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Chemical Composition and Bioactivities of the Marine Alga *Isochrysis galbana* CCMP 1324 from Taiwan

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The present study investigated the chemical composition of *Isochrysis galbana*, a species of marine microalgae which is frequently and widely used as a feedstock in aquaculture. The *I. galbana* alga contained hot-water extractable polysaccharides (11.17%); after hydrolysis the carbohydrate components were treated with trimethylsilane for derivatization and subsequent GC-MS analysis. The mono-sugar compositions of *I. galbana* were 2.07% fucose, 2.50% rhamnose, 2.72% arabinose, 8.49% xylose, 15.7% mannose, 32.73% galactose and 35.79% glucose. Using the murine macrophage cell line J774A.1, we found that polysaccharides of *I. galbana* were able to induce prointerleukin-1β (pro-IL-1β) protein expression. Furthermore, the composition of *I. galbana* was determined to consist of 8.58% ash, 38.16% extractable by 95% alcohol and 21.75% extractable by alcohol/hexane (1/1). Five kinds of chlorophyll and one sterol were separated from the alcoholic extractives of *I. galbana*, including pheophorbide-a, ethyl pheophorbide-a, 10S-10-hydroxypheophytin-a, 10R-10-hydroxypheophytin-a, (13^2-R)-pheophyin-a, and brassicasterol. In addition, the major soluble components of alcohol/hexane were found to be 9-octadecenoic acid (E) (38.41%), hexadecanoic acid (23.34%), tetradecanoic acid (15.70%), and octadecanoic acid (7.16%) – but only a few polyunsaturated fatty acids such as 9,12,15-octadecatrienoic acid (1.95%), 9,12-octadecadienoic acid (Z,Z) (3.44%), and docosahexaenoic acid (0.21%). This is the first occasion that polysaccharides from *I. galbana* have been demonstrated to exert immunomodulatory properties by the induction of IL-1 within macrophages.

Keywords: *Isochrysis galbana*, extractives, chemical compositions, polysaccharides, bioactivity, pro-IL-1β.

Marine microalgal biomasses play an important role as primary producers in the animal food chain. *Isochrysis galbana*, a golden-brown flagellate marine microalga, is widely used as an aquaculture feed for young fish and in bivalve hatcheries owing to its being rich in PUFA [1]. Many studies have focused on the relationship between fatty acids and algal growth [2-4]. In addition, because of the high content of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), *I. galbana* is recognized for its beneficial effects on human health, and is considered to be a good substitute for fish oil in the human diet. However, only a few studies have shown the detailed chemical composition of *I. galbana*. The purpose of the present study was to analyze the chemical composition of *I. galbana*, including: hexane/alcohol extracts; alcohol extracts; total carbon, hydrogen, oxygen, nitrogen and sulfur; and total polysaccharides.

First we analyzed the ratio of carbon, hydrogen, oxygen, nitrogen, sulfur and ash of *I. galbana* cultured in the laboratory. The results showed that *I. galbana* contains: C (56.35%), H (7.55%), O (20.29%), N (6.25%), S (0.97%) and ash (8.59%), based on dry weight. *I. galbana* also has a high content of hexane/alcohol (1/1) extracts, as well as extracts of alcohol only which amount to 21.75% and 38.16%.

Sixteen components of fatty acids from *I. galbana* are identified and presented in Table 1, where all compounds are listed in order of their elution from the DB-5HT column. The major component of fatty acids is 9-octadecenoic acid (E) (38.41% of total fatty acids), followed by hexadecanoic acid (23.34%), tetradecanoic acid (15.70%), octadecanoic acid (7.16%), and 9-octadecenoic acid (Z) (3.16%). The compositions of fatty acids from *I. galbana* in this study are not similar to an earlier study, which showed higher DHA (22:6n-3) (7.91%) [5]. Also, some differences were found from the study by Lin et al., who determined DHA to be a major fatty acid at every growth phase [4]. It is interesting that we found a few fatty acids of odd carbons such as pentadecanoic acid, heptadecanoic acid, and heneicosanoic acid, which had not been previously found [4]. This could be a result of different growth conditions.

Table 1: Chemical composition of *L. galbana*, alcohol/hexane soluble.

Compound ID	RT [a]	Conc. (%)	Identification
Tetradecanoic acid, methyl ester	14.51	15.70	MS, KI, ST
Pentadecanoic acid, methyl ester	16.52	0.800	MS, KI, ST
11,14,17-Eicosatrienoic acid, methyl ester	17.76	0.11	MS, KI
(Z)-9-Hexadecenoic acid, methyl ester	18.04	2.52	MS, KI
Hexadecanoic acid, methyl ester	18.50	23.34	MS, KI, ST
Heptadecanoic acid, methyl ester	20.53	0.18	MS, KI, ST
9,12,15-Octadecatrienoic acid, methyl ester,	21.22	1.95	MS, KI, ST
9,12-Octadecadienoic acid (Z,Z)-, methyl	21.54	3.44	MS, KI,
9-Octadecenoic acid (E)-, methyl ester	21.66	38.41	MS, KI, ST
9-Octadecenoic acid (Z)-, methyl ester	21.77	3.16	MS, KI,ST
Octadecanoic acid, methyl ester	22.16	7.16	MS, KI, ST
Arachidonic acid, ethyl ester	24.30	0.09	MS, KI,
Heneicosanoic acid, methyl ester	27.14	0.12	MS, KI
4,7,10,13,16,19-Docosahexaenoic acid,	27.40	0.21	MS, KI,
Docosanoic acid, methyl ester	28.67	2.81	MS, KI
Total saturated		50.11	
Total monoenoic		44.09	
Total PUFA		5.8	

[a] Retention time on a DB-5 HT column with reference to *n*-alkanes.
[b] MS, NIST and Wiley library spectra and the literature; RI, Retention index; ST, authentic standard compounds.

In general, chlorophyll compounds are used for estimating the biomass of microalgae and can be used to measure growth. Few existing studies have focused on the variety of pigments from *I. galbana*. Herein we have separated and identified the pigments from 1.65 g of alcoholic extracts of *I. galbana*. As shown in Figure 1, *I. galbana* contains at least five kinds of chlorophyll compounds. They include (13^2-R)-pheophytin-a (1.5 mg; RT = 8.03 min), pheophorbide-a (1 mg; RT = 15.82 min), 10R-10-hydroxypheophytin-a (3 mg; RT = 22.54 min), 10S-10-hydroxypheophytin-a (1 mg; RT = 23.51 min), and ethyl pheophorbide-a (4 mg; RT = 34.92 min). We also found brassicasterol (2 mg; RT = 15.10 min). In 1981, Volkman et al. [6] were the first to report that 24-methyl-22-dehydrocholesterol was the dominant sterol from *Isochrysis galbana* [6]. Our experimental results were similar, in that brassicasterol was found to be the principal sterol from *I. galbana*. Park et al. (2002) [7] demonstrated that autotrophically grown *I. galbana* contains three major sterols (24-oxocholesterol acetate, ergost-5-en-3-ol, and cholest-5-en-24-1,3-(acetyloxy)-,3-ol), with 24-methylcholesta-5,22-dien-3-ol as a minor sterol [7]. Meanwhile they found that the total sterol content will clearly decrease during dark culture, with such decreases being particularly evident in two major sterols, 24-oxocholesterol acetate and ergost-5-en-3-ol.

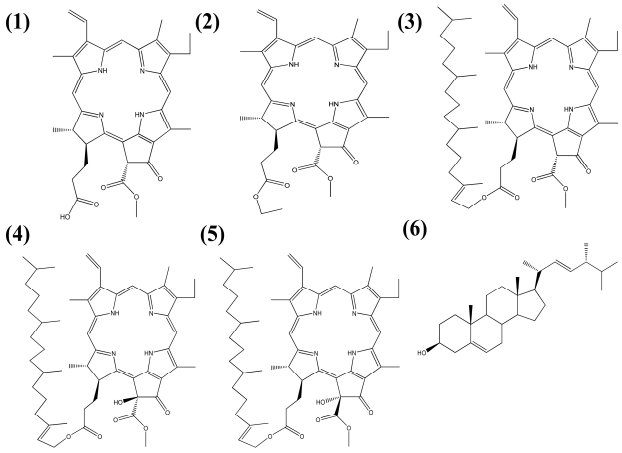

Figure 1. The chemical structure of alcoholic extracts from *I. galbana*.of (1). Pheophorbide-a (2). Ethyl pheophorbide-a (3). (13^2-R)-pheophyin-a (4). 10R-10-Hydroxypheophytin-a (5). 10S-10-Hydroxypheophytin-a (6). Brassicasterol

Our study shows that the mono-sugar compositions of Isochrysis polysaccharide (IP) from *I. galbana* were 2.07% fucose, 2.50% rhamnose, 2.72% arabinose, 8.49% xylose, 15.7% mannose, 32.73% galactose and 35.79% glucose. Furthermore, no cytotoxic effect was observed after J7774A.1 cells were treated with various concentrations of IP for a period of 24 h, as measured by MTT assay (Figure 2).

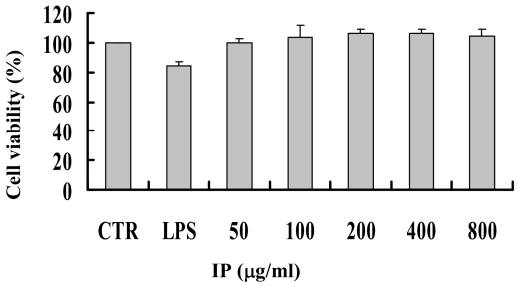

Figure 2: Effect of Isochrysis polysaccharide (IP) on cell viability. J774A.1 macrophages (5×10^3/well) were treated with IP or DMSO (control) for 24 h, followed by incubating with MTT reagent The absorbance (A550-A690) was measured by spectrophotometry. Data were expressed as mean ± SE from three separate experiments

An IP-induced IL-1 precursor, pro-IL-1, was detected in the whole cell lysates after *I. galbana* alga (IP) stimulation by means of Western-blotting analysis. In this time course study, the expression of pro-IL-1 protein within IP-stimulated cells was detected at 6 h post-

stimulation. In addition, the expression of pro-IL-1 increased with increasing IP concentrations in a dosage-dependent manner (Figure 3). Such results demonstrate that IP stimulates pro-IL-1 expression, a result that would appear to be similar to the ability of polysaccharides isolated from algae of *Rhizoclonium riparium* or *Chlorella pyrenoidosa* to stimulate pro-IL-1 expression within murine macrophages [8-9]. Fabregas et al. (1999) found that endocellular extracts from *I. galbana* could clearly inhibit viral hemorrhagic septicemia virus (VHSV) replication at a dose of 20 μg/mL, and that *I. galbana* contained sulfated soluble exopolysaccharides [10].

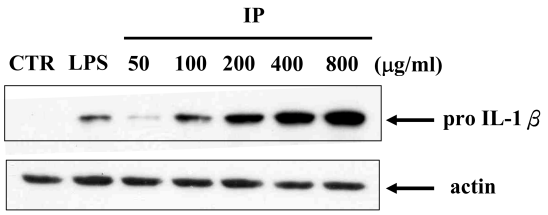

Figure 3: IP induces pro-IL-1 protein expression in J774A.1 cells. Dose response of Western-blotting analyses of pro-IL-1 protein expression in IP-treated cells: cells were treated with various concentrations of IP as indicated (0 to 800 μg/mL) for 6h, and whole cell lysates were analyzed by Western blotting (LPS as positive control). Pro-IL-1 and actin (as an internal control) are indicated by arrows on the right.

Experimental

Algal culture and collection: *I. galbana* alga used in this study was obtained from the Tungkang Biotechnology Research Center, Fisheries Research Institute, Republic of China. Purified *I. galbana* CCMP 1324 (0.9 L, 680 nm, OD 1.10) was inoculated and cultured in Walne's medium [9]. This included nutrient solution, vitamin solution and trace metal solution in a 10 L PET tank containing 8.1 L seawater autoclaved at 120 °C for 20 min, to which was added 9.0 mL of nutrient solution and 0.9 mL of vitamin solution. The culture medium was agitated gently by bubbling air with a flow rate of 4.7 L/min and a culture time of 7 d. Continuous illumination at an irradiance of 5900 lx was provided by fluorescent lamps. The culture medium was then centrifuged (Himac CR22-GII, Hitachi, Japan) continuously at 12000 rpm, 25 ºC. After lyophilization the yields of the alga were 0.1 g/L (dry weight) culture medium.

Total C、H、O、N、S : Total carbon, hydrogen, oxygen, nitrogen and sulfur contents were determined by CHN elemental analysis. Freeze-dried samples (15 g each) were combusted in a 2400 CHN/O elemental analyzer (Perkin-Elmer, Waltham MA, USA) [11].

Extraction and compositions of hexane/alcohol extracts of I. galbana: Ten g of the sample was extracted in a Soxhlet apparatus with *n*-hexane:alcohol (95% v/v) (50:50) for 48 h. The solution was rotary-evaporated at 65 ºC to provide hydroalcoholic extracts (HAE) of 2.175 g. HAE (0.5 g) was tested for further trimethylsilylation with Sylon HTP (HMDS/TMCS/pyridine, 3:1:9) trimethylsilylation reagent (Supelco, Bellefonte PA, USA). The final trimethylsilylated (TMS) derivatives were kept in *n*-hexane for gas chromatography–mass spectrometry (GC-MS) analysis. A Hewlett-Packard HP 6890 gas chromatograph equipped with a DB-5HT fused silica capillary column (30 m x 0.25 mm x 0.25 μm film thickness; Agilent Technologies, Santa Clara CA, USA) and a FID detector was used for quantitative determination of the components. Oven temperature was programmed as follows: 100 ºC for 2 min, rising to 275 ºC at 5 ºC/min; injector temperature, 270 ºC; carrier gas, He with a flow rate of 1 mL/min; detector temperature, 250 ºC; split ratio 50.1:1. One μL sample was injected. Identification of the oil components was based on their retention indices and MS results. The GC analysis parameters listed above and the MS were obtained (full scan mode; scan time, 0.3 s; mass range, MHz 30–500) in the electron impact (EI) mode at 70 eV.

Extraction, purification and identification of alcoholic extracts of I. galbana: Dry alga (5 g) was treated with ethanol (95% v/v for 10 d, repeated 3 times) at room temperature; then the extract was concentrated to provide alcoholic extract (AE). AE (1.65 g) was applied on a silica gel column (Si 60) for separation. Then it was eluted with acetone/*n*-hexane to give 43 subfractions. Each eluted volume of the fraction was 150 ml. The chlorophyll compounds were purified by preparative HPLC (KNAUER RI detector 2400, pump 100; KNAUER, Germany) on a Merck (Germany) Hibar Fertigsaule RT column Si 60 (25 cm length, 1 cm i.d., 5.0 μm). The separation conditions were as follows: flow rate 4 mL/min; mobile phase, acetone/hexane = 1/9. The sterol was separated by a Phenomenex Luna silica (2) column (25 cm length, 1 cm i.d., 5.0 μm) under the following conditions: flow rate 4 mL/min; mobile phase, acetone/hexane = 1/15. The structures of the compounds were confirmed by comparison of physical and spectral data (including optical rotation, EI-MS, ^{1}H-NMR) with previously reported values.

Extraction of the polysaccharides from I. galbana: Five g dry alga were extracted by hexane/alcohol, ground into a fine powder, and then suspended in 100 mL of distilled water. After autoclaving at 121 °C for 30 min, the extracts were filtered by a 0.2 μm membrane. The extracts were then vacuum-concentrated at 50 °C, giving a final volume of 30 mL to which five volumes of 95% ethanol was added slowly at 4 °C. Then the mixture was centrifuged to produce precipitates of ca. 1.910 g (d/w). Sixty mg of the precipitates were treated further with 3 mg proteinase K for removal of the peptide part, and

dialyzed against H_2O (Spectra/Por® membrane, molecular weight cutoff 1000 Da), resulting in 17.5 mg polysaccharide of *I. galbana* (IP).

Sugar compositions *analysis:* Sugar composition was determined by GC-MS. Polysaccharide content of *I. galbana* was determined by methanolysis with 0.5 M methanolic HCl at 80 °C for 16 h and trimethylsilylation with Sylon HTP. The final trimethylsilylated (TMS) derivatives were kept in *n*-hexane for GC-MS analysis [12]. Carbohydrate analysis was done with inositol as the internal standard; its integrated peak area was used to establish the relative amounts of the constituents. The compounds were identified by comparing their mass spectrometric fragmentation patterns with those of authentic standards, and the quantity of compounds was obtained by integrating the peak area of the spectrograms.

Microculture tetrazolium (MTT) assay for cell viability: J774A.1 macrophages were seeded in 96-well plates at a density of 5×10^3 cells/well. Cells were incubated with IP for 24 h. Cell viability was determined using colorimetric MTT assays.

Cell cultures: Murine J774A.1 macrophages were obtained from the American Type Culture Collection (ATCC) (Rockville MD, USA). All cells were propagated in RPMI-1640 medium supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine (Life Technologies, Carlsbad CA, USA), and cultured in a 37 °C, 5% CO_2 incubator [8,9].

Western Blotting: Whole cell lysates were separated by 12% SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated in blocking solution (5% nonfat milk in PBS with 0.1% Tween 20) at room temperature for 1 h. The membrane was incubated with anti-IL-1β antibody at room temperature for 2 h. After washing three times in PBS with 0.1% Tween 20, the membrane was incubated with an HRP-conjugated secondary antibody directed against the primary antibody. The membrane was developed by an enhanced chemiluminescence Western-blotting detection system (DuPont NEN® Research Products, Boston MA, USA) according to the manufacturer's instructions [8,9].

Statistical analysis: All values are given as mean ± SE. Data analysis involved one-way ANOVA with subsequent Scheffé test.

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