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*Original Article*

# Effects of the ethanol extract of *Neopyropia yezoensis*, cultivated in the Seto Inland Sea (Setonaikai), on the viability of 10 human cancer cells including endocrine therapy-resistant breast cancer cells

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**ABSTRACT** — Here, we established an ethanol extraction method and obtained extracts of *Neopyropia yezoensis* cultivated in three different locations (extracts A-C) in the Seto Inland Sea (Setonaikai). The effects of the extracts on 10 human cancer cells derived from seven different organs were investigated. Extract A exerted the strongest anti-proliferative effects on all types of cancer cells, including an endocrine therapy-resistant aggressive breast cancer model, LTED cells. We analyzed the effects of the extracts on MCF-7 (parental cells for producing LTED cells)/LTED cells, along with four established anti-proliferative agents (etoposide, LY2835219, paclitaxel, and trichostatin A) with different action mechanisms. The inhibitory effects of extract A on both breast cancer cells were comparable with those of paclitaxel, although the other agents showed a preferable reduction in MCF-7 cell viability. We provide evidence of the involvement of component(s), especially those of extract A of *N. yezoensis*, which exerted anti-proliferative effects on cancer cells.

**Key words:** *Neopyropia yezoensis*, LTED cells, Anti-proliferative activity, Setonaikai

## INTRODUCTION

*Neopyropia yezoensis* (nori), a seaweed (also known as 'sea vegetable'), previously known as *Pyropia yezoensis* (Yang *et al*., 2020), is an important edible seaweed in Asia. It is extensively cultivated in Asian countries including Japan, China, and Korea (Yang *et al*., 2020). The nori sheets prepared from dried *N. yezoensis* can be used for making Japanese traditional sushi or California rolls. In addition to the nutritional and industrial merits,

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some extracts of *N. yezoensis* have been reported to exert biological properties, such as *in vitro* anti-proliferative activity, against some human cancer cells, Hep3B, HeLa, MCF-7, and MDA-MB-231 cells (Park *et al*., 2014; He *et al*., 2019). To perform animal testing, it is important to obtain *in vitro* information on whether the extract of *N. yezoensis* can exert i) cancer type-specific anti-proliferative effects and ii) killing effects on endocrine therapy-resistant cell models such as long-term estrogen-deprived (LTED) cells that are derived from parent MCF-7 cells (Katzenellenbogen *et al*., 1987; Yue *et al*., 2003; Takeda *et al*., 2016; Hirao-Suzuki *et al*., 2020). Generally, the components of plant products produced under natural environment can vary with the environment. Accordingly, the biological properties of *N. yezoensis* may change with the sampling sites.

In the present study, we established an ethanol extraction method for *N. yezoensis*. We then performed a cell viability analysis with three ethanol extracts (A-C) of samples collected in three different areas in the Seto Inland Sea (Setonaikai) using 10 human cancer cells.

## MATERIALS AND METHODS

### Reagents

Etoposide, paclitaxel, and trichostatin A were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) (purity: ≥ 98%). LY2835219 was purchased from AdooQ BioScience (Irvine, CA, USA) (purity: ≥ 98%). All chemicals were dissolved in dimethyl sulfoxide (DMSO).

### Seaweed material

We obtained three samples of *N. yezoensis* (samples A-C), which were cultivated at a depth of approximately 1 m and sampled in three different locations in Setonaikai (Fig. 1). The samples were collected in January 2016 at the following coordinates: sample A, 34°22.0381′N/133°22.4810′E; sample B, 34°20.7152′N/133°19.4698′E; and sample C, 34°20.1848′N/133°18.3024′E (Fig. 1; Maruko Suisan Co., Ltd.). Voucher specimens are maintained in Fukuyama University's Natural Products Laboratory of the Satoyama Satoumi Project. For morphological observations, images of live thallus collected from these sites were captured using a digital camera, and then small portions of the thallus were excised and observed under a light microscope.

### Extraction procedure

The seaweed was washed with water and dried for 24 hr in a drying machine at 25°C (approximately 500 mg; dried material). Thereafter, freshly prepared cold 60% ethanol was added to the dried seaweed, to a concentration of 5 mg/mL; then, the sample was homogenised for 10 min under ice-cold conditions using a Polytron tissue homogeniser (Kinematica AG, Littau, Switzerland). The obtained homogenised sample was collected in a shading tube, incubated for 24 hr at room temperature, and then centrifuged at 200 *g* for 1 min at 15°C. The obtained supernatant was subjected to vacuum evaporation for 120 min at 30°C, followed by deep-freezing for 24 hr at -80°C. Finally, the extract was subjected to freeze-drying (approximately 25 mg; final extract) (percentage yield, approximately 5%). Before the experiments, DMSO was added to each extract.

### Cell culture and cell viability analysis

MCF-7, SK-BR-3, HEC-1-B, SK-OV-3, HCT116, HT-29, HepG2, A549, and PANC-1 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). LTED cells was derived from parent MCF-7 cells as previously mentioned (Katzenellenbogen *et al*., 1987; Jeng *et al*., 1998; Chan *et al*., 2002; Takeda *et al*., 2016; Hirao-Suzuki *et al*., 2020). The cell culture conditions were based on previously reported procedures (Takeda *et al*., 2016; Hirao-Suzuki *et al*., 2020). Cell viability analysis was performed using MTS assay as previously mentioned (Hirao-Suzuki *et al*., 2020).

### Data analysis

$IC_{50}$ values were obtained using SigmaPlot 11 software (Systat Software, Inc., San Jose, CA, USA). The statical significance was determined using one-way ANOVA followed by Tukey-Kramer's post-hoc test using StatView 5.0J software (SAS Institute Inc., Cary, NC, USA). A $P$ value $< 0.05$ was considered significant.

## RESULTS AND DISCUSSION

We obtained three samples (A-C) of *N. yezoensis*, cultivated in Setonaikai (Fig. 1). Sample A was collected from a site close to Tsugarujima/Fukuyama and samples B/C were collected from sites close to Tashima/Fukuyama. There were no morphological differences in the thallus of the seaweed among the samples (Fig. 1B, upper panel). In addition, the morphology of cells and chloroplasts (each cell contains one chloroplast) was similar among the samples (Fig. 1B, lower panel). We established an ethanol extraction method for raw samples of *N. yezoensis* and analyzed the effects of the obtained extracts on the viability of 10 human cancer cells.

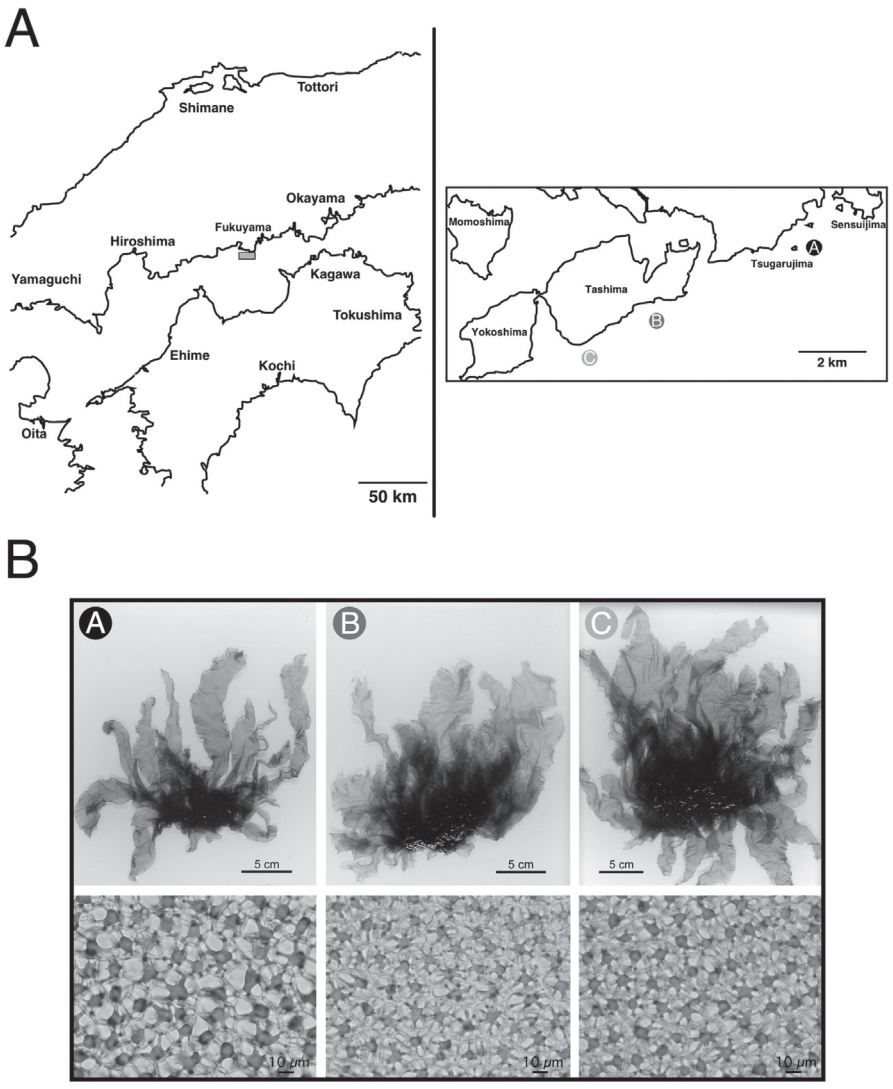


**Fig. 1.** Map showing the locations of original sampling sites and morphology of live thalli of *Neopyropia yezoensis*. (A) Map showing the locations of original sampling sites (A-C) of *N. yezoensis* in Setonaikai. An enlarged map of the square filled with grey shade indicated in the left panel is shown in the right panel. Sample A was obtained from a site close to Tsugarujima/Fukuyama and samples B/C were obtained from sites close to Tashima/Fukuyama. (B) Live thalli of cultivated *N. yezoensis* (A-C). The upper panels indicate the morphology of live thalli (each scale bar is 5 cm). The lower panels present the surface view of live blades showing vegetative cells with a stellate chloroplast (each scale bar is 10 μm).

We first focused on the effects of the three extracts on endocrine-related cancers: breast (MCF-7, LTED, and SK-BR-3), endometrial (HEC-1-B), and ovarian (SK-OV-3) cancer cells. Among the three extracts, extract A exhibited the strongest anti-proliferative activity with the $IC_{50}$ value of 3.8 ± 0.49 μg/mL for MCF-7, 4.7 ± 0.36 μg/mL for LTED, and 5.7 ± 0.20 μg/mL for SK-BR-3, followed by B/C in MCF-7 (Fig. 2A). The $IC_{50}$ values of extracts B/C for LTED and SK-BR-3 cells were not calculated owing to the weak inhibitory effects of the samples. The viability of HEC-1-B and SK-OV-3 cells were significantly reduced by extract A, with the $IC_{50}$ value of 6.8 ± 0.43 μg/mL for HEC-1-B and 3.5 ± 0.19 μg/mL for SK-OV-3, followed by extracts B in SK-OV-3, but not significantly by extract B/C for HEC-1-B and extracts C for SK-OV-3 ($IC_{50}$: > 50 μg/mL) (Fig. 2B and C). Overall, among the three ethanol extracts of *N. yezoensis*, extract A exerted the most potent inhibitory effect on sev-

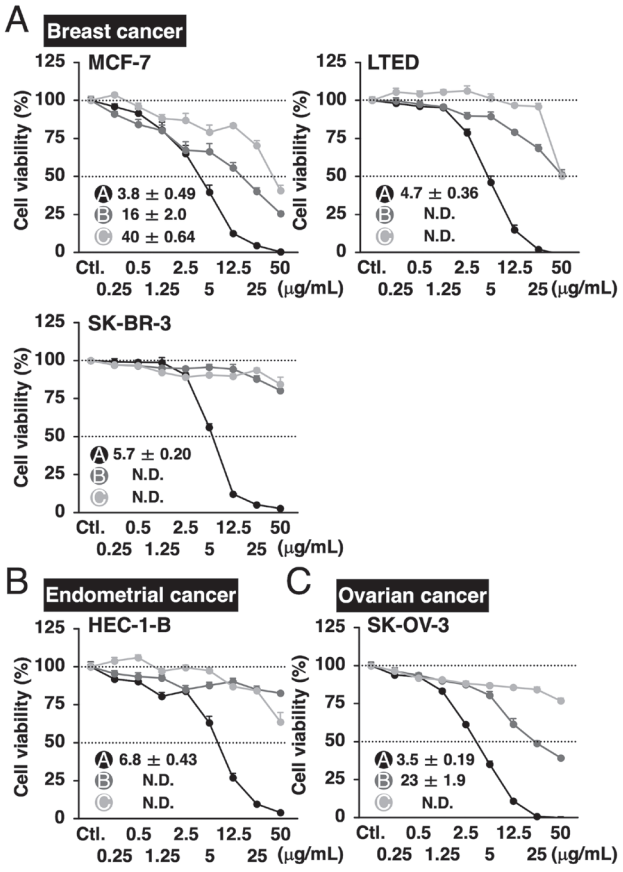


**Fig. 2.** Effects of the ethanol extracts of *Neopyropia yezoensis* on the viability of MCF-7, LTED, SK-BR-3, HEC-1-B, and SK-OV-3 cells. The cells were treated with the ethanol extracts of *N. yezoensis* (0.25 to 50 μg/mL) for 48 hr. Data are expressed as mean ± S.E. (n = 6) percentage of the vehicle-treated control (Ctl).

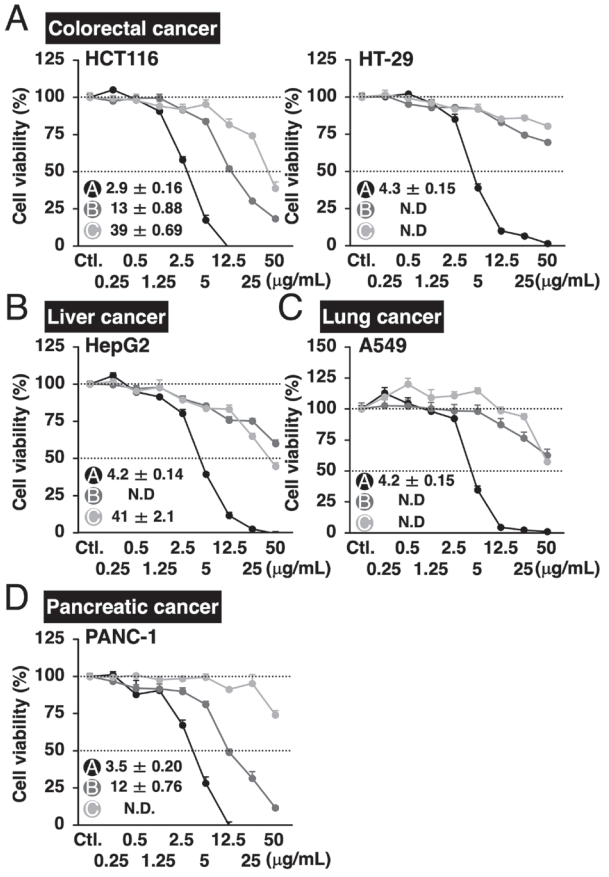


**Fig. 3.** Effects of the ethanol extracts of *Neopyropia yezoensis* on the viability of HCT116, HT-29, HepG2, A549, and PANC-1 cells. The cells were treated with the ethanol extracts of *N. yezoensis* (0.25 to 50 μg/mL) for 48 hr. Data are expressed as mean ± S.E. (n = 6) percentage of the vehicle-treated control (Ctl).

eral lines of human cancer.

We evaluated the effects of the three extracts on the viability of colorectal (HCT116 and HT-29), liver (HepG2), lung (A549), and pancreatic (PANC-1) cancer cells. The viability of the cancer cells was significantly reduced by extract A (followed by extract B or C), with an $IC_{50}$ value of 3-4 μg/mL (Fig. 3). Taken together with the findings presented in Fig. 2, extract A exerted the strongest anti-proliferative effect on cancer cells irrespective of the cancer type.

LTED cells were derived from parent estrogen-dependent MCF-7 cells, through culture under estrogen-deprived conditions for more than 6 months. The LTED cells exhibited resistance to aromatase inhibitors (*i.e.*, endocrine therapy resistance) due to the acquisition of estrogen-independent growth (Katzenellenbogen *et al*., 1987; Yue *et al*., 2003; Takeda *et al*., 2016; Hirao-Suzuki *et al*., 2020). Furthermore, these cells can be used as an *in vitro* model of postmenopausal tumorigenesis, exhibiting a more aggressive behaviour (Katzenellenbogen *et al*., 1987; Jeng *et al*., 1998; Chan *et al*., 2002; Hirao-Suzuki *et al*., 2020). We evaluated the sensitivity of the cells to four anti-proliferative agents with different action mechanisms, namely, etoposide, a topoisomerase IIα inhibitor (Solovyan *et al*., 2002; Takeda *et al*., 2013), LY2835219, a cyclin-dependent kinase 4/6 inhibitor (Gelbert *et al*., 2014), paclitaxel, a microtubule-stabilising agent (Lu *et al*., 2012), and trichostatin A, a histone deacetylase inhibitor (Yoshida *et al*., 1990). As expected, the LTED cells required higher concentrations ($IC_{50}$) of the agents than

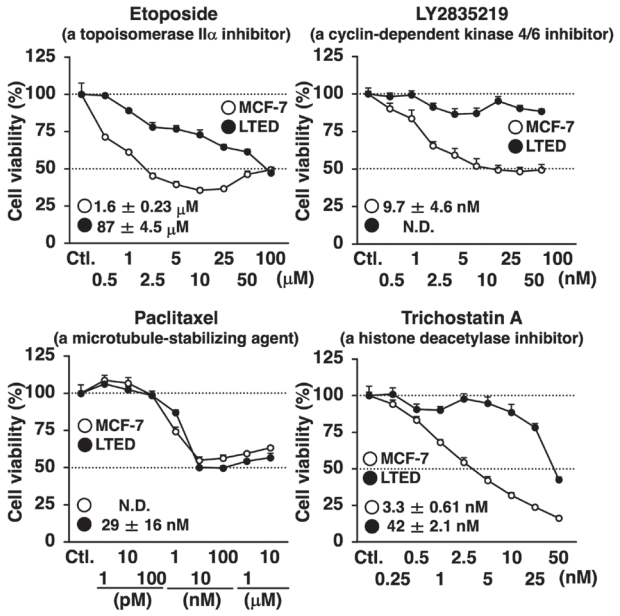


**Fig. 4.** Effects of the established anti-proliferative agents on the viability of MCF-7 and LTED cells. The cells were treated with etoposide (0.5 to 100 μM), LY2835219 (0.5 to 100 nM), paclitaxel (1 pM to 10 μM), trichostatin A (0.25 to 50 nM) for 48 hr. Data are expressed as mean ± S.E. (n = 6) percentage of the vehicle-treated control (Ctl).

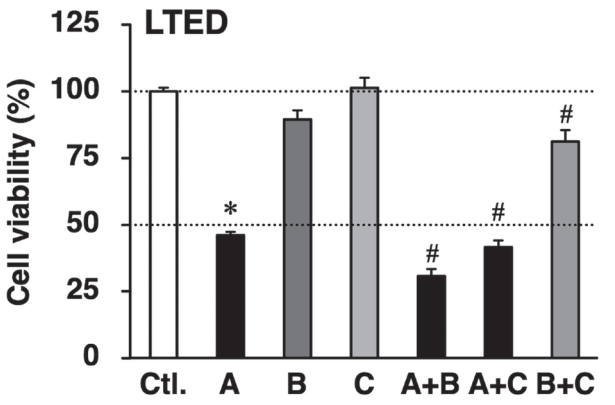


**Fig. 5.** Effects of co-treatment with two ethanol extracts of *Neopyropia yezoensis* on the viability of LTED cells. LTED cells were treated with extracts A, B, C, A/B, A/C, or B/C of *N. yezoensis* (5 μg/mL) for 48 hr. Data are expressed as mean ± S.E. (n = 6) percentage of the vehicle-treated control (Ctl). Significant differences compared with Ctl. and the singly treated samples are marked with asterisks (*$P < 0.05$) and hashes ($^{\#}P < 0.05$), respectively.

MCF-7 cells, although there was no considerable difference in the effect of paclitaxel on the two breast cancer cells (Fig. 4). It should be noted that extract A, but not extracts B/C, exhibited comparable inhibitory effects on MCF-7 and LTED cells (Fig. 2A), as paclitaxel. To examine mechanism(s) underlying the anti-proliferative effects of extract A, a cell-cycle progression analysis using a flow cytometer was performed. G2/M phase arrest was detected in MCF-7 and LTED cells treated with extract A (5 μg/mL), as well as paclitaxel (data not shown), which is known to cause G2/M phase arrest in MCF-7 cells (Bacus *et al*., 2001). Thus, extract A might contain component(s) similar to paclitaxel. Compared with extract A, extracts B/C showed a very weak inhibitory effect on the viability of cancer cells including LTED cells (Figs. 2 and 3). This could be because extracts B/C contain certain inhibitory molecule(s) against molecules responsible for the anti-proliferative activity, commonly present in the three extracts. As shown in Fig. 5, compared with treatments with extract A, B, or C (5 μg/mL), the viability of LTED cells was additively reduced by the co-treatment with A+B, A+C, or B+C. This suggested that the ethanol extract (A-C)-mediated anti-proliferation relies on the amount of molecule(s) presented in *N. yezoensis*.

As we used the crude extracts of *N. yezoensis*, and not pure samples, there may have been an artifact during the course of extraction. To exclude this possibility, we further analyzed the effect of another lot of sample A (*i.e.* A') on the viability of cancer cells. The experiments were performed as indicated in Figs. 2 and 3. The percent difference in the obtained $IC_{50}$ values between samples A and A' was below 15%, which was not significant (data not shown).

In this study, among the three extracts, extract A presented the strongest anti-proliferative effects on the 10 human cancer cells regardless of the cancer type; however, it is not clear why extract A showed the strongest potential over extracts B/C. One possible explanation might be the existence of inter-regional discrepancies between the samples (see Fig. 1). The concentration of nutrients in seawater varied between sites A and B; nutrient salts in site A were 2.5 times lower than those in site B (approximately 0.04 mg/L); although this finding is preliminary, environmental conditions, such as nutrient salts, of regions where *N. yezoensis* is cultivated might be involved in the action of extract A.

To validate the anti-proliferative effects of *N. yezoensis* on cancer cells, additional experiments are required to

identify the component(s) involved. Studies should also verify whether there are inter-regional discrepancies in *N. yezoensis* cultivated beyond Setonaikai.

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**Conflict of interest----** The authors declare that there is no conflict of interest.

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