



Research article

Processing-induced changes in components that affect the radical scavenging activity of ethanolic extracts from *Neopyropia yezoensis*

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Abstract: Many marine red algae species are used as food sources around the world. Here, the study focuses on free radical scavenging properties as a beneficial activity of the red alga *Neopyropia yezoensis* (Susabi-nori), which is consumed in Japan, and investigates quantitative changes in the compounds that could affect the activity due to processing steps. It was found that the dried and roasted samples showed more active radical scavenging properties detected by the 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay compared to the fresh samples. The contents of phenolic compounds, mycosporine-like amino acids (MAAs), chlorophylls, and carotenoids were evaluated; it was suggested that an increase in phenolic compounds was responsible for the enhancement of the activity. On the other hand, no correlation with the activity was found for MAAs, chlorophylls, and carotenoids. Furthermore, by comparing three different sampling times (early December, early January, and early March), it was found that the sample taken in early December showed the highest radical scavenging activity. These results suggest that the useful activities of *N. yezoensis*, including its antioxidant activity, can be influenced by its processing steps and harvest time.

Keywords: *Neopyropia yezoensis*; red alga; antioxidant; phenolic compounds; mycosporine-like amino acids; carotenoids

1. Introduction

The red alga *Neopyropia yezoensis* (Susabi-nori) is one of the commonly eaten seaweeds in Japan, and it is generally known as 'nori'. The harvest season for *N. yezoensis* in nori farms is from November to approximately March of the following year. When harvested fresh, *N. yezoensis* may be cooked and eaten; however, in most cases, it is processed into 'yaki-nori'. The process for manufacturing yaki-nori is as follows. First, fresh *N. yezoensis* is cut into small pieces. Then, it is formed into sheets and dried at approximately 40°C for several hours to form a product called 'kan-nori'. Yaki-nori, which is commonly available in Japan, is made by roasting kan-nori for a few seconds at 180–200°C [1].

Seaweed is known as an excellent source of dietary fiber, vitamins, polyunsaturated fatty acids, minerals, amino acids, and antioxidants [2]. *N. yezoensis* is no exception; it has been reported that extracts of *N. yezoensis* prepared using methanol, acetone, ethyl acetate, hexane, or chloroform-methanol exhibited antioxidant effects [3]. The components that exhibited antioxidant activities in the *N. yezoensis* extracts have been thought to be related to usujirene, which is a type of mycosporine-like amino acid (MAA), the carotenoid β -carotene, and chlorophyll analogs [3]. However, to our knowledge, there have been no reports that evaluated the effects of processing on the antioxidant activity of *N. yezoensis*. To evaluate this, we sought to utilize *N. yezoensis* harvested on 4 December 2023 in Ise Bay, Aichi Prefecture (Figure 1), which is one of the leading nori aquaculture production areas in Japan. We sought to study the relationship between the antioxidant power of the *N. yezoensis* ethanol extract and its content of antioxidants, including phenolic compounds, MAAs, chlorophylls, and carotenoids.



Figure 1. Location where *N. yezoensis* samples were collected. The points denoted by the black circles indicate the location where *N. yezoensis* samples were collected. The figure was created by editing the digital map of the Geospatial Information Authority of Japan (<https://www.gsi.go.jp/>).

2. Materials and methods

2.1. Preparation of *N. yezoensis* ethanol extract

Fresh *N. yezoensis* thalli were collected at a farm near Onizaki Fishing Port in Tokoname City, Aichi Prefecture, Japan (34.9°N, 136.8°E, Figure 1) on 4 December 2023, 10 January 2024, and 3 March 2024. For the preparation of ethanol extracts, 1.33 g of fresh *N. yezoensis* thalli were mixed with 20 mL of ethanol (Sigma-Aldrich Japan, Tokyo, Japan). Then, the thalli were disrupted by homogenization for 2 iterations with an FSH-2A homogenizer (Mxbaoheng, purchased via Amazon.co.jp) at 15k rpm for 3 min at 25°C. For dried kan-nori and roasted yaki-nori, 0.10 g of the samples were processed in a similar manner as the fresh samples, except for prewetting the samples with 1.23 g of H₂O. The kan-nori was prepared by drying fresh *N. yezoensis* formed into a sheet at approximately 40°C for 2.5 hours. In this process, 40 g of fresh sample became approximately 3 g of kan-nori. As for yaki-nori, the conditions were unknown as it was outsourced to a processing company; generally, the process is carried out at 180–200°C for a few seconds [1]. The weight of each sheet of yaki-nori was approximately 3 g. Absorption spectrum analyses were immediately performed using the prepared ethanol extracts, and the chlorophyll and carotenoid contents were calculated. For antioxidative assays and measurement of the total phenolic content (TPC), total flavonoid content (TFC), and MAA content, aqueous solutions of ethanol extracts dried under a reduced pressure were used. At that time, 1.0 mL of the dried ethanol extracts were dissolved in 1.0 mL of water, and the supernatants obtained by centrifugation at 22,900 x g for 15 minutes at 25°C were used.

2.2. Evaluation of free radical scavenging properties of *N. yezoensis* ethanol extract

The antioxidative power of the ethanol extracts were evaluated by the 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay. The ABTS assay was performed as previously described [4] with minor modifications. Briefly, the ABTS radical solution was generated by mixing a 7 mM ABTS stock solution in water with 2.45 mM of potassium persulfate in water. Then, this mixture was incubated in the dark for 16 h at 25°C. Prior to the assay, the ABTS solution was diluted in water to generate an absorbance of approximately 0.5 at 415 nm. Either test samples (10 µL) or trolox standards were added to 90 µL of the diluted ABTS solution and mixed. After incubation for 15 min at 25°C, the absorbance was measured at 734 nm. A quantification was performed based on the trolox standard curve. The antioxidative power values were expressed in Trolox equivalents (TEs) µg/mgFW.

2.3. Assays for total phenolic content (TPC) and total flavonoid content

The TPC and TFC were quantified using previously described colorimetric methods [5]. Briefly, for the TPC, 40 µL of each cell-free extract was mixed with 25 µL of distilled water and 105 µL of a 10% (v/v) Folin-Ciocalteu reagent. After incubation at 25°C for 3 min, 80 µL of a 7.5% (w/v) sodium carbonate solution was added and the mixture was incubated at 25°C for 15 min. Next, the mixtures were diluted with an equal volume (250 µL) of distilled water, and the absorbance was measured at 760 nm. A quantification was performed based upon the gallic acid standard curve. The TPC values were expressed in gallic acid equivalents (GAEs) µg/mgDW. For the TFC, 25 µL of each cell-free extract was mixed with 7.5 µL of a 5% (w/v) sodium nitrate solution and 100 µL of water. After incubation

at 25°C for 5 min, 7.5 µL of a 10% (w/v) aluminium chloride solution was added and the mixture was incubated at 25°C for 5 min. Next, 100 µL of a 4% (w/v) sodium hydroxide solution and 10 µL of water were added and the mixture was incubated at 25°C for 15 min. The absorbance of the mixtures was measured at 510 nm. A quantification was performed based upon the quercetin standard curve. The TFC values were expressed in quercetin equivalents (QEs) µg/mgDW.

2.4. Quantification and identification of MAAs

For quantification of the MAA content in the extracts, a high-performance liquid chromatography (HPLC) analysis was performed [6]. For identification and quantification of shinorine and porphyra-334, authentic standards were used. In addition, liquid chromatography-mass spectrometry (LC-MS) analysis was performed at the Global Facility Center, Hokkaido University, using an Accela LC System (Thermo Scientific, Massachusetts, USA) and an LTQ Orbitrap Discovery (Thermo Scientific, Massachusetts, USA). Separation was conducted using an InertSustain AQ-C18 (1.9 µm; 150 by 2.1 mm). The mobile phase was run at a flow rate of 0.1 mL/min using a 0.1% formic acid solution. The MS analysis was performed in positive electrospray ionization (ESI) mode with a scan range of m/z 200–1000. In addition to the LC-MS analysis, the absorption spectra of the MAAs were confirmed by a photodiode array detector.

2.5. Quantification of chlorophylls and carotenoids

The amounts of chlorophyll *a* (*Ca*), chlorophyll *b* (*Cb*), and carotenoids (*Car*) contained in the ethanol extract were calculated using the following equations proposed by Lichtenthaler and Wellburn using the absorbance values at 470, 665, and 649 nm [7]:

$$Ca = 13.95A_{663} - 6.88A_{649},$$

$$Cb = 24.96A_{649} - 7.32A_{665},$$

$$Car = (1000A_{470} - 2.05Ca - 114.8Cb)/245.$$

3. Results and discussion

3.1. Antioxidative power and antioxidant content of *N. yezoensis* ethanol extracts prepared using fresh thalli, dried kan-nori and roasted yaki-nori

As shown in Table 1, the ethanol extracts prepared from processed *N. yezoensis* samples showed higher free radical scavenging activities than extracts prepared from fresh samples. The activities increased by 1.22- and 2.24-fold during processing from fresh to dried and from dried to roasted, respectively. Although it is known that the radical scavenging activities of aqueous extracts prepared from *Neopyropia tenera* (Asakusa-nori), a species closely related to *N. yezoensis*, was increased by roasting [8]; to the best of our knowledge, this is the first report showing that the antioxidative activity was enhanced by drying fresh nori samples.

Next, in order to explore the origins of the nori processing-dependent variations in antioxidant activity, we investigated changes in the phenolic compounds, MAAs, chlorophylls, and carotenoids, all of which are compounds that can exhibit antioxidant activity. These antioxidant compounds were

evaluated under different processing conditions (Table 1).

Table 1. Antioxidative power and antioxidant content of *N. yezoensis* ethanol extracts prepared using fresh thalli, dried kan-nori, and roasted yaki-nori.

Sample	Antioxidative power		Phenolic content		
	ABTS assay* ¹ (TEs $\mu\text{mol}/\text{mgDW}$)* ⁴		TPC* ² (GAEs $\mu\text{g}/\text{mgDW}$)* ⁵		TFC* ³ (QEs $\mu\text{g}/\text{mgDW}$)* ⁶
Fresh	10.71 \pm 0.04 ^a		2.25 \pm 0.06 ^a		42.17 \pm 2.76 ^a
Dried	13.07 \pm 0.10 ^b		2.44 \pm 0.07 ^b		75.90 \pm 1.84 ^b
Roasted	29.34 \pm 1.54 ^c		4.38 \pm 0.08 ^c		96.27 \pm 10.57 ^b
Sample	MAA content		Chlorophyll (Chl) content		Carotenoid content
	Shinorine ($\mu\text{g}/\text{mgDW}$)	Porphyra-334 ($\mu\text{g}/\text{mgDW}$)	Chl <i>a</i> ($\mu\text{g}/\text{mgDW}$)	Chl <i>b</i> ($\mu\text{g}/\text{mgDW}$)	($\mu\text{g}/\text{mgDW}$)
Fresh	0.26 \pm 0.03 ^a	4.60 \pm 0.42 ^a	8.22 \pm 0.47 ^a	0.47 \pm 0.10 ^a	2.09 \pm 0.05 ^a
Dried	0.46 \pm 0.01 ^b	6.17 \pm 0.08 ^b	8.47 \pm 0.37 ^a	0.45 \pm 0.11 ^a	2.02 \pm 0.06 ^a
Roasted	0.40 \pm 0.02 ^c	5.45 \pm 0.28 ^a	9.80 \pm 0.10 ^b	0.63 \pm 0.03 ^a	2.36 \pm 0.02 ^b

Note: Values are presented as mean \pm deviation ($n = 3$).

Means with different letters indicate statistical significance when comparing among sample processing states (Student's *t*-test, $p < 0.05$).

*¹The ABTS assay was used to measure antioxidative activity.

*²The total phenolic content (TPC) is shown.

*³The total flavonoid content (TFC) is shown.

*⁴The antioxidative power values were expressed in Trolox equivalents (TEs) $\mu\text{mol}/\text{mgDW}$.

*⁵The TPC values were expressed in gallic acid equivalents (GAEs) $\mu\text{g}/\text{mgDW}$.

*⁶The TFC values were expressed in quercetin equivalents (QEs) $\mu\text{g}/\text{mgDW}$.

The most consistent changes in antioxidant activity and phenolic content were for the TPC and the TFC (Table 1). In fact, a phenolic compound, gallotannin, has been reported to exhibit strong radical scavenging activity in *N. tenera* [8]. On the other hand, the changes in the MAA, chlorophyll, and carotenoid contents did not correlate with the antioxidative activity, that is, their amounts did not show statistically significant increases through both the drying and roasting processes. Regarding MAAs, in addition to porphyra-334 and shinorine, we found an unidentified MAA candidate with a molecular weight of 244 in the ethanol extract (Figure 2). Among these three, porphyra-334 was the most abundant in the extract. Although porphyra-334 is known to exhibit an antioxidant activity, its activity was lower than that of mycorporine-2-glycine and shinorine [6], and in one case, no activity was detected [9]. In *N. yezoensis* extracts, the fluctuation of porphyra-334 may not significantly contribute to the total antioxidant activity. It should also be noted that an MAA referred to as usujirene was previously reported to show a strong antioxidant effect in *N. yezoensis* [3]; however, usujirene was not detected in this study. Thus, the increase in the antioxidative activity of the *N. yezoensis* ethanol extract, which was enhanced by the drying and roasting processes, is thought to be due to the increase in phenolic compounds. It would be an interesting research topic to determine the structures of the increased phenolic compounds in *N. yezoensis* during processing. Although it is currently unknown what molecular mechanism induced phenolic compounds by processing in *N. yezoensis*, in addition to

N. tenera mentioned above, it has been shown very recently that the related species *Porphyra dentata* also increased the radical scavenging activity along with the induction of the TPC and the TFC by the roasting treatment [10]. Regarding the drying process, it has been reported that the content of phenolic compounds in some *Porphyra* species decreased by natural drying stress at 14°C [11]. Considering that the drying process in this study was carried out at a relatively high temperature of 40°C, it is possible that high temperature stress affected the induction of the TPC in *N. yezoensis*.

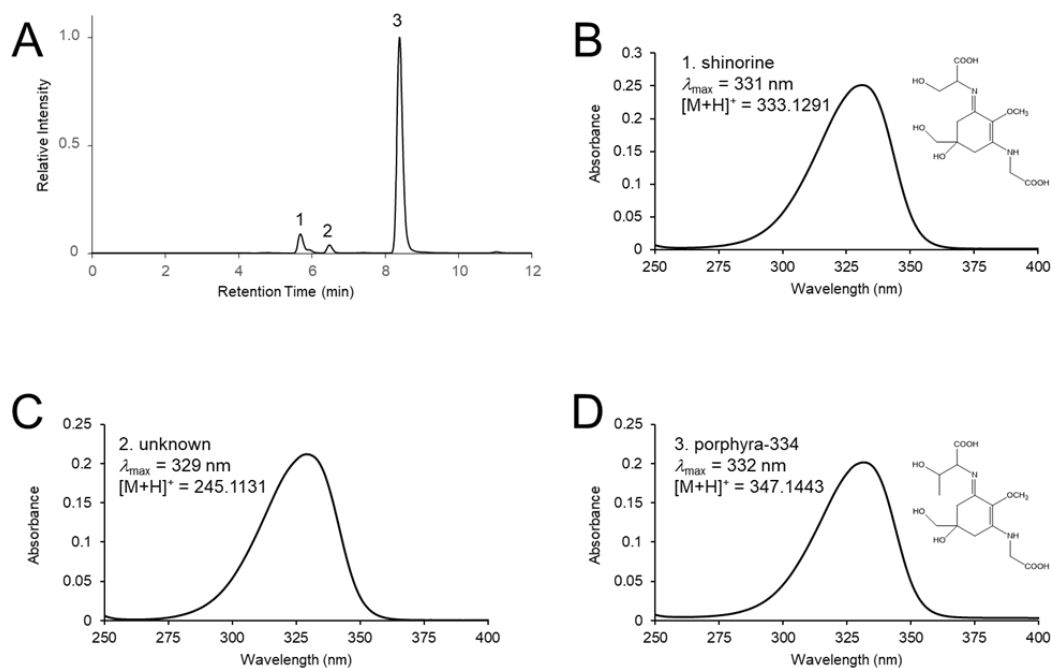


Figure 2. MAAs in *N. yezoensis* ethanol extracts. (A) Representative HPLC chromatogram of MAAs. (B-D) The data of absorption spectrum, absorption maximum (λ_{max}), and $[\text{M}+\text{H}]^+$ for each component in the peaks detected by HPLC analysis. The components in the peaks # 1 and 3 were identified as shinorine and porphyra-334, respectively. The component in the peak #2 has not yet characterized.

3.2. Antioxidative power and antioxidant content of *N. yezoensis* ethanol extracts prepared using fresh thalli at different collection dates

In addition to the effects of processing, we investigated changes in the antioxidative activity and the antioxidant content in the ethanol extract of *N. yezoensis* depending on the time of collection. Besides 4 December 2023, we collected fresh *N. yezoensis* thalli on 10 January 2024 and 3 March 2024 at the same location. The ABTS assay showed that the extract prepared from the sample collected in December 2023 exhibited the highest activity (Table 2). The activity of the January 2024 sample was decreased to less than half of the December 2023 level, and the activity of the March 2024 sample was not significantly different from that of the January 2024 sample (Table 2). The most consistent change in antioxidant activity and antioxidant content was for the TPC. Since no correlation was found between the TFC and the antioxidant activity, it is possible that phenolic compounds other than flavonoids are primarily responsible for the seasonal fluctuation in antioxidant activity of the *N.*

yezoensis ethanolic extract. Alternatively, although not investigated in this study, red algal polysaccharides are known to have biological and medicinal properties, including antioxidant activities [12,13]. It may be possible that polysaccharides increase in *N. yezoensis* with seasonal changes. Figure 3 shows the following abiotic environmental factors of the *N. yezoensis* collection site: seasonal changes in air temperature, seawater temperature, nutrient concentrations in the seawater, and sunshine hours. The decrease in the antioxidative activity and the antioxidant content from December 2023 to January 2024 appeared to be correlated with decreases in the seawater temperature and the nutrient concentrations. It has been reported that *N. yezoensis* grew normally at temperatures between 10–17°C [14], though its biological activity may decrease at the low temperatures (around 12°C) seen in January 2024 (Figure 3B). Additionally, it is thought that a decrease in the nutrient concentrations is directly related to the biological activity of *N. yezoensis* (Figure 3C and D). The relatively high temperature and high nutrient concentration may be the factors that accumulate higher concentrations of phenolic compounds with antioxidant activities seen in December 2023. Since multiple environmental factors may have a complex effect, it might be necessary to accumulate more data by conducting analyses over multiple years in the future.

Table 2. Antioxidative power and antioxidant content of *N. yezoensis* ethanol extracts prepared using fresh thalli at different collection dates.

Collected Date of Fresh Samples		December 4, 2023	January 10, 2024	March 3, 2024
Antioxidative power	ABTS assay ^{*1}	10.71 ± 0.04 ^a	5.26 ± 0.02 ^b	9.23 ± 0.44 ^{ab}
	(TEs µmol/mgDW) ^{*4}			
Phenolic content	TPC ^{*2}	2.25 ± 0.06 ^a	1.67 ± 0.19 ^b	1.84 ± 0.26 ^{ab}
	(GAEs µg/mgDW) ^{*5}			
	TFC ^{*3}	42.17 ± 5.71 ^a	28.56 ± 4.20 ^a	13.37 ± 1.76 ^b
MAA content	(QEs µg/mgDW) ^{*6}			
	Shinorine (µg/mgDW)	0.26 ± 0.03 ^a	0.29 ± 0.01 ^b	0.20 ± 0.06 ^{ab}
Chlorophyll (Chl) content	Porphyra-334 (µg/mgDW)	4.60 ± 0.42 ^a	6.35 ± 0.22 ^b	4.73 ± 1.06 ^{ab}
	Chl <i>a</i> (µg/mgDW)	8.22 ± 0.47 ^a	6.99 ± 0.06 ^b	5.84 ± 0.30 ^c
Carotenoid content	Chl <i>b</i> (µg/mgDW)	0.47 ± 0.10 ^a	0.35 ± 0.16 ^{ab}	0.17 ± 0.07 ^b
	(µg/mgDW)	2.09 ± 0.05 ^a	1.77 ± 0.08 ^b	1.65 ± 0.05 ^c

Note: Values are presented as mean ± deviation ($n = 3$).

Means with different letters indicate statistical significance when comparing among different collection dates (Student's *t*-test, $p < 0.05$).

^{*1}The ABTS assay was used to measure antioxidative activity.

^{*2}The total phenolic content (TPC) is shown.

^{*3}The total flavonoid content (TFC) is shown.

^{*4} The antioxidative power values were expressed in Trolox equivalents (TEs) µmol/mgDW.

^{*5} The TPC values were expressed in gallic acid equivalents (GAEs) µg/mgDW.

^{*6} The TFC values were expressed in quercetin equivalents (QEs) µg/mgDW.

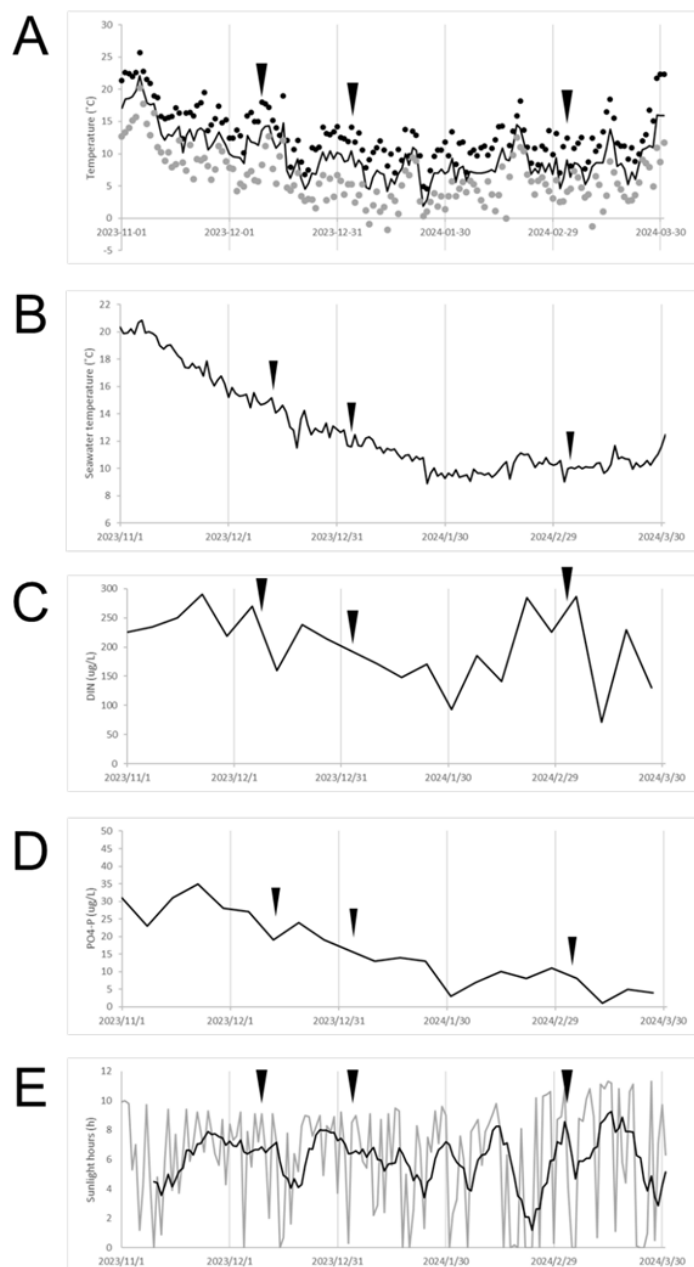


Figure 3. Abiotic conditions at the nori farm. (A) Air temperature. The air temperature data at Chubu Centrair International Airport (34.5°N, 136.5°E) was obtained from the Japan Meteorological Agency (JMA) website (<https://www.jma.go.jp/jma/index.html>) and represented temperature data near the nori farm. (B) Seawater temperature. The seawater temperature data at a depth of 1 m near the nori farm at 6 am was obtained from the Ise Bay Environmental Database (34.4°N, 136.6°N, <https://www.isewan-db.go.jp/>). (C) Concentration of dissolved inorganic nitrogen (DIN). (D) Concentration of phosphate ion. (E) Daily sunshine hours (gray line) and their one-week average value (black line). The daily sunshine hours at the observation point (34.4°N, 136.6°N) were obtained from the JMA website. (F) Erythemal UV intensity. The arrow heads indicate the sampling date.

4. Conclusions and future perspectives

In conclusion, this study revealed that the antioxidant activity of *N. yezoensis* was enhanced by drying and roasting. Roasting kan-nori to process it into yaki-nori is intended to brighten its color and give it a unique aroma. In this study, the antioxidant activity of *N. yezoensis* increased during the processing process, especially the roasting process, which indicates that this processing also affected the content of active ingredients. In the future, it will be important to investigate not only the antioxidant activity, but also the efficacy against aging and diseases, such as anti-inflammatory and anti-glycation effects. In addition, *N. yezoensis* exhibited higher antioxidative activities when it was collected early in the collection season, when the seawater temperature was high and nutrients were relatively abundant. Our data suggested that phenolic compounds significantly contributed to the enhancement of the antioxidant effect. In Japan, a variety of seaweeds are eaten, so it is of a great interest to explore whether drying or roasting edible seaweeds other than nori alters their antioxidant content. For example, there is a report that showed that roasting the edible red alga *N. tenera* (Asakusanori) [8] and brown alga *Laminaria japonica* (Ma-konbu) [15] enhanced the radical scavenging activities of their aqueous extracts. In this case, it was proposed that the increase in phenolic compounds mediated by the roasting process was related to the enhanced activities. Finally, we would like to make some points about the methodology in this paper. First, we used an ethanol extract of *N. yezoensis* because it efficiently dissolved the chlorophylls and carotenoids that were measured; additionally, these extracts had high solubilities in water-soluble molecules such as polyphenols and MAAs. However, in the future, an analysis using aqueous extracts might help to explore different active ingredients in *N. yezoensis*. Additionally, in this study, we conducted an ABTS assay, which is commonly used to measure free radical scavenging activity; however, it should be noted that more significant results may be obtained by using other methods to measure the antioxidant activity, such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, the singlet oxygen quenching activity, and the hydrogen peroxide quenching activity.

Author contribution

H.K., M.H., and R.W.S. performed the experiments, analyzed the data, and wrote the manuscript. R.N. collected the fresh *N. yezoensis* samples. S.K. connected H.K. and R.N. and made it possible to carry out this study.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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