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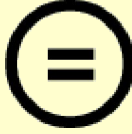

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A THESIS

FOR THE DEGREE OF MASTER OF PHILOSOPHY

Preparative isolation and purification of bioactive compounds from marine algae using centrifugal partition chromatography and theiranti-inflammatory activity

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Department of Marine Life Science

GRADUATE SCHOOL

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FEBRUARY, 2012

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Preparative isolation and purification of bioactive compounds from marine algae using centrifugal partition chromatography and their anti-inflammatory activity

Ji-Hyeok Lee

(Superbvised by Professor You-Jin Jeon)

A thesis submitted in partial fulfillment of the requirement for the degree of

MASTER OF PHILOSOPHY

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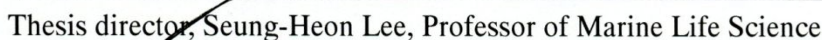
This thesis has been examined and approved by



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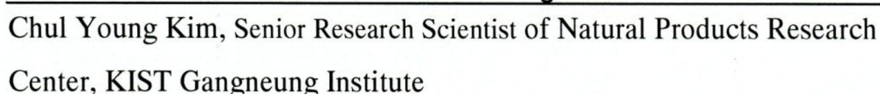

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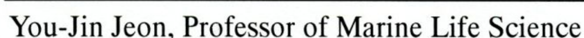

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국문초록

고속원심분배크로마토그래피는액체-액체크로마토그래피의일종으로서섞이지않는두층간의 용매에서성분의분배의차에의해분리되는크로마토그래피기술로서 90%이상의순수한물질을 대량으로빠르게, 단일공정으로정제하는데용이하다. 또한기존의분리법이갖는용매의소비가 많고, 정제소요시간이길며, 반복적인분리공정과생리활성물질의낮은수집과시료의고정상흡착등과같은문제에대한대체방법으로서제시되어지고있다. 최근고속원심분배크로마토그래피는육상식물로부터생리활성물질을분리정제하는데널리이용되어지고있는반면, 해조류의경우에는 *Ascophyllumnodosum* 와같은 소수의 종에서만 이용되어졌다. 그러므로 우리는 제주도 해안에 널리 서식 하는 갈조류의 일종인 감태와 꽈배기모자반을 고속원심분배크로마토그래피를 이용하여 생리활성 물질을 분리 정제하였다.

1. 감태(*Ecklonia cava*)는 70% EtOH로 3시간 동안 3번에 걸쳐 초음파 추출을 하여, 10,000rpm으로 10분간 원심분리 하여 상층액을 농축 후, *n*-hexane, chloroform, ethyl acetate, *n*-butanol, water fraction으로 나누었으며, 기존 보고에서 가장 많은 생리활성 물질인 phlorotannins이 많다고 알려진 ethyl acetate fraction을 가지고, n-hexane / ethyl acetate / methanol / water 용매를 비율 별에 따라 분배 계수 값인 K-value를 구하였다. 그리고 가장 우수한 분배 계수 값을 갖는 용매 조건인 n-hexane, ethyl acetate, methanol / water (2:8:3:7)의 조건으로 하강모드 (Descending mode)

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로 하여 2ml/min의 유속에 1000rpm의 원심속도에서 고속원심분배크로마토그래피를 작동하였다. 그 결과, 6개의 fraction을 얻었고, 90%이상의 순도를 갖는 Dieckol과 6,6-bieckol은 *E. cava* ethylacetate 층 500mg 중 각각 34.7mg과 41.2mg을 얻었고, 나머지 fraction에서는 phloroeckol, 2,7-phloroglucino-6,6-bieckol과 분자량 527m/z의 Unknown compound 1, 분자량 866m/z의 Unknown compound 2,분자량 744m/z의 Unknown compound 3,분자량 974m/z의 Unknown compound 4를 각각 획득하였다. 그리고 n-hexane, ethyl acetate, methanol / water (2:7:3:7)의 조건의 fraction 6에서는 90%이상의 순도의 phlorofucofuroeckol-A를 31.1mg을 수집할 수 있었다.

2. 꽈배기 모자반(*Sargassumsiliquastrum*)은 80% MeOH 로 3 시간 동안 3 번에 걸쳐 초음파 추출을 하여, 10,000rpm 으로 10 분간 원심분리 하여 상층액을 농축 후, *n*-hexane, chloroform, ethyl acetate, *n*-buthanol, water fraction 으로 나누었으며, online HPLC 분석 결과 높은 활성 물질이 다량 함유된 chloroforum fraction 을 가지고, n-hexane / ethyl acetate / methanol / water 용매를 비율 별에 따라 분배 계수 값인 K-value 를 구하였다. 그리고 가장 우수한 분배 계수 값을 갖는 용매 조건인 n-hexane, ethyl acetate, methanol / water (5:5:7:3)의 조건으로 하강모드 (Descending mode)로 하여 2ml/min 의 유속에 1000rpm 의 원심속도에서 고속원심분배크로마토그래피를수행하였다. 그 결과, 5 개의 fraction 을 얻었고,

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80%이상의 순도를 갖는 Unknown compound 1 과 푸코잔틴을 꽈배기 모자반 chloroforum 층 500mg 중 각각 105 mg 과 10 mg 을 얻었고, 나머지 fraction 에서는 분자량 408 *m/z* 을 갖는 Unknown compound 2 와 분자량 408 *m/z* 의 Unknown compound 3, 분자량 424m/z 의 Unknown compound 4, 분자량 428m/z 의 Unknown compound 5 를 각각 획득하였다.

3. 꽈배기 모자반에서 분리된 fraction 중, 푸코잔틴을 제외한 4 개의 fraction 을 가지고 LPS 에 의해 유도된 RAW 264.7 세포에서 염증성 매개인자 중 NO 생성의 억제에 대한 활성을 측정하 결과, fraction 1 (5573F1)에서가 가장 우수한 저해 활성을 보여, 5573F1 의 H-NMR 과 C-NMR 분석결과 Jang et al. (2005) 에 의해 보고된바 있는 Sargacromanol E 로 확인되어 졌다. Sargacromanol E 을 가지고 염증성 매개인자인 NO, PGE2, IL-1b 와 TNF-α 의 생성 저해 활성을 측정한 결과, 농도의존적으로 억제하는 것을 확인 하였다, 항염증 활성의 작용기전 규명을 위해, LPS 로 자극된 세포 내 p38, JNK, ERK $_{1/2}$ 와 같은 MAPKs 활성화에 미치는 영향을 조사한 결과, MAPKs 인산화를 억제함을 western blot 을 통해 확인하였다. 이러한 결과는 Sargacromanol E 이가 MAPKs 를 조절함으로써 항염증활성을 나타내는 것으로 사려된다.

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Part I .

Preparative isolation and purification of phlorotannins from *Ecklonia cava* using centrifugal partition chromatography

1. ABSTRACTS

Ecklonia cava is widely distributed in Jejuisland. Main constituents of *E. cava*have been reportedphlorotannins such asdieckol, eckol, 6-6 bieckol, phloroglucinol, phloroeckol, phlorofucofuroeckol-A. Although many phlorotannins were purified from E. cava, isolation and purification of those compounds required time consuming, tedious and repeated chromatographic steps. Centrifugal partition chromatography (CPC) can be used to purify various bioactive-compounds efficiently from *E. cava* by one-step.Phlorotannins were successfully separated from the crude extracts of *E. cava* by preparative centrifugal partition chromatography with a two-phase solventsystem composed of *n*-hexane:ethylacetate:methanol:water (2:8:3:7, v/v). Dieckol (40.2 mg), and 6,6-bieckol (27 mg)were purified from the500 mg crude extract by one step.

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Phloroeckol and 2,7-phloroglucinol-6,6-bieckol were also isolated from *E. cava* extracts.And unknown compounds 1~3 which haven't reported from *E. cava* were also isolated. Purified phlorofucofuroeckol-A was isolated from fraction 6 of 2:8:3:7(H:E:M:W, v/v/v/v) solvent condition.The purities of the isolated dieckol, 6,6-bieckol and phlorofucofuroeckol-A wereover 90% accordingto HPLC analysis and electrospray ionization multi stage tandem mass spectrometry (ESI-MS) in negative and positive ion mode. Also 2,7-phloroglucinol-6,6-bieckol and pyrolgallol-phloroglucinol-6,6-bieckol could be collected by recycle HPLC and unknown compound 1~3 was purified by prep-HPLC. Unknown compounds1~3are going to be confirmed by ^{1}H-NMR, ^{13}C-NMR and 2D-NMR.

2. INTRODUCTION

Marine algae are considered to be a rich source of antioxidants. The potential antioxidant compounds from these marine algae have been identified as some pigments (fucoxanthin, astaxanthin, carotenoids) and polyphenols (phenolic acids, flavonoids, tannins) (Heo*et al*., 2005). Phlorotannin among these bioactive compounds are a class of compounds with polymerized phloroglucinol units found in brown algae. Especially, phlorotannins such as dieckol, eckol, 6,6-bieckol and phlorofucofuroeckol-Awhich are rich in an edible brown algae*Ecklonia cava***(Fig. 1-1)**,

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were known to have various biological activities such as anticancer, antioxidant, immune-modulation, anti-allergic disease, anti-neurodegenerative disease, anti-diabete and anti-HIV-1, etc(Athukorala*et al*., 2006, Hyun *et al*., 2011, Artan*et al*., 2008, Shim *et al*., 2009).But, toisolatethese phlorotannins from *E. cava,*due to be demanded repetitive chromatography processes on Sephadex LH-20 column chromatography and reversed-phase HPLC, it is difficult to isolate them in large quantities and use in industrial application. (Kim*et al*., 2006, Heo*et al*., 2009).Support-free liquid-liquid chromatographic techniques such as counter-current chromatography (CCC) and centrifugal partition chromatography (CPC) are useful to isolate large quantities of these polar compounds.

One of liquid-liquid chromatographic techniques, preparative CPC systemis a non-solid support preparative liquid-liquid separation process chromatographic technique which is based on the difference in distribution of components over two immiscible liquid phases and is possible to large isolate and purify large quantities of the compounds with a purity of over 90% by one step process (Michel*et al*., 1997,Delannay*et al*., 2006, Bourdat-Deschamps*et al*., 2004). In addition, CPCsystem also offers thefollowing technological advantages such as versatile products, faster, less expensive product development, retention of bioactivity integrity, higher throughput, higher yields and reduced operating costs. The solutes are separated according to their partition coefficient (*K*) expressed as the ratio of their concentration in the stationary phase to their

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concentration in the mobile phase (Berthod*et al*., 1988). CPC system has been widely used at separation of bioactive compounds from land plants (Marston*et al*., 1988, Bourdat – Deschamps*et al*., 2004, Kim*et al*., 2006). But, in case of seaweeds, only a few algae such as *Ascophyllumnodosum* have been subjected to CPC (Chevolot*et al*.,1998, 2000).

Therefore, in this study, we isolated and purified biological active compounds from *E. cava*usingpreparative CPCas a quick and effective method.

Fig. 1-1. The photography of *E. cava*

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3. MATERIALS & METHODS

3.1. Materials

Ecklonia cava collected on the coast of Jeju island, South Korea in June 2009, was ground and shifted through a 50 mesh standard testing sieve after dried by freeze dryer SFDSMO6, and then the dried *E. cava* was stored in refrigerator until use. All solvents used for preparation of crude sample andCPC separation were of analytical grade (Daejung Chemicals&Metals Co., Seoul, Korea). HPLC grade solvents werepurchased from Burdick & Jackson (MI, USA).

3.2. Apparatus

LLB-M high performance CPC (Sanki Engineering, Kyoto,Japan) was used in preparative CPC. The total cell volumeis 240 mL. A four-way switching valve incorporated inthe CPC apparatus allows operating in either thedescending or the ascending mode. This CPC system wasequipped with a Hitachi 6000 pump (Hitachi, Japan), anL-4000 UV detector (Hitachi), and a Gilson FC 203B fractioncollector (Gilson, France). The samples were manuallyinjected through a

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Rheodynevalve (Rheodyne, CA,USA) with a 2 mL sample loop.

3.3. Preparation of crude sample from *E. cava*

The dried *E. cava* (20 g) was extracted three times for 3hr with 70% EtOH under sonication at room temperature. The extract, concentrated in a rotary vacuum evaporator, partitioned with ethyl acetate, and then the dried ethyl acetate fraction was stored in a refrigeratorfor CPC separation. The whole process was illustrated in **Fig. 1-2**.

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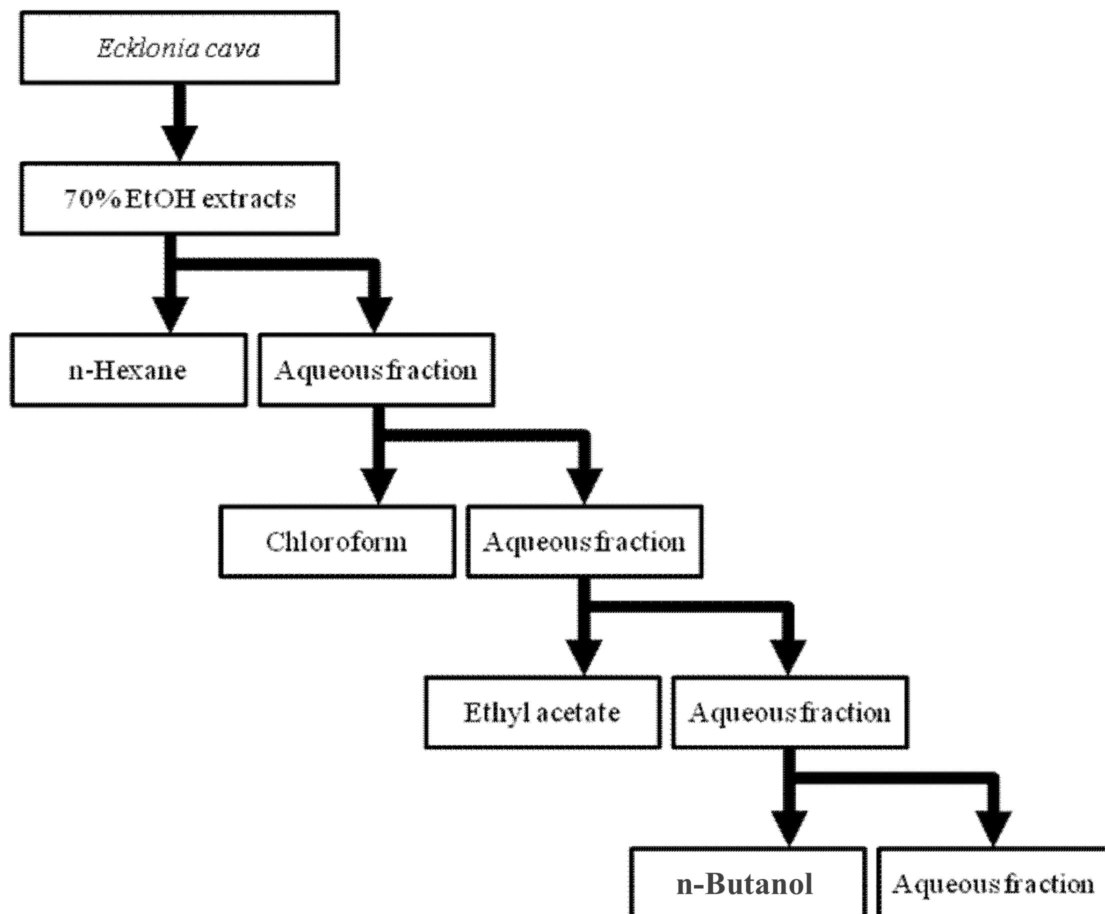

Fig. 1-2.Fractionation scheme of crude extracts from *Ecklonia cava*

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3. 4. Preparation of two-phase solvent system

The CPCexperiments were performed using a two-phase solventsystem composed of n-hexane:ethylacetate:methanol:water(2:8:3:7, v/v). The two phases were separated afterthoroughly equilibrating the mixture in a separatingfunnel at room temperature. The upper organicphase was used as the stationary phase, whereas thelower aqueous phase was employed as the mobilephase.

3. 5. CPC separation procedure

The CPC column wasinitially filled with the organic stationary phase andthen rotated at 1000 rpm while the mobile phase was pumped into the column in the descending mode at the flow rate used for the separation (2 mL/min). When the mobile phase emerged from the column, indicating that hydrostatic equilibrium had been reached (back pressure : 29MPa), The concentrated ethyl acetate fraction (500mg) of 70% EtOH extracts from *E. cava* was dissolved in 6 mL of a 1:1 (v/v) mixture of the two CPC solvent system was injected through the Rheodyne injectionvalve. The effluent from the CPC was monitored in theUV at 290 nm and fractions were collected with 6ml in 10ml tube by a Gilson FC 203 B fraction collector.

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3. 6. HPLC analysis

The HPLC system in this experiment consisted of a binary FLEXARUHPLC pump, a FLEXARPDA detector, a FLEXARPDA auto sampler (PERKIN ELMER,USA). A 10ul of 5mg/ml sample solution was directly injected on Atlantis T3 3um 3.0 X 150mm column (Waters, USA) using a gradient acetonitrile–water solvent system. The mobile phase was acetonitrile – water in gradient mode as follows: acetonitrile with 0.1% formic acid – water with 0.1% formic acid (0 min ~ 40 min : 10:90 v/v ~ 40:60 v/v, ~ 50 min : ~ 50:50 v/v, ~60min : ~ 100:0 v/v). The flow rate was 0.2 mL/min with UV absorbance detection at 290 nm.

3. 7. HPLC–DAD–ESI/MS analysis of purified compounds

HPLC–DAD–ESI/MS analyses were carried out using aHewlett-Packard 1100 series HPLC system equipped withan autosampler, a column oven, a binary pump, a DADdetector, and a degasser (Hewlett –Packard, Waldbronn,Germany) coupled to a Finnigan MAT LCQ ion-trap massspectrometer (Finnigan MAT, San Jose, CA, USA)equipped with a Finnigan electrospray source and capableof analyzing ions up to *m/z* 2000. Xcalibur software(Finnigan MAT) was used for the operation. The chromatographicconditions are identical to those described inSection 3.6

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and the outlet of the flow cell was connectedto a splitting valve, from which a flow of 0.2 mL/min wasdiverted to the electrospray ion source via a short lengthof fused silica tubing. Negative ion mass spectra of thecolumn eluate were recorded in the range m/z 100~2000.The source voltage was set to 4.5 kV and the capillarytemperature to 250℃. The other conditions were as follows:capillary voltage, –36.5 V; inter-octapole lens voltage,10 V; sheath gas, 80 psi (551.6 kPa); auxiliary gas,20 psi (137.9 kPa).

3. RESULTS& DISCUSSIONS

4. 1. HPLCand LC/MS-DAD-ESI analysis of EtOAc fraction of *E. cava*

The EtOAc fraction from *E. cava* has been informed to have various bioactive compounds such as dieckol, phloroeckol, 6,6-bieckol in previous studies (Kang*et al*., 2005a, 2005b). Therefore,the EtOAc fraction was selected in further experiments. The EtOAc fraction was analyzed by described HPLC condition (Atlantis T3 C18 column, 3μm 3.0 x 150mm) and its chromatogram depicted in **Fig. 1-3**. And HPLC peak 1 ~3were suggested as unknown comound (1), dieckol(2) and 2,7-phloroglucinol-6,6-bieckol (3), respectively by both LC/MS-DAD-ESI

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and pervious reports (Lee*et al*., 2009,Kang*et al*., 2011). Phlorofucofuroeckol-A and pyrolgallol-

phloroglucinol-6,6-bieckol shared in HPLC peak 4 (No described in this paper).

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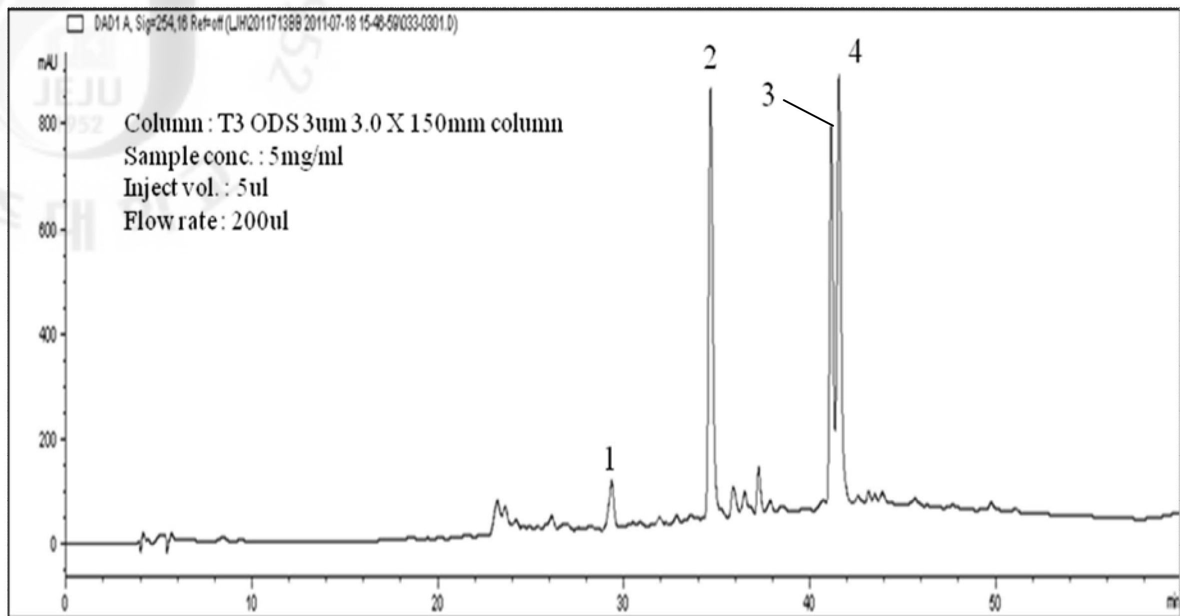

Fig. 1-3. HPLC chromatogram of EtAOC fraction from *E. cava*

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4. 2. Optimization of two-phase solvent system

Partition coefficient (*K*) for selection of a suitable two phase solvent systems were the most important for successful separation of target samples by preparative CPC. In order to choice efficient separation, several two-phase solvent system was performed through different compositions and volume ratios of two immiscible solvents such as *n*-hexane:ethylacetate:methanol:water, and then their*K* values was calculated and showed in **Table 1-1**. 3:7:3:7 (*n*-hexane:ethylacetate:methanol:water, v/v), 2:8:3:7 and 2:8:2:8 (*n*-hexane:ethylacetate:methanol:water, v/v) among all solvent conditions showed appropriate *K* values to isolate phlorotannins from *E. cava*. But, the phlorotannins were not seperated efficiently by CPC under two-phase solventsystem composed of 3:7:3:7 (H:E:M:W, v/v/v/v) and 2:8:2:8 (H:E:M:W, v/v/v/v).Two-phase solventsystem composed of 2:8:3:7 (H:E:M:W, v/v/v/v) showed good separation performance to isolate6,6 bieckol, dieckol and 2,7-phloroglucinol-6,6-bieckol, etc.

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Table1-1.*K*-valuesas solvent condition of EtAOc fractionfrom *E. cava*

Solvents	*K*-value			
H:E:M:W	Unknown compound	Dieckol	2,7-phloroglucinol-6,6-bieckol	Phlorofucofuroeckol A
5:5:5:5	0.00	0.00	0.00	0.00
4:5:4:5	0.00	0.00	0.00	0.01
4:6:4:6	0.44	0.49	0.52	0.55
3:7:3:7	0.16	0.25	0.44	1.65
2:8:3:7	0.36	0.51	0.85	3.21
2:8:2:8	1.52	2.11	6.89	7.30
1:9:1:9	-	28.43	73.65	67.66

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4. 3. Separation of phlorotannins by CPC

The EtOAcfraction (500 mg) of *E. cava* was dissolvedin a 1:1 (v/v) mixture of two-phase solvent composedof 2:8:3:7 (H:E:M:W, v/v/v/v). Because the partitioncoefficient of each HPLC peaks (1~4) was 0.36, 0.51, 0.85 and3.21, respectively, preparative CPC was operated on descending mode selected upper phase as stationary phase and lower phase as mobile phase. The retention of the stationary phase in the coil retained69.5% and pressure exhibited 29MPa during operating. Preparative CPC chromatogram was described in **Fig. 1-4**.In HPLC chromatogram and MS data of each CPC fractions measured by HPLC and LC-DAD-ESI/MS (**Fig. 1-5**), the compounds existed in fraction 3 and 5 was suggested as 6,6-bieckol and dieckol, respectively. Fraction 1 showed unknown compound 1 withmolecular weight (MW) 527*m/z*, fraction 2 showed unknown compound 2 with MW 866 *m/z*, fraction 4 showed phloroeckol(Li *et al*., 2009) and unknown compound 3 had MW 744 *m/z* and fraction 6 showed 2,7-phloroglucino-6,6-bieckol and pyrolgallol-phloroglucinol-6,6-bieckol(Kang *et al*., 2011). And purified plorofucofuroeckol-A (Li *et al*., 2009)was showed in fraction 6 isolated by 2:7:3:7 solvent condition (H:E:M:W, v/v/v/v). 6,6-bieckol, dieckol and plorofucofuroeckol-A showed purity with up to 90% according to analysis by HPLC peak area. And yields of 6,6-bieckol, dieckol and plorofucofuroeckol-A isolated from 500mg of the EtOAc fraction in one-step of CPC system was 40.2 mg, 34.7mg and 31.1mg, respectively. Purified unknown compound 1~3

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and phloroeckolwith up to 90% was gained by prep-HPLC. Also 2,7-phloroglucinol-6,6-bieckol and pyrolgallol-phloroglucinol-6,6-bieckol could be collected by recycle HPLC. All unknown compounds and previous reported compounds suggested by HPLC-DAD-ESI/MS need to be definitely identified using ^{1}H-NMR and ^{13}C-NMR spectra.

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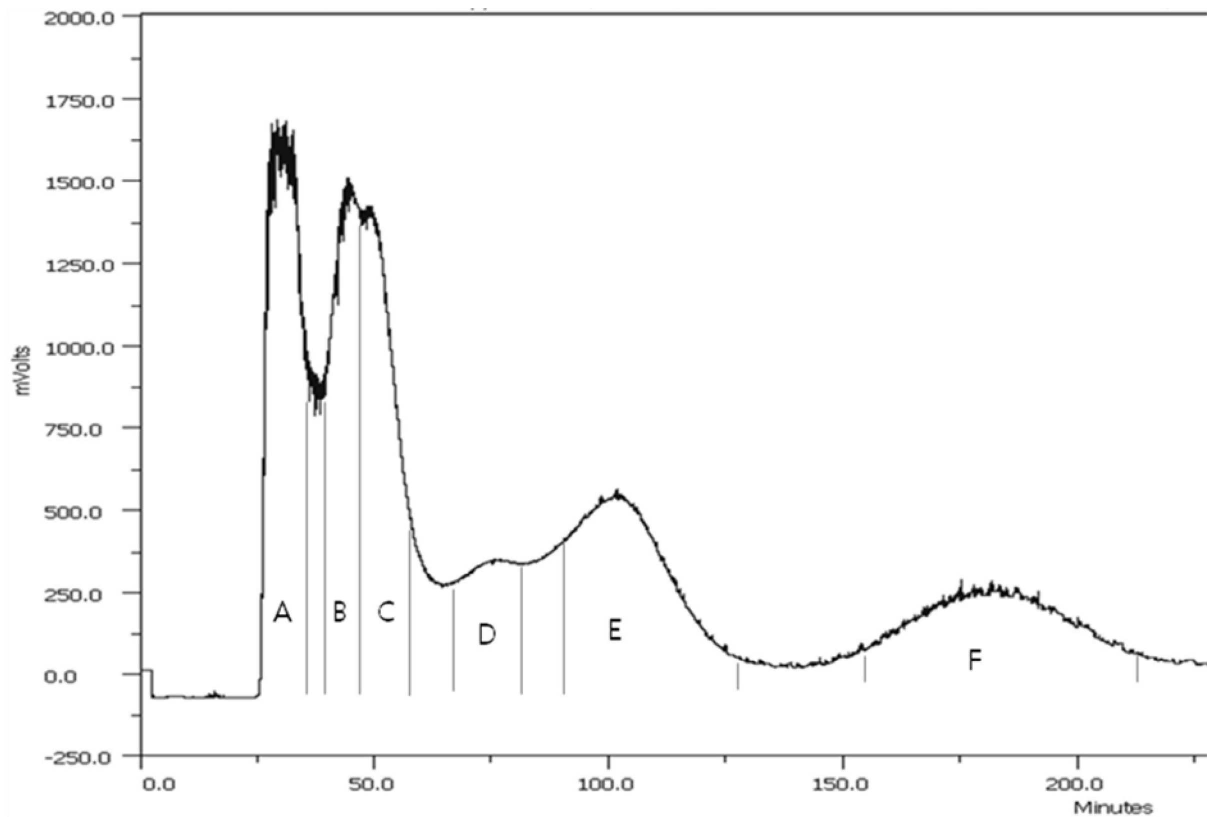

Fig. 1-4.CPC chromatogram of EtAOc fractionfrom *E. cava*(*n*-hexane:EtAOc:MeOH:water– 2:8:3:7, v/v)

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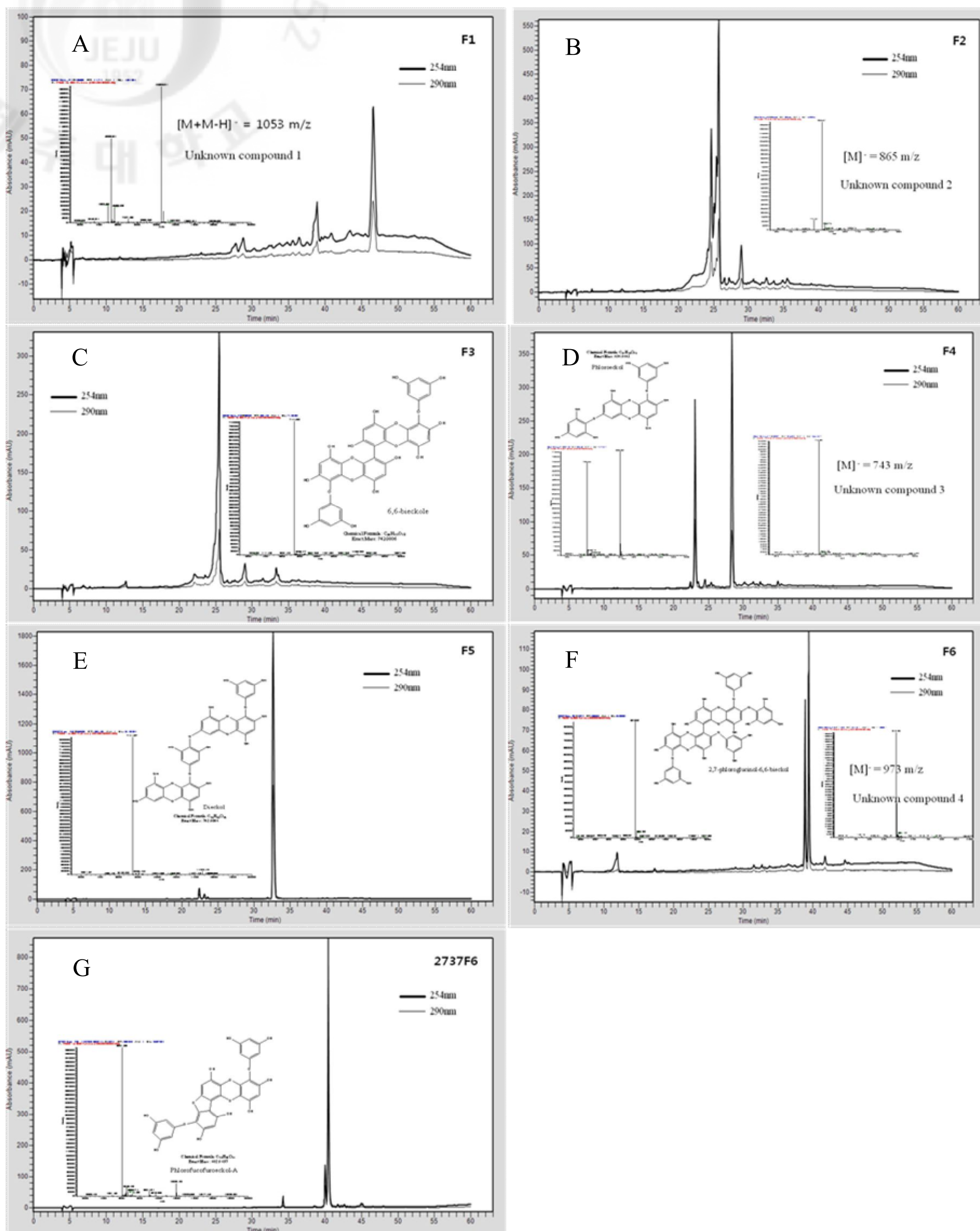

Fig. 1-5.Chromatogram and MS data of each fractions of CPC by HPLC and HPLC/MS

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4. CONCLUSION

In this study, we could purifycompounds such as 6,6-bieckol, dieckol and plorofucofuroeckol-A with high yield by one-step CPC operation. Also, we could isolate phloroeckol, 2,7-phloroglucinol-6,6-bieckol, pyrolgallol-phloroglucinol-6,6-bieckol and three unknown compounds. In conclusion, we demonstrated CPC system is a useful process to isolate and purify phlorotannins from *E. cava***(Fig 1-6)**.

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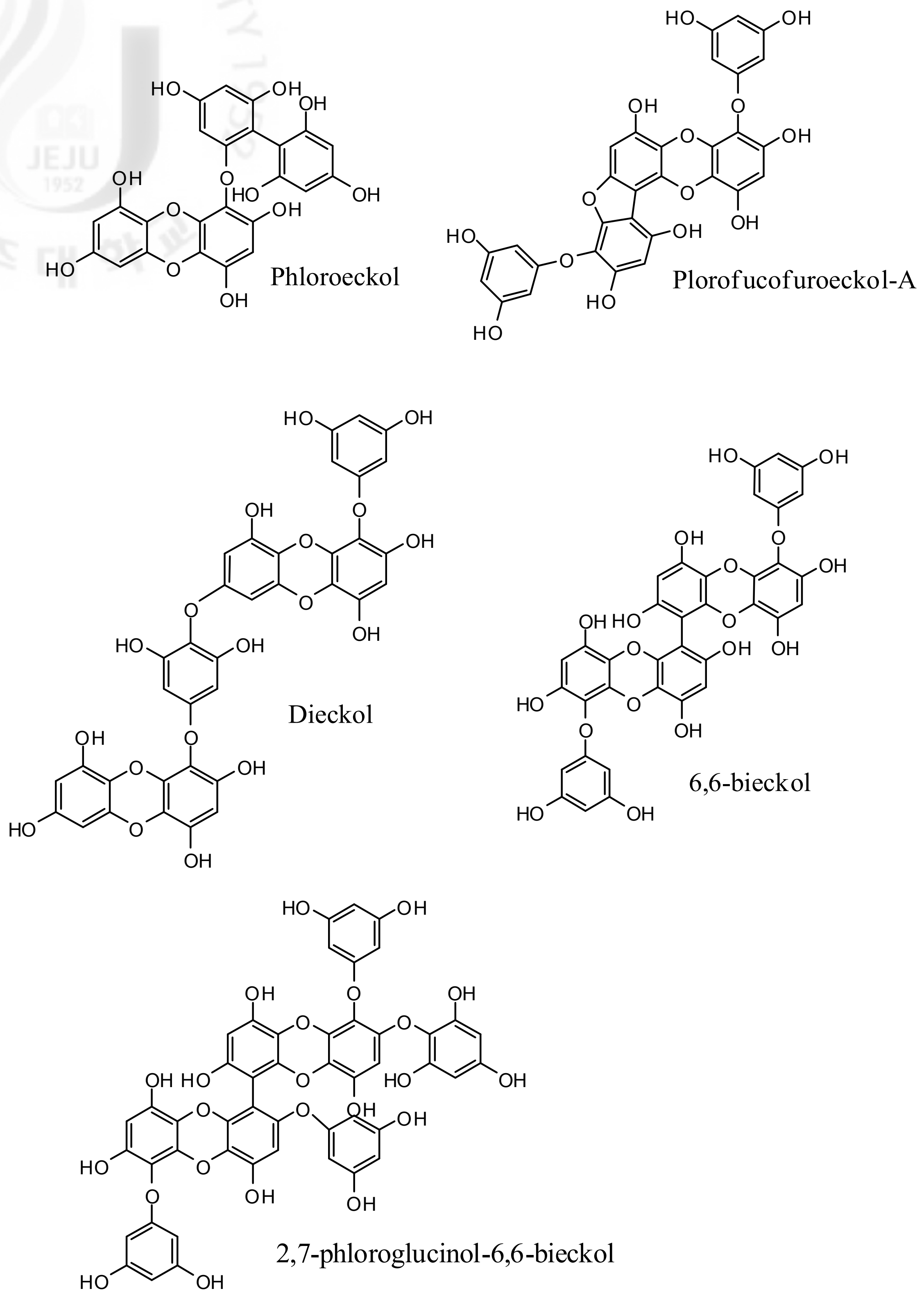

Fig. 1-6.Chemical structures of isolated phlorotannins from *E. cava* using CPC

Part II .

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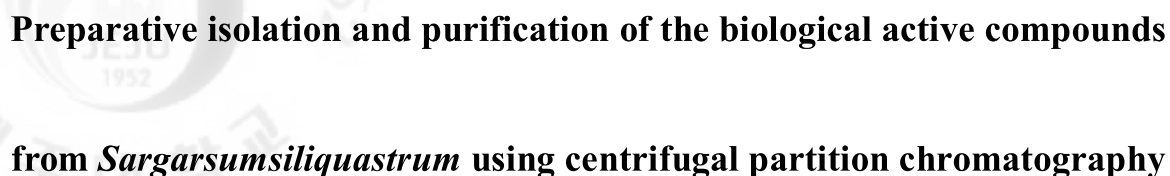

Preparative isolation and purification of the biological active compounds from *Sargarsumsiliquastrum* using centrifugal partition chromatography

1. ABSTRACTS

Sargarsumsliquastrum, brown algae,is widely distributed in Jejuisland. 10E-farnesylacetone, 10*Z*-farnesylacetone, sargachromenol E and fucoxanthin have been knownas active compounds from *Sargarsumsliquastrum*. Centrifugal partition chromatography (CPC) can be used to purify various bioactive compounds easily from natural materals by one-step. We confirmed antioxidative compounds existed in $CHCl_3$ fraction of *S. sliquastrum*by online-HPLC which could measure antioxidant activity through decease of $ABTS^+$. Active compounds were successfully separated from the $CHCl_3$ fraction of *S.sliquastrum*by preparative CPC with a two-phase solventsystem composed of *n*-hexane:ethylacetate:methanol:water (5:5:7:3, v/v). Unknown compound 1 (105 mg), unknown compound 2 (50 mg), unknown compound 3 (12mg) and fucoxanthin (10mg)wasisolated from the500 mg crude extract by one step. Unknown compound

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4, 5 existed in CPC fraction 5. And unknown compound 1~5 which haven't reported from *S. sliquastrum* were also isolated. The purity of the isolated fucoxanthin and unknown compound 1wereover 80% accordingto HPLC analysis and electrospray ionization multi stage tandem mass spectrometry (ESI-MS) in negative and positive ion mode. Andpurityof unknown compound 2and 3were up to 70%.And unknown compound 4 and 5 with over 90% purity were separated by prep-HPLC.

2. INTRODUCTION

Sargarsum spp., brown algae,are found throughout tropical and subtropical areas of the world and are reported to produce metabolites of structural classes such as plastoquinones (Segawa and Shirahama, 1987; Mori *et al*., 2005; Ishitsuka*et al*., 1979), chromanols (Kato *et al*., 1975), chromenes (Jang *et al*., 2005; Kikuchi *et al*., 1975), steroids (Tang *et al*., 2002a) and glycerides (Tang *et al*., 2002b). And then, recently, active compounds from*Sargassumsiliquasturm* has been reported that chromeneinduces apoptosis via caspase-3 activation in human leukemiaHL-60 cells (Heo*et al*., 2011), farnesylacetones has vasodilatation effect on the basilar and carotid arteries of rabbits (Park*et al*., 2008) and fucoxanthin has protective effect on UV-Binduced cell damage

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(Heo*et al*., 2009). For rapid selection of unknown compounds on antioxidant activity from some crude extracts, sensitive on-line HPLC methods (on-line HPLC-DPPHand on-line HPLC-ABTS assays) for analysing free radical scavengingactivity have been developed (Koleva, Niederländer, & VanBeek, 2000, 2001). Therefore, antioxidative compounds from *S. siliquastrum* could be corfirmed easily and rapidly by on-line HPLC-ABTS (**Fig. 2-1**). But, traditional methods to purify antioxidative compounds such as chromanols and fucoxhantin were demanded repetitive chromatography processes on Sephadex LH-20 column chromatography and reversed-phase HPLC (Zang*et al*., 2005). Also, because of traditional methods which had problem such as complex process and limited amount of compounds, it was difficult to use them in industrial application. Therefore, we purified biological active compounds using centrifugal partition chromatography (CPC) as a fast and effective method.

One of liquid-liquid chromatographic techniques, preparative CPC systemis a non-solid support preparative liquid-liquid separation process chromatographic technique which is based on the difference in distribution of components over two immiscible liquid phases and is possible to large isolate and purify large quantities of the compounds with a purity of over 90% by one step process (Michel*et al*., 1997,Delannay*et al*., 2006, Bourdat-Deschamps*et al*., 2004). In addition, CPCsystem also offers thefollowing technological advantages such as versatile products, faster, less expensive product development, retention of bioactivity integrity, higher throughput, higher yields and

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reduced operating costs. The solutes are separated according to their partition coefficient (*K*) expressed as the ratio of their concentration in the stationary phase to their concentration in the mobile phase (Berthod*et al*., 1988). CPC system has been widely used at separation of bioactive compounds from land plants (Martston*et al*., 1988, Bourdat – Deschamps*et al*., 2004, Kim*et al*., 2006). But, in case of seaweeds, only a few algae such as *Ascophyllumnodosum* have been subjected to CPC (Chevolota*et al*., 1998, 2000).

Therefore, in this study, we applied CPC system to large isolateactive compounds from *S. siliquastrum*confirmed by online HPLC-ABTS^{+} system.

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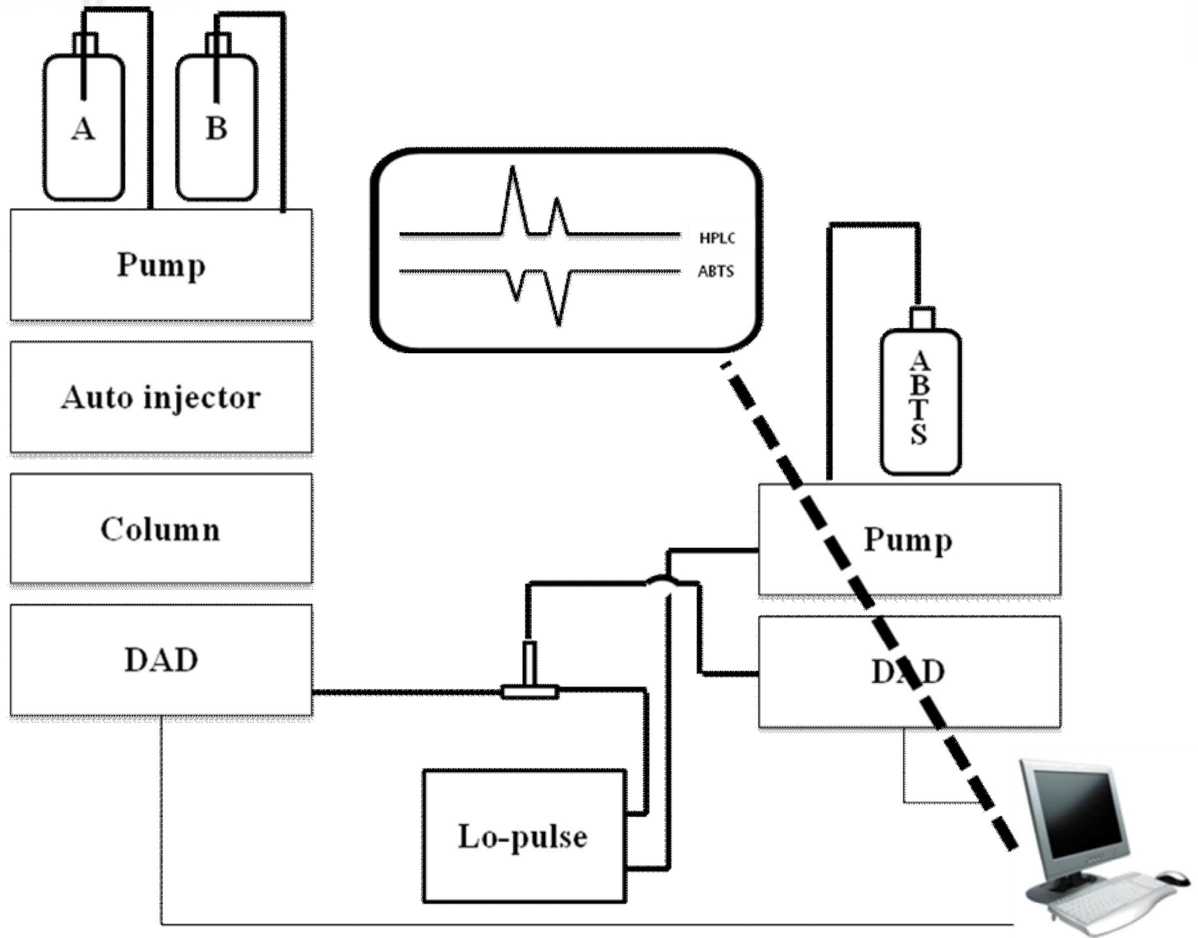

Fig. 2-1. Scheme of online-HPLC-ABTS⁺system

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3. MATERIALS & METHODS

3.1. Materials

S. siliquastrum collected on the coast of Jejuisland, south Korea in June 2009, was ground and shifted through a 50 mesh standard testing sieve after dried by freeze dryer, and then the dried *S. siliquastrum* was stored in refrigerator until use. All solvents used for preparation of crude sample andCPC separation were of analytical grade (Daejung Chemicals&Metals Co., Seoul, Korea). HPLC grade solvents werepurchased from Burdick & Jackson (MI, USA).

3.2. Apparatus

LLB-M high performance CPC (Sanki Engineering, Kyoto,Japan) was used in preparative CPC. The total cell volumeis 240 mL. A four-way switching valve incorporated inthe CPC apparatus allows operating in either thedescending or the ascending mode. This CPC system wasequipped with a Hitachi 6000 pump (Hitachi, Japan), anL-4000 UV detector (Hitachi), and a Gilson FC 203B fractioncollector (Gilson, France). The samples were manuallyinjected through a

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Rheodynevalve (Rheodyne, CA,USA) with a 2 mL sample loop.

^{1}H-NMR spectra were measured with a JEOL JNM-LA300 spectrometer and ^{13}C-NMR spectra with a BrukerAVANCE 400 spectrometer. Mass spectra (FAB-MS and EIMS)were recorded on a JEOL JMS 700 spectrometer. TheHPLC system in this experiment consisted of a binary Gilson321 pump, a Gilson UV–Vis 151 detector, a Gilson234 auto-injector, and a 506C interface module (Gilson).

3.3. Preparation of crude extracts from *S. siliquastrum*

Dried *S. siliquastrum*(**Fig. 2-2**, 600 g) was extracted three times for 3hr with 80% MeOH under sonication at room temperature. The extract, concentrated in a rotary vacuum evaporator, partitioned with $CHCl_3$, and then the dried $CHCl_3$ fraction (62 g) was stored in a refrigeratorfor CPC separation. The whole process was illustrated in **Fig. 2-3**.

3.4. Preparation of two-phase solvent system andsample solution

The CPCexperiments were performed using a two-phase solventsystem composed of *n*-

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hexane:ethylacetate:methanol:water(5:5:7:3, v/v). The two phases were separated afterthoroughly equilibrating the mixture in a separatingfunnel at room temperature. The upper organicphase was used as the stationary phase, whereas thelower aqueous phase was employed as the mobilephase.

3.5. CPC separation procedure

The CPC column was initially filled with the organic stationary phase and then rotated at 1000 rpm while the mobile phase was pumped into the column in the descending mode at the flow rate used for the separation (2 mL/min). When the mobile phase emerged from the column, indicating that hydrostatic equilibrium had been reached (back pressure : 3.9 MPa), The concentrated ethyl acetate fraction (500mg) of 80% MeOH extracts from *S. siliquastrum* was dissolved in 6 mL of a 1:1 (v/v) mixture of the two CPC solvent system phases was injected through the Rheodyne injection valve. The effluent from the CPC was monitored in the UV at 254 nm and fractions were collected with 6ml in 10ml tube by a Gilson FC 203 B fraction collector.

3.6. HPLC analysis

The HPLC system in this experiment consisted of a binary FLEXARUHPLC pump, a FLEXARPDA detector, a FLEXARPDA auto sampler (PERKIN ELMER, USA). A 10ul of

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5mg/ml sample solution was directly injected on Atlantis T3 3um 3.0 X 150mm column (Waters, USA) using a gradient acetonitrile–water solvent system. The mobile phase was acetonitrile – water in gradient mode as follows: acetonitrile with 0.1% formic acid – water with 0.1% formic acid (0 min ~ 10 min : 10:90 v/v ~ 60:40 v/v, ~ 60 min : ~ 100:0 v/v). The flow rate was 0.2 mL/min with UV absorbance detection at 254 nm.

3.7. HPLC–DAD–ESI/MS analysis of purified compounds

HPLC–DAD–ESI/MS analyses were carried out using aHewlett-Packard 1100 series HPLC system equipped withan autosampler, a column oven, a binary pump, a DADdetector, and a degasser (Hewlett –Packard, Waldbronn,Germany) coupled to a Finnigan MAT LCQ ion-trap massspectrometer (Finnigan MAT, San Jose, CA, USA)equipped with a Finnigan electrospray source and capableof analyzing ions up to m/z 2000. Xcalibur software(Finnigan MAT) was used for the operation. The chromatographicconditions are identical to those described inSection 2.4 and the outlet of the flow cell was connectedto a splitting valve, from which a flow of 0.2 mL/min wasdiverted to the electrospray ion source via a short lengthof fused silica tubing. Negative ion mass spectra of thecolumn eluate were recorded in the range m/z 100–2000. The source voltage was set to 4.5 kV and the capillary temperature to 250℃. The other conditions were as

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follows:capillary voltage, –36.5 V; inter-octapole lens voltage,10 V; sheath gas, 80 psi (551.6 kPa); auxiliary gas,20 psi (137.9 kPa).

3.8. On-line HPLC–ABTS^{+} assay

HPLC coupled with ABTS assay was performed by using themethod developed by Koleva*et al.* (2001) with some modifications. A stock solution containing 3.5 mM potassium persulphateand 2 mM ABTS was prepared and kept at room temperaturein darkness for 12 h in order to stabilize the radical. Theradical reagent was prepared by diluting the stock solution withpure water to an absorbance of 0.70 ± 0.02 at 680 nm. The extracts(10 uL) were injected into an Waters HPLC system. HPLC separationwas carried out as described in the previous section. HPLC eluatesfrom the column then arrived at a T-junction, where the ABTSreagent was added. The ABTS reagent flow rate was 0.7 mL/mindelivered by a Waters Reagent Pump (Waters Corporation, USA).After the eluates mixed with ABTS reagent in a reaction coil(15 m x 0.25 mm i.d. PEEK tubing), the negative peaks were measured by UV/Vis spectrometer at 680 nm. Water wasused as the control by replacing ABTS^{+} in terms of above procedure.Data were analysed using Empower Software.

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Fig. 2-2. Figure of *Sargassumsiliquastrum*

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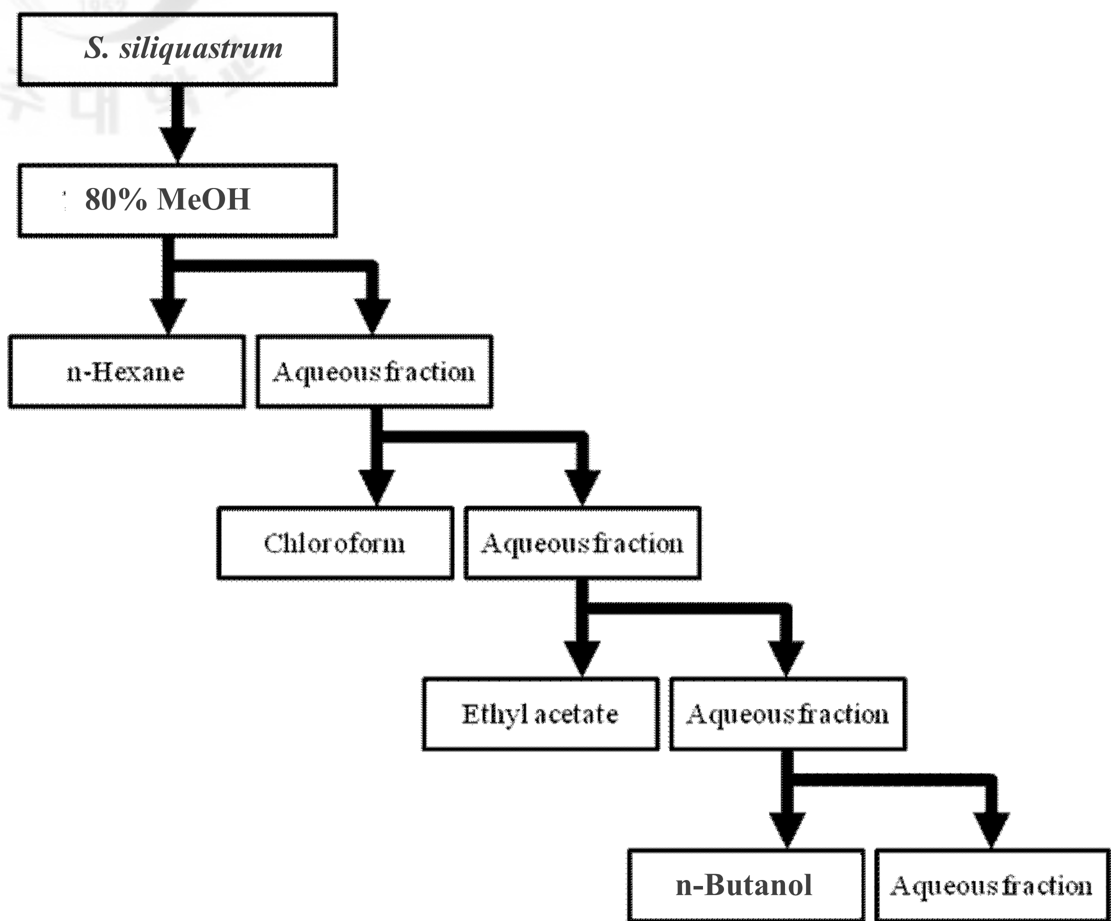

Fig. 2-3. Fractionation scheme of crude extracts from *S. siliquastrum*

4. RESULTS & DISCUSSIONS

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4. 1. HPLC and on-line HPLC-ABTS analysis of $CHCl_3$ fraction of *S. siliquastrum*

In HPLC analysis results as each fractions of *S. siliquastrum*,we suggested that compounds of CHCl3 fraction could beseparated efficiently due to the best separation performance compared with other fractions (No described in this study). $CHCl_3$ fraction of *S. siliquastrum* was analyzed by described on-line HPLC-$ABTS^+$ condition and chromatogram depicted in **Fig. 2-4**. The determination of antioxidant activity on on-line HPLC wasbased on a decrease in absorbance680 or 734 nm after postcolumnreaction of HPLC separated antioxidants with the $ABTS^+$. Therefore, themethod are focused on the analyses of free radical scavengingactivities of complex mixtures, especially the various plant or seaweed extracts.In these results, HPLC peak 2 and 3 had the highestantioxidant activity than other peaks. HPLC peak 5 and 8 also showed good free radical scavenging activity against $ABTS^+$. Therefore, we separated and collected the active compounds to operate preparative CPC by focusing on these peaks.

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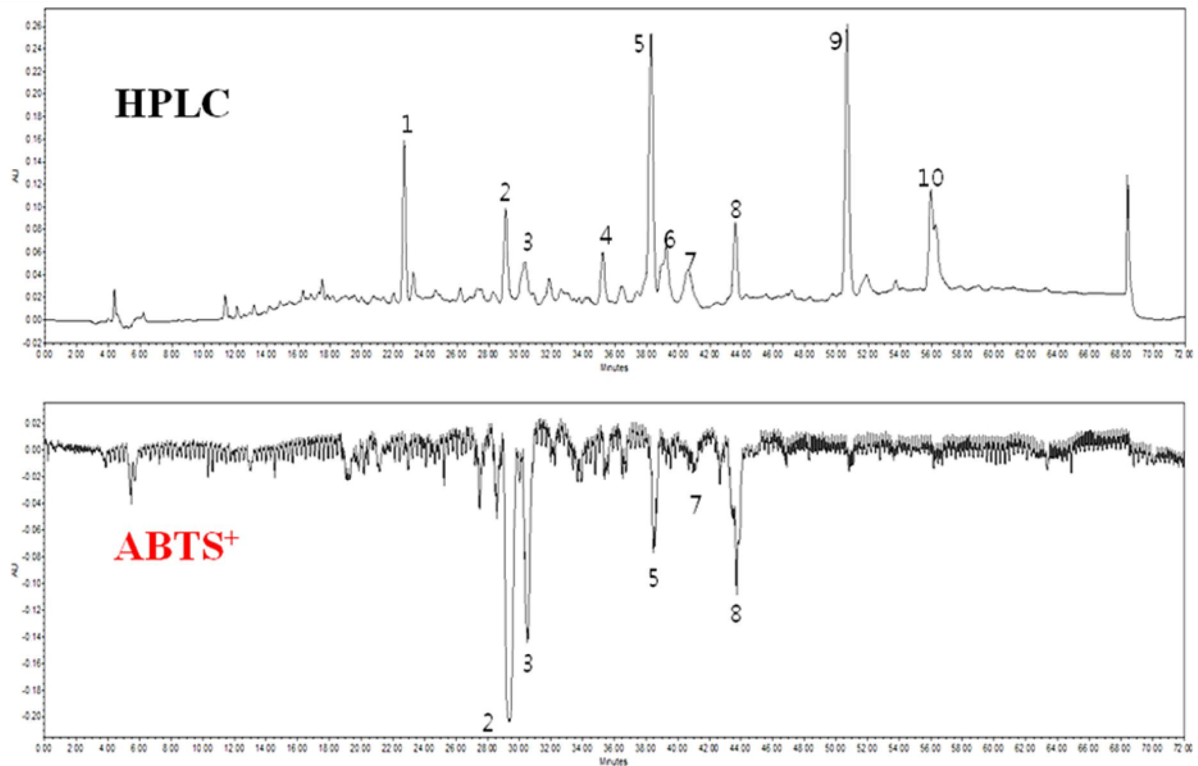

Fig. 2-4. On-line HPLC-ABTS^{+} chromatogram of $CHCl_3$ fraction from *S. siliquastrum*

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4. 2. Optimization of two-phase solvent system

Partition coefficient (*K*) for selection of a suitable two phase solvent systems were the most important for successful separation of target samples by preparative CPC. In order to choice efficient separation, several two-phase solvent system was performed through different compositions and volume ratios of two immiscible solvents such as *n*-hexane:ethylacetate:methanol:water, and then their *K* values was calculated and showed in **Table 2-1**. Two-phase solventsystem composed of 5:5:7:3 (*n*-hexane:ethylacetate:methanol:water, v/v) exhibitedgood *K* values toseparate antioxidative compounds confirmed by on-line HPLC. The most efficient separation of each compoundswas perfumed under solvent condition, *n*-hexane:ethylacetate:methanol:water (5:5:7:3, v/v) by CPC.

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Table2-1. K-valuesas solvent condition of $CHCl_3$ fraction from *S. siliquastrum*

HEMW	1	2	3	4	5	6	7	8	9	10
9:1:9:1	0	0	0	0	0	0	0	0	0	0
8:2:8:2	0	0	0	0	0.005	0.37	0	0	0	0
7:3:7:3	0	0.12	0.09	0	3.87	1.57	0	-	0.31	0
5:5:7:3	0	0.25	0.23	18.18	0.39	0.01	0.81	0	0.36	0.32
6:4:7:2	0.52	0.12	0.09	0.3	0.47	1.08	0.70	0	0.15	0
6:4:6:4	0	0.82	0.85	3.67	5.88	30.68	0	-	4.34	0
5:5:5:5	0.58	4.41	-	-	-	-	-	5.07	-	1.01

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4. 3. Separation of antioxidative compounds by CPC

The *K*-values of most of target compounds exhibited lower numerical values than1. Therefore, preparative CPC was operated on descending mode selected upper phase as stationary phase and lower phase as mobile phase. The retention of the stationary phase in the coil retained75% and pressure exhibited 39MPa during operating. Preparative CPC chromatogram was described in **Fig. 2-5**. Fraction 1 and 2 among all fractions showed purified compound with up to 80% according to analysis by HPLC peak area. And fraction 3 and 4 showed purity with up to 75%.The yields of fraction 1~ 4 isolated from 500mg of $CHCl_3$ fraction from *S. siliquastrum*by one-step of CPC system was 105 mg,10mg, 50mg and 12mg, respectively. In analysis results of each CPC fraction measured by HPLC-DAD-ESI/MS, the compoundexisted in fraction 2 was confirmed as fucoxanthin, andthe compounds existed in fraction 1, 3 and 4were suggestedas unknown compound 1(molecular weight (MW)428*m/z*),2 (MW 408*m/z*) and 3 (MW 408*m/z*), respectively. Two compounds existed in fraction 5 had yield of 60mg were suggested as unknown compound 4 (MW 424 *m/z*) and 5 (MW 428*m/z*), respectively. Purified unknown compound 4 and 5 with up to 90% was gained by prep-HPLC.Isolation of HPLC peak 2, 7 among antioxidative peaks were possible by CPC and they were suggested as unknown compound 1 and 3 by retention time of

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HPLC.All unknown compounds and fucoxhantin suggested by HPLC-DAD-ESI/MS need to be definitely identified using ^{1}H-NMR and ^{13}C-NMR spectra.

5. CONCLUSION

In this study, we could isolate the fucoxhantin andantioxidative compounds with high yield by one-step CPC operation. In conclusion, we demonstrated CPC system is a useful process to isolate and purify active compounds from *S. siliquastrum.*

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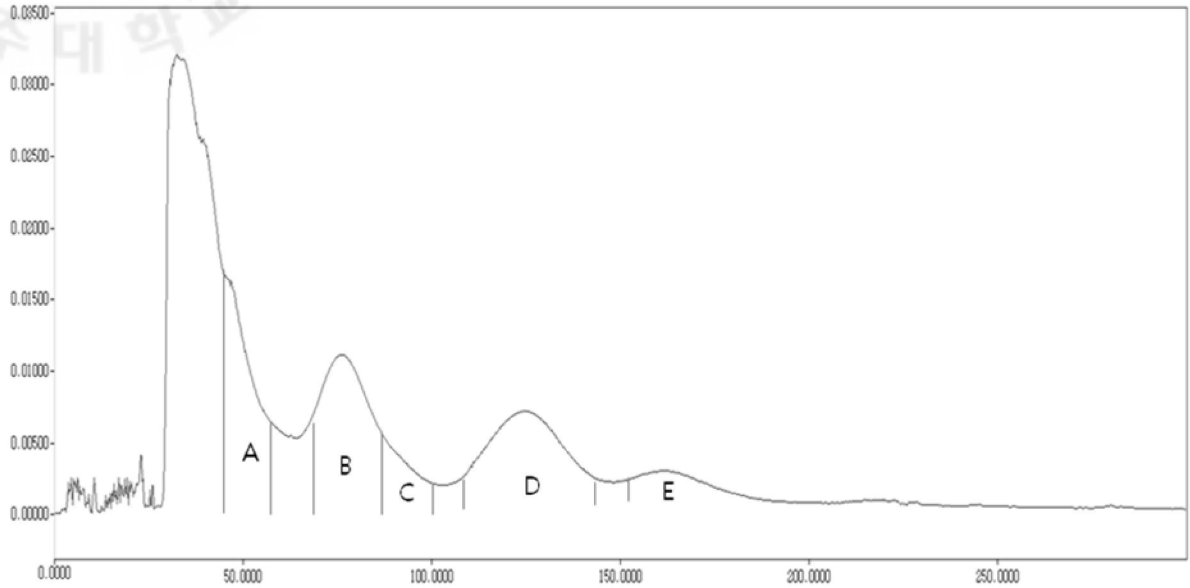

Fig. 2-5. CPC chromatogram of *S. siliquastrum*$CHCl_3$ fraction (*n*-hexane:EtAOc:MeOH : water –5:5:7:3, v/v)

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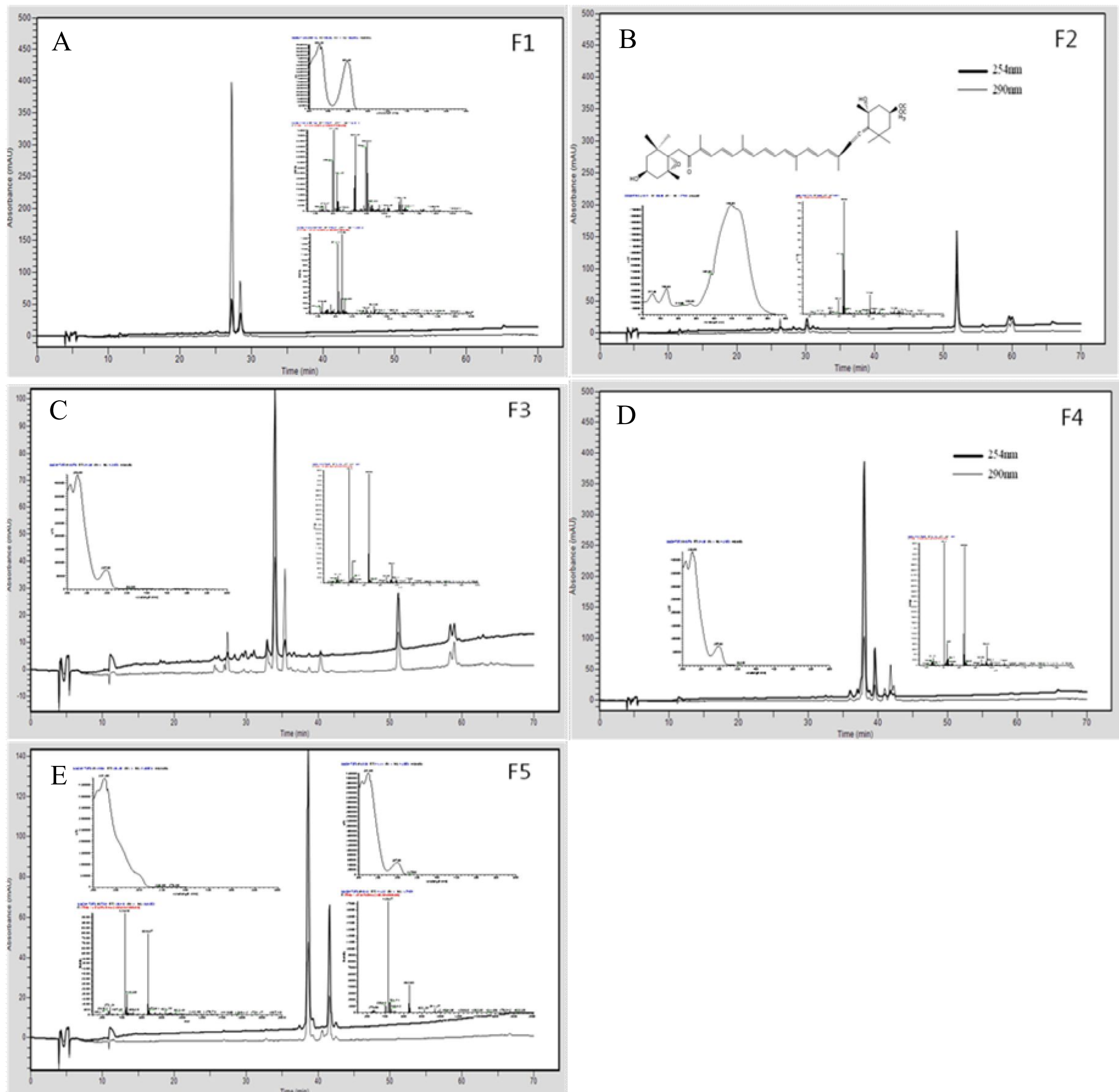

Fig. 2-6. Chromatogram and MS data of each fractions of CPC by HPLC and HPLC/MS

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Part III .

Anti-inflammatory activity of sargachromanol E isolated from *Sargarssumsiliquasum*on LPS induced 264.7 RAW cell via MAPK pathway

1. ABSTRACTS

Sargarssumsiliquasum belongs toSargassaceae, has been inhabitedin a coast on Jeju island, South Korea. In this study, we have evaluated the anti-inflammatory of compounds isolated from*S. siliquasum*by centrifugal partition chromatography (CPC). To evaluate anti-inflammatory activity, it measured a coefficient of NO in lipopolysaccharide (LPS) induced RAW264.7cell line and a cytotoxicity by MTT assay. And then, IL1β and TNF-αwhich are main cytokine of inflammation, iNOS and COX-2 was evaluated by the ELISA kit. In these results, 5573F1 isolated from *S. siliquasum*by CPC showed lowest NO production (IC_{50} value : 6.99 ug/ml). The 5573F1 confirmed as

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sargachromanol Eby HPLC, LC-MS ESI, ^{1}H-NMR and^{13}C-NMR data. sargachromanol Ealso inhibited production of IL1β, TNF-α and PGE_2. In western-blot assay, sargachromanol Einhibited express of iNOS, COX-2, phosphate P38 andphosphate $ERK_{1/2}$.In conclusion, sargachromanol E inhibited inflammation in LPS induced RAW 264.7 cells via MAPK pathway.

2. INTRODUCTION

Inflammation represents a highly co-ordinated set of events thatallow tissues to respond against injury or infection. It involves theparticipation of various cell types expressing and reacting to diversemediators along a very precise sequence of events (Babu*et al*.,2009). Usually, inflammation is initiated through the productionof specific cytokines or chemokines characterized by recruitmentof leukocytes to the damage site. However, sustained or excessiveinflammation can lead to numerous diseases including rheumatoidarthritis, psoriasis and inflammatory bowel disease (Simon andGreen, 2005).Macrophages play a key role in inflammatory and immune reactionsby releasing a variety of inflammatory mediators such ascytokines, chemokines, growth factors, iNOS, COX-2 (Ramanaand Srivastava.,2006) and increased circulating levels of lipopolysaccharide (LPS)lead to increased mitochondrial activity and the formation of reactiveoxygen species (ROS), resulting in disturbed

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redox homeostasisin macrophages that activate redox-sensitive transcription factors,such as NF-B and AP-1 (Woo *et al*., 2004; Liu and Malik, 2006),and lead to apoptotic cell death of macrophages (Woo *et al*., 2004;Asehnoune*et al*., 2004; Liu and Malik, 2006). Therefore, interferingwith signalling pathways that lead to LPS-mediated apoptosis mayrepresent an important therapeutic target for suppressing inflammatoryresponses.Inflammatory stimuli, such as LPS activation of macrophages,lead to the activation of the transcription factors NF-kB and AP-1, which promote the expression of several pro-inflammatorycytokines, including TNF-α, IL-1, and IL-6 (Nunez Miguel*et al*., 2007), as well as other inflammatory mediators, includingnitric oxide (NO) and prostaglandin E_2 (PGE_2), which are synthesised byinducible nitric oxide synthase (iNOS) and cyclooxygenase (COX),respectively.

Brown algae are under-exploited plant resources that are well known as producers of a great variety of secondary metabolites with different carbon skeletons (Blunt *et al*., 2006).*Sargassum* sp. are found throughout tropical and subtropical areas of the world and are reported to produce metabolites of structural classes such as plastoquinones (Segawa and Shirahama, 1987; Mori *et al*., 2005; Ishitsuka*et al*., 1979), chromanols (Kato *et al*., 1975), chromenes (Jang *et al*., 2005; Kikuchi et al., 1975), steroids (Tang *et al*., 2002a) and glycerides (Tang *et al*., 2002b). And then, recently, active compounds from *Sargarssumsiliquasum*(described in Fig. 2.) has been reported that chromenehad antioxidant activity (Cho *et al*., 2008) and induces apoptosis via caspase-3 activation in human

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leukemiaHL-60 cells (Heo*et al*., 2011), farnesylacetones has vasodilatation effect on the basilar and carotid arteries of rabbits (Park*et al*., 2008) and fucoxanthin has protective effect on UV-Binduced cell damage (Heo*et al*., 2009). However, active compounds excepted of fucoxanthin from *S.siliquasum*have not been published on anti-inflammatory effect.

Therefore, we evaluated that active compounds isolated from *S.siliquastrum* induced anti-inflammatory activities via NFkB and MAPK pathways on LPS induced RAW 264.7 cells.

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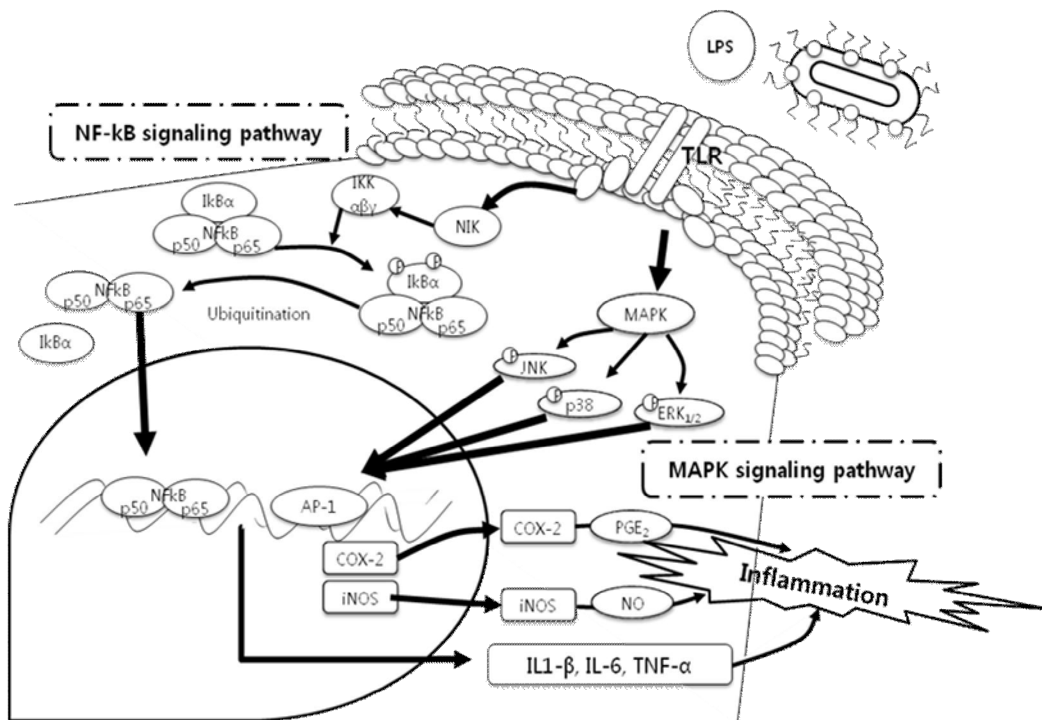

Fig. 3-1. Signaling pathway of LPS-induced inflammation

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3. MATERIALS & METHODS

3.1. Materials

Lipopolysaccharide (LPS) was purchased from sigma chemical Co., Ltd (ST. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) penicillin-strptomycin and trypsine-EDTA were obtained from Gibco/BRL (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, dimethyl sulfoxide (DMSO)werepurchased from Sigma (St. Louis, MO, USA).M-MuLV reverse transcriptase were purchased from Promega (Madison, WI, USA). The enzyme-linked immunosorbent assay (ELISA) kit for IL-1β, TNF-α and Prostaglandin E_2 (PGE_2) were purchased from R & D Systems Inc. (Minneapolis, MN, USA). Protein assay kit and ECL detection reagent were bought from Bio-Rad (Richmond, CA, USA) and Amercham Biosciences (Piscataway, NJ, USA), respectively. Other all reagents and solvents were purchased from Sigma (St. Louis, MO, USA).

3. 2. Cell culture

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The murine macrophage cell line RAW 264.7 cells was grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% (v/v) heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 lg/ml). Cultureswere maintained at 37 ℃ in a 5% CO_2 incubator.

3.3. Determination of nitric oxide (NO) production

After a 24 h pre-incubation of RAW 264.7 cells (1.5×105 cells/ml)with LPS (1 μg/ml), the quantity of nitrite accumulated in the culturemedium was measured as an indicator of NO production. In brief,100 μl of cell culture medium was mixed with 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediaminedihydrochlorideìn 2.5% phosphoric acid), the mixture was incubated at roomtemperature for 10 min, and the absorbance at 540 nm was measuredin a microplate reader. Fresh culture medium was employed as ablank in every experiment. The quantity of nitrite was determinedfrom a sodium nitrite standard curve.

3.4. Measurement of pro-inflammatory cytokines (TNF-α, IL-1β) and PGE_2 production

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All sampls solubilized with DMSO was diluted with PBS before treatment. The inhibitory effect of samples on the pro-inflammatory cytokines (IL-1β, TNF-α) and PGE_2 production from LPS induced RAW 264.7 cells was determined using a competitive enzyme immunoassay (ELISA) kit according to the manufacturer's instructions.

3.5. Western blot analysis

RAW 264.7 cells plated at 2 x 10^5 cells/ml were treated with HPCPC fractions from *S. siliquastrum*and harvested. Thecell lysates were prepared with lysis buffer (50 mmol/l Tris–HCl (pH 7.4),150 mmol/l NaCl, 1% Triton X-100, 0.1% SDS and 1 mmol/l EDTA). The cell lysateswere washed via centrifugation, and the protein concentrations in the lysates weredetermined using a BCA™ protein assay kit. The lysates containing 30ug of proteinwere subjected to electrophoresis on 10% or 15% sodium dodecyl sulfate–polyacrylamidegels, and the gels were transferred onto nitrocellulose membranes (Bio-Rad,Hercules, CA, USA). The membranes were incubated with primary antibody againstCOX-2, iNOS, p38, pp38, JNK, pJNK, ERK, pERK, IkBα, pIkBα, NFkB p50 and p65and β-actin in TTBS (25 mmol/lTris–HCl, 137 mmol/l NaCl, 0.1% Tween 20, pH 7.4) containing 0.5% non-fat dry milkfor 1 hr. The membranes were then washed with TTBS and incubated with secondaryantibodies. Signals were developed

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using an ECL Western blotting detection kit andexposed on X-ray films.

3. 6. ^{1}H-NMR and ^{13}C -NMR analysis of purified compound

^{1}H-NMR spectra and ^{13}C-NMR spectra were measured with a JEOL JNM-LA300 spectrometer. Mass spectra (FAB-MS and EIMS)were recorded on a JEOL JMS 700 spectrometer.

3. 7. Statistical analysis

All the measurements were made in triplicate and all values were representedas means ± standard error. The results were subjected to an analysis of the variance(ANOVA) using the Turkey test to analyze the difference. A value of $p < 0.05$ was consideredto indicate statistical significance.

4. RESULTS & DISCUSSIONS

4. 1. NO and PGE_2production inhibitory effect of compounds isolated from *S. siliquastrum*in LPS-induced 264.7 RAWcells

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To evaluate anti-inflammatory activity of unknown compound 1~5 isolated from *S. siliquastrum* in Part II, each fractions included unknown compound 1~5 were measured on inhibitiory activity against NO production in lipopolysaccharide (LPS) induced 267.4 RAW cells (**Fig. 3-2**).After the fraction 1, 3, 4 and 5 isolated from *S. siliquastrum*(named as 5573-F1, 5573-F3, 5573-F4 and 5573-F5, respectively) by centrifugal partition chromatography (CPC) were treated in RAW 264.7 cells for 2hr, and then LPS was treated for 24hr. Culture supernatants were used for evaluation of NO production by Griess reaction.The 5573-F1 included with unknown compound 1 among fractions from *S. siliquastrum*showed the strongest inhibitory activity against NO production and its IC_{50} value exhibited 6.99 μg/ml. Although 5573-F1 showed high cytotoxicity at 25 μg/ml, cytotoxicity wasn't showed at below 25 μg/ml, therefore next all experiments were progressed at concentrations of below 25 μg/ml. Results about inhibitory activityof 5573-F1 against PGE_2 production exhibited in **Fig. 3-3**. 5573-F1 inhibited PGE_2 production in LPS-induced 264.7 RAW cells in a dose-dependently manner (IC_{50} value : 11.7 μg/ml) and exhibited maximum 80% inhibitory activity at 25 μg/ml.

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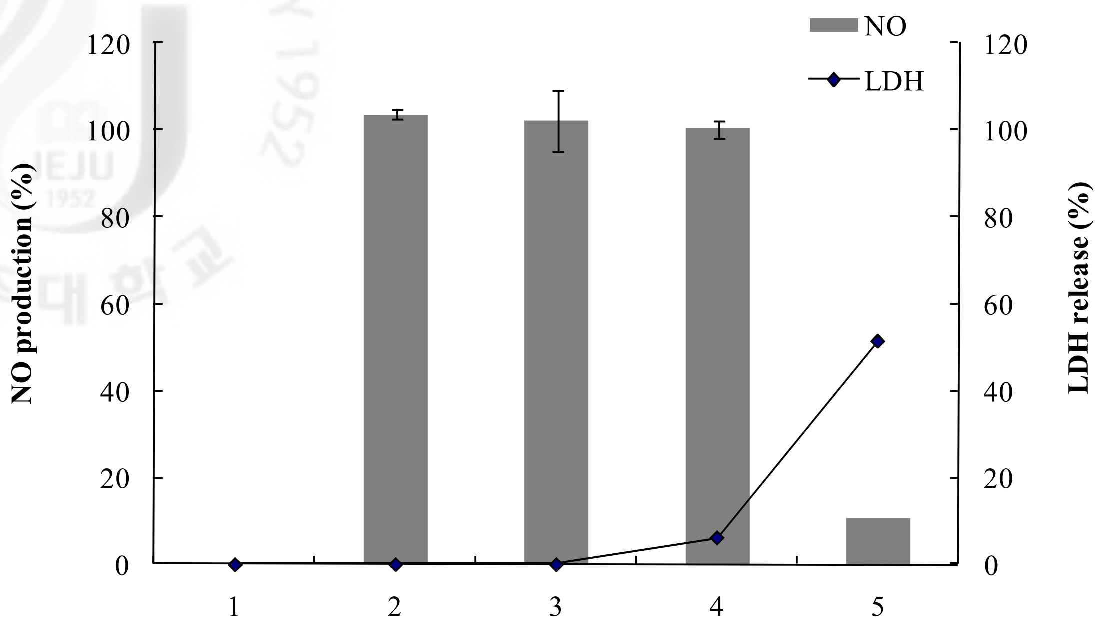

Sample	-	5573-F1	5573-F3	5573-F4	5573-F5
LPS (1ug/ml)	-	+	+	+	+
IC_{50} (ug/ml)		6.99	19.46	21	10.31

Fig. 3-2. Inhibitory effect of eachfractionsfrom *S. siliquastrum*against the NO production in LPS-induced RAW 264.7 cells.

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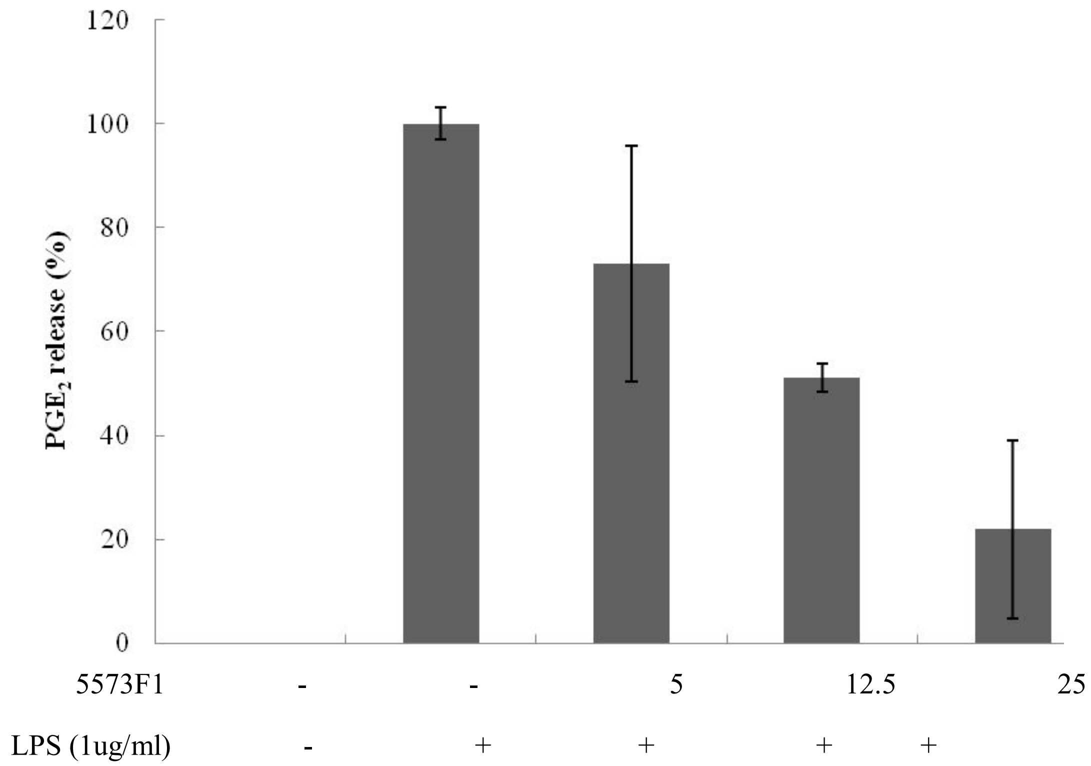

Fig. 3-3. Inhibitory effect of 5573-F1 against the PGE_2 production in LPS-induced RAW 264.7 cells

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4. 2. Structural identification of anti-inflammatory compound

Identification of fraction 1 (5573-F1) among CPC fractions was carried out by ^{1}H NMR, ^{13}C NMR and HPLC–DAD–ESI/MS (positive ion mode)(**Fig. 3-4, Table 3-1**) and purified unknown compound in 5573-F1 was confirmed as sargachromanol E reported by Jang *et al* (2005).

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Table 3-1. ^{1}H and ^{13}C NMR Assignments for 5573-F1

C/H#	dHmult	dC
2		75.0
3	1.78, 1.72	31.42
4	2.65	22.10
4a		121.2
5	6.30	112.6
6	6.39	147.8
7		115.7
8		127.4
8a		146.0
1’	1.60, 1.52	39.2
2’	2.13	21.9
3’	5.19	124.7
4’		135.9
5’	1.97	39.2
6’	2.08	26.0
7’	5.36	127.6
8’		131.6
9’	3.81	80.3
10’	4.23	67.2
11’	5.03	124.5
12’		135.9
13’	1.67	24.8
14’	1.66	17.2
15’	1.55	11.0
16’	1.57	15.8
17’	1.25	23.1
18’	2.06	

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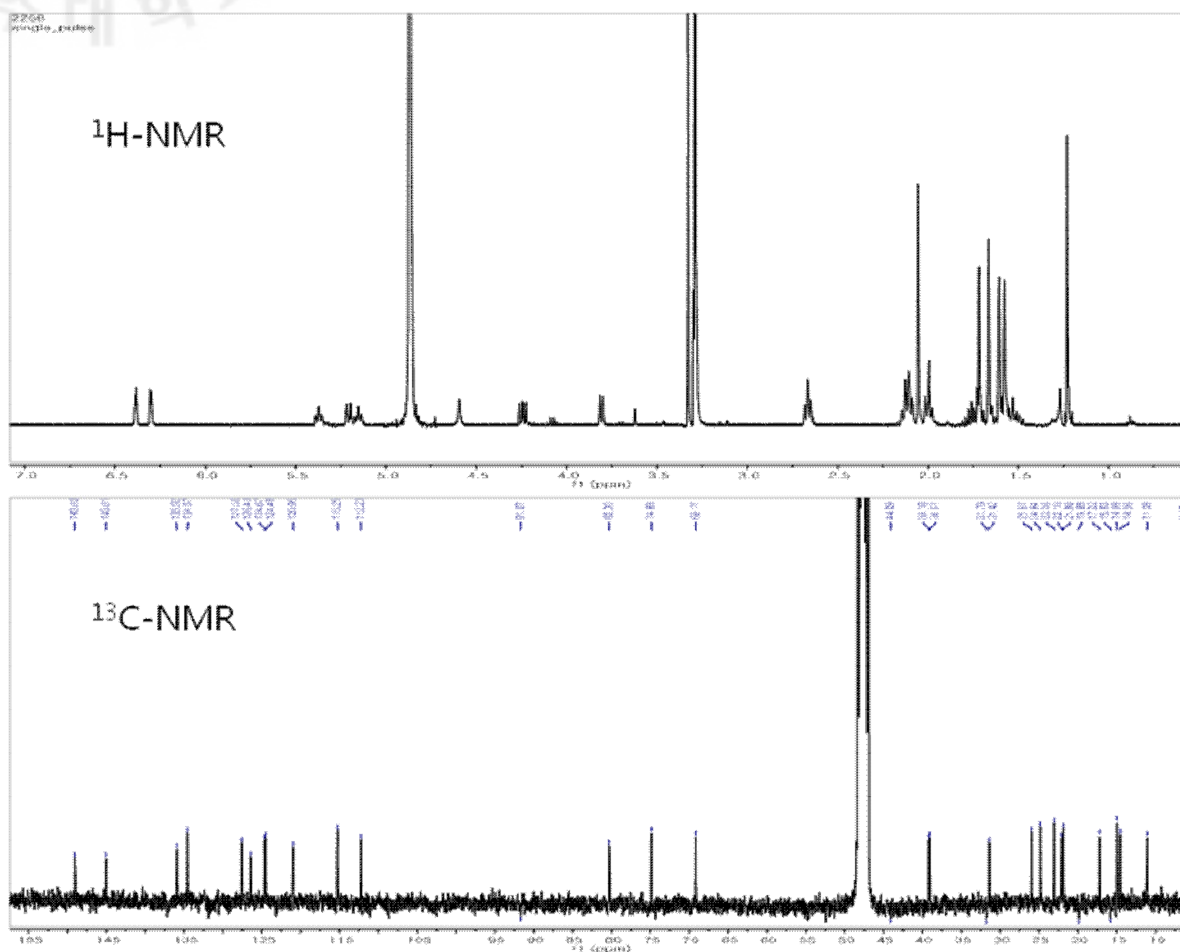

Fig. 3-4. ^{1}H and ^{13}C-NMR spectra of 5573F1

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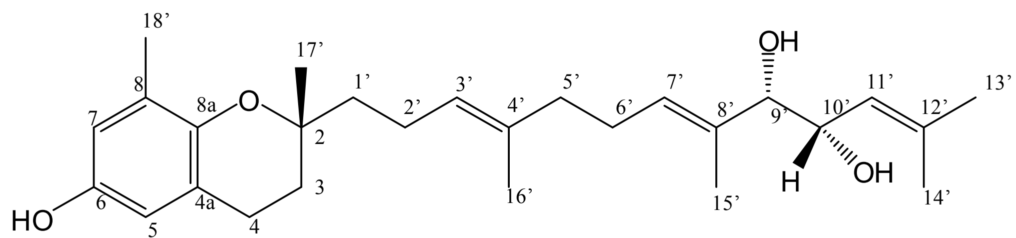

Fig. 3-5. Structure of sargachromanol E isolated from *S. siliquastrum* by CPC

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4. 3. Inhibitory effect of sargachromanol E against expression of iNOS and COX-2 proteins in LPS-induced 264.7 RAWcells

Inflammatory processes are mediated by multiple molecular mechanisms. Two of the most prominent are the production of NO by iNOS and the formation of PGE_2 by COX-2 (Kim *et al.*, 2005). To evaluate mechanism of anti-inflammatory activity of sargachromanol E isolated from *S. siliquastrum*, we measured inhibitory effects against up-regulation of iNOS and COX-2 protein in LPS induced 264.7 RAW cells by western-blot (**Fig. 3-6**). In this result, sargachromanol E showed to inhibitdose-dependently expression of iNOS and COX-2 protein induced strongly by LPS.Especially, we confirmed sargachromanol E inhibited drivingly expression of COX-2 at above 12.5 mg/ml by western blot.

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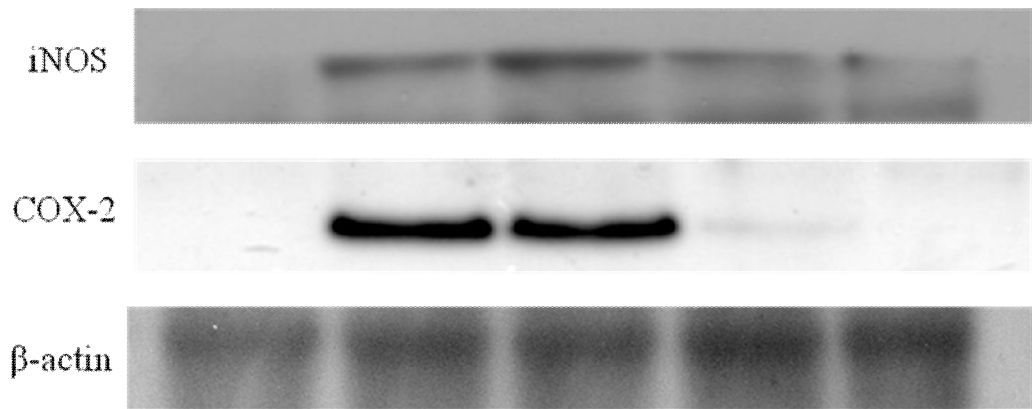

Fig. 3-6. Inhibitory effect of sargachromanol Eon expression of iNOS and COX-2 protein in LPS-induced RAW 264.7 cells

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4. 4. Inhibitory effect of sargachromanol Eagainst the production of proinflammatory cytokines and phosphorylation of MAPKin LPS-induced 264.7 RAWcells

Macrophage induced by LPS released from bacterium has two pathways such as NFkB and MAPK pathway. In NFkB pathway, IkBα binding with NFkB was phosphorylated by IKK and then, NFkBseparated with phosphate IkBαtransfer to nuclear. Due to transcription of NFkB in nuclear, pro-inflammatory cytokines (TNF-α and IL-1β)and PGE_2were released. In MAPK pathway, MAPKs are a highly conserved family of protein serine/threonine kinase and include the $ERK_{1/2}$, JNK and p38 subgroups.Therefore, to confirm anti-inflammatory pathway of sargachromanol E,we measuredTNF-αand IL-1β production in LPS-induced RAW 264.7 cells using ELISA kit (**Fig. 3-7**).In addition, expression of the $ERK_{1/2}$, JNK and p38 were also evaluated by western-blot assay (**Fig. 3-8**). Levels of released TNF-αand IL-1β was increasedby LPS. Sargachromanol E showed to inhibit pro-inflammatory cytokines (TNF-α and IL-1β) in dose-dependent manners.Especially, group treated with Sargachromanol Eshowed to be similar to those ofthe control group.In addition, Sargachromanol Einhibited dose-dependently subgroups such as p-P38 and $ERK_{1/2}$ expressed

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strongly by LPS.

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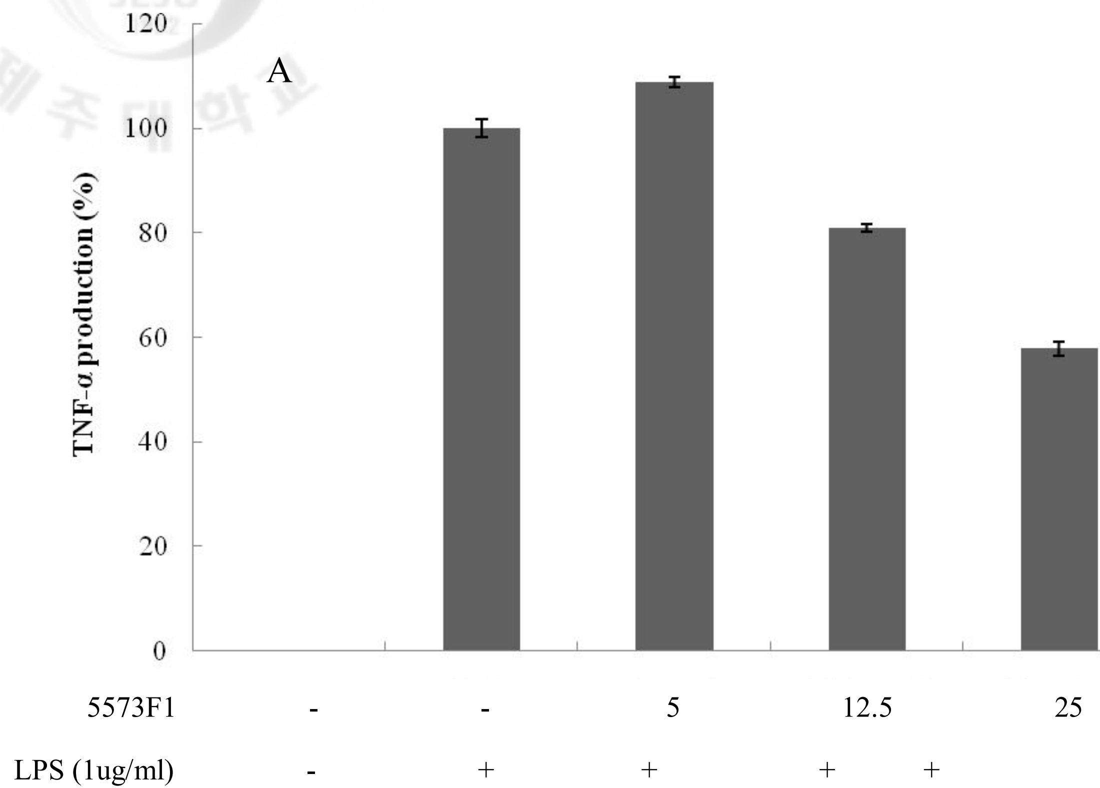

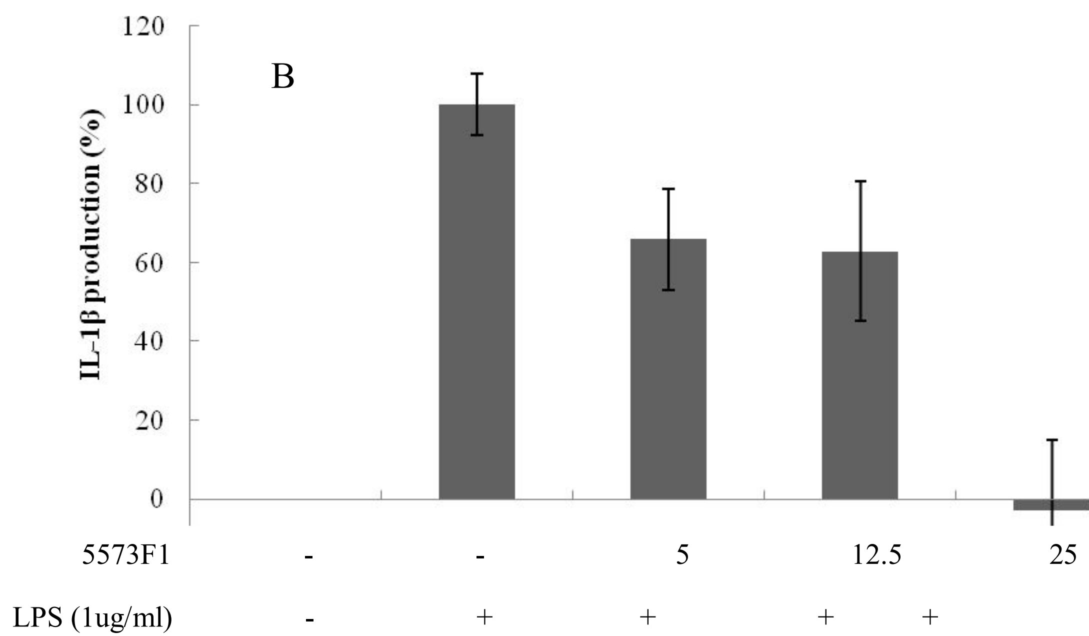

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3-7. Inhibitory effect of sargachromanol E on the TNF-α(A) and IL-1β (B) production in LPS-induced RAW 264.7 cells

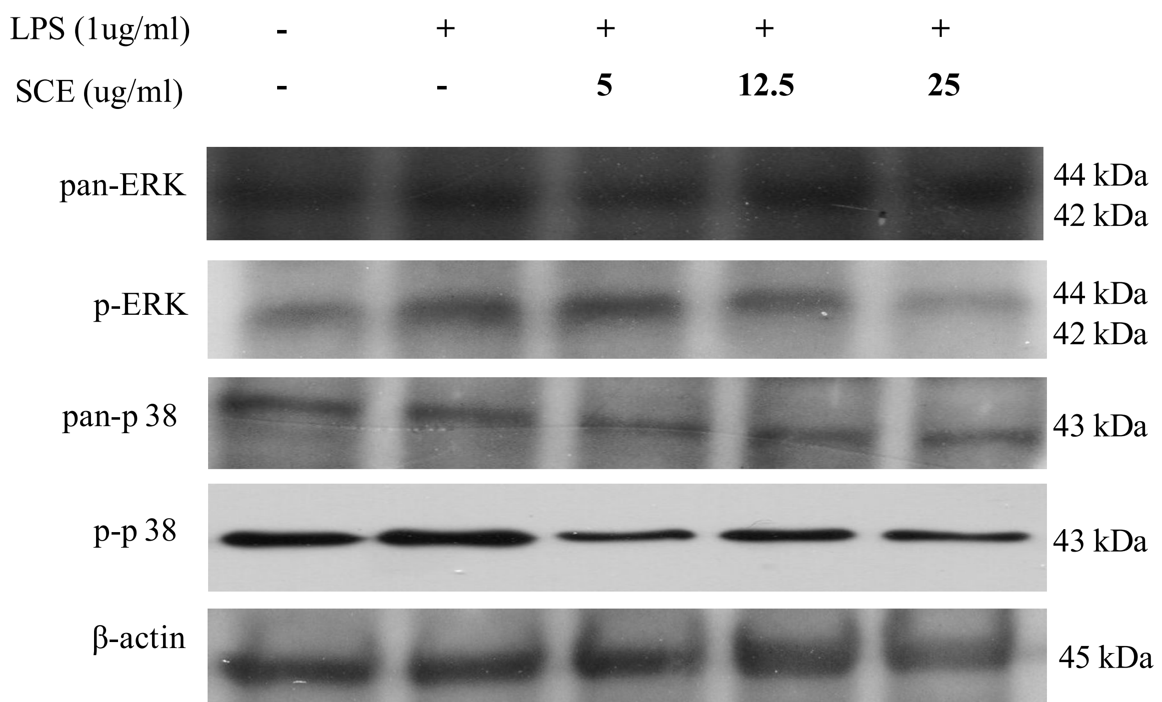

Fig. 3-8. Inhibitory effect of sargachromanol E on the MAPKs protein in LPS-induced RAW 264.7 cells

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5. CONCLUSION

In this study, we could confirm that sargacromanol E isolated from S. siliquastrum had the strongest anti-inflammatory activity. Also we could know that sargacromanol E had anti-imflammatory effect via MAPK pathway as inhibiting expression of p-p38 and $ERK_{1/2}$ induced by LPS (**Fig 3-9**).

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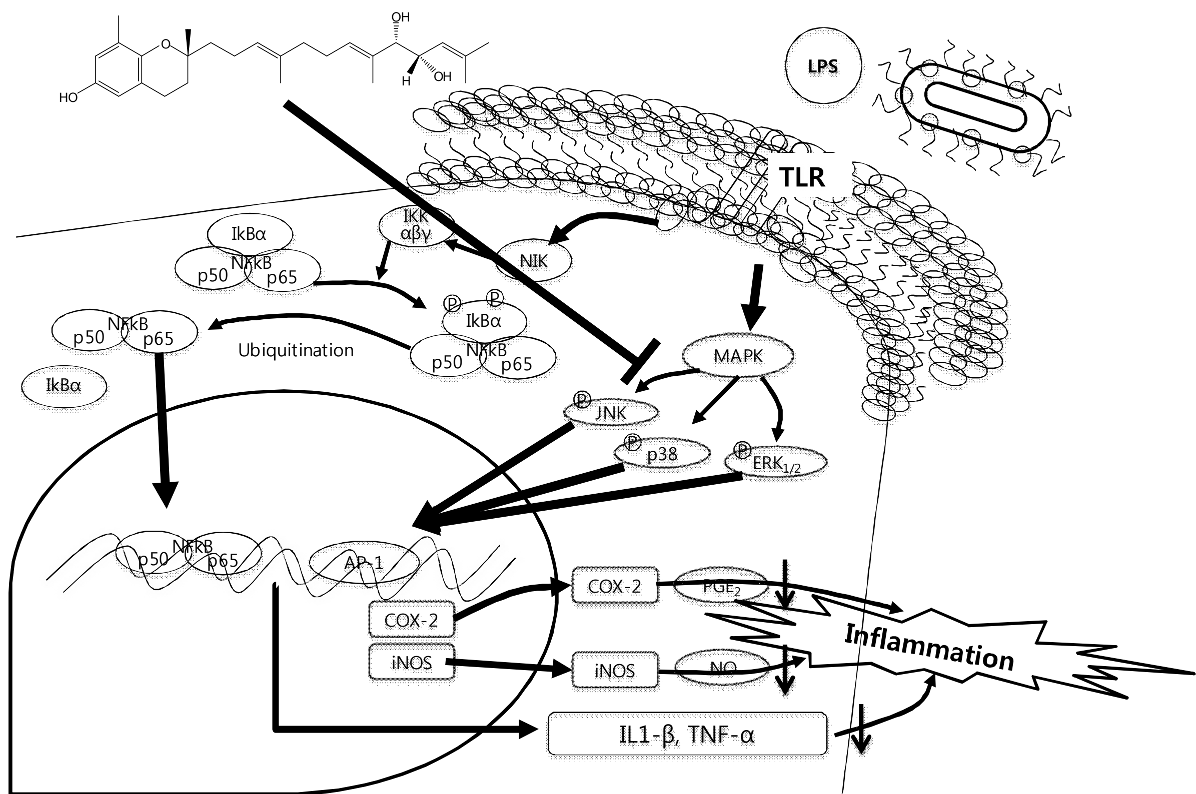

Fig. 3-9. Signaling pathway for anti-inflammation of sargachromanol E in LPS-induced RAW 264.7 cells

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