A THESIS

FOR THE DIGREE OF DOCTOR OF PHILOSOPHY

Search of new bioactive components and exploitation of their health promoting potentials as functional ingredients from brown seaweed *Ecklonia cava* and edible sea cucumber *Holothuria edulis*

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GRADUATE SCHOOL

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AUGUST, 2012

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A thesis submitted in partial fulfillment of the requirement for the degree of

DOCTOR OF PHYLOSOPHY

AUGUST, 2012

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SUMMARY

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.

It is well known that marine organisms are not only very important resources as food, feed, and energy rich source, but they are also rich sources of structurally novel and biologically active metabolites with valuable industrial potentials. Thus, efforts at discovery of novel active components from marine bioresources over the past years have yielded a considerable amount of new active metabolites which may not available in terrestrial environment. Therefore, marine derived active components, whose immense biochemically diversity looks like to become a rich source of novel chemical entities for the use as functional ingredients in many industrial applications such as functional foods, pharmaceuticals and nutraceuticals. This report deals with the antioxidant, anti-inflammatory and anticancer potentials of biologically active components from the brown seaweed *Ecklonia cave* and edible sea cucumber *Holothuria edulis.*

The protective effect of bioactive components recovered from brown seaweed *E. cava* processing by-product on H_2O_2-mediated DNA damage in Vero cells was evaluated. *E. cava* processing by-product was fermented by edible yeast *Candida utilis* and its antioxidant activities were evaluated via radical scavenging using electron spin resonance

(ESR) spectrometer. Effective fermentation duration was discovered as 24 h prior to being extracted with 80% EtOH. Major bioactive components in the extract were phlorotannins including triphlorethol-A, eckol, dieckol and eckstolonol. The phlorotannin rich fermented *E. cava* processing by-product extract (FEPBE) strongly enhanced the cell viability against H_2O_2-induced oxidative damage in Vero cells and exhibited good protective properties against H_2O_2-induced cell apoptosis as demonstrated by nuclear staining with Hoechst 33342 and flow cytometric analysis.

The anti-inflammatory potential of the FEPBE was evaluated *in vitro.* The bioactive components recovered from *E. cava* processing by-product attenuate production of nitric oxide (NO) and prostaglandin-E_2 (PGE_2) by suppressing inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) protein expressions in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The release of pro-inflammatory cytokines, interleukin-1β (IL-1β) and interleukin-6 (IL-6) was also significantly suppressed. Hence, it could be confirmed that the bioactive components responsible for the antioxidant and anti-inflammatory activity are still remaining in the *E. cava* processing by-product and can be recovered as a value-added bio-mass fraction after fermentation with *C. utilis*.

In vitro anticancer and anti-inflammatory potentials of edible sea cucumber *H. edulis* were evaluated. The anticancer effect of aqueous fraction of edible sea cucumber *H. edulis* (ESC-AQ) on human leukemia HL-60 cells was assessed. ESC-AQ induced apoptosis in HL-60 cells as evidenced by the formation of apoptotic bodies and the accumulation of DNA in the sub-G1 phase of the cell cycle. The induced apoptosis was accompanied by down regulation of Bcl-xL, up regulation of Bax and activation of caspase-3. In this study, EtOAc solvent fraction of edible sea cucumber *H. edulis* (ESC-EA) modulated LPS-

induced inflammatory responses in murine macrophages. ESC-EA significantly suppressed the NO production and PGE_2 production by suppressing iNOS and COX-2 protein extrusions in LPS-induced RAW 264.7 macrophages. In addition, ESC-EA suppressed the release of pro-inflammatory cytokines TNF-α, IL-1β and IL-6. According to the results, it could be suggested that ESC-AQ and ESC-EA could be incorporated in food formulations as functional ingredients. Besides, they might be further developed as potential therapeutic agents in the particular applications.

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electrophoresed and the expression levels of iNOS and COX-2 were detected with specific antibodies.

Fig. III-9. Inhibitory effect of ESC-EA on LPS-induced TNF-α production in RAW 264.7 macrophages. After incubation of cells with LPS for 24 h in the presence or absence of the extract, the concentration of TNF-α in the medium was measured. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

Fig. III-10. Inhibitory effect of ESC-EA on LPS-induced IL-1β production in RAW 264.7 macrophages. After incubation of cells with LPS for 24 h in the presence or absence of the extract, the concentration of IL-1β in the medium was measured. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

Fig. III-11. Inhibitory effect of ESC-EA on LPS-induced IL-6 production in RAW 264.7 macrophages. After incubation of cells with LPS for 24 h in the presence or absence of the extract, the concentration of IL-6 in the medium was measured. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

Fig. III-12. The anticancer activity of ESC-AQ is associated with the up regulation of Bax, down regulation of Bcl-xL, and activation of caspase-3 in HL-60 cells.

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INTRODUCTION

What are natural products? The term is usually reserved for secondary metabolites produced by an organism. Secondary metabolites are very broad group of metabolites, with no distinct boundaries, and grouped under no single unifying definition. In most cases we do not really know what biological role these compounds play, except that they represent a treasure trove of chemistry that can be both interest and benefit to us. Despite a very promising field, it is yet to achieve its bloom. However, science is the intellectual activity carried on by humans that is formulated to discover new information about the natural world in which we thrive. Moreover, natural product science is one branch of this never-ending curiosity of humans to design the best out of the existing framework of resources in the natural environment. To date, a large number of natural ingredients have been extracted and purified from both territorial and marine resources. A vast exploration of various natural resources in search of bioactive metabolites has led to some efficient functional ingredients. These ingredients have been extensively screened for bioactivities with a broad number of therapeutic indications. The World Health Organization (WHO) has estimated that approximately 65% of the world's population relies mainly on plant-derived traditional medicines for their primary health care (Kvalheim et al., 2011). Moreover, natural products play an essential role in many areas in the society such as nutritional and therapeutic agents to prevent or cure diseases.

The marine environment is believed to be the original source of life on the Earth (Wallace, 1997). It is becoming the most explored habitat because of its chemical and biological diversity. The art by which marine organisms elaborate bioactive molecules is fascinating. Marine environment provides different biosynthetic conditions to organisms that live in it. It is not surprising that marine organisms are a wonderful source of biologically active

natural products and hence, they have attracted the attention of scientist over the years. The biological activities of extracts or isolated compounds of marine organisms could be assessed in several ways. To date, a large number of bioactive chemical components have been discovered from different marine organisms with various biological properties (Manilal et al., 2009). However, remains a huge amount of potential locked beneath the waves. These bioactive natural products are likely open the opportunities towards the development of entirely novel classes of pharmaceuticals, nutraceuticals, cosmeceuticals and functional foods (Fig. 1).

Natural products, especially in medicinal plants, are a valuable source of lead compounds in modern drug discovery. The use of foods and medicinal plants to improve health is nearly as old as humanity (Lansky et al., 2008). It was estimated that bioactive natural ingredients contributed to approximately one third of the top-selling drugs currently on the market (Zhou et al., 2010). Over the years, there are significant developments in drug design and highly active chemical compounds can be synthesized. However, natural products are considered as great sources for the development of new pharmaceuticals which are more effective with fewer adverse effects. Therefore, use of natural products as disease remedies has a long history and natural products research continues to provide a tremendous variety of lead structures which are used as templates for the development of new drugs by the pharmaceutical industry (Boris et al., 1996). The ocean is considered to be a source of potential drugs. Therefore, in recent years, marine natural products have been recognized as effective drug candidates. In this regard, the selected marine organisms and their derivatives might be employed as useful natural products for the development of effective pharmaceuticals. However, a fair understanding of biological, toxicological and clinical evaluation is essential to those interested in searching potential drugs from the

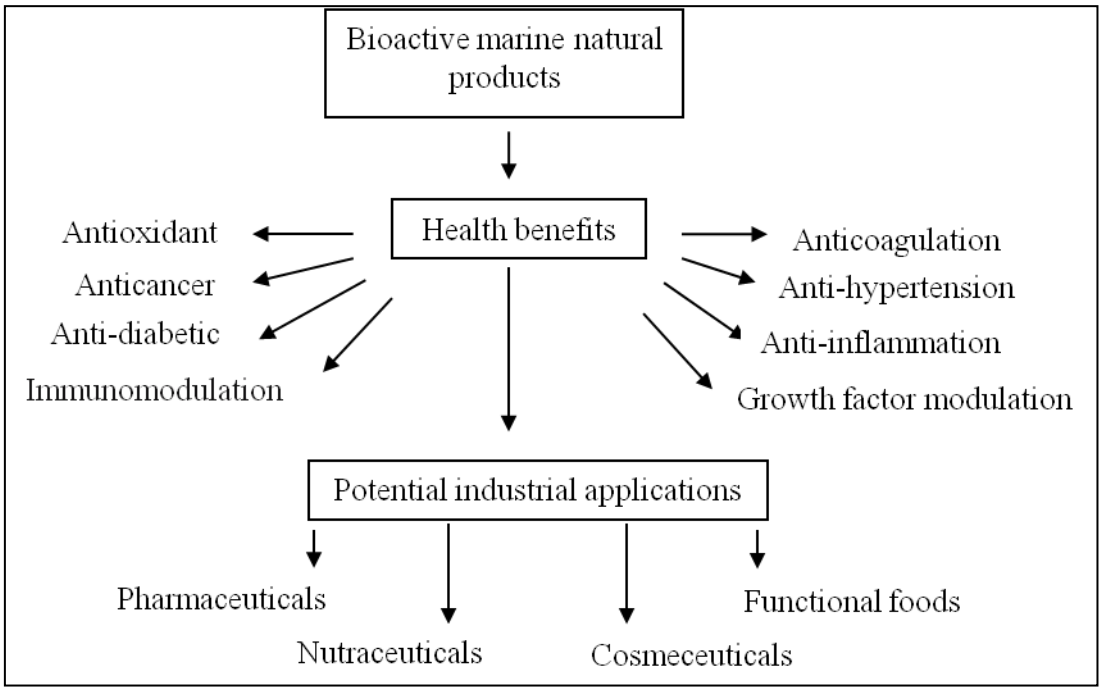

Fig. 1. Schematic showing potential industrial uses of functional and biological properties of bioactive marine natural products

marine organisms.

Nowadays, natural ingredients are becoming more and more attractive for the above mentioned industrial-related applications. This interesting trend is believed to be a better alternative approach to the use of fully synthetic materials in the highlighted fields. In addition, this consumer-driven demand has led to the development of useful natural products. Many academic, scientific and regulatory organizations are considering ways to establish a scientific basis to support and further validate claims of functional components or foods containing them. More recently, consumer interest in the relationship between diet and health has increased the demand for information about functional foods. Hence, the food industry, agricultural community and now consumers have all shown growing interest in natural functional ingredients.

While food has long been used to improve health, our knowledge of the relationship between food components and health is now being used to improve food. Functional foods are one of the top trends in the current food industry. Functional Foods have bioactive food components that can potentially enhance health when eaten on a regular basis as part of a varied diet. Functional foods are an emerging field of food science due to their increasing popularity with health-conscious consumers and therefore, there is a growing interest among producers as well as the public in functional foods or foods that may provide health benefits beyond basic nutrition (Marinangeli and Jones, 2010; Spence, 2006). Food and nutraceutical science has moved from identifying and correcting nutritional deficiencies to designing foods and nutraceuticals that promote optimal health and reduce the risk of diseases (Hold and Kraan, 2011). Epidemiological studies and clinical trials carried out in different countries have demonstrated and suggested, functional foods typically include

health claims such as reduction of cancer risk, improvement of heart health, stimulation of immune system, improvement of gastrointestinal health, maintenance of urinary tract health, anti-inflammatory effects, reduction of high blood pressure, maintenance of vision, antibacterial and antiviral activities and antiobese effects related to the particular functional ingredients. Today's science and technology can be used to provide many additional functional foods, and future scientific and technological advances promise an even greater range of health benefits for consumers. The current study elucidates the key success of the selected marine organisms influencing the development of functional foods or dietary supplements with reference to the functional properties of the bioactive components.

Seaweeds belong to a group of organisms that has enormous ecological importance and represent a significant proportion of the world's biodiversity. They are a large and diverse group of organisms which play vital ecological roles in marine communities. It falls into three broad categories as brown, red and green seaweed based on pigmentation (Dawczynski et al., 2007). Seaweeds have always been of great interest in Asian culture as marine food sources (Rioux et al., 2009). By contrast, use of seaweeds as a food has strong roots in Asian countries such as China, Japan and Korea. In addition, the food industries consume a wide range of algae, which are well known to have high contents of fiber, minerals, vitamins and different antioxidants in many countries (Cardozo et al., 2007). In contrast, seaweeds represent an important economic resource mostly in the countries of East and South Asia where they are not only largely harvested but also intensively and largely employed in the human nutrition (Caliceti et al., 2002).

Seaweeds or marine macro algae are potential renewable resource in the marine environment and known to be an extremely rich source of bioactive compounds (Chandini

et al., 2008; Kladi et al., 2004). Therefore, among the marine sources, seaweeds are the most valuable sources of structurally diverse bioactive compounds. Thus, algae can be a very interesting natural source of new metabolites with various biological activities that could be used as functional ingredients (Kashman and Rudi, 2004; Plaza et al., 2008). Biological activities correlate to the presence of chemical compounds, particularly secondary metabolites. The presence of these compounds may assist in predicting some traditional uses of medicinal plants (Kamatou et al., 2008). Previous researches have proved their potential medicinal value against various diseases such as allergy, cancer, diabetes, hypertension, oxidative stress, inflammation, thrombosis, obesity, lipidemia, and other degenerative disorders (Wijesinghe and Jeon, 2012a). Moreover, phytochemicals produced by algal species may potentially be bioactive compounds of interests in the food, drug and nutraceutical industries. Thus, the value of seaweeds as new functional and health ingredients is gaining popularity. Further, they have the potential to provide the cosmetics and food market with valuable biomolecules such as highly unsaturated fatty acids, tannins, carotenoids, and sulfated polysaccharides. Hence, a large number of seaweed bioactives have been identified with potential uses in various areas including functional food, pharmaceutical, and cosmeceutical. However, novel potential areas have to be explored in order to maximize the effective utilization of seaweeds.

The Phaeophyceae or brown seaweeds are a large group of multicellular algae, and they play an important role in marine environments both as food and for the habitats they form. Most brown seaweeds contain the pigment fucoxanthin and various pheophycean tannins which are responsible for the distinctive greenish-brown colour as the name indicated. Worldwide there are about 1500 species of brown seaweeds and they produce vast numbers of useful active components (Davis et al., 2003; Reddy and Urban, 2009). Some

species are of sufficient commercial importance, such that they have become subjects of extensive research in their own right.

Ecklonia cava (Fig. 2), a kind of brown alga (Laminariaceae) that is found abundantly in the sub tidal regions of Jeju Island, South Korea and Japan (Kim et al., 2006; Kim et al., 2008). It is plentifully produced Jeju Island of South Korea (30,000 tons per year) for commercial purposes. This brown seaweed is popular in Korea and Japan as a food ingredient and supplement of animal feed (Lee et al., 2010). In addition, *E. cava* has long been utilized as a traditional food and also as a traditional folk herb (Li et al., 2009; Shim et al., 2009). The brown alga *E. cava*, for instance, has been the target of special attention. *E. cava* has a variety of bio active compounds including peptides, polysaccharides, carotenoids, fucoidans, and phlorotannins showing different biological activities (Heo et al., 2009). Even this valuable seaweed is not available around the world; *E. cava* may expand its value in various industries due to its wide range of biological activities in whole over the world (Ahn et al., 2007). In addition, an increasing number of scientific papers published for the last few years highlighting that various biological activities and their possible industrial applications of the brown seaweed *E. cava*. Once the structures and functional properties of these biologically active compounds from *E. cava* are understood, they may serve as potential source materials for functional food, nutraceutical, pharmaceutical or cosmeceutical industries. The unique properties of secondary metabolites that are found in this seaweed are especially interesting, which emphasizes the importance of obtaining further involvement in such areas.

Holothurians (sea cucumbers) are traditionally consumed raw, dried, and boiled as food for human consumption in many tropical and subtropical countries. Major consuming

countries are China, Hong Kong, South Korea, Singapore and Japan (Ozer et al., 2004). In some Pacific countries such as Japan and Korea, the body wall and viscera of sea cucumbers are eaten as raw or pickled. In some other countries, dried sea cucumbers are widely used in soups or fried with meat or vegetables (Conand and Byrne, 1993). Moreover, sea cucumbers are well known to exert beneficial effects on human health (Aminin, 2001). These echinoderms are used in Asian traditional medicine to maintain fitness during long fishing travels, to prevent, reduce or cure several ailments like constipation, renal deficiency or arthritis. Several papers published in the last two decades came in support of these medicinal purposes showing multiple biological activities of sea cucumber extracts as wound healing promoter and exhibiting antimicrobial, anticancer, and immunomodulatory properties (Mamelona et al., 2007).

Holothuria edulis, an edible sea cucumber, is a holothurian echinoderm (Fig. 3). *H. edulis* is distributed throughout the Indo-Pacific, predominantly residing in the shallow waters of coral reef flats and fore reef slopes, to depths of around 20 m. *H. edulis* plays an important role in coral reef ecosystems by aiding bioturbation. They are nocturnally active and prefer to shelter under coral boulders during the day. They are one of the most conspicuous members of the benthic community and are found to be constantly deposited feeding in sand and rubble (Uthicke, 1997).

Taken together, the objectives of this work are to evaluate the health promoting potentials of bioactive components of the brown seaweed *E. cava* and edible sea cucumber *H. edulis*. Hence, herein, in this report discusses the importance of biologically active components from brown seaweed *E. cava* and edible sea cucumber *H. edulis* under three sections. Part I deals with the protective effect of bioactive components recovered from brown seaweed

Ecklonia cava processing by-product on H_2O_2-mediated DNA damage in Vero cells; Part II demonstrates the anti-inflammatory potential of phlorotannin rich fermented *E. cava* processing by-product extract; and Part-III deals with the anticancer and anti-inflammatory potentials of bioactive components from edible sea cucumber *H. edulis*. The findings of this work might be useful in the applications of the bioactive components derived from the selected marine organisms in medicinal, nutraceutical and functional food applications in relation to human health.

Fig. 2. The brown seaweed *Ecklonia cava*

Fig. 3. The edible sea cucumber *Holothuria edulis*

Part I

Value-added fermentation of *Ecklonia cava* processing by-product and its antioxidant effect

ABSTRACT

The interest in the extraction of polyphenolic compounds from plant materials is nowadays focused on upgrading of the large amount of by-products coming from food or cosmetics industries from which the press residues have particularly high contents of polyphenols. In this study, processing by-product of a brown seaweed *Ecklonia cava* which can be obtained after polyphenolic extraction was fermented by edible yeast *Candida utilis* and its antioxidant potentials were evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl and alkyl radical scavenging using electron spin resonance (ESR) spectrometer. *E. cava* processing by-product was fermented for 1~4 days prior to being extracted with 80% EtOH and significant differences were observed in extraction yields, total phenolic contents (TPC) and radical scavenging activities with the fermentation time. Extract from the *E. cava* processing by-product fermented for 1 day exhibited the highest TPC and also found to be the strongest antioxidant. One-day fermented *E. cava* processing by-product extract (FEPBE) strongly enhanced the cell viability against H_2O_2-induced oxidative damage in Vero cells. Phlorotannin rich FEPBE exhibited good protective properties against H_2O_2-induced cell apoptosis as was demonstrated by a decreased quantity of sub-G1 hypodiploid cells and decreased apoptotic body formation on the flow cytometry analysis and nuclear staining. The results of this study demonstrated that, the fermentation elevated functionally important polyphenolic contents of *E. cava* processing by-product and resultant antioxidant activities were enhanced. Therefore, the fermentation could offer a tool to further increase the bioactive potential of low-valued *E. cava* processing by-product.

1. INTRODUCTION

Reactive oxygen species (ROS) form as a natural by-product of the normal metabolism of oxygen and play important roles in cell signaling. (Seifried et al., 2007). However, these compounds, when present in a high enough concentration, can attack biological molecules and leading to cell or tissue injury associated with degenerative diseases such as atherosclerosis, cancer, diabetes mellitus and inflammation (Fig. I-1) (Amarowicz et al., 2004; Choi et al., 2002; Shibata et al., 2008). Cells are normally able to defend themselves against ROS damage with the use of enzymes. Small molecule antioxidants such as vitamin C, vitamin E, uric acid, and glutathione also play important roles as cellular antioxidants. Similarly, polyphenol antioxidants assist in preventing ROS damage by scavenging free radicals (Cai et al., 2002; Jacob et al., 2008; Lotito and Frei, 2004; Zubia et al., 2007).

The scientific interest in the isolation and characterization of natural active metabolites for use in pharmaceuticals, nutraceuticals, cosmeceuticals and functional foods has considerably increased in last recent years (Byeon et al., 2012). Use of synthetic additives especially in food applications is limited in various countries by specific regulations (Kintzios et al., 2010). In addition, several studies have been conducted using synthetic drugs, which can cause undesirable side effects (Mohamed et al., 2012; Park and Jhon, 2010). Therefore, a large number of scientific studies have been carried out to demonstrate various biological properties of extracts or isolated compounds from various plant materials which can be used in many industrial applications as active ingredients. In the last recent years, studies about the extraction of polyphenolic compounds from natural products have attracted special attention (Shahidi et al., 2006). The antioxidant activity of plasma has been shown to increase after consumption of foods high in antioxidants (Araujo

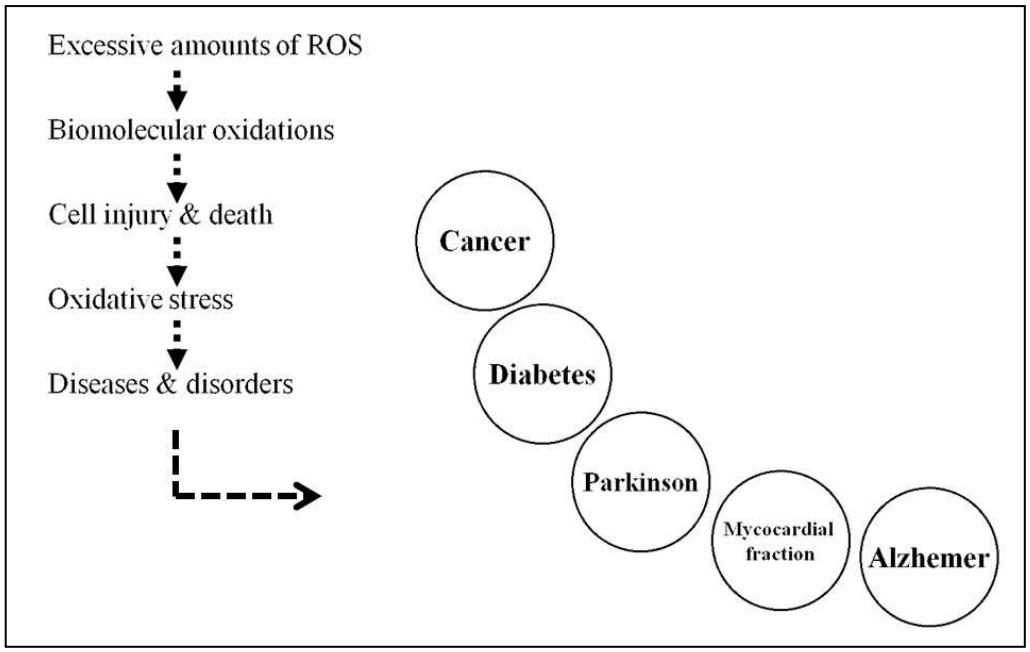

Fig. I-1. ROS can attack biological molecules and leading to cell or tissue injury associated with degenerative diseases.

et al., 2011; Liyana-Pathirana and Shahidi, 2007). Thus, phytochemicals may combat oxidative stress in the human body by maintaining a balance between oxidants and antioxidants (Temple, 2000).

It is well established that brown algae contain polyphenolic compounds with strong antioxidant activity (Heo et al., 2009). Furthermore, it has been reported that the brown seaweed *E. cava* has numerous biological activities including antioxidant (Kim et al., 2006; Li et al., 2009), anticoagulative (Athukorala et al., 2006a), anti inflammatory (Kim and Bae, 2010) and anticancer (Athukorala et al., 2006b; Kong et al., 2009). These beneficial effects have been attributed to its variety of chemical ingredients, including polyphenols. Thus, the exploitation of bioactive metabolites of *E. cava* for relevant applications as a source of functional ingredients is highly considerable (Fig. I-2).

Fermentation is a very interesting process used in plant foods to increase the nutritional quality and remove undesirable compounds (Martha et al., 2005). In addition, several products of microbial fermentation are also incorporated into food as additives and supplements such as antioxidants, flavours, colourants, preservatives and sweeteners (Couto and Sanroman, 2006). Mould fermented foods play an important role, especially in Asian countries where the production process for many foods includes a fungal fermentation (Geisen and Farber, 2001). Microorganisms play a central role in the production of a wide range of primary and secondary metabolites. The edible yeast *Candida utilis* (Fig. I-3) is generally recognized as a safe (GRAS) substance by the Food and Drug Administration and it is an industrially important microorganism (Miura et al., 1998). It is used in the production of several biologically useful materials, such as glutathione, and certain amino acids and enzymes (Kondo et al., 1995).

Recycling of the by-products or food wastes has been a very interesting research field. However, as yet little practical effort to utilize the food wastes for phenolic recovery has been reported from seaweeds. In addition, only a few by-product derived antioxidants have been developed successfully from the vast quantities of plant residues produced by the food processing industry (Peschel et al., 2006). However, currently after polyphenol extraction, by-product of *E. cava* has been disposed as fertilizer or directly discarded as a processing waste. But, this *E. cava* processing by-product still seems to contain useful components which can be recycled after improve the quality by fermentation. Hence, it is interesting to find a use for low-valued or wasted residue as a bio-resource for the development of functional foods, nutraceuticals and functional cosmetics. Considering these aspects, part I aims towards the assessment of the antioxidant activity of *E. cava* processing by-product fermented by *C. utilis*. Attempts have also been made to quantify the potential bioactive compounds which can be potential candidates for the industrial applications as functional ingredients.

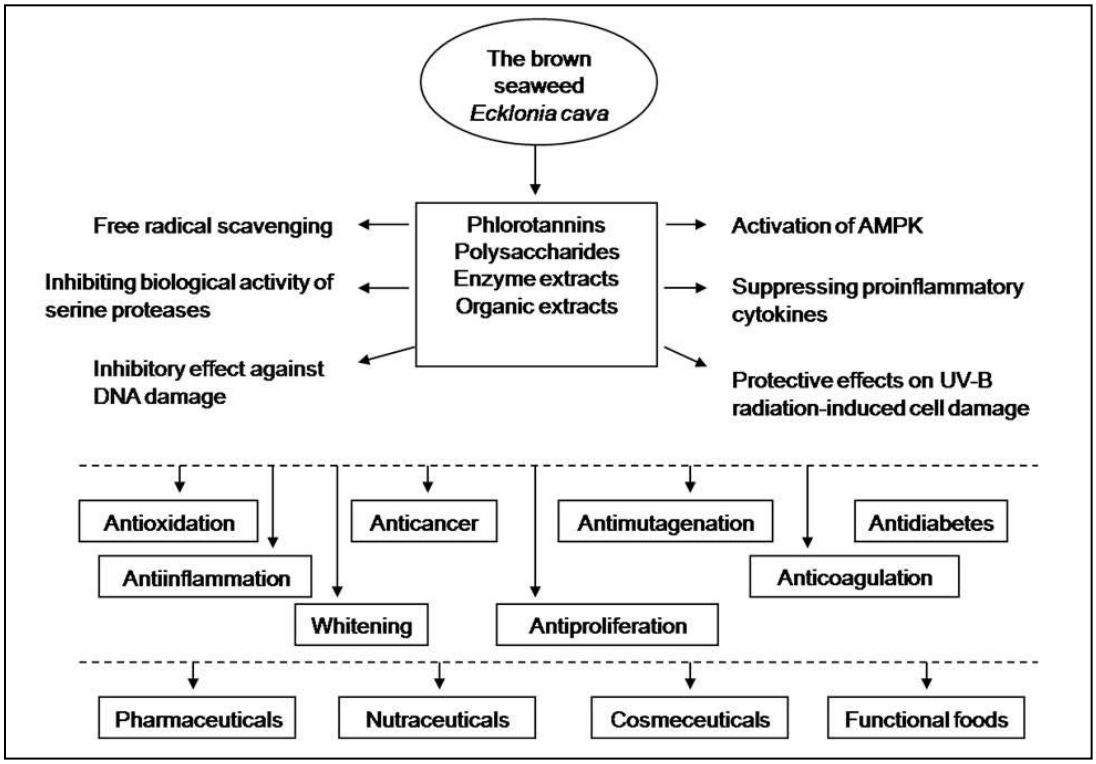

Fig. I-2. Schematic presentation of biological properties and potential industrial uses of functional ingredients from brown seaweed *E. cava.*

Fig. I-3. The edible yeast *C. utilis.*

2. MATERIALS AND METHODS

2.1. Materials and reagents

E. cava residue which is a processing by-product obtained after polyphenolic extraction was provided by Aqua Green Tech Co., Ltd., Jeju, South Korea. The yeast *C. utilis* ATCC 9950 originated from a pure culture was used as the microorganism for the fermentation process.

1, 1-diphenyl-2-picrylhydrazyl (DPPH), 5,5-Dimethyl-1-pyrrolin N-oxide (DMPO), 2, 2-azobis (2- amidinopropane) hydrochloride (AAPH), α-(4-pyridyl-1- oxide)-N-t-butylnitrone (4-POBN), 2', 7'-dichlorodi-hydrofluorescein diacetate (DCHF-DA), 3- (4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl tetrazolium bromide (MTT), Dulbecco's modified eagle medium (DMEM), dimethyl sulfoxide (DMSO), propidium iodide (PI) and Hoechst 33342 were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other reagents were of the highest grade available commercially.

2.2. Fermentation of *E. cava* processing by-product

The fermentation process was carried out according to the method described by Dordevic et al. (2010) with slight modifications. *E. cava* processing by-product powder (150 g) was homogenized with 3 L of distilled water in a 10 L flask. Then the mixture was autoclaved and allowed to cool before the addition of the fungus. After that the mixture was inoculated with 1% (w/w) of *C. utilis*. Then the sample was allowed to ferment under 120 rpm at 30°C for 1~4 days. Control sample was prepared without any inoculation. Each sample was prepared in triplicates. Fig. I-4 shows the essential steps involved in the fermentation of *E. cava* processing by-product.

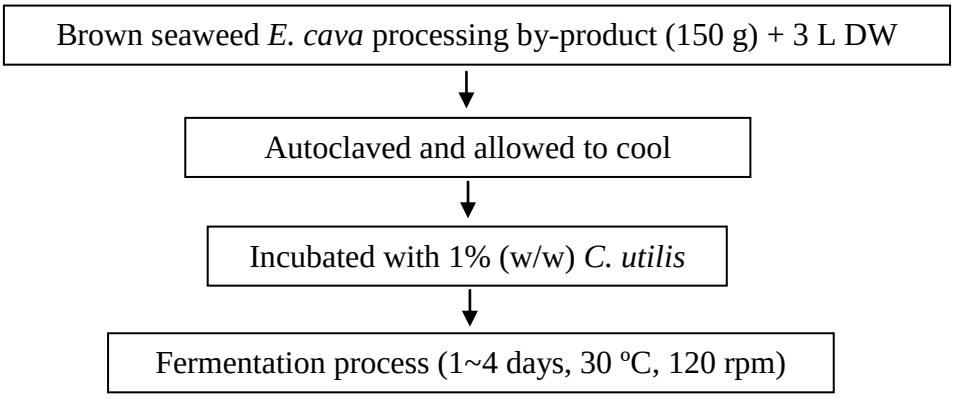

Fig. I-4. Essential steps involved in the fermentation process of *E. cava* processing by-product.

2.3. Preparation of sample extract from fermented *E. cava* processing by-product

Fermented *E. cava* processing by-product was freeze-dried and homogenized prior to extraction. Sample extracts were prepared using 80% EtOH. The fermented sample (10 g) was extracted using 80% EtOH (1L) at room temperature for 24 h in a shaking incubator (120 rmp). Then it was filtered and the filtrate was evaporated under vacuum at 40 °C to obtain a dry extract. Fig. I-5 shows the essential steps involved in the preparation of fermented *E. cava* processing by-product extract.

2.4. Determination of total phenolic content

Total phenolic content was determined according to the protocol described by Chandler and Dodds (1983). 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.

2.5. DPPH radical scavenging assay

DPPH radical scavenging activity was measured according to the method described by Nanjo et al. (1996) using electron spin resonance (ESR) spectrometer (Fig. I-6). Eighty percent EtOH solution of 60 μL of each sample (or 80% EtOH itself as a control) was added to 60 μL of DPPH (60 μM) in methanol. After mixing vigorously for 10 sec, the solutions were transferred into a 100 μL Teflon capillary tube and fitted into the cavity of the ESR spectrometer (JES-FA; Jeol, Tokyo, Japan). The spin adduct was measured on the

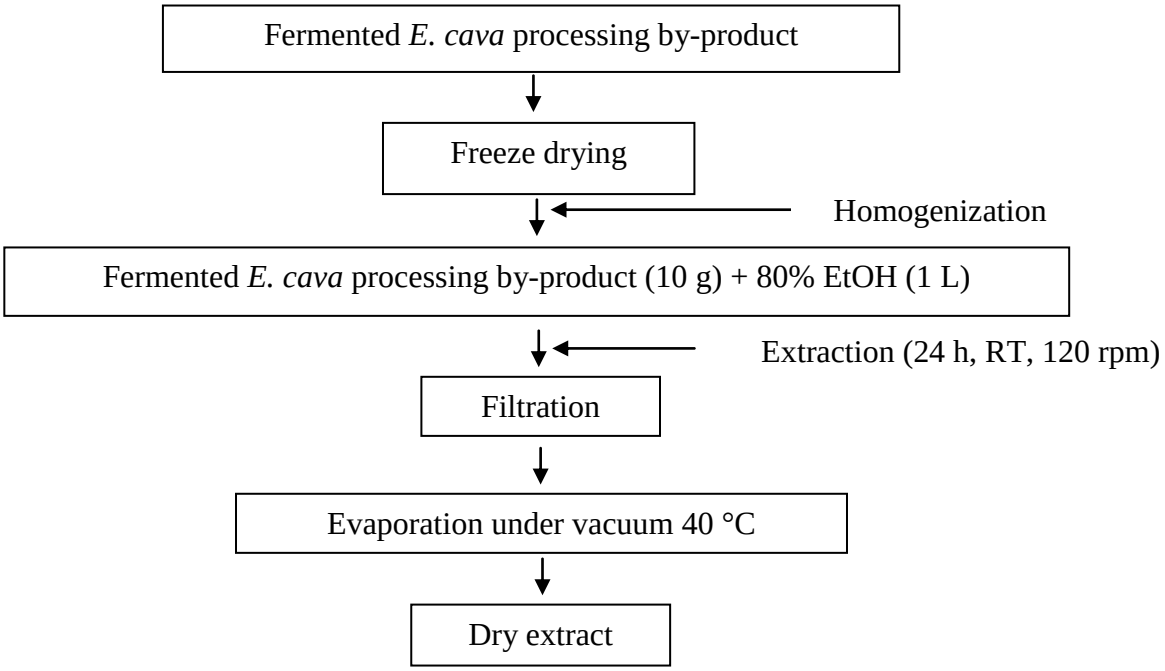

Fig. I-5. Essential steps involved in the preparation of sample extract from fermented *E. cava* processing by-product.

Fig. I-6. Electron spin resonance (ESR) spectrometer.

ESR spectrometer exactly 2 min later. Measurement conditions were as follows. Central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5, and temperature 298 K.

2.6. Hydroxyl radical scavenging assay

Hydroxyl radicals were generated by Fenton reaction, and reacted rapidly with nitrone spin trap DMPO; the resultant DMPO-OH adducts were detectable with an ESR spectrometer (Rosen and Rauckman, 1980). The ESR spectrum was recorded 2.5 min after mixing in a phosphate buffer solution (pH 7.4) with sample 20 μL, 0.3 M DMPO 20 μL, 10 mM $FeSO_4$ 20 μL and 10 mM H_2O_2 20 μL using an ESR spectrometer set at the following conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 1 mW, gain 6.3×10^5, and temperature 298 K.

2.7. Alkyl radical scavenging assay

Alkyl radicals were generated by AAPH. The phosphate buffered solution (pH 7.4) reaction mixtures containing 10 mm/L AAPH, 10 mM/4-POBN and indicated concentrations of testing samples, were incubated at 37˚C in a water bath for 30 min (Hiramoto et al., 1993), and then transferred to a 100 μL Teflon capillary tube. The spin adduct was recorded on a JESFA ESR spectrometer. Measurement conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 10 mW, gain 6.3×10^5, temperature 298 K.

2.8. Determination of triphlorethol-A, eckol, dieckol and eckstolonol contents

Each phlorotannin content of 80% EtOH extracts from non-fermented and fermented *E. cava* processing by-product were analyzed according to the method described by Lee et al. (2010). The HPLC system (YoungLin Instrument, Gyeonggi, Korea) comprising the following components: Acme 9000 UV/Vis spectrometer, gradient pump, vacuum degasser, and mixer were used for the determination of each phlorotannin. The column used a RP-C18 column (250×4.6 mm, 5 μM, Waters, Milford, MA, USA). For HPLC analysis, mobile phases used in the gradient elution consisted of primary eluant (A) consisting of 10% methanol and 0.04% trifluoroacetic acid (TFA), and a secondary eluant (B) consisting of 100% methanol and 0.04% TFA. Ten percent solvent A changed in the linear gradient to 100% of B 40 min after injection. The flow rate was 1 mL/min, the column temperature was at room temperature (20°C), and sample volume injected was 20 μL. The absorbance was measured at a wavelength 230 nm for the detection of each phlorotannin. Identical standard curves were obtained for the calibration each phlorotannin contents.

2.9. Cell culture

A Vero cell line was cultured in DMEM containing 10% heat-inactivated calf serum, streptomycin (100 μg/ml) and penicillin (100 unit/ml) at 37°C in an incubator, under a humidified atmosphere containing 5% CO_2.

2.10. Determination of intracellular ROS generation (DCHF-DA assay)

For the cell based assays, the sample was dissolved in DMSO and further diluted in culture media. Intracellular ROS generation was determined using oxidation-sensitive dye DCHF-DA according to the method described by Engelmann et al. (2005). The Vero cells were seeded in 96-well plate at a concentration of 1.0×10^5 cells/mL. After 16 h of incubation time at 37°C the cells were treated with 10 μL of different concentrations of the sample (25,

50 and 100 µg/mL) and incubated at 37°C under a humidified atmosphere. After 30 min H_2O_2 was added at a concentration of 1mM and then the cells were incubated for an additional 30 min in the same conditions. Finally DCHF-DA was introduced to the cells and DCHF-DA fluorescence was detected at an excitation wavelength of 485 nm and the emission wavelength of 535 nm, using a Perkin-Elmer LS-5B spectrofluorometer (Walthman, MA, USA). The percentage of intracellular ROS scavenging activity was calculated in accordance with the following equation:

Intracellular ROS scavenging activity (%) = $[1-(C_1/C_0)] \times 100$

Where; C_1 is the fluorescence intensity of cells treated with H_2O_2 and sample extract, and C_0 is the fluorescence intensity of cells treated with H_2O_2 and distilled water instead of the sample extract.

2.11. Determination of cell viability (MTT assay)

Cell viability was estimated using MTT assay according to the method described by Mosmann (1983). The Vero cells were seeded in 96-well plate at a concentration of 1.0×10^5 cells/mL. After 16 h of incubation time at 37°C the cells were treated with 10 µL of the sample at different concentrations (25, 50 and 100 µg/mL) and incubated at 37°C under a humidified atmosphere for 1 h. Then, 10 µL of H_2O_2 (1 mM) was added and incubated for 24 h at 37°C. After that MTT stock solution (50 µL; 2 mg/mL) was added to each of the wells, to a total reaction volume of 200 µL. After 4 h of incubation time the plates were centrifuged for 5 min at 800 rpm and the supernatant was aspirated. The formazan crystals in each well were dissolved in 150 µL of dimethylsulfoxide (DMSO) and absorbance was measured using enzyme linked immune sorbent assay (ELISA) reader (Sunrise; Tecan Co. Ltd., Australia) at 540 nm. Relative cell viability was evaluated in accordance with the

quantity of MTT converted to the insoluble formazan salt. The optical density of the formazan generated in the control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

2.12. Nuclear staining with Hoechst 33342

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33342. Cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis (Gschwind and Huber, 1995; Lizard et al., 1995). The Vero cells were seeded in 24-well plates at a concentration of 1.0×10^5 cells/mL. Sixteen hours after seeding, the cells were treated with various concentrations of the sample (25, 50 and 100 μg/mL), and further incubated for 1 h at 37°C in a humidified atmosphere prior to exposure to H_2O_2 (1 mM). After 24 h, 1.5 μL of Hoechst 33342 (stock 10 mg/mL), a DNA-specific fluorescent dye, was added to each well, followed by 10 min of incubation at 37°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro colour digital camera, in order to examine the degree of nuclear condensation.

2.13. Flow cytometry analysis

Flow cytometry analyses were conducted in order to determine the proportion of apoptotic sub-G1 hypodiploid cells (Lizard et al., 1995). The Vero cells were placed in 6-well plates at a concentration of 1.0×10^5 cells/mL, and 16 h after plating the cells were treated with various concentrations of the sample (25, 50 and 100 μg/mL). After 1 h additional incubation, H_2O_2 (1 mM) was added to the culture. Then after 24 h, the cells were harvested at the indicated time, and fixed for 30 min in 1 mL of 70% EtOH at 4°C. The

cells were then washed twice with PBS, and incubated for 30 min in darkness in 1 mL of PBS containing 100 μg PI and 100 μg RNase A, at 37°C. Flow cytometric analysis was conducted using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA). Effects on the cell cycle were determined by measuring changes in the percentage of cell distribution at each phase of the cell cycle, and were assessed by histograms generated by the Cell Quest and Mod-Fit computer programs (Nicoletti et al., 1991).

2.14. Statistical analysis

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

3. RESULTS

3.1. Effect of fermentation on extraction yield and TPC

It was found that 80% EtOH extract from brown seaweed *E. cava* has a significantly higher extraction yield compared to the extracts from *E. cava* processing by-product. However, among the extracts from fermented *E. cava* processing by-product, significantly higher yield was obtained from the extract fermented for 1 day (Fig. I-7). According to the results, prolonged fermentation reduced the extraction yields.

In addition, considerable variations were found in TPC, ranging from 20.80 to 36.5% (Fig. I-8). The extract from *E. cava* showed significantly higher TPC compared to the extracts from fermented *E. cava* processing by-product. However, among the extracts from *E. cava* processing by-product, the fermented samples exhibited higher TPC than non-fermented sample indicating that fermentation has a significant effect on TPC. In contrast, the highest TPC was observed in the extract fermented for 1 day. This is in agreement with the extraction yield because the same extract exhibited the highest yield among the *E. cava* processing by-product samples.

3.2. Effect of fermentation on radical scavenging activities

All the fermented samples examined were found to possess higher radical scavenging activities than the non fermented sample. Among the fermented samples, the strongest radical scavenging activities with the lowest IC_{50} values were obtained from the 1-day fermented sample while the weakest radical scavenging activities with the highest IC_{50} values were obtained from the sample fermented for 4 days (Table I-1). It was observed that the extract containing the highest level of TPC was also the strongest potent radical

scavenger. By contrast, in DPPH radical scavenging activities, 1-day fermented sample showed almost 3 fold stronger activity than the non-fermented sample while in hydroxyl radical scavenging activity, the same sample showed 2.7 fold stronger activity than the non-fermented sample. In addition, 1.8 fold stronger activity was observed for the 1-day fermented sample than non-fermented sample in alkyl radical scavenging activities.

Vitamin C was used as the reference antioxidant in this study and respective radical scavenging values of vitamin C were superior to those of the fermented and non-fermented samples. However, the DPPH radical scavenging activity of the 1-day fermented sample was comparable with the activity of vitamin C.

The 1-day fermented *E. cava* processing by-product extract (FEPBE) was used in the further experiments due to its profound radical scavenging properties.

3.3. Effect of fermentation on the phlorotannin content

According to some previous reports associated with biological properties of *E. cava*, phlorotannins are surely the key compounds. Therefore, in the present study we determined the each phlorotannin content of FEPBE using HPLC analysis. Figure I-9 shows the HPLC chromatogram and the chemical structures of the corresponding phlorotannin compounds. As shown in the table I-2, except eckstolonol all the other phlorotannins were considerably higher in the FEPBE than the extract obtained from non-fermented *E. cava* processing by-product. Ultimately, fermentation found to be playing a crucial role in the elevation of total phlorotannin content in the FEPBE. In addition, dieckol is the prominent phlorotannin and triphlorethol-A found to be the least in both fermented and non-fermented extracts.

3.4. Effect of FEPBE on intracellular ROS scavenging activity

In the present work, we investigated the intracellular ROS scavenging activity of FEPBE. The DCF fluorescence was reached to the peak in the cells treated only with H_2O_2. However, treatment with the extracts from FEPBE decreased the fluorescence in the Vero cells (Fig. I-10). In contrast, the fluorescence intensity decreased gradually, when the sample concentration increased indicating that the activity is dose dependant. Moreover, the scavenging activity of the FEPBE on intracellular ROS dose dependently increased as 29.05, 38.92 and 52.02 % at the concentrations of 25, 50 and 100 μg/mL respectively.

3.5. Protective effect of FEPBE on H_2O_2-induced cell damage

The protective effect of FEPBE on H_2O_2-induced cellular damage is shown in Fig. I-11. As shown in the figure, H_2O_2 treatment without the sample decreased cell viability to 51.94 %, while the FEPBE prevented cells from H_2O_2-induced cellular damage, restoring cell survival to 79.75, 88.71 and 95.32% at the concentrations of 25, 50 and 100 μg/mL respectively. In contrast, the results of MTT assay showed that formazan content was reduced due to H_2O_2 treatment, however, significantly increased with the addition of the phlorotannin rich FEPBE.

3.6. Protective effect of FEPBE on H_2O_2-induced apoptosis in Vero cells

Due to the profound antioxidative activity evidenced by FEPBE; it was further evaluated with regard to its ability to protect against H_2O_2-induced cell apoptosis. Clear image of the negative control exhibited no cell apoptosis (Fig. I-12A). But, typical fluorescence photographs of shrunken nuclei, chromatic condensation and appearance of apoptotic bodies were taken of the Vero cells after 24 h of H_2O_2 treatment (Fig. I-12B). However,

when the cells were treated with the sample at the concentrations of 25 and 50 μg/mL 1 h prior to H_2O_2 treatment, a considerable reduction for apoptotic bodies was observed (Fig. I-12C and D).

3.7. Effect of FEPBE on cell cycle pattern

As shown in the Fig. I-13, in the cell cycle analysis of DNA contents, control showed little sub-G_1 phase (9.7%). But, DNA content analysis conducted following the H_2O_2 treatment of Vero cells revealed an increase in the proportion of cells with sub-G_1 DNA content to 47%. However, the cells that pre-treated with the sample were evidenced significantly reduced sub-G_1 DNA contents (23.17% and 16.43%).

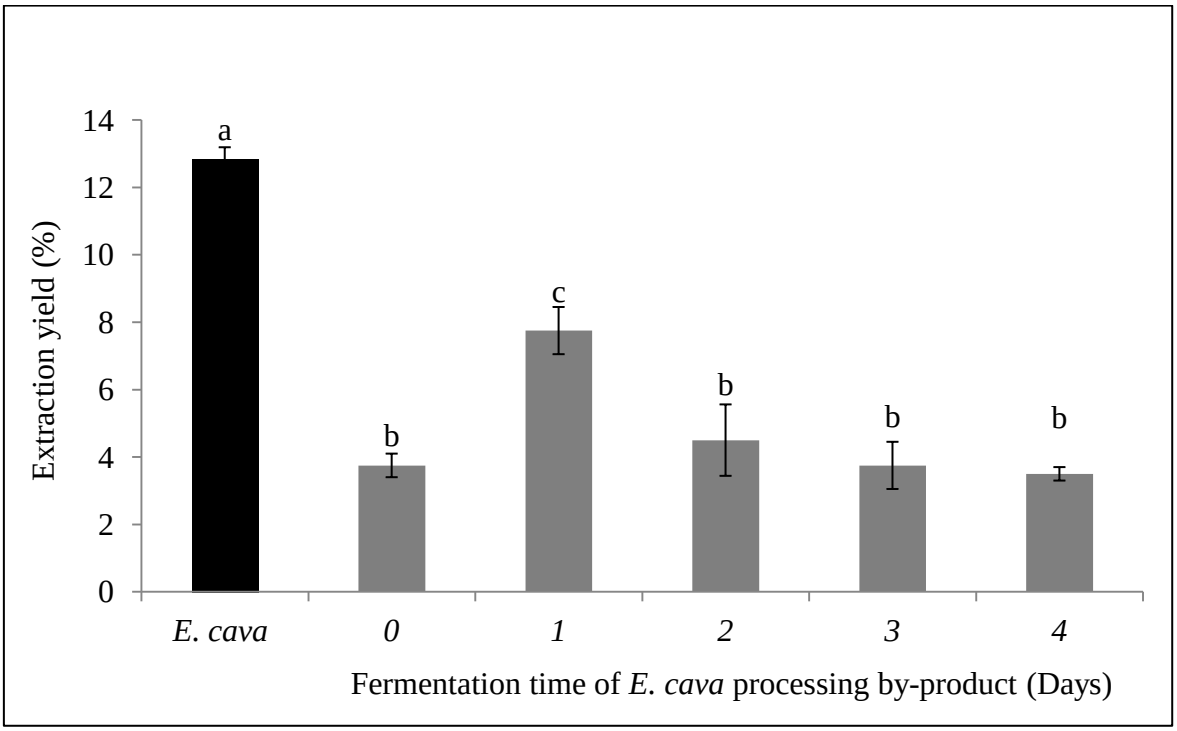

Fig. I-7. Extraction yields in 80% EtOH extracts from brown seaweed *E. cava* and fermented *E. cava* processing by-product. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

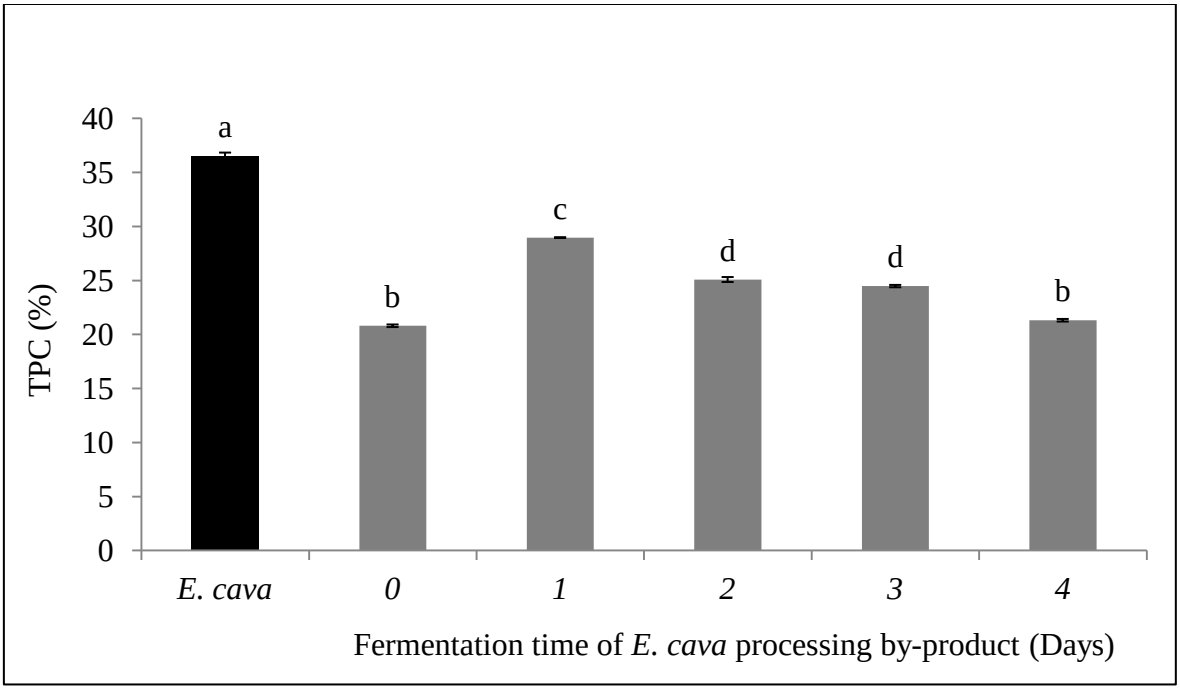

Fig. I-8. Total phenolic content in 80% EtOH extracts from brown seaweed *E. cava* and fermented *E. cava* processing by-product. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

Table I-1. Radical scavenging activities of the 80% EtOH extracts from *E. cava* processing by-product.

Fermentation time (Days)	IC_{50} values[a] (μg/mL)		
	DPPH radical	Hydroxyl radical	Alkyl radical
0	31.17 ± 0.23	271.86 ± 7.31	68.50 ± 2.06
1	10.58 ± 1.87	99.21 ± 0.53	35.46 ± 2.00
2	12.48 ± 1.72	113.31 ± 3.91	47.51 ± 2.21
3	13.95 ± 1.06	123.76 ± 7.10	40.20 ± 2.60
4	20.83 ± 1.89	171.52 ± 4.48	48.99 ± 0.53
Ascorbic acid[b]	9.28 ± 1.48	30.28 ± 1.98	21.44 ± 2.31

[a]IC_{50} value was defined as the necessary concentration at which the radicals generated by reaction systems were scavenged by 50%, respectively.

[b] Standard antioxidant.

Values are mean ± SD of three determinations.

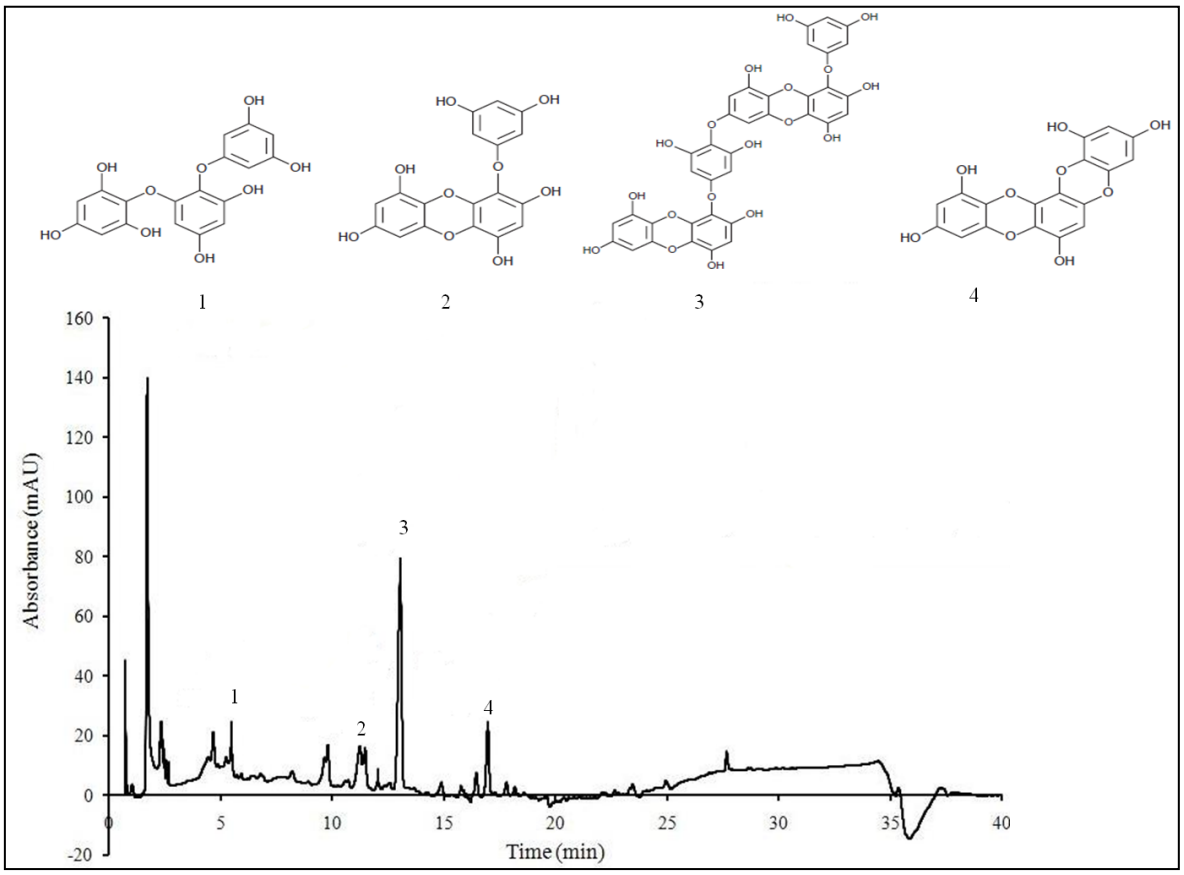

Fig. I-9. HPLC chromatogram of FEPBE and the chemical structures of the corresponding phlorotannin compounds. Column: 150 × 2.9 mm. Separation was performed with a gradient from 10-100% methanol in 40 min at a flow rate of 0.8 mL/min. Elution was monitored at 230 nm. Injection volume 10 µL (1 mg/mL). (1) - triphlorethol-A, (2) - eckol, (3) - dieckol and (4) - eckstolonol.

Table I-2. Phlorotannin contents of non-FEPBE and FEPBE.

Sample	Phlorotannin content (mg/g)				
	TA	EK	DK	ES	Total
Non-FEPBE	2.6	13.0	41.1	10.3	67.0
FEPBE	7.1	18.8	49.4	8.6	83.9

TA - triphlorethol-A, EK - eckol, DK - dieckol and ES - eckstolonol.

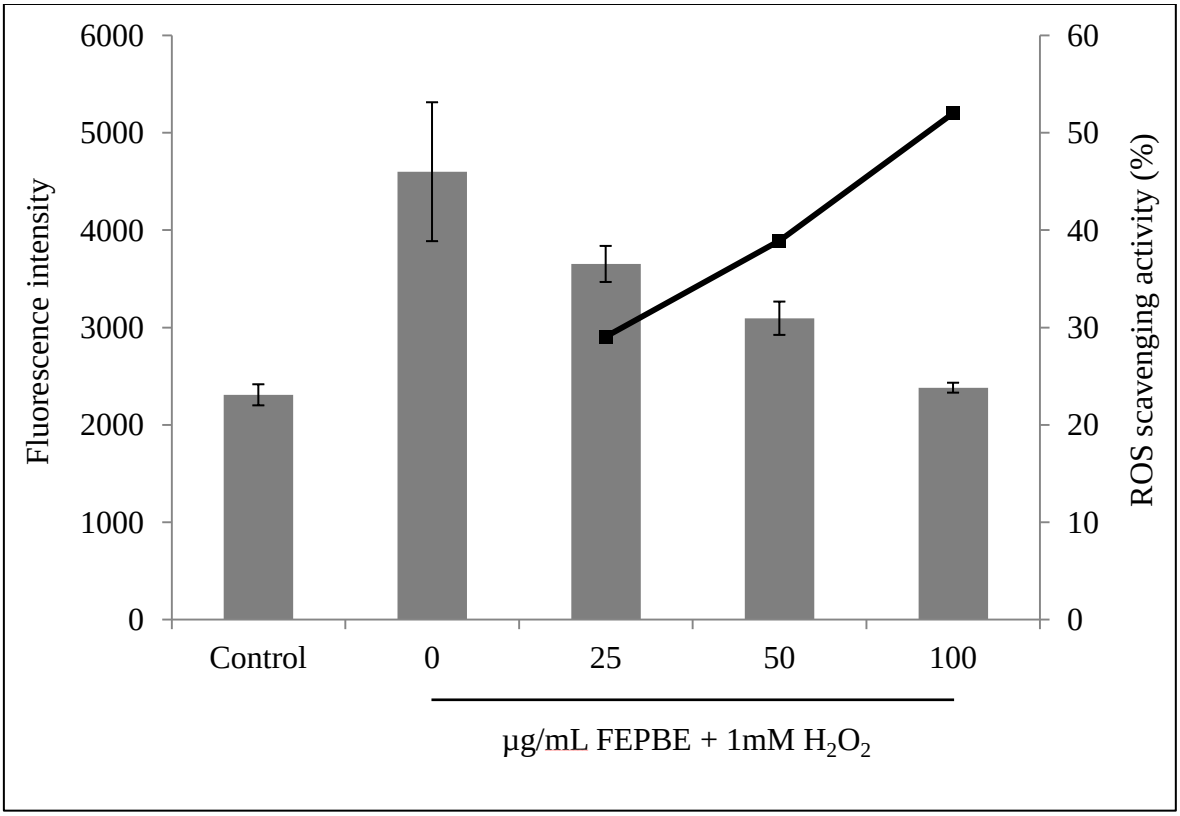

Fig. I-10. Intercellular ROS scavenging activity of FEPBE against H_2O_2-induced oxidative damage in Vero cells. The intercellular ROS generation was determined by DCHF-DA assay using spectrofluorometry. Values are mean ± SD of three determinations.

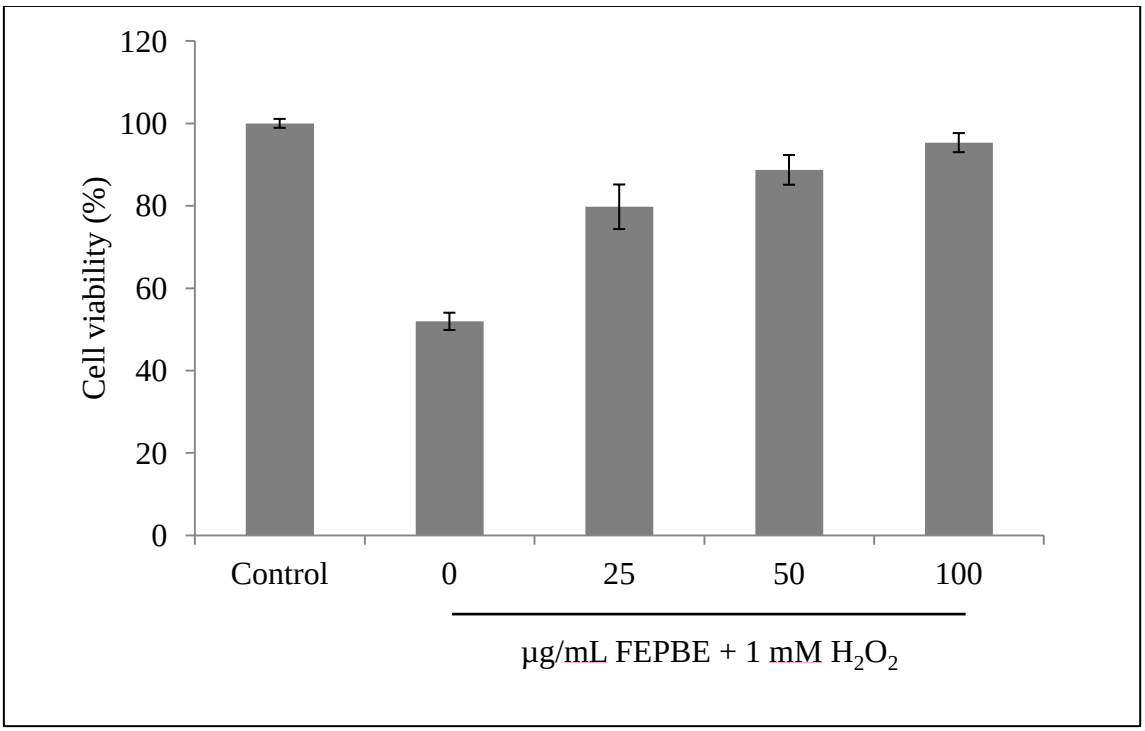

Fig. I-11. Protective effect FEPBE against H_2O_2-induced oxidative damage in Vero cells. The viability of cells on H_2O_2 treatment was determined by MTT assay. Values are mean ± SD of three determinations.

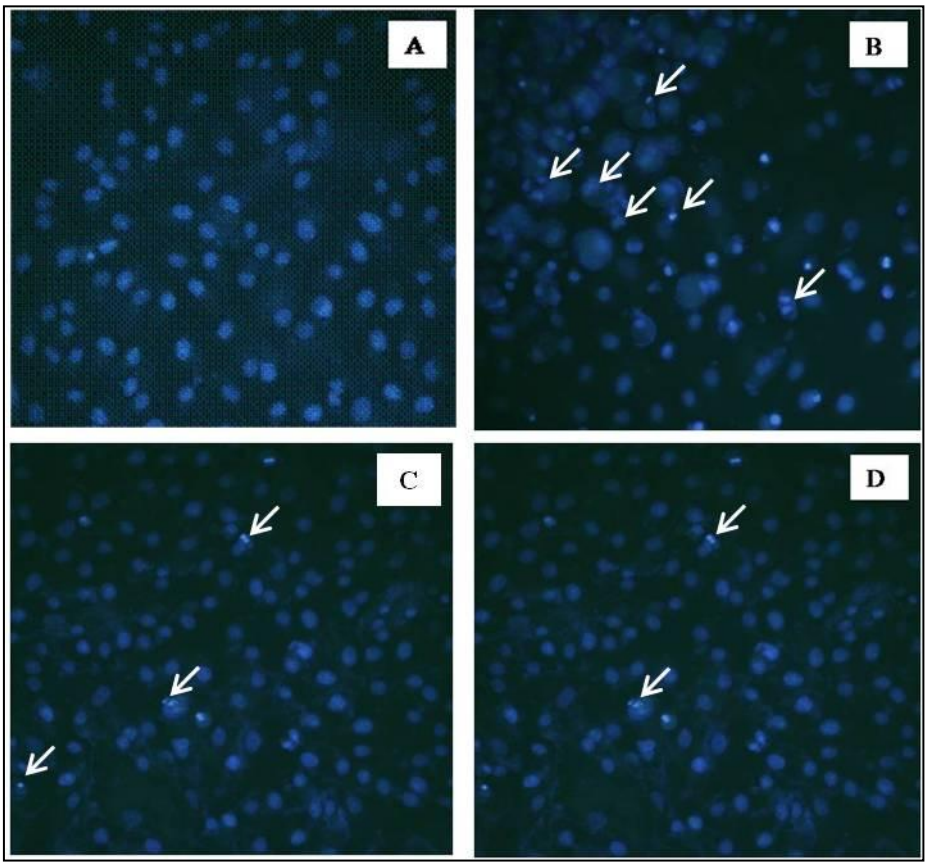

Fig. I-12. Protective effect of FEPBEagainst H_2O_2-induced cell apoptosis in Vero cells. Apoptotic body formation was observed under a fluorescent microscope after Hoechst 33342 staining and is indicated by arrows. (A) Control, (B) H_2O_2-treated, (C) H_2O_2 + 50 μg/mL and (D) H_2O_2 + 100 μg/mL.

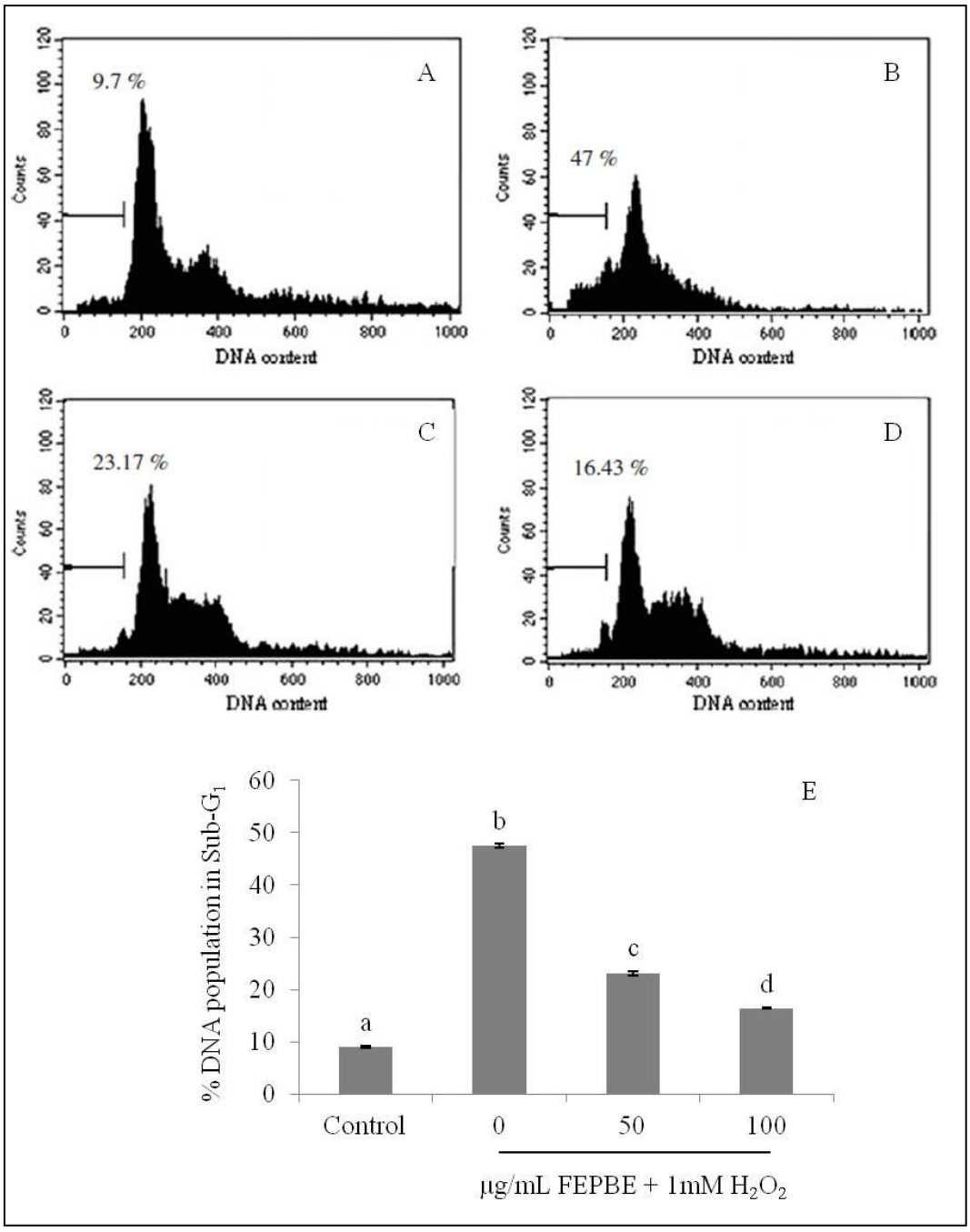

Fig. I-13. Effect of FEPBE on cell cycle pattern and apoptotic cell proportion in Vero cells. Apoptotic sub-G1 DNA content was detected by flow cytometry after propidium iodide staining. (A) Control, (B) H_2O_2-treated, (C) H_2O_2 + 50 μg/mL, (D) H_2O_2 + 100 μg/mL and (E) bar graph for sub-G1 peach patterns of Vero cells treated with FEPBE against H_2O_2mediated cell damage.

4. DISCUSSION

The present work made the first attempt to demonstrate antioxidant activity of the brown seaweed *E. cava* processing by-product. Hence, these results provide the first report of potential antioxidant activity of FEPBE. The results of the present study demonstrated that the *E. cava* processing by-product still retained important polyphenolic concentrations and thus might be considered as a potential source of antioxidants.

Naturally occurring polyphenols are today among the most talked about classes of phytochemicals. In the last decade, much work has been presented by the scientific community, which focuses on plant polyphenols (Dimitrios, 2006). Extraction is the first step in the isolation of phenolic compounds from plant materials. In addition, extraction is influenced by the chemical nature of the compounds, the extraction method employed and the presence of interfering substances (Chirinos et al., 2007). Polyphenolic compounds are generally more soluble in polar organic solvents than in water. In contrast, the effective extractants recommended are aqueous mixtures of methanol, ethanol and acetone (Wang et al., 2009).

Spin tapping is the most direct method to detect highly reactive free radicals generated for short times (Ahn et al., 2007, Kopani et al., 2006). In this study, radical scavenging activities FEPBE were evaluated against DPPH, hydroxyl and alkyl free radicals using ESR technique. This method consequently provides an excellent way to examine the potential antioxidant ability in the tested samples. DPPH is a stable free radical and has been commonly used to screen phenolic compounds containing high free radical scavenging ability (Lee et al., 2007a). Rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction. Therefore, it has been

widely used to evaluate the antioxidant activity of natural antioxidants. Hydroxyl radicals are highly reactive free radicals which are capable of biological substrates such as carbohydrates, polyunsaturated fatty acids, proteins and DNA. Hydroxyl radical generated in the Fenton system (Fe^{2+}/H_2O_2) was tapped by DMPO, forming spin adduct which could be detected by ESR spectrometer (Li et al., 2009). The alkyl radical spin adduct was observed when AAPH was incubated with the spin trap 4-POBN at 37˚C for 30 min and these radicals were measured using an ESR spectrometer.

In this study, phlorotannin rich FEPBE found to be the most potent radical scavenger among the tested samples. Federica et al. (2004) reported that, DPPH radical scavenging activity has positive correlation with those of total phenolic compounds. In addition, it is reported that remarkable alkyl radical scavenging activity of phlorotannins purified from the brown seaweed *E. cava* (Ahn et al., 2007). Hence, it can be suggested that, phlorotannins of the FEPBE may be the principal constituents responsible for the antiradical properties of the extracts. Moreover, with these results it is clear that fermentation has a significant effect on induced antioxidant activity.

To date, the DCHF-DA assay has been extensively used to measure the levels of oxidative stress and ROS generation in many cell types in response to a variety of stimuli or environmental stresses (Loetchutinat et al., 2005). It has been demonstrated that in the presence of ROS, DCHF is oxidized to highly fluorescent DCF. It is well known that H_2O_2 is the principal ROS responsible for the oxidation of DCHF to DCF (LeBel et al., 1992). The results of this study indicate that FEPBE has efficient antioxidant properties. In addition, one of the previous experiments evidenced H_2O_2 scavenging ability of enzymatic extracts of the brown seaweed *E. cava* (Kim et al., 2006).

In this work, to evaluate whether FEPBE protect from cellular damage induced by H_2O_2, Vero cells were pretreated with sample for 24 h in the absence or presence of oxidative stress. MTT is commonly used to measure the number of metabolically active cells in *in vitro* assays (Young et al., 2005). It is a colourimetric assay and based on the conversion of yellow, water-soluble MTT to the purple, water insoluble end product, formazan, by mitochondrial dehydrogenase. The amount of formazan formed is proportional to the number of metabolically active cells (Mossman, 1983). The results of the current study imply that the sample has the ability to protect Vero cells from oxidative stress-related cellular damages. By contrast, FEPBE is very much responsible as an antioxidant resource. Recently, Kang et al., (2007) reported that phlorotannins isolated from *E. cave* showed antioxidant and cytoprotective effects against oxidative stress; triphlorethol-A protected cell damage from H_2O_2 and radiation induced oxidative stress via radical quenching effect. In the present study, in addition to the morphological evaluations, the protective effects against H_2O_2-induced cell apoptosis exerted by the FEPBE were confirmed via flow cytometry. The obtained results indicated that cell apoptosis was induced by the treated H_2O_2 and the inhibition activities on the H_2O_2-induced cell apoptosis and reduced sub-G_1 DNA contents might be related to the antioxidant properties of phlorotannins. One of the previous studies also reported that, phlorotannins purified from *E. cava* have the potential inhibitory effect on H_2O_2-mediated DNA damage and harmful free radicals (Ahn et al., 2007).

5. CONCLUSION

In conclusion, *E. cava* processing by-product can be recovered as a value-added fraction after fermentation with *C. utilis*. Further, the by-product exhibited the highest TPC and antioxidant activity after fermenting for 1 day. Antioxidant properties were reduced by prolonged fermentation. However, this study demonstrated that, *E. cava* processing by-product still retained important phenolic concentrations and it is a good source of phlorotannins and antioxidants. Moreover, their recovery and further application would provide commercial value to the waste fraction. Thus, the phlorotannin rich FEPBE becomes an attractive value-added segment, with many industrial applications including functional foods nutraceuticals and functional cosmetics.

Part II

Bioactive components recovered from *Ecklonia cava* processing by-product attenuate inflammatory response by suppressing inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and pro-inflammatory cytokines in lipopolysaccharide-stimulated RAW 264.7 macrophages

ABSTRACT

In this study, fermentation was employed as a tool to further increase the bioactive potential of processing by-product from a brown seaweed *Ecklonia cava*, which can be obtained from food and cosmetic industries after its polyphenol extraction. The fermentation process was done for 24 h using an industrially important microorganism *Candida utilis* prior to being extracted with 80% EtOH. Part I confirmed that the fermentation process can be employed in order to enhance the availability of bioactive compounds in low valued *E. cava* residue. Major bioactive components in the fermented *E. cava* processing by-product extract (FEPBE) are phlorotannins including triphlorethol-A (7.1 mg/g), eckol (18.8 mg/g), dieckol (49.4 mg/g) and eckstolonol (8.6 mg/g). Dieckol is the most prominent phlorotannin among them. The anti-inflammatory potential of the FEPBE was evaluated *in vitro.* The phlorotannin rich FEPBE dose-dependently inhibited the nitric oxide (NO) production, prostaglandin-E_2 (PGE_2) production and suppressed the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions in lipopolysaccharide-stimulated RAW 264.7 cells. The release of pro-inflammatory cytokines, interleukin-1β (IL-1β) and interleukin-6 (IL-6) was significantly reduced by the extract in a dose-dependent manner. However, the extract did not show any inhibitory effect on the release of cytokine tumor necrosis factor (TNF-α) at the tested concentrations. Due to the profound anti-inflammatory activity, FEPBE appears as a value-added biomass fraction that can be exploited in numerous industrial applications as a source of functional ingredients.

1. INTRODUCTION

Inflammation is a complex biological response of vascular tissues to harmful stimuli and it is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue. Macrophages play a key role in inflammation (Kazlowska et al., 2010). As an inflammatory response activated cells such as macrophages release increased amounts of NO, PGE_2 and cytokines such as TNF-α, IL-1ß and IL-6. NO production is controlled by the nitric oxide synthases (NOS), which include iNOS, endothelial NOS (eNOS) and neuronal NOS (nNOS). In contrast, iNOS is highly expressed in macrophages; its activation leads to organ destruction in some inflammatory and autoimmune diseases (Yoon et al., 2009). In addition, PGE_2 is another important inflammatory mediator and is produced from arachidonic acid metabolites by the catalysis of COX-2. A number of inflammatory stimuli, such as LPS, activate immune cells to up-regulate such inflammatory states, and these are therefore useful targets in the development of new anti-inflammatory therapeutic agents and exploration of the molecular anti-inflammatory mechanisms of a potential drug (Zeilhofer and Brune, 2006).

Anti-inflammatory agents can be used to treat inflammatory reactions and most of these agents act by preventing the release of inflammatory mediators or inhibiting the actions of releasing mediators on their target cells (Fig. II-1). Nowadays, research has been focused on finding anti-inflammatory agents with less toxicity. As a result of increasing demand, vital research attempts are on the way for discovering bioactive agents from natural resources, especially from marine organisms (Chandini et al., 2008; Mohsin and Kuruo, 2011).

Polyphenols have emerged as one major category of natural products important to human health. Increasing scientific evidence shows that polyphenols are good antioxidants, are effective in preventing cardiovascular and inflammatory diseases, and can also be used as chemo-preventative agents for cancer (Zhang et al., 2006). Phlorotannins (brown algal polyphenols) derived from the members of the Phaeophyta have long been considered important secondary metabolites with a role in biological functions (Wijesinghe and Jeon, 2012a).

The fermentation process can be defined as the breakdown of complex substrates into a mixture of simple organic compounds without net oxidation (Lucas et al., 2007). Over the history, fermentation has been employed to improve the nutritional quality, flavour, aroma and functional properties of foods (Ibanoglu and Ibanoglu, 2001). Many biochemical changes occur during fermentation, leading to an altered ratio of plant metabolites, which affect product properties such as extractability and bioactivity (Dordevic et al., 2010; Xu et al., 2008).

In the previous section, it was successfully demonstrated the value-added fermentation of *E. cava* processing by product and its antioxidant effect (Part I). In this study, as a part of search for plant based bioactive components, the potential anti-inflammatory activity of the fermented *E. cava* processing by-product extract (FEPBE) was evaluated for the first time. Hence, in this study, FEPBE has been evaluated as a potential source of phlorotannin compounds with anti-inflammatory activity. Thus, the aim of part II is to evaluate the anti-inflammatory potential of the phlorotannin rich FEPBE and explore the use of this value-added by-product as a source of functional ingredients.

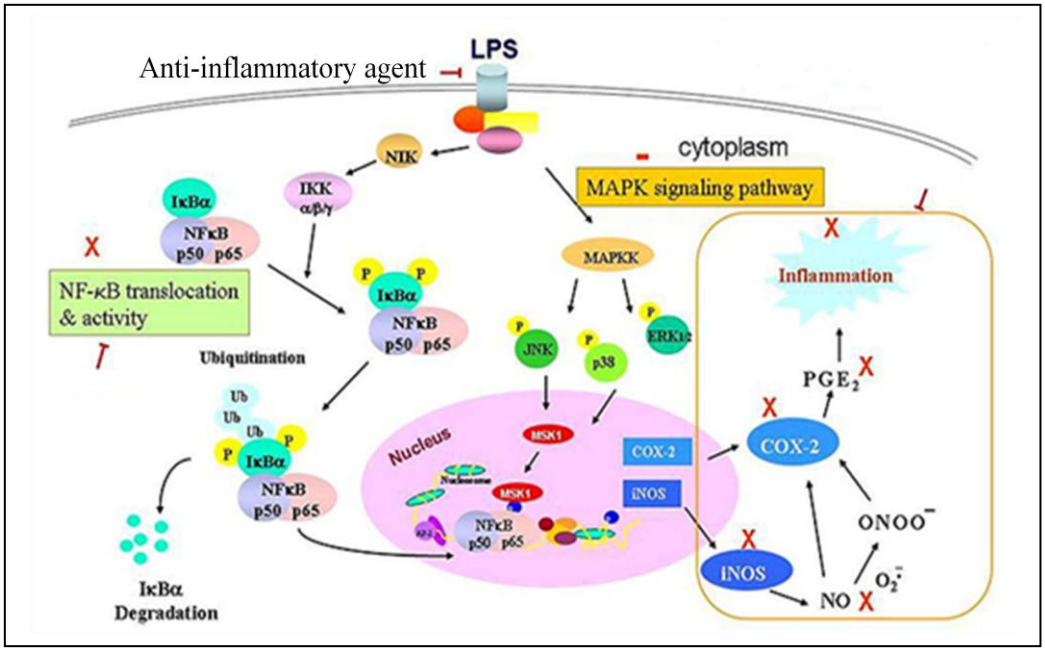

Fig. II-1. Inhibitory mechanism of an anti-inflammatory agent on LPS-induced inflammation.

2. MATERIALS AND METHODS

2.1. Materials and reagents

E. cava residue which is a processing by-product obtained after polyphenolic extraction was provided by Aqua Green Tech Co., Ltd., Jeju, South Korea. The yeast *Candida utilis* ATCC 9950 originated from a pure culture was used as the microorganism for the fermentation process.

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO Inc., NY, USA. All other chemicals and reagents were analytical grade.

2.2. Fermentation of *E. cava* processing by-product and preparation of FEPBE

The fermentation of *E. cava* processing by-product was done according to the previously described method (Dordevic et al., 2010; Wijesinghe et al., 2012). Briefly, 150 g of the *E. cava* processing by-product powder was homogenized with 3 L of distilled water in a 10 L flask and the mixture was autoclaved prior to the addition of the yeast. Then it was allowed to cool and inoculated with 1% (w/w) *C. utilis*. After that the mixture was fermented in a shaking incubator (120 rpm) at 30 °C for 24 h. Fermented *E. cava* processing by-product was lyophilized and homogenized prior to extraction. The fermented sample (10 g) was extracted using 80% ethanol (1L) at room temperature for 24 h in a shaking incubator (120 rmp). Then it was filtered and the filtrate was evaporated under vacuum at 40 °C to obtain the dry extract (Fig. II-2). As reported in the part I total phlorotannin content in the extract is 83.9 mg/g and triphlorethol-A (7.1 mg/g), eckol (18.8 mg/g), dieckol (49.4 mg/g) and

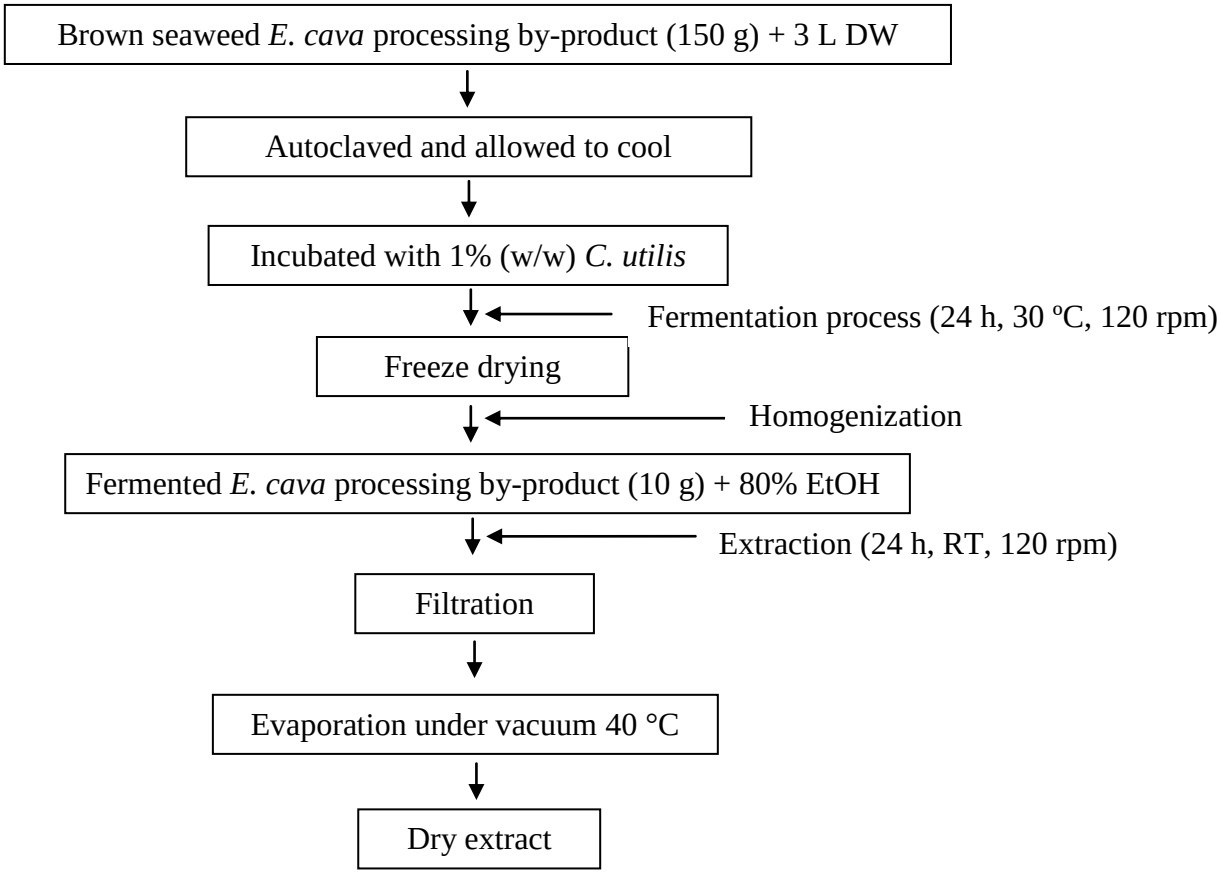

Fig. II-2. Essential steps involved in the fermentation of *E. cava* processing by-product and preparation of the sample extract.

eckstolonol (8.6 mg/g) are the major bioactive phlorotannin compounds in the FEPBE.

2.3. Cell culture

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). 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 °C. The cells were sub cultured every 2 days and exponential phase cells were used throughout the experiments.

2.4. Determination of NO production

RAW 264.7 cells (1.0×10^5 cell/mL) were plated in a 24-well plate and after 16 h the cells were pre-incubated with various concentrations of the sample at 37 °C 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 (Lee et al., 2007b). 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 enzyme-linked immunosorbent assay (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 °C. 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 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 (1μg/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 °C. Then protein contents in the supernatant were measured using the BCATM 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 (Cho et al., 2000). Briefly, RAW 264.7 cells (1.0×10^5 cells/mL) were pretreated with the sample for 2 h and then treated with LPS (1μg/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

3.1. Effect of FEPBE on LPS-induced NO production and LDH release in RAW 264.7 cells

Since NO produced by iNOS is one of the inflammatory mediators, the effects of various concentrations of FEPBE on NO production in LPS-activated RAW 264.7 cells 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 extract decreased the NO production at all the tested concentrations (Fig. II-3). In addition, as confirmed by the LDH assay the extract did not show any cytotoxic effect on RAW 264.7 cells at the tested concentrations. Thus, FEPBE can be considered as a potential agent for suppressing NO production without any cytotoxic effect.

3.2. Effect of FEPBE on LPS-induced PGE_2 production

According to the results of the present study, FEPBE strongly suppressed LPS-induced PGE_2 production in a dose-dependent manner (Fig. II-4). In contrast, the extract suppressed the PGE_2 production by 74.4% at the concentration of 10 μg/mL. This result indicated that FEPBE might induce the anti-inflammatory activity by strongly suppressing the PGE_2 production in RAW 264.7 macrophages.

3.3. Effect of FEPBE on LPS-induced iNOS and COX-2 protein expression

In order to further characterize the inhibitory effects of FEPBE on NO and PGE_2 production, Western blot analysis was performed. Therefore, in this study, the inhibitory effects of FEPBE on iNOS and COX-2 proteins were confirmed by means of Western blot analysis. The iNOS and COX-2 protein expression were markedly increased when the

macrophages treat only with LPS compared to the control without LPS and the sample (Fig. II-5). It is strikingly noticeable both in iNOS and COX-2. However, pretreatment with the sample significantly inhibited both iNOS and COX-2 protein expression in a dose-dependent manner. Moreover, iNOS and COX-2 protein expressions were completely suppressed at the concentration of 10 μg/mL. All these results clearly demonstrated that FEPBE inhibited the production of NO by decreasing iNOS protein expression and inhibited the production of PGE_2 by decreasing COX-2 protein expression.

3.4. Effect of FEPBE on LPS-induced pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) production

The inhibition of cytokine production or function is a key mechanism in the control of inflammation because inflammatory stimulators such as LPS induce cytokines in the process of macrophage activation, which mediates tissue response in different phases of inflammation. Therefore, we evaluated the inhibitory effects of FEPBE on the production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in LPS-stimulated RAW 264.7 macrophages. However, the extract did not show any inhibitory effect on the production of cytokine TNF-α at the tested concentrations (Fig. II-6). But, it was found that the pretreatment of macrophages with the sample strongly inhibited the production of cytokines IL-1β and IL-6 (Fig. II-7 and II-8). In contrast, the extract inhibited the production of IL-1β and IL-6 by 70. 49% and 91.6% respectively at the concentration of 10 μg/mL. Therefore, our results suggest that the inhibition pathway of iNOS by the extract might be associated with the attenuation of IL-1β and IL-6 formation.

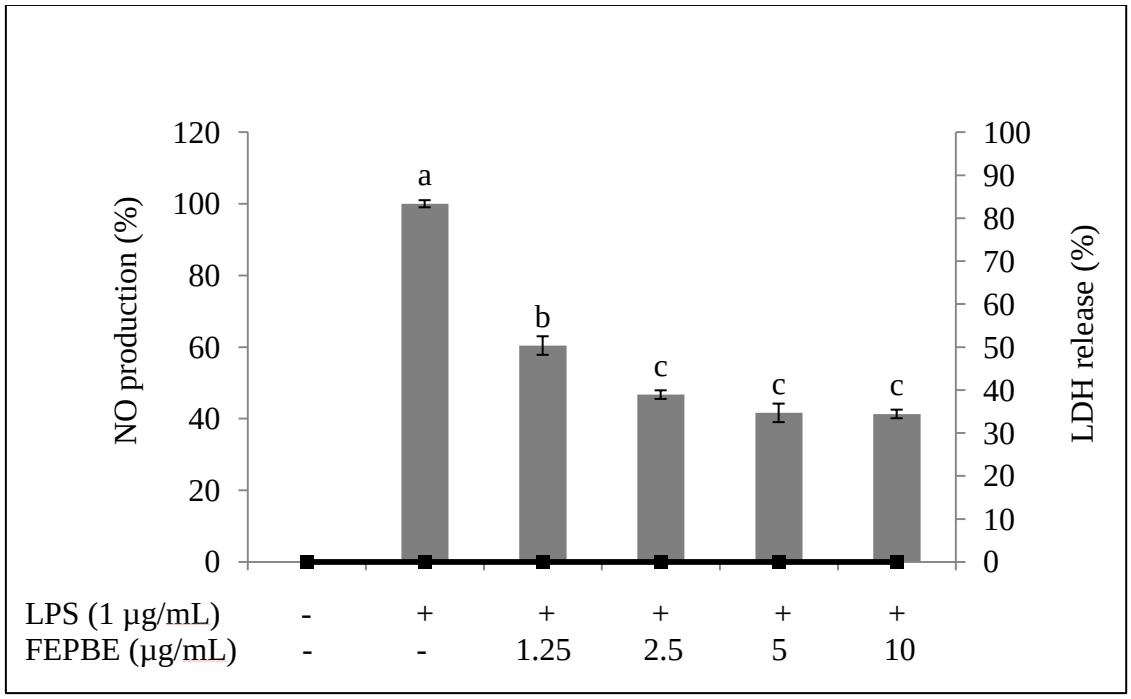

Fig. II-3. Inhibitory effect of FEPBE on LPS-induced NO production and LDH release in RAW 264.7 macrophages. Incubation of the extract with cells in response to LPS (1 μg/mL) for 24 h, the NO levels in the medium were measured. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

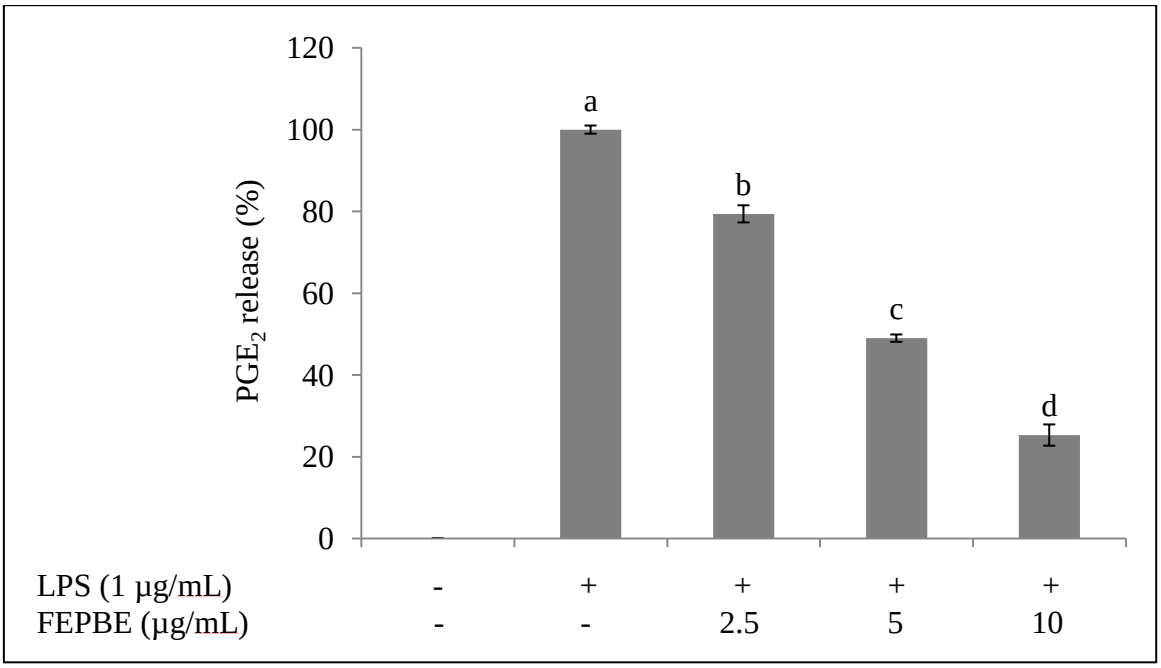

Fig.II-4. Inhibitory effect of FEPBE on LPS-induced PGE_2 production in RAW 264.7 macrophages. After incubation of cells with LPS for 24 h in the presence or absence of the extract, the concentrations of PGE_2 in the medium were measured. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

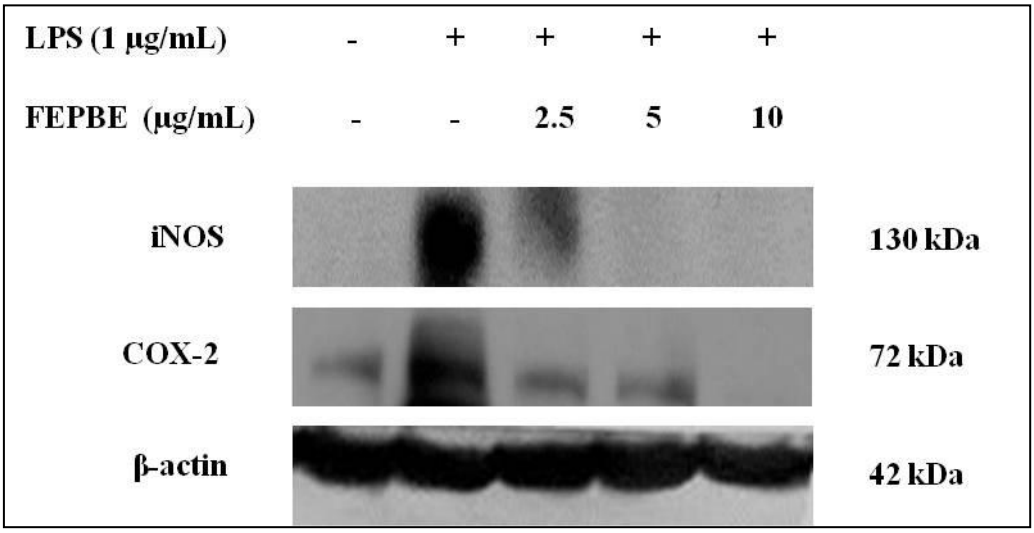

Fig. II-5. Inhibitory effect of FEPBE on LPS-induced iNOS and COX-2 protein expression in RAW 264.7 macrophages. The cells were incubated with LPS for 24 h in the presence or absence of the extract. Then, cell lysates were electrophoresed and the expression levels of iNOS and COX-2 were detected with specific antibodies.

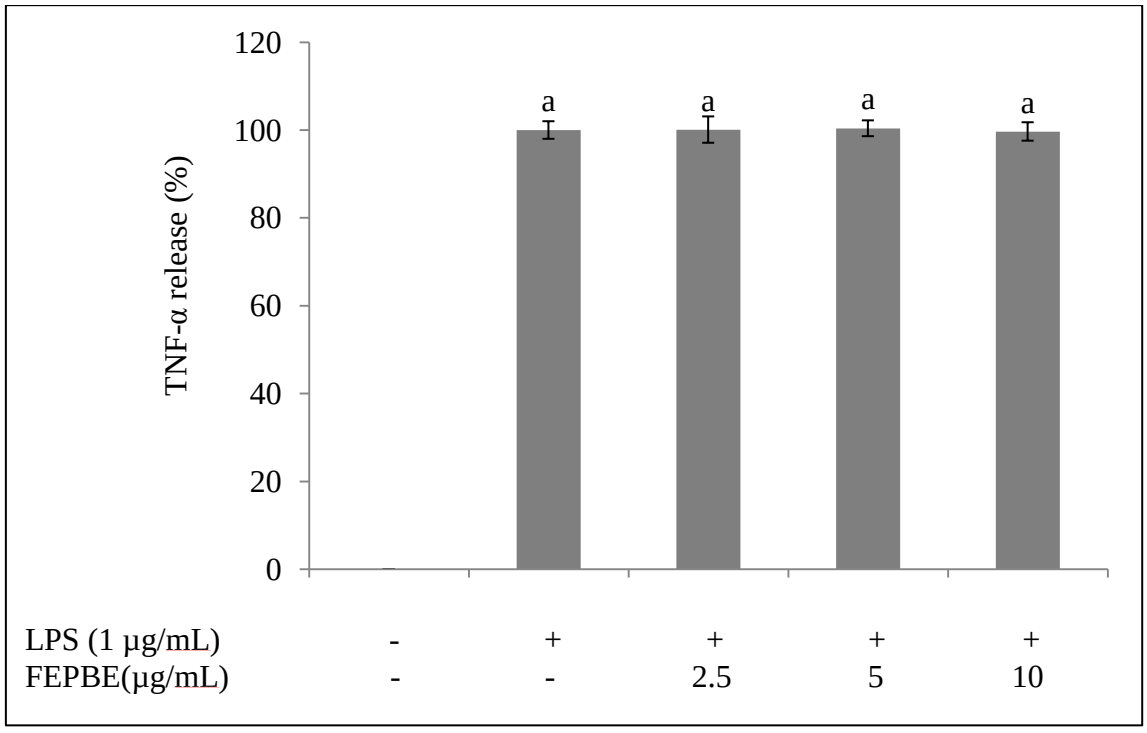

Fig. II-6. Inhibitory effect of FEPBE on LPS-induced TNF-α production in RAW 264.7 macrophages. After incubation of cells with LPS for 24 h in the presence or absence of the extract, the concentration of TNF-α in the medium was measured. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

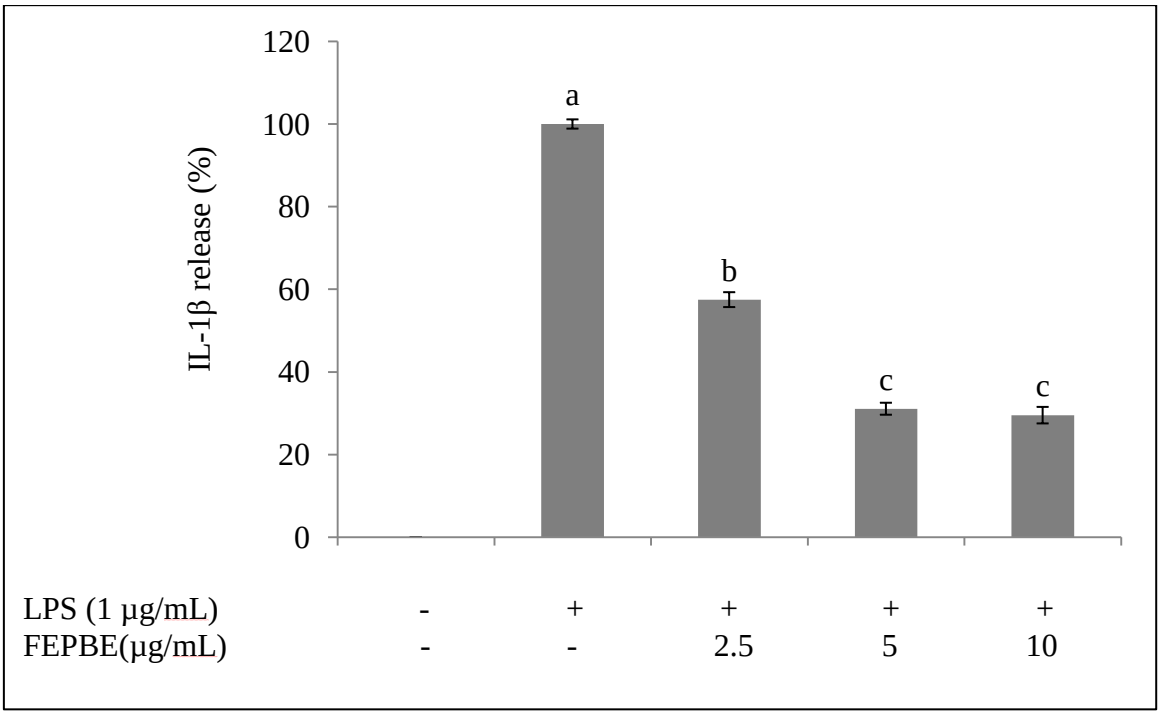

Fig. II-7. Inhibitory effect of FEPBE on LPS-induced IL-1β production in RAW 264.7 macrophages. After incubation of cells with LPS for 24 h in the presence or absence of the extract, the concentration of IL-1β in the medium was measured. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

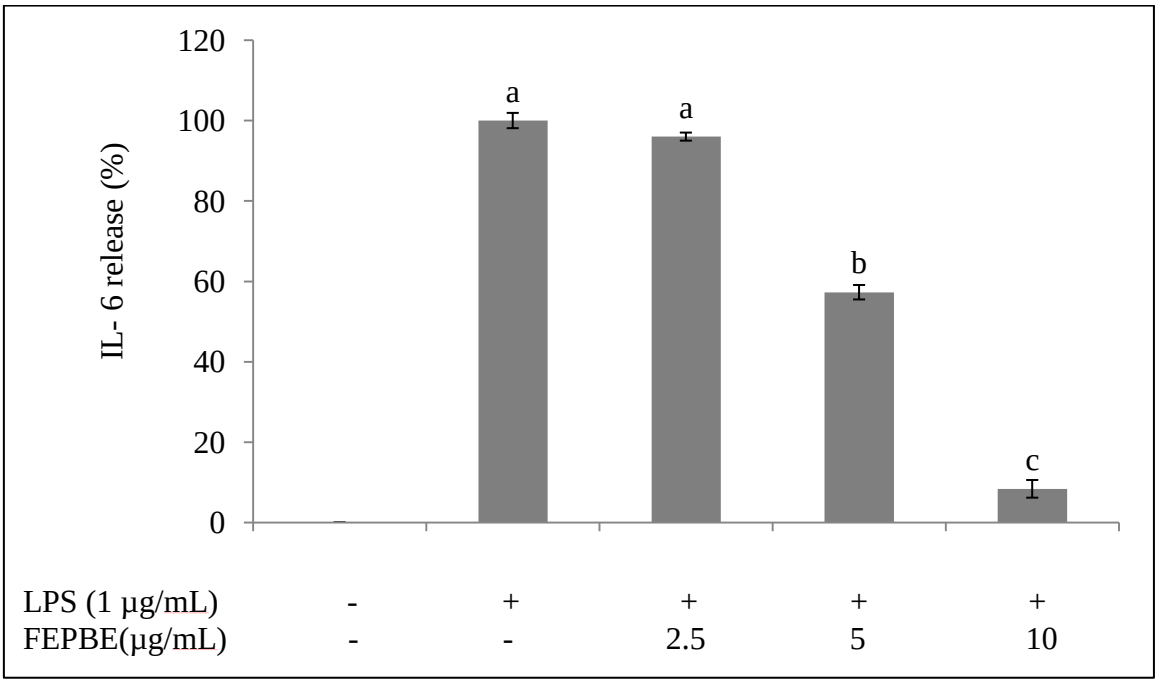

Fig. II-8. Inhibitory effect of FEPBE on LPS-induced IL-6 production in RAW 264.7 macrophages. After incubation of cells with LPS for 24 h in the presence or absence of the extract, the concentration of IL-6 in the medium was measured. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

4. DISCUSSION

Natural bioactive metabolites in both terrestrial and marine environment have received much attention from pharmaceutical, nutraceutical, cosmeceutical and functional food interest. Therefore, the biological activities of these bioactive components have been assessed in numerous studies both *in vitro* and *in vivo*. With today's interest in bioactive ingredients, the seaweeds represent potential source to be explored (Vishchuk et al., 2011). During the past years, brown seaweeds attracted much attention as a source of phlorotannins and polysaccharides with various biological activities (Eldeen et al., 2009, Heo et al., 2009). Brown seaweed *E. cava* attracted extensive interest due to its multiple biological activities. This brown seaweed has been identified as a potential producer of a wide spectrum of natural substances. In addition, studies have identified a great number of biologically active phlorotannin compounds from the brown seaweed *E. cava* such as eckol, dieckol, 6, 6,'bieckol, eckstolonol and triphlorethol-A (Wijesinghe and Jeon, 2012b). Therefore, brown algae *E. cava* may expand its value in various industries due to its wide range of biological activities (Ahn et al., 2007).

The results of the part I confirmed that FEPBE contain considerable amount of polyphenolic compounds. In addition, in the part I also determined the phlorotannin content in the FEPBE. In contrast, as previously reported, triphlorethol-A, eckol, dieckol, and eckstolonol are the major phlorotannin compounds in the extract and dieckol is the most prominent phlorotannin among them. Moreover, the fermentation process played a crucial role to enhance the availability of bioactive constituents of the *E. cava* processing by-product. Since the phlorotannin content of the FEPBE is considerably high, further use of this value-added by-product is also apparent. Thus, in the present section, we evaluate the potential anti-inflammatory activity of the phlorotannin rich FEPBE.

Nitric oxide plays crucial roles in many cellular functions in the nervous, cardiac, vascular, and immune systems, and also acts as an intracellular and intracellular signal molecule (Wang et al., 2006). Low levels of NO production are important in protecting organs such as liver from ischemic damage. However, the chronic expression of NO is associated with various carcinomas and inflammatory conditions, and NO is also generated by macrophages as a part of the human immune responses (Kassim et al., 2010). Moreover, under pathological conditions, NO production is increased by iNOS (Kim et al., 1999; Suh et al., 2003). Therefore, inhibition of NO production by suppression of iNOS expression may have potential therapeutic value when related to inflammation.

With the profound inhibitory effect on NO production evidenced by FEPBE, it was further evaluated with regard to its ability to inhibit the LPS-induced PGE_2 production in RAW 264.7 macrophages. Prostaglandin synthesis requires the coordinated activity of multiple enzymes and PGE_2 is the most abundant prostanoid in humans which is involved in regulating many different fundamental biological functions (Nakagawa, 2011; Stebulis et al., 2008). In addition to their important mediator role in the inflammatory process, prostaglandins play a pivotal role in maintaining the homeostasis of various tissues (Filaretova et al., 2002). Induction of COX-2 activity and subsequent generation of PGE_2 are closely related to the NO production (Cahng et al., 2006). COX-2 enzymatic activity catalyzes the first committed step in prostaglandin synthesis (Savonenko et al., 2009). Inhibition of iNOS, the enzyme mediating macrophage NO production has been shown to block prostaglandin release in RAW264.7 macrophages (Ahmed et al., 2002). Some of the previous reports demonstrated that certain active compounds might have the potential to affect NO production and iNOS enzyme activity (Kim et al., 1999). Inhibition of iNOS, the enzyme mediating macrophage NO production has been shown to block prostaglandin

release in RAW264.7 macrophages (Ahmed et al., 2002). In addition, COX-2 enzymatic activity catalyzes the first committed step in prostaglandin synthesis (Savonenko et al., 2009). Some of the previous reports demonstrated that certain active compounds might have the potential to affect NO production and iNOS enzyme activity (Kim et al., 1999). Hence, the inhibitory effects of FEPBE on NO and PGE_2 production was further characterized using Western blot analysis. According to our results, it could be suggested that FEPBE inhibited the NO production by decreasing both iNOS and COX-2 protein expression in RAW 264.7 cells.

Cytokines, the chemical messengers between immune cells, play crucial roles in mediating inflammatory and immune responses. They comprise a group of small polypeptides possessing tremendous diversity in their potential actions. These peptides have typically been classified as either pro-inflammatory or anti-inflammatory, based on their actions in peripheral tissues (Smith et al., 2012; You et al., 2011). The pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 which are mainly produced by activated monocytes or macrophages, stimulate bone resorption and also enhance the production of PGE_2 in several types of cells (Park et al., 2004). It is well known that a high concentration of pro-inflammatory cytokines plays a critical role in the induction of iNOS through activation of NF-κB (D'Orazio et al., 2012; Fernandes et al., 2010). Thiemermann et al. (1993) investigated the endogenous production of TNF-α contributes to the induction of iNOS in response to LPS in a rat model. Further, they reported the significance of the inhibitors of iNOS as therapeutically important agents. Since the inflammatory stimulators such as LPS induce cytokines in the process of macrophage activation, the inhibition of cytokine production or function is a key mechanism in the control of inflammation (Hseu et al., 2005).

Phenolic antioxidants inhibit the induction of inflammatory cytokines by inflammatory stimuli. In addition, according to the previous reports, antioxidant and anti-inflammatory potentials of phlorotannins directly associated with the inhibition of cytokines, iNOS and COX-2 (Lee et al., 2010; Ma et al., 2003; Ryu et al., 2009). Moreover, recently, Kang et al. (2007) reported that phlorotannins isolated from *E. cave* showed antioxidant and cytoprotective effects against oxidative stress. Besides, part I demonstrated the antioxidant potential of the phlorotannin rich extract from fermented *E. cava* processing by-product *in vitro*. Therefore, it is possible that the profound ability of phlorotannin rich FEPBE to suppress reactive oxygen species (ROS) might be associated with the inhibition of iNOS and COX-2 protein expression as well as the inhibition of IL-1β and IL-6 pro-inflammatory cytokines; hence the extract gained anti-inflammatory potential.

5. CONCLUSION

In this study, we investigated the *in vitro* anti-inflammatory potency of the brown seaweed *E. cava* processing by-product fermented by *C. utilis*. Our results demonstrate the profound inhibitory effect of phlorotannin rich FEPBE on NO and PGE_2 production in LPS-stimulated RAW 264.7 macrophages. In addition, the extract suppressed the iNOS and COX-2 protein expression and the IL-1β and IL-6 pro-inflammatory cytokines. Hence, we can confirm that the bioactive components responsible for the anti-inflammatory activity are still remaining in the *E. cava* processing by-product. Moreover this study demonstrates the possibility of recovering bioactive components from the residual source which could be commercially exploited as functional ingredients.

Part III

Investigation of the potential bioactive components from edible sea cucumber *Holothuria edulis* that mediate apoptosis of human HL-60 leukemia cells and suppress inflammatory responses in lipopolysaccharide-stimulated RAW 264.7 macrophages

ABSTRACT

Natural products have played an important role in drug discovery and are source of scaffolds for the development of new functional ingredients. 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* anticancer and anti-inflammatory potentials. Aqueous fraction of the edible sea cucumber (ESC-AQ) has shown a high cytotoxicity against human HL-60 leukemia cell line while EtOAc fraction of the edible sea cucumber (ESC-EA) exhibited profound anti-inflammatory potentials in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. An induction effect of apoptotic body formation in response to ESC-AQ treatment was confirmed in HL-60 cells stained with Hoechst 33342 and flow cytometry analysis. The up regulation of Bax and caspase-3 protein expression was observed while the expression of Bcl-xL protein was down regulated in ESC-AQ treated HL-60 cells. 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) expressions in LPS-stimulated RAW 264.7 cells. ESC-EA significantly suppressed PGE_2 release in addition to pro inflammatory cytokines TNF-α, IL-1β and IL-6. Due to the profound anticancer and anti-inflammatory activities, ESC-AQ and ESC-EA appear as economically important biomass fractions that can be exploited in numerous industrial applications as a source of functional ingredients.

1. INTRODUCTION

At present major health problems faced by mankind are cancer and inflammatory diseases. Cancer, a generic term for a large group of diseases that can affect any part of the body, is a leading cause of death worldwide (Bandgar and Gawande, 2010). The major interventions of conventional medicine include surgery and chemotherapy which aim at eliminating the cancer or prolonging life span of the patients (Dong et al., 2011). Moreover, limited numbers of anticancer drugs for the treatment of cancer are available in the market. However, it is often of those interventions failed to reach their expected results. In addition, various drugs available in the market for the treatment of inflammatory diseases have serious side effects such as dyspepsia, gastro duodenal ulcers and gastritis bleeding, hence cannot be used continuously for a long time. Thus, there is an urgent need to search new anticancer agents and to develop new safer anti-inflammatory drugs (Sondhi et al., 2010). Therefore, researchers are looking elsewhere for effective therapeutics, particularly from natural sources (Kong et al., 2009). 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 (Awang et al., 2010).

Sea cucumbers, also known as Holothuroids, are marine animals belonging to the class *Holothuroidea*. These marine invertebrates are habitually found in the benthic areas and deep seas across the world. They are usually soft-bodied echinoderms comprising a diverse group of flexible, elongated, worm-like organisms, with a leathery skin and gelatinous body, looking like a cucumber (Bordbar et al., 2011). Sea cucumbers are one of the marine animals which are important as a human food source, particularly in some parts of Asia (Taiyeb-Ali et al., 2003). Sea cucumbers are traditionally consumed raw, dried, and boiled as food for human consumption in many tropical and subtropical countries (Ozer et al.,

2004). Specially, sea cucumbers have been used as a traditional tonic food in China and other Asian countries for thousands of years (Chen et al., 2011; Wu et al., 2012). In addition, as seafood, sea cucumbers are usually processed into a dried product which can be ranked as of high, medium or low commercial value based on species, abundance, appearance, odor, color, thickness of the body wall and main market demand (Wen et al., 2010). Therefore, taken together, they have high commercial value coupled with increasing global production and trade.

Since sea cucumbers are well known to exert beneficial effects on human health, these echinoderms are used in Asian folk medicine (Aminin, 2001; Mamelona et al., 2007). 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 (Zhong et al., 2007). As a mean of defense, most sea cucumbers contain, in their body wall and viscera, secondary metabolites (Dyck et al., 2010). 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 sulfates, glycosaminoglycan, sulfated polysaccharides, sterols, phenolics, cerberosides, lectins, peptides, glycoprotein, glycosphingolipids and essential fatty acids (Bordbar et al., 2011).

There are many different varieties of sea cucumber in the world. Recently, many researchers have studied the various biological properties of these species. However, to the best of our knowledge, this is the first report in the existing literature on the evaluation of anticancer and anti-inflammatory potentials of the edible sea cucumber *H. edulis*. In addition, this research also provides the information on potential applications of the *H.*

edulis and/or its extracts as a health-promoting commodity or as functional ingredients. Taken together, part III is mainly designed to evaluate the anticancer and anti-inflammatory potentials of edible sea cucumber *H. edulis* with regard to exploring its bioactive components as functional ingredients for functional foods, nutraceuticals and pharmaceuticals.

2. MATERIALS AND METHODS

2.1. Materials and reagents

RPMI-1640 medium, DMEM, FBS and trypsine–EDTA were purchased from Gibco/BRL (Burlington, Ont, Canada). MTT, RNase A, dihydroethidium (DE), PI, ethidium bromide (EtBr), DMSO and Hoechst 33342 were purchased from Sigma (St. Louis, MO, USA). Antibodies against Bax, Bcl-xL, cleaved caspase-3 and β-actin were purchased from Cell Signaling Technology (Bedford, Massachusetts, USA). All other chemicals and reagents used herein were of analytical grade.

2.2. Preparation of the sea cucumber extract and fractionation

Collected fresh edible sea cucumbers (*H. edulis*) were clean and freeze dried. Extraction procedure was done using 80% MeOH 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 EtOAc and partitioned with double distilled water (DDW) 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). The viscous aqueous fraction (54 g) was freeze dried (ESC-AQ). For the cell based assays, samples were dissolved in DMSO and further diluted in culture media.

2.3. Determination of approximate chemical composition

Approximate chemical composition of ESC-AQ was determined according to the AOAC method (1990). Crude carbohydrate was determined by phenol-sulfuric acid reaction

(absorbance at 480 nm; using glucose as the calibration standard), crude lipid was quantified by Soxhlet method and crude ash was prepared at 550 ºC in a dry-type furnace. Moisture content was determined by keeping the sample in a dry oven and the crude protein content was determined by Kjeldahl method. Content of the each component was expressed as a percentage value.

2.4. Determination of total phenolic content

Total phenolic content of ESC-EA was determined according to the protocol described by Chandler and Dodds (1983). 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.

2.5. Cell culture

Human promyelocytic leukemia cell line (HL-60) was grown on RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 lg/mL). The culture was maintained at 37 ºC in a 5% CO_2 incubator.

A RAW 264.7 cell line was cultured in DMEM supplemented with 100 U/mL of penicillin, 100 µg/mL of streptomycin and 10% FBS. The cells were incubated and maintained in an atmosphere of 5% CO_2 at 37 °C. The cells were sub cultured every 2 days and exponential phase cells were used throughout the experiments.

2.6. Cell growth inhibitory assay

The cytotoxicity of the ESC-AQ against the tumor cells was assessed via a colorimetric MTT assay. HL-60 cells were seeded (1.0×10^5 cells/mL) together with various concentrations of the sample (25, 50 and 100 μg/mL) and incubated for up to 48 h prior to MTT treatment. MTT stock solution (50 μL; 2 mg/mL in PBS) was added to each well to achieve a total reaction volume of 250 μL. After 3 h of incubation, the plates were centrifuged for 10 min at 2000 rpm and the supernatants were aspirated. The formazan crystals in each well were dissolved in DMSO. The amount of purple formazan was assessed by measuring the absorbance at 540 nm using an ELISA reader (Sunrise; Tecan Co. Ltd., Australia).

2.7. Nuclear staining with Hoechst 33342

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33342. Cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis (Gschwind and Huber, 1995; Lizard et al., 1995). The HL-60 cells were seeded in 24-well plates at a concentration of 1.0×10^5 cells/mL. Sixteen hours after seeding, the cells were treated with various concentrations of the sample (25, 50 and 100 μg/mL), and further incubated for 12 h at 37°C in a humidified atmosphere. Then, 1.5 μL of Hoechst 33342, a DNA-specific fluorescent dye, was added at a final concentration of 10 μg/mL, followed by 10 min of incubation at 37°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro colour digital camera, in order to examine the degree of nuclear condensation.

2.8. Cell cycle analysis

Flow cytometry analyses were conducted in order to determine the proportion of apoptotic sub-G1 hypodiploid cells (Lizard et al., 1995). The HL-60 cells were placed in 6-well plates at a concentration of 4.0×10^5 cells/mL, and 16 h after plating the cells were treated with various concentrations of the sample (25, 50 and 100 μg/mL). Then after 12 h, the cells were harvested at the indicated time, and fixed for 30 min in 1 mL of 70% EtOH at 4°C. The cells were then washed twice with PBS, and incubated for 30 min in darkness in 1 mL of PBS containing 100 μg PI and 100 μg RNase A, at 37°C. Flow cytometric analysis was conducted using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA). Effects on the cell cycle were determined by measuring changes in the percentage of cell distribution at each phase of the cell cycle, and were assessed by histograms generated by the Cell Quest and Mod-Fit computer programs (Nicoletti et al., 1991).

2.9. Western blot analysis (anticancer)

Cells (2.0×10^5 cells/mL) were treated with different concentrations of ESC-AQ, and then harvested. The cell lysates were prepared with lysis buffer (50 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS and 1 mmol/L EDTA). Cell lysates were washed via centrifugation, and the protein concentrations were determined using a BCA™ protein assay kit. The lysates containing 30 μg of protein were subjected to electrophoresis on 10% or 12% sodium dodecyl sulfate–polyacrylamide gels, and the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated with primary antibody against Bax, Bcl-xL, cleaved caspase-3, and β-actin in TTBS (25 mmol/L Tris–HCl, 137 mmol/L NaCl, 0.1% Tween 20, pH 7.4) containing 0.5% nonfat dry milk for 1 h. The membranes were then washed with TTBS and incubated with

secondary antibodies. Signals were developed using an ECL Western blotting detection kit and exposed to X-ray films.

2.10. Determination of NO production

RAW 264.7 cells (1.0×10^5 cell/mL) were plated in a 24-well plate and after 16 h the cells were pre-incubated with various concentrations of the sample (ESC-EA) at 37 °C 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 (Lee et al., 2007b). 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.

2.11. 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 °C. 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).

2.12. Determination of 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 (1μg/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.

2.13. Western blot analysis (anti-inflammation)

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).

2.14. 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 (Cho et al., 2000). Briefly, RAW 264.7 cells (1.0×10^5 cells/mL) were pretreated with the sample for 2 h and then treated with LPS (1μg/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.

2.15. 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

3.1. Approximate chemical composition and total phenolic content

During fractionation, ESC-AQ gained higher quantity than ESC-EA. However, polyphenolic compounds are generally more soluble in polar organic solvents than in water. Therefore, total phenolic content of the ESC-EA was quantified and it was found that ESC-EA contain 10.32% total phenolic content. In addition, the approximate chemical composition of the ESC-AQ was determined. Ash content accounted for 55.74% of the total dry weight. Moisture, carbohydrate, crude protein, crude lipid contents were 23.27%, 12.15%, 5.42% and 3.41% respectively.

3.2. Inhibitory effect of ESC-AQ on the growth of HL-60 cells

To evaluate whether edible sea cucumber *H. edulis* possess potential anticancer activity on HL-60 cells, in the preliminary studies determined the growth inhibitory effects of different fractions using MTT assay (data not shown). Among the tested samples aqueous fraction of the edible sea cucumber (ESC-AQ) exhibited the profound anticancer effect on HL-70 cells. In addition, ESC-AQ strongly inhibited HL-60 cell growth in a dose dependant manner (Fig. III-1). Moreover, ESC-AQ showed more than 82% cell growth inhibition at the concentration of 100 μg/mL. Then the effect of ESC-AQ on cell viability in normal (Vero) cells was tested. As shown in the Fig. III-2, slight toxicity was observed in Vero cells at the tested concentrations.

3.3. Induction effect of ESC-AQ on apoptosis in HL-60 cells

To examine nuclear morphological changes in response to ESC-AQ treatment, HL-60 cells were stained with the cell-permeable DNA dye Hoechst 33342 and visualized by

fluorescent microscopy. The determination of apoptosis in different concentrations of ESC-AQ-treated HL-60 cells was carried out. As can be seen in Fig. III-2A a typical image of untreated cells featuring round intact nuclei was observed. By way of contrast, the ESC-AQ-treated cells evidenced morphological changes characteristic of apoptosis, including chromatin condensation, bleb formation around the nucleus, and phase bright nuclear fragmentation (Fig. III-2B-D). In addition, Fig. III-2B-D showed that the proportion of apoptotic cells increased gradually in a dose-dependent manner and evidenced typical morphological changes, including reductions in cellular volume, bright staining, and condensed or fragmented nuclei.

3.4. Effect of ESC-AQ on sub-G_1 DNA contents in cell cycle analysis

In order to quantitatively measure the apoptosis induction by ESC-AQ treatment, flow cytometry analysis was carried out. As shown in Fig. III-3A, the sub-G_1 DNA content was 7.02% in control cells, however, as demonstrated in Fig. III-3B-D, ESC-AQ treatment in the HL-60 cells at different concentrations (25, 50 and 100 μg/mL) induced dose-dependent increases in the proportion of apoptotic cells, which was reflected by the sub-G_1 populations (7.81%, 59.85% and 68.97%). These results demonstrate that ESC-AQ-induced cell death was caused principally by apoptosis induction.

3.5. Effect of ESC-AQ on apoptosis-related protein expressions in HL-60 cells

Expressions of the intracellular proteins related to apoptosis, such as Bax, Bcl-xL and caspase-3 were investigated to understand the mechanisms by which ESC-AQ induces apoptosis in HL-60 cells. As shown in Fig. III-4, the level of the pro-apoptotic protein Bax was markedly increased and the level of an anti-apoptotic protein Bcl-xL was decreased

markedly in ESC-AQ treated HL-60 cells. In contrast, expression of Bcl-xL was completely suppressed by ESC-AQ at the concentration of 100 μg/mL. The expression of the active form of caspase-3 was increased with the presence of 100 μg/mL of ESC-AQ in HL-60 cells.

3.6. Effect of ESC-EA on LPS-induced NO production and LDH release in murine macrophages

To evaluate whether edible sea cucumber *H. edulis* possess potential anti-inflammatory activity, in the preliminary studies determined the inhibitory effects of different fractions on NO production in LPS-stimulated RAW 264.7 cells (data not shown). Among the tested samples AtOAc fraction of the edible sea cucumber (ESC-EA) exhibited the profound anti-inflammatory effect. Therefore, ESC-EA was selected for further evaluation. 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. III-5). 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.

3.7. Effect of ESC-EA on LPS-induced PGE_2 production

According to the results of the present study, 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. III-6). This result 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.

3.8. Effect of ESC-EA on LPS-induced iNOS and COX-2 protein expression

Fig. III-7 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 treat only with LPS compared to the control. However, ESC-EA suppressed the iNOS and COX-2 protein expression, compared with that of LPS-treated alone cells.

3.9. Effect of ESC-EA on LPS-induced pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) production

The inhibitory effects of ESC-EA on the production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 were further evaluated in LPS-stimulated RAW 264.7 macrophages. It was found that pretreatment of macrophages with ESC-EA considerably inhibited the production of cytokines TNF-α, IL-1β and IL-6 in a similar pattern (Fig. III-8-10). Release of the cytokines was significantly influenced by ESC-EA at the concentrations of 50 and 100 μg/mL. 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.

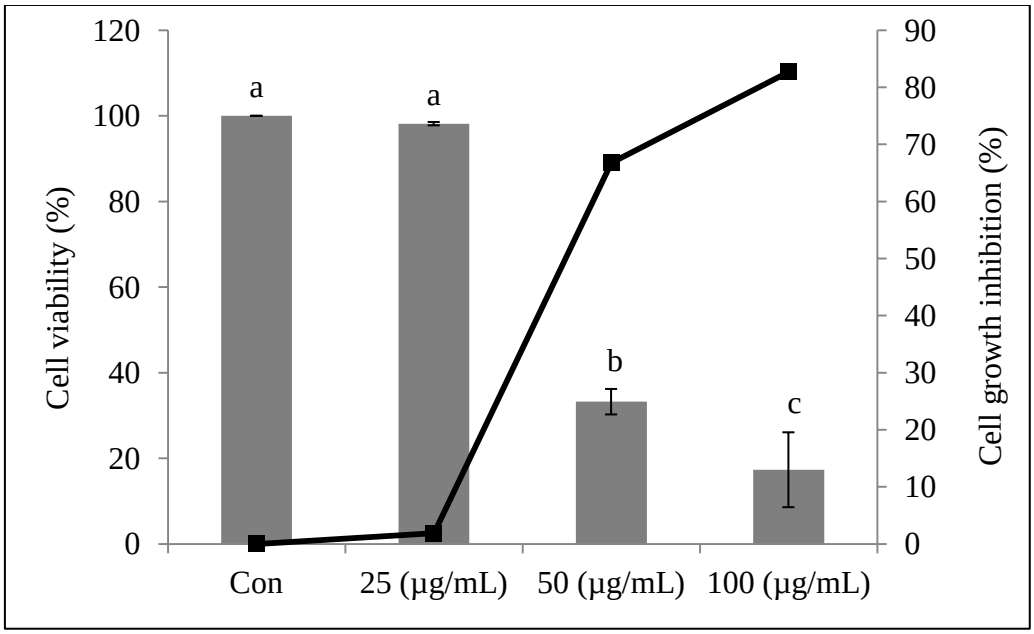

Fig. III-1. Cell grown inhibitory effect of ESC-AQ in HL-60 cells. Cells were treated with ESC-AQ of *H. edulis* and cell viability was measured by MTT assay at 48 h. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

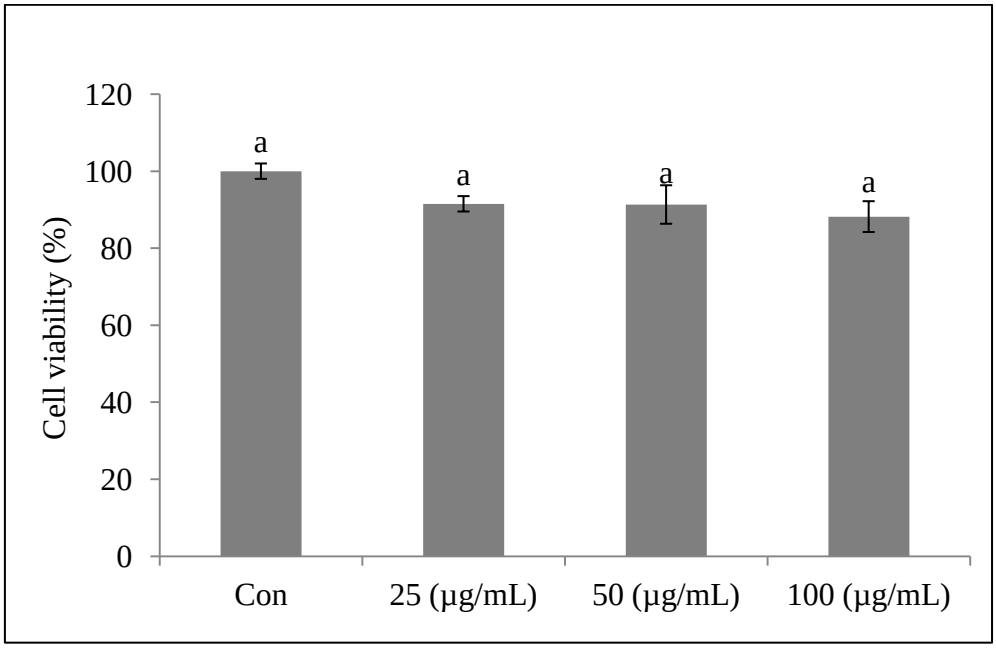

Fig. III-2. Effect of ESC-AQ of *H. edulis* on cell viability in normal (Vero) cells. Cells were treated with ESC-AQ and cell viability was measured by MTT assay at 48 h. Values are mean ± SD of three determinations.

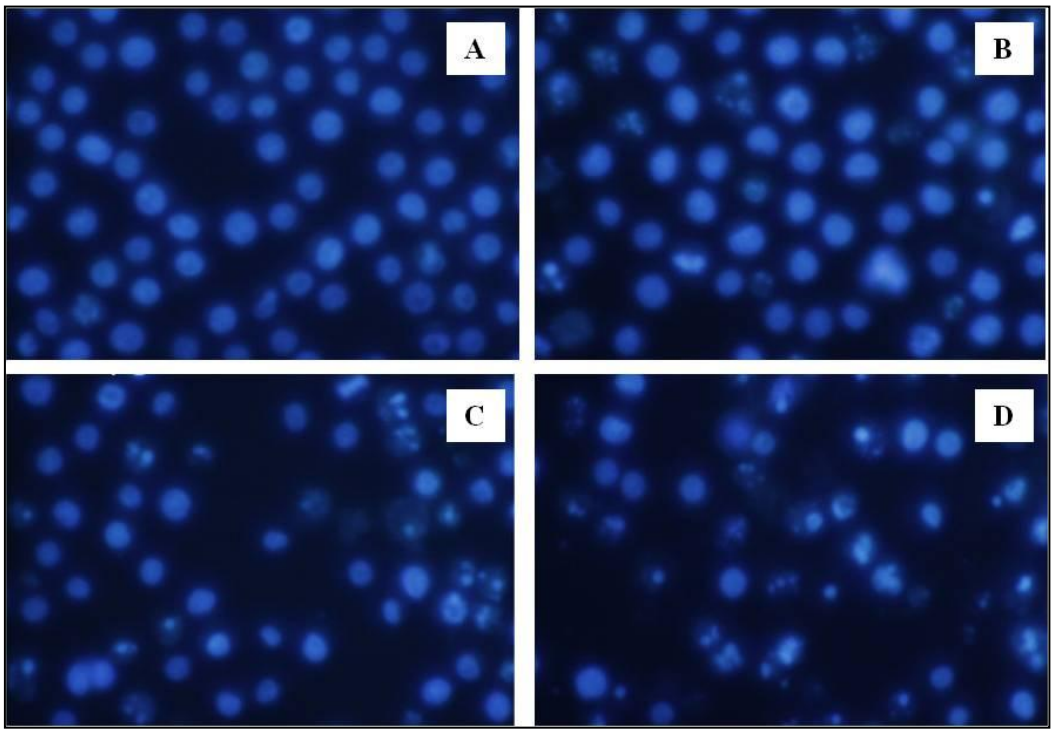

Fig. III-3. Induction effect of ESC-AQ on apoptotic body formation in HL-60 cells. The cells were treated with different doses of ESC-AQ for 12 h and visualized for apoptotic bodies under fluorescent microscope (400×) using a blue filter after staining with Hoechst 33342. (A) Control, (B) 25 μg/mL, (C) 50 μg/mL and (D) 100 μg/mL.

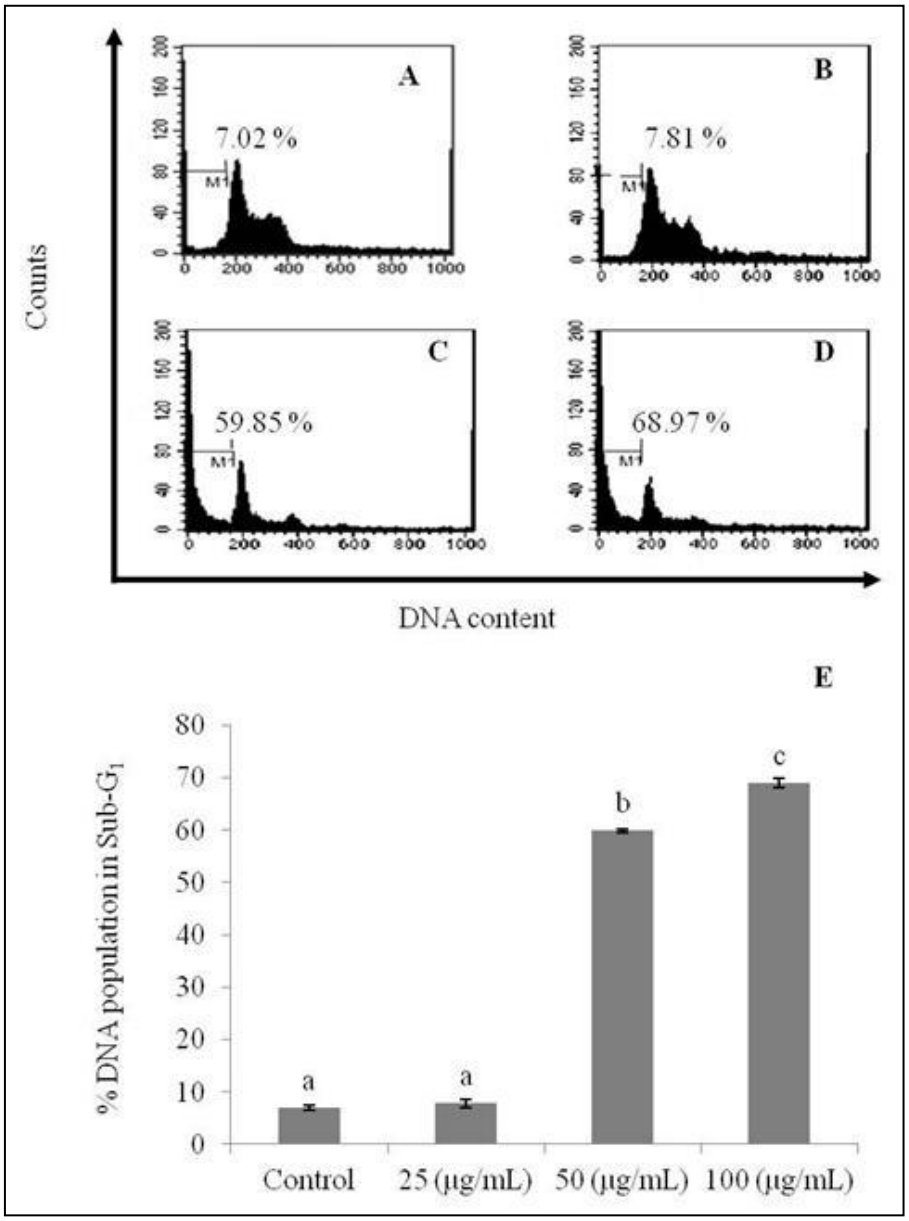

Fig. III-4. Effect of ESC-AQ on cell cycle pattern and apoptotic cell proportion in HL-60 cells. Apoptotic sub-G1 DNA content was detected by flow cytometry after propidium iodide staining. (A) Control, (B) 25 μg/mL, (C) 50 μg/mL, (D) 100 μg/mL and (E) bar graph for sub-G1 peak patterns of HL-60 cells treated with different concentrations of ESC-AQ.

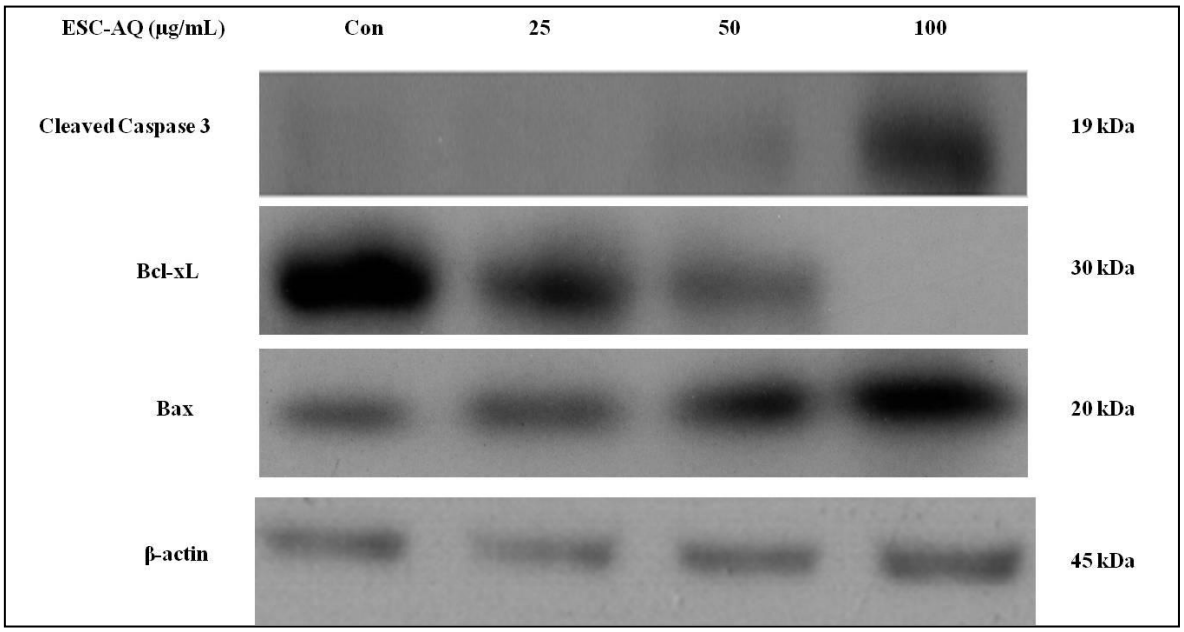

Fig. III-5. Effect of ESC-AQ on apoptosis-related protein expressions in HL-60 cells. Cells were treated with ESC-AQ at the indicated concentration for 12 h. Whole cell lysates were subjected to Western blot analysis of anti-Bax, -Bcl-xL and cleaved caspase-3 monoclonal antibodies. β-actin was used as internal control.

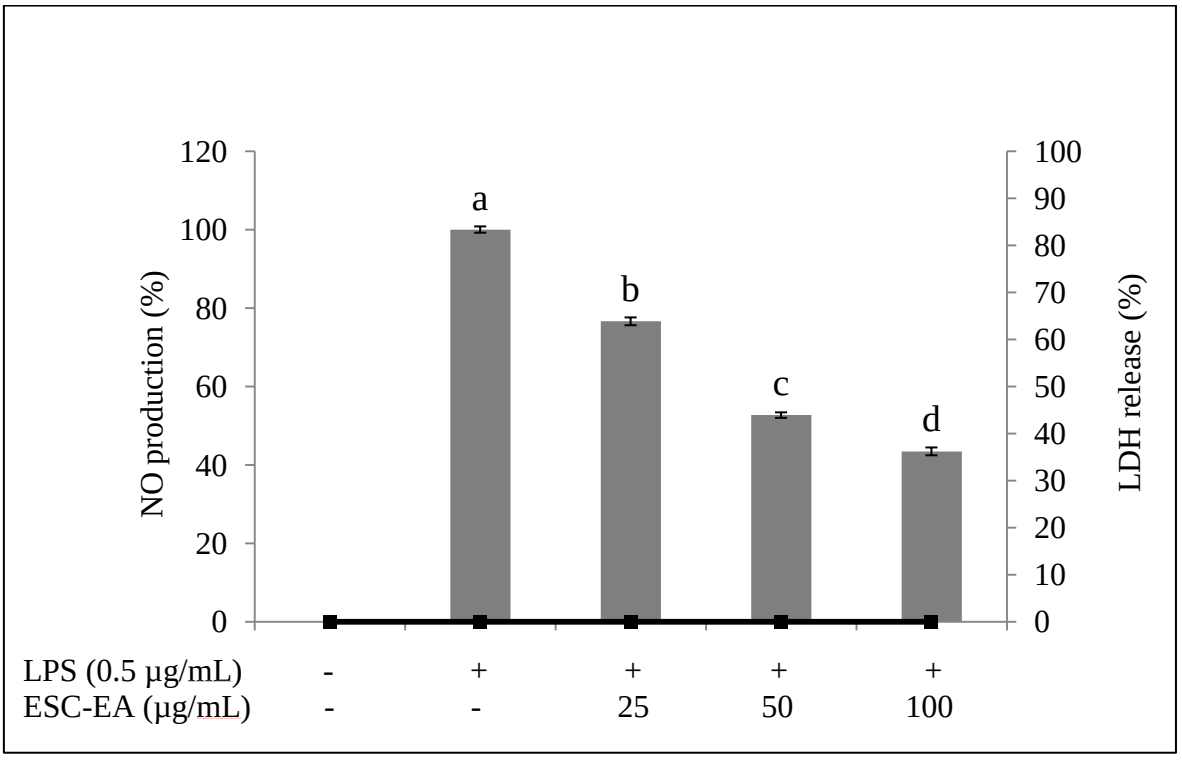

Fig. III-6. Inhibitory effect of ESC-EA on LPS-induced NO production and LDH release in RAW 264.7 macrophages. Incubation of the extract with cells in response to LPS (1 μg/mL) for 24 h, the NO levels in the medium were measured. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

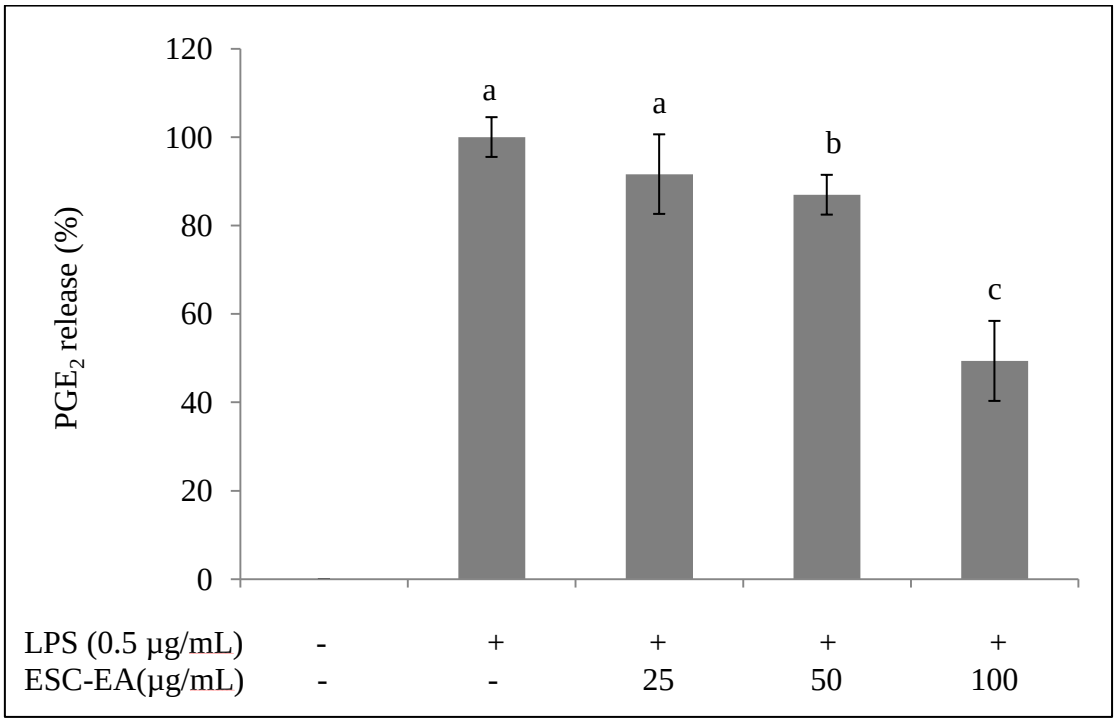

Fig. III-7. Inhibitory effect of ESC-EA on LPS-induced PGE_2 production in RAW 264.7 macrophages. After incubation of cells with LPS for 24 h in the presence or absence of the extract, the concentrations of PGE_2 in the medium were measured. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

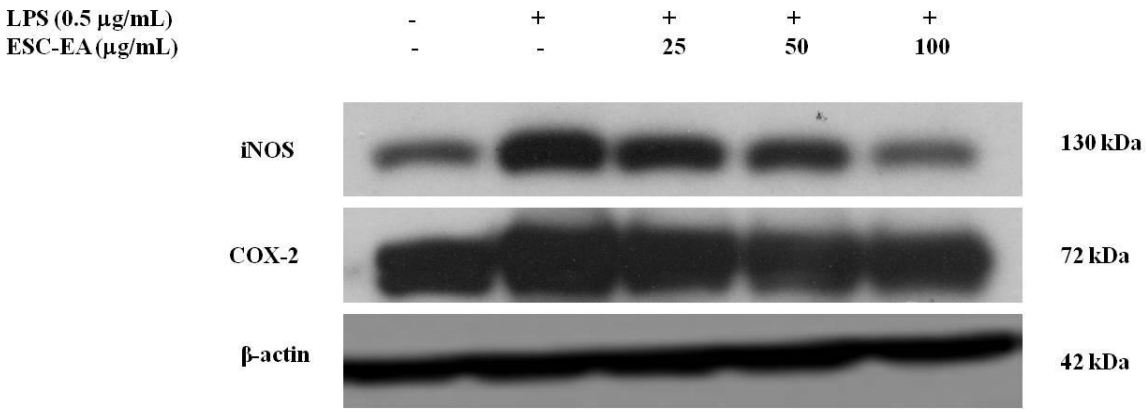

Fig. III-8. Inhibitory effect of ESC-EA on LPS-induced iNOS and COX-2 protein expression in RAW 264.7 macrophages. The cells were incubated with LPS for 24 h in the presence or absence of the extract. Then, cell lysates were electrophoresed and the expression levels of iNOS and COX-2 were detected with specific antibodies.

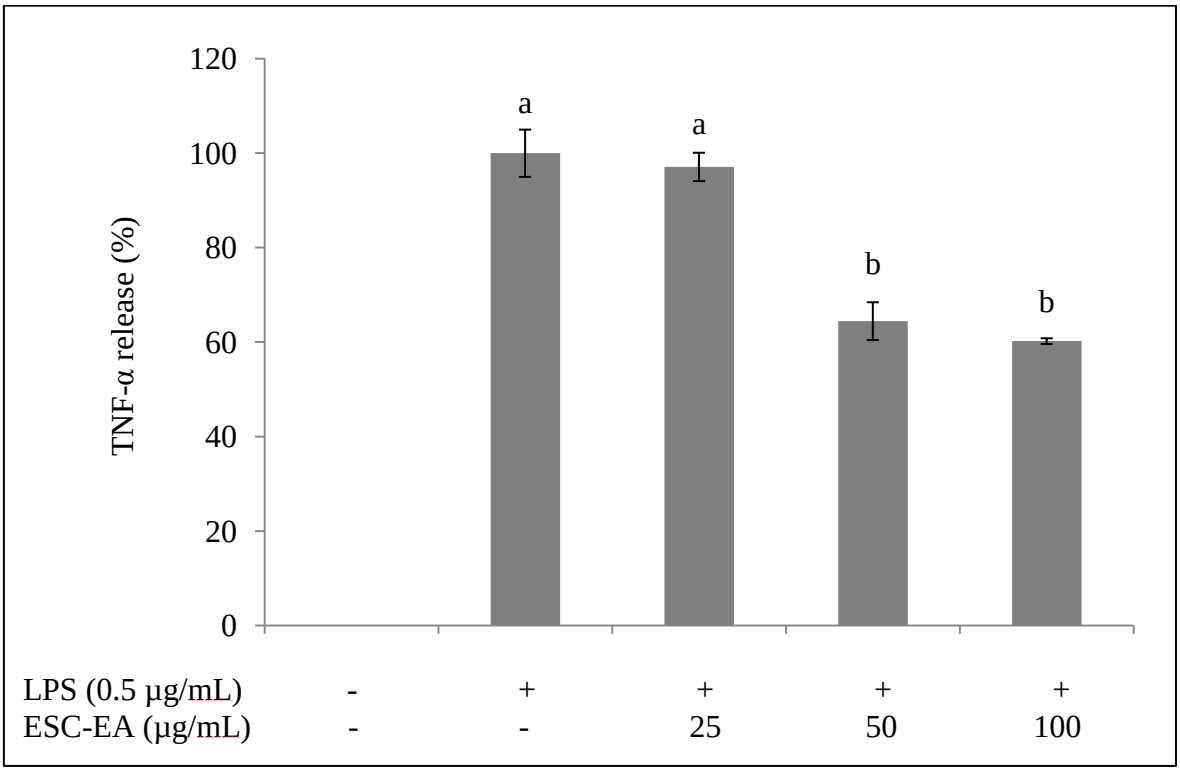

Fig. III-9. Inhibitory effect of ESC-EA on LPS-induced TNF-α production in RAW 264.7 macrophages. After incubation of cells with LPS for 24 h in the presence or absence of the extract, the concentration of TNF-α in the medium was measured. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

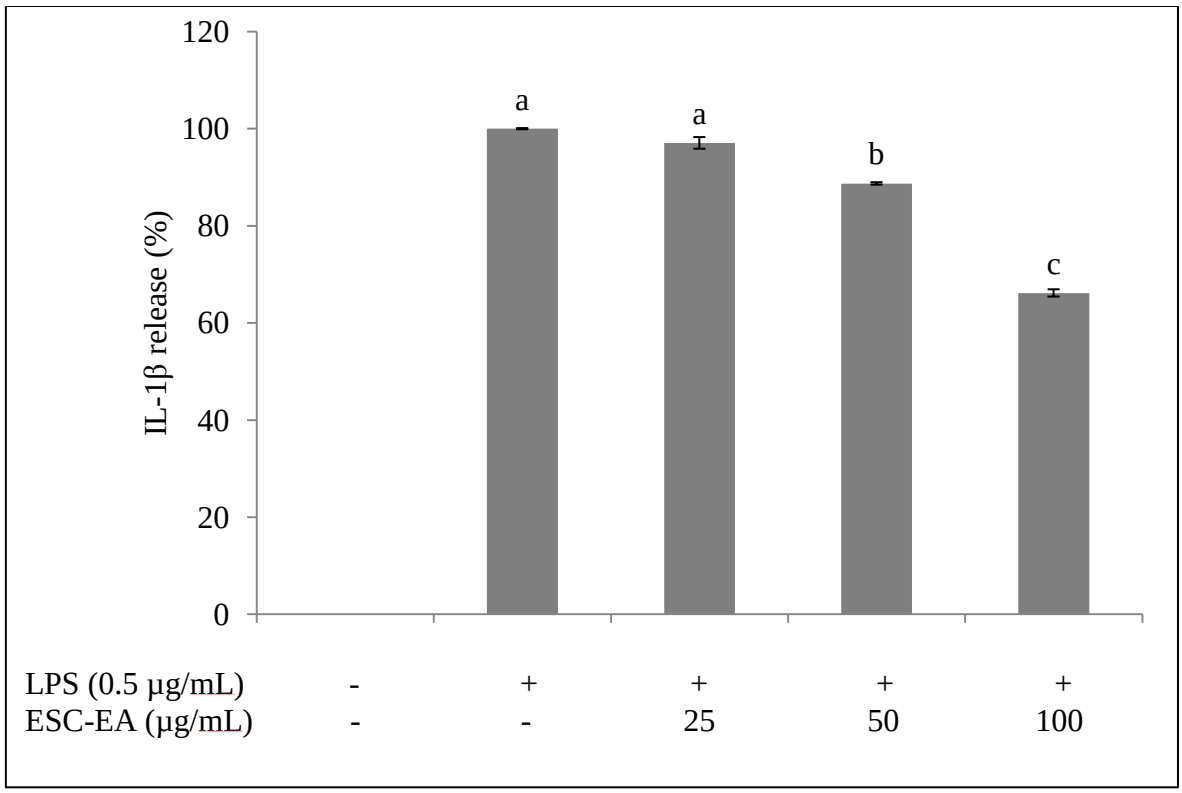

Fig. III-10. Inhibitory effect of ESC-EA on LPS-induced IL-1β production in RAW 264.7 macrophages. After incubation of cells with LPS for 24 h in the presence or absence of the extract, the concentration of IL-1β in the medium was measured. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

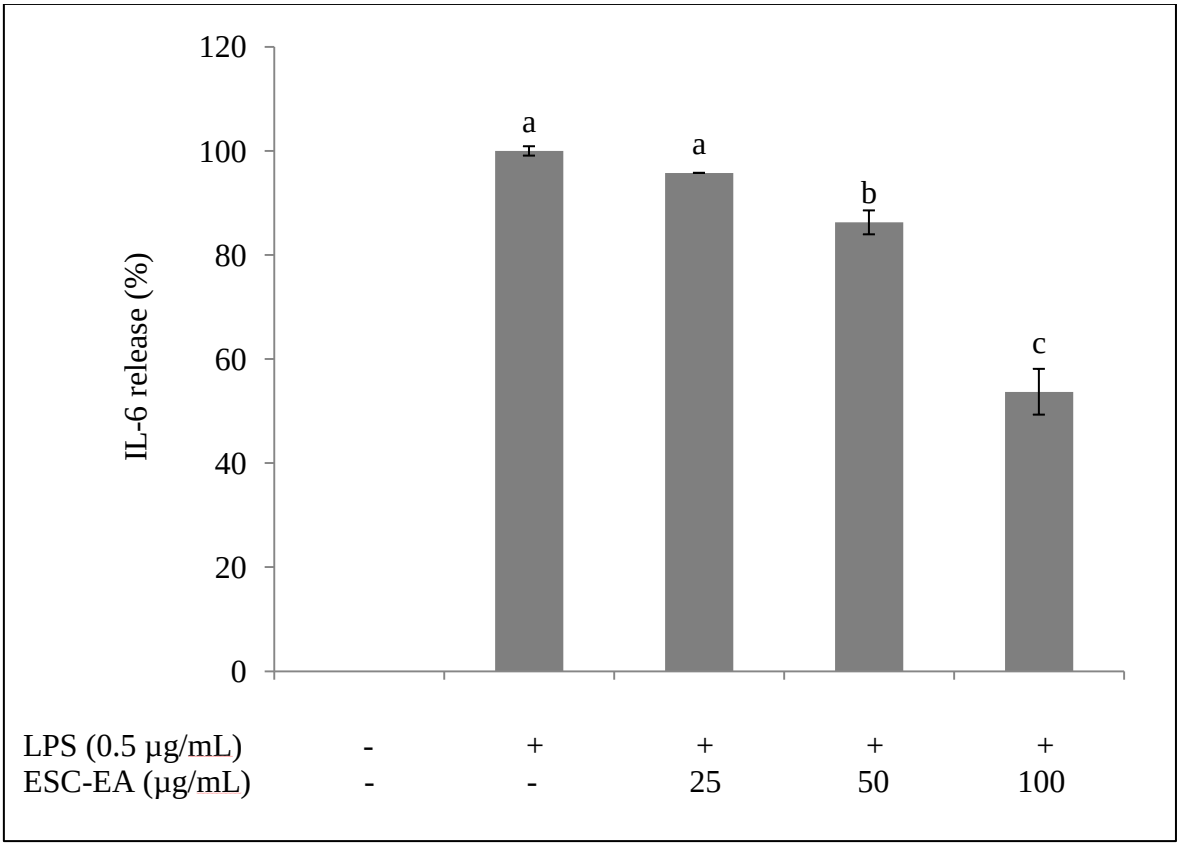

Fig. III-11. Inhibitory effect of ESC-EA on LPS-induced IL-6 production in RAW 264.7 macrophages. After incubation of cells with LPS for 24 h in the presence or absence of the extract, the concentration of IL-6 in the medium was measured. Values are mean ± SD of three determinations. Values with different alphabets are significantly different at $P < 0.05$ as analyzed by DMRT.

4. DISCUSSION

Sea cucumbers are considered to be beneficial for health by many consumers around the world. Since the wide range of useful biological activities possessed by sea cucumbers and their derivatives, in continuation of efforts in search of potential bio active agents, the edible sea cucumber *H. edulis* evaluated for anti-inflammatory and anticancer activities for the first time.

At present major health problems faced by mankind are cancer and inflammatory diseases (Sondhi et al., 2010). In addition, Cancer is the second leading cause of death in the present society after cardiovascular diseases (Bandgar et al., 2010). A great deal of efforts has been underway to treat various forms of cancer for decades; and until recently, chemoprevention of cancer is receiving its due to sharing of attention. Natural product chemistry and high-throughput screening against cancer cells are established methods for primary anticancer drug discovery.

Many chemotherapeutic agents are reported to exert their anticancer effects by inducing apoptosis of cancer cells (Kamesaki, 1998). Apoptosis, also known as programmed cell death, is characterized by typical cellular morphology and biochemical features including cell shrinkage, cytoplasm vacuolization, chromatin condensation, DNA fragmentation, and finally cellular breakdown into apoptotic bodies (Heo et al., 2011). It is an important biological mechanism that contributes to the maintenance of the integrity of multi-cellular organisms, and is dependent on the expression of cell-intrinsicsuicide machinery. The mitochondrial permeability transition is an important step in the induction of cellular apoptosis (Kang et al., 2012).

In this study, the cytotoxic effect of ESC-AQ was investigated in human HL-60 leukemia cell line. Due to the promising cytotoxic effect showed by ESC-AQ, it was further evaluated on cell apoptosis, cell cycle analysis and apoptosis related protein expressions. The results of this study showed typical morphological characteristics of apoptosis, such as nuclear condensation and apoptotic body formation, in HL-60 cells treated with ESC-AQ. In addition, the apoptosis of HL-60 cells induced by ESC-AQ was also confirmed by the sub-G_1 DNA accumulation. These results suggest that ESC-AQ might mediate its growth inhibitory effects on HL-60 cells by mechanisms involving the induction of apoptosis.

Many studies have shown that the regulation of apoptosis involved a host of molecules, in particular, the expression of proteins such as Bcl-xL and Bax are altered with the induction of apoptosis (Ren et al., 2008). Bcl-xL is a transmembrane molecule in the mitochondria. It is one of several anti-apoptotic proteins which are members of the Bcl-2 family of proteins. It has been implicated in the survival of cancer cells. Bax is a pro-apoptotic Bcl-2 protein and it promotes apoptosis by competing with Bcl-2 proper. In addition, sequential activation of caspases plays a central role in the execution-phase of cell apoptosis (Heo et al., 2011). Chemopreventive agents help to interrupt or reverse the progresses of cancer to prevent carcinogenesis and reverse precancerosis (Namvar et al., 2012). The ESC-AQ suppressed the growth of human HL-60 leukemia cells via apoptosis induction through modulation of apoptosis related protein expression. According to the observed results it could be suggested that, ESC-AQ could inhibit the proliferation of HL-60 cells by mechanisms involving the induction of apoptosis. Furthermore, the apoptosis of HL-60 cells induced by ESC-AQ is associated with the up regulation of Bax, down regulation of Bcl-xL, and activation of caspase-3 (Fig. III-11).

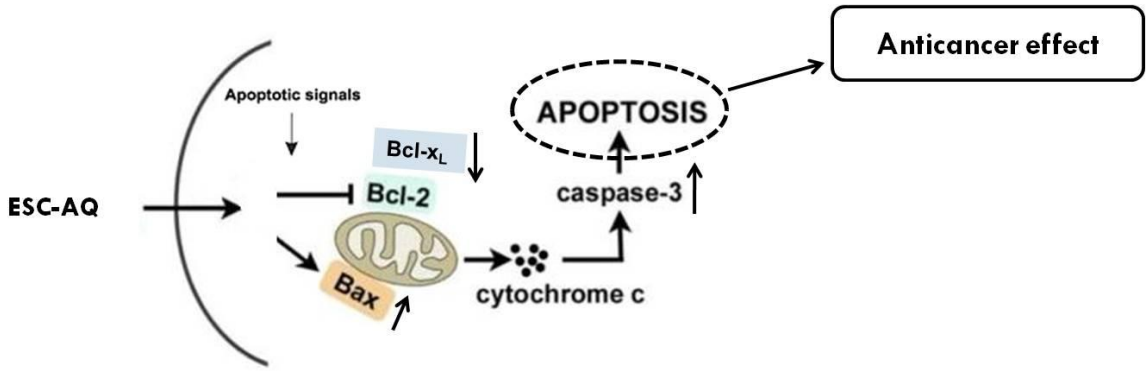

Fig. III-12. The anticancer activity of ESC-AQ is associated with the up regulation of Bax, down regulation of Bcl-xL, and activation of caspase-3 in HL-60 cells.

In addition to the anticancer activity, anti-inflammatory potential of the edible sea cucumber *H. edulis* was evaluated in this study. 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 (Kazlowska et al., 2010; Speranza et al., 2012). If they are not controlled or resolved, inflammation cascades can lead to the development of diseases such as chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and psoriasis. 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 (Kim et al., 1999). Therefore, it is clear that inhibition of NO production may have therapeutic value over inflammatory diseases. In the present work, since ESC-EA exhibited potent activity and further experiments were conducted to determine its potential.

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 (Ahmed et al., 2002; Savonenko et al., 2009). 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 (Bandgar et al., 2010).

The results of this study indicated that ESC-EA could induce the anti-inflammatory activity by suppressing the PGE_2 production in LPS-stimulated RAW 264.7 macrophages. In addition, the inhibitory effects of ESC-EA on NO and PGE_2 production was further characterized using Western blot analysis. According to the results, it could be suggested that ESC-EA inhibited the NO production by decreasing both iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 macrophages. In this study, also the results showed that ESC-EA suppressed the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 up to a significant extent, suggesting that the suppression of iNOS by ESC-EA might be associated with the attenuation of the cytokine release.

5. CONCLUSION

This report revealed the anticancer and anti-inflammatory potentials of the edible sea cucumber *H. edulis* for the first time. Collectively, the possessed biological activities of the edible sea cucumber *H. eludis* indicate that their consumption would be beneficial to health. In addition, the findings of this study may facilitate awareness about anticancer and anti-inflammatory properties of *H. edulis* and help future developments in possible industrial applications. Finally, in addition to the pharmacological potential for anticancer and anti-inflammatory applications, rich opportunities for edible sea cucumber *H. edulis* exist within the arena for functional foods and nutraceuticals. These opportunities derive primarily from the extremely benign safety profile of the edible sea cucumber products.

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ACKNOWLEDGEMENT

Foremost, I would like to express my sincere gratitude to my supervisor Prof. You-Jin Jeon for the continuous support, motivation, encouragement and patience during my Ph.D. study. His valuable guidance helped me in all the time of research work as well as the writing of this thesis. It has been an honor to be one of his Ph.D. students. I highly appreciated his contributions of time, ideas and funding since September, 2009.

Besides my advisor, I would like to convey my thanks to the rest of my thesis committee: Prof. Gi-Young Kim, Prof. Sang-Rul Park, Prof. Seung-Heon Lee and Dr. Daekyung Kim for their encouragement and insightful comments.

I would like to thank people and the government of Republic of Korea for giving me the opportunity to come here and reading the Ph.D. In addition, I gratefully acknowledged Brain Korea (BK)-21 project, the funding sources that made my Ph.D. work possible.

Part of my study was a collaborative work with the Laboratory of Natural Products Chemistry, Institute for Tropical Biology and Conservation, University Malaysia Sabah, Malaysia. The active fractions of edible sea cucumber *Holothuria edulis* were provided by Prof. Charles S. Vairappan. I highly appreciated the contribution of Prof. Charles and his research team.

My sincere thanks also go to Dr. Mahinda Senevirathne, Dr. Mahanama de Zoysa, Dr. Chamilani Nikapitiya and Dr. K.H. Sarananda who worked hard for introducing me to Jeju National University.

My time at Jeju National University was enjoyable due to the many friends and groups that became a part of my life. I am grateful for the time spent with them. Help came in all forms, I don't think I would be able to say thank you enough to everyone who had

contributed in this success. However, special thanks should go to all my lab mates in the Marine Bioresource Technology Lab for their great help not only throughout my study, but also in my personal life in Jeju, Republic of Korea. I am not overstating, if I say that the last three years would be quite a challenge for me without those helping hands. I highly appreciated all the assistance during last recent years.

My deepest gratitude goes to my family for their unflagging love and support throughout my life; this dissertation is simply impossible without them. I cannot ask for more from my parents, as they are simply perfect. I have no suitable word that can fully describe their everlasting love for me. Besides my parents, I would like to thank my loving sister for supporting me spiritually throughout my life.

Last, but not the least my deepest appreciation goes to my ever loving and encouraging wife Deepani for her support all the way of my life. Your patience and understanding is amazing. You cheered me on and on.

Thank you very much.

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August, 2012.