Thesis for the Degree of Doctor of Engineering

Anti-inflammatory activities of the brown alga *Undaria pinnatifida* (Harvey) Suringar

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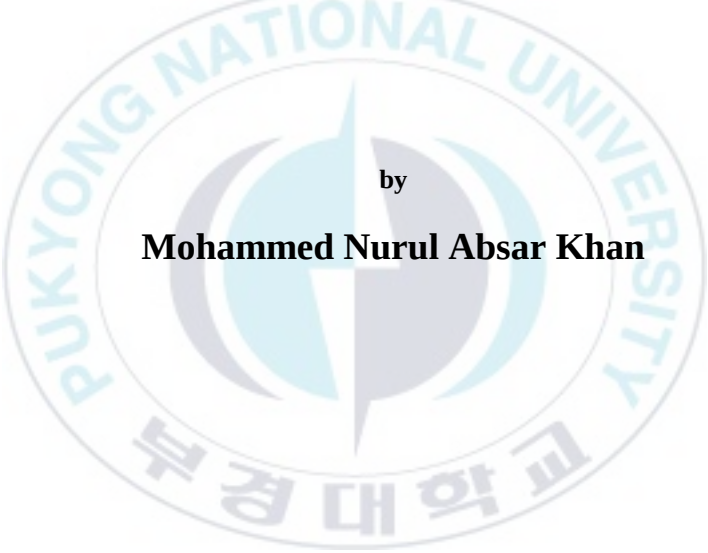
갈조류 미역 *Undaria pinnatifida* (Harvey) Suringar 의 항염증 활성

Advisor: Prof. Yong-Ki Hong



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A dissertation
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Abstract

In this study, 37 seaweeds species have been screened to investigate the anti-inflammatory activity of common seaweeds available in Korean peninsula. The seaweed extracts were tested in laboratory assays against in vivo phorbol-12-myristate 13 acetate (PMA) induced mouse ear inflammation model. A total of 14 species displayed anti-inflammatory activity, of which two showed strong suppressive activity against inflammation. The brown alga *Undaria pinnatifida* had shown the strongest inhibition activity (80%) against edema and also erythema (78%).

The second part of this study was focused on the anti-inflammatory activities of a methanol extract of *U. pinnatifida*. Methanol extract showed a 50% inhibitory concentration (IC_{50}) value of 10.3 mg/ml against mouse ear edema induced by PMA. A 40-mg/ml methanol extract maintained a 50% or higher suppression when applied either 3 h before or 2 h after PMA application. With the extract application, edema diminished to half maximal levels after 23 h compared to 31 h with vehicle. The blade part of the thallus showed the highest activity, while the northern forma of *U. pinnatifida* had a slightly higher activity than the southern forma. In the analgesic test, the methanol extract showed a suppressive effect on acetic acid-induced writhing response, with an IC_{50} of 0.48 g/kg body weight. The extract also showed antipyretic activity when tested in yeast-induced hyperthermic mice. The methanol extract showed no toxicity to mice after an oral administration of 10 g/ kg.

The methanol extract of *U. pinnatifida* exhibited potent inhibition of erythematous inflammation. Assessed by digital photo analysis, the IC_{50} of the extract on mouse ear erythema induced by PMA was 15.6 mg/ml. The IC_{50} value of the extract when erythema was measured using laser speckle flowgraphy was 18.1 mg/ml. In an *in vitro* preparation, a 10 mg/ml concentration of extract

contracted blood vessels to approximately 20% of the maximal contraction induced by 10^{-6} M noradrenaline. Erythema reduction to half-maximal values took 12 h with 40 mg/ml extract, compared to 25 h with vehicle application. The methanol extracts suppressed erythema by 50% when applied within 1 h before or 15 min after PMA application. Extracts prepared from different seaweed tissues varied in effect, with the blade showing the greatest anti-erythema activity. In addition, the northern forma of *U. pinnatifida* had slightly more activity than the southern forma.

Finally, two anti-inflammatory omega-3 polyunsaturated fatty acids (PUFAs) of stearidonic acid (SA) and eicosapentaenoic acid (EPA) and one pro-inflammatory ω6 PUFA of arachidonic acid (AA) were isolated from the edible brown alga *Undaria pinnatifida*. SA was active against mouse ear inflammation induced by phorbol myristate acetate, with IC_{50} values of 58, 114, and 85 mM against the inflammatory symptoms of edema, erythema, and blood flow, respectively. EPA was also active against edema, erythema, and blood flow, with IC_{50} values of 76, 153, and 78 mM, respectively. Although AA at low concentrations showed anti-inflammatory activities when 10 h later, AA doses of more than 80 mM measured induced inflammatory symptoms 1 h later. Mature thalli generally had larger amounts of PUFAs than young thalli. The algal blade contained more ω3 PUFAs than were found in other parts, while the holdfast contained extremely high amounts of AA. Late-season thalli showed increased amounts of PUFAs, especially AA.

***Keywords*:**Aanalgesic; anti-edema; anti-erythema; anti-inflammatory; antipyretic; arachidonic acid; brown alga; digital photo analysis; eicosapentanoic acid; laser speckle flowgraphy; Phaeophyta; stearidonic acid; *Undaria pinnatifida*

Chapter 1

General introduction

One quarter of the world's drugs come from natural sources, primarily from microorganisms and plants. As terrestrial resources become over explored, attention has turned to the marine environment as an alternative source of novel bioactive metabolites. The Ocean, which is called the 'mother of origin of life', is also the source of structurally unique natural products that are mainly accumulated in living organisms. Several of these compounds show pharmacological activities and are helpful for the invention and discovery of bioactive compounds. Modern technologies have opened vast areas of research for the extraction of biomedical compounds from oceans and seas. In recent years, many bioactive compounds have been extracted from various marine resources (Donia et al., 2003) but marine algae have received comparatively less bioassay attention although there are a number of seaweeds with economic potential (Critchley et al., 1998). The ancient tradition and everyday habit of Asian people have made possible a large number of epidemiological researches showing the health benefits linked to seaweed consumption (Teas 1981; Hiqashi et al., 1999; Funahashi et al., 1999). Assuming that the regular intake of seaweeds is useful for the prophylaxis of heart and colon disease, and it may also be effective to prevent other western-risk disease. It will be of great if these species could be the major role players in drug development.

Inflammatory disease

Despite progress in medical research during the past decades, the treatment of many serious diseases remains problematic (Bohlin, 1995). Inflammatory diseases remain one of the world's major health problems (Yesilada et al., 1997). The symptoms of many chronic diseases include inflammation and pain. The occurrence of some of these diseases, such as osteoarthritis, which afflicts 1 in 3 Americans

over 60 years of age, continues to rise as life expectancies increase (Jensen and Fenical, 2000). Others, including the autoimmune inflammatory diseases remain serious problems that are difficult to treat with current medicines. Because much of the discomfort associated with inflammatory diseases is the result of the inflammation process itself, there have been aggressive efforts to search for new and more effective anti-inflammatory drugs. Although anti-inflammatory activities have been reported for only a few marine microbial metabolites, the numbers could increase dramatically if there were to become a more common target.

An inflammatory response implicates macrophages and neutrophils, which secrete a number of mediators (eicosanoids, oxidants, cytokine and lytic enzymes) responsible for initiation, progression and persistence of acute or chronic state of inflammation (Lefkowitz et al., 1999). Prostaglandin E_2 (PGE_2) and nitric oxide (NO) are most important amongst these mediators and are produced in macrophages by cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), respectively (Harris et al., 2002 and MacMicking et al., 1997). PGE_2 is implicated in inducing the production of various chemo-attractants and pro-inflammatory cytokines (Harris et al., 2002), while NO is responsible for vasodilatation, increase in vascular permeability and edema formation at the site of inflammation (Moncada et al., 1991). NO along with superoxide ($O_2^{\cdot-}$) and the products of their interaction, also initiates a wide range of toxic oxidative reactions causing tissue injury (Hogg, 1998). Likewise, the neutrophils too produce oxidants and release granular constituents comprising of lytic enzymes performing important role in inflammatory injury (Yoshikawa and Naito, 2000). Inhibition in the release of these mediators is a potential strategy to control inflammation and is implicated in mechanism of action of a number of anti-inflammatory drugs including the representative ones like dexamethasone (Bourke and Moynagh, 1999).

Topical application of phorbol 13-myristate acetate (PMA) offers a skin inflammation model appropriate for evaluating anti-inflammatory agents. PMA induces inflammation by activating PLA_2, which subsequently activates the release and metabolism of arachidonic acid. The COX-2 inhibitors

are very effective in suppressing PMA induced ear edema, indicating the role of prostaglandins. It is of interest also to note that studies have shown that regular taking of aspirin and other non-conventional non-steroid anti-inflammatory drugs (NSAIDs) provide 40-50% reduction in relation to risk of death by colon cancer, thus indicating that the inhibitory effect of these compounds on COX enzymes has chemo-preventive effects (Crosby and Dubois, 2003; Young, 2004).

The focus of research in inflammation was maintained with the credo "elucidate the chemical mediators that evoke the cardinal signs of inflammation- heat, redness, swelling, pain, and loss of function (Winyard and Willoughby, 2003)-so that inhibitors could be prepared as new treatments to control the side effects of inflammation during disease". As depicted on the February 23, 2004 cover of *Time* magazine, inflammation has emerged as playing a central role in many prevalent diseases not believed previously to involve inflammation. These include Alzimer's disease, cardiovascular disease (Helgadottir et al., 2004), and cancer (Erlinger et al., 2004) in addition to those well appreciated and associated with inflammation, such as arthritis and periodontal diseases (Gallin et al., 1999; Van Dyke and Serhan, 2003). Efforts to understand the molecular basis chronic disease prevention and health promotion by certain nutraceuticals have intensified over the past few decades (Ohigashi et al., 1997). Nutraceuticals that may inhibit any of the molecular targets (e.g., cytokines, tumor necrosis factor-α TNF- α, interleukin-1 IL-1) have potential to inhibit or reduce the inflammatory process, via the following mechanisms:

(1) inhibition of the activation of NF_kB,
(2) blocking the overexpression of pro-inflammatory cytokines such as TNF- α, IL-1, and IL-6,
(3) down-regulation of the overexpression of CAMs and enzymes (phospholipase A_2, COX-2, and 5-LOX, iNOS and myeloperoxidase),
(4) inhibit enzyme activity such as phospholipase A_2, COX-2 and 5-LOX, iNOS and myeloperoxidase,
(5) inhibit ROS generating enzyme activity, and
(6) increase ability to scavenge ROS.

The omega-3 family of polyunsaturated fatty acids (PUFAs) possesses anti-inflammatory activity in vitro and in vivo (Pacht et al., 2003; Simopoulos 2002a; Hong et al., 2003). Frequent intake of omega-3 PUFAs in the diet is associated with significant beneficial effect for health promotion and reduction of the occurrence of certain chronic diseases (Jho et al., 2003; Calder et al., 2002). Reddy and Lokesh (1994) reported that including high amounts of omega-3 PUFAs in the diet of rats lowered carrageenan-induced edema in the foot pad of rats. Polymorphonuclear leukocytes from rats fed a menhaden oil diet incorporated omega-3 PUFAs into phospholipids membranes at the expense of arachidonic acid showed diminished cytokine production and reduced the generation of reactive oxygen species (Utsunomiya et al., 1994; Joe and Lokesh 1994). Several laboratories reported that omega-3 PUFAs is able to modulate inflammation and cytokine production (De Caterina et al., 2000; Rothman and others 1997). It suppresses nitric production in vitro and in vivo systems (Komatsu et al., 2003). Omega-3 PUFAs modulate prostaglandin metabolism and decrease triglycerides, lower cholesterol, and have anti-thrombotic and anti-inflammatory properties (De Caterina et al., 1994).

Intense interest in nutraceuticals and their potential benefits has created the need to review the existing scientific information on their effect in preventing and managing inflammation that accompanies most inflammatory chronic diseases. Currently, both steroidal and non-steroidal drugs are used in the relief of inflammation but prolonged use of these drugs associates with severe side effects. Consequently, there is a need to develop new varieties of anti-inflammatory agent with fewer side effects.

Medicinal properties of seaweed were restricted to traditional and folk medicines (Lincoln et al., 1991). Research into the active ingredients of seaweeds used in folk remedies underlies another area of drug discovery. A number of seaweed species are used as traditional medicine and health care belief as well as food in various regions of the world (Matsuzaki and Iwamura, 1981). Macroalgae is rich in 20-carbon atom PUFAs, chiefly eicosapentaenoic and docosahexanoic acids (Stefanov et al., 1988; Gerwick and Bernart, 1993). Seaweeds are capable of metabolising various C20-PUFAs via

oxidative pathways (Gerwick et al., 1993). The first involves fatty acid cyclooxygenase acting on arachidonic acid, as in mammalian systems (Noguchi et al., 1994). The other mechanism uses lipoxygenase, also acting on arachidonic acid (Gregson et al., 1979). In many red algae, the metabolised products of PUFAs, called oxylipins, resemble eicosanoid hormones in higher 254 plants and humans which fulfill a range of physiologically important functions (Gerwick et al., 1993; Imbs et al., 2001). The anomalous production of these compounds underlies a number of diseases related to inflammation (Gerwick and Bernart, 1993), and so eicosanoids and their derivatives have received much research attention in the search for development of new classes of anti-inflammatory drugs (Jacobs et al., 1993).

Therefore, omega-3 PUFAs are the most talked about nutraceutical and is in the process of obtaining a value for recommended daily allowance. Some edible marine algae contain omega-3 PUFAs at a high percentage, reaching 40% of the total fatty acids (Takagi et al., 1985). More recent studies assessed that omega-3 PUFA supplementation could be helpful against many inflammatory diseases. The omega-3 PUFAs are mainly known for their anti-inflammatory effects related to their competition as substrates for cyclooxygenase (COX) and lipoxygenase (LOX) leading to the formation of less active prostaglandins and leukotrienes (James et al., 2000). Omega-3 PUFAs contained diet UVB erythemal sensitivity (Rhodes et al., 1994), probably by decreasing prostaglandin E_2 (PGE_2) levels into the skin.

Prior to the 1950s, the medicinal properties of seaweeds were restricted to traditional and folk medicines (Lincoln et al., 1991). However, in recent years pharmaceutical firms have started looking towards marine organisms, including seaweeds, in their search for new drugs from natural sources, to produce more cost effective remedies that are affordable by the population (Farnsworth, 1994). These products are also increasingly being used in medical and biochemical research. According to Ireland et al. (1993), algae have been the source of about 35% of the newly discovered chemicals between 1977–1987, followed by sponges (29%) and cnidarians (22%). Research into the active ingredients of

seaweeds used in folk 246 remedies underlies another area of drug discovery. Since pharmaceutical companies have access to extensive libraries of natural products, and many compounds are of marine origin, high-throughput automated systems can be used for rapid screening in the search for new drugs (Cordell, 2000). An obstacle to the drug development of marine natural products is the lack of sufficient raw materials for comprehensive pharmacological evaluation (Jensen and Fenical, 2000). More often than not, compounds are isolated in mg quantities, and their structures are elucidated and published. For any pharmaceutical lead from a marine source, supply issues will always be a problem. Unless supply can be addressed in an economically feasible fashion, the dream of new effective drugs from the sea will be falter. Thus, one of the candidates for pharmaceutical source would be seaweed that is abundant or easily aquaculturable.

Biology of *Undaria pinnatifida*

Undaria pinnatifida (Harvey) Suringar: Alariaceae, Laminariales, Phaeophyceae is a popular brown alga native to Korea (Miyok), Japan (Wakame) and China, where it is extensively cultivated as food. Populations of *U. pinnatifida* are established throughout Europe, New Zealand, Argentina and Australia. In Korea, *U. pinnatifida* cultivation began with artificial seeding and experimental cultivation which started in1964. Thereafter, the annual production of cultured *U. pinnatifida* rapidly increased from 110 000 metric tons (wet wt.) in 1973 to 286,611 metric tons (wet wt.) in 2005 which market value was 44,000,000 US$ (44,326,784,000 won).

The sporophyte reaches a length of 1-2 or even 3 m in most areas (Casas and Piriz, 1996) but is usually less than 1 metre in the Mediterranean Sea (Haefner, 2003). The species includes at least two morphological forms f. *typica* and f. *distans*, the latter with a longer stipe and sporophylls often not reaching all way up to the lamina (Okamura, 1926).

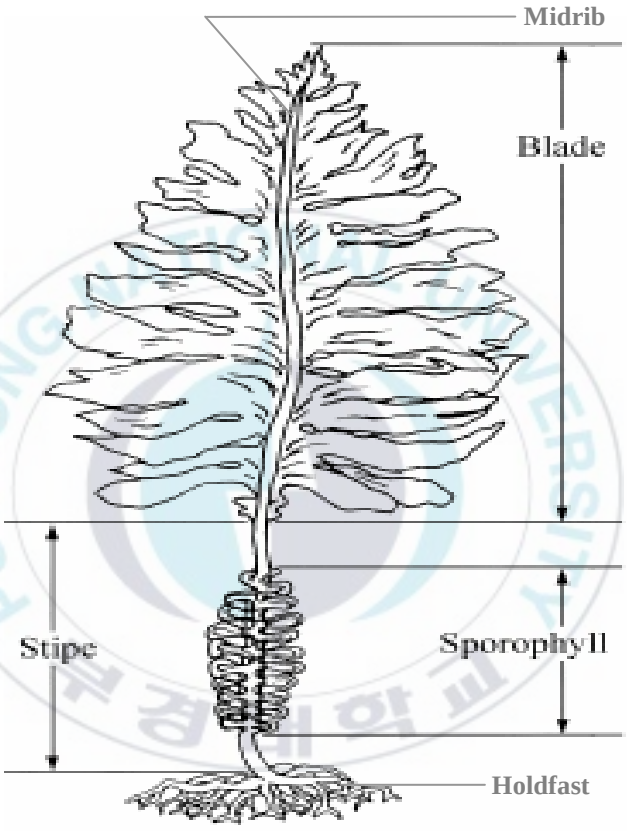

Figure 1.1. Blade, midrib, stipe, sporophyl and holdfast of a mature *Undaria pinnatifida.*

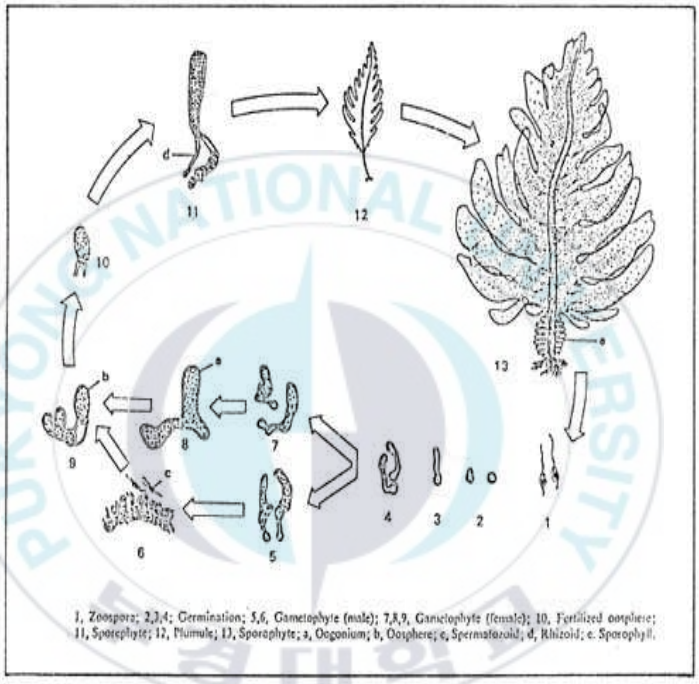

Figure 1.2. Life cycle of *Undaria pinnatifida.*

The species has a heteromorphic life cycle with a large sporophyte and separate microscopic female and male gametophytes. The growing zone is located between the top of the stipe and the lamina, making the top of the lamina the oldest part. The basal part of the sporophyte develops two undulated, wing like sporophylls (one along each side of the stipe, but they may become interleaved and look like one unit) with zoosporangial sori, producing millions of spores per gram tissue (Saito, 1975). Photosynthesis slows down and growth stops in most areas at high water temperatures when most of the lamina deteriorates, and stipes and holdfasts usually disappear during the end of summer (Saito, 1975), but may also persist (Hay and Villouta, 1993). Some introduced populations have successive recruitment during the year, i.e. both small and large sporophytes occur together (Hay and Villouta, 1993). The microscopic gametophytes are very difficult to spot in the field. The female plants consist of only a few cells bearing the oogonia and the males of some more, smaller cells with antheridia. The gametophytes may have a dormancy period especially at low light, and are capable of surviving adverse conditions as thick-walled resting stages (Saito, 1975). After fertilization sporophytes develop, at first attached to the female gametophyte. The species is not known to reproduce vegetatively by fragmentation, but asexual reproduction through unfertilized eggs, developing into parthenogenetic sporophytes, has been seen in laboratory experiments. The relationship of water temperature and light to the various life stages of *U. pinnatifida* and its nutrient compositions are shown in Table 1.1, Table 1.2 and Table 1.3.

Table 1.1. Water temperature and light in relation to the various life stages of *U. pinnatifida* (Jung, 1988).

Stage	Water temperature	Light	Month
Sporophylls-sporangium			Apr-Jul
Sporangium-zoospore discharge	14-22^{0}C (opt.17-20^{0}C)		Apr-Jul
Zoospore-gametophyte	17-20^{0}C (no growth at 23^{0}C)	2000-6000 Lux	May-Jul
Gametophyte resting phase	25-30^{0}C	500 Lux	Jul-Aug
Gametophyte maturation & germination of germling	Drops under 20^{0}C	1000 Lux	Sep-Oct
Germling growth phase	17-10^{0}C (opt. 15^{0}C)		Oct-Nov
Growing fronds of thallus	13-5^{0}C (opt. 10^{0}C)		Nov-Apr

Table 1.2. Nutritional contents of *U. pinnatifida* (Matsuzaki and Iwamura, 1981).

	Weight of dry products (%)	Weight of reference products (%)
Water	16.0	
Protein	12.7	34.2 in *Porphyra*
Lipid	1.5	1.6 in *Laminaria*
Carbohydrate	47.8	61.5 in *Enteromorpha*
Cellulose	3.6	13.0 in *Hizikia*
Mineral	18.4	34.0 in *Hizikia*
	Per 100g of dry products	Per 100g of reference products
Vitamin A (I.U.)	140	38400 in *Porphyra*
Vitamin B_1 (mg)	0.11	0.21 in *Porphyra*
Vitamin B_2 (mg)	0.14	0.32 in *Laminaria*
Vitamin C (mg)	15	20 in *Porphyra*
Niacin (mg)	10	8 in *Enteromorpha*
Calcium (mg)	1300	1400 in *laminaria*
Iodine (mg)	7.9	260 in *Laminaria*
Iron (mg)	13	106 in *Enteromorpha*

Table 1.3. Arsenic, selenium, fluorine and iodine contents in *U. pinnatifida* (Adachi et al., 1978).

Different parts (ppm)	Arsenic	Selenium	Fluoine	Iodine
Top	7.55-54.6	0.03-0.04	3.9	13.1
Blade	8.98-32.7	0.02-0.05	2.3-8.7	59.3-127.2
	(40.5)	(0.02)	(2.7)	(576)
Stipe	4.78-56.1	0.02-0.04	2.6	67.8

* () is for *Laminaria japonica*

The brown alga *U. pinnatifida* is a constituent of traditional Korean and Japanese cuisine. Almost all Korean women, even immigrated to foreign countries (Park and Peterson, 1991), eat the *U. pinnatifida* soup after childbirth for a month because of the belief that it cleans the blood and milk production. Use of the *U. pinnatifida* for curing fever, urination, lump or swelling is recorded in an oriental medical textbook Donguibogam published in 1613 (Donguibogam committee, 1999). It is also known to contain ingredients that contract the uterus after childbirth (Huh et al., 1992). For herbal medicine in China, it is used to treat urinary diseases and dropsy (Tseng and Chang, 1984). All of these symptoms are related to anti-inflammation reaction. Considering this traditional concept, several studies have focused on the beneficial effects of *U. pinnatifida* on cardiovascular risks, such as hypertension (Hata et al., 2002), hypercholesterolemia (Murata et al., 1999), and anti-viral activity and antiobesity (Meda et al., 2005). As far as we are aware, however, little work has been conducted on the anti-inflammatory effect of *U. pinnatifida*. In an attempt to validate anti-inflammatory properties from the more prevailing and potent *U. pinnatifida*, the following specific tasks have been set out:

- Screening of common seaweeds with anti-inflammatory properties.
- Evaluate the medicinal activity of *U. pinnatifida*, measuring anti-inflammatory activity of a methanol extract against mouse ear edema, as an analgesic and antipyretic, and for toxicity.
- Investigation of anti-erythematous inflammatory activity of methanol extract and development of a simple, easy and cost effective method of digital photo analysis to measure the effectiveness of *U. pinnatifida* on erythematous inflammation.
- Isolation and characterization of anti-inflammatory constituents, and evaluate potential of isolated constituents of *U. pinnatifida*.
- Study of the distribution of bioactive compounds in *U. pinnatifida*.

The detailed experimental procedures and the findings of the above mentioned tasks are discussed in the subsequent chapters.

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Chapter 2

Anti-inflammatory activities of a methanol extract of the brown seaweed *Undaria pinnatifida*

Abstract

Anti-inflammatory activities of a methanol extract of the brown seaweed *Undaria pinnatifida* Suringar were measured. *U. pinnatifida* has been used as a traditional medicine in the treatment of fever, urination problems, lumps and swelling, and as a health-care diet for women after childbirth. Methanol extract showed an IC_{50} value of 10.3 mg/ml against mouse ear edema induced by phorbol myristate acetate (PMA). A 40-mg/ml methanol extract maintained a 50% or higher suppression when applied either 3 h before or 2 h after PMA application. With the extract application, edema diminished to half maximal levels after 23 h compared to 31 h with vehicle. The blade part of the thallus showed the highest activity, while the northern forma of *U. pinnatifida* had a slightly higher activity. Compared with 37 common seaweeds, *U. pinnatifida* showed the greatest suppression. In the analgesic test, the methanol extract showed a suppressive effect on acetic acid-induced writhing response, with an IC_{50} of 0.48 g/kg body weight. The extract also showed antipyretic activity when tested in yeast-induced hyperthermic mice. The methanol extract showed no toxicity to mice after an oral administration of 10 g/kg.

Keywords**:** Analgesic; anti-edema; anti-inflammatory; antipyretic; brown seaweed; *Undaria pinnatifida*

Introduction

Many studies have concentrated on the contribution of marine organisms, including seaweeds, to the search for new drugs from natural products. For drug development, selection of samples for biological activity assays is often based on ecological observations of species with unique chemical mechanisms for coping with environmental pressures (Smit, 2004). Another approach in this search is through assaying for active ingredients of organisms used in folk remedies. A number of seaweed species are used as traditional medicines, foods and health-care in various regions of the world. The use of the brown alga, *U. pinnatifida* (Harvey) Suringar (known as Miyok), as a cure for fever, urination problems, lumps and swelling, is recorded in the Oriental medical textbook Donguibogam published in 1613 (Donguibogam Committee, 1999). As an herbal medicine in China, it has been used to treat urinary diseases and dropsy (Tseng and Chang, 1984). It is also known to have an 18:4 n-3 PUFA that inhibits leukotriene production in inflammation (Ishihara et al., 1998). Most of these effects are directly or indirectly related to the anti-inflammatory action of the seaweed.

U. pinnatifida is common along temperate coastal regions of the northeast Pacific including Korea, Japan and northern China (Ohno and Matsuoka, 1993). Nowadays, it occurs in temperate regions all over the world as an invasive species (Aguilar-Rosas et al., 2004). It is annual brown seaweed belonging to the Family Alariaceae and grows on rocks and reefs to a depth of 1–10m below tide-level in open seas or within bays near the open sea. Length of mature fronds is 1-2m. In 2004, production was estimated at 262,000 tons (wet wt) by aquaculture and 700 tons (wet wt) by natural collection in Korea (http://fs.fips.go.kr).

Thus, to evaluate the medicinal activity of *U. pinnatifida*, an abundant species with immense aquaculture potential, we measured anti-inflammatory activity of a methanol extract against mouse ear edema, as an analgesic and antipyretic, and for toxicity.

Materials and methods

Animals

BALB/c mice (8–10 weeks old; 25–30 g body weight), purchased from Daehan Biolink Co. (Chungbuk, Korea), were used for anti-inflammatory and other assays. Animals were kept in a room at temperature of 24±1^{o}C on a 12h light/dark cycle and with free access to food and water. All animals were acclimatized for at least 1 week before experimentation. The study was performed in accordance with the U.S. NIH Guidelines for the Care and Use of Laboratory Animals.

Seaweed extracts

The brown alga, *U. pinnatifida* f. *distans* (Harvey) Suringar(northern forma), was collected from aquaculture farm in Kijang (Busan, Korea) in May 2004 and 2005. Other seaweed species, for activity comparative purposes, were collected from the coast of Korea between October 2003 and June 2005. Voucher specimens have been deposited in the Biochemistry laboratory, Biotechnology & Bioengineering Department, Pukyong National University, Korea. The whole body of *U. pinnatifida* was ground into make powder form using a coffee grinder. For each 20 g seaweed powder, 1 litre of 100% methanol was used to extract the methanol-soluble fraction at room temperature for 1 day. To remove salt from the seaweed extracts, methanol extraction was repeated several times (from the previous methanol-soluble fraction) until, to the eye, the amount of salt were negligible. In case of *U. pinnatifida* powder (1 kg), it gave a dark brown residue (11.2 g) – a yield of 1.1% (Figure-2.1).

Anti-inflammatory test

Stock solutions of seaweed for anti-inflammatory assays were prepared by adding 1 ml of ethanol to every 40 mg of dried seaweed extract. Phorbol myristate acetate (PMA; Sigma, St. Louis, MO, USA) was topically applied to the inner side of the mouse ear at 0.2 μg in 10 μl acetone with an equal

volume of seaweed extracts in ethanol (0.4 mg/10 μl). Ear edema (swelling) was measured 10 h later using a spring-loaded micrometer (Mitutoyo Corp., Tokyo, Japan). Edema value was expressed as $(T_{10}-T_0)/T_0$, where T_{10} is ear thickness 10 h after PMA application and T_0 is ear thickness at 0 h. The edema value with ethanol vehicle was 0.81 ± 0.04. Relative inhibition (%) is expressed as (1– edema value of extract/edema value of vehicle) × 100.

Analgesic test

The analgesic test by acetic acid-induced writhing was followed essentially as described by Nakamura et al. (1986). The writhing response was elicited by an intraperitoneal injection of 0.7% acetic acid in saline at a dose of 10 ml/kg body weight. The *U. pinnatifida* extract and ethanol vehicle were intraperitoneally injected into the mice 30 min before acetic acid. Number of writhes was counted for 15 min, beginning 5 min after acetic acid injection.

Antipyretic test

The antipyretic property of the extract was tested in mice in which hyperthermia had been induced following the method of Teotino et al. (1963). Mice were made hyperthermic by subcutaneous injection of 15% brewer's yeast in a saline suspension at a dose of 10 ml/kg body weight. Rectal temperature was recorded using an electric thermometer connected to a probe. The temperature peaked 24 h later. At this peak, the extract (10 g in 10 ml of 5% Tween-80) and control (10 ml of 5% Tween-80) per kg body weight were given by oral administration and the rectal temperature recorded over a 2.5h period.

Toxicity test

Mice were fasted for 6 h with water *ad libitum*. The extract (10 g or 15 g in 10 ml of 5% Tween-80) per kg body weight was administered orally to mice (n = 10 or 19, respectively). The animals were observed for any abnormal behavior for 3 h, and mortality was noted up to 2 weeks after oral administration. The weight of major organs from dead mice were measured and checked for abnormalities. Cytotoxicity was observed with a human HaCaT cell line using the crystal violet proliferation assay.

Statistical analysis

All animal experiments were performed with at least seven mice for each group. Data are reported as mean ± S.E., and the statistical analysis was performed using Student's *t*-test.

Results

Edema suppression

Mouse ear edema was caused by topical application of PMA (0.05–1.0 μg in 10 μl acetone) to determine the optimal concentration. A swelling response was noted 10 h after application with a concentration of 0.2 μg or lower, at 15 h with 0.5 μg and at 20 h with 1.0 μg, with increasingly higher edema values (data not shown). To assay edema suppression in the short term, the ear thickness was measured 10 h after 0.2 μg PMA application. Edema value at 10 h reached 0.81 ± 0.04. To measure inhibition rate of the methanol extract of *U. pinnatifida* against 0.2 μg PMA in 10 μl acetone, 10 μl of different concentrations of the extract were mixed and applied to the ear. The concentration giving a 50% inhibition (IC_{50}) was 10.3 mg/ml (Figure 2.2). A concentration of 40 mg/ml inhibited 85%, i.e. suppressed edema almost completely. The 80 mg/ml extract was too dense to be used, thus, the concentration of 40 mg/ml was used for further experiments.

To elucidate its preventative effect, the extract was applied before PMA application. The 40 mg/ml methanol extract completely prevented edema when applied 30 min prior to PMA application (Figure 2.3). The preventative effect was maintained at 50% or higher when applied at least 3 h earlier. Application of the extract earlier than 3 h showed a weak effect with time. To test its curative effect, the extract was applied after PMA application. With the 40 mg/ml methanol extract, the curative effect was maintained at 50% or higher when applied within 2 h after PMA application (Figure 2.3). Thus, the extract had a potent preventative activity against inflammation, and results were better if the extract was applied at or near the time of PMA application.

Edema was maximal at 10 h after PMA application and diminished slowly with time. To ascertain the effect of extract on diminishing edema, it was applied at the maximal edema time of 10 h, and the edema measured for a further 48 h (Figure 2.4). Edema decreased quickly with the application of the

extract – diminution to half maximal value was 23 h with the extract, compared to 31 h with the vehicle application.

A mature thallus of *U. pinnatifida* consists of a pinnately divided blade with midrib, a compressed stipe containing mature undulated sporophyll frill and sporophyll midrib, and a fibrous holdfast. When an extract from each part of the thallus was compared for anti-edema activity, the blade showed the highest inhibition of 96% against edema (Table 2.1). Mature thalli (approximately 1 m in height) had a higher activity than young thalli (approximately 20 cm in height), in general. *U. pinnatifida* can be distinguished as two morphological forma: *U. pinnatifida* f. *distans* (northern forma) and *U. pinnatifida* f. *typica* (southern forma). When an extract from each forma was compared for anti-edema activity, northern forma showed a slightly higher activity against edema (Table 2.2). To compare the relative activity of *U. pinnatifida* with other seaweed species, 37 species of common seaweed were collected and anti-edema activity compared under the same assay conditions. Of the 37 seaweed extracts tested, *U. pinnatifida* and *Enteromorpha linza* showed the highest inhibition activity (>80%) against edema (Table 2.3). Isolation of the main active compounds from both *U. pinnatifida* and *E. linza* are in progress. Of the remaining seaweeds, *Colpomenia sinuosa*, *Ecklonia stolonifera*, *Sargassum thunbergii*, *Gracilaria verrucosa*, and *Pachymeniopsis elliptica* showed inhibition activity higher than 70%.

Analgesic activity

With an intraperitoneal (i.p.) injection of 0.7% acetic acid, mice showed writhing behavior 33 ± 0.7 times over the observation period of 15 min. When methanol extract of *U. pinnatifida* was i.p. injected to the other side of the abdominal cavity 30 min prior to acetic acid, it caused inhibition of the writhing response with an IC_{50} of 0.48 g/kg-bw (Figure 2.5). IC_{50} of aspirin as a standard analgesic was 0.07 g/kg-bw.

Antipyretic activity

When mice were subcutaneously (s.c.) injected in the back with 15% brewer's yeast, the rectal temperature reached a peak of 39.19±0.07^{o}C from the normal 38.45±0.06 ^{o}C after 24 h. At this peak, the methanol extract was administered orally at a dose of 10 g/kg body weight. It resulted in a significant lowering of rectal temperature in hyperthermic mice, similar to aspirin (Figure 2.6). The decrease in rectal temperature was still evident when assessed 2.5 h after administration of the extract.

Toxicity

Even though *U. pinnatifida* is very common in the daily diet in Korea and Japan, acute toxicity of the methanol extract was evaluated by oral administration to mice at 10 and 15 g/kg body weight. There was no mortality or obvious symptoms in 10 mice during the 2-weeks after the administration of 10 g/kg. At 15 g/kg, six of 19 mice died within 1 day and the other 13 mice were alive after 2 weeks. Immediately after oral administration at 15 g/kg, most mice showed backward movement, trying to vomit, jumping, sleeping, scaling and writhing for 5–10 min. The behavior seems to have originated from the large dose and the high density of the extract in the stomach. From the six mice that died within 1 day, the major organs were removed, weighed and examined (Table 2.4). Weight of stomach and heart had increased, while most of the extract remained in the stomach without digestion. The other organs showed no significant changes. Thus, a safe concentration of 10 g/kg body weight and no problems with the traditional *U. pinnatifida* diet, suggests that the extract can be used safely by humans at moderate doses. When the methanol extract was tested for cytotoxicity with a human HaCaT cell line using the crystal violet proliferation assay, 1% of the methanol extract in cell culture medium showed 57% proliferation. After removing the hexane-soluble fraction from the *U. pinnatifida* powder in advance, the methanol extract showed a 91% proliferation. Thus, even the weakly cytotoxic-like component can be easily removed by extracting with hexane solvent.

Discussion

The seaweed *U. pinnatifida* is well known as a health-care diet for nursing Korean mothers. Almost all Korean women, even those who have immigrated to foreign countries (Park and Peterson, 1991), eat *U. pinnatifida* soup for a month or so after childbirth in the belief that it helps postpartum convalescence and cleanses the blood. In an encyclopedia published in the Chinese Dang Dynasty, there is a record that Koreans observed whales eating *U. pinnatifida* after giving birth and, thereafter, used it as a postpartum health food (Kim, 2006). Traditionally, Japanese also eat *Undaria* during the postpartal period, as well as promote good hair and skin condition (Matsuzaki and Iwamura, 1980). It has also been used traditionally to treat fever, urination problems, lumps or swelling (Donguibogam Committee, 1999). In herbal medicine in China, *U. pinnatifida* is used to treat urinary diseases, dropsy, stomach ailments, hemorrhoids, anal fistulas, leucorrhea in women and nocturnal emission in men (Tseng and Chang, 1984). The seaweed is also known to affect contraction of the uterus (Huh et al., 1992), to stimulate hepatic fatty acid oxidation (Murata et al., 1999), and to have antioxidation (Yoo et al., 2004), antitumor (Hosokawa et al., 2004), antihypertension (Suetsuna et al., 2004), antivirus (Thompson and Dragar, 2004), and antiobesity properties (Maeda et al., 2005).

Mature thalli of *U. pinnatifida* showed higher anti-inflammatory activity than young thalli (Table 1). Usually, young thalli produce more primary metabolites in growing fast, while mature thalli would have more mechanisms to modify biocompounds or protect cellular functions (Chen et al., 1998). Damaged thalli had the same activity as normal thalli (Table 1). Occasionally, when seaweed is damaged by herbivores or others, the tissues produce secondary metabolites to prevent attack and repair tissue damage (Arnold and Targett, 2002). From the results, the active component was not produced via such an inducible pathway. According to WHO (1992), herbal medicine is said to be nontoxic if the LD_{50} is lower than 5 g/kg body weight. From this assertion, it can be said that the methanol extract is not toxic, since no mortality at 10 g/kg was recorded.

Recently, three active compounds have been purified from *U. pinnatifida* tissue and their chemical identification is in progress. They appear to be similar to omega-3 PUFAs. The omega-3 PUFAs are mainly known for their anti-inflammatory effects, related to their competition as substrates for cyclooxygenase and lipoxygenase, leading to decreased production of prostaglandins and leukotrienes (James et al., 2000). Diet supplementation with n-3 PUFAs causes a reduction in the expression and activity of aggrecanases, inflammation-inducible cytokines and cyclooxygenase-2, but not the constitutively expressed cyclooxygenase-1 (Curtis et al., 2000).

In conclusion, the present investigation clearly demonstrates that the methanol extract of the brown seaweed *U. pinnatifida* showed significant anti-inflammatory activity without any serious toxic side-effects at moderate dose. *U. pinnatifida* has been used in health care and indigenous medicine as a remedy for inflammation-related symptoms, and this study supports these claims.

Table 2.1. Anti-edema activity of methanol extracts from different parts of *U. pinnatifida* thalli. Reference with PMA showed edema value of 0.81 ± 0.04, i.e. relative inhibition of 0%.

	Mature thalli		Young thalli	
	Edema value	Relative inhibition (%)	Edema value	Relative inhibition (%)
Blade	0.03 ± 0.02	96	0.16 ± 0.04	80
Midrib	0.16 ± 0.06	80	0.26 ± 0.03	68
Stipe	ND	ND	0.24 ± 0.03	70
Sporophyll frill	0.08 ± 0.05	91	ND	ND
Sporophyll midrib	0.45 ± 0.05	44	ND	ND
Holdfast	0.37 ± 0.12	54	0.23 ± 0.06	72

Mean ± S.E. ($n \geq 7$); $P < 0.001$.

ND, not determined.

Table 2.2. Anti-edema activity of methanol extracts from northern and southern formas of *U. pinnatifida* thalli. Reference with PMA showed edema value of 0.81 ± 0.04, i.e. relative inhibition of 0%.

Forma	Edema value	Relative inhibition (%)
Undaria pinnatifida f. *distans* (Northern forma)	0.06 ± 0.03	92
Undaria pinnatifida f. *typica* (Southern forma)	0.14 ± 0.03	83

Mean ± S.E. ($n \geq 7$); $P < 0.001$.

Table 2.3. Comparison of anti-edema activity of methanol extracts from various seaweed species.

Species	Edema value	Relative inhibition (%)
CHLOROPHYTA		
Capsosiphon fulvescens	0.40 ± 0.05	51
Codium fragile	0.53 ± 0.04	33
Enteromorpha compressa	0.28 ± 0.01*	65
Enteromorpha linza	0.13 ± 0.00*	84
Scytosiphon lomentaria	0.26 ± 0.04*	68
Ulva pertusa	0.37 ± 0.02	54
PHAEOPHYTA		
Colpomenia bullosa	0.39 ± 0.03	52
Colpomenia sinuosa	0.23 ± 0.01*	72
Costaria costata	0.38 ± 0.04	53
Dictyota dichotoma	0.39 ± 0.03	52
Ecklonia cava	0.28 ± 0.04*	65
Ecklonia stolonifera	0.20 ± 0.03*	75
Hizikia fusiformis	0.30 ± 0.01*	63
Ishige okamurae	0.45 ± 0.03	44
Ishige sinicola	0.70 ± 0.04	14
Laminaria japonica	0.79 ± 0.10	01
Sargassum confusum	0.48 ± 0.04	41
Sargassum fulvellum	0.72 ± 0.10	11
Sargassum horneri	0.58 ± 0.03	28
Sargassum ringgoldianum	0.62 ± 0.03	23
Sargassum sagamianum	0.32 ± 0.04*	60
Sargassum thunbergii	0.20 ± 0.01*	75
Undaria pinnatifida	0.12 ± 0.00*	85
RHODOPHYTA		
Carpopeltis cornea	0.21 ± 0.02	25
Chondrus ocellatus	0.33 ± 0.02	59
Corallina pilulifera	0.66 ± 0.02	19
Gigartina tenella	0.38 ± 0.20	53
Gracilaria verrucosa	0.22 ± 0.02*	73
Gymnogongrus flabelliformis	0.56 ± 0.02	31
Hypnea charoides	0.70 ± 0.02	14
Helminthocladia australis	0.31 ± 0.06*	62
Lomentaria catenata	0.76 ± 0.00	06
Meristotheca papulosa	0.50 ± 0.00	38
Pachymeniopsis elliptica	0.18 ± 0.02*	78
Pachymeniopsis lanceolata	0.42 ± 0.00	48
Porphyra yezoensis	0.29 ± 0.04*	64
Symphyocladia latiuscula	0.60 ± 0.07	30
Control	0.81 ± 0.04	0

Mean ± S.E. ($n \geq 8$); *$P < 0.001$.

Table 2.4. Weight (g/kg body weight) of major organs of mice that died within 1 day after oral administration of a 15-g extract/kg body weight. Control was administered with 5% Tween-80.

Name of organs	Control	Extract (15 g/kg)
Heart	4.7 ± 0.3	7.9 ± 0.5*
Kidney	19.8 ± 0.7	16.1 ± 1.3
Liver	66.7 ± 3.3	61.0 ± 4.5
Lung	7.4 ± 0.1	7.2 ± 0.2
Pancreas	4.0 ± 0.1	5.0 ± 0.5
Spleen	2.9 ± 0.2	3.6 ± 0.5
Stomach	57.3 ± 6.9	89.4 ± 2.8*

Mean ± S.E. (n = 6); *P < 0.01.

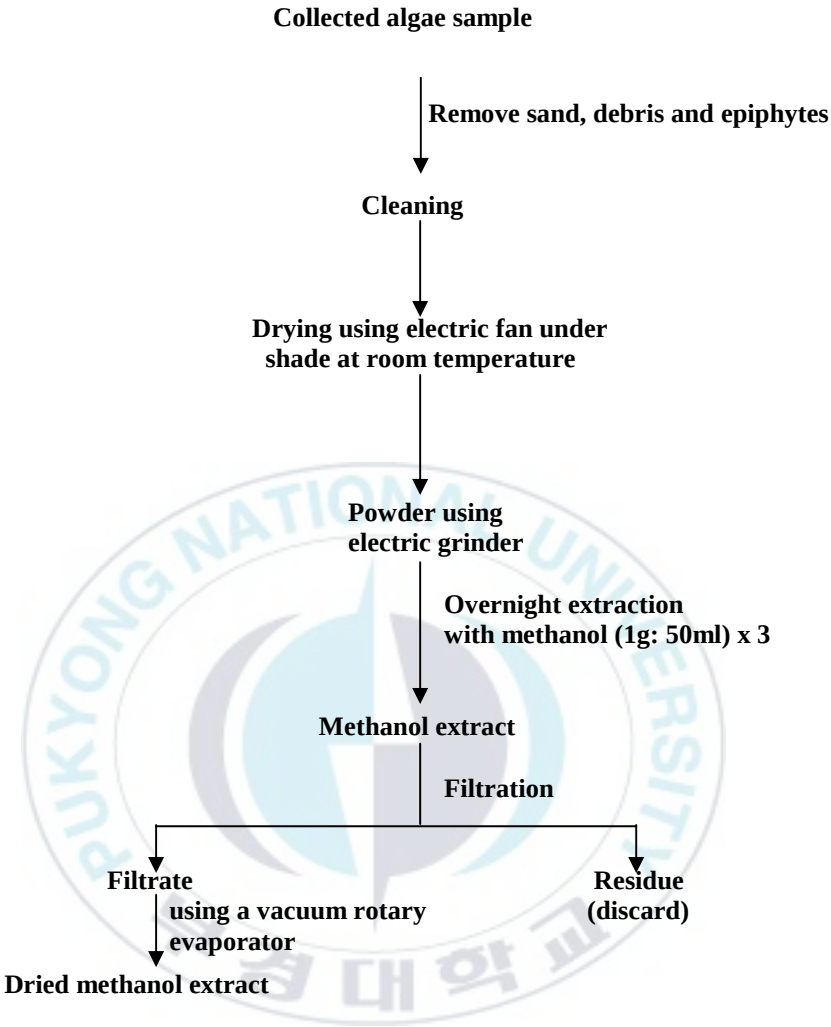

Figure 2.1. Experimental protocol of preparing crude algal extracts for preliminary screening.

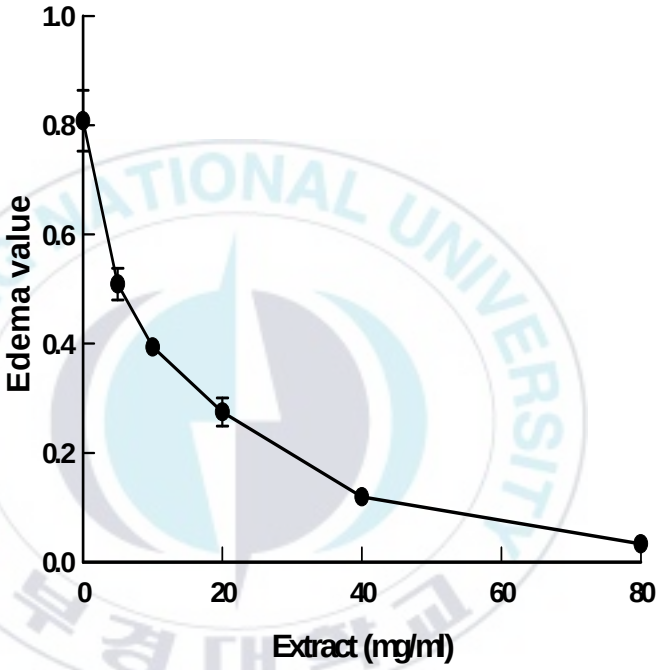

Figure 2.2. Suppression of edema by different concentrations of methanol extract of *Undaria pinnatifida*. Mean ± S.E. ($n \geq 7$).

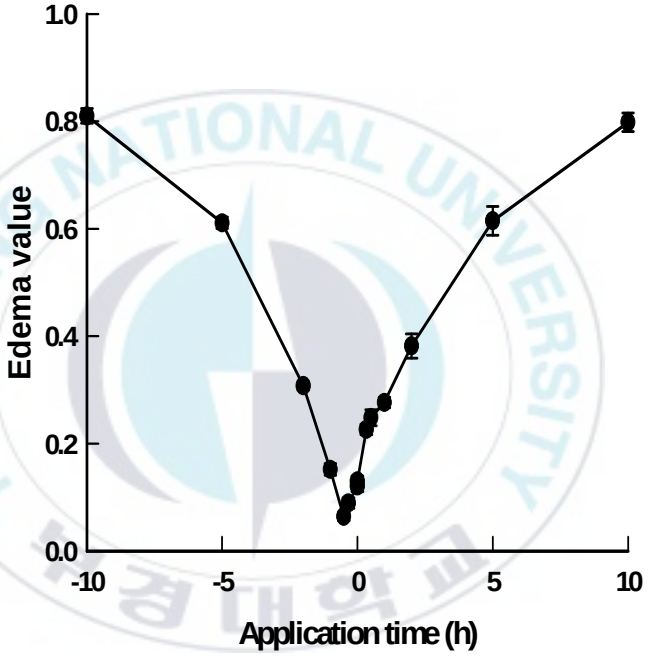

Figure 2.3. Effect of application of methanol extract before (–10 to 0 h) and after PMA application (0 to 10 h). Mean ± S.E. (*n* ≥ 7).

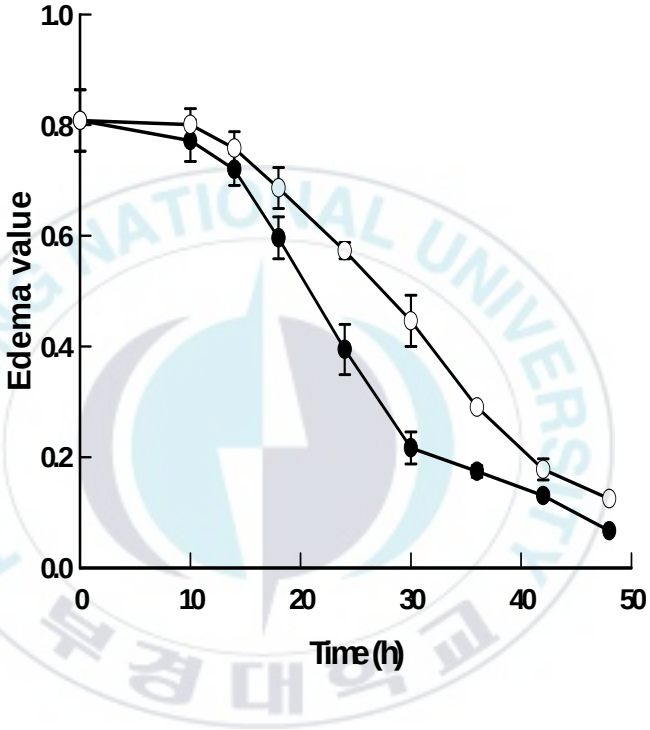

Figure 2.4. Diminution time for edema with methanol extract. Edema was induced maximally and an extract of 0.4 mg/10 μl (●) applied on the swollen ear. Vehicle only (o). Mean ± S.E. (*n* ≥ 7).

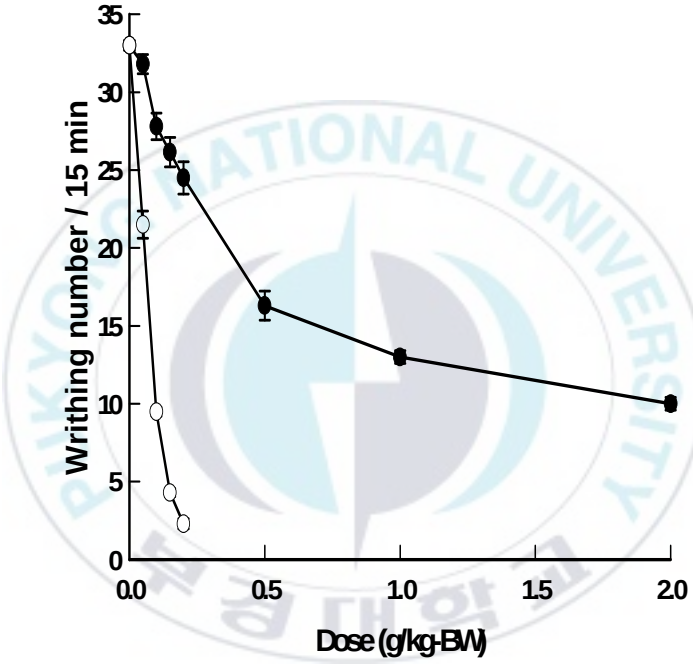

Figure 2.5. Effect of methanol extract on acetic acid-induced writhing response in mice. Methanol extract (●). Aspirin (o). Mean ± S.E. ($n \geq 10$).

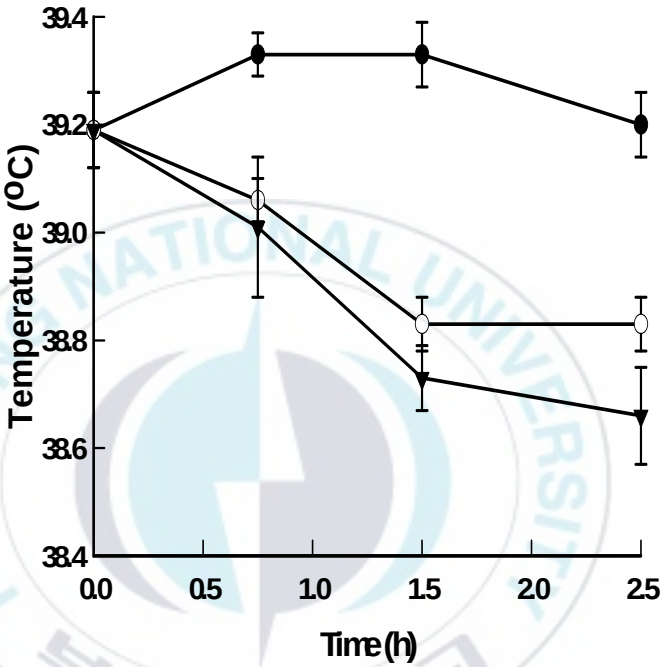

Figure 2.6. Effect of methanol extract on antipyretic activity in hyperthermic mice induced by yeast. Vehicle only (●). Extract at 10 mg/kg body weight (o). Aspirin at 150 mg/kg body weight (▼). Mean ± S.E. ($n \geq 10$). $P < 0.1$.

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Chapter 3

Effects of a methanol extract of the brown seaweed *Undaria pinnatifida* on erythematous inflammation

Abstract

A methanol extract of the brown seaweed *Undaria pinnatifida* produced potent inhibition of erythematous inflammation. Assessed by digital photo analysis, the IC_{50} of the extract on mouse ear erythema induced by PMA was 15.6 mg/ml. The IC_{50} value of the extract when erythema was measured using laser speckle flowgraphy was 18.1 mg/ml. In an *in vitro* preparation, a 10 mg/ml concentration of extract contracted blood vessels to approximately 20% of the maximal contraction induced by 10^{-6} M noradrenaline. Erythema reduction to half-maximal values took 12 h with 40 mg/ml extract, compared to 25 h with vehicle application. The methanol extract suppressed erythema by 50% when applied within 1 h before or 15 min after PMA application. Extracts prepared from different seaweed tissues varied in effect, with the blade showing the greatest anti-erythema activity. In addition, the northern forma of *U. pinnatifida* had slightly more activity than the southern forma.

Key words: Anti-erythema; anti-inflammation; digital photo analysis; laser speckle flowgraphy; Phaeophyta, *Undaria pinnatifida*

Introduction

The edible brown seaweed *Undaria pinnatifida* is often used as a health-care diet and in traditional medicine preparations in East Asia. It has been reported as a cure for fever, urination problems, lumps and swelling as early as 1613 in the Oriental medical textbook Donguibogam (Donguibogam Committee, 1999). In Japan, *U. pinnatifida* is eaten during the postnatal period as well, and is also thought to promote hair and skin health (Matsuzaki and Iwamura, 1980). It is used in China as an herbal medicine to treat several ailments, including urinary diseases, dropsy, stomach ailments, hemorrhoids, anal fistula, leucorrhea in women, and nocturnal emission in men (Tseng and Chang, 1984). *U. pinnatifida* is also been reported to affect uterine contractions (Huh et al., 1992) and hepatic fatty acid oxidation (Murata et al., 1999). Most of these claims are thought to be directly or indirectly related to the anti-inflammatory properties of this seaweed, which is also known to contain various potentially therapeutic compounds, including the antiviral compound fucoidan (Thompson and Dragar, 2004), antihypertensive dipeptide (Suetsuna et al., 2004), and fucoxanthin, known to possess stroke-preventative (Ikeda et al., 2003), antitumor (Hosokawa et al., 2004), and antiobese properties (Maeda et al., 2005).

Erythema, the clinical description of redness of the skin, is one of the major visible symptoms of inflammation. It is influenced by several factors, such as vascular structure and amount of erythrocytes in the tissue, in addition to thickness and pigmentation of the epidermis and dermis (Mattsson et al., 1997). Clinical grading of skin erythema is subjectively variable when performed using the naked eye. As such, common methods for comparative analysis of erythema or irritancy include skin reflectance spectrophotometer (Bjerring and Andersen, 1987), use of an erythema meter (Dykes et al., 1991), digital image analysis (Mattsson et al., 1997), and blood flow analysis using laser speckle flowgraphy (Lee et al., 2003).

In the present study, we developed a simple method of digital photo analysis to measure the effectiveness of *U. pinnatifida* on erythematous inflammation. This method was validated by comparing erythema values obtained using digital photo analysis with those recorded using laser speckle flowgraphy. The anti-erythema effect of the extract was confirmed by measuring arterial contraction in vitro. Furthermore, using digital photo analysis, we assayed the anti-erythema activity of various concentrations of *U. pinnatifida* extract, and the effects of different application times, different tissue parts, and different forma species on PMA-induced erythema.

Materials and methods

Seaweed extracts

Undaria pinnatifida f. *distans* (Harvey) Suringar was harvested from Kijang Aquaculture Farm (Busan, Korea) in April 2005 and April 2006. A methanol extract was prepared by allowing dried seaweed powder to sit in 100% methanol (1 litre methanol per 20 g seaweed powder) for 1 day at room temperature. To remove salt from the extract, the methanol-soluble fraction was re-extracted into 100% methanol several times until the amount of salt was negligible upon visual inspection. The methanol was then removed by evaporation, leaving the dried brown residue. This extraction resulted in a yield of 1.1% (i.e., 1 kg dried *U. pinnatifida* powder produced 11.2 g of extract). Stock solutions of seaweed extract for anti-erythema assays were prepared by solubilizing 40 mg of methanol extract in 1 ml of ethanol.

Animals

BALB/c mice (8–10 weeks old; 20–25 g body weight) and adult female Sprague-Dawley rats (200–250 g) purchased from Daehan Biolink Co. (Chungbuk, Korea) were used in anti-erythema assays. Animals were housed at 24 ± 1 ^{o}C on a 12-h light/dark cycle with ad libitum access to food and water. All animals were acclimatized to housing conditions for at least 1 week before experimentation. The study was performed in accordance with NIH Guidelines for the Care and Use of Laboratory Animals (Bethesda, MD, USA).

Anti-erythema assay

Phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO, USA) was used to produce erythema. In these experiments, PMA was topically applied to the inner side of the mouse ear (0.2 µg in 10 µl

acetone) with an equal volume of seaweed extract in ethanol (0.4 mg per 10 µl). Ear erythema was measured immediately following treatment and again 10 h later by digital photo analysis and laser speckle flowgraphy. Erythema values were expressed as $(E_{10} - E_0)/E_0$, where E_{10} is ear erythema 10 h following PMA applications, and E_0 is ear erythema at 0 h (immediately after treatment). Relative inhibition (%) was expressed as [1 – (erythema value of extract / erythema value of PMA)] × 100.

Digital photo analysis

Erythema was measured by analyzing digital still photographs taken at 0 h (immediately after treatment) and 10 h later. Photographs were white balance-adjusted using a digital camera (E330; Olympus, Tokyo, Japan), then transferred to a computer in JPEG format for color analysis. Photographs were analyzed using Adobe Photoshop 7.0 (Adobe; San Jose, CA, USA) at 100% image view. The color intensity was measured for the color magenta (0–100%), and read at five different areas diagonally across each ear. Erythema scores for each ear were calculated by averaging these five measurements.

Laser speckle flowgraphy

Local blood flow in the mouse ear was measured using laser speckle flowgraphy (Inflameter LFG-1; SoftCare, Fukuoka, Japan). The ear skin was scanned by moving the laser beam over the inner surface of the mouse ear. The distance between the scanner and ear surface was 0.5 cm. The recorded perfusion values were analyzed on a personal computer, and local blood flow calculated for a skin area 5 mm in diameter according to Lee et al. (2003). Relative blood flow was calculated in a similar fashion to relative erythema values (see above).

Measurement of blood vessel contraction

In these experiments, a physiograph was used to measure blood vessel contraction in descending thoracic aortas from adult rats (Katoue et al., 2006). For isometric tension recording, artery ring segments (3–4 mm in length) were set up in a 5.0 ml organ bath containing oxygenated (95% O_2 – 5% CO_2) Krebs solution at 37 ^{o}C. The preparations were allowed to equilibrate for up to 60 min at normal 1.0 gravity, during which the bath fluid was changed every 15 min. Contractions were recorded using force transducers (Ufer AP-5; Iwashiya, Kyoto, Japan) on a physiograph recorder (SS 250F; Sekonic, Tokyo, Japan). After equilibration, arterial contraction was first induced with 10^{-6} M noradrenaline to provide a baseline contraction value. We then measured contraction produced by varying concentrations of *U. pinnatifida* extract. Arterial contraction was measured in centimeters, and values are expressed as a percentage of noradrenaline-induced contraction.

Statistical analysis

The experiments were replicated at least seven times for each independent assay, and the highest and lowest values discarded. The mean values for each assay were compared with controls using Student's *t*-test, and *P*-values of less than 0.05 were considered statistically significant.

Results

Digital photo analysis

Mouse ear erythema was induced by topical application of PMA. A range of concentrations (0.05–1.0 μg in 10 μl acetone) was used to determine the optimal PMA dose. A redness response was maximally noted 10 h after application with 0.2 μg PMA or lower, at 15 h with 0.5 μg PMA, and at 22 h with 1.0 μg PMA, with increasingly higher erythema values (data not shown). For short-term erythema suppression experiments, we measured erythema 10 h after 0.2 μg PMA application. The average erythema value at this time point was 0.27 ± 0.01 (Table 3.1). Different concentrations of the methanol extract of *U. pinnatifida* were topically applied to the ear (10 μl volume) to measure the inhibitory effects of the extract on erythema produced by 0.2 μg PMA. The IC_{50} for the seaweed extract suppression of erythema was 15.6 mg/ml (Figure 3.1). An extract concentration of 40 mg/ml produced a robust inhibition of erythema (78% reduction). The 80 mg/ml extract was too dense to be used, and thus, the concentration of 40 mg/ml was applied for the digital photo analysis experiments.

Laser speckle flowgraphy

To compare different measures of erythema suppression, ear blood flow was measured using laser speckle flowgraphy at 0 h and 10 h following application of 0.2 μg PMA. The average blood flow value at 10 h was 0.086 ± 0.004. Different concentrations of methanol extract of *U. pinnatifida* (10 μl volume) were co-applied with 0.2 μg PMA to measure the blood flow-suppressing effects of the extract. The IC_{50} for the extract on blood flow was 18.1 mg/ml (Figure 3.2). Extract concentrations of 40 mg/ml and 80 mg/ml suppressed blood flow by 69% and 85%, respectively. These results show a potent suppression of blood flow, with similar patterns of change and a similar IC_{50} value to those recorded using digital photo analysis. A linear correlation of laser speckle flowgraphy values (Y) was

observed with those obtained using digital photo analysis (X); the relationship is described by Y = 0.2550X + 0.0145 with a confidence of 0.96.

Blood vessel contraction

To confirm suppression of erythema and blood flow, we measured blood vessel contraction using a rat thoracic aorta preparation in a 5 ml organ bath containing Krebs solution. Artery rings were first contracted maximally (6.5 ± 0.1 cm) with 10^{-6} M noradrenaline. After relaxation with 10^{-5} M acetylcholine, different concentrations of *U. pinnatifida* extract (0.5–30 mg/ml in Krebs solution) were applied and contraction measured. The extracts produced arterial contraction in a concentration-dependent manner (Figure 3.3). Extract concentrations of 10 mg/ml and 30 mg/ml induced contractions that were 20% and 23%, respectively, of the maximal contraction induced by 10^{-6} M noradrenaline. These results show that the seaweed extract promotes blood vessel contraction, and suggest that this effect is responsible for the observed reductions in blood flow and redness produced by the extract. Although this in vitro blood vessel measurement is more sensitive than the in vivo methods of digital photo analysis and laser speckle flowgraphy, the blood vessels are obtained from rats postmortem and as such can only be used once.

Application time

The above results indicate that erythema (and anti-erythema activity) is easily measured by digital photo analysis, without the need for special instrumentation. Thus, in further experiments investigating the effects of application time, different plant tissue parts, and different seaweed species, erythema was quantified using digital photography. To determine the preventative effects of *U. pinnatifida* extract on erythematous inflammation, the extract was applied to the mouse ear at various times prior to PMA application. The 40 mg/ml extract significantly inhibited erythema when applied 30 min prior to PMA application (Figure 3.4), and inhibition of erythema by 50% or more was

observed with pretreatment times up to 1 h. Curative effects of the extract were assessed by applying the seaweed extract after PMA application. The 40 mg/ml extract produced a 50% or greater reduction of erythema when applied within 15 min of PMA application (Figure 3.4). Even though the extract showed potent curative activity against erythema, results were more pronounced if the extract was applied either 30 min before or at the same time as PMA application. Erythema was maximal at 10 h after PMA application and diminished slowly with time. To ascertain the effects of *U. pinnatifida* on diminishing erythema, the extract was applied at the maximal erythema time point of 10 h, and redness measured for a further 38 h (Figure 3.5). Erythema decreased quickly upon application of the extract; reduction to half-maximal erythema values took 12 h with the *U. pinnatifida* extract, compared to 25 h with vehicle application.

Different tissues and species

A mature thallus of *U. pinnatifida* consists of a pinnately divided blade with a midrib, a compressed stipe containing a mature undulated sporophyll frill and a sporophyll midrib, and a fibrous holdfast. A comparison of extracts produced from separate parts of the thallus showed that the blade produced the greatest inhibition against erythema (86%) (Table 3.1). In general, mature thalli (approximately 1 m in height) had a higher activity than young thalli (approximately 20 cm in height). *U. pinnatifida* can be distinguished as two morphological forma: *U. pinnatifida* f. *distans* (northern forma) and *U. pinnatifida* f. *typica* (southern forma). When extracts from each forma were compared, the northern forma showed slightly greater anti-erythema activity (Table 3.2). The relative activity of *U. pinnatifida* was compared with 37 other species of common seaweed under the same assay conditions. Of the seaweed species tested, *U. pinnatifida* produced the most inhibition (78%) of erythema. Other species showed some anti-erythema effects; of the remaining seaweeds, *Costaria costata*, *Carpopeltis cornea*, *Ecklonia cava*, *Ecklonia stolonifera*, *Enteromorpha linza*, *Pachymeniopsis elliptica*, and *Porphyra yezoensis* produced more than 55% inhibition of erythema (Table 3.3).

Discussion

Here we report that erythematous inflammation induced by topical application of PMA was significantly suppressed by a methanol extract of *U. pinnatifida*. This extract contracted blood vessels and reduced blood flow, presumably reducing the aggregation of red blood cells in the inflamed area for a short period. We demonstrate that the presence of red blood cells can be simply measured by using digital photography and analysis with the Adobe Photoshop software program. In an earlier study employing digital image analysis, Mattsson et al. (1997) used "red, green, blue" (RGB) and "hue, saturation, intensity" (HSI) color model values from digitized images to measure erythema. The present results suggest that magenta color values (0–100%) obtained from Photoshop are sufficient to provide an accurate measurement of erythema, as we observed a linear relationship between magenta color values and concentration of applied PMA. Digital image analysis has also been used successfully to measure skin erythema due to radiotherapy (Wengström et al., 2004), further supporting the validity and reliability of this technique. Laser speckle flowgraphy directly reflects the movement of blood cells in subepithelial tissue (Lee et al., 2003). We detected a linear correlation of measured erythema values obtained using digital photo analysis and laser speckle flowgraphy. Digital photo analysis evaluates visible light reflected from the skin and is therefore influenced by factors such as differences in chromophores and illumination (Anderson and Parrish, 1982). These factors are accounted for in the present study as all experiments were performed under the same illumination and photo conditions. The current results suggest that these two noninvasive methods can be used in a complementary fashion. The benefits of digital photography are certainly numerous and include rapid image production, easy and rapid deletion of poor images, decreased costs for enlargements, ease of editing and image storage, and effortless placement in presentation and publications (Smith, 2002).

The results of this study indicate that *U. pinnatifida* extract was more effective in preventing erythema than curing existing erythema, as the greatest effects were seen when the extract was applied within 1 h before or up to 15 min after PMA treatment. The extract also showed an inhibitory

effect on PMA-induced edema (data not shown). The extract may exert its effects by inhibiting production of some inflammatory mediators. Three bioactive compounds have recently been purified from *U. pinnatifida* tissues and their chemical identification is in progress. They appear to be similar to omega-3 PUFAs, which are mainly known for their anti-inflammatory effects. It is believed these compounds competitively inhibit the enzymes cyclooxygenase and lipoxygenase, leading to decreased prostaglandins and leukotrienes production (James et al., 2000). Diet supplementation with n-3 PUFAs has also been shown to cause a reduction in the expression and activity of aggrecanases, inflammation-inducible cytokines, and cyclooxygenase-2, but not the constitutively expressed cyclooxygenase-1 (Curtis et al., 2000). We also report that mature thalli exhibit higher anti-erythema activity than young thalli (Table 1). It is generally thought that young thalli produce more primary metabolites due to rapid growth, while mature thalli possess more mechanisms to modify bioactive compounds or protect cellular functions (Chen et al., 1998). Partially damaged thalli showed similar activity as undamaged plants (data not shown). As such, the compound responsible for the observed anti-erythema effects is unlikely to be a secondary metabolite produced to prevent attack or repair tissues damaged by herbivores or other factors. Further studies will be needed to determine the specific identity of the active components.

In conclusion, these results may explain some of the medicinal properties attributed to *U. pinnatifida*, long employed in health care and traditional medicine as a remedy for symptoms of erythematous inflammation. This study also shows that the digital photo analysis technique is a relatively simple and inexpensive tool for noninvasive erythema assessment in biological research as well as in clinical practice.

Table 3.1. Anti-erythema activity of methanol extracts from different tissue parts of *Undaria pinnatifida* thalli. PMA alone resulted in a mean erythema value of 0.27 ± 0.01 (i.e., 0% relative inhibition).

	Mature thalli		Young thalli	
	Erythema value	Relative inhibition (%)	Erythema value	Relative inhibition (%)
Blade	0.04 ± 0.01	86	0.19 ± 0.06	29
Midrib	0.12 ± 0.03	56	0.16 ± 0.00	40
Stipe	0.15 ± 0.00	46	0.09 ± 0.02	67
Sporophyll frill	0.05 ± 0.02	80	ND	ND
Sporophyll midrib	0.17 ± 0.03	37	ND	ND
Holdfast	0.14 ± 0.04	48	0.17 ± 0.04	36

Values represent the mean ± SE ($n \geq 7$). $p < 0.001$

ND, not determined

Table 3.2. Anti-erythema activity of methanol extracts from northern forma and southern forma of *Undaria pinnatifida* thalli. PMA alone resulted in a mean erythema value of 0.27 ± 0.01 (i.e., 0% relative inhibition).

Forma	Erythema value	Relative inhibition (%)
U. pinnatifida f. *distans* (northern forma)	0.05 ± 0.03	79
U. pinnatifida f. *typica* (southern forma)	0.06 ± 0.01	77

Values represent the mean ± SE ($n \geq 7$). $p < 0.001$

Table 3.3. Comparison of anti-erythema activity of methanol extracts from various seaweed species.

Species	Erythema value	Relative inhibition (%)
CHLOROPHYTA		
Capsosiphon fulvescens	0.21 ±0.00	22
Codium fragile	0.20 ± 0.01	26
Enteromorpha compressa	0.15 ± 0.02	44
Enteromorpha linza	0.08 ± 0.00*	70
Scytosiphon lomentaria	0.16± 0.02	41
Ulva pertusa	0.15 ± 0.03	44
PHAEOPHYTA		
Colpomenia bullosa	0.13 ± 0.02	52
Colpomenia sinuosa	0.13 ± 0.02	52
Costaria costata	0.12 ± 0.00	56
Dictyota dichotoma	0.16 ± 0.00	41
Ecklonia cava	0.12 ± 0.00	56
Ecklonia stolonifera	0.11 ± 0.00	59
Hizikia fusiformis	0.17 ± 0.02	37
Ishige okamurae	0.19 ± 0.00	30
Ishige sinicola	0.16 ± 0.00	41
Laminaria japonica	0.23 ± 0.02	15
Sargassum confusum	0.13 ± 0.01	52
Sargassum fulvellum	0.20 ± 0.02	26
Sargassum horneri	0.15 ± 0.03	44
Sargassum ringgoldianum	0.21 ± 0.00	22
Sargassum sagamianum	0.13 ± 0.00	52
Sargassum thunbergii	0.13 ± 0.01	52
Undaria pinnatifida	0.06 ± 0.00*	78
RHODOPHYTA		
Carpopeltis cornea	0.12 ± 0.00	56
Chondrus ocellatus	0.14 ± 0.04	48
Corallina pilulifera	0.22 ± 0.01	19
Gigartina tenella	0.15 ± 0.00	44
Gracilaria verrucosa	0.13 ± 0.00	52
Gymnogongrus flabelliformis	0.18 ± 0.02	33
Hypnea charoides	0.17 ± 0.02	37
Helminthocladia australis	0.14 ± 0.02	48
Lomentaria catenata	0.23 ± 0.03	15
Meristotheca papulosa	0.13 ± 0.01	52
Pachymeniopsis elliptica	0.10 ± 0.02	63
Pachymeniopsis lanceolata	0.14 ± 0.01	48
Porphyra yezoensis	0.12 ± 0.02	56
Symphyocladia latiuscula	0.17 ± 0.00	37
Control	0.27 ± 0.01	0

Mean ± S.E. ($n \geq 8$); *$P < 0.001$

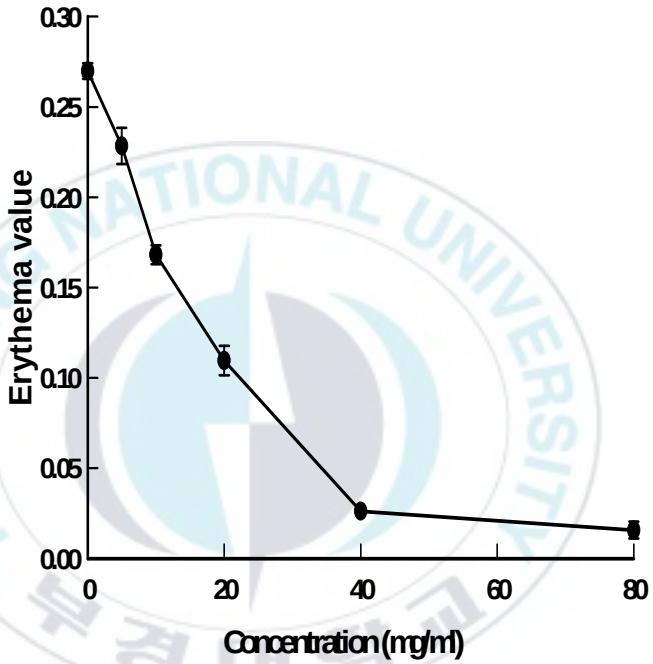

Figure 3.1. Supression of erythema by different concentrations of methanol extract of *Undaria pinnatifida*. Erythema was measured using digital photo analysis. Values represent the mean ± SE ($n \geq 7$).

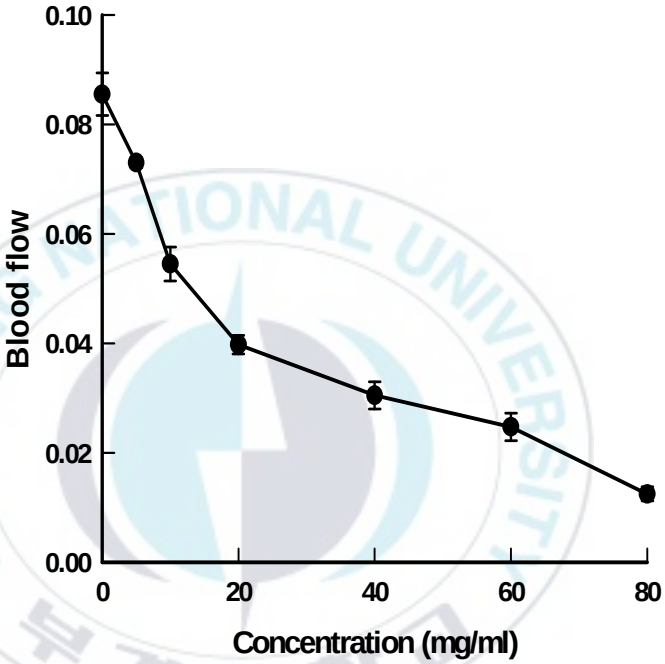

Figure 3.2. Suppression of blood flow by different concentrations of methanol extract of *Undaria pinnatifida*. Blood flow was measured using laser speckle flowgraphy. Values represent the mean ± SE ($n \geq 7$).

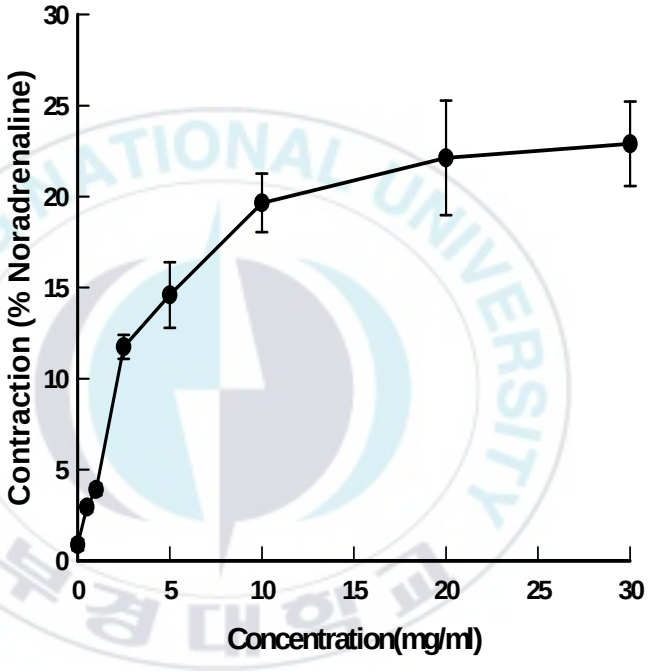

Figure 3.3. Arterial contraction produced by different concentrations of methanol extract of *Undaria pinnatifida*. Contraction values are expressed as a percentage of maximal contraction as produced by 10^{-6} M noradrenaline (6.5 ± 0.1 cm). Values represent the mean ± SE ($n \geq 7$).

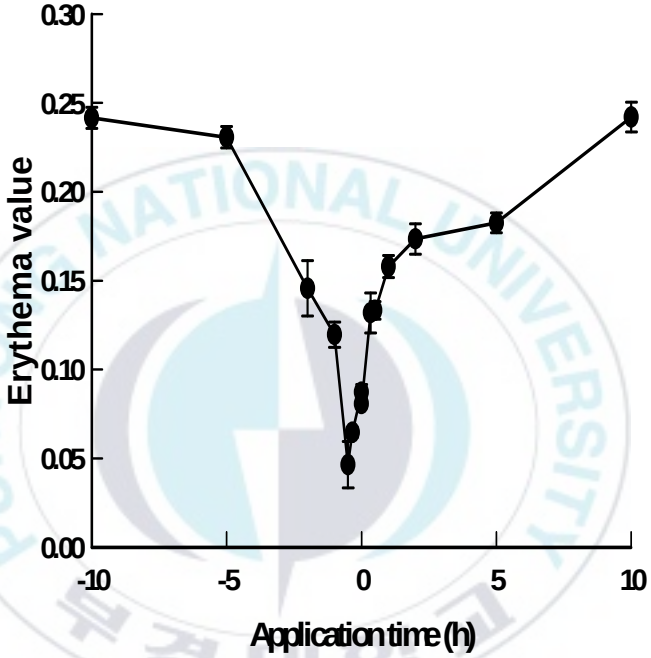

Figure 3.4. Effects of application time on suppression of erythema by methanol extract of *Undaria pinnatifida.* The extract was applied both before (–10 to 0 h) and after PMA application (0 to 10 h). Values represent the mean ± SE ($n \geq 7$).

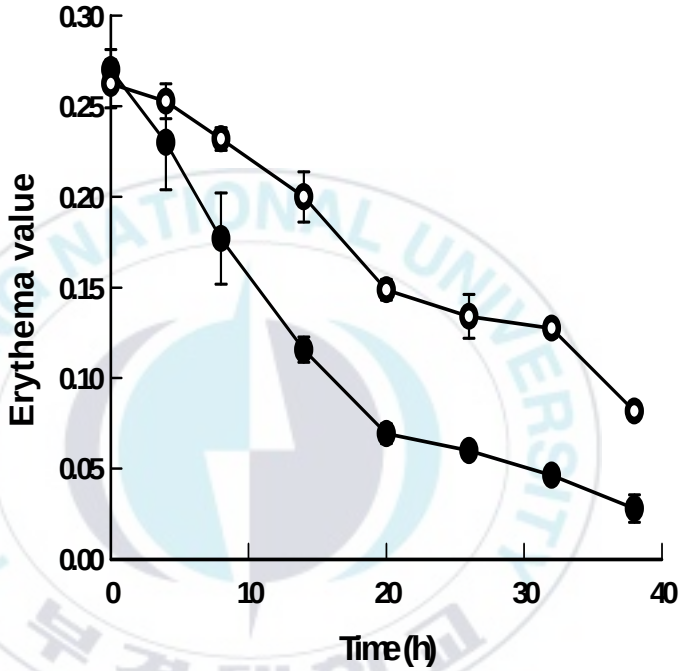

Figure 3.5. Effects of methanol extract of *Undaria pinnatifida* on reduction of erythema over time. Extract at a concentration of 0.4 mg per 10 μL (●) or vehicle (○) was applied to the mouse ear at the time of maximal erythema. Values represent the mean ± SE ($n \geq 7$).

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Chapter 4

Isolation of two anti-inflammatory and one pro-inflammatory PUFAs from the brown seaweed *Undaria pinnatifida*

Abstract

Two anti-inflammatory ω3 polyunsaturated fatty acids (PUFAs) of stearidonic acid (SA) and eicosapentaenoic acid (EPA) and one pro-inflammatory ω6 PUFA of arachidonic acid (AA) were isolated from the edible brown seaweed *Undaria pinnatifida*. SA was active against mouse ear inflammation induced by phorbol myristate acetate, with IC_{50} values of 58, 114, and 85 mM against the inflammatory symptoms of edema, erythema, and blood flow, respectively. EPA was also active against edema, erythema, and blood flow, with IC_{50} values of 76, 153, and 78 mM, respectively. Although AA at low concentrations showed anti-inflammatory activities when 10 h later, AA doses of more than 80 mM measured induced inflammatory symptoms 1 h later. Mature thalli generally had larger amounts of PUFAs than young thalli. The algal blade contained more ω3 PUFAs than were found in other parts, while the holdfast contained extremely high amounts of AA. Late-season thalli showed increased amounts of PUFAs, especially AA.

Key words: anti-inflammation; arachidonic acid; eicosapentaenoic acid; Phaeophyta; stearidonic acid; *Undaria pinnatifida*

Introduction

A number of seaweed species are consumed as food in various regions of the world. *Undaria pinnatifida* (Harvey) Suringer, commonly known as miyok in Korea and wakame in Japan, is an edible annual brown seaweed, growing up to 2 m long. In 2006, Korean aquaculture produced 305,000 tons (wet wt) (MOMAF, 2006). *Undaria pinnatifida* is popularly known as a health food among East Asian people. Almost all Korean women, even those who have migrated to other countries, consume miyok soup during a month-long postnatal period, as traditional belief holds that it helps the postnatal recovery, cleans the blood, and increases breast milk production. It has been used traditionally to treat fever, urination problems, lumps, and swelling (Donguibogam Committee, 1999). This seaweed is also used as an herbal medicine in China to treat urinary diseases, dropsy, stomach ailments, hemorrhoids, and fistulas (Tseng and Chang, 1984). Apart from its traditional uses, *U. pinnatifida* has been reported to alter uterine contraction (Huh et al., 1992) and hepatic fatty acid oxidation (Murata et al., 1999). In addition, *U. pinnatifida* inhibits eicosanoid production (Ishihara et al., 1998); has a preventive effect on cerebrovascular diseases (Ikeda et al., 2003); exhibits antitumor (Hosokawa et al., 2004), antiviral (Thompson and Dragar, 2004), and antihypertensive activities (Suetsuna et al., 2004); and shows an antiobesity effect (Maeda et al., 2005).

In our previous study (Khan et al., 2005), a methanol extract of *U. pinnatifida* was demonstrated to display potent anti-inflammatory activities against phorbol myristate acetate (PMA)-induced mouse ear inflammation. In an attempt to identify the anti-inflammatory compounds from the seaweed, we isolated two polyunsaturated fatty acids (PUFAs) of stearidonic acid (SA) and eicosapentaenoic acid (EPA) that showed inhibitory effects against the inflammatory symptoms of edema, erythema, and blood flow. We also isolated one pro-inflammatory compound of arachidonic acid (AA) from *U. pinnatifida* thalli.

Materials and methods

Algal material

The brown seaweed *Undaria pinnatifida* f. *distans* (Harvey) Suringar (northern type) was harvested from Kijang aquaculture farm, Korea, in January 2005 and January 2006, with the voucher specimen deposited in our laboratory (Y. K. Hong). For convenience, the seaweed tissue was completely dried for 1 week at room temperature, and then ground to powder for 5 min using a coffee grinder. The powder was stored at –20^{o}C until use.

Isolation of anti- and pro-inflammatory compounds

To isolate the anti- and pro-inflammatory compounds from *U. pinnatifida* thalli, the algal powder (1.0 kg) was extracted three times with 17.5 L acetonitrile, and the crude extract was evaporated under vacuum to give a dark brown residue (4.8 g). The acetonitrile extract was chromatographed on a silica gel column (70–230 mesh, 22g, Ø 4.5 cm × 40 cm) and successively eluted with 90 mL each of n-hexane, methylene chloride, acetonitrile, and methanol. The active methylene chloride eluent (1.9 g) was dried and dissolved in 4.75 mL of methanol for reverse-phase high performance liquid chromatography (RP-HPLC). Each 300-µL (120mg) aliquot was separated on a C18 column (10 mm ID × 25 cm) (Ultrasphere; Beckman Coulter, Fullerton, CA, USA). The analysis was performed on a Waters 600 gradient liquid chromatograph (Waters, Milford, MA, USA) monitored at 213 nm. The mobile phase consisted of two solvent systems: acetonitrile with 0.1% TFA and distilled water with 0.1% TFA. Elution was performed with a linear gradient of 0 to 100% v/v acetonitrile over 33 min for compound ATD-2 (stearidonic acid: SA), and with 100% v/v acetonitrile over 40 min for compounds ATD-4 (eicosapentanoic acid: EPA) and ATD-9 (arachidonic acid: AA), at a flow rate of 2 mL min^{-1} (Figure 4.1). Each eluted compound was dried under a stream of nitrogen gas.

Analytical methods

The purified compounds were analyzed on a GC-MS-QP5050A (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector, and compared to the spectral data from the database. Electron Impact Mass Spectrometric (EIMS) and HR-FABMS data were obtained from a JMS-700 spectrometer (JEOL, Tokyo, Japan) and a JMS HX 110 Tandem mass spectrometer (JEOL), respectively. The 1-D Nuclear Magnetic Resonance (NMR; ^{1}H, ^{13}C, and DEPT) and 2-D NMR (HMQC, HMBC, and COSY) spectra were taken on a JNM-ECP 400 NMR spectrometer (JEOL), using methanol-d (CD_3OD) for ATD-2, ATD-4, and ATD-9. The structures of the purified compounds were identified and confirmed to be identical to the spectral data in Fu et al. (2004).

Inflammatory bioassays

BALB/c mice (8–10 weeks old; 20–25 g body weight) were used for inflammatory assays. The animals were housed at 24 ± 1°C on a 12-h light/dark cycle, with free access to food and water. Animal experiments were performed in accordance with the U.S. NIH Guidelines for the Care and Use of Laboratory Animals (Bethesda, MD, USA). Various concentrations of the purified compounds and indomethacin as a reference were prepared in 10 μL of 100% ethanol and applied topically to the inner side of the mouse ear. Phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO, USA; 0.2 μg in 10 μL acetone) was applied topically to the same side of the ear 30 min later. Ear edema (swelling) was measured 10 h after the PMA application, using a spring-loaded micrometer (Mitutoyo Corp., Tokyo, Japan). The edema value was expressed as $(S_{10} - S_0)/S_0$, where S_{10} is the ear thickness 10 h after PMA application, and S_0 is the ear thickness at 0 h. The edema value was 0.81 ± 0.04 with the ethanol vehicle. Ear erythema (redness) was determined at 10 h, using digital photography adjusted to balance white and Photoshop 7.0 (Adobe, San Jose, CA, USA) to measure the magenta value. The erythema value was expressed as $(R_{10} - R_0)/R_0$, where R_{10} is ear redness 10 h after PMA application, and R_0 is ear redness at 0 h. Local blood flow in the mouse ear was measured

using laser speckle flowgraphy (Inflameter LFG-1; SoftCare, Fukuoka, Japan). The ear skin was scanned by moving the laser beam over the inner surface of the mouse ear. The distance between the scanner and ear surface was 0.5 cm. Blood flow was analyzed for a skin area 5 mm in diameter, after the method of Lee et al. (2003). Blood flow was calculated as $(B_{10} - B_0)/B_0$, where B_{10} is blood flow 10 h following PMA application, and B_0 is blood flow at 0 h.

Quantification of anti- and pro-inflammatory compounds

To measure the amounts of anti- and pro-inflammatory compounds in *U. pinnatifida*, the thalli were completely dried in shade at room temperature for a week, and then ground for 5 min to powder. The powder (0.4 g) was extracted with 8 mL dichloromethane on a rotator for 1 h at 30 rpm. After centrifugation at 2000 × *g* for 5 min, 4 mL of the clean supernatant was evaporated to 5 mg mL^{-1} for RP-HPLC. Each 100-µL aliquot was separated on an Ultrasphere C18 column, using the same isolation procedure as that for SA, EPA, and AA. Each isolated compound was reconfirmed by ^{1}H and ^{13}C NMR. The amount of each compound was assessed by measuring the dimensions of HPLC peaks, using the standard curve of each pure compound.

Statistical analysis

The experiments were replicated at least seven times for each independent assay, and the highest and lowest values were discarded. The mean values of the indices were compared to the control using Student's *t*-test.

Results

Identification of compounds

To identify anti- and pro-inflammatory compounds from *U. pinnatifida*, 1 kg of seaweed powder was extracted with acetonitrile and chromatographed on a silica gel column. The active methylene chloride fraction was then separated by RP-HPLC and generated 11 peaks, using a gradient of acetonitrile in water and isocratic 100% acetonitrile (Figure 4.2). Of the two anti-inflammatories, the ATD-2 peak was eluted at 98% (on 32.4 min) (Figure 4.3) and the ATD-4 peak at 100% (on 33.6 min) acetonitrile (Figure 4.4). They appeared as oily compounds, weighing 8.4 mg and 9.2 mg, respectively, and yielding 8.4×10^{-4}% and 9.2×10^{-4}%, respectively, from the seaweed powder. A pro-inflammatory peak of ATD-9 was eluted at 100% (on 35.5 min) acetonitrile (Figure 4.5), as an oily compound weighing 19.3 mg, which amounted to a 1.9×10^{-3}% yield.

The molecular composition of ATD-2 is $C_{18}H_{28}O_2$ based on high-resolution fast atom bombardment mass spectra (HRFABMS) data (negative mode, $[M-H]^-$ at m/z 275.3), which indicated that ATD-2 contained five double-bond equivalents, comprising four carbon–carbon double bonds and one carbonyl carbon. The ^{1}H NMR spectrum revealed the presence of a methyl proton at δ_H 0.96 (H-18), eight methylene protons, and eight methine proton signals. From the ^{13}C NMR spectrum, we observed one carbonyl carbon (C-1), one methyl carbon at δ_C 14.6 (C-18), eight methylene carbons, and eight methine carbons. From the correlation spectroscopy (COSY) spectrum, we determined the first double-bond position from the terminal methyl carbon (C-18). The terminal methyl proton (H-18), methylene proton (H-17), and methine proton (H-16) showed a series of COSY correlations with each other, demonstrating that the first double bond was at the third position. From these spectral data, we identified ATD-2 as octadeca-6,9,12,15-tetraenoic acid (C18:4 ω3), stearidonic acid (SA), or moroctic acid (Figure 4.6A).

We identified the molecular composition of ATD-4 as $C_{20}H_{30}O_2$ from the HRFABMS spectrum (negative mode, $[M-H]^-$ at m/z 301.3), which indicated that ATD-4 contained six double-bond equivalents, comprising five carbon–carbon double bonds and one carbonyl carbon. The ^1H NMR spectrum revealed the presence of a methyl proton at δ_H 0.90 (H-20), eight methylene protons, and ten methine protons. The ^{13}C NMR spectrum revealed one carbonyl carbon at δ_C 174.9 (C-1), one methyl carbon at δ_C 15.0 (H-20), eight methylene carbons, and ten methine carbons. From these spectral data, ATD-4 was identified as eicosa-5,8,11,14,17-pentaenoic acid (EPA) (C20:5 ω3), or timnodonic acid (Figure 4.6B).

The molecular composition of ATD-9 is $C_{20}H_{32}O_2$ based on the HRFABMS data (negative mode, $[M-H]^-$ at m/z 303.4). This data indicated that ATD-9 contained five double-bond equivalents, comprising four carbon–carbon double bonds and one carbonyl carbon. The ^1H NMR spectrum revealed the presence of a methyl proton at δ_H 0.83 (H-20), ten methylene protons, and eight methine proton signals. From the ^{13}C NMR spectrum, we observed one carbonyl carbon (C-1), one methyl carbon at δ_C 14.8 (C-20), eight methylene carbons, and eight methine carbons. From the COSY and heteronuclear multiple bond correlation (HMBC) spectra, we determined the first double-bond position from the terminal methyl carbon (C-20). The terminal methyl proton (H-20) showed a COSY correlation to H-19 and a HMBC correlation to C-18, and the proton-attached C-18 (H-18) showed a HMBC correlation to C-17. Two methylene protons (H-16, H-17) and a methine proton (H-15) showed a series of COSY correlations with each other, demonstrating that the first double bond was at the sixth position. From these spectral data, ATD-9 was identified as eicosa-5,8,11,14-tetraenoic acid (C20:4 ω6), or arachidonic acid (AA) (Figure 4.6C).

Anti- and pro-inflammatory activities

Purified compounds of SA and EPA were tested for anti-inflammatory activities against the PMA-induced mouse ear inflammation symptoms of edema, erythema, and blood flow. The inhibitory effects of different concentrations of SA topically applied to mouse ears were dose-dependent. The SA concentrations producing 50% inhibition (IC_{50}) were 58, 114, and 85 mM for edema, erythema, and blood flow, respectively (Figure 4.7). The inhibitory effects of different concentrations of EPA topically applied to mouse ears were also dose-dependent. The EPA concentrations producing IC_{50} were 76, 153, and 78 mM for edema, erythema, and blood flow, respectively (Figure 4.8). The topical application of indomethacin as a reference significantly decreased the PMA-induced inflammation and yielded IC_{50} of 25, 48, and 50 mM for edema, erythema, and blood flow, respectively (data not shown). Thus, pure SA and EPA showed almost half the anti-inflammatory activity of indomethacin. Additionally, when purified AA without PMA was topically applied to mouse ears, edema, erythema, and blood flow reached maximal values 1 h later (Figure 4.9). Low AA concentrations showed anti-inflammatory activities against PMA-induced edema, erythema, and blood flow when measured 10 h later (data not shown). Concentrations >80 mM demonstrated pro-inflammation effects.

Amount of anti- and pro-inflammatory compounds in thalli

We examined the amounts of SA, EPA, and AA in different parts of *U. pinnatifida* thalli. A mature thallus consists of a pinnately divided blade with midrib, a mature undulated sporophyll, and a fibrous holdfast. The blade contained the highest amounts of ω3 PUFAs: SA and EPA (Table 4.1). The mature sporophyll had very low amounts of SA, EPA, and AA, whereas holdfasts from mature and young thalli contained extremely high amounts of ω6 AA, with 2.4 and 1.3 mg, respectively, per 1 g dry powder. Mature thalli (~1 m in height) generally had higher amounts of SA, EPA, and AA than young thalli (~20 cm in height). Additionally, four representative local types of *U. pinnatifida*

from the east (Kijang), west (Taean), south (Wando) seas, and the Sanriku Sea of Japan were cultured at Kijang aquaculture farm. The amounts of SA, EPA, and AA from mature blades of each local type were measured at different collection times during the harvest season. The Taean and Sanriku types generally had more SA, EPA, and AA (Table 4.2). The Taean cultivar showed relatively high amounts of ω3 PUFAs (SA and EPA) compared to ω6 AA at the 11 January harvest. Thalli collected late in the season had higher amounts of SA, EPA, and AA, especially the latter. Generally, *U. pinnatifida* gathered early in the harvest season yielded more ω3 PUFAs than ω6 PUFA.

Discussion

From the *U. pinnatifida* extract, we isolated two ω3 PUFAs, SA and EPA, with anti-inflammatory activity and one ω6 PUFA, AA, with pro-inflammatory activity. The two anti-inflammatory fatty acids reduced edema, erythema, and blood flow potently. The use of this seaweed as a cure for fever, urination problems, lumps, and swelling is recorded in the Oriental medical textbook *Donguibogam*, published in 1613 (Donguibogam Committee, 1999). As an herbal medicine in China, *U. pinnatifida* has been used to treat urinary diseases and dropsy (Tseng and Chang, 1984). The seaweed is also known to contain SA, which inhibits leukotriene production in inflammation (Ishihara et al., 1998). Most of these effects are directly or indirectly related to the anti-inflammatory action of the seaweed. Furthermore, the ω3 PUFA of SA is reported as a 5-lipoxygenase inhibitor (Guichardant et al., 1993). EPA also suppresses inflammation and is associated with a reduction in arachidonic acid levels (Raederstorff et al., 1996). Ear inflammations induced by arachidonic acid and ultraviolet-B irradiation was significantly suppressed in mice at a dose of 300 mg EPA kg^{-1} body weight per day for 2 weeks (Danno et al., 1993). SA and EPA inhibit UV-induced dermal fibroblasts (Kim et al., 2005), leukocyte–endothelial interactions (Sethi, 2002), and inflammatory mediator release in blood and splenocytes of mice (Ishihara et al., 2002). Supplementing diets with ω3 PUFAs reduces the expression and activity of aggrecanases and inflammation-inducible cytokines and cyclooxygenase-2 (Curtis et al., 2000). However, they had no effect on constitutively expressed cyclooxygenase-1. Thus, these findings for SA and EPA from *U. pinnatifida* reinforce the claims of the health-care industry and indigenous medicine that the seaweed can be used as a remedy for inflammation-related symptoms. In addition, the amounts of SA and EPA in *U. pinnatifida* can be used as criteria for quality assessment of the seaweed products and strain improvement. However, this seaweed also contains AA, a pro-inflammatory compound. Serhan (2005) found that AA is not only a precursor to pro-inflammatory lipid mediators but can also be converted to anti-inflammatory lipid mediators, such as the lipoxins, in the resolution phase. In this study, we also observed anti-inflammatory

activities of AA at low concentrations against edema, erythema, and blood flow when measured 10 h later, but not 1 h later.

Eskra et al. (1986) quantified leukotrienes and hydroxyeicosatetraenoic acids in biological samples using RP-HPLC. We modified the solvent extraction to enhance the purity of PUFAs from *U. pinnatifida* and quantified yields using RP-HPLC. Mature thalli of *U. pinnatifida* contained greater amounts of SA, EPA, and AA than young thalli. Generally, young thalli produce more primary metabolites for fast growth, whereas mature thalli have more mechanisms to modify biocompounds or protect cellular functions (Chen et al., 1998). *Undaria pinnatifida* is a common brown seaweed along temperate coastal regions of the northeast Pacific, including Korea, Japan, and northern China (Ohno and Matsuoka, 1993). Currently, it occurs in temperate regions worldwide as an invasive species (Aguilar-Rosas et al., 2004). We plan to increase the amount of ω3 PUFAs and reduce ω6 PUFA by modifying after-harvest processing.

Table 4.1. Amount of stearidonic acid (SA), eicosapentaenoic acid (EPA), and arachidonic acid (AA) extracted from different parts of *U. pinnatifida* thalli.

	Mature thalli			Young thalli		
	SA	EPA	AA	SA	EPA	AA
Injured blade	329 ± 4	218 ± 4	447 ± 4	200 ± 4	132 ± 4	551 ± 9
Blade	730 ± 12	433 ± 19	930 ± 19	673 ± 13	365 ± 6	744 ± 16
Midrib	211 ± 7	204 ± 6	154 ± 9	122 ± 4	79 ± 4	762 ± 11
Sporophyll	0 ± 0	36 ± 4	261 ± 9	ND	ND	ND
Holdfast	11 ± 0	72 ± 4	2,394 ± 12	50 ± 4	200 ± 4	1,263 ± 4

∗Data are microgram amounts of the mean ± S.E. ($n \geq 5$) from 1 g dry weight of thalli.

∗ND, not determined.

Table 4.2. Amount of stearidonic acid (SA), eicosapentaenoic acid (EPA), and arachidonic acid (AA) from blades of different local types of *U. pinnatifida*, all cultured at Kijang aquaculture farm, Korea, and harvested at different times.

Local types	Harvested 11 Jan. 2006			Harvested 18 Jan. 2006			Harvested 26 Jan. 2006		
	SA	EPA	AA	SA	EPA	AA	SA	EPA	AA
Kijang, Korea	712 ± 7	411 ± 4	873 ± 7	1,227 ± 7	733 ± 7	1,699 ± 4	712 ± 7	569 ± 6	1,528 ± 25
Taean, Korea	973 ± 4	1,062 ± 22	819 ± 4	1,943 ± 6	1,306 ± 4	2,504 ± 7	1,173 ± 9	758 ± 7	1,696 ± 12
Wando, Korea	261 ± 7	229 ± 9	623 ± 11	1,055 ± 4	948 ± 7	969 ± 7	1,692 ± 9	1,084 ± 12	2,097 ± 13
Sanriku, Japan	1,231 ± 9	991 ± 13	1,617 ± 4	1,767 ± 9	1,374 ± 6	2,751 ± 7	1,012 ± 9	737 ± 9	1,953 ± 11

∗Data are microgram amounts of the mean ± S.E. ($n \geq 5$) from 1 g dry weight of blades.

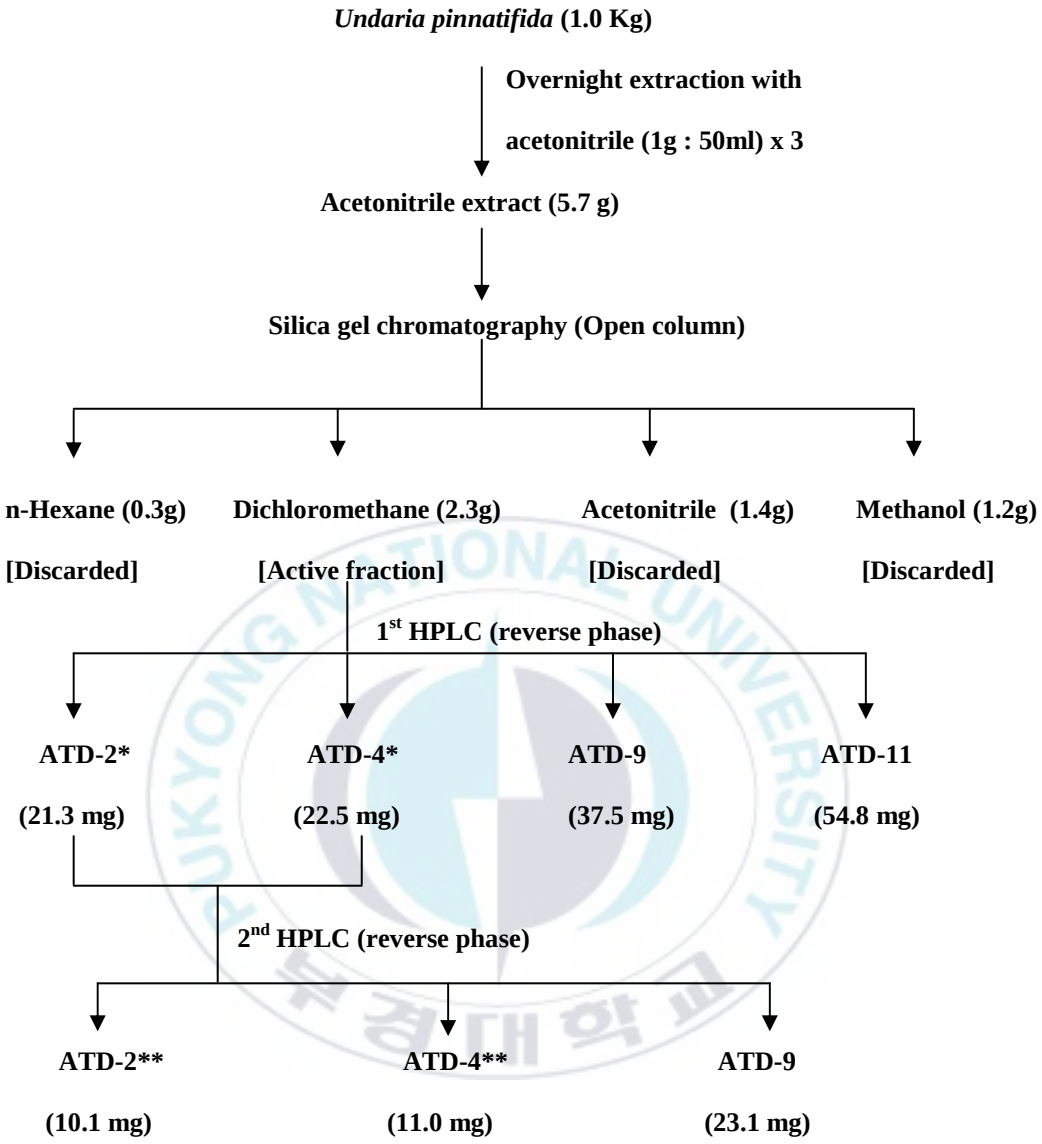


Figure 4.1. Purification procedure of anti-inflammatory compounds from *U. pinnatifida*.
*** Inhibitory fraction; ** Inhibitory compound.**

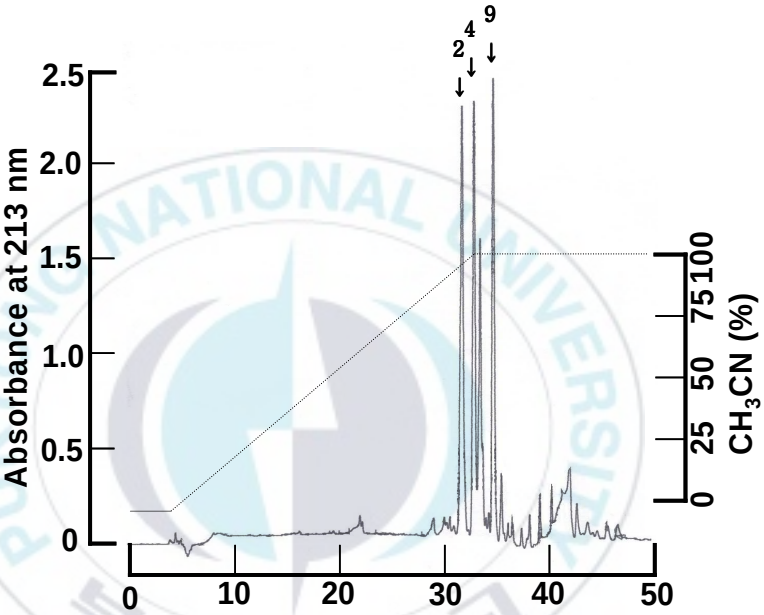


Figure 4.2. First HPLC profile for the isolation of anti-inflammatory substances (ATD-2, ATD-4 and ATD-9) using Beckman C18 column. Arrow 2 and 4 indicate the fractions that have anti-inflammatory activity, and arrow 9 indicates the fraction that has inflammatory activity.

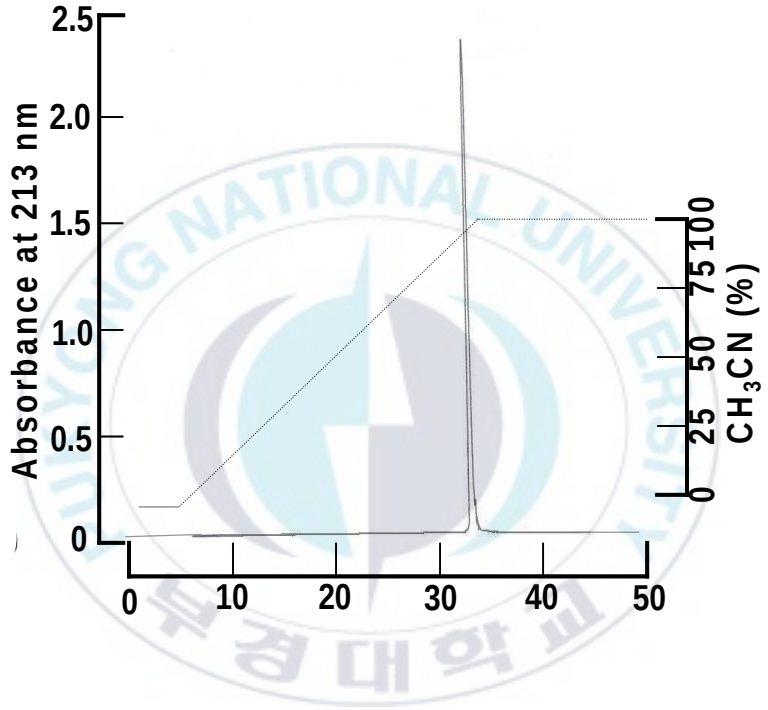


Figure 4.3. Second HPLC profile for the isolation of anti-inflammatory substance (ATD-2) using Alltech C18 column.

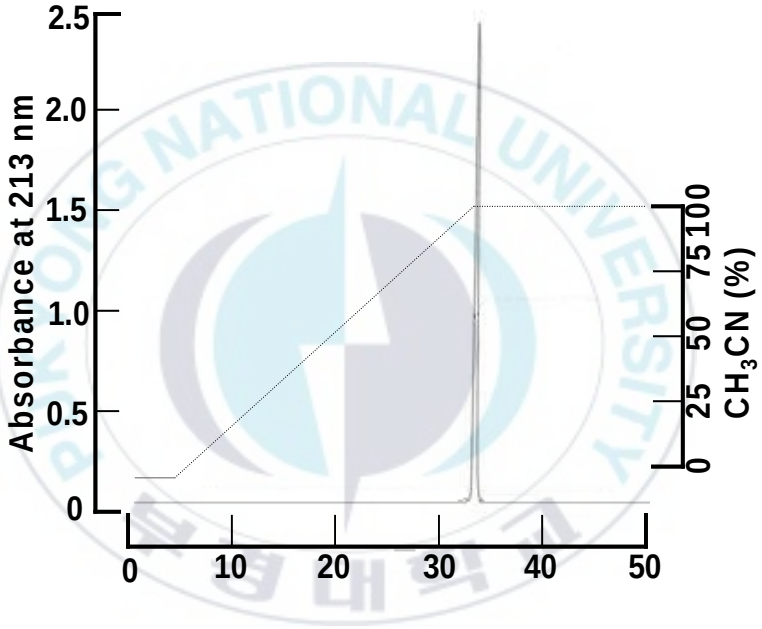


Figure 4.4. Second HPLC profile for the isolation of anti-inflammatory substance (ATD-4) using Alltech C18 column.

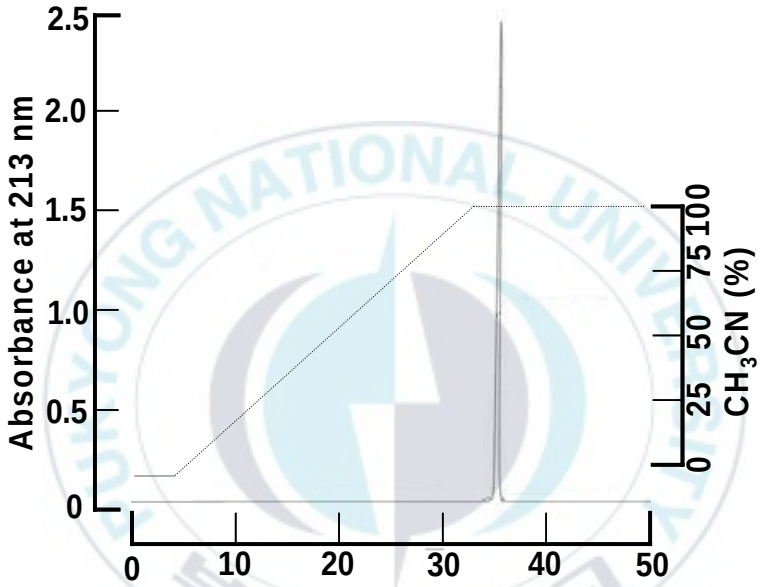
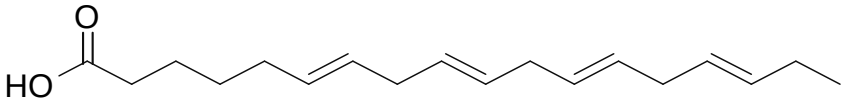
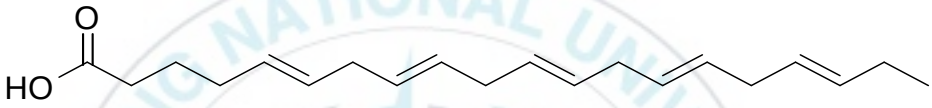


Figure 4.5. Second HPLC profile for the isolation of inflammatory compound (ATD-9) using Alltech C18 column.

(A)



(B)



(C)

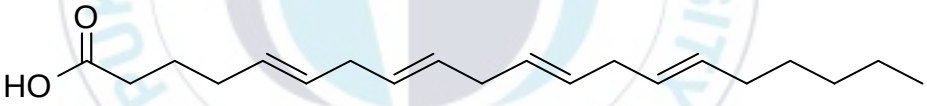


Figure 4.6. Chemical structure of stearidonic acid (A), eicosapentaenoic acid (B), and arachidonic acid (C) isolated from *U. pinnatifida.*

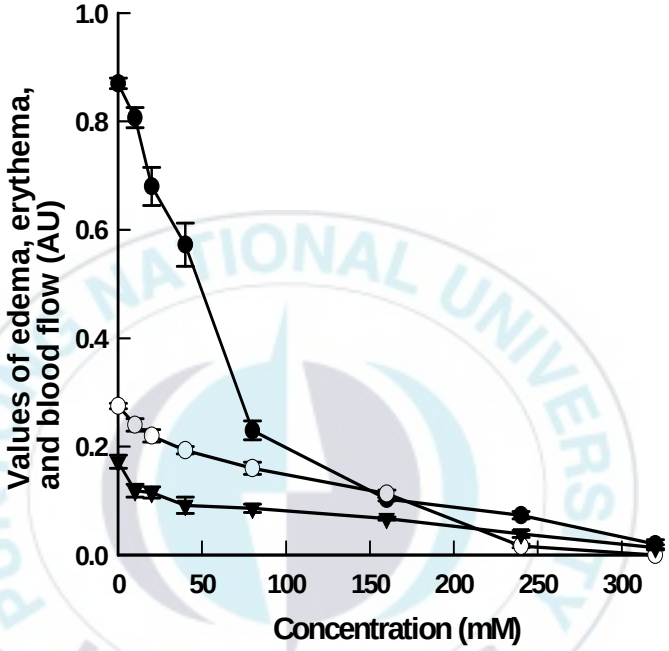


Figure 4.7. Anti-inflammatory activity of stearidonic acid (SA) on mouse ear. Edema (●), erythema (○), and blood flow (▼) were measured with different concentrations of SA plus PMA (0.2 μg/ear). Indomethacin, used as a reference, showed 50% inhibition at doses of 25, 48, and 50 mM for edema, erythema, and blood flow, respectively. Values represent the mean ± S.E. ($n \geq 5$).

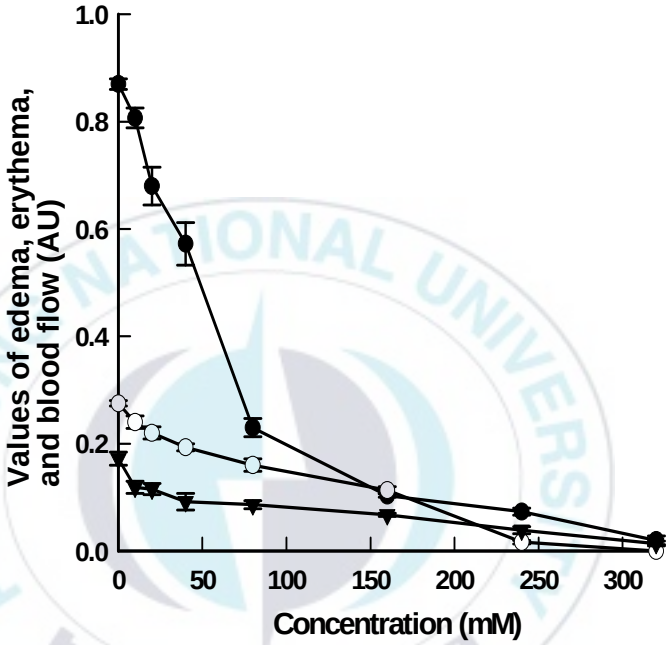


Figure 4.8. Anti-inflammatory activity of eicosapentaenoic acid (EPA) on mouse ear. Edema (●), erythema (○), and blood flow (▼) were measured with different concentrations of EPA plus PMA (0.2 μg/ear). Indomethacin, used as a reference, showed 50% inhibition at doses of 25, 48, and 50 mM for edema, erythema, and blood flow, respectively. Values represent the mean ± S.E. ($n \geq 5$).

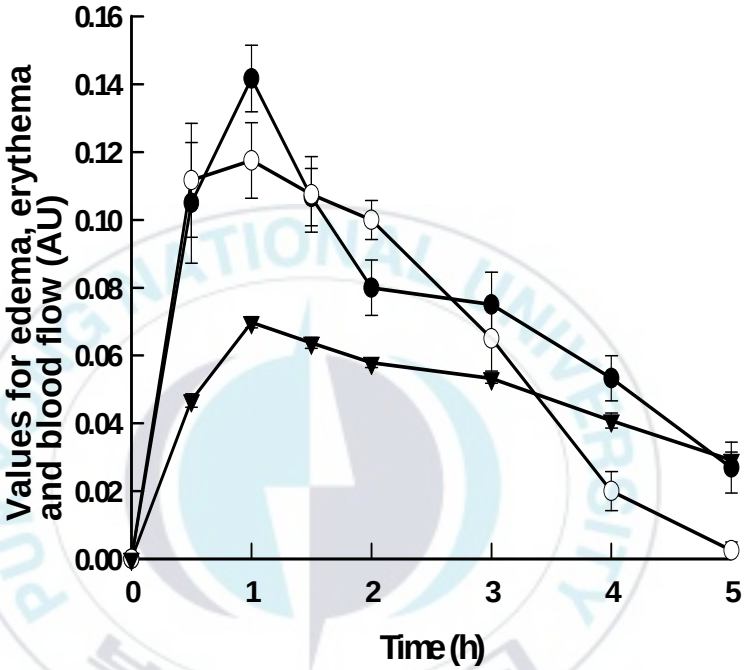


Figure 4.9. Pro-inflammatory activity of arachidonic acid (AA) on mouse ear. Edema (●), erythema (○), and blood flow (▼) were measured with 100 mM AA. Values represent the mean ± S.E. (*n* ≥ 5).

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Conclusions

- The present investigation claims that the popular brown alga *Undaria pinnatifida* possesses anti-inflammatory properties among all common seaweeds available in Korean coast.

- The present study clearly demonstrates that the methanol extract of the brown seaweed *U. pinnatifida* showed significant anti-inflammatory activity without any serious toxic side-effects at moderate dose.

- The work reports that erythematous inflammation induced by PMA was significantly suppressed by a methanol extract of *U. pinnatifida*.

- The study also shows that the digital photo analysis technique is a relatively simple and inexpensive tool for noninvasive erythema assessment in biological research as well as in clinical practice.

- The study reveals that the brown seaweed contains two PUFAs, stearidonic acid (SA) and eicosapentaenoic acid (EPA) which provide scientific supporting evidence for the use of *U. pinnatifida* as an anti-inflammatory remedy in folk medicine.

Finally, it may be concluded, the brown alga *Undaria pinnatifida* possesses potent anti-inflammatory constituents which explore a new opportunity for this alga to be employed as ingredients of pharmaceutical and cosmeceutical products employed in the treatment of inflammatory diseases.

Summary (in Korean)

갈조류 미역 *Undaria pinnatifida* (Harvey) Suringar 의 항염증 활성

모하메드 누를 압사르 칸

부경대학교 일반대학원 생물공학과

본 연구는 한국 연안에 많이 분포하는 37 종의 해조류들을 대상으로 하여 항염증 활성을 조사하였다. 해조류 추출물들은 in vivo 에서 쥐의 귀에 염증을 유도하는 phorbol-12-myristate 13-acetate(PMA)에 대해서 억제 반응을 측정하였다. 모두 14 종이 항염증 활성을 나타냈고, 그 중 갈조류 미역(*Undaria pinnatifida*)이 부종과 홍반에 대해서 각각 80%와 78%의 가장 강한 저해 활성을 보였다.

이 연구의 두 번째 부분에서는 미역의 메탄올 추출물의 항염증 활성에 초점을 맞추었다. 메탄올 추출물은 PMA 에 의해서 유도되는 쥐의 귀 부종에 대해 10.3mg/ml 값에서 50%의 저해농도(IC_{50})값을 보였다. 40-mg/ml 메탄올 추출물은 PMA 접종 3 시간 전 또는 PMA 접종 2 시간 후에 주입했을 때 50% 또는 그 보다 더 높은 억제를 유지했다. 엽상체의 잎 부분이 가장 높은 활성을 보였으며, 북방형의 미역이 남방형의 미역보다 약간 더 높은 활성을 가지고 있었다. 통증억제 시험에서는, 메탄올 추출물은 통증을 유도하는 acetic acid 에 대해 억제효과를 나타냈으며, 그 IC_{50} 은 몸무게 kg 에 대해 0.48g 이었다. 추출물은 또한 효모로 고열이 유도된 쥐에 시험되었을 때 해열의 활성을 보였다. 메탄올 추출물은 10g/kg 의 구강 투여 후에도 쥐에 아무런 독성을 나타내지 않았다.

미역의 메탄올 추출물은 홍반 반응에 대해서 강력한 저해를 보였다. 디지털 사진 분석에 의해 측정했을 때, PMA 에 의해 유도되어진 쥐의 귀 홍반에 대한 추출물의 IC_{50} 값은 15.6mg/ml 였다. 홍반을 laser speckle flowgraphy 을 이용하여 측정했을 때 추출물의 IC_{50} 값은 18.1mg/ml 였다. In vivo 상에서, 10mg/ml 의 추출물 농도는 10^{-6}M noradrenaline 에 의해서 유도되어진 혈관 수축의 약 20%까지 혈관을 수축시켰다.

이 연구에서 미역으로부터 stearidonic acid(SA)와 eicosapentaenoic acid(EPA)의 2 개의 항염증적인 omega-3 고도불포화지방산(PUFAs)과 arachidonic acid(AA)의 pro-inflammatory ω6 PUFA 을 분리하였다. SA 는 phorbol myristate acetate 에 의해 유도되어진 쥐의 귀 염증 증상인 부종, 홍반, 혈류에 대해서 각각 58, 114, 85mM 의 IC_{50} 값을 나타내었다. EPA 는 부종, 홍반, 혈류에 대해 IC_{50} 값은 각각 76, 153, 78mM 였다. 비록 AA 가 낮은 농도에서 10 시간 후에 항염증 활성을 보였지만, 80mM 이상의 AA 투여는 1 시간 후에 염증 증상을 유도했다. 성숙한 엽상체는 일반적으로 어린 엽상체보다 더 많은 양의 PUFAs 를 가지고 있었다. 해조류 잎은 다른 부분에서 발견되어진 것보다 더 많은 ω3 PUFAs 를 가지고 있었다. 반면 가근부위는 매우 높은 양의 AA 를 포함했다. 늦은 수확시기의 엽상체는 PUFAs 중 특히 AA 의 양이 증가된 것으로 보였다.

결론적으로, 갈조류 미역은 염증 질병의 예방 및 치료에 이용되어지는 약품 및 화장품의 첨가제 재료로써도 이용될 수 있는 강력한 항염증성분을 가지고 있다고 볼 수 있다.

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