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Efficacy of Seaweed (Sargassum sp.) Extract to Prevent Vibriosis in White Shrimp (Litopenaeus vannamei) Juvenile

Indriyani Nur Mulyadi
Halu Oleo University

Wa Iba
Halu Oleo University and University of Rhode Island, wa.iba@uho.ac.id

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Research Article
Efficacy of Seaweed (*Sargassum* sp.) Extract to Prevent Vibriosis in White Shrimp (*Litopenaeus vannamei*) Juvenile

Mulyadi, Indriyani Nur and Wa Iba

Department of Aquaculture, Faculty of Fisheries and Marine Science, Halu Oleo University, 93232 Kendari, JL. HEA Mokodompit, Anduonohu, South East Sulawesi, Indonesia

Abstract

Background and Objectives: Marine algae, especially brown algae (*Sargassum* sp.), is a natural compound plant capable of being prophylactic and immunostimulant. This study was aimed to evaluate the efficacy of seaweed (*Sargassum* sp.) extract to improve resistance and immune response of juvenile white shrimps (*Litopenaeus vannamei*) infected by *Vibrio algynolyticus*. **Materials and Methods:** The ethanolic extract of seaweed was used to evaluate its antibacterial effect by immerse the juvenile shrimp at a dose level of 0 (control), 150, 250 and 350 ppm for 3 h. Shrimp immune response was observed based on total haemocyte count (THC) and differential haemocyte count (DHC). Moreover, the bacterial challenge test was used for the evaluation of shrimp resistance. **Results:** The immersed shrimp in 150 ppm extract showed a significant increase in the number of THC and improve DHC value. During bacterial challenge test, shrimp juveniles immersed in 150 and 250 ppm had a 100% relative percent survival (RPS) which was higher than those in 350 ppm that had only 50% survival. Moreover, there were no significant histological changes of the hepatopancreas organ following infection in shrimps immersed in 150 and 250 ppm seaweed extract groups, whereas shrimps in control group showed hyperplasia and necrotic in nucleus cells. In addition, changes in the form of excess fat infiltration occured in tissues indicates the vulnerability of shrimp in control group. **Conclusion:** The study indicates that extract of brown seaweed *Sargassum* sp. is a potential immunostimulant to be used in juvenile white shrimps culture to control vibriosis.

Key words: Antibacterial, immunostimulant, white leg shrimp, *Sargassum* sp., survival, hemolymph

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Corresponding Author: Indriyani Nur, Department of Aquaculture, Faculty of Fisheries and Marine Science, Halu Oleo University, 93232 Kendari, JL. HEA Mokodompit, Anduonohu, South East Sulawesi, Indonesia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

White leg shrimp (*Litopenaeus vannamei*) is one of the world largest cultivated shrimp species[1,2]. Over the next few years, the increasing demand for white shrimps compelled intensive cultivation of this species, causing many issues due to increased disease outbreaks induced by microorganisms resulting in mass mortality[3]. Disease in white leg shrimp larvae and early juveniles caused by bacteria is an adverse problem in hatchery or nursery phase and will imminently affect its culture sustainability and productivity[4,5].

The high mortality of larvae and early juveniles in shrimp hatchery as a result of vibrio bacterial strains infection or known as vibriosis disease is caused by bacteria *V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus*[5-8]. While most of bacterial species were less pathogenic in nature, it has been recorded that vibrio bacterial diseases in penaeid shrimp cultivation systems involving at least 14 species, include *Vibrio harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, *V. campbelli*, *V. fischeri*, *V. damsella*, *V. pelagicus*, *V. orientalis*, *V. ordalii*, *V. mediterrani* and *V. logei*[9]. Some control measures that may overcome such diseases including supervision of water quality, proper feeding and dosing appropriate immunostimulants.

Pathogen control is essential to prevent vibriosis outbreak in shrimp hatchery and nursery phase. Generally, the common practices in vibrio bacterial control is the application of synthetic chemicals such as antibiotics and drugs compounds during hatchery phase of the shrimp. However, owing to technical, operational, ecological and financial variables and constraints, this was not a very effective approach. In the last few years, the use of many forms of synthetic chemicals in the pathogen control program has been restricted. It is partly due to high cost of synthetic antibiotics, concern about negative effects over the environment, harmful effects on human health and other non-target populations, their non-biodegradable nature, high rate of ecosystem biological magnification and increasing antibiotic resistance[10]. This indiscriminate and unabated use of chemicals and antibiotics, however, has resulted an intense discussion among environmentalist and government agencies to prohibit these products completely. These factors put forward for the growth of a broad range of shrimp aquaculture sustainability approaches. Thus, the concept of pathogen disease control in aquaculture, particularly using herbal and phytopharmaca for disease prevention has received wide spread attention during the last decade[11,12].

Similar to their terrestrial counterpart, marine plants (seaweeds or macroalgae) are also regarded as a wealthy source of bioactive molecules[13,14]. Some seeweed species were recognized and extracted for a broad range of bioactive compounds with various pharmacological functions, including antioxidants, soluble nutritional fibres, proteins, minerals, vitamins, phytochemicals and polyunsaturated fatty acids[15]. Marine algae, particularly brown algae (*Sargassum* sp.), is a natural compound factory that has the capacity as a prophylactic and immunostimulant material. The active ingredients in *Sargassum* sp. were described in our previous study including tannin, saponin, β-carotene, flavonoids, alkaloids, phenolic, steroid and glycose[16]. Alginic acid[17] and fucoidan[18] from brown algae and K-carrageenan from red algae[19] showed a role as an immune trigger which helps aquatic animals in preventing various diseases[20]. Provision of natural immunity triggers proven to be able to optimize the health profile of aquatic animals including various immunity parameters such as total hemocyte count (THC) and phagocytic activity[21,22].

The research conducted by Yeh *et al.*[22] showed that administration of *S. duplicatum* extract on white leg shrimp juvenile could strengthen its immunity during challenge test with pathogenic bacteria. The increase in immunity was due to the polysaccharides extract containing immunostimulants, specifically fucoidan and alginic acid. The intake of bioactive materials also reduces the vulnerability of aquatic animals to various types of diseases such as vibriosis and viral diseases such as white syndrome viruses[18].

Application of immunostimulants to mitigate vibriosis infections in white shrimps juveniles needs to take into account the appropriate dosages and administration time. Yeh *et al.*[22] and Huynh *et al.*[23] found that the control of *Vibrio* sp. in a hatchery with the use of *Sargassum* sp. extract from Penghu Island, Taiwan, by soaking shrimp larvae for 3 h at a dose of 300 ppm, was able to enhance the immunity of white leg shrimp larvae whereas higher doses did not affect the immune parameters. The study implied that the use of high doses may affect the shrimp immunity and less economical. Therefore, in this study lower dosage of *Sargassum* sp. extract from Buton Straits, Indonesia was used to determine its efficacy as immunostimulant in white shrimp juveniles.

MATERIALS AND METHODS

Preparation of seaweed extract: Samples of seaweed *Sargassum* sp. were collected from Bonerita waters, Buton Strait of South East Sulawesi Province between April and May,

2018. Seaweed were air dried at room temperature without sunlight exposure. Samples were subsequently chopped and ground using food grade blender into powder form and stored in a cool and dry place before further processing. The dried and ground seaweed was extracted using ethanol solution (1:4, w /v). After the maceration process with ethanol for 24 h, the extract was filtered and dried. Then, the extract was filtered using filter paper and the solvent evaporated to dryness under reduced pressure at 35°C using a rotary vacuum[24]. The dried and powdered crude extracts were kept at 4°C and protected from light and moisture until further use.

Experimental shrimp and rearing activity: Juvenile white shrimps were acquired from the hatchery of Benur Kita, Barru, South Sulawesi, Indonesia and was raised at shrimp hatchery in Mata, Kendari, SE-Sulawesi, Indonesia until they reached juvenile size of 4-5 cm. Shrimps from the hatchery were reared for 2 weeks for acclimatization purpose to experiment condition. They fed 5 times a day (every 4 h) using *Artemia salina* and artificial commercial feed combination at 5% of the shrimp body weight. Furthermore, experimental shrimps were randomly distributed into 12 aquariums (40×25×30 cm) with density of 15 shrimps/aquarium, referring to the optimal density in intensive ponds between[25] 100-300 shrimps m^{-2}. Continuous aeration, siphoned and saline water renewal were applied to maintain clean and dissolved oxygen. During the experiment, water quality parameters were maintained on a normal level (temperature range was 28-30°C, DO>3 ppm and salinity was 33-35 ppt).

Experimental design: The experimental design of the present study was a complete randomized consisting of 3 treatments in triplicates. *Sargassum* extract was added to the water at levels of 0.0 (control), 150, 250 and 350 ppm. Juvenile shrimps were immersed for 3 h in aquarium with seaweed extract addition, then the shrimps were returned to normal water medium (Fig. 1).

Challenge test: After the 3 h immersion trial, the shrimps were challenged with *V. algynolyticus*. Infection was performed via an intramuscular injection of *V. algynolyticus* at a concentration of 10^7 CFU mL^{-1} using a 1 mL syringe and negative control 0.1 mL/individual shrimps were injected with Phosphate Buffered Saline. The clinical symptoms and mortality rates of the shrimps were observed for 72 h after injection.

Observed parameters: The experimental parameters observed were immune response, relative percent survival (RPS), hemolymph glucose, histopathology and clinical signs. The immune response parameters measured at 72 h post challenge test included total hemocyte count (THC) and differensial hemocyte count (DHC). The THC was calculated to discover the number of the shrimp's hemocyte in reference to the method of Blaxhall and Daisley[26], whereas, DHC calculation was based on Martin and Graves[27].

Relative percent survival (RPS) versus control was calculated at the end of 72 h of infection using the formula by Amend[28]:

$$RPS\ (\%) = 1-\frac{\text{Number of shrimp mortality at the treatment group}}{\text{Number of shrimp mortality at the control group}}\times 100$$

Clinical signs were observed following 6 h of challenge test. The level of glucose in the hemolymph was measured by applying a drop of hemolymph to a chemically treated, disposable 'test-strip', which was then inserted into an electronic blood glucose meter. The reaction between the test strip and the hemolymph is detected by the glucose meter and presented in mg dL^{-1} units[29].

Following the challenge test, histological examination was done through microscopic identification of the tissues. Hepatopancreas were removed from each shrimp with clinical indications. Tissues were cut into tiny parts and fixed in 10% formaldehyde. Fixed tissues then were processed for histological preparation (following procedures of

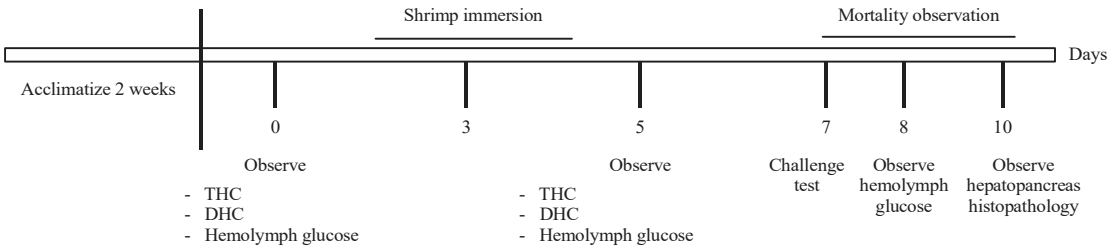

Fig. 1: Experimental design of the study

Takashima and Hibiya[30] and Munford *et al.*[31]. Dehydration was used to remove water from the tissues with a series of alcohols: 70-95-100% and then followed by clearing with xylene. Finally, the embedding agent was infiltrated the tissue. Embedding process was performed by placing tissue in fresh paraffin wax and allowing paraffin to cool, then they were cut into sections that can be put on a slide. Slides were stained with haematoxylin and eosin (H and E). Prepared sections and stained were examined under a light microscope (Olympus BX53), photograph was taken (Olympus DP21 camera with Stream program) and histopathological changes were assessed.

Statistical analysis: The immune parameters obtained and RPS data were analyzed using the analysis of variance (ANOVA). Duncan's test was used to determine significant differences ($p<0.05$) using SPSS Statistics 20.0 software. Histopathologal cause of bacterial infection was descriptively analyzed.

RESULTS

Hemolymph parameters: The levels of immune response were measured after 5 days of immersion treatment through recording total hemocyte count (THC) and differential hemocyte count (DHC). The highest number of THC was found in blood of shrimp juvenile immersed in 150 ppm of seaweed extract whereas, the lowest THC was in 350 ppm. It is suggested that the higher the concentration, the smaller the THC value (Fig. 2).

Observation on DHC directed at defining patterns of change of hyalin cells, semi-granular cells and granular cells proportion in hemolymph after extract *Sargassum* treatment. The H cells were the smallest cells with few granules, their nucleus occupied most of the space in cells. G cells were the biggest cells and contained abundant granules, while SG cells were smaller and contained less granules than G cells and were more round in common. Hyalin and semi-granular cells were the two dominant types of cells that demonstrate no distinctions in each treatments, whereas, semi-granular cells reduced significantly in elevated extract concentration (250 and 350 ppm) (Fig. 3, 4).

The data of haemolymph glucose in shrimps before and after treatments showed no significant difference. The average level of haemolymph glucose was 25 mg dL^{-1} in the initial of experiment and increased slightly at the end of extract exposure treatment at 33 mg dL^{-1}. Juveniles shrimp immersion in the *Sargassum* extract at lower concentration did not increased the glucose levels, but pathogenic infection of *Vibrio* sp. caused significant shrimp stress which was characterized by a very dramatic rose in glucose level (107 mg dL^{-1}) (Fig. 5).

Survival: Higher percentage of survived shrimp juveniles was found in those that were immersed in lower seaweed concentration compared to higher one. Survival of shrimps immersed in *Sargassum* extract showed that at 150 and 250 ppm treatments was 100% whereas, at 350 ppm, it was only 50% at 72 h post-challenge (Fig. 6).

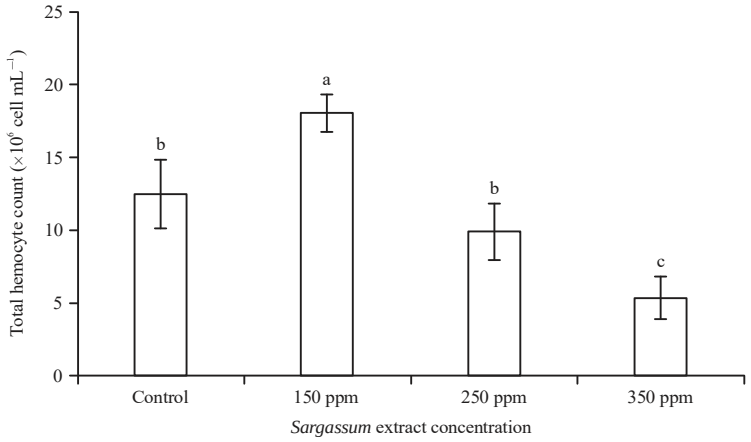

Fig. 2: THC of white leg shrimp juveniles after immersed in seaweed extract at different concentration (Mean±SE)
Different superscript letters indicated a significant difference among treatments ($p<0.05$)

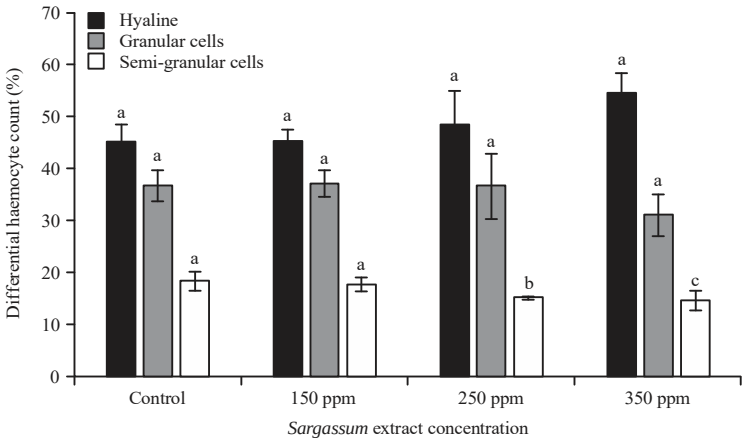

Fig. 3: DHC of immersed vannamei shrimp in the different diluted extract dosages
Different superscript letters indicated significant difference among treatments ($p<0.05$)

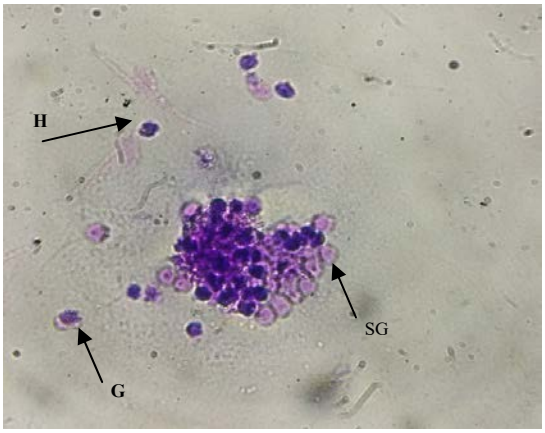

Fig. 4: Morphology of shrimp hemocyte cells
H: Hyaline, G: Granular, SG: Semi-granular

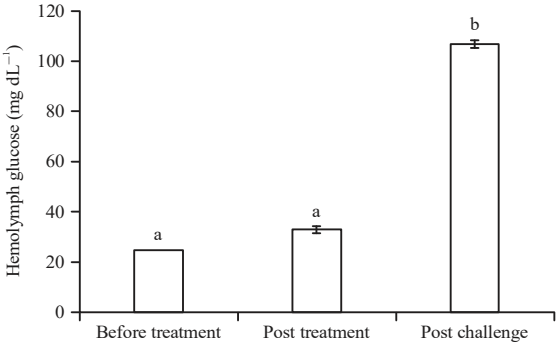

Fig. 5: Level of hemolymph glucose of shrimps exposed by immersion in *Sargassum* extracts 150 ppm (treatment A) and challenged with *Vibrio* bacteria

Histopathology: White leg shrimp juveniles that were challenged with *Vibrio* sp. infection without seaweed extract

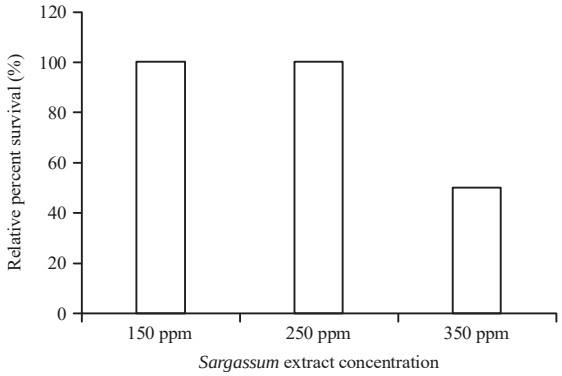

Fig. 6: Relative percent survival (RPS) (%) of juvenile white leg shrimps after immersion and challenged with *Vibrio* sp., at different concentration of seaweed extract

immersion treatment showed some clinical signs such as necrosis of the tail and empty intestine (Fig. 7).

Histopathology analysis confirmed the clinical signs observation of shrimp juveniles without seaweed extract treatment. Hepatopancreas histopathology showed that those shrimp exhibited severe damage in their tissues include hyperplasia of cells (H), necrotic cells in nucleus (N) and excessive lipid cell infiltration/lipid degeneration (DL) (Fig. 8). Conversely, hepatopancreas of juvenile shrimps immersed in 150 ppm of seaweed extract showed no change or normal-appearing tissues (Fig. 9) whereas, those 250 ppm showed tissues with minimal damage as indicated by minimal amount of lipid degeneration (DL) and hyperplasia (H) without accompanied by necrotic cells found in tissues (Fig. 10). However, shrimp juveniles immersed in 350 ppm of seaweed extract showed a more moderate damage of tissues as shown by hyperplasia (H) and necrotic (N) with moderate intensity in cells (Fig. 11).

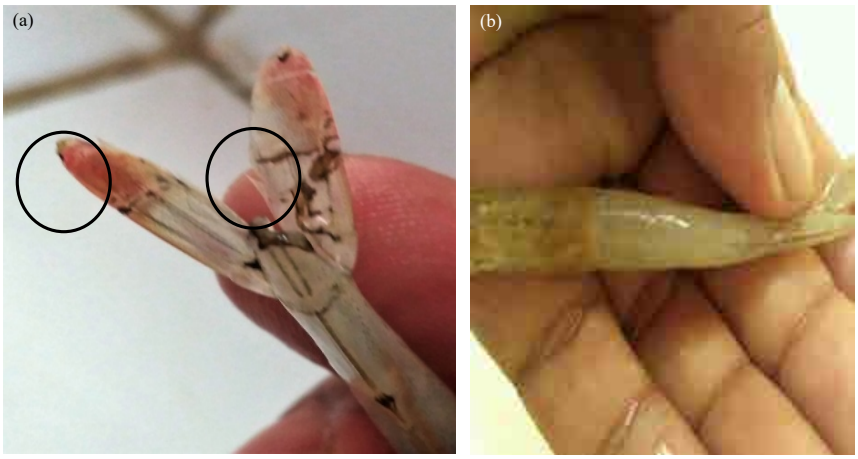

Fig. 7(a-b): Clinical signs of shrimp in control group after 72 h shrimps were challenged with *Vibrio* bacteria. Infected shrimp showing necrosis of the (a) Tail and (b) Pale and empty intestine

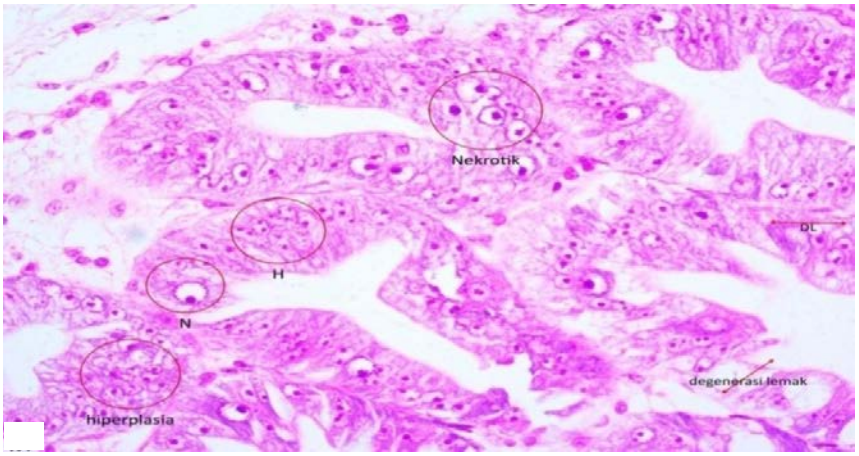

Fig. 8: Histopathology of hepatopancreas in control group (shrimp juveniles without *Sargassum* sp. extract)
H: Hyperplasia, N: Necrotic, DL: Lipid degeneration

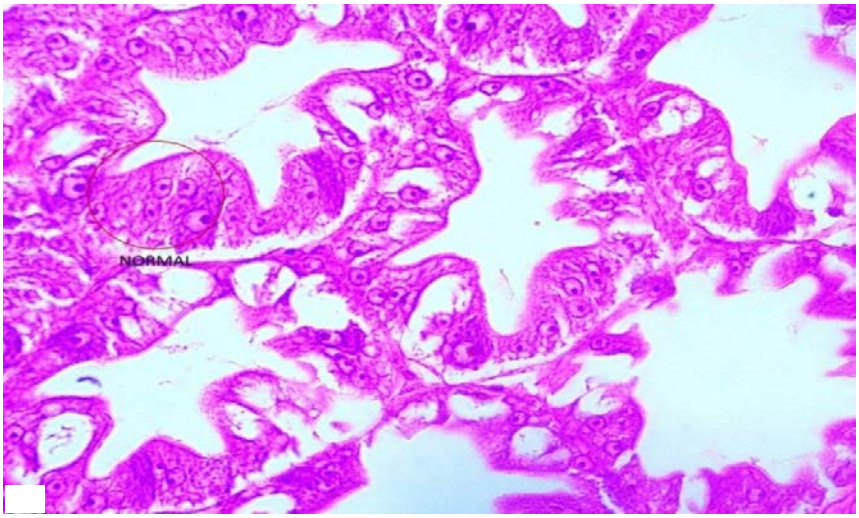

Fig. 9: Hepatopancreas tissues in shrimp immersed with *Sargassum* sp. extract at 150 ppm

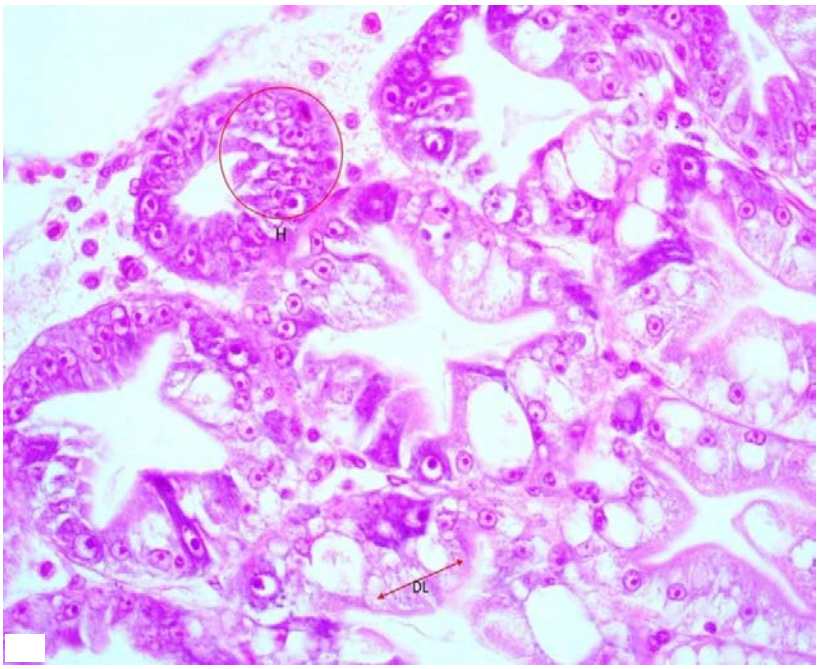

Fig. 10: Histopathology of hepatopancreas in treatment B (shrimp with *Sargassum* sp. extract dose of 250 ppm)
H: Hyperplasia, DL: Lipid degeneration

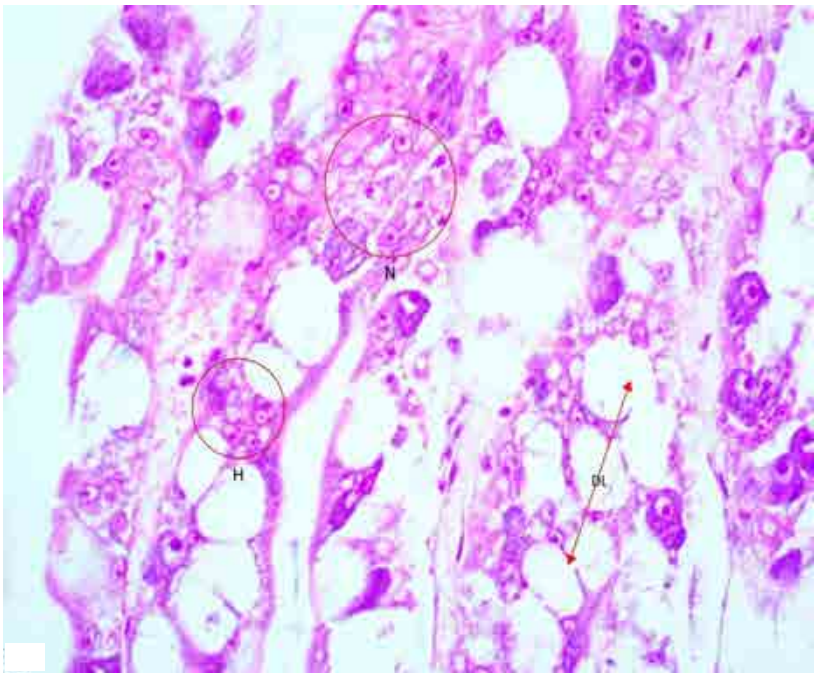

Fig. 11: Histopathology of hepatopancreas in shrimp with *Sargassum* sp. extract at 350 ppm
H: Hyperplasia, N: Necrotic, DL: Lipid degeneration

DISCUSSION

This study showed that lower concentration of seaweed extract may have a better effect on juvenile shrimps blood parameters after immersion in considerably short time compared to higher concentration or without seaweed extract. The higher number of THC of shrimp immersed in 150 ppm seaweed extract indicated that an appropriate

concentration, *Sargassum* sp. bioactive compounds are capable of enhancing immune response due to the higher chance of creation of phagocytic cells, which is very important in controlling microorganism attacks[32]. Van de Braak[33], Felix *et al.*[21], Wang and Chen[34] and Maftuch *et al.*[35] stated that the increasing number of THC indicated a rise in both cellular and humoral immune response. In the current research, shrimp receiving the *Sargassum* extract through immersion showed an enhanced number of haemocytes that indicates haemocyte proliferation. This implied that immune system of white leg shrimps juvenile was ready to deal with pathogenic infections. However, THC significantly decreased at high concentration of *Sargassum* extract (350 ppm), suggesting that high concentration could paradoxically suppress immune responses (Fig. 2), thus resulting in being easily defeated by pathogens that ultimately leads to low survival (Fig. 6).

Stress is an animal disorder due to sources of inconvenient conditions. There is an increase in glucose hemolymph at the moment of stress to overcome the elevated demands for energy. In the view of Cuzon *et al.*[36], if level of glucose in shrimp exceeds 150 mg dL^{-1}, this indicates that the shrimp is stressed and requires higher energy. Herbal compounds have the capacity to inhibit oxygen anion generation and scavenge free radicals. It has been shown that the herbal antioxidant impact is comparable to that of superoxide dismutase, metal-ion chelators and xanthine oxidase inhibitors. *Picrorhiza kurroa* herb used as an antistress compound for shrimps is the finest example[37]. Stress increases susceptibility to diseases, possibly due to chronic elevated cortisol levels, resulting in immunosuppressive. Stress in crayfish can be indicated from the level of glucose in hemolymph[29]. This study obviously demonstrated a rise in glucose level up to 107 mg dL^{-1} due to infection with vibriosis, but stress has not escalated mortality as it was supported by the active compound of *Sargassum* for anti-stress (Fig. 5).

The hepatopancreas histological analysis showed the structural abnormalities in hepatopancreas infected with bacteria. Abnormalities could be seen in the control that was shrimp without immersion extraction treatment *Sargasssum* sp. Hepatopancreas is the most important organ for a shrimp organism. Hepatopancreas is a target organ for pathogen infection and environmental changes. Histopathological changes in hepatopancreas may be used as indicators for the assessment of the health of crustaceans especially stress levels and shrimp susceptibility[38]. Necrosis, haemorrhage and vacuolization of hepatopancreas tubule epithelial reduced cells were among the major disease-related histopathological changes observed in infected shrimps. Necrotic cells and tissues would experience decreased activity and eventually die. Munford *et al.*[31] stated that necrotic tissue in an organism can be caused by several factors including bacteria that invade, injury to body parts, trauma, stress and the toxins in the waters. Necrotic is a morphological change which can lead to tissue cell death and shrinking of the nucleus size.

Symptoms of shrimp affected by vibriosis can be identified visually by observing directly both the physical and behavior of shrimps because a sick shrimp will show symptoms that are different from healthy shrimp. The results of the observation of *V. alginolyticus* bacteria on vannamei shrimp showed clinical symptoms and behavior of shrimp. The first day there was a decrease in shrimp appetite, marked shrimp intestinal contents were not full or intermittent and a lot of leftover feed on the basis of maintenance, but not accompanied by clinical symptoms that were significant from pathogenic infections. On day 2, the infection symptoms were seen in Fig. 7, the larvae looked weak and inactive, the head always faced up and the movements were uncontrolled, the shrimp tail was necrotizing, melanosis on the skin, the body of the shrimp looked pale, swimming legs, telson and uropod were reddish. Clinical symptoms detected in test shrimp indicated the presence of the *Vibrio* genus bacterial infection. Similar clinical symptoms have also been reported in shrimps with vibriosis[39].

Biologically active substances in seaweed *Sargassum* sp. extracts in this study are tannin, saponin, β-carotene, flavonoids, alkaloids, phenolic, steroid and glycose[16]. Naturally, the main functions of plant secondary metabolites are to protect plants from attack by insects, herbivores and pathogens, or to survive from other biotic and abiotic stresses. Flavonoids are one of the compounds that play an important part in boosting the immune system. Flavonoids are plant secondary metabolites which have shown protective effects on cancer, heart diseases and retinal inflamation in human[40,41]. Other important metabolite in seaweed extract is β-carotene that is regarded as damaged cells protector by converting the β-carotene into vitamin A which in turn repairs the cells[42]. Alkaloids compounds in seaweed may damage the bacteria nucleic acids (DNA and RNA) as the basic structure of these alkaloids are alkylating agents and other substances that react covalently with purine and pyrimidine bases[43]. Saponins work in shrimp as antimicrobials, tannins function to inhibit bacteria by denaturing proteins and damaging membranes of bacterial cells by dissolving lipid contained in cell walls. Phenolic acts as a reduction agent, a supplier of hydrogen and a potential agent of chelation. These natural bioactive compounds were assumed have various pharmacologic activities of shrimps[32,44]. Through a simple method, immersion with the right dose of immunostimulants could be improve the phagocytic capacity response.

The ability of *Sargassum* sp. used in this study to increase the immune system and prevent the emergence of juvenile shrimp disease might also be attributed to several important polysaccharides in the extract. The major polysaccharides that commonly found in seaweed are agar, carrageenan and alginates[45]. When lipopolysaccharides, peptidoglycans and β-1,3-glucan molecules are present-commonly found in bacteria and fungi[46], cellular reaction, responsible for phagocytosis, melanisation, encapsulation and coagulation, is triggered. This has been proven in Arizo *et al.*[47] study who found that properties of β-glucan present in seaweed *Gracillaria edulis* reacted with binding β-glucan proteins (βGBP) leading to haemocyte degranulation. Furthermore, the phytochemical pathway of brown seaweed *Sargassum* sp. in this study might provide additional protection for shrimps juvenile as shown by DHC number and cytoplasmic granules cells when administered at lower concentration (Fig. 3, 4).

The findings of this study showed that shrimp survival, hemolymph parameters and the histology of hepatopancreas were better when treated with 150 and 250 ppm *Sargassum* sp. This suggests that the sufficient amount of extract of *Sargassum* sp. to enhance immunity thus survival is between 150 and 250 ppm. More studies are needed regarding the inexpensive extraction methods for commercial applications of *Sargassum* extract, extract administration of either oral or immersion methods, its concentrations and the exposure time of shrimp to these extracts (*in vivo*).

CONCLUSION

In conclusion, white shrimp that immersed in the seawater containing the *Sargassum* extract 150 ppm showed significantly increased immune response by increasing the haemocyte count, proportional differential haemocyte, stabilizing hemolymph glucose level and high resistance against *V. alginolyticus* infection.

SIGNIFICANCE STATEMENT

This study discovered the beneficial effects of ethanolic extract of brown marine algae *Sargassum* sp. for the preventi on of vibriosis in the culture of juvenile white shrimp. This study will help the researchers to uncover the potential of marine algae to be used as natural antibiotic and immune stimulant in shrimp culture that many researchers were not able to explore. Thus a new concentration on delivering the marine algae extract may be arrived at.

ACKNOWLEDGMENTS

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