Journal of Environmental Biology **July 2008,** 29(4) 479-484 (2008)

Antioxidant activity of mojabanchromanol, a novel chromene, isolated from brown alga *Sargassum siliquastrum*

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(Received: March 13, 2007; Revised received: December 18, 2007; Accepted: December 19, 2007)

Abstract: *Silica gel chromatography, HPLC, EI -Mass, and NMR analyses were performed in order to determine the structure obtained from the separation and refinement of the main components of an ethanol extraction of Sargassum siliquastrum, which indicated its antioxidant activity. An ethanol extraction of Sargassum siliquastrum demonstrated the strongest antioxidant activities of ethyl acetate when isolated by silica gel chromatography. This amounts to 84.9±1.2% of its 0.5 mg ml^{-1} concentration. The thiobarbituric acid reactive substances (TBARS) measurements from 3 isolations of an HPLC separation of ethyl acetate showed that the antioxidant activity of peak 2 was the most dominant at 84.08% in a 0.5 mg ml^{-1} concentration. Peak 2 was verified as a type of chromene through EI-Mass and NMR analysis. Its metal sealing characteristic was low while its characteristic of TBARS and DPPH erasure indicated similar or higher levels of metal sealing characteristic. The NMR spectroscopic data was used to elucidate the structure of this new compound, which showed strong antioxidant activity in the assay.*

Key words: *Antioxidant activity, Chromene, Brown alga, Sargassum siliquastrum*
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Introduction

Several free-radicals within the human body provoke DNA duplication by attacking the organic molecules or cause DNA deformation by changing the normal function or properties of transcription (Brawn *et al*., 1981). Free-radicals, in turn, alters the membrane permeability due to the formation of polyvalent unsaturated fatty acid substrates and cholesterol peroxides (Halliwell *et al*., 1984).

While having a toxic effect on the cells, free-radicals can also directly or indirectly lead to other disorders, including cancer (Bomzon *et al*., 2001), cerebral diseases such as brain tumors, Parkinson's disease, and cerebrovascular diseases such as arteriosclerosis, cardiac disorders, etc. Its activity is also implicated in various diseases such as skin disorders or as a cause of senility (Yagi, 1987), *etc.* Moreover, if the substrate is oxidized during storage or processing, its taste deteriorates due to the presence of bad smells, discoloration, coloration or a change in its material properties. It also causes distinct toxic properties within certain types of carbonyl compounds, which result from the continual oxidation and destruction of peroxides (Kaunitz, 1967). Thus, antioxidants such as radical scavengers, which remove free-radicals by restraining free-radical generation or suspending each chain reaction by giving up its own hydrogen or electron, are added to protect against oxidation by taking in external antioxidant materials. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydropuinone (TBHQ) *etc.,* are recently used as synthesized antioxidants with strong antioxidant properties. However, a number of safety concerns are associated with the use of such antioxidants such as their production of teratogenic factors and carcinogens due to the toxic properties of a part of intake substance in the body (Ito *et al*., 1983). This increased hepatomegaly and microsomal enzymatic activity in the liver resulted in pathological damage to the lung, kidney, and other internal organs (Branen, 1975). The interest in finding natural antioxidants is increasing with increasing safety awareness and the desire for a healthy modern life because a steady intake of foods that contain a large amount of natural antioxidants has been found to have adverse effects.

A large number of antioxidant elements have been found as a result of steady research investigating the antioxidant effects of natural plants. Tocopherol, flavone derivatives, sesamol, gossypol, the hydrolysate of proteins and some spices *etc.,* are currently being used as natural antioxidants. Isoflavone, antioxidants such as phenolic acids, tocopherol, phytic acid, saponin, trypsin inhibitors, amino acid, peptide, *etc.* were found in yellow beans. Additionally, chlorogenic acid and 1,4-di-o-caffcoylquinic acid were extracted from araliaceous shrubs and their antioxidant effects were investigated (Kim *et al*., 2005). Also, the usage and properties of antioxident materials of herbage are well-known and the basic clinical research has been partially performed (Lee and Shibamoto, 2002). But, the research on the antioxidant effect and material separation of algae material is still deficient. There are approximately 8,000 species of marine algae in the world. It has been reported that their antioxidation, antimicrobial, antifouling effects (Heo *et al.,* 2005, Bansemir *et al.*, 2006, Sidharthan *et al.,* 2004). The antioxidant effects of *Sargassum siliquastrum* were reported by Lee *et al.* (1996) and the research on the separation of bromophenols and chromenes from *Sargassum siliquastrum* was reported (Jang *et al*., 2005).

This research was carried out to determine the structure and mechanism of antioxidation by the separation and purification of

its main components, which demonstrated the antioxidant effects within an ethanol extract of *Sargassum siliquastrum*.

Materials and Methods

Sargassum siliquastrum has been used in experiments in which the specimens were collected from the seashore of Pusan, and was used at a freezing storage of -70°C after dried pulverization.

Extraction was performed twice after extracting supernatant by mixing for 24 hr at room temperature, using 10 times stronger n-haxane, chloroform, ethanol and distilled water for *Sargassum siliquastrum*. The second extraction was performed using the same method as the first extraction using the same solvent for the dregs, which remained after the extraction of supernatant. After the filtration of supernatant with filter paper (Advantec 5A), it was used as a reagent with full dry and storage at 37°C after concentration via a rotary evaporator (RE200, Yamato Co., Japan).

The amount of malonedialdehyde was measured for the Thiobarbituric acid reactive substances (TBARS) analysis according to the method of Buege and Aust (1978). Oil emulsion mixing was carried out by adding 50 µl of tween-20 and 0.25 ml of tuna eyeball oil to 1 M of potassium phosphate buffer (pH 6.5) 8 ml. And then, all of reaction in oil emulsion is performed according to the method of Buege and Aust (1978). The absorbency was then measured for supernatant at 531 nm using a UV/visible spectrophotometer (GENESYS 10 UV, Rochester NY, USA) after centrifugation for 10 minutes at 3000 rpm in a centrifugal separator (UNION 32R, Hanil Co., Korea).

The removal effects of DPPH radicals were measured using a modification of the Blois (1958) method. A 1:1 ratio of sample to 0.2 mM DPPH solution were mixed by vortexing, allowed to incubate for 30 min at room temperature, and the absorbency was then measured at 517 nm by a UV/visible spectrophotometer (GENESYS 10 UV, Rochester NY, USA) with a sampling of 1 ml.

The metal blockade power was measured in accordance with the method discribed by Shimada (1992). After 0.3 ml of distilled water, 0.1 ml of iron (II) chloride solution at 2 mM and 0.2 ml of a ferrozine solution at 5 mM were added to 1 ml of sample, the mixture was permitted to react for 20 minutes at room temperature and its absorbency was then measured at 562 nm wave length.

The reducing power was measured using a slight modification of the method described by Oyaizu (1986). A 2.5 ml aliquot of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of a 1% potassium ferricyanide solution were added to 0.5 ml of sample. The mixture was reacted for 20 min at 50°C in a water bath after full mixing. It was then centrifuged for 10 minutes at a speed of 3000 rpm after the addition of 2.5 ml of 10% TCA solution to the reacting mixed liquid, and 2 ml of distilled water and 0.4 ml of 0.1% iron(III) chloride solution were added and mixed at 2 ml of upper level liquid. Its reducing power was then determined to measure its absorbency at 700 nm.

Silica gel column chromatography was used in the first refinement of the *Sargassum siliquastrum* ethanol extract. The slurry was made from activated silica gel (230~400 mes, Merck Co., Germany) by n-hexane and charged to a glass column (50×65 mm, 120 ml). It was then washed 3 times in strong n-hexane. The *Sargassum siliquastrum* ethanol extract was diluted with methanol, and 3 ml of it was loaded. A 360 ml volume of n-hexane, chloroform, ethyl acetate, ethanol and methanol were added and orderly fractioned by increasing the polarity. The fractioned material was concentrated by reducing the pressure and was completely dried at 37°C.

Water systems were applied to the fraction of the ethyl acetate division. The reagent was used after filtering the dried *Sargassum siliquastrum* ethanol extract ethyl acetate division and diluted with methanol for HPLC using a 0.5 µm membrane filter (Advantec MFS, Inc., Japan). The conditions used in the analysis were as follows; µBondapak C_{18} 125 Å10 µm (Waters Ltd., Ireland) was applied to the column and methanol-0.1% TFA for HPLC was applied for the moving status with a flow velocity of 1.0 ml/min. A Linear UVIS 204 detector was used to measure the maximum absorption wavelength 248 nm.

Peak 2 was identified by EI-Mass (JMS 700, JEOL Co., Japan) analysis after complete drying and melting with methanol. The high resolution and low resolution were measured using the DIP (direct inject prove) method. The ion source temperature for the analysis was 280°C and the ionizing voltage was 70 eV.

^{13}C-NMR (100MHz), ^{1}H-NMR (400MHz), DEPT (distortion enhanced by polarization transfer), COSY (correlation spectroscopy), HMBC and HMQC were measured using a Fourier transform nuclear magnetic resonance spectrometer (JNM-ECP 400, JEOL Co., Japan) by melting the sample in a deuterated solvent (CD_3OD), in order to determine the structure of Peak 2. Deuterated solvent (CD_3OD), which is an internal standard material, was applied to a chemical shift, and methanol was used as a solvent and expressed using the unit of ppm.

Results and Discussion

The *Sargassum siliquastrum* ethanol extract was separated using silica gel column chromatography. As a result of the separation by enhancing the polarity and collecting the n-hexane, chloroform, ethyl acetate, ethanol and methanol as solvents, the fraction separated by hexane was not shown (Table 1). The chloroform division showed the best rate at 37.8%. It was diluted to a concentration of 5.0, 1.0 and 0.5 mg ml^{-1} after each separated division was concentrated. The TBARS generation restraint effect was then measured (Table 2). Its antioxidation effects at a concentration of 0.5 mg ml^{-1}, in descending, order were ethyl acetate > chloroform > ethanol > methanol. This was especially true for the ethyl acetate division, which exhibited a higher antioxidative effect than the ethanol extract of *Sargassum siliquastrum* at 84.86%, which was similar to the effect of BHT at 87.9%. It is similar with Mori and Lee reports. Mori's report have

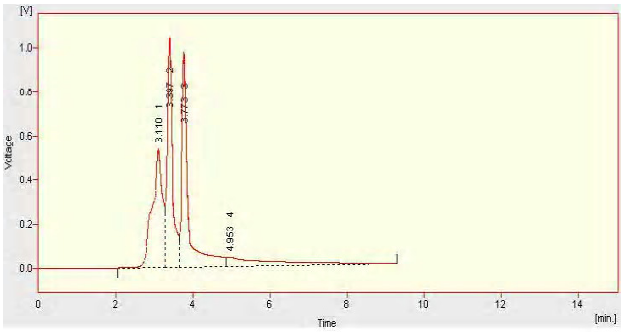

Fig. 1: HPLC chromatogram of the ethyl acetate fraction from the ethanol extract of *S. siliquastrum*

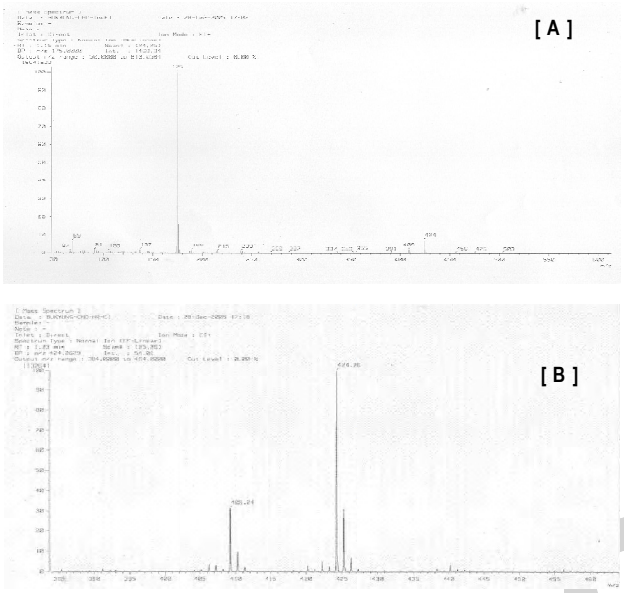

Fig. 2: Profile of EI-Mass spectrum from the peak 2 (A) Low resolution, (B) High resolution

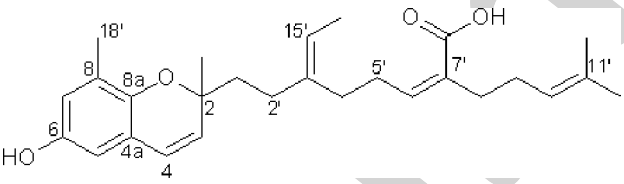

Fig. 3: Mojabanchromanol

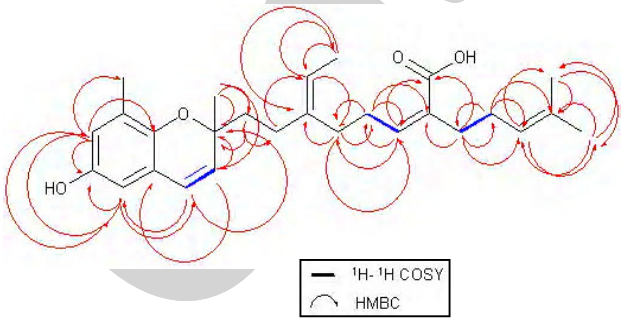

Fig. 4: Key ^{1}H-^{1}H COSY and HMBC data of Mojabanchromanol

Table - 1: Yields of various solvent fractions from the ethanol extract of *S. siliquastrum*

Solvent	Yields (%)
n-Hexane	0
Chloroform	37.8
Ethyl acetate	31.5
Ethanol	27.0
Methanol	4.3

Table - 2: Comparison of the antioxidant ability of various solvent fractions from the *S. siliquastrum* ethanol extract

Solvent	% Concentrations (mg ml^{-1})		
	5	1	0.5
n-Hexane	0	0	0
Chloroform	88.34 ± 1.1	81.85 ± 2.5	71.07 ± 3.5
Ethyl acetate	88.33 ± 0.6	86.69 ± 0.3	84.86 ± 1.2
Ethanol	74.36 ± 4.2	40.97 ± 6.7	31.61 ± 5.7
Methanol	42.51 ± 5.6	6.86 ± 1.0	1.94 ± 4.9

Table - 3: Comparison of the antioxidant ability with the HPLC fraction of ethl acetate depending on the concentration from the *S. siliquastrum* ethanol extract

	% Concentrations (mg ml^{-1})	
	1	0.5
Fraction 1	88.47 ± 2.0	72.96 ± 3.5
Fraction 2	84.39 ± 1.6	84.08 ± 2.7
Fraction 3	81.72 ± 0.9	65.60 ± 2.7

revealed that ethyl acetate fraction of *Sargassum micracathum* had the highest antioxidation effects on lipid perosidation system (Mori *et al*., 2003). Lee's study have reported that ethyl acetate division of *Ecklonia stolonifera* showed the strongest antioxidative activities on lipid peroxidation of a mouse liver homogenate (Lee *et al*., 1996).

After performing silica gel column chromatography, the ethyl acetate division, which demonstrated the best antioxidant activity, was separated using a μ-Bondapak C_{18} reverse-phase column on HPLC. Three typical fractions were obtained by separating methanol-0.1% TFA for the moving phase(Fig. 1). Each fraction was measured by a TBARS generation restraint effect to determine the antioxidant effects by the complete drying and melting of methanol (Table 3). The results of the antioxidant effect measurements for each fraction showed that 3 fractions had similar effects at 83.47±2.0%, 84.39±1.6%, and 81.72±0.9% to the ethyl acetate fractional material, which showed an effect of 86.69±0.3%. Fraction 2, in particular, maintained the effect of 84.08±2.7% even at a concentration of 0.5 mg ml^{-1}, which indicated that the antioxidant material of the *Sargassum siliquastrum* ethanol extract exists at all fractions; however, the major antioxidant materials exist only in fraction 2.

The molecular weight was determined by EI-mass in order to elucidate the properties of fraction 2(Fig. 2). The result of the EI-Mass verified that fraction 2 was a single material and that the molecular

weight of this material was 424.263 MW, measured at low and high resolutions.

Fraction 2 was isolated as yellow oil and the molecular formula of $C_{27}H_{36}O_4$ was confirmed by the high-resolution mass spectrum (424.263). This data indicated that fraction 2 contained 10 double bond equivalents that could be attributed to seven C=C double bonds (C-3/C-4, C-4a/C-5, C-6/C-7, C-8/C-8a, C-3´/C-15´, C-6´/C-7´ and C-10´/C-11´), one carbonyl group at dC 171.6 (C-14´) and two ring systems (Fig. 3). The structure was elucidated by analyzing the 1H, 13C, 1H-1H COSY, HMBC, and HMQC NMR spectral data that was recorded in CD3OD (Table 4). Four partial structures were established from the COSY and HMBC spectra (Fig. 4). The first fragment was constructed starting with the methyl proton (H-18´) which showed HMBC correlations to C-8a, C-8 and C-7. Two methine proton signals (H-7 and H-5) showed HMBC correlations to the quaternary C-6. The proton signal at 6.13 ppm (H-5) showed another HMBC correlation to C-4a. This moiety was expanded to C-4 based on the HMBC correlation from the H-5 proton of methane to C-4. The proton attached to C-4 (H-4) showed a COSY correlation with H-3 and this proton (H-3) showed HMBC correlations to the oxygenated quaternary carbon (C-2) and methyl carbon (C-17´). This data established the dimethyl-chromen moiety. This moiety was expanded to a second fragment by the HMBC correlation from H-17' to C-1'. HMBC correlations for the second fragment were observed from the methylene protons H-1' to C-2' and C-3'. The proton attached to C-3' (H-3') showed HMBC correlations to C-5' and C-6', which indicated a five carbon-fragment. A third fragment consisted of two methylene carbons (C-4´ to C-5´), one methine (C-6´) and two quaternary carbons (C-7´ and C-14´). The proton attached to C-4´ (H-4´) was the HMBC correlation to C-5´ and C-6´. The proton signal at 2.42 ppm (H-5´) showed correlations to H-6´, which in turn showed HMBC correlations to C-7' and C-14', which was clearly oxygenated based on the carbon chemical shift (dC 171.6). This data indicated the presence of a four-carbon fragment that was attached to a hydroxyl group. The left end of the third fragment (H-4´) was connected by the HMBC correlation to C-3´ and the right end (C-7´) was connected to the last fragment by the HMBC correlation from H-8´ to C-7´. The last fragment was constructed starting with the methylene proton (H-8´), which showed COSY correlations to H-9´. This proton (H-9´) showed an HMBC correlation to the methine carbon (C-10´) and the quaternary carbon (C-11´). The proton attached to C-10´ (H-10´) showed an HMBC correlation to the quaternary carbon (C-11´) and two methyl carbons (C-12´ and C-13´) to generate a six-carbon fragment. This information allowed the structure of mojabanchromanol to be established as shown in Fig. 3. Mojabanchromanol is similar with sargachromanols, isolated from *Sargassum siliquastrum* by Jang *et al.* (2005), which are kind of chromenes. It has been recently reported that chromenes has strong antiviral and antioxidation effects (Mori *et al*., 2003). Besides, many studies have been done to synthesize chromene derivatives (Mori *et al.*, 2006; Kwak *et al*., 2006).

Table - 4: NMR Spectral data for mojabanchromanol in CD_3OD

C/H#	dH mult (J in Hz)	dC	COSY		HMBC
2		78.7			
3	5.62, d (9.9)	131.5	C H	H-4	C-2, C-4a
4	6.25, d (9.9)	124.2	C H	H-3	C-2, C-5
4a		122.6			
5	6.24, s	111.3	C H		C-4, C-6, C-7
6		145.3			
7	6.45, s	117.9	C H		C-5, C-6, C-18´
8		126.8			
8a		151.3			
1´	1.61, m	41.9	CH2		C-2, C-2´
2´	2.02, m	23.7	CH2		
3´		135.5			
4´	2.03, m	40.2	CH2	H-5´	C-3´, C-5´, C-6´
5´	2.51, m	28.8	CH2	H-6´, H-4´	C-4´, C-6´, C-7´
6´	5.81, t (7.52)	142.6	C H	H-5´	C-4´, C-5´, C-14´
7´		133.3			
8´	2.23, m	35.9	CH2	H-9´	C-7´,C-9´,C-10´,C-14´
9´	1.64, m	28.9	CH2	H-8´, H-10´	C-8´, C-10´, C-11´
10´	5.08, m	124.7	C H	H-9´	C-12´, C-13´
11´		132.9			
12´	1.67, s	25.9	CH3		C-10´, C-11´, C-13´
13´	1.54, s	17.7	CH3		C-10´, C-11´, C-12´
14´		171			
15´	5.15, m	126.1	C H	H-16´	C-2´, C-4´, C-16´
16´	1.54, d (1.36)	15.6	CH3	H-15´	C-3´, C-15'
17´	1.33, s	26.2	CH3		C-2, C-3, C-1´
18'	2.05, s	15.8	CH3		C-6, C-7

Table - 5: Comparison of antioxidant ability with fraction 2 of ethyl acetate from *S. siliquastrum* ethanol extract

	TBARS (%)	DPPH radical scavenging effect (%)	Chelating effect (%)	Reducing power (absorbance at 700 nm)
BHT	87.90±0.8	93.02±0.1	---[a]	---
α-Tocopherol	58.57±3.0	95.47±3.7	---	---
Ascorbic acid	32.18±3.9	14.50±0.6	---	0.68±0.0
EDTA	---	---	99.76±0.2	---
Ethanol extract	72.11±4.0	94.75±0.7	4.80±0.8	0.13±0.0
Fraction 2	84.08±2.7	96.07±0.1	1.21±0.9	0.17±0.0

Concentration is 0.5 mg ml^{-1}, [a]not tested

Mojabanchromanol was diluted to a concentration of 0.5 mg $ml^{-1}$with methanol, and the DPPH radical elimination ability, metal blockade power and reducing power were measured in order to determine the properties of antioxidants and an antioxidation mechanism (Table 5). Levels of 84.08%, 96.07% were shown in the TBARS analysis and DPPH radical elimination ability, respectively, which demonstrated a similar or higher effect than BHT, ±-tocopherol and ascorbic acid, which moved to control group. However, the metal blockade power was 1.12%, which is dramatically decreased. This was also reduced to about 20% with ascorbic acid, which was slightly higher than the effect of the extract. Radical scavengers, which restrained the rancidity by terminating the acid chain reaction by antioxidant acting as hydrogen or electron donators to free radicals to stabilize non-radical compounds, exhibited antioxidant activity. Lim *et al.* (2002) reported that the *Sargassum siliquastrum* methanol extract demonstrated an antioxidant effect since the hydroxyl group and aromatic ring of the antioxidant components in the *Sargassum siliquastrum* functioned as nonpolar chain-breaking antioxidants. Additionally, Mori *et al.* (2006) reported that the phenol moiety of the chromene derivative separated from *Sargassum micracanthumchromenes* reacts with radicals and demonstrates antioxidant effects by stabilizing phenoxyl radicals, which coincides with Lim's report. Many studies have indicated that marine algae extracts and their phenol compound have antioxidant activities. Yan *et al.* (1999) showed that the major active compound from a *Hijikia fussiforms* was identified as fucoxanthin. Cahyana *et al.*(1992) reported that pyropheophytin a, that was one of the chlorophyll a-related compounds in *Eisenia biscyclis*, had an strong antioxidative activities. Recently, phlorotannin was isolated as an effective antioxidant component from *Sargassum kjellmanianum*, *Ecklonia stolonifera* and *Ecklonia cava* (Yan *et al*., 1996; Lee *et al*., 1996; Ham *et al.,* 2007).

As a result of separating the *Sargassum siliquastrum* ethanol extract by silica gel column chromatography, the chloroform fractional material showed the best rate at 37.8%. The ethyl acetate fractional material showed high antioxidative effects at 84.86% at a concentration of 0.5 mg ml^{-1}, which is similar to the 87.9% of BHT. The results of the TBARS measurements showed that there were 3 fractions obtained from the separation of the ethyl acetate fractional material that showed the best antioxidant activities using a μ-Bondapak C_{18} reverse-phase column on HPLC. Fraction 2 showed high antioxidative effects at 84.08±2.7% at 0.5 mg ml^{-1}. The major antioxidant material of the *Sargassum siliquastrum* ethanol extract was estimated to exist in peak 2. Peak 2 was verified as mojabanchromanol, a type of chromene which has a molecular weight of 424.263 MV, from the result of the structural analysis of El-Mass and NMR used to verify the components of peak 2. The measured results of the DPPH radical elimination power, metal blockade power and reducing power performed to verify the antioxidant mechanism of mojabanchromanol showed that the DPPH radical elimination power was 96.07%. This demonstrates a high antioxidative effect with the metal blockade power showing levels as dramatically low as 1.12%. The reducing power was approximately 20% with ascorbic acid. It was found that radical scavengers, which restrained the rancidity by terminating the acid chain reaction with antioxidant acting as hydrogen or electron donators to free radicals to stabilize non-radical compounds. All of this functioned as antioxidant activity.

Acknowledgments

This research was supported by a grant (B-2005-04) from Marine Bioprocess Research Center of the Marine Bio 21 Center funded by the Ministry of Maritime Affairs and Fisheries, Republic of Korea and Engineering Foundation and the Ministry of Commerce, Industry and Energy(MOCIE) and Korea Industrial Technology Foundation(KOTEF) through the Human Resource Training Project for Regional Innovation.

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