Thesis for the Degree of Master of Engineering

Study on Antioxidant and Antimicrobial Activity of Oil Extracted from Brown Seaweed (*Laminaria japonica*) Added Wheat Germ using Supercritical Carbon Dioxide

by

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Pukyong National University

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By

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A dissertation

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이 주 희

부 경 대 학 교 대 학 원 식 품 공 학 과

요 약

우리나라는 매년 60만톤 이상의 다양한 종류의 해조류가 생산되며 그 생산량은 전 세계 4위를 달할 만큼 해조류 양식 및 이용 산업이 매우 발달해 있다. 다시마 (*Laminaria japonica*) 는 갈조식물군 중 다시마과에 속하며 수용성 식이섬유인 알긴산이 풍부하여 혈중 콜레스테롤 수치를 저하시키는 효과가 있고, 카드뮴과 같은 유해 중금속을 방출하는 작용을 한다. 또한 산성 다당류인 푸코이단이 풍부하게 함유되어 있어 항혈액응고 작용과 항암효과 등 다양한 생리작용을 한다. 밀배아는 밀가루 제조시 순수한 배아만을 분리하여 얻어지는 부산물로 밀에 10% 정도 함유되어 있다. 밀배아에는 천연 항산화제 역할을 하는 토코페롤, 잔토필, 카로텐으로 구성된 카로티노이드 색소 등이 많이 함유되어 있다. 특히 밀배아 오일에는 토코페롤 함량이 많기 때문에 건강식품으로도 그 가치가 있으며, 불포화지방산 및 필수지방산의 함량이 매우 높아 의약품, 화장품 및 건강기능성 식품 소재로서 높은 가치를 지닌다. 이산화탄소는 낮은 임계점과 무독성, 그리고 용매의 사용량이 적고 부식성이 없어 친환경적이기 때문에 가장 널리 이용되는 초임계 유체이며 기존의 추출법이 가지는 어려움을 해결할 수 있는 새로운 혁신기술로서 주목 받고 있다.

다시마는 밀배아와 비교하였을 때, 낮은 지질 함량으로 인해 영양적 에너지가 적음에도 불구하고, 지질이 높음 수준의 필수 불포화 지방산을 함유하고 있어 그 가치가 높다. 따라서 다시마에 밀배아를 첨가하여 다양한 지방산 조성 및 폴리페놀, 토코페롤, 푸코잔틴을 함유하는 새로운 오일을 만들어서 항산화 및 항균 효과에 어떤 영향을 미치는지 알아보고자 하였다. 다시마와 밀배아 샘플은 두 가지 형태로 혼합되었다. 첫

번째 방법은 파우더 형태의 다시마와 밀배아를 혼합한 뒤 SC-$C0_2$ 및 hexane을 이용하여 혼합물의 오일을 추출 하는 것이고, 두 번째 방법은 SC-$C0_2$ 및 hexane을 이용하여 각각 추출한 다시마 및 밀배아 오일을 일정한 비율로 섞는 방법으로, 추출된 오일의 항산화 및 항균특성에 대하여 연구하였다.

초임계 이산화탄소를 이용하여 다시마, 밀배아, 첨가 비율을 달리한 다시마와 밀배아 혼합물로부터 오일의 추출은 온도는 40 ℃로 고정하였으며 압력조건은 300-400 bar에서 시행하였으며, 이산화탄소 유량은 54.55 g/min로서 총 추출 시간인 1시간 동안 지속적으로 흐르게 하였다. 오일의 지방산 분석을 위해 gas chromatography (GC)를 이용하였고, 다시마와 밀배아를 혼합한 경우에 단일 물질보다 다양한 조성의 지방산을 얻을 수 있었으며, 불포화지방산의 함량 또한 높게 나타났다. 특히 palmitic acid, oleic acid, linoleic acid 함량이 높게 나타났으며, Arachidonic acid 및 eicosapentaenoic acid도 동정되었다. 총 페놀, 푸코잔틴 및 토코페롤 함량에 있어서 파우더 형태로 혼합하여 추출한 오일보다 각각 추출한 오일을 혼합하였을 때 함량이 높게 나타났으며, 특히 α-토코페롤의 경우 함량이 상당히 증가하였음을 확인할 수 있었다. 산화 안정성 및 항산화 효과를 측정한 실험에서도 각각 추출한 오일을 혼합한 경우에 대체적으로 효과가 뛰어 났다. 항균활성을 측정한 결과 또한 각각 추출한 밀배아 오일보다 다시마 오일의 함량을 높여 혼합한 경우에 효과가 가장 뛰어남을 확인할 수 있었다. 대부부의 실험에서 해조류 및 밀배아를 각각 추출한 뒤, 밀배아 오일 함량보다 다시마 오일 함량을 높게 하였을 때 항산화 및 항균 활성 효과가 가장 높게 나타났다.

Introduction

Seaweeds are an important unconventional source of vitamins (liposoluble and hydrosoluble), commonly consumed fresh or dried in many coastal areas (Honya et al., 1994; Li, 1989; Mchug, 1991; Osse, 1990; Sanchez-Machado et al., 2004). Nevertheless, there has been little exploitation and exploration of seaweeds, despite potential industrial and agricultural applications. At the present time, seaweeds are used worldwide for different purposes (Chapman, V. J., and Chapman, D. J., 1980). Brown seaweeds are the most common edible seaweeds in Korea. Interested in seaweed lipid has been on the rise owing to the recognition of important bioactive molecules like conjugated fatty acids, pigments (especially fucoxanthin), that have profound physiological effects in the treatment of tumors and other cancer related problems (Hosokawa et al., 2004; Kohono et al., 2004). Furthermore, pigments in seaweeds have important nutraceutical properties, including antioxidant and biological response modifying qualities (Holt, 2008). In addition, polyunsaturated fatty acids (PUFAs) are reported to share more than 30% of total fatty acids in diatom or brown algae (Nomura et al., 1997).

Wheat germ which is the by-product of flour-milling industry is a unique source of highly concentrated nutrients. It contains about 52% carbohydrate, 23% protein, 11% water, 10% oil and 4% ash (NAL, 2008). Wheat germ oil is used in

products such as foods, biological insect control agents, pharmaceuticals and cosmetic formulations, and has been shown to reduce plasma and liver cholesterol in animals and to delay aging (T.S. Kahlon, 1989).

The use of supercritical fluid extraction (SFE) offers numerous potential advantages over conventional extraction processes, such as reduced extraction time, reduced organic solvent volume, and more selective extractions (L.T. Taylor, 1996). Supercritical fluids have a relatively high liquid-like density as well as a relatively low viscosity and high diffusivity (Q. Lang and C.M. Wai, 2001). These properties provide a unique solvent that is both effective at dissolving materials as well as penetrating solid matrices.

Supercritical carbon dioxide (SC-CO_2), in particular, is an attractive supercritical solvent, low critical temperature (31.1℃), and the fact that it is both non-toxic and inert. Because of these properties, SC-CO_2 can be useful when applied to food and pharmaceutical industry. This technology can be extracted high purity materials and economical. In recent years, the use of SFE for the removal of organic compounds from different liquid and solid matrices has attracted much attention. This technique has some advantages over more conventional separation techniques, largely due to the unique physical properties of SFs. SFE using CO_2 is a promising process for the extraction and fractionation of edible oils containing labile PUFAs and lipid soluble bioactive compounds.

Brown seaweeds have a low lipid content compared with wheat germ, thus being a low source of nutritional energy. Nevertheless, it is worth mentioning that the lipid fraction might contain higher levels of essential polyunsaturated fatty acids compared with traditional vegetables such as wheat germ, which might be of interest if we consider the large amount brown seaweeds. Also, wheat germ oil has a lot of nutritional ingredients. Thus, when adding the wheat germ oil to brown seaweed oil can make new oils contained a variety of fatty acids and phytochemicals to improve the antioxidant and antimicrobial activity. Combination of brown seaweed and wheat germ was progressed two ways. One way was brown seaweed powder added wheat germ powder before extraction. Brown seaweed and wheat germ were combined with optimal proportion, at ratio 7:3 and 3:7. Then it was extracted at pressure ranges of (300-400 bar) and constant temperature (40 ℃). Another one way was brown seaweed oil and wheat germ oil extracted respectively by SC-CO_2 and hexane. And then, two oil type samples were mixed to same ratio (7:3 and 3:7).

The objective of this study was to extract oil from mixture of brown seaweed (*Laminaria japonica*) and wheat germ by SC-CO_2 with lipid soluble bioactive compounds, and the fatty acids compositions of extracts at different extraction condition and ratio of mixing were also analyzed. And then, through the new composition of oil from brown seaweed added wheat germ by two different types

of combination using supercritical carbon dioxide and organic solvents, the improvement effect of antioxidant activity and antimicrobial activity were investigated.

Materials and Methods

1. Materials

The brown seaweed (*Laminaria japonica*) was collected from Geumil-eup, Wando-gun, Jeollanam-do, and the wheat germ was provided from Young-Nam Flour Mills Company (Busan, Republic of Korea). Carbon dioxide (99.99 % pure) was supplied by KOSEM (Yangsan, Republic of Korea). Standards of 2,2-diphenyl-1-picryl-hydrazyl (DPPH), tocopherols, fucoxanthin, FAMEmix and Mueller-Hinton agar were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A). Microorganisms were supplied by Korean Culture Center of Microorganism (KCCM). All other reagents used in different analysis were analytical or HPLC grade.

2. Sample preparation

After drying in an oven (DONGWON SCIENCE SYSTEM, Korea) at low temperature (40 °C), brown seaweed and wheat germ samples were crushed in a mechanical blender (PHLIPS HR 1727 Mixer) and sieved by a mesh. Brown seaweed samples were used 710 μm mesh, and wheat germ samples were used 500 μm mesh. The sieved samples were then stored at -30 °C and used for SC-CO_2 and organic solvent extraction.

3. Method

3.1. Supercritical carbon dioxide extraction

A plant scale supercritical fluid extraction unit was used for extracting oil from brown seaweed and wheat germ. The flow diagram of the equipment is shown in Fig.1.

Mixture of brown seaweed and wheat germ sample, 700 g, was packed into a stainless steel extraction vessel which was 5 L in volume. Liquefied carbon dioxide was pumped to the extraction vessel up to the desired pressure which was regulated by a back-pressure regulator. The pressure of CO_2 was automatically maintained by the pump. The temperature of vessel was maintained heat by itself. Oil extraction by SC-CO_2 was performed at temperature of 40 ℃ and pressure of 300-400 bar. The total extraction time was 1 hr. The flow rate of CO_2 was kept constant at 54.55 g/min for all extraction conditions and CO_2 volume passing through the apparatus were measured using a dry gas meter. The extracted oil was collected on the glass separation vessels. After SC-CO_2 extraction, the extracted oil was then stored at -40 ℃ until further analysis. The condition of extracts was shown in Table 2.

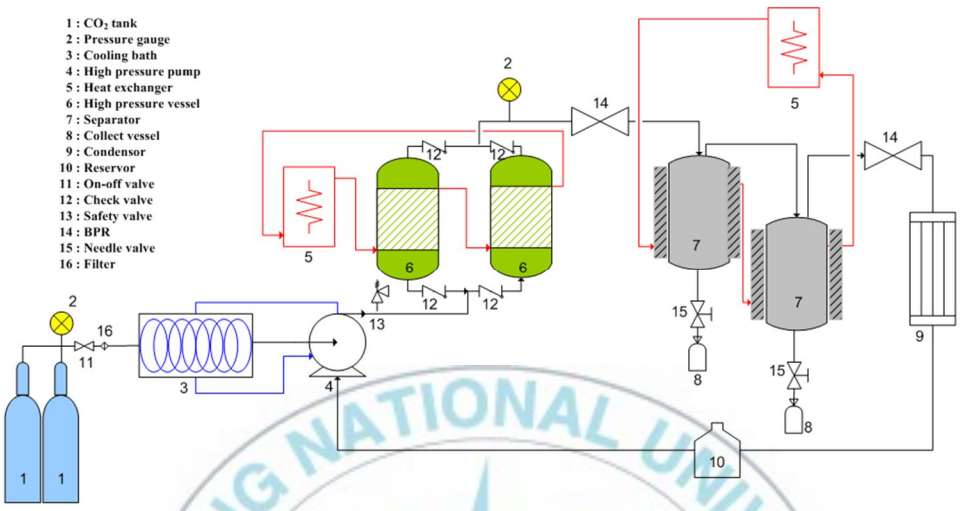


Fig. 1. Schematic diagram of supercritical carbon dioxide extraction.

Table 1. Operating conditions used in the experiments of supercritical carbon dioxide extraction

Parameter	Conditions
Sample	Brown seaweed or wheat germ (700 g)
Solvent	Carbon dioxide
Temperature	40 ℃
Pressure	300 bar, 400 bar
Operating time	1 hr
Flow rate of carbon dioxide	54.55 g/min

3.2 Organic solvent extraction

The extraction was carried out with or without soxhlet apparatus using hexane as solvent. During soxhlet apparatus using, 2 g of freeze dried brown seaweed and wheat germ sample was placed into the extraction thimble and the extraction was run 24 hr until the color of the condensed solvent at the top of the apparatus was clear. During without soxhlet apparatus using, 400 g of dried brown seaweed and wheat germ sample with 2 L hexane was placed into the beaker and stirred 24 rh by digital-heating mantle (MS-DMB 607/5L) at 40 °C and 400 RPM. After extraction, the hexane solution was evaporated in a rotary vacuum evaporator at 40 °C. The extracted oil was collected in the vial and stored at -20 °C until further use and analysis.

Table 2. Condition of extracts of brown seaweed and wheat germ samples

Sample form	Condition	
	Pressures	Temperature
SFC-300B	Brown seaweed oil extracted at 300 bar	40 ℃
SFC-300BW	The oil of brown seaweed and wheat germ (the ratio of 7:3) extracted at 300 bar	
SFC-300WB	The oil of brown seaweed and wheat germ (the ratio of 3:7) extracted at 300 bar	
SFC-300W	Wheat germ oil in extracted at 300 bar	
SFC-300B+W	Brown seaweed oil added wheat germ oil (the ratio of 7:3) extracted at 300 bar	
SFC-300W+B	Brown seaweed oil added wheat germ oil (the ratio of 3:7) extracted at 300 bar	
SFC-400B	Brown seaweed oil extracted at 400 bar	
SFC-400BW	The oil of brown seaweed and wheat germ (the ratio of 7:3) extracted at 400 bar	
SFC-400WB	The oil of brown seaweed and wheat germ (the ratio of 3:7) extracted at 400 bar	
SFC-400W	Wheat germ oil extracted at 400 bar	
SFC-400B+W	Brown seaweed oil added wheat germ oil (the ratio of 7:3) extracted at 400 bar	
SFC-400W+B	Brown seaweed oil added wheat germ oil (the ratio of 3:7) extracted at 400 bar	
H-B	Brown seaweed oil extracted by hexane	
H-BW	The oil of brown seaweed and wheat germ (the ratio of 7:3) extracted by hexane	
H-WB	The oil of brown seaweed and wheat germ (the ratio of 3:7) extracted by hexane	
H-W	Wheat germ oil extracted by hexane	
H-B+W	Brown seaweed oil added wheat germ oil (the ratio of 7:3) extracted by hexane	
H-W+B	Brown seaweed oil added wheat germ oil (the ratio of 3:7) extracted by hexane	

3.3 Analysis of fatty acid composition by GC

The fatty acid compositions of extracts of brown seaweed and wheat germ obtained by SC-CO_2 extraction and organic solvent extraction were determined by gas chromatography using a Hewlett Packard gas chromatograph (6890 Series II GC system). The fatty acid methyl esters were prepared firstly according to AOCS official method Ce 2-66 (AOCS, 1998) and then separated using an Agilent Supleco fused silica capillary column. GC conditions for the detection of fatty acids were shown in Table 3. Fatty acid methyl esters were identified by comparison of retention time with standard fatty acid methyl esters mixture.

Table 3. GC conditions for the detection of fatty acids

Parameter	Conditions
Instrument	Agilent 6890N
Split	Splitless
Inject time	250 ℃
Detect time	260 ℃
Carrier gas flow	He, 1mL/min
Oven time	140 ℃ (5 min) → 4 ℃/min → 250 ℃ (15 min)
Column	Agilent supleco fused silica capillary column 100.0 m×0.25 μm ×0.20 μm

3.4 Analysis of total phenolic content (TPC)

Total phenolic content of extract was determined using Folin-Ciocalteu colorimetric method according to Li et al. (2008) and Wong et al. (2006) with slight modification. 1 mL of 100 times diluted (v/v) extract was mixed with 1 mL of 1/10 (v/v, in deionized water) diluted Folin-Ciocalteu reagent (FCR). After 4 min, 800 μL of sodium carbonate solution (7.5% w/v) was added. Then, the mixture was allowed to stand at room temperature in dark environment for 2 hr. The absorbance of mixture was read at 765 nm with UV-spectrophotometer (UVIKON933, KONTRON INSTRUMENTS, Italy). The measurements were carried out in triplicate and gallic acid was used for calibration of standard curve.

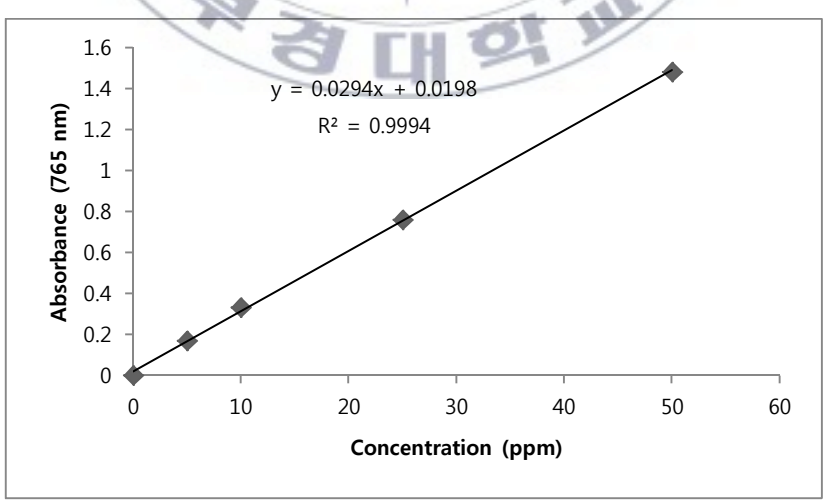


Fig. 2. Calibration curve of gallic acid for estimation of total phenolic contents.

3.5 Analysis of fucoxanthin by HPLC

3.5.1. Solvent fractionation of extracts

Prior to HPLC analysis, the solvent fractionation procedure was used as a step to simplify the separation by removing other substances. Solvent fractionation of extracts was separated as described previously Sachindra et al. with a slight modification. Approximately 1 g of extract obtained from mixture of brown seaweed and wheat germ was dissolved in 100 mL of methanol. 100 mL of n-hexane was added to the methanol solution in a separatory funnel. After shaking the funnel vigorously, the lower layer was transferred into another separating funnel. The upper layer (hexane layer) was evaporated under vacuum in a rotary evaporator at 30 ℃ to obtain the hexane extracted fraction. The lower layer in the funnel was mixed with added 100 mL of ethyl acetate and 50 mL of water. After shaking the mixture vigorously, the upper layer was concentrated to obtain the ethyl acetate extracted fraction. 200 mL of butanol and 50 mL of water were added to the lower layer and mixed vigorously. After removal of the upper layer, 50 mL butanol and 10 mL of water were added to the lower layer. The butanol layer was taken and combined with the first butanol layer and the solvent again

removed by rotary evaporation. The remaining aqueous solution in the separatory funnel after butanol removal was concentrated to obtain the residual fraction. The fractions except for the residual part were dissolved in ethanol. Residual fraction was dissolved in ethanol/water (70:30, v/v). All samples were kept in the dark and stored at −30 ℃ under nitrogen for further analysis.

3.5.2. Determination of fucoxanthin

Fucoxanthin was analyzed by high-performance liquid chromatography (HPLC), as described previously Maeda et al. (2005). The HPLC analysis was carried out with a Waters HPLC equipped with a model 600E system controller, a model 484 UV/VIS detector and a XTerra®MS C18 column (5 μm, 4.6 x 250 mm, Waters, USA). The mobile phase was methanol-acetonitrile (70:30, v/v) and the flow rate was 1 mL/min. Fucoxanthin was detected at 450 nm and its content was estimated by the standard calibration curve using purified fucoxanthin (purity > 98 %). Purified fucoxanthin was isolated from the extracts including the brown seaweed.

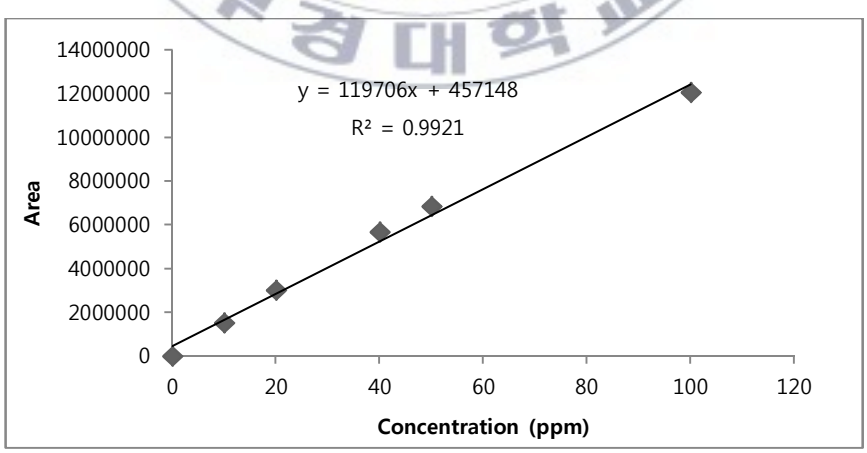
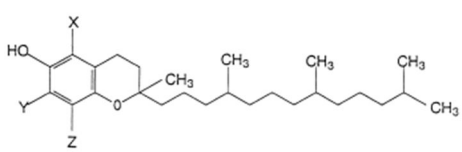


Fig. 3. Calibration curve of fucoxanthin standard.

3.6. Analysis of tocopherols by HPLC

Tocopherols analysis was determined using a high performance liquid chromatography (LC-NETⅡ/ADC, LG-2080-04, DG-2080-54, Jasco, Japan) equipped with a pump (PU-2080Plus, Jasco, Japan), an evaporative light scattering detector (400 ELSD, Softa Corporation) and a Eclipse Plus C18 column (5 μm, 4.6 x 250 mm, Aglient, USA). About 10 mg, exactly weighed, of oil was dissolved in 10 ml of methanol. Aliquots of this solution were injected for HPLC analysis. The contents of α-, β- and γ- tocopherol in the extracts were obtained from calibration graphs and expressed in μg/g of sample (dry base). The calibration equation for α- and β- + γ- tocopherol was y = 31349x-428548 (R^2=0.9997) and y = 68898x–271271 (R^2 = 0.9991)



X = Y = Z = CH_3 α - tocopherol
X = Z = CH_3 ; Y = H β - tocopherol
X = H ; Y = Z = CH_3 γ - tocopherol

Fig. 4. Structure of tocopherols.

3.7. Oxidative stability of extracted oil

3.7.1. Peroxide value

Peroxide value was determined by Y. K. Wang et al. 1 g of sample was dissolved in 30 mL of chloroform : acetic acid (2:3). Peroxide value of oil was analyzed by titration with 0.1 N $Na_2S_2O_3$ solution and using an iodometry. The peroxide value was calculated using the following equation.

$$POV = \frac{(A-B) \times F}{c} \times 10 \ (0.01 \times 1000 \ mg/kg)$$

POV is the peroxide value of the sample, A is the volume of the 0.1 N $Na_2S_2O_3$ solution of the titration (mL), B is blank, F is the concentration of the 0.1 N $Na_2S_2O_3$ factor and c is the weight of the sample (g).

3.7.2. Free fatty acid contents (FFA)

FFA of extracted oil from each sample was analyzed as described by Benardez et al. (2005). Accurately, 50 mg of oil was placed into pyrex tubes tubes with the addition of 3 mL of cyclohexane and then 1 mL of cupric acetate-pyridine reagent was added. Tubes were vortexed for 30 sec. After centrifugation at 2000 g for 10 min, the upper layer was read at 710 nm. The FFA content of oil was measured on a calibration curve constructed from oleic acid standard. Copper reagent was prepared according to Lowry and Tinsley (1976). Briefly, 5 % (w/v) aqueous solution of cupric acetate was prepared and filtered. Then the pH of cupric acetate solution was adjusted to 6.1 using pyridine.

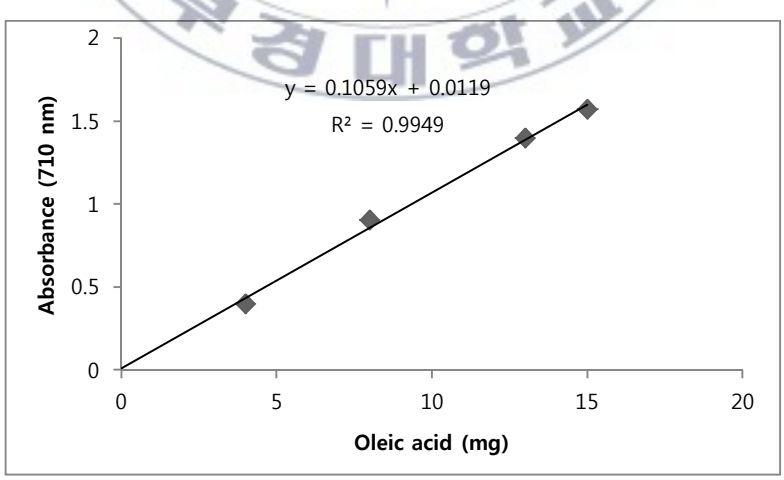


Fig. 5. Calibration curve of oleic acid for estimation of free fatty acids in oil.

3.8. Antioxidant activity of extracted oil

3.8.1. DPPH radical scavenging activity

The DPPH radical scavenging capacity of extracts was determined based on the method described Shimada et al. (1992) and Hsu et al. (2003) with some modifications. Aliquots of 1 mL methanolic sample solution added 5 mL of freshly prepared 0.1 mM DPPH methanolic solutions. After thoroughly mixed, and kept for 50 min in the dark place. Subsequently, the absorbance of sample was measured at 517 nm using UV-spectrophotometer (UVIKON933, KONTRON INSTRUMENTS, Italy). 1 mL of Ascorbic acid in methanol (10,000 ppm) was used for control. 1 mL of the Methanol, replacing the extracted oil, was used as the blank. The percentage of DPPH radical scavenging capacity was calculated using this equation:

$$\text{Scavenging effect (\%)} = [1-(A_{517nm,sample}/A_{517nm,blank})] \times 100\ \%$$

All tests were carried out in triplicate.

3.8.2. ABTS radical scavenging activity

The radical scavenging capacity of extracts against ABTS radical cation was assessed according to the method described by Wetwitakyaklung et al. (2006), Li et al. (2008) and Chew et al. (2011) with slight modifications. An ABTS radical solution was prepared by mixing 7 mM ABTS solution and 2.45 mM potassium persulfate solution at a ratio 1:1 (v/v).The mixture was vortexed for 10 sec and then allowed to stand in dark condition at room temperature for 24 hr to give a dark blue. The absorbance of ABTS radical solution was equilibrated to an absorbance of 0.7 ± 0.02 at 734 nm by diluting with ethanol before used. 0.1 mL of extract was mixed with 3.9 mL of ABTS radical solution. Blank was also prepared by replacing 0.1 mL deionized water with extract. The absorbance of mixture was read immediately at 734 nm by using UV-spectrophotometer (UVIKON933, KONTRON INSTRUMENTS, Italy) against blank after incubation at room temperature for 6 min. The radical scavenging capacity of ABTS (%) was calculated as [1- (As / Ac)] x 100% (As = Absorbance of sample at 734 nm; Ac = Absorbance of negative control at 734 nm). Trolox solution was used to calibrate the standard curve. Each extract was analyzed in triplicate and the results were expressed as μmol trolox equivalent per 100 g dried weight (μmol TEAC / 100 g DW).

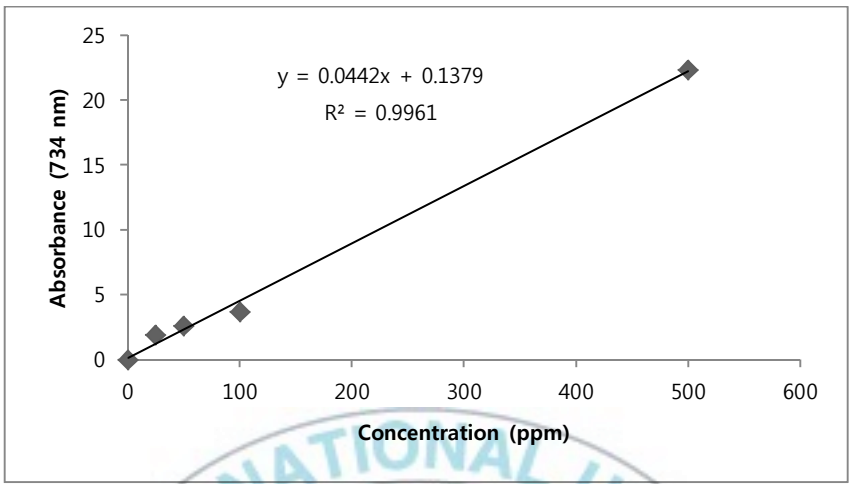


Fig. 6. Calibration curve of trolox for ABTS radical scavenging activity of oil.

3.8.3. Rancimat test

A Metrohm Rancimat model 743 (Methrom Instruments, Herusau, Swizerland) was utilized. A steam of filtered and dried air at flow rate 20 L/hr is bubbled into samples (3 g) contained in reaction vessel. Vessels are placed in an electric heating block which is set at 110 ℃. Effluent air containing volatile organic acids from the oil are collected in a measuring vessel with 60 mL of distilled water. The conductivity of the water is continuously recorded and the OSIs of the oil samples were automatically recorded at 120 ℃ for acceleration test. The oil for all determinations was randomized to determine their position in the heating block.

3.9. Antimicrobial activity of extracted oil

3.9.1. Paper disc diffusion assay

The antimicrobial activities of the oils were determined by paper disc diffusion assay with slight modification (Hun et al. 1994). Shown in Table 4, six microorganisms were used in this study. All strains were obtained from the Korean Culture Center of Microorganisms (KCCM). McFarland standard No. 0.5 was used in the preparation of suspension of microorganism. The turbidity of bacterial suspension was adjusted according McFarland standard. The accurate turbidity of the bacterial suspension was confirmed by UV-spectrophotometer (UVIKON933, KONTRON INSTRUMENTS, Italy) on 625 nm, with approximate cell density of each bacterial strain was 10^7 CFU/mL. Mueller-Hinton agar was used as growth medium for bacterial strains and sterilized at 121 ^oC for 15 min. The agar was poured into sterile glass petri dishes and allowed for several times until stiff agar forming. Bacterial suspension was spread out on agar surface with sterile cotton. After then, Advantec paper disc (10 mm) contains oil or control (methanol) was impregnated at the surface of agar. The soaked discs were then placed in the middle of the plates and incubated for 24 hr at 37 ^oC and the diameter of each inhibitory zone was measured (in mm).

3.9.2. Minimum inhibition concentration (MIC) assay

The values of Minimum Inhibitory Concentration (MIC) were measured against bacteria tested with slight modification (G. S. El-Baroty et al. 2010). The disc diffusion assay was performed with oils on different concentration. Sterile Mueller-Hinton agar was poured into glass petri dishes and allowed for 30 min to make solid agar. The inoculums of bacteria then spread out at the surface of agar. After then, holes of 6.0 mm diameter were punched into the agar to create wells, wherein the certain concentration of oils was placed there. The MIC was the lowest concentration that inhibited the microorganisms, i.e. with an inhibition zone greater than 6.0 mm after 24 hr incubation period at 37 ºC.

Table 4. Microorganisms and culture medium used for antimicrobial activity tests

Microorganisms	Strain	Medium
Gram positive		
Bacillus cereus	KCCM 40022	Nutrient agar
Listeria monocytogenes	KCCM 40307	Nutrient agar
Staphylococcus aureus	KCCM 40050	Nutrient agar
Gram negative		
Escherichia coli	KCCM 11234	Nutrient agar
Pseudomonas aeruginosa	KCCM 11803	Nutrient agar
Salmonella typhimurium	KCCM 11862	Nutrient agar

Results and Discussion

1. Comparison of oil yield by SC-CO_2 and hexane extraction

The total amount of oil obtained by soxhlet extraction using hexane from brown seaweed and wheat germ was 1.0 to 11.2 % (w/w in hot-air dried raw sample) shown in Fig.7. On the other hand, the amount of total oil in SC-CO_2 extraction was 0.9 to 10.3 % for the experiment conducted at 300 bar 40 ºC. Comparing to the amount oil of brown seaweed, those of wheat germ was high. By considering that the extraction of oil using hexane was complete, the highest yield by SC-CO_2 extraction was almost 92 %. The maximum oil yield obtained by SC-CO_2 was 70 % from peach seed (Sanchez-Vicente et al., 2009) and 87 % from squid viscera (Md. Salim Uddin, 2011). The differences in maximum yield may be occurred due to variation of processing unit, operating conditions, sample size, percentage of lipid in sample etc (Md. Salim Uddin, 2011).

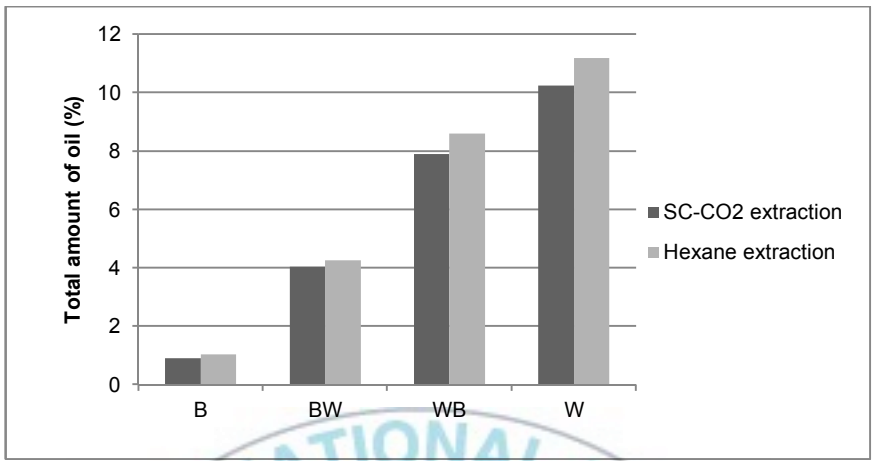


Fig. 7. The percentage of total amount of oil from brown seaweed (B), mixture of brown seaweed and wheat germ at ratio of 7:3 (BW), mixture of brown seaweed and wheat germ at ratio of 3:7 (WB) and wheat germ (W) at SC-CO_2 extraction at 300 bar and 40 ºC and hexane extraction.

2. Fatty acid compositions

Fatty acids are merely carboxylic acids with long hydrocarbon chains which are either saturated or unsaturated. It can be an indicator of its stability, physical properties, and nutritional value. The fatty acid composition of oil was presented in Table 5. There were no differences in fatty acid composition of oils obtained SC-CO_2 and hexane extraction and a high content of unsaturated fatty acids in oil were identified. The components of wheat germ oil are palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and γ-linolenic acid (C18:3). Brown seaweed oil contains these components as well as palmitoleic acid (C16:1), stearic acid (C18:0), arachidonic acid (C20:4), erucic aic (C22:1) and EPA (C20:5). The most predominant result is that oil of brown seaweed added wheat germ had lots of linoleic acid. Another unsaturated fatty acid, EPA was also present. Oils obtained from brown seaweed and wheat germ could have a various compositions of fatty acids. Variations in fatty acid contents are attributable both to environmental and genetic differences. Otherwise, though EPA is nutritionally superior by itself, it can be synthesized to DHA with linoleic acid. Therefore, it is observed that combination of wheat germ oil containing high content of linoleic acid is efficient to improve the nutritional functionality of brown seaweed oil.

Table 5. Fatty acid compositions of extracts

Fatty acids			C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:4	C22:1	C20:5
$SC-CO_2$ extraction	300 bar	SFC-300B	26.0	3.3	3.1	39.7	8.9	1.2	0.6	11.8	5.6
		SFC-300W	18.4	-	0.7	17.1	62.3	1.5	-	-	-
		SFC-300BW	19.6	0.7	1.1	20.0	54.7	0.5	0.3	2.1	1.0
		SFC-300WB	19.5	0.9	0.9	17.5	58.5	1.4	0.2	0.7	0.3
		SFC-300B+W	22.1	2.0	30.8	3.0	31.2	0.9	0.3	6.7	3.0
		SFC-300W+B	19.9	0.9	23.4	1.3	48.1	0.4	1.3	3.2	1.5
	400 bar	SFC-400B	23.2	3.0	3.1	38.5	9.0	2.8	0.6	13.1	6.6
		SFC-400W	18.1	-	0.9	17.0	62.3	1.6	-	-	-
		SFC-400BW	18.8	0.6	0.8	20.5	54.6	1.6	0.4	1.9	0.8
		SFC-400WB	17.9	0.3	0.9	18.3	60.1	1.6	0.2	0.5	0.2
		SFC-400B+W	21.5	2.0	31.3	3.5	30.8	0.9	0.3	6.7	3.0
		SFC-400W+B	19.7	0.9	23.5	1.4	48.1	0.5	1.3	3.1	1.5
Hexane extraction		H-B	22.2	2.9	2.9	37.8	9.0	1.2	4.6	13.4	5.9
		H-W	14.6	-	1.0	18.1	64.4	2.0	-	-	-
		H-BW	18.8	0.6	1.0	20.4	54.0	1.9	0.8	1.8	0.8
		H-WB	17.8	0.2	0.9	18.3	59.2	1.8	0.6	0.7	0.5
		H-B+W	19.8	2.1	29.0	2.8	28.6	1.2	3.7	9.0	3.8
		H-W+B	19.0	1.5	25.9	2.0	40.1	0.8	2.2	5.9	2.6

3. Total phenolic contents

TPC analysis was based on measuring the color change caused by reduction of the Folin-Ciocalteu reagent by phenolates in the presence of sodium carbonate. Phenolics have been associated with color, sensory qualities, and nutritional and antioxidant properties of food (GW Jung, 2011). Phenolic compounds have been reported to have several biological activities including antioxidant activity (S. KUMAR CHANDIMI et al., 2008). TPC of oil of brown seaweed and wheat germ was shown in Table 6. Comparing to the oil obtained by hexane extraction, the oil obtained by SC-CO_2 extraction had high phenolic content value. TPC value range from 61.63 to 194.29 mg GAE/100g DW in SC-CO_2 extraction and it was maximum at 300 bar and 40℃. In terms of combination effect, addition of Wheat germ oil to brown seaweed oil after extraction was more effective than addition of wheat germ powder before extraction in total phenolic contents. It means that the oil contained various fatty acid compositions from brown seaweed mixed with wheat germ also had lots of phenolic compounds similar to brown seaweed oil. Phenolic compounds in plants possess antioxidant activity, and may help protect cells against the oxidative damage caused by free radicals (A. KIRAKOSYAN et al., 2003).

Table 6. Total phenolic content of extracts at different extraction conditions

Conditions		Total phenolic content (mg/100g DW)
$SC\text{-}CO_2$ extraction	SCF-300B	214.6
	SCF-300BW	99.5
	SCF-300WB	71.6
	SCF-300W	39.4
	SCF-300B+W	171.9
	SCF-300W+B	105.4
	SCF-400B	209.7
	SCF-400BW	75.1
	SCF-400WB	70.6
	SCF-400W	54.4
	SCF-400B+W	160.8
	SCF-400W+B	77.8
Hexane extraction	H-B	183.9
	H-BW	101.4
	H-WB	104.9
	H-W	85.7
	H-B+W	179.8
	H-W+B	105.5

4. Fucoxanthin contents

By the successive solvent fractionation, fucoxanthin was concentrated in ethyl acetate fraction. Hexnae fraction and butanol fraction didn't indicate the reasonable value. Table 7 was shown to the fucoxanthin content in ethyl acetate fraction. Though there was no significant difference between SC-CO_2 and hexane extraction, results showed that brown seaweed (*Laminaria japonica*) contained a considerable amount of fucoxanthin (10.6 to 12.2 mg/g oil) at all conditions. These results are relatively similar to the several kinds of brown seaweeds (0.28 to 12.71 mg/g extracts) as reported by M.K. Widjaja-Adhi Airanthi et al. (2011). Furthermore, the fucoxanthin contents of oil mixture such as SFC-300B+W, SFC-300W+B, SFC-400B+W, SFC-400W+B, H-B+W and H-W+B (7.1, 5.3, 8.7, 5.8, 8.4 and 5.5 mg/g oil, respectively) was significantly comparable with fucoxanthin contents of SFC-300BW, SFC-300WB, SFC-400BW, SFC-400WB, H-BW and H-WB (2.7, 0.9, 2.3, 0.7, 2.2 and 0.6 mg/g oil, respectively) The combination of brown seaweed oil and wheat germ oil after SC-CO_2 extraction showed higher fucoxanthin contents than oil obtained mixture of brown seaweed powder and wheat germ powder. This means that oil mixture of brown seaweed and wheat germ was more effective to obtain fucoxanthin than oil obtained from powder mixture of brown seaweed and wheat germ.

Table 7. The fucoxanthin content of extracts at different extraction conditions

Conditions		Fucoxanthin (mg/g oil)
$SC-CO_2$ extraction	SCF-300B	10.6
	SCF-300BW	2.7
	SCF-300WB	0.9
	SCF-300W	-
	SCF-300B+W	7.1
	SCF-300W+B	5.3
	SCF-400B	12.2
	SCF-400BW	2.3
	SCF-400WB	0.7
	SCF-400W	-
	SCF-400B+W	8.7
	SCF-400W+B	5.8
Hexane extraction	H-B	12.1
	H-BW	2.2
	H-WB	0.6
	H-W	-
	H-B+W	8.4
	H-W+B	5.5

5. Tocopherol contents

By comparing retention times of the standard solutions, α-, β- and γ-tocopherols were identified in the oils. The quantitative data on tocopherols are presented in Table 8. The contents of tocopherols at 300 bar and 40 ^{o}C was indicated similar results in previous studies (Go-woon Jung, 2011). Roasted wheat germ oil using SC-CO_2 at high pressure contained high contents of tocopherol (Gelmez et al., 2009). The highest amount of α- and β + γ-tocopherols were respectively SCF-400W+B and SCF-400B (3786.1 and 1052.7 μg/g oil). Brown seaweed oil added wheat germ oil after SC-CO_2 and hexane extraction showed higher α-tocopherol content than the oil extracted from mixture of brown seaweed powder and wheat germ powder. Especially, α-tocopherol content was improved than each brown seaweed oil and wheat germ oil. It means that combination of brown seaweed oil and wheat germ oil was very effective and successive to improve the antioxidant activity by tocopherols. In case of β+ γ – tocopherol, the oil obtained from mixture of brown seaweed powder and wheat germ powder was higher than brown seaweed oil added wheat germ oil.

Table 8. The α-Tocopherol and β+ γ -Tocopherol contents of extracts at different extraction conditions

Conditions		α-Tocopherol (μg/g oil)	β+ γ -Tocopherol (μg/g oil)
$SC-CO_2$ extraction	SCF-300B	874.7	709.4
	SCF-300BW	1491.8	561.1
	SCF-300WB	1246.9	484.2
	SCF-300W	1159.5	367.4
	SCF-300B+W	2175.3	468.3
	SCF-300W+B	3725.7	299.0
	SCF-400B	1276.4	1052.7
	SCF-400BW	824.2	721.4
	SCF-400WB	1067.4	491.2
	SCF-400W	1336.4	458.4
	SCF-400B+W	2416.4	338.4
	SCF-400W+B	3786.1	241.6
Hexane extraction	H-B	1515.8	978.2
	H-BW	597.8	955.1
	H-WB	1690.5	523.7
	H-W	2496.5	391.1
	H-B+W	2215.1	291.3
	H-W+B	3187.4	338.9

6. Oxidative stability

6.1. Comparison of POV of SC-CO_2 and hexane extraction

POV was the indicator to measure the oxidative stability and quality of oil. It was carried out to measure the rancidity which occurs by auto oxidation state of oil. It was found that oil obtained from mixture of brown seaweed and wheat germ showed low value in Table 9. These results associated with FFA contents.

6.2. Free fatty acid contents

The quality of oil is deteriorated at production and storage conditions. FFA contents of oil extracted by SC-CO_2 and hexane extraction was shown in Table 9. The highest content of FFA was found in the oil by hexane extraction. The FFA contents of oil extracted by hexane ranged from 4.0 to 28.2 g/100g oil. Comparing to FFA contents of brown seaweed oil, those of wheat germ oil was remarkably low. When adding the wheat germ oil to brown seaweed oil, the contents of FFA was shown to low value than only brown seaweed oil. Thus, mixing the two samples was more effective to protect oil oxidation.

Table 9. Analysis of free fatty acids and peroxide value of extract

Conditions		Free fatty acids (g/100g oil)	Peroxide value (meq/1000g)
$SC-CO_2$ extraction	SCF-300B	23.8	17.6
	SCF-300BW	12.6	15.0
	SCF-300WB	5.6	7.2
	SCF-300W	5.0	6.9
	SCF-300B+W	19.6	15.8
	SCF-300W+B	6.8	8.1
	SCF-400B	20.8	16.2
	SCF-400BW	10.2	12.0
	SCF-400WB	5.0	6.3
	SCF-400W	3.0	4.8
	SCF-400B+W	17.8	14.3
	SCF-400W+B	3.8	5.8
Hexane extraction	H-B	28.2	19.6
	H-BW	16.0	16.5
	H-WB	6.8	8.1
	H-W	4.0	5.8
	H-B+W	23.2	17.8
	H-W+B	7.6	9.7

7. Antioxidant activity

7.1. DPPH free radical scavenging effect

The DPPH radical scavenging effects of oils of brown seaweed and wheat germ obtained after SC-CO_2 are shown in Fig. 8. Generally, brown seaweed oil and wheat germ oil with SC-CO_2 showed considerable free radical scavenging activity. When extracting the two samples together by SC-CO_2, oils also indicated similar values. No significant difference of the DPPH radical scavenging activities of oil obtained by SC-CO_2 and hexane was found. Therefore, such dietary antioxidants from oil may be particularly important in fighting diseases by conferring protection against free radical damage to cellular DNA and lipids (Jung GW et al. 2012).

7.2. ABTS free radical scavenging effect

Fig. 9 was showed ABTS free radical scavenging effect of oils. The oil obtained by SC-CO_2 extraction at 400 bar and 40 ℃ showed the strongest ABTS radical scavenging activity compared to the oil obtained at other extraction conditions and also hexane extraction. When the oil extracted from brown seaweed powder added wheat germ powder indicated low ABTS free radical scavenging activity than each brown seaweed oil and wheat germ oil. Otherwise, brown seaweed oil added wheat germ oil after extraction was showed appropriative results for effect of each brown seaweed and wheat germ oil. It was found that mixing the brown seaweed oil and wheat germ oil after extraction was more effective to obtain high ABTS free radical scavenging activity.

7.3. Rancimat test

The rancimat test is commonly used to evaluate the oxidative stability of fatty acid methyl ester. It is determined measuring the induction period of each sample. The induction period provides the starting point of lipid oxidation. Rancimat machine measures the ability of samples to resist oxidation under conditions of heat and continuous air flow. The instrument is used extensively to determine the detrimental effects of metallic contaminants and the ameliorating effects of chelators and antioxidants. Fig. 10 was showed the induction period of oils. The induction period of the mixture of brown seaweed and wheat germ oils was longer than other conditions. The oil (B+W) obtained by SC-CO_2 extraction at 400 bar and 40 ^{o}C showed longest induction period. Otherwise, the oil obtained by hexane extraction showed the induction period of 3.52 to 5.15 hr. When comparing the result of SC-CO_2 extraction, the oil extracted by SC-CO_2 was more efficient to delay the oxidation in experimental environments. It was observed that SC-CO_2 was less exposed the air than hexane for extraction.

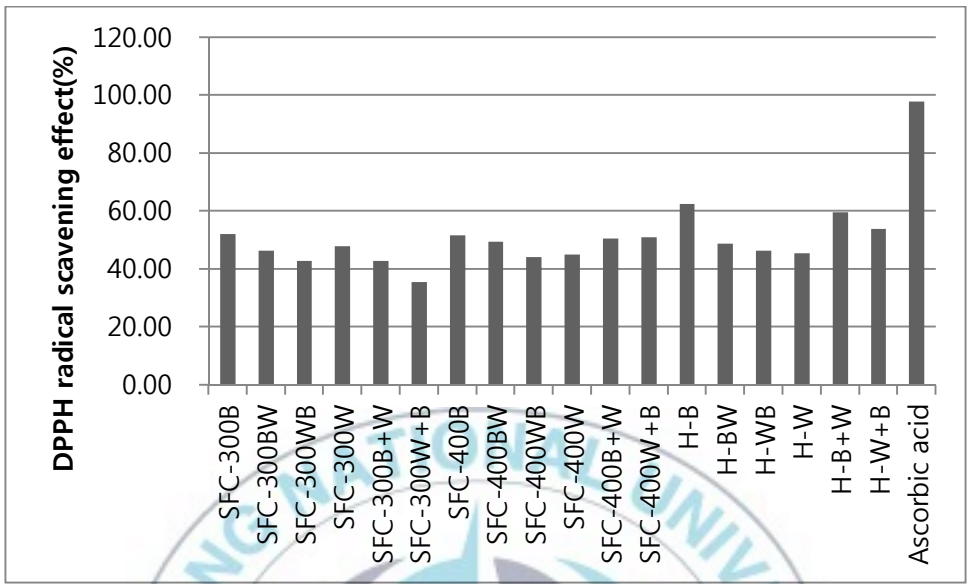


Fig. 8. DPPH radical scavenging activity of oil of brown seaweed and wheat germ obtained by SC-CO_2 extraction and organic extraction: concentration of ascorbic acid was 10,000 ppm.

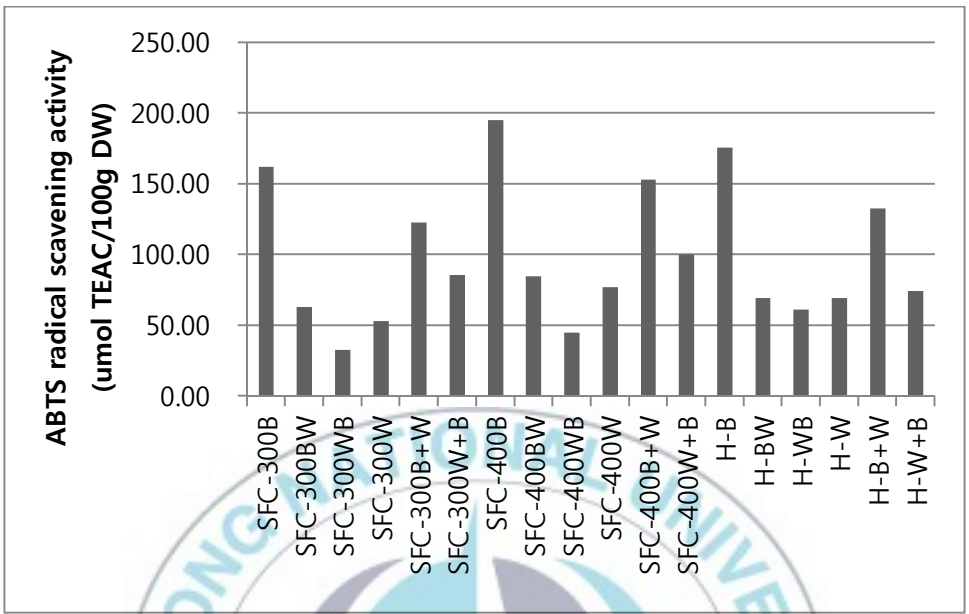


Fig. 9. ABTS radical scavenging activity of oil of brown seaweed and wheat germ obtained by SC-CO_2 extraction and organic extraction.

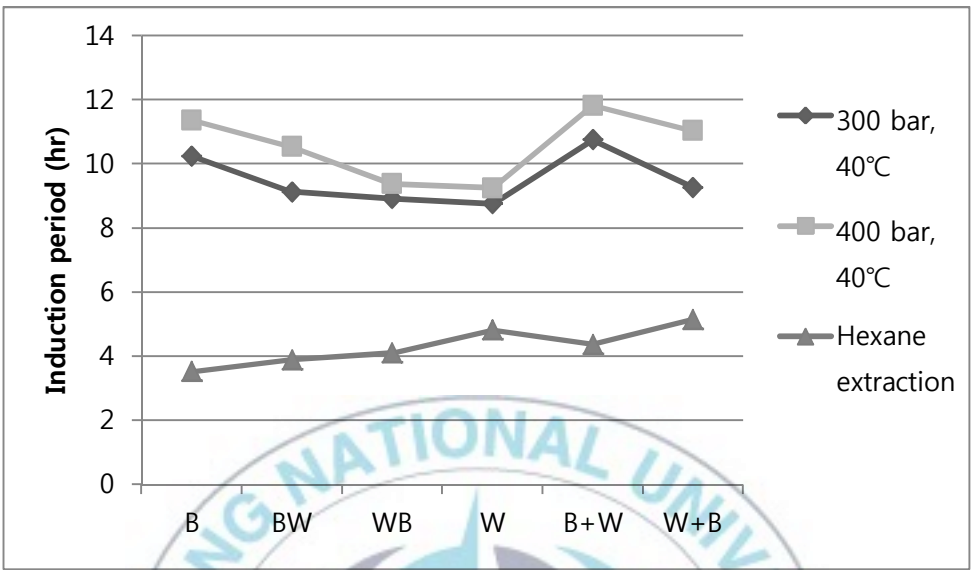


Fig. 10. Induction period of oil of brown seaweed and wheat germ obtained by SC-CO_2 extraction and organic extraction.

8. Antimicrobial activity

8.1. Paper disc diffusion

As a result of antioxidant activity of oil at different conditions, the condition of 400 bar and 40 ^{o}C by SC-CO_2 was efficient than other conditions. So, for antimicrobial activity test, the oil obtained at 400 bar and 40 ^{o}C by SC-CO_2 extraction was used.

The antimicrobial activity in various oil of mixture was assessed by paper disc diffusion assay. The results indicated variation in the antimicrobial properties of the oil (Table 10). The oil extracted from brown seaweed and wheat germ showed the antimicrobial activity against *L. monocytogenes*, *S. aureus, E. coli* and *P. aeruginosa* .The most effective oil was SFC-400B+W, its inhibitory zones for each of the microorganism that were sensitive. Greater inhibition was observed in the case of the SFC-400B+W against *L. monocytogenes* and *P. aeruginosa.* The mixture of brown seaweed and wheat germ oils had more high level of antimicrobial activity than mixture oil obtained from brown seaweed and wheat germ powders. Antimicrobial activity in plant extracts depends not only on the presence of phenolic compounds but also on the presence of various secondary metabolites (Gordana et al., 2007).

8.2. Minimum Inhibition Concentration

Through the paper disc diffusion assay, it was confirmed that the oil obtained from brown seaweed and wheat germ has antimicrobial activity. In case of SFC-400B+W, *B. cereus, L. monocytogenes, P. aeruginosa* and *S. typhimurium* did not appear the growth in 5,000 μg/ml. About those bacteria, the oil had a MIC of 5,000 μg/ml. Otherwise, about *E. coli*, it had a MIC of 2,000 μg/ml. SFC-400B, SFC-400BW, SFC-400B+W and SFC400-W+B were showed high level of antimicrobial activity, especially against *P. aeruginosa*, Shown in Table 11, the most effective oil was SFC-400 B+W against most of bacteria. The mixture of brown seaweed and wheat germ oils obtained after SC-CO_2 extraction have a value as potential sources of antimicrobial agents against food-borne bacteria.

Table 10. Clear zone of the oil from of brown seaweed and wheat germ obtained by SC-CO_2 and organic extraction

Microorganisms	Clear zone of the oil					
	SFC-400B	SFC-400BW	SFC-400WB	SFC-400W	SFC-400B+W	SFC-400W+B
B. cereus	-	-	-	-	-	-
L. monocytogenes	±	±	-	±	+	±
S. aureus	±	-	-	±	±	±
E. coli	-	-	-	-	+	-
P. aeruginosa	+	±	-	-	+	-
S. typhimurium	-	-	-	-	-	-

- No inhibition (10 mm)
± Very slight inhibition (10~11 mm)
+ Slight inhibition (11~12 mm)
++ Moderate inhibition (12~16 mm)

Table 11. Minimal inhibitory concentration of the oil from brown seaweed and wheat germ obtained by SC-CO_2 and organic extraction

Microorganisms	MIC (μg/ml)					
	SFC-400B	SFC-400BW	SFC-400WB	SFC-400W	SFC-400B+W	SFC-400W+B
B. cereus	>10,000	10,000	>10,000	>10,000	5,000	>10,000
L. monocytogenes	>10,000	>10,000	>10,000	>10,000	5,000	10,000
S. aureus	5,000	10,000	>10,000	>10,000	10,000	>10,000
E. coli	>10,000	>10,000	>10,000	>10,000	2,000	10,000
P. aeruginosa	2,000	5,000	>10,000	>10,000	5,000	5,000
S. typhimurium	>10,000	>10,000	>10,000	>10,000	5,000	>10,000

Conclusion

In SC-CO_2 and hexane extraction, both brown seaweed oil mixed wheat germ oil after extraction and the oil extracted from mixture of brown seaweed and wheat germ powders had a diverse and outstanding compositions of fatty acid. The oil contained new and various fatty acid compositions from brown seaweed mixed with wheat germ also had lots of phenolic compounds and fucoxanthin, and it was similar to only brown seaweed oil. In antioxidant activity for DPPH and ABTS free radical scavenging activity and rancimat test, mixture of brown seaweed and wheat germ oils showed comparable results with each samples. Especially, α-tocopherol content of mixture of brown seaweed and wheat germ oils was increased remarkably than that of each sample. It means that that oil mixture of brown seaweed and wheat germ after extraction was more effective than powder mixture of brown seaweed and wheat germ in antioxidant activity. And, when the oil was extracted at 400 bar and 40 ^{o}C by SC-CO_2, results was desirable. Otherwise, in the antimicrobial activity, oil mixture of brown seaweed and wheat germ after extraction was more effective than powder mixture of brown seaweed and wheat germ (B+W). It caused by high amount of antioxidant materials such as phenolic compounds, tocopherols, fucoxanthin and

phytochemicals. As a result, brown seaweed oil added wheat germ oil (ratio of 7:3) was excellent in antioxidant and antimicrobial activity. Thus, the mixture of brown seaweed oil and wheat germ oil with good quality and diverse nutrient ingredients using SC-CO_2 extraction will be more effective for human and other living organisms.

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