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Antimicrobial, Antioxidant and Hemolytic Activity of Water-soluble Extract of Mottled Anemone *Urticina crassicornis*

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Abstract

We evaluated the biological activities of five water extracts of tissue of the mottled anemone *Urticina crassicornis*. Most extracts exhibited broad-spectrum antimicrobial activity as determined by ultrasensitive radial diffusion assay (URDA) against gram-positive and -negative bacteria, including a fish pathogen, *Aeromonas hydrophila*, but no activity against fungi. The activity of the extracts was abolished by tryptic digestion, indicating that protein compounds were responsible for the antimicrobial activity. Furthermore, in a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity assay, only the visceral tissue extract showed activity. However, no extract had hemolytic activity against human red blood cells. Consequently, this study suggests the water-soluble extract of mottled anemone to be a promising source of proteinaceous antimicrobial compounds that can be utilized for development of novel antibiotics.

Key words: *Urticina crassicornis*, Water-soluble extract, Antimicrobial activity, Antioxidant activity, Hemolysis

Introduction

The marine environment is highly exigent, competitive, and aggressive, which leads to the production of specific and potent bioactive compounds by marine organisms. Marine organisms are regarded as the largest unexplored source of bioactive compounds, and their biotechnological potential has resulted in extensive evaluation of their biological activities (Bhakuni and Rawat, 2006; Rocha et al., 2015; Suleria et al., 2015). However, the number of compounds isolated from marine organisms is heavily weighted in favor of lipid (or organic solvent)-soluble agents or compounds with medium polarity (Bhakuni and Rawat, 2006; Mehbub et al., 2014; Rocha et al., 2015). This might be due to the difficulties associated with the isolation and purification of water-soluble compounds. Despite these difficulties, the water-soluble fractions cannot be disregarded if the objective is to isolate compounds with biological activity. Furthermore, recent progress in separation techniques has enabled extraction of water-soluble components from marine organisms, which are frequently responsible for biological activities (Díaz López and Montalvo, 2015; Fellenberg et al., 2010; Gross et al., 2010).

The phylum cnidarian (such as sea anemones, jellyfish, and corals) has been the subject of efforts to discover novel bioactive compounds, which involves considerable bioprospecting and screening (Leal et al., 2012; Rocha et al., 2011). The phylum cnidarian is a large, diverse group of > 11,000 species, 7,500 of which, including sea anemones, belong to the class anthozoa (Rocha et al., 2011).

Many studies of bioactive compounds from sea anemones have been reported in the last two decades. However, the majority focused on antitumor activity and the factors responsible, such as toxins and terpenoid compounds; moreover, few sea anemone species were involved (Rocha et al., 2011; Rocha et al., 2015).

The mottled anemone *Urticina crassicornis* is a large,

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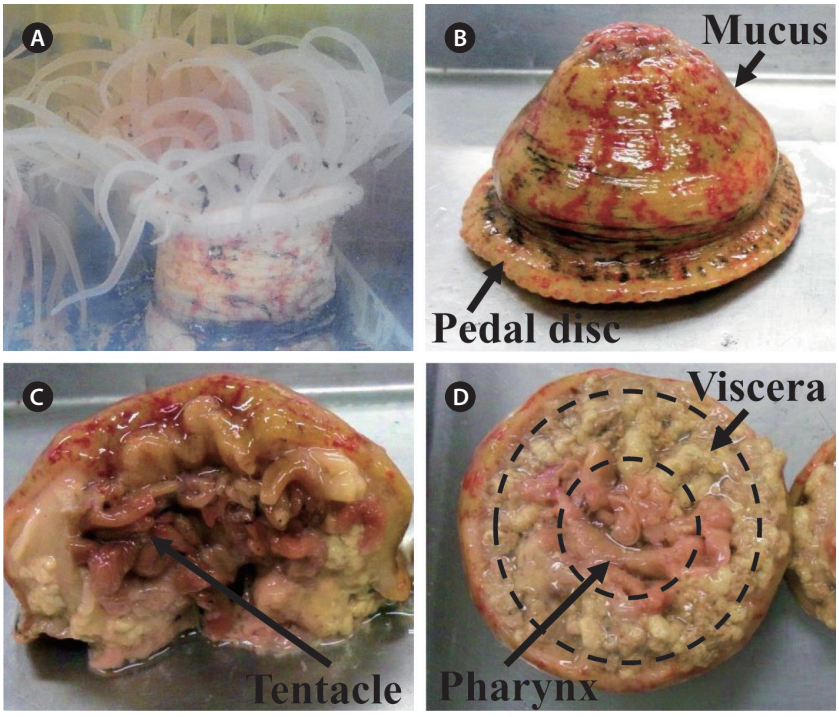

Fig. 1. The mottled anemone, *Urticina crassicornis*. *U. crassicornis* temporary attached re-circulating aquarium (A) and tissues, pedal disc, mucus, tentacle, viscera and pharynx (B-D), used in this study are illustrated.

common intertidal and subtidal sea anemone species that lives in coastal areas worldwide, such as the coast of Pacific Rim countries, including Korea (Song and Cha, 2002). This animal attaches to the sea bottom and has locomotion ability; thus, it is vulnerable to predation, the surrounding pathogenic microbiota, and exposure to ultraviolet radiation (UV). It is unsurprising that the mottled anemone, like other marine invertebrates, has evolved an effective defense system to protect against harmful environmental factors (Aneiros and Garateix, 2004; Derby, 2007; Hagiwara et al., 2015; Mayer et al., 2013; Pietrzyk et al., 2015). Materials extracted from two sea anemones (*Stichodactyla mertensii* and *Stichodactyla gigante*an) in India using an organic solvent were investigated in terms of their antibacterial and antifungal activities; all extracts exhibited broad-spectrum antimicrobial activity (Thangaraj et al., 2011). To date, sea anemones have received little attention regarding antimicrobial and antioxidant factors.

In the present study, we investigated the antimicrobial and antioxidant activities of water-soluble materials from tissues of the mottled anemone, *U. crassicornis*, extracted using acidified water.

Materials and Methods

Animals

Mottled anemone *Urticina crassicornis* (diameter, 7–9 cm; height, 5–6 cm; wet weight, 400–500 g) was commercially obtained from Gijang in Busan, Korea. Live sea anemones were transported to the laboratory in seawater in an air-tight package and maintained in a recirculating seawater system at 15°C until sample extraction (Fig. 1A). The sea anemones were divided and dissected into mucus, viscera, pharynx, tentacle, and pedal discs (Fig. 1B–1D). All tissues were collected, immediately frozen in liquid nitrogen, and stored at -75°C until use.

Tissue extraction

Organs were added to four volumes of pre-heated water containing 1% acetic acid (v/v) and boiled for 5 min to inhibit proteolytic degradation. The boiled tissues were then homogenized on ice (Polytron PT1200C homogenizer, Kinematica AG, Switzerland), and homogenates were centrifuged at 13,000 *g* for 25 min at 4°C. Supernatants were lyophilized and dissolved in 0.01% acetic acid at a concentration of 6 mg/mL. Extracted samples were stored at -75°C until use.

Ultrasensitive radial diffusion assay (URDA)

The antimicrobial activity of all extracts was evaluated by URDA as described previously (Seo et al., 2014). Briefly, fish pathogens and other microbial strains were precultured overnight in tryptic soy broth (TSB) at 25°C and 37°C, respectively. Precultured microbial strains were diluted with 10 mM phosphate buffer (PB, pH 6.6) to a McFarland turbidity standard of 0.5 (Vitek Colorimeter #52-1210; Hach, Loveland, CO, USA) corresponding to ~10^8 CFU/mL, and 0.5 mL of the diluted strains was mixed with 9.5 mL of underlay gel containing 0.03% TSB and 1% Type I (low EEO) agarose in 10 mM PB (pH 6.6).

The extracts were diluted twofold, and 15 μg of each extract in 5 μL of 0.01% acetic acid were added to 2.5-mm-diameter wells made in the 1-mm-thick underlay gel. After complete diffusion of each extract for 3 h at room temperature, underlay gels containing microbial strains were overlaid with 10 mL of 6% TSB and 1% agarose in 10 mM PB (pH 6.6). The plates were incubated for 16 h at the appropriate temperature for growth of microbial strains, and then the diameters of the clear zones were measured. The antimicrobial assay was performed in triplicate and the results were averaged.

The bacterial and fungal strains used in this study were as follows: gram-positive bacteria—*Bacillus subtilis* KCTC1021, *Micrococcus luteus* KCTC1071, and *Staphylococcus aureus* RN4220; and gram-negative bacteria—*Escherichia coli* D31, *Pseudomonas aeruginosa* KCTC2004, *Salmonella enterica* ATCC13311 and *Shigella sonnei* KCTC2009, and the fish pathogens *Aeromonas hydrophila* KCTC2358 and *Edwardsiella tarda* KCTC12267; and the fungus, *Candida albicans* KCTC7965. *E. coli* D31 and *S. aureus* RN4220 were provided by Professor E. J. Noga (NCSU, Raleigh, NC, USA) and H. D. Jeong (PKNU, Busan, KR), respectively. The other microbial strains were purchased from the Korean Collection for Type Cultures (KCTC) or American Type Culture Collection (ATCC).

Antioxidant assay

The antioxidant activity of the extracts was measured in terms of hydrogen-donating or radical-scavenging ability, using the DPPH method with slight modification (Alma et al., 2003). Briefly, 100 μL of DPPH solution (0.2 mM in methanol) was incubated with 100 μL of each extract and serially diluted twofold with 0.01% acetic acid at a concentration of 0.09–3.0 mg/mL. The reaction mixture was shaken well and incubated in the dark for 30 min at room temperature. The reference standard or control was prepared as above with 1 mM ascorbic acid or without extract, respectively. The absorbance of the solution at 517 nm was measured using a microplate reader (Sunrise™, TECAN, Swiss) against a blank. The radical scavenging activity was determined as a decrease in the absorbance of DPPH and was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [1 - (A_S - A_1) / A_0] \times 100;$$

Where A_S is the absorbance of the sample with DPPH-methanol solution, A_0 is the absorbance control of the DPPH-methanol solution, and A_1 is the absorbance of the sample with methanol. Scavenging activity values were the means ± standard deviation of three parallel measurements.

Effect of proteolytic digestion on antimicrobial activity

Reaction mixtures containing 10 μg of each extract and 1 μg of crystalline trypsin in 5 μL of water were incubated for 60 min at 37°C. The antimicrobial activities against gram-positive and -negative bacteria, *B. subtilis* KCTC1021 and *E. coli* D31, which were highly susceptible to all extracts, were tested by URDA. The effect of proteolytic digestion on the antimicrobial activity of each extract was compared with that of a mock experiment using only 10 μg of each extract in 5 μL water without trypsin under identical conditions.

Hemolytic assay

Hemolytic activity of extracts was determined using human red blood cells (HRBCs) (Park et al., 2011). HRBCs were collected from citric acid-treated blood by centrifugation at 8,000 *g* for 1 min, and washed three times with 5 mM Tris-HCl (pH 7.4) containing 150 mM NaCl to remove the plasma and buffy coat. Washed HRBCs were resuspended to 3% with washing buffer, and 45 μL of the suspension with 5 μL extract (15 μg) was incubated for 60 min at 37°C. Hemolytic activity values were expressed as the hemoglobin content calculated from the absorbance at 405 nm of the supernatant after centrifugation at 13,000 *g* for 10 min. Baseline and 100% hemolysis values were obtained by incubating HRBCs with buffer and 0.1% Triton X-100, respectively.

The percentage of hemolysis was calculated using the following formula:

$$\text{\% Hemolysis} = [(\text{Abs}_{405} \text{ in the extract solution} - \text{Abs}_{405} \text{ in buffer}) / (\text{Abs}_{405} \text{ in 0.1\% Triton X-100} - \text{Abs}_{405} \text{ in buffer})] \times 100$$

Hemolysis assays were performed in triplicate and the results were averaged.

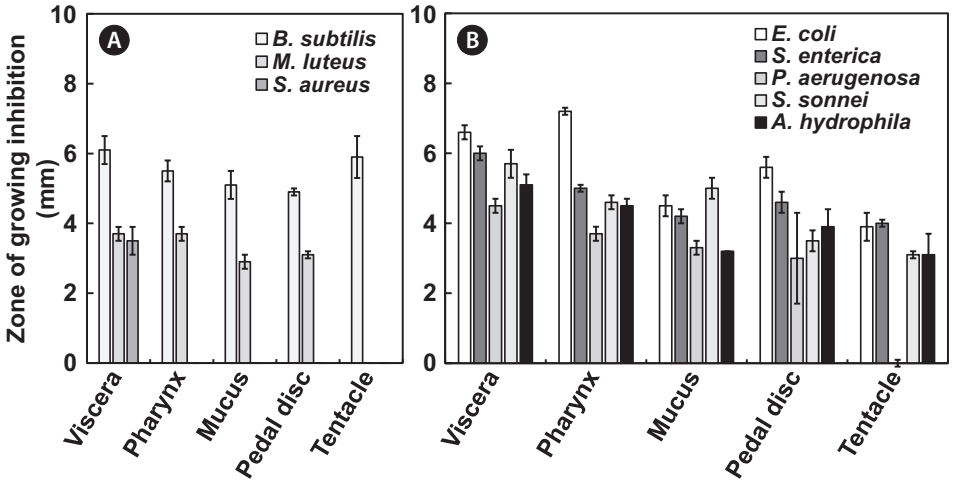

Fig. 2. Antimicrobial activity of water-soluble extracts from various tissues of *Urticina crassicornis* against gram-positive bacteria (A) and gram-negative bacteria (B).

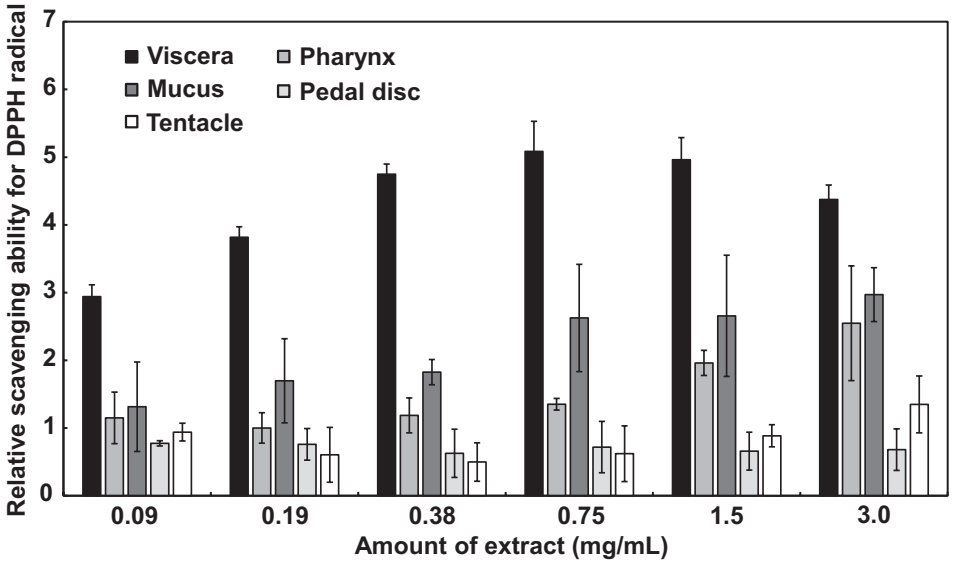

Fig. 3. DPPH radical scavenging activity of extracts of *Urticina crassicornis* obtained using acidify water. Each value represents the relative activities compared with the scavenging activity by 1 mM ascorbic acid and a Mean ± SD (n = 3).

Results

Antimicrobial activity of *U. crassicornis* tissue extracts

The antimicrobial activity of extracts from *U. crassicornis* is shown in Fig. 2. Among the tissue extracts, the visceral extract exhibited the broadest-spectrum antimicrobial activity against gram-positive and -negative bacteria, including one fish pathogen, *A. hydrophila*, but no activity against *C. albicans* or *E. tarda*. Extracts of the pharynx, mucus and pedal disc also had broad antimicrobial activity, but no activity against the gram-positive *S. aureus*. The tentacle extract showed a narrower spectrum of antimicrobial activity compared with other tissue extracts; however, activity against one gram-positive bacterium, *B. subtilis*, and four gram-negative bacteria—*E. coli*, *S. enterica*, *S. sonnei*, and *A. hydrophila*—was evident. Interestingly, the specificity of the antimicrobial activity varied among the tissue extracts. The visceral, pharynx, and pedal disc extracts exhibited highly specific activity against *E. coli*; the tentacle extract exhibited highly specific activity against *B. subtilis*; and the mucus extract exhibited highly specific activity against both *B. subtilis*, and *S. sonnei*. Collectively, these data suggest that tissue extracts from *U. crassicornis* might be a promising source of potent antimicrobial compounds.

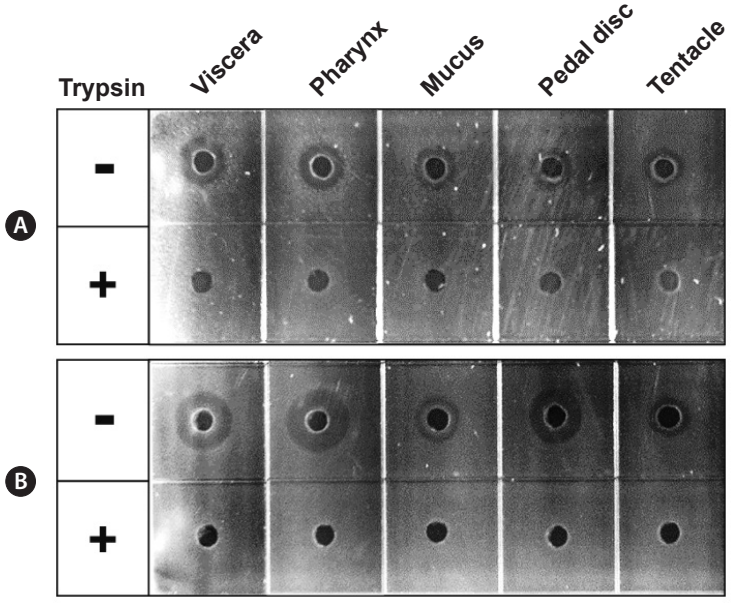

Fig. 4. Effect of tryptic digestion of extracts to antimicrobial activity against *Bacillus subtilis* (A) and *Escherichia coli* (B)

Antioxidant activity of the extracts

The antioxidant activity of each extract was evaluated with the scavenging ability of DPPH radical. Unfortunately, the remarkable ability to scavenge DPPH radical was not observed by most extracts, though only the visceral extract at 0.38–1.5 mg displayed a meaningful DPPH radical-scavenging activity comparable to that of 1 mM ascorbic acid (Fig. 3). These data indicate that the procedures used did not facilitate extraction of antioxidant compounds from mottled anemone.

Effect of tryptic digestion on antimicrobial activity

We next identified the components of the extracts by tryptic digestion. All extracts lost the antimicrobial activity against both gram-positive and -negative bacteria after digestion (Fig. 4), indicating that proteinaceous factors are responsible for the broad antimicrobial activity of mottled anemone extracts; therefore, further studies are warranted.

Measurement of hemolytic activity

Hemolysis of RBCs is regarded as a problem during the development of antimicrobial drugs (Juvvadi et al., 1996; Ruiz et al., 2014). Therefore, the hemolytic activity of the extracts was measured using HRBCs. The extracts with broad-spectrum antimicrobial activity exhibited little or no hemolytic activity against HRBCs (Table 1). These data suggest mottled anemone extracts have potential for various applications.

Discussion

The rapid emergence of drug-resistant bacteria, and the increasing concern regarding the toxicity and adverse effects of synthetic antioxidants, have resulted in efforts to develop new classes of antimicrobial agents and antioxidants derived from natural sources (Dantas et al., 2008; Grundmann et al., 2006; Kahl and Kappus, 1993). Our findings suggest that extracts from various *U. crassicornis* tissues exhibited broad-spectrum antimicrobial activity, the specificity of which differed (and was abolished by tryptic digestion) (Figs. 2 and 4). Interestingly, the extracts showed antimicrobial activity against *A. hydrophila*, a gram-negative bacterium that can cause severe, and even fatal, symptoms in fish and humans (Alhazmi 2015; Jacobs and Chenia, 2007). Moreover, none of the tissue extracts affected HRBCs (Table 1).

Antimicrobial peptides or proteins (AMPs) are components of both the vertebrate and invertebrate immune systems, but

Table 1. Hemolytic activity of extracts of *Urticina crassicornis* to human red blood cells (HRBCs)

Extracts	Hemolysis %
Viscera	3.23 ± 1.63
Pharynx	2.15 ± 0.86
Mucus	2.38 ± 0.54
Pedal disc	2.01 ± 0.53
Tentacle	1.91 ± 0.66

are especially important in invertebrates, in which they play an essential role in innate immunity. Due to their broad spectrum of activity against a range of bacteria, fungi, enveloped viruses and parasites (Brown and Hancock, 2006; Izadpanah and Gallo, 2005; Mohan et al., 2010; Vizioli and Salzet, 2002), as well as their selectivity, speed of action and low propensity for the development of bacterial resistance, AMPs are ideal candidates for development of clinical, aquacultural and agricultural agents (Bradshaw, 2003; Keymanesh et al., 2009; Noga et al., 2011). However, their susceptibility to proteolytic degradation and toxicity to mammalian cells must be overcome. Recently, several antimicrobial peptides have been identified from acidified water extract of cnidarian—Pd-AMP1 from coral and aurelin from jellyfish (de Lima et al., 2013; Ovchinnikova et al., 2006). These peptides revealed broad-spectrum antimicrobial activity against gram-positive and -negative bacteria, and no structural homology with other known antimicrobial peptides. The antimicrobial activities of water extracts of the venom of four sea anemone species—*Paracondactylis indidus, Paracondactylis sinensis, Heteractis magnifica and Stichodactyla haddoni*—have been reported (Subramanian et al., 2011). However, these water extracts caused hemolysis of chicken and goat RBCs (Subramanian et al., 2011). No antimicrobial factors from sea anemone (Anthozoa class) extracts have been reported to date. Consequently, the antimicrobial activity of water-soluble material extracted from *U. crassicornis* might facilitate discovery of novel antimicrobial peptides or proteins.

Only the visceral extract of *U. crassicornis* displayed notable antioxidant activity comparable with that of ascorbic acid (Fig. 3). The viscera, which comprise the gonad and intestine, are within the pharynx and gastrovascular cavity (Fig. 1D) and may produce antioxidant molecules to regulate imbalance in reactive oxygen species during oxidative stress, breakdown of nutrients and production of energy. This is similar to other aquatic animals in which the antioxidant system comprises water-soluble low-molecular-mass and high-molecular-mass antioxidants (Livingstone 2001). Low-molecular-mass antioxidants include reduced glutathione, ascorbic acid and others, while high-molecular-mass antioxidants include antioxidant enzymes and other proteins/enzymes such as ferritin and metallothioneins (Livingstone, 2001; Lushchak, 2011). In contrast, most *U. crassicornis* extracts exhibited little or no activity, indicating that either the extraction procedure was inappropriate, or the extracts contain few or no antioxidant agents.

In conclusion, we investigated the antimicrobial and antioxidant activity of water-soluble extracts from various tissues of *U. crassicornis* and evaluated their hemolytic activity against HRBCs. We thus suggest that water-soluble extracts of mottled anemone are promising sources of antimicrobial peptides or protein, and can be applied to economic perspective together with developing studies.

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