Article

Effects of Dietary *Bacillus natto* Productive Protein on the Skin: In Vitro Efficacy Test and Single-Armed Trial

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Abstract: The dietary bacillus natto productive protein (BNPP) is a functional food ingredient that contains Bacillopeptidase F. BNPP is reported to improve blood flow. Based on previous research, we investigated the effect of BNPP on the skin. In vitro tests were performed to evaluate BNPP for its inhibitory effects on tyrosinase, elastase, and active oxygen (2,2-diphenyl-1-picrylhydrazyl (DPPH)) radical scavenging activities. In addition, a small-scale, single-armed trial of 15 female participants aged 40–65 years were conducted to assess the effects on human skin of BNPP, administered 250 mg/day orally, for 6 weeks. The beneficial effects of BNPP on the skin were shown by the evaluation of the tyrosinase inhibitory (0.01% and 0.1%), elastase inhibitory (0.00001% to 0.001%), and DPPH radical scavenging (1% and 10%) activities. In addition, the results suggested that the oral administration of BNPP may significantly enhance skin rosiness and also achieve significant improvement in skin conditions, defined as complexion, skin elasticity and resilience, moist feeling, skin texture, cosmetic adhesion, fine lines, under-eye darkness, eye bags, sagging cheeks, and sagging mouth. Furthermore, to investigate the use of BNPP as cosmetics, a skin irritation study was conducted using a cultured human skin model. The results showed that BNPP is non-irritant. In addition, to confirm the stability of BNPP, the quality of BNPP at the time of manufacture and three years and six months after manufacture was examined. The results showed no quality problems. These results suggest that the BNPP could be used as cosmetic purposes.

Keywords: Bacillopeptidase F; *Bacillus subtilis* var. *natto*; tyrosinase; elastase; DPPH

1. Introduction

Japanese traditional foods are known to be healthy overall and have the effects of reducing all-cause mortality, cardiovascular mortality, and combined cancer mortality [1]. Natto is a traditional Japanese fermented food rich in nutrients, including vitamins, proteins, amino acids, and dietary fiber. It has a long history in the traditional diet of Japanese people. However, some people are unwilling to eat natto because of its distinctive odor. To address this issue, Daiwa Pharmaceutical Co., Ltd. (Tokyo, Japan) developed BNPP as a functional food ingredient containing Bacillopeptidase F (EC 3.4.21.), one of the proteases produced by natto bacteria, and produces it under the trade name NKCP®. BNPP is a white or light-yellow powder with a slight fermented odor. The natto bacteria are removed from BNPP. The distinctive odor and vitamin K produced by natto are also removed from BNPP to negligible levels, creating a product that is easier to ingest than natto. BNPP has anti-coagulation, thrombolytic, and blood viscosity-lowering effects [2], and the oral administration of BNPP to rats showed an effect of promoting thrombolysis [3]. In addition, in a four-week study of oral BNPP administration, participants reported symptom improvements associated with blood circulation, such as blood flow; elevation in the skin surface temperature of the neck and shoulders; an improvement of neck muscle stiffness,

shoulder stiffness, and low back pain; the amelioration of coldness of the extremities and headache; and decreased blood pressure [4–7].

The causal association between skin aging and blood flow has been demonstrated [8]; therefore, maintaining good blood flow is essential to skin care. Since BNPP is known to be effective in improving blood flow [7], it is expected to exhibit a skincare effect as well. To date, however, there has been no study demonstrating the effect of BNPP on skin. With this background, the present study evaluated the inhibitory effects on tyrosinase (EC 1.14.18.1), elastase (EC 3.4.21.36), and active oxygen (2,2-diphenyl-1-picrylhydrazyl (DPPH)) radical scavenging activities of BNPP. Furthermore, a small-scale trial with adult female participants was also performed to evaluate the effect of BNPP on the skin. The potential for the future use of BNPP as cosmetics was also investigated.

2. Materials and Methods

2.1. Measurement of Tyrosinase Inhibitory Activity

The effect of tyrosinase inhibitory activity on the BNPP test samples was evaluated using dihydroxyphenylalanine (DOPA) as a substrate [9,10]. The test samples were prepared as follows. First, 80 μL of BNPP solution or 80 μL of the control were put into individual 1.5 mL test tubes, and 160 μL of tyrosinase (40 units/mL) (CAS No. 9002-10-2, Sigma-Aldrich, St. Louis, MO, USA) solution and 400 μL of 100 mM phosphate buffer (pH 6.8) were added. The solution was vortexed and incubated at 23 °C for 3 min. For the blank, 100 mM phosphate buffer was used. Next, 200 μL of 2.5 mM 3,4-L-DOPA (CAS No. 59-92-7, Wako, Japan) solution was added, mixed with the vortex mixer, and incubated at 23 °C for 10 min. The solution was then centrifuged at 12,000× *g* for 3 min. The supernatant was recovered into a 1.5 mL test tube, and 210 μL of the recovered supernatant was dispensed into each well of a 96-well plate in triplicate. The absorbance (optical density (OD) 490) was measured at 490 nm with a microplate reader. The activity rate of tyrosinase was calculated from the OD 490 values of each sample well and control using the following formula:

$$\text{Tyrosinase activity rate (\%)} = (S - SB)/(C - CB) \times 100$$

where C is the OD 490 of the control; CB is the OD 490 of the blank for the control; S is the OD 490 of the test sample; SB is the OD 490 of the blank for the test sample.

2.2. Measurement of Elastase Inhibitory Activity

The effect of BNPP on the elastase activity was evaluated using N-succinyl-ala-ala-ala-p-nitroanilide as a substrate [11]. First, 200 μL of BNPP solution or control, 200 μL of elastase (1.25 μg/mL) (CAS No. 39445-21-1, Sigma-Aldrich, St. Louis, MO, USA) solution, and 400 μL of 0.555 mM *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (CAS No. 52299-14-6, Sigma-Aldrich, St. Louis, MO, USA) solution were put into a 1.5 mL test tube. The solution was vortexed and incubated at 37 °C for 15 min. For the blank, 0.05 M Tris-HCl buffer (pH 8.0) was used. The solution was then centrifuged at 12,000× *g* for 3 min. The supernatant was recovered into a 1.5 mL test tube, and 200 μL of the recovered supernatant was dispensed into each well of a 96-well plate in triplicate. The absorbance (OD 415) was measured at 415 nm with a microplate reader.

The activity rate of elastase was calculated from OD 490 values of each sample well and control using the following formula:

$$\text{Elastase activity rate (\%)} = (S - SB)/(C - CB) \times 100$$

where C is the OD 415 of the control; CB is the OD 415 of the blank for the control; S is the OD 415 of the test sample; SB is the OD 415 of the blank for the test sample.

2.3. Evaluation of DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of BNPP was evaluated as follows [12]. First, 240 μL of BNPP solution or control, 240 μL of ethanol (CAS No. 64-17-5, Japan Alcohol,

Japan), and 160 µL of 0.25 M acetate buffer (pH 5.5) were put into a 1.5 mL test tube. The solution was vortexed and incubated at 37 °C for 5 min. Ethanol was used for the blank. Next, 160 µL of 78.4 µM DPPH (CAS No. 1898-66-4, Sigma-Aldrich, St. Louis, MO, USA) solution was added into the test tube, and the solution was mixed with the vortex mixer and incubated at 37 °C for 30 min. The solution was then centrifuged at $12{,}000 \times g$ for 3 min. The supernatant was recovered into a 1.5 mL test tube, and 200 µL of the recovered supernatant was dispensed into each well of a 96-well plate in triplicate. The absorbance (OD 517) was measured at 517 nm with a microplate reader.

Active oxygen residual rate was calculated from the OD 517 values of each sample well and control using the following formula:

$$\text{Active oxygen residual rate (\%)} = (S - SB)/(C - CB) \times 100$$

where C is the OD 517 of the control; CB is the OD 517 of the blank for the control; S is the OD 517 of the test sample; SB is the OD 517 of the blank for the test sample.

2.4. Skin Irritation Test

A skin irritation test was performed using a LabCyte EPI-MODEL24 (Japan Tissue Engineering Co., Ltd., Aichi, Japan) [13]. LabCyte EPI-MODEL24 tissues were transferred into 24-well plates with the assay medium (500 µL) and incubated overnight (37 °C, 5% CO_2, humidified atmosphere). Three tissues were used to assess of BNPP. To ensure good contact with the epidermal surface, the epidermal surface was moistened with 25 µL distilled water and then 25 mg of BNPP was added. Three tissues serving as negative controls were treated with 25 µL distilled water, and three tissues serving as positive controls were exposed to 5% sodium lauryl sulfate (SLS, CAS No. 151-21-3, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). After 15 min, each tissue was washed with phosphate buffered saline (PBS, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The tissues were then transferred to new wells containing 1 mL of fresh assay medium. The treated and control tissues were post-incubated for 42 h (37 °C, 5% CO_2, humidified atmosphere). After 42 h post-incubation, tissues were transferred to new wells on 24-well plates containing 500 µL of fresh prepared MTT medium (0.5 mg/mL) for the MTT assay. Tissues were incubated for three hours (37 °C, 5% CO_2, humidified atmosphere) and were then transferred to microtubes containing 300 µL isopropanol, completely immersing the tissue. Each tissue was refrigerated overnight and the pigment was extracted. Subsequently, 200 µL of extracts were transferred to a 96-well plate. The optical density was measured at 570 nm and at 650 nm as a reference absorbance, with isopropanol as a blank.

Cell viability was calculated from the absorbance of the test substance relative to the absorbance of the negative control using the following formula:

$$\text{Cell viability (\%)} = Sa/NC \times 100$$

where Sa is the absorbance of BNPP or positive control; NC is the mean absorbance of the negative control (n = 3).

Cell viability was assessed as irritating if the mean value of cell viability (n = 3) was less than 50%, and non-irritating if the mean value of cell viability (n = 3) was more than 50%.

2.5. Clinical Study

2.5.1. Inclusion/Exclusion Criteria for Participants

Participants included were healthy women aged 40–65 who were concerned about the "darkness" of their skin, such as under-eye pigmentation or bags (hereafter referred to as darkness).

The exclusion criteria were as follows:

a.   Dermatological diseases, such as atopic dermatitis, contact dermatitis, and/or cutaneous hypersensitivity;

b. Use of an oral drug or topical drug applied to the test skin site within two weeks before starting the study with a potential impact on the study intent;
c. Injury or tanned area that may interfere with measurement;
d. Past medical history of malignant tumors, heart failure, and/or myocardial infarction;
e. Chronic diseases under medical treatment, as follows: atrial fibrillation, arrhythmia, liver failure, kidney disease, cerebrovascular disease, rheumatoid arthritis, diabetes mellitus, dyslipidemia, hypertension, and other chronic diseases;
f. Regular intake of medicines (herbal medicines), food for specified health uses, foods with functional claims, health foods, and supplements;
g. Allergies to medicines or test food-related foods; and
h. Pregnancy, breastfeeding, or intention to become pregnant during the study period.

Based on these criteria, 15 participants were evaluated for this study.

2.5.2. Ethics Approval and Informed Consent

The present study was conducted in consideration of medical ethics in accordance with the Declaration of Helsinki and the Ethical Guidelines for Life Sciences and Medical Research Involving Human Subjects, and was approved by the Ethics Committees of NISHI-UMEDA Clinic for Asian Medical Collaboration (Japan, IRB No. 20000026). The study protocol was registered with the University Hospital Medical Information Network (UMIN-CTR) (UMIN000049095). The study was conducted from October 2022 to January 2023 in Japan.

Prior to the study, the participants received explanation on the study orally and in writing, and signed the informed consent to participate in the study. The participants could withdraw their consent at any time.

2.5.3. Test Food Samples

The test food samples were supplied by Daiwa Pharmaceutical Co., Ltd. (Tokyo, Japan). The samples were formulated as tablets containing 125 mg of BNPP per tablet and compounded ingredients. These ingredients included BNPP, maltodextrin, crystalline cellulose, sucrose fatty acid ester, silicon dioxide granules, and shellac.

2.5.4. Study Design

In this single-arm study, the participants were instructed to take 2 BNPP-containing tablets per day after dinner (250 mg/day) for the 6-week intervention period. All outcome variables were assessed at baseline and at the end of 6 weeks. The primary endpoints were defined as skin brightness, hue, and chroma. The secondary endpoints were defined as dark circle status, stratum corneum moisture content, skin texture, and skin viscoelasticity and responses to the questionnaire about skin conditions. Before skin site testing, participants were asked to wash their test skin sites and faces, followed by 15 min habituation in an environmental chamber controlled at 20 ± 2 °C, 50 ± 5% humidity. The measurements were taken after habituation.

Skin lightness, hue, and chroma were calculated by measuring Lab values under the left eye using a CM-26d® spectrophotometer (Konica Minolta Japan, Inc., Tokyo, Japan). A higher value of L* means brighter; a value of +a* indicates a red tint and −a* indicates a green tint; a value of +b* indicates a yellow tint and −b* indicates a blue tint; a higher value of c* means brighter shades; and a lower value of c* means duller shades.

The dark circles status score was based on the naked eye observation of the degree of dark circles under the left eye and rated by the physician responsible, on a seven-point scale: very prominent, 1; prominent, 2; somewhat prominent, 3; neither prominent nor not prominent, 4; not very prominent, 5; not prominent, 6; and not prominent at all, 7.

The stratum corneum moisture content was measured five times at the same location on the inner left forearm using a Corneometer CM825® (Courage + Khazaka Electronic, Cologne, Germany) and the average of the measurements was used.

The skin texture was measured using the VISIA Evolution (Canfield Scientific, Parsippany-Troy Hills, NJ, USA) to determine the number of texture marks on the left side of the face.

The skin viscoelasticity was measured five times at the same location on the inner left forearm using a Cutometer MPA580® (Courage + Khazaka Electronic, Cologne, Germany) and the average of the measured values was used. R2 (Ua/Uf, width of return after release of negative pressure/maximum elongation value at negative pressure = skin restoration rate), R5 (Ur/Ue, immediate width of return after release of negative pressure/elastic part of relaxation phase = elastic modulus at suction phase), and R7 (Ur/Uf, immediate width of return after release of negative pressure/maximum elongation value at negative pressure = elastic modulus at contraction).

After a macroscopic self-observation of their skin conditions, participants answered the survey questions about skin conditions, defined as complexion, skin elasticity and resilience, moist feeling, skin texture, cosmetic adhesion, fine lines, under-eye darkness, eye bags, sagging cheeks, and sagging mouth on the following 7-point scale: 1 = very bad; 2 = bad; 3 = slightly bad; 4 = acceptable; 5 = good; 6 = fairly good; and 7 = very good.

2.6. Statistical Analysis

The statistical analysis of tyrosinase inhibitory activity, elastase inhibitory activity, and DPPH radical scavenging activity was performed by Student *t*-test of parametric tests. The statistical analysis of skin lightness, hue, and chroma, stratum corneum moisture content, skin texture, and skin viscoelasticity was performed by Student-paired samples *t*-test of parametric tests. For dark circles status score and skin condition, the statistical analysis was performed using the nonparametric Wilcoxon signed-rank test. For all items, $p < 0.05$ was considered significant.

3. Results

3.1. Tyrosinase Inhibitory Activity, Elastase Inhibitory Activity, and DPPH Radical Scavenging Activity

Tyrosinase is an enzyme that induces skin melanogenesis. Elastase is an enzyme that degrades elastin, which plays a key role in keeping skin resilient (termed elasticity). In addition, active oxygen is involved in melanogenesis and photoaging by ultraviolet rays. BNPP exhibited significant inhibitory activity on tyrosinase at 0.01% and 0.1% (Figure 1a), while on elastase at 0.00001% to 0.001% (Figure 1b), and DPPH radical scavenging at 1% and 10% (Figure 1c).

3.2. Participant Tests

All fifteen participants (all female; average age 52.5 ± 7.29 years) completed the study without any issues, such as failing to remember to take the tablets or developing a poor physical condition.

3.2.1. Primary Endpoints

Brightness (L*) enhanced from 67.24 to 68.56 with no significant difference, and the complexion became slightly brighter, showing a trend toward improvement in skin conditions. Whereas skin hue a* significantly increased from 16.27 to 17.04, skin hue b* significantly decreased from 22.07 to 21.11. An increase in skin hue a* value indicates an increase in the skin's red tone, whereas a decrease in skin hue b* indicates a reduced yellow tone. Croma (c*) slightly decreased from 27.46 to 27.20, with no significant difference (Table 1).
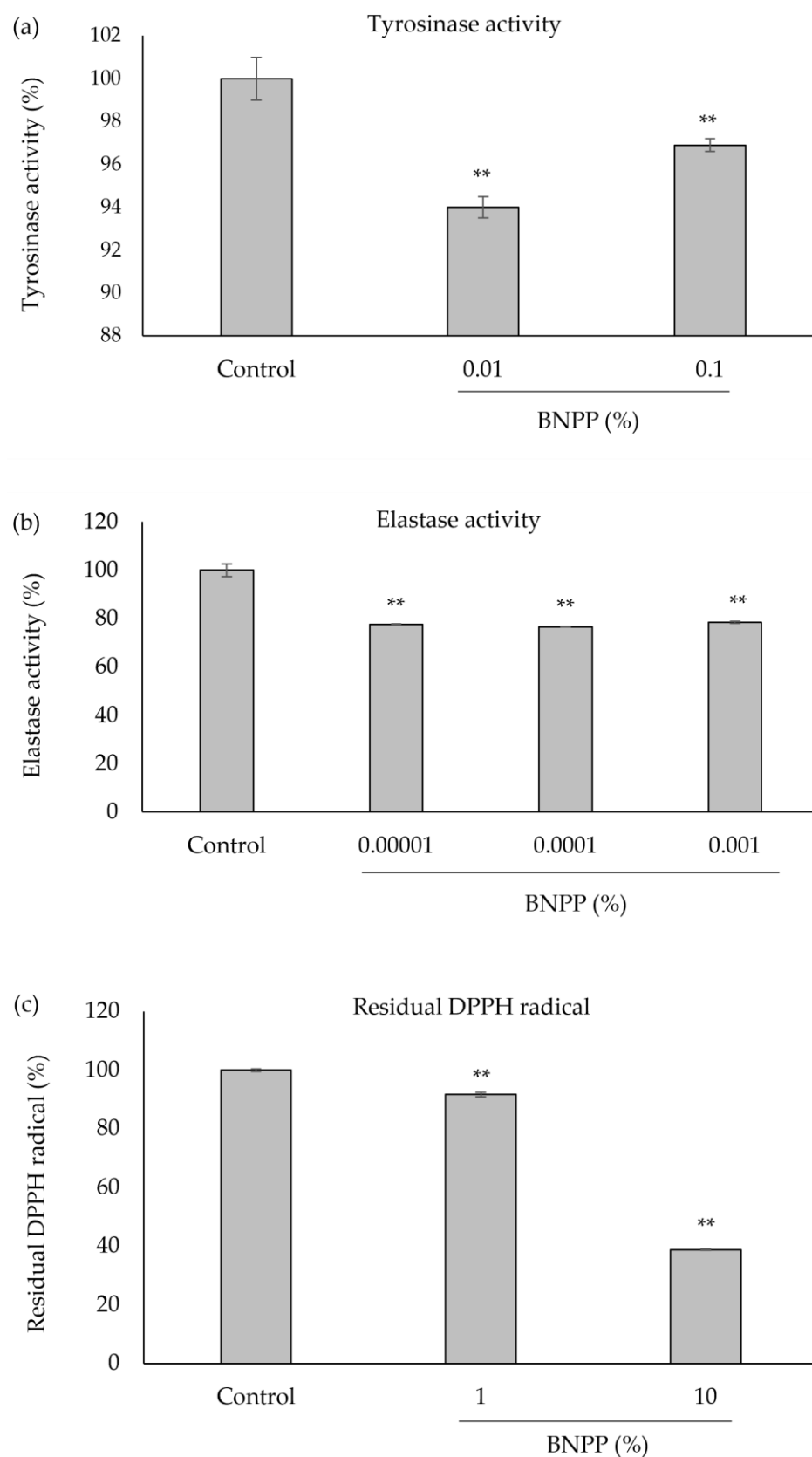
(a)

(b)

(c)

Figure 1. Inhibitory effect of BNPP on tyrosinase, elastase, and reactive oxygen species (DPPH) radical scavenging activity. (**a**) Tyrosinase activity; (**b**) elastase activity; (**c**) residual DPPH radical. Vertical bars indicate standard deviations. **: Compared with control, $p < 0.01$.

Table 1. Effects of BNPP on skin brightness, hue, and chroma.

Item		Baseline	6 Weeks After	*p* Value
		Mean ± Standard Deviation	Mean ± Standard Deviation	
Brightness (L)		67.24 ± 3.66	68.56 ± 3.72	0.0879
Hue	a*	16.27 ± 1.49	17.04 ± 1.84	0.0158
	b*	22.07 ± 3.14	21.11 ± 2.91	0.0024
Chroma (c*)		27.46 ± 3.03	27.20 ± 2.77	0.4398

3.2.2. Secondary Endpoints

The dark circles status score slightly improved from 2.93 to 3.07, with no significant difference. Also, the stratum corneum moisture content increased from 25.9 to 27.3, with no significant difference. The skin texture was slightly enhanced from 1327.5 to 1335.0, with no significant difference. The skin viscoelasticity was slightly reduced in R2 (0.745 to 0.723) and in R7 (0.546 to 0.517), with no significant difference, whereas skin viscoelasticity was slightly but significantly reduced in R5 (0.753 to 0.707) ($p = 0.025$). A significant enhancement was observed in all responses to the questions of the questionnaire survey, suggesting that the skin conditions were improved (Table 2).

Table 2. Effects of BNPP on skin conditions reported by participants on the ten-question survey.

Item	Baseline	6 Weeks After	*p* Value
	Mean ± Standard Deviation	Mean ± Standard Deviation	
Complexion	3.27 ± 1.03	4.67 ± 0.90	0.005
Skin elasticity and resilience	3.07 ± 1.22	4.27 ± 1.03	0.003
Moist feeling	3.20 ± 1.15	4.53 ± 1.36	0.007
Skin texture	2.80 ± 0.94	4.33 ± 0.90	0.002
Cosmetic adhesion	3.67 ± 1.05	4.67 ± 1.11	0.026
Fine lines	2.93 ± 1.03	4.00 ± 1.13	0.029
Under-eye darkness	2.27 ± 0.88	3.93 ± 1.28	0.001
Eye bags	2.47 ± 0.92	3.87 ± 0.99	0.005
Sagging cheeks	2.47 ± 0.99	3.60 ± 0.99	0.012
Sagging mouth	2.33 ± 0.98	3.53 ± 1.13	0.011

3.3. Skin Irritation Test

The cell viability was assessed as irritating if the mean value of cell viability was less than 50%, and non-irritating if the mean value of cell viability was more than 50%. The mean value of cell viability for BNPP was 93.6%, and therefore, the skin irritancy was assessed as non-irritant (Figure 2).

3.4. Stability of BNPP

To investigate the stability of BNPP, properties (color, odor), aerobic plate count, and coliform bacteria of BNPP (Lot No. B2HJ0101) at production and after 3 years and 6 months of storage at room temperature were tested. The results showed little difference between the two test results, confirming their high stability (Table 3).
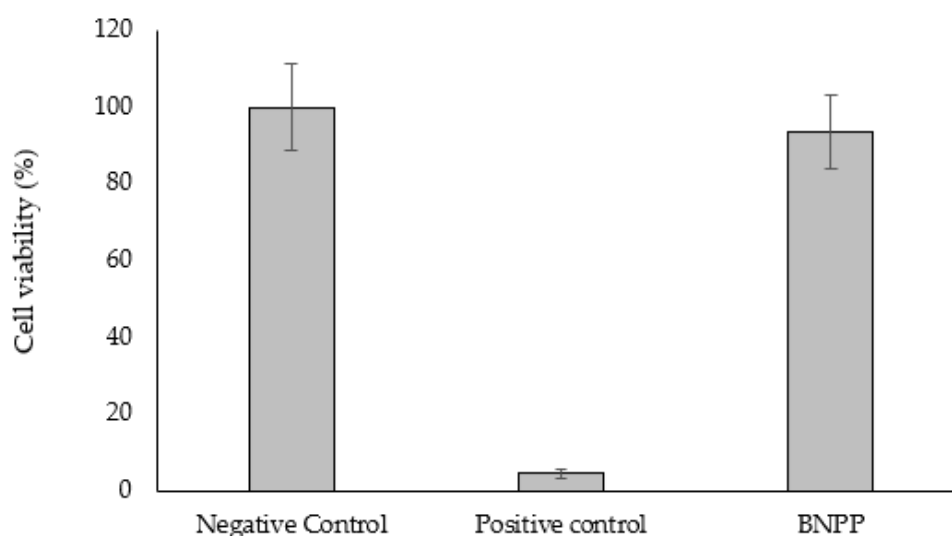
Figure 2. Skin irritation test of BNPP. The skin irritancy of BNPP was assessed using the LabCyte EPI-MODEL24. Cell viability was assessed as irritating if the mean value of cell viability (n = 3) was less than 50%, and non-irritating if the mean value of cell viability (n = 3) was more than 50%.

Table 3. The stability of BNPP (Lot No. B2HJ0101).

Test Item		Specification	At Production	After 3 Years and 6 Months
Properties	Color	White or light yellow	Light yellow	Light yellow
	Odor	Odorless or slight fermented odor	Odorless	Odorless
Aerobic plate count (cfu/g)		3×10^3 or less	Less than 1×10^2	Less than 1×10^2
Coliform bacteria		Negative	Negative	Negative

4. Discussion

In previous studies, BNPP has demonstrated an effect of improving blood flow, an increase in skin surface temperature of the neck and shoulders, an amelioration of muscle stiffness in the neck, an amelioration of shoulder stiffness, low back pain improvement, improvement in the coldness of the extremities, an amelioration of headaches, and a decrease in blood pressure [4–7]. The present study demonstrated that BNPP has tyrosinase inhibitory, elastase inhibitory, and DPPH radical scavenging activities.

Melanin is the major pigment present in human skin and is the most important photoprotective factor in response to UV damage from the sun [14]. Melanin is synthesized in melanosomes of melanocytes [15]. Tyrosinase is a multifunctional copper-containing polyphenol oxidase and is key to melanin biosynthesis [16]. Tyrosinase is closely involved in melanin synthesis via the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (l-DOPA) and the oxidation of l-DOPA to dopaquinone [17]. As tyrosinase overactivation is correlated with pigmentation, the inhibition of this enzyme is the most effective approach to control melanin overproduction and its dangerous effects. Therefore, tyrosinase inhibitors are being developed for use as whitening agents [18].

Elastin is a protein with elastic properties that exists in connective tissue, including the skin, and plays an important role in maintaining tissue structure after stretching or recoil. Excessive UV irradiation and the excessive production of active oxygen both trigger the expression of elastase, which reduces skin elasticity and causes wrinkles and sagging [19,20]. Elastase inhibitors have been reported to increase the linearity of dermal elastin fibers and skin elasticity, thereby preventing wrinkles and sagging [19].

A major factor in exogenous skin ageing is exposure to UV radiation [21–23]. Oxidative stress is considered to have a major influence on extrinsic skin ageing, with reactive oxygen

species (ROS) being one of the main contributors [24]. Excessive sun exposure to the skin induces the production of large amounts of ROS, reduces the production of peroxidase and glutathione reductase and, in addition, triggers oxidative damage to elastin and collagen, resulting in reduced skin elasticity, rough skin, and wrinkle formation. Research is underway to reduce photo-ageing of the skin by reducing reactive oxygen species [25].

In this study, we found that BNPP has tyrosinase inhibitory, elastase inhibitory, and DPPH radical scavenging activities in vitro. It is reasonable to suggest that BNPP has the effects of preventing brown spot formation, lightening the skin, and improving fine lines/wrinkles. Based on these findings, we have further examined the efficacy of BNPP in humans. Participants who took oral BNPP 250 mg/day for 6 weeks achieved a significant increase in skin hue a* but a significant decrease in skin hue b* compared to baseline. These results indicate the increased red and decreased yellow tones in the skin. The brightness appeared to be enhanced, leading to a brighter skin tone, although no significant difference was observed. The red tone of the skin reflects the hemoglobin level in the microvascular system of the skin [26]. In a clinical study on healthy subjects, Sunagawa et al. showed that the ingestion of BNPP for four weeks increased skin surface temperature [6]. Fujita et al. also showed in a clinical study on healthy subjects that the ingestion of BNPP for four weeks improved blood flow after cold water immersion [7]. It is suggested that the blood flow rate of the skin may be increased by the supplementation of BNPP and thus enhance the skin's red tone. Blood flow in the skin decreases after the age of 40, and there is a link between rough skin and blood flow, with rough skin reported to have less blood flow than normal skin [27]. BNPP intake was suggested to improve blood flow and improve skin condition.

The values obtained from evaluating the secondary endpoints—dark circles status, stratum corneum moisture content, and skin texture—all increased, but showed no significant differences. Although the skin viscoelasticity decreased in R2, R5, and R7, only a decrease in R5 level was statistically significant. Notably, viscoelasticity is reported to deteriorate in the medial forearm skin because of dry air from autumn to winter [28]. Because this study was conducted during the period from fall to winter (October 2022 to January 2023), this finding may have been subject to seasonal influences; therefore, a further controlled study is needed to address this issue.

In the questionnaire survey, significant improvement was observed in all endpoints, which reflected the participant's responses to the ten survey questions about skin conditions, defined as complexion, skin elasticity and resilience, moist feeling, skin texture, cosmetic adhesion, fine lines, under-eye darkness, eye bags, sagging cheeks, and sagging mouth. The *p*-values for all items were very low, suggesting that participants clearly felt that the consumption of BNPP had a positive effect on their skin.

Skin irritation and stability tests were conducted to explore the potential use of BNPP as cosmetics. The results showed that BNPP was not irritating to the skin and could be applied to the skin. In addition, a high stability of more than three years was confirmed.

5. Conclusions

In this study, we revealed that BNPP has tyrosinase inhibitory, elastase inhibitory, and DPPH radical scavenging activities, suggesting that it has the effects of preventing brown spot formation, lightening the skin, and improving fine lines/wrinkles and sagging. In addition, from the participant tests for BNPP, we showed a statistically significant increase in the skin red tone and a decrease in yellow tone, as well as a significant improvement of skin conditions: complexion, skin elasticity and resilience, moist feeling, skin texture, cosmetic adhesion, fine lines, under-eye darkness, eye bags, sagging cheeks, and sagging mouth. As described above, this study suggests that BNPP has a positive effect on the skin. However, this study has several limitations. The small number of participants could have limited the findings of our study. It should be also noted that the current results of the skin tests are difficult to be extrapolated to other skin complexion types. Further large-scale trials with various skin complexion types are required. The results of the skin irritation test

and the stability test suggest that BNPP could be used for cosmetic purposes. However, further tests are required for its use as a cosmetic.

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