The present study investigated the chemical composition of *Isochrysis galbana* Parke, a marine microalga which is widely used as a feedstock in aquaculture. From gas chromatography/mass spectrometric analysis the mono-sugar compositions of *I. galbana* were 2.1% fucose, 2.5% rhamnose, 2.7% arabinose, 8.5% xylose, 15.7% mannose, 32.7% galactose and 35.8% glucose. The polysaccharides of *I. galbana* were able to induce prointerleukin-1β (pro-IL-1β) protein expression within murine macrophages. Furthermore, five kinds of chlorophyll and one sterol were separated from the ethanolic extracts, including pheophorbide-a, ethyl pheophorbide-a, 10S-10-hydroxypheophytin-a, 10R-10-hydroxypheophytin-a, (132-R)-pheophytin-a, and brassicasterol. In addition, the major soluble components of the ethanol/n-hexane extract were 9-octadecenoic acid (E) (38.4%), hexadecanoic acid (23.3%), tetradecanoic acid (15.7%), and octadecanoic acid (7.2%), but only a few polyunsaturated fatty acids were found, such as 9,12,15-octadecatrienoic acid (1.9%), 9,12-octadecadienoic acid (Z,Z) (3.4%), and docosahexaenoic acid (0.2%). 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*, extracts, chemical compositions, polysaccharides, bioactivity, pro-IL-1β.

Marine microalgal biomasses play an important role as primary producers in the animal food chain. *Isochrysis galbana* Parke, a golden-brown flagellate marine microalga, is widely used as an aquaculture feed for young fish and in bivalve hatcheries because it is rich in polyunsaturated fatty acids (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. The purpose of the present study was to analyze the chemical composition of *I. galbana*, including n-hexane/ethanol extracts; ethanol extracts; total carbon, hydrogen, oxygen, nitrogen and sulfur; and total polysaccharides.

First we analyzed the ratio of carbon, hydrogen, oxygen, nitrogen, sulfur and ash in *I. galbana* cultured in the laboratory. Based on dry weight, the values obtained were: C (56.3%), H (7.5%), O (20.3%), N (6.25%), S (1.0%) and ash (8.6%). *I. galbana* also yielded a high content of n-hexane/ethanol (1/1) and ethanol extractive (21.7% and 38.2%, respectively).

Sixteen fatty acids were identified. These are given in Table 1, where all compounds are listed in order of their elution from the DB-5HT column. The major fatty acid was 9-octadecenoic acid (E) (38.4% of total), followed by hexadecanoic acid (23.3%), tetradecanoic acid (15.7%), octadecanoic acid (7.2%), and 9-octadecenoic acid (Z) (3.2%). The composition of fatty acids in *I. galbana* found in this study differs from the results of
Parkin 24-methyl-22-dehydrocholesterol is the dominant sterol 24-oxocholesterol acetate and ergost-5-en-3-ol. decreases being particularly evident in two major sterols, content clearly decreased during dark culture, with such results found that the total sterol (acetyloxy)-,3-ol), with 24-methylcholesta-5,22-dien- 3-ol, ergost-5-en-3-ol, and cholest-5- en-24-1, 3- (acetoxy)-,3-ol), with 24-methylcholesta-5,22-dien- 3-ol as a minor sterol [7]. They found that the total sterol content clearly decreased during dark culture, with such decreases being particularly evident in two major sterols, 24-oxocholesterol acetate and ergost-5-en-3-ol.

Our study showed that the monosaccharide composition of a polysaccharide (IP) from *I. galbana* was 2.07% 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, 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.

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 an ethanolic extract of *I. galbana*. At least five chlorophyll compounds (Figure 1) were found including (13Z)-pheophytin-a (1.5 mg; RT = 8.03 min), pheophorbide-a (1 mg; RT = 15.82 min), 10R-10-hydroxypheophytin-a (3 mg; RT = 23.51 min), and ethyl pheophorbide-a (4 mg; RT = 22.54 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 is the dominant sterol in *I. galbana* [6]. Our experimental results were similar. Park et al. [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- (acetoxy)-,3-ol), with 24-methylcholesta-5,22-dien- 3-ol as a minor sterol [7]. They found that the total sterol content clearly decreased 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 (1) pheophorbide-a; (2) ethyl pheophorbide-a; (3) (13Z)-pheophytin-a; (4) 10R-10-hydroxypheophytin-a; (5) 10S-10-hydroxypheophytin-a; (6) brassicasterol.](Image)

![Figure 2: Effect of *Isochrysis* polysaccharide (IP) on cell viability.](Image)

It is well known that IL-1β is secreted mainly from activated macrophages; this could activate other immune cells and modulate immune responses. An IP-induced IL-1β precursor, pro-IL-1β, was detected in whole cell lysates after IP stimulation by means of Western-blotting analysis. In this time course study, the expression of pro-IL-1β protein within IP-stimulated cells was detected at six hours post-stimulation. In addition, the expression of pro-IL-1β increased with increasing IP concentrations in a dose-dependent manner. Such results demonstrate that IP stimulates pro-IL-1β expression, a result that would appear to be similar to the ability of polysaccharides isolated from the algae Rhizoclonium riparium (Roth) Harvey and Chlorella pyrenoidosa Chick to stimulate pro-IL-1β expression within murine macrophages [8,9].
Fabregas et al. found that endocellular extracts of *I. galbana* clearly inhibited viral hemorrhagic septicemia virus (VHSV) replication at a dose of 20 μg/mL, and that *I. galbana* contained sulfated soluble exopolysaccharides [10].

**Experimental**

**Algal culture and collection:** *I. galbana* 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, vitamin and trace metal solutions 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 at 25°C. After lyophilization the yield of alga was 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 composition of n-hexane/ethanol extracts of *I. galbana***: Ten grams of sample was extracted in a Soxhlet apparatus with *n*-hexane: ethanol (95% v/v) (50:50) for 48 h. The solution was rotary-evaporated at 65 °C to provide a hydro-ethanolic extractive (HAE) (2.175 g). The HAE (0.5 g) was trimethylsilylated with Sylon HTP (HMDS/TMCS/pyridine, 3:1:9) trimethylsilylation reagent (Supelco, Bellefonte PA, USA). The final derivatives were kept in *n*-hexane for gas chromatography–mass spectrometric (GC-MS) analysis. A Hewlett-Packard HR 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 were used for quantitative determination of the components. The oven temperature was programmed as follows: 100°C for 2 min, rising to 275°C at 5°C/min; injector temperature, 270°C; carrier gas, He with a flow rate of 1 mL/min; detector temperature, 250°C; 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 ethanolic 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 the ethanolic extract (AE). AE (1.65 g) was applied to a silica gel column (Si 60) and eluted with acetone/*n*-hexane to give 43 sub-fractions. Each eluted fraction was obtained by integrating the peak area of the spectra. Authentic standards, and the quantity of compounds was established with previously reported values.

**Extraction of polysaccharides from *I. galbana***: Five grams dry alga was extracted with *n*-hexane/ethanol. The extractive was ground into a fine powder, and then suspended in 100 mL distilled water. After autoclaving at 121°C for 30 min, the extract was filtered through a 0.2 μm membrane. The extract was then vacuum-concentrated at 50°C, giving a final volume of 30 mL to which 5 volumes of 95% ethanol was added slowly at 4°C. Then the mixture was centrifuged to produce a precipitate of ca. 1.910 g, dry wt. Sixty mg of the precipitate was treated further with 3 mg proteinase K for removal of the peptide part, and dialyzed against H2O (Spectra/Por® membrane, molecular weight cutoff 1,000 Da), resulting in 17.5 mg polysaccharide [*I. galbana* (IP)].

**Sugar composition analysis**: Sugar composition was determined by GC-MS. The 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; integrated peak area was used to establish the relative amounts of the constituents. 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 spectra.

**Microculture tetrazolium (MTT) assay for cell viability**: J774A.1 macrophages were seeded in 96-well plates at a density of 5 × 10³ 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% CO₂ 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 then incubated with anti-IL-1β antibody at room temperature for 2 h. After washing 3 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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