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Immunomodulatory activities of ethyl acetate extracts of two marine sponges *Gelliodes fibrosa* and *Tedania anhelans* and brown algae *Sargassum ilicifolium* with reference to phagocytosis

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ABSTRACT

In vitro and *In vivo* effect of ethyl acetate fraction (ETF) of two marine sponges *Gelliodes fibrosa* and *Tedania anhelans* and brown algae *Sargassum ilicifolium* were applied on neutrophil phagocytosis activity. The different concentrations of 10, 25, 50 and 100 µg/ml ETF was tested for phagocytosis as neutrophil locomotion and chemotaxis test and qualitative nitroblue tetrazolium test using human neutrophils. Further on Swiss albino mice, of either sex weighing 18-25g, selected dose of ETF concentrations of 25, 50, 100 mg/kg body weight were administrated po for macrophage phagocytosis activity by carbon clearance test. *In vitro* study revealed that *Tedania anhelans* and *Sargassum ilicifolium* has stimulated chemotatic, phagocytic and intracellular killing of human neutrophils at a dose of 50 µg/ml and 100µg/ml respectively. *In vivo* studies of both sponge species have shown moderate immunostimulator activity, whereas algal species have shown prominent immunostimulator activity.

Keywords: *Gelliodes fibrosa*, *Tedania anhelans*, *Sargassum ilicifolium* Macrophage phagocytosis, Nitroblue tetrazolium test, Carbon clearance test.

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INTRODUCTION

The Marine sponges and seaweeds have created a promising significance in the biomedical area, mainly because of their contents of bioactive substances which show great potential as anti-inflammatory, antimicrobial, antiviral, and anti-tumoral drugs [1]. Indeed, several species of sponges and brown algae have been found to have immune-stimulant, antitumoral, or antiviral activity [2] and these bioactive compounds are identified as steroids, terpenoids, isoprenoids, sesquiterpenes.

Immunomodulation is explained as any change in the immune response and may involve induction, expression, amplification of any part or phase in the immune response. Modulation may be very specific limited to a given antigen/agent or non-specific, with a great effect on immune response. Stimulation of the immune response is preferred for certain people such as immunocompromised patient. Whereas, suppression of immune response is consideration for others such as transplant recipient or patient autoallergic or inflammatory diseases [3]. Several *in vivo* and *in vitro* models are available for screening immunomodulation activity [4]. Phagocytosis is one of the extensively used method for screening immune response.

Sargassum ilicifolium, *Gelliodes fibrosa* and *Tedania anhelans* are extensively distributed in Western coast of India. However, little information about its chemical constituents and biological activity is available compared with those of other sponges and brown algae. Therefore, in the present study an attempt has been made to evaluate these marine extracts for their immunomodulatory potencies using *in vitro* and *in vivo* models for phagocytosis.

EXPERIMENTAL

Materials

Test organisms

Gelliodes fibrosa and *Tedania anhelans* and *Sargassum ilicifolium* were widely distributed intertidal organisms and was collected manually from Western coast of India. *Gelliodes fibrosa* and *Tedania anhelans* were authenticated by Dr P.A.Thomas Retd. Principal Scientist ICAR, Trivandrum while *Sargassum ilicifolium* was authenticated by Dr K.Baligereranga. Dept of Botany, Govt Arts and science college Karwar. Voucher specimens (GF-2003, TA-2003 and SI-2003) are deposited in our laboratory (BLDEA COP Bijapur).

Animals used

Swiss albino mice of either sex, weighing 18–25 g each, were used. They were housed under standard conditions of temperature (25±10ºC) and relative humidity (60±10%), 12/12 h light/dark cycle, and fed with standard pellets and water *ad libitum*. Animal experiments were performed in accordance with the CPCSEA norms and obtained from BLDEA Medical College Bijapur Animals House. (REF: 1076/c/07/ CPCSEA).

Methods

Preparation of extract

After removing all adhered materials with knife *Gelliodes fibrosa* and *Tedania anhelans* were extracted with methanol. Whereas *Sargassum ilicifolium* was washed with sea water and later repeatedly by tap water to remove epiphytic growth. All the species were then dried under shade for about 4 to 5 days, powdered and was then subjected to methanol extraction (maceration). All the methanolic extracts were

subjected for partitioning with ethyl acetate. ETF were then concentrated and dried under reduced pressure separately.

Preparation of samples

Stock solutions for *in vitro* and *in vivo* studies were prepared by dissolving ETF in Dimethylsulphoxide (DMSO) and diluted with normal saline to obtain required concentration.

In-vitro Immunomodulation study

Qualitative Nitrobluetetrazolium (NBT) Test

A suspension of leucocytes (5 x 10^6 / ml) was prepared in 0.5 ml PBS solution in five test tubes. 0.1 ml endotoxin-activated plasma (standard) and 0.1 ml of PBS solution (control) was added in first two test tubes, while to remaining test tubes 0.1 ml different concentrations of test samples (25, 50, 100 µg/ml) was added. A 0.2 ml of freshly made-up 0.15% NBT solution was added to each tube and was incubated at 37 ^{o}C for 20 min and centrifuged gently at 400 rpm for 3-4 min to discard the supernatant. A drop of PBS was added for resuspending the cells in small volume.

A thin film was prepared with a drop on a microscope slide, dried, heat fixed, and counter stained with dilute carbol-fuschin for 15 sec. The sildes were washed under tap water, dried and observed using a oil immersion objective. Cells were scored as positive when ingested particles were stained blue-black by precipitated formazan, the oxygen dependent reduction product of NBT. At least 300 cells were scored for each determination [5].

Neutrophil locomotion and chemotaxis test under agarose

The 0.2 g of agarose was dissolved in 10 ml distilled water by careful boiling in a water bath. 10 ml of prewarmed double strength Eagle's MEM was then added to agarose solution and around 5 ml of this mixture was added to each culture plate. The medium was then allowed to cool to 56 °C. After solidifying, appropriate wells in agarose were made. Using Pasteur pipette, wells were prepared measuring 3 mm in the diameter and spaced apart

Middle well of each set was filled with 10 µl neutrophils, 5×10^7/ml and 10 µl of control solution (MEM medium) was added to left outer well. Similarly, added 10 µl of test solution or 10^{-8} M. Fm-leu-phe to right outer well. Plates were then incubated in humidified chamber at 37°C for 2 h. Migration was stopped by flooding plates with 2.5 % glutaraldehyde for 1 h at room temperature. Agarose layer was carefully removed with a small spatula. Then the cells were stained with Giemsa solutions. Calculated migration of neutrophils by measuring the distance migrated by the cells.

The migration distance for the positive samples is corrected for random migration and subtracting the distance moved by the control well. The result is then expressed as corrected distance moved arithmetic mean ± SE of four migrations in sqmm [6].

In-vivo Immunomodulation study

Carbon clearance test

Swiss albino mice were divided into 4 groups, each containing 6 animals. Group I (control) was given 1.0% sodium carboxy methyl cellulose in water (0.3 ml/mouse) for 5 days. Group II-IV were given different

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concentrations of ETF (25, 50 and 100 mg/kg, p.o.) for 7 days. At the end of 7th day, after 48 h, mice were injected via the tail vein with carbon ink suspension (1:50 dilution of Indian ink, Camel, 10 µl/gm body wt.). Blood samples were withdrawn (in EDTA solution 5 µl) from the retro-orbital vein at 0 and 15 min, a 25-µl sample was mixed with 0.1% sodium carbonate solution (2 ml) and its absorbance at 650 nm was determined. The rate of carbon clearance (phagocytic index k) was calculated from the slope of each time-concentration curve drawn by plotting (100-mean transmittance value) as ordinate on semilogarithmic paper against time as abscissa. Results were expressed as the arithmetic mean ± S.E.M. of six mice [7,8].

Statistical analysis

All experiments were repeated thrice and results are expressed as mean±SD. Statistical analysis of data was done using student's '*t*' test.

RESULTS

Preliminary phytochemical investigation reveals the presence of terpenes, steroids and lipids. The *In vitro* study of ETF has caused a significant increase in the percentage of NBT-positive cells containing the reduced NBT dye (Table 1) and movement in agarose medium in a dose dependent manner (Table 2). Therefore it reveals that *Tedania anhelans* and *Sargassum ilicifolium* has stimulated chemotatic, phagocytic and intracellular killing of human neutrophils at a dose of 50 µg/ml and 100µg/ml respectively. *In vivo* studies of both sponge species have shown moderate immunostimulator activity at a concentration of 100 mg/ml as compared with control, whereas algal species have shown prominent immunostimulator activity at the same dose. From Table 3, ETFSI showed dose dependent manner immunostimulatory activity as compared to sponge species.

Table 1. Effect of ETF on qualitative nitroblue tetrazolium test.

Sl No	Groups	Concentration mg/ml	% NBT positive cells
1	Control (PBS)		22.85 ± 1.12
2	Endotoxin activated plasma		75.85 ± 0.82
3	ETFGF	10%	26.12 ± 0.15*
		25%	32.88 ± 1.02*
		50%	35.26 ± 0.61*
		100%	39.15 ± 0.68*
3	ETFTA	10%	35.42 ± 0.85*
		25%	51.21 ± 0.71**
		50%	73.12 ± 0.25**
		100%	61.39 ± 0.42**
3	ETFSI	10%	28.15 ± 0.09*
		25%	39.82 ± 0.67*
		50%	56.64 ± 0.34**
		100%	71.06 ± 0.01**

Values are mean ± SEM (n=6) * P < 0.05 ** P < 0.001

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Table 2. Effect of ETF on neutrophil locomotion and chemotaxis.

Sl No	Groups	Concentration mg/ml	Arithmetic mean ± SEM in mm^2
1	Control (PBS)		0.02 ± 0.06
2	Fm-leu-phe		7.1 ± 0.12
3	ETFGF	10%	2.1 ± 0.18
		25%	2.4 ± 0.04
		50%	2.8 ± 0.21*
		100%	3.2 ± 0.11**
3	ETFTA	10%	2.3 ± 0.24
		25%	3.6 ± 0.31
		50%	4.2 ± 0.01*
		100%	3.9 ± 0.26**
3	ETFSI	10%	2.1 ± 0.16
		25%	3.2 ± 0.05
		50%	4.4 ± 0.18*
		100%	5.1 ± 0.06**

Values are mean ± SEM (n=6) * P < 0.05, ** P < 0.001

Table 3. Effect of ETF on Carbon clearance test.

Time (min)	Species	Control	ETF 25 mg/kg	ETF 50 mg/kg	ETF 100 mg/kg
3	GF	0.55 ± 0.22	0.61 ± 0.07	0.89 ± 0.12	1.23 ± 0.09
	TA	0.37 ± 0.07	1.02 ± 0.09	1.10 ± 0.18	1.45 ± 0.06
	SI	0.45 ± 0.14	1.26 ± 0.11	1.35 ± 0.32	1.55 ± 0.19
6	GF	0.48 ± 0.20	0.52 ± 0.16	0.72 ± 0.03	0.98 ± 0.01
	TA	0.31 ± 0.35	0.89 ± 0.29	0.98 ± 0.11	1.16 ± 0.15
	SI	0.40 ± 0.13	0.95 ± 0.32	1.17 ± 0.02	1.29 ± 0.06
9	GF	0.34 ± 0.32	0.45 ± 0.08	0.61 ± 0.10	0.81 ± 0.09
	TA	0.29 ± 0.18	0.65 ± 0.05	0.86 ± 0.15	0.92 ± 0.02
	SI	0.31 ± 0.28	0.83 ± 0.12	1.01 ± 0.22	1.13 ± 0.09
12	GF	0.37 ± 0.25	0.35 ± 0.12	0.49 ± 0.06	0.76 ± 0.01
	TA	0.21 ± 0.19	0.49 ± 0.11	0.66 ± 0.22	0.78 ± 0.11
	SI	0.21 ± 0.05	0.69 ± 0.19	0.81 ± 0.22	0.96 ± 0.05
15	GF	0.25 ± 0.02	0.30 ± 0.12	0.37 ± 0.03	0.52 ± 0.08
	TA	0.19 ± 0.32	0.38 ± 0.18	0.53 ± 0.07	0.61 ± 0.04
	SI	0.18 ± 0.18	0.53 ± 0.06	0.72 ± 0.03	0.84 ± 0.05
Mean phagocytic activity/Mean slope	GF	0.024 ± 0.008	0.031 ± 0.002	0.032 ± 0.008	0.041 ± 0.009*
	TA	0.029 ± 0.036	0.059 ± 0.022	0.071 ± 0.014*	0.085 ± 0.011*
	SI	0.027 ± 0.005	0.065 ± 0.010*	0.088 ± 0.004*	0.099 ± 0.012**

Values are mean ± SEM (n=6) * P < 0.05, ** P < 0.001

DISCUSSION

The use of immunostimulants, mostly as adjuvant to chemotherapy, to control and prevention of infection holds great promise [9]. Significant interest has now been generated in research on bioactive

molecules from marine drugs designated as immunomodulatory agents in alternate systems of medicine. The ethyl acetate fraction of both the sponges and algae were tested in the present study to determine their possible effect on immune function at three dose levels.

In vitro models such as Neutrophil locomotion and chemotaxis test and Qualitative NBT test showed significant immunostimulant activity of sponge *Tedania anhelans* and algae *Sargassum ilicifolium* at a dose 50 µg/ml and 100µg/ml respectively. This investigation reveals the ETF fractions have significantly increased the phagocytic function of human neutrophils, when compared with control and increase the movement of neutrophils towords the foreign body which is the most important step in the phagocytosis process or activity. Therefore, the ETF of *Tedania anhelans* and *Sargassum ilicifolium* have significant chemoattractant property. ETF of the above species has significantly increased in the intracellular reduction of nitroblue tetrazolium dye to formazan by the neutrophils confirming the intracellular killing and preserves the integrity of neutrophils.

The ethyl acetate extracts of marine sponges *Gelliodes fibrosa* and *Tedania anhelans* on *in vivo* carbon clearance test have shown moderate immunostimulantory effect. Whereas an algal species *Sargassum ilicifolium* have shown prominient immunostimulatory effect at a dose of 100 mg/kg body weight po route. This may suggest that macrophage probably stimulate production of cytokines which in turn stimulates other immunocytes which may help in defence mechanisms of body to counter various infections.

Preliminary observations of these marine animal extracts also requires further investigations for exact mechanism of immunomodulation.

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