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Study on the Antiinflammatory Activity of Methanol Extract from Seagrass *Zostera japonica*

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Methanolic extracts from the seagrass *Zostera japonica* were extracted successively using *n*-hexane (*n*-C_6H_{14}), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and water to give the *n*-C_6H_{14} (16.8%), CH_2Cl_2 (40.6%), EtOAc (34.1%), and H_2O (8.5%) soluble fractions, respectively. We have demonstrated that the hexane fraction has the highest capacity to inhibit proIL-1β expression as compared to other fractions in lipopolysaccharides (LPS)-stimulated J774A.1 murine macrophages. Further analysis of the composition and antiinflammatory activity of the subfraction H5 from hexane fraction showed that it had the best antiinflammatory capacity and that it's major constituents were fatty acids, including palmitic acid methyl ester (21.5%), palmitic acid (24.02%), linoleic acid methyl ester (13.09%), oleic acid methyl ester (8.41%), and linoleic acid (7.93%), respectively. H5 inhibited LPS-induced TNFα, IL-1β, and IL-6 in a dose-dependent manner, suggesting that H5 is bioactive in antiinflammation in vitro. This study is the first to report the antiinflammatory activity of extracts obtained from the seagrass *Z. japonica*.

KEYWORDS: ***Zostera japonica*; extract; seagrass; antiinflammatory capacity; fatty acid; macrophage**

INTRODUCTION

The *Zostera* spp. are widely distributed seagrasses that inhabit intertidal zones in the northern hemisphere (*1*−*4*). In recent decades, seagrasses have played an important role in helping to estimate the ecological and economic value of various ecosystems; yet, only a very few studies have investigated the utility of seagrass bioactivity. One report (*5*) demonstrated a toxicity against larvae of the mosquito *Culex quinquefasciatus*, and another (*6*) demonstrated strong antibacterial properties. Although marine seagrasses are abundant, their bioactivity has been rarely considered. Lipopolysaccharides (LPS) or endotoxin is one of the cell wall components of Gram-negative bacteria (*7*). LPS is antigenic and able to activate monocytes/macrophages to secrete various inflammatory cytokines, including TNFα, IL-1β, and IL-6 protein (*8*−*12*). While mediation of inflammation against pathogen infection by these inflammatory cytokines could be beneficial to the host, overexpression of these cytokines may cause serious disease, including septic shock. Hence, suppression of TNFα, IL-1β, and IL-6 production could aid in the treatment of septic shock.

Zostera japonica is an abundant seagrass species along the Penghu coast in Taiwan but is an underutilized marine resource. To explore its potential as a source of natural drugs, this study investigated the effective composition and antiinflammatory capacity of the methanolic extract (ME) from the seagrass of *Z. japonica* by using a model of murine macrophage J774A.1 cells. This investigation included producing a ME from *Z. japonica*, the separation and chromatography of this extract, and an analysis of the chemical composition and bioactivity. This is the first report to demonstrate that the extract of *Z. japonica* has antiinflammatory activity in macrophages. However, if it is to be used as a source of natural drugs in the future, the efficacy and safety of the extract need to be further investigated.

MATERIALS AND METHODS

Materials. The samples of *Z. japonica* were collected from the Penghu coast. Murine macrophage J774A.1 cells were obtained from ATCC (Rockville, MD), and LPS (from *Escherichia coli* 0111:B4) was obtained from Sigma Co. The mouse IL-1β, IL-6, and TNFα enzyme-linked immunosorbent assay Kit were purchased from R & D Systems, Inc. (Minneapolis, MN). Anti-IL-1β polyclonal antibody, anti-rabbit IgG-HRP, and anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Linoleic acid methyl ester, linoleic acid, oleic acid methyl ester, and testosterone were purchased from Acros Organics (PA). The other chemicals and reagents used in this study were of the highest grade commercially available.

Extraction and Fraction. The samples of *Z. japonica* were collected from the Penghu coast. These specimens were rinsed thoroughly and

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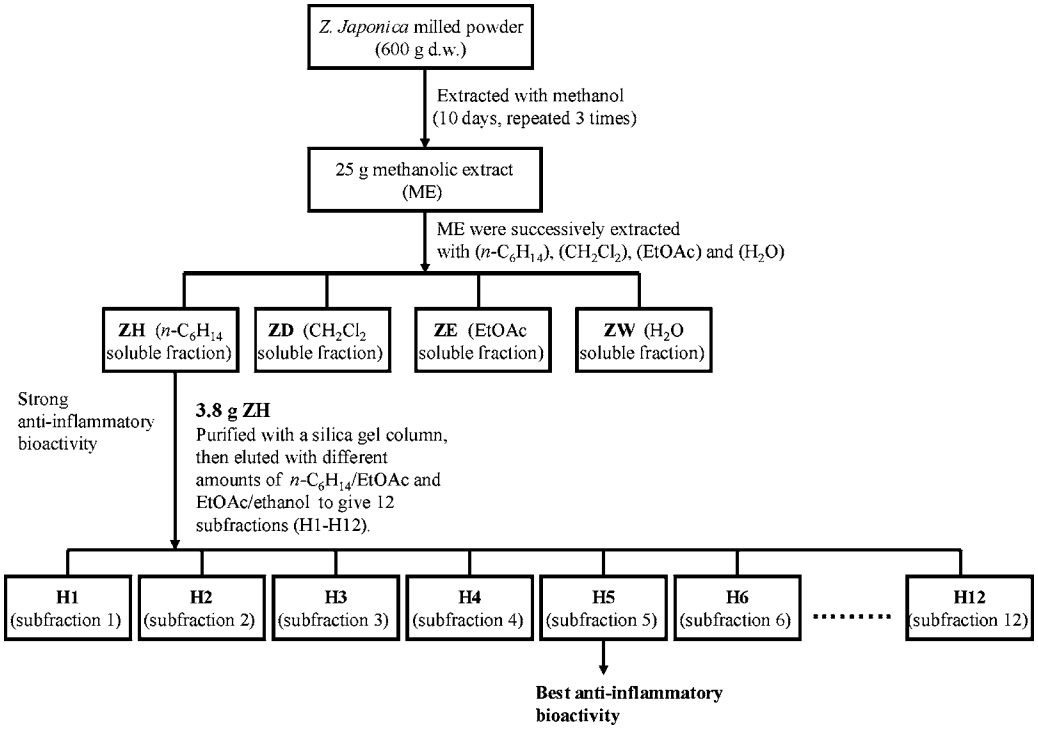

Figure 1. Preparation scheme of ME from seagrass *Z. japonica*.

air-dried before being milled. The powdered *Z. japonica* (600 g d.w.) was extracted with methanol (10 days, repeated three times) at room temperature. The extract was concentrated to give a ME of ca. 25 g. To yield soluble fractions of the hexane (n-C_6H_{14}), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and water (H_2O), 20 g of ME was successively liquid−liquid partitioned with hexane, CH_2Cl_2, EtOAc, and H_2O (each solvent volume was 300 mL and was repeated three times). The collected soluble fractions of the hexane, CH_2Cl_2, EtOAc, and H_2O and the subfractions of hexane were then dried, which then yielded about 3.8 g of hexane soluble fraction. The hexane fraction was applied on the top of a 60 g silica gel column (120 cm in length, 1.8 cm i.d.), then eluted with hexane/EtOAc (95/5, 85/15, 70/30, 50/50, 20/80, 0/100) followed by elution with EtOAc/ethanol (100/0, 80/20, 70/30, 60/40, 50/50, 20/80, 0/100) to give 12 subfractions (H1−H12). Each eluted volume of subfraction was 200 mL, except for the H1 subfraction, which was 400 mL, and the flow rate was 20 mL/min. Finally, we got about 487 mg from subfraction H5. All of the sample preparation procedures are shown in **Figure 1**.

Gas Chromatography−Mass Spectrometry (GC-MS) Analysis. The sampling of the subfractions was performed by GC-MS (HP 6890N, with MS detector model HP 5973) and using a DB-5HT column, 15 m in length, 0.25 mm i.d., 0.1 μm film. The oven temperature was increased from 85 to 275 °C at a rate of 10 °C min^{-1}. The injection temperature was 300 °C, the detector temperature was 280 °C, and helium was used as a carrier gas at a split ratio of 20:1. The compounds were identified by comparison of their mass spectrometric fragmentation patterns with those of authentic standards.

Cell Culture. J774A.1 cells (1×10^6/mL) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Co., Logan, UT) and 2 mM L-glutamine (Life Technologies, Inc., MD) in a 37 °C, 5% CO_2 incubator (the cultured medium volume was 2 mL), unless otherwise indicated. J774A.1 cells were pretreated with the extract (dissolved in dimethyl sulfoxide, DMSO) for 30 min, followed by LPS (1 μg/mL) treatment for an additional 6 h. The sample was dissolved in DMSO, and the final DMSO concentration was 0.1% in all cultures containing this agent; the same amount of vehicle (2 μL) was added to the control cultures. The ProIL-1β protein expression level was analyzed by Western blot using anti-IL-1β antibody as described by Hsu et al. (*13*).

Enzyme-Linked Immunosorbent Assay (ELISA). To investigate the inhibitory effect of the subfraction H5 on IL-1β, IL-6, and TNFα protein secretion from LPS-stimulated J774A.1 cells, cells were pretreated with subfraction H5 (5−50 μg/mL) for 30 min at 37 °C, followed by LPS (1 μg/mL) treatment for the indicated times, and the cell culture medium was collected. For assaying purposes, 50 μL of biotinylated antibodies reagent was added to anti-mouse TNFα, IL-1β, and IL-6 precoated stripwell plates, respectively, with 50 μL of supernatant concentrate from tested samples for various times and incubated at room temperature for 3−6 h. After the plate was washed three times with washing buffer provided in kit components, 100 μL of diluted streptavidin-HRP concentrate was added to each well and incubated at room temperature for 30 min. The washing process was repeated, and 100 μL of premixed TMB substrate solution was added to each well and developed at room temperature in the dark for 30 min. Following the addition of 100 μL of provided stop solution to each well to stop the reaction, the absorbance of the plate was measured by MRX microplate reader (Dynex Tech. Inc., VA) at 450−550 nm wavelengths. The calculation of the relative absorbance units and the IL-1β concentration for each sample, as well as the construction of the standard curve of recombinant mouse TNFα, IL-1β, and IL-6 calibration curves, were performed as described in the manual from R & D Systems.

Western Blot. To investigate the inhibitory effect of H5 on proIL-1β expression in LPS-stimulated J774A.1, the cells were pretreated with H5 (5−50 μg/mL) for 30 min at 37 °C, followed by LPS (1 μg/mL) treatment for 6 h. The reaction was stopped by the addition of ice-cold phosphate-buffered saline (PBS) containing 5 mM Na_3VO_4. Cells were immediately pelleted at 4 °C and lysed with 100 μL of ice-cold lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton, 205 mM pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 2 μg/mL leupeptin, and 1 mM PMSF) on ice for 10−15 min. Insoluble material was removed by centrifuging at 4 °C for 15 min at 12000*g*. The protein concentrations were determined using Bio-Rad protein assay. Samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene fluoride filter (Millipore Inc., Bedford, MA). Filters were incubated in blocking solution (5% nonfat milk in PBS with 0.1% Tween 20). These blocking steps were performed at room temperature for 1 h. Filters were incubated with primary anti-IL-1β antibody for 2 h. After they were washed three times in PBS with 0.1% Tween 20, filters were incubated with an HRP-conjugated secondary antibody directed against the primary antibody. Blots were developed by an

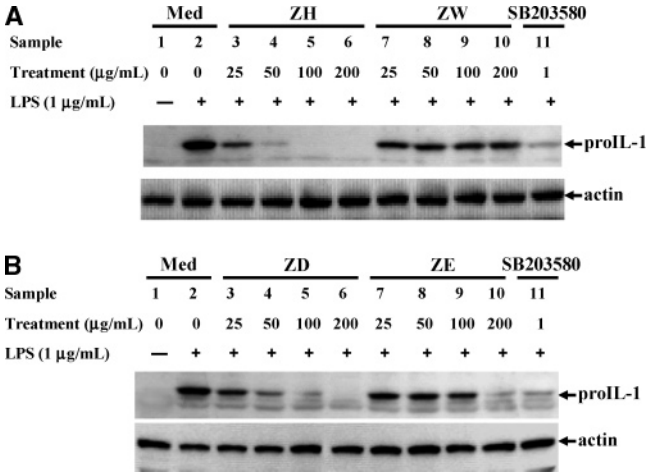

Figure 2. Effects of different fractions of ME from the seagrass of *Z. japonica* on proIL-1β protein expression in LPS-stimulated J774A.1 cells. Cells were pretreated with the indicated concentrations of fractions of sample for 30 min, followed by LPS (1 μg/mL) treatment for an additional 6 h. ProIL-1β and actin (as an internal control) expression were analyzed by Western blot. These experiments were repeated three times, and a representative result is shown. (**A**) ZH (hexane soluble fraction), ZW (water soluble fraction), Med (medium as negative control), and SB203580 (1 μM) as positive control. (**B**) ZD (dichloromethane soluble fraction), ZE (ethyl acetate soluble fraction), Med (medium as negative control), and SB203580 (1 μM) as positive control.

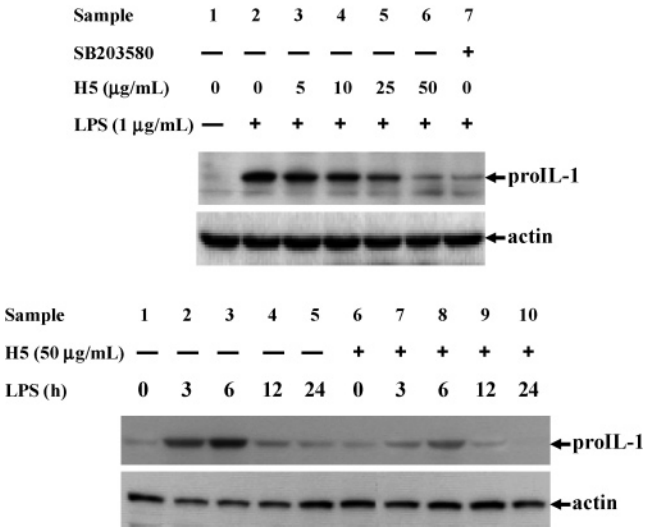

Figure 3. Effects of subfraction 5 (H5) of the hexane fraction on proIL-1β expression in LPS-stimulated J774A.1 cells. (**A**) Cells were pretreated with 5, 10, 25, and 50 μg/mL of H5, respectively, for 30 min, followed by LPS (1 μg/mL) treatment for an additional 6 h and SB203580 (1 μM) added as a positive control. ProIL-1β and actin (as an internal control) expression were analyzed by Western blot. These experiments were repeated three times, and a representative result is shown. (**B**) Cells were pretreated with or without H5 (50 μg/mL) for 30 min, followed by LPS (1 μg/mL) treatment for 0–24 h as indicated. ProIL-1β and actin (as an internal control) expression were analyzed by Western blot. These experiments were repeated three times, and a representative result is shown.

enhanced chemiluminescence Western blotting detection system (DuPont NEN Research Product Co., Boston, MA) according to the manufacturer's instructions. Histograms represent quantification by PhosphorImager of proIL-1β in J774A.1 cells with ImageQuaNT software from Molecular Dynamics.

Microculture Tetrazolium (MTT) Assay for Cell Viability. The cytotoxicity of the subfraction H5 was assessed using the MTT assay (*14*). After they were cultured on 96 well plates for 6 h, the cells were washed twice and incubated with 100 μL of 1 mg/mL of MTT for 2 h at 37 °C. The medium was discarded, and 100 μL of lysis buffer was then added. After 30 min of incubation, the absorbance was measured at 570 nm using a microplate reader.

Statistical Analysis. Statistical differences between the experimental groups were examined by analysis of variance, and statistical significance was determined at $p < 0.05$. The experiments were conducted three times or as indicated, and all data are expressed as means ± SD.

RESULTS AND DISCUSSION

Screening of the Bioactive Fraction. The CH_2Cl_2 soluble fraction had the highest yields, about 40.6%, and the next was the EtOAc fraction at 34.1%, hexane fraction at 16.8%, and H_2O fraction at 8.5%. As can be seen in **Figure 2**, various dosages of the different fractions from ME of *Z. japonica* inhibited proIL-1β protein expression in LPS-stimulated J774A.1 cells.

Macrophages play important roles in regulating cell-mediated immune response. In addition to the well-known function of endocytosis, macrophages can be induced to secrete a series of cytokines such as TNFα, IL-1β, and IL-6, which promote inflammatory responses. In this study, we utilized J774A.1 cells as a model to test the antiinflammatory capacity of the ME from *Z. japonica* in LPS-stimulated cells. It was obvious that the hexane fraction had a more efficient ability to restrain the expression of proIL-1β; approximately 90% of proIL-1β expression was inhibited by 25 μg/mL of hexane soluble fraction (**Figure 2A**, ZH). In addition, 50 μg/mL of CH_2Cl_2 soluble fraction also significantly inhibited proIL-1β expression (**Figure 2B**, ZD). However, the fraction of H_2O or EtOAc had no effect on LPS-induced proIL-1β expression even at high concentrations (**Figure 2A**, ZW, and **Figure 2B**, ZE). To further clarify the antiinflammatory activity of the hexane fraction, we continuously separated by using different polarity solvents to achieve 12 subfractions (H1−H12) from the hexane fraction. We found that the subfraction of the hexane fraction (H5) reduced proIL-1β expression at a dose-dependent manner in LPS-stimulated cells (**Figure 3A**). In addition, to ascertain that H5 has clear antiinflammatory potential, we conducted a time-dependent experiment. As can be seen in **Figure 3B**, proIL-1β expression was detectable at 3 h after LPS stimulation, peaked at 6 h, and returned to basal level after 12 h (samples 1−5). In contrast, LPS-induced proIL-1β expression was reduced in the presence of H5 at each time point (samples 6−10).

ELISA. To investigate whether the subfraction H5 isolated from *Z. japonica* exhibits antiinflammatory activity on macrophages, J774A.1 cells were pretreated with various concentrations of H5 for 30 min and challenged with LPS for an additional 6 h. As shown in **Figure 4**, about 56 ng/mL of TNFα was secreted from J774A.1 cells in response to LPS stimulation as measured by ELISA. Interestingly, LPS-induced TNFα secretion was obviously reduced in H5 pretreated cells. Specifically, LPS-induced TNFα secretion was reduced to 42 and 36 ng/mL by 25 and 50 μg/mL of H5, respectively. In addition, pretreatment with H5 also resulted in a dose-dependent inhibitory effect on LPS-induced IL-1β secretion (**Figure 5**). Specifically, LPS induced about 112 pg/mL of IL-1β secretion in the absence of H5, and IL-1β secretion was reduced to 97, 84, 50, and 26 ng/mL by 5, 10, 25, and 50 μg/mL of H5 pretreatment, respectively (**Figure 5**). IL-6 plays an important role in immune and inflammatory responses, which have been considered proinflammatory cytokines (*15*−*17*). We found that pretreatment with

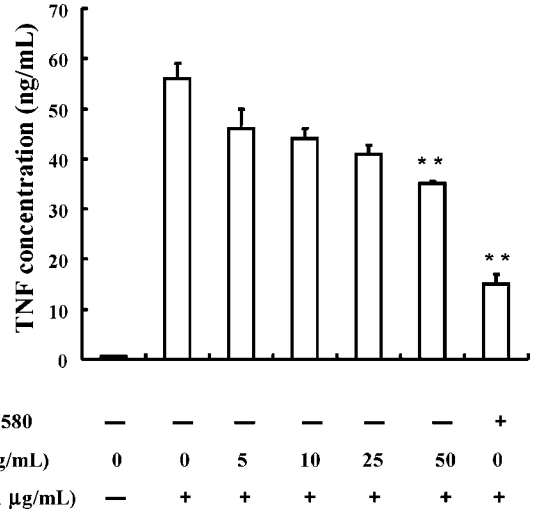

Figure 4. H5 inhibits TNFα secretion in LPS-stimulated J774A.1 cells. Cells were pretreated with the indicated concentrations of H5 for 30 min prior to incubation with LPS (1 μg/mL) for 6 h. The concentration of TNFα in condition medium was analyzed by ELISA. Data shown here are expressed as the mean ± SD, $n = 4$. *$p < 0.05$; **$p < 0.01$ vs LPS alone.

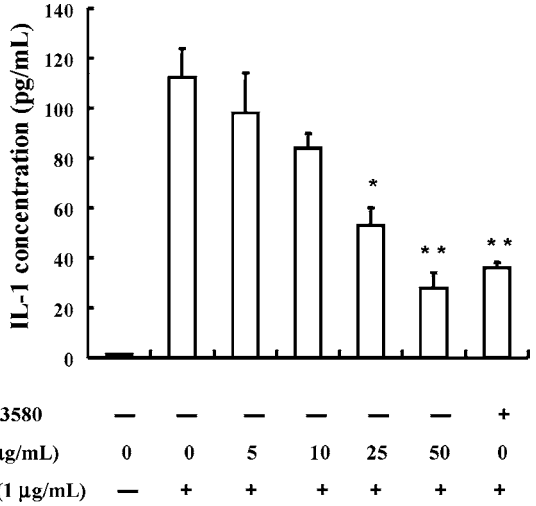

Figure 5. H5 inhibits IL-1β secretion in LPS-stimulated J774A.1 cells. Cells were pretreated with the indicated concentrations of H5 for 30 min prior to incubation with LPS (1 μg/mL) for 24 h. The concentration of IL-1β in condition medium was analyzed by ELISA. Data shown here are expressed as the mean ± SD, $n = 4$. *$p < 0.05$; **$p < 0.01$ vs LPS alone.

50 μg/mL of H5 could inhibit IL-6 secretion by 74% as compared with the unpretreated control in LPS-stimulated macrophages (**Figure 6**). Similar research has been observed in a diterpene tanshinone IIA, which was isolated from the medicinal herb *Salvia miltiorrhiza*. The tanshinone IIA could markedly inhibit TNFα, IL-1β, and IL-6 production in murine macrophages (*18*). Our current results suggest that H5 also has antiinflammatory bioactivity potential. Furthermore, no cytotoxic effect was observed after cells were treated with various concentrations of H5 or DMSO (0.1%) for 24 h as measured by MTT assay (**Figure 7**). In contrast, PD98059, a specific inhibitor of MEK, significantly inhibited cell viability. These results indicated that the inhibition of these cytokines by H5 was not due to cell death.

Chemical Compositions of Bioactive Subfraction. After further analyzing the chemical compositions of different sub-

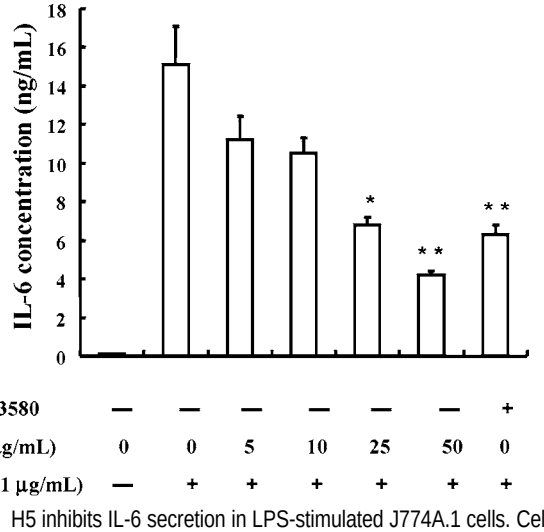

Figure 6. H5 inhibits IL-6 secretion in LPS-stimulated J774A.1 cells. Cells were pretreated with the indicated concentrations of H5 for 30 min prior to incubation with LPS (1 μg/mL) for 6 h. The concentration of IL-6 in condition medium was analyzed by ELISA. Data shown here are expressed as the mean ± SD, $n = 4$. *$p < 0.05$; **$p < 0.01$ vs LPS alone.

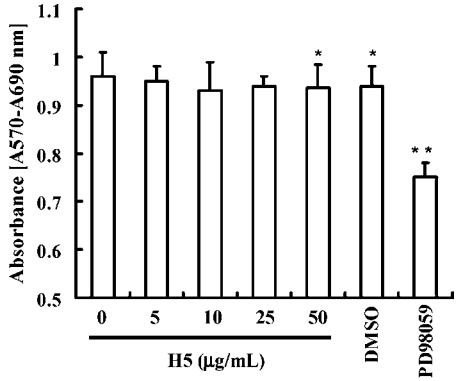

Figure 7. Effect of H5 on J774A.1 cells viability. Cells were either treated with H5 (0–50 μg/mL), DMSO vehicle (concentration is 0.1% as negative control), or PD98059 (concentration is 100 μM as positive control) for 24 h, followed by incubation with the MTT reagent and precipitate solubilized, and the absorbance ($A_{550} - A_{690}$) was measured by spectrophotometry. The bar graph with the mean absorbance values of three separate experiments is shown. Data shown here are expressed as the mean ± SD, $n = 3$. *$p < 0.05$; **$p < 0.01$ vs LPS alone.

fractions by using GC-MS, which are reported in **Table 1**, a total of 11 compounds were identified in the H5 subfraction, constituting 90% of the fatty acid (FA). We demonstrated that the major constituents of H5 were 21.50% palmitic acid methyl ester, 24.02% palmitic acid, 13.09% linoleic acid methyl ester, 8.41% oleic acid methyl ester, 7.93% linoleic acid, 5.90% stearic acid, and 5.66% stearic acid methyl ester, respectively. The methyl ester could be a result of the esterification of FA with methanol and ethanol. According to our results, the 16:0 FA was the dominant composition in H5, followed by 18:2 FA and 18:1 FA, respectively.

A similar result was obtained by Sanina et al. (*19*), who demonstrated that there were only three major FAs, 16:0, 18:2, and 18:3, from the seagrass *Z. marina* and that the polyunsaturated fatty acids (PUFAs) of *Z. marina* mostly belonged to C_{18} series. In recent studies that point out that unsaturated FAs could decrease the production of proinflammatory cytokines, Harbige demonstrated that the γ-linolenic acid (18:3n-6) reduced IL-1 and TNF production (*20*), and Yu et al. demonstrated that conjugated linoleic acid has the capacity to inhibit IL-1 and

Table 1. Major Chemical Compositions of Subfraction H5 of ME from Seagrass of *Z. japonica*

retention time (min)	chemical compound	%	primary fragments in the mass spectra
9.06	β-elemene	1.35	41, 67, 81, 93,[a] 107, 121, 133, 189
10.26	tetradecanoic acid, methyl ester	1.54	43, 55, 74,[a] 87, 101, 143, 199
12.32	hexadecanoic acid, methyl ester	21.5	43, 55, 74,[a] 87, 101, 143, 227, 270
12.79	hexadecanoic acid	24.02	43, 60, 73,[a] 83, 129, 157, 213, 256
12.97	hexadecanoic acid, ethyl ester	2.91	43, 55, 73, 88,[a] 101, 115, 157, 284
13.86	linoleic acid, methyl ester	13.09	41, 55, 67,[a] 81, 95, 109, 115, 263, 294
13.93	oleic acid	8.41	41, 55,[a] 69, 83, 97, 123, 180, 222, 264, 296
14.18	stearic acid, methyl ester	5.66	43, 55, 74,[a] 87, 143, 199, 255, 298
14.35	linoleic acid	7.93	41, 55,[a] 61, 67, 73, 81, 95, 109, 280
14.55	stearic acid	5.90	43,[a] 55, 73, 129, 185, 241, 284
20.21	testosterone	2.84	41, 55, 67, 91, 105, 124,[a] 147, 288

[a] Base peak with 100% intensity.

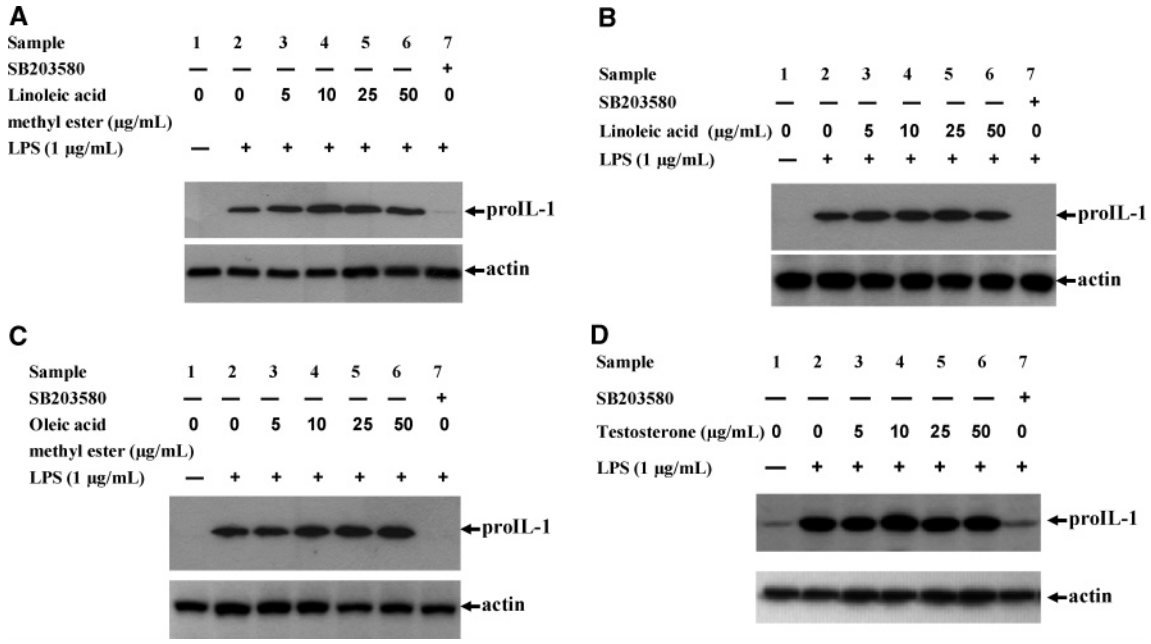

Figure 8. Effects of linoleic acid methyl ester, linoleic acid, oleic acid methyl ester, and testosterone on proIL-1β expression in LPS-stimulated J774A.1 cells. Cells were pretreated with indicated concentration of fractions of sample for 30 min, followed by LPS (1 μg/mL) treatment for an additional 6 h and SB203580 (concentration is 1 μM as positive control); sample 1 is the negative control. ProIL-1β and actin (as an internal control) expression were analyzed by Western blot. These experiments were repeated three times, and a representative result is shown. (**A**) Dose-dependent incubation of linoleic acid methyl ester (0–50 μg/mL). (**B**) Dose-dependent incubation of linoleic acid (0–50 μg/mL). (**C**) Dose-dependent incubation of oleic acid methyl ester (0–50 μg/mL). (**D**) Dose-dependent incubation of testosterone (0–50 μg/mL).

IL-6 in murine macrophages (*21*). Bergé et al. isolated PUFAs from the red microalga *Porphyidium cruentum*, which had strongly inhibited the production of superoxide anions generated by peritoneal leukocytes primed with phorbol myristate acetate, demonstrating that they were accompanied by good antiinflammatory activity (*22*). A lot of studies have demonstrated that conjugated linoleic acid CLA may inhibit LPS-induced inflammatory mediator such as iNOS and COX2 in RAW 264.7 murine macrophages (*23*, *24*). In addition, Malkin et al. found that testosterone could suppress the expression of the proinflammatory cytokines TNFα, IL-1β, and IL-6 in vitro (*25*). It is interesting that there was no obvious antiinflammatory activity found for the FAs or testosterone in this model (**Figure 8A**−**D**). We found that LPS-induced proIL-1β expression in J774A.1 macrophages was not inhibited by linoleic acid methyl ester, linoleic acid, oleic acid ester, or testosterone, which were the major components exhibited in ME of *Z. japonica*. In our study, the FAs that were the major constituents of subfraction H5 did not contribute to antiinflammatory activity, as they did in previous studies, but this may be due to differences in the experimental model used. Our results strongly suggest that the antiinflammatory activities of extract of *Z. japonica* probably came from other minor and unidentified components (above 4.85%). Further research to identify the functions of the other bioactive components are in progress, and the results will be reported soon.

ACKNOWLEDGMENT

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