Note

Effects of Extracts from Tropical Seaweeds on DPPH Radicals and Caco-2 Cells Treated with Hydrogen Peroxide

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Edible seaweeds were collected from Indonesia, a tropical country that does not show seasonal variations in temperature, to evaluate their health-related activities. Ethanol and acetone extracts were prepared from 3 green and 3 brown algae. The ethanol and acetone extracts from *Padina australis* showed the strongest DPPH radical scavenging activity. These extracts also had the highest concentrations of total phenol and flavonoid. Both the ethanol and acetone extracts of the 6 Indonesian seaweeds decreased Caco-2 cell viability when such cells were treated with $600\,\mu$M hydrogen peroxide. However, when Caco-2 cells were treated with 700 or $800\,\mu$M hydrogen peroxide, the ethanol and acetone extracts from *P. australis* increased cell viability significantly more than those from the other seaweeds. This study indicates that organic extracts of seaweed have useful health-related functions.

Keywords: Seaweed, ethanol extract, acetone extract, DPPH radical scavenging activity, Caco-2, cell viability

Introduction

Seaweed is rich in minerals and dietary fiber, and seaweed is a traditional food in Asia. In some reports, the health aspects of seaweed in relation to its soluble dietary fiber and mineral contents was mentioned (Wang *et al.,* 2001, Santoso *et al.,* 2006). Dietary fiber is found in the high-molecular-weight fraction of seaweed, while the low-molecular-weight fraction of seaweed is considered to contain substances such as free amino acids, solubilized minerals, and polyphenols (Wong and Cheung, 2000, Yoshie *et al.,* 2000). In this study, we focused on evaluating the health-related activity of seaweed extracts mainly consisting of low-molecular-weight compounds.

Many studies have shown the radical scavenging and ACE inhibition activities of the water-soluble and organic-solvent-soluble fractions from seaweed (Jung *et al.,* 2006; Sato *et al.,* 2002; Nahas *et al.,* 2007; Hou *et al.,* 2003). Yuan *et al.* (2005b) and Yuan and Walsh (2006) reported that seaweed extracts affect the proliferation of colon cancer cells. Radicals are present around tumors and inflammatory tissue, among others. Therefore, radical scavenging activity is considered to be useful for helping to repair damage caused by inflammation.

The organic solvent-soluble fraction from seaweed contains compounds such as polyphenols and flavonoids. In addition, radicals are present throughout the human body.

Thus, the *in vitro* radical scavenging activity of organic extracts from seaweed and the effects of organic extracts from seaweed on the viability of cells treated with hydrogen peroxide were selected to be evaluated in this study.

Materials and Methods

Materials Six edible Indonesian seaweed species were used in this study: three green algae (*Caulerpa sertularoides, Halimeda macroloba,* and *Ulva reticulata*), and three brown algae (*Padina australis, Sargassum polycystum,* and *Turbinaria conoides*). *C. sertularoides* is known as *Umibudo* in Japan, and *U. reticulata* is similar to the Japanese *Aosa. P. australis* is similar to the Japanese seaweed *Umiuchiwa,* but this tropical seaweed has a thinner and softer body than Japanese one. All seaweed samples used were obtained from the Seribu Island, Jakarta Prefecture, Indonesia. They were washed with clean seawater and transported to the laboratory under refrigeration. They were then washed with tap water, wiped with paper towels, and then minced with a food processor (MK-K75; Matsushita Electric Co., Osaka, Japan), and stored at -20℃.

DPPH radical was purchased from Sigma Chemicals (St Louis, MO, USA). All the other reagents used in the experiments were of analytical grade.

Proximate analysis The chemical composition, i.e., moisture, ash, fat, and protein contents of the dried seaweed samples, was analyzed in accordance with the AOAC method (AOAC, 1995).

Preparation of ethanol and acetone extracts Two sol-

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vents, ethanol and acetone were used as solvents to prepare seaweed extracts. A five-fold volume of solvent (100% ethanol or 100% acetone) was added to a seaweed sample, the mixture was homogenized and collected supernatants (extracts) were evaporated, and then redissolved in the same solvent.

Determination of total phenol and flavonoid contents The acetone in the extracts was removed using nitrogen gas, and the extracts were then redissolved in ethanol prior to analysis. Total phenol contents in the ethanol and acetone extracts from seaweed samples were determined following the method of Singleton and Rossi (1965) and Lim *et al.* (2002), with some modifications. Gallic acid was used as the standard and the result was calculated as gallic acid equivalent (mg GAE/g dry sample). Total flavonoid content was analyzed following the method of Eberhardt *et al.* (2000) and Liu *et al.* (2002), with some modifications. As seaweeds have a high morin content (Yoshie-Stark *et al.,* 2003), therefore, morin was used as a standard. The results are expressed as miligrams of morin equivalents.

DPPH radical scavenging activity DPPH radical scavenging activity was analyzed following the method explained in the report of Yoshie-Stark *et al.* (2004).

Caco-2 cell culture Human colon adenocarcinoma Caco-2 cells were obtained from the Institute of Physical and Chemical Research (RIKEN Cell Bank, Ibaraki, Japan). Caco-2 cells were cultured and maintained following the report of Sugawara *et al.* (2001).

Treatment of Caco-2 cells with hydrogen peroxide A hydrogen peroxide (H_2O_2) stock solution (9 mol/L) was kept at 4°C and diluted just before use in PBS buffer (Ca and Mg free). Cells (passages 20–40, 5.0×10^4 cells/mL, 100 μL/well) were seeded onto 96-well microplates and incubated for 24 h prior to H_2O_2 treatment. The monolayer of cells was treated with 6 μL of H_2O_2 (to make final concentrations of 600, 700, and 800 μmol/L) for 24 h.

Effect of seaweed extract on hydrogen peroxide treated Caco-2 cells Cells (5.0×10^3 cells/well) were seeded onto 96-well microplates (100 μL/ each well) and incubated for 24 h. Before H_2O_2 treatment, the monolayer of cells was treated with 6 μL of a sample extract (2.5 mg sample equivalent/mL at final concentration) for 30 min. Then, 6 μL of H_2O_2 (to make final concentrations of 600, 700, and 800 μmol/L) were added and the plates were incubated for 24 h.

Cell viability Cell counting kit-8 (Wako Pure Chemicals, Osaka, Japan) was used for the cell viability assay. As mentioned above, cells were seeded onto 96-well plates and incubated for 24 h before the test treatment. Then, treatment with sample extracts and hydrogen peroxide or hydrogen peroxide alone was performed for 24 h. For a positive control (PC), MTT assay was performed with the addition of PBS instead of the sample extract. Ethanol was added to the treatment blank (B) instead of sample extract. After incubation, a water-soluble tetrazolium salt-based assay was performed as follows: 10 μL of the cell counting kit-8 solution was added to each well and incubated for 4 h at 37°C under 5% CO_2. The absorbance at 450 nm was read using a microplate reader. Cell viability

Table 1. Proximate compositions of seaweed samples (means±SD g/100 g).

Seaweed samples	Moisture	Ash	Fat	Protein
Green algae				
Caulerpa sertularoides	82.4 ± 0.6[b]	2.9 ± 0.2[a]	2.3 ± 0.1[c]	3.1 ± 0.2[d]
Halimeda macroloba	73.1 ± 1.4[a]	22.5 ± 0.3[d]	0.4 ± 0.1[a]	1.6 ± 0.1[c]
Ulva reticulata	83.9 ± 0.3[cd]	2.9 ± 0.1[a]	1.2 ± 0.2[c]	1.2 ± 0.2[bc]
Brown algae				
Padina australis	83.1 ± 1.0[bc]	5.5 ± 0.4[c]	0.8 ± 0.1[b]	1.5 ± 0.1[c]
Sargassum polycystum	84.7 ± 0.4[d]	3.8 ± 0.3[b]	0.3 ± 0.0[a]	0.9 ± 0.1[a]
Turbinaria conoides	85.1 ± 0.3[d]	2.5 ± 0.1[a]	0.8 ± 0.1[b]	1.0 ± 0.1[ab]

Values with different superscript letters are significantly different ($p < 0.05$, $n = 3$).

was calculated as follows:

Cell viability (%)=(S–B)/(PC–B)×100
- S: Absorbance from test sample
- B: Absorbance of Blank (Ethanol was added instead of sample extract)
- PC: Absorbance of positive control (PBS was added instead of sample extract).

Statistical analysis Results are presented as mean ± S.D. (n=3–5). ANOVA was used to calculate significant differences.

Results and Discussion

Proximate analysis The proximate compositions of the seaweed samples are shown in Table 1. *H. macroloba* had a significantly higher ash content than the other seaweeds at a concentration of 22.5 g/100 g. The reason for this high concentration of ash in *H. macroloba* is considered to be the high calcium content needed to maintain this plant's hard body. Among all of tested samples, only *H. macroloba* lives in a sandy shallow area and has a hard plant body. Green algae had a composition of 73.1–83.9 g/100 g moisture, 2.9 g/100 g ash, 0.4–2.3 g/100 g fat, and 1.2–3.1 g/100 g protein. Brown algae had a composition of 83.1–85.1 g/100 g moisture, 2.5–5.5 g/100 g, 0.3–0.8 g/100 g fat, and 0.9–1.5 g/100 g protein.

Phenol and flavonoid contents Figures 1 and 2 summarize the total polyphenol and flavonoid contents of the seaweed extracts. As shown in Fig. 1, ethanol extracts from green algae had a total phenol content of 0–10 mg gallic acid equivalent (GAE)/g dry matter, whereas those from brown algae had a total phenol content of 5–45 mg GAE/g dry matter. Ethanol extracts from green and brown algae showed total flavonoid contents of 0.5–3 and 3.5–4.5 mg morin equivalent (ME)/g, respectively. *P. australis* showed a significantly higher phenol content than other seaweeds. Brown algae showed higher polyphenol and flavonoid contents than green algae. The total polyphenol content (expressed as gallic acid equivalents) of the dulse and *Sargassum* methanol extracts were reported
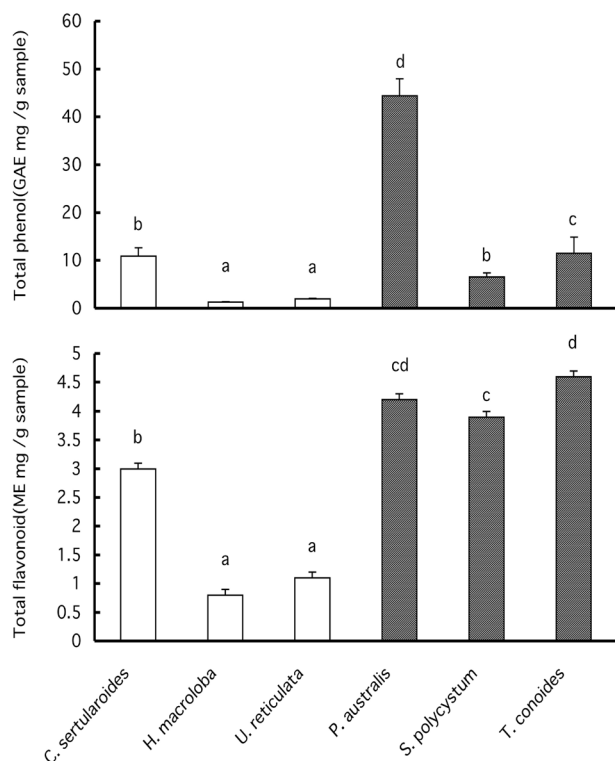
Fig. 1. Phenol (top) and flavonoid (bottom) contents of ethanol extracts from 6 Indonesian seaweeds.
Total phenol content was calculated as gallic acid equivalent, GAE (mg/g dry sample), and total flavonoid content was calculated as morin equivalent, ME (mg/g dry sample). Letters over each column not sharing the same are significantly different ($P<0.05$).
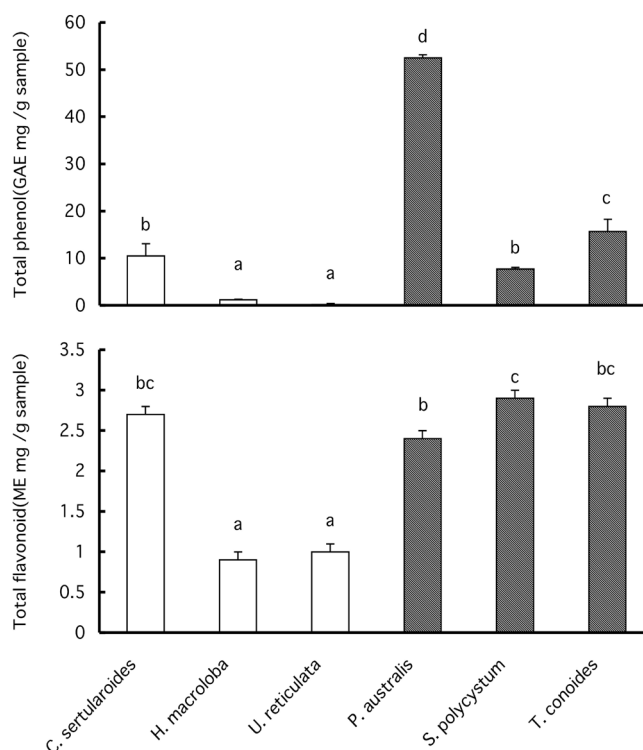
Fig. 2. Phenol (top) and flavonoid (bottom) contents of acetone extracts from 6 Indonesian seaweeds.
Total phenol content was calculated as gallic acid equivalent, GAE (mg/g dry sample), and total flavonoid content was calculated as morin equivalent, ME (mg/g dry sample). Letters over each column not sharing the same are significantly different ($P<0.05$).

to be 10.3 mg GAE/g (Yuan *et al.,* 2005a) and 51.0 mg GAE/g (Lim *et al.,* 2002). With respect to the result of these studies, *P. australis* showed a similar polyphenol content. As shown in Fig. 2, acetone extracts from green and brown algae showed total polyphenol contents of 0–10 mg GAE/g and 7–50 mg GAE/g, respectively. Acetone extracts from green and brown algae showed total flavonoid contents of 1–2.8 mg ME/g, and 2.5–3 mg ME/g, respectively. *P. australis* showed a polyphenol content that was significantly higher than those of the other seaweeds. Jimenez-Escrig *et al.* (2001) reported that hydrolyzed and acetone/water extracts showed phenol contents of 41.4 mg GAE/g for *Fucus,* 7.3 mg/g for *Laminaria,* 6.6 mg/g for *Undaria,* and 5.7 mg/g for *Porphyra.* Compared with these values, *P. australis* showed significantly high contents of both polyphenol and flavonoid.

DPPH radical scavenging activity The DPPH radical scavenging activities of the ethanol extracts and acetone extracts from the seaweeds are shown in Fig. 3. Ethanol and acetone extracts from *P. australis* showed significantly higher DPPH radical scavenging activities than those from the other seaweed samples. Total polyphenol content and radical scavenging activity did not show a clear correlation; however samples with a higher polyphenol content showed a higher radical scavenging activity.

Jimenez-Escrig *et al.* (2001) reported the correlation between polyphenol content and radical scavenging activity. They also reported DPPH radical scavenging activity to have a median effective dose of 3.07 mg sample/g DPPH with a polyphenol content of 41.4 mg GAE. Their polyphenol content/DPPH radical scavenging activity ratio was similar to that in our results for *P. australis,* since the ethanol extract of *P. australis* showed a median effective dose (ED_{50}) of 1.3 mg of sample with a polyphenol content of 45 mg GAE/g, and the acetone extract of *P. australis* showed an ED_{50} of 3 mg of sample with a polyphenol content of 50 mg GAE/g. Ethanol extracts of green seaweeds showed significantly stronger radical scavenging activities than acetone extracts, whereas extracts from brown seaweeds did not show clear difference in radical scavenging activity between the two extraction solvents.

Cell viability The viabilities of cells treated with 600–800 μM H_2O_2 with and without treatment with ethanol extracts from seaweeds are shown in Fig. 4. When cells were treated with 600 μM H_2O_2, cell viability became 24%. Additional treatment with seaweed extracts did not increase cell viability; it actually significantly decreased cell viability. However, when cells were treated with 700 μM H_2O_2, cell viability showed a significant increase with *P. australis* extract treatment. A higher concentration of
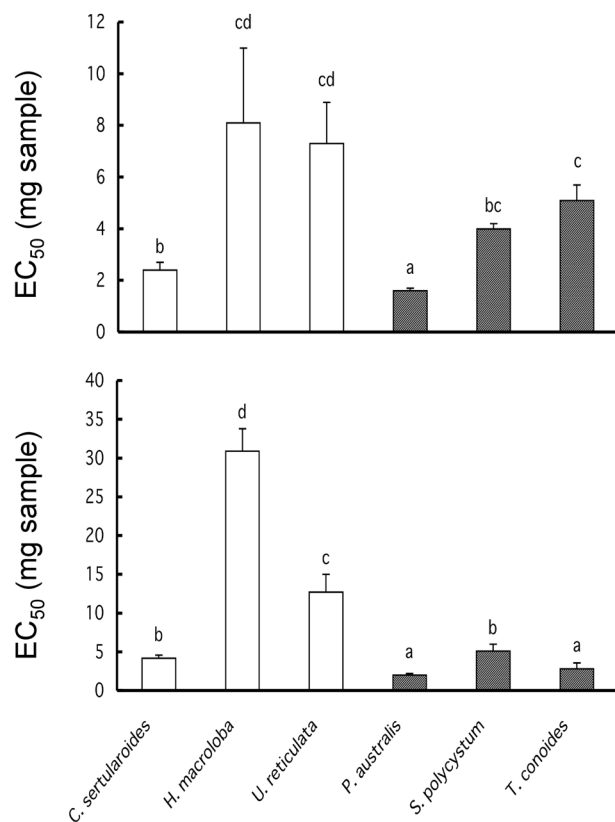
Fig. 3. DPPH radical scavenging activities of ethanol (top) and acetone (bottom) extracts from seaweeds. ED_{50} was defined as 50% of the radical scavenging concentrations of dried sample (mg/mL).

Letters over each column not sharing the same are significantly different ($P<0.05$).

Fig. 4. Changes in H_2O_2-treated Caco-2 cell viability induced by ethanol extracts from 6 Indonesian seaweeds. Cells were treated with $600\,\mu$mol/L H_2O_2 (top), $700\,\mu$mol/L H_2O_2 (middle), and $800\,\mu$mol/L H_2O_2 (bottom).

Letters over each column not sharing the same are significantly different ($P<0.05$).

H_2O_2 ($800\,\mu$M) decreased cell viability; however, in this case, the ethanol extracts from all the seaweeds showed a protective effect on the cells. In particular, ethanol extracts from *P. australis* did not decrease cell viability during $600\,\mu$M H_2O_2 treatment, and induced the largest increase in cell viability during $800\,\mu$M H_2O_2 treatment. Similar results were found also with acetone extracts from the other seaweeds as shown in Fig. 5. The dulse, kelp, *Porphyra, Gelidium* extracts were reported to inhibit cancer cell proliferation in a dose-dependent manner up to 5.0 mg/mL seaweed extract (Yuan and Walsh, 2006; Cho et al., 1997). A correlation between the antiproliferative effects of dulse and kelp extracts and the total polyphenol content of the extracts has also been mentioned (Yuan and Walsh, 2006). In our results, cells treated with a low concentration of hydrogen peroxide showed a decrease in cell viability during concurrent incubation with seaweed extracts. However, when cells showed a low cell viability owing to treatment with a high concentration of hydrogen peroxide, the presence of seaweed extracts increased cell viability. Some studies have used methanol extracts of seaweed; others used butanol extracts. The results from these reports and our study indicate that seaweed is a possible source of useful anti-
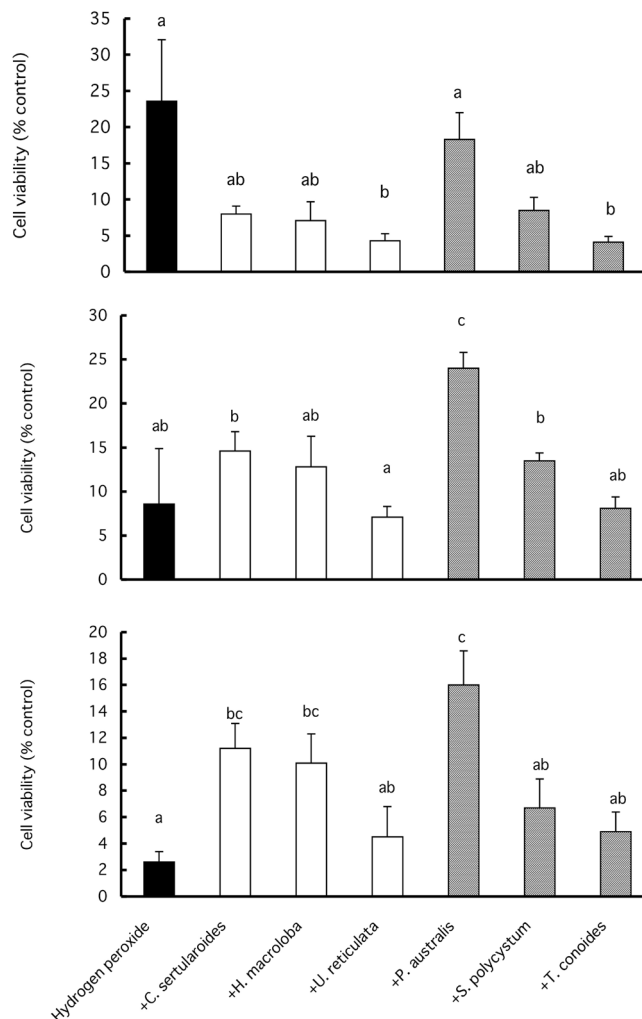
oxidative compounds and that different compounds can be obtained as a result of the selection of the extraction solvent.

Conclusions Ethanol extracts from seaweeds were found to have interesting health properties. Specific fractions of seaweeds rich in polyphenol may affect DPPH radical scavenging activity. However, the effect of such fractions on cancer cell proliferation depend on the concentration of hydrogen peroxide; some effectively enhance cell proliferation, and some through a combination of oxidative stress strength and extract concentration, reduce cell proliferation. Further research on organic extracts from seaweeds is necessary, to analyze not only with an analysis of the polyphenolic composition of each organic extract from the seaweeds, but also with an analysis of their effects on normal cells.
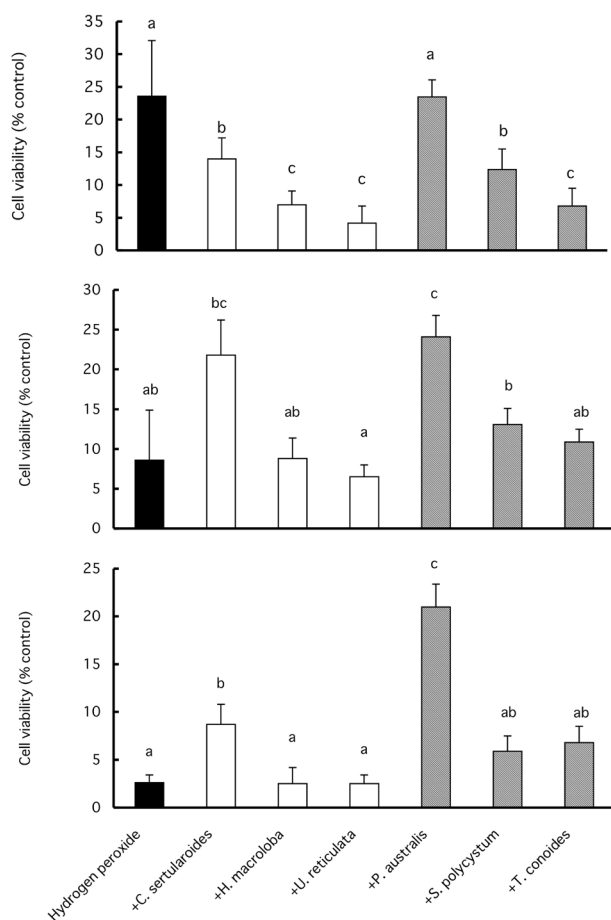
Fig. 5. Changes in H_2O_2-treated Caco-2 cell viability by acetone extracts from 6 Indonesian seaweeds. Cells were treated with 600μmol/L H_2O_2 (top), 700μmol/L H_2O_2 (middle), and 800μmol/L H_2O_2 (bottom).

Letters over each column not sharing the same are significantly different ($P < 0.05$).

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