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NOVEL IMMUNOMODULATORY EFFECT OF *GRACILARIA VERRUCOSA* AND *POTAMOGETON PECTINATUS* EXTRACTS ON *IN VITRO* ACTIVATION OF T CELLS

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ABSTRACT

Plants are a rich source for immunomodulatory substances which are capable of activating or regulating mammalian immune response. Here we have studied the effects of aqueous crude extracts of two weed plants inhabiting the Chilika lake ecosystem of Orissa, India, *Gracilaria verrucosa* and *Potamogeton pectinatus* on T cell activation status. Flow cytometric analysis of T cell activation status by analysing cell viability and expression of early activation marker CD69 indicated that crude extracts (CE) of both plants possessed significant immunomodulatory properties. These were associated with spectroscopic analysis of the extracts by nuclear magnetic resonance (NMR) spectroscopy and UV-Visible spectroscopy. From the results, we propose that while both the extracts are immunostimulatory in nature, their higher doses lead to cellular cytotoxicity. However, in low doses, the extracts are found to be activating T cell responses as depicted by elevated CD69 expression on $CD3^+$ T cells. The immunomodulatory effect is apparently related to the presence of carbohydrate moieties in the extracts as suggested by NMR spectral analysis. Further studies are warranted to elucidate the detailed mechanism of action of the extracts and to pin down the exact active components responsible for the activity.

Key words: Immunomodulation, Chilika Lake, *Potamogeton pectinatus*, *Gracilaria verrucosa*, T cell activation

INTRODUCTION

Immunomodulators are substances which exert pharmacological or biological effects on immune response (Bin-Hafeez *et al.* 2003, Schepetkin *et al*. 2006). Those agents or compounds which can regulate individual mechanisms of immunity have the potential of being developed into drugs. The term immunomodulation usually represents a change in the parameters associated with the immune system activity by an agent when it acts under varying dose or time regimens (Abd-Alla *et al.* 2009). Immunomodulating action of biologically active substances can manifest either as immunostimulation or immunoactivation which is strengthening of the immune response or as immunosuppression which is the regulation of immune reactions.

Several plants and their isolated constituents have been reported to possess the ability to stimulate the

immune system. This is mostly because plants produce a large number of natural products that have antimicrobial and immunomodulatory effects, in order to ward off natural enemies and to adapt to environmental conditions. Some of these compounds include isoflavonoids, phytosterols, polysaccharides, alkaloids, sesquiterpenes, glucans, tanins, vitamins, and a variety of other phytochemical substances (Taleb-Contini *et al.* 2006, Chiang *et al.* 2003). Several medicinal plants have been reported to exert anti-inflammatory, anti-stress and anti-cancer effects by modulating the immune functions (Desai *et al*. 2002, Kumar *et al.* 2006). In addition to this, immunological activity of plants and immune system of mammals have many features in common (Schepetkin *et al.* 2006). These characteristics allow for the potential use of plant extracts and compounds in immunomodulation. Currently, a number of plant products are being investigated for immune response modifying activity (Sacerdote, 2006). Hence the modulation of immune response with the aid of various bioactives is an area of considerable interest.

Marine and brackish water algae contain various biologically active compounds which have been used as source of food, feed and medicine. Until now, more than 2400 marine natural products have been isolated from seaweeds of subtropical and tropical populations (Manilal *et al*. 2009). There are regional plant species which are not reported in medicinal literature but possess significant ability to modulate mammalian immune responses. Ecosystems like those present in the Chilika region (Orissa, India) harbour rare plant species which possess beneficial effects in terms of immunomodulation. Some of the flora which are posing a threat to the ecosystem biodiversity (for e.g., weeds like *Phragmites karka*) may turn out to be economically important if these can be used as potential immunomodulators. Even plants for which immunomodulatory effects have been cited, the mechanisms are poorly understood. Hence the use of Chilika as a model ecosystem to evaluate plants for medicinal and immunomodulatory activities can reveal the most likely candidates and elucidate the mechanisms by which these effects are mediated. Moreover, our studies are suggestive that this might serve as a plausible alternative to convert the menace of invasive weeds in Chilika to a blessing in disguise.

MATERIALS AND METHODS

Preparation of crude extracts

The plant species, *Gracilaria verrucosa,* a member of red algae (Rhodophycaeae) and *Potamogeton pectinatus*, an underwater angiosperm, were collected from the backwaters of the Chilika Lake (Orissa, India) during February 2011. The plant tissue was rinsed with distilled water thoroughly to remove dirt and other attached particles. The crude extract was obtained from the dry whole plant tissue of the plants. 1 g of shade dried plants was weighed and powdered using mortar and pestle by adding liquid nitrogen. This powder was homogenized completely in 10 ml double distilled water. The resultant mixture was autoclaved in 50 ml Falcon tubes and the warm extract was filtered repeatedly using cheesecloth. The aqueous extract was then aliquoted into 1.5 ml Eppendorf microfuge tubes and stored at 4 °C for future use.

UV-Vis Spectra analysis

The UV-Vis spectra of aqueous extracts were measured in the range of 200 – 800 nm using a NanoDrop2000 Analyzer (Fischer Scientific Ltd.)

NMR analysis of the plant extracts

The plant extracts were dried by evaporation on a dry bath at 60°C and the residues were dissolved in 500 µl of D_2O (Heavy water). The $_1H$ NMR of the solutions was performed using a 400 M Hz NMR spectrometer.

Cell lines

Jurkat (JKT cells, a human T cell line) cell line was used to perform *in vitro* T cell activation assay.

Cell culture

The cells were cultured in 75 ml cell culture flasks first and later in 24 well cell culture plates for treatment with crude extracts by using Iscove's Modified Dulbecco's Medium containing 10% fetal bovine serum and were incubated at 37°C in the presence of 5% CO_2 (Morgan *et al*. 1992).

Treatment of cells with crude extracts

The clarified crude extracts were sterilized by millipore filter with 0.2 µm pore size inside the laminar flow before treatment. The cells were treated with three different concentrations of either of the extracts on a log scale. 1 µl, 10 µl and 100 µl of either *Gracilaria* or *Potamogeton* extract were added to the corresponding wells in a 24 well plate and the plate was incubated overnight inside a CO_2 incubator. As a positive control, Concanavalin A (ConA) 5µg/ml was used for cell culture.

Trypan blue exclusion test

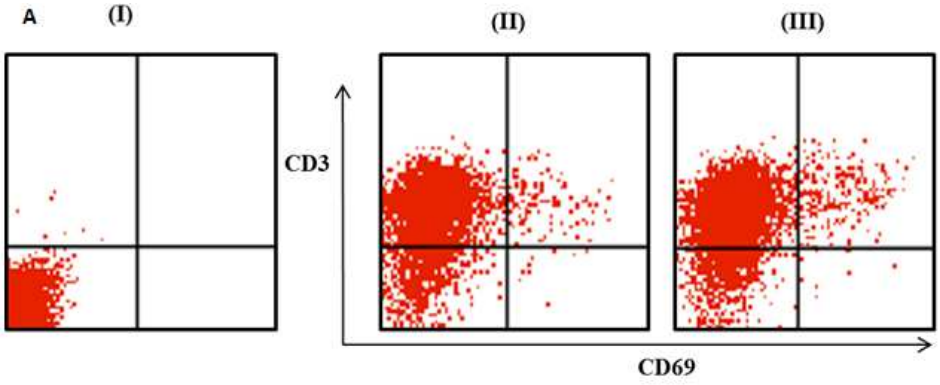
In the first step to determine the viable cell number before and after treatment by the aqueous crude extracts, trypan blue exclusion test was used as a semi quantitative method. Briefly, the cells were seeded at a density of 1.15×10^6 cells/well and they were treated with different concentrations of the aqueous extract for 72 h at 37°C in the presence of 5% CO_2. After 24 h, 10 µl of medium and equal volume of trypan blue were mixed and viable and dead cells were counted by a haemacytometer (Morgan *et al*., 1992).

Flow cytometry assay

Microfuge tubes containing 24 h cell cultures were added with lysing reagent and were incubated 15 min at 37°C. Following centrifugation, the supernatant was removed by aspiration. Each cell pellet was resuspended in phosphate-buffered saline (PBS). Single cell suspensions of Jurkat cells thus obtained were stained with phycoerythrin-tagged anti-CD69 and allophycocyanin (APC)-tagged anti-CD3 for 30 min at room temperature along with isotype as negative control. The cells were then washed once with PBS containing 1% fetal bovine serum and resuspended in PBS. Cell-associated fluorescence was assessed with a BD FACSCalibur flow cytometer equipped with CellQuestPro software. A gate including live cell populations was drawn by correlated analysis of forward and right-angle scatter characteristics of lymphocytes. Under this cursor setting, the cell samples were then analyzed for CD3 and CD69 expression. A total of more than 10,000 cells were analyzed for each sample.

RESULTS AND DISCUSSION

T cell activation is promoted by low doses of Gracilaria and Potamogeton extracts: CD69 is a lymphoid activation antigen whose rapid expression (< 2 h post activation) makes it amenable for the early detection of T-cell activation (Cambiaggi *et al.*, 1992). In the study, using flow cytometry, we evaluated the CD69 expression on T cells treated with crude extracts. Cells treated with Concanavalin A (ConA), an established T cell mitogen, were used as positive control for T cell activation (Fig. 1). In the present experimental set up, we have used Jurkat T cell line, where CD69 expression was found to be increased (Fig. 1) in a time dependent manner. Here we have studied the effect of aqueous extracts of *Gracilaria verucosa* and *Potamogeton pectinatus* for 24h to study T cell-associated immunomodulation. CD69 expression levels in cell samples treated with low doses of both the plant extracts at 24 h time point (as an early effect) were found to be comparable to ConA-driven T cell activation (Fig. 2).



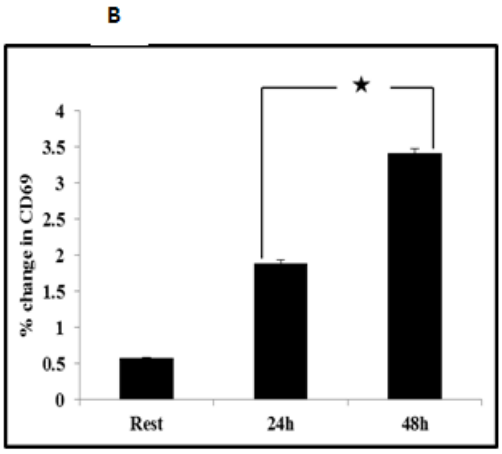


Figure 1. ***ConA driven in vitro T cell activation by elevation of $CD3^+$ $CD69^+$ T cells.***
A. Flow cytometric analysis (Dot-plots) demonstrating CD69 expression on $CD3^+$ JKT T cell line. The numbers [i-iii] in the upper right quadrant of each panel represents the frequency of CD3 cells expressing CD69 (i) Negative control of unstained cells with autofluorescence. (ii)CD3 and CD69 fluorescent conjugated antibody stained unstimulated/resting T cells ((iii) ConA activated T cells
B. Histogram showing T cell activation by ConA, as mentioned in materials and methods. Bar graph represents mean value, Error line represents SD value, ANOVA Statistical test was performed to assess the significance of the differences between the mean values from control and experimental groups and to calculate p value (⋆ P<0.05) (n=3)

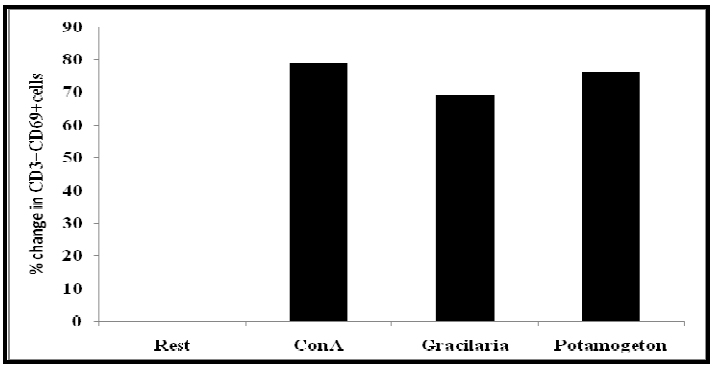


Figure ***2 In vitro treatment with low doses of the plant extracts promote T cell activation. Histograms showing the % changes in CD69 expression in treated samples. CD69 expression of cells treated with Gracilaria and Potamogeton extracts at a dose of 1 µl is similar to 5µg/ml ConA activated cells. Above result is the representative of two independent experiments (n=2).***

The plant extracts induce cytotoxicity at higher concentrations:

Trypan blue exclusion test provides insight on the viability of the cells under treatment and thus about cytotoxicity of the treating agent (Morgan *et al.,* 1992). The variation in expression of CD69 marker with concentration of the crude extract of *Potamogeton pectinatus* was in correlation with cell viability data from trypan blue exclusion test. Cells treated with both ConA and *Gracilaria verrucosa* extract together showed increased CD69 expression at lower and moderate concentrations of the extract but the marker expression decreased at higher doses and this correlated with the trypan blue count (Fig.3).

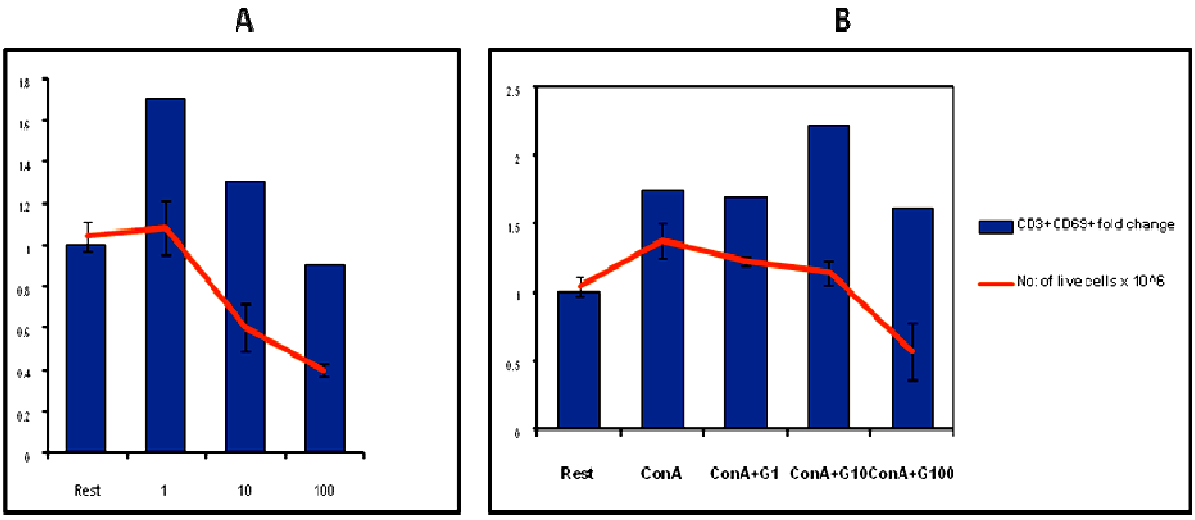


Figure 3. ***Graphs showing the fold changes in CD69 expression, and number of live cells in treated samples. Blue bars indicate fold changes and the red line plots represent the number of live cells per treated sample from trypan blue exclusion tests. A. Higher doses of Potamogeton (P100) lead to decreased CD69 expression associated with the induction of cellular cytotoxicity. B. Combined treatment with ConA and Gracilaria extract leads to elevated expression of CD69 (ConA + G1, ConA + G10) while at higher doses (ConA + G100), expression decreases due to cytotoxicity***

Major components in the plant extracts include carbohydrate and amino acid residues: The analysis of UV-Vis spectra of *Potamogeton pectinatus* shows the presence of characteristic peaks associated with aldehyde, keto and aromatic amino acid groups. Similarly, the NMR spectrum also portrayed peaks in the 3-4 ppm chemical shift region (Fig. 4) which suggests the presence of carbohydrate moieties (Jansson *et al*., 2006). These compounds might be the agents behind the immunomodulatory effects possessed by the extracts.

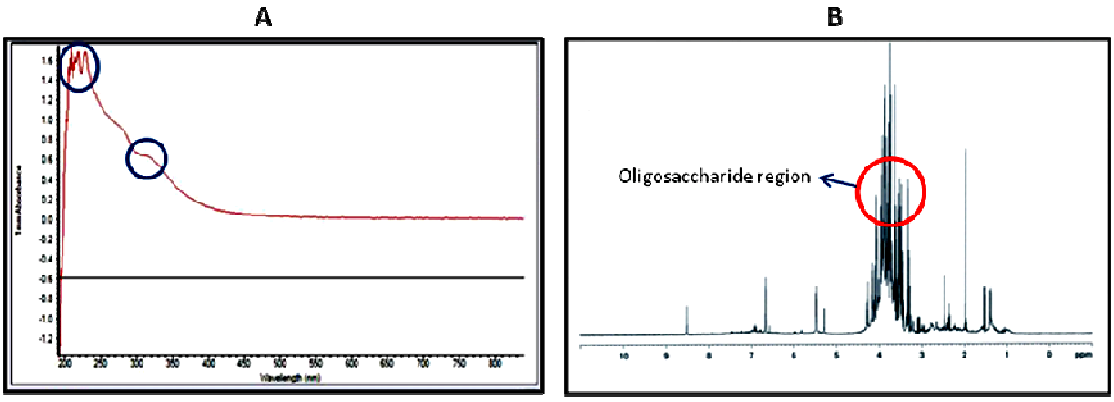


Figure 4. ***UV-Vis absorption and nuclear magnetic resonance spectra of Potamogeton pectinatus. A. Absorption spectrum shows peaks indicative of presence of aldehyde, keto and aromatic carboxyl groups. B. NMR spectrum shows messy peaks in the 3-4 ppm region, suggestive of the presence of carbohydrate residues.***

CONCLUSIONS

The crude extracts of *Gracilaria verrucosa* and *Potamogeton pectinatus* possess the ability to enhance *In vitro* T cell activation and hence show potential immunomodulatory activity(Fig. 1 and 2). However, they were found to be cytotoxic at higher concentrations (Fig. 3). There is a possibility that this toxicity arises as the extracts induce activation induced cell death (AICD) of T cells at elevated doses. Analysis of NMR and UV-Vis spectra of the extracts reveal the availability of sugar and amino acid groups suggestive of the presence of carbohydrates or glycoproteins which might have lectin-like functionality. We speculate that these compounds in the plant extracts might be responsible for the immunomodulatory effects. Isolation and characterization of these components and further evaluation of their action can be the initial steps towards unravelling the detailed mechanisms behind immune activation by these newly identified plant products.

To the best of our knowledge, this is the first study to report the immunomodulatory property of these plant species from Chilika lagoon, Orissa. We hope that this would lead to more such studies on the Chilika ecosystem and shed light on novel plant products with future possibilities of translational research towards host cell immune activation.

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