



Biological Characterization of Natto Extract from *Bacillus subtilis* Fermented Soybeans: Effects on Cell Proliferation and Enzyme Inhibition for Cosmetic Applications

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Article info

Article history:

Received : 13 June 2025

Revised : 2 September 2025

Accepted : 11 September 2025

Keywords:

Bacillus subtilis var. natto, Natto extract, Cell proliferation, Emulsion serum

Abstract

Natto fermentation by *Bacillus subtilis* var. *natto* is a well-established process that enhances the nutritional profile of soybeans by producing bioactive compounds, including proteins, vitamins, peptides, and antioxidant-rich phytochemicals. These phytochemicals, especially those with antioxidant properties and inhibitors of collagenase and tyrosinase, hold promise as bioactive agents for cosmetic formulations. The present study aimed to extract bioactive compounds from natto powder (NP) using ethanol, assess their phenolic and flavonoid contents, and determine their inhibitory activities against collagenase, elastase, and tyrosinase. The natto powder extract (NPE) yielded $19.00 \pm 0.67\%$, with total phenolic and flavonoid contents of 20.64 ± 0.20 mg gallic acid equivalent (GAE)/g extract and 22.34 ± 3.28 mg quercetin equivalents (QE)/g extract, respectively. It exhibited significant antioxidant activities, with IC_{50} values of 2.84 ± 0.19 mg/mL for 2,2-diphenyl-1-picrylhydrazyl (DPPH), 0.76 ± 0.09 mg/mL for 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 94.04 ± 1.18 mg TE/g extract for ferric reducing antioxidant power (FRAP). The extract also inhibits collagenase, elastase, and tyrosinase activities. NPE at noncytotoxic concentrations (62.5 and 125 μ g/mL) significantly promoted human fibroblast proliferation. An emulsion serum containing NPE was developed and tested for stability over 6 heating-cooling cycles. The emulsion serum exhibited good stability with no phase separation and maintained an acceptable pH, viscosity, and color. These findings highlight the potential of NPE as a promising ingredient in cosmeceutical skin care formulations.

Introduction

Skin aging is a complex biological process influenced by both intrinsic and extrinsic factors.

Although intrinsic aging is genetically determined and progressive, extrinsic factors, such as ultraviolet (UV) radiation, environmental pollutants, and sleep deprivation, accelerate the deterioration of skin structure and function

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(Wang et al., 2024; Hussein et al., 2025). These external stressors induce the degradation of dermal components such as collagen and elastin by upregulating matrix-degrading enzymes, including collagenase and elastase. Simultaneously, tyrosinase, a key enzyme in melanogenesis, becomes overactive, leading to hyperpigmentation and a reduced skin luminosity. Collectively, these enzymatic actions contribute to wrinkle formation, loss of elasticity, and uneven skin pigmentation. Consequently, the inhibition of these enzymes is considered a key strategy for the development of anti-aging and skin-brightening cosmetics (Jiratchayamaethasakul et al., 2020; Kim et al., 2024; He et al., 2024).

In recent years, growing consumer demand for cosmetic products formulated with natural plant-derived extracts has been observed (Persistence Market Research, 2025), particularly for those rich in phenolic compounds and flavonoids. These compounds have shown potent antioxidant activity and the ability to modulate key skin-aging enzymes (Xie et al., 2024). Natto is a traditional Japanese fermented food produced by fermenting soybeans with *Bacillus subtilis* var. natto, a Gram-positive endospore-forming bacterium (Miao et al., 2024). This fermentation process produces the fibrinolytic enzyme nattokinase, which has been widely studied for its cardiovascular benefits (Li et al., 2023), as well as enhances the release and bioavailability of isoflavones, phenolic compounds, and flavonoids. These bioactive compounds exhibit antioxidant, anti-inflammatory, and enzyme-inhibitory properties, with potential applications in promoting blood circulation and preventing skin aging (Xiang et al., 2022; Domaszewska-Szostek et al., 2021; Dou et al., 2023; Igari et al., 2023; Xu et al., 2024).

Therefore, the present study aimed to investigate the phenolic and flavonoid contents, antioxidant potential and inhibitory activities against collagenase, elastase, and tyrosinase of natto extract, with the objective of developing cosmetic formulations targeting skin care and rejuvenation.

Materials and methods

1. Preparation and extraction of natto

Briefly, soybeans were cleaned, soaked overnight in water, and autoclaved at 121°C for 15 min. *B. subtilis* var. natto was prepared by culturing in Luria-Bertani (LB) broth at 37°C for 18 h with agitation (200 rpm), followed by centrifugation (8,000×g, 10 min) and resuspension in sterile saline to a final concentration of

1×10^8 colony-forming units (CFU)/mL. After cooling to approximately 40°C, *B. subtilis* var. natto was added to the sterilized soybeans to achieve a final concentration of 1×10^6 CFU/g of soybeans. The mixture was incubated at 37°C for 24 h for fermentation. After fermentation, natto was freeze-dried to obtain natto powder (NP). The freeze-drying process lasted for 48 h until the final moisture content was below 5%, which was used as the endpoint to indicate completion. The powder was extracted with 80% ethanol at a sample-to-solvent ratio of 1:20 (w/v) for 3 h on a rotary shaker (250 rpm) (Liu et al., 2022; Rocchi et al., 2024). The solvent was evaporated using a rotary evaporator, and the extract was freeze-dried. The % yield was calculated as:

$$\% \text{ yield} = (\text{weight of dried extract} / \text{weight of NP used for extraction}) \times 100$$

The resulting natto powder extract (NPE) was stored at -20°C until further analysis.

2. Total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu method (Rungruang et al., 2022). A 20 µL sample of NPE (1 mg/mL) was mixed with 100 µL of 10% Folin-Ciocalteu solution. After adding 80 µL of 7.5% Na₂CO₃ (w/v), the mixture was incubated for 60 min. Absorbance was measured at 760 nm using a microplate reader (Infinite® 200 Pro, Tecan, Switzerland). The total phenolic content was expressed as gallic acid equivalent (mg GAE/g extract).

3. Total flavonoid content

Total flavonoid content was quantified using the aluminum chloride colorimetric method (Rungruang et al., 2022). NPE (30 µL) was mixed with 100 µL MeOH and 20 µL of NaNO₂ (1:5, w/v) and agitated for 6 min. After adding 15 µL of 1:10 AlCl₃ solution, the mixture was incubated for 6 min. Finally, 55 µL of 1 M NaOH was added, and the solution was incubated for a further 10 min at 25°C. The absorbance was measured at 510 nm. The results were expressed as quercetin equivalents (mg QE/g extract).

4. In vitro antioxidant assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH)

DPPH• assay was performed as described by Rungruang et al. (2022). A 150 µL DPPH• solution (Sigma-Aldrich, USA) was mixed with 50 µL of NPE and incubated in the dark for 30 min. The absorbance was measured at 515 nm. The antioxidant activity of the extract was calculated as the IC₅₀ value (the concentration of the extract capable of scavenging 50% of the DPPH radicals).

5. *In vitro* antioxidant assay using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+})

The ABTS^{•+} assay was performed as described by Rungruang et al. (2022). ABTS^{•+} radical was generated by mixing 2.45 mM K₂S₂O₈ and 7.0 mM ABTS (Sigma-Aldrich, USA). The mixture was stored in the dark for 16 h. Before analysis, the ABTS solution was diluted with 95% EtOH to an absorbance of 0.70±0.05 at 734 nm. NPE (40 µL) was mixed with 160 µL of the diluted ABTS radical solution and incubated in the dark for 6 min. The absorbance was measured at 734 nm, and the results were expressed as IC₅₀ values.

6. *In vitro* antioxidant assay using ferric reducing antioxidant power (FRAP)

FRAP assay was performed as described by Rungruang et al. (2022). The ferric reducing antioxidant power of NPE (1 mg/mL) was determined by incubating 150 µL of freshly prepared FRAP reagent with 20 µL of extract at 37°C for 8 min. Absorbance was measured at 593 nm, and FRAP values were expressed as Trolox (TE)/g extract based on a Trolox standard curve.

7. Tyrosinase inhibitory activity

Tyrosinase inhibition was assessed as previously dissolved by Rungruang et al. (2022). The extracts were dissolved in 50 mM sodium phosphate buffer (pH 6.8). The positive control (A) contained 160 µL of 50 mM buffer, 20 µL of 2.5 mM L-3,4-dihydroxyphenylalanine (L-DOPA), and 20 µL of 100 units/mL tyrosinase (Sigma-Aldrich, USA). The negative control (B) included 180 µL of buffer and 20 µL of 2.5 mM L-DOPA (Sigma-Aldrich, USA). For each NPE (C), 20 µL of NPE was mixed with 140 µL buffer, 20 µL of tyrosinase, and 20 µL of L-DOPA. The blank (D) consisted of 20 µL extract, 160 µL buffer, and 20 µL L-DOPA. All reactions were incubated at 37°C in 96-well plate, and the absorbance was measured at 475 nm. Kojic acid was used as the standard. The percentage tyrosinase inhibitory activity was calculated using the following equation:

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

Where, A = without test sample; B = without test sample, without enzyme; C = test sample; D = test sample, without enzyme.

8. Collagenase inhibitory activity

Collagenase inhibition was determined using a fluorometric assay (Pientaweeratch et al., 2016) with the EnzChek® Gelatinase/Collagenase assay kit. In a

96-well plate, 20 µL of NPE was mixed with 100 µL collagenase (*Clostridium histolyticum*), and 20 µL DQ™ collagen I. The reaction mixture was then incubated at 25°C for 90 min in the dark. The fluorescence intensity was measured at 485 nm (excitation) and 538 nm (emission). Epigallocatechin gallate (EGCG) was used as a positive control. Collagenase inhibitory activity was calculated using the following equation:

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

Where, A = without test sample; B = without test sample, without enzyme; C = test sample; D = test sample, without enzyme.

9. Elastase inhibitory activity

Elastase inhibition was measured as described by Thring et al. (2009) with some modifications. In a 96-well plate, 20 µL of NPE was pre-incubated with 1 µg/mL final concentration of porcine pancreatic elastase (Sigma-Aldrich, USA) for 15 min at 25°C. The reaction was then initiated by adding 1.6 mM N-succinyl-Ala-Ala-Ala-p-nitroanilide (Sigma-Aldrich, USA), followed by incubation at 25°C. Absorbance was measured at 381 and 402 nm every 30 sec for 20 min using a microplate reader (Infinite® 200 Pro, Tecan, Switzerland). Epigallocatechin (EGCG) was used as a standard.

10. Cell viability assay

The cytotoxicity of NPE was evaluated using the PrestoBlue® assay, following the method of Panichakul et al. (2022) with slight modifications. Human dermal fibroblasts (CCD-986sk, ATCC® CRL-1947™) were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Gibco®, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS; Gibco®, Thermo Fisher Scientific, USA) and maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cells were seeded at a density of 7×10³ cells/well in 96-well plates and incubated for 24 h to allow cell attachment. Subsequently, cells were treated with NPE at various concentrations ranging 31.25–1,000 µg/mL and incubated for an additional 48 h. After treatment, the medium was removed, and 90 µL of fresh medium, along with 10 µL of PrestoBlue® reagent, was added to each well. Plates were incubated for 3 h, and absorbance was measured at 570 and 600 nm using a microplate reader (Infinite® 200 Pro, Tecan, Switzerland). Cell viability was calculated as a percentage relative to the untreated control cells.

11. Cell proliferation assay

The proliferative effect of NPE on human dermal fibroblasts (CCD-986sk; ATCC® CRL-1947™) was evaluated using the PrestoBlue® Cell Viability Reagent (Panichakul et al., 2022). Cells were seeded at a density of 7×10^3 cells/well in 96-well plates containing 100 μ L of IMDM supplemented with 10% FBS and 1% penicillin-streptomycin, followed by incubation at 37°C in a humidified atmosphere with 5% CO₂ for 24 h. Subsequently, the medium was replaced with IMDM containing 2% FBS, and cells were treated with NPE at concentrations of 31.25, 62.50, and 125 μ g/mL for 72 h. After treatment, the medium was removed and replaced with 90 μ L of fresh medium and 10 μ L of PrestoBlue® reagent. The plates were incubated for an additional 3 h, and the absorbance was measured at 570 and 600 nm using a microplate reader. Cell proliferation was expressed as a percentage relative to the untreated control cells.

12. Microorganisms and heavy metals testing

Microbial contamination of NPE was assessed using the total aerobic plate count (ISO 21149:2017; ISO 16212: 2017) (International Organization for Standardization, 2017a; 2017b) and testing for *Pseudomonas aeruginosa* (ISO 22717:2015) (International Organization for Standardization, 2015c), *Staphylococcus aureus* (ISO 22718:2015) (International Organization for Standardization, 2015a), and *Candida albicans* (ISO 18416:2015) (International Organization for Standardization, 2015b). Heavy metals (Hg, Pb, As, and Cd) were quantified by inductively coupled plasma-optical emission spectroscopy (ICP-OES) using an Agilent ICP-OES (Model 5110, Agilent Technologies, USA), based on the in-house method TC7.2-12 based on ACM 005 (ASEAN Cosmetic Committee, 2013).

13. Emulsion serum formulation

The emulsion serum formulation shown in Table 1 was prepared by mixing DI water and butylene glycol (Part A). Part B ingredients were combined and added to Part A, followed by homogenization at 500 rpm for 5 min using a homogenizer (IKA, Germany). Parts C, D, and E were subsequently added. F1 and F2 formulas were the emulsion serum base and emulsion serum with NPE, respectively.

14. Heating - Cooling stability test

Stability was assessed using a cycling test (Rungruang et al., 2019). 1 cycle involved storing the emulsion serum at 45 $\pm$ 2°C for 24 h, followed by 24 h at 4°C. This process was repeated for 6 cycles. The serum

Table 1 Ingredients of emulsion serum containing NPE

Part	Ingredient	Function	F1 (%)	F2 (%)
A	Deionized (DI) water	Diluent	89.55	88.55
	Butylene glycol	Humectant	5.00	5.00
B	Mineral oil	Emollient	3.00	3.00
	Polyacrylamide, C13-14	Emulsifier	0.50	0.50
	Isoparaffin, and Laureth-7	Emollient	1.00	1.00
	Isopropyl myristate			
	Vitamin E	Antioxidant	0.10	0.10
C	Natto powder extract (NPE)	Active ingredient	–	1.00
D	Fragrance	Perfuming agent	0.10	0.10
E	Phenoxyethanol	Preservative	0.75	0.75

Remark: F1 = Base emulsion serum formulation; F2 = Emulsion serum formulation containing NPE.

was evaluated for changes in its appearance, color, pH, and viscosity.

15. Statistical analysis

All experiments were performed in triplicate. Data were analyzed using SPSS 28 (Version 28, IBM Corp., USA). The values were expressed as mean $\pm$ standard deviation (SD). Analysis of variance (ANOVA) was used to analyze the data. Duncan's multiple range test was used to determine significant differences between means ($p < 0.05$).

Results and discussion

1. Percentage yield, appearance, total phenolic and total flavonoid contents of NPE

The fermented and freeze-dried natto powder (NP) appeared as fine yellow powder (Fig. 1A). Extraction with 80% ethanol followed by freeze-drying resulted in a reddish-brown extract (Fig. 1B), with a yield of $19.00 \pm 0.67\%$. Ethanol extraction of natto, similar to that of other fermented legumes such as soybean natto, enhances antioxidant activity by concentrating bioactive phenolic compounds. The darker color of the extract, ranging from yellow to reddish-brown, was likely due to oxidation and the presence of tannins and polyphenols, as previously reported (Mwangwela et al., 2007; Li et al., 2008; Sutikno, 2019). The total phenolic content of NPE, expressed as mg gallic acid equivalent (GAE)/g of dry extract, is listed in Table 2. NPE exhibited a phenolic content of 20.64 ± 0.20 mg GAE/g extract. In addition, the total flavonoid content was 22.34 ± 3.28 mg quercetin equivalent (QE)/g extract. Fermentation of soybeans with *B. subtilis* var. natto significantly increased the total phenolic and flavonoid contents. This increase was primarily attributed to microbial enzymatic activity, particularly that of beta-glucosidase, which hydrolyzes



Fig. 1 Visual characteristics of (A) freeze-dried NP and (B) freeze-dried NPE

isoflavone glycosides into their aglycone forms (Ping et al., 2012; Chen et al., 2022). This enzymatic process enhances the availability of phenolic compounds and boosts antioxidant activity (Ping et al., 2012; Huang, 2023). In addition to enhancing the phenolic and flavonoid contents, fermentation alters other biochemical profiles, including polysaccharides, proteins, fatty acids, and volatile compounds, which contribute to the unique properties of natto (Chen et al., 2022).

Table 2 Yield, total phenolic content, and total flavonoid content of NPE

Sample	%Yield	Total phenolic (mg GAE/g extract)	Total flavonoid (mg QE/g extract)
NPE	19.00±0.67	20.64±0.20	22.34±3.28

Remark: The results show the mean ± SD of 3 independent trials.

2. Antioxidant activity of NPE

The antioxidant activity of NPE was assessed using DPPH[•] and ABTS^{•+} radical scavenging assays, with IC₅₀ values indicating the concentration required to neutralize 50% of the free radicals. Lower IC₅₀ values correspond to higher antioxidant activities. The IC₅₀ values for DPPH[•] and ABTS^{•+} in NPE were 2.840±0.1901 mg/mL and 0.765±0.0980 mg/mL, respectively (Table 3). In a previous study, Zhang et al. (2021) reported that *B. subtilis* natto-fermented soy protein isolate at a concentration of 10 mg/mL inhibited DPPH and ABTS radicals by approximately 35% and 70%, respectively. Similarly, Kulprachakarn et al. (2021) showed that Thai fermented soybean (Thua-Nao) extract exhibited antioxidant capacities of 61.03±0.97 μmol Trolox/g of dry extract and 66.54±3.97 μM FeSO₄/g of dry extract for ABTS

and FRAP, respectively, further supporting the relatively high radical scavenging potential of fermented soybean-based extracts. These results correlated with the phenolic and flavonoid contents of the extract. The presence of an orthodihydroxy structure in the B-ring of phenolic compounds enhances their effectiveness as hydrogen donors, which is consistent with previous studies showing a positive correlation between bioactive compounds in crude extracts and antioxidant activity (Gris et al., 2011). Specifically, phenolic compounds contribute significantly owing to their –OH groups, which are potent hydrogen donors (Rungruang et al., 2021a). These findings are supported by those of Zhang et al. (2021), where soy protein isolates fermented with *B. subtilis* var. natto exhibited higher DPPH and ABTS radical scavenging activities than unfermented isolates. The FRAP value of NPE was 94.042±1.1865 mg TE/g extract, which is consistent with previous reports by Dajanta et al. (2013), indicating that the fermentation of black soybeans with *B. subtilis* significantly increased FRAP values, which correlate with enhanced phenolic and flavonoid contents.

Table 3 Antioxidant activities (DPPH, ABTS, and FRAP) of NPE

Sample/Standard	IC ₅₀ (mg/mL)		FRAP (mg TE/g Extract)
	DPPH	ABTS	
NPE	2.840±0.1901 ^a	0.765±0.0980 ^a	94.042±1.1865
L-ascorbic acid	0.031±0.0060 ^b	0.001±0.0002 ^b	–

Remark: The results show the mean ± SD of 3 independent trials. Values with different superscript letters in the same column are significantly different ($p < 0.05$).

3. Anti-tyrosinase, anti-elastase, and anti-collagenase activities of NPE

Matrix metalloproteinases (MMPs), including collagenase and elastase, play a crucial role in the degradation of collagen and elastin in human skin and are essential for maintaining skin structure and elasticity (Philips et al., 2011; Thring et al., 2009). In this study, NPE exhibited significant inhibition of collagenase and elastase, with IC_{50} values of 2.64 ± 0.27 mg/mL and 0.98 ± 0.101 mg/mL, respectively (Table 4). In a previous study, Hsu and Chiang (2009) reported that *B. subtilis* natto-fermented *Radix astragali* at a concentration of 800 mg/mL inhibited collagenase, gelatinase, and elastase activities by 26.55%, 24.87%, and 12.58%, respectively, indicating that the inhibitory potential of NPE in the present study is within the active range observed for other *B. subtilis* natto-fermented herbal extracts. The inhibition of collagenase by NPE may involve multiple mechanisms, including interactions between the polyphenol hydroxyl groups and the functional groups in collagenase. Hydrophobic interactions between the polyphenol benzene ring and collagenase may induce conformational changes and deactivate the enzyme (Pientaweeratch et al., 2016). Polyphenols in NPE, such as genistein and daidzein, are metal chelators that bind to the Zn active site of collagenase, thereby preventing substrate digestion (McDonald et al., 1996). Previous studies have shown that even low concentrations (10 ppm) of soy isoflavones can inhibit the UVB-induced expression of MMP-1 in fibroblasts (Kim et al., 2004). Fermentation of soybeans with *B. subtilis* var. natto significantly increased the total phenolic and flavonoid contents. These bioactive compounds, including polyphenols and flavonoids, are known for their inhibitory effects on skin aging enzymes such as elastase and collagenase in various plant extracts. Similarly, fermentation of *Radix astragali* with *B. subtilis* var. natto enhanced pro-collagen biosynthesis in human dermal fibroblasts, indicating potential anti-aging benefits related to collagen production (Hsu & Chiang, 2009). For tyrosinase inhibition, NPE had an IC_{50} value of 10.91 ± 0.242 mg/mL (Table 4). Tyrosinase, a copper-containing enzyme, catalyzes melanin production by converting L-DOPA to dopaquinone, which subsequently forms melanin and brown pigments (Rungruang et al., 2021b). Previous studies have reported that soybeans fermented with *B. subtilis* var. natto show an increased production of bioactive compounds, including polyphenols and peptides, which inhibit tyrosinase, a key enzyme in melanin synthesis. In previous studies, Jin et al. (2020)

reported that fermentation can influence tyrosinase inhibitory activity by altering the profile and concentration of bioactive compounds.

Table 4 Anti-tyrosinase, anti-elastase, and anti-collagenase inhibition of NPE

Sample/Standard	IC_{50} (mg/mL)		Tyrosinase
	Collagenase	Elastase	
NPE	2.64 ± 0.272^a	0.98 ± 0.101^a	10.91 ± 0.242^a
EGCG	0.07 ± 0.001^b	0.21 ± 0.002^b	—
Kojic acid	—	—	0.02 ± 0.007^b

Remark: The results show the mean $\pm$ SD of 3 independent trials. Values with different superscript letters in the same column are significantly different ($p < 0.05$).

4. Cytotoxicity of NPE on human fibroblasts

The cytotoxicity of NPE was assessed using the PrestoBlue® assay in CCD-986sk. Cells were treated with NPE at concentrations ranging 31.25–1,000 μ g/mL for 48 h, and cell viability was calculated relative to the untreated control. As shown in Fig. 2, treatment with NPE at concentrations up to 500 μ g/mL did not significantly reduce cell viability ($p < 0.05$), indicating no notable cytotoxic effect under these conditions. Therefore, concentrations ≤ 500 μ g/mL were considered non-cytotoxic.

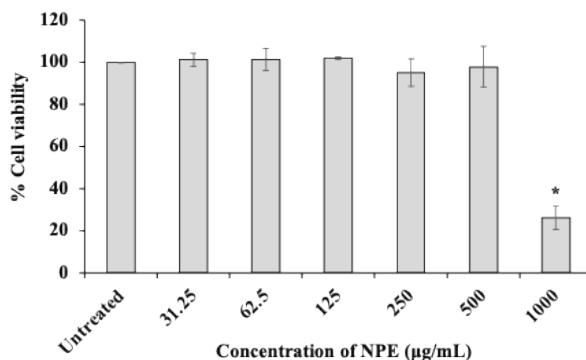


Fig. 2 Cell viability of human dermal fibroblasts (CCD-986sk) treated with NPE. Results are expressed as mean $\pm$ SD from 3 independent experiments. Cell viability in the untreated group was set at 100%. Asterisk (*) indicates $p < 0.05$.

5. Natto powder extract induced cell proliferation in human fibroblasts

The proliferative effects of NPE on CCD-986sk human fibroblasts were evaluated using the PrestoBlue® cell viability assay. As shown in Fig. 3, treatment with NPE at concentrations of 62.5 and 125 μ g/mL significantly enhanced fibroblast proliferation, with viability values of $111.08 \pm 4.09\%$ and $111.39 \pm 0.72\%$, respectively,

compared to the untreated control (100.00%, $p < 0.05$). In contrast, treatment with 31.25 $\mu\text{g/mL}$ of NPE resulted in a slight increase in cell viability ($103.55 \pm 1.45\%$), although this was not statistically significant. These results indicate that NPE concentrations of 62.5 and 125 $\mu\text{g/mL}$ effectively stimulate fibroblast proliferation in CCD-986sk cells. The observed enhancement in cell proliferation can be attributed to the bioactive compounds present in NPE, which were enhanced through fermentation by *B. subtilis* var. natto. Fermentation processes have been shown to promote collagen synthesis. For instance, the fermentation of *Radix astragali* by *B. subtilis* upregulates collagen production by increasing the expression of transforming growth factor- $\beta 1$ (TGF- $\beta 1$), a key regulator of collagen biosynthesis in human dermal fibroblasts (Hsu & Chiang, 2009; Zhang et al., 2017). Furthermore, a recent study by Olenic et al. (2025) highlighted the role of nattokinase, a protease derived from *B. subtilis* var. natto, in supporting the long-term subculturing of fibroblasts without impairing their proliferative capacity.

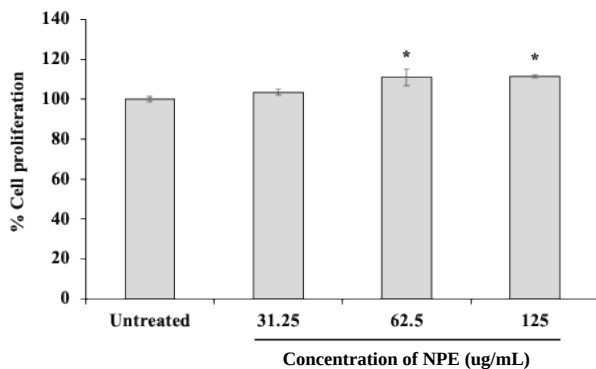


Fig. 3 Cell proliferation of human dermal fibroblast (CCD-986sk) treated with NPE at concentrations of 31.25, 62.5, and 125 $\mu\text{g/mL}$ for 72 h. Results are expressed as the mean $\pm$ SD from 3 independent experiments. The untreated control group is set at 100% for comparison. Asterisk (*) indicates $p < 0.05$ compared to the untreated control group.

6. Microorganisms and heavy metals testing

Microbial contamination and heavy metals in NPE were evaluated in accordance with the Ministry of Public Health. (2016) and applicable heavy metal standards (Table 5). The results showed that NPE was free from contamination by both microorganisms and heavy metals, indicating its safety for cosmetic use. These findings confirm that NPE complies with safety standards for both microbial and chemical contaminants, supporting its potential for use in cosmetic formulations.

Table 5 Microbial contamination and heavy metals testing in NPE

Tests	Method	LOD	Results
Lead (Pb)	In house method TC7.2-12	0.24	Not detected (mg/kg)
Arsenic (As)	In house method TC7.2-12	0.10	Not detected (mg/kg)
Mercury (Hg)	In house method TC7.2-12	0.03	Not detected (mg/kg)
Cadmium (Cd)	In house method TC7.2-12	0.06	Not detected (mg/kg)
Total aerobic plate count	ISO 21149:2017, ISO 16212:2017	–	<10 CFU/mL
<i>Pseudomonas aeruginosa</i>	ISO 22717:2015	–	Not detected
<i>Staphylococcus aureus</i>	ISO 22718:2015	–	Not detected
<i>Candida albicans</i>	ISO 18416:2015	–	Not detected

Remark: In-house method TC7.2-12 based on ACM 005 determination of heavy metals, 2013.

7. Stability test of emulsion serum containing NPE products

In this study, two emulsion serum formulations were developed: F1 (emulsion serum base) without NPE and F2 (NPE-enriched emulsion serum). Both formulations underwent 6 cycles of heating-cooling testing to assess their stability, including appearance, color (L^* , a^* , b^*), pH, and viscosity. Neither F1 nor F2 exhibited phase separation (Fig. 4), indicating a good overall stability. For color, F2 showed a slight change, with a ΔE of 2.38 ± 0.98 , showing a noticeable difference in color after 6 cycles (Mokrzycki & Tatol, 2011). This change is likely owing to the yellow hue of NPE in F2 (Table 6). pH values of both formulations decreased slightly, with F1 dropping from 5.78 ± 0.02 to 5.33 ± 0.03 and F2 from 5.82 ± 0.01 to 5.33 ± 0.02 (Table 6). These pH values (5.0–6.0) remain within the acceptable range for human skin (Waqas et al., 2010), indicating that both F1 and F2 formulations-maintained stability under thermal stress. Gallarate et al. (2013) noted that such variations in pH can result from the breakdown of oil-phase components, such as mineral oil, leading to the formation of free fatty acids. Regarding viscosity, F2 exhibited higher viscosity (763.33 ± 9.87 cP) compared to F1 (554.00 ± 5.29 cP), likely because of the presence of NPE. Although both formulations experienced slight decreases in viscosity after the heating-cooling cycles, the values remained within acceptable ranges, confirming that NPE did not significantly affect the stability of the emulsion. It has been reported that heating may lower the viscosity of emulsion, and this effect was not fully reversed after cooling (Babcock, 1931). These results indicate that the inclusion of NPE did not significantly alter the physical properties of the emulsion serum, supporting its potential for use in cosmetic applications.



Fig. 4 Appearance of (A) F1, emulsion serum base and (B) F2, emulsion serum containing NPE after 6 cycles heating-cooling test

Table 6 Color, pH, and viscosity of emulsion serum base (F1) and emulsion serum containing NPE (F2) after Heating-Cooling test

Formulation	Cycle	Color				pH	Viscosity (cP)
		L*	a*	b*	ΔE		
F1	0	55.22±0.37 ^b	-0.67±0.36 ^a	2.66±0.30 ^a	—	5.78±0.02 ^a	554.00±5.29 ^a
	6	56.94±0.29 ^a	-0.67±0.31 ^a	2.73±0.44 ^a	1.72±0.21	5.33±0.03 ^b	500.67±10.26 ^b
F2	0	56.15±0.93 ^b	-0.57±0.21 ^a	4.51±0.04 ^a	—	5.82±0.01 ^a	763.33±9.87 ^a
	6	58.41±0.11 ^a	-0.88±0.15 ^b	3.86±0.15 ^b	2.38±0.98	5.33±0.02 ^b	701.33±4.16 ^b

Remark: The results show the mean ± SD of 3 independent trials. Statistical comparisons were performed using each formula (cycle 0 vs. cycle 6). Values with different superscript letters in the same row are significantly different ($p<0.05$). The viscosity was measured using a Brookfield DV2T Prime Viscometer with a No. 4 spindle set to 100 rpm and a % torque > 80%. The pH was determined using a pH meter with a standard pH calibration. L*, a*, and b* represent the lightness, red-green, and yellow-blue color coordinates, respectively, measured using the CIELAB color system.

Conclusion

The ethanal extract of natto powder exhibited significant antioxidant activity and potent inhibition of key skin-aging enzymes, including collagenase, elastase, and tyrosinase. NPE stimulated the proliferation of human dermal fibroblasts. These findings indicate its potential for development as an active ingredient in cosmeceutical formulations with anti-aging, skin-whitening, and antioxidant properties. Furthermore, NPE-containing emulsion serum showed satisfactory stability and maintained favorable physical properties under various storage conditions, indicating its viability for cosmetic product development. Future studies should investigate its long-term efficacy and clinical safety.

Acknowledgments

The authors gratefully acknowledge Komatsu Foods Products and Distributions Co., Ltd., for providing the natto powder used in this study. We also extend our sincere appreciation to the Cosmeceutical Bioactivity Analysis and Testing Laboratory (CosBAT), Faculty of Science and Technology, Suan Dusit University, for

supplying the essential facilities and instrumentation required for the analyses conducted in this research.

Conflicts of interest

The authors declare no conflict of interest.

Declaration of Generative Artificial Intelligence in the Writing Process

During the preparation of this manuscript, the authors used ChatGPT for enhancing language clarity and improving grammar. The final content was reviewed and edited by the authors, who take full responsibility for the accuracy and integrity of the publication.

References

ASEAN Cosmetic Committee. (2013). *Determination of heavy metals (Arsenic, Cadmium, Lead and Mercury) in cosmetic products* (No. ACM 005). ASEAN Cosmetic Committee.

Babcock, C.J. (1931). *Some factors affecting the viscosity of cream* (No. 249). United States Department of Agriculture.

- Chen, X., Lu, Y., Zhao, A., Wu, Y., Zhang, Y., & Yang, X. (2022). Quantitative analyses for several nutrients and volatile components during fermentation of soybean by *Bacillus subtilis* natto. *Food Chemistry*, 374, 131725. <https://doi.org/10.1016/j.foodchem.2021.131725>
- Dajanta, K., Janpum, P., & Leksing, W. (2013). Antioxidant capacities, total phenolics and flavonoids in black and yellow soybeans fermented by *Bacillus subtilis*: A comparative study of Thai fermented soybeans (*thua nao*). *International Food Research Journal*, 20(6), 3125–3132.
- Domaszewska-Szostek, A., Puzianowska-Kuźnicka, M., & Kurylowicz, A. (2021). Flavonoids in skin senescence prevention and treatment. *International Journal of Molecular Sciences*, 22(13), 6814. <https://doi.org/10.3390/ijms22136814>
- Dou, J., Feng, N., Guo, F., Chen, Z., Liang, J., Wang, T., ... Xu, Z. (2023). Applications of probiotic constituents in cosmetics. *Molecules*, 28(19), 6765. <https://doi.org/10.3390/molecules28196765>
- Gallarate, M., Chirio, D., Bussano, R., Peira, E., Battaglia, L., Baratta, F., & Trotta, M. (2013). Development of O/W nanoemulsions for ophthalmic administration of timolol. *International Journal of Pharmaceutics*, 440(2), 126–134. <https://doi.org/10.1016/j.ijpharm.2012.10.015>
- Gris, E.F., Mattivi, F., Ferreira, E.A., Vrhovsek, U., Filho, D.W., Pedrosa, R.C., & Bordignon-Luiz, M.T. (2011). Stilbenes and tyrosol as target compounds in the assessment of antioxidant and hypolipidemic activity of *Vitis vinifera* red wines from Southern Brazil. *Journal of Agricultural and Food Chemistry*, 59(14), 7954–7961. <https://doi.org/10.1021/jf2008056>
- He, X., Gao, X., Guo, Y., & Xie, W. (2024). Research progress on bioactive factors against skin aging. *International Journal of Molecular Sciences*, 25(7), 3797. <https://doi.org/10.3390/ijms25073797>
- Hsu, M.F., & Chiang, B.H. (2009). Effect of *Bacillus subtilis* natto-fermented *Radix astragali* on collagen production in human skin fibroblasts. *Process Biochemistry*, 44(1), 83–90. <https://doi.org/10.3390/ijms25073797>
- Huang, C.L. (2023). *Effect of fermentation by Bacillus subtilis natto on phenolic contents, composition and antioxidant activities of four types of common bean (Phaseolus vulgaris L.)* [Doctoral dissertation]. University of Guelph.
- Hussein, R.S., Bin Dayel, S., Abahussein, O., & El-Sherbiny, A.A. (2025). Influences on skin and intrinsic aging: Biological, environmental, and therapeutic insights. *Journal of Cosmetic Dermatology*, 24(2), e16688. <https://doi.org/10.1111/jocd.16688>
- Igari, N., Ninomiya, R., & Kawakami, S. (2023). Effects of dietary *Bacillus natto* productive protein on the skin: In *Vitro* efficacy test and single-armed trial. *Cosmetics*, 10(5), 135. <https://doi.org/10.3390/cosmetics10050135>
- International Organization for Standardization. (2015a). *Cosmetics-Microbiology-Detection of Staphylococcus aureus* (ISO Standard No. 22718:2015). <https://www.iso.org/standard/68313.html>
- International Organization for Standardization. (2015b). *Cosmetics-Microbiology-Detection of Candida albicans* (ISO Standard No. 18416:2015). <https://www.iso.org/standard/68314.html>
- International Organization for Standardization. (2015c). *Cosmetics-Microbiology-Detection of Pseudomonas aeruginosa* (ISO Standard No. 22717:2015). <https://www.iso.org/standard/68312.html>
- International Organization for Standardization. (2017a). *Cosmetics-Microbiology-Enumeration and detection of aerobic mesophilic bacteria* (ISO Standard No. 21149:2017). <https://www.iso.org/standard/72240.html>
- International Organization for Standardization. (2017b). *Cosmetics-Microbiology-Enumeration of yeast and mould* (ISO Standard No. 16212:2017). <https://www.iso.org/standard/72241.html>
- Jin, Y.H., Jeon, A.R., & Mah, J.H. (2020). Tyrosinase inhibitory activity of soybeans fermented with *Bacillus subtilis* capable of producing a phenolic glycoside, arbutin. *Antioxidants*, 9(12), 1301. <https://doi.org/10.3390/antiox9121301>
- Jiratchayamaethasakul, C., Ding, Y., Hwang, O., Im, S.T., Jang, Y., Myung, S.W., ... Lee, S.H. (2020). In vitro screening of elastase, collagenase, hyaluronidase, and tyrosinase inhibitory and antioxidant activities of 22 halophyte plant extracts for novel cosmeceuticals. *Fisheries and Aquatic Sciences*, 23(1), 1–9. <https://doi.org/10.1186/s41240-020-00149-8>
- Kim, H.B., Kim, E.S., Kim, K.T., Kim, Y.M., & Eom, S.H. (2024). In vitro study on the inhibitory effects of Korean brown, green, and red seaweed extracts on collagenase, elastase, and hyaluronidase. *Fisheries and Aquatic Sciences*, 27(11), 783–790. <https://doi.org/10.47853/FAS.2024.e72>
- Kim, S.Y., Kim, S.J., Lee, J.Y., Kim, W.G., Park, W.S., Sim, Y.C., & Lee, S.J. (2004). Protective effects of dietary soy isoflavones against UV-induced skin-aging in hairless mouse model. *Journal of the American College of Nutrition*, 23(2), 157–162. <https://doi.org/10.1080/07315724.2004.10719356>
- Kulprachakarn, K., Chaipoot, S., Phongphisutthinant, R., Paradee, N., Prommaban, A., Ounjaijean, S., ... Boonyapranai, K. (2021). Antioxidant potential and cytotoxic effect of isoflavones extract from Thai fermented soybean (*Thua-Nao*). *Molecules*, 26(24), 7432. <https://doi.org/10.3390/molecules26247432>
- Li, H., Guo, A., & Wang, H. (2008). Mechanisms of oxidative browning of wine. *Food Chemistry*, 108(1), 1–13. <https://doi.org/10.1016/j.foodchem.2007.10.065>
- Li, X., Long, J., Gao, Q., Pan, M., Wang, J., Yang, F., & Zhang, Y. (2023). Nattokinase supplementation and cardiovascular risk factors: A systematic review and meta-analysis of randomized controlled trials. *Reviews in Cardiovascular Medicine*, 24(8), 234. <https://doi.org/10.31083/j.rcm2408234>

- Liu, W., Dun, M., Liu, X., Zhang, G., & Ling, J. (2022). Effects on total phenolic and flavonoid content, antioxidant properties, and angiotensin I-converting enzyme inhibitory activity of beans by solid-state fermentation with *Cordyceps militaris*. *International Journal of Food Properties*, 25(1), 477–491. <https://doi.org/10.1080/10942912.2022.2048009>
- McDonald, M., Mila, I., & Scalbert, A. (1996). Precipitation of metal ions by plant polyphenols: Optimal conditions and origin of precipitation. *Journal of Agricultural and Food Chemistry*, 44(2), 599–606. <https://doi.org/10.1021/jf950459q>
- Miao, X., Niu, H., Sun, M., Dong, X., Hua, M., Su, Y., ... Li, D. (2024). A comparative study on the nutritional composition, protein structure and effects on gut microbiota of 5 fermented soybean products (FSPs). *Food Research International*, 183, 114199. <https://doi.org/10.1016/j.foodres.2024.114199>
- Ministry of Public Health. (2016). *Government gazette, notification of the Ministry of Public Health* (Volume 133, Special Part 72 Ngor). Royal Thai Government Gazette.
- Mokrzycki, W.S., & Tatol, M. (2011). Colour difference ΔE^* survey. *Machine Graphics and Vision*, 20(4), 383–411.
- Mwangwela, A.M., Waniska, R.D., McDonough, C., & Minnaar, A. (2007). Cowpea cooking characteristics as affected by micronisation temperature: A study of the physicochemical and functional properties of starch. *Journal of the Science of Food and Agriculture*, 87(3), 399–410. <https://doi.org/10.1002/jsfa.2711>
- Olenic, M., Deelkens, C., van Hengel, J., Schauwer, C., & Thorrez, L. (2025). Nattokinase as a novel food-grade enzyme for long term cell subculture. *Food Research International*, 203, 115800. <https://doi.org/10.1016/j.foodres.2025.115800>
- Panichakul, T., Ponnikorn, S., Tupchiangmai, W., Haritakun, W., & Srisanga, K. (2022). Skin anti-aging potential of *Ipomoea pes-caprae* ethanolic extracts on promoting cell proliferation and collagen production in human fibroblasts (CCD-986sk cells). *Pharmaceuticals*, 15(8), 969. <https://doi.org/10.3390/ph15080969>
- Persistence Market Research. (2025). *Natural and organic personal care market: Global industry analysis 2017–2021 and forecast 2022–2032*. <https://www.persistencemarketresearch.com/market-research/natural-and-organic-personal-care-market.asp>
- Philips, N., Auler, S., Hugo, R., & Gonzalez, S. (2011). Beneficial regulation of matrix metalloproteinases for skin health. *Enzyme Research*, 2011(1), 427285. <https://doi.org/10.4061/2011/427285>
- Pientaweeratch, S., Panapisal, V., & Tansirikongkol, A. (2016). Antioxidant, anti-collagenase and anti-elastase activities of *Phyllanthus emblica*, *Manilkara zapota* and silymarin: An *in vitro* comparative study for anti-aging applications. *Pharmaceutical Biology*, 54(9), 1865–1872. <https://doi.org/10.3109/13880209.2015.1133658>
- Ping, S.P., Shih, S.C., Rong, C.T., & King, W.Q. (2012). Effect of isoflavone aglycone content and antioxidation activity in natto by various cultures of *Bacillus subtilis* during the fermentation period. *Journal of Nutrition & Food Sciences*, 2(7), 1000153. <https://doi.org/10.4172/2155-9600.1000153>
- Rocchi, R., Zwinkels, J., Kooijman, M., Garre, A., & Smid, E. J. (2024). Development of novel natto using legumes produced in Europe. *Heliyon*, 10(5), e26849. <https://doi.org/10.1016/j.heliyon.2024.e26849>
- Rungruang, R., Modsuwan, J., & Srisukong, A. (2019). Antioxidant and Anti-wrinkle Activities of Tubtim Chumphae Rice Extract for Application in Facial Cosmetics. *KMUTT Research and Development Journal*, 42(1), 95–107.
- Rungruang, R., Panichakul, T., Rattanathavorn, W., Kaisangsri, N., Kerdchoechnuen, O., Laohakunjit, N., & Chao, L.K. (2021a). Effects of extraction methods on the flavonoid and phenolic contents and anti-aging properties of *Rhyncholaeliocattleya* Haw Yuan beauty extracts. *ScienceAsia*, 47(6), 698–706. <https://doi.org/10.2306/scienceasia1513-1874.2021.100>
- Rungruang, R., Peasura, N., & Kaisangsri, N. (2022). Phytochemical screening, quantitative analysis of cyanidin-3-*O*-glucoside content, and anticancer activity of novel rice bran (Tubtim Chumphae rice). *Journal of Advanced Pharmaceutical Technology & Research*, 13(4), 312–316. https://doi.org/10.4103/japtr.japtr_458_22
- Rungruang, R., Ratanathavorn, W., Boohuad, N., Selamassakul, O., & Kaisangsri, N. (2021b). Antioxidant and anti-aging enzyme activities of bioactive compounds isolated from selected Zingiberaceae plants. *Agriculture and Natural Resources*, 55(1), 153–160. <https://doi.org/10.34044/j.anres.2021.55.1.20>
- Sutikno, N. (2019). *Effect of fermentation time and ratio of cocultures using Bacillus subtilis var. natto and Lactobacillus delbrueckii KSM 10 on physicochemical and sensory properties of cowpea (Vigna unguiculata L. Walp) natto* [Doctoral dissertation]. Soegijapranata Catholic University.
- Thring, T.S., Hili, P., & Naughton, D.P. (2009). Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants. *BMC Complementary and Alternative Medicine*, 9(1), 27. <https://doi.org/10.1186/1472-6882-9-27>
- Wang, J., Huang, H., Tao, K., Guo, L., Hu, X., & Chang, H. (2024). Novel *Thermus thermophilus* and *Bacillus subtilis* mixed-culture ferment extract provides potent skin benefits *in vitro* and protects skin from aging. *Journal of Cosmetic Dermatology*, 23(12), 4334–4342. <https://doi.org/10.1111/jocd.16531>
- Waqas, M.K., Akhtar, N., Ahmad, M., Murtaza, G., Khan, H.M.S., Iqbal, M., ... Bhatti, N.S. (2010). Formulation and characterization of a cream containing extract of fenugreek seeds. *Acta Poloniae Pharmaceutica - Drug Research*, 67(2), 173–178.

- Xiang, A., Wang, J., Xie, B., Hu, K., Chen, M., & Sun, Z. (2022). Determination of 14 isoflavone isomers in natto by UPLC-ESI-MS/MS and antioxidation and antiglycation profiles. *Foods*, 11(15), 2229. <https://doi.org/10.3390/foods11152229>
- Xie, M., Jiang, Z., Lin, X., & Wei, X. (2024). Application of plant extracts cosmetics in the field of anti-aging. *Journal of Dermatologic Science and Cosmetic Technology*, 1(2), 100014. <https://doi.org/10.1016/j.jdsct.2024.100014>
- Xu, C., Sun, S., & Han, A. (2024). Effects of fermentation process and *in vitro* digestion on the content of total phenols and isoflavones and antioxidant properties in natto. *Agricultural Science and Food Processing*, 1(1), 38–47. <https://doi.org/10.62762/ASFP.2024.541137>
- Zhang, B., Liu, Y., Ji, Q., Zhao, M., Zeng, J., Liu, L., Gong, A. (2017). Nattokinase crude extract enhances cutaneous wound healing. *Journal of Biomaterials and Tissue Engineering*, 7(12), 1281–1286. <https://doi.org/10.1166/jbte.2017.1697>
- Zhang, X., Sun, X., Li, W., Huang, X., Tao, L., Li, T., & Li, S. (2021). *In vitro* and *in vivo* antioxidant activities of soy protein isolate fermented with *Bacillus subtilis* natto. *Journal of Food Science and Technology*, 58(8), 3199–3204. <https://doi.org/10.1007/s13197-020-04823-6>