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Research Article

A SIMPLE AND RELIABLE ASSAY TO DETECT IMMUNOSUPPRESSANT ACTIVITY IN MACROALGAE

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ABSTRACT

The possible immunosuppressant activity of extracts of *Ulva fasciata, Hypnea valentiae* and *Hypnea musciformis* was evaluated by fish scale transplantation assay. The crude *Hypnea* extracts exhibited delayed acceptance of transplanted scale from 4th day onwards in the F_2 progeny of *Poecilia reticulata* at 0.001% level compared to fractionated extract of *Ulva fasciata*. No rejection of transplanted scale was noticed at 0.01% of Ethanol-fresh extract of *U. fasciata* (EF) fraction up to 10th day of experiment. Rejection of transplanted scale was noticed in the case of acetone and ethanol - fresh extract among the F_2 progeny from 3rd and 4th day respectively.

Keywords: *Ulva fasciata, Poecilia reticulata, Hypnea valentiae, Hypnea musciformis.*

INTRODUCTION

Pharmaceutical market is growing rapidly and continuously and the reason behind this motivation can be the growing numbers of drug-resistant infectious disease and more and more upcoming disorders. The terrestrial resources have been greatly explored and thus academic and industry researchers are striving to get lead molecules from the inner space of oceans[1]. Marine organisms are rich sources of secondary or primary metabolites and are potential bioactive compounds of interest in the pharmaceutical industry. To date, many chemically unique compounds of marine origin with various biological activities have been isolated, and some of them are under investigation and are being used to develop new pharmaceuticals. The secondary metabolites of macroalgal sources from oceans are also equally effective like the marine fauna. Macroalgae are considered as a source of bioactive compounds as they are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities. Research and utilization of marine algal community have increased markedly that directly offers enormous untapped reservoir of novel drug leads endowed with ingenious structure and potential biological activities. In our previous studies, antifouling, anticoagulant, glucosidase inhibitory properties, antimicrobial activities of aqueous extract of macroalgae, growth responses of microalgae and selective cytotoxic activities of macroalgae from the Indian coast have already been reported[2-7].

Usage of cyclosporins (Neoral, Sandimmune, SangCya), Azathioprines (Imuran), corticosteroids such as prednisolone (Deltasone, Orasone) and commercially available other immunosuppressant therapeutants is of great significance of improved graft survival rates with corresponding decrease in rate of rejection due to their immunosuppressive action. Immunosuppressant drugs also called anti-rejection drugs are used to prevent the body from rejecting a transplanted organ. Such immunosuppressant drugs find application in the management of various autoimmune diseases. However, its clinical and experimental use is hampered by several side effects[8]. Nephrotoxicity is one of the main side effects of cyclosporine therapy. Other common metabolic abnormalities include: hyperleukemia, hypomagnesemia, hyperlipidemia, hypertension

and hirsutism. Increased risk of infection is a common side effect of all the immunosuppressant drugs. Immunosuppressant drugs also lead to increased risk of cancer because the immune system also plays a role in protecting the body against some forms of cancer. Other minor side effects of immunosuppressant drugs include: loss of appetite, nausea and trembling or shaking of the hands[9]. Although macroalgae were widely used for various biological studies, its immunosuppressant properties through fish scale transplantation assay have not yet been pharmacologically evaluated. Hence, the present study was undertaken to evaluate a rapid scale acceptance or rejection test using the guppy, *Poecilia reticulata* as the model and to examine whether marine macroalgal metabolites do have immunosuppressant activity.

MATERIALS AND METHODS

Collection and Extraction of macroalgae

The Chlorophyta (green) macroalgae, *Ulva fasciata* Delile, 1813 and Rhodophyta (red) macroalgae, *Hypnea valentiae* (Turner) Montagne were collected from the intertidal rocks of Vizhinjam (Lat. 08° 22' N; Long. 76° 59'E) Southwest coast of India and *Hypnea musciformis* Lamour (Rhodophyta) was collected along the Rameswaram/Mandapam (79° 20'E Long; 09° 25' N Lat) regions, Southeast coast of India (Figure 1). Immediately after collection, the macroalgae were washed in filtered fresh seawater to remove the epiphytes, sand and other extraneous matter. After draining off the water, the algae were wiped with a blotting sheet and air-dried under shade. They were then cut into small pieces and dried in an incubator at 37°C. Completely dried material was weighed and ground finely in a mechanical grinder. The crude macroalgal secondary metabolites were extracted using methanol[10]. In this process, 500 g of finely powdered algal material 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. Finally, it was reduced to a thick viscous crude extract in a rotary vacuum evaporator (Buchi) at 40°C. Crude extract (200 mg) was further fractionated using normal phase column chromatography, C-40 silica columns (Length-600 mm, Bore-30 mm) of mesh size (60-120 μ) with sintered disc and screw cock following step gradient solvent system from low to high polarity. The fractions obtained were once again evaporated and concentrated using rotary vacuum evaporator.

Experimental fish - Guppies, *Poecilia reticulata* (Peters, 1860)

To evaluate the immunosuppressant activity of these macroalgal extracts, the scale transplantation experiments were designed using the guppy, *Poecilia reticulata*[11] (Figure 2). *Poecilia reticulata* females with round tail and males with delta-fan tail (each 20 nos.) obtained from ornamental fish retailer from Trivandrum, Kerala were transported to the laboratory in oxygenated water in polythene bags. Fishes were transferred to 50 L glass aquaria and acclimatized to the laboratory conditions. Rearing and breeding tank were set with lighting for 10 h at an ambient temperature range of 28.0 ± 2.5°C and with 50% water exchange/day. In the breeding tanks, the fishes were maintained at the standard density of one female and two males. All fish were fed twice daily with pellet feed (CP feeds) and young ones were fed with freshly hatched *Artemia* nauplii. The newly born F_1 & F_2 progenies were separated from their respective mother and maintained in separate glass aquaria.

Standardization of anesthesia

Anaesthetic dose was standardized with commercially available clove oil containing 70-80% eugenol [2-methoxy-4-2-(2-prophenyl)-phenol][12] through bath treatment experiment and conducted in glass aquaria (200 × 300 × 200 mm) with 2 litres of aerated fresh water at 27°C. The dose of 100 ppm was found to be effective for the guppy giving an induction time of 3.0 ± 0.4 minutes and with a recovery period of 1.0 ± 0.1 minute. The induction and recovery periods were recorded using stopwatch. Fishes were individually tested thrice to find out the sedation time and finalize the effective dose. After recovery period, the anaesthetised fishes were transferred to experimental tank containing fresh aerated water.

Transplantation experiment

Fishes were divided in to 4 batches of 5 individuals each for experiments to record the possible immunosuppressant activity. Each group was maintained in separate tank. The *Ulva fasciata* fractions (Ac05- acetone extract, DM2- Dichloromethane extract, EF- Ethanol-fresh extract) and methanol extracts of *Hypnea valentiae* and *H. musciformis* were applied at 0.1%, 0.01% and 0.001% to experimental group of fish through 'hyper-osmotic infiltration method', in which individual fish were dipped in to the hypersaline solution for 10 seconds (1.5% salt added to water), taken outside and dipped in to the extract appropriate at the requisite concentration for 1 minute.

The scale transplantation was made from female to female guppy (allograft) of F_1 & F_2 generation (weight range of 1.4 ± 0.5 g and length range of 5.2 ± 1.0 cm). Experimental guppies were anaesthetised with clove oil and placed side-by-side on wet cotton in a sterilized petri dish under a dissection microscope. Using specially prepared watchmaker forceps, from the donor guppy, the 13th single scute scale was carefully removed from the lateral line and stained by safranin solution for 2 seconds and immediately taken outside, washed in distilled water to remove excess stain (for easy identification of transplanted scale) and directly fixed (implanted) into the 13th scute scale portion of recipient guppy in the vacant place (13th scute scale was already removed from recipient guppy during the time of experiment). Immediately after grafting, the fishes were allowed to recuperate quietly in a petri dish on soaked cotton. After 5 min, when the grafts got settled enough for the fish to swim, the guppies were returned to their respective aquaria. In addition, 0.001 ppm Chloramphenicol bath was given as a measure of post-operative care. The scale transplanted fish were kept under complete observation for first 24 h and every day thereafter for 10 days and the transplanted scales were observed under a stereo microscope (Olympus make) for detecting evidence of healing, inflammation and other gross signs of incompatibility.

RESULTS AND DISCUSSION

In all the experiments using guppies (*Poecilia reticulata*), complete acceptance of transplanted scale was not noted (Table1,2,3). Dose-dependent activity was recorded for the different extract doses tested. Comparing the fractionated extract of *Ulva fasciata,* the crude *Hypnea* extract exhibited delayed acceptance of transplanted scale from 4th day onwards in the F_2 progeny at 0.001% level. In short, delayed acceptance of transplanted scale started slowly and continued up to 10th day of experiment. On 9th and 10th day, the F_2 progeny in DM2 fraction did not exhibit delayed acceptance of transplanted scale. The Dichloromethane (DM2) fraction induced the rejection of transplanted scale from 4th day of F_1 progeny at 0.01% level.

No rejection of transplanted scale was noticed at 0.01% of EF fraction up to 10th day of experiment. Rejection of transplanted scale was noticed in the case of acetone and ethanol - fresh extract of F_2 progeny from 3rd and 4th day onwards respectively. During the experiment, appearance of white cloudy patches around all the transplanted scales was noted. The morphological changes observed in transplanted scales consisted of: swelling, cloudiness and disruption and disappearance of melanocytes. In the control group, there was no evidence of acceptance of transplanted allograft scales. Cloudiness and disruption of melanocytes and rejection of scales were noted from the 2nd day of experiment.

Scale or fin transplantation experiments in fish has been studied in a limited number of fish species. Cooper reported the rejection of allografts using melanophores in the transplanted scales of *Fundulus*[11]. In the macroalga, *Sargassum kjellmanianum* Yendo, the immunosuppressant compound, 3, 4-dideoxyglucosone-3-ene (DGE) was isolated and evaluated using mice model[13]. The DGE had marked suppressive effects on proliferation of bone marrow cells, L929 fibroblastoma and B16 melanoma. Treatment with DGE was effective on not only the delayed-type hypersensitivity model in mouse but also in the collagen-induced mouse arthritis model. The suppressive activity could be inferred as selective one for the immune cells.

Immunosuppressant drugs such as Cyclosporins act by inhibiting T-cell activation and preventing T-cells from attacking the transplanted organ. Azathioprine drugs disrupt the synthesis of DNA, RNA and cell division. While Corticosteroids drugs suppress the inflammation associated with transplant rejection. The results of guppy scale transplantation experiment indicated minimal immunosuppressant activity of macroalgal extracts except that of the crude *Hypnea* extract. The immunosuppressive activity of aqueous extract of *Eisenia bicyclis*, *Sargassum sagamiamum*, *Amphiroa aberram* and *Gracilaria verrucosa* were earlier reported[14]. At 0.1% concentration of macroalgal extract, mortality of guppies were noted indicating the toxicity of the extract. However, the results obtained from 0.001% concentration indicated the immunosuppressant potentiality of tested samples.

The results of the present study as well as previous report of the presence of immunosuppressants in *Sargassum kjellmanianum* (Yendo)[12] suggested that tested macroalgae are potential sources of immunosuppressant compounds and warrants further specific studies on characterization of compounds. The present scale transplantation assay has evident advantages such as: (i) Guppy is a common, small, live-bearing fish available. (ii) The test could be performed with minimum quantity of the test compound. (iii) Results can be noticed/observed within few days. (iv) The relative toxicity of test compound could also be

estimated based on the scale acceptance or rejection rate. Thus considering these merits compared to the 'mouse model assay', the scale transplantation technique could form one reliable rapid low cost one to detect the potent immunosuppressant activity from marine macroalgal extracts.

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Table 1: Immunosuppressant studies in guppies exposed to *Hypnea musciformis*

	Day 4		Day 5		Day 6		Day 7		Day 8		Day 9		Day 10	
Con (%)	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2
0.001	-	+	-	+	-	++	-	++	-	++	-	++	-	+
0.01	-	-	-	-	-	+	-	+	-	+	-	+	-	-
0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Complete Acceptance: +++; No complete acceptance: -; Delayed acceptance: ++; No rejection: +;
F1: First progeny; F2: Second progeny

Table 2: Immunosuppressant studies in guppies exposed to *H. valentiae*

	Day 4		Day 5		Day 6		Day 7		Day 8		Day 9		Day 10	
Con (%)	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2
0.001	-	++	-	++	-	++	-	++	-	++	-	++	-	+
0.01	-	+	-	+	-	+	-	+	-	+	-	+	-	-
0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-

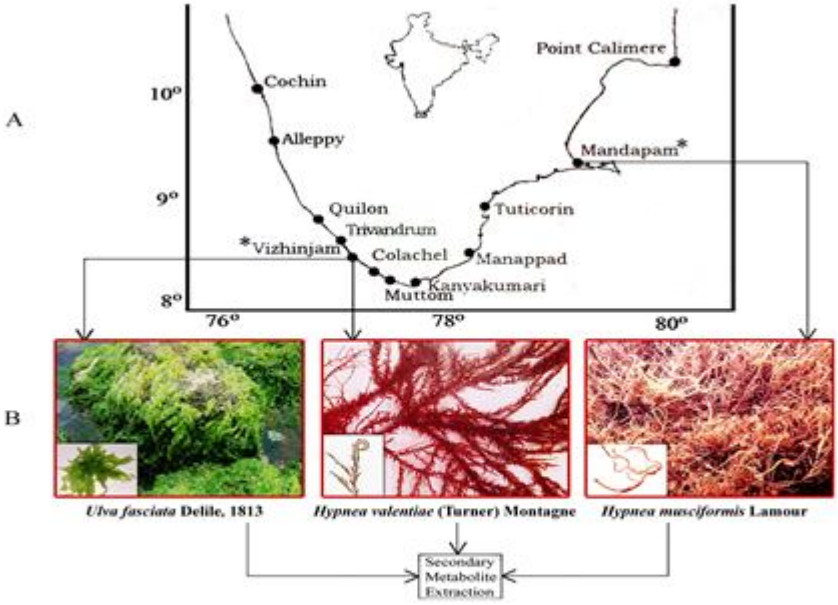
Complete Acceptance: +++; No complete acceptance: -; Delayed acceptance: ++;
F1: First progeny; F2: Second progeny

Table 3: Immunosuppressant studies in guppies exposed to fractions of *Ulva fasciata*

		Day 3		Day 4		Day 5		Day 6		Day 7		Day 8		Day 9		Day 10	
Extract	Con (%)	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2
Aco5	0.001	-	R	-	R	-	R	-	R	-	R	-	R	-	R	-	R
	0.01	-	-	-	-	-	R	-	R	-	R	-	R	-	R	-	R
	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DM2	0.001	-	-	-	-	-	-	-	-	-	-	-	-	-	NDA	-	NDA
	0.01	-	-	R	R	R	R	R	R	R	R	R	R	R	R	R	R
	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EF	0.001	-	-	-	R	-	R	-	R	-	R	-	R	-	R	-	R
	0.01	-	-	+	R	+	R	+	R	+	R	+	R	+	R	+	R
	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Complete Acceptance: +++; No complete acceptance: -; Delayed acceptance: ++; No delayed acceptance: NDA; Rejection: R; Ac05: Acetone extract (U. fasciata); DM2: Dichloromethane extract (U. fasciata); EF: Ethanol-fresh extract (U. fasciata)

Fig.1 Flow chart for screening of secondary metabolites from macroalgae



A. *Collection centers of marine macroalgae along the peninsular coast of India
B. Collected macroalgae (*Hypnea musciformis* Lamour, *Hypnea valentiae* (Turner) Montagne and *Ulva fasciata* Delile, 1813)

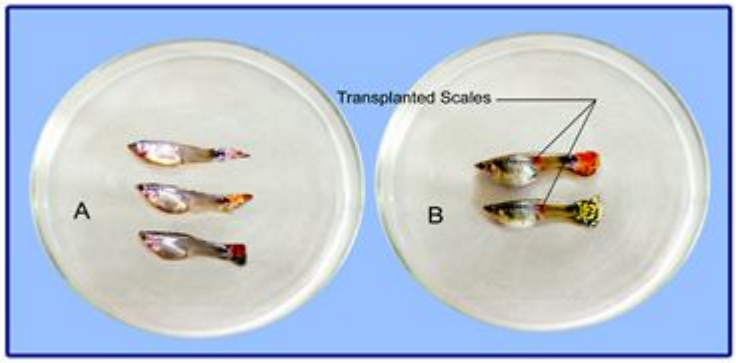


Figure 1. Anaesthetised guppies (*Poecilia reticulata*) before (A) and after (B) scale transplantation

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