Anti-inflammatory activity of a sulfated polysaccharide isolated from an enzymatic digest of brown seaweed *Sargassum horneri* in RAW 264.7 cells

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BACKGROUND/OBJECTIVES: *Sargassum horneri* is an edible brown alga that grows in the subtidal zone as an annual species along the coasts of South Korea, China, and Japan. Recently, an extreme amount of *S. horneri* moved into the coasts of Jeju Island from the east coast of China, which made huge economic and environmental loss to the Jeju Island. Thus, utilization of this biomass becomes a big issue with the local authorities. Therefore, the present study was performed to evaluate the anti-inflammatory potential of crude polysaccharides (CPs) extracted from *S. horneri* China strain in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.
MATERIALS/METHODS: CPs were precipitated from *S. horneri* digests prepared by enzyme assistant extraction using four food-grade enzymes (AMG, Celluclast, Viscozyme, and Alcalase). The production levels of nitric oxide (NO) and pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-1β were measured by Griess assay and enzyme-linked immunosorbent assay, respectively. The levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), nuclear factor (NF)-κB, and mitogen-activated protein kinases (MAPKs) were measured by using western blot. The IR spectrums of the CPs were recorded using a fourier transform infrared spectroscopy (FT-IR) spectrometer.
RESULTS: The polysaccharides from the Celluclast enzyme digest (CCP) showed the highest inhibition of NO production in LPS-stimulated RAW 264.7 cells (IC$_{50}$ value: 95.7 μg/mL). Also, CCP dose-dependently down-regulated the protein expression levels of iNOS and COX-2 as well as the production of inflammatory cytokines, including TNF-α and IL-1β, compared to the only LPS-treated cells. In addition, CCP inhibited the activation of NF-κB p50 and p65 and the phosphorylation of MAPKs, including p38 and extracellular signal-regulated kinase, in LPS-stimulated RAW 264.7 cells. Furthermore, FT-IR analysis showed that the FT-IR spectrum of CCP is similar to that of commercial fucoidan.
CONCLUSIONS: Our results suggest that CCP has anti-inflammatory activities and is a potential candidate for the formulation of a functional food ingredient or/and drug to treat inflammatory diseases.

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INTRODUCTION

Macrophages play an important role in inflammatory responses through the release of various inflammatory factors such as nitric oxide (NO), prostaglandins, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 and cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 [1,2]. Especially, macrophages can be stimulated by pro-inflammatory cytokines and endotoxin such as lipopolysaccharide (LPS) and it causes inflammatory responses [1]. In addition, it has been demonstrated that during the inflammatory process, the abnormal release of NO and prostaglandin E2 (PGE$_2$) is caused by the activation of iNOS and COX-2 known as two pleiotropic inflammatory mediators which regulates platelet aggregation, vascular permeability, and thrombus formation [2]. Also, LPS stimulation can regulate the expression of COX-2 and iNOS and the secretions of TNF-α, IL-1β, and IL-6 known as the major pro-inflammatory cytokines by activating the nuclear factor-κB

(NF-κB) signaling pathway as well as mitogen- activated protein kinase (MAPK) known as its upstream molecule [2,3]. Therefore, inhibition of these inflammatory mediators and cytokines might be an useful approach for the treatment of inflammatory diseases.

Marine environments are considered an invaluable treasure trove of bioactive compounds since they have large chemical and biological variations [4]. Among the studied marine organisms, brown seaweeds have numerous compounds with a broad spectrum of bioactivities, including anti-inflammatory, antioxidant, anti-microbial, and neuroprotective effects [5]. Recent *in vitro* and *in vivo* studies have shown that polysaccharides isolated from brown seaweeds have excellent therapeutic potential against inflammatory reactions [6]. The isolation of bioactive compounds from seaweed depends heavily on the extraction conditions. However, a large amount of cell wall polysaccharides in these seaweeds acts as a physical barrier that limits the use of conventional chemical and mechanical extraction methods. Therefore, enzyme-assisted extraction methods (EEMs) that can digest cell walls are often used to isolate bioactive compounds from seaweed [7]. These enzymatic extracts show increased food safety and water-solubility compared to organic solvent extracts, which is an added advantage of using EEMs over solvent extraction [8].

Sargassum horneri (*S. horneri*) is an edible brown alga that grows in the subtidal zone as an annual species along the coasts of China, Japan, and South Korea. The thallus of *S. horneri* can reach a length of more than 7 m and a fresh weight of 3 kg [9]. Previous reports have indicated that *S. horneri* consisted of vitamins, amino acids, and polysaccharides has utilized as a food source as well as an ingredient in traditional medicine for thousands of years in Asian countries including South Korea, Japan, and China [10,11]. According to previous reports, the sulfated polysaccharide and some extracts from *S. horneri* China strain exerted potent bioactive properties in *in vitro* conditions such as antioxidant and anticancer properties [12]. However, there is a little information on anti-inflammatory potentials of polysaccharides isolated from *S. horneri* China strain and its biological mechanism. Moreover, within last few years, a huge amount of *S. horneri* moved into the coasts of Jeju Island from the east coast of China and caused considerable damages in the fishing industry and the beauty of the shores around Jeju Island. At this point, utilization of *S. horneri* is important to resolve the problems in South Korea have caused from this seaweed. Therefore, in this study, we prepared four crude polysaccharides (CPs) from *S. horneri* China strain by using four food grade enzymes including amyloglucosidase (AMG), Celluclast, Viscozyme, and Alcalase and evaluated their anti-inflammatory activities and its biological mechanism.

MATERIALS AND METHODS

Sample collection

S. horneri China strain was collected during May to June 2015 along the coast of Jeju Island in South Korea. *S. horneri* China strain was identified by Jeju Biodiversity Research Institute (Jeju, Korea).

Chemicals

The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Gibco BRL (Burlington, ON, Canada). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), fucoidan (Product No. F5631-1G), and FT-IR-grade KBR powder were purchased from Sigma-Aldrich (St. Louis, MO, USA). Three carbohydrate-degrading enzymes (AMG, Celluclast, and Viscozyme) and one protease (Alcalase) were donated by Novo Nordisk (Bagsvaerd, Denmark). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α, IL-1β and PGE$_2$ were purchased from R&D Systems Inc. (Minneapolis, MN, USA). All other chemicals and reagents used in these experiments were of analytical grade.

Preparation of enzymatic digests from S. horneri

The freeze-dried *S. horneri* samples were homogenized with a grinder to obtain a fine powder. The enzymatic hydrolytic reactions were performed by using the methods of Heo *et al*. [8]. Briefly, 2 g of *S. horneri* powder was incubated in 100 mL of distilled water (DW) with 20 μl or 20 mg of AMG, Celluclast, Viscozyme, or Alcalase at the optimum temperature and pH as described by Heo *et al*. [8]. After 24 h, the samples were centrifuged at 2,774 × g for 10 min and the supernatants were filtered with Watman No.4 (GE Healthcare, Buckinghamshire, UK). Then, the supernatants were freeze-dried and used as enzymatic digests.

Separation of crude polysaccharides from the enzymatic digests

Crude polysaccharides were prepared from the four enzymatic extracts according to the method described by Shao *et al*. [13] with slight modifications. Briefly, the enzymatic digests were mixed with 95% ethanol to a final concentration of 66%. The mixture was stored at 4℃ for 24 h, and then the precipitates were collected by centrifugation at 10,000 × g for 20 min at 4℃. The precipitates were freeze-dried and used as crude polysaccharides (CPs). The freeze-dried CPs were dissolved in PBS for use in the experiments. The CPs separated from the four enzymatic digests with AMG, Celluclast, Viscozyme, and Alcalase were nominated as follow. AMGCP, crude polysaccharides from AMG enzymatic digestion; CCP, crude polysaccharides from Celluclast enzymatic digestion; VCP, crude polysaccharides from Viscozyme enzymatic digestion; AlCP, crude polysaccharides from Alcalase enzymatic digestion.

Analysis of chemical composition

Total polysaccharide content was measured by the phenol-sulfuric acid method as described by method described by DuBois *et al*. [14] using glucose as the standard. Total phenol content was quantified by using a protocol described by Chandler and Dodds [15] using gallic acid as the standard. The protein content of all samples was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as the standard. Finally, the sulfate content of the CPs was checked by the BaCl$_2$ gelatin method using Na$_2$SO$_4$ as the standard as described by Saito *et al*. [16] with slight modifications.

Fourier transform infrared (FT-IR) spectroscopy

The IR spectrums of the CPs were recorded using a FT-IR spectrometer (Nicolet™ 6700 FT-IR spectrometer; Madison, WI, USA). The CPs were homogenized with KBr powder and then pressed into pellets for FT-IR measurement in the frequency range of 500-4,000 cm^{-1}.

Cell culture

RAW 264.7 cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 1% streptomycin (100 µg/mL), and penicillin (100 unit/mL). RAW 264.7 cells were incubated under 5% CO_2 at 37°C (Sanyo MCO-18AIC CO_2 Incubator; Moriguchi, Japan). Cultured cells from passage 4-6 were used for the experiments.

Cell viability assay

The cytotoxicity of the CPs to RAW 264.7 cells was evaluated via the colorimetric MTT assay as described by Mosmann [17], with slight modifications. Briefly, the cells (1×10^5 cells/mL) were seeded in a 24-well plate and incubated for 24 h. Then, the cells were treated with CPs (50 µg/mL and 100 µg/mL) for 24 h. MTT reagent (200 µg/mL) was added to each well. After 3 h of incubation, the formasan crystals were dissolved in DMSO, and the amount of blue-black formazan was determined by measuring the absorbance at 540 nm. The optical density of the formazan generated in non-treated control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

Determination of NO production

To determine the effect of CPs on NO production in LPS-stimulated RAW 264.7 cells, we performed griess assay described by Leiro *et al.* [18] with slight modifications. Briefly, the cells (1×10^5 cells/mL) were seeded in 24-well plates and incubated for 24 h. Then, the cells were treated with CPs (50 µg/mL and 100 µg/mL) for 1 h and stimulated with LPS (1 µg/mL) for 24 h. Finally, equal amounts of the culture medium and Griess reagent were reacted in a 96-well plate for 10 min, and the absorbance was measured at 540 nm using an ELISA plate reader machine (BioTek Instruments, Inc., Winooski, USA). The optical density of NO produced in only LPS-treated cells was considered to represent 100%. The data are expressed as mean percentages of the NO production versus the NO production of only LPS-treated cells.

Determination of PGE$_2$ production

Prostaglandins play an important role in regulating inflammatory responses [19]. Cultured RAW-264.7 cells (1×10^5 cells/mL) were treated with measured concentrations of polysaccharides, and after a 1 h incubation, the cells were stimulated with LPS (1 µg/mL). After 24 h of incubation, the PGE$_2$ concentration in the supernatant was quantified by using a competitive enzyme immunoassay kit, according to the manufacturer's instruction.

Measurement of TNF-α and IL-1β production

RAW 264.7 cells (1×10^5 cells/well) were pretreated with CCP (25 µg/mL, 50 µg/mL, and 100 µg/mL) for 1 h and then incubated with LPS (1 µg/mL) for 24 h. The production levels of TNF-α and IL-1β in macrophage cytoplasm were quantified using ELISA kits, according to the manufacturer's instructions.

Western blot analysis

To determine the effect of CCP on the protein expression levels of iNOS, COX-2, NF-κB, and MAPK in LPS-stimulated RAW 264.7 cells, western blot analysis was performed. Briefly, RAW 264.7 cells (1×10^5 cells/mL) were seeded in 6-well plates and incubated for 24 h. The cells were treated with CCP (25 µg/mL, 50 µg/mL and 100 µg/mL) for 1 h. And then, the cells were stimulated with LPS (1 µg/mL) for 10 min or 20 min and 24 h. Nucleic and cytosolic proteins were extracted from the cells with the NE-PER® Nuclear and Cytoplasmic extraction kit (Thermo scientific, Rockford, USA).

After separation on a 10% SDS-polyacrylamide gel under denaturing conditions, the cytoplasmic proteins (40 µg) were electrotransferred onto a nitrocellulose membrane. After blocking with 5% nonfat milk for 1 h, the blots were separately incubated with the following primary antibodies: rabbit polyclonal antibodies (iNOS, p44/42 (ERK, extracellular signal-regulated kinase), phosphorylated p44/42 (ERK), p38, phosphorylated p38, NF-κB p65, NF-κB p50, and nucleolin), and mouse monoclonal antibodies (COX-2, and β-actin) (Cell Signaling Technology, Beverly, MA, USA) for 1 h. The blots were washed twice with Tween 20/Tris-buffered saline (TTBS) and then incubated with HRP-conjugated anti-mouse or anti-rabbit IgG for 45 min. Antibody binding was visualized by using enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, IL, USA). The basal levels of the each proteins were normalized by analyzing the level of β-actin or nucleolin protein by using ImageJ program.

Statistical analysis

All the data were expressed as the mean ± standard of three determinations. The collected data were analyzed by analysis of variance using the SPSS v20 statistical analysis package. The mean values of each experiment were compared using one-way analysis of variance. Duncan's multiple range test (DMRT) was used to determine mean separation. A *P*-value < 0.05 was considered to be statistically significant.

RESULTS

Extraction yields and general components.

First of all, we prepared the four kinds of enzymatic extracts from *S. honeri* and checked their extraction yields. The results showed the extraction yields of enzymatic digests (AMG digest; 16.00 ± 0.50%, Celluclast digest; 20.17 ± 0.76%, Viscozyme digest; 21.0 ± 1.00%, and Alcalase digest; 22.17 ± 0.76%) were significantly increased, compared to that of the DW extract (9.50 ± 0.87%).

Next, we isolated crude polysaccharides from the enzymatic digests (CPs) and analyzed their isolation yields and proximate composition. Among the CPs, CCP led to the highest isolation yield (88.7%), compared to the others (AMGCP, VCP, and AICP). As indicated in Table 1, the four kinds of CPs contained the

Table 1. General components of crude polysaccharide

Samples	Polysaccharide (g/100 g)	Protein (g/100 g)	Polyphenol (g/100 g)	Sulfate content (g/100 g)
AMGCP	71.63 ± 2.17[a]	8.80 ± 0.82[b]	4.63 ± 0.30[c]	11.51 ± 0.07[b]
CCP	88.7 ± 2.44[b]	4.01 ± 1.06[a]	3.90 ± 0.15[b]	12.01 ± 0.98[b]
VCP	84.68 ± 1.63[b]	11.45 ± 0.00[c]	3.68 ± 0.15[a,b]	11.34 ± 0.18[b]
AlCP	81.25 ± 2.44[b]	15.77 ± 0.57[d]	3.37 ± 0.00[a]	2.20 ± 0.07[a]

All the values are mean ± SD (n = 3). Different letters in the same column mean significant differences between samples at *P* < 0.05 level by Duncan's multiple range test. AlCP, crude polysaccharides from alcalase enzymatic digestion; AMGCP, crude polysaccharides from AMG enzymatic digestion; CCP, crude polysaccharides from celluclast enzymatic digestion; VCP, crude polysaccharides from viscozyme enzymatic digestion.

major polysaccharide contents and minor protein and polyphenolic contents. Especially, CCP showed the highest polysaccharide and sulfate content (88.7 ± 2.44% and 12.01 ± 0.98%, respectively). From these results, we indicate that the isolated CCPs might be sulfated polysaccharides due to its plentiful polysaccharide and sulfate group contents.

FT-IR spectrums of CPs

FT-IR spectroscopy is a useful analytical approach to identify the vibrations between the different atoms in molecules. The spectra obtained between 400-4,000 cm^{-1} can be used to analyze the structural features of polysaccharides, including glucosidic bonds and functional groups [20,21]. In the present study, the FT-IR spectra of the four CPs were compared to that of a fucoidan purchased from Sigma-Aldrich. Interestingly, CCP had an IR spectrum similar to that of the commercial fucoidan (Fig. 1). In contrast, AlCP showed a completely different pattern than that of the other CPs and the commercial fucoidan. It has been reported that strong absorption at 840 cm^{-1} (a sulfate group at axial C-4) and shoulder absorption at 820 cm^{-1} (a sulfate group at C-2) are unique to native fucoidans. In addition, absorption at 1,240 cm^{-1} represents the sulfate ester groups found in commercial fucoidan [22-24]. Moreover, the bands developed at 1,646-1,652 cm^{-1} were due to the bending vibrations of HOH and the broad bands centered at 2920-2940 cm^{-1} were assigned to C-H stretching vibrations. In addition, the bands in the region between 3,400-3,410 cm^{-1} are developed due to the stretching vibration of O-H which indicated hydroxyl
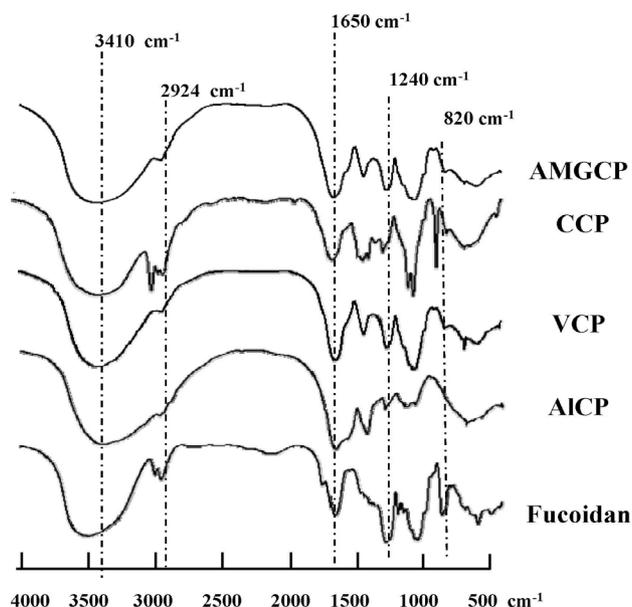

Fig. 1. FT-IR spectra of crude polysaccharides extracted from the brown seaweed *S. horneri* and a commercial fucoidan. AMGCP, crude polysaccharides from AMG enzymatic digestion; CCP, crude polysaccharides from Celluclast enzymatic digestion; VCP, crude polysaccharides from Viscozyme enzymatic digestion; AlCP, crude polysaccharides from Alcalase enzymatic digestion.

groups existed in the fucoidan [25,26].

CCP showed the highest inhibitory effect on the NO production in LPS-stimulated RAW 264.7 cells without cytotoxicity

Cell viability experiments showed no cytotoxicity in RAW 264.7 cells for any of the CPs, with more than 90% viability (Fig. 2A). In addition, 100 μg/mL of AMGCP, CCP, VCP, and AlCP inhibited the NO production of RAW 264.7 cells induced by LPS stimulation (Fig. 2B). In particular, among the CPs, CCP had the highest inhibitory effect on the NO production in LPS-stimulated RAW 264.7 cells, with an IC$_{50}$ value of 95.7 μg/mL and it was dose-dependent. Our results indicated that CCP has the inhibitory effect on the NO production in LPS-stimulated RAW 264.7 cells, without cytotoxicity. Therefore, CCP was used for all subsequent experiments.
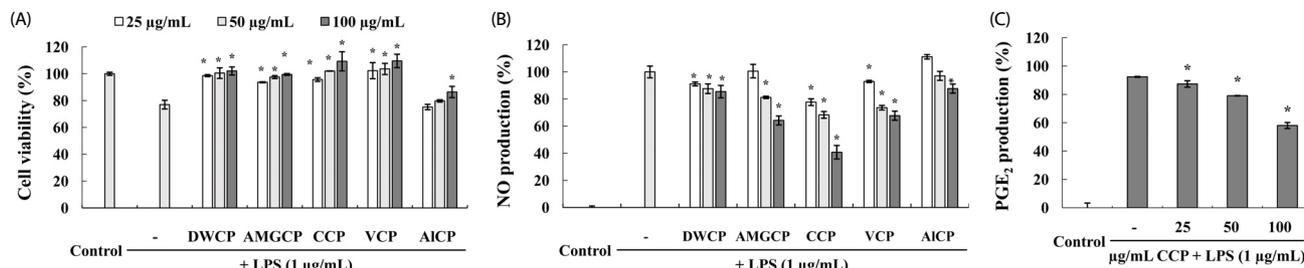

Fig. 2. (A) Cytotoxicity of crude polysaccharides (CP) on RAW 264.7 cells in the presence of LPS. The viability of cells without sample and LPS has been taken as reference (100%). (B) Dose-dependent inhibition of NO production by CPs in LPS-stimulated RAW264.7 macrophages. The level of NO production is expressed as percentages of that of the group treated with LPS alone. (C) The inhibitory effects of the crude polysaccharides from the Celluclast enzyme digest (CCP) on the production of PGE$_2$ in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1 × 10^5) were stimulated with LPS (1 μg/mL) for 24 h with or without CCP. Supernatants were collected and levels of PGE$_2$ in the culture supernatant were determined by ELISA according to the manufacturer's instructions. Data points and bars represent the arithmetic means ± SD (n = 3). Each data point represents the mean ± SD (* *P* < 0.05 by Duncan's multiple range test). DWCP, crude polysaccharide from distilled water digestion; AMGCP, crude polysaccharides from AMG enzymatic digestion; CCP, crude polysaccharides from celluclast enzymatic digestion; VCP, crude polysaccharides from viscozyme enzymatic digestion; AlCP, crude polysaccharides from alcalase enzymatic digestion.
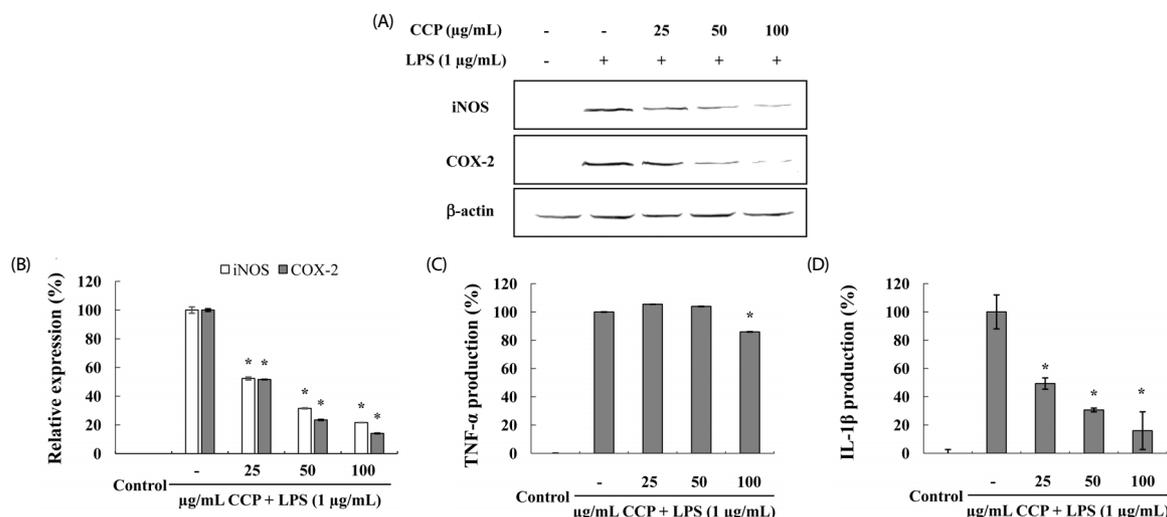
Fig. 3. Effects of crude polysaccharides from the Celluclast enzyme digest (CCP) on LPS-induced iNOS and COX-2 protein expression in RAW 264.7 cells. Cells (1 × 10⁵ cells/mL) were treated with indicated concentrations of CCP (25, 50, or 100 μg/mL) for 1 h before LPS (1 μg/mL) treatment for 24 h. Cell lysates (40 μg) were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against iNOS and COX-2. (A) The proteins were then visualized by ECL. (B) The intensity of the bands was measured by ImageJ software. Relative amounts of iNOS and COX-2 compared to β-actin and the density ratio represented the relative intensity of each band against that of the β-actin. The gel shown is a representative of the results from three separate experiments. The inhibitory effect of Effects of the crude polysaccharides from the CCP on the production of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (1 × 10⁵) were stimulated with LPS (1 μg/mL) for 24 h with or without CCP. Supernatants were collected, and TNF-α (C) and IL-1β (D) levels in the culture supernatant were determined by ELISA according to the manufacturer's instructions. Data points and bars represent the arithmetic means ± SD (n = 3). Each data point represents the mean ± SEM (* *P* < 0.05).
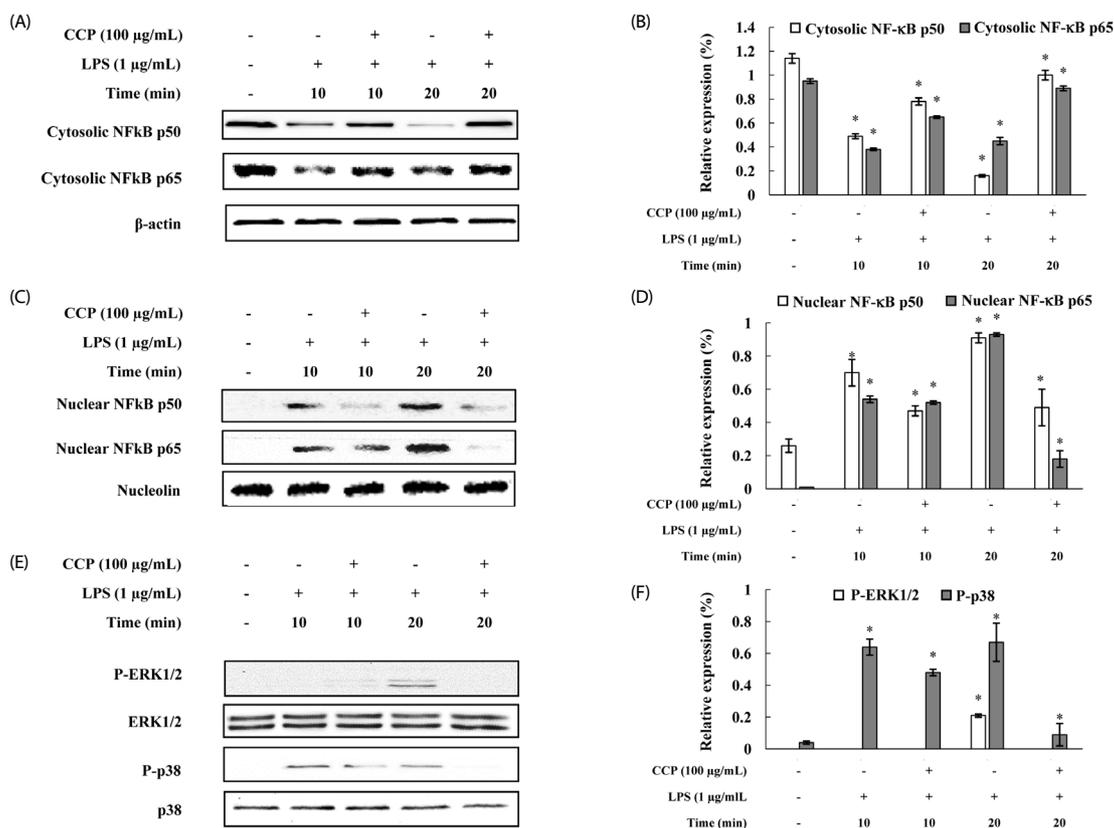

Fig. 4. Inhibitory effects of the crude polysaccharides from the Celluclast enzyme digest (CCP) on NF-κB and MAPK activation in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with LPS (1 μg/mL) or CCP (100 μg/mL) for 10 or 20 min. (A) and (B) Cytosolic cell lysates, (C) and (D) nuclear protein extracts (40 μg) were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against NF-κB p50 and p65. The proteins were then visualized by ECL. (E) and (F) Effects of CCP on MAPK activation induced by LPS in RAW 264.7 cells. RAW 264.7 cells were treated with LPS (1 μg/mL) or CCP (100 μg/mL) for 10 or 20 min. Total protein (40 μg) was separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against ERK and p38. The proteins were then visualized by ECL. The intensity of the bands was measured by ImageJ software. Relative amounts of density ratio represented the relative intensity of each band against that of the standard protein. The results shown are representative of those obtained from three independent experiments. Each data point represents the mean ± SEM (* *P* < 0.05).

CCP dose-dependently reduced the secretion of PGE₂ from LPS-stimulated RAW 264.7 cells

Next, we evaluated effect of CCP on PGE_2 production in LPS-stimulated RAW 264.7 cells. As shown in Fig. 2C, the LPS stimulation induced the PGE_2 production in RAW 264.7 cells, whereas it was dose-dependently inhibited by the treatment of CCP.

CCP reduced the expression levels of iNOS and COX-2 proteins in LPS-stimulated RAW 264.7 cells

The suppressive effect of CCP on the protein expression of iNOS and COX-2 was measured by western blot analysis. As shown in Fig. 3A and 3B, CCP dose-dependently down-regulated the expression levels of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells, compared to the levels in the only LPS-treated cells.

CCP reduced the secretion of pro-inflammatory cytokines from LPS-stimulated RAW 264.7 cells

It is a well-established fact that the overproduction of pro-inflammatory cytokines, including TNF-α and IL-1β, has an important role in the pathogenesis of various inflammatory diseases [27-29]. Therefore, compounds with inhibitory properties against TNF-α and IL-1β are considered anti-inflammatory agents. To examine the potential anti-inflammatory effects of CCP in LPS-stimulated RAW 264.7 cells, the levels of inflammatory cytokines, such as TNF-α and IL-1β, in culture supernatants were measured by ELISA. The results clearly demonstrated that the secretion of TNF-α and IL-1β was significantly increased by stimulation with LPS (Fig. 3C, 3D). Interestingly, the secretion of pro-inflammatory cytokines was inhibited by treatment with CCP in a dose-dependent manner.

CCP inhibited the NF-κB and MAPK signaling in LPS-stimulated RAW 264.7 cells

Using western blot analysis, we investigated the effect of CCP on NF-κB and MAPK activities in LPS-stimulated RAW 264.7 cells. As shown in Fig. 4A and 4B, the results demonstrated that the cytosolic NF-κB p50 and p65 protein levels were reduced by the stimulation of LPS in RAW 264.7 cells, compared to the non-treated control cells. However, the treatment of CCP (100 µg/mL) inhibited the reduction of cytosolic p50 and p65 protein levels in LPS-stimulated RAW 264.7 cells. CCP also inhibited the translocation of p50 and p65 to the nucleus compared to the only LPS-stimulated RAW 264.7 cells (Fig. 4C and 4D). In further study, we examined the effect of CCP on MAPK signal pathway known as an upstream molecule. Interestingly, CCP down-regulated the phosphorylation of p38 and ERK increased by LPS stimulation in RAW 264.7 cells (Fig. 4E and 4F). From these results, we suggest that CCP has anti-inflammatory effects via inhibiting the activation of MAPK and NF-κB signaling by down-regulating the phosphorylation of p38 and ERK and the translocation of NF-κB p50 and p65 into nucleus.

DISCUSSION

EEM is a popular green extraction method due to the increased extraction efficiency compared to water extraction and nontoxic nature compared to the organic solvent extraction methods. Thus, compounds from EEM are cheaper and safer to use as material in functional foods, cosmeceuticals or nutraceuticals [7]. According to the extraction data, we also observed that EEM techniques significantly increased the extraction efficiency without alerting cell viability and showed better NO inhibition in LPS-stimulated RAW 264.7 cells in tested concentrations compared to water extraction (data not shown).

Numerous anti-inflammatory polysaccharides have been isolated from marine brown algae and their role as anti-inflammatory agents has been well documented [30]. Fucoidan is a natural sulfated polysaccharide found in brown algae. Recently, a number of studies demonstrated that the anti-inflammatory activity of fucoidan occurred by suppressing the activation of NF-κB and MAPK pathways [31,32]. However, the anti-inflammatory potential of sulfate-rich polysaccharides separated from *S. horneri* via enzymatic digestion has not yet been reported. Therefore, in the present study, we evaluated the anti-inflammatory activities of sulfate-rich CPs separated from the enzymatic digests of *S. horneri* on LPS-stimulated RAW 264.7 cells. Interestingly, the crude sulfated polysaccharides separated from the enzymatic digests exhibited the higher NO inhibitory activities than the CP isolated from DW extract. Based on these results, our results indicate that the enzyme extraction technique improved the extraction yields and the polysaccharide contents as well as the inhibition of NO production in LPS-stimulated macrophages. Our results were also similar to the suggestions of Wijesinghe *et al.* [7] that CP obtained from enzymatic digestions had better performance and high polysaccharide yield compared to the CPs obtained from DW extract.

During inflammatory responses, the expression of iNOS and COX-2 in macrophages leads to the production of NO and PGE_2. Additionally, NO and PGE_2 produced during inflammation can be cytotoxic to the host cells, and prolonged production of NO and PGE_2 in inflammatory cells can cause a range of inflammatory diseases and cancers [33,34]. Moreover, recent studies have shown the exposure of macrophages into inflammatory stimulant can cause significant up-regulation of pro-inflammatory cytokines such as IL-1β and TNF-α [35]. Finally, the excessive production of pro-inflammatory cytokines by activated macrophages play an important role in pathogenesis inflammatory diseases such as rheumatoid arthritis, Alzheimer's disease, and Parkinson's disease as well as inflammatory mediators [36]. Therefore, reducing levels of these mediators might be an effective strategy for treat inflammatory diseases [37]. Indeed, previous studies have demonstrated that sulfated polysaccharides including fucoidan derived from *E. cava* and *Fucus vesiculosus* inhibited the expression of inflammatory mediators such as iNOS and COX-2 in activated RAW 264.7 cells and BV2 microglia [31]. In this study, we revealed that CCP decreased the NO and PGE_2 productions via reducing the expression levels of inflammatory mediators such as iNOS and COX2 as well as the production of inflammatory cytokines such as TNF-α and IL-1β. These results suggest that the inhibitory effect of CCP on the inflammatory mediators and cytokines is one of the mechanisms responsible for its anti-inflammatory activities and CCP might have potential to develop as a functional food ingredient or as a therapeutic agent to treat

inflammatory diseases.

Normally, LPS stimulation leads to inflammation via activation of the NF-κB and MAPK signaling pathway [31,38]. The NF-κB transcription factor family comprises five members, namely c-Rel, RelB, p50, p52, and p65. NF-κB is an important transcription factor involved in inflammatory responses. NF-κB is normally sequestered in the cytosol and is activated by inflammatory stimulants, such as LPS [31]. After stimulation of macrophages, the cytosolic NF-κB proteins are transported into the nucleus where they induce the transcription of pro-inflammatory genes, including iNOS and COX-2, as well as the genes encoding pro-inflammatory cytokines [31]. Furthermore, altered NF-κB activation is caused by deregulated, and often constitutive, NF-κB phosphorylation, which is major contributor of the pathogenesis of chronic inflammatory diseases and cancer. Therefore, inhibition of NF-κB signaling cascade related proteins found to be a promising target for treatment of inflammation associated diseases [39]. Interestingly, our results indicated that CCP suppressed the activation of NF-κB signaling via the inhibiting the phosphorylation of NF-κB as well as its translocation to the nucleus in the LPS-stimulated RAW 264.7 cells.

Exposure of RAW 264.7 cells into the LPS can activate the MAPK signaling cascade associated proteins such as ERK, p38, and JNK [40]. Moreover, a number of studies have reported that the MAPK signaling pathway regulates the activation of NF-κB [41,42]. In addition, studies have demonstrated that the phosphorylation of p38 kinase and ERK1/2 are involved in the transcriptional regulation of pro-inflammatory cytokines such as IL-1β and TNF-α [43,44]. Thus, inhibition of MAPK related protein expression is also considered a useful approach to treating inflammatory diseases as MAPK signaling cascade capable of regulating NF-κB and pro-inflammatory cytokine production [41,42,45]. Our results also demonstrated that CCP inhibited the phosphorylation of ERK1/2 and p38. Recently, many researchers have reported that various polysaccharides, including fucoidan derived from seaweeds, exert anti-inflammatory effects by inhibiting activation of the NF-κB and MAPK signaling pathway in LPS-stimulated mouse macrophages [30,46,47]. With these results, we demonstrated that the anti-inflammatory capacity of CCP, a sulfated polysaccharide via the inhibition of NF-κB signaling may be due to its inhibitory effect on the MAPK signaling.

In conclusion, this study suggests that CCP led to the anti-inflammatory effects by blocking the activation of the NF-κB and MAPK signaling pathway. Based on these findings, we conclude that CCP is a potential candidate for the formulation of a functional food ingredient or/and drug to treat inflammatory diseases.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interests.

REFERENCES

1. Nathan C. Nitric oxide as a secretory product of mammalian cells. FASEB J 1992;6:3051-64.
2. Knöferl MW, Diodato MD, Schwacha MG, Cioffi WG, Bland KI, Chaudry IH. Cyclooxygenase-2-mediated regulation of Kupffer cell interleukin-6 production following trauma-hemorrhage and subsequent sepsis. Shock 2001;16:479-83.
3. Louis E, Franchimont D, Piron A, Gevaert Y, Schaaf-Lafontaine N, Roland S, Mahieu P, Malaise M, De Groote D, Louis R, Belaiche J. Tumour necrosis factor (TNF) gene polymorphism influences TNF-α production in lipopolysaccharide (LPS)-stimulated whole blood cell culture in healthy humans. Clin Exp Immunol 1998;113:401-6.
4. Simmons TL, Andrianasolo E, McPhail K, Flatt P, Gerwick WH. Marine natural products as anticancer drugs. Mol Cancer Ther 2005;4: 333-42.
5. Sanjeewa KK, Kim EA, Son KT, Jeon YJ. Bioactive properties and potentials cosmeceutical applications of phlorotannins isolated from brown seaweeds: a review. J Photochem Photobiol B 2016; 162:100-5.
6. Wijesekara I, Pangestuti R, Kim SK. Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. Carbohydr Polym 2011;84:14-21.
7. Wijesinghe WA, Jeon YJ. Enzyme-assistant extraction (EAE) of bioactive components: a useful approach for recovery of industrially important metabolites from seaweeds: a review. Fitoterapia 2012;83:6-12.
8. Heo SJ, Jeon YJ, Lee JH, Kim HT, Lee KW. Antioxidant effect of enzymatic hydrolyzate from a Kelp, Ecklonia cava. Algae 2003;18: 341-7.
9. Pang SJ, Liu F, Shan TF, Gao SQ, Zhang ZH. Cultivation of the brown alga Sargassum horneri: sexual reproduction and seedling production in tank culture under reduced solar irradiance in ambient temperature. J Appl Phycol 2009;21:413-22.
10. Liu L, Heinrich M, Myers S, Dworjanyn SA. Towards a better understanding of medicinal uses of the brown seaweed Sargassum in traditional Chinese medicine: a phytochemical and pharmacological review. J Ethnopharmacol 2012;142:591-619.
11. Preeprame S, Hayashi K, Lee JB, Sankawa U, Hayashi T. A novel antivirally active fucan sulfate derived from an edible brown alga, Sargassum horneri. Chem Pharm Bull (Tokyo) 2001;49:484-5.
12. Shao P, Chen X, Sun P. Chemical characterization, antioxidant and antitumor activity of sulfated polysaccharide from Sargassum horneri. Carbohydr Polym 2014;105:260-9.
13. Shao P, Chen X, Sun P. Improvement of antioxidant and moisture-preserving activities of Sargassum horneri polysaccharide enzymatic hydrolyzates. Int J Biol Macromol 2015;74:420-7.
14. DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350-6.
15. Chandler SF, Dodds JH. The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasodine in callus cultures of solanum laciniatum. Plant Cell Rep 1983;2:205-8.
16. Saito H, Yamagata T, Suzuki S. Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. J Biol Chem 1968;243:1536-42.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
18. Leiro J, Alvarez E, García D, Orallo F. Resveratrol modulates rat macrophage functions. Int Immunopharmacol 2002;2:767-74.
19. Park JW, Choi YJ, Suh SI, Kwon TK. Involvement of ERK and protein tyrosine phosphatase signaling pathways in EGCG-induced cycloox-

ygenase-2 expression in Raw 264.7 cells. Biochem Biophys Res Commun 2001;286:721-5.

20. Yang L, Zhang LM. Chemical structural and chain conformational characterization of some bioactive polysaccharides isolated from natural sources. Carbohydr Polym 2009;76:349-61.

21. Patankar MS, Oehninger S, Barnett T, Williams RL, Clark GF. A revised structure for fucoidan may explain some of its biological activities. J Biol Chem 1993;268:21770-6.

22. Bernardi G, Springer GF. Properties of highly purified fucan. J Biol Chem 1962;237:75-80.

23. Qiu X, Amarasekara A, Doctor V. Effect of oversulfation on the chemical and biological properties of fucoidan. Carbohydr Polym 2006;63:224-8.

24. Wijesinghe WA, Athukorala Y, Jeon YJ. Effect of anticoagulative sulfated polysaccharide purified from enzyme-assistant extract of a brown seaweed Ecklonia cava on Wistar rats. Carbohydr Polym 2011;86:917-21.

25. Gómez-Ordóñez E, Rupérez P. FTIR-ATR spectroscopy as a tool for polysaccharide identification in edible brown and red seaweeds. Food Hydrocoll 2011;25:1514-20.

26. Chen Y, Mao W, Gao Y, Teng X, Zhu W, Chen Y, Zhao C, Li N, Wang C, Yan M, Shan J, Lin C, Guo T. Structural elucidation of an extracellular polysaccharide produced by the marine fungus Aspergillus versicolor. Carbohydr Polym 2013;93:478-83.

27. Bertolini A, Ottani A, Sandrini M. Dual acting anti-inflammatory drugs: a reappraisal. Pharmacol Res 2001;44:437-50.

28. Tilg H, Wilmer A, Vogel W, Herold M, Nölchen B, Judmaier G, Huber C. Serum levels of cytokines in chronic liver diseases. Gastroenterology 1992;103:264-74.

29. Sestini P, Armetti L, Gambaro G, Pieroni MG, Refini RM, Sala A, Vaghi A, Folco GC, Bianco S, Robuschi M. Inhaled PGE2 prevents aspirin-induced bronchoconstriction and urinary LTE4 excretion in aspirin-sensitive asthma. Am J Respir Crit Care Med 1996;153:572-5.

30. Wijesinghe WA, Jeon YJ. Biological activities and potential industrial applications of fucose rich sulfated polysaccharides and fucoidans isolated from brown seaweeds: a review. Carbohydr Polym 2012;88:13-20.

31. Park HY, Han MH, Park C, Jin CY, Kim GY, Choi IW, Kim ND, Nam TJ, Kwon TK, Choi YH. Anti-inflammatory effects of fucoidan through inhibition of NF-κB, MAPK and Akt activation in lipopolysaccharide-induced BV2 microglia cells. Food Chem Toxicol 2011;49:1745-52.

32. Wu GJ, Shiu SM, Hsieh MC, Tsai GJ. Anti-inflammatory activity of a sulfated polysaccharide from the brown alga Sargassum cristaefolium. Food Hydrocoll 2016;53:16-23.

33. Posadas I, Terencio MC, Guillén I, Ferrándiz ML, Coloma J, Payá M, Alcaraz MJ. Co-regulation between cyclo-oxygenase-2 and inducible nitric oxide synthase expression in the time-course of murine inflammation. Naunyn Schmiedebergs Arch Pharmacol 2000;361:98-106.

34. Attur MG, Patel R, Thakker G, Vyas P, Levartovsky D, Patel P, Naqvi S, Raza R, Patel K, Abramson D, Bruno G, Abramson SB, Amin AR.
Differential anti-inflammatory effects of immunosuppressive drugs: cyclosporin, rapamycin and FK-506 on inducible nitric oxide synthase, nitric oxide, cyclooxygenase-2 and PGE2 production. Inflamm Res 2000;49:20-6.

35. Heo SJ, Yoon WJ, Kim KN, Ahn GN, Kang SM, Kang DH, Affan A, Oh C, Jung WK, Jeon YJ. Evaluation of anti-inflammatory effect of fucoxanthin isolated from brown algae in lipopolysaccharide-stimulated RAW 264.7 macrophages. Food Chem Toxicol 2010;48:2045-51.

36. Tews DS, Goebel HH. Cytokine expression profile in idiopathic inflammatory myopathies. J Neuropathol Exp Neurol 1996;55:342-7.

37. Jung WK, Ahn YW, Lee SH, Choi YH, Kim SK, Yea SS, Choi I, Park SG, Seo SK, Lee SW, Choi IW. Ecklonia cava ethanolic extracts inhibit lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in BV2 microglia via the MAP kinase and NF-κB pathways. Food Chem Toxicol 2009;47:410-7.

38. Kang JS, Yoon YD, Lee KH, Park SK, Kim HM. Costunolide inhibits interleukin-1β expression by down-regulation of AP-1 and MAPK activity in LPS-stimulated RAW 264.7 cells. Biochem Biophys Res Commun 2004;313:171-7.

39. Viatour P, Merville MP, Bours V, Chariot A. Phosphorylation of NF-κB and IκB proteins: implications in cancer and inflammation. Trends Biochem Sci 2005;30:43-52.

40. Xiao YQ, Malcolm K, Worthen GS, Gardai S, Schiemann WP, Fadok VA, Bratton DL, Henson PM. Cross-talk between ERK and p38 MAPK mediates selective suppression of pro-inflammatory cytokines by transforming growth factor-β. J Biol Chem 2002;277:14884-93.

41. Aga M, Watters JJ, Pfeiffer ZA, Wiepz GJ, Sommer JA, Bertics PJ. Evidence for nucleotide receptor modulation of cross talk between MAP kinase and NF-κB signaling pathways in murine RAW 264.7 macrophages. Am J Physiol Cell Physiol 2004;286:C923-30.

42. DeFranco AL, Hambleton J, McMahon M, Weinstein SL. Examination of the role of MAP kinase in the response of macrophages to lipopolysaccharide. Prog Clin Biol Res 1995;392:407-20.

43. Mahtani KR, Brook M, Dean JL, Sully G, Saklatvala J, Clark AR. Mitogen-activated protein kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability. Mol Cell Biol 2001;21:6461-9.

44. Koistinaho M, Koistinaho J. Role of p38 and p44/42 mitogen-activated protein kinases in microglia. Glia 2002;40:175-83.

45. Tegeder I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J 2001;15:2057-72.

46. Yang JW, Yoon SY, Oh SJ, Kim SK, Kang KW. Bifunctional effects of fucoidan on the expression of inducible nitric oxide synthase. Biochem Biophys Res Commun 2006;346:345-50.

47. Ale MT, Mikkelsen JD, Meyer AS. Important determinants for fucoidan bioactivity: a critical review of structure-function relations and extraction methods for fucose-containing sulfated polysaccharides from brown seaweeds. Mar Drugs 2011;9:2106-30.