Original Article

# Stimulation of Cell Mediated Immune Response by Protein Hydrolysate from *Porphyra yezoensis*

**Kalkooru Lakshminarayanan Venkatraman, Azeemullah Azmathullah Syed, Alka Mehta***

Department of Integrative Biology, School of Biosciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, INDIA.

## ABSTRACT

**Aim:** To investigate the immunomodulatory effects of the protein hydrolysate from *Porphyra yezoensis*. **Objectives:** In this study, the *in vitro* immunomodulatory effects of protein hydrolysate of *Porphyra yezoensis* was studied by assay of phagocytosis and Nitric oxide production in RAW 264.7 macrophages and DTH (Delayed type hypersensitivity), NBT (Nitroblue tetrazolium) and Neutrophil adhesion assay in mice. **Results:** Hydrolysate treatment was found to be non-toxic in both *in vitro* and *in vivo* models. *In vitro* results suggested that hydrolysate could enhance the phagocytic activity and also inhibit the production of nitric oxide. Oral administration of hydrolysate (500 mg/kg) evoked an increase in the percent neutrophil adhesion to nylon fibers as well as potentiated the DTH reaction induced by SRBC (Sheep red blood cells). A phagocytic response was observed with significant changes in formation of formazan crystals. **Conclusion:** The results indicated that enzymatic hydrolysate from *P. yezoensis* could enhance the cell mediated immune response. Thus, the Nori protein hydrolysate could be explored as a possible natural immune stimulant to be used as nutraceuticals.

**Key words:** *Porphyra yezoensis*, Enzymatic hydrolysate, Immunomodulation, Macrophages Nitrobluetetrazolium, Delayed-type hypersensitivity, Phagocytic index.

## INTRODUCTION

Immunization, infection and various other factors trigger immunity, which acts as a defense mechanism against diseases. Activation of a number of specific cells and mediators against foreign bodies constitutes the immune response.[1] Various exogenous and endogenous factors termed immunomodulators are capable of stimulating the immune response and modulate the pathophysiological processes.[2] Immunomodulators from dietary components have been reported to be effective and play a significant role as health promoting factors.[3] Protein hydrolysates derived from the enzymatic hydrolysis have been reported as a good source of protein for human health, owing to their better gastrointestinal absorption. Nowadays, protein hydrolysates have gained attention and it is being extracted from many natural sources for its varied bioactivity.[4,5] Protein hydrolysates have also been proven to increase immune cell functions like lymphocyte proliferation, natural killer cell activity, antibody synthesis and cytokine regulation.[6] In recent years, the quest for searching novel bioactive metabolites from aquatic sources has gained a commendable amount of interest. *Porphyra* (Rhodophyta) is red algae, consumed widely as traditional food for their health benefits. It is well known for its high protein, polysaccharide, fiber content, polyunsaturated fatty acids and wide range of pharmacological benefits.[7] Previously, anticoagulant and antioxidant studies of the enzymatic hydrolysate from *P. yezoensis* have been reported from our laboratory.[8,9] Immunomodulatory activities of *Porphyra*n.[10] and Phycoerythrin.[11] have been discussed earlier but the immunomodulatory activity of enzymatic hydrolysates and peptides from *P. yezoensis* remain unclear. Bioactive protein hydrolysates or peptides by enzymatic hydrolysis from food source are gaining more attention because

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**Correspondence:**
***Dr. Alka Mehta***
Department of Integrative Biology, School of Biosciences and Technology, Vellore Institute of Technology, Vellore 632014, Tamil Nadu, INDIA.
Phone no: +91-0416-2202479
Email id: alkamehta@vit.ac.in

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they are easily digested and absorbed in the intestine. They normally exhibit more bioactivity than their parent protein. Different cleavage site of the enzyme can affect the release of peptides possessing immunomodulatory activities.[3] Though *Porphyra* species had been extensively studied to have a variety of bioactivities, biologically important molecules from *P. yezoensis* are not yet studied widely. The aim of this study is to evaluate the immunomodulatory properties of the enzymatic hydrolysate from *P. yezoensis*.

## MATERIALS AND METHODS

Commercially available Nori sheets were purchased from Japan and stored at −80°C until used. Cell culture media and other supplements were procured from HiMedia, Mumbai, India. Levamisole was procured from Johnson and Johnson (Ethnor Ltd.,), Mumbai, India. Porcine pepsin (EC 3.4.23.1), Dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT),Neutral Red Solution were obtained from Sigma Chemical Co. (St Louis, Missouri, USA). All chemicals and reagents used in this study were of analytical grade.

### Preparation of enzymatic hydrolysate

*P. yezoensis*, commercially available in dried form as Nori sheets, was shattered to fine powder using pestle and mortar. The enzymatic hydrolysis was performed according to previously described method.[8] The obtained hydrolysate was lyophilized (Micromodulyo freeze dryer, Thermo Scientific, USA) and stored at -80°C until further analysis.

### SDS-PAGE analysis

The electrophoretic pattern of the crude and hydrolysate was determined by SDS-PAGE (12%). Molecular weight markers (BSA- 66.5 kDa, Lysozyme – 14.4 kDa, Insulin – 3.0 kDa) were also run along with the crude protein extract and hydrolysate.

### In vitro Immunomodulatory Assays

Mouse macrophage cell line, RAW 264.7, was purchased from National Centre for Cell Sciences (NCCS), Pune, India and cultured in monolayer in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 100 IU/mL penicillin. The cells were incubated at 37°C in 5% $CO_2$ incubator. The adherent cells were kept for cell passage and sub cultured routinely.

### Assay of RAW 264.7 cell viability

The cell viability was assessed according to the conventional MTT-based colorimetric method.[12] 10 µL of RAW 264.7 cells, at a concentration of 1 x $10^7$ cells/ml in complete DMEM, were seeded into a 96-well flat bottom plate and incubated at 37°C in a 5% $CO_2$ incubator for 24 hr. 50 µL of hydrolysate was added in different concentration (25, 100 and 500 µg/mL) in each well. Complete DMEM was used as control and LPS (final concentration of 1mg/mL) was used as a positive control. The cells were incubated for 24 hr. After incubation, 10 µL of MTT solution (5 mg/mL) was added to the wells and the plates were again incubated for 4 hr at 37°C. After removing the medium, 100µL of DMSO was added to completely dissolve the formazan crystals and the absorbance was read photometrically at 570nm using microtiter plate reader (Bio-Rad, USA).

### Assay of Phagocytosis of RAW 264.7 cells

The neutral red uptake assay was performed to assess the phagocytic ability of macrophage.[13] The macrophages were cultured as previously described and were treated with varying concentrations of hydrolysate (each 25, 100, 500 µg/mL) in a 96 well plate and incubated for 24 hr at 37°C in $CO_2$ incubator. Neutral red solution (100 µL) was added to each well at a concentration of 0.075%. After 1hr incubation, the cells were washed twice with PBS to remove excess neutral red that was not phagocytized. 100 µL of Lysis buffer (0.1M glacial acetic acid and ethanol at the ratio of 1:1 (v/v)) was added and the plate was incubated again overnight at 37°C. The phagocytic index was calculated at 540nm using micro titer plate reader.

### Determination of nitric oxide (NO) levels

The nitric oxide level released by macrophages was determined by measuring the nitrite level in the cell supernatant by Griess reaction.[12,14] The collected cell supernatant from the assay of phagocytosis of macrophages was mixed with equal volume of Griess reagent (1% sulfanilamide and 0.1% N-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid) in 96-well cell plates and incubated at room temperature for 10 min. The absorbance was measured at 540 nm.A sodium nitrite ($NaNO_2$) standard curve was used to calculate nitrite concentration.

### *In vivo* Immunomodulatory Assays

Swiss albino male mice weighing 20-25g were maintained in the institute's animal house. The animals received food and water *ad libitum* and acclimatized to standard laboratory conditions of 12 hr light/dark cycle and temperature 25±2°C. The experimental protocol was approved by the Institutional Animal Ethical Committee of Vellore Institute of Technology, Vellore. All the procedure performed in this study involving animals

were in accordance with the ethical standards of the institution (Reference No.: VIT/IAEC/Feb13/19).

### Antigen preparation

Sheep Red Blood Cells (SRBC) were collected in Alsever's solution from a local slaughter house and were used to immunize the animals. It was washed thrice with pyrogen free 0.9% normal saline and adjusted to 0.5x $10^9$ cells/mL for immunization and challenge.[15]

### Immunization and Experimental Design

Mice were randomly divided into four groups of six animals each. Group I served as control and received 0.9% saline. Group II received Levamisole (50 mg/kg body weight.), an established immune stimulant agent, as positive control.[16] Groups III served as experimental group and received hydrolysate (500 mg/kg body weight). All the groups received respective treatments for 21 days prior to immunization. The animals were immunized by intra-peritoneal injection of 100 µl freshly prepared SRBC solution containing 0.5 x $10^9$ cells and the day of immunization was considered as Day 0 (Reference No.: VIT/IAEC/Feb13/19).

### Neutrophil adhesion test

The main function of the Neutrophils is to adhere to the endothelium and eliminate foreign threats. Neutrophil adhesion test is done to determine the percentage of neutrophil which adhere to the nylon fibre. This test was determined using the method of Srikumar *et al.*[17] On the last day of drug treatment, the blood samples were withdrawn in citrated tubes. After the analysis of initial hematological parameters and neutrophil percentage, blood samples were incubated with 80 mg/ml of nylon fibre for 15 min at 37°C. The incubated blood samples were again analyzed for Total Leukocyte Count (TLC) and Differential Leukocyte Count (DLC). The product of TLC and DLC indicates neutrophil index of blood samples. Percent neutrophil adhesion was calculated as below:

$$\text{Neutrophil Index (NI)} = \text{Total leukocyte count} \times \text{Neutrophil adehesion (\%)}$$

$$\text{Neutrophil adhesion (\%)} = ((\text{NI of control} - \text{NI of test}) \div \text{NI of control}) \times 100$$

### Delayed-type hypersensitivity (DTH)

The animals were treated and immunized as mentioned above. DTH response was determined using the method of Doherty.[18] The day of immunization was designated as day 0, after which the hydrolysate was administered for seven days. On the 7th day of immunization, the foot paw thickness was measured and the animals were challenged by injecting 20 µl of 0.5x$10^9$ SRBC cells in the right hind paw of the mice. 24 and 48 hr post challenge, the paw thickness of the mice was measured in terms of dorso-ventral thickness using Vernier caliper and the differences were taken for measurement.

### Nitrobluetetrazolium reduction test

The killing ability of the neutrophil was assessed by Nitroblue Tetrazolium reduction test (NBT).[17] Blood samples were collected and incubated at 37°C for 30 min and blood smear was fixed. To the smear, 0.4ml of NBT medium (0.2 ml of 0.34% sucrose solution, 0.2 ml of 0.28% NBT and 0.2 ml of inactivated foetal calf serum) was added and incubated at 37°C for 30 min. Post incubation, the slides were washed with saline and stained with safranin and observed under microscope.

### Histopathological analysis

The animals were weighed and sacrificed. The spleen and thymus were collected andwere fixed with 10% formalin for histopathological analysis. Thin sections of the samples were made using microtome and then stained with hematoxylin and eosin and observed under microscope (40x magnification) for histological changes.

### Partial purification of Phycoerythrin (R-PE)

Initially, the dried Nori was dispersed and homogenized in 50mM phosphate buffer (pH 7.0) with liquid nitrogen. Then the homogenate was subjected to two step ammonium sulfate precipitation (25% and 55%). Further the precipitate was centrifuged and dialyzed against distilled water to obtain partially purified Phycoerythrin.[17,19]

### Statistical analysis

All experiments were performed in triplicate (or more) and results were expressed as mean ± standard deviation. The statistical analysis of the data was carried out using GraphPad prism software for Windows OS. The statistical significance between the groups was determined by one way analysis of variance (ANOVA) followed by Dunnett's test. Significant differences were evaluated at $p>0.05$, $p>0.01$ and $p>0.0001$.

### Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The ethical standards of experiments are in accordance with the guidelines provided by the CPCSEA and World Medical Association Declaration of Helsinki on Ethical Principles for Medical Research Involving involving

experimental animals for studies. Approval Number: Reference No.: VIT/IAEC/Feb13/19.

## RESULTS AND DISCUSSION

The electrophoretic pattern of the crude extract and enzymatic hydrolysate obtained from Nori after pepsin hydrolysis is shown in Figure 1. A number of high molecular weight bands and protein streak was observed in crude (Figure 1: Lane 2 and 3) but after enzymatic hydrolysis, only low molecular weight fragments were observed (Figure 1: Lane 4 and 5). The obtained enzymatic hydrolysate, after lyophilization and appropriate dilution was used for immunomodulatory assay.

### Assessment of *in vitro* Immunomodulatory Activity

#### Effect of hydrolysate on cytotoxicity of macrophages

To use any compound/preparation, synthetic or natural for therapeutic purpose, the first condition is its non-toxic nature. To evaluate the safety of the hydrolysate, the cytotoxicity assay on RAW 264.7 cell line was performed. Active cells convert MTT to purple color formazan and the absorbance at 570 nm can be correlated with the live activity of cells and hence an indication of viability. After 24 hr of growth, RAW 264.7 macrophages were treated with different concentrations (25, 100 and 500µg/ml) of hydrolysate for another 24 hr and then MTT assay was performed. As shown in Figure 2A, exposure of different concentrations of the hydrolysate did not exhibit significant change in the absorbance at 570 nm. No statistical difference was found between the control and test groups. These results indicate that the protein hydrolysate was not toxic to the RAW 264.7 cells.

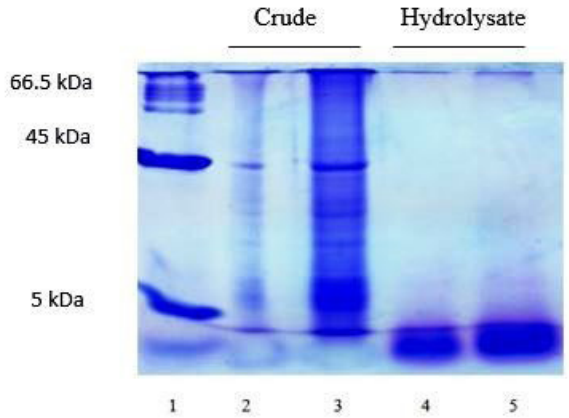


**Figure 1: SDS Electrophoretic pattern of crude aqueous extract and protein hydrolysate. From the left: Lane 1 - molecular weight marker; Lane 2 and 3 – different concentrations of crude aqueous extract; lane 4 and 5 different concentrations of pepsin hydrolysate.**

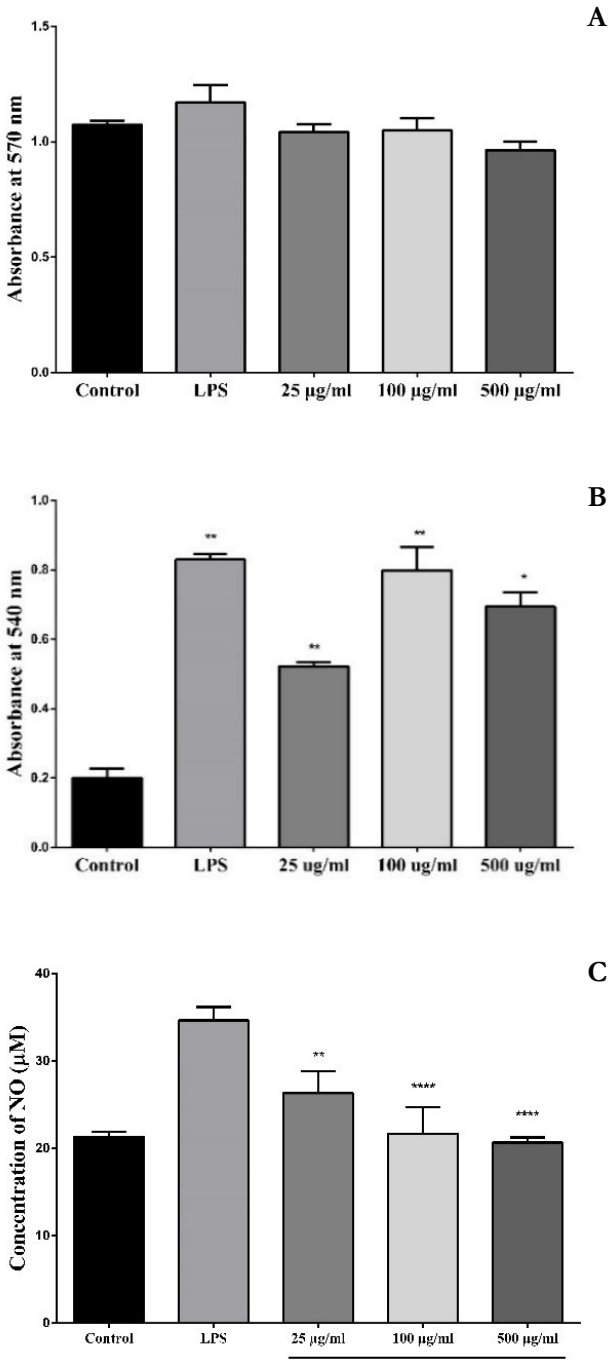


**Figure 2: Effect of protein hydrolysate on A cell viability, B phagocytic activity and C Nitric Oxide production of RAW 264.7 macrophages. RAW 264.7 cells were treated with various concentrations of hydrolysate for 24 h. The results are expressed as mean±SD (*n*=3).**

### Effect of hydrolysate on the phagocytic activity of macrophages

Phagocytosis being the initial step of the immune response is a prodigious characteristic of activated macrophages.[20] The phagocytic ability was measured by the neutral red uptake assay. RAW 264.7 macrophages were treated with different concentrations (25, 100

and 500µg/mL) of hydrolysate. As shown in Figure 2B, a significant increase in the phagocytic activity was observed in hydrolysate treated group when compared with untreated control group. The results demonstrated that lower concentrations of the hydrolysate (25 and 100µg/mL) significantly stimulated phagocytosis whereas higher concentration (500µg/mL) was significantly lower ($p$<0.5), which confirmed that hydrolysate exhibited its maximum activity at a concentration of 100µg/mL.

### Effect of hydrolysate on NO production

Inhibition of NO production is fundamental in therapeutic management of inflammatory diseases. The Griess assay was performed to investigate whether hydrolysate inhibits NO production of LPS induced RAW 264.7 cells. As shown in the Figure 2C, treatment with hydrolysate at concentrations 25, 100 and 500 µg/mL significantly inhibited the NO production. The NO inhibitory effect was most potent ($p$<0.0001) at 100 and 500µg/mL.

### Assessment of *in vivo* Immunomodulatory Activity

Mice were given the respective treatments as mentioned in the materials and methods section and were immunized with SRBC to evaluate cell mediated immune response by Neutrophil adhesion assays, DTH and NBT.

### Effect of hydrolysate on neutrophil adherence

As depicted in Figure 3A, the adhesion in the control group was 13.8 % whereas the hydrolysate treated groups showed 21.3% adhesion. Increase in the adhesion indicates that hydrolysate would have triggered the cell mediated immune response. Administration of the hydrolysate could have stimulated the neutrophils towards the inflammatory site since neutrophils play an important role in the initial stage of defense mechanism and enhances the leukocyte extravasation.[17] Neutrophil adhesion describes the margination of the neutrophils in blood vessels and the macrophages reaching the site of the inflammation. Beta 2 integrins mediate and regulate the neutrophil from the blood stream and adhere to the surface. Increase in the adhesion increases the immunity of the body against the microbial infection.

### Delayed-type hypersensitivity reaction

Antigen specific DTH effector T-lymphocytes, monocytes, regulatory B cells and basophils are the cells involved in DTH reactions. The animals were sensitized and then elicited to measure the foot pad thickness which gives the DTH response.[21] The Figure 3B shows the DTH activity of the hydrolysate and positive control levamisole. The treatment with hydrolysate significantly inhibited the foot paw edema of the mice compared to the control group after 24 hr of the secondary immunization. At the concentration of 500 mg/kg, the pepsin hydrolysate used in this study displayed better activity comparable to that of positive control levamisole through cell-mediated immune response, as assessed by DTH assay.

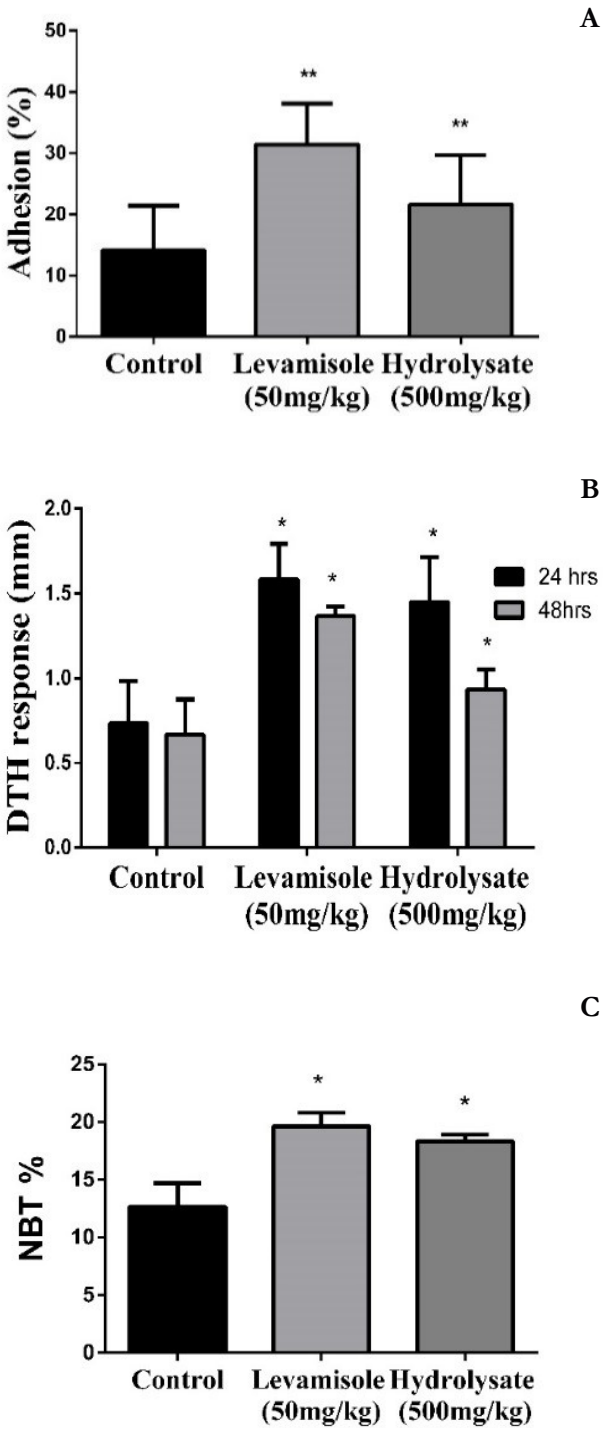


**Figure 3: Effect of protein hydrolysate on A Neutrophil adhesion, B Delayed-type hypersensitivity and C Nitroblue tetrazolium reduction. The results are expressed as mean±SD ($n$=3).**

### Effect of hydrolysate on metabolic activity of Neutrophil

In NBT test, the metabolic activity of the neutrophil is revealed which shows the capacity of the neutrophils to ingest NBT and form formazan crystals. The test showed significant reduction of the dye which indicates the activity of neutrophils against the pathogens.[22] The results in Figure 3C indicate that NBT reduction was significantly enhanced in hydrolysate treated groups when compared with the control group. The stimulation of phagocytosis by hydrolysate was comparable to that of positive control, Levamisole.

### Histopathological Analysis

The morphological evaluations revealed that no differences were found in the total splenic area between the control and the hydrolysate treated groups. Furthermore, no differences were found in the white and red pulp region between the groups (Figure 4A and 4B). In case of thymus, no destruction of the thymocytes and thymic involution was observed (Figure 4C and 4D). Thus, this shows that administration of hydrolysate did not show any apparent change in the cellular architecture of spleen and thymus.

Previously, Cian *et al.*[23] reported that protein fraction obtained from water extract of *P. columbina* using ultrafiltration contained Phycoerythrin showing immunomodulatory activities. Phycoerythrin is a characteristic pigment of red algae so to rule out the possible interference of Phycoerythrin on the protein hydrolysate, R-PE was partially purified from *P. yezoensis* and its absorption in the visible spectrum was measured and compared with the hydrolysate. The spectra thus obtained from purified crude protein extract, protein hydrolysate and R-PE are compared in the Figure 5. Three characteristic absorption peaks of R-PE at 566, 545 and 495 nm were observed in absorption spectrum (Figure 5). In comparison, the absorption spectrum corresponding to crude extract and hydrolysate were significantly different from that of R-PE suggesting its absence in both. Further the SDS-PAGE profile also implied that the crude (Figure 1. Lane 2 and 3) did not show the presence of two major subunits with 18 kDa and 20 kDa and minor subunit of 30 kDa of R-PE respectively.[23]

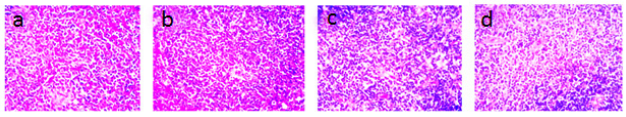


**Figure 4: Photomicrography of control and hydrolysate treated groups, A,B Spleen and C,D thymus stained with hematoxylin and eosin at 40x original magnification showing no destruction in the cellular architecture after administration of the protein hydrolysate.**

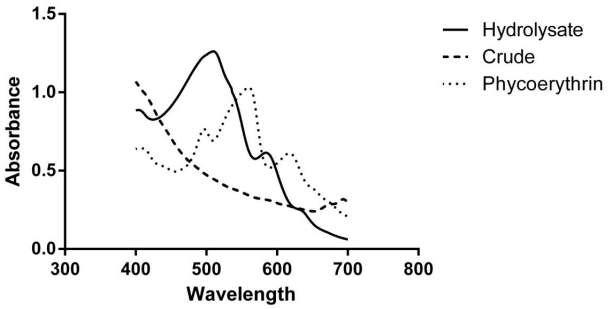


**Figure 5: Absorption Spectrum (400-800 nm) of crude extract and hydrolysate from *P. yezoensis* compared with partially purified R-Phycoerythrin (R-PE).**

## CONCLUSION

In conclusion, the current investigation suggests that enzymatic hydrolysate from *P. yezoensis* could enhance the peritoneal macrophages to devour neutral red uptake, could inhibit the production of NO and oral administration of hydrolysate in animal models stimulated the cell mediated immunity. Results thus obtained in the current study suggest the immunomodulating effects of the enzymatic hydrolysate. Since *P. yezoensis* is edible seaweed and consumed worldwide, the hydrolysate is devoid of side effects and could be used as an effective and potential immunomodulating agent.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

### Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Approval Number Reference No.: VIT/IAEC/Feb13/19.

## ABBREVIATIONS

**DTH:** Delayed type hypersensitivity; **LPS:** Lipopolysacchharide; **NBT:** Nitroblue Tetrazolium; **NI:** Neutrophil Index; **NO:** Nitric Oxide; **R-PE:** Phycoerythrin; **SRBC:** Sheep Red Blood Cells.

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## PICTORIAL ABSTRACT

## SUMMARY

Protein hydrolysate was tested for cytotoxicity and phagocytic effect using RAW 264.7 macrophage cell lines. It was evident that the hydrolysate did not have any toxicity and it induced the phagocytic activity. It also inhibited the LPS induced NO production. Protein hydrolysate was lyophilised and it was orally administered to mice and the neutrophil adhesion test, delayed-type hypersensitivity and nitroblue tetrazolium assay were done. The results as mentioned above inferred that the enzymatic hydrolysate from *Porphyra yezoensis* enhanced the cell-mediated immune response.

## About Authors

**Alka Mehta** is the Professor of School of Biosciences and Technology at Vellore Institute of Technology, Vellore. Her field of interest is study of bioactive peptides from macro algae with therapeutic activity. She also works in the area of food safety. Her focus is on degradation and detoxification of mycotoxins in food and feed. The goal of this work is to make food safe for consumption and reduce economic losses due to fungal infestation and mycotoxin contamination. She has more than 50 research publications in the reputed journal.

**Venkatraman KL,** Research Scholar in School of Biosciences and Technology at Vellore Institute of Technology, Vellore. His field of interest is about protein purification, bioactive peptides, anticoagulant and Immunomodulatory activity.

**Azeemullah Azmathullah Syed,** Research Scholar in School of Biosciences and Technology at Vellore Institute of Technology, Vellore. His field of interest is about protein purification, bioactive peptides, anticoagulant and Immunomodulatory activity.

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