



## - RESEARCH ARTICLE -

### Immunostimulatory and Immunomodulatory Effects of *Nitzschia navis-varingica*, *Heterocapsa pygmaea* and *Chrysochromulina alifera* Whole Cell Extracts on Mammalian Macrophage Cells

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#### Abstract

Microalgal species have been used as a food source and as medicine since ancient times in Africa, Mexico and China. In our study we focused on the whole cell extracts of three microalgal species that have not been studied before: the diatom *Nitzschia navis-varingica*, the haptophyte *Chrysochromulina alifera* and the dinoflagellate *Heterocapsa pygmaea*. Effects of the diatom *Nitzschia navis-varingica*, the haptophyte *Chrysochromulina alifera* and the dinoflagellate *Heterocapsa pygmaea* on mammalian macrophage cells were investigated first time in this study. A significant anti-inflammatory impact of cell extracts in distilled water was observed. Direct anti-inflammatory influence of the extracts without any solvents or further processes facilitates its usage in biotechnology. Furthermore, the ability to include these species in the human diet brings out new opportunities in terms of increasing the tolerance to certain allergic food products in patients with allergic reactions as well as inflammatory bowel disease. Moreover, these species can be used in the creams for the atopic applications on the skin to prevent skin allergies. With this study, we are presenting data supporting the biotechnological potential of the diatom *Nitzschia navis-varingica*, the haptophyte *Chrysochromulina alifera* and the dinoflagellate *Heterocapsa pygmaea* species against inflammatory and allergic reactions.

#### Keywords:

Microalgae, macrophages, inflammation, natural drugs, immunomodulation

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#### Introduction

Excessive inflammatory responses are associated with numerous different disorders such as atherosclerosis, insulin resistance, rheumatoid arthritis, cancer and dementia (Chung et al., 2009;

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Del Prete et al., 2011; Ku et al., 2013). Several nonsteroidal anti-inflammatory drugs have undesirable side-effects and raise the risk of cardiovascular diseases (Ku et al., 2013; Jugdutt, 2007). Thus, alternative natural products with anti-inflammatory effects and less of the side effects would create an immense potential for the field of inflammatory diseases as well as other disorders associated with inflammation. Anti-inflammatory impacts of microalgal species have been related to their pigments, polyunsaturated fatty acids (PUFA) and sulphated polysaccharides (Priyadarshani & Rath, 2012; Gigova & Marinova, 2016; Calder, 1996; Talero et al., 2015). In addition to these components, rich contents of protein, vitamin, and mineral of microalgae prompt them to be used for nutraceutical and therapeutical purposes (Priyadarshani & Rath, 2012; Gigova & Marinova, 2016; Calder, 1996).

Omega-3 polyunsaturated fatty acids were reported to restrain the proliferation of human and rodent lymphocytes (Calder, 1996; Calder, 2012). Thus, they were suggested as a therapeutical agent in chronic obstructive pulmonary disease, rheumatoid arthritis, inflammatory bowel disease and asthma (Calder, 2012; Miyata & Arita, 2015; Yates et al., 2014). Microalgae species have reservoirs of Omega-3 as well as other lipids. For example, lipid extracts of the cyanophyte *Spirulina platensis* was reported to reduce the production of proinflammatory cytokines in macrophages and splenocytes (Ku et al., 2013).

Fucoxanthin is found in diatoms and it has been shown to exert an anti-inflammatory impact by suppressing mast cell degranulation (Sakai et al., 2011). Although bioavailability of this pigment has been reported as low in humans, its usage associated with edible oil or lipid could increase its absorption rate (Peng et al., 2011).

Sulphated polysaccharides are found in several different microalgal species and groups including diatoms, dinoflagellates and haptophytes and these polysaccharides have anti-inflammatory activity (Talero et al., 2015). Sulphated polysaccharides have also been reported to prevent adhesion of *Helicobacter pylori* on gastric surfaces, which can preclude carcinogenesis (Amaro et al., 2013). Polysaccharides of *Chlorella stigmatophora* was shown to have an immunosuppressive effect in the carrageenan-induced paw edema test in rats (Guzman, 2003). Moreover, oral administration of hot water extracts of *Chlorella vulgaris* in mice suppressed the production of immunoglobulin (Ig)E against casein antigen (Hasegawa et al., 1999).

Purpose of our investigation was testing the immunomodulatory and immunostimulatory effects of microalgal extracts belonging to three distinct taxonomical groups on mammalian macrophage cells. All three species, *Nitzschia navis-varingica* Lundholm & Moestrup, *Heterocapsa pygmaea* Lobelich III, R.J.Schmidt & Sherley and *Chrysochromulina alifera* Parke & Manton exhibited a significant immunosuppressive impact on macrophage cells. These results suggest that extracts of these microalgae could be used in the treatment of inflammatory disorders as well as in the induction of diet associated allergies.

## Material and Methods

### *Microalgal cell cultures and obtaining their extracts*

Cultures of *Nitzschia navis-varingica*, *Heterocapsa pygmaea* and *Chrysochromulina alifera* were isolated from the surface water of Mersin (36°46'N, 34°35'E) and Erdemli coast (36°60' N, 34°31'E) in the north-eastern Mediterranean Sea. References used for the identification of species

were Lundholm & Moestrup, 2000; Iwataki et al., 2004; Parke & Manton, 1956; Puigserver et al., 2003. Cultures were grown in 100 mL Erlen-Meyer flasks with f/20-Si medium prepared with autoclaved seawater filtered through 0.45  $\mu\text{m}$  pore size nitrocellulose membrane filters and kept in a climate chamber at  $23 \pm 2$  °C under 12-h light, 12-h dark cycle, emitted from OSAKA TL2001:6W cool white fluorescent tubes. Cell volumes were calculated according to morphometry of each species considering cell diameter, length and width. One  $\mu\text{m}^3$  volume was assumed equivalent to 1 pg wet wt (Gasiunaite et al., 2005). 1mg of cells were pelleted and burst in 1 mL of sterile distilled water. After 15 minutes of sonication at least with 20 kHz ultrasound, samples were further filter sterilized with 0.2 $\mu\text{M}$  Whatman Syringe filters.

#### ***In vitro cell activation studies:***

- ***Cell Culture:*** RAW 264.7 cells were purchased from ATCC and grown in Roswell Park Memorial Institute media (RPMI 1640) media with 10% fetal bovine serum, 1% antibiotics (100  $\mu\text{g}\text{mL}^{-1}$  penicillin and 100  $\mu\text{g}\text{mL}^{-1}$  streptomycin) and sodium pyruvate. Cells were incubated in 37 °C 5%  $\text{CO}_2$  incubator. Cell media was refreshed once in every 4 days until they reach confluency to be used in the experiments.

- ***Microalgal cell extract and Lipopolysachharide (LPS) stimulation to mimick danger signal:*** RAW 264.7 cells were put in  $10^6$  cells/well concentration in 1mL fresh complete RPMI as described above into 24-well plates, then they were rested overnight in 37 °C 5%  $\text{CO}_2$  incubator. We tested 100 $\mu\text{g}\text{mL}^{-1}$  algal cell extracts' effect on RAW 264.7 cells in the presence and absence of inflammatory stimulator LPS. 1 $\mu\text{L}$  of LPS (1mg/mL, Enzo Life Sciences, Salmonella minnesotaR595) was put into 1 mL media of overnight rested cells. The same volume of distilled water was put into control negative and LPS only control wells. Then cells were treated with the algal cell extracts and LPS for 24 hours in 37 C 5% incubator. Afterwards supernatants of each well were collected into eppendorf tubes and centrifuged at 2000RPM to get rid of any cellular debris, then supernatants were transferred into fresh eppendorf tubes and kept at -80°C before further examination. All experimental conditions were tested as a triplicate. In order to measure IL1b production by RAW 264.7 cells, freshly prepared 5mM ATP (Fisher Scientific) was put into each well 2 hours before the harvest. The same experimental set up as stated above was used.

- ***TNF $\alpha$ , IL6 and IL1b ELISAs:*** TNF $\alpha$ , IL6 and IL1b production was measured by using enzyme-linked immunosorbent assay (ELISA). For each cytokine type (BD Biosciences, CA, USA) ELISA kit was used by following the manufacturer's instructions. Maxisorb 96 well plate (Krackeler) was first coated overnight with hamster anti-mouse cytokine ( $0.5 \mu\text{g}\text{mL}^{-1}$  in bicarbonate buffer pH=9.5, 100  $\mu\text{L}$  /well). After getting rid of the solution, the plate was washed 3 times with 0.05% Tween 20 PBS. Then plate was blocked with 200  $\mu\text{L}$  blocking buffer (1% BSA PBS) in each well after 3 hours of incubation at room temperature. After washing the plate 3 times samples were put in 100  $\mu\text{L}$  into each well and incubated overnight at 4 °C. After washing the plate 3 times 100  $\mu\text{L}$  biotin human anti-mouse cytokine (0.5  $\mu\text{g}/\text{mL}$  10% FBS PBS) was put into each well and plate was incubated at room temperature for 2 hours. After discarding the solution the plate was washed 3 times, and 100  $\mu\text{L}$  of Streptavidin HRP solution was put into each well and plate was incubated for 2 hours at room temperature. Then plate was washed 3 times and 100  $\mu\text{L}$  TMB substrate (BD OptEIA) was put into each well and 50  $\mu\text{L}$  of 1 M sulfuric acid was used to stop the reaction and absorbance was measured at 450nm. By using known concentrations of each cytokine's as standard the concentration of TNF $\alpha$ , IL1b and IL6 in each sample was calculated.

- **Cell counting and proliferation:** After removing the supernatant media the cells are resuspended in 1ml PBS and counted by mixing 10  $\mu\text{L}$  of cells and 90  $\mu\text{L}$  of Trypan Blue (0.1  $\mu\text{M}$ ) and counting them by a hemacytometer and microscope.

**Statistical analysis:** GraphPad Prism Software version 5 was used for statistical analysis and for each data set there was nine independent results and unpaired two-tailed t-test was executed to draw statistical significance.

## Results

Whole cell extracts of microalgal species *Nitzschia navis-varingica*, *Heterocapsa pygmaea* and *Chrysochromulina aliferadid* not change the cell viability of macrophages: Trypan Blue was used in order to differentiate between live and dead cells after stimulation of the macrophages.  $100\mu\text{g mL}^{-1}$  concentration of microalgal cell extracts was used. In negative control wells sterile water was used and in positive control wells  $1\mu\text{g mL}^{-1}$  of LPS was applied to the cells. After 24 hours of incubation, cell viability was 100% under all conditions (Figure 1). Therefore, microalgal cell extracts did not cause cytotoxicity based on cell viability (Figure 1). This data suggests that potential application of these microalgal extracts would not lead to toxicity on macrophages in terms of live cell percentages.

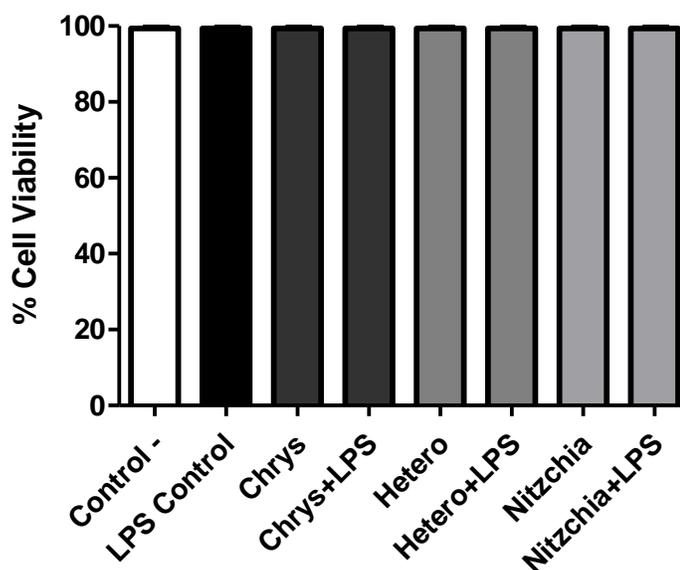


Figure 1. Percentage of viable cells were counted with Trypan blue staining after stimulating RAW macrophage cells for 24 hours with 100  $\mu\text{g/ml}$  of the diatom *Nitzschia navis-varingica*, the haptophyte *Chrysochromulina alifera* and the dinoflagellate *Heterocapsa pygmaea* species extracts in sterile distilled water. Student t test was applied for statistical analysis, N=9.

Whole cell extracts of microalgal species *Chrysochromulina alifera* did not have any immunostimulatory effect on macrophages whereas *Nitzschia navis-varingica* and *Heterocapsa pygmaea* stimulated the macrophages even in the absence of the danger signal: *In vitro* activation of macrophages was done by using LPS from gram-negative

bacteria. LPS mimics the danger signal to stimulate macrophages and lead production of pro-inflammatory cytokines. LPS stimulate macrophages secreted a substantial amount of TNF $\alpha$ , IL1b and IL6 and these wells were specified as positive control wells. In the absence of this danger molecule, we used the whole cell extracts of microalgal species *Chrysochromulina alifera*, *Nitzschia navis-varingica* and *Heterocapsa pygmaea* at 100 $\mu\text{g mL}^{-1}$  concentration in order to examine their immuno-stimulatory potential. As shown in Figures 2-4, based on pro-inflammatory TNF $\alpha$  and IL1b production levels *Nitzschia navis-varingica* and *Heterocapsa pygmaea* mildly stimulated the macrophages after 24 hours of incubation. There was a significant production of these cytokines after 24 hours stimulation of macrophages with *Nitzschia navis-varingica* and *Heterocapsa pygmaea* whole cell extracts, but this production was not as substantial as those of positive control wells. These extracts could not stimulate macrophages to produce IL6 cytokine. Moreover, *Chrysochromulina alifera* whole cell extracts did not cause production of TNF $\alpha$ , IL1b and IL6 cytokines by the macrophages (Figures 2-4).

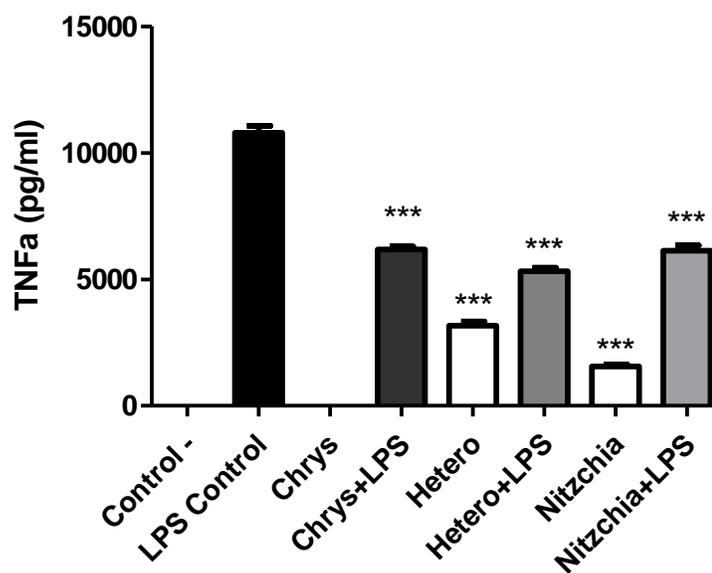


Figure 2. TNF $\alpha$  ELISA for the supernatants of RAW macrophage cells stimulated for 24 hours with 100  $\mu\text{g/ml}$  of the diatom *Nitzschia navis-varingica*, the haptophyte *Chrysochromulina alifera* and the dinoflagellate *Heterocapsa pygmaea* species extracts in sterile distilled water. Student t test was applied for statistical analysis,  $p < 0.0001$   $N = 9$ .

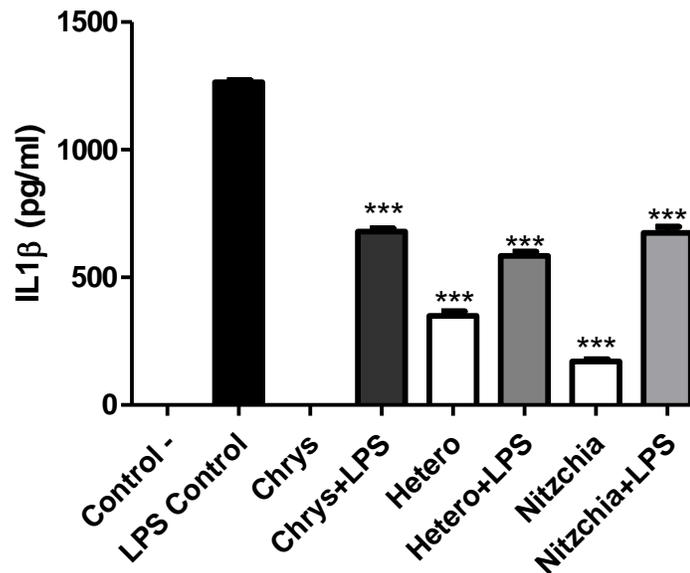


Figure 3. IL1 $\beta$  ELISA for the supernatants of RAW macrophage cells stimulated for 24 hours with 100  $\mu$ g/ml of the diatom *Nitzschia navis-varingica*, the haptophyte *Chrysochromulina alifera* and the dinoflagellate *Heterocapsa pygmaea* species extracts in sterile distilled water. Student t test was applied for statistical analysis,  $p < 0.0001$  N=9.

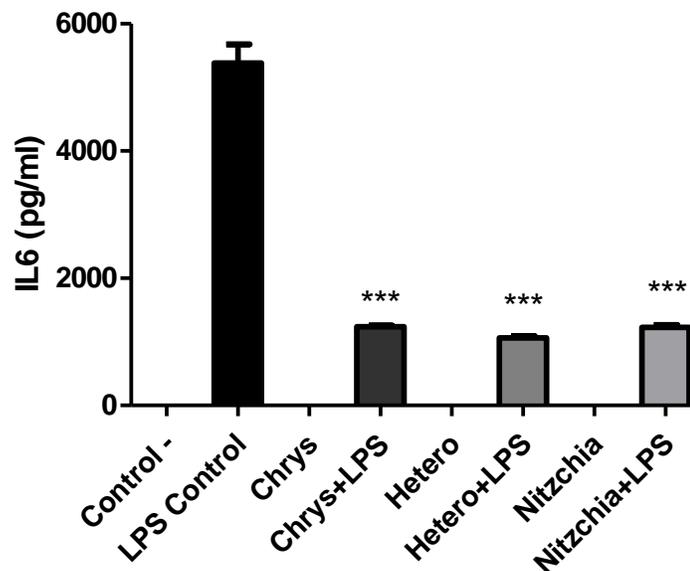


Figure 4. IL6 ELISA for the supernatants of RAW macrophage cells stimulated for 24 hours with 100  $\mu$ g/ml of the diatom *Nitzschia navis-varingica*, the haptophyte *Chrysochromulina alifera* and the dinoflagellate *Heterocapsa pygmaea* species extracts in sterile distilled water. Student t test was applied for statistical analysis,  $p < 0.0001$  N=9.

Whole cell extracts of microalgal species *Nitzschia navis-varingica*, *Heterocapsa pygmaea* and *Chrysochromulina alifera* had anti-inflammatory role based on decreases in TNF $\alpha$ ,

*IL1b and IL6 production by LPS stimulated macrophages:* In the presence of danger signal mimic LPS, immunomodulatory roles of *Nitzschia navis-varingica*, *Heterocapsa pygmaea* and *Chrysochromulina alifera* whole cell extracts were examined on the macrophages. As shown in Figures 2-4, LPS stimulated mouse macrophages produced a significant amount of TNF $\alpha$ , IL6 and IL1b compared to untreated negative control (with no danger signal). All of the microalgal extracts caused a significant decrease in the production of TNF $\alpha$ , IL1b and IL6 by LPS danger signal stimulated macrophages compared to positive control wells (Figure 2-4). This decrease was more robust in IL6 production (Figure 4). Due to their negative impact on pro-inflammatory cytokine production by danger signal LPS stimulated macrophages, whole cell extracts of *Nitzschia navis-varingica*, *Heterocapsa pygmaea* and *Chrysochromulina alifera* have immunomodulatory effect (Figure 2-4).

## Discussion

Microalgal species have been used as a food source and as medicine since ancient times in Africa, Mexico and China (Capelli, 2010; Ciferri, 1983; Bisen, 2012; Pugh, 2001). *Spirulinaplantensis* (*Arthrospira plantensis*) and *Nostoc flagelliforme* are among these microalgal species. *Nostoc flagelliforme* has been utilized for the treatment of diarrhea, hypertension and hepatitis since 400 years in China (Capelli, 2010). In our study we focused on the whole cell extracts of three microalgal species that have not been studied before: the diatom *Nitzschia navis-varingica*, the haptophyte *Chrysochromulina alifera* and the dinoflagellate *Heterocapsa pygmaea*.

Due to indispensable role of immune cells in different disease conditions ranging from inflammatory diseases to autoimmune diseases, allergies and cancer; there has been an increasing urge to discover and develop natural or synthetic molecules that can regulate the function of immune system cells (Broide, 2009; Iwalewa et al., 2007; Hancock et al., 2012; Kaufmann & Simon, 2015; Julier et al., 2017; Khalil et al., 2016; Tan & Coussens, 2007). These molecules are known as immunomodulatory molecules (Broide, 2009; Iwalewa et al., 2007; Hancock et al., 2012; Kaufmann & Simon, 2015; Julier et al., 2017; Khalil et al., 2016; Tan & Coussens, 2007).

Previous studies have shown that extracts and different fractions of different microalgal species have immunomodulatory or chemotherapeutic potentials (Dantas et al., 1999; Azamai et al., 2009). Oral administration of 500 mg/Kg of *Chlorella vulgaris* provided 55% survival rate of *Listeria monocytogene* infected mice (Dantas et al., 1999). Oral administration of *Chlorella vulgaris* extracts had definite chemopreventive effect by inducing apoptosis in hepatocarcinogenesis-induced rats (Azamai et al., 2009).

In our study, we aimed to decipher immunostimulatory and immunomodulatory potential of the diatom *Nitzschia navis-varingica*, the haptophyte *Chrysochromulina alifera* and the dinoflagellate *Heterocapsa pygmaea* whole cell extracts on mouse macrophage cells. We used 100ug/ml of these microalgal whole cell extracts and in the absence of any danger signal, only *Nitzschia navis-varingica* and *Heterocapsa pygmaea* cell extracts were able to stimulate macrophages whereas *Chrysochromulina alifera* extracts did not activate the macrophages based on pro-inflammatory cytokines TNF $\alpha$  and IL1b secretion (Figures 2-4). These extracts did not have any cytotoxic effect based on Trypan Blue staining (Figure 1), cell viability was 100% under all experimental conditions. Therefore, based on macrophage cell viability these microalgal cell extracts are safe to use.

When we examined the immunomodulatory role of these microalgal species' whole cell extracts, there was a significant and substantial decrease in pro-inflammatory TNF $\alpha$ , IL1 $\beta$  and IL6 production in LPS stimulated macrophages treated with 100 $\mu$ g mL<sup>-1</sup> of each microalgal species cell extracts compared to only LPS stimulated positive control wells (Figures 2-4). Whole cell extracts of used species have a strong immunomodulatory potential that can be utilized as anti-inflammatory drug in inflammatory bowel disease or it can be given as a supplement with the allergen foods in order to develop tolerance in patients with food allergies. Since the whole cell extracts were used and there was no extra extraction and fractionation, large scale production of these microalgal species would be relatively cheaper and economical.

In conclusion, our results support a potent immunostimulatory (adjuvant) and anti-inflammatory roles for the whole cell extracts of the diatom *Nitzschia navis-varingica*, the haptophyte *Chrysochromulina alifera* and the dinoflagellate *Heterocapsa pygmaea*. None of the extracts led to a substantial or significant change in cell viability at the used concentration. Therefore, extracts of these species could be used safely on the macrophages. In our current studies, we are deciphering their mechanism of action at molecular level in terms of different transcription factors and signal transduction pathway components. In our future studies, we will be presenting data on their in vivo efficacy and further effect on different immune system cell types.

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