-003). This research was conducted after it was previously registered on the clinical test registration system, a medical information network for university hospitals (UMIN-CTR) (Registration Number: UMIN000022273).

Results

Accomplishment status of test

At the start of this test, there were 12 subjects in each group, 24 in total. However, 2 subjects and one subject dropped out from test group and control, respectively. In either case, the reason for dropping out from the test was personal and there was no relation with the test products. Safety analysis (FAS analysis) targeted at 24 subjects of 12 subjects of test group (55.1 ± 5.2 years) and 12 subjects of control (55.1 ± 4.0 years), and the analysis of the effectiveness (PPS analysis) targeted at 10 subjects of test group (56.0 ± 5.1 years) and 11 subjects of control (55.0 ± 4.2 years) excluding dropouts, 21 subjects in total.

Subjective symptom

No significant difference in the score of either item concerning physical or mental symptoms by AAQOL 4 weeks, 8 weeks and 12 weeks after the start of intake between the 2 groups.

No significant change was observed in their lifestyle including the number of cigarettes smoked per day, amount of drinking, amount of exercise, sleeping hours, water intake and TV watching hours.

Evaluation of skin condition

Image analysis of face skin

The results of image analysis of face skin by VISIA are shown in ***Table 3***. Although there was no significant difference in wrinkle scored between before and after in test group, significant score increase was observed in control. There was no significant difference in the change of wrinkle scored between the groups. Although there was no significant change in skin pore score in test group, there was significant improvement in control. There was no significant difference in the change of skin pore score between the groups. There was significant decrease in UV spot scores both in test group and control and no significant difference between the groups. There was no significant change in any other item.

Skin AGEs fluorescence

Skin AGE fluorescence on the inside of upper arm

Table 3. Image analysis of the face skin by VISIA.

Image analysis (Score)	Group	n	0w	12w
Skin spots	Test	10	2.10 ± 0.35	2.26 ± 0.38
	Control	11	2.40 ± 0.23	2.34 ± 0.24
Wrinkle	Test	10	1.50 ± 0.38	1.80 ± 0.62
	Control	11	1.12 ± 0.20	1.59 ± 0.24*
Texture	Test	10	0.91 ± 0.17	1.10 ± 0.25
	Control	11	0.92 ± 0.29	0.90 ± 0.24
Skin pores	Test	10	1.14 ± 0.22	1.10 ± 0.21
	Control	11	1.09 ± 0.24	0.99 ± 0.22*
UV spots	Test	10	2.91 ± 0.57	2.53 ± 0.45*
	Control	11	2.68 ± 0.45	2.22 ± 0.37*
Brown spots	Test	10	8.49 ± 0.81	8.45 ± 0.88
	Control	11	7.78 ± 0.59	8.16 ± 0.51
Red spots	Test	10	0.95 ± 0.12	1.02 ± 0.13
	Control	11	1.02 ± 0.15	1.09 ± 0.13
Porphyrin	Test	10	0.12 ± 0.10	0.12 ± 0.09
	Control	11	0.13 ± 0.10	0.10 ± 0.06

Results are expressed as mean ± SEM. * $p < 0.05$ by Paired t-test vs 0w and inter-group analysis by Student's t test. UV, ultraviolet; SEM, standard error mean.

significantly decreased 8 weeks after the start of intake in test group, and significant differences in the change rates 4, 8 and 12 weeks after were observed between the groups ($p < 0.05$, ***Table 4***). Although no significant change was observed in the intensity of skin AGEs fluorescence on the inside of forearm in test group through test period, the analysis between the groups showed that it tended to decline more in test group than control ($p = 0.097$, ***Fig. 1***). No significant difference in the intensity of AGE florescence on the tip of finger (change rate) was observed between the 2 groups.

Skin elasticity

Skin elasticity index R6 significantly decreased 12 weeks after in test group and significant difference was observed by an inter-group analysis of the amount of change ($p = 0.028$, ***Table 4***, ***Fig. 2***). No significant difference was observed in R2, R5, or R7 between the groups.

Skin moisture and transepidermal water loss

Skin moisture significantly increased 8 weeks after (37.4%, $p = 0.006$) and 12 weeks after (37.7%, $p = 0.004$) in test group compared with before the intake (***Table 4***, ***Fig. 3***). There was no significant change prior to the intake in the control. There was no significant difference between the groups. There was no significant difference in transepidermal water loss in both groups.

Color difference

There was no significant difference in index L*, a*, b*, Melanin index, Hb index or $HbSP_2$ index in color difference test between the groups (***Table 4***).

Glycative stress markers

There was no significant difference in FPG, HbA1c, insulin, LDL-C, HDL-C or TG between test group and control (***Table 5***). There was no significant difference in 3DG (an intermediate in generating process of AGEs), CML (one of AGEs) or pentosidine between the 2 groups.

Oxidative stress markers

There was no significant difference in d-ROM, BAP, oxidized LDL, 8-OHdG or isoprostane between test group and control (***Table 6***).

Safety evaluation indices

Although temporary symptoms of cold, diarrhea and others were confirmed in subject's diary, they disappeared in short-term and they were temporary symptoms. No harmful event which was clearly considered to be caused by the test product was confirmed.

No serious and harmful event considered to be caused by the test product was recognized during the observation period.

Table 4. Skin examination.

Skin examination	Group	n	0w	4w	8w	12w	Inter-group analysis p value 4w	8w	12w
Skin AGE fluorescnce (Upperarm)	Test	10	250.7 ± 7.4	243.0 ± 6.7	224.1 ± 5.4*	231.6 ± 8.9	**0.021**	**0.012**	**0.023**
	Control	11	241.0 ± 12.8	266.3 ± 13.0	264.6 ± 16.5	264.0 ± 17.7			
(Fore arm)	Test	10	225.3 ± 7.2	223.8 ± 8.4	216.0 ± 7.7	218.7 ± 9.1	0.365	0.159	0.097
	Control	11	221.6 ± 7.4	226.7 ± 9.6	223.7 ± 8.6	232.7 ± 10.9			
(Fingertip)	Test	10	2.55 ± 0.12	2.46 ± 0.15	2.57 ± 0.12	2.33 ± 0.11*	0.806	0.578	0.664
	Control	11	2.48 ± 0.09	2.31 ± 0.13	2.41 ± 0.11	2.32 ± 0.14			
Skin elasticity (R2)	Test	10	0.77 ± 0.04	0.84 ± 0.02**	0.86 ± 0.02**	0.84 ± 0.03**	0.069	0.105	0.224
	Control	11	0.80 ± 0.02	0.83 ± 0.02	0.86 ± 0.01**	0.84 ± 0.03*			
(R5)	Test	10	0.70 ± 0.06	0.77 ± 0.05**	0.78 ± 0.04**	0.73 ± 0.05	0.054	0.368	0.434
	Control	11	0.72 ± 0.02	0.74 ± 0.03	0.79 ± 0.03**	0.74 ± 0.03			
(R6)	Test	10	0.36 ± 0.02	0.34 ± 0.02	0.34 ± 0.02	0.29 ± 0.01**	0.774	0.887	**0.028**
	Control	11	0.36 ± 0.02	0.35 ± 0.02	0.34 ± 0.02	0.34 ± 0.01			
(R7)	Test	10	0.52 ± 0.05	0.57 ± 0.04**	0.58 ± 0.03**	0.57 ± 0.04**	0.101	0.32	0.178
	Control	11	0.53 ± 0.02	0.55 ± 0.03	0.59 ± 0.02**	0.55 ± 0.02			
Skin moisture	Test	10	32.0 ± 1.5	32.0 ± 1.7	37.4 ± 1.8**	37.7 ± 2.0**	0.341	0.277	0.376
	Control	11	31.3 ± 1.3	33.1 ± 1.0	33.6 ± 1.6	34.9 ± 1.8			
TEWL g/hm^2	Test	10	6.90 ± 0.41	7.67 ± 0.47	7.31 ± 0.44	6.70 ± 0.42	0.866	0.85	0.699
	Control	11	6.80 ± 0.41	7.52 ± 0.31	7.05 ± 0.33	6.80 ± 0.30			
Colour difference (L*)	Test	10	66.8 ± 0.4	66.6 ± 0.5	66.7 ± 0.4	66.3 ± 0.5	0.429	0.336	0.653
	Control	11	66.8 ± 0.9	66.8 ± 0.8	66.4 ± 1.0	66.5 ± 1.0			
(a*)	Test	10	4.53 ± 0.27	4.55 ± 0.30	4.53 ± 0.24	4.65 ± 0.32	0.883	0.455	0.321
	Control	11	4.72 ± 0.48	4.63 ± 0.42	4.82 ± 0.46	4.63 ± 0.47			
(b*)	Test	10	14.6 ± 0.5	14.1 ± 0.6	14.5 ± 0.6	14.6 ± 0.7	0.158	0.614	0.600
	Control	11	14.8 ± 0.7	14.7 ± 0.7	14.8 ± 0.8	14.9 ± 0.7			
Melanin Index	Test	10	0.68 ± 0.04	0.68 ± 0.04	0.68 ± 0.04	0.69 ± 0.04	0.488	0.396	0.372
	Control	11	0.72 ± 0.07	0.73 ± 0.06	0.74 ± 0.07	0.76 ± 0.07			
Hb Index	Test	10	0.81 ± 0.05	0.80 ± 0.06	0.79 ± 0.05	0.83 ± 0.05	0.767	0.682	0.083
	Control	11	0.79 ± 0.05	0.75 ± 0.04	0.78 ± 0.05	0.74 ± 0.05			
Hb SO_2 Index	Test	10	54.14 ± 1.62	53.65 ± 2.90	54.78 ± 2.52	52.23 ± 2.30	0.687	0.717	0.969
	Control	11	54.55 ± 2.32	54.45 ± 1.82	54.21 ± 2.37	52.27 ± 2.23			

Results are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ by Dunnett's test vs 0W and inter-group analysis by Student's t test. AGE, advanced glycation end product; TEWL, transepidermal water loss; Hb, hemoglobin; SO_2, oxygen saturation; SEM, standard error mean.

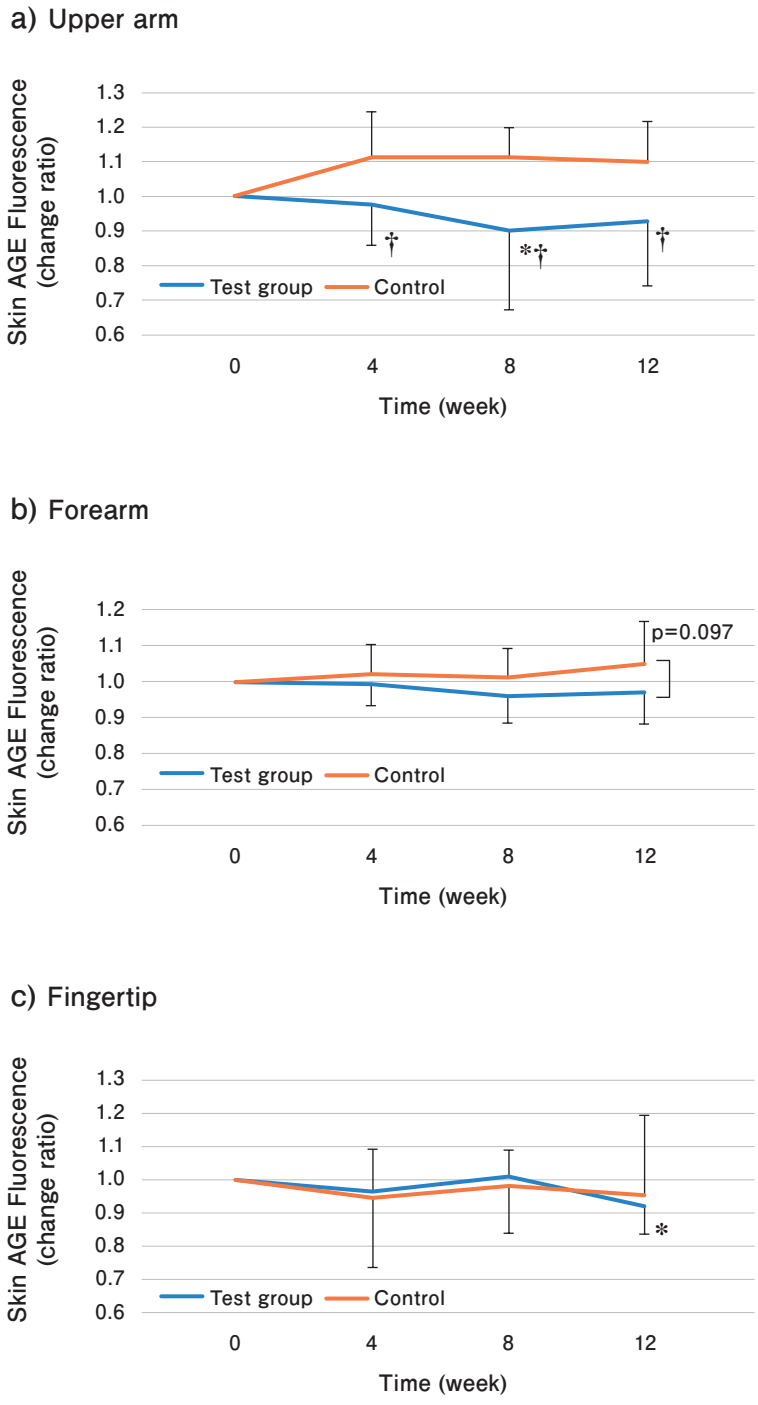

Fig. 1. **Skin AGE fluorescence.**

Skin AGE fluorescence intensity was measured at upper arm (**a**) and fore arm (**b**) by Truage scanner, and at finger tip by Age Sensor (**c**). Results of change ratio are expressed as mean ± SEM. * $p < 0.05$ by Dunnett's test vs 0W, † $p < 0.05$ vs. control (n = 11) by Student's t test. Change values from 0W are analyzed by inter-group analysis. Control: n = 11, Test group: n = 10. AGE, advanced glycation end product; SEM, standard error mean.

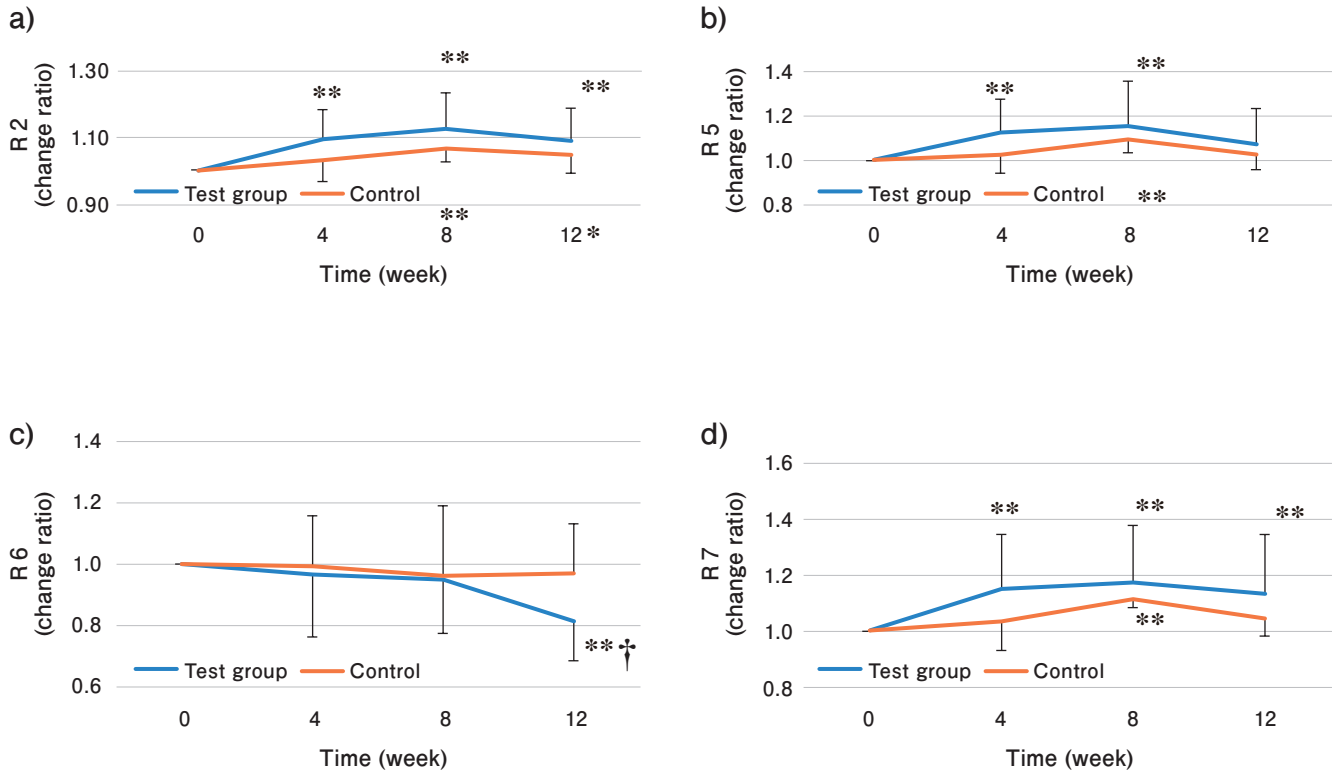

Fig. 2. **Skin elasticity.**

Skin elasticity was measured at upper arm. **a**: R2, **b**: R5, **c**: R6, **d**: R7. Resultsof change are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ by Dunnett's test vs 0W, † $p < 0.05$ vs. control by Student's t test. Change values from 0W are analyzed by inter-group analysis. Control: n = 11, Test group: n = 10. SEM, standard error mean.

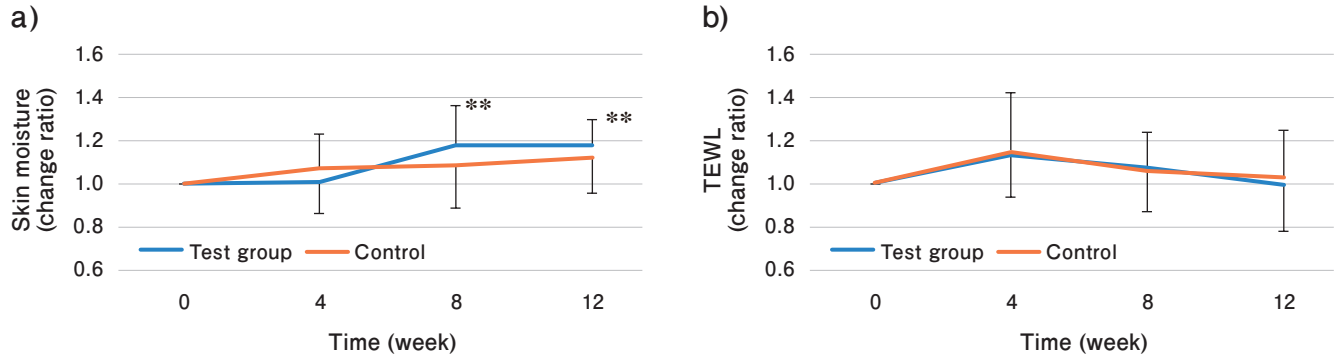

Fig. 3. **Skin moisture (a) and TEWL (b).**

Results of change ratio are expressed as mean ± SEM. ** $p < 0.01$ by Dunnett's test vs 0w. Control: n = 11, Test group: n = 10. TEWL, transepidermal water loss; SEM, standard error mean.

Table 5. Gltcative stress markers.

Glycative stress index		Group	n	0w	4w	8w	12w	Inter-group analysis p value 4w	8w	12w
3DG	ng/mL	Test	10	10.93 ± 0.68	10.96 ± 0.39	10.57 ± 0.35	10.04 ± 0.26	0.480	0.967	0.95
		Control	11	11.04 ± 0.47	11.89 ± 0.57	10.80 ± 0.43	10.32 ± 0.47			
CML	μg/mL	Test	10	5.70 ± 0.25	5.34 ± 0.21	4.79 ± 0.27**	4.45 ± 0.23**	0.995	0.447	0.310
		Control	11	5.72 ± 0.16	5.38 ± 0.17	5.02 ± 0.19**	4.65 ± 0.11**			
Pentosidine (plasma)	μg/mL	Test	10	0.043 ± 0.002	0.046 ± 0.001	0.049 ± 0.003	0.043 ± 0.003	0.194	0.994	0.773
		Control	11	0.042 ± 0.002	0.042 ± 0.000	0.049 ± 0.003*	0.042 ± 0.003			
Pentosidine (urine)	μg/mL	Test	10	0.012 ± 0.002	0.009 ± 0.001	0.011 ± 0.001	0.010 ± 0.002	0.883	0.389	0.489
		Control	11	0.015 ± 0.002	0.011 ± 0.001	0.011 ± 0.001	0.010 ± 0.001			
Pentosidine / Creatinine	μg/mg crea.	Test	10	0.014 ± 0.001	0.013 ± 0.001	0.015 ± 0.002	0.015 ± 0.002	0.518	0.942	0.135
		Control	11	0.016 ± 0.002	0.015 ± 0.001	0.015 ± 0.001	0.012 ± 0.001			
Insulin	μU/mL	Test	10	3.18 ± 0.53	2.83 ± 0.38	2.75 ± 0.33	2.79 ± 0.39	0.506	0.980	0.216
		Control	11	2.46 ± 0.30	2.42 ± 0.25	2.24 ± 0.28	2.87 ± 0.36			
FPG	mg/dL	Test	12	88.8 ± 1.8	88.4 ± 1.4	89.8 ± 1.3	87.9 ± 1.7	**0.03**	0.440	0.166
		Control	12	87.9 ± 1.3	90.1 ± 1.4	86.5 ± 0.7	89.2 ± 1.9			
HbA1c [NGSP]	%	Test	12	5.33 ± 0.05	5.23 ± 0.04**	5.38 ± 0.05	5.28 ± 0.06	0.92	0.821	0.852
		Control	12	5.44 ± 0.05	5.30 ± 0.06**	5.44 ± 0.06	5.34 ± 0.05*			
TG	mg/dL	Test	12	66.6 ± 6.6	71.7 ± 8.0	77.9 ± 13.4	80.1 ± 8.9	0.545	0.357	0.47
		Control	12	67.7 ± 7.6	89.5 ± 22.9	71.3 ± 11.9	73.5 ± 11.7			
LDL-C	mg/dL	Test	12	129.9 ± 6.1	132.9 ± 8.6	127.5 ± 8.6	124.4 ± 9.6	0.454	0.082	0.488
		Control	12	126.5 ± 11.5	126.0 ± 12.5	112.2 ± 10.9*	113.1 ± 11.2*			
HDL-C	mg/dL	Test	12	81.8 ± 5.2	79.5 ± 5.1	78.2 ± 5.8	72.7 ± 5.6**	0.716	0.354	0.973
		Control	12	91.4 ± 5.9	91.0 ± 6.1	87.1 ± 5.8*	83.6 ± 6.2*			

Results are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ by Dunnett's test vs 0W and inter-group analysis by Student's t test. 3DG, 3-deoxyglucosone; CML, CML, N^{ε}-carboxymethyllysine; FPG, fasting plasma glucose; TG, triglyceride; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; SEM, standard error mean.

Table 6. Oxidative stress markers.

Oxidative Stress		Group	n	0w	4w	8w	12w	Inter-group analysis p value 4w	8w	12w
d-ROM		Test	10	412.2 ± 23.9	392.0 ± 20.1	364.4 ± 13.1	377.3 ± 16.7	0.561	0.585	0.933
		Control	11	403.2 ± 13.9	377.2 ± 18.3	375.5 ± 15.6*	379.6 ± 18.6			
BAP		Test	10	2300.2 ± 44.0	2302.8 ± 47.0	2263.2 ± 48.2	2231.6 ± 33.7	0.523	0.239	0.486
		Control	11	2403.0 ± 34.3	2380.4 ± 48.2	2286.5 ± 44.1	2262.0 ± 60.8			
BAP / dROM ratio		Test	10	0.77 ± 0.05	0.80 ± 0.04	0.84 ± 0.04	0.80 ± 0.04	0.713	0.357	0.543
		Control	11	0.80 ± 0.03	0.86 ± 0.05	0.82 ± 0.03	0.81 ± 0.04			
Oxidized LDL	U/L	Test	10	70.9 ± 7.3	72.0 ± 4.9	74.4 ± 8.2	71.8 ± 6.8	0.297	0.613	0.661
		Control	11	58.5 ± 7.0	64.8 ± 7.2	62.8 ± 6.5	60.8 ± 6.5			
8-OHdG	ng/mL	Test	10	9.03 ± 2.05	6.71 ± 1.16	8.03 ± 1.10	7.54 ± 1.53	0.611	0.368	0.955
		Control	11	11.76 ± 1.74	9.40 ± 1.78	9.16 ± 1.72	10.18 ± 1.62			
8-OHdG / Cre	ng/mg crea.	Test	10	11.1 ± 0.75	10.40 ± 0.89	11.42 ± 0.99	10.12 ± 0.79	0.757	0.448	0.419
		Control	11	12.69 ± 0.89	11.65 ± 1.18	12.21 ± 1.17	12.26 ± 1.10			
Isoprostane	ng/mL	Test	10	2.49 ± 0.65	1.62 ± 0.37	1.92 ± 0.31	1.97 ± 0.32	0.859	0.438	0.919
		Control	11	2.87 ± 0.47	2.53 ± 0.45	2.19 ± 0.41	2.49 ± 0.40			
Isoprostane / Cre	ng/mg crea.	Test	10	2.91 ± 0.28	2.44 ± 0.22	2.66 ± 0.24	2.92 ± 0.35	0.149	0.520	0.896
		Control	11	3.07 ± 0.26	3.23 ± 0.34	3.07 ± 0.34	3.22 ± 0.36			

Results are expressed as mean ± SEM. * $p < 0.05$ by Dunnett's test vs 0W and inter-group analysis by Student's t test. d-ROM, Diacron-reactive oxygen metabolites; BAP, biological anti-oxident potential; LDL, low-density lipoprotein; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; Cre, creatinine; SEM, standard error mean.

Discussion

Evaluation of data

A double-blind placebo-controlled parallel-group comparison test was conducted for the purpose of evaluating anti-glycative effects and skin quality improving effects by the intake of black vinegar blended with mangosteen pericarp extract for 12 consecutive weeks, targeting subjects whose skin AGE fluorescence intensity was strong, selected through screening from postmenopausal females and the following results were obtained. The differences in the composition between test product and control product were mangosteen pericarp extract, *Kinkotsuso* extract, tian-tea extract, indigestible dextrin and apple juice. A small amount of apple juice was added for the purpose of adding taste. A significant improving effect on the skin AGE fluorescence intensity (inside of upper arm) by the test product not affecting glycolipid metabolism was recognized. There was a significant improving effect on skin elasticity (R6 by cutometer) by the test product. There was no significant change in skin moisture in the control but significant improvement was recognized only in the test group.

Glucose metabolism indices (FPG and Hba1c) and lipid metabolism indices (LDL-C, HDL-C and TG) were included in the indices of safety evaluation. However, as the results of the comparison of these indices between the 0th week and the 12th week, there was no change accompanying significant difference in any item between the groups. No effect or efficacy of the components contained in the test products on these indices was confirmed. However, there were many cases where the values before the test (the 0th week) were within the normal range: Among the 12 subjects of test group, the number of subjects whose FPG was 100 mg/dL or more was 0, that of those whose HbA1c was 5.8% or more was 0, that of those whose LDL-C was 130 mg/dL or more was six, that of those whose 140 mg/dL or more was three, that of those whose 150 mg/dL or more was two, and that of those whose TG was 130 mg/dL or more was 0. In the first place, those without high blood pressure or hyperlipidemia (high LDL-C level and high TG level) are considered not to have the room for improvement.

As for oxidative stress markers, none of oxidation degree (dROM), antioxidant potential (BAP) and oxidized LDL in blood, 8-OHdG and isoprostane in urine was influenced by the test product. Although parts of functional components included in mangosteen and tian-tea have the antioxidant potential, it was not demonstrated in this test.

The most notable thing in this test is that the intensity of skin AGE fluorescence was significantly improved in the test group. As the cause of the generation of AGEs, in addition to aging, high blood pressure, lipid abnormality, smoking, drinking, dietary habit (eating speed and order and proportion of carbohydrate), quality and length of sleep, mental and physical stresses and others are considered [14, 15]. There surely exist those who's accumulation of AGEs is high even though their standard glycolipid metabolism indices are normal, so that it can be interpreted that their higher accumulation of AGEs was improved by the intake of test product.

As the leverage for decreasing skin AGE accumulation, 1) inhibition of sugar absorption and correction of postprandial high blood glucose, 2) inhibition of the generation of AGEs and 3) promotion of decomposition and excretion of AGEs are considered. Because the test product includes a number of functional materials, it is required to discuss how each material functions.

Among glycative stress markers, there was no significant changes in 3DG, CML or pentosidine in plasma or pentosidine in urine (spot urine), and there was a significant difference only in the amount of skin AGE florescence between the groups. As the first reason, it is considered to be because the subjects whose intensity of skin AGE fluoresce was higher were selected through screening. In these cases, it is presumed that there was enough room for the improvement in the intensity of skin AGE fluorescence. In the report concerning the correlation among the amount of skin AGE fluorescence and AGEs and AGE intermediates in blood, it is reported that the correlation between CML and 3DG is not high [16, 17]. In the screening by skin AGEs, there are possibly many cases where CML and 3DG are close to normal ranges, and it is presumed that in these cases, these indices will not fluctuate any more through intervention.

It is very significant to determine the quantity of pentosidine in blood for the purpose of evaluating glycative stress in a living body. According to the report concerning the correlation between the amount of skin fluorescence AGEs and plasma pentosidine has a stronger correlation than CML [16]. There are two methods, the ELISA method and the HPLC method for the measurement of pentosidine in plasma; however, both methods have their own various problems. For the measurement of pentosidine in this test, a measurement kit using the ELISA method on the market [18, 19] was used. However, because it requires heat treatment at the pre-processing stage of the blood specimen, it is pointed out that as glycation occurs at the time of heat treatment, the value of pentosidine may possibly rise [20]. The method that has been conventionally used as HPLC method is ion-pair method, which experimental manipulation is complex and takes a long time in pre-processing of samples and the possibility of sample loss is pointed out. In order to correctly evaluate glycative stress in a living body, it is required to establish an accurate and effective pentosidine measuring method.

Pentosidine in urine or using spot urine is affected by water-intake situation before the test and body composition (water volume, in particular). The use of preserved urine specimen or urine accumulated in the bladder at night is desirable.

As a result of this test of skin elasticity, elasticity indices R2, R5 and R7 did not result in significant differences between the groups. However, in the comparison between the groups (comparison of the change rate based on the value before the intake), an improvement tendency was observed in index R2 in test group at the 4th week ($p = 0.069$), significant improvement in index R5 in test group at the 4th week ($p = 0.0054$) and significant decline in index R5 at the 12th week ($p=0.028$). Generally, skin elasticity decreases with age and elasticity indices R2 and R7 decreases [21]. It cannot be said that the evaluation method of R6 has been adequately established; however, in the previous reports [22-24], the decline in R6 has been evaluated as a positive effect. The test product in this research includes ingredients inhibiting the generation of AGEs. There has been no information regarding the relationship between the decline of R6 and the inhibition on the generation of AGEs so far. Further discussion is expected on this matter.

As a cause of the decline of skin elasticity, there was a decrease of production of extracellular matrix components including fibronectin caused by the deterioration of function of fibroblast and the low production of elastic fibers including collagen and elastin [2]. Collagen protein deteriorated by oxidation and glycation affects skin elasticity. In the case of

patients with type 2 diabetes, whose glycative stress is strong, skin elasticity curve sifts downwardly compared with healthy people and the skin elasticity sharply decreases [21], which is the reason why glycative stress becomes a large reason of decrease of skin elasticity.

The mechanism where the decline in skin elasticity is caused by the glycation of collagen fibers is as follows: Collagen fibers are triple-helically structured and play a role of maintaining the elasticity of skin together with elastic fibers. Lysine and arginine residue that constitute collagen protein are easily affected by glycation reaction, and they change to AGEs and form crosslink between fibers. When collagen fibers are fixed with each other by the bridge, they lose mobility and skin elasticity declines [25].

Black vinega

Black vinegar is contained in both control product (0.167 mL) and test product (16.7 mL); however, only a very small amount (1% of that in test product) is contained in control product. The effects of acetic acid, a main ingredient of black vinegar, on postprandial blood glucose level is discussed [7-10], and it is reported that the intake of rice added with pure rice vinegar decreases AUC more than rice only [7, 8], that the same effect is observed in apple vinegar and tomato vinegar [9] and they have the effect to lower cholesterol level [26, 27]. As a result of this test, the acetic acid contained in test product possibly contributed to the decrease of AUC.

In the randomized double-blind placebo-controlled parallel-group comparison tests that were conducted in this research, the results of the intake of acetic drink including indigestible dextrin and mixed herb extracts for 8 consecutive weeks showed the tendency to decrease CML of stratum corneum, a glycative stress marker [28]. The acetic acid contained in black vinegar is expected to bring about favorable effects without exacerbating glycative stress.

Mangosteen

The main component of test product is mangosteen pericarp extract. Mangosteen (*Garcinia mangostana* is the botanical name) is a tall evergreen tree belonging to genus Garcinia in the family Guttiferae and grows up to 20 m. Southeast Asia is the main production area of mangosteen. The fruit of mangosteen is 5-7 cm in diameter and covered with a thick peal. Vitamin B1 (0.11 mg) and mangan (0.35 mg) are contained in its edible part. As a folk medicine, the powdered outer peel of mangosteen fruit has been used for the treatment of diarrhea, dysentery and skin diseases. The leaves of mangosteen are dried and used as tea. The red pigment contained in the peal can be used as yellow dye. Its outer peel contains polyphenol of xanthone structure such as α-, β-, γ-mangostin, garicinone, mangostanol, gartinin and others [29].

Mangosteen pericarp extract inhibits the generation of AGEs [30-32]. It inhibits the generation of pentosidine, in particular, and alleviates the lowering of skin elasticity by preventing the crosslink formation caused by collagen glycation [30]. Mangosteen pericarp extract has the effect of delaying the breakdown of starch to convert it to maltose, oligosaccharide and glucose, by inhibiting amylase activity [33] and α-glucosidase activity [34]. Mangosteen pericarp extract has been probed to have blood glucose-lowering action by the test using normal blood-glucose rats and streptozotocin-induced diabetic rats [35, 36]. The activation of AMPK caused by mangosteen pericarp extract is involved in the improvement of glycolipid metabolism [37]. Because the diabetic indices were nearly normal in this test, no blood glucose lowering or the impact on insulin resistance by test product was observed.

In our previous research, as the result of comparison of the inhibitory effects of 74 kinds of fruits (IC_{50}) on the generations of in-vitro fluorescence AGEs of HSA-glucose reaction system and I-type collagen-glucose reaction system (***Table 7***), the IC_{50} of mangosteen pericarp extract were 0.040 mg/mL and 0.074 mg/mL and it showed the activity as strong as aminoguanidine for positive control (0.063 mg/mL and 0.232 mg/mL) or stronger [3]. When the above results are taken into consideration, the ingredients of mangosteen pericarp extract possibly contributed to the decrease of the intensity of skin AGE fluorescence of the test group, which was recognized in this test.

Tian-tea

Tian-tea (Chinese blackberry, *Rubus suavissimus* in botanical name) is used for allergic diseases including hay fever as folk medicine at times [38]. In our precedent research, the inhibitory effects of tian-tea (IC_{50}) on the generation of in-vitro fluorescence AGEs of HSA-glucose reaction system and that of I-type collagen-glucose reaction system were compared (***Table 7***). As a result, the IC_{50} of tian-tea was 0.046 mg/mL and 0.010 mg/mL and it showed the activity as strong as aminoguanidine for positive control (0.063 mg/mL and 0.232 mg/mL) or stronger [39].

The inhibitory effect of tian-tea on the generation of AGEs possibly contributed to the decrease in the intensity of skin AGE fluorescence of test group that was recognized this time.

Table 7. Inhibitory actions of AGE formation (IC_{50})

Reaction model	Collagen I / Glucose		HSA / Glucose			Ref. number
	Fluorescent AGEs	CML	Fluorescent AGEs	Pentosidine	3DG	
Mangosteen pericarp	0.074		0.04			3
fruit	1.224		0.697			3
Tian cha (Chinese blackberry tea)	0.010	0.011	0.046	0.141	0.021	39
Apple rind	0.098 ～ 1.461		0.262 ～ 1.620			3
fruit	0.645 ～ > 5.0		1.077 ～ > 5.0			3
Aminoguanidine	0.4	0.180	0.068	> 1.0	0.320	39

Unit: mg/dL; AGE, advanced glycation end product; IC_{50}, 50% inhibitory concentration; HSA, human serum albumin; CML, N^{ε}-carboxymethyllysine; 3DG, 3-deoxyglucosone; Ref., reference.

Safety

No adverse event was recognized in the clinical test during the test or after the completion of test of this time. Mangosteen pericarp extract that was used in this time was subjected to cytotoxicity testing [40] and animal toxicity testing [41]. The safety of mangosteen pericarp extract in the volume used in this test is assured. Tian-tea has a long and rich history as food since ancient time and its safety is assured. *Tekkotsuso* has been used for long time as an herbal medicine and no serious harmful event was observed within the range of the quantity used in this test.

Conclusion

The double-blind placebo-controlled parallel-group comparison tests by the intake of black vinegar drink blended with mangosteen pericarp extract for 12 weeks were conducted targeting postmenopausal females with higher accumulation of skin AGEs and a significant improving effect on skin AGE accumulation and skin elasticity (R6) were recognized. No significant serious side effect was recognized during test period and the safety of the test was confirmed. The test product can be expected to be safe functional food improving skin condition by its anti-glycative effect.

In this test, the subjects with higher AGE fluorescence intensity were selected from 73 females. These subjects were supposed to have lifestyle heightening glycative stress. It may be the reason why the skin AGE fluorescent intensity of the control tended to increase during the observation period of the 0th week, the 4th week, the 8th week and the 12th week. It is significant that test product decreased the skin AGE fluorescent intensity of those who had had lifestyle habits heightening glycative stress.

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Conflict of Interest Statement

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