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Exploitation of Health Promoting Potentials of Edible Sea Cucumber *(Holothuria Edulis)*: Search of New Bioactive Components as Functional Ingredients

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Abstract. Though sea cucumbers have been a dietary delicacy and a medicinal cure for Asians over many centuries, biological properties of the edible sea cucumber *Holothuria edulis* were not revealed yet. In this study, edible sea cucumber *H. edulis* was evaluated for its *in vitro* anti-inflammatory potential via the determination of pro-inflammatory mediators. Ethyl acetate fraction (EtOAc) of the edible sea cucumber (ESC-EA) exhibited profound anti-inflammatory potentials in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. ESC-EA dose-dependently inhibited the nitric oxide (NO) production, and showed significant down regulation of the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in LPS-stimulated RAW 264.7 cells. ESC-EA significantly suppressed prostaglandin E_2 (PGE_2) release in addition to pro inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6. Due to the profound anti-inflammatory activity, ESC-EA appears as economically important biomass fractions that can be exploited in numerous industrial applications as a source of functional ingredients.

Keywords: anti-inflammation, bioactive component, edible sea cucumber, functional food.

1. Introduction

Researchers are looking elsewhere for effective bioactive metabolites, particularly from natural sources [1]. Especially in Asian countries, over the last 20 years, there have been rapid changes in the popularity of the use of natural systems to maintain health and for alternative therapy [2]. Functional foods, nutraceuticals, and dietary supplements are important for health promotion and disease risk reduction. Although a myriad of bioactive components are known to render the expected beneficial effects, the mechanisms involved are varied and may work individually or collectively in providing the effects. Hence, over the years, the biological activities of natural products could have gained a considerable research interest and studies about the extraction and isolation of active components from natural resources have attracted special attention in last recent years. In addition, due to their potential therapeutic value, natural bioactive ingredients nowadays attracted considerable interest among food processors and nutritionist [3].

Since sea cucumbers are well known to exert beneficial effects on human health, these echinoderms are used in Asian folk medicine [4]. In addition, recently, scientific evidence supporting their importance as nutraceuticals and functional foods has attracted growing interest from nutritionists and pharmacologists as well as the general public [5]. As a mean of defense, most sea cucumbers contain, in their body wall and viscera, secondary metabolites [6]. Therapeutic properties and medicinal benefits of sea cucumbers can be linked to the presence of a wide array of bioactives especially triterpene glycosides (saponins), chondroitin

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sulfates, glycosaminoglycan, sulfated polysaccharides, sterols, phenolics, cerberosides, lectins, peptides, glycoprotein, glycosphingolipids and essential fatty acids [7]. Taken together, objective of the current work is to evaluate the anti-inflammatory potentials of the edible sea cucumber *H. edulis* in lipopolysaccharide-stimulated RAW 264.7 macrophages. The findings of the present study might be useful in further exploitation of the bioactive components of the edible sea cucumber *H. edulis* as functional ingredients in possible industrial applications.

2. Materials and Methods

2.1. Preparation of Crude Extract and Fractionation

Collected fresh edible sea cucumbers (*H. edulis*) were clean and freeze dried. Extraction procedure was done using 80% methanol for 3 days at room temperature. Then the mixture was homogenized and further extracted for another 3 days at room temperature. After the extraction, solvent was filtered out and vacuum evaporated to obtain the concentrated edible sea cucumber methanolic extract. The resulting crude extract was redissolved in ethyl acetate (EtOAc) and partitioned with double distilled water in 3:1 ratio. The EtOAc fraction (ESC-EA) was dehydrated in sodium sulphate anhydrous, and then concentrated to leave an oily red crude (1.1 g). For the cell based assays, samples were dissolved in DMSO and further diluted in culture media.

2.2. Determination of Total Phenolic Content

Total phenolic content of ESC-EA was determined according to the protocol described by Chandler and Dodds (1983) [8]. One milliliter of sample was mixed in a test tube containing 1 mL of 95% EtOH, 5 mL of distilled water, and 0.5 mL of 50% Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min, and then 1 mL of 5% Na_2CO_3 was added and it was mixed thoroughly and placed in the dark for 1 h. Absorbance was measured at 725 nm using a UV-VIS spectrometer (Opron 3000 Hansan Tech. Co Ltd., Korea). A gallic acid standard curve was obtained for the calibration of phenolic content.

2.3. Cell Culture

A RAW 264.7 cell line was cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin and 10% Fetal bovine serum (FBS). The cells were incubated and maintained in an atmosphere of 5% CO_2 at 37 ℃. The cells were sub cultured every 2 days and exponential phase cells were used throughout the experiments.

2.4. Determination of Nitric Oxide (NO) Production

RAW 264.7 cells (1.0×10^5 cell/mL) were cultured in a 24-well plate and after 16 h the cells were pre-incubated with various concentrations of the sample (ESC-EA) at 37 ℃ for 1 h. Then further incubated for another 24 h with LPS (1 μg/mL) at the same temperature. After the incubation, quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production [9]. Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min, and the optical density at 540 nm was measured using an ELISA microplate reader (Sunrise, Tecan Co. Ltd., Australia). The fresh culture medium was used as a blank in every experiment.

2.5. Lactate Dehydrogenase (LDH) Cytotoxicity Assay

RAW 264.7 cells (1.5×10^5 cells/mL) were plated in 96-well plate and after 16 h the cells were pre-incubated with various concentrations of the sample for 1 h at 37 ℃. Then the cells were further incubated for another 24 h with LPS (1 μg/mL) at the same temperature. After the incubation, LDH level in the culture medium was determined using an LDH cytotoxicity detection kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 50 μL of reaction mixture was added to each well, and the reaction was incubated for 30 min at room temperature in the dark. Then, 50 μL of stop solution was added to each well and absorbance was measured at 490 nm using a microplate reader (Sunrise, Tecan Co. Ltd., Australia).

2.6. Determination of Prostaglandin E_2 (PGE_2) Production

RAW 264.7 cells (1.0×10^5 cells/mL) were pretreated with the sample for 2 h and then treated with LPS (1 μg/mL) to allow cytokine production for 24 h. The PGE_2 levels in the culture medium were quantified using a competitive enzyme immunoassay kit (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The release of PGE_2 was measured relative to that of the control value.

2.7. Western Blot Analysis

RAW 264.7 cells (1.0×10^5 cells/mL) were pre-incubated for 16 h and then treated with LPS (1 μg/mL) in the presence or absence of the sample. After incubation for 24 h, the cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and the cell lysates were prepared with lysis buffer (50 mM/L Tris-HCl (pH 7.4), 150mM/L NaCl, 1% Triton X-100, 0.1% SDS and 1 mM/L EDTA) for 20 min on ice. Cell lysates were centrifuged at 14,000 ×g for 20 min at 4 ℃. Then protein contents in the supernatant were measured using the BCA™ protein assay kit. Cell lysates (30-50 μg) were subjected to electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels (8-12%), and the separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was pre-incubated with blocking solution (5% skim milk in Tris buffered saline containing Tween-20) for 90 min at room temperature. Then the membrane incubated with anti-mouse iNOS (1:1000; Calbiochem, La Jolla, CA, USA) and anti-mouse COX-2 (1:1000; BD Biosciences Pharmingen, San Jose, CA, USA) for overnight at room temperature. After washing, the blots were incubated with horseradish peroxidase conjugated goat anti-mouse IgG secondary antibody (1:5000; Amersham Pharmacia Biotech, Little Chalfont, UK) for 90 min at room temperature. The bands were visualized on X-ray film using ECL detection reagent (Amersham Biosciences, Piscataway, NJ, USA).

2.8. Determination of Pro-Inflammatory Cytokines (TNF-α, IL-1β and IL-6) Production

The inhibitory effect of the sample on the production of pro-inflammatory cytokines from LPS stimulated RAW 264.7 cells was determined according to a previously described method [10]. Briefly, RAW 264.7 cells (1.0×10^5 cells/mL) were pretreated with the sample for 2 h and then treated with LPS (1 μg/mL) to allow production of pre-inflammatory cytokines for 24 h. Supernatants were used for the assay using an ELISA kit (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.9. Statistical Analysis

All the data are expressed as mean ± standard deviation of three determinations. Statistical comparison was performed via a one-way analysis of variance followed by Duncan's multiple range test (DMRT). P values of less than 0.05 ($P<0.05$) were considered as significant.

3. Results and Discussion

Inflammation is the body's way of dealing with infections and tissue damage, but there is a fine balance between the beneficial effects of inflammatory cascades and their potential for long-term tissue destruction [11]. However, pronged inflammation is a common underlying factor contributing to the exacerbation of a wide variety of diseases such as arthritis, asthma, and cancer which represent a serious health problem. Inflammatory responses are generally characterized by abundant productions of NO and PGE_2, and of cytokines such as IL-1β, IL-6, and TNF-α; therefore, these pro-inflammatory mediators are regarded as essential anti-inflammatory targets. However the use of almost all the anti-inflammatory drugs currently available for the treatment of inflammatory diseases causes serious side effects [12]. Thus, there is an increasing interest in the search of new anti-inflammatory therapeutic agents with minimum side effects from natural resources [13].

Polyphenolic compounds are generally more soluble in polar organic solvents than in water. In this work, total phenolic content of the ESC-EA was quantified and it was found that ESC-EA contain 10.32% total phenolic content.

The effects of various concentrations of ESC-EA on NO production in LPS-stimulated RAW 264.7 macrophages were evaluated *in vitro*. Stimulation of the cells with LPS resulted in an enhancement of NO concentration in the medium. However, the pretreatment with the ESC-EA significantly decreased the NO production at all the tested concentrations (Fig. 1). In addition, as confirmed by the LDH assay the ESC-EA

did not show any cytotoxic effect on RAW 264.7 cells at the tested concentrations. Thus, ESC-EA can be considered as a potential agent for suppressing NO production without any cytotoxic effect.

The results indicated that ESC-EA might induce the anti-inflammatory activity by suppressing the PGE_2 release in a reasonable manner in RAW 264.7 macrophages. As shown in the figure, ESC-EA slightly suppressed LPS-induced PGE_2 production at the lower concentrations. However, the extract suppressed the PGE_2 production by 50% at the concentration of 100 µg/mL (Fig. 2).

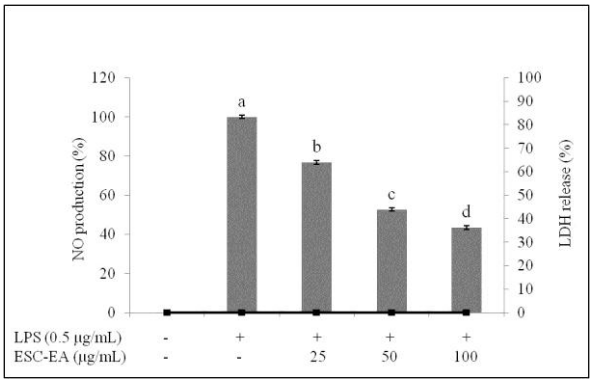


Fig. 1: Inhibitory effect of ESC-EA on LPS-induced NO production and LDH release in RAW 264.7 macrophages. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

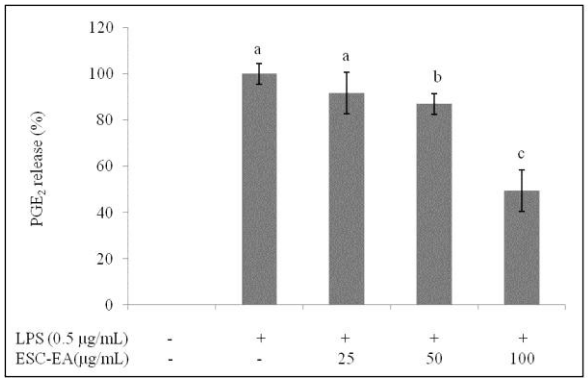


Fig. 2: Inhibitory effect of ESC-EA on LPS-induced PGE_2 production in RAW 264.7 macrophages. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

3.1. Effect of ESC-EA on LPS-induced iNOS and COX-2 Protein Expression

Fig. 3 shows the influence of ESC-EA on iNOS and COX-2 protein expression in RAW 264.7 macrophages. The iNOS and COX-2 protein expressions were markedly increased when the macrophages treated only with LPS compared to the control. However, ESC-EA suppressed the iNOS and COX-2 protein expressions, compared with that of LPS-treated alone cells.

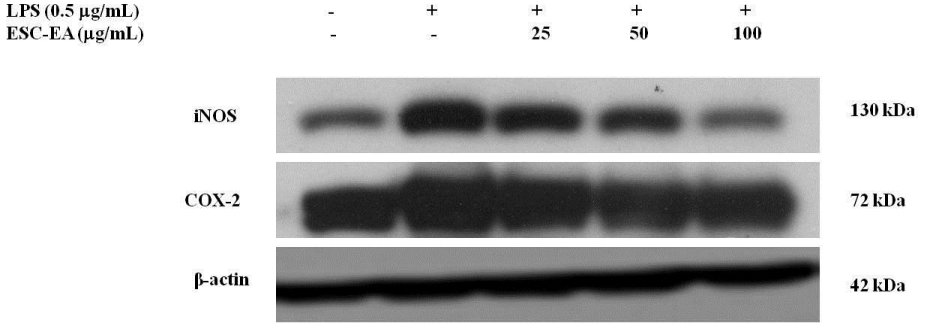


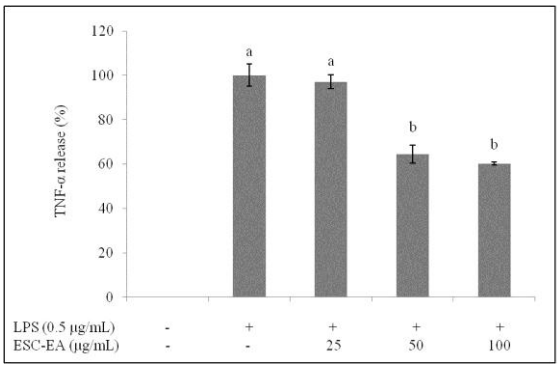
Fig. 3: Inhibitory effect of ESC-EA on LPS-induced iNOS and COX-2 protein expression in RAW 264.7 macrophages.

It was found that ESC-EA considerably inhibited the production of cytokines TNF-α, IL-1β and IL-6 in a similar pattern (Fig. 4). Decrease in cytokines showed a concentration dependent profile with the increase in ESC-EA. It could be suggested that ESC-EA exerts anti-inflammatory effects like decreasing NO and /or PGE_2 productions by down-regulating the expression level of pro-inflammatory mediators such as iNOS and/or COX-2 or pro-inflammatory cytokines such as TNF-α,, IL-1β and IL-6 in LPS stimulated macrophages.

NO is usually generated by macrophages as part of the human immune responses. The chronic expression of NO could also be associated with various carcinomas and inflammatory conditions. In addition, NO production could also be increased by the production of iNOS under pathological conditions. Therefore, it is clear that inhibition of NO production may have therapeutic value over inflammatory diseases. Within many inflammation cascades or pathways, there are often pivotal molecular targets that, when antagonized or

neutralized, block the output of the pathway. A relatively small number of pivotal targets have been identified that have yielded many successful anti-inflammatory drugs. These targets include the enzymes such as COX-1 and COX-2, cytokines including TNF-α, IL-1β and IL-6 [14]. Therefore, inhibition of these targets has become a major focus of current drug discovery and development, and an important *in vitro* method for evaluating the bioactivity of drugs [15].

a.



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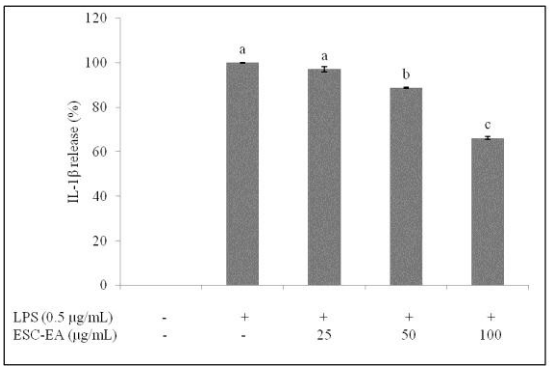

c.

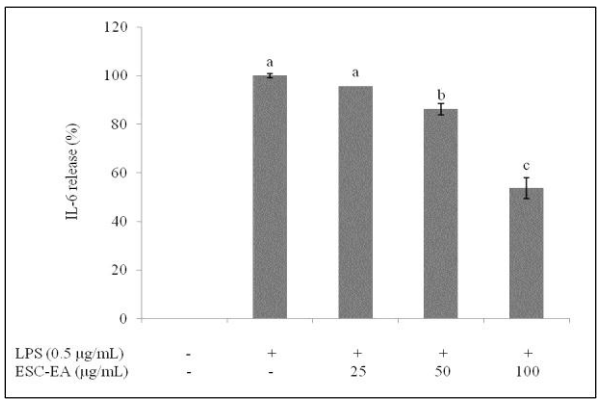

Fig. 4: Inhibitory effect of ESC-EA on LPS-induced (a) TNF-α (b) IL-1β and (c) IL-6 production in RAW 264.7 macrophages. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

4. Conclusion

The possessed anti-inflammatory activity of the edible sea cucumber *H. edulis* indicates that their consumption would be beneficial to health. In addition, the findings of this study may facilitate awareness about anti-inflammatory properties of *H. edulis* and help future developments with possible industrial applications. Hence, rich opportunities for edible sea cucumber *H. edulis* exist within the arena for functional foods and nutraceuticals.

5. References

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