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Antioxidant and anti-norovirus properties of aqueous acetic acid macromolecular extracts of edible brown macroalgae

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ABSTRACT

Previous studies reported the activity of fucoidans and polyphenols (phlorotannins) extracted from brown algae against enveloped viruses. The aim of this study was to investigate the effects of brown macroalgae on the non-enveloped norovirus. Aqueous acetic acid macromolecular (>3 kDa) extracts (AAEs) were obtained from ten samples of eight brown macroalgal species. Saccharides and sulphate composition, weight-averaged molecular weight, and total phenolic content (TPC) of the AAEs were measured to estimate their fucoidan and polyphenol content. Murine RAW264.7 macrophage cells and the human norovirus surrogate murine norovirus-1 (MNV-1) were used for the anti-norovirus assay. One hour of direct exposure to TPC-rich AAEs from *Sargassum horneri* (Sh), *Eisenia bicyclis* (Eb), *Cladosiphon okamuranus* (Co), and *Undaria pinnatifida* inactivated MNV-1. In contrast, pre-treatment of RAW264.7 cells for 20 h with AAEs from another *U. pinnatifida* sample, *Analipus japonicus,* and Co protected RAW264.7 cells against MNV-1 infection. The AAEs from Co contained high TPC and sulphated fucans (fucoidans) might be the most promising anti-norovirus agents as they directly inactivated MNV-1 and protected RAW264.7 cells from it. The AAEs of phlorotannin-rich Sh and Eb are also potentially effective at direct inactivating MNV-1.

1. Introduction

Human noroviruses (HuNoV) are non-enveloped RNA viruses of the Caliciviridae. They are major causative agents of food-borne illnesses (Hardstaff et al., 2018). HuNoV cause ~700 million infections and > ~200,000 deaths worldwide annually (Netzler, Tuipulotu, & White, 2018). Shellfish and other ready-to-eat foods are sources of HuNoV infections, which manifest as diarrhoea, nausea, and vomiting. Cases of this disease are reported globally. Epidemiological and genetic studies have been conducted on it in China, India, several European countries, the United States, and Japan (Angelo, Nisler, Hall, Brown, & Gould, 2016; Iritani et al., 2019; Li et al., 2020; Sharma et al., 2020). Outbreaks in hospitals and nursing homes are serious as they may be fatal in immunocompromised patients (Fraenkel, Inghammar, Söderlund-Strand, Johansson, & Böttiger, 2018; Parrón et al., 2019). In the United States, person-to-person transmission is the most common route and accounts for 66–77% of all norovirus outbreaks. In contrast, foodborne transmission accounts for 17–26% of all norovirus outbreaks (Burke et al., 2019).

Seven norovirus genotypes (GI–GVII) have been reported. The GI, GII, and GIV genogroups are human pathogens (Netzler et al., 2018). Acquired immunity against human noroviruses lasts only from several months to 2 years (Rouhani et al., 2016). Moreover, no drugs with efficacy against norovirus have been developed. Norovirus infections must be prevented through proper sanitation and hygiene. Several recent studies reported the antiviral efficacy of natural materials such as heat-denatured lysozyme derived from certain food products (Takahashi et al., 2018). Certain functional food components have inhibitory efficacy against norovirus infection (Chéron, Yu, Kolawole, Shakhnovich, & Wobus, 2015). Murine norovirus (MNV) has often served as a surrogate for HuNoV because no culture method has been established for HuNoV (Netzler et al., 2018; Wobus, Thackray, & Virgin, 2006). There are numerous reports on drugs with anti-norovirus efficacy (Netzler et al., 2018). However, there is no silver bullet against the infection itself. Therefore, prevention of the infection by sanitation and dietary immunomodulation are vital.

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Brown algae such as makombu (*Saccharina japonica*), wakame (*Undaria pinnatifida*), akamoku (*Sargassum horneri*), okinawamozuku (*Cladosiphon okamuranus*), and arame (*Eisenia bicyclis*) are very popular in Japanese cuisine. Brown algae are rich in alginates (polyglucuronate and polymannuronate), fucoidans (sulphated fucans), laminarins (β-1,3-glucans), and phlorotannins (algal polyphenols) (Kuda & Ikemori, 2009). However, the relative quantities of these natural products vary with season and algal species. Brown macroalgae may contain 10–50% (w/v) alkali-extractable alginates that are used as thickeners, bioreactor membrane beds, and dental impression material (Porse & Rudolph, 2017). Acid-extractable fucoidans and phlorotannins are alginate by-products and have antioxidant and antitumor activity (Jacobsen, Sørensen, Holdt, Akoh, & Hermund, 2019; Palanisamy, Vinosha, Marudhupandi, Rajasekar, & Prabhu, 2017).

Previous studies reported the efficacy of fucoidans against enveloped DNA viruses including herpes simplex (HSV) and hepatitis B and enveloped RNA viruses such as influenza A, Newcastle disease (NDV), HIV-1, and dengue-2 (DEN2) (Dinesh et al., 2016; Hidari et al., 2008; Lee, Hayashi, Hashimoto, Nakano, & Hayashi, 2004; Li et al., 2017; Trejo-Avila et al., 2016; Wang et al., 2017). Other studies investigated the efficacy of phlorotannins extracted from the edible brown alga *Ecklonia cava* against HIV-1, SARS-CoV, and influenza virus (Karadeniz, Kang, Park, Park, & Kim, 2014; Park et al., 2013; Ryu et al., 2011). However, no prior studies evaluated the efficacy of fucoidan against norovirus.

Here, we assessed the effects of brown algal extracts containing fucoidans, laminarins, and phlorotannins against non-enveloped norovirus. We infected cultured murine RAW264.7 macrophages with the human norovirus surrogate murine norovirus (MNV-1) (Hirneisen et al., 2010; Kamarasu, Hsu, & Moore, 2018) and subjected them to various macromolecular fractions obtained from aqueous acetic acid extracts (AAEs) of eight traditional edible brown algal species containing fucoidans, laminarins, and phlorotannins and determined their anti-norovirus efficacy. We also evaluated the antioxidant and immunomodulatory effects of these AAEs.

2. Materials and methods

2.1. Aqueous acetic acid extracts (AAEs) of edible brown macroalgae

Ten products derived from eight traditional edible brown algal (Table 1) species were purchased from retail shops in Hokkaido, Japan. The secondary metabolites were extracted with aqueous acetic acid. This method is traditionally used in industry to obtain fucoidans (Ale & Meyer, 2013). Acidified solutions cannot dissolve the alginic acids that are abundant in brown macroalgae. For each product, 500 g fresh algae or 100 g dried algae or crude fucoidan (Ups2) was added to 1.5 L of 2.5% (w/v) aqueous acetic acid, heated at 75 °C for 3 h, and passed through a No. 2 filter paper (Toyo Roshi Ksisha, Tokyo, Japan). Low-MW (<3 kDa) compounds in the filtrate were eliminated with a cross-flow ultrafiltration module (Asahi Kasei Corp., Tokyo, Japan). The macromolecular fraction was washed with 5 L tap water and lyophilised. Three grams of powder was dissolved in 300 mL distilled water (DW) and passed through a microfiltration module with 0.45 μm pore size (Microza MF Lab Module, ULP-143, Asahi Kasei Corp., Tokyo, Japan). The filter was washed four times with 200 mL DW and the filtrate was lyophilised and used as an AAE.

2.2. Saccharide and sulphate composition and weight-averaged molecular weight (WAMW)

2.2.1. Saccharide and sulphate composition

For the saccharide composition analysis, 2.5 mL of 1% (w/v) AAE and 1 mol/L H_2SO_4 were mixed and the solution was autoclaved at 120 °C for 1 h and labelled with 3-methyl-1-phenyl-5-pyrazolone (PMP; Fujifilm Wako Pure Chemical Industries Ltd., Osaka, Japan) (Sumiyoshi et al., 2003). The PMP-labelled saccharides were detected under the following conditions: column, TSKgel ODS 80TM (Tosoh, Tokyo, Japan); column temperature, 40 °C; eluent, 0.1 mol/L CH_3CN; flow rate, 0.9 mL/min; and UV detection at 245 nm. The sulphate product was measured by the method of Dodgson (1961).

2.2.2. Gel permeation chromatography

To estimate the WAMW of the compounds in the AAEs, the samples were diluted with DW and subjected to gel permeation chromatography (GPC). The GPC conditions were as follows: column, TSKgel $GMPW_{XL}$ (Tosoh, Tokyo, Japan); column temperature, 40 °C; eluent, 0.3 M $NaNO_3$ + 50 mM CH_3COOH; flow rate, 1 mL/min; and refractive index detection. The WAMW reference curve was plotted using a pullulan standard.

2.3. Total phenolic content (TPC) and antioxidant properties

The AAEs were re-dissolved in DW to form a 10 mg/mL solution and the TPC and antioxidant properties were determined as previously described (Takei et al., 2017; Taniguchi et al., 2019), with slight modifications. The AAE solutions were serially diluted with DW to 0.156–10 mg/mL to determine their antioxidant capacity.

Table 1
Edible brown macroalgae samples used in this study.

Name of edible algae Japanese name			Production Area	part	Product form	Purchase year	Abbreviation
Akamoku	*Fucales*	*Sargassum horneri (Turner) C. Agardh*	Akita, Japan	Whole[a]	Boiled and frozen	2017	Sh
Makombu	*Laminariales*	*Saccharina japonica (Areschoug) C. E. Lane, C. Mayes, Druehl & G. W. Saunders*	Donan, Hokkaido, Japan	Frond	Dried under the sun	2018	Sj
Tororokombu	*Laminariales*	*Saccharina gyrata (Kjellman) C. E. Lane, C. Mayes, Druehl & G. W. Saunders*	Rausu, Hokkaido, Japan	Frond	Dried under the sun	2006	Sg
Gagome	*Laminariales*	*Kjellmaniella crassifolia Miyabe*	Donan, Hokkaido, Japan		Dried	2016	Kc
Mekabu	*Laminariales*	*Undaria pinnatifida (Harvey) Suringar*	Miyagi, Japan	Sporophyll	Frozen	2018	Ups1
Mekabu	*Laminariales*	*Undaria pinnatifida (Harvey) Suringar*	China	Sporophyll	Crude fucoidan	2017	Ups2
Arame	*Laminariales*	*Eisenia bicyclis (Kjellman) Setchell*	Oki Is., Shimane, Japan	Frond	Dried	2018	Eb1
Arame (Tororome)	*Laminariales*	*Eisenia bicyclis (Kjellman) Setchell*	Chiba, Japan	Frond of young body	Dried	2018	Eb2
Okinawa-mozuku	*Ectocarpales*	*Cladosiphon okamuranus Tokida*	Okinawa, Japan	Whole	Frozen	2017	Co
Matsumo	*Ralfsiales*	*Analipus japonicus (Harvey) M. J. Wynne*	Donan, Hokkaido, Japan	Wholle	Frozen	2018	Aj

[a] Besides holdfast.

2.3.1. TPC

Each 0.03-mL AAE aliquot was placed in a 96-well microplate ($n = 3$), combined with 0.06 mL of 10% (w/v) Folin-Ciocalteu phenol reagent (Sigma-Aldrich Corp., St. Louis, MO, USA), and incubated for 3 min at room temperature (approximately 20 °C). Then 0.12 mL of 10% (w/v) Na_2NO_3 was added and the mixture was incubated at room temperature for 60 min. The optical density was measured at 750 nm in a grating microplate reader (SH-1000 Lab; Corona Electric, Hitachinaka, Ibaraki, Japan). TPC was expressed as phloroglucinol (PGEq)/g AAE equivalents.

2.3.2. Ferric-reducing power

Serially diluted AAE, catechin positive control, and DW negative control (0.05 mL) were placed in a 96-well microplate ($n = 3$), combined with 0.25 mL of 0.1 mol/L phosphate buffer (pH 7.2) and 0.025 mL of 1% (w/v) potassium ferricyanide (Sigma-Aldrich Corp., St. Louis, MO, USA), and incubated at 37 °C for 60 min. Then 0.025 mL of 10% (w/v) trichloroacetic acid (TCA) and 0.1 mL DW were added and the absorbance was measured at 700 nm (Abs1). Then 0.025 mL of 0.1% (w/v) $FeCl_3$ was added and the absorbance was measured again at 700 nm (Abs2). Increase in absorbance of each serial AAE dilution or catechin was determined as follows:

$$\text{Increase in absorbance (OD700nm)} = (\text{Abs2} - \text{Abs1}) \text{ of sample} - (\text{Abs2} - \text{Abs1}) \text{ of DW} \quad (1)$$

Based on the sample and catechin concentrations required to increases the absorbance by 0.5 unit, the Fe-reducing power of sample was calculated and recorded as μmol catechin (CatEq)/g AAE equivalents.

2.3.3. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity

Serially diluted AAE, catechin, or DW (0.1 mL) was placed in a 96-well microplate ($n = 3$) and combined with 0.1 mL of 99.5% (v/v) ethanol. Absorbance was read at 517 nm (Abs1). Then 0.025 mL of 1 mM L DPPH (Sigma-Aldrich Corp., St. Louis, MO, USA) ethanolic solution was added and the mixture was incubated at 37 °C in the dark for 30 min. Then, absorbance was measured again at 517 nm (Abs2). The percentage DPPH scavenging was estimated as follows:

$$\% \text{ scavenging} = \left(1 - \frac{(\text{Abs2} - \text{Abs1}) \text{ of sample}}{(\text{Abs2} - \text{Abs1}) \text{ of DW}}\right) \times 100 \quad (2)$$

Based on the IC_{50} defined as the concentrations of sample and catechin required to reduce the initial DPPH by 50%, sample activity of sample was calculated and recorded as μmol catechin (CatEq)/g AAE equivalents.

2.3.4. Superoxide anion (O_2^-) radical-scavenging activity

Serially diluted AAE, catechin, or DW (0.1 mL) was placed in a 96-well microplate ($n = 3$) and combined with 0.05 mL of 0.25 M phosphate buffer (pH 7.2), 0.025 mL of 2 mM reduced β-nicotinamide adenine dinucleotide (Sigma-Aldrich Corp., St. Louis, MO, USA), and 0.025 mL of 0.5 mM nitro blue tetrazolium (Sigma-Aldrich Corp., St. Louis, MO, USA). Absorbance was measured at 560 nm (Abs1). Then 0.025 mL of 0.03 mM phenazine methosulphate (Sigma-Aldrich Corp., St. Louis, MO, USA) was added and the mixture was incubated in the dark at room temperature for 5 min. Absorbance was measured again at 560 nm (Abs2). The radical-scavenging capacity was calculated and recorded in the same manner as the DPPH radical-scavenging capacity.

2.4. Anti-norovirus effect

2.4.1. Virus and viral culture

The anti-norovirus efficacies of the AAE samples were determined using a previously reported method (Takahashi et al., 2018), with slight modifications. Murine norovirus 1 (MNV-1, Wobus et al., 2006) served as a surrogate for human norovirus (Hirneisen et al., 2010; Kamarasu et al., 2018). Murine macrophage-like RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Fujifilm Wako Pure Chemical Industries Ltd., Osaka, Japan) containing 5% (v/v) foetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were infected with MNV-1 at 0.1 multiplicity of infection (MoI) and incubated for at 37 °C in a 5% CO_2 atmosphere for 3 d. After cytopathy, the cells were frozen/thawed four times and centrifuged at 8, 000 × g for 20 min at 4 °C for viral particle purification. Infectivity was determined by plaque assay. The extracted viruses and cell cultures were stored at −80 °C until subsequent use as MNV-1 suspensions.

2.4.2. Direct effect on MNV-1

Aliquots (0.12 mL) of 5 mg/L or 10 mg/L AAE solution were mixed with MNV-1 suspensions of 6 log plaque-forming units (PFUs)/mL. After 60 min incubation at room temperature, the mixtures were combined with 1.08 mL of DMEM and decimally diluted with it.

The plaque formation assay was performed according to the method of Wobus et al. (2006). Approximately 3 mL RAW264.7 cells at 10^5/mL density was placed in each well of a six-well plate (BD Bioscience, Franklin Lakes, NJ, USA) and incubated at 37 °C under 5% CO_2 for 18 h. The medium was removed and serially diluted samples and MNV-1 suspension (0.5 mL) were added ($n = 3$). After 60-min incubation with gentle shaking at room temperature, the MNV suspensions were removed and combined with 2 mL of 1.5% (w/v) See Plaque Agarose (Lonza, Tokyo, Japan) plus DMEM and incubated at 37 °C in a 5% CO_2 atmosphere for 48 h.

To detect the plaques, 0.33% (w/v) neutral red solution (Sigma-Aldrich Corp., St. Louis, MO, USA) was diluted with nine volumes of phosphate-buffered saline (PBS; Nissui Pharmaceutical, Tokyo Japan). Then 2 mL diluted neutral red solution was added to each well and the cultures were incubated at 37 °C under 5% CO_2 for 60 min. The staining solution was then removed and the plaques were counted.

2.4.3. Effects on host RAW264.7 cells

Aliquots of 3 mL RAW264.7 cells at 10^5/mL density were placed in each well of a six-well plate (BD Bioscience, Franklin Lakes, NJ, USA). AAE solutions were added to a final concentration of 0.3 mg/mL ($n = 3$) and the mixtures were incubated at 37 °C under 5% CO_2 for 20 h. Then, the MNV-1 infection assay was conducted as described above.

2.5. Nitric oxide (NO) generation and cytotoxicity in RAW264.7 culture media

To identify immunomodulation in the AAE samples, the NO concentrations in the RAW264.7 cell culture media were measured as previously described (Taniguchi et al., 2019). Briefly, 0.1 mL of 6 log RAW264.7 cells/mL was placed into each well of a 96-well microplate and incubated at 37 °C in a 5% CO_2 atmosphere for 48 h. The culture media were replaced with 0.2 mL fresh DMEM containing 0.3 mg/mL AAEs sample ($n = 3$) and incubated at 37 °C under 5% CO_2 for 18 h. The nitric oxide in 0.1 mL culture medium was mixed with 0.1 mL of 10% (w/v) Griess-Romijn nitrite reagent (Fujifilm Wako Pure Chemical Industries Ltd., Osaka, Japan) in the dark for 20 min and the optical density was measured at 540 nm. The NO concentrations were interpolated from a standard aqueous $NaNO_2$ curve. Lipopolysaccharide (LPS) from *Escherichia coli* O111 (final concentration 0.19 μg/mL; Sigma-Aldrich Corp., St. Louis, MO, USA) served as the positive control. The cytotoxicity of AAE concentrations resembling those used in the previous assays was determined with Cell Counting Kit-8 (Dojindo Laboratories, Mashiki, Japan) in accordance with the manufacturer's instructions.

2.6. Statistical analysis

TPC, antioxidant capacity, and antiviral activity are presented as means ± standard error (SE; $n = 3$ measurements per analysis). Data were subjected to ANOVA and Tukey's or Dunnett's post-hoc tests in

Excel Statistic v. 6 (Microsoft Corp., Redmond, WA, USA). $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Saccharide and sulphate composition and WAMW of AAE

The saccharide and sulphate compositions of the AAEs are shown in Table 2. Of the ten AAEs analysed, five (akamoku *Sargassum horneri* [Sh], makombu *Saccharina japonica* [Sj], gagome *Kjellmaniella crassifolia* [Kc], okinawamozuku *Cladosiphon okamuranus* [Co], and matsumo *Analipus japonicus* [Aj]) had relatively high fucose content (24–37% w/w) and sulphate content (11–32%) and low galactose content (<4%). These AAEs might have contained fucose-rich fucoidan (Silchenko et al., 2017; Song et al., 2018). The approximate fucose:galactose ratio in the mekabu sporophyll of *Undaria pinnatifida* (Ups1 and Ups2) and the arame *Eisenia bicyclis* (Eb2) was 3:2 and the sulphate content was in the range of 20–22%. Co contained 7% glucuronic acid. A high glucose concentration range (25–56%) was detected in tororokombu *Saccharina gyrata* (Sg) and both *E. bicyclis* (Eb1 and Eb2). However, none of these algae had substantial fucose. The primary polysaccharide in the Sg, Eb1, and Eb2 extracts might have been laminarin (Maeda & Nishizawa, 1968). Eb1 had low fucose and sulphate content.

The GPC of the AAEs are shown in Fig. 1. WAMW is also shown in Table 2. The WAMW of all samples was in the range of 18–191 kDa. In the three samples thought to be rich in laminarin (Sg, Eb1, and Eb2), WAMW was generally low (18–41 kDa). However, a fucose-rich Kc sample had low WAMW (23 kDa). Although Kc had high viscosity, it also had both low-MW (4 kDa) and high-MW (200–300 kDa) fucoidans (Katayama, Nishio, Kishimura, & Saeki, 2012). The arrows in Fig. 1 indicate that Sh and Aj exhibited two primary GPC peaks. Of these, the low-WAMW peaks could correspond to laminarin (Kuda & Ikemori, 2009). The Ups1 AAE showed a broad GPC peak that might correspond to various compounds with different MW.

3.2. TPC and antioxidant properties

TPC (Fig. 2A) was in the range of 9–128 μmol PGEq/g. The highest TPC were determined for Sh followed by Eb2 (104 μmol PGEq/g AAE), Eb1 (85 μmol PGEq/g AAE), Sj (67 μmol PGEq/g AAE), and Aj (31 μmol PGEq/g AAE). The Fe-reducing power (Fig. 2B) and the DPPH radical-scavenging capacity (Fig. 2C) of the five AAEs were ~25 μmol PGEq/g AAE and 34 μmol PGEq/g AAE, respectively. These values correlated with TPC (Fig. 2B' and 2C'; $r = 0.995$ for Fe-reducing capacity *vs.* TPC; $r = 0.928$ for DPPH radical-scavenging activity *vs.* TPC).

Co had the highest O_2^- radical-scavenging capacity (55 μmol CatEq/g) (Fig. 2D) followed by Kc, Ups2, Sh, Sj, Eb2, Ups1, Ha, and Eb1 (35–22 μmol CatEq/g). Hence, Co, Kc, Ups1, Ups2, and Kc were not rich in polyphenols (35–22 μmol CatEq/g; Fig. 2A) or glucans (Table 2). Therefore, fucoidans might have explained the observed high O_2^- radical-scavenging capacity. Conversely, polyphenols may have accounted for the observed O_2^- radical-scavenging capacities of Sh, Eb1, and Eb2. The O_2^- radical-scavenging capacity was low in Sg possibly because the AAE was rich in laminarin but not polyphenols.

The observed correlation between TPC and antioxidant capacity especially in terms of DPPH radical-scavenging activity and Fe-reducing power resemble those reported for hot water extracts of dried brown algae such as *S. horneri, E. bicyclis*, and *U. pinnatifida* (Kuda & Ikemori, 2009; Takei et al., 2017). Several brown algae are rich in phlorotannins and 95% of their TPCs have high MWs (>3 kDa) (Wei, Li, Hu, & Xu, 2003). Though crude fucoidans have O_2^- radical-scavenging activity, their Fe-reducing powers and DPPH radical-scavenging capacities are relatively low (Kuda, Tsunekawa, Hishi, & Araki, 2005).

3.3. Direct anti-norovirus effect on MNV-1

The numbers of PFUs in RAW264.7 cells inoculated with MNV-1 and directly treated with 5 mg/mL AAEs are shown in Fig. 3A. Relative to the no-treatment group (NT, 100%), the Co, Ups2, Eb1, Sh, and Kc treatments reduced MNV-1 virulence to 20%, 25%, 32%, 36%, and 57%, respectively. No plaques were detected in the cells treated with 10 mg/mL Eb1 AAEs s or 10 mg/mL Sj AAEs (<0.5%; Fig. 3B). Compared to NT, MNV-1 virulence in RAW264.7 cells treated with 10 mg/mL Sh, Eb2, Co, Ups2, and Kc was reduced to 3.1%, 4.1%, 4.6%, 11.2%, and 17.3%, respectively. However, the mechanism underlying this inactivation effect was not elucidated here. The saccharide composition and TPC were similar for both Ups1 and Ups2. However, the WAMW for Ups1 was 3 times higher than that of Ups2 (Table 2; Fig. 1). To clarify the mechanisms involved, the active compounds of Ups1 and Ups2 must be isolated and their interactions should be identified.

Of the seven AAE samples obtained from brown algae displaying strong anti-MNV activity, Sh, Eb1, Eb2, and Sj had high TPC. This property may be correlated with their Fe-reducing powers and DPPH radical-scavenging capacities (Fig. 2A–C). In contrast, the Fe-reducing powers and DPPH radical-scavenging capacities were low for Kc, Co, and Ups2. Several studies focused on the immunomodulatory activity of these algal extracts but few have investigated their direct viral inactivation. For example, *E. cava* phlorotannins inactivated haemorrhagic septicaemia virus and induced reverse transcription in HIV-1 (Yang et al., 2008; Karadeniz et al., 2014). The low fucoidan content and high TPC could explain the ability of Eb1 and Eb2 to inactivate MNV-1 (Table 2) (Fig. 2A). The fucoidan in Co bound dengue virus (Hidari et al., 2008). As MNV-1 has no envelope, however, further studies are required to determine the mechanism by which Co directly inactivates it. Fucoidan and TPC could explain the ability of Sh and Sj to inactivate MNV-1.

Table 2
Composition of saccharides and sulphate and weight-average molecular weight (WAMW) of acetic acid-aqueous macromolecule extracts (AAEs) of edible brown macroalgae.

Name of edible algae	Abbreviation	Saccharides and sulphate* (g/100g AAE)								WAMW
Scientific name		Fuc	Glu	Gal	Man	Rha	Xyl	Glc	Sulp	(kDa)
Sargassum horneri	Sh	35	7	1	0	0	0	0	19	41.8[b]
Saccharina japonica	Sj	24	0	2	1	0	0	2	11	61.8
Saccharina gyrata	Sg	10	56	0	0	0	0	0	8	18.2
Kjellmaniella crassifolia	Kc	32	0	0	0	0	0	0	32	23.0
Undaria pinnatifida[a]	Ups1	23	0	16	1	0	0	1	20	191.0
Undaria pinnatifida[a]	Ups2	22	0	16	0	0	0	1	20	57.0
Eisenia bicyclis	Eb1	3	25	0	1	0	0	1	3	41.0
Eisenia bicyclis	Eb2	14	46	8	0	0	0	0	22	34.8
Cladosiphon okamuranus	Co	37	0	0	0	0	2	7	12	60.4
Analipus japonicus	Aj	35	13	4	1	1	5	3	13	116.5**

[a] Fuc, fucose; Glu, glucose; Gal, galactose; Man, mannose; Rha, rhamnose; Glc, glucuronic acid; Sulp, sulphate.
[b] Samples had two main peaks (Fig. 1). Data are means of triplicate measurements.

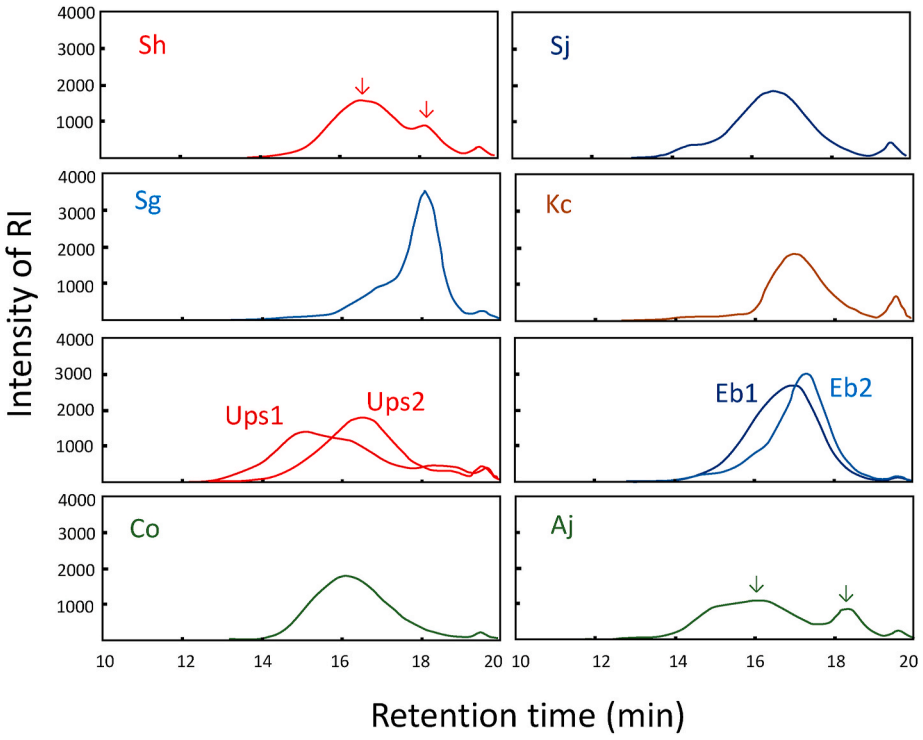

Fig. 1. Gel permeation chromatogram (GPC) of the macromolecular fractions obtained from aqueous acetic acid macromolecular extracts (AAEs) of edible brown algae. Sh, *Sargassum horneri*; Sj, *Saccharina japonica*; Sg, *S. gyrata*; Kc, *Kjellmaniella crassifolia*; Ups1 and 2, *Undaria pinnatifida* (sporophyll); Eb1 and 2, *Eisenia bicyclis*; Co, *Cladosiphon okamuranus*; Aj, *Analipus japonicus*. Sh and Aj exhibited two primary GPC peaks (arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

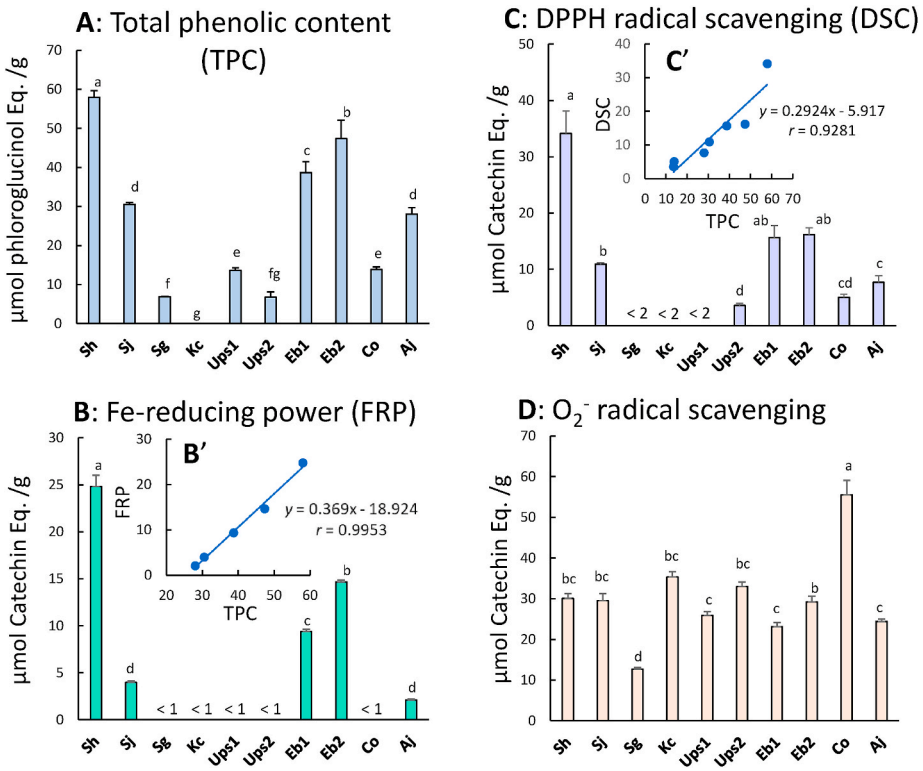

Fig. 2. Total phenolic content (A), Fe-reducing power (B), DPPH radical-scavenging capacity (C), and superoxide anion radical-scavenging capacity (D) of aqueous acetic acid macromolecular extracts. Data are means ± SD of triplicate measurements and were subjected to ANOVA and Tukey's post-hoc test. [a-b] Different superscript letters indicate significant differences between treatment means ($p < 0.05$). B′ and C′ show coefficients of correlation between total phenolic content (TPC) and Fe-reducing (FRP) or DPPH radical-scavenging (DSC), respectively, capacities.

3.4. Effect on host RAW264.7 cells

Before MNV-1 infection, host murine macrophage RAW264.7 cells were treated with 0.3 mg/mL AAE in DMEM for 20 h. No RAW264.7 cytotoxicity was observed at this AAE concentration (data not shown). After the treatment, the culture medium was replaced with fresh DMEM and the cells were inoculated with MNV-1. MNV-1 infection was dramatically reduced in response to Ups1 (14%), Co (21%), and Aj (25%) (Fig. 4A). In contrast, Ups2 had no apparent antiviral activity. This observation contradicted the direct inactivating effect of Ups2 (Fig. 3).

Host cell pre-treatment with fucoidan confers anti-infection activity because this substance has immunomodulatory capacity (Sun et al., 2020). We measured NO production in RAW264.7 cells as an indicator

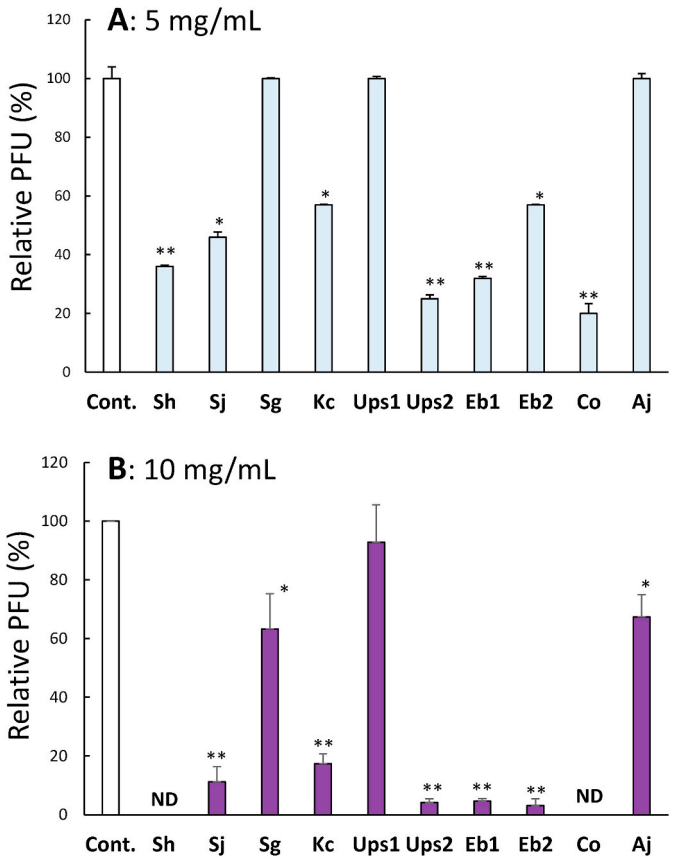

Fig. 3. Direct inactivation efficacy of 5 mg/mL (A) and 10 mg/mL (B) aqueous acetic acid macromolecular extracts (AAEs) against murine norovirus-1 (MNV-1). RAW264.7 murine macrophages were subjected to AA for 60 min and infected with MNV-1. Relative PFU (%) = (PFU with AAE treatment)/(PFU without AAE treatment (NT)). ND: not detected. Data are means ± SD of triplicate measurements and were subjected to ANOVA and Dunnett's post-hoc test. *, ** significantly different from the control (*$p < 0.05$, **$p < 0.01$).

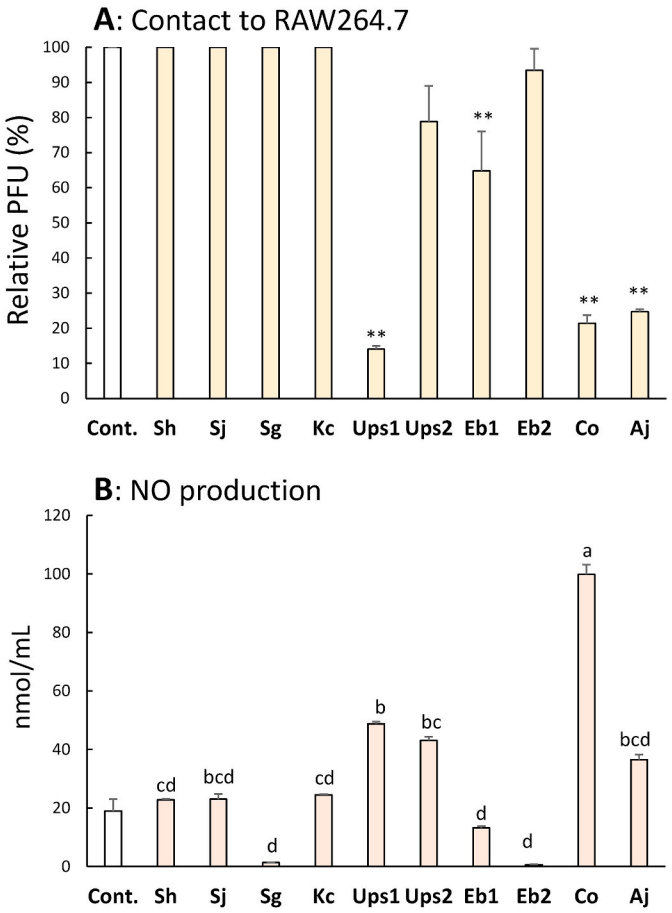

Fig. 4. Effects of 20 h pre-treatment with 0.3 mg/mL aqueous acetic acid macromolecular extracts (AAE) on RAW264.7 cells infected with MNV-1 (A) and on NO production in RAW264.7 cells (B). Relative PFU (%) = (PFU with AAE treatment)/(PFU without AAE treatment (NT)). LPS: lipopolysaccharide from *Escherichia coli* O111 (0.19 μg/mL). Data are means ± SD of triplicate measurements and were subjected to ANOVA and Dunnett's post-hoc test. **Significantly different from the control (**$p < 0.01$). [a-d] Different superscript letters indicate significant differences between treatment means ($p < 0.05$).

of immunostimulation (Yoon et al., 2011) (Fig. 4B). No correlation was observed between PFU and NO production. Nevertheless, Ups1, Aj, and especially Co presented with high NO content in the culture medium (36–100 nmol/mL). Ups1, Aj, and Co might be rich in fucoidans rather than laminarins (Table 2). Neither Co nor Ups1 had high TPC. Thus, their protective effect against MNV-1 could be explained by their fucoidan content. The O_2^- radical-scavenging capacity was high for Co (Fig. 2D) and might be correlated with NO immunomodulation (Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015).

Three edible algae tested in the present study demonstrated antiviral efficacy (Figs. 3 and 4A). Ups fucoidans have activity against HSV and influenza A virus (Hayashi, Lee, Nakano, & Hayashi, 2013; Hayashi, Nakano, Hashimoto, Kanekiyo, & Haytashi, 2008). Previous studies reported the efficacy of Co fucoidans against NDV, DEN2, and HCV (Hidari et al., 2008; Mori, Nakasone, Tomimori, & Ishikawa, 2012; Trejo-Avila et al., 2016). However, few studies have reported any antiviral efficacy for Aj.

The present study indicated that Co AAE is a promising functional material as it directly inactivates MNV-1 and affects host macrophages. The AAE of phlorotannin-rich Sh, Eb1, and Eb2 are also potentially effective at inactivating MNV-1. Further experimentation is required to elucidate the antiviral efficacy of purified fucoidan and phlorotannin both *in vitro* and *in vivo*. Another research objective is to optimise the conditions for acetic acid extraction and extract microfiltration as these processes will have practical applications in the food industry.

4. Conclusion

The present study revealed that certain AAEs from edible brown macroalgae inactivate non-enveloped norovirus. Fucoidan and TPC could explain the ability of the AAEs to directly inactivate MNV-1. The protective effect against MNV-1 could be explained by their fucoidan content. Co is an especially promising as an anti-norovirus agent. Ups1 and Aj displayed immunomodulatory efficacy in macrophages and are desirable functional food items. The AAEs of TPC-rich Sh, Eb1, and Eb2 could be valuable because they directly inactivate MNV-1. Further experimentation is required to elucidate the antiviral efficacy of purified fucoidan and phlorotannin.

CRediT authorship contribution statement

Takashi Kuda: Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing - review & editing, Visualization, Supervision, Project administration. **Makoto Nishizawa:** Conceptualization, Methodology, Resources, Formal analysis, Supervision. **Daiki Toshima:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Keiichiro Matsushima:** Formal analysis. **Seiichiro Yoshida:** Formal analysis. **Hajime Takahashi:** Conceptualization, Methodology, Supervision. **Bon Kimura:** Conceptualization,

Methodology, Supervision. **Takashi Yamagishi:** Conceptualization, Methodology, Resources, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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