A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Anti-inflammatory and anti-tumor activity of fucoxanthin from *Ishige okamurae* through MAPKs regulation

Kil-Nam Kim

Department of Aquatic life medicine

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

2010. 02

CONTENTS

Part Ⅱ. Fucoxanthin inhibits the inflammatory response by suppressing NF-κB and MAPKs activation in lipopolysaccharide-induced RAW 264.7 macrophages

Part Ⅲ. Fucoxanthin induces apoptosis in human leukemia HL-60 cells through a ROS-mediated MAPK pathway

Part Ⅳ. Fucoxanthin induces G_0/G_1 phase arrest and apoptosis in melanoma

B16F10 cells through Akt and Bcl-xL signaling pathway

국문초록

푸코잔틴은 카로티노이드 색소류 잔토필의 일종으로 갈조식물과 황색식물에만 함유되어 있는 물질이다. 푸코잔틴은 항암과 항산화 및 항비만 등 다양한 생리활성이 있다고 알려져 있으나, 항염증과 항암활성의 메커니즘에 대한 연구는 미흡한 실정이다. 이 연구에서는 패(갈조류)로부터 푸코잔틴을 분리하고, 분리된 푸코잔틴이 항염증활성과 HL-60(백혈병 세포)과 B16F10(피부암 세포) 세포에 대한 항암활성을 측정하였고, 그 작용 메커니즘을 조사하였다.

1. 푸코잔틴은 LPS로 자극된 RAW 264.7 세포에서 염증성 매개 인자의 생성에 미치는 영향을 조사 한 결과, 푸코잔틴은 염증성 매개인자인 TNF-α, IL-1β, IL-6와 NO의 생성을 농도의존적으로 억제함을 알 수 있었다. 항염증 활성의 작용기전 규명을 위해, LPS로 자극된 NF-κB와 MAPK 활성화에 미치는 영향을 조사한 결과는 NF-κB의 전사활성 억제와 MAPK 인산화를 억제함을 확인 되었다. 이러한 결과는 푸코잔틴이 NF-κB와 MAPK 조절함으로써 항염증활성을 나타내는 것으로 생각된다.
2. 푸코잔틴의 HL-60 세포에 대한 항암활성은 세포성장을 억제시키고 apoptosis로 세포사멸을 일으켰다. 세포사멸의 작용기전은 푸코잔틴

을 처리 하였을 때 MAPK 활성화를 조사한 결과, p38과 JNK가 활성화를 보였다. 그리고, 암세포 사멸에 영향을 미치는 ROS의 발생은 푸코잔틴을 처리하였을 때 ROS가 발생하는 것을 확인하였다. 이 ROS 발생이 apoptosis와 MAPK에 미치는 영향은 항산화제인 NAC을 푸코잔티과 같이 처리하였을 때 apoptosis 유도가 억제되고 MAPK이 활성이 억제되는 것을 확인하였다. 이러한 결과는 ROS발생에 의해 MAPK가 조절되면서 HL-60 세포를 apoptosis로 유도하여 항암활성이 나타나는 것으로 생각된다.

3. 푸코잔틴의 B16F10 세포에 대한 항암활성은 푸코잔틴을 처리하였을 때 B16F10 세포의 증식이 유의적으로 감소하였다. 세포 증식은 cell arrest 또는 apoptosis에 의해 억제되는데, 푸코잔틴을 처리하였을 때 G_0/G_1기 arrest와 apoptosis가 유도 되는 것이 cell cycle과 형태적 관찰을 통해 확인되었다. G_0/G_1기 arrest 관련 단백질 측정으로 확인된 arrest 작용 기전은 푸코잔틴을 처리 하였을 때 Akt 활성화가 억제되고 p15와 p27 단백질이 증가 되었다. 또한 apoptosis유도 기전으로 Bcl-xL 단백질이 감소 함으로써 apoptosis가 유도 되는 것을 확인 할 수 있었다. 이와 같은 결과로 볼 때 Akt와 Bcl-xL 조절에 의해 B16F10 세포의 cell arrest와 apoptosis가 유도 됨으로써 항암활성을

나타내는 것으로 생각된다.

이 모든 결과를 종합해 볼 때, 푸코잔틴이 항염과 항암활성에 의해 산업적 용도가 매우 다양할 것으로 생각되며 특히 식품산업으로서의 이용 가능성을 높일 수 있을 것이라 판단된다.

LIST OF FIGURES

Fig. 2-2. Inhibitory effects of fucoxanthin on PGE2 production in RAW 264.7 cells. Cells (1.5×10^5 cells/ml) were stimulated by LPS (1 μg/ml) for 24 h in the presence of fucoxanthin (10, 20, and 40 μM). Supernatants were collected, and the PGE2 production in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments.

Fig. 2-3. Inhibitory effect of fucoxanthin on the protein level of iNOS and COX-2 in RAW264.7 cells. RAW 264.7 cells (1.0×10^6 cells/ml) were pre-incubated for 18 h, and the cells were stimulated with LPS (1 μg/ml) in the presence of fucoxanthin (10, 20, and 40 μM) for 24 h. iNOS and COX-2 protein level were determined using immunoblotting method. iNOS and COX-2 versus β-actin was measured by densitometry. Values are the mean ± SD of triplicate experiments. **,P<0.05* and ***,P<0.01* indicate significant differences from the LPS-stimulated group.

Fig. 2-4. Inhibitory effects of fucoxanthin on the iNOS and COX-2 mRNA expression in RAW 264.7 cells. RAW 264.7 cells (1.0×10^6 cells/ml) were pre-incubated for 18 h, and the iNOS and COX-2 mRNA expressions were determined from 24 h culture of cells stimulated with LPS (1 μg/ml) in the presence of fucoxanthhin (10, 20, and 40 μM). iNOS and COX-2 versus β-actin was measured by densitometry. Values are the mean ± SD of triplicate experiments. **,P<0.05* and ***,P<0.01* indicate significant

differences from the LPS-stimulated group.

Fig. 2-5. Inhibitory effect of fucoxanthin on the TNF-α production in RAW264.7 cells. The production of TNF-α was assayed in the culture medium of cells stimulated with LPS (1 μg/ml) for 24 h in the presence of fucoxanthin (10, 20, and 40 μM). Supernatants were collected, and the TNF-α concentration in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments. *,$P<0.05$; **,$P<0.01$.

Fig. 2-6. Inhibitory effect of fucoxanthin on the IL-1β production in RAW264.7 cells. The production of IL-1β was assayed in the culture medium of cells stimulated with LPS (1 μg/ml) for 24 h in the presence of fucoxanthin (10, 20, and 40 μM). Supernatants were collected, and the IL-1β concentration in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments. *,$P<0.05$; **,$P<0.01$.

Fig. 2-7. Inhibitory effect of fucoxanthin on the IL-6 production in RAW264.7 cells. The production of IL-6 was assayed in the culture medium of cells stimulated with LPS (1 μg/ml) for 24 h in the presence of fucoxanthin (10, 20, and 40 μM). Supernatants were collected, and the IL-6 concentration in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments. *,$P<0.05$; **,$P<0.01$.

Fig. 2-8. Inhibitory effects of fucoxanthin on the IL-6, IL-1β and TNF-α mRNA expression in RAW 264.7 cells. RAW 264.7 cells (1.0×10^6 cells/ml) were pre-incubated for 18 hr,

and the IL-6, IL-1β and TNF-α mRNA expressions were determined from 24 h culture of cells stimulated with LPS (1 μg/ml) in the presence of fucoxanthhin (10, 20, and 40 μM). IL-6, IL-1β and TNF-α versus β-actin was measured by densitometry. Values are the mean ± SD of triplicate experiments. *,*P<0.05* and **,*P<0.01* indicate significant differences from the LPS-stimulated group.

Fig. 2-9. Inhibitory effect of fucoxanthine on the protein level of IκB-α in RAW264.7 cells. RAW 264.7 cells (1.0×10^6 cells/ml) were pre-incubated for 18 h, and the cells were pre-incubated for 2 h with PDCT (20 μM) and oil extract of fucoxanthine (12.5, 25, and 50 uM) at indicated concentrations and then stimulated for 15 min with LPS (1 μg/ml). The levels of p-IκB-α (phosphorylated-IκB-α) and pan-IκB-α were determined using immunoblotting method.

Fig. 2-10. Inhibitory effect of fucoxanthine on the protein level of NF-κB in RAW264.7 cells. RAW 264.7 cells (1.0×10^6 cells/ml) were pre-incubated for 18 h, and the cells were pre-incubated for 2 h with PDCT (20 μM) and oil extract of fucoxanthine (12.5, 25, and 50 uM) at indicated concentrations and then stimulated for 15 min with LPS (1 μg/ml). The levels of P-p65 (phosphorylated-p65) and P-p50 were determined using immunoblotting method.

Fig. 2-11. Inhibitory effect of fucoxanthine on the protein level of ERK in RAW264.7 cells.

RAW 264.7 cells (1.0 × 10^6 cells/ml) were pre-incubated for 18 h, and the cells were pre-incubated for 2 h with PD98059 (20 μM) and fucoxanthine (12.5, 25, and 50 uM) at indicated concentrations and then stimulated for 15 min with LPS (1 μg/ml). The levels of p-ERK (phosphorylated-ERK) and pan-ERK were determined using immunoblotting method.

Fig. 2-12. Inhibitory effect of fucoxanthie on the protein level of p-38 in RAW264.7 cells.

RAW 264.7 cells (1.0 × 10^6 cells/ml) were pre-incubated for 18 h, and the cells were pre-incubated for 2 h with SB203580 (20 μM) and fucoxanthine (12.5, 25, and 50 uM) at indicated concentrations and then stimulated for 15 min with LPS (1 μg/ml). The levels of p-p38 (phosphorylated-p-38) and pan-p-38 were determined using immunoblotting method.

Fig. 2-13. Inhibitory effect of fucoxanthine on the protein level of JNK in RAW264.7 cells.

RAW 264.7 cells (1.0 × 10^6 cells/ml) were pre-incubated for 18 h, and the cells were pre-incubated for 2 h with SP600125 (20 μM) and fucoxanthine (12.5, 25, and 50 uM) at indicated concentrations and then stimulated for 15 min with LPS (1 μg/ml). The levels of p-JNK (phosphorylated-JNK) and pan-JNK were determined using immunoblotting method.

Fig. 2-14. Inhibitory mechanism of fucoxanthin on LPS-induced inflammation.

Fig. 3-6. Effect of fucoxanthin on phosphorylation of p38 in HL-60 cells. The cells were stimulated with 15 μM of fucoxanthin for 24 h. The levels p-p38 (phosphorylated-p38) and pan-p38 were determined using immunoblotting method.

Fig. 3-7. The generation of ROS by fucoxanthin in tumor cells. (A) HL-60, HCT-15, and HepG2 cells were seeded at 4×10^5 and treated with 30 μM of fucoxanthin (B) HL-60 cells were seeded at 4×10^5 and treated with the difference concentrations (8, 15, and 30 μM) of fucoxanthin. After 2 h, the cells were labeled with 5 μM HE or 10 μM DCFH2-DA for 30 min at 37℃ and subsequent FACs analysis for intracellular accumulation of ROS. FI: fluorescence intensity.

Fig. 3-8. NAC pre-treatment protected against the Fucoxanthin-induced apoptosis through suppression of ROS generation. HL-60 cells were pretreated with 2 mM NAC for 1 h prior to fucoxanthin exposure for 24 h. (A) The cells were stained with PI and analyzed by flow cytometry. (B) Apoptotic bodies were stained with Hoechst 33342 solution and then observed under a fluorescent microscop using a blue filter. (C) Fragmented DNA was extracted and analyzed on 1.5% agarose gel containing EtBr. (D) HL-60 cells were pretreated with 2 mM NAC for 1 h prior to fucoxanthin exposure for 48 h. Cells viability was measured using MTT assay.

Fig. 3-9. Effect of NAC on phosphorylation of MAPKs. The cells were pre-treated with 2mM

NAC, and then treated with 15 μM fucoxanthin for 12 h. Equal amounts of cell lysates (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-p-p38, anti-p38, anti-p-JNK, and anti-JNK).

Fig. 3-10. Effect of NAC on down-regulation of Bcl-xL, caspase-3, -7, and PARP cleavage activation by fucoxanthin. The cells were pre-treated with 2mM NAC, and then treated with 15 μM fucoxanthin for 12 h. Equal amounts of cell lysates (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-Bax, anti-Bcl-xL, anti-casapse-3, -7, and anti-PARP).

Fig. 3-11. Signaling pathway responsible for fucoxanthin-induced apoptotic death of HL-60 cells.

Fig. 4-1. Inhibitory effect of fucoxanthin against growth of B16F10 cells. Each point represents the mean ± SD of three independent experiments. Means with different letters within a column are significantly different (*, $p<0.05$, **, $p<0.01$).

Fig. 4-2. Effect of fucoxanthin on cell cycle pattern and apoptotic portion in B16F10 cells by flow cytometric analysis. B16F10 cells were seeded at 1×10^5 and treated with the difference concentrations of fucoxanthin for 24 h. The cells were stained with PI and analyzed by flow cytometry. The cells were stained with PI and analyzed by flow

cytometry. (A) Histogram of cell cycle patterns of B16F10 cells. (B) Bar graph of cell cycle patterns of B16F10 cells.

Fig. 4-3. Effect of fucoxanthin on cell cycle pattern and apoptotic portion in B16F10 cells by flow cytometric analysis. B16F10 cells were seeded at 1×10^5 and treated with the difference concentrations of fucoxanthin for 48 h. The cells were stained with PI and analyzed by flow cytometry. The cells were stained with PI and analyzed by flow cytometry. (A) Histogram of cell cycle patterns of B16F10 cells. (B) Bar graph of cell cycle patterns of B16F10 cells.

Fig. 4-4. Effect of fucoxanthin on morphological changes in B16F10 cells. (A) B16F10 cells were stimulated with 200 μM of fucoxanthin for the indicated periods (0-48 h) at 37°C. (B) B16F10 cells were stimulated with the indicated concentrations (50-200 μM) of fucoxanthin for 24 h at 37 °C. The cells were double stained with Hoechst 33342 and PI solution and then observed under a fluorescent microscop using a blue filter.

Fig. 4-5. Effect of fucoxanthin on protein expression related cell cycle arrest of B16F10 cells. The cells were treated with 50 and 100 μM fucoxanthin for 24 h. Equal amounts of cell lysates (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-p15, anti-p27, anti-CDK4, anti-CyclinD1, D2, and Anti-p-Rb (phosphorylated-Rb)).

Fig. 4-6. Effect of fucoxanthin on phosphorylation of Akt in B16F10 cells. The cells were stimulated with 100 μM of fucoxanthin for 24 and 48 h. The levels p-Akt (phosphorylated-Akt) and pan-Akt were determined using immunoblotting method.

Fig. 4-7. Effect of Triciribine (a specific Akt inhibitor) against the Fucoxanthin-induced cell arrest. (A) The cells were stimulated with 100 μM of Fucoxanthin for 24 h after pretreatment with 40 μM of Triciribine for 1 h. The cells were stained with PI and analyzed by flow cytometry. (B) The cells were stimulated with 100 μM of Fucoxanthin for 48 h after pretreatment with 40 μM of Triciribine for 1 h. by using MTT assays. Means with different letters within a column are significantly different (**, $p<0.01$).

Fig. 4-8. Fucoxanthin treatment results in modulation of anti- and proapoptotic proteins in B16F10 cells. The cells were treated with 200 μM fucoxanthin for 24 h. Equal amounts of cell lysates (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-Bax, -Bcl-xL, -pro-caspase-9, -cleaved caspase-3, and -PARP).

Fig. 4-9. Fucoxanthin treatment results in modulation of anti-apoptotic proteins in B16F10 cells. The cells were treated with 200 μM fucoxanthin for 24 h. Equal amounts of cell lysates (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-c-IAP-1, -2, and anti-XIAP).

LIST OF TABLES

INTRODUCTION

Marine algae are classified as unicellular microalgae and macroalgae, which are macroscopic plants of marine benthoses (Ricketts and Calvin, 1962). Macroalgae, also known as seaweed, are distinguished according to the nature of their pigments: brown algae (phaeophyta), red algae (rhodophyta) and green algae (chlorophyta). In Asian countries, several species of seaweed are used as human food, to provide nutrition and a peculiar taste. Fresh dried seaweed is extensively consumed, especially by people living in coastal areas. They are of nutritional interest as they are low calories foods but rich in vitamins, minerals and dietary fiber (Jensen, 1993; Noda, 1993; Oohusa, 1993). Currently, human consumption of brown algae (66.5%), red algae (33%) and green algae (5%) is high in Asia, mainly Korea, Japan, and China (Dawes, 1998). The marine algae have mainly been used in western countries as raw material to extract alginates (from brown algae) and agar and carragenates (from red algae) which have been used as ingredients in food, pharmaceuticals and diverse consumer products and industrial processes (Skjak-Braek and Martinsen, 1991; Lewis et al., 1998). The marine algae have recently been identified as an under-exploited plant resource and functional food (Nisizawa et al., 1987; Heo et al., 2005a,b). They have also proven to be rich sources of structurally diverse bioactive compounds with valuable pharmaceutical and

biomedical potential. In particular, brown algae are plentifully present around Jeju Island, Korea, where these valuable brown algae have various biological compounds, such as xanthopophyll, pigments, fucoidans, phycocolloids, phlorotannins, and fucoxanthin (Halliwell and Gutteridge, 1999). Several researches on those kinds of compounds have pointed out a variety of biological benefits including antioxidant, anticoagulant, antihypertention, antibacterial and antitumor activities (Nagayama et al., 2002; Mayer and Hamann, 2004; Athukorala and Jeon, 2005; Kotake-Nara et al., 2005; Heo et al., 2008). Among the brown algae, *Ishige okamurae* (Phylum Phaeophyta, Class Phaeophyceae, Order Chordariales, Family Ishigeaceae) is found throughout the temperate coastal zone of the Korean peninsula, and generally forms highly persistent populations in clear waters (Lee and Kang, 1986). It is abundant along the coast of Jeju Island of Korea, and is regarded as an edible brown alga This algae is a brown algae and is reported to contain fucoxanthin, diphlorethohydroxycarmalol, stearic acid, methyl myristate and palmitic acid, (2S)-1-O-palmitoyl-2-O-myristoyl-3-O-(6- sulfo-alpha-D-quinovo pyranosyl) glycerol and its derivatives (Tang et al., 2002, Heo et al., 2008). However, relatively few biological studies have been conducted on these algae.

Carotenoids are natural pigments derived from five-carbon isoprene units that are enzymatically polymerized to form regular highly conjugated 40-carbon structures (with up

to 15 conjugated double bonds). One or both ends of the carbon skeleton may undergo cyclization to form ring β-ionone end groups, which additionally may be substituted by oxo, hydroxyl or epoxy groups at different positions to form the different xanthophylls (Solomons and Bulux, 1994). At least 600 different carotenoids exercising important biological functions in bacteria, algae, plants and animals have been identified to date (Polivka and Sundström, 2004) (**Fig.** Ⅰ). Fucoxanthin is a major carotenoid of edible brown algae and has a unique structure including allenic, conjugated carbonyl, epoxide, and acetyl group in its molecule. Fucoxanthin contributes >10% of the estimated total production of carotenoids in nature (Liaeen-Jensen, 1978, 1998). Hosokawa et al. (2004) reported that fucoxanthin, isolated from the edible brown algae *Undria pinnatifida*, induced apoptosis of Caco-2 colon cancer cells. Also, fucoxanthin has been reported to induce apoptosis in prostate cancer PC-3, DU145, LNCaP cells (Kotake-Nara et al., 2001), and leukemia (Kotake-Nara et al., 2005). In addition, fucoxanthin has been shown to cause cell cycle arrest in neuroblastoma cells (Okuzumi et al., 1990) and human hepatoma HepG2 cells (Das et al., 2008). Futhermor, fucoxanthin reported to have several functions including antioxidant, anti-inflammatory, and aniobesity (Okuzumi et al., 1993; Shimidzu et al., 1996; Yan et al., 1999; Hosokawa et al., 2004; Maeda et al., 2005). However, the possible mechanism responsible for its anti-inflammatory effects is still unknown. In this study, This results show for the first

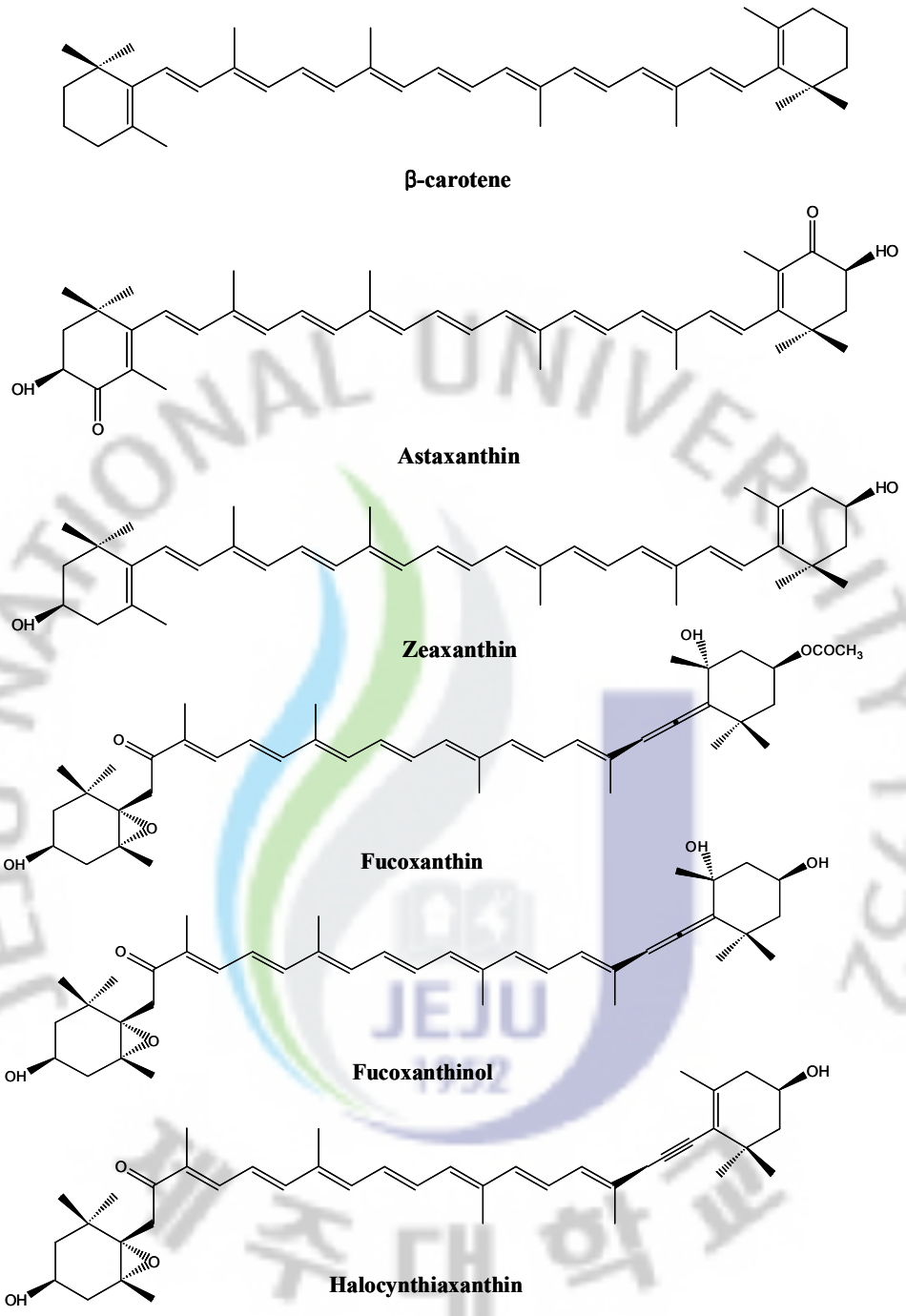

Fig. Ⅰ. Chemical structures of carotenoids isolated from nature.

time that fucoxanthin inhibits NF-κB activity and phosphorylation of MAP kinases in LPS stimulated RAW 264.7 macrophages.

Inflammation is an essential aspect of host response to infection and injury, and is required to maintain our health state against bacterial and viral infections. However, excessive or prolonged inflammation can be harmful, contributing to the pathogenesis of many diseases, such as arthritis, asthma, multiple sclerosis, inflammatory bowel disease, and atherosclerosis (Guzik et al., 2003; Nathan, 2002; Rankin, 2004). The inflammatory process is activated inflammatory cells (neutrophils, eosinophils, mononuclear phagocytes and macrophages) secrete increased amounts of NO, prostaglandin and cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α), and thus, these pro-inflammatory mediators are important anti-inflammatory targets (Lawrence et al., 2002; Larsen and Henson, 1983).

Bacterial endotoxin (lipopolysaccharide, LPS) is a major component of the outer membrane of Gram-negative bacteria and one of the most potent microbial initiators of inflammation (Cohen, 2002; Fujihara et al., 2003; Guha and Mackman, 2001) (**Fig. Ⅱ**). LPS activates monocytes and macrophages to produce proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-1. Although cytokine production is important for the efficient control of growth and dissemination of invading

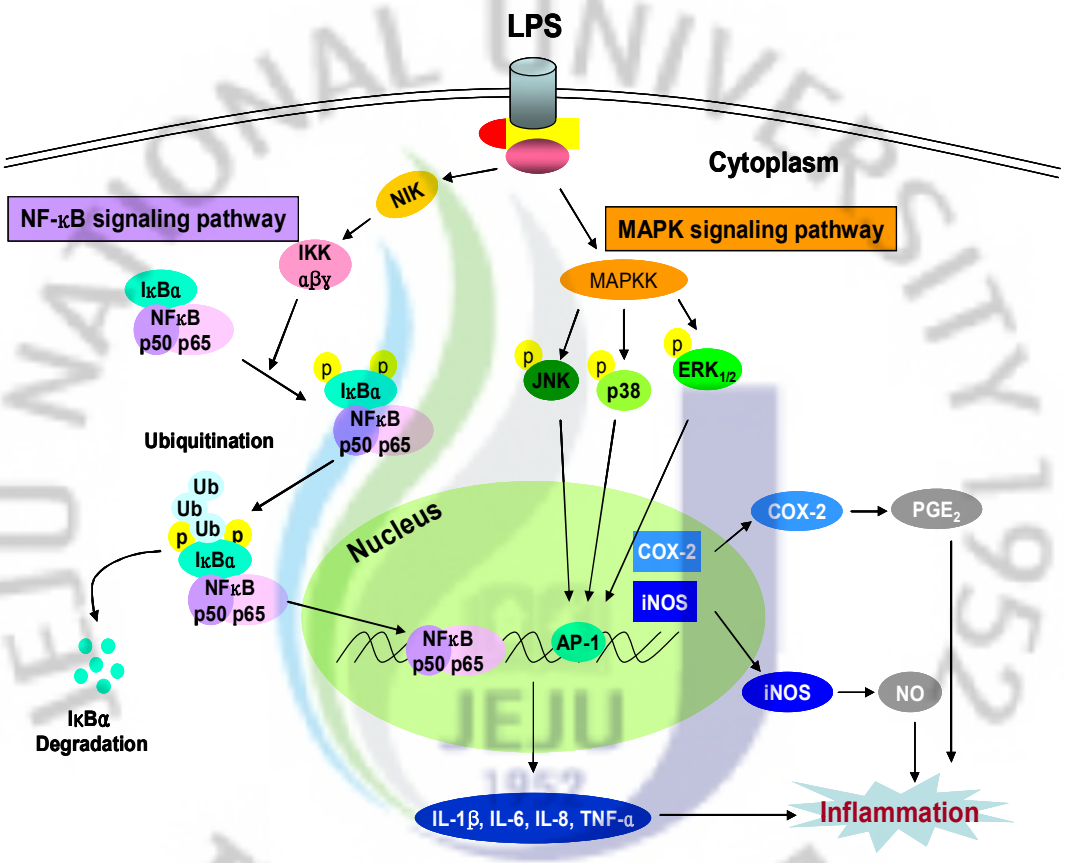

Fig. Ⅱ. LPS-induced inflammation.

pathogens, overproduction of cytokines have been implicated in many human diseases such as rheumatoid arthritis, septic shock and other chronic inflammatory diseases (Dobrovolskaia and Vogel, 2002; Ishii et al., 2003; Lee et al., 2004). TNF-α and IL-1 are associated with osteoclast differentiation and activation, resulting in bone loss. In patients with rheumatoid arthritis, treatment with TNF and IL-1 blockers significantly reduced erosions and joint space narrowing (Strand and Kavanaugh, 2004). Also, IL-1 and TNF are produced by microglia during CNS inflammation. Pathogens or pathogen components such as LPS can immediately induce IL-1 and TNF-α in both cultured microglia and LPS-injected rats (Appel et al., 1995; Buttini and Boddeke, 1995).

Nitric oxide (NO) is also an important mediator and effector molecule with various biological functions (Cross and Wilson, 2003; Stepien et al., 2005; Bove and Vliet, 2006). NO produced by activated macrophages has been shown to mediate host defense functions such as antimicrobial and antitumor activities, but excess production causes tissue damage associated with acute and chronic inflammation (Macmicking et al., 1997). LPS-induced NO production is mediated by a series of signalling pathways, such as nuclear factor (NF)-κB and mitogen-activated protein (MAP) kinases (Islam et al., 2004; Lee and Schorey, 2005).

NF-kB is a major transcription factor involved in the release of proteins that mediate cardiovascular growth, stress response, and inflammation by controlled gene expression. The

degradation or phosphorylation of IkB-a are necessary to release NF-kB from the cytoplasmic NF-kB/ IkB-a complex and allow its subsequent translocation to the nucleus of the cell (Kim et al., 2006; Murakami et al., 2005; Inoue et al., 2007). Many compounds isolated from natural plant exhibit anti-inflammatory activity associated with their potent NF-κB inhibition. For example, a green tea polypenol (epigallocatechin-3-gallate) and rosemary phytopolyphenol (Carnosol) were found to directly block the activity of I-κB kinase (Yang et al., 2001; Lo et al., 2002). Various upstream activators are involved in LPS signalling may mediate activation of the MAPK pathway, including extracellular signalregulated kinase 1/2 (ERK 1/2), p38 and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) in monocytes and macrophages. ERK 1/2 belongs to a group of serine/threonine-specific mitogen-activated protein kinases (MAPKs). ERK 1/2 is activated by various extracellular stimuli, including LPS, and connects the extracellular signal to intracellular transcription factors and other regulatory proteins, thus participating in regulation of gene expression. LPS-induced inflammatory gene expression inhibited by PD98059, a specific inhibitor of MEK that is an upstream activator of ERK 1/2 (Lahti et al., 2000; Su and Karin, 1996). The p38 kinase is an important mediator of stress-induced gene expression (Raingeaud et al., 1995). In particular, the p38 kinase plays a key role in the LPSinduced signal transduction pathway (Rafi et al., 2007; Lee and Young, 1996). Also,

stimulation of RAW 264.7 cells rapidly activates the JNK pathway (Hambleton et al., 1997). This pathway contains the c-Jun N-terminal kinase (JNK), which is also known as stressactivated protein kinase (SAPK1). Two isoforms of JNK (54 and 46 kDa) phosphorylate the N-terminus of c-Jun (Swantek et al., 1997). Treatment of either SB203580 (inhibitor of p38 MAPK) or SP600125 (inhibitor of JNK) suppressed NO, TNF-α, and IL-1β production (Kwak et al., 2005).

Cancer is a disease manifested by uncontrolled cell growth that presents over 100 distinct clinical pathologies (Kufe et al., 2003). Cancer is the largest single cause of death in both men and women, claiming over 6 million lives each year in the world. In the last few decades, basic cancer researches have produced remarkable advances in understanding of cancer biology and cancer genetics (Lowe and Lin, 2000). Recently, many anti-cancer drugs have been developed and applied by clinical doctors. Chemotherapeutic agents and radiation which cause DNA mutation in actively dividing cells, were intended to selectively kill cancer cells while having limited effect on normal cells. Unfortunately, these cytotoxic agents, while effective in managing certain types of cancer, were limited in their utility due to their toxicity on normal dividing cell populations resulting in adverse side effects. Therefore, the researches and developments of new and safe drugs have become one of the interest areas in the pharmaceutical industry (Yang et al., 2000).

Apoptosis is a selective process of physiological cell deletion that regulates the balance between cell proliferation and cell death. The failure of apoptosis is considered to contribute generally to the development of human malignancies (Shinkai et al., 1996). Because it was recently suggested that cancer chemotherapeutics exert part of their pharmacological effects by triggering apoptotic cell death, the induction of apoptosis in cancer cells has become a target of cancer treatment (Ahmad et al., 1997; Kim et al., 1999).

Reactive oxygen species (ROS), most particularly the superoxide anion ($\cdot O_2^-$), hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2), are unwanted metabolic by-products of normal aerobic metabolism. ROS normally exist in all aerobic cells in balance with biochemical antioxidants, which convert ROS into water (Feig et al., 1994; Kondo et al., 2006; Ham et al., 2006). High levels of ROS lead to apoptosis and necrosis, which are implicated in cancer, aging, and neurodegenerative disorder (Martindale and Holbrook, 2002; Hussain et al., 2003). Therefore, recently, increasing studies have indicated that ROS are involved in anticancer drug-mediated apoptosis (Huan et al., 2000; Kotamraju et al., 2004). However, the role of ROS generation with respect to the anticancer effects of fucoxanthin is not fully understood.

Many studies have shown that reproductive toxicants can regulate the activity of mitogen-activated protein kinase (MAPK) signaling pathways, which are involved in such cellular

responses as proliferation, differentiation, and apoptosis (Iseki et al., 2005; Jin et al., 2008; LaChapelle et al., 2007; Valbonesi et al., 2008; Wollenhaupt et al., 2005). The MAPKs include three major kinases: extracellular signal-regulated kinase (ERK), p38 kinase, and c-Jun N-terminal kinase (JNK). ERK1/2 activation has been generally accepted as a promoter cell survival, activating anti-apoptotic signaling pathways; whereas activation of JNK1/2 and p38 MAPK are associated with cell apoptosis (Harper and LoGrasso, 2001; Xia et al., 1995; Wang et al., 2008). Many studies have demonstrated that activation of the MAPKs signaling pathway plays a promoting role in the apoptosis induced by chemotherapeutic drugs (Valbonesi et al., 2008; Wollenhaupt et al., 2005; Lee et al., 2009; Jung et al., 2008). Therefore, this paper aim was to further explore the mechanisms underlying fucoxanthin-induced apoptosis in human leukemia HL-60 cells by studying the role of fucoxanthin-induced ROS in MAPKs phosphorylation.

Cell cycle control mechanisms serve major regulatory functions for cell growth. Many cytotoxic agents and/or DNA-damaging agents induce apoptosis by arresting the cell cycle (Kaina, 2003; Nakanishi et al., 2006). In fact, the anti-cancer properties of many anti-cancer agents act through the induction of cell cycle arrest and/or apoptotic cell death. Cell cycle progression is regulated by sequential activation and subsequent inactivation of a series of cyclin-dependent kinases (cdks) during the corresponding phases. The activities of cdks are

positively regulated by cyclins and negatively regulated by cdk inhibitors, such as the INK4 family, $p15^{INK4B}$ and the Kip family, $p27^{Kip1}$ (Bai et al., 2003; Lloyd et al., 1999). During progression of the G_0/G_1 phase and transition of G_1 to the S phases, a complex of cyclin and the cdk hyper-phosphorylates retinoblastoma protein (pRb), leading to the release of transcription factors such as E2F (Puri et al., 1999). The released E2F then promotes the expression of genes, which is required for cellular proliferation (Weinberg, 1995). Therefore, cell cycle arrest and the induction of apoptosis in cancer cells become the major indicators of anticancer effects. Previous study has reported that fucoxanthin inhibited tumor cell growth by inducing cell cycle arrest at the G_1 phase and/or apoptosis by modulating the expression of cell cycle and apoptosis-related genes (Kotake-Nara et al., 2001; Satomi and Nishino, 2007; Das et al., 2005). However, there is not information available concerning the ability of fucoxanthin to inhibit melanoma cancer. Therefore, in this study investigated the molecular mechanism of fucoxanthin-induced growth arrest and apoptosis in B16F10 melanoma cells

Part Ⅰ.

Isolation of fucoxanthin from *Ishige okamurae*

Part Ⅰ.

Isolation of fucoxanthin from *Ishige okamurae*

1. ABSTRACT

Fucoxanthin contents of 16 species of the brown algae collected from Jeju Island area were measured by HPLC fingerfrinting analysis. A variety of chloroform ($CHCl_3$) extracts of brown algae showed fucoxanthin contents. Among them, *Ishige okamurae* showed relatively higher fucoxanthin contents of 11.4 mg/g of powder, when compared to other brown algae. Therefore, the samples of *I. okamurae* were selected for use in further isolation fucoxanthin. Fucoxanthin was isolated from the methanolic extract from *I. okamurae*. This structure was elucidated based on NMR spectroscopic data.

2. MATERIALS AND METHODS

2.1. General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter. The UV and FT-IR spectra were recorded on a Pharmacia Biotech Ultrospec 3000 UV/Visible spectrometer and a SHIMAZU 8400s FT-IR spectrometer, respectively. NMR spectra were recorded on a Bruker 500 MHz and Varian INOVA 400 MHz NMR spectrometer. CD_3OD and $CDCl_3$ were used as a solvent for the NMR experiments, and the solvent signals were used as an internal reference. ESI and HREI mass spectra acquired using a Finnigan Navigator 30086 and JMS-700 MSTATION high resolution mass spectrometer system, respectively. The HPLC was carried out on a Waters HPLC system equipped with a Waters 996 photodiode array detector and Millenium32 software using C_{18} column (J'sphere ODS-H80, 150×20 mm, 4 μm, YMC Co.).

2.2. Materials

Sixteen species of brown algae were collected along the coast of Jeju Island, Korea,

between October 2007 and March 2008 (**Table 1-1**). The samples were washed three times with tap water to remove salt, sand, and epiphytes attached to the surface, then carefully rinsed with fresh water, and maintained in a medical refrigerator at –20℃. Therefore, the frozen samples were lyophilized and homogenized with a grinder prior to extraction.

2.3. Extraction procedure of chloroform extracts from brown algae

The brown algae samples were pulverized into powder using a grinder. The algae powder (1 g) was extracted with chlorofrom (100 ml) at a room temperature for 24 h and filtrated. After filtration, the methanolic extracts were evaporated to dryness under vacuum. This extracts were used for further HPLC-fingerfrint analysis.

2.4. HPLC-fingerfrint analysis

HPLC analysis were carried out using an ACCELA system (ThermoFisher Scientific, USA), which included pump, auto sampler, column oven, and PDA detector, connected to Xcalibur software. A Hypersil-Gold C18 column (100 mm × 2.1 mm i.d., 1.9 um) from Thermo was used. The column temperature was maintained at 25 ℃. The standards and

Table 1-1. The list of brown algae.

No.	Scientific name
1	*Sargassum thunbergii* (지충이)
2	*Sargassum patens* (쌍발이모자반)
3	*Myagropsis myagroides* (외톨개모자반)
4	*Undaria pinnatifida* (미역)
5	*Sargassum hemiphyllum* (짝잎모자반)
6	*Myelophycus caespitosus* (바위수염)
7	*Sargassum piluliferum* (구슬모자반)
8	*Ishige foliacea* (넓패)
9	*Padina arborescens* (부챗말)
10	*Ishige okamurae* (패)
11	*Dictyota coriacea* (참가죽그물바탕말)
12	*Sargassum horneri* (괭생이모자반)
13	*Sargassum tortile* (큰열매모자반)
14	*Sargassum ringgoldianum* (큰잎모자반)
15	*Hizikia fusiforme* (톳)
16	*Ecklonia cava* (감태)

samples were separated using an isocratic mobile phase consisting of 80% methanol. The flow rate was set at 0.2 ml/min and the injection volume was 5 µl. The detection wavelength was set at 450 nm. Identification and quantification of fucoxanthin was based on retention time when co-injected with standards.

2.5. Extraction and isolation of fucoxanthin

Extraction was performed from the powder of *I. okamurae* (**Fig. 1-1**) with 80% aqueous MeOH, and was evaporated under vacuo. Then, the MeOH extract was partitioned with $CHCl_3$. The chloroform extract was fractionated by silica column chromatography with stepwise elution of $CHCl_3$-MeOH mixture (100:1-1:1) to afford separated active fractions. A combined active fraction was further subjected to a Sephadex LH-20 column saturated with 100 % MeOH, and then finally purified by reversed-phase HPLC (90% aqueous MeOH) to give compound **1**.

Fucoxanthin (1): bright orange solid; $[\alpha]_D$ +18° (*c* 0.2, $CHCl_3$); IR (KBr) ν_{max} 3438, 2361, 2332, 1723, 1654, 1605, 1251, 1030 cm^{-1}; UV (MeOH): 468 nm (ε 51,000) (sh), 446 (56,000), 332 (17,000), 267 (24,000); ^1H and ^{13}C NMR, see **Table 1-2**; LREIMS *m/z* 658 $[M]^+$ (calcd for $C_{42}H_{58}O_6$, 658).

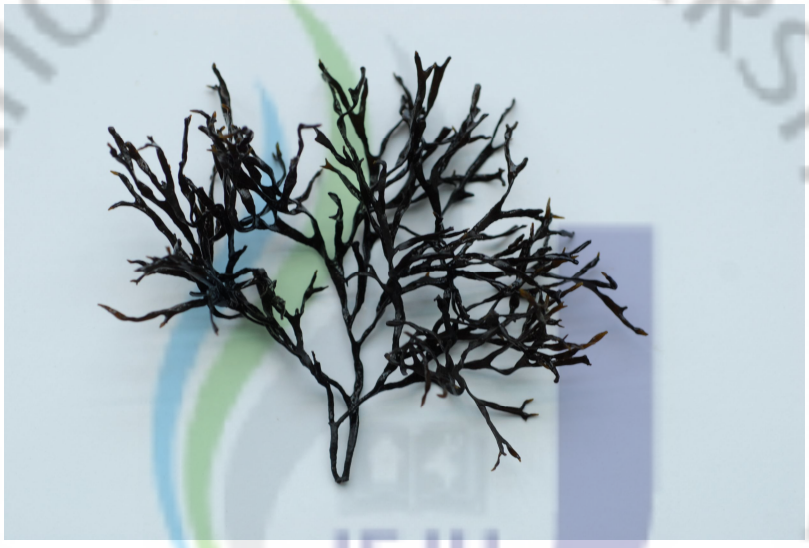

Fig. 1-1. The photography of the alga, *Ishige okamurae*.

Table 1-2. ^{1}H and ^{13}C NMR assignments for fucoxanthin.

Position	^{13}C	^{1}H (mult. J=Hz)	Position	^{13}C	^{1}H (mult. J=Hz)
1	35.8		1'	35.2	
2	47.1	1.36 (1H, dd, J=8.7, 14.2) 1.49 (1H, dd, J=14.2)	2'	45.4	1.41 (1H, dd, J=10.4, 14.9) 2.00 (1H, dd, J=2.9, 14.9)
3	64.3	3.80 (1H, m)	3'	68.0	5.37 (1H, tt, J=8.8, 12.0)
4	41.6	1.77 (1H, dd, J=8.7, 14.2) 2.29 (1H, dd, J=2.9, 17.8)	4'	45.2	1.53 (1H, dd, J=10.4, 14.9) 2.29 (1H, dd, J=2.9, 17.8)
5	66.2		5'	72.7	
6	67.1		6'	117.5	
7	40.8	2.59, 3.64 (2H, d, J=20.4)	7'	202.4	
8	170.5		8'	103.4	6.04 (1H, s)
9	134.5		9'	132.5	
10	139.1	7.14 (1H, d, J=12.8)	10'	128.5	6.12 (1H, d, J=11.6)
11	123.4	6.58 (1H, m)	11'	125.7	6.71 (1H, t, J=12.0)
12	145.0	6.66 (1H, t, J=12.8)	12'	137.1	6.34 (1H, d, J=11.6)
13	135.4		13'	138.1	
14	136.6	6.40 (1H, d, J=11.6)	14'	132.2	6.26 (1H, d, J=11.6)
15	129.4	6.67 (1H, m)	15'	132.5	6.71 (1H, t, J=12.0, 14.2)
16	25.0	1.02 (3H, s)	16'	29.2	1.37 (3H, s)
17	28.1	0.95 (3H, s)	17'	32.1	1.06 (3H, s)
18	21.2	1.21 (3H, s)	18'	31.3	1.34 (3H, s)
19	11.8	1.93 (3H, s)	19'	14.0	1.80 (3H, s)
20	12.8	1.98 (3H, s)	20'	12.9	1.98 (3H, s)
			3'OAc, CH_3	21.4	2.03 (3H, s)
			3'OAc, C=O	197.9	

* 400 MHz for ^{1}H and 100 MHz for ^{13}C

3. RESULTS AND DISCUSSIONS

Fucoxanthin is a major carotenoid of edible brown algae and has a unique structure including allenic, conjugated carbonyl, epoxide, and acetyl group in its molecule. There have recently been several reports that fucoxanthin gave biological activities such as antioxidant, antiobesity, antitumor, and anticarcinogenic activities (Okuzumi et al., 1993; Shimidzu et al., 1996; Yan et al., 1999; Hosokawa et al., 2004; Maeda et al., 2005). However, the possible mechanism responsible for its anti-inflammatory and anti-tumor effects are remains unknown. Therefore, this author was fucoxanthin isolated from brown algae, *I. okamurae*.

The chromatographic conditions used by Heo (2008) were initially used to run standard fucoxanthin samples and then further optimised. A chromatogram obtained from the analysis of standard fucoxanthin and extract of brown seasseds are shown in **Fig 1-2A and B**, respectively. The HPLC profile of fucoxanthin content is shown in **Table 1-3**. Among the brown algae screened, *I. okamurae* (11.4 mg/g dry sample) contained higher level of fucoxanthin followed by *Sargassum thunbergii* (10.0 mg/g dry sample). The level of fucoxanthin in other brown algae screened ranged between 1.8 and 9.0 mg/g. Therefore, the *I. okamurae* was selected for use in further isolation fucoxanthin.

The isolation of the fucoxanthin compounds from *I. okamurae* was carried out as **Fig. 1-3**.

The dried *I. okamurae* (700 g) was extracted twice with 80% aqueous MeOH at roomtempe rature. The MeOH extract was filtered and concentrated under reduced pressure. The residue was subjected to successive extraction with organic solvents of *n*- hexane and chloroform. The chloroform layer was concentrated under vacuum and the crude residue (13.8 g) was applied to a silica gel column and eluted with $CHCl_3$ and $CHCl_3$-MeOH mixture (100:1-1:1) with increas ing proportion of MeOH. The active fractions were collected and combined by monitoring with analytical thinlayer chromatography to yield six fractions. Sephdex LH- 20 column chromatography (100% MeOH) of the fraction of IO.CS3 (700 mg) resulted in the pure fucoxanthin (**1**)(90.9 mg). **1** was isolated as a bright orange solid and the IR spectrum of **1** showed the presence of the hydroxyl (3438 cm^{-1}), sp-hybrid carbon (allenic) (2361, 2332 cm^{-1}), ester (1723, 1251 cm^{-1}), and polyene (1654, 1605 cm^{-1}). The ^{1}H and ^{13}C NMR spectra of **1** revealed signals assignable to polyene having acetyl, conjugated ketone, two quaternary geminal dimethyls, two quaternary geminal methyls of oxygen, four olefinic methyls, and allene functionalities (**Fig. 1-4**). The physicochemical features outlined above suggested that **1** was a carotenoid in which one of the hydroxyl groups was acetylated. From detailed comparison of the data for **1** with those of fucoxanthin, **1** was in agreement with an authentic fucoxanthin in all aspects (Palermo et al., 1991; Choi et al., 2000). Based on the above evidence, **1** was determined as fucoxanthin.

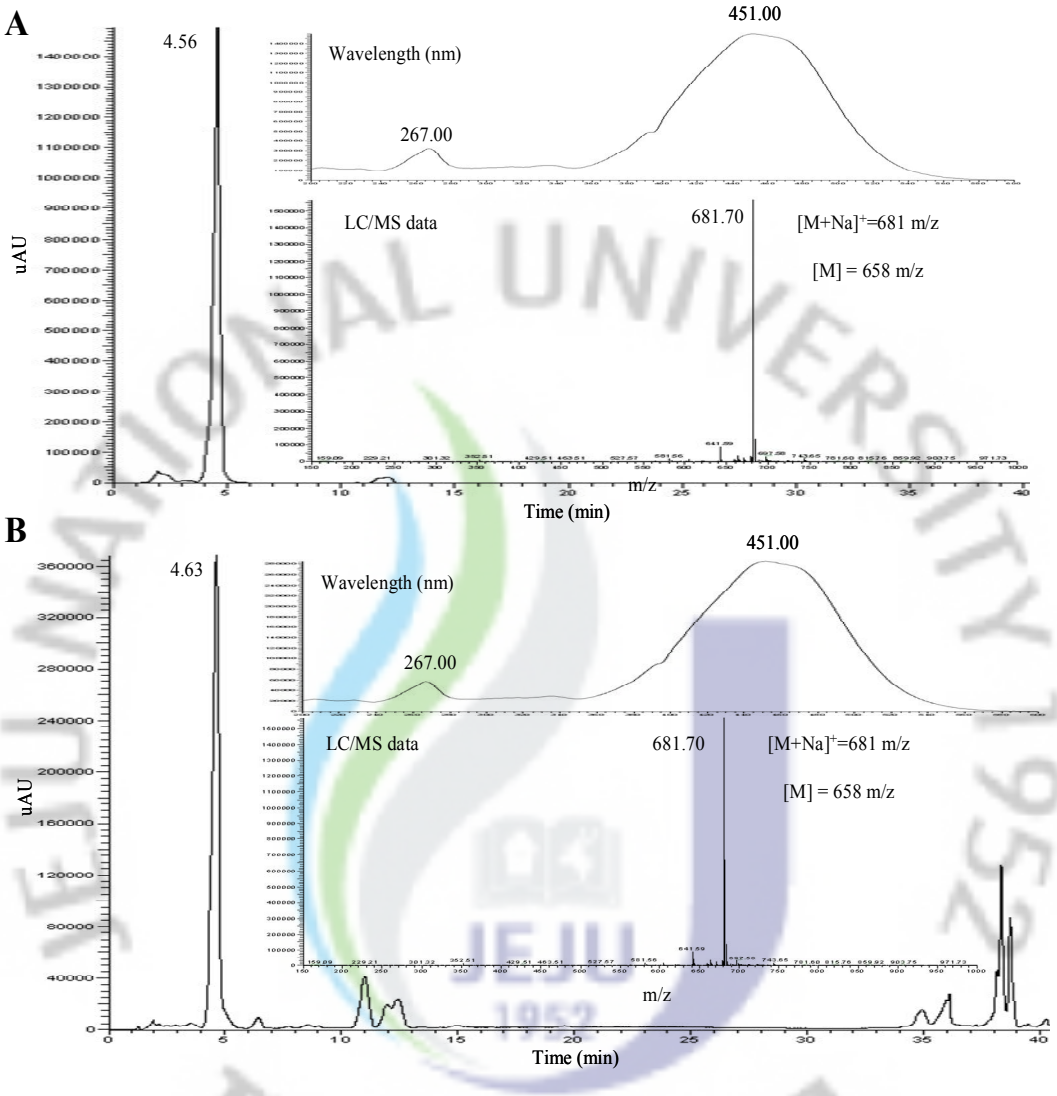

Fig. 1-2. HPLC fingerprinting analysis of the *I. okamurae* chloroform extract. HPLC profiles of authentic fucoxanthin and *I. okamurae* extract are shown in (A) and (B), respectively.

Table 1-3. Fucoxanthin contents of brown algae.

No.	Scientific name	Chloroform ex. (mg)	Fucoxanthin Contents	
			mg/mg ex.	mg/g powder
1	*Sargassum thunbergii* (지충이)	46.7	0.2145	10.02
2	*Sargassum patens* (쌍발이모자반)	20.1	0.2169	4.36
3	*Myagropsis myagroides* (외톨개모자반)	44.4	0.2030	9.01
4	*Undaria pinnatifida* (미역)	29.3	0.2214	6.49
5	*Sargassum hemiphyllum* (짝잎모자반)	21.4	0.2232	4.78
6	*Myelophycus caespitosus* (바위수염)	32.1	0.1930	6.20
7	*Sargassum piluliferum* (구슬모자반)	19.2	0.2081	4.00
8	*Ishige foliacea* (넓패)	32.4	0.1981	6.42
9	*Padina arborescens* (부챗말)	21.2	0.2138	4.53
10	*Ishige okamurae* (패)	58.79	0.1932	11.36
11	*Dictyota coriacea* (참가죽그물바탕말)	31.5	0.2038	6.42
12	*Sargassum horneri* (괭생이모자반)	10.5	0.2248	2.36
13	*Sargassum tortile* (큰열매모자반)	8.29	0.2182	1.81
14	*Sargassum ringgoldianum* (큰잎모자반)	8.3	0.3420	2.84
15	*Hizikia fusiforme* (톳)	11.1	0.2308	2.56
16	*Ecklonia cava* (감태)	15.8	0.2282	3.61

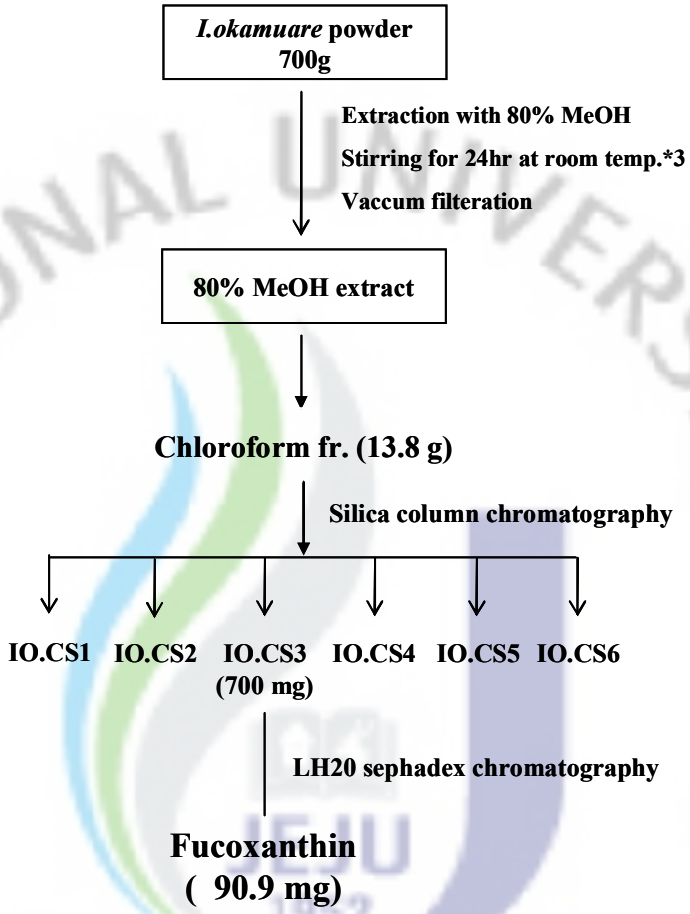

Fig. 1-3. Isolation scheme of the fucoxanthin from the alga *I. okamurae*.

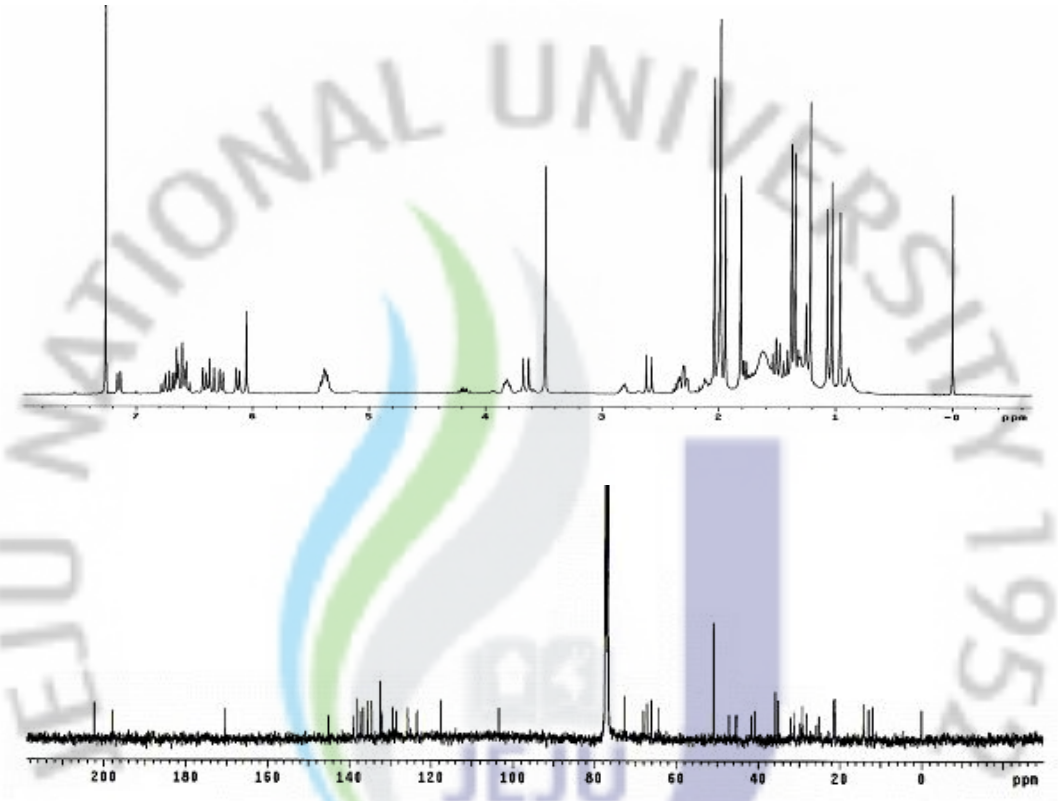

Fig. 1-4. Proton and Carbon NMR spectrum of fucoxanthin (1).

Part Ⅱ.

Fucoxanthin inhibits the inflammatory response by suppressing NF-κB and MAPKs activation in lipopolysaccharide-induced RAW 264.7 macrophages

Part Ⅱ.

Fucoxanthin inhibits the inflammatory response by suppressing NF-κB and MAPKs activation in lipopolysaccharide-induced RAW 264.7 macrophages

1. ABSTRACT

It is well known that pro-inflammatory mediators like nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 contribute to the courses of many inflammatory diseases. In the present study was investigated the anti-inflammatory effects of fucoxanthin, a natural biologically active substance isolated from *Ishige okamurae* by examining its inhibitory effects on pro-inflammatory mediators in lipopolysaccharide (LPS)-stimulated murine macrophage RAW 264.7 cells. Fucoxanthin caused dose-dependent reductions in the levels of inducible nitric oxide (iNOS) at protein and mRNA levels and concomitant decreases in NO production. In addition, it was found that fucoxanthin suppressed the production and mRNA expressions of inflammatory

cytokines, such as, IL-1β, TNF-α and IL-6. Furthermore, fucoxanthin inhibited the cytoplasmic degradation of IκB-α and the nuclear translocation of p50 and p65 protein, leading to less NF-κB transactivation. In addition, fucoxanthin dose-dependently inhibited the phosphorylations of JNK, ERK and p38. Taken together, these results suggest that fucoxanthin reduces levels of the pro-inflammatory mediators, such as, NO, iNOS, IL-1β, TNF-α, and IL-6 through the inhibition of NF-κB activation and suppression of JNK, ERK and p38 phosphorylation in RAW 264.7 cells. These findings reveal in part the molecular basis for the anti-inflammatory properties of fucoxanthin.

2. MATERIAL AND METHODS

2.1. Materials

Lipopolysaccharide (LPS) was purchased from sigma Chemical Co (St. Louis, MO) Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) penicillin–streptomycin and trypsine–EDTA were obtained from Gibco/BRL (Grand Island, NY, USA). The lactate dehydrogenase (LDH) cytotoxicity detection kit and M-MuLV reverse transcriptase were purchased from Promega (Madison, WI, USA). The enzyme-linked immunosorbent assay (ELISA) kit for IL-1β, IL-6, TNF-α and Prostaglandin E_2 (PGE_2) were purchased from R & D Systems Inc (Minneapolis, MN, USA). Protein assay kit and ECL detection reagent were bought from Bio-Rad (Richmond, CA, USA) and Amersham Biosicences (Piscataway, NJ, USA), respectivly Antibodies against extracellular signal-regulated kinase (ERK), p38 kinase, c-Jun N-terminal kinase (JNK), IκB-α, phospho-ERK, phospho-p38, phosphor-JNK, phospho-IκB-α, phosphor-p65, phosphor-p50, and β-actin were purchased from Cell Signaling Technology (Bedford, Massachusetts, USA). Antibodies against iNOS and COX-2 were obtained from Calbiochem (La Jolla, CA, USA)

and BD Biosciences Pharmingen (San Jose, CA, USA), respectively. The other chemicals and reagents used were of analytical grade.

2.2. Cell Culture

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB; Seoul, KOREA). RAW 264.7 cells were cultured in DMEM supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin and 10% FBS. The cells were incubated in an atmosphere of 5% CO_2 at 37°C and were subcultured every 3 days.

2.3. LDH Cytotoxicity Assay

RAW 264.7 cells (1.5×10^5 cells/ml) plated in 96-well plates were pre-incubated and then treated with LPS (1 μg/ml) plus aliquots of fucoxanthin at 37°C for 24 h. The medium was carefully removed from each well, and the LDH activity in the medium was determined using an LDH cytotoxicity detection kit. Briefly, 100 μl of reaction mixture were added to each well, and the reaction was incubated for 30 min at room temperature in the dark. The absorbance of each well was measured at 490 nm using a UV spectrophotometer.

2.4. Determination of Nitric oxide (NO) Production

After pre-incubation of RAW 264.7 cells (1.5×10^5 cells/ml) with LPS (1 μg/ml) for 24 hours, the quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent [1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid], the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was used as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve.

2.5. Measurement of pro-inflammatory cytokines (TNF-α IL-1β and IL-6) production

Fucoxanthin solubilized with DMSO was diluted with DMEM before treatment. The inhibitory effect of fucoxanthin on the pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) production from LPS treated RAW 264.7 cells was determined as described in the Cho et

al. (2000) protocols. Supernatants were used for pro-inflammatory cytokines assay using mouse ELISA kit.

2.6. Determination of PGE_2 Production

Fucoxanthin was diluted with DMEM before treatment. Cells were treated with LPS (1 μg/ml) to allow cytokine production for 24 h. The PGE_2 concentration in the culture medium was quantified using a competitive enzyme immunoassay kit according to the manufacturer's instructions. The production of PGE_2 was measured relative to that following control treatment.

2.7. RNA Isolation and RT-PCR Analysis

Total RNA from LPS-treated RAW 264.7 cells was prepared with Tri-Reagent (MRC, Cincinnati, OH, USA), according to the manufacturer's protocol. RNA was stored at -70°C until used. The reverse transcription of 1 μg RNA was carried out with M-MuLV reverse transcriptase, oligo dT-18 primer, dNTP (0.5 μM) and 1 U RNase inhibitor. After this reaction cocktail was incubated at 70°C for 5 min, 25°C for 5 min, and 37°C for 60 min in

series, M-MuLV reverse transcriptase was inactivated by heating at 70°C for 10 min. Polymerase chain reaction (PCR) was performed in reaction buffer [cDNA, 1.25 U Taq DNA polymerase (Promega, WI, USA), 3'- and 5'-primer (50 μM each) and 200 mM dNTP in 200 mM Tris-HCl buffer (pH 8.4) containing 500 mM KCl and 1-4 mM $MgCl_2$]. The PCR was performed in a DNA gene cycler with amplification by 30 cycles of 94°C for 45 sec (denaturing), 60 - 65°C for 45 sec (annealing) and 72°C for 1 min (primer extension). The primers (Bioneer, Seoul, Korea) used in this experiment were indicated in **Table 2-1** (F: forward, R: reverse). The PCR products were electrophoresed in 1.2% agarose gels and stained with ethidium bromide.

2.8. Western blot analysis

Murine macrophage cell line RAW 264.7 were pre-incubated for 18 h, and then stimulated with LPS (1 μg/ml) in the presence of fucoxanthin for the indicated time. After incubation, the cells were collected and washed twice with cold-PBS. The cells were lysed in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM NaVO3, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 25 μg/ml leupeptin] and kept on ice for 30 min. Cell lysate

were washed by centrifugation, and protein concentrations were determined by using BCA™ protein assay kit.. Aliquots of the lysates (30-50 μg of protein) were separated on a 12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)]. After blocking the nonspecific site with 1% bovine serum albumin (BSA), the membrane was then incubated with specific primary antibody at 4℃ for overnight. The membrane was further incubated for 60 min with a peroxidase-conjugated secondary antibody (1:5000, Vector Laboratories, Burlingame, USA) at room temperature. The immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit.

2.9. Statistical Analysis

All data are presented as mean ± SD. Significant differences among the groups were determined using the unpaired Student's *t*-test. A value of $*p<0.05$ was accepted as an indication of statistical significance.

Table 2-1. Sequences of primers and fragment sizes of the investigated genes in RT-PCR analysis.

Gene		Primer sequences	Fragment size(bp)
TNF-α	F	5'-TTGACCTCAGCGCTGAGTTG-3'	364
	R	5'-CCTGTAGCCCACGTCGTAGC-3'	
IL-1β	F	5'-CAGGATGAGGACATGAGCACC-3'	447
	R	5'-CTCTGCAGACTCAAACTCCAC-3'	
IL-6	F	5'-GTACTCCAGAAGACCAGAGG-3'	308
	R	5'-TGCTGGTGACAACCACGGCC-3'	
iNOS	F	5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'	496
	R	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'	
COX-2	F	5'-CACTACATCCTGACCCACTT-3'	696
	R	5'-ATGCTCCTGCTTGAGTATGT-3'	
β-Actin	F	5'-GTGGGCCGCCCTAGGCACCAG-3'	603
	R	5'-GGAGGAAGAGGATGCGGCAGT-3'	

3. RESULT

3.1. Effects of fucoxanthin on NO and PGE2 productions in LPS-stimulated RAW264.7 cells

To examine the potential anti-inflammatory properties of fucoxanthin on LPS-induced NO and PGE_2 production in RAW 264.7 cells, cells were treated with or without fucoxanthin (10, 20, and 40 μM) for 1 h and then treated with LPS (1 μg/ml) for 16 h. NO and PGE_2 concentrations were measured in the culture supernatants by the Griess reaction and ELISA assays, respectively. LPS treatment significantly increased the concentrations of NO and PGE_2. Fucoxanthin inhibited the LPS-induced production of NO in a concentration-dependent manner: 31%, 65%, and 80% at 10, 20, and 40 μM, respectively (**Fig 2-1**) whereas LPS-induced PGE_2 increase was not affected by fucoxanthin (**Fig. 2-2**). The cytotoxic effect of fucoxanthin was evaluated in the presence or absence of LPS using the LDH assay (**Fig. 2-1**). Fucoxanthin did not affect the cytotoxic of RAW 264.7 cells at the concentrations used (10, 20, and 40 μM) to inhibit NO. Thus, the inhibitory effects were not attributable to cytotoxic effects.

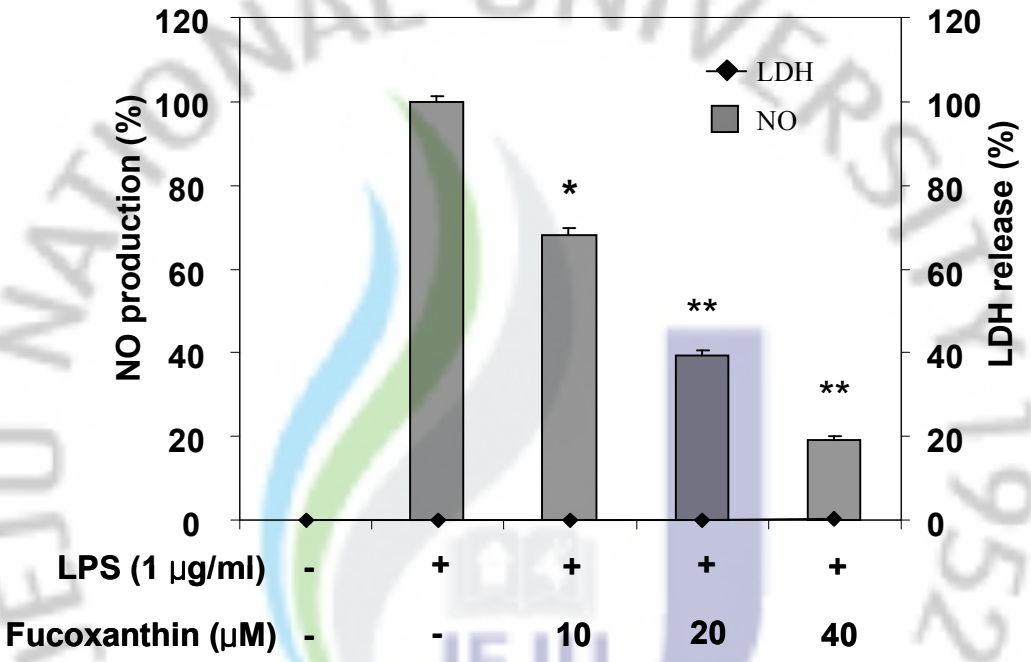

Fig. 2-1. Inhibitory effect of fucoxanthin on the NO production in RAW264.7 cells. The production of nitric oxide was assayed in the culture medium of cells stimulated with LPS (1 μg/ml) for 24 h in the presence of fucoxanthin (10, 20, and 40 μM). Cytotoxicity was determined using the LDH method. Values are the mean ± SD of triplicate experiments. *,*P<0.05* and **,*P<0.01* indicate significant differences from the LPS-stimulated group.

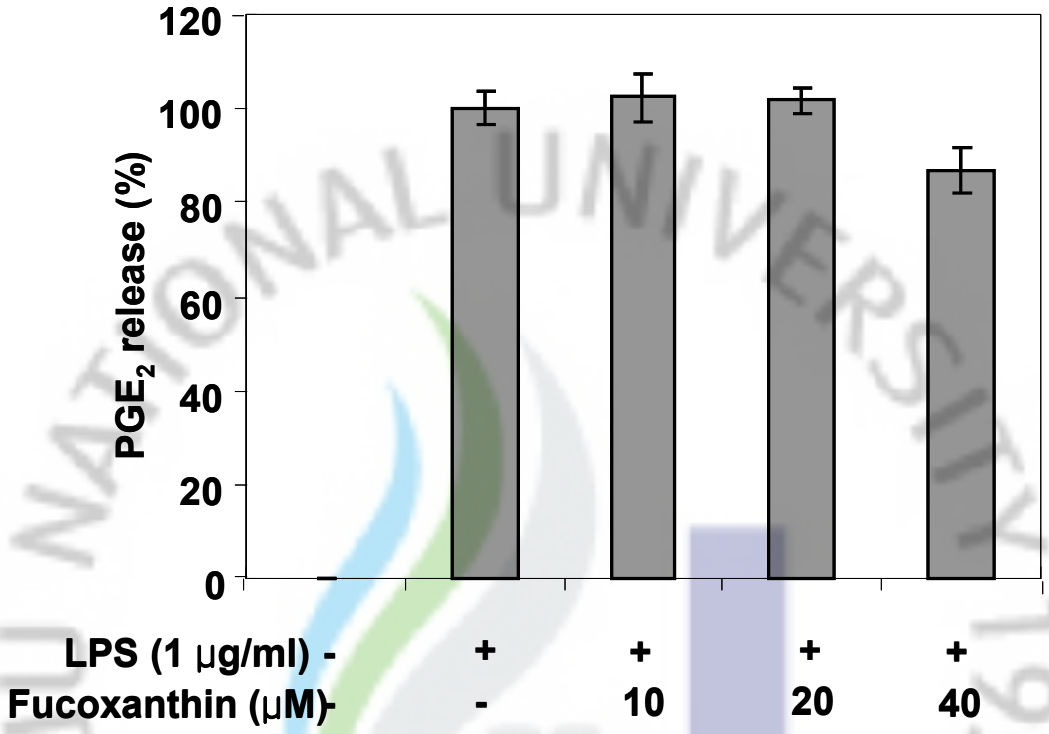

Fig. 2-2. Inhibitory effects of fucoxanthin on PGE2 production in RAW 264.7 cells. Cells (1.5×10^5 cells/ml) were stimulated by LPS (1 μg/ml) for 24 h in the presence of fucoxanthin (10, 20, and 40 μM). Supernatants were collected, and the PGE2 production in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments.

3.2. Effects of fucoxanthin on increase of iNOS and COX-2 protein and mRNA in LPS-stimulated RAW264.7 cells

Inflammatory processes are mediated by multiple molecular mechanisms. Two of the most prominent are the production of NO by iNOS and the formation of prostaglandins by COX-2 (Kim et al., 2005). To investigate the anti-inflammatory activity of fucoxanthin, this author tested the effects of fucoxanthin on LPS-induced iNOS and COX-2 protein up-regulation in RAW264.7 cells by Western blotting and RT-PCR analysis. The expression levels of iNOS and COX-2 were strongly induced by LPS. However, fucoxanthin inhibited iNOS protein induction in a dose-dependent manner, the densitometric analysis of three different experiments demonstrated that iNOS protein expression induced by LPS was inhibited by 64% and 93% by fucoxanthin (20 and 40 μM), respectively (**Fig. 2-3**). RT-PCR analysis showed that the content of iNOS mRNA was correlated with its protein level, approximately 54% and 85% reduction was observed compared to that of the cells treated with LPS only (**Fig. 2-4**). In contrast, fucoxanthin had no effect on the increased of COX-2 protein and mRNA level by LPS (**Fig 2-3, 4**). These data indicated that fucoxanthin inhibited LPS-induced iNOS but not COX-2 up-regulation in RAW264.7 cells suggesting its anti-inflammatory activity.

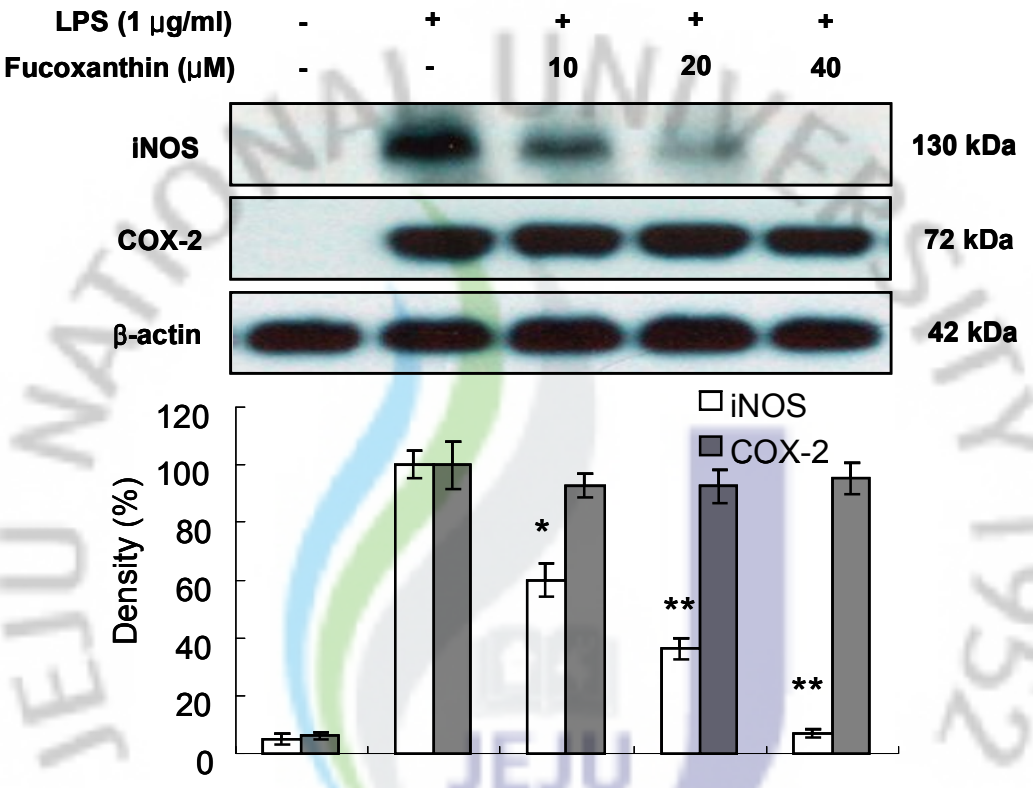

Fig. 2-3. Inhibitory effect of fucoxanthin on the protein level of iNOS and COX-2 in RAW264.7 cells. RAW 264.7 cells (1.0×10^6 cells/ml) were pre-incubated for 18 h, and the cells were stimulated with LPS (1 μg/ml) in the presence of fucoxanthin (10, 20, and 40 μM) for 24 h. iNOS and COX-2 protein level were determined using immunoblotting method. iNOS and COX-2 versus β-actin was measured by densitometry. Values are the mean ± SD of triplicate experiments. **,P<0.05* and ***,P<0.01* indicate significant differences from the LPS-stimulated group.

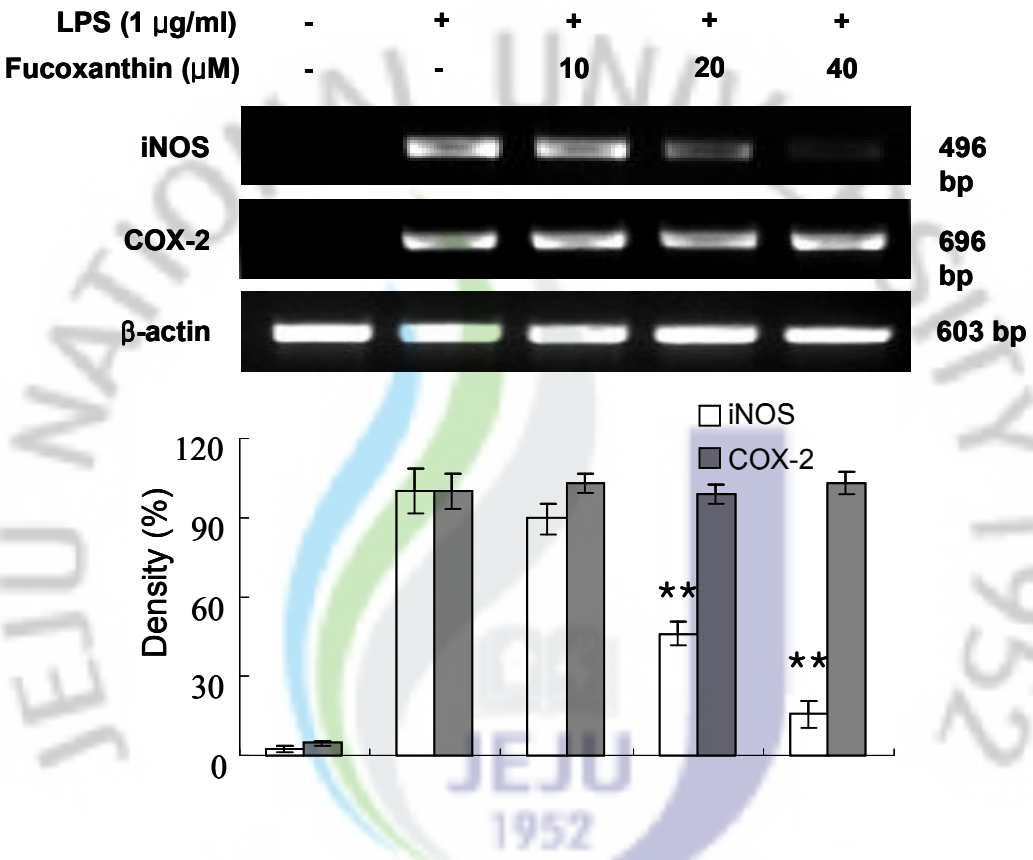

Fig. 2-4. Inhibitory effects of fucoxanthin on the iNOS and COX-2 mRNA expression in RAW 264.7 cells. RAW 264.7 cells (1.0×10^6 cells/ml) were pre-incubated for 18 h, and the iNOS and COX-2 mRNA expressions were determined from 24 h culture of cells stimulated with LPS (1 μg/ml) in the presence of fucoxanthhin (10, 20, and 40 μM). iNOS and COX-2 versus β-actin was measured by densitometry. Values are the mean ± SD of triplicate experiments. **,P<0.05* and ***,P<0.01* indicate significant differences from the LPS-stimulated group.

3.3. Effects of fucoxanthin on the production of proinflammatory cytokines and relative mRNA expressions in LPS-stimulated RAW 264.7 cells

TNF-α, IL-1β, and IL-6 play important roles in the immune response to many inflammatory stimuli (Bertolini et al., 2001; Lind, 2003; Tilg et al., 1992). Therefore, the effects of fucoxanthin on the inhibition of TNF-α, IL-1β, and IL-6 were investigated. Levels of TNF-α, IL-1β, and IL-6 in the culture supernatants were measured using ELISA kits. Treatment of RAW 264.7 cells with LPS alone resulted in significant increases in cytokine production as compared to the control group. However, TNF-α (**Fig. 2-5**), IL-1β (**Fig. 2-6**), and IL-6 (**Fig. 2-7**) levels in the supernatant from the cells treated with fucoxanthin were significantly decreased as compared to the LPS group in a dose-dependent manner.

The expressions of mRNAs relative to the production of proinflammatory cytokines were detected using RT-PCR assay. As shown in **Fig. 2-8**, the amounts of TNF-α, IL-1β, and IL-6 transcripts in the RAW 264.7 cells in the steady state were markedly increased by the LPS treatment. However, treatment with 40 μM fucoxanthin inhibited the expression of the TNF-α, IL-1β, and IL-6 transcripts by 64.3%, 78.7%, and 60.8%, respectively.

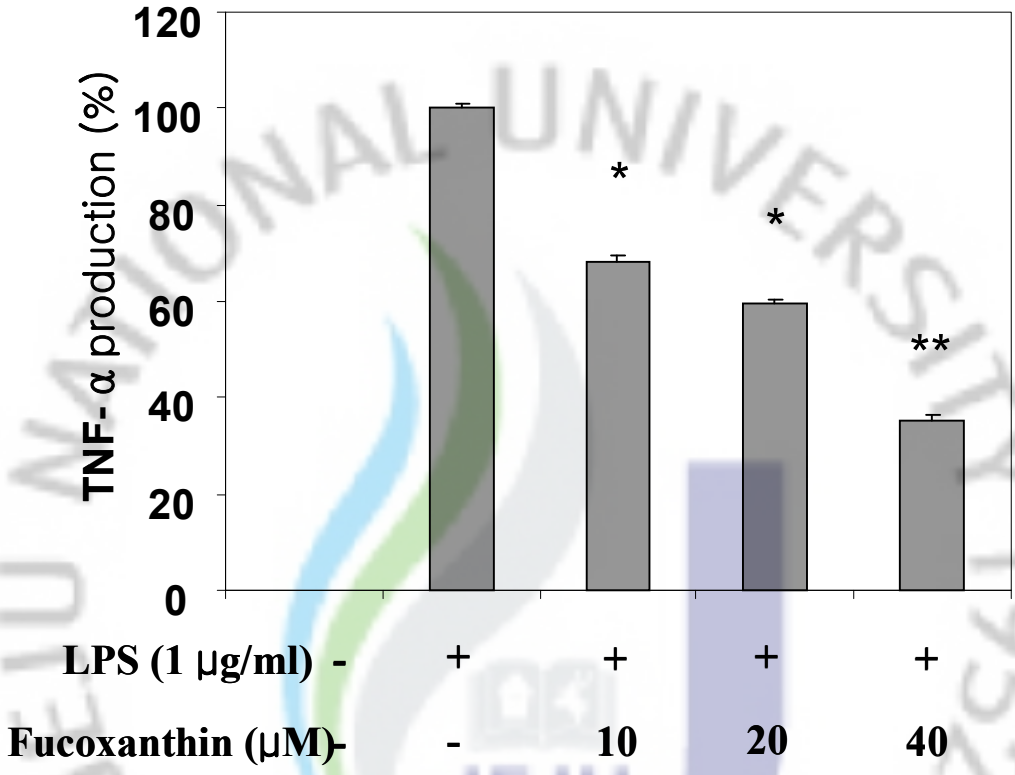

Fig. 2-5. Inhibitory effect of fucoxanthin on the TNF-α production in RAW264.7 cells. The production of TNF-α was assayed in the culture medium of cells stimulated with LPS (1 μg/ml) for 24 h in the presence of fucoxanthin (10, 20, and 40 μM). Supernatants were collected, and the TNF-α concentration in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments. **,P<0.05; **,P<0.01*.

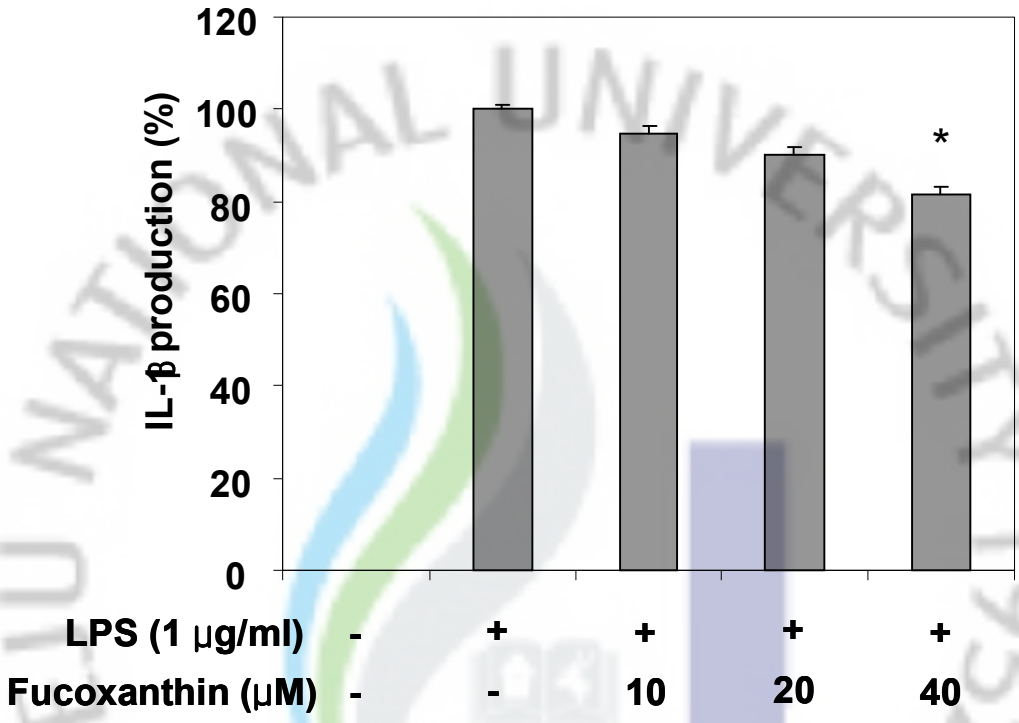

Fig. 2-6. Inhibitory effect of fucoxanthin on the IL-1β production in RAW264.7 cells. The production of IL-1β was assayed in the culture medium of cells stimulated with LPS (1 μg/ml) for 24 h in the presence of fucoxanthin (10, 20, and 40 μM). Supernatants were collected, and the IL-1β concentration in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments. *,*P<0.05;* **,*P<0.01*.

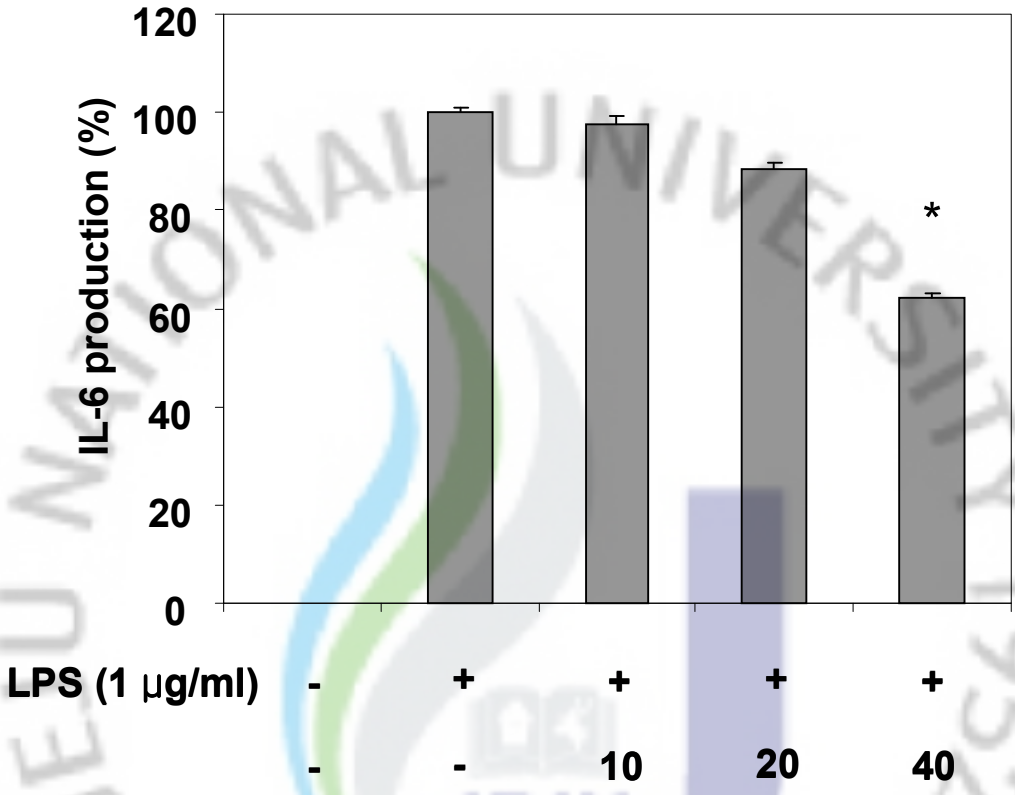

Fig. 2-7. Inhibitory effect of fucoxanthin on the IL-6 production in RAW264.7 cells. The production of IL-6 was assayed in the culture medium of cells stimulated with LPS (1 μg/ml) for 24 h in the presence of fucoxanthin (10, 20, and 40 μM). Supernatants were collected, and the IL-6 concentration in the supernatants was determined by ELISA. Values are the mean ± SD of triplicate experiments. **,P<0.05; **,P<0.01*.

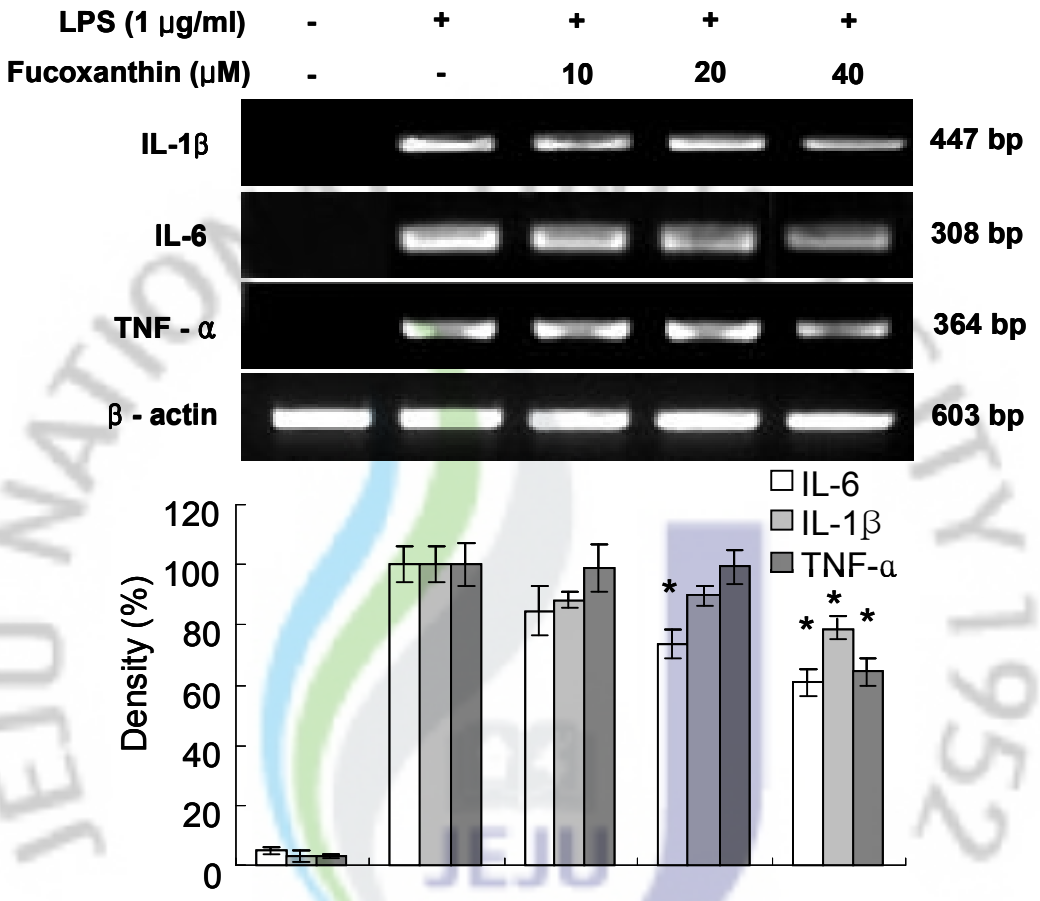

Fig. 2-8. Inhibitory effects of fucoxanthin on the IL-6, IL-1β and TNF-α mRNA expression in RAW 264.7 cells. RAW 264.7 cells (1.0×10^6 cells/ml) were pre-incubated for 18 hr, and the IL-6, IL-1β and TNF-α mRNA expressions were determined from 24 h culture of cells stimulated with LPS (1 μg/ml) in the presence of fucoxanthhin (10, 20, and 40 μM). IL-6, IL-1β and TNF-α versus β-actin was measured by densitometry. Values are the mean ± SD of triplicate experiments. **,P<0.05* and ***,P<0.01* indicate significant differences from the LPS-stimulated group.

3.4. Effects of fucoxanthin on degradation of IκB-α and phosphorylation of p50, and p60 in LPS-stimulated RAW 264.7 cells

NF-κB is inactive in the cytosol because it is bound to IκB, and becomes active after IκB has been phosphorylated and subsequently degraded (Ghosh and Karin, 2002). Thus, this author next examined whether fucoxanthin inhibits the phosphorylation and degradation of IκB. Accordingly, RAW264.7 cells were pretreated with fucoxanthin for 30 min, and IκB-α protein levels were determined after 15 min of further exposure to LPS (1 μg/ml). Fucoxanthin was found to significantly suppress the LPS-induced phosphorylation and degradation of IκB-α (**Fig. 2-9**). These results demonstrate that fucoxanthin inhibits LPS-induced NF-κB activation through prevention of IκB-α phosphorylation degradation, To confirm this, author further examined the nuclear translocation of p65 and p50, a part of p65/p50 heterodimer of NF-κB. LPS was found to induce p65 and p50 translocation from the cytosol to the nucleus after 1 h of treatment, and fucoxanthin markedly suppressed the nuclear translocation of p65 and p50 (**Fig. 2-10**).

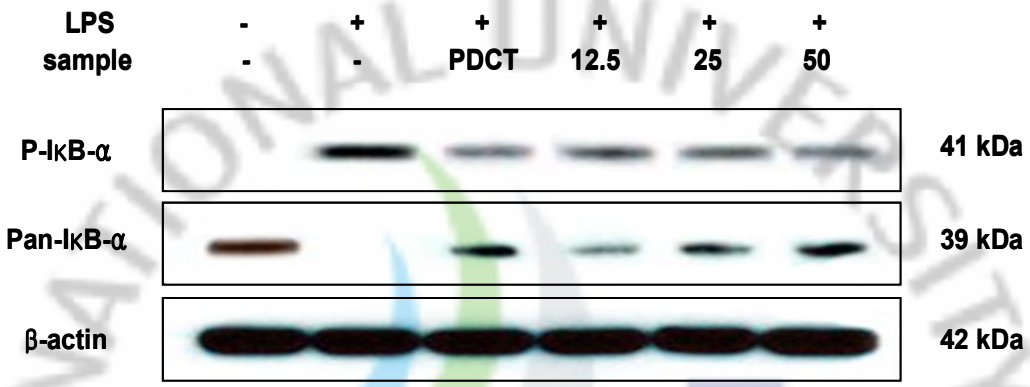

Fig. 2-9. Inhibitory effect of fucoxanthine on the protein level of IκB-α in RAW264.7 cells. RAW 264.7 cells (1.0×10^6 cells/ml) were pre-incubated for 18 h, and the cells were pre-incubated for 2 h with PDCT (20 μM) and oil extract of fucoxanthine (12.5, 25, and 50 uM) at indicated concentrations and then stimulated for 15 min with LPS (1 μg/ml). The levels of p-IκB-α (phosphorylated-IκB-α) and pan-IκB-α were determined using immunoblotting method.

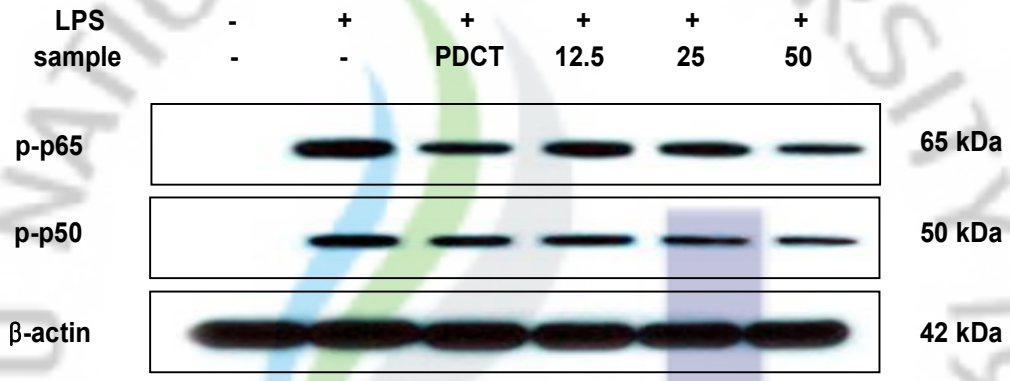

Figure 2-10. Inhibitory effect of fucoxanthine on the protein level of NF-κB in RAW264.7 cells. RAW 264.7 cells (1.0×10^6 cells/ml) were pre-incubated for 18 h, and the cells were pre-incubated for 2 h with PDCT (20 μM) and oil extract of fucoxanthine (12.5, 25, and 50 uM) at indicated concentrations and then stimulated for 15 min with LPS (1 μg/ml). The levels of P-p65 (phosphorylated-p65) and P-p50 were determined using immunoblotting method.

3.5. Effects of fucoxanthin on phosphorylation of MAPKs in the LPS-stimulated RAW 264.7 cells

To investigate whether the inhibition of inflammatory response by fucoxanthin is mediated through a MAPK pathway, next evaluated the effects of fucoxanthin on LPS-induced phosphorylation of ERK1/2, p38 and JNK MAPKs in RAW 264.7 cells by Western blot. As shown in **Fig. 2-11, 12, 13**, LPS (1 μg/ml) significantly promoted the phosphorylations of ERK1/2, JNK and p38 MAP kinase in RAW 264.7 cells. Fucoxanthin (12.5, 25, and 50 μM) dramatically decreased the phosphorylations of ERK1/2, p38, JNK MAPKs in a dose-dependent manner. However, nonphosphorylated ERK1/2, p38 and JNK kinase expressions were unaffected by LPS or LPS plus fucoxanthin.

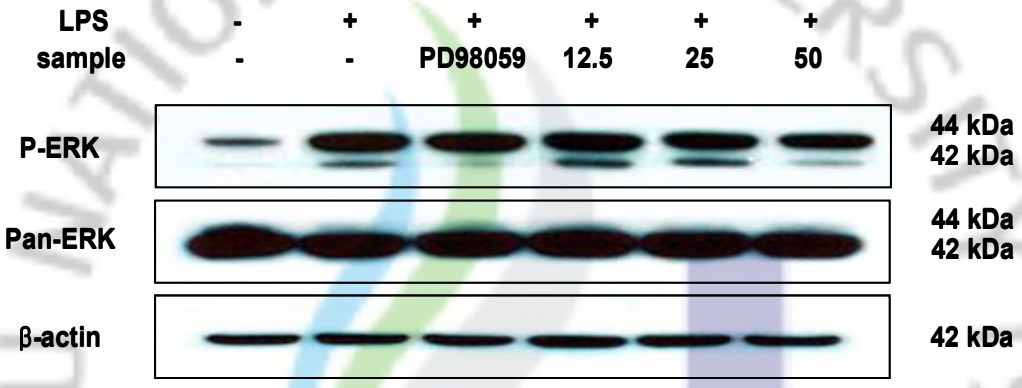

Fig. 2-11. Inhibitory effect of fucoxanthine on the protein level of ERK in RAW264.7 cells. RAW 264.7 cells (1.0×10^6 cells/ml) were pre-incubated for 18 h, and the cells were pre-incubated for 2 h with PD98059 (20 μM) and fucoxanthine (12.5, 25, and 50 uM) at indicated concentrations and then stimulated for 15 min with LPS (1 μg/ml). The levels of p-ERK (phosphorylated-ERK) and pan-ERK were determined using immunoblotting method.

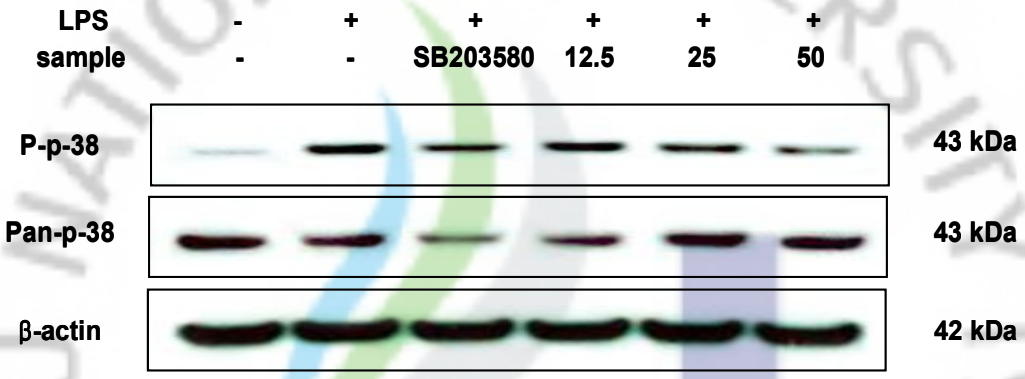

Fig. 2-12. Inhibitory effect of fucoxanthie on the protein level of p-38 in RAW264.7 cells. RAW 264.7 cells (1.0×10^6 cells/ml) were pre-incubated for 18 h, and the cells were pre-incubated for 2 h with SB203580 (20 μM) and fucoxanthine (12.5, 25, and 50 uM) at indicated concentrations and then stimulated for 15 min with LPS (1 μg/ml). The levels of p-p38 (phosphorylated-p-38) and pan-p-38 were determined using immunoblotting method.

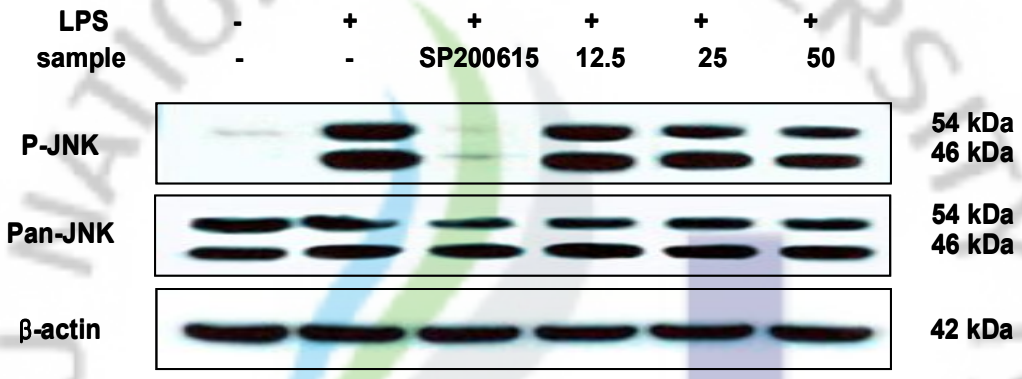

Fig. 2-13. Inhibitory effect of fucoxanthine on the protein level of JNK in RAW264.7 cells. RAW 264.7 cells (1.0×10^6 cells/ml) were pre-incubated for 18 h, and the cells were pre-incubated for 2 h with SP600125 (20 μM) and fucoxanthine (12.5, 25, and 50 uM) at indicated concentrations and then stimulated for 15 min with LPS (1 μg/ml). The levels of p-JNK (phosphorylated-JNK) and pan-JNK were determined using immunoblotting method.

4. DISCUTION

The present study demonstrates for the first time that fucoxanthin, isolated from *I. okamurae*, possesses anti-inflammatory properties that are due to the down-regulation of the MAPKs signal pathways and inhibition of NF-κB activation in LPS-stimulated RAW 264.7 cells. The excessive amounts of NO produced by activation of iNOS in response to LPS play an important role in inflammation (McCann, 2005). Furthermore, Pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are known to contribute to tissue damage and multiple organ failure. They are considered to be important initiators of the inflammatory response and mediators of the development of various inflammatory diseases (Mannel and Echtenacher, 2000; Glauser, 1996). Fucoxanthin inhibited NO production via inhibiting expression of iNOS protein and mRNA. Fucoxanthin also inhibited LPS-induced production of TNF-α, IL-1β and IL-6. Marcus et al. (2003) reported that iNOS expression is stimulated by pro-inflammatory cytokines such as IL-1β and TNF-α. In addition, IL-6 is stimulated by TNF-α and is essential for increases in NO (Schrader et al., 2007). PGE2, another pro-inflammatory mediator involved in inflammatory responses, is generated by the sequential metabolism of arachidonic acid by COX-2. However, this data show fucoxanthin not affects COX-2 protein level. Therefore, the above results suggest that the inhibition of TNF-α, IL-1β

and IL-6 production may also be related to the inhibitory effects of fucoxanthin on the LPS-induced NO production.

LPS induces its inflammatory effects through the activation of both the MAPK signaling and the classical NF-κB pathway. Thus, inhibition of the production of these signaling pathways may explain the potent activity of fucoxanthin as a suppressor of inflammatory mediators and cytokines. NF-κB is a mammalian transcription factor that controls a number of genes, such as, iNOS, COX-2, TNF-α, IL-1β, and IL-6, which are important for immunity and inflammation, and NF-κB is activated by LPS (Barnes and Karin, 1997). NF-κB usually exists as a homodimer or heterodimer, and a heterodimer of p50 and one of Rel family p65 is known to mediate the expression of genes related with innate immune response (Baeuerle and Baltimore, 1996). In unstimulated cells, NF-κB dimers are bound to inhibitory κB (IκBs), and as a result are retained in the cytoplasm. However, when the cells are stimulated with pro-inflammatory stimuli, IκBs are rapidly phosphorylated and degraded via IκB kinase (IKK) complex, and the freed NF-κB is translocated to the nucleus, where it binds to target sites and induces the transcriptions of pro-inflammatory mediators (Li and Verma, 2002; Karin, 1999). These results showed that fucoxanthin inhibited the cytoplasmic degradation of IκB-α and the nuclear translocation of p50 and p65 protein, leading to less NF-κB transactivation. This result suggests that the effects of fucoxanthin on the production of

inflammatory mediators and cytokines are at least partially mediated by the suppression of the NF-κB signaling pathway.

MAPKs are a highly conserved family of protein serine/threonine kinases and include the p38, ERK1/ 2, and JNK subgroups (Ruland and Mak, 2003). In addition, MAPKs have been implicated in the signaling pathway for LPS-induced inflammation. LPS activates all three types of MAPKs (ERK, JNK, and p38) in mouse macrophages (Hommes et al., 2003). p38 is activated by LPS stimulation and has been postulated to play and important role in the control of iNOS and TNF-α gene expression (Bhat et al, 1998). In addition, the activation of ERK is thought to be involved in LPS induced macrophage responses, such as, in the increased production of pro-inflammatory cytokines and iNOS (Bhat et al, 1998; Ajizian et al., 1999). Furthermore, JNK regulates the expression of LPS-induced iNOS (Uto et al., 2005). In the present study, this author examined the effects of fucoxanthin on the activation of these MAPKs, and found that fucoxanthin inhibited the phosphorylations of ERK1/2, JNK and p38. These results suggest that the phosphorylations of ERK1/2, JNK and p38 may contribute to the anti-inflammatory effects of fucoxanthin in LPS-stimulated RAW 264.7 macrophage cells.

In conclusion, the present study shows that fucoxanthin has anti-inflammatory activity, which depends on its ability to regulate the production of NO and other cytokines (TNF-α,

IL-1β, and IL-6) by suppressing the activation of NF-κB and the phosphorylation of MAPKs in LPS stimulated RAW 264.7 cells (**Fig. 2-14**). Fucoxanthin may be useful for the treatment of inflammatory diseases.

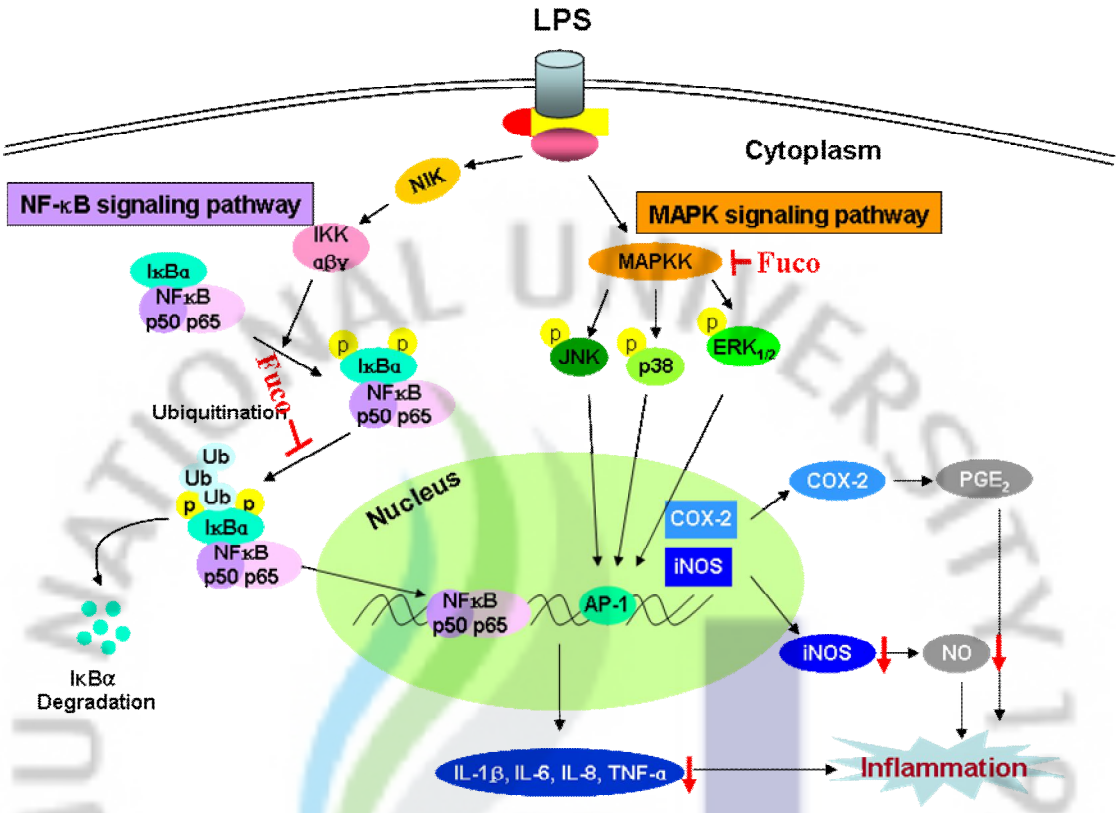

Fig. 2-14. Inhibitory mechanism of fucoxanthin on LPS-induced inflammation.

Part Ⅲ.

Fucoxanthin induces apoptosis in human leukemia HL-60 cells through a ROS-mediated MAPK pathway

Part Ⅲ.

Fucoxanthin induces apoptosis in human leukemia HL-60 cells through a ROS-mediated MAPK pathway

1. ABSTRACT

Fucoxanthin, a natural biologically active substance isolated from *Ishige okamurae*, has potent antitumor activity of human leukemia cell HL-60 cells via apoptosis induction. However, the mechanism underlying fucoxanthin-induced apoptosis in HL-60 cells remains unclear. In the present study focused on the effect of fucoxanthin induction on reactive oxygen species (ROS) accumulation, and triggering the mitogen-activated protein kinase (MAPKs) signaling pathway in HL-60 cells. This paper found that ROS are generated during fucoxanthin-induced cytotoxicity and apoptosis in HL-60 cells, and N-acetylcysteine (NAC) which is a scavenger of ROS, suppressed fucoxanthin-induced cytotoxicity and apoptosis. Moreover, fucoxanthin dramatically increased phosphorylation of c-Jun N-terminal kinase (JNK) and p38 kinase when compared to the treatment without fucoxanthin but the treatment

with NAC dramatically inhibited fucoxanthin-induced phosphorylation of JNK and p38 kinase in HL-60 cells. In addition, fucoxanthin induced the cleavage of caspase-3, -7, and poly-ADP-ribose polymerase (PARP) and decrease of Bcl-xL levels, whereas NAC pre-treatment significantly inhibited the cleavage of caspase-3, -7, and PARP and decrease of Bcl-xL levels. In this study it was firstly demonstrated that fucoxanthin generated ROS and the accumulation of ROS played an important role in fucoxanthin-induced JNK/p38 MAPK signaling pathway, which provided further theoretical support for the application of fucoxanthin as a promising anticancer agent.

2. MATERIALS AND METHODS

2.1. Materials

RPMI-1640 medium, fetal bovine serum (FBS) penicillin–streptomycin and trypsine–EDTA were purchased from Gibco/BRL (Burlington, Ont, Canada). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, Dihydroethidium (DE), 2' 7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), propidium iodide (PI), Ethidium bromide (EtBr), dimethyl sulfoxide (DMSO) and Hoechst 33342 were purchased from Sigma (St. Louis, MO, USA). DNA ladder size markers were bought from Invitrogen (Carlsbad, CA, USA). Antibodies against Bax, Bcl-xL, cleaved caspase-3, -7, PARP, Akt, extracellular signal-regulated kinase (ERK), p38 kinase, c-Jun N-terminal kinase (JNK), phosphor-Akt, phospho-ERK, phosphor-p38, phosphor-JNK, and β-actin were purchased from Cell Signaling Technology (Bedford, Massachusetts, USA). The other chemicals and reagents used were of analytical grade.

2.2. Cell culture

HL-60 (human promyelocytic leukemia cell line), HT-29 (human colon carcinoma cell line) and HepG-2 (human hepatoma cell line) cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37°C in 5 % CO_2 incubator.

2.3. Cell growth inhibitory assay

The cytotoxicity of fucoxanthin against the tumor cells were determined by a colorimetric MTT assay. Suspension cells (HL-60 cells) were seeded (5×10^4 cells/ml) together with the various concentrations (7.5, 15, and 30 μM or 15 μM) and incubated up to 72 h before MTT treatment. Attached cells (HT-29 and HepG-2 cells) were seeded in a 96-well plate at a concentration of 2×10^4 cells/ml. Sixteen hours after seeding, the cells were treated with various concentrations (8, 15, and 30 μM) of the fucoxanthin. The cells were then incubated for an additional 72 h at 37°C. MTT stock solution (50 μl; 2 mg/ml in PBS) was then added to each well for a total reaction volume of 250 μl. After incubating for 4 h, the plate was centrifuged at 2,000 rpm for 10 min and the supernatant was aspirated. The formazan crystals in each well were dissolved in DMSO. The amount of purple formazan was determined by measuring the absorbance at 540 nm.

2.4. Measurement of ROS

Accumulation of intracellular H_2O_2 and $\cdot O_2^-$ was detected with the probes $DCFH_2$-DA and HE, respectively, as described previously (Wen et al., 2002; Zhang et al., 2003). Briefly, The HL-60, HCT-15 and HepG2 cells were placed in a 6-well plate at a concentration of 4×10^5 cells/mL. The cells were treated with various concentrations (8, 15, and 30 μM or only 30 μM) of the fucoxanthin. After 2 h, the cells were labeled with 5 μM HE or 10 μM $DCFH_2$-DA for 30 min at 37℃. The labeled cells were washed with phosphate-buffer saline (PBS), and the fluorescence was analyzed using flow cytometer.

2.5. Nuclear staining with Hoechst 33342

The nuclear morphology of cells was studied by using cell-permeable DNA dye Hoechst 33342. Cells having homogeneously stained nuclei were considered to be viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis (Gschwind and Huber, 1995; Lizard et al., 1995). HL-60 cells were placed in 24-well plate at a concentration of 4×10^5 cells/ml. The cells were treated with various concentrations of the fucoxanthin and further incubated 24 h. Then, Hoechst 33342, a DNA specific fluorescent

dye was added into the culture medium at final concentration of 10 μg/ml and plate was incubated for another 10 min at 37°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera to examine the degree of nuclear condensation.

2.6. Determination of DNA fragmentation

The characteristic ladder pattern of DNA breakage was analyzed by agarose gel electrophoresis. The HL-60 cells were placed in 6-well plate at a concentration of 4×10^5 cells/ml. The cells were treated with various concentrations of the fucoxanthin and further incubated 24 h. The DNA was isolated using a Promega Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) and analyzed electrophoretically on 1.2% agarose gel containing 0.1 μg/mL ethidium bromide.

2.7. Cell cycle analysis

Cell cycle analysis was performed to determine the proportion of apoptotic sub-G_1 hypodiploid cells (Nicoletti et al., 1991). The HL-60 cells were placed in a 6-well plate at a

concentration of 4.0×10^5 cells/ml. The cells were treated with fucoxanthin or NAC. After 24 h, the cells were harvested at the indicated time and fixed in 1 ml of 70% ethanol for 30 min at 4°C. The cells were washed twice with PBS and incubated in the dark in 1 ml of PBS containing 100 μg PI and 100 μg RNase A for 30 min at 37°C. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The effect on cell cycle was determined by changes in the percentage of cell distribution at each phase of the cell cycle and assessed by histograms generated by the computer program Cell Quest and Mod-Fit.

2.8. Western blot analysis

Cells (2×10^5 cells/ml) were treated with NAC (2 mM) for 1 h before a challenge with 15 μM fucoxanthin for 24 h and harvested. The cell lysates were prepared with lysis buffer (50 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 1% Triton X-100, 0.1% SDS and 1 mmol/l EDTA). Cell lysate were washed by centrifugation, and protein concentrations were determined by using BCA™ protein assay kit. The lysate containing 30 μg of protein were subjected to electrophoresis on 10% or 12% sodium dodecyl sulfate-polyacrylamide gel, and the gel was transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The

membranes were incubated with primary antibody against Bax, Bcl-xL, cleaved caspase-3, -7, PARP, JNK, ERK, p38 kinase, and β-actin in TTBS (25 mmol/l Tris-HCl, 137 mmol/l NaCl, 0.1% Tween 20, pH 7.4) containing 0.5% nonfat dry milk at 1 h. Membranes were washed with TTBS and incubated with secondary antibodies. Signals were developed using an ECL western blotting detection kit and exposed to X-ray films.

2.9. Statistical analysis

All data are presented as mean ± SD. Significant differences among the groups were determined using the unpaired Student's *t*-test. A value of $*p<0.05$ was accepted as an indication of statistical significance.

3. RESULTS

3.1. Inhibitory effect of fucoxanthin on the growth of tumor cells

In this study, three tumor cell lines, namely HL-60, HT-29, and HepG-2 cells were used to determine the cell growth inhibition activity of fucoxanthin (**Fig. 3-1**). The fucoxanthin showed significant inhibition on the proliferation of HL-60 cells with an inhibition rate of 86.0% at 30 μM. However, growth inhibition of fucoxanthin on HepG-2 and HT-29 cells evidenced lower levels of effect than was observed with HL-60 cells. Hence, HL-60 cell line was selected for further experiments.

3.2. Induction effects of apoptosis in HL-60 cells by fucoxanthin

To determine whether fucoxanthin induced apoptosis in HL-60 cells, this author investigated DNA fragmentation, which is a biochemical hallmark for apoptosis. The results demonstrated that fucoxanthin induced endonucleolytic DNA cleavage in a dose-dependent manner (**Fig. 3-2A**). The nuclear morphology changes of HL-60 cells were investigated by using the cell-permeable DNA dye Hoechst 33342. The control, without the fucoxanthin

showed clear image and exhibited no DNA damage. However, obvious cell damage was observed in the cells treated with fucoxanthin (**Fig. 3-2B**). This suggests that the HL-60 cells may undergo apoptosis after fucoxanthin pretreatment, and there is a good correlation between extent of apoptosis and inhibition of the cell growth.

3.3. Effects of fucoxanthin on phosphoryation of MAPKs and Akt

In order to characterize the fucoxanthin-induced changes in the kinase signaling pathways, this author analyzed phosphorylation status of Akt and MAPKs during the fucoxanthin-induced apoptosis of HL-60 cells. As shown in **Fig. 3-3,4,5,6**, fucoxanthin had little effect on the phosphorylation status of Akt and ERK but dramatically increased phosphorylation of JNK and p38 kinase when compared to the treatment without fucoxanthin. This result implies that JNK and p38 MAPK activated by fucoxanthin could play a critical role in inducing apoptotic cell death in HL-60 cells.

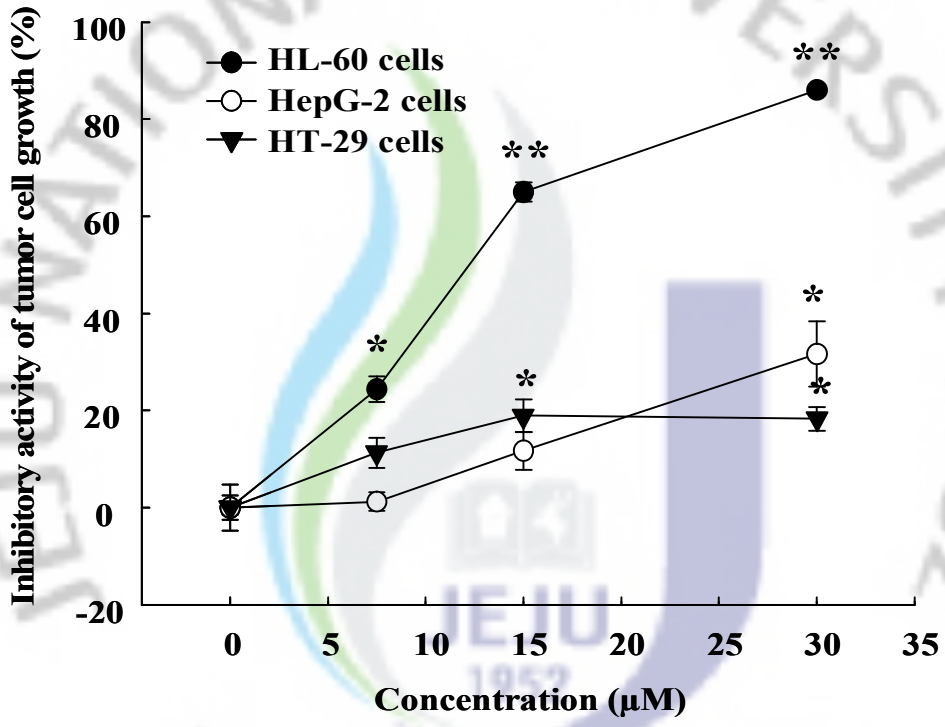

Fig. 3-1. Inhibitory effect of fucoxanthin against growth of the tumor cells (HL-60, HepG-2, and HT-29 cells). Means with different letters within a column are significantly different (*,p<0.05, **, p<0.01).

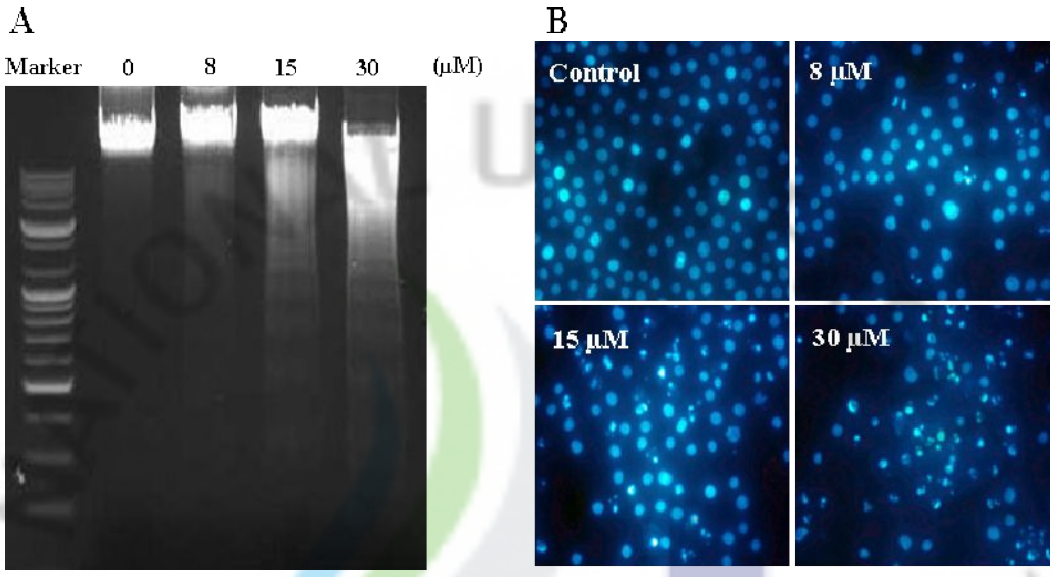

Fig. 3-2. Induction of apoptosis by fucoxanthin treatment of HL-60 cells. HL-60 cells were seeded at 4×10^5 and treated with the difference concentrations of fucoxanthin for 24 h. (A) Fragmented DNA was extracted and analyzed on 1.5% agarose gel containing EtBr. (B) Apoptotic bodies were stained with Hoechst 33342 solution and then observed under a fluorescent microscop using a blue filter.

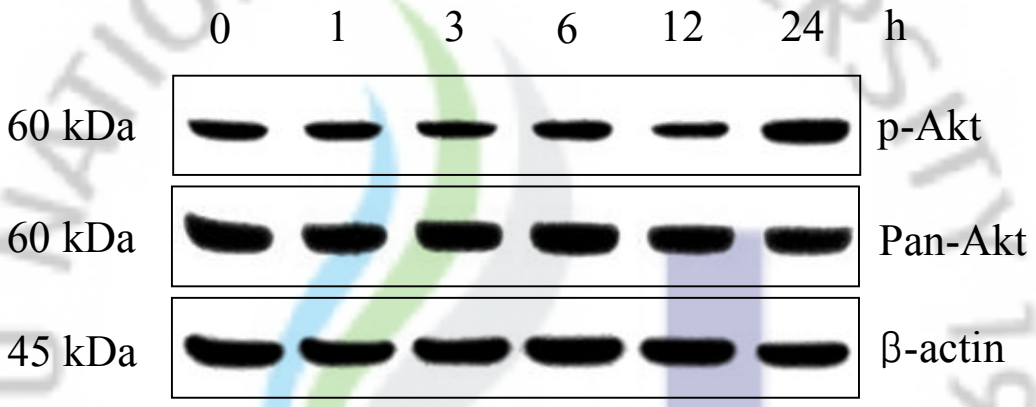

Fig. 3-3. Effect of fucoxanthin on phosphorylation of Akt in HL-60 cells. The cells were stimulated with 15 μM of fucoxanthin for 24 h. The levels p-Akt (phosphorylated-Akt) and pan-Akt were determined using immunoblotting method.

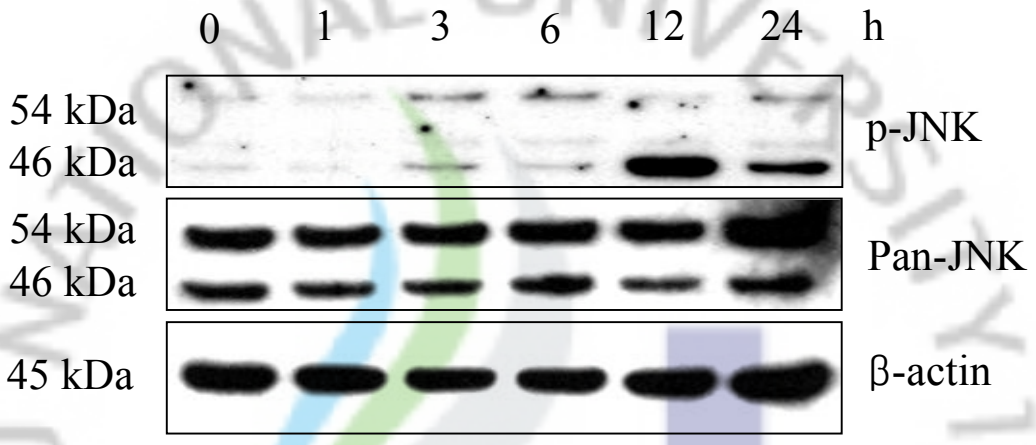

Fig. 3-4. Effect of fucoxanthin on phosphorylation of JNK in HL-60 cells. The cells were stimulated with 15 μM of fucoxanthin for 24 h. The levels p-JNK (phosphorylated-JNK) and pan-JNK were determined using immunoblotting method.

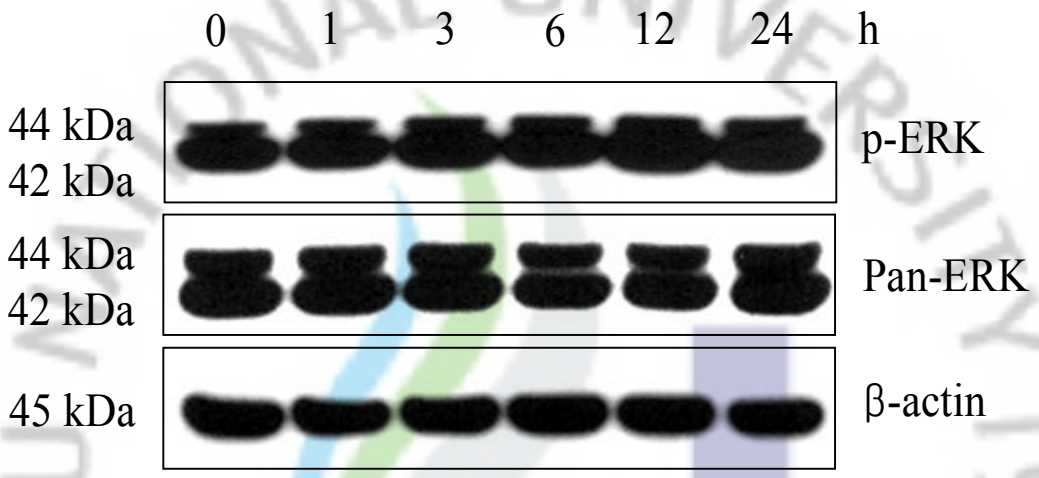

Fig. 3-5. Effect of fucoxanthin on phosphorylation of ERK in HL-60 cells. The cells were stimulated with 15 μM of fucoxanthin for 24 h. The levels p-ERK (phosphorylated-ERK) and pan-ERK were determined using immunoblotting method.

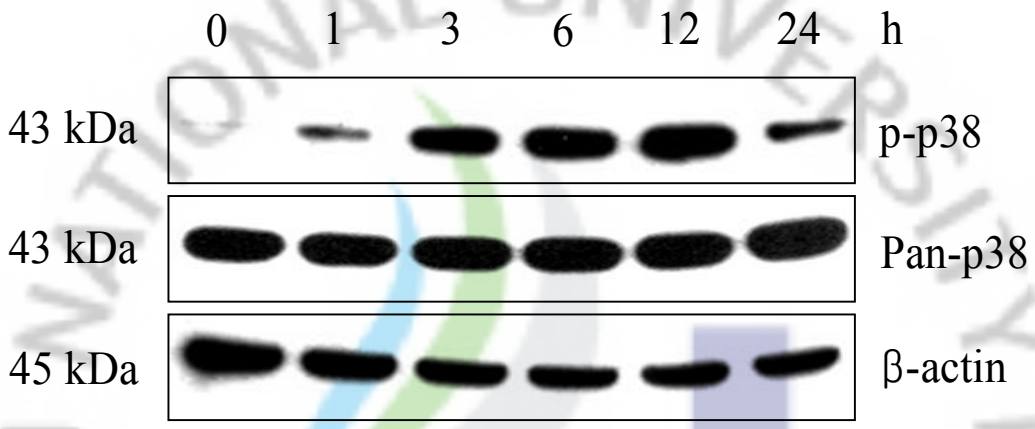

Fig. 3-6. Effect of fucoxanthin on phosphorylation of p38 in HL-60 cells. The cells were stimulated with 15 μM of fucoxanthin for 24 h. The levels p-p38 (phosphorylated-p38) and pan-p38 were determined using immunoblotting method.

3.4. ROS generation triggers fucoxanthin-induced apoptosis

It has been reported that ROS generation plays an important role in the proapoptotic activities of various anticancer agents (Zhang and Chen, 2004; Kim et al., 2005). Therefore, this author tested the possibility that fucoxanthin induce apoptosis allowing for ROS accumulation in HL-60 cells. DCFH-DA (for H_2O_2) and DE (for $\cdot O_2^-$) were used to measure ROS production in the cells. As shown in **Fig. 3-7A**, Fucoxanthin treatment increases H_2O_2 and $\cdot O_2^-$ production as this author observed an increase in fluorescence intensity (FI) from 132 and 99 in vehicle treated HL-60 cells to 227 and 138 in fucoxanthin (30 μM) treated HL-60 cells, respectively. However, no significant increase in intracellular ROS was observed in HCT-15 and HepG2 cells with fucoxanthin (30 μM). This result indicated that ROS generation is an impotent role in growth inhibition by fucoxanthin in HL-60 cells. Hence, HL-60 cells were selected for further experiments. Fucoxanthin treatment induced a dose-dependent increase in mean HE and DCF fluorescence, when compared with the control cells (**Fig. 3-7B**). To further illustrate the relationship between fucoxanthin-induced ROS generation and apoptosis, HL-60 cells were treated with fucoxanthin in the presence or absence of antioxidant, NAC. As shown in Fig. 6A, the treatment with 15 μM of fucoxanthin for 24 h resulted in a significant accumulation of cells with sub-G_1 DNA content (70.4%),

but NAC-pretreated cells significantly inhibited the sub-G_1 phase (18.4%). In addition, pretreatment of cells with NAC reduced the fucoxanthin-induced apoptotic bodies (**Fig. 3-8B**) and DNA fragmentation (**Fig. 3-8C**). NAC also prevented the fucoxanthin-reduced cell viability in HL-60 cells (**Fig. 3-8D**). Taken together, these results suggest that ROS generation is an important target in apoptosis by fucoxanthin in HL-60 cells.

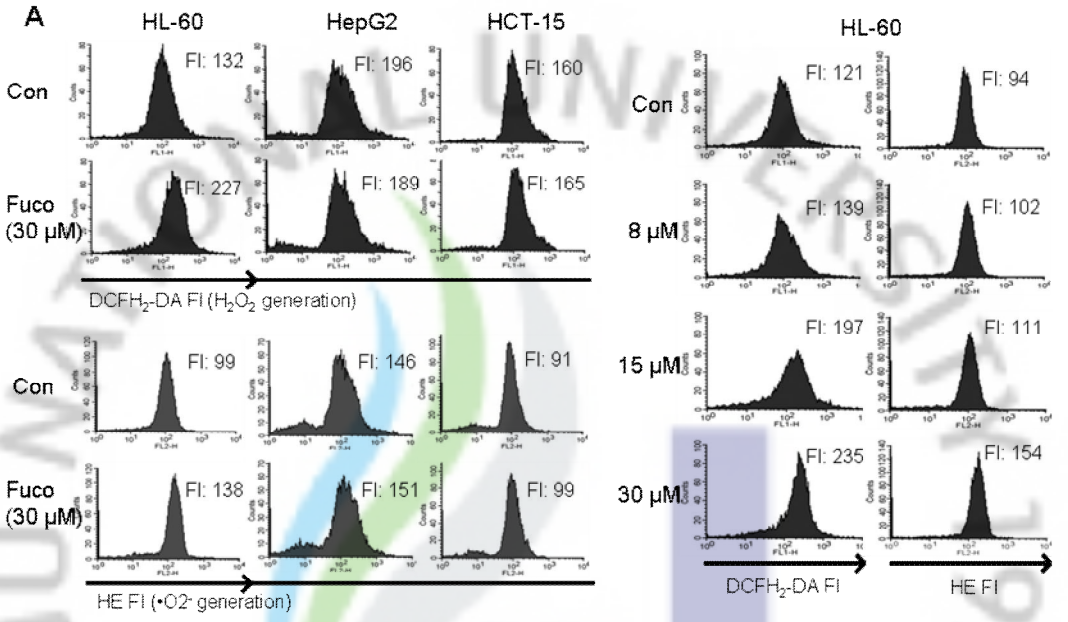

Fig. 3-7. The generation of ROS by fucoxanthin in tumor cells. (A) HL-60, HCT-15, and HepG2 cells were seeded at 4×10^5 and treated with 30 μM of fucoxanthin (B) HL-60 cells were seeded at 4×10^5 and treated with the difference concentrations (8, 15, and 30 μM) of fucoxanthin. After 2 h, the cells were labeled with 5 μM HE or 10 μM DCFH2-DA for 30 min at 37℃ and subsequent FACs analysis for intracellular accumulation of ROS. FI: fluorescence intensity.

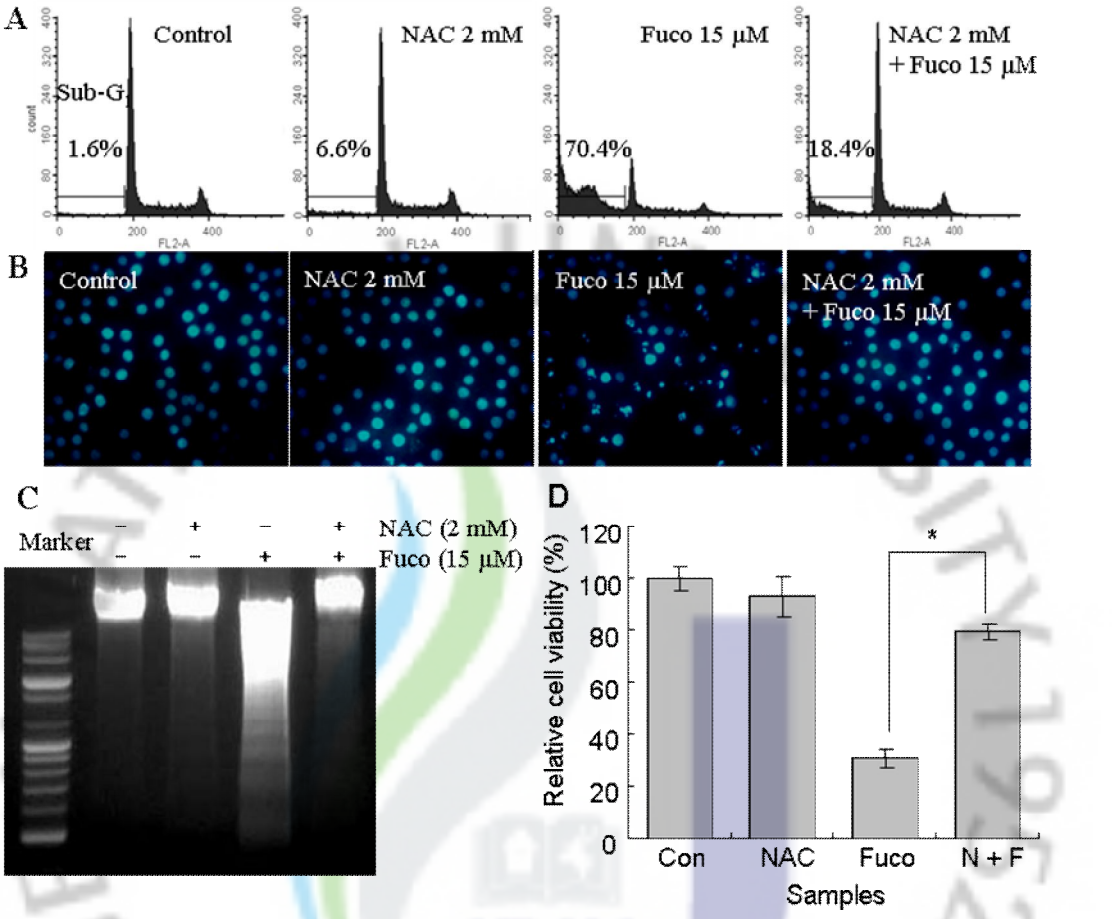

Fig. 3-8. NAC pre-treatment protected against the Fucoxanthin-induced apoptosis through suppression of ROS generation. HL-60 cells were pretreated with 2 mM NAC for 1 h prior to fucoxanthin exposure for 24 h. (A) The cells were stained with PI and analyzed by flow cytometry. (B) Apoptotic bodies were stained with Hoechst 33342 solution and then observed under a fluorescent microscop using a blue filter. (C) Fragmented DNA was extracted and analyzed on 1.5% agarose gel containing EtBr. (D) HL-60 cells were pretreated with 2 mM NAC for 1 h prior to fucoxanthin exposure for 48 h. Cells viability was measured using MTT assay.

3.5. Effects of NAC on the activation of MAPKs by fucoxanthin

Results in **Fig. 3-4, 5, 6, 7, and 8** clearly showed that both MAPKs and ROS targets are essential for fucoxanthin-induced apoptosis, although the causal relationship between MAPKs and ROS target is unclear. Therefore, this author evaluated the relationship between fucoxanthin-induced ROS generation and activation of JNK and p38 MAPK. HL-60 cells were pretreated with or without NAC for 1 h, followed by exposure to fucoxanthin for 12 h. This paper found that treatment with NAC dramatically inhibited fucoxanthin-induced phosphorylation of JNK, and p38 MAPK in the cells (**Fig. 3-9**). Collectively, the findings supported the notion that fucoxanthin induced ROS generation activates JNK/p38 MAPK pathway, triggering apoptosis of the HL-60 cells.

3.6. Effect of NAC on down-regulation of Bcl-xL, caspase-3, -7, and PARP cleavage activation by fucoxanthin

The expression level of Bcl-xL, antiapoptotic protein was decreased with fucoxanthin (15 μM), however, the blocking of the ROS generation by pretreatment of the cells with NAC effectively prevented the fucoxanthin-induced down-regulation of Bcl-xL expression.

Furthermore, Fucoxanthin induced the cleavage of caspase-3, -7, and PARP, whereas NAC pre-treatment significantly inhibited the cleavage of caspase-3, -7, and PARP (**Fig. 3-10**). This finding indicates that fucoxanthin induced ROS can also lead to Bcl-xL regulation of apoptosis in HL-60 cells.

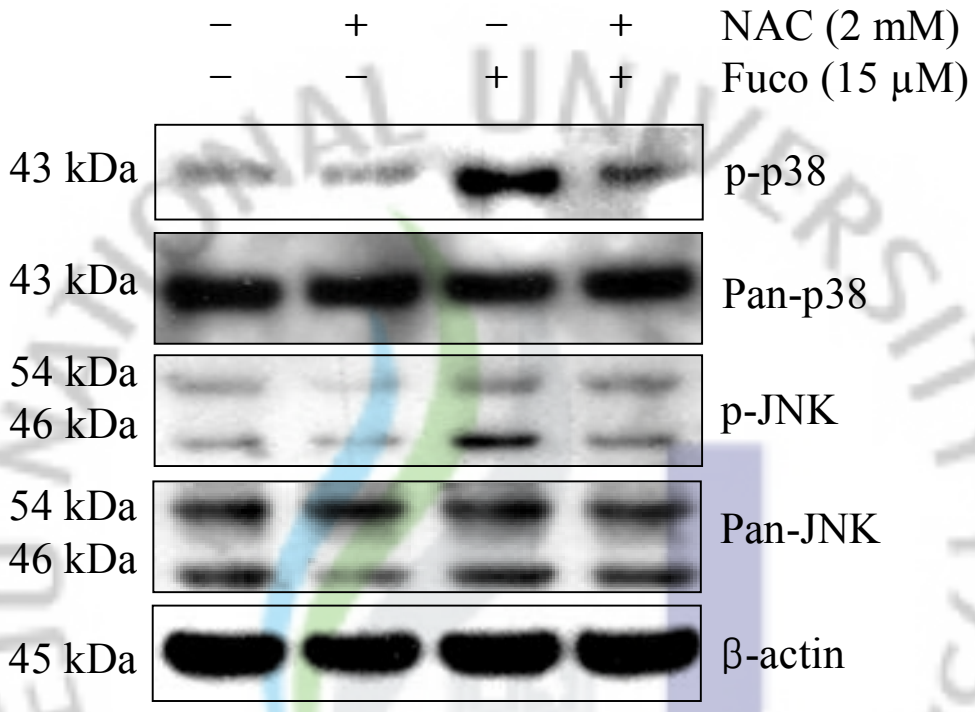

Fig. 3-9. Effect of NAC on phosphorylation of MAPKs. The cells were pre-treated with 2mM NAC, and then treated with 15 μM fucoxanthin for 12 h. Equal amounts of cell lysates (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-p-p38, anti-p38, anti-p-JNK, and anti-JNK).

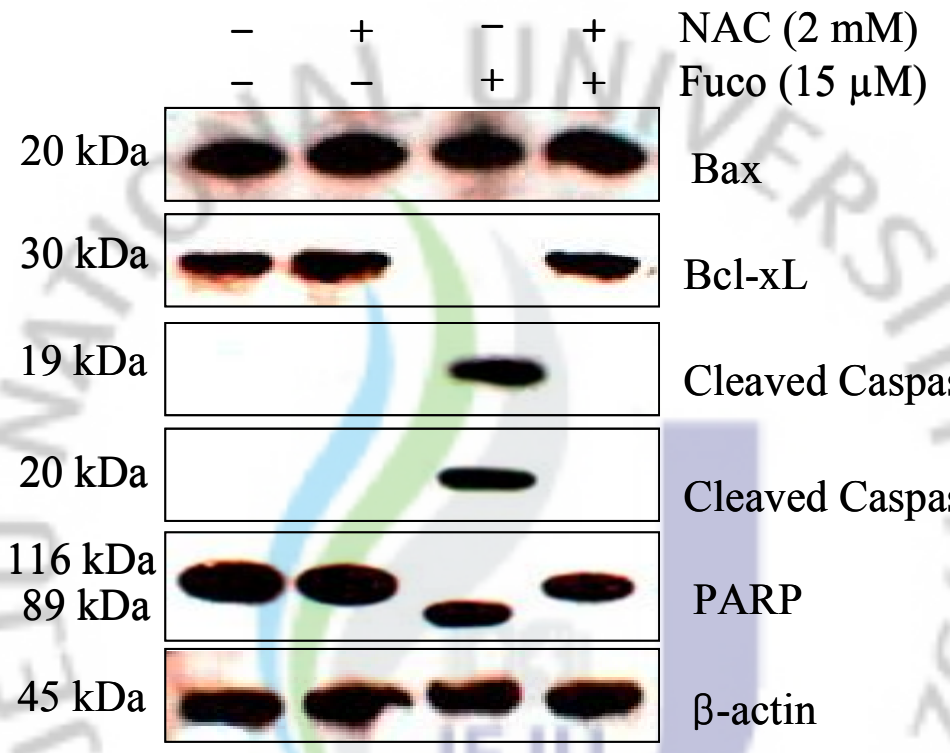

Fig. 3-10. Effect of NAC on down-regulation of Bcl-xL, caspase-3, -7, and PARP cleavage activation by fucoxanthin. The cells were pre-treated with 2mM NAC, and then treated with 15 μM fucoxanthin for 12 h. Equal amounts of cell lysates (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-Bax, anti-Bcl-xL, anti-casapse-3, -7, and anti-PARP).

4. DISCUSSION

ROS are by-products of normal cellular oxidative processes, and they are generated in the mitochondria and from other sources. They inflict serious damage to lipids, protein, and DNA, and they have been suggested as regulating the process involved in the initiation of apoptotic signaling. Recent reports have been documented that ROS generation plays an important role in the proapoptotic activities of various anticancer agents. In this paper first reported that fucoxanthin induced apoptosis through ROS-mediated MAPKs regulation.

This study showed that fucoxanthin has cytotoxic effects on HL-60 cells, and then demonstrated that fucoxanthin could induce apoptosis of HL-60 cells in a dose-dependent manner. Previous studies have reported that fucoxanthin-induced cytotoxic and apoptosis in human colon cancer cells, human leukemia cell, and prostate cancer cells (Hosokawa et al., 1999, 2004; Kotake-Nara et al., 2004, 2005). Some reports mentioned that fucoxanthin-induce apoptosis through down-regulation of Bcl-2 or activation of caspase-3 in HL-60 cells (Kotake-Nara et al., 2005; Nakazawa et al., 2009). However, the mechanism underlying fucoxanthin-induced apoptosis in HL-60 cells remains unclear. Fucoxanthin can induce apoptosis in HL-60 cells through other pathways.

MAPK signaling pathways play important roles in cell growth and death. MAPK

cascades consist of a core of three protein kinases such as ERK1/2, p38 and JNK pathways (Harper and LoGrasso, 2001; Xia et al., 1995; Wang et al., 2008; Lee et al., 2009; Jung et al., 2008). ERK is activated primarily by mitogenic stimuli, such as growth factors, leading to cell growth and survival (Chang and Karin, 2001). On the other hand, JNK and p38 are activated by UV irradiation, DNA damage, hydrogen peroxide, and heat and osmotic shock, resulting apoptotic cell death (Chen et al., 1996; Kawasaki et al; 1997; Zanke et al., 1996). Many studies have demonstrated that activation of JNK and p38 MAPK pathway plays a promoting role in the apoptosis induced by chemotherapeutic drugs (Valbonesi et al., 2008; Wollenhaupt et al., 2005; Lee et al., 2009; Jung et al., 2008). Thus, to understand the molecular mechanism of fucoxanthin, the potential involvement of MAPK pathway was investigated during fucoxanthin-induced apoptosis by Western blotting. This paper observed increased levels of phosphorylated JNK and p38 MAPK in HL-60 cells after 12 h of fucoxanthin treatment. This results indicated that the fucoxanthin induce apoptosis through activation of JNK and p38 MAPK.

One major biochemical change in cancer cells after treatments with anti-cancer agents is the increase in ROS generation which is often considered as a cancer-promoting factor (Szatrowski and Nathan, 1991; Schumacker, 2006; Trachootham et al., 2006). Studies have been demonstrated that high levels of ROS can cause cellular damage (Pelicano et al., 2004;

Valko et al., 2006; Li et al., 2007; Zhao et al., 2006; Hseu et al., 2008) and play an important role in mediating apoptosis (Garcia-Ruiz et al., 1997; Coyle and Puttfarcken, 1993). Interestingly, ROS has been demonstrated to selectively kill cancer cells (Zhang and Chen, 2004; Schumacker, 2006; Kuo et al., 2007). For instance, Hileman et al. (2004) showed that ROS generated by 2-methoxyestradiol (2-ME) preferentially kill human leukemia cells without exhibiting significant cytotoxicity to normal lymphocytes (Hileman et al., 2004). In the present study found that ROS are generated during fucoxanthin-induced apoptosis in HL-60 cells, and that oxidant scavenger, such as NAC, suppressed fucoxanthin-induced cytotoxicity and apoptosis. Kotake-Nara et al. (2005) have showed that apoptosis induced by fucoxanthin was not associated with the generation of ROS, but this results showed that apoptosis induced by fucoxanthin is associated with ROS generation. This author believes that the reason for this difference might be the ROS determination assays. In this study focused onto both intracellular H_2O_2 and superoxide generation using FACS as well as for further clarification of the relationship between ROS generation and apoptotic body formation, when HL-60 cells were treated fucoxanthin with or without an commercial antioxidant, NAC. Additionally, this author also found that the treatment with NAC dramatically inhibited fucoxanthin-induced phosphorylation of JNK, and p38 kinase in the cells. This fact is clearly to prove that fucoxanthin generate ROS. Oxidant injury is known

to activate all MAPK cascades in response to ROS generation; and ROS can interfere with various signaling pathways (Lee and Esselman, 2002; Levinthal and Defranco, 2005). The generation of ROS by some chemicals plays an important role in MAPK activation and apoptosis (Fernandes et al., 2008). This strongly supports a key role of fucoxanthin-induced JNK and p38 MAPK activation in ROS generation. Next, this author tried to determine whether Bcl-xL regulation depends on the production of ROS in fucoxanthin-treated cells. The Bcl-2 family, comprising anti-apoptotic (Bcl-2 and Bcl-xL) and pro-apoptotic (Bax and Bad) members, are regulators that function as molecular rheostats to control cell survival (Yang and Korsmeyer, 1996; White, 1996). Quenching of ROS by NAC prevented the down-regulation of Bcl-xL, activation of caspase-7 and caspase-3-mediated PARP cleavage, indicating that ROS are required for fucoxanthin-induced Bcl-xL-mediated apoptosis in HL-60 cells. Generally, various chemotherapeutic drugs induce apoptotic cell death through caspase-mediated pathways in cancer cells (Aprigliano et al., 2008; Kim and Chung, 2008; Umit Bagriacik et al., 2007; Aquino Esperanza et al., 2008; Koike et al., 2006).

In conclusion, this data indicate that HL-60 cells are highly sensitive to growth inhibition and apoptosis induction by fucoxanthin. Fucoxanthin-induced apoptosis is associated with JNK/p38 MAPK signaling pathways, which is mediated by ROS generation (Fig. 9). Hence, the present results suggest that fucoxanthin isolated from *I. okamurae* could be a potential

candidate for the development of anti-cancer drug for the treatment of leukemia.

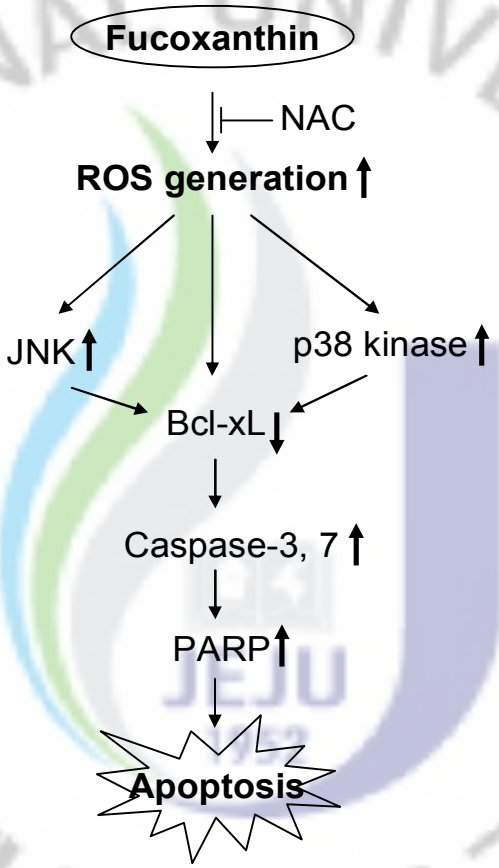

Fig. 3-11. Signaling pathway responsible for fucoxanthin-induced apoptotic death of HL-60 cells.

Part Ⅳ.

Fucoxanthin induces G_0/G_1 phase arrest and apoptosis in melanoma B16F10 cells through Akt and Bcl-xL signaling pathway

Part Ⅳ.

Fucoxanthin induces G_0/G_1 phase arrest and apoptosis in melanoma B16F10 cells through Akt and Bcl-xL signaling pathway

1 ABSTRACT

The present study was designed to evaluate the molecular mechanisms of fucoxanthin against melanoma cell lines (B16F10 cells). Fucoxanthin reduced the viability of B16F10 cells in a dose-dependent manner accompanied by the induction of cell cycle arrest during the G_0/G_1 phase and apoptosis. Fucoxanthin-induced G_0/G_1 arrest was associated with a marked decrease in the protein expressions of phosphorylated-Rb (retinoblastoma protein), cyclin D(1 and 2) and cyclin-dependent kinase (Cdk)4 and up-regulation of the protein levels of $p15^{INK4B}$ and $p27^{Kip1}$. The induction of cell arrest was also associated with the inactivation of Akt, and Akt inhibitor Triciribine significantly increases fucoxanthin-induced cell death. Fucoxanthin-induced apoptosis was accompanied with down-regulation of the protein levels of Bcl-xL, and inhibitor of apoptosis protein (IAPs), resulting in sequential activation of caspase-9, caspase-3, and PARP. Furthermore, the anti-tumor effect of fucoxanthin was

assessed *in vivo* in Balb/c mice. Intraperitoneal administration of fucoxanthin significantly inhibited the growth of tumor mass in B16F10 cells implanted mice. These results suggest that fucoxanthin has anti-tumor properties on melanoma cells by inducing selectively the genes related to cell cycle arrest and apoptosis.

2. MATERIAL AND METHODS

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) penicillin–streptomycin and trypsine–EDTA were purchased from Gibco/BRL (Burlington, Ont, Canada). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNase A, propidium iodide (PI), dimethyl sulfoxide (DMSO) and Hoechst 33342 were purchased from Sigma (St. Louis, MO, USA). Antibodies against Bax, Bcl-xL, cleaved caspase-3, -7, PARP, Akt, phosphor-Akt, and β-actin were purchased from Cell Signaling Technology (Bedford, Massachusetts, USA). The other chemicals and reagents used were of analytical grade.

2.2. Cell culture

B16F10 (murine melanoma cell line) cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37°C in 5 % CO_2 incubator.

2.3. Cell growth inhibitory assay

The cytotoxicity of fucoxanthin against the B16F10 cells were determined by a colorimetric MTT assay. B16F10 cells were seeded in a 96-well plate at a concentration of 2×10^4 cells/ml. Sixteen hours after seeding, the cells were treated with various concentrations (12, 25, 50, and 100 μM) of the fucoxanthin. The cells were then incubated for an additional 72 h at 37°C. MTT stock solution (50 μl; 2 mg/ml in PBS) was then added to each well for a total reaction volume of 250 μl. After incubating for 4 h, the plate was centrifuged at 2,000 rpm for 10 min and the supernatant was aspirated. The formazan crystals in each well were dissolved in DMSO. The amount of purple formazan was determined by measuring the absorbance at 540 nm.

2.4. Nuclear double staining with Hoechst 33342 and PI

The nuclear morphology of cells was studied by using cell-permeable DNA dye Hoechst 33342 and PI. Cells having homogeneously stained nuclei were considered to be viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis (Gschwind and Huber, 1995; Lizard et al., 1995). B16F10 cells were placed in 24-well plate at a concentration of 1×10^5 cells/ml. Sixteen hours after seeding, the cells were

treated with various concentrations (50, 100, and 200 μM or 200 μM) of the fucoxanthin and further incubated for difference times (0, 8, 24, and 48 h). Then, Hoechst 33342 and PI, a DNA specific fluorescent dye was added into the culture medium at final concentration of 10 and 5 μg/ml, respectively, and plate was incubated for another 10 min at 37°C. The stained cells were then observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera to examine the degree of nuclear condensation.

2.5. Cell cycle analysis

Cell cycle analysis was performed to determine the proportion of apoptotic sub-G_1 hypodiploid cells (Nicoletti et al., 1991). B16F10 cells were placed in a 6-well plate at a concentration of 1.0×10^5 cells/ml. Sixteen hours after seeding, the cells were treated with with various concentrations (50, 100, and 200 μM) of the fucoxanthin. After 24 or 48 h, the cells were harvested at the indicated time and fixed in 1 ml of 70% ethanol for 30 min at 4°C. The cells were washed twice with PBS and incubated in the dark in 1 ml of PBS containing 100 μg PI and 100 μg RNase A for 30 min at 37°C. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The effect on cell cycle was determined by changes in the percentage of cell distribution at each phase of

the cell cycle and assessed by histograms generated by the computer program Cell Quest and Mod-Fit (Wang et al., 1999).

2.6. Western blot analysis

Cells (2×10^5 cells/ml) were treated with fucoxanthin and harvested. The cell lysates were prepared with lysis buffer (50 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 1% Triton X-100, 0.1% SDS and 1 mmol/l EDTA). Cell lysate were washed by centrifugation, and protein concentrations were determined by using BCA™ protein assay kit. The lysate containing 40 μg of protein were subjected to electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel, and the gel was transferred onto a nitrocellulose membrane Murine macrophage cell line B16F10 Cells were pre-incubated for 18 h, and then stimulated with LPS (1 μg/mL) in the presence of fucoxanthin for the indicated time. After incubation, the cells were collected and washed twice with cold-PBS. The cells were lysed in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM NaVO3, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 25 μg/ml leupeptin] and kept on ice for 30 min. Cell lysate were washed by centrifugation, and protein concentrations were determined by using BCA™ protein assay

kit.. Aliquots of the lysates (30-50 μg of protein) were separated on a 12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA) with a glycine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)]. After blocking the nonspecific site with 1% bovine serum albumin (BSA), the membrane was then incubated with specific primary antibody at 4℃ for overnight. The membrane was further incubated for 60 min with a peroxidase-conjugated secondary antibody (1:5000, Vector Laboratories, Burlingame, USA) at room temperature. The immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit.

2.7. Fucoxanthin inhibits tumor growth in mice

Balb/c mice weighting 20-22 g (6 weeks old) were purchased from Orientbio, Inc. (Sungnam, Korea) and housed in conventional animal facilities with a NIH-07-approved diet and water ad libitum at a constant temperature (23 ± 1°C) according to the guidelines for the Care and Use of Laboratory Animals of the Institutional Ethical Committee of Jeju National University.

Mice were randomly separated into three groups: a sham B16F10 melanoma cells-

implanted group, B16F10 melanoma cells-implanted control group, a fucoxanthin plus B16F10 melanoma cells-implanted group.

For a fucoxanthin plus B16F10 melanoma cells-implanted group, this paper first injected intraperitoneal (i. p.) into test mice with fucoxanthin (300 μg) dissolved in saline (pH 7.4) (300 μg/100 μl/mouse), that is at 24 h and 2 h before the implantation of B16F10 melanoma cells. Then, for experimental tumor induction of B16F10 melanoma tumor, this author assessed by intradermal (i.d.) inoculation of tumor cells (5×10^5 cells/100 μl/mouse) into mice of both B16F10 melanoma cells-implanted control group and a fucoxanthin plus B16F10 melanoma cells-implanted group (5 mice per a group). After the induction of B16F10 melanoma tumor, this author also injected fucoxanthin (300 μg/100 μl/mouse) into mice once every 5 days by intraperitoneal injection for 20 days. Additionally, as controls, mice of sham B16F10 melanoma cells-implanted and B16F10 melanoma cells-implanted groups were injected i.p. with saline (100 μl).

2.8. Statistical analysis

All data are presented as mean ± SD. Significant differences among the groups were determined using the unpaired Student's *t*-test. A value of $*p<0.05$ was accepted as an

indication of statistical significance.

3. RESULTS

3.1. Inhibitory effect of fucoxanthin on the growth of B16F10 cells

This author investigated the effects of fucoxanthin on the growth of B16F10 cells. Cells were exposed to increasing doses of fucoxanthin for 72 h and cell viability was determined by MTT assay. As shown in **Fig. 4-1**, cell growth inhibition activity was markedly increased 72 h after exposure to fucoxnathin in a dose-dependent manner. The proliferation of B16F10 cells was reduced by 82% upon a 72 h exposure to 100 μM fucoxanthin. Thus, fucoxanthin seems to be capable of exerting inhibition of cell proliferation.

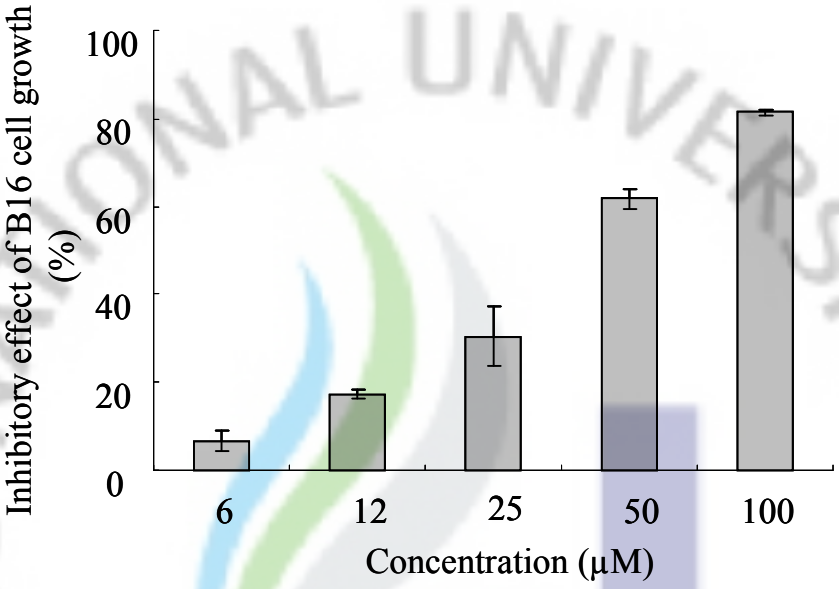

Fig. 4-1. Inhibitory effect of fucoxanthin against growth of B16F10. Each point represents the mean ± SD of three independent experiments. Means with different letters within a column are significantly different (*,p<0.05, **, *p*<0.01).

3.2. Fucoxanthin induces cycle arrest at G_0/G_1 phase in B16F10 cells

The induction of apoptosis and cell cycle arrest has been considered as the major cause of anti-proliferation (Hsiao et al., 2007). Flow cytometry was used for further investigation on the underlying mechanisms accounting for the antiproliferative action of fucoxanthin. In vitro DNA detection assay-flow cytometry was carried out to determine whether fucoxanthin-induced cell growth inhibition was the result of induction of apoptosis or cell cycle arrest or a combination of these two modes. **Fig 4-2A, 3A** shows the representative DNA distribution histograms of B16F10 cells incubated in the absence or presence of fucoxanthin (50, 100, and 200 μM) for 24h or 48 h. **Fig 4-2, 3B** summarizes the relative percentages of cells at each phase of the cell cycle following treatment with different fucoxanthin concentrations (50, 100, and 200 μM). The results reveal that fucoxanthin treatments for 24 h caused an increase in the percentage of cells at G_0/G_1 phase, which was accompanied by a corresponding reduction in the percentages of cells at S and G_2/M phase (**Fig. 4-2**). In addition, the presence of a distinct sub-G_1 peak was observed in cells treated with fucoxanthin for 48 h, suggesting the induction of apoptotic cell death (**Fig. 4-3**).

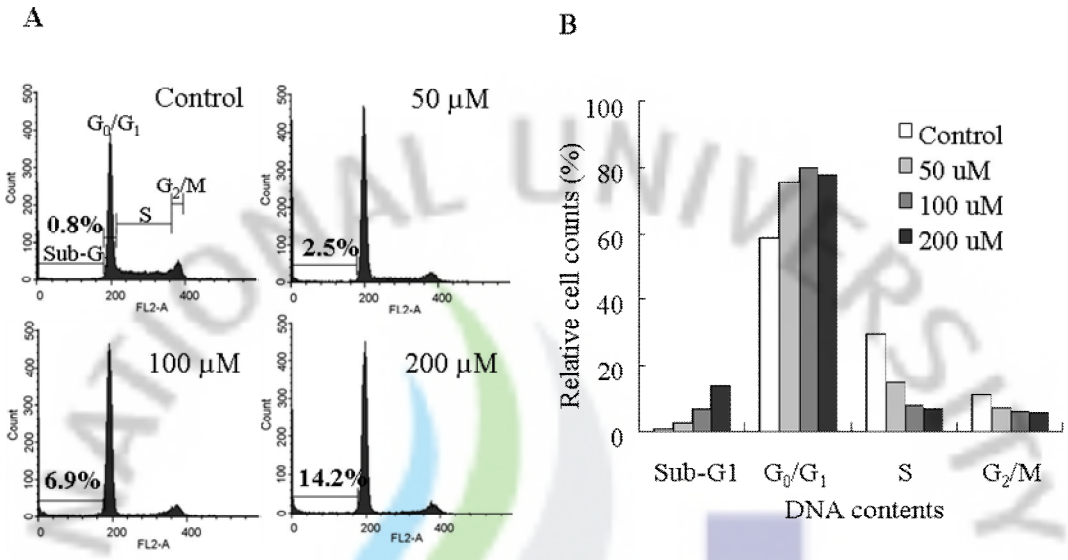

Fig. 4-2. Effect of fucoxanthin on cell cycle pattern and apoptotic portion in B16F10 cells by flow cytometric analysis. B16F10 cells were seeded at 1×10^5 and treated with the difference concentrations of fucoxanthin for 24 h. The cells were stained with PI and analyzed by flow cytometry. The cells were stained with PI and analyzed by flow cytometry. (A) Histogram of cell cycle patterns of B16F10 cells. (B) Bar graph of cell cycle patterns of B16F10 cells.

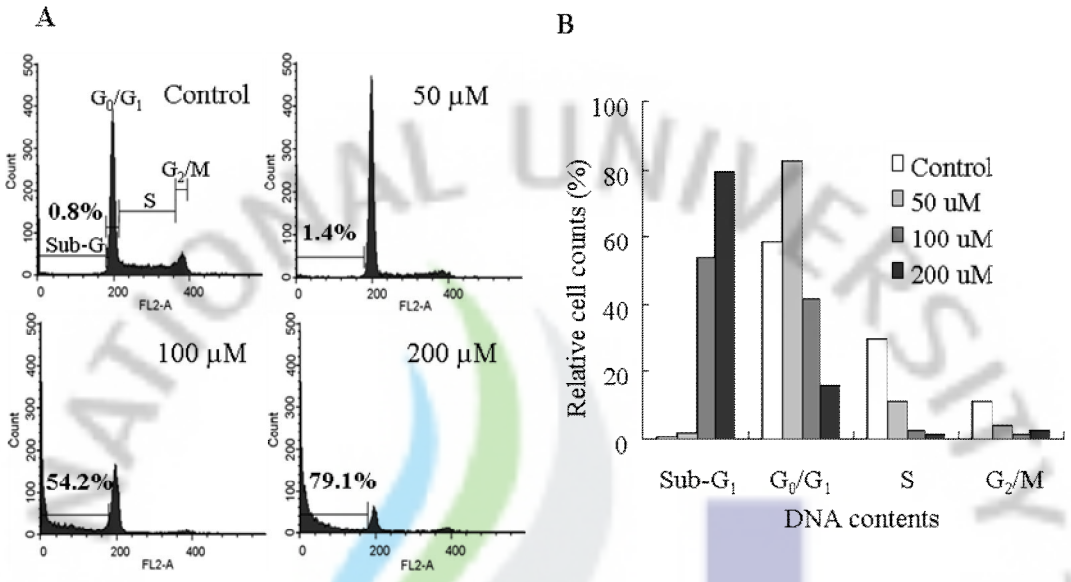

Fig. 4-3. Effect of fucoxanthin on cell cycle pattern and apoptotic portion in B16F10 cells by flow cytometric analysis. B16F10 cells were seeded at 1×10^5 and treated with the difference concentrations of fucoxanthin for 48 h. The cells were stained with PI and analyzed by flow cytometry. The cells were stained with PI and analyzed by flow cytometry. (A) Histogram of cell cycle patterns of B16F10 cells. (B) Bar graph of cell cycle patterns of B16F10 cells.

3.3. Induction effects of apoptosis in B16F10 cells by fucoxanthin

Induction of cell death and apoptosis by fucoxanthin was further studied by Hoechst-PI double staining assay. Apoptotic cell death was confirmed by apoptotic body and nuclear condensation as detected by Hoechst 33342 staining assay. Co-staining of the cells with PI allows the discrimination of dead cells from apoptotic cells. The control, without the fucoxanthin showed clear image and exhibited no DNA damage. However, obvious cell damage was observed in the cells treated with fucoxanthin (**Fig. 4-4**). Cells treated with fucoxanthin at difference concentration (**Fig. 4-4A**) dramatically increased nuclear condensation and apoptotic bodies. Similar results were obtained depending on incubation time of fucoxanthin at 200 μM (**Fig. 4-4B**). These data indicate that fucoxnathin may have notable apoptosis inducing activity against B16F10 cells.

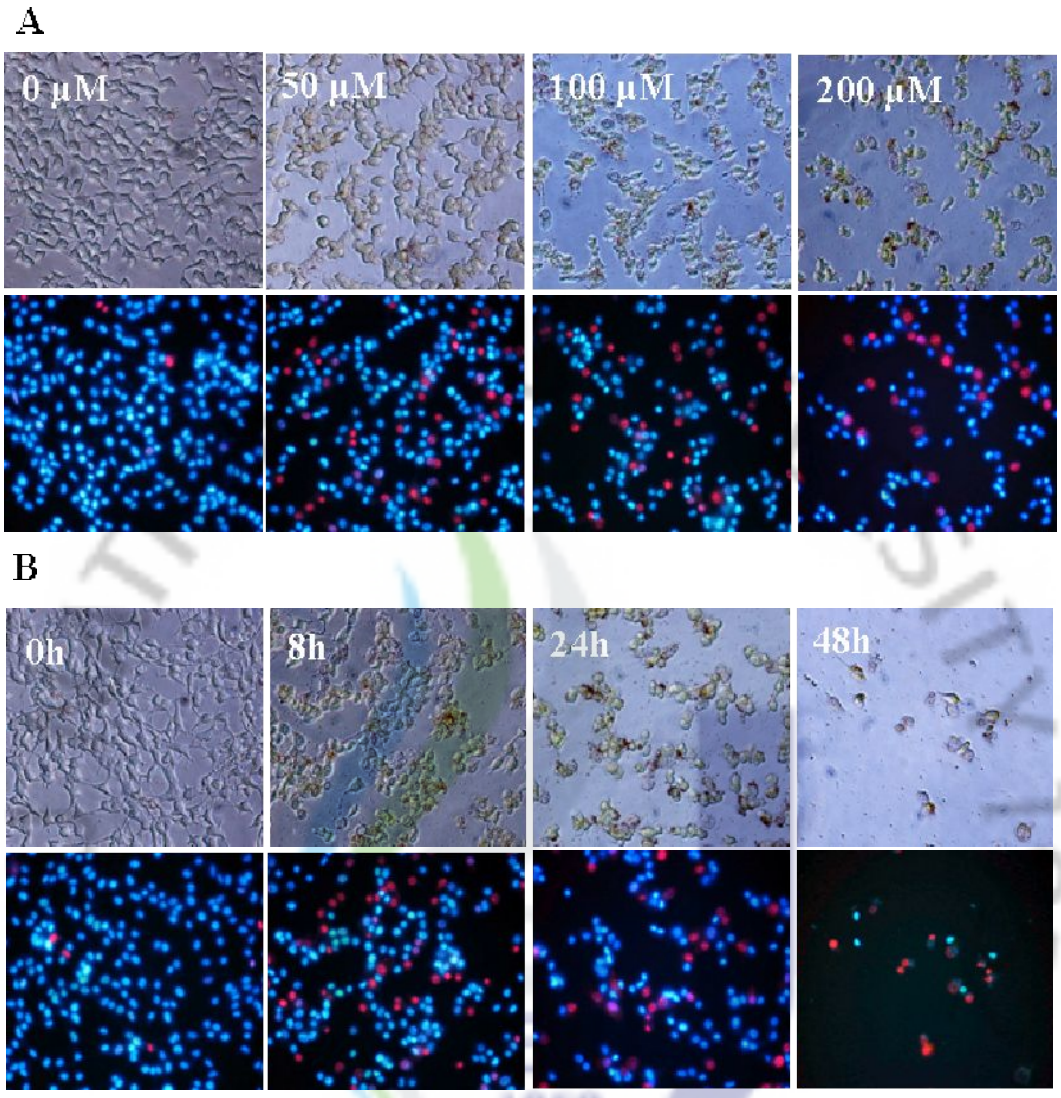

Fig. 4-4. Effect of fucoxanthin on morphological changes in B16F10 cells. (A) B16F10 cells were stimulated with the indicated concentrations (50-200 μM) of fucoxanthin for 24 h at 37 °C. (B) B16F10 cells were stimulated with 200 μM of fucoxanthin for the indicated periods (0-48 h) at 37°C. The cells were double stained with Hoechst 33342 and PI solution and then observed under a fluorescent microscop using a blue filter.

3.4. Effects of fucoxanthin on cell cycle regulatory proteins

Since fucoxanthin-induced G_0/G_1 arrest in B16F10 cells, this author next investigated the effect of fucoxanthin on cell cycle regulatory molecules involved in G_0/G_1 phase of the cell cycle progression. Phosphorylated-Rb, $p15^{INK4B}$ and $p27^{Kip1}$ have been known to play a critical role in the G_1 to S phase cell cycle transition (Bai et al., 2003; Lloyd et al., 1999; Chang et al., 2004a,b). As shown in **Fig. 4-4**, this paper found that pRb proteins were obviously decreased by fucoxanthin in a dose-dependent manner in B16F10 cells. The $p15^{INK4B}$ and $p27^{Kip1}$, a specific inhibitor of CDKs, are also mainly involved in G_1/S transition of cell cycle. The $p15^{INK4B}$ and $p27^{Kip1}$ was markedly decreased by treatment with fucoxanthin in a dose-dependent manner (**Fig .4-4**). As many studies have shown that CDKs and cyclins play crucial roles in the regulation of cell cycle progression (Morgan, 1995), this paper observed a dose-dependent decrease in cyclin D1 and D2 **(Fig. 4-4)**. Meanwhile, a decrease in cyclin D1 and D2 expression observed in fucoxanthin-treated cells was accompanied by a reduction in the amount of CDK4 that is associated with cyclin D1 and D2. CDK4 was rarely detectable in these cells, especially at 100 μM fucoxanthin.

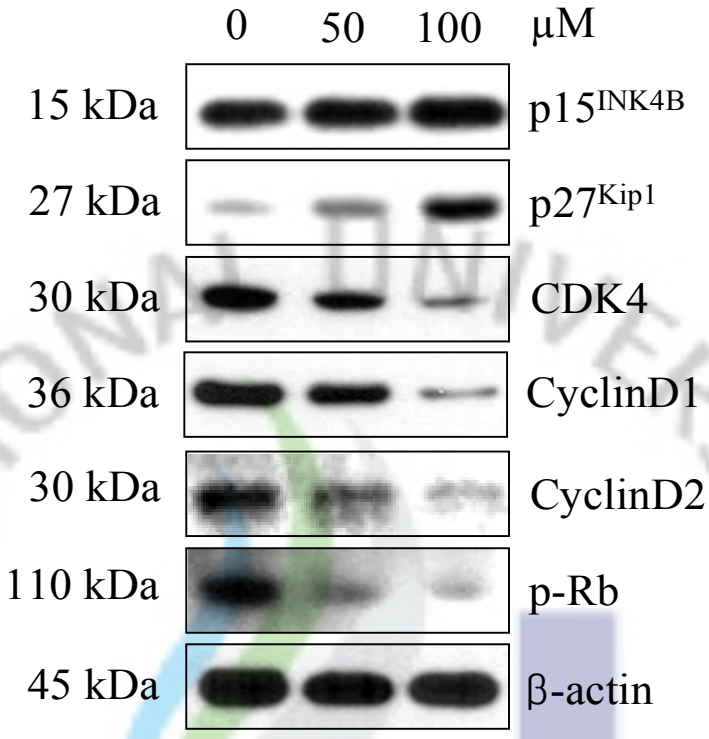

Fig. 4-5. Effect of fucoxanthin on protein expression related cell cycle arrest of B16F10 cells. The cells were treated with 50 and 100 μM fucoxanthin for 24 h. Equal amounts of cell lysates (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-p15, anti-p27, anti-CDK4, anti-CyclinD1, D2, and Anti-p-Rb (phosphorylated-Rb)).

3.5. Effects of fucoxanthin on phosphoryation of Akt

In order to characterize the fucoxanthin-induced changes in the kinase signaling pathways, this author analyzed phosphorylation status of Akt during the fucoxanthin-induced cell arrest of B16F10 cells. As shown in **Fig 4-6,** Akt phosphorylation (Thr-308) was down-regulated by fucoxanthin treatment in a time-dependent manner, but total Akt expression was not affected. Next, to evaluate the relationship between Akt activity and cell arrest, this author investigated whether fucoxanthin induces cell arrest in the presence of Triciribine (a specific Akt inhibitor) by cell cycle and an MTT assay. As shown in **Fig 4-7A and B**, treatment with Triciribine markedly increased G_0/G_1 phase (from 75.8% to 81.4%) and decreased cell viability (from 61.1± 3.1% to 35.7 ± 1.2%) in the presence fucoxanthin. Taken together, these results indicate that the Akt pathway plays an important role in regulating fucoxanthin-induced cell arrest in B16F10 cells.

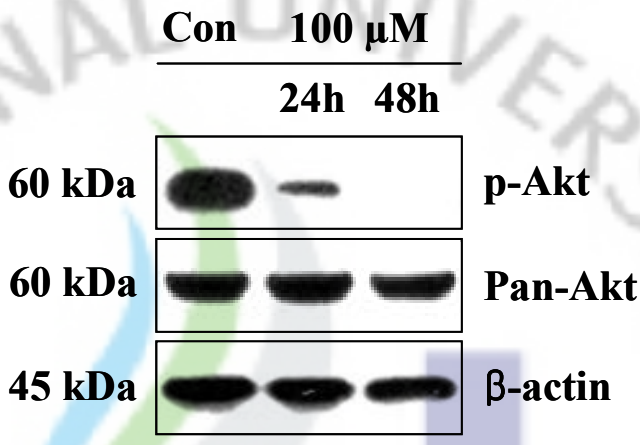

Fig. 4-6. Effect of fucoxanthin on phosphorylation of Akt in B16F10. The cells were stimulated with 100 μM of fucoxanthin for 24 and 48 h. The levels p-Akt (phosphorylated-Akt) and pan-Akt were determined using immunoblotting method.

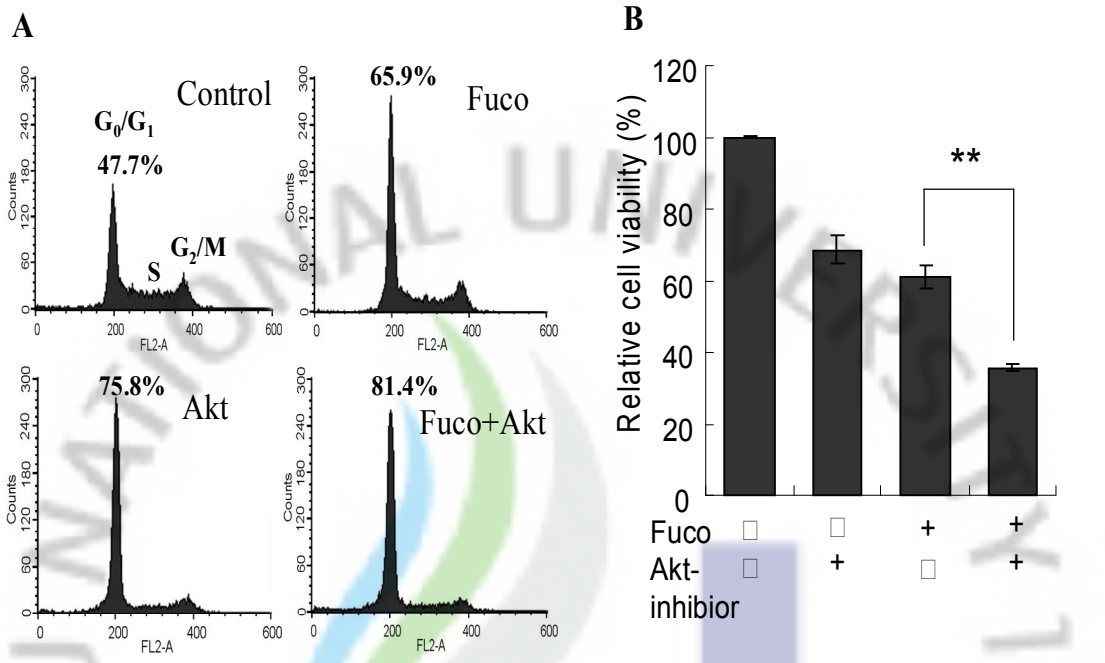

Fig. 4-7. Effect of Triciribine (a specific Akt inhibitor) against the Fucoxanthin-induced cell arrest. (A) The cells were stimulated with 100 μM of Fucoxanthin for 24 h after pretreatment with 40 μM of Triciribine for 1 h. The cells were stained with PI and analyzed by flow cytometry. (B) The cells were stimulated with 100 μM of Fucoxanthin for 48 h after pretreatment with 40 μM of Triciribine for 1 h. by using MTT assays. Means with different letters within a column are significantly different (**, $p<0.01$).

3.6. Effects of fucoxanthin on apoptosis-related protein levels of B16F10 cells

To study the mechanism of fucoxanthin induced apoptosis, this author evaluated the expressions of Bax, Bcl-xL, caspase-3, -9, IAP family and PARP by Western blot analysis. The expression level of Bcl-xL, antiapoptotic protein was decreased gradually with fucoxanthin. However, the expression of Bax, a pro-apoptotic protein, did not change (**Fig. 4-8**). The expression of the active form of caspase-3 and -9 was increased at 200 μM, and PARP was also cleaved at a concentration of 200 μM (**Fig. 4-8**). It has also been known that the IAP family proteins bind to caspases, which leads to caspase inactivation for an antiapoptotic effect in eukaryotic cells (Deveraux and Reed, 1999). This author further examined the involvement of the IAP family (XIAP, cIAP-1 and cIAP-2) in fucoxanthin-treated cells. Fucoxanthin treated cells dramatically resulted in a time-dependent downregulation of IAP family members (**Fig. 4-9**).

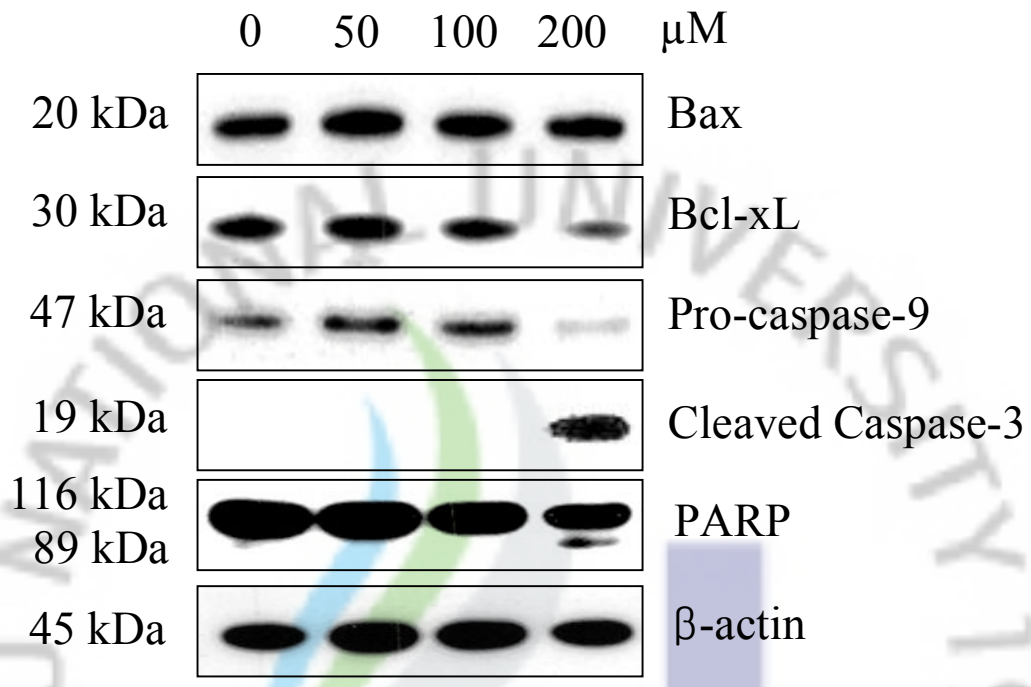

Fig. 4-8. Fucoxanthin treatment results in modulation of anti- and pro-apoptotic proteins in B16F10 cells. The cells were treated with 200 μM fucoxanthin for 24 h. Equal amounts of cell lysates (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-Bax, -Bcl-xL, -pro-caspase-9, -cleaved caspase-3, and -PARP).

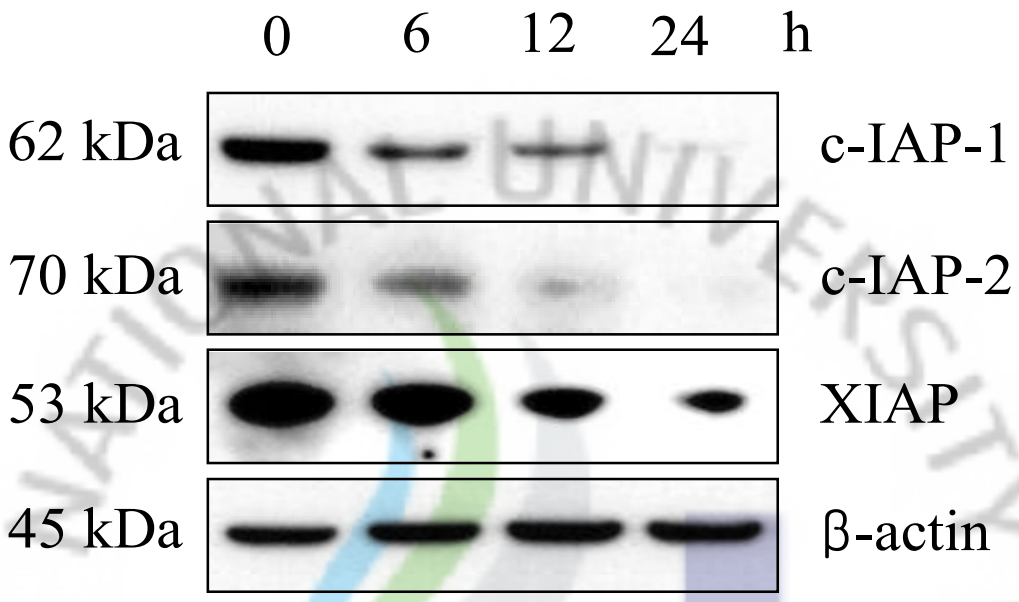

Fig. 4-9. Fucoxanthin treatment results in modulation of anti-apoptotic proteins in B16F10 cells. The cells were treated with 200 μM fucoxanthin for 24 h. Equal amounts of cell lysates (30 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies (anti-c-IAP-1, -2, and anti-XIAP).

3.7. Effect of fucoxanthin on tumor growth *in vivo*

The effects of the i.p. administration of fucoxanthin on tumor growth in Balb/c mice are shown in **Fig. 4-10**. Peritoneally disseminated melanoma tumor mass was markedly retarded by i.p. treatment of fucoxanthin compared with the positive control mice. In the positive control group, mean of tumor weight amounted to 124 mg, whereas the mean of tumor burden in fucoxanthin treated group was only 27 mg, a reduction of 5 folds. These results suggest that fucoxanthin also has *in vivo* anti-tumor effect.

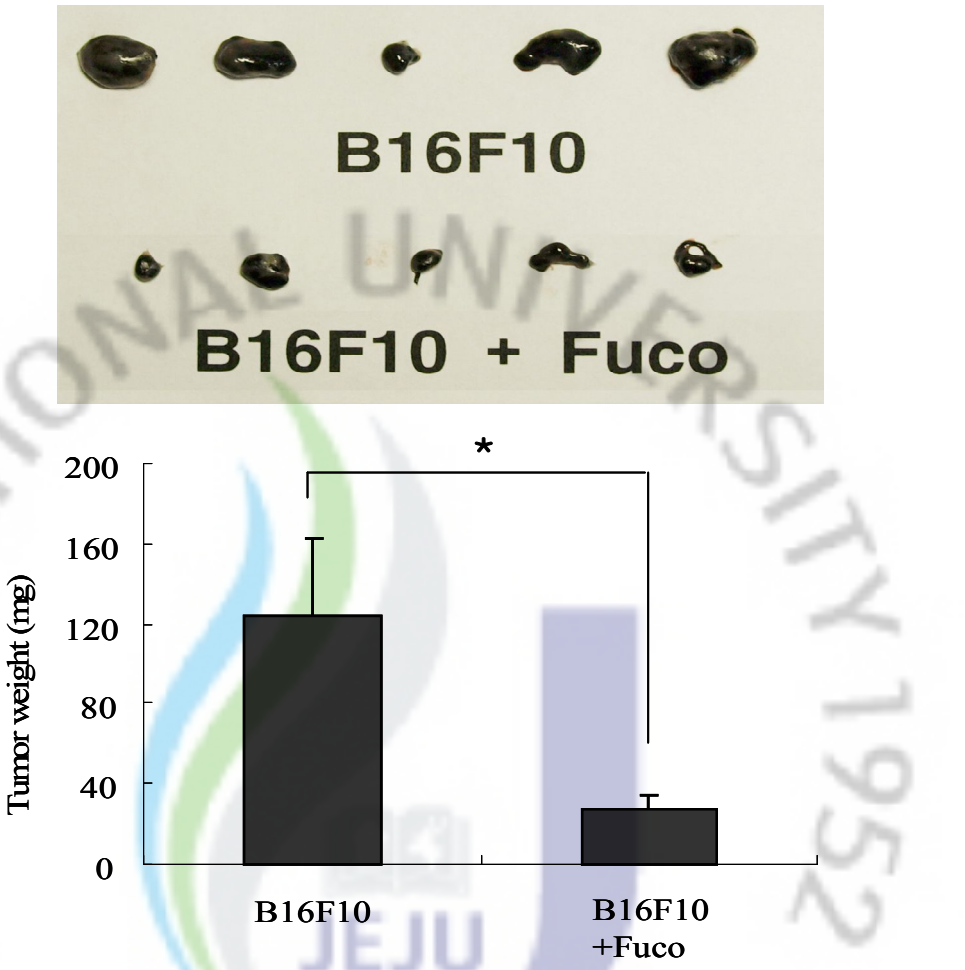

Fig. 4-10. Effect of the i.p. administration of fucoxnathin on the inhibition of tumor (B16F10) growth in Balb/c mice. Results are expressed as the mean ±S.D. Means with different letters within a column are significantly different (*, $p<0.05$).

4. DISCUSSION

Fucoxanthin has been reported to induce apoptosis in prostate cancer PC-3, DU145, LNCaP cells (Kotake-Nara et al., 2001), and leukemia (Kotake-Nara et al., 2005). In addition, fucoxanthin has been shown to cause cell cycle arrest in neuroblastoma cells (Okuzumi et al., 1990) and human hepatoma HepG2 cells (Das et al., 2008). However, there is not information available concerning the ability of fucoxanthin to inhibit B16F10 melanoma cells. Here, for the first time demonstrates that the mechanism of fucoxanthin-induced growth inhibition of B16F10 cells is due to a G_0/G_1 cell cycle arrest and apoptosis via down-regulation of Akt and Bcl-xL. Futhermore, this author also fist showed that fucoxanthin suppressed *in vivo* growth of B16F10 melanoma in Balb/c mice.

In the present study first examined the antiproliferative effect of fucoxanthin B16F10 melnoma cells at concentrations ranging from 6 to 100 μM for 72 h. Fucoxanthin significantly decreased the proliferation of B16F10 cells in a dose-dependent manner. More importantly, the fucoxanthin also exhibits effective anticancer properties in Balb/c mice. This was consistent with previous reports that fucoxanthin inhibits the growth of human prostate, leukemia, Neuroblastoma, and hepatoma cells (Das et al., 2008; Kotake-Nara et al., 2001; Kotake-Nara et al., 2005). Suppression of growth by fucoxanthin can be partially

explained by the occurrence of arrest during the cell cycle. Cell cycle analysis using flow cytometry revealed that treatment with 50 μM fucoxanthin induced G_0/G_1 phase arrest of the cell cycle at 24 h. However, these concentrations did not induced similar level of cell death as evidenced by sub-G_1 cells. These imply that the G_0/G_1 phase arrest of the cell cycle can be considered as one of the pathways by which the growth of B16F10 cells is inhibited. On the other hand, 100 and 200 μM of fucoxanthin caused the cell cycle arrest at 24 h and induced the apoptosis 48 h after treatment. Thus, this compound seems likely to cause cell cycle arrest at the low concentration (50 μM) and apoptosis at the high concentrations (100 and 200 μM) in B16F10 cells.

pRb is the master switch regulating cell cycle progression, and its continual phosphorylation parallels cell transition through G_1 and S (Weinberg, 1995). The overwhelming majority of invasive and metastatic melanoma specimens and cell lines express normal Rb protein (Albino et al., 2000). In the hypophosphorylated state, Rb family proteins associate with and inhibit the activity of E2F family transcription factors, which are involved in the transcription of key cell cycle regulators. Upon growth stimulation, the G_1-specific CDKs/cyclins phosphorylate Rb proteins on multiple residues, resulting in the release of E2F family transcription factors (Roy et al., 2007). In the present study showed that hinokitiol treatment causes a dose-dependent decrease in the level of total Rb protein

and inhibits Rb phosphorylation. Many studies have showed that the cyclins and CDKs control the G_1/S transition in the cell cycle. In addition, the regulation for the cyclins and CDKs activity has turned out to be the most productive strategy for the discovery and design of novel anticancer agents targeting the cell cycle progression (Wu et al., 2006). Weinstein (2000) also reported that a family of CDK inhibitors plays a major role in the cell cycle regulation. In contrast, activation of CDKs at G_1 phase are negatively regulated by two families of CDK inhibitors (CKIs): the kinase inhibitor protein (KIP) family including $p21^{WAF1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, and the inhibitors of CDK4 (INK4) family including $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$ acting to inhibit CDK4 and CDK6 (Pei and Xiong, 2005). The cytostatic mechanism of fucoxanthin in B16F10 cells appeared to be related to the induction of cell cycle arrest at G_1 phase. This arrest is associated with a negative control of the cell cycle machinery that inhibits G_1-S transition. This results also indicate that fucoxanthin caused down-regulation of cyclin D1 and cyclin D2 expression, which was well correlated with decrease in expression levels of CDK 4. Concomitantly, the expression levels of $p15^{INK4B}$ and $p27^{Kip1}$ were upregulated in cells exposed to fucoxanthin. Elevation of these CDK inhibitors is suggested to act as negative regulators of G_1 cell cycle progression by inhibiting the CDK activation.

The Akt signal pathway plays critical roles in regulating cell survival and death in many

physiological and pathological settings. Akt is involved in cell cycle regulation by preventing GSK-3β mediated phosphorylation and degradation of cyclin D1 (14) and by negatively regulating the cyclin dependent kinase inhibitors $p27^{Kip}$ (15) and $p21^{Waf1/CIP1}$ (16). The present study sought to determine effects of Akt on fucoxanthin-induced cell arrest. This results demonstrated that fucoxanthin downregulates the Akt signaling pathway, and that the Akt pathway inhibition significantly increased fucoxanthin-induced cell arrest. These data strongly suggest that fucxoanthin-induced cell arrest is associated with the Akt pathway

Apoptosis is an important way to maintain cellular homeostasis between cell division and cell death (Green and Reed, 1998; Hengartner, 2000; Kaufmann and Hengartner, 2001). Apoptosis is a cellular suicide or a programmed cell death that is mediated by the activation of an evolutionary conserved intracellular pathway (Bold, 1997). So, induction of apoptosis in cancer cells is one of the useful strategies for the development of anticancer drug (Hu and Kavanagh, 2003). Apoptosis is a tightly regulated process, which involves changes in the expression of distinct genes. Members of the Bcl-2 family (such as Bcl-2 and Bcl-xL) of proteins are critical regulators of the apoptotic pathway (Korsmeyer, 1999). Bcl-2 and Bcl-xL is an upstream molecule in the apoptotic pathway and are identified as potent suppressors of apoptosis (Hockenbery, 1993). This data clearly demonstrated that fucoxanthin treatment to B16F10 cells resulted in a concentration dependent decrease in Bcl-xL levels. In addition,

caspase activation is often regulated by various cellular proteins including members of the inhibitor of apoptosis (IAP; Deveraux and Reed 1999) or Bcl-2 families (Adams and Cory 1998; Antonsson and Martinou 2000). This data reveal that the protein expression of c-IAP-1, c-IAP-2, and XIAP proteins was decreased by fucoxanthin treatment. The cleavage of caspase-3 and -9 appears to be correlated with fucoxanthin-induced apoptosis in B16F10 cells. Caspase-3 and -9 are key components in the mitochondrial initiated pathway (Budihardjo et al., 1999). Once caspase is activated, a variety of cellular proteins are targeted, leading ultimately to apoptosis. PARP is the best known substrate of caspase and cleaved from 116 kDa intact form into 85 kDa fragment (Konopleva et al., 1999). This is important for cells to maintain their viaility; cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (Oliver et al., 1998). The intact form of 116 kDa and cleaved form of 85 kDa of PARP were detected in fucoxanthine treated B16F10 cells. Hence, these data fist demonstrate that fucoxanthin induce apoptosis can be occurred through Bcl-xL and IAPs regulation.

In conclusion, fucoxanthin had antiproliferative effects by inducing apoptosis and cell cycle arrest in melanoma B16F10 cells. Fucoxanthin increased the proportion of cells in the G1 phase of the cell cycle, which is associated with a decrease in Akt, cyclinD1, D2, and CDK4 expression and the induction of p15 and p27. Fucoxanthin-induced apoptosis might

be related to caspase-3 and -9 activation and the down-regulation of Bcl-xL and IAPs expression (**Fig. 4-11**). Taken together, the data presented in this *in vitro* and *in vivo* study provide important insights into this cell-cycle-based therapeutic strategy and form a strong basis for the development of fucoxanthin as an anticancer agent.

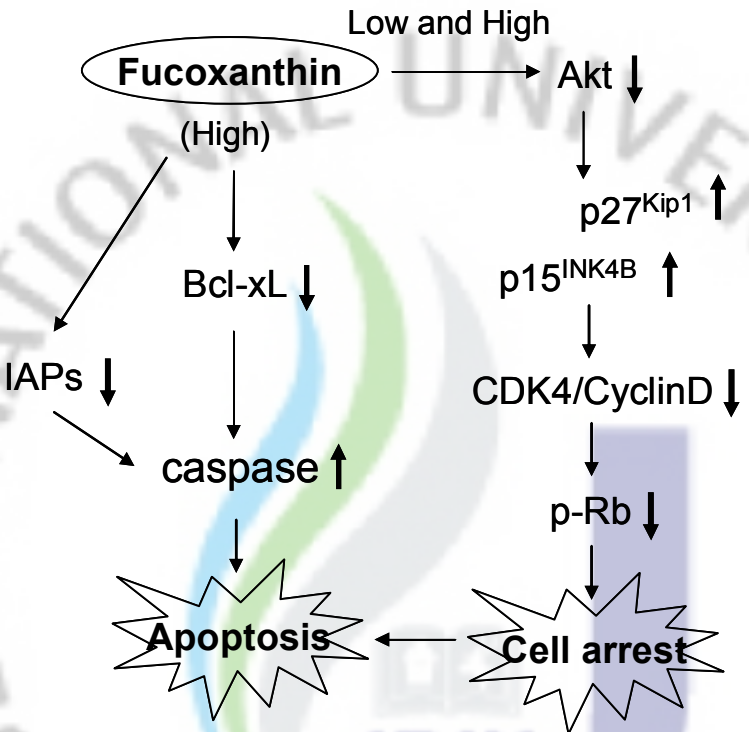

Fig. 4-11. Signaling pathway responsible for fucoxanthin-induced apoptosis and cell arrest of B16F10 cells.

REFERENCES

Adams, J. M , Cory, S. 1998. The Bcl-2 protein family: arbiters of cell survival. *Science* **281:** 1322-1326.

Ahmad, N, Feyes, D. K., Nieminen, A.L., Agarwal, R., Mukhtar, H. 1997. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J. Natl. Cancer Inst.* **89:** 1881-1886.

Ajizian, S. J., English, B. K., Meals, E.A. 1999. Specific inhibitors of p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways block inducible nitric oxide synthase and tumor necrosis factor accumulation in murine macrophages stimulated with lipopolysaccharide and interferon-gamma. *J. Infect. Dis.* **179:** 939-44.

Albino, A. P., Juan, G., Traganos, F., Reinhart, L., Connolly, J., Rose, D. R., Darzynkiewicz, Z. 2000. Cell cycle arrest and apoptosis of melanoma cells by docosahexaenoic acid: association with decreased pRb phosphorylation. *Cancer Res.* **60:** 4139-4145.

Antonsson, B., Martinou, J. C. 2000. The Bcl-2 protein family. *Exp. Cell Res.* **256:** 50-57.

Appel, K., Honegger, P., Gebicke-Haerter, P. J. 1995. Expression of interleukin-3 and tumor necrosis factor-beta mRNAs in cultured microglia. *J. Neuroimmunol*. **60:** 83-91.

Aprigliano, I., Dudas, J., Ramadori, G., Saile, B. 2008. Atorvastatin induces apoptosis by a

caspase-9-dependent pathway: an in vitro study on activated rat hepatic stellate cells. *Liver Int.* **28:** 546-557.

Aquino Esperanza, J. A., Aguirre, M. V., Aispuru, G. R., Lettieri, C .N., Juaristi, J. A., Alvarez, M. A., Brandan, N. C. 2008. In vivo 5-fluorouracil-[corrected]induced apoptosis on murine thymocytes: involvement of FAS, Bax and Caspase3. *Cell Biol. Toxicol*. **24:** 411-422.

Athukorala, Y., Jeon, Y. J. 2005. Screening for angiotensin 1-converting enzyme inhibitory activity of *Ecklonia cava*. *J. Food Sci. Nutr.* **10:** 134-139.

Baeuerle, P. A., Baltimore, D. 1996. NF-kappa B: ten years after. *Cell* **87:**13-20.

Bai, F., Pei, X. H., Godfrey, V. L., Xiong Y. 2003. Haploinsufficiency of p18(INK4c) sensitizes mice to carcinogen-induced tumorigenesis. *Mol. Cell Biol.* **23:** 1269-1277.

Barnes, P.J., Karin, M. 1997. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* **336:** 1066–1071.

Bertolini, A., Ottani, A., Sandrini, M., 2001. Dual acting anti-inflammatory drugs: a reappraisal. *Pharmacol. Res.* **44:** 437-450.

Bhat, N. R., Zhang, P, Lee, J. C, Hogan, E. L. 1998. Extracellular signal regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factoralpha gene expression in endotoxin-stimulated

primary glial cultures. *J. Neurosci.* **18:** 1633-1641.

Bold, R. J., Termuhlen, P. M., McConkey, D. J. 1997. Apoptosis, cancer and cancer therapy. *Surgical Oncology Oxford* **6:** 133-142.

Bove, P. F., Van, A. 2006. Nitric oxide and reactive nitrogen species in airway epithelial signaling and inflammation. *Free Radic. Biol. Med.* **41:** 515-527.

Budihardjo, I., Oliver, H., Lutter M., Luo, X., Wang, X. 1999. Biochemical pathways of caspase activation during apoptosis. *Annu. Rev. Cell Dev. Biol*. **15:** 269-290.

Buttini, M., Boddeke, H. 1995. Peripheral lipopolysaccharide stimulation induces interleukin-1 beta messenger RNA in rat brain microglial cells. *Neurosci.* **65:** 523-530.

Chang, L., Karin, M. 2001. Mammalian MAP kinase signalling cascades. *Nature* **410:** 37-40.

Chen, Y. R., Meyer, C. F., Tan, T. H. 1996. Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in gamma radiation-induced apoptosis. *J. Biol. Chem.* **271:** 631-634.

Cho, J. Y., Baik, K. U., Jung, J. H., Park, M. H. 2000. In vitro anti-inflammatory effects of cynaropicrin, a sesquiterpene lactone, from *Saussurea lappa*. *Eur. J. Pharmacol*., **398:** 399-407.

Choi, J. S., Lee, W. K., Cho, Y. J., Kim, D. S., Kim, A., Chung, H. Y., Jung, J. H., Im, K. S., Choi, W. C., Choi, H. D., Son, B. W. 2000. Bioactive carotenoids, Fucoxanthin as chemotaxonomic marker and antioxidative agent from the marine bacillariophycean

microalga *Hantzschia marina*. *Nat. Prod. Sci.* **6:** 122-125.

Cohen, J. 2002. The immunopathogenesis of sepsis. *Nature* **420:** 885-891.

Coyle, J. T., Puttfarcken, P. 1993. Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262:** 689-695.

Cross, R. K., Wilson, K. T. 2003. Nitric Oxide in Inflammatory Bowel Disease. *Inflamm. Bowel. Dis*. **9:** 179-189.

Das, S. K. Hashimoto, T. Kanazawa, K. 2008. Growth inhibition of human hepatic carcinoma HepG2 cells by fucoxanthin is associated with down-regulation of cyclin D. *Biochim. Biophys. Acta* **1780:** 743-749.

Das, S. K., Hashimoto, T., Shimizu, K., Yoshida, T., Sakai, T., Sowa, Y., Komoto, A., Kanazawa, K. 2005. Fucoxanthin induces cell cycle arrest at G0/G1 phase in human colon carcinoma cells through up-regulation of p21WAF1/Cip1. *Biochem. Biophys. Acta* **1726:** 328-335.

Dawes, C. J. 1998. Marine Botany. John Wiley & Sons, Inc., New York, p. 480.

Deveraux, Q. L., Reed, J. C. 1999. IAP family proteins: suppressors of apoptosis. *Genes Develop*. **13:** 239-252.

Diehl, J. A., Chenq, M., Roussel M. F., Sherr, C. J. 1998. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* **12:** 3499-3511.

Dobrovolskaia, M. A., Vogel, S. N. 2002. Toll receptors, CD14, and macrophage activation anddeactivation by LPS. *Microbes. Infect.* **4:** 903-914.

Feig, D. I., Reid, T. M., Loeb, L. A. 1994. Reactive oxygen species in tumorigenesis. *Cancer Res.* **54:** 1890-1894.

Fernandes, A. F., Zhou, J., Zhang, X., Bian, Q., Sparrow, J., Taylor, A., Pereira, P., Shang, F. 2008. Oxidative inactivation of the proteasome inretinal pigment epithelial cells. A potential link between oxidative stress and up-regulation of interleukin-8. *J. Biol. Chem.* **283:** 20745-20753.

Fujihara, M., Muroi, M., Tanamoto, K., Suzuki, T., Azuma, H., Ikeda, H. 2003. Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. *Pharmacol. Ther.* **100:** 171-194.

Garcia-Ruiz, C., Colell, A., Mari, M., Morales, A., Fernandez-Checa, J. C. 1997. Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. *J. Biol. Chem*. **272:** 11369-11377.

Gesbert F., Sellers W. R., Siqnoretti S., Loda, M., Griffin J. D. 2000. BCR/ABL regulates expression of the cyclin-dependent kinase inhibitor p27Kip1 through the phosphatidylinositol 3-Kinase/AKT pathway. *J. Biol. Chem.* **275:** 39223-39230.

Ghosh S, Karin M. 2002. Missing pieces in the NF-kappaB puzzle. *Cell* **109:** 81-96.

Glauser, M. P. 1996. The inflammatory cytokines. New developments in the pathophysiology and treatment of septic shock. *Drugs* **52:** 9-17.

Green, D. R., Reed, J. C. 1998. Mitochondria and apoptosis. *Science* **281:** 1309-1312.

Gschwind, M., Huber, G. 1995. Apoptotic cell death induced by β-amyloid 1-42 peptide is cell type dependent. *J. Neurochem*. **65:** 292-300.

Guha, M., Mackman, N. 2001. LPS induction of gene expression in human monocytes. *Cell Signal* **13:** 85-94.

Guzik, T. J., Korbut, R., Adamek-Guzik, T. 2003. Nitric oxide and superoxide in inflammation and immune regulation. *J. Physiol. Pharmacol.* **54:** 469-487.

Halliwell, B., Gutteridge, J. M. C. 1999. Antioxidant defenses. In: Free radicals in biology and medicine, 3rd ed. pp. 105-159. Oxford Science Publications, Oxford, UK.

Ham, Y. M., Lim, J. H. Na, H. K., Choi, J. S., Park, B. D., Yim, H., Lee, S. K. 2006. Ginsenoside-Rh2-induced mitochondrial depolarization and apoptosis are associated with reactive oxygen species- and Ca_2^+-mediated c-Jun NH2-terminal kinase 1 activation in HeLa cells. *J. Pharmacol. Exp. Ther.* **319:** 1276-1285.

Hambleton, J., Weinstein, S. L., Lem, L., DeFranco, A. L. 1997. Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. *Proc. Natl.*

Acad. Sci. U S A **93:** 2774-2778.

Harper, S. J., LoGrasso, P. 2001. Signaling for survival and death in neurones: the role of stress-activated kinases, JNK and p38. *Cell Signal* 13: 299-310.

Hengartner, M. O. 2000. The biochemistry of apoptosis. *Nature* **407:** 770-776.

Heo, S. J. 2008. Development of functional cosmetic materials using the bioactive compounds from brown seaweeds. A thesis for the degree of Ph.D, Jeju National Univesity.

Heo, S. J., Kim, J. P., Jung, W. K., Lee, N. H., Kang, H. S., Jun, E. M., Park, S. H., Kang, S. M., Lee, Y. J., Park, P. J., Jeon, Y. J. 2008. Identification of chemical structure and free radical scavenging activity of diphlorethohydroxycarmalol isolated from brown alga, *Ishige okamurae*. *J. Micobiol. Biotechnol.* **18:** 676-681.

Heo, S. J., Pak, E. J., Lee, K. W., Jeon, Y. J. 2005a. Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresource Technol.* **96:** 1613-1623.

Heo, S. J., Park, P. J., Park, E. J., Kim, S. K., Jeon, Y. J. 2005b. Antioxidant activity of enzymatic extracts from a brown seaweed *Ecklonia cava* by electron spin resonance spectrometry and comet assay. *Eur. Food Res. Technol.* **221:** 41-47.

Hileman, E. O., Liu, J. S., Albitar, M., Keating, M. J., Huang, P. 2004. Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. *Cancer Chemother.*

Pharmacol. **53:** 209-219.

Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Milliman, C. L., Korsmeyer, S. J. 1993. Bcl-2 function in an antioxidant pathway to prevent apoptosis. *Cell* **75:** 241-251.

Hommes, D. W., Peppelenbosch, M. P., van Deventer, S. J., 2003. Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory argets. *Gut*. **52:** 144-151

Hosokawa, M., Kudo, M., Maeda, H., Kohno, H., Tanaka, T., Miyashita, K. 2004. Fucoxanthin induces apoptosis and enhances the antiproliferative effect on the PPARγ ligand, troglitazone, on colon cancer cells. *Biochim. Biophys. Acta* **1675:** 113-119.

Hosokawa, M., Wanezaki, S., Miyauchi, K., Kurihara, H., Kohno, H., Kawabata, J., Odashima, S., Takahashi, K. 1999. Apoptosis-inducing effect of fucoxanthin on human leukemia cell HL-60. *Food Sci. Technol. Res.* **5:** 243-246.

Hseu, Y. C., Chang, W. H., Chen, C. S., Liao, J. W., Huang, C. J., Lu, F. J., Chia, Y. C., Hsu, H. K., Wu, J. J., Yang, H.,L. 2008. Antioxidant activities of Toona Sinensis leaves extracts using different antioxidant models. *Food Chem. Toxicol*. **46:** 105-114.

Hsiao, Y.C., Hsieh, Y. S., Kuo,W. H., Chiou, H. L., Yang, S. F., Chiang,W. L., Chu, S. C. 2007. The tumor-growth inhibitory activity of flavanone and 2′-OH flavanone *in vitro* and *in vivo* through induction of cell cycle arrest and suppression of cyclins and CDKs. *J.*

Biomed. Sci. **14:** 107-119.

Huan, P., Feng, L., Oldham, E. A., Keating, M. J., Plunkett, W. 2000. Superoxide dismutase as a target for the selective killing of cancer cells. *Nature* **407:** 390-395.

Hussain, S. P., Hofseth, L. J., Harris, C. C. 2003 Radical causes of cancer. *Nat. Rev. Cancer* **3:** 276-284.

Inoue, J., Gohda, J., Akiyama, T., Semba, K. 2007. NF-κB activation in development and progression of cancer. *Cancer sci.* **98:** 268-274.

Iseki, M., Ikuta, T., Kobayashi, T., Kawajiri, K. 2005. Growth suppression of LeydigTM3 cells mediated by aryl hydrocarbon receptor. *Biochem. Biophys. Res. Commun.* **331:** 902-908.

Ishii, R., Horie, M., Saito, K., Arisawa, M., Kitanaka, S. 2003. Inhibition of 53 lipopolysaccharide-induced pro-inflammatory cytokine expression via suppression of nuclear factor-κB activation by Mallotus japonicus phloroglucinol derivatives. *Biochim. Biophys. Acta* **1620:** 108-118.

Islam, S., Hassan, F., Mu, M. M., Ito, H., Koide, N., Mori, I., Yoshida, T., Yokochi, T. 2004. Piceatannol prevents lipopolysaccharide (LPS)-induced nitric oxide (NO) production and nuclear factor (NF)-kappaB activation by inhibiting IkappaB kinase (IKK). *Microbiol. Immunol.* **48:** 729-736.

Jensen, A. 1993. Present and future needs for alga and algal products. *Hydrobiol.***260/261:** 15-21.

Jin, M. H., Hong, C. H., Lee, H. Y., Kang, H. J., Han, S. W. 2008. Enhanced TGF-beta1 is involved in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced oxidative stress in C57BL/6 mouse testis. *Toxicol. Lett.* 178: 202-209.

Jung, Y. S., Jeong, E. M., Park, E. K., Kim Y. M., Sohn, S., Lee, S. H., Baik, E. J., Moon, C. H. 2008. Cadmium induces apoptotic cell death through p38 MAPK in brain microvessel endothelial cells. *Eur. J. Pharmacol.* **578:** 11-18.

Kaina, B. 2003. DNA damage-triggered apoptosis: critical role of DNA repair, double-strand breaks, cell proliferation and signaling. *Biochem. Pharmacol.* **66:** 1547-1554.

Karin, M. 1999. The beginning of the end: IkappaB kinase (IKK) and NF-kappaB activation. *J. Biol. Chem.* **274:** 27339-9342.

Kaufmann, S., Hengartner, M. O. 2001. Programmed cell death: alive and well in the new millennium.*Trends Genet.* **11**: 526-534.

Kawasaki, H., Morooka, T., Shimohama, S., Kimura, J., Hirano, T., Gotoh, Y., Nishida, E. 1997. Activation and involvement of p38 mitogenactivated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells. *J. Biol. Chem*. **272:** 18518-18521.

Kim, B. C., Kim, H. G., Lee, S. A., Lim, S., Park, E. H., Kim, S. J., Lim, C. J. 2005. Genipin-induced apoptosis in hepatoma cells is mediated by reactive oxygen species/c-Jun NH2-terminal kinase-dependent activation of mitochondrial pathway. *Biochem. Pharmacol.* **70:** 1398-1407.

Kim, B. M., Chung, H. W. 2008. Desferrioxamine (DFX) induces apoptosis through the p38-caspase8-Bid-Bax pathway in PHA-stimulated human lymphocytes. *Toxicol. Appl. Pharmacol.* **228:** 24-31.

Kim, J. K., Oh, S. M., Kwon, H. S., Oh, Y. S., Lim, S. S., Sin, H. K. 2006. Anti-inflammatory effect of roasted licorice extracts on lipopolysaccharide-induced inflammatory responses in murine macrophages. *Biochem. Biophys. Res. Commun.* **345:** 1215-1223.

Kim, S. F., Huri, D. A., Snyder, S. H. 2005. Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. *Science* **310:** 1966-70.

Kim, Y. S., Jin, S.H., Lee, Y. H., Kim, S. I., Park, J. D. 1999. Ginsenoside Rh2 induces apoptosis independently of Bcl-2 Bcl-xL, or Bax in C6Bu-1 cells. *Arch. Pharm. Res.* **22:** 448-453.

Koike, K., Takaki, A., Tatsukawa, M., Suzuki, M., Shiraha, H., Iwasaki, Y., Sakaguchi, K., Shiratori, Y. 2006. Combination of 5-FU and IFNalpha enhances IFN signaling pathway

and caspase-8 activity, resulting in marked apoptosis in hepatoma cell lines. *Int. J. Oncol.* **29:** 1253-1261.

Kondo, N. Nakamura, H. Masutani, H. Yodoi, J. 2006. Redox regulation of human thioredoxin network. *Antioxid. Redox. Signal.* **8:** 1881-1890.

Konopleva, M., Zhao, S., Xie, Z., Segall, H., Younes, A., Claxton, D. F., Estrov, Z., Kornblau, S. M., Andreeff, M. 1999. Apoptosis: Molecules and mechanisms. *Adv. Exp. Med. Biol.* **457:** 217-236.

Korsmeyer, S. J. 1999. Bcl-2 gene family and the regulation of programmed cell death. *Cancer Res.* **59:** 1693-1700.

Kotake-Nara, E., Asai, A., Nagao, A. 2005. Neoxanthin and fucoxanthin induce apoptosis in PC-3 human prostate cancer cells. *Cancer lett.* **220:** 75-84.

Kotake-Nara, E., Kushiro, M., Zhang, H., Sugawara, T., Miyashita, K., Nagao, A. 2001. Carotenoids affect proliferation of human prostate cancer cells. *J. Nutr.* **131:** 3303-3306.

Kotake-Nara, E., Terasaki, M., Nagao, A. 2005. Charaterization of apoptosis induced by fucoxanthin in human promyelocytic leukemia cells. *Biosic. Biotechnol. Biochem.* **69:** 224-227.

Kotamraju, S., Kalivendi, S. V., Konorev, E., Chitambar, C. R., Joseph, J., Kalyanaraman B. 2004. Oxidant-induced iron signaling in doxorubicin-mediated apoptosis. *Methods*

Enzymol. **378:** 362-382.

Kufe, D. W., Pollock, R. E., Weichselbaum, R. R., Bast Jr, R. C., Gansier, T. S., Holland, J. F. 2003. Cancer medicine, 6th ed. Hamiton, Ontario: American Cancer Society Inc. and B.C. Decker, Inc.

Kuo, P. L., Chen, C. Y., Hsu, Y. L. 2007. Isoobtusilactone A induces cell cycle arrest and apoptosis through reactive oxygen species/apoptosis signal-regulating kinase 1 signaling pathway in human breast cancer cells. *Cancer Res.* **67:** 7406-7420.

Kwak, H. J., Song, J. S., Heo, J. H., Yang, S. D., Nam, J. Y., Cheon, H. G. 2005. Roflumilast inhibits lipopolysaccharided-induced inflammatory mediators via suppression of Nuclear Factor-κB, p38 mitogen-activated protein kinase, and c-Jun NH2-terminal kinase activation. *J. Pharmacol. Exp. Ther.* **315:** 1188-1195.

LaChapelle, A. M., Ruygrok, M. L., Toomer, M. Oost, J. J. Monnie, M. L. Swenson, J. A., Compton, A., Stebbins-Boaz, A. B. 2007. The hormonal herbicide, 2,4-dichlorophenoxyacetic acid, inhibits *Xenopus* oocyte maturation by targeting translational and post-translational mechanisms. *Reprod. Toxicol.* **23:** 20-31.

Lahti, A., Lahde, M., Kankaanranta, H., Moilanen E. 2000. Inhibition of extracellular signalregulated kinase suppresses endotoxin-induced nitric oxide synthesis in mouse macrophages and in human colon epithelial cells. *J. Pharmacol. Exp. Ther.* **294:** 1188-

1194.

Lee, E. S., Ju, H. K., Moon, T. C., Lee, E. K., Jahng, Y. D., Lee, S. H., Son, J. K., Baek, S. H., Chang, H. W. 2004. Inhibition of Nitric Oxide and Tumor Necrosis Factor-α (TNF-α) 54 Production by Propenone Compound through Blockade of Nuclear Factor (NF)-κB Activation in Cultured Murine Macrophages. *Biol. Pharm. Bull.* **27:** 617-620.

Lee, I. K., Kang, J. W. 1986. A check list of marine algae in Korea. *Algae* **1:** 311–325.

Lee, J. C., Young, P. R. 1996. Role of CSB/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. *J. Leukoc. Biol.* **59:** 152-157.

Lee, K., Esselman, W. J. 2002. Inhibition of PTPs by H_2O_2 regulates the activation of distinct MAPK pathways. *Free. Radic. Biol. Med.* **33:** 1121-1132.

Lee, S. B., Schorey, J. S. 2005. Activation and mitogen-activated protein kinase regulation of transcription factors Ets and NF-kappaB in Mycobacterium-infected macrophages and role of these factors in tumor necrosis factor alpha and nitric oxide synthase 2 promoter function. *Infect. Immun.* **73:** 6499-6507.

Lee. Y. S., Yoon, H. J., Oh, J. H., Park, H. J., Lee, E. H., Song, C. W., Yoon, S. 2009. 1,3-Dinitrobenzene induces apoptosis in TM4 moust Sertoli cells: Involvement of the c-Jun N-termical kinase (JNK) MAPK pathway. *Toxicol. Lett.* **189:** 145-151.

Levinthal, D. J., Defranco, D. B. 2005. Reversible oxidation of ERK-directed protein

phosphatases drives oxidative toxicity in neurons. *J. Biol. Chem.* **280:** 5875-5883.

Lewis, J. G., Stanley, N. F., Guist, G. G. 1988. In: Lembi, C. A., Waaland, J. R. (Eds.). Algae and Human Affairs. Cambridge University Press, New York, PP. 205-236.

Li, G. X., Hu, H., Jiang, C., Schuster, T., Lu, J. 2007. Differential involvement of reactive oxygen species in apoptosis induced by two classes of selenium compounds in human prostate cancer cells. *Int. J. Cancer* **120:** 2034-2043.

Li, Q., Verma, I. M. 2002. NF-kappaB regulation in the immune system. *Nat. Rev. Immunol.* **2:** 725-34.

Liaeen-Jensen, S. 1978. Marine carotenoids. In Marine Natural Products, *Chemical and Biochemical Perspective* Vol. 2; Scheuer, P. J., Ed.; Academic Press: New York, pp. 1-73.

Liaeen-Jensen, S. 1998. Carotenoids in chemosystematics. In Carotenoids, Vol. 3, *Biosynthesis and Metabolism*; Britton, G., Liaaen-Jensen, S., Pfander, H., Eds.; Birkhauser: Basel, Switzerland, pp. 217-247.

Lind, L. 2003. Circulating markers of inflammation and atherosclerosis. *Atherosclerosis* **169:** 203-214.

Lizard, G., Fournel, S., Genestier, L., Dhedin, N., Chaput, C., Flacher, M., Mutin, M., Panaye, G., Revillard, J. P. 1995. Kinetics of plasma membrane and mitochondrial

alterations in the cells undergoing apoptosis. *Cytometry* **21:** 275-283.

Lloyd, R. V., Erickson, L. A., Jin, L., Kulig, E., Qian, X., Cheville, J. C., Scheithauer, B. W. 1999. p27kip1: a multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers. *Am. J. Pathol.* **154:** 313-323.

Lo, A. H., Liang, Y. C., Lin-Shiau, S. Y., Ho, C. T., Lin, J. K. 2002. Carnosol, an antioxidant in rosemary, suppresses inducible nitric oxide synthase through down-regulating nuclear factor-κB in mouse macrophages. *Carcinogenesis* **23:** 983-991.

Lowe, S. W., Lin, A. W. 2000. apoptosis in cancer. *Carcinogenesis* **21:** 485-495.

MacMicking, J., Xie, Q. W., Nathan, C. 1997. Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15:** 323-350.

Maeda, H., Hosokawa, M., Sashima, T. Funayama, K., Miyashita, K. 2005. Fucoxanthin from edible seaweed, *Undaria pinnatifida*, shows antiobesity effect through UCP1 expression in white adipose tissues. *Biochem. Biophys. Res. Commum.* **332:** 392-371.

Mannel, D. N., Echtenacher, B. 2000 TNF in the inflammatory response. *Chem. Immunol.* **74:** 141-61.

Marcus, J. S., Karackattu, S. L., Fleegal, M. A., Sumners, C. 2003. Cytokine-stimulated inducible nitric oxide synthase expression in astroglia: role of Erk mitogenactivated protein kinase and NF-kappaB. *Glia*. **41:** 152-160.

Martindale, J. L., Holbrook, N. J. 2002. Cellular response to oxidative stress: signaling for suicide and survival, *J. Cell Physiol.* **192:** 1-15.

Mayer, A. M. S., Hamann, M. T. 2005. Marine pharmacology in 2001-2002: marine compounds with anthelmintic, antibacterial, anticoagulant, antidiabetic, antifungal, anti-inflammatory, antimalarial, antiplatelet, antiprotozoal, anti-tuberculosis, and antiviral activities; affecting the cardiovascular, immune and nervous systems and other miscellaneous mechanisms of action. *Comp. Biochem. Physiol. C.* **140:** 265-286.

McCann, S. M., Mastronardi, C., de Laurentiis, A., Rettori, V. 2005. The nitric oxide theory of aging revisited. *Annals of New York Academy of Sciences* **1057:** 64-84.

Murakami, A., Nishizawa, T., Egawa, K., Kawada, T., Nishikawa, Y., Uenakai, K., Ohigashi, H. 2005. New class of linoleic acid metabolites biosynthesized by corn and rice lipoxygenase: Suppression of proinflammatory mediator expression via attenuation of MAPK- and Akt-, but not PPARg-, dependent pathways in stimulated macrophage. *Biochem. Pharmacol.* **70:** 1330-1342.

Nagayama, K., Iwamura, Y., Shibata, T., Hirayama, I., Nakamura, T. 2002. Bactericidal activity of phlorotannins from the brown alga *Ecklonia kurome*. *J. Antimicrob. Chemother.* **50:** 889-893.

Nakanishi, M. Shimada, M. Niida, H. 2006. Genetic instability in cancer cells by impaired

cell cycle checkpoints. *Cancer Sci.* **97:** 984-989.

Nakazawa, Y., Sashima, T., Hosokawa, M., Miyashita, K. 2009. Comparative evaluation of growth inhibitory effect of stereoisomers of fucoxanthin in human cancer cell lines. *J. Fucnct. Foods* **1:** 88-97.

Nathan, C. 2002. Points of control in inflammation. *Nature* **420:** 846-852.

Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., Riccardi, C. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods* **139:** 271-279.

Nisizawa, K., Noda, H., Kikuchi, R., Watamaba, T. 1987. The main seaweed foods in Japan. *Hydrobiol.* **151/152:** 5-29.

Noda, H. 1993. Health benefits and nutritional properties of Nori. *J. Appl. Phycol.* **5:** 255-258.

Oilver, F. J., Rubia G., Rolli V., Ruiz-Ruiz M. C., Murcia G., Murcia J. M. 1998. Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis. Lesson from an uncleavable mutant. *J. Biol. Chem.* **273:** 33533-33539.

Okuzumi, J. Nishino, H. Murakoshi, M. Iwashima, A. Tanaka, Y. Yamane, T. Fujita, Y. Takahashi T. 1990. Inhibitory effects of fucoxanthin, a natural carotenoid, on N-myc expression and cell cycle progression in human malignant tumor cells. *Cancer Lett.*

55: 75-81.

Okuzumi, J., Takahashi, T., Yamane, T., Kitao, Y., Inagake, M., Ohya, K., Nishino, H., Tanaka, Y. 1993. Inhibitory effects of fucoxanthin, a natural carotenoid, on *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine-induced mouse duodenal carcinogenesis. *Cancer Lett.* **68:** 159-168.

Oohusa, T. 1993. Recent trend in Nori products and market in Asia. *J. Appl. Phycol.* **5:** 155-159.

Palermo, J. A., Gros, E. G., Seldes, A. M. 1991. Carotenoids from three red algae of the Corallinaceae. *Phytochem.* **30:** 2983-2986.

Pei X. H., Xiong, Y. 2005. Biochemical and cellular mechanisms of mammalian CDK inhibitors: a few unresolved issues. *Oncogene* **24:** 2787-2795.

Pelicano, H., Carney, D., Huang, P. 2004. ROS stress in cancer cells and therapeutic implications. *Drug Resist. Update* **7:** 97-110.

Polivka, T., Sundström, V. 2004. Ultrafast dynamics of carotenoid excited states-from solution to natural and artificial systems. *Chem. Rev.* **104:** 2021-2071.

Puri, P. L., MacLachlan, T. K., Levrero, M., Giordano, A. 1999. The intrinsic cell cycle: from yeast to mammals, in: G.S. Stein, R. Baserga, A. Giordano, D.T. Denhardt (Eds.), The Molecular Basis of Cell Cycle and Growth Control. *Wiley-Liss, New York* pp. 15-79.

Rafi, M. M., Yadav, P. N., Rossi, A. O. 2007. Glucosamine inhibits LPS-induced COX-2 and iNOS expression in mouse macrophage cells (RAW 264.7) by inhibition of p38-MAP kinase and transcription factor NF-kappaB. *Mol. Nutr. Food Res.* **51:** 587-593.

Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., Davis R. J., 1995. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *Biol. Chem.* **270:** 7420-7426.

Rankin, J. A. 2004. Biological mediators of acute inflammation. *AACN Clin Issues* **15:** 3-17.

Ricketts, E., Calvin, J. 1962. *Between pacific tides*. 3rd et. Revised by Hedgpeth, J. Stanford, California: Stanford University Press. Smith, G. M. 1944. *Marine algae of the monterey peninsula*. Stanford, California: Stanford University Press.

Roy, S., Kaur, M., Agarwal, C., Tecklenburg, M., Sclafani, R. A., Agarwal, R. 2007. p21 and p27 induction by silibinin is essential for its cell cycle arrest effect in prostate carcinoma cells. *Mol. Cancer Ther.* **6:** 2696-2707.

Ruland, J., Mak, T. W. 2003. Transducing signals from antigen receptors to nuclear factor kappaB. *Immunol. Rev.* **193:** 93-100.

Satomi, Y., Nishino, H. 2007. Fucoxanthin, a natural carotenoid, induces G1 arrest and GADD45 gene expression in human cancer cells. *In Vivo* **21:** 305-309.

Schrader, L. I., Kinzenbaw, D. A., Johnson, A. W., Faraci, F. M., Didion, S. P. 2007. IL-6 deficiency protects against angiotensin II induced endothelial dysfunction and hypertrophy. *Arterioscler. Thromb. Vasc. Biol.* **27:** 2576- 2581.

Schumacker, P. T. 2006. Reactive oxygen species in cancer cells: live by the sword, die by the sword. *Cancer Cell* **10:** 175-176.

Shimidzu, N., Goto, M., Miki, W. 1996. Carotenoid as singlet oxygen quenchers in marine organisms. *Fish. Sci.* **62:** 134-137.

Shinkai, K., Akedo, H., Mukai, M., Imamura, F., Isoai, A. 1996. Kobayashi M, Kitagawa I. Inhibition of in vitro tumor cell invasion by ginsenoside Rg3. *Jpn. J. Cancer Res.* **87:** 357-362.

Skjak-Braek, G., Martinsen, A. 1991. In: Guiry, M. D., Blunden, G. (Eds.). Seaweed Resources in Europe: Uses and Potential. John Wiley & Sons, pp. 219-256.

Solomons, N. W., Bulux, J. 1994. Plant sources of pro-vitamin A and human nutriture. *Nutr. Rev.* **51:** 199-204.

Stepien, K., Tomaszewski, M., Czuczwar, S. J. 2005. Neuroprotective properties of statins. *Pharmacol. Rep.* **57:** 561-569.

Strand, V., Kavanaugh, A. F. 2004. The role of interleukin-1 in bone resorption in rheumatoid arthritis. *Rheumatol.* **43:** 10-16.

Su, B., Karin, M. 1996. Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr. Opin. Immunol.* **8:** 402-411.

Swantek, J. L., Cobb, M. H., Geppert, T. D. 1997. Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK. *Mol. Cell Biol.* **17:** 6274-6282.

Szatrowski, T. P., Nathan, C. F. 1991. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res.* **51:** 794-798.

Tang, H. F., Yi, Y.H., Yao, X.S., Wu, J. H , Zhang, S.Y., Xu, Q.Z. 2002. Studies on the chemical constituents from marine brown alga Ishige okamurai. *Zhongguo Zhong Yao Za Zhi.* **27:** 269- 273.

Tilg, H., Wilmer, A., Vogel, W., Herold, M., Nolchen, B., Judmaier, G., Huber, C. 1992. Serum levels of cytokines in chronic liver diseases. *Gastroenterol.* **103:** 264-274.

Trachootham, D., Zhou, Y., Zhang, H., Demizu, Y., Chen, Z., Pelicano, H., Chiao, P. J., Achanta, G., Arlinghaus, R. B., Liu, J., Huang, P. 2006. Selective killing of ncogenically transformed cells through a ROS mediated mechanism by b-phenylethyl isothiocyanate. *Cancer Cell* **10:** 241-252.

Umit Bagriacik, E. Uslu, K., Yurtcu, E., Stefek, M., Karasu, C. 2007. Stobadine inhibits

doxorubicin-induced apoptosis through a caspase-9 dependent pathway in P815 mastocytoma cells. *Cell Biol. Int.* **31:** 979-984.

Uto, T., Fujii, M., Hou D. X. 2005. 6-(Methylsulfinyl)hexyl isothiocyanate suppresses inducible nitric oxide synthase expression through the inhibition of Janus kinase 2-mediated JNK pathway in lipopolysaccharide-activated murine macrophages. *Biochem. Pharmacol.* **70:**1211-21.

Valbonesi, P., RicciFranzellitti, L. S., Biondi, C., Fabbri, E. 2008. Effects of cadmium on MAPK signalling pathways and HSP70 expression in a human trophoblast cell line *Placenta* **29:** 725-733.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., Mazur, M. 2006. Free radical, metals and antioxidants in oxidative stress-induce cancer. *Chem. Biol. Interact*. **160:** 1-40.

Wang, X. W., Zhan, Q., Coursen, J. D., Khan, M. A., Kontny, H. U., Yu, L., Hollander, M. C., O'Connor, P. M., Fornace, A. J., Harris, C. C. 1993. GADD45 induction of a G2/M cell cycle checkpoint. *Proc. Natl. Acad. Sci. U.S.A*. **96:** 3706-3711.

Wang, Z., Cao, N., Nantajit, D., Fan, M., Liu, Y., Li, J. J. 2008. Mitogen-activated protein kinase phosphatase-1 represses c-Jun NH2-terminal kinase-mediated apoptosis via NF-kappaB regulation. *J. Biol. Chem.* 283: 21011-21023.

Weinberg, R. A. 1995. The retinoblastoma protein and cell cycle control. *Cell* **81:** 323–330.

Weinstein, I. B. 2000. Disorders in cell circuitry during multistage carcinogenesis: the role of homeostasis. *Carcinogenesis* **21:** 857-864.

Wen, J., You, K. R., Lee, S. Y., Song, C. H., Kim, D.G. 2002. Oxidative stress-mediated apoptosis: the anticancer effect of the sesquiterpene lactone parthenolide. *J. Biol. Chem.* **277:** 38954-38964.

White, E. 1996. Life, death, and the pursuit of apoptosis. *Genes Dev.* **10:** 1-15.

Wollenhaupt, K., Danicke, S., Brussow, K. P., Tiemann, U. 2005. *In vitro* and *in vivo* effects of deoxynivalenol (DNV) on regulators of cap dependent translation control in porcine endometrium. *Reprod. Toxicol.* 21: 60-73.

Wu, C. C., Lin, J. P., Yang, J. S., Chou, S. T., Chen, S. C., Lin, Y. T., Lin, H. L., Chung, J. G. 2006. Capsaicin induced cell cycle arrest and apoptosis in human esophagus epidermoid carcinoma CE81T/VGH cells through the elevation of intracellular reactive oxygen species and Ca2+ productions and caspase-3 activation. *Mut. Res.* **601:** 71-82.

Xia, Z., Dickens, M. Raingeaud, J. Davis, R. J. Greenberg, M. E. 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 270: 1326-1331.

Yan, X., Chuda, Y., Suzuki, M., Nagata, T. 1999. Fucoxanthin as the major antioxidant in *Hijikia fusiformis*, a common edible seaweed. *Biosci. Biotechnol. Biochem.* **63:** 605-607.

Yang, E., Korsmeyer, S. J. 1996. Molecular thanatopsis: a discourse on the BCL2 family and cell death. *Blood* **88:** 386-401.

Yang, F., OZ, H. S., Barve, S., de Villiers, W. J., McClain, C. J., Varilek, G. W. 2001. The green tea polyphenol (-)-Epigallocatechin-3-Gallate blocks nuclear factor-κB activation by 58 inhibition IκB kinase activity in the intestinal epithelial cell line IEC-6. *Mol. Pharmacol*. **60:** 528-533.

Yang, L. L., Lee, C. Y., Yen, K. Y. 2000. Induction of apoptosis by hydrolysable tannins from *Eugenia jambos* L. on human leukemia cells. *Cancer lett.* **157:** 65-75.

Zanke, B. W., Boudreau, K., Rubie, E., Winnett, E., Tibbles, L. A., Zon, L., Kyriakis, J., Liu, F. F., Woodgett, J.R. 1996. The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. *Curr. Biol.* **6:** 606-13.

Zhang, Y., Chen, F. 2004. Reactive oxygen species (ROS), troublemakers between nuclear factor-kappaB (NF-kappaB) and c-Jun NH(2)-terminal kinase (JNK). *Cancer Res.* **64:** 1902-1905.

Zhang, Z., Leonard, S. S., Huang, C., Vallyathan, V., Castranova, V. Shi, X. 2003. Role of reactive oxygen species and MAPKs in vanadate-induced G_2/M phase arrest, Free Radic. *Biol. Med*. **34:** 1333-1342.

Zhao, R., Xiang, N., Domann, F. E., Zhong, W. 2006. Expression of p53 enhances selenite-induced superoxide production and apoptosis in human prostate cancer cells. *Cancer Res.* **66:** 2296-2304.

Zhou, B. P., Liao, Y., Xia W., Spohn, B., Lee M. H., Hunq, M. C. 2001. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat. Cell Biol.* **3:** 245-252.

ACKNOWLEDGEMENT

박사졸업 논문을 쓰면서 학부와 석•박사 10 년의 시간동안을 돌아보며 이 논문이 있기까지 도움을 주신 많은 분들께 감사의 인사를 드리고자 합니다. 진로를 제대로 잡지 못했던 학부시절부터 대학원생활을 거쳐 본 논문이 있기까지 세심한 가르침으로 절 이끌어주신 전유진 교수님께 머리숙여 진심으로 감사드립니다. 또한 바쁘신 중에도 이 논문이 좀 더 나은 방향으로 나아 갈 수 있게 심사해 주시고 관심을 보여주신 이기완 교수님, 김수현 교수님, 허문수 교수님, 김기영 교수님, 학부시절부터 대학원기간 동안 늘 많은 관심과 조언을 해주셨던 송춘복 교수님, 이제희 교수님 그리고 여인규 교수님께 진심으로 감사드립니다. 생리활성 실험을 처음 가르쳐 주신 현진원 교수님과 강경아 박사님 그리고 연구에 전념 할 수 있도록 많은 도움을 주신 하진환 교수님께도 감사의 마음을 전합니다.

대학원생활 동안 항상 옆에서 힘이 되어 주었던 우리 실험실원 수진이 형, 마힌다, 선희, 긴내, 승홍, 성명, 석천, 아름다슬, 원우, 창익, 민철, 상원, 주영, 혜미, 재영, 자나카, 양수동에게 감사인사드립니다. 대학원 생활하면서 많은 도움을 준 맹진이 형, 철홍이 형, 영건이 형, 문휴 형, 언제나 항상 옆에 있어주었던 동기 만철이에게 감사하다는 말을 전합니다. 또한 인생선배로서 많은 조언을 해주신 진창남 선배님, 김원석 선배님, 양현필 선배님, 강도형 선배님, 박경일 선배님, 양병규 선배님께도 감사의 말씀을 드립니다.

현재 (재) 제주하이테크산업진흥원에 근무하면서 박사학위를 무사히 마칠 수 있도록 시간을 허락해주시고 격려를 해주신 고유봉원장님께 감사드리며, 저희 생물종다양성연구소 이욱재실장님, 박수영팀장님, 정용환팀장님외 많은 박사님들과 직원분들에게 고마운 마음을 전합니다. 특히, 이 논문이 있기까지 옆자리에서 많은 도움을 준 원종이 형에게 감사의 말을 전합니다.

항상 장남인 큰아들이 잘되기만을 바라시는 아버지, 이젠 두 아이에 엄마가 된 누나, 새로운 가족이 된 매형, 든든한 남동생 그리고 아빠라는 이름으로 불릴 수 있게해준 나에 에너지 형규 마지막으로 힘들때나 즐거울때나 같이하고 연구에만 몰두할 수 있도록 내조해준 내조에 여왕 효정에게 이 논문을 바칩니다. 그리고 이 글을 보고 계시는 모든 분들께 감사드리며 항상 건강하시고 좋은 일만 있기를 바랍니다. 그리고 사랑합니다.