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The Anti-Rotaviral and Anti-Inflammatory Effects of *Hyrtios* and *Haliclona* Species

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The marine sponges *Hyrtios* and *Haliclona* species, both of which are known to produce secondary bioactive metabolites, were used to extract 1304KO-327 and 1304KO-328. Such secondary metabolites are potentially antibacterial, antiviral, anti-inflammatory, antitumoral, antifungal, and antiplasmodial. In the present study, the effects of 1304KO-327 and 1304KO-328 were studied for their clinical and pathological importance. The cytotoxicity of 1304KO-327 and 1304KO-328 was assessed via MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on HT-29, Caco-2, and Raw 264.7 cells. Rotavirus-infected Caco-2 cells were used to prove the antiviral effects of the marine sponge extracts. The test results cogently proved that the virus-inhibiting effects of the sponge extracts improved with extract concentration. Anti-inflammatory effects of the marine sponge extracts were tested on Lipopolysaccharide-treated Raw 264.7 cells. Nitric oxide and cytokine were produced by treatment of the cells with LPS and the inhibiting effects of the sponge extracts on IL-1β formation were investigated. This study found that the NO production was decreased dose dependently, and IL-1β formation was significantly reduced by the marine sponge extracts.

Keywords: *Hyrtios* species, *Haliclona* species, marine sponge, anti-rotaviral, anti-inflammation

Introduction

The ocean covers approximately 70% of Earth [14]. Recent research results cast a very promising potential for many marine organisms as sources of medically and pathologically beneficial bioactive compounds [5]. Thus, the sponges are attracting attention for their many interesting bioactive substances in such simplest and primitive form of metazoan life [6, 17, 30]. In fact, physical evidence proved that the sponge existed for more than 600 million years [21, 22]. Such a long term existence of the sessile, simple, and multicellular animal sponges is because they could defend themselves via production of secondary metabolites against predators and pathogens alike [1, 2, 24 25, 35]. These bioactive compounds are known to have antibacterial [3, 15, 23], antifungal [10, 36], anti-inflammatory [28], antiviral [10, 31] and antitumoral [4, 29, 32] activities. However, a dearth of literature on the marine sponges warrants their further study as a potential source to extract bioactive compounds for medical and pharmaceutical purposes [18].

Most sponges are closely associated with a variety of microbes [9, 25], which comprise approximately 40% of total cellular contents of a sponge and are a major source of biologically active secondary metabolites [27, 33]. For example, one such endosymbiotic bacterium contributes to an increased spectrum of the sponge's inherent capability for internal waste removal, provision of photosynthate, digestion, increased structural rigidity, and production of bioactive metabolites [9].

Rotavirus is an icosahedral genus of double-stranded RNA virus in the family *Reoviridae*, causing severe gastroenteritis in children under five years of age worldwide. Although this causes an insignificant death rate of 300 deaths a year in developed countries [11], the basic treatment for diarrhea of oral rehydration solution does not effectively shorten the duration of rotavirus-induced diarrhea [12, 13, 16, 26]. The Rotarix and Rotateq for prevention of rotavirus

gastroenteritis could cause a risk of intussusception for approximately 1 in 12,000 vaccinated children [8], warranting further research on the efficient treatment of rotavirus-induced diarrhea.

The sponge-extracted secondary metabolites have various features. The antibacterial and antitumoral effects of various sponges have been studied scientifically, but anti-inflammatory and antiviral effects should be more intensively studied. Therefore, the anti-inflammatory and antiviral effects of the methanol extracts derived from the marine sponges collected from the Kosrae Island, Micronesia, Central Pacific were comprehensibly investigated in the present study.

Materials and Methods

Marine Sponge Specimens

Marine sponge specimens were manually collected with skin scuba diving equipment at Kosrae Island in the Federated States of Micronesia in April 2013. The specimens thus collected were immediately washed with sterilized artificial seawater and instantly frozen by storage at −20°C until use. These specimens were similarly extracted with methanol (3 × 3 L) at the Korea Institute of Ocean Science & Technology as detailed in previous publications [7, 20]. The methanol-extracted specimens thus provided were designated as 1304KO-327 and 1304KO-328 for subsequent dissolution in sterile distilled water with a final concentration of 50 mg/ml.

Cells and Viruses

Murine macrophage Raw 264.7 cells, the human intestinal epithelial cell line Caco-2, and the rhesus monkey kidney cell line Vero were obtained from the Korea Cell Line Bank. The Raw 264.7 cells were cultured in Dulbeco's Modified Eagle's Medium (Corning, USA) supplemented with 10% fetal bovine serum (Corning, USA) and 1% penicillin/streptomycin (Gibco Invitrogen, USA) at 37°C in a humidified 5% CO_2 incubator. Caco-2 and Vero cells were grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640) containing 10% fetal bovine serum (Corning, USA) and 1% penicillin/streptomycin (Gibco) at 37°C in a humidified 5% CO_2 incubator.

Human rotavirus Wa strain (HRV; KBPV-VR-47) was also obtained from the Korea Cell Line Bank for use as the pathogenic virus. Viruses were multiplied in the Vero cells and harvested by freeze thawing 3 times. HRVs were concentrated by ultracentrifugation, and the titer was selected with 1.5×10^5 plaque forming unit/ml by plaque assay on Vero cells as described in a previous study [19]. The virus stock was stored at −80°C until use.

Cytotoxicity Assay

The cell cytotoxicity was determined using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma, USA) assay. Raw 264.7 cells (2×10^4 cells/well) were individually seeded on 96-well plates (Caco-2 cells, 2.5×10^4 cells/well). After 24 h incubation, cells were treated with the 1304KO-327 and 1304KO-328 extracts for 24 h. Cells with medium only were used as the control. The medium was removed after 100 µl of MTT solution was added to each well. The plates were subsequently incubated for 4 h at 37°C in a humidified 5% CO_2 incubator. Then the supernatant was removed and 100 µl of dimethylsulfoxide (DMSO) was added. Finally, the absorbance was measured at 540 nm using a microplate reader (BMG Labtech, Germany). In the subsequent assay preparations, cell treatment and the absorbance measurement were performed in the same manner as described in this section, except for the cell treatment for the ELISA.

Antiviral MTT Assay

The antiviral assay was conducted by the MTT method: Caco-2 cells (1.0×10^6 cells/well) were seeded on 6-well plates and HRVs were activated for 50 min by treatment with 5 µl/ml trypsin at 37°C. After 24 h incubation, the cells were inoculated with trypsin-activated HRV for 2 h at 37°C in a humidified 5% CO_2 incubator. The supernatant was removed and the cells were washed with phosphate buffer saline solution (Gibco). The infection medium (trypsin 5 µl/ml, RPMI-1640) was added to each well and the cells were similarly treated as described above. The cells with medium only and the cells with HRV only were used as the positive and the negative controls, respectively. The medium was subsequently removed and 1 ml of MTT solution was added to each well, and the plates were incubated for 4 h at 37°C in humidified 5% CO_2. Then, the supernatant was removed and 1.5 ml of DMSO was added.

Anti-Inflammatory Nitric Oxide (NO) Assay

The murine macrophage Raw 264.7 cells (2×10^4 cells/well) were seeded in 96-well plates. Cells were stimulated with 98 µl/well of lipopolysaccharide (LPS, 1 µg/ml) (Sigma Aldrich, USA) and incubated for 30 min at 37°C and 5% CO_2, and 2 µl/well of 1304KO-327 or 1304KO-328 was added. The mixture was shaken for 10 min to react and the optical density was measured at 540 nm wavelength using an ELISA microplate reader. In order to quantify the NO value, the standard calibration curve was drawn up by means of the serial dilution method. Cells were similarly treated as described above and the cells with medium only were used as the control. After incubation for 24 h, 100 µl of Griess reagent was mixed with an equal volume of cell supernatant.

Assay of IL-1β Production

After stimulation with LPS, cells were treated with sponge extracts and incubated for 24 h. The concentrations of IL-1β in the culture media were assessed using an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, USA) as per the manufacturer's instructions.

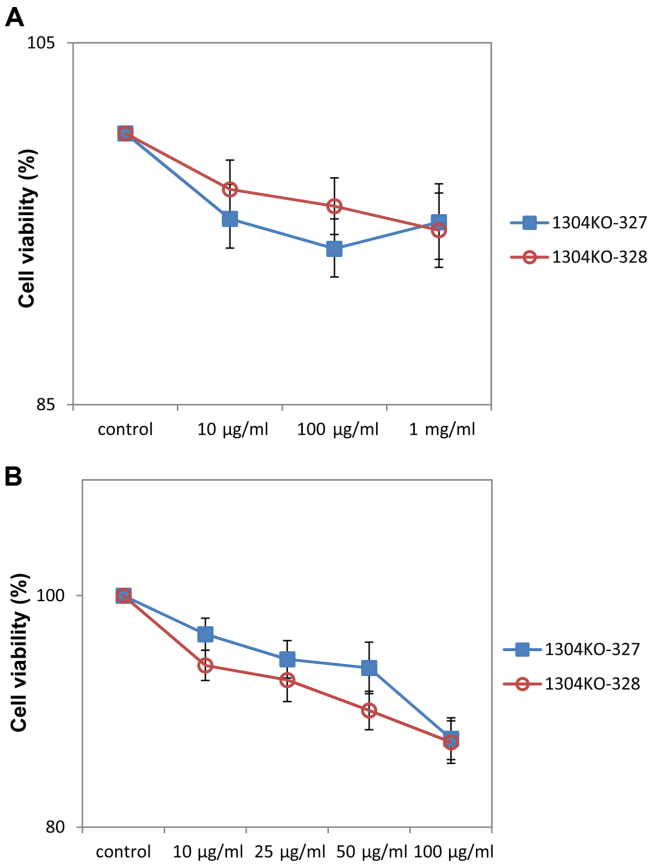

Fig. 1. Effects of 1304KO-327 (*Hyrtios* sp.) and 1304KO-328 (*Haliclona* sp.) on the viability of Raw 264.7 cells (**A**) and Caco-2 cells (**B**).

Cells were treated with 1304KO-327 or 1304KO-328 for 24 h. Cell viability was determined by MTT assay. Data are expressed as the percentage of the control. Error bars represent the mean ± standard deviation.

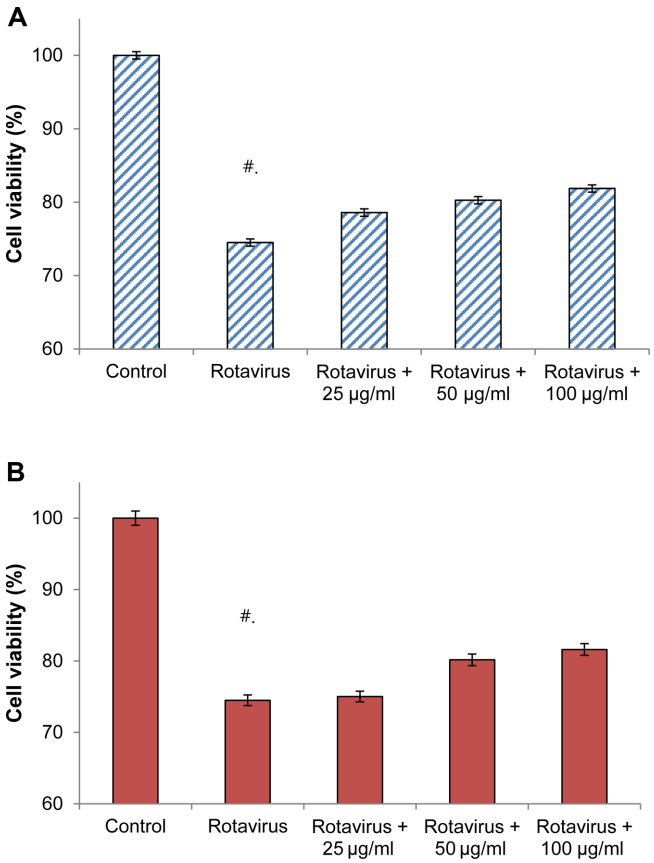

Fig. 2. Antiviral effects of 1304KO-327 (**A**) and 1304KO-328 (**B**) on rotavirus-infected Caco-2 cells.

Caco-2 cells (1.0×10^6 cells/well) were infected with 500 μl of rotavirus for 2 h. Then, sponge extracts were treated for 24 h. Antiviral activity was determined by MTT assay. Data are presented as means ± SDs of three independent experiments. ($^{\#}p < 0.05$ vs. control, $^{*}p < 0.05$ vs. rotavirus).

Results and Discussion

Cytotoxicity of 1304KO-327 (*Hyrtios* Species) and 1304KO-328 (*Haliclona* Species)

The cell viability was evaluated by MTT assay, especially related to toxicity of the 1304KO-327 and 1304KO-328 extracts. The individual extract was serially diluted for treatment of the Raw 264.7 and Caco-2 cells for 24 h. Raw 264.7 cells treated with 1304KO-327 (1 mg/ml) and 1304KO-328 (1 mg/ml) indicated 93.62 ± 1.65% and 94.64 ± 1.62% of cell viability, respectively, compared with untreated cells (Fig. 1A). Caco-2 cells treated with the 1304KO-327 (100 μg/ml) and 1304KO-328 (100 μg/ml) extracts inhibited 87.64 ± 1.81% and 87.34 ± 1.83% of cell viability, respectively, as shown in Fig. 1B. Results on cell viability of the Caco-2 cells showedd dose-dependent effect. Therefore, it is strongly inferred that treatment of the Raw 264.7 and Caco-2 cells with the 1304KO-327 (*Hyrtios* sp.) and 1304KO-328 (*Haliclona* sp.) extracts showed slight cytotoxicity as a result of no significance.

Antiviral Effects of the 1304KO-327 and 1304KO-328 Extracts on HRV-Infected Caco-2 Cells

The antiviral effect of the sponge extracts was also evaluated by the MTT assay for comparison of the cell viability. For this purpose, an antiviral assay was used on Caco-2 cells. The *Hyrtios* sp. and *Haliclona* sp. extracts showed rotavirus inhibitory effects on the rotavirus-infected Caco-2 cells. When the viability of the positive control, not treated with rotavirus, was set on a scale of

100%, the negative control, treated with virus only, was set at 74.50 ± 0.05% of viability (Fig. 2), experimentally substantiating the cell damage caused by the HRV via cytopathic effect. The (100 μg/ml) maximum concentration of the 1304KO-327 extract resulted in 81.87 ± 0.09% of cell viability, whereas the (10 μg/ml) minimum concentration inhibited cell viability to the level of 78.58 ± 0.08% (Fig. 2A). On the other hand, the maximum concentration (100 μg/ml) of 1304KO-328 extract rendered 81.61 ± 0.06% of viability and the (10 μg/ml) minimum concentration inhibited such viability to the level of 75.02 ± 0.07% (Fig. 2B). In view of this, the increased extract concentrations resulted in higher cell viability than the negative control and the viability of the Caco-2 cells increased in a dose-dependent fashion. All in all, the 1304KO-327 (*Hyrtios* sp.) and 1304KO-328 (*Haliclona* sp.) extracts efficiently inhibited rotavirus multiplication in the Caco-2 cells.

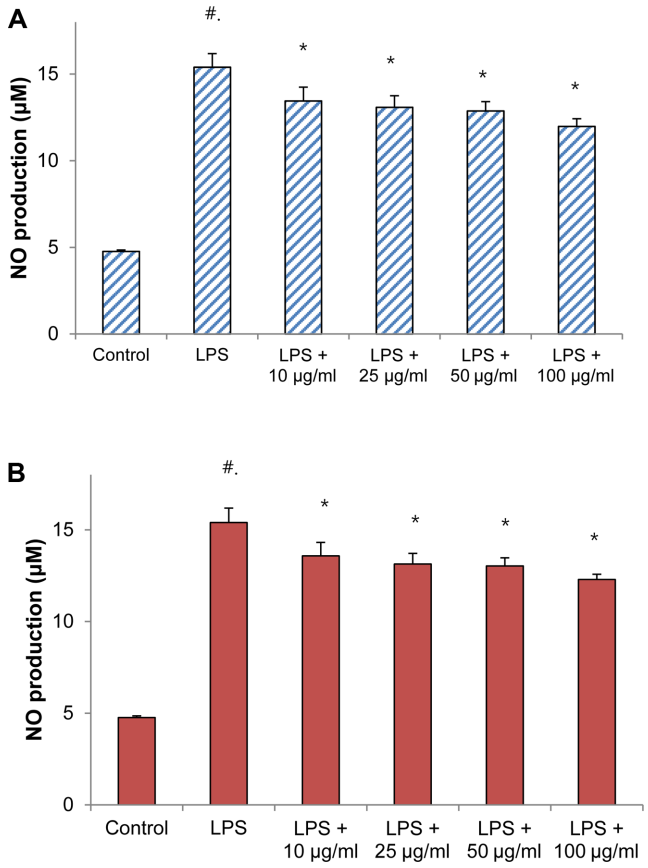

Fig. 3. Effects of 1304KO-327 (**A**) and 1304KO-328 (**B**) on the production of nitric oxide (NO) in LPS-stimulated Raw 264.7 cells.

Raw 264.7 cells were treated with the sponge extracts. Nitrite was measured using the Griess reaction. Data are presented as means ± SDs of four independent experiments. ($^{\#}p < 0.0001$ vs. control, $^{*}p < 0.0001$ vs. LPS).

Inhibition of NO Production by the 1304KO-327 and 1304KO-328 Extracts in LPS-Stimulated Raw 264.7 Cells

Inflammation is rendered by inflammatory mediators, such as NO. To evaluate the anti-inflammatory effect of the 1304KO-327 and 1304KO-328 sponge extracts, Raw 264.7 cells were stimulated with LPS (1 μg/ml) and treated with and without the aforementioned sponge extracts. The resulting NO concentration was measured using the Griess reagent. The LPS-stimulated Raw 264.7 cells resulted in 15.39 ± 0.78 μM of NO (Fig. 3). The significant disparity between the LPS-stimulated and control Raw 264.7 cells was attributed to the inflammatory response induced by the LPS-stimulated Raw 264.7 cells. Figs. 3A and 3B convincingly show inhibitory effects of the 1304KO-327 and 1304KO-328 extracts on NO production at all concentrations. NO production was observed to decrease in a dose-dependent manner by the 1304KO-327 and 1304KO-328 extracts.

Inhibition of Cytokine Production in LPS-Stimulated Raw 264.7 Cells by the 1304KO-327 and 1304KO-328 Extracts

Inflammation is usually accompanied by the pro-inflammatory cytokines. To examine the anti-inflammatory effect of the 1304KO-327 and 1304KO-328 extracts, the concentration of the IL-1β in LPS (1 μg/ml)-stimulated Raw 264.7 cells treated with the sponge extracts was measured. IL-1β production in the culture medium was measured using the ELISA. Fig. 4 shows inflammation of

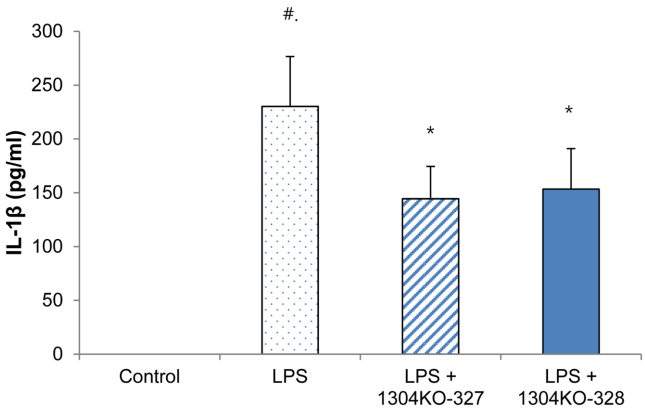

Fig. 4. Effects of 1304KO-327 (*Hyrtios* sp.) and 1304KO-328 (*Haliclona* sp.) on the production of IL-1β in Raw 264.7 cells.

Raw 264.7 cells were treated with 10 μg/ml of 1304KO-327 and 1304KO-328. Data are presented as means ± SDs of three independent experiments. ($^{\#}p < 0.05$ vs. control, $^{*}p < 0.05$ vs. LPS).

the LPS (1 μg/ml)-stimulated Raw 264.7 cells as corroborated by the IL-1β concentration of 230.1 ± 46.4 pg/ml, which exceeded the acceptable level of the control group. It is strongly inferred with reasonable certainty that such inflammation was induced by the LPS (1 μg/ml). On the other hand, 10 μg/ml each of the 1304KO-327 and 1304KO-328 extracts significantly reduced IL-1β production, as shown in Fig. 4.

We investigated the anti-inflammatory and antiviral effects of marine sponges from crude extracts. Marine sponges take in nutrients through body pores and produce secondary metabolites with bioactivity. As reported by Skariyachan *et al*. [33], *Hyrtios* spp. were reported to have cytotoxic and antioxidant activities. Our results were consistent with this study and indicated that 1304KO-327 and 1304KO-328 extracts significantly reduced the production of IL-1β, pro-inflammatory cytokine. 1304KO-327 (*Hyrtios* sp.) and 1304KO-328 (*Haliclona* sp.) extracts also efficiently inhibited rotavirus multiplication depending on their concentration in Caco-2 cells. Consequently, the present research showed the promising and beneficial potential of the 1304KO-327 and 1304KO-328 sponge extracts as anti-inflammatory drug candidates.

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