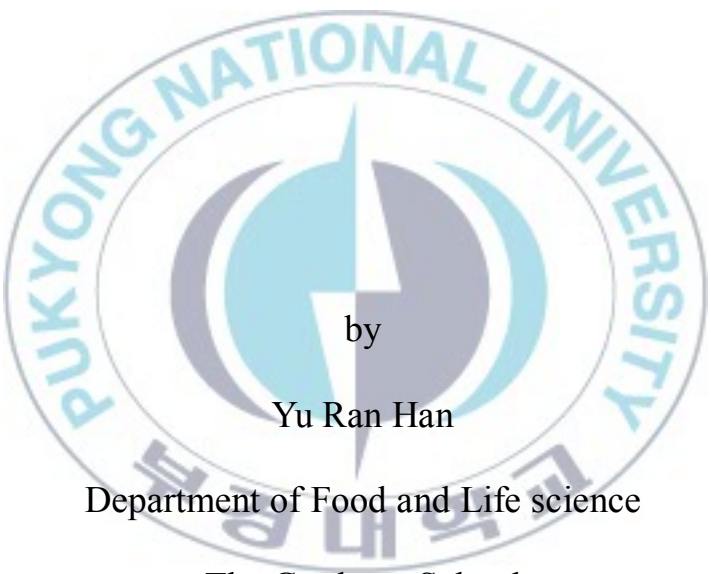
Thesis for the Degree of Master of Science

Antioxidant and anti-inflammatory activities of *Hizikia fusiformis*



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Antioxidant and anti-inflammatory activities of *Hizikia fusiformis*

(톳의 항산화 및 항염증 효과)

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Contents

LIST OF TABLES ---------- i

LIST OF FIGURES ---------- ii

LIST OF ABBREVIATIONS ---------- iii

Abstract ---------- iv

Ⅰ. Introduction ---------- 1

Ⅱ. Materials and Methods ---------- 4

1. Material ---------- 4

2. Chemicals and Instruments ---------- 4

2-1. Chmicals ---------- 4

2-2. General experimental procedures ---------- 5

2-3. Instruments ---------- 6

2-4. Cell line ---------- 6

3. Method ---------- 7

3-1. Extraction, fractionation, and isolation of *Hizikia fusiformis* ---------- 7

3-2. Spectroscopic properties of compounds isolated from CH_2Cl_2 fraction of *H.*

fusiformis---------- 10

3-3. Evaluation of Antioxidant activity ---------- 11

3-3-1. Determination of total phenolic contents (TPCs) ---------- 11

3-3-2. Assay for $ONOO^-$ scavenging activity ---------- 11

3-3-3. Assay for DPPH radical scavenging activity ---------- 12

3-3-4. Assay for $ABTS^{\bullet+}$ radical scavenging activity ---------- 13

3-3-5. Inhibition of $ONOO^-$ mediated tysorine nitration ---------- 14

3-4. Evaluation of anti-inflammatory activity ---------- 15

3-4-1. Cell culture ---------- 15

3-4-2. Assay for cell viability ---------- 16

3-4-3. Assay for measurement of cellular NO production ---------- 16

3-4-4. Assay for inhibition of intracellular ROS generation ---------- 17

3-4-5. Analysis for inhibition of iNOS and COX-2 protein expression ----- 17

3-5. Statics ---------- 18

Ⅲ. Result ---------- 19

1. Antioxidant activities of MeOH of *H. fusiformis* extract and its solvent-soluble fractions ---------- 19

1-1. Total phenolic content ---------- 19

1-2. $ONOO^-$ scavenging activity ---------- 21

1-3. DPPH radical scavenging activity ---------- 21

1-4. ABTS radical scavenging activity ---------- 22

1-5. Inhibitory activity of $ONOO^-$ mediated tyrosine nitration ---------- 25

2. Anti-inflammatory activities of MeOH extract of *H. fusiformis* and its solvent-soluble fractions on RAW 264.7 cells ---------- 28

2-1. Effect of cell viability ---------- 28

2-2. Inhibitory activity of NO production ---------- 28

2-3. Inhibitory activity of intracellular ROS generation ---------- 32

2-4. Effects of the CH_2Cl_2 and EtOAC fractions of *H. fusiformis* on LPS- induced iNOS and COX-2 protein expression ---------- 37

3. Isolation of compounds from the CH_2Cl_2 fraction of *H. fusiformis* ---------- 39

Ⅳ. Discussion ---------- 41

Ⅴ. Conclusion ---------- 55

Ⅵ. References ---------- 56

감사의 글

LIST OF TABLES

Table **Page**

Table 1. Yeild and total phenolic content MeOH extract and its fractions from *H. fusiformis* ---------- 20
Table 2. Antioxidant activities of MeOH extract and its fractions from *H. fusiformis* ---------- 24

LIST OF FIGURES

Figure **Page**

Fig. 1. Extraction and fractionation of *H. fusiformis* ---------- 9
Fig. 2. Effects of the MeOH extract and its solvent soluble fractions from *H. fusiformis* on the nitration of BSA by $ONOO^-$ ---------- 26, 27
Fig. 3. Effects of the MeOH extract and its solvent soluble fractions from *H. fusiformis* on LPS-induced NO production and cell viability in RAW 264.7 cells ---------- 30
Fig. 4. Effect of the CH_2Cl_2 fraction from *H. fusiformis* on LPS-induced NO production and cell viability in RAW 264.7 cells ---------- 31
Fig. 5. Effects of the MeOH extract and its solvent soluble fractions from *H. fusiformis* on the *t*-BHP-induced ROS generation in RAW 264.7 cells -- 34
Fig. 6. Effect of the CH_2Cl_2 fraction from *H. fusiformis* on the *t*-BHP-induced ROS generation in RAW 264.7 cells ---------- 35
Fig. 7. Effect of the EtOAc fraction from *H. fusiformis* on the *t*-BHP-induced ROS generation in RAW 264.7 cells ---------- 36
Fig. 8. Effects of the CH_2Cl_2 and EtOAc fractions on LPS-induced iNOS and COX-2 expression in RAW 264.7 cells ---------- 38
Fig. 9. Structures of 24-methylene cholesterol and fucosterol ---------- 40

LIST OF ABBREVIATIONS

ABTS	: 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
AMT	: 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride
BSA	: bovine serum albumin
COX-2	: cyclooxygenase-2
^{13}C-NMR	: carbon 13 nuclear magnetic resonance
DCFH-DA	: 2',7'-dichlorodihydro-fluorescein diacetate
DMEM	: Dulbecco's modified Eagle's medium
DMSO	: dimethyl sulfoxide
DPPH	: 1,1-diphenyl-2-picrylhydrazyl
DTPA	: diethylene pentaacetate
FBS	: fetal bovine serum
^{1}H-NMR	: proton nuclear magnetic resonance
iNOS	: inducible nitric oxide synthase
IC_{50}	: 50% inhibitory concentration
LPS	: lipopolysaccharide
MTT	: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
t-BHP	: *tert*-butylhydroperoxide
PVDF	: polyvinylidene fluoride
Si gel	: silica gel
TLC	: thin-layer chromatography.

톳의 항산화 및 항염증 효과

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초 록

다양한 프리 라디컬들에 의해 생성되는 산화적 스트레스는 염증질환 및 노화, 암, 당뇨등의 다양한 질병들을 일으키는 주요원인으로 작용한다. 체내의 항산화 방어시스템의 불균형으로 인해 Reactive oxygen species (ROS), reactive nitrogen species (RNS), superoxide ($\bullet O_2$), hydrogen peroxide (H_2O_2) 와 같은 프리 라디컬들이 과다하게 생성되면 면역체계는 무너지게 되고 이에 따라 각종 염증 사이토카인과 염증 관련 효소 및 유전자들이 발현되며 결국 염증 질환 및 염증 관련 질병들을 일으키게 된다. 또한 증가된 프리 라디컬은 nitrite oxide (NO)와 연쇄반응을 일으켜 peroxynitrite ($ONOO^-$) 와 nitro tyrosine 같은 반응생성물을 생성한다. 특히 $ONOO^-$는 반응성이 높아 세포독성뿐 아니라 세포막의 지질과산화를 일으며 세포죽음과 조직손상을 야기시키게 된다. 따라서 질병들의 근원이되는 산화적 스트레스를 감소시키고 예방하는 것은 중요한 의미를 가질 것이다.

톳은 한국, 일본 그리고 중국 등지의 해안에서 풍부하게 서식하고 있는 식용 갈조류이다. 아시아 지역에서 건강기능 식품으로서 널리 이용되고 있으며 다양한 건강적 이점들 때문에 그 활성들에 대한 집중적 연구가 진행되어 오고있다. 이번 연구에서는 톳의 메탄올 추출물과 그 분획물을 이용하여 총 페놀함량 측정, peroxynitrite ($ONOO^-$), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) 라디컬, 그리고 $ONOO^-$를 매개로 하여 생성되는 tyrosine nitration의 소거 및 억제에 대한 효과를 *in vitro* assay를 통하여 항산화 활성을 평가하였다. 뿐만 아니라 마우스의 대식세포로부터 얻어지는 RAW 264.7 세포주를 사용하여 lipopolysaccharide (LPS) 에 의해 유도되는 NO의 생성, *tert*-

butylhydroperoxide (*t*-BHP) 로 유도되는 세포내 ROS의 생성, 그리고 LPS의 유도로 발현되는 inducible nitric oxide synthase (iNOS) 와 cyclooxygenase-2 (COX-2) 단백질 발현의 억제효과를 평가함으로서 항염증 활성을 조사하였다. 그 결과, 실험에 사용된 톳의 메탄올 추출물 및 그 분획물들 중에서 ethyl acetate (EtOAc) 분획물이 55.86 GAE, mg/g of fraction으로 가장 높은 페놀함량을 가지고 있었고 $ONOO^-$, DPPH, ABTS 라디컬에 대해 가장 높은 프리 라디컬 소거활성을 보였으며 그들의 IC_{50} 값은 각각 9.54, 216.75, 그리고 55.71 μg/ml 이였다. 게다가 EtOAc 분획물은 3.12, 6.25, 12.5, 그리고 25 μg/ml의 농도범위에서 $ONOO^-$에 의해 생성되는 tyrosine nitration을 효과적으로 억제하였다. 그리고 dichloromethane (CH_2Cl_2) 분획물 또한 $ONOO^-$에 의해 생성되는 tyrosine nitration을 12.5 에서 100 μg/ml 농도범위 에서억제활성을 보였다. 그리고 RAW 264.7 세포에서 LPS로 유도되는 NO의 생성을 가장 효과적으로 억제하였으며 그의 IC_{50} 값은 4.46 μg/ml로 나타났고 CH_2Cl_2 분획물의 처리는 LPS에 의해 발현량이 높아지는 iNOS 단백질을 2.5, 5, 그리고 10 μg/ml 농도에서 상당히 억제하였다. *t*-BHP로 유도되는 세포 내 ROS의 생성 실험의 경우에서는 CH_2Cl_2와 EtOAc 분획물 모두 세포독성을 띄지 않는 12.5 μg/ml 그리고 12.5 에서 100 μg/ml 처리 농도 사이에서 각각 ROS의 생성을 감소 시켰다.

이번 연구결과를 통해 톳의 CH_2Cl_2와 EtOAc 분획물이 상당한 항산화 및 항염증 활성을 가짐을 확인하였으며 이들 분획물에서 생리활성물질을 찾아 그 활성을 연구함으로서 앞으로 산화적스트레스와 연관되는 염증적 질환에 대해 효과적인 예방 및 치료제가 될 수 있을 것이라 기대된다.

Ⅰ. Introduction

Oxidative stress is characterized by the excessive production of reactive oxygen species (ROS), including the superoxide radicals ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and the hydroxyl (OH) radicals, all of which can attack proteins, lipid membranes, and nucleic acids, causing cell injury and apoptosis (Simon *et al*., 2000). ROS and reactive nitrogen species (RNS) are produced in cells or tissues under physiological and pathological conditions and the interaction between ROS and RNS can lead to the production of highly reactive species such as peroxynitrite ($ONOO^-$), a product of NO and superoxide (Ischiropoulos *et al*., 1992). $ONOO^-$ is able to induce oxidative damage and may cause harm to cellular targets (Epe *et al*., 1996). The damaging influences of oxidative stress also cause inflammation accompanied by overproduction of pro-inflammatory mediators. Inflammation, complex response to injury caused by noxious physical or chemical stimuli, is considered a typical component of many diseases such as arthritis, asthma, multiple sclerosis, inflammatory bowel disease and atherosclerosis. (Nathan, 2002; Guzic *et al*., 2003; Rankin, 2004). The inflammatory responses are controlled by excessive release of soluble pro-inflammatory mediators such as ROS, NO, prostaglandin E2 (PGE_2) generated by activated iNOS and COX-2, respectively (Walsh *et al*., 2005;). Overproduction of several cytokines and pro-

inflammatory molecules interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α), and NO can modulate inflammation. It is well known that various cytokines play important roles in the regulation of the immune response (Bonizzi and Karine, 2004). Thus inhibition of the production of these inflammatory mediators is an important target in the treatment of inflammatory disease. And scavenging of free radical and inhibitory activation of NO production might be a useful therapeutic strategy in the treatment of inflammatory diseases (Hobbs *et al*., 1999; Sautebin, 2000). The diverse and unique chemical structures of marine algae have been of great interest, as they are associated with a wide array of pharmacological activities. Numerous crude extracts and pure compounds obtained from algae have been reported to possess antioxidant and radical scavenging activities (Lim *et al*., 2002; Kang *et al*., 2004; Kuda *et al*., 2005; Duan *et al*., 2006; Ganesan *et al*., 2008; Zou *et al*., 2008). Therefore, we carried out several tests in radical-scavenging assays to assess the antioxidant and NO inhibition assays to evaluate the anti-inflammatory properties of *H. fusiformis* and also to reveal the interrelationship of the antioxidant and anti-inflammatory effect.

H. fusiformis, a representative edible brown seaweed, belongs to the family Sargassaceae and is distributed in the temperate seaside areas of the northwest Pacific, including Korea, Japan, and China, has been used widely as a health food for hundreds of years (Dobashi *et al*., 1989; Tokudome *et al*., 2001). In Korea, it is commonly found on the west, and south coasts and on Jeju Island. *H. fusiformis*

is well known for its distinctive flavor and high contents of calcium, vitamin A, inorganic salt, iodine and dietary fiber, all of which help to prevent different diseases such as diabetic, high blood pressure, colorectal cancer, constipation, thyroid cancer, beriberi (Arasaki and Arasaki, 1983), arteriosclerosis, and postmenopausal disorders like osteoporosis (Watanabe *et al*., 1979; Li *et al*., 2006), also possesses a variety of biological activities including, antioxidant (Ryu *et al*., 1989; Lee *et al*., 1996; Ko *et al*., 2002; Park *et al*,. 2005), antimicrobial (Abdussalam, 1990; Kim *et al*., 1994; Lim *et al*., 1995), anticancer (Kim *et al*., 1990; Bae, 2004; Kim *et al*., 2005), anti-mutagen (Okai *et al*., 1993; Yasuji *et al*., 1994), and anticoagulation activities (Kim *et al*., 1998; Yoon *et al*., 2000; Koo *et al*., 2001) as well as modulator of lipid metabolism (Jung *et al*., 2001). Owing to its high therapeutic value, extensive research has been focused on the isolation of bioactive substances from *H. fusiformis*. Previously, laminaran, fucoidan and fucoxanthin isolated from *H. fusiformis*, which have been reported as potential immunomodulator (Church *et al*., 1989; Shan *et al*., 1999), an anti coagulant (Kim *et al*., 1998; Li *et al*., 2006) and a free radical scavenging activity (Yan *et al*., 1999). However, the biochemical mechanisms underlying *H. fusiformis*-mediated antioxidant and anti-inflammatory activities have not yet been clarified in detail. The aim of this research was to investigate the potential antioxidant and anti-inflammatory activities from *H. fusiformis* MeOH extract and its solvent-soluble fractions.

Ⅱ. Materials and Method

1. Material

The *H. fusiformis* used in this study was purchased from Wando, Republic of Korea, in April 2012. A whole plant voucher specimen was registered and deposited at the Department of Food and Life Science, Pukyong National University, Busan, South Korea (Professor Choi, J. S.).

2. Chemicals and Instruments

2-1. Chemicals

Phosphomolybdic-phosphotungstic acid reagent (Folin-Ciocalteu reagent), sodium carbonate (Na_2CO_3), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), potassium persulfate ($K_2S_4O_8$), dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS) from *Escherichia coli*, Griess reagent, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), *t*-butylhydroperoxide (*t*-BHP), dihydrorhodamine 123 (DHR123), diethylene pentaacetate (DTPA), 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxyl acid

(trolox), bovine serum albumin (BSA), and fetal bovine serum (FBS) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Hyclone (Logan, UT, USA). Primary antibodies (iNOS, COX-2, and β-actin) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was obtained from Millipore Co. (Billerica, MA, USA). Supersignal® West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). All chemicals and solvents used were purchased from E. Merck, Fluka, or Sigma-Aldrich Co., unless stated otherwise.

2-2. General experimental procedures

All ^{1}H- and ^{13}C-NMR spectra were measured in deuterated chloroform ($CDCl_3$) using a JEOL JNM ECP-400 spectrometer at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR. Chemical shifts were referenced to the respective residual solvent peak (2.50 ppm for ^{1}H- and 39.5 ppm for ^{13}C-NMR). Column chromatography was performed using silica (Si) gel 60 (70-230 mesh, Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was conducted on precoated Merck Kieselgel 60 F254 plates (20 × 20 cm, 0.25 mm) using 50% H_2SO_4 as a spray reagent. All solvents for column chromatography were of reagent grade and were acquired from commercial sources.

2-3. Instruments

Absorbace for DPPH, ABTS radical scavenging assay and cell viability, NO production assay was measured by microplate spectrophotometer (Molecular Devices, CA, USA). Ultrospec®2100pro UV/visible spectrophotometer (Amersham Biosciences, NJ, USA) was used for total phenolic content assay. And measurement of ROS and $ONOO^-$ was measured by microplate fluorescence reader (Bio-Tek Instruments Inc., FLx 800, UT, USA).

2-4. Cell line

RAW 264.7 cells which are originated from mouse leukaemic monocyte macrophage was used for experiment and it was parceled out through American Tissue Culture Collection (ATCC, Rockville, MD, USA).

3. Method

3-1. Extraction, fractionation, and isolation of *Hizikia fusiformis*

The lyophilized powder of *H. fusiformis* (338 g) was refluxed in methanol (MeOH) for 3 h (1L × 3). The total filtrate was concentrated to dryness *in vacuo* at 40°C to acquire the MeOH extract. The extract (52.35g) was suspended in distilled water and then successively partitioned with dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) to yield the CH_2Cl_2 (4.53 g), EtOAc (0.44 g), and *n*-BuOH (2.55 g) fractions, respectively, as well as an aqueous residue (43.03 g) (Fig. 1).

To isolate the compounds, *H. fusiformis* (5 kg) was refluxed in MeOH for 3 h (3L × 5). The extract (1.68 kg) was suspended in distilled water and then partitioned with CH_2Cl_2 to yield the CH_2Cl_2 fraction (134.3 g). The CH_2Cl_2 fraction was subjected to column chromatography over a Si-gel column using hexane-acetone (100:0 → 1:1 → MeOH, gradient) to obtain 15 subfractions (F-1 to F-15). Among them, F-4 (13.46 g) was chromatographed over a Si-gel column using hexane-acetone (8:1 → MeOH) to afford 15 subfractions (F-4-1 to F-4-15). Subfraction F-4-5 (2 g) was chromatographed over a Si-gel column using hexane-acetone (40:1 → 35:1 → 25:1 → 10:1 → 1:1 → 1:5 → Acetone → MeOH) to obtain 15 subfractions (F-4-5-1 to F-4-5-15). Subfraction F-4-5-2 (96.7 mg) was chromatographed over a Si-gel column using hexane-acetone (25:1 → MeOH) to

obtain ten subfractions (F-4-5-2-1 to F-4-5-2-10). Among them, subfraction F-4-5-2-4 (69.6 mg) was chromatographed over a Si-gel column using hexane-acetone (18:1 → MeOH) to afford six subfractions (F-4-5-2-4-1 to F-4-5-2-4-6). The filtrate of subfraction F-4-5-2-4-3 (15.1 mg) was then chromatographed over a Si-gel column with hexane-acetone (50:1 → MeOH) to yield 24-methylene cholesterol (7.9 mg). F-5 (16.24 g) was chromatographed over a Si-gel column using hexane-acetone (25:1 → 20:1 → acetone → MeOH) to afford eight subfractions (F-5-1 to F-5-8). Subfraction F-5-4 (10.08 g) was chromatographed over a Si-gel column using hexane-acetone (30:1 →1:1 → acetone → MeOH, gradient) to obtain four subfractions (F-5-4-1 to F-5-4-4). F-5-4-2 and F-5-4-3 were recombined and recrystallized with MeOH to afford fucosterol (1.05 g). Those two compounds, 24-methylene cholesterol and fucosterol, were identified by spectroscopic analysis, including ^{1}H- and ^{13}C NMR, as well as by comparison with published spectral data and TLC analysis (Lu *et al*., 2004; Jung *et al*., 2013).

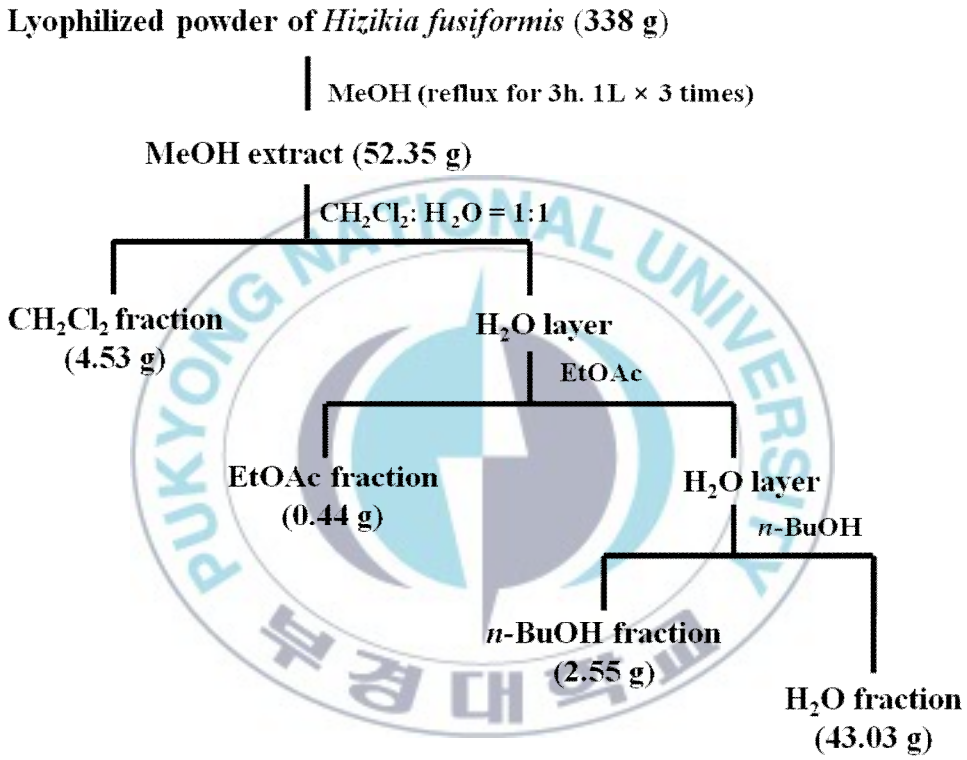


Fig. 1. Extraction and fractionation of *H. fusiformis*

3-2. Spectroscopic properties of compounds isolated from CH_2Cl_2 fraction of *H. fusiformis*

24-Methylene cholesterol – White amorphous powder: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.35 (1H, br d, *J* = 5.2 Hz, H-6), 4.63 (2H, s, CH_2-28), 3.53 (1H, m, H-3), 1.03 (3H, s, H-19), 1.01 (3H, d, *J* = 6.8 Hz, H-21), 0.99 (3H, d, *J* = 6.8 Hz, H-27), 0.98 (3H, d, *J* = 6.4 Hz, H-26), 0.68 (3H, s, H-18). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 156.8 (C-24), 140.7 (C-5), 121.7 (C-6), 105.9 (C-28), 71.8 (C-3), 56.7 (C-14), 55.9 (C-17), 50.1 (C-9), 42.3 (C-13), 42.2 (C-4), 39.7 (C-12), 37.2 (C-1), 36.4 (C-10), 35.7 (C-22), 34.6 (C-25), 33.8 (C-23), 31.8 (C-7), 31.8 (C-8), 31.6 (C-2), 30.9 (C-20), 28.2 (C-16), 24.3 (C-15), 21.9 (C-26), 21.8 (C-27), 21.0 (C-11), 19.3 (C-19), 18.7 (C-21), 11.9 (C-18).

Fucosterol - White amorphous powder: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.34 (1H, br d, *J* = 5.2 Hz, H-6), 5.19 (1H, q, *J* = 6.8 Hz, H-28), 3.53 (1H, m, H-3), 1.58 (3H, d, *J* = 6.8 Hz, H-29), 1.03 (3H, s, H-19), 1.01 (3H, d, *J* = 6.8 Hz, H-21), 0.99 (3H, d, *J* = 6.8 Hz, H-27), 0.98 (3H, d, *J* = 6.4 Hz, H-26), 0.68 (3H, s, H-18). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 146.9 (C-24), 140.7 (C-5), 121.7 (C-6), 115.5 (C-28), 71.8 (C-3), 56.7 (C-14), 55.7 (C-17), 50.1 (C-9), 42.3 (C-13), 42.2 (C-4), 39.7 (C-12), 37.2 (C-1), 36.5 (C-10), 36.4 (C-20), 35.2 (C-22), 34.7 (C-25), 31.9 (C-7), 31.9 (C-8), 31.6 (C-2), 28.2 (C-16), 25.6 (C-23), 24.3 (C-15), 22.2 (C-26), 22.1 (C-27), 21.0 (C-11), 19.4 (C-19), 18.7 (C-21), 13.2 (C-29), 11.8 (C-18).

3-3. Evaluation of antioxidant activity

3-3-1. Determination of total phenolic contents (TPCs)

TPC values of the MeOH extract and its different solvent-soluble fractions were determined using the Folin–Ciocalteu assay with minor modifications (Iqbal & Bhanger, 2006). The reaction mixtures contained 250 µl of each sample at final concentration 400 µg/ml and 750 µl of Folin–Ciocalteu reagent, and were kept at ambient conditions for 5 min, followed by the addition of 2 ml of 7.5% Na_2CO_3. The final mixture was diluted to a 7 ml total volume with deionized H_2O. The reaction mixtures were kept in the dark at ambient conditions for 1 h to complete the reaction, and the absorbance was measured at 765 nm on an Ultrospec®2100pro UV/visible spectrophotometer (Amersham Biosciences, NJ, USA). All experiments were conducted using gallic acid as a calibration standard, and the results were recorded as mg of gallic acid equivalent per g of dried extract or fraction (GAE, mg/g of each extract or fraction).

3-3-2. Assay for $ONOO^-$ scavenging activity

Peroxynitrite ($ONOO^-$) scavenging activity was assessed by a modified Kooy's

method (Kooy *et al*., 1994) that involved the monitoring of fluorescent rhodamine 123 produced from non-fluorescent DHR 123 in the presence of $ONOO^-$. The rhodamine buffer (pH 7.4) consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 μM DTPA. The final DHR 123 concentration was 5 μM. The buffer in this assay was prepared prior to use and placed on ice. The samples were dissolved in 10% DMSO (final concentration 50 μg/ml). Authentic $ONOO^-$ (10 μM) dissolved in 0.3 N sodium hydroxide was or was not added, and after 5 minutes, the background and final fluorescent intensities were measured. The fluorescence intensity of oxidized DHR 123 was evaluated using a microplate fluorescence reader (Bio-Tek Instruments Inc., FLx 800, Winooski, UT, USA) at excitation and emission wavelengths of 480 and 530 nm, respectively. Peroxynitrite-scavenging values were calculated as the final fluorescence intensity minus the background fluorescence *via* the detection of DHR 123 oxidation and were expressed as mean ± S.D. L-Penicillamine was used as a positive control.

3-3-3. Assay for DPPH radical scavenging activity

DPPH radical scavenging activity was evaluated using the method of Blois (1958), with slight modifications. *H. fusiformis* samples and DPPH were dissolved in MeOH. After mixing gently and standing at room temperature for 30

min, the optical density of the reactant was measured at 520 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The DPPH radical scavenging activity of the *H. fusiformis* MeOH extract and its solvent-soluble fractions were expressed in terms of IC_{50} value (μg/ml, required to scavenge DPPH radical formation by 50%), which was calculated from the log-dose inhibition curve and expressed as mean ± S.D. L-Ascorbic acid was used as a positive control.

3-3-4. Assay for $ABTS^{\bullet+}$ radical scavenging activity

This assay is based on the ability of different substances to scavenge ABTS radical cations ($ABTS^{\bullet+}$) as compared to the positive control trolox according to the method developed by Pellegrini (1999). $ABTS^{\bullet+}$ radical cations were produced by reacting ABTS with potassium persulfate ($K_2S_4O_8$). ABTS was dissolved at a 7 mM concentration in 10 ml H_2O, and ABTS radical cation was produced by adding 400 μl of 60 mM $K_2S_4O_8$ (final concentration 2.45 mM). The mixture was stored in the dark at room temperature for 16 h. The radical was stable in this form for more than two days when stored in the dark at room temperature. The photometric assay was carried out with 180 μl of $ABTS^{\bullet+}$ solution with 20 μl of *H. fusiformis* MeOH extract and its solvent-soluble fractions, which were dissolved in EtOH solution (final concentration 100 μg/ml). All extract and fractions were

stirred for 30 seconds. The optical density was measured at 734 nm after 2 min using a microplate spectrophotometer (Molecular Devices). The $ABTS^{\bullet+}$ radical scavenging of *H. fusiformis* was calculated by determining the decrease in absorbance at different concentrations and was expressed as mean ± S.D. Trolox and L-ascorbic acid were used as positive controls.

3-3-5. Inhibition of $ONOO^-$ mediated tyrosine nitration

$ONOO^-$-mediated protein tyrosine nitration was evaluated using the method of Aulak *et al* (2001), with slight modifications. In order to examine the inhibition of $ONOO^-$-induced BSA nitration, 2.5 μl of various concentrations of *H. fusiformis* MeOH extract and its solvent-soluble fractions dissolved in 10% DMSO were added to 95 μl of BSA (0.4 mg protein/ml) and mixed with 2.5 μl of $ONOO^-$ (200 μM). After incubation with shaking at 37°C for 20 min, the mixed sample was added to Bio-Rad gel buffer in a ratio of 1:1 and boiled for 5 min to denature the proteins. The total protein equivalent for the reactant was separated on 10% SDS-polyacrylamide minigel at 80V for 30 min, followed by 100V for 1 h and then transferred to a PVDF membrane at 80V for 110 min in a wet transfer system (Bio-Rad, Hercules, CA, USA). The membrane was immediately placed in a blocking solution (5% non-fat dry milk in TBS-Tween buffer (w/v), Bio-Rad TBS, and 0.1% Tween-20, pH 7.4) at room temperature for 1 h. The membrane was

washed three times (10 min) in TBS-Tween buffer and incubated with a monoclonal anti-nitrotyrosine antibody (5% non-fat dry milk), diluted 1:2500 in TBS-Tween buffers at 4°C overnight. After three more washes in TBST buffer (10 and 5 min), the membrane was incubated with horseradish peroxidase-conjugated sheep-anti-mouse secondary antibody diluted 1:2000 in TBST buffer at room temperature for 1 h. After three washes in TBST buffers, antibody labeling was visualized using the Supersignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions, and the membrane was exposed to X-ray film (Kodak, Rochester, NY, USA). Pre-stained blue protein markers were used for molecular-weight determination.

3-4. Evaluation of anti-inflammatory activity

3-4-1. Cell culture

RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml amphotericin B at 37°C under 5% CO_2 -humidified air.

3-4-2. Assay for cell viability

Cell viability was assessed using the MTT assay as described previously (Mossman, 1983). In brief, RAW 264.7 cells were seeded into a 96-well plate at a density of 1×10^5 cells per well and incubated at 37°C for 24 h. The cells were then treated with various concentrations of the samples (MeOH extract and its solvent-soluble fraction). After an additional 24 h incubation at 37°C, 100 μl of MTT (0.5 mg/ml in PBS) was added to each well, and the incubation was continued for another 2 h. The resulting color was assayed at 570 nm using a microplate spectrophotometer (Molecular Devices).

3-4-3. Assay for measurement of cellular NO production

The nitrite concentration in the medium was measured using the Griess reagent as an indicator of NO production as previously described (Shin *et al*., 2008). Briefly, RAW 264.7 cells (2.0×10^5 cells/well in a 24-well plate with 500 μl of culture medium) were pretreated with samples for 2 h and incubated for 18 h with LPS (1.0 μg/ml). After incubation, the nitrite concentration of the supernatants (100 μl/well) was measured after adding 100 μl Griess reagent. To quantify the nitrite concentration, standard nitrite solutions were prepared, and the absorbance of the mixtures was determined using a microplate spectrophotometer (Molecular

Devices) at a wavelength of 540 nm. The iNOS inhibitor AMT was used as a positive control.

3-4-4. Assay for inhibition of intracellular ROS generation

ROS generation was assessed using a ROS-sensitive fluorescence indicator, DCFH-DA (Lebel & Bondy, 1990). To determine intracellular ROS scavenging activity, RAW 264.7 cells (2.0×10^4 cells/well) were seeded in black 96-well plates. After 24 h, the cells were treated with samples (MeOH extract and its solvent-soluble fractions at various concentration) for 1 h and incubated with DCFH-DA (20 μM) and *t*-BHP (200 μM) for 30 min to induce ROS generation. Fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (Bio-Tek Instruments Inc., FLx 800).

3-4-5. Analysis for inhibition of iNOS and COX-2 protein expression

To measure the protein levels of iNOS and COX-2, a Western blotting technique was used. RAW 264.7 cells were cultured in 100 mm culture dishes in the presence or absence of LPS (1.0 μg/ml) and with/without test samples for 18 h. Afterward, cells were washed twice with ice-cold PBS and lysed with a buffer on

ice for 30 min. Cell extracts were obtained by centrifugation at 14000×g at 4°C for 20 min. Cytosolic proteins were electrophoretically separated on SDS-PAGE and transferred onto PVDF membranes. The membranes were immediately blocked with 5% (w/v) non-fat dry milk in TBST buffer [Tris-buffered saline containing 0.1% Tween 20 (pH 7.4)] at room temperature for 1 h. The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody, diluted 1:2000 in 5% (w/v) non-fat dry milk in TBST buffers (10 min), and the antibody labeling was visualized with a Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions and exposed to X-ray film (GE Healthcare Ltd., UK). Pre-stained blue protein markers were used for molecular-weight determination.

3-5. Statistics

Statistical differences between the control and test groups were determined using Student's *t*-test. Results on anti-inflammatory and antioxidant effects were expressed as the mean ± S.D. of triplicate samples.

Ⅲ. Result

1. Antioxidant activities of MeOH extract of *H. fusiformis* and its solvent-soluble fractions

1-1. Total phenolic content

Folin–Ciocalteu reagent and $AlCl_3$ were used to determine the TPCs of the *H. fusiformis* MeOH extract and its solvent-soluble fractions, as shown in Table 1. Folin–Ciocalteu reagent reacts with all phenolic compounds to form a blue color complex that can be detected with a maximum absorbance at 765 nm (Singleton and Rossi, 1965). The TPCs result was recorded as mg of gallic acid equivalent per g of dried extract or fraction (GAE, mg/g extract or fractions). As shown in Table 1, the MeOH extract exhibited a poor TPC of 1.41 GAE mg/g. However, the EtOAc fraction derived from the MeOH extract exhibited the highest TPCs of 55.86 GAE mg/g. Both CH_2Cl_2 and *n*-BuOH fractions showed similar TPCs of 12.13 and 13.52 GAE mg/g, respectively while the H_2O fraction showed the lowest TPC of 0.54 GAE mg/g, respectively.

Table 1. Yield and total phenol content of MeOH extract and its fractions from *H. fusiformis*

Samples	Yeild (%) [a]	Total phenolics [b]
MeOH ext.	15.49	1.41
CH_2Cl_2 fr.	1.34	12.13
EtOAc fr.	0.13	55.86
n-BuOH fr.	0.75	13.52
H_2O fr.	12.73	0.54

[a] The yield (w/w) percentage of the MeOH extract and fractions from raw material (338 g).
[b] Gallic acid equivalents (GAE, mg/g of extract or fractions) for the total phenol content.

1-2. $ONOO^-$ scavenging activity

Peroxynitrite is an oxidant and nitrating agent because of its oxidizing properties. Formation of peroxynitrite *in vivo* has been ascribed to the reaction of the free radical superoxide with the free radical nitric oxide (Pacher *et al*., 2007; Csaba *et al*., 2007). The scavenging activity of the MeOH extract and its solvent-soluble fractions against $ONOO^-$ was evaluated *via* cell-free based $ONOO^-$ assay. As demonstrated in Table 2, MeOH extract as well as its different solvent-soluble fractions showed potential $ONOO^-$ scavenging activity. Among the fractions, the EtOAc fraction showed the most potent $ONOO^-$ scavenging activity with an IC_{50} value of 9.54 μg/ml compared to the positive control L-penicillamine with an IC_{50} value of 0.93 μg/ml. On the other hand, the CH_2Cl_2 and *n*-BuOH fractions displayed moderate scavenging activity with IC_{50} values of 31.22 and 46.59 μg/ml, respectively, compared to the EtOAc fraction. In contrast, H_2O fraction did not show any scavenging activity up to the concentration of 50 μg/ml.

1-3. DPPH radical scavenging activity

The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to be decolorized in the presence of antioxidants. The DPPH radical which contains an odd electron can be changed to a stable diamagnetic molecule

after accepting an electron or hydrogen radical in the presence of antioxidants (Blois, 1958). The DPPH free radical scavenging activity of the MeOH extract and its solvent-soluble fractions was evaluated and summarized in the Table 2. Similar to ABTS radical scavenging assay, the MeOH extract as well as its different solvent soluble fractions including CH_2Cl_2, *n*-BuOH, and H_2O did not exhibit DPPH radical scavenging activity at the concentrations tested. Interestingly, only the EtOAc fraction showed potential DPPH radical scavenging activity with an IC_{50} value of 216.75 μg/ml compared to positive control L-ascorbic acid with an IC_{50} value of 1.46 μg/ml.

1-4. ABTS radical scavenging activity

ABTS scavenging assay is a decolorization technique in that the radical is generated directly in a stable form prior to reaction with putative antioxidants. When an antioxidant is added to the reaction, there is a degree of decolorization owing to the reversal of the formation of ABTS radical cations, which leads to decreases in absorbance that can be measured quantitatively at 734 nm to evaluate the potential of the antioxidants (Pellegrini *et al*., 1999; Lee *et al*., 2006). The ABTS radical scavenging activity of the MeOH extract as well as its different solvent-soluble fractions is summarized in the Table 2. It is evident from the Table 2, the MeOH extract as well as its different solvent-soluble fractions

including CH_2Cl_2, *n*-BuOH, and H_2O did not exhibit ABTS radical scavenging activity at the concentrations tested. Interestingly, only the EtOAc fraction exhibited moderate ABTS radical scavenging activity with an IC_{50} value of 55.71 μg/ml compared to the positive control Trolox with an IC_{50} value of 1.84 μg/ml.

Table 2. Antioxidant activities of MeOH extract and its fractions from *H. fusiformis*

Samples	Peroxynitrite	DPPH	ABTS
	IC_{50} (μg/ml) Mean ± S.D		
MeOH ext.	361.32 ± 11.87	> 800	> 2000
CH_2Cl_2 fr.	31.22 ± 2.29	> 800	> 100
EtOAc fr.	9.54 ± 0.45	216.75 ± 5.37	55.71 ± 0.28
n-BuOH fr.	45.69 ± 0.81	> 800	> 100
H_2O fr.	> 50	> 800	> 100
L-Penicillamine [a]	0.93 ± 0.03		
Trolox [b]			1.84 ± 0.01
L- Ascorbic acid [c]		1.46 ± 0.03	3.32 ± 0.11

a. b. c **Positive controls used in peroxynitrite, DPPH, and ABTS radical scavenging activity assays.**

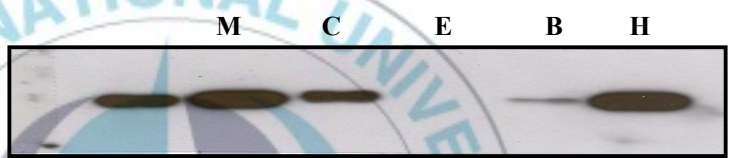
1-5. Inhibitory activity of $ONOO^-$ mediated tyrosine nitration

In order to determine the inhibition against $ONOO^-$-induced tyrosine nitration of *H. fusiformis*, the western blot analysis was performed using the 3-nitrotyrosine antibody. The inhibitory activity of MeOH extract and its solvent-soluble fractions was evaluated as demonstrated in Fig. 2. As shown in Fig. 2-(a), pretreatment of the MeOH extract with different concentrations range of 200 to 1600 μg/ml resulted in moderate dose-dependent inhibitory activities against $ONOO^-$-mediated tyrosine nitration. And we pretreated the MeOH extract and its different solvent soluble fractions at the concentration of 50 μg/ml (Fig. 2-(b)). The results showed that pretreatment with the CH_2Cl_2, EtOAc, and *n*-BuOH fractions at 50 μg/ml significantly inhibited the nitration of tyrosine while pretreatment with MeOH extract and H_2O fraction at same concentration did not inhibit tyrosine nitration. As demonstrated in Fig. 2-(c, d, e), treatment of CH_2Cl_2, EtOAc, and *n*-BuOH fractions inhibited nitration of tyrosine in a concentration dependant manner at the range of 12.5 ~ 100 μg/ml, 3.125 ~ 25 μg/ml, and 6.25 ~ 50 μg/ml, respectively. Among them, EtOAc fraction showed significant inhibitory effect at even lower concentration and tyrosine nitration was barely detectable at the concentration of 25 μg/ml. In the contrast, the H_2O fraction did not show any inhibitory activity up to a concentration of 400 μg/ml, as shown in Fig. 2-(f).

(a)

MeOH extract (μg/ml)	—	—	200	400	800	1600
$ONOO^-$ (200 μM)	—	+	+	+	+	+

(b)



Sample (50 μg/ml)	—	—	+	+	+	+	+
$ONOO^-$ (200 μM)	—	+	+	+	+	+	+

(c)

CH_2Cl_2 fraction (μg/ml)	—	—	12.5	25	50	100
$ONOO^-$ (200 μM)	—	+	+	+	+	+

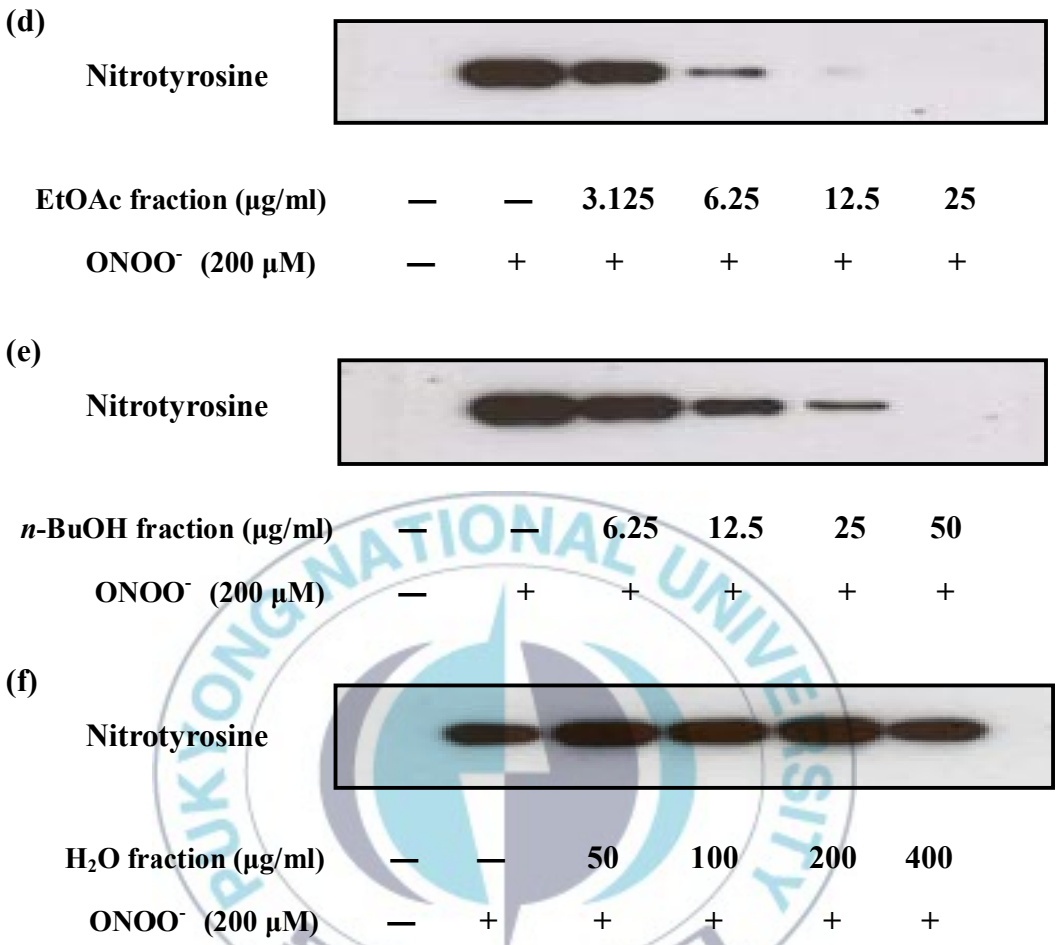


Fig. 2. Effects of the MeOH extract and its solvent-soluble fractions from *H. fusiformis* on the nitration of BSA by $ONOO^-$.

The mixtures of the samples and BSA were incubated at 25 ˚C for 10 min. And the reactants were resolved by electrophoresis in 10% SDS-polyacrylamide gel. (a) Effect of *H. fusiformis* MeOH extract at various concentration, (b) Effects of *H. fusiformis* MeOH extract and its solvent soluble fractions at 50 μg/ml, (c) Effects of *H. fusiformis* CH_2Cl_2, (d) EtOAc, (e) *n*-BuOH, (f) H_2O fractions at various concentrations.

2. Anti-inflammatory activities of the MeOH extract of *H. fusiformis* and its solvent-soluble fractions on RAW 264.7 cells

2-1. Effect of cell viability

The cytotoxicity of the MeOH extract and its solvent soluble fractions obtained from *H. fusiformis* was measured by MTT assay. As shown in Fig. 3, the MeOH extract and its solvent soluble fractions were tested at of 50 μg/ml concentration; except for the CH_2Cl_2 fraction, they exerted no cytotoxic effects. The MeOH extract, EtOAc, *n*-BuOH, and H_2O fractions displayed with 93.51, 108.0, 92.70, and 98.22 % of cell viability, respectively. In case of CH_2Cl_2 fraction, it showed cytotoxicity with only 18.07 % of cell viability at 50 μg/ml. For this reason, the CH_2Cl_2 fraction was tested at a lower concentration and was found to be nontoxic up to 10 μg/ml, as shown in Fig. 4.

2-2. Inhibitory activity of NO production

To evaluate whether the *H. fusiformis* MeOH extract and its solvent soluble fractions possess potential anti-inflammatory effects, we investigated the inhibitory effect on NO production in LPS-induced RAW 264.7 cells. As illustrated in Fig. 3, NO production was increased dramatically up to 19.05 μM

upon stimulation with LPS; however, pretreatment with the MeOH extract and its solvent-soluble fractions at concentration of 50 μg/ml reduced NO production. Although the MeOH extract showed moderate NO production inhibitory activity (43.58%), two of its fractions, the CH_2Cl_2 and EtOAc fractions, showed strong NO production inhibitory activity with inhibition percentages of 91.44 and 61.39%, respectively. Both the *n*-BuOH, and H_2O fractions displayed moderate inhibitory activities on NO production with inhibition percentages of 33.69 and 21.19 %, respectively. Because the CH_2Cl_2 fraction showed high cytotoxicity at a concentration of 50 μg/ml we tested its effects on cell viability and NO production at lower concentrations (2.5 ~ 10 μg/ml). Interestingly, we found that the CH_2Cl_2 fraction showed strong NO production inhibitory activity even at the lower concentrations of 2.5, 5 and 10 μg/ml, with inhibition percentages of 37.27, 62.83, and 85.39 %, respectively. Moreover, at 10 μg/ml of the CH_2Cl_2 fraction, the NO production inhibitory activity was similar to that of AMT, a positive control used in the assay; these results are illustrated in Fig. 4.

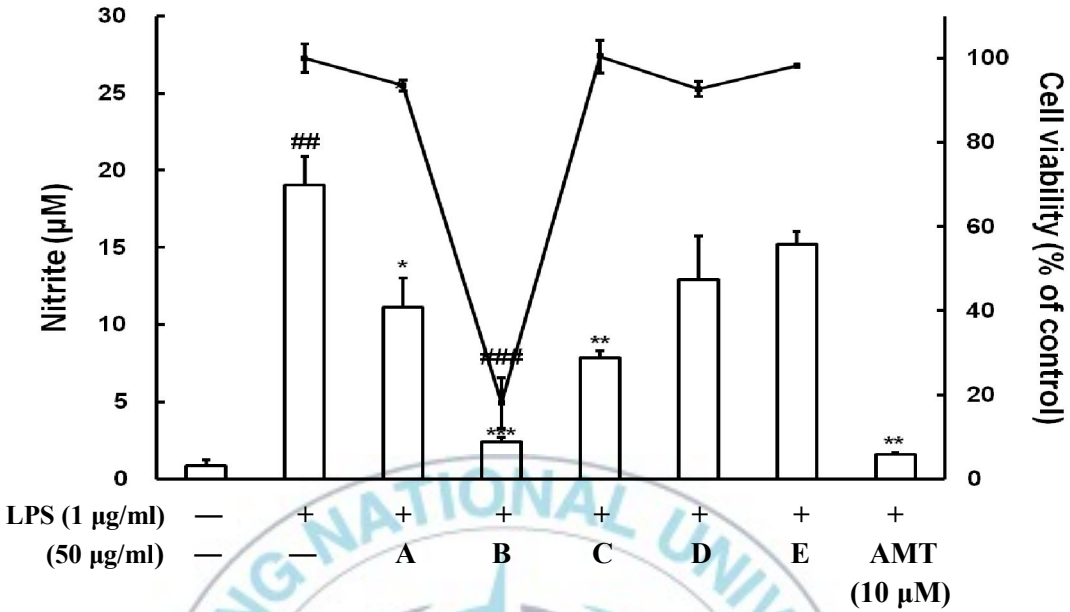


Fig. 3. Effects of the MeOH extract and its solvent-soluble fractions from *H. fusiformis* on LPS-induced NO production and cell viability in RAW 264.7 cells.

RAW 264.7 cells were pretreated with a 50 μg/ml concentration of MeOH extract of *H. fusiformis* (A) and its solvent-soluble fractions (CH_2Cl_2 (B), EtOAc (C), *n*-BuOH (D), and H_2O (E)) and LPS (1.0 μg/ml) for 2 h. After a further 18 h of incubation, the amounts of NO in the culture supernatants were measured by the Griess reaction assay. Cell viability was determined using the MTT method. Data represent mean ± STDEV of triplicate experiments. Result of one-factor analysis of variance $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus untreated control, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus LPS-treated group.

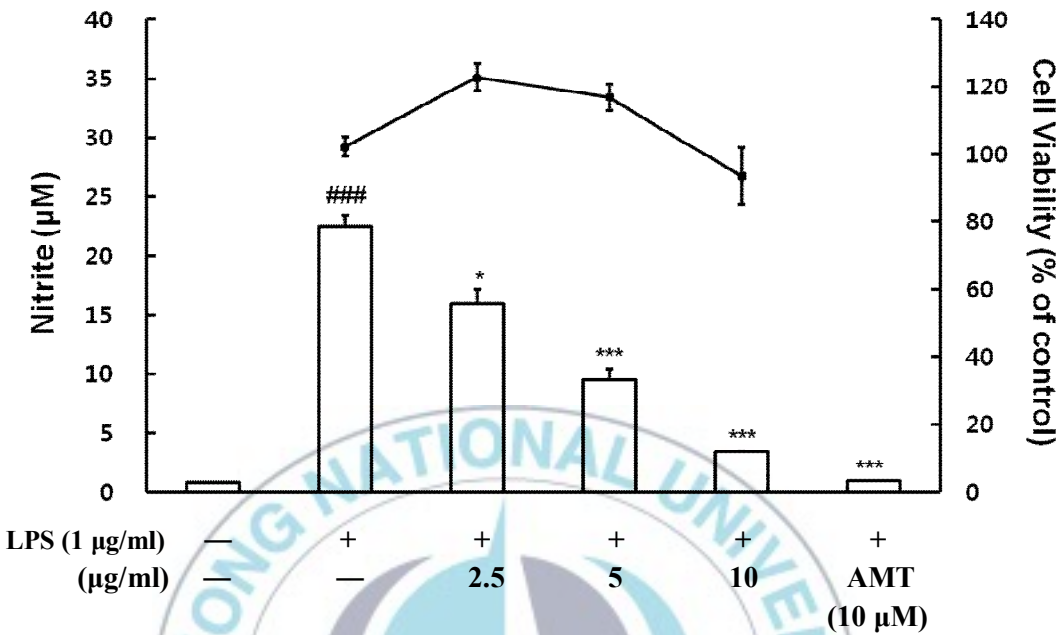


Fig. 4. Effect of the CH_2Cl_2 fraction from *H. fusiformis* on LPS-induced NO production and cell viability in RAW 264.7 cells.

RAW 264.7 cells were pretreated with the indicated concentrations of CH_2Cl_2 fraction of *H. fusiformis* and LPS (1.0 ug/ml) for 2 h. After another 18 h of incubation, the amounts of NO in the culture supernatants were measured by the Griess reaction assay. Cell viability was determined using the MTT method. The data represent mean ± STDEV of triplicate experiments. Results of one-factor analysis of variance $^{###}P < 0.001$ versus untreated control, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus LPS-treated group.

2-3. Inhibitory activity of intracellular ROS generation

We investigated the inhibitory effect of *H. fusiformis* on *t*-BHP induced ROS generation in RAW 264.7 cells. The cells were treated with *t*-BHP to induce oxidative stress and ROS generation was assessed using the ROS-sensitive fluorescene indicator DCFH-DA (Lebel and Bondy, 1990). When DCFH-DA is applied to viable cells, it penetrates into the cell membrane and is deacetylated by intracellular esterase to form non-fluorescent DCFH. In the presence of ROS, DCFH is oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF), which is readily detected by fluorescent spectrophotometer. The cells were pretreated with 50 μg/ml of the MeOH extract and its different solvent-soluble fractions before induction of intracellular ROS generation by *t*-BHP, as shown in Fig. 5. It is evident from our result in Fig. 5 that *t*-BHP dramatically increased ROS generation in cells compared to untreated cells. However, pretreatment with MeOH extract and its different solvent soluble-fractions reduced ROS generation. The ROS generation inhibitory activity of the MeOH extract as well as individual fraction at the concentration of 50 μg/ml was in the following order: EtOAc > CH_2Cl_2 > H_2O > MeOH > *n*-BuOH fractions. In particular, both the CH_2Cl_2 and EtOAc fractions showed strong inhibitory activities by ROS generation up to 50.0 and 49.85 %, respectively. The H_2O fractions showed weak ROS generation inhibitory activity, while *n*-BuOH fraction did not inhibit ROS generation at the

tested concentration. Since CH_2Cl_2 at concentration of 50 μg/ml was found toxic, we tested it at lower concentration (12.5 ~ 100) μg/ml. Interestingly, it showed significant inhibitory activity against *t*-BHP induced ROS generation at nontoxic concentration of 12.5 μg/ml by as much as 70.32 % (Fig. 6). The EtOAc fraction at concentrations of 12.5, 25, 50, and 100 μg/ml also reduced ROS generation in a concentration dependant manner 25.2, 34.55, 49.38, and 57.17%, respectively.

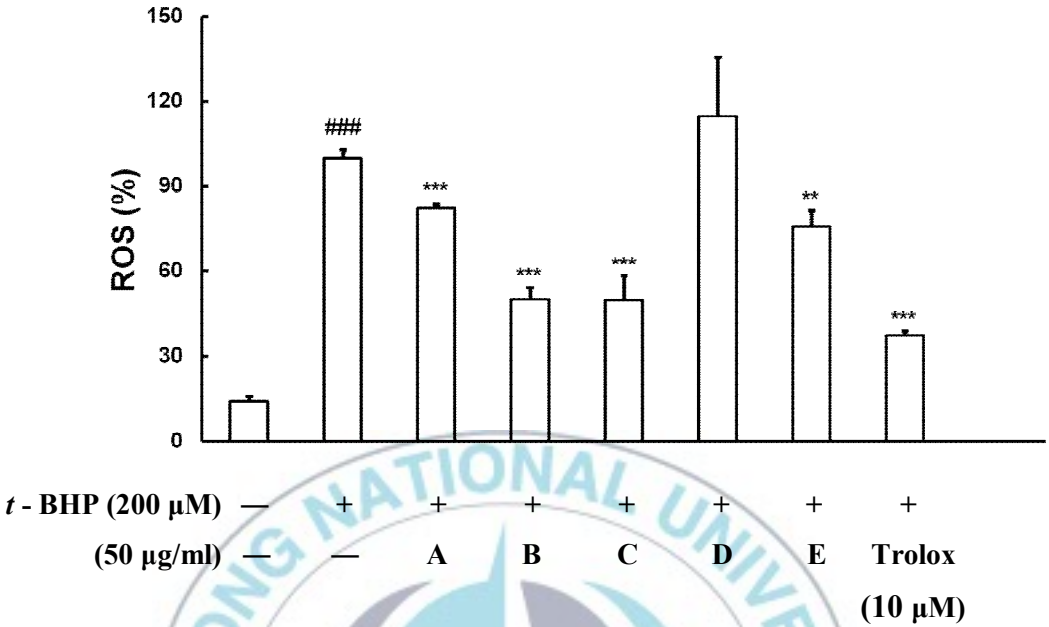


Fig. 5. Effects of the MeOH extract and its solvent-soluble fractions from *H. fusiformis* on the *t*-BHP-induced ROS generation in RAW 264.7 cells.

Cells were pre-treated with the indicated concentration of MeOH extract (A) and its solvent soluble fractions (CH_2Cl_2 (B), EtOAc (C), *n*-BuOH (D), and H_2O (E)) and incubated for 1 hour and then treated with *t*-BHP (200 μM) and DCFH-DA (20 μM) for 30 min to induce ROS generation. The control values were obtained in the absence of *t*-BHP (200 μM). The data represent mean ± STDEV of triplicate experiments. $^{###}p < 0.001$ indicates a significant difference from the control group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant differences from the control group.

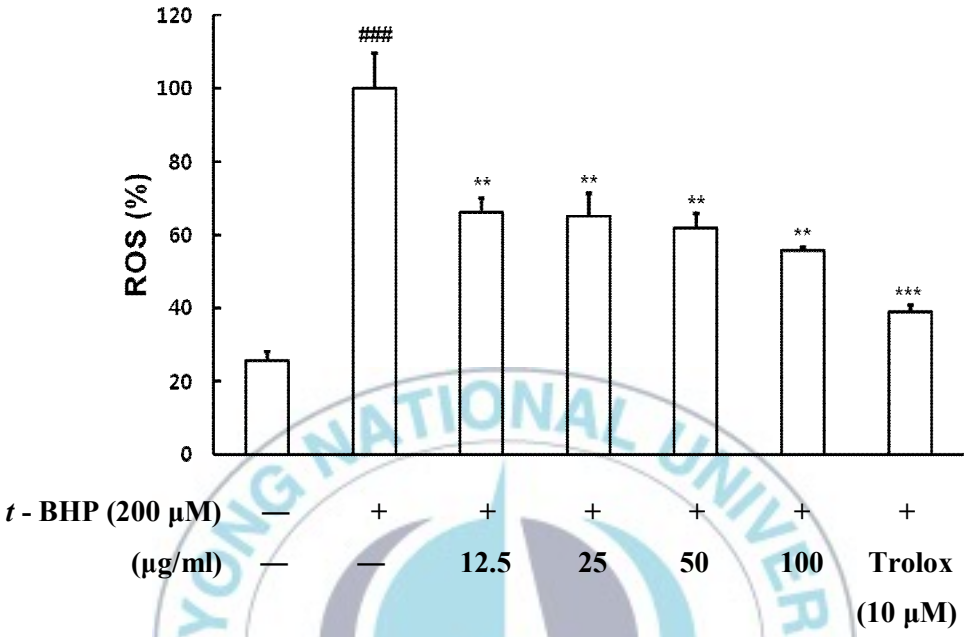


Fig. 6. Effect of the CH_2Cl_2 fraction from *H. fusiformis* on the *t*-BHP-induced ROS generation in RAW 264.7 cells.

Cells were pretreated with the indicated concentrations (12.5 25, 50, and 100 μg/ml) of CH_2Cl_2 fraction for 1 hour and then treated with *t*-BHP (200 μM) and DCFH-DA (20 μM) for 30 min to induce ROS generation. Control values were obtained in the absence of *t*-BHP (200 μM). $^{###}p < 0.001$ indicates a significant difference from the control group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant differences from the *t*-BHP group.

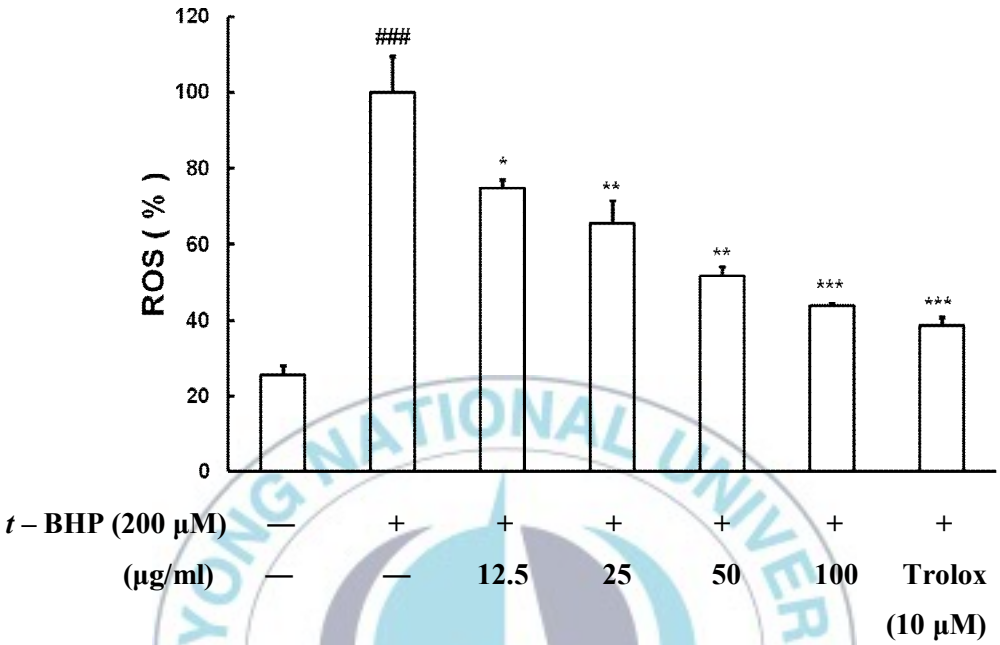


Fig. 7. Effect of the EtOAc fraction from *H. fusiformis* on the *t*-BHP-induced ROS generation in RAW 264.7 cells.

Cells were pretreated with the indicated concentrations (12.5 25, 50, and 100 μg/ml) of EtOAc fraction for 1 hour and then treated with *t*-BHP (200 μM) and DCFH-DA (20 μM) for 30 min to induce ROS generation. Control values were obtained in the absence of *t*-BHP (200 μM). $^{###}p < 0.001$ indicates a significant difference from the control group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant differences from the *t*-BHP group.

2-4. Effects of the CH_2Cl_2 and EtOAc fractions of *H. fusiformis* on LPS-induced iNOS and COX-2 protein expression

On the basis of the NO production inhibitory activity results, we also investigated the inhibitory effects of the CH_2Cl_2 and EtOAc fractions on NO-mediated gene expression of inflammatory mediators such as iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells by Western blot analysis. The iNOS and COX-2 protein expression was significantly increased when macrophages were treated only with LPS (1 μg/ml), while the expression of iNOS and COX-2 proteins by unstimulated RAW 264.7 cells was almost undetectable. Consistent with our NO inhibitory results, pretreatment with the CH_2Cl_2 and EtOAc fractions inhibited iNOS protein expression in a concentration-dependent manner, as shown in Fig. 5. However, neither fraction inhibited COX-2 protein expression. Therefore, it can be speculated that the inhibition of cellular NO production by CH_2Cl_2 and EtOAc fractions was mediated by suppression of iNOS protein expression.

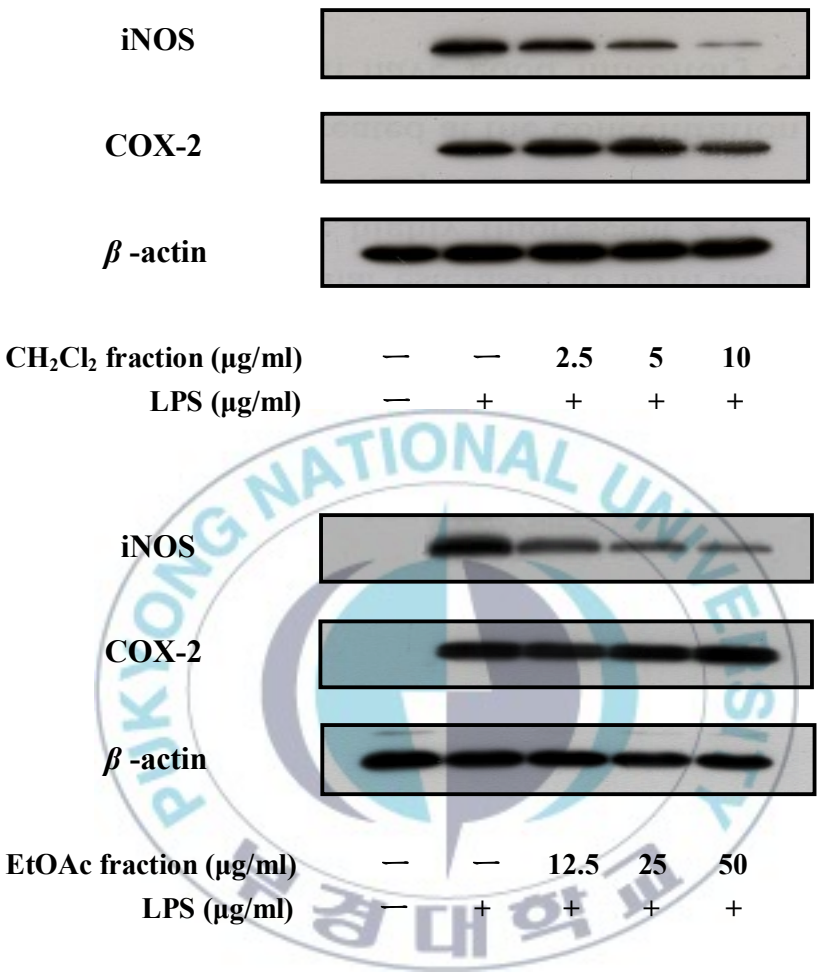
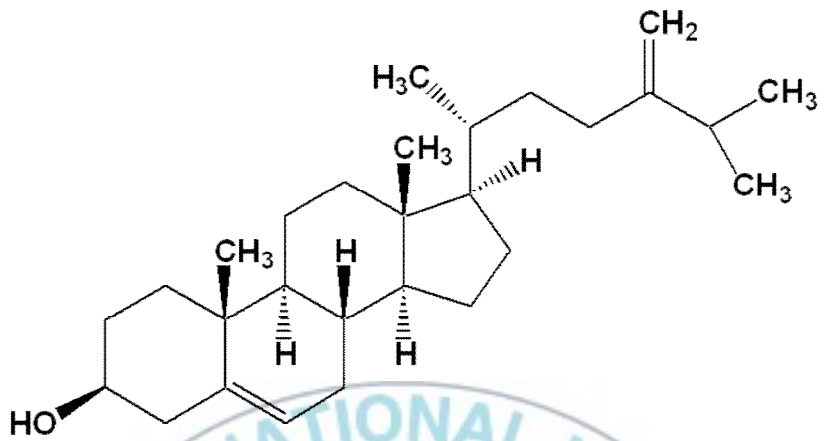


Fig. 8. Effects of the CH_2Cl_2 fraction on LPS-induced COX-2 and iNOS expression in RAW 264.7 cells.

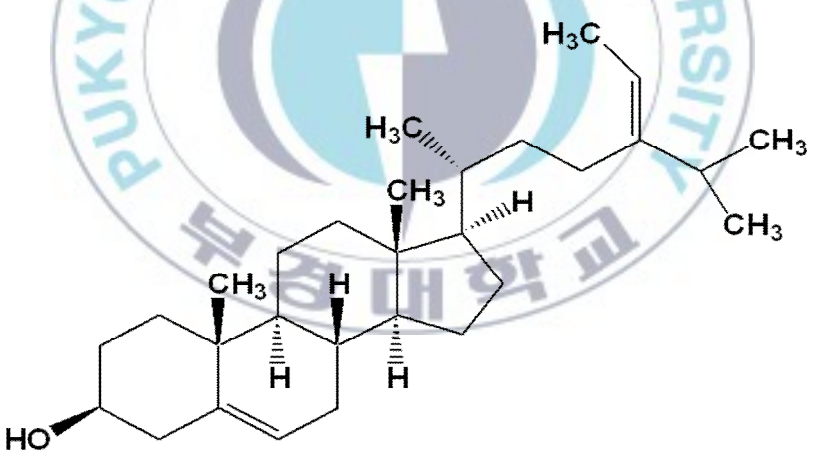
The cells were pretreated with the indicated concentration (2.5, 5, and 10 μg/ml) of the CH_2Cl_2 fraction for 2 hours and LPS (1.0 μg/ml) for 18 hours. Cytosolic lysates were seperated on SDS-PAGE. COX-2, iNOS, and β-actin were detected by western blot analysis.

3. Isolation of compounds from the CH_2Cl_2 fraction of *H. fusiformis*

The MeOH extract was subjected to solvent partitioning between the CH_2Cl_2, EtOAc, *n*-BuOH, and H_2O fractions. Among the several solvent soluble fractions, the CH_2Cl_2 fraction exhibited the highest antioxidant and anti-inflammatory activities followed by the EtOAc fraction, and both fractions showed dual inhibitory activities. Repeated column chromatography was undertaken to isolate fucosterol and 24-methylene cholesterol from the CH_2Cl_2 fraction. The fucosterol and 24-methylene cholesterol compounds were characterized and identified by ^{1}H- and ^{13}C-NMR, as well as through comparison with data from published literature and TLC (Lu *et al*., 2004; Jung *et al*., 2013).



24-Methylene cholesterol



Fucosterol

Fig. 9. Structures of 24-methylene cholesterol and fucosterol.

Ⅳ. Discussion

The ocean environment contains over 80% of world's plant and animal species with more than 150,000 seaweeds (Jha and Zi-rong, 2004). The marine environment is an extraordinary reservoir of bioactive natural products, which exhibit unusual chemical and structural features not found in terrestrial natural products (Almeida *et al*., 2011; Queiroz *et al*., 2011; Souza *et al*., 2009). It is because of the marine organisms have evolved physiological and biochemical mechanisms to help them survive in hostile environment (Kijjoa and Sawangwong, 2004; La Barre *et al*., 2010; Lira *et al*., 2011). For this reason, many marine resources have been attracted attention in the search for natural bioactive compounds to develop new drugs and health foods. Also, several marine natural products are currently in pre-clinical and clinical evaluation, especially in the areas of cancer, pain and inflammatory diseases (Proksch *et al*., 2002; Mayer *et al*., 2010; Zhang *et al*., 2010; O'Sullivan *et al*., 2010; Matta *et al*., 2011). Seaweeds are found in abundance of the coastal areas. And it is represented as a huge yet untapped potential for new source of therapeutics in many parts of the world. Also, seaweed has been widely used as a food source and in medicine as natural sources of highly bio-available trace elements due to their beneficial potential (Booth, 1964; Dhargalkar and Pereira, 2005; Smit, 2004).

In recent years, active compounds from seaweeds have been propounded for nutraceuticals and functional foods. Because it is revealed that they are a valuable food resource which contains many phytochemicals with various bioactivities such as carotenoids, terpenoids, xanthophylls, chlorophyll, vitamins, saturated and polyunsaturated fatty acids, amino acids, acetogenins, polyphenols, alkaloids, halogenated compounds, polysaccharides, proteoglycans, alginate, laminaran, rhamnan sulfate, galactosyl glycerol and fucoidan (Paniagua-Michel *et al*., 2009; Cen-Pacheco *et al*., 2010; Klisch and Hader, 2008; Pallela *et al*., 2010; D'Ayala *et al*., 2008; Kellmann *et al*., 2010; Souza *et al*., 2009; Guven *et al*., 2010; Cabrita *et al*., 2010; La Barre *et al*., 2010). Moreover, seaweeds are important ecologically and commercially to many regions of the world, especially in Asian countries such as China, Japan and Korea (Smit, 2004). The Japanese and Chinese use brown seaweed especially, as treatment of hyperthyroidism and other disorders (Elena *et al*., 2001; Kim *et al*., 1997; Okai *et al*., 1997; Premila *et al*., 1996). Seaweed can be divided as three different classes: red algae (Rhodophyta), brown algae (Phaeophyta), and green algae (Chlorophyta). Each type of seaweed gets its name based on the color appeared to us. Each kind has additional distinguishing characteristics as well. Among them, it has been reported that brown seaweed present higher antioxidant potential in comparison with red and green families and contain compounds not found in terrestrial sources algae (Al-Amoudi *et al*., 2009; Costa *et al*., 2010; Cox *et al*., 2010; Kang *et al*., 2004;

Kindleysides *et al*., 2012). Considering this, in the present study, we investigated *H. fusiformis* (class Phaeophyceae, order Fucales, family Sargassaceae), consuming as a popular dish in both countries in Korea and Japan. Several evidences suggested that *H. fusiformis* possesses a number of biologically active compounds with potential therapeutic value as antioxidants, immuno-modulators and anticoagulants (Kim *et al*. 1998; Okai *et al*. 1998; Yan *et al*. 1999; Nagai and and Yukimoto, 2003). Considering these result, we evaluated the activities of antioxidant and anti-inflammatory with *H. fusiformis* MeOH and its solvent soluble fractions by several experimental methods.

Humans are impacted by many free radicals, including ROS) and RNS originating from both internal and external sources (Boonchum *et al*., 2011). Generally, ROS includes oxygen radicals and non-radical derivatives, which can be converted into radicals or as oxidizing agents. RNS includes nitrogen radicals and non-radical species. Oxygen is necessary for aerobic organisms and nitrogen is usually existed in the food. And their intermediates are essential for cellular functions, immune responses and redox regulation of signal transduction in animals (Michael et al., 2002). ROS can be generated in cells by several metabolic pathways, ionizing radiation, UV light, cigarette smoke, industrial waste and pollutants. Furthermore, this ROS constitutes superoxide ($\bullet O_2^-$), hydroxyl (HO•), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), and they are generated in cells as aerobic products of normal cellular respiration (Boonchum *et*

al., 2011). Both ROS and their reaction products can damage essential bio-molecules like proteins, DNA, and lipids to cause various human diseases (Wu *et al.*, 1998). The steady state levels of ROS and free radicals are maintained in cells by the activity of a cellular antioxidant defense system including superoxide dismutase, catalase, and peroxidase. However, under stressful conditions, this delicate defense system balance is perturbed, causing enhanced production of ROS, which leads to physiological and pathological conditions such as inflammation, diabetes, shock, arthritis, carcinogenesis, and aging (Betteridge, 2000; Chae *et al*., 2004; Kang *et al*., 2005; Saha *et al*., 2004). In other words, overproduction of ROS and free radicals resulting from an imbalance between ROS generation and scavenging systems might causes other kinds of diseases (Smith *et al*., 1996; Diaz *et al*., 1997; Aruoma, 1998; Burns *et al*., 2001; Huang *et al*., 2006). However, the ROS and free radicals can be reduced by the defensive activity of antioxidants present in tissues (Halliwell, 1991). $ONOO^-$, highly reactive oxidant, can be generated *in vivo* under pathological conditions by a rapid reaction between a superoxide anion radical ($\bullet O_2$) and nitric oxide (NO). It is now well reported that endothelial cells, neutrophils, and macrophages are capable of producing $ONOO^-$, which can lead to oxidation of lipid membranes, which in turn contributes to cell death and tissue injury (Salvemini *et al*., 1998). Furthermore, high levels of $ONOO^-$ cause tyrosine nitration in target proteins which often leads to loss of protein activity (Frears *et al*., 1996; Stadtman *et al*.,

1998). Immunological studies have revealed that protein tyrosine nitration may be involved in a variety of cellular functions including cell signaling pathways and protein structure alterations (Lee *et al*., 2009). These evidences support the involvement of oxidative/nitrosative stress in the initiation and progression of various inflammatory diseases. In this regard, we evaluated the antioxidant activities of *H. fusiformis* MeOH extract and its solvent soluble fractions through several experimental method including total phenolic contents, scavenging activities of $ONOO^-$, DPPH, ABTS radical, and inhibition of $ONOO^-$ mediated tyrosine nitration. As a result, we confirmed that there is significant antioxidant activity on EtOAc fraction by total phenolic content, scavenging activities of $ONOO^-$, DPPH, ABTS radical, and inhibition of $ONOO^-$ mediated tyrosine nitration. In all kind of antioxidant assays, EtOAc fraction showed most potent scavenging activity with highest content of phenolics among the four fractions even though MeOH extract showed weak activity. Its total phenolic content was 55.85 GAE, mg/g of fraction and IC_{50} values were 9.54 μg/ml, 216.75 μg/ml, and 55.71 μg/ml on the $ONOO^-$, DPPH, and ABTS radical scavenging assay, respectively. It is thought that EtOAc fraction probably has differences in chemical composition compared to the other fractions. The EtOAc fraction contains polar compounds relatively and according to these result, it can be guessed that a number of antioxidants components present in EtOAc fraction. Various studies showed strong correlations between total phenolic content and

multiple biological functions such as antioxidant, anticancer, anti-aging effects of plants. Antioxidant capacity and health benefits are often ascribed to their total phenolic contents (Hua *et al*., 2008; Nagai *et al*., 2003). Thus, it can be explained that the strong antioxidant activity of the EtOAc fraction is due to its high content of phenolic compounds (Table 1). In other word, these various phenolic compounds in the EtOAc fraction may lead to good antioxidant activity with their synergistic effect. And although the CH_2Cl_2 fraction didn't show as much active as the EtOAc fraction, it also showed moderate scavenging activity against $ONOO^-$ scavenging assay. In case of $ONOO^-$ mediated tyrosine nitration found by western blot analysis, the EtOAc farction showed the strongest inhibitory activity as similar to previous result. Tyrosine nitration was barely detectable at the concentration of 25 μg/ml. The MeOH extract showed weak inhibitory activity, however, the CH_2Cl_2 and the *n*-BuOH fraction presented good inhibitory activity in a concentration dependent manner at the range of 12.6 ~ 100 μg/ml and 6.25 ~ 50 μg/ml concentration, respectively. Therefore, it is possible that *H. fusiformis,* particularly the EtOAc fraction, might be a potential source of novel antioxidants.

Inflammation is a major cause of many diseases. Over the past few decades, it was realized that the process of inflammation is practically the same in different disorders. Inflammation is the activation of the immune system's response to infection and injury and has been implicated in the pathogeneses of arthritis,

cancer, stroke, as well as in neurodegenerative and cardiovascular disease. And it is characterized by a complex of interaction between mediators of inflammation and inflammatory cells directed toward removing irritants and healing of tissue injuries (Halliwell *et al*., 1995). In fact, in the inflammatory diseases, the antioxidant defense system is compromised by increased markers of oxidative stress and decreased levels of protective antioxidant enzymes (D'Orazio *et al*., 2012**)**. The association of antioxidants with inflammation stems from the recognition that free radicals are produced during the inflammatory process by macrophages. Although the pathophysiological basis of those conditions is not yet fully understood, ROS have often been implicated in the inflammation. Because high levels of ROS is produced to exert a defense against pathogens. Also it has been reported that ROS are involved in the cyclooxygenase- and lipoxygenase-mediated conversion of arachidonic acid into proinflammatory intermediates. On this basis, several natural and synthetic antioxidants have been tested and shown to possess anti-inflammatory properties (Halliwell *et al*., 1995).

Nitric oxide (NO) is a multifunctional biomolecule involved in many physiological and pathological processes. In the immune system, NO plays an important role as a vasodilator, neurotransmitter under normal physiological conditions (Nakagawa and Yokozawa, 2002). NO production is mainly catalyzed by nitric oxide synthase (NOS) which exists in three isoforms, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). eNOS and nNOS

are constitutively expressed and play an important role in normal physiological activities (Huang *et al*., 2012). On the contrary, iNOS catalyzes the formation if NO from L-arginine increases during progress of the inflammation. In other words, the iNOS-mediated NO production can promote pathological inflammation. During pathological condition, NO production is increased by the iNOS, and more over, it gives the opportunity to react with superoxide, resulting in peroxynitirite formation and cell toxicity, which have been found in inflammation. After all, excessive production of iNOS lead to other disease and it has been reported that iNOS-mediated overproduction of NO implicated in epithelia carcinogenesis (Ohshima and Bartsch, 1994), and cause mutagenesis, DNA structural damaging, and *N*-nitrosoamine formation (Arroyo *et al*., 1992; Miwa *et al*., 1987; Wink *et al*., 1991). Therefore, selective inhibition on iNOS activity has been established as a therapeutic approach for treating inflammation (Huang *et al*., 2012). Macrophages play an important role in inflammatory diseases relating to over production of pro-inflammatory cytokines, and inflammatory mediators. Production of macrophage mediators has been determined in many inflammatory tissues, along with increased expression of their mRNAs, following exposure to immune stimulants including bacterial endotoxin lipopolysaccharide (LPS). In RAW 264.7 cells, LPS stimulation induced iNOS transcription and its protein synthesis, and increased NO production (Henkel *et al*., 1993; Xie *et al*., 1994). Here, we evaluated the NO

production inhibitory activity of the MeOH extract of *H. fusiformis* and its different solvent-soluble fractions in LPS-induced Raw 264.7 cells *via* the Griess reaction, a spectrophotometric determination for nitrite. As depicted in Fig. 3, pretreatment of LPS-stimulated RAW 264.7 cells with MeOH extract and its CH_2Cl_2- and EtOAc-soluble fractions at a concentration of 50 μg/ml significantly inhibited NO production. Considering the number of viable cells obtained from cell viability assay after treatment with a 50 μg/ml concentration of the CH_2Cl_2 fraction indicated that the inhibition of NO production by the CH_2Cl_2 fraction might be attributed to its cytotoxic effect. To confirm this, we tested the CH_2Cl_2 fraction at a lower concentration range (2.5 - 10 μg/ml) using a cell viability assay and LPS-induced NO production in RAW 264.7 cells. Interestingly, the CH_2Cl_2 fraction at this lower concentration range strongly inhibited NO production in LPS-stimulated RAW 264.7 cells without affecting cell viability, as shown in Fig. 4. To investigate whether the inhibitory effects on NO production by the CH_2Cl_2 and EtOAc fractions were mediated *via* inhibition of iNOS protein expression, Western blot analysis was performed. Consistent with our previous results, both the CH_2Cl_2 and EtOAc fractions at nontoxic concentrations dose-dependently inhibited the expression of iNOS protein in LPS-stimulated RAW 264.7 cells.

Similar to iNOS, cyclooxygenase-2 (COX-2) is also an inducible pro-inflammatory enzyme. There are two COX isoforms, which differ mainly in their

pattern of expression. COX-1 is constitutively expressed in most tissues including kidney, lung, stomach, duodenum, jejunum, ileum, colon, and cecum a322 (Kargman *et al*., 1996). While, COX-2 is produced during inflammatory condition and not expressed consistently in normal state (Harris *et al*., 1994; Hirst *et al*., 1995). It has been reported that COX-2 is implicated to prostaglandin biosynthesis in inflammation. Thus COX-2 is induced by numerous extracellular and intracellular physiologic stimuli. These stimuli include lipopoly-saccharide (LPS) (Fu *et al*., 1990; Lee *et al*., 1992; O'Sullivan *et al*., 1992), interleukin-1 (IL-1), tumor necrosis factor (TNF) (Coyne *et al*., 1992; Jones *et al*., 1993), epidermal growth factor (EGF) (Hamasaki *et al*., 1993), transforming growth factor alpha (TGFa) (Du Bois *et al*., 1994), interferon-g (Riese *et al*., 1994), retinoic acid, platelet activating factor (PAF) (Bazan *et al*., 1994), and arachidonic acid. Thus, selective inhibitors of COX-2 have been demonstrated to provide anti-inflammatory effect with a marked reduction in gastrointestinal toxicity as compared to traditional NSAIDs (non-steroidal anti-inflammatory drugs) (Liao *et al*., 2012). It has been found that NSAIDs directly affect cyclooxygenase activity, either by covalently modifying the enzyme (as in the case of aspirin and the selective COX-2 inhibitor APHS), or by competing with the substrate for the active site (as with virtually all other NSAIDs). Aspirin and indomethacin, two commonly used NSAIDs, inhibited prostaglandin production by blocking COX-2 enzyme activity (Vane, 1971). As we know prostaglandins

participate in a number of normal physiologic functions, it is predictable that chronic blockade of cyclooxygenase leads to some undesirable side effects including gastrointestinal ulceration, bleeding and perforation. Therefore, inhibitors which could distinguish between the two cyclooxygenase isoforms might achieve analgesic and anti-inflammatory benefits without the accompanying undesirable gastrointestinal side effects. Therefore, idebtifying the natural inhibitors which can reduce the expression of iNOS and COX-2 are important targets for anti-inflammatory remedy. In the present study, since the CH_2Cl_2 and EtOAc fractions showed promising anti-inflammatory potential, we also evaluated their inhibitory effects on COX-2 protein expression in LPS-stimulated RAW 264.7 cells. Interestingly, although these two fractions strongly inhibited NO production as well as its corresponding iNOS protein expression, they did not inhibit COX-2 protein expression in LPS-stimulated RAW 264.7 cells at the tested concentrations. Accordingly, it is possible that the ability of *H. fusiformis* to inhibit NO production may be involved in the inhibition of iNOS expression, and thus reduced inflammation. Also in the treatment of the EtOAc fraction, we found the considerable inhibition of NO production without any toxicity. Through the *t*-BHP-induced ROS generation assay, we observed that both of the CH_2Cl_2 and EtOAc fractions have inhibitory effect against intracellular ROS generation induced *t*-BHP. In case of the CH_2Cl_2 fraction, it exhibited moderate effect than EtOAc fraction and it showed cytotoxicity at

around or above 50 μg/ml concentration. But we confirmed it has scavenging activity of ROS generation at nontoxic concentration, 12.5 μg/ml. On the other hand, EtOAc fraction showed good inhibitory activity against *t*-BHP-induced ROS generation in a dose dependant manner at nontoxic concentration. At the highest concentration 100 μg/ml, it showed strong scavenging effect compare to CH_2Cl_2 fraction. The comparative inhibitory activities of several fractions derived from the MeOH extract of *H. fusiformis* on both NO production and ROS generation can be explained on the basis of compositional and differences in the content of the active compounds. In recent years, large amounts of evidences have been accumulated, demonstrating that free radicals as well as reactive oxygen species generated from oxidative/nitrosative stress are important components of inflammation. It has been previously reported that compounds with antioxidant properties could be expected to have anti-inflammatory activity (Melagraki *et al*., 2009; Conforti *et al*., 2009). Taken together, all the results from the present study clearly demonstrate the potential antioxidant and anti-inflammatory activities of *H. fusiformis*.

In the search for bioactive compounds, extensive chromatography of the CH_2Cl_2 fraction yielded two known compounds: 24-methylene cholesterol and fucosterol. Fucosterol has been recognized as the predominant sterol of brown seaweed and is commonly found together with small amounts of cholesterol, 24-methylene cholesterol, and saringosterol (Carter *et al*., 1939; Ikekawa *et al*.,

1966). It was previously reported that fucosterol has antioxidant and anti-inflammatory activities (Yoo *et al*., 2012; Jung *et al*., 2013). 24-methylene cholesterol is the intermediate product of fucosterol biosynthesis from desmosterol (Patterson, 1968). Based on this evidence, we speculate that fucosterol present in the CH_2Cl_2 fraction contributed to the antioxidant and anti-inflammatory activities of the CH_2Cl_2 fraction.

In conclusion, it has been led to many investigations of the anti-inflammatory activity of antioxidant components (Omisore *et al*., 2005) and the association of antioxidants and inflammation stems from the recognition that free radicals are produced during the inflammatory process by macrophages. Thus, antioxidants that can scavenge free radicals or suppressed ROS generation are expected to treat of inflammation associated disorders (Backhouse *et al*., 1994). In addition, natural product derived antioxidants are more acceptable than synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroxyquinone (TBHQ) and propyl gallate, and display a variety of beneficial functions. Therefore, plant constituents having antioxidant activities together with anti-inflammatory activities may provide better opportunities to develop anti-inflammatory agents. In recent time, many studies have thought powerful, nontoxic natural antioxidants with anti-inflammatory potentials from edible seaweed to prevent reactive species related disorders. And also seaweed has been attracted an interest for their bioactive substances which

have chances to be used as antioxidant (Nagai and Yukimoto, 2003; Nakai *et al*., 2006). Seaweed produces various types of antioxidant to counteract environmental stresses (Lesser, 2006). In agreement with this circumstance, ingredients of the CH_2Cl_2 and EtOAc fractions could be considerable as multiple pharmacological properties. Especially, the CH_2Cl_2 fraction is expected to contain lots of non-polar elements like sterols and fatty acid. These elements are possible to have good anti-inflammatory effect in terms of their permeability to move in and out easily between cell membrane and cytoplasm, even though they usually show cell cytotoxicity. However, the application of limit on not showing cytotoxicity will give us good therapeutic effect. And also there would be a great expectation as antioxidant and anti-inflammatory activity for the EtOAc fraction. Consequently, *H. fusiformis* can be used as a potential source to act as an effective multiple sources on the inhibition of inflammatory mediator and scavenging of free radicals.

Ⅴ. Conclusion

The antioxidant and anti-inflammatory activities of the MeOH extract of *H. fusiformis* and its different solvent-soluble fractions were explored using several *in vitro* experimental methods. We confirmed that *H. fusiformis* exhibited both antioxidant and anti-inflammatory effects. In particular, the EtOAc fraction exhibited strong antioxidant activity together with significant anti-inflammatory activity and was able to suppress $OONO^-$-mediated protein tyrosine nitration. The CH_2Cl_2 fraction showed the most potent anti-inflammatory effect *via* inhibition of LPS-induced NO production, as well as iNOS protein expression in LPS-stimulated RAW 264.7 cells. Fucosterol was isolated from the CH_2Cl_2 fraction; fucosterol was previously reported to possess antioxidant and anti-inflammatory activities. Therefore, fucosterol was expected to be an important constituent contributing to the strong anti-inflammatory activity of the CH_2Cl_2 fraction. Taken together, our results clearly demonstrate the potential antioxidant and anti-inflammatory activities of *H. fusiformis*. *H. fusiformis* is thus as a potential source of natural antioxidants as well as a therapeutic remedy for the treatment of inflammation and oxidative stress-related diseases. However, further studies are required to isolate more specific bioactive compounds and their mechanisms of action toward antioxidant and anti-inflammatory activities.

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본 논문이 완성되기까지 제게 많은 힘이 되어주시고 도움을 주셨던 분들께 이 자리를 빌어 감사의 마음을 전하고자 합니다.

먼저, 부족한 저를 이 자리까지 올 수 있도록 학문의 길을 열어주시고 올바른 연구자의 삶과 연구에 대한 열정과 보람을 가르쳐 주신 최재수 교수님께 존경과 감사의 마음을 전합니다. 앞으로도 그 가르침 잊지않고 매 순간 최선을 다해 살아가도록 하겠습니다. 또한, 바쁘신 중에도 귀중한 시간 내어주셔서 논문 심사뿐 만 아니라 진심어린 조언을 해주셨던 류은순 교수님, 남택정 교수님께 깊은 감사를 드립니다. 그리고 학부시절부터 석사과정까지 많은 가르침 주신 김형락 교수님, 류홍수 교수님, 김재일 교수님, 변대석 교수님께도 진심으로 감사 드립니다. 실험적으로 많은 도움과 격려를 해주셨던 전북대학교 정현아 교수님께도 깊은 감사를 드립니다.

실험실 생활을 하는동안 가장 가까이서 제게 힘이 되주었던 실험실 선후배님들께도 감사의 마음을 전합니다. 한 학기밖에 함께 하지 못했지만 처음 실험실 생활을 시작할 무렵 많이 가르쳐 주고 힘이 되주었던, 학부때부터 지금까지 언제나 든든한 친구이자 동생인 보라에게 고맙다는 말을 전하고 싶습니다. 그리고 꼼꼼하고 참한 혜은이, 힘들었던 저를 넓은 마음으로 이해해주시고 지켜봐주셨던 희진언니, 가장 힘들 때 함께 힘듦을 공유했던 찬미, 친구처럼 곁에서 토닥여 주고 의지가 됐던 예쁜 언지, 동생이지만 세심하게 잘챙겨줬던 순수하고 해맑은 정수, 그리고 실험실 막내이자 우리의 기쁨이 되어주는 아람이에게도 고맙다는 말을 전하고 싶습니다. 2년이 조금 넘는 시간동안 힘든일도 많았고 또 소소한 즐거움도 있었는데 그런 시간들을 소중한 사람들과 함께 할 수 있어서 감사했습니다. 또, 먼나라에서 와 열심히 공부하고 연구하는, 그리고 실험실 선배로서 저를 많이 도와준 Nurul에게 고맙다는 말 전하고 싶습니다. Nurul의 아내이자

후배인 Ishita, 그리고 Sabiha, Subash, Ali 에게도 고맙다는말 전하고 싶습니다. 그리고 석사과정 동안 바쁘다는 핑계로 잘 챙겨주지 못하고 소홀히 했지만 이해해주고 격려해 준 사랑하는 친구들에게도 고맙다는 말을 전하고 싶습니다. 그리고 여러가지로 챙겨주시고 도와주신 식품생명학과 조교 선생님 경진언니, 슬기, 인선언니께도 감사의 마음을 전합니다.

끝으로 사랑하는 가족들에게 깊은 감사의 마음 전하고 싶습니다. 언제나 저를 믿어주시고 끊임없는 격려와 조언을 해주신 부모님께 감사드립니다. 남들보다 조금 늦은 저이지만 한 걸음 뒤에서 든든히 지켜봐주시고 지원을 아끼지 않으시며 묵묵히 기다려주시는 마음 감사합니다. 앞으로 한 계단, 한계단 더 성장하는 모습으로 보답하겠습니다. 누나로서 잘 챙겨주지 못하고 짜증만 내서 미안한 동생에게도 고마운 마음 전하고 싶습니다.

지금 이 순간이 학문의 길을 가는 첫 시작이라고 생각하며 많은 분들의 도움과 가르침을 통해 무사히 첫 걸음을 뗄 수 있었습니다. 저에게 따뜻한 사랑과 관심 주셨던 많은 분들께 다시 한번 감사드리며 앞으로 이 가르침 마음 속 깊이 새겨 저 넓은 세상에서도 훌륭한 연구자로서 최선을 다해 살아가도록 하겠습니다.

2014년 1월

한유란 올림