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Growth responses of microalgae, *Chlorella salina* and *Isochrysis galbana* exposed to extracts of the macroalga, *Hypnea musciformis*

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ABSTRACT

The influence of methanol extract and its fractions of the marine macroalga, *Hypnea musciformis* on aquaculturally important microalgae *viz*., *Chlorella salina* and *Isochrysis galbana* were investigated. Lower concentrations of the extract of *H. musciformis* resulted in marginal growth gain for *C. salina* and *I. galbana*. However, the fractionated extract inhibited the growth of both microalgae tested, suggesting toxicity. Results signify the importance of macroalgal extract in regulating the growth of aquaculturally important microalgae.

Keywords: *Chlorella salina*, *Hypnea musciformis, Isochrysis galbana,* Methanol extract

Introduction

Marine macroalgae represent great source of majority of complex marine natural products and could be a promising source of novel bioactive compounds. These macroalgae, better known as seaweeds, synthesize a variety of secondary metabolites with complex chemical structures and different bioactivity spectra (Kannathasan *et al*., 2008). Heat stable allelochemicals present in the extracts are reported to exhibit allelopathic effects and this could be one of the promising mitigation strategies for harmful algal blooms (Tang and Gobler, 2011). Macroalgae are reported to be rich sources of organic and inorganic nutrients like calcium, magnesium, sodium, potassium and plant growth regulators like auxin, cytokinin and gibberlins which are found to be highly essential for the metabolic activities of microalgae and some of these compounds are further used as enrichers for the growth of microalgae in aquaculture ventures (Christobel and Lipton, 2010).

Worldwide, about 30 microalgal species are widely used in mariculture as live feed, due to the presence of long chain polyunsaturated fatty acids (PUFAs) *viz.*, eicosapentaenoic acid (EPA) and docosa hexaenoic acid (DHA) (Watanabe *et al*., 1989; Takeuchi *et al*., 1990). Microalgae are indispensable for early larval stages of finfish as well as shellfish species with fastidious dietary requirements that cannot be met by formulations incorporating traditional agricultural commodity products (Sorgeloos, 1996). Species of microalgae belonging to the genera *Chlorella* and *Isochrysis* have been widely utilized in marine fish hatcheries in view of transferring the essential fatty acids from the algae to fish larvae (James *et al*., 1987). Considering these facts, an investigation was undertaken to evaluate the effect of *Hypnea musciformis* extract on the growth performance of the microalgae, *Chlorella salina* and *Isochrysis galbana* and the results are presented.

Materials and methods

The red macroalga, *Hypnea musciformis* (Rhodophyta) was collected from Rameswaram/Mandapam (lat. 09° 25' N; long. 79° 20' E) regions along the south-east coast of India. Live specimens were dislodged using a metal scraper and then hand-picked. The freshly collected macroalgae were washed in filtered seawater to remove the epiphytes, sand and other extraneous materials and air-dried under shade. Completely dried material was weighed and finely ground in a mechanical grinder. The crude macroalgal secondary metabolites were extracted using methanol as per the method given in Selvin and Lipton (2004). For this, 500 g of finely powdered of *H. musciformis* was refluxed with methanol in a 5 l capacity round bottom flask. The extract was filtered and concentrated to recover the excess solvents in another distillation system and subsequently reduced to a thick viscous crude extract in a rotary vacuum evaporator (Buchi) at 40 °C. The crude methanol extract (200 mg) was further fractionated using normal phase column chromatography, C-40 silica columns (Length - 600 mm, Bore - 30 mm) of 60 - 120 µ mesh size with sintered disc and screw cock following the step-gradient solvent system from low to high polarity. The fractions obtained were again evaporated and concentrated in the rotary vacuum evaporator (Fig. 1).

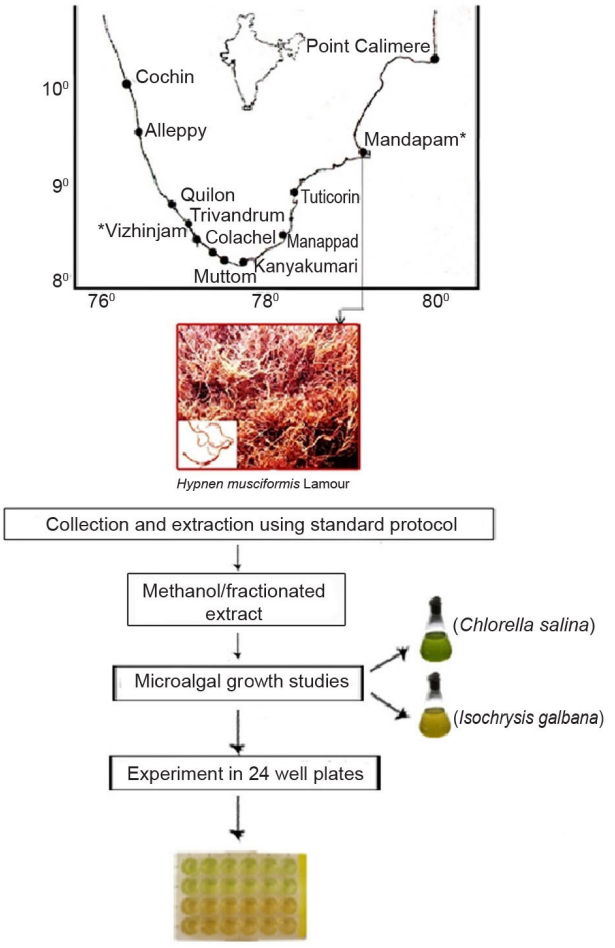

Fig. 1. Macroalgal extraction and assay procedure

Starter cultures of *Chlorella salina* and *Isochrysis galbana* were obtained from the Research Centre of the Central Marine Fisheries Research Institute, Vizhinjam and maintained in Walne's medium at a proportion of 1 ml l^{-1} Walne's A, 0.5 ml l^{-1} Walne's B and 0.05 ml of Vitamin B_1 and B_{12} (Walne, 1974). From this, 500 ml of enriched media was taken in 1 l Erlenmeyer flask and inoculated with the appropriate algal starter culture and maintained under optimum light (1000 lux) and temperature (28 ± 0.66 °C) conditions. The entire density of cells was recorded from stock culture for comparative studies.

The assay was performed in 18 mm dia 24 well plates. Selected concentrations of the extract were added to each well along with 0.2 ml of culture and the plates were incubated under illumination (1000 lux from either sides of the incubation rack) at an ambient temperature of 28 ± 0.66 °C for a period of 8 days. Walne's medium with microalgal culture without extract served as the control. From the 1st to 8th day, the contents were stirred periodically and subsequently allowed to set for 1 to 2 h. Separate microtips were used for each sampling. The density of cells were recorded using a haemocytometer on the 1st day, 2nd day and subsequently on every alternate days to determine the growth response *i.e.,* either proliferation or inhibition. The results were analyzed using one-way ANOVA using Microsoft Statistica Software Version 2.01.

Results

Addition of *Hypnea musciformis* extracts at 0.01% and 0.001% to the culture of *Chlorella salina* and *Isochrysis galbana* enhanced the growth response of cells ($p<0.05$). However, at 0.1% and 1.0%, inhibition of cell growth was noted (Fig. 2 and 3). The cell density of *C. salina* increased to 7.1 x 10^5 cells ml^{-1} and 7.8 x 10^5 cells ml^{-1} at the log phase at 0.01% and 0.001% respectively on 2nd day of culture compared to the reference culture cell density of 6.2 x 10^5 cells ml^{-1} and an increase of 15 to 25% viable cell numbers was noted as compared to the control group. At the stationary phase on 4th day, in *C. salina*, 2 to 12.5% and on 6th day 9 to 13% growth enhancement was observed at

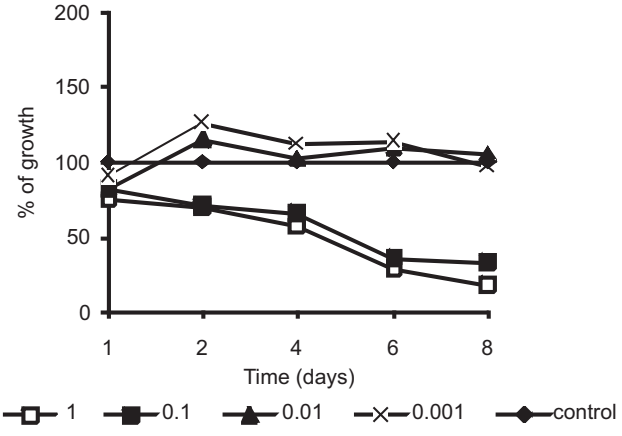

Fig. 2. Growth of *Chlorella salina* exposed to methanol extract of *Hypnea musciformis*

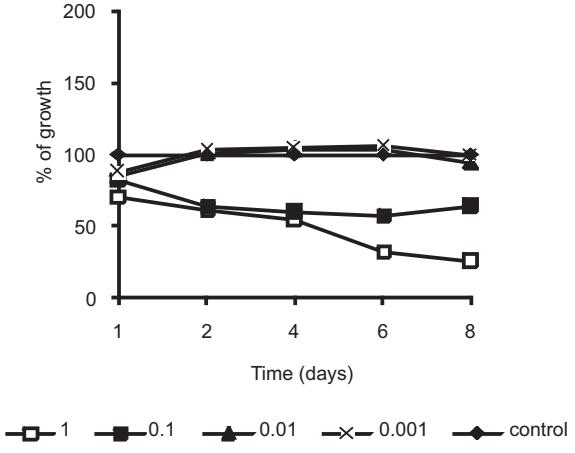

Fig. 3. Growth of *Isochrysis galbana* exposed to methanol extract of *Hypnea musciformis*

0.01 and 0.001% respectively. *I. galbana* exhibited 3 to 6% growth enhancement during the stationary phase and then declined. In *C. salina*, 1% extract level inhibited the cell growth to the extent of 24.5, 30.64, 42.22, 71.81 and 82.92% on 1st, 2nd, 4th, 6th and 8th day respectively. More or less 80% inhibition was noted at 0.1% in the stationary phase on 8th day. Upto 74.7% and 42.5% inhibition was noted in *I. galbana* at 1.0% and 0.1% level respectively.

Fractionated extract of *H. musciformis* at 0.001% and 0.01% level, inhibited the growth of *C. salina* and *I. galbana*. The 0.01% fractions of H_2 (Hexane - 100%), HB5a (Hexane:Benzene -80:20%), HB1a (Hexane: Benzene - 70:30%), HB2b (Hexane; Benzene 60:40%) and H_4 (Hexane -100%) obtained from *H. musciformis* inhibited the growth of *C. salina* to the extent of 93.44, 87.19, 80.63% on 8th day and 73 and 66.67% on 6th day (Fig. 4). The 0.001% of HB1a fraction reached declining level on 8th day post-exposure while H_2 showed a regaining trend from 6th day onwards towards the control level. Same trend was also shown by the fraction of HB2b at 0.001% level. H_4 fractions also exhibited lower level of toxicity and the growth was restored near the control group at 0.001% level (Fig. 5). For *I. galbana*, the fractions of *H. musciformis viz*., H_4, HB2b, HB5a, HB1a and H_2, induced inhibition to the extent of 95, 90.5, 90, 77.11% on 8th day and 36.5% on 2nd day at 0.01% (Fig. 6). A drastic decline in growth of *I. galbana* with HB2b and H_4 at 0.01 and 0.001% was noticed from 6th day onwards. In case of H_2, the growth was reduced to 7.69% on the 4th day and restored to that of the control values at 0.001% for *C. salina*. The 0.01and 0.001% of the HB1a fraction from the 4th day onwards indicated a steady state of stationary phase up to the end of 8th day (Fig. 7).

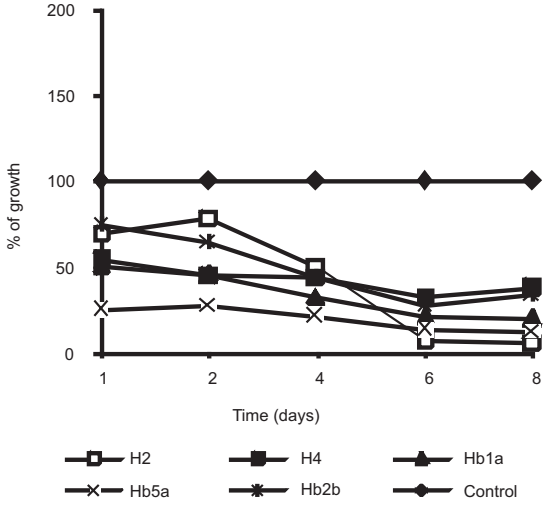

Fig. 4. Growth response of *Chlorella salina* towards fractionated extract of *Hypnea musciformis* at 0.01%.

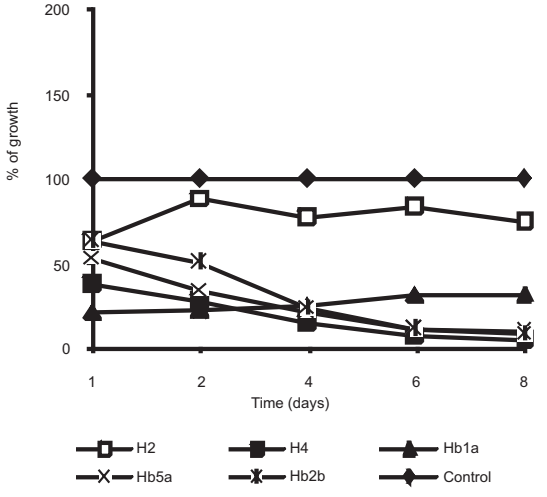

Fig. 6. Growth response of *Isochrysis galbana* towards fractionated extract of *Hypnea musciformis* at 0.01%.

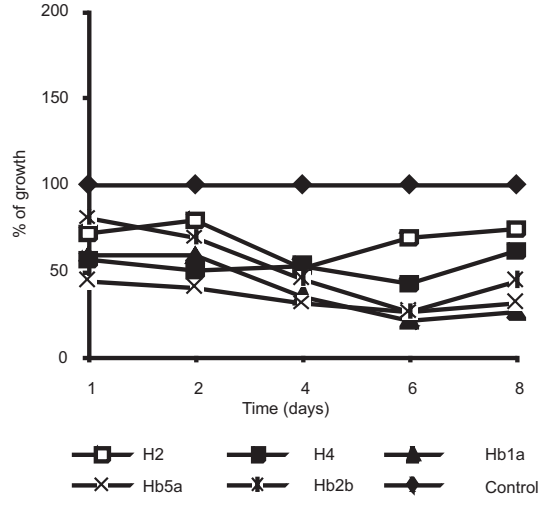

Fig. 5. Growth response of *Chlorella salina* towards fractionated extract of *Hypnea musciformis* at 0.001%.

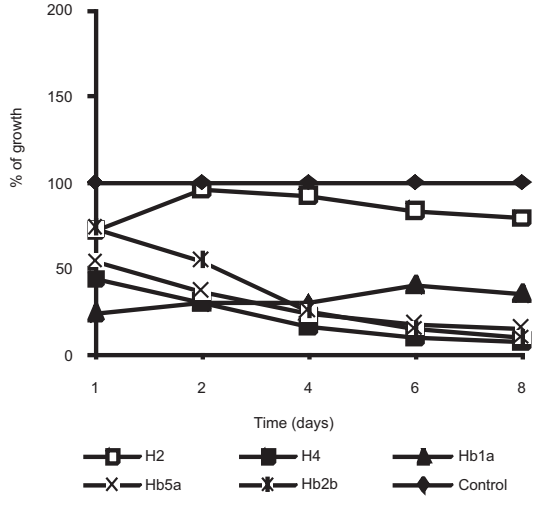

Fig. 7. Growth response of *Isochrysis galbana* towards fractionated extract of *Hypnea musciformis* at 0.001%.

Discussion

The macroalga *H. musciformis* has been proven as a rich source of minerals and plant growth regulators (Mumford and Miura, 1998). The use of this marine alga with high protein and mineral content could be a promising way for the utilization of this marine resource that remain under-exploited along the Indian coasts (Johri and Mitra, 2001) The study was initially mooted to gain insights into the toxicity as well as difference in growth characteristics of microalgae up on supplementation of macroalgal extract in the media.

From the results, it is evident that the lower concentrations of methanol extracts of *H. musciformis* could enhance the growth of microalgae, *C. salina* and *I. galbana* at very low levels of 0.01 and 0.001% incorporation. Previous studies in our laboratory have shown that the methanol extracts of *H. musciformis* influenced the growth response of *Nannochloropsis* sp. and *C. salina* at 1 mg ml^{-1} (Selvin, 2001). The possibilities of seaweed-based cytokinins, with the functional roles of promoting terrestrial plant growth have been reported by Sun *et al*. (1998). Cell growth stimulation of marine microalgae, *Nitzschia closterium* and *Platymonas subcordiformis* at lower concentrations of toxicants were also discussed by Huang *et al*. (2002). However, fractionated extracts of *H. musciformis* completely inhibited *C. salina* and *I. galbana* at 0.01 and 0.001%. This could be attributed to the growth regulation substances available in the extracts and the possible presence of organic compounds of pharmaceutical importance which could inhibit the growth of microalgae in the fractionated extract of *H. musciformis*.

Tang and Gobler (2011) examined the effects of the macroalga, *Ulva lactuca*, collected from estuaries of New York, USA, on the growth of seven common HAB (Harmful Algal Bloom) species *viz., Aureococcus anophagefferens*, *Chattonella marina*, *Cochlodinium polykrikoides*, *Karlodinium veneficum*, *Karenia brevis*, *Prorocentrum minimum* and *Pseudo-Nitzschia multiseries*. Their results indicated that fresh thalli of *U. lactuca* added at environmentally realistic levels (mg l^{-1}) were capable of lysing or strongly inhibiting the growth of all seven HAB species in a dose-dependent manner within controlled laboratory experiments. The extracts of dried and powdered *U. lactuca* exhibited dramatic allelopathic effect on the HAB species.

Growth inhibitory effect of the extract of *Corallina pilulifera* towards *Cochlodinium polykrikoides* at a concentration of 50 µg ml^{-1} compared to the reference culture level was reported by Jeong *et al*. (2000). According to Jin *et al*. (2006), the acetone and chloroform extracts of *U. pertusa* had no apparent effect on the growth of *Alexandrium tamarense* and *Prorocentrum micans*. In the present study, all the tested fractions of macroalgal extract had inhibitory role. Kakisawa *et al*. (1988) isolated microalgal growth inhibitory substances from brown alga *Cladosiphon okamuranus* and reported that the isolated substances were active against phytoplankton without cell coverings and inactive to ones with rigid cell walls. Therefore, the difference in outermost layer structures among *Chlorella salina* and *Isochrysis galbana* cells could be attributed to their sensitivity differences to the bioactive substances of *Hypnea* sp. Kroes (1972) fractionated *Chlorococcum ellipsoideum* and found that only the lipophylic fractions had an initial inhibiting effect on *Chlamydomonas globosa* whereas the high molecular weight fraction exhibited an initial promoting effect with a subsequent inhibiting effect. Hence, the varied response characteristics of microalgae exposed to *H. musciformis* extracts could be important from the pharmaceutical as well as aquacutlural point of view.

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