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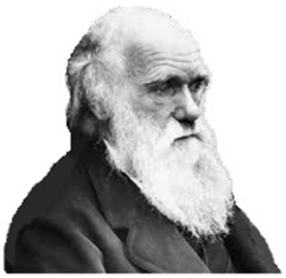

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Mucuna pruriens-seed extract stimulating immune system in *Litopenaeus vannamei* against *Vibrio harveyi*

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ABSTRACT

Objective: To evaluate the immunostimulant (ISt) role of *Mucuna pruriens*-seed (*Mp*-seed) enriched with Artemia (*Ar*) in *Litopenaeus vannamei* (*L. vannamei*) infected by *Vibrio harveyi* (*V. harveyi*). Method: Fifteen shrimp (30±5g) were stocked per 250 L tank in 3 replicates and fed twice daily with *Ar* enriched with *Mp*-seed methanolic extracts at 100 ppm and 200 ppm for 21 days. Infected *L. vannamei* shows altered immunoparameter (IPM) and antioxidant enzyme status due to the *V. harveyi* infection induced stress. Results: Significantly increased ($p<0.05$) total haemocyte count, respiratory burst, phenoloxidase and superoxide dismutase activities were observed in shrimp fed with *Mp* enriched *Ar* diets compared to infected group. Supplementation of *Mp* enriched *Ar* in diet could enhance the immunity of shrimp against *V. harveyi* infection by reverses the antioxidant level. The histology of hepatopancreas (HPC) also shows *Mp*-fed groups has restores the normal structure. Conclusion: The study demonstrated that *Mp* enriched *Ar* in diet have a potentially role in ISt and an effective antioxidant by regulating infectious stress against vibriosis.

***Keywords*:** *Mucuna pruriens*, Vibriosis, *Litopenaeus vannamei*, immunostimulant, *Vibrio harveyi*

1. INTRODUCTION

The use of biofloc has been reported to improve the nutritional and reproductive development of organisms,[1,2] further its ability to prevent disease outbreaks has been attributed

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to its ability to stability water quality parameters, increasing the presence of bacteria that produce short chain fatty acids to protect the intestinal epithelium and prevent diseases, disrupting the quorum sensing of pathogenic *Vibriosis* by biofloc microorganisms, is one of the predominant bacterial diseases responsible for mass mortality of farmed shrimp globally, and producing a probiotic effect similar to commercial products used in aquaculture.[3,4] Antibiotics are widely used in shrimp farming to treat infectious diseases at both larval and growth phases of production cycle, and aquaculture farmers depend on different antibiotics at both to treat and prevent bacterial infections in shrimps.[5] It's usage is associated with ecological and human health effects which comprise pathogens resistance, spreading of antibiotic resistance to other organism, disease persistence in the aquatic environment and also effects on the biogeochemical composition, which antibiotic residues accumulates in the shrimp tissues might alter intestinal flora of human cause allergy problems and food poisoning.[6] Vibrio species are increasing resistance levels towards many of the clinically used antibiotics.[7]

Medicinal plants have been used as traditional medicines for centuries in India.[8,9] It offers new source of organic chemical compounds as antibacterial agents. It also used as remedies for infectious diseases. Recently, more interest is gained by herb-drug in shrimp industries due to their easy availability, biodegradability, cost-effectiveness and broad-spectrum activity as well as the non-hazardous nature of the phyto-chemicals to the environment. Additionally, they are free from toxic accumulation of the chemicals and moreover safe to surroundings and human during extensive application. *Mucuna pruriens* (*Mp*) is one of the well accepted medicinal plants of India, widely utilized in the multiple pharmacological and is phytoconstituents (MPCs) of numerous indigenous drug formulations. The aimed of present study to evaluate ISt influence of *Mp*-seed methanol extract against vibriosis caused by *V. harveyi* in *L. vannamei*.

2. MATERIAL AND METHODS

2. 1. Animal collection and acclimatization

Healthy adult *L. vannamei* of both sexes were collected from culture pond near Kaliveli Lake (12º 7' 11.02' N, 79º 51' 27.66' E) located 25 km north of Pondicherry, south-east coast of India during harvesting period. Shrimps were maintained in the wet laboratory for one month prior to experiment in a controlled environment. They were kept at a density of 0.2 shrimp per liter in 500-liter tanks containing 150 L of water salinity 35 ppt, 28-32 °C, 7.5–8.5 pH, and 4.5 mg/l minimum dissolved oxygen. The tanks were run on a recirculation system including bio-filter and sedimentation tank. The sedimentation tanks were siphoned once every 3–4 days and approximately 20% of the water was replaced every week. Sea water collected through a sand filter (25mm cartridge filter) and an UV sterilizer. Shrimps were fed two times a day with commercial shrimp pellets CP-Aquaculture, India.

2. 2. Experimental shrimps

Adult healthy *L. vannamei* (body weight of 30 ± 5g) of 15 (n = 15) individuals per group were used in the present study. Shrimps were shifted to the individual tank of 250 L capacity. The water parameters such as dissolved oxygen, salinity, pH and water temperature were maintained at > 5.5 mg L^{-1}, 35 ppt, 7.5 ± 0.3 and 32 ± 0.5 °C respectively. The healthy shrimps were selected for the experimental purpose. Experimental infection dose are derived from calculated LD_{50} value. Shrimps are infected experimentally by injection of *V. harveyi* (isolated

from infected shrimp) suspension at 0.5 ml dose of 4.0 x 10^6 CFU/mL for two consecutive days through the ventral sinus of the cephalothorax were subjected to experimental purposes.[10]

2. 3. Collection and Preparation of plant extract

Mp-seeds were purchased from the ayurvedic shop in Puducherry and its identification was confirmed by traditional and experts of plant biologist. The *Mp*-seeds were made to shade dried for a month time to minimize its moisture content. Using electric blender, seeds were pulverized into fine powder. Finely powdered seed materials were then sieved using fine mesh size of forty. Finely sieved seed powder were packed tightly and subjected for extraction process. *Mp*-seed powder was subjected for methanolic extraction (Analytical grade) (Merck, Inc) by the method.[11] 100 g powder of *Mp*-seed was soaked in 1000 ml of methanol sequentially, stirred for about 6 minutes and left overnight. Thereafter, the solution was filtered using filter paper (Whatman No. 1) and the extract was evaporated to dryness under reduced pressure and temperature (below 40 °C) in rotary vacuum evaporator. The concentrated *Mp*-seed methanol extract was dried and stored at 4 °C until use.

2. 4. Enrichment of *Artemia* (*Ar*) for experiment diets

The *Ar* enrichment with herbal extracts procedure was followed as described[12] with some modification. *Artemia franciscana* (*A. franciscana*) (Tuticorin, India) were acclimatized for 5 hrs in normal seawater. *Mp*-seed methanolic extract was emulsified with oil (Super-Selco, Artemia systems, Belgium) then enriched at concentration of 100 ppm and 200 ppm in enrichment tank with *Ar*-biomass (5000/L seawater). After 4 hrs of enrichment, the enriched *Ar* were rinsed in seawater at temperature of 32±2 °C and salinity of 30 ppt sequentially dip in fresh water then frozen into 1 cm^3 blocks. *Ar* was enriched without herbal extracts for the normal and infected groups.

2. 5. Experimental design

Eight groups of adult healthy *L. vannamei* were stocked in tank of 250-litre capacity for 21 days in three replicates (Table 1). The shrimps were fed two times a day at 8.00 and 18.00 h at 10% of the body weight (BW). Waste matters and uneaten feed and were cleared before feeding on every experimental study. During the feeding time, the water exchange was stopped for1 hour to avoid the loss of feed.

Table 1. Segregation of shrimps into different groups.

	Male	**Female**
L. vannamei	*Ar* (Normal group)	*Ar* (Normal group)
	V. harveyi infected	*V. harveyi* infected
	Ar+*Mp* (100 ppm)	*Ar*+*Mp* (100 ppm)
	Ar+*Mp* (200 ppm)	*Ar*+*Mp* (200 ppm)

2. 6. Collection of hemolymph

Haemolymph (HmL) was collected from the ventral sinus of shrimp, using a 1 ml sterile syringe (25 gauge, hypodermic needle) containing 0.9 ml of an anticoagulant solution (0.34 M sodium chloride, 30 mM tri-sodium citrate, 0.12 M glucose, 10 mM EDTA, osmolality adjusted with glucose to 780 mOsm kg^{-1}, pH 7.55). Hemolymph was collected from randomly selected shrimps in each group.

2. 6. 1. Total hemocyte (THmC) count

A 100 µL HmL was gently mixed with anticoagulant then transferred to a sterile 1.5 mL eppendorf tubes. HmL was fixed with an equal volume of 10% buffered formalin in using the following ratio: 3 parts anticoagulant, 1 part HmL, 5 parts formalin. A 20 µL aliquot was stained using 1.2% Rose Bengal in 50% ethanol and allowed to stain for 20 min. THmC was counted using a Neubauer haemocytometer (NHM) under a compound microscope at 40x magnification. Obtained values were expressed as THmC mL^{-1} HmL[13] using the following formula:

THmC count = (A xdcf)/(B x (4 x 10^{-6})

Dilution correction factor (dcf) = Vh/(Vh+ Vac)

Where: A = total number of cell counted; B = total number of squares counted; Vh= volume of HmL; Vac= volume of anticoagulant.

2. 6. 2. Phenoloxidase (PO) activity

The HmL samples were transferred to a cold tissue homogenizer, homogenized and centrifuged at 700 ×g at 4 °C for 20 min; the supernatant fluid was then discarded and the pellet was rinsed, re-suspended gently in cacodylate citrate buffer and centrifuged again. The pellet was then re-suspended with 200 µl cacodylate buffer and a 100 µl aliquot was incubated with 50 µl trypsin (Sigma, 1 mg ml^{-1}), which served as an activator. Then, 50 µl of L-DOPA was added for 10 min at 25–26 °C, followed by 800 µl of cacodylate buffer 5 min later. The optical density at 490 nm was measured using a UV-vis spectrometer (1800, Shimadzu, Japan). The control solution, which consisted of 100 µl cell suspension, 50 µl cacodylate buffer (to replace the trypsin), and 50 µl L-DOPA, was used to measure the background phenoloxidase activity in all test solutions. The optical density of the shrimp's PO activity for all test groups was expressed as dopachrome formation in 50 µl of HmL.[14]

2. 6. 3. Respiratory burst (RB) activity

HmL samples 100 µl was collected in an Eppendorf tube and centrifuged at 1600 rpm for 5 min in a refrigerated centrifuge. After centrifugation the supernatant was discarded and 100 µl of laminarin ($C_6H_{10}O_5$) (2mg/ ml in HBSS) was added to the HmC and allowed to react for 30 min at 37 ºC. Then $C_6H_{10}O_5$ was discarded and the HmL were washed 3 times with 100 µl HBSS, and then stained with 100 µl NBT solution (0.3%) for 30 min at room temperature. The staining reaction was terminated by removing NBT solution and adding absolute methanol. After three washings with 70% methanol, the HmC were air dried and finally 120 µl of 2M KOH and 140 µl of DMSO was added to dissolve the formazan (CH_4N_4). The optical density

of the dissolved (CH_4N_4) was read at 630 nm in a spectrophotometer. RB were expressed as NBT-reduction in 50 µl of HmL.[15]

2. 6. 4. Superoxide dismutase (SOD) activity

To 0.1 ml of 1:10 diluted cell extract, 0.25 ml of absolute ethanol and 0.15 ml of chloroform were added. After 15 minutes of shaking in a mechanical shaker, the suspension was centrifuged for 10 minutes at 2500 rpm, and the supernatant was used for the assay. The reaction mixture for auto-oxidation consisted of 2 ml of Tris-HCl buffer (pH 8.2), 0.5 ml of 2 mM pyrogallol and 1.5 mL of water. Initially, the rate of auto-oxidation of pyrogallol was noted at an interval of 1min. for 3 min. this was considered as 100% auto-oxidation. The assay mixture for the enzyme contained 2ml of Tris. HCl buffer (pH 8.2), 0.5 mL to 2 mM pyrogallal a final volume of 4 mL. The rate of inhibition of pyrogallol auto-oxidation after the addition of enzyme was noted. The percentage inhibition in the auto-oxidation of pyrogallol in the presence of tissue extract was converted to units of inhibition. The amount of enzyme required to give 50% inhibition of enzyme activity. The enzyme activity was measured reading against reagent blank at 470 nm. The exact unit of enzyme activity was then calculated using the formula. The SOD activity was expressed as Units/mg protein.[16]

$$\text{Units of inhibition} = \frac{\text{assay volume}}{\text{volume of tissue extract}} \text{X dilution factor X} \frac{1}{\text{mg protein}}$$

2. 7. Antioxidant enzyme

Antioxidant enzyme assay were evaluated spectrophotometrically in tissue homogenates (HmG) by the following methods:

2. 7. 1. Superoxide dismutase

The SOD was assayed according to the method[16] has been described above in 2.6.4.

2. 7. 2. Catalase

The assay mixture contained 0.5 ml of Hydrogen peroxide (H_2O_2), 1 ml of buffer and 0.4 ml of water, 0.1 ml of 1:10 diluted tissue extract was added to initiate the reaction. 2 ml of dichromate acetic acid ($Na_2Cr_2O_7$) reagent was added after 15, 30, 45 and 60 seconds, to arrest the reaction. To the control tube, the enzyme was added after the addition of the $Na_2Cr_2O_7$ reagent. The tubes were then heated for 10 minutes, allowed to cool, and the green color developed was read at 570 nm. The activity of catalase (CAT) was expressed as µ moles of H_2O_2 consumed/min/mg protein.[17]

2. 7. 3. Glutathione peroxidase (GPx)

0.5 ml of buffer, 0.1 ml of Sodium azide (NaN_3), 0.2 ml or reduced Glutathione ($C_{10}H_{17}N_3O_6S$), 0.1 ml of H_2O_2 and 0.5 ml of 1:1 diluted aliquot of the enzyme were taken and the total volume was made up to 2 ml with distilled water. The tubes were incubated at 37 °C for 3 minutes and the reaction was terminated by the addition of 0.5 ml of 10% TCA. To determine the residual $C_{10}H_{17}N_3O_6S$ content, the supernatant was removed after centrifugation (1500 rpm for 8 minutes) and to this 4.0 ml of Disodium hydrogen phosphate (Na_2HPO_4)

(0.3M) solution and 1ml of the DTNB reagent were added. The color developed was read at 412 nm against a suitable reagent blank containing only phosphate (PO_4^{3-}) solution and DTNB reagent. The enzyme activity was expressed as µ moles of GSH Consumed/min/mg protein.[18]

2. 7. 4. Glutathione reductase

The assay mixture containing 0.2 ml of tissue extract, 1.5 ml of Sodium phosphate (Na_3PO_4) buffer, 0.5 ml of EDTA 0.2 ml of oxidized glutathione and 0.1 ml of NADPH were added. The decreased in optical density at 340nm was then monitored for 2 minutes at 30 seconds interval in a spectrophotometer against a reagent blank. Glutathione reductase activity was expressed as µ moles of NADPH oxidized/min/mg protein.[19]

2. 8. Histology

After 21 days of experiment period, shrimps from each group taken for histology studies. Hepatopancreas tissue (HPCT) samples were preserved in Davidson fixative for 48 h and then processed by routine histology.[20] The tissue sections were prepared for Haematoxylin-Eosin staining (HmTES) and analyzed under optical microscopy for histological studies.

2. 9. Statistical analysis

The resulting data obtained from the experiment was analyzed by means of one-way analysis of variance (ANOVA) and Duncan's multiple comparison of the means. The level of Significance for the analysis was set to $P<0.05$. Statistical analyses were carried out using the software SPSS (version 20) software package.

3. RESULTS

The immunity of the shrimp was evaluated by examine THmC, PO, RB and SOD activity of HmL in male and female *L. vannamei* shown in Figure 1.

The THmL Count of *V. harveyi* infected both sexes was significantly lower than normal control and *Mp*-fed groups. At the end of experiment *Mp* enriched *Ar* fed groups of both sexes shows significant increase in THmC whereas infected groups shows lesser THmC than normal control (Fig. 1A). The experimental diet fed to both sexes of *L. vannamei* with PO-activity was relatively matched with normal control group value. However, the *V. harveyi* infected shrimp has low PO-activity in both male and female. The results inferred that the PO-activity was high in *Mp* 200 pm enriched *Ar* groups when compared to infected shrimps of *L. vannamei* (Fig. 1B). The high RB-activity was registered by *Mp*-seed extract enriched ARM fed groups. RB-activity decreased significantly in *V. harveyi* infected shrimp of both sexes. The results indicate that RB-activities of 200 ppm *Mp* enriched groups were significantly higher than that of control shrimp (Fig. 1C). Shrimps fed on *Mp* 100 ppm and 200 ppm enriched diet displayed higher SOD activity in both sexes. SOD-activity decreased significantly in infected male and female *L. vannamei*. The SOD level of 200 ppm *Mp* enriched fed group was 1.7 fold higher than the infected group, whereas in 100 ppm fed groups reaches to normal control level. As a concluding remark *Mp* fed groups shows significant increase in immunoparameter whereas infected group shows lower than normal control. This indicates that *Mp* has IST role in *L. vannamei* against *V. harveyi* infection (Fig. 1D).

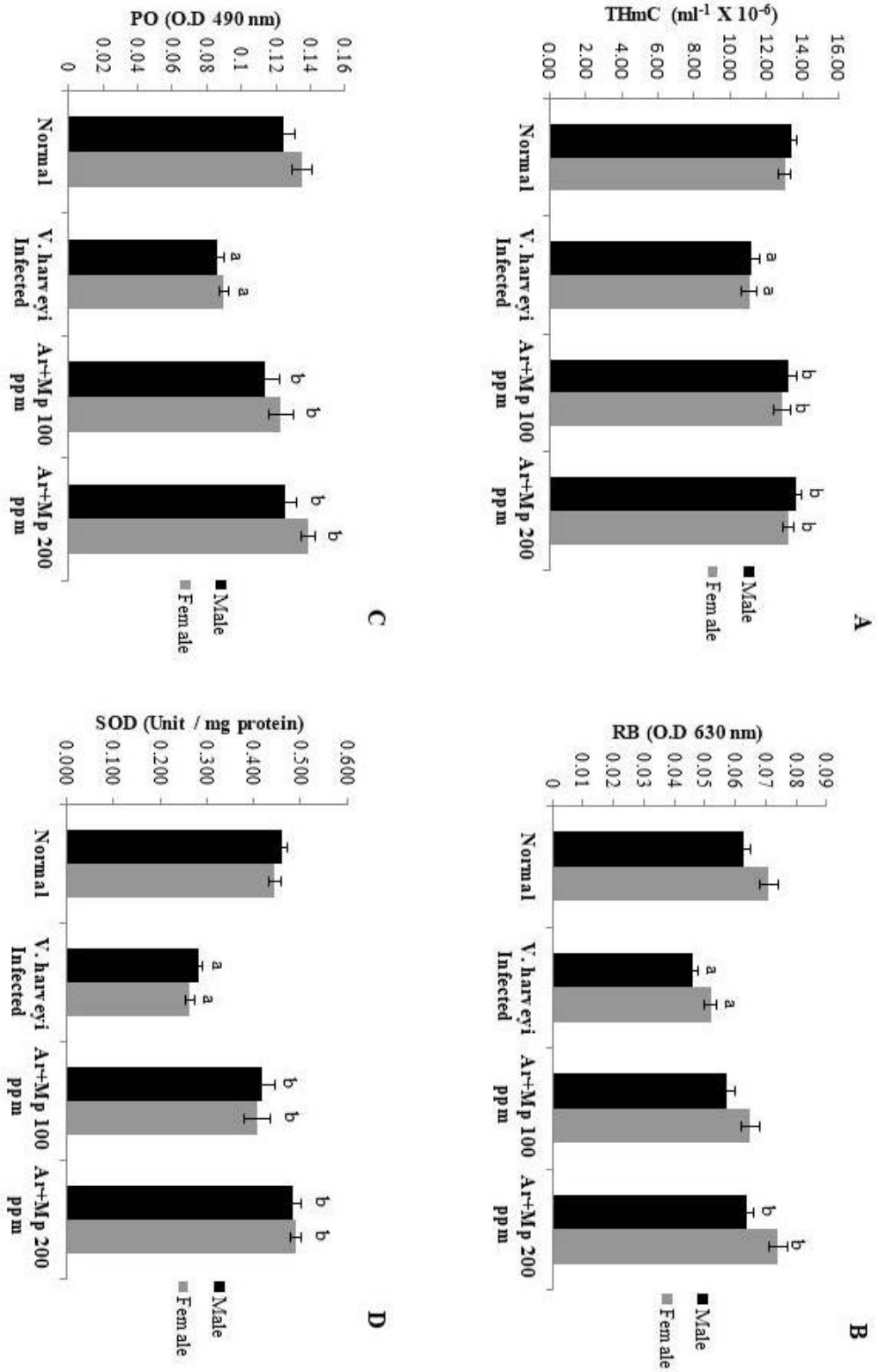


Figure 1. (A) HmL THmC count, **(B)** PO activity, **(C)** RB activity, **(D)** SOD activity.

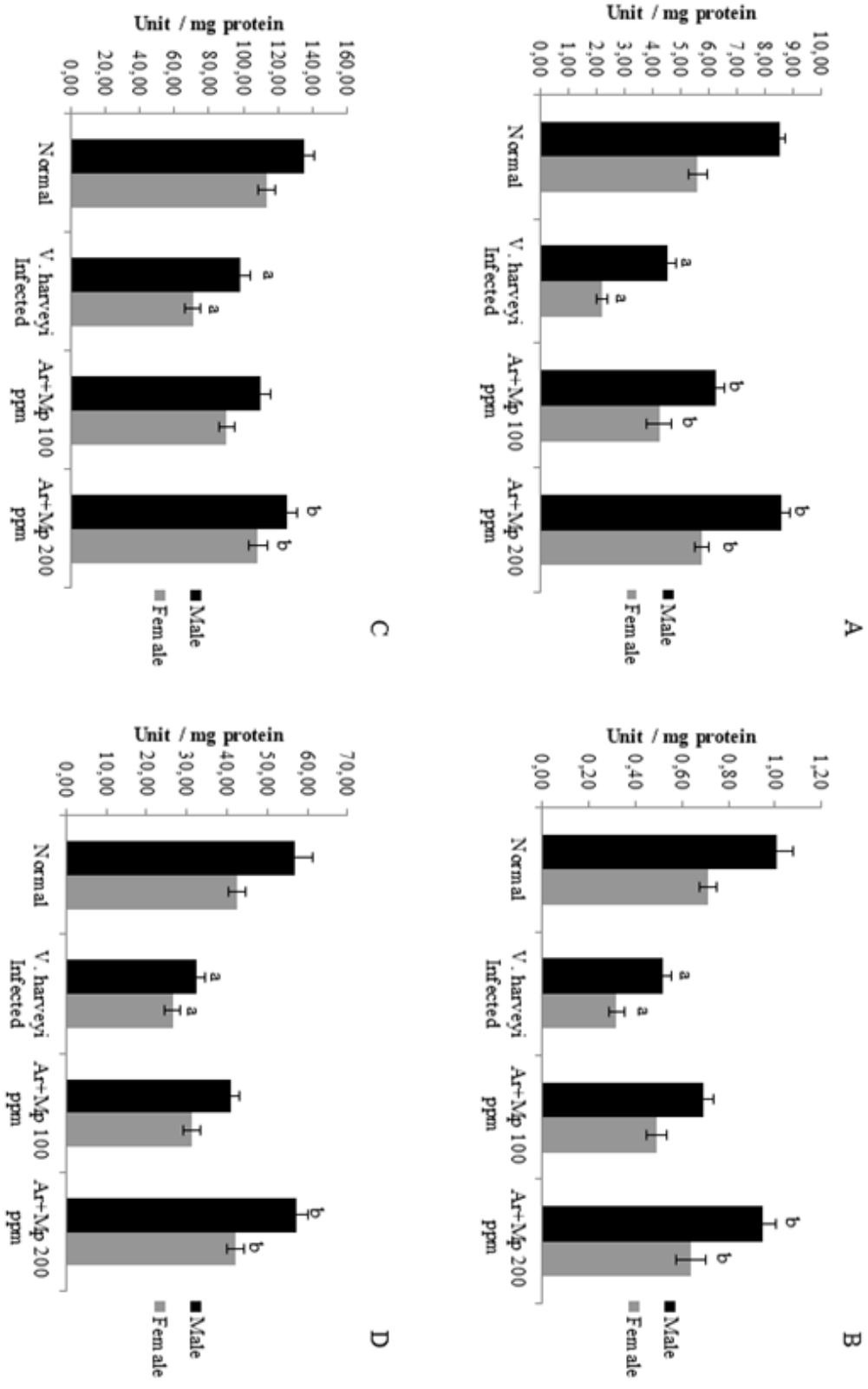
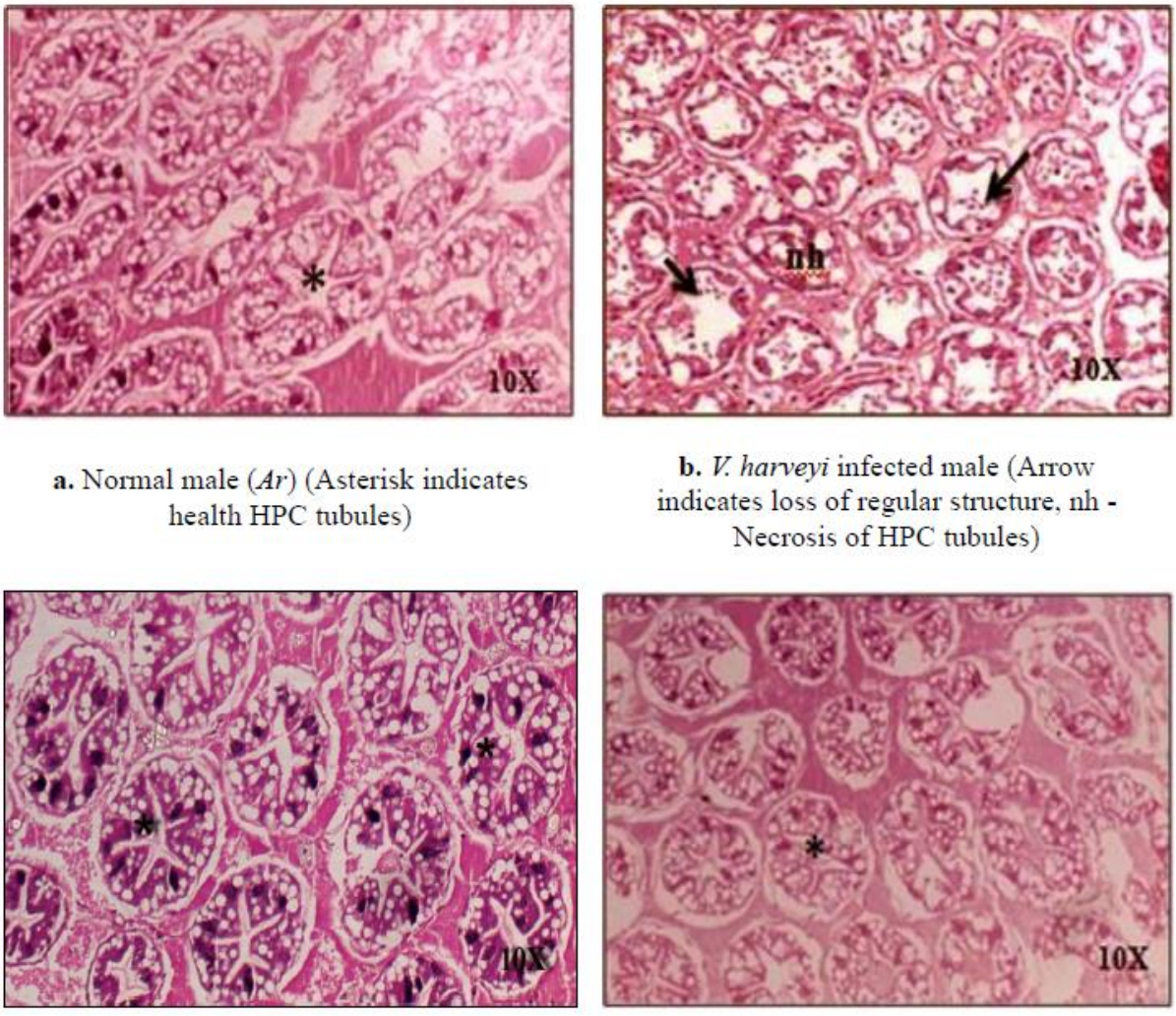


Figure 2. (A) Muscle SOD activity, **(B)** CAT activity, **(C)** GPx activity, **(D)** GSH activity.

Shrimps muscle tissues of each group are utilized for the estimation of antioxidant enzyme in *L. vannamei*- male and female shown in figure 2. The SOD level in muscle tissues was high in *Mp*-seed extract enriched *Ar* fed groups, when compare to *V. harveyi* infected shrimps. *Mp* fed group recovered the SOD to a significant state (Fig. 2A). CAT enzyme level was totally low in infected shrimps when compared to normal control CAT enzyme level reaches normal in *Mp* fed group in both sexes (Fig. 2B).

GPx level was low in infected group when compare to normal control. *Mp* fed group exerted recovery level of GPx (Fig. 2C). GSH level in infected shrimps was considerably lower than that of normal control. *Mp* fed group adjusted the GSH level to normal level (Fig. 2D). Significantly increased SOD, CAT, GPx and GSH activities were observed in shrimp fed with *Mp* enriched *Ar* diets compared to *V. harveyi* infected. Higher level of antioxidant status indicates that shrimps acquired resistance to *V. harveyi* infection.



a. Normal male (*Ar*) (Asterisk indicates health HPC tubules)

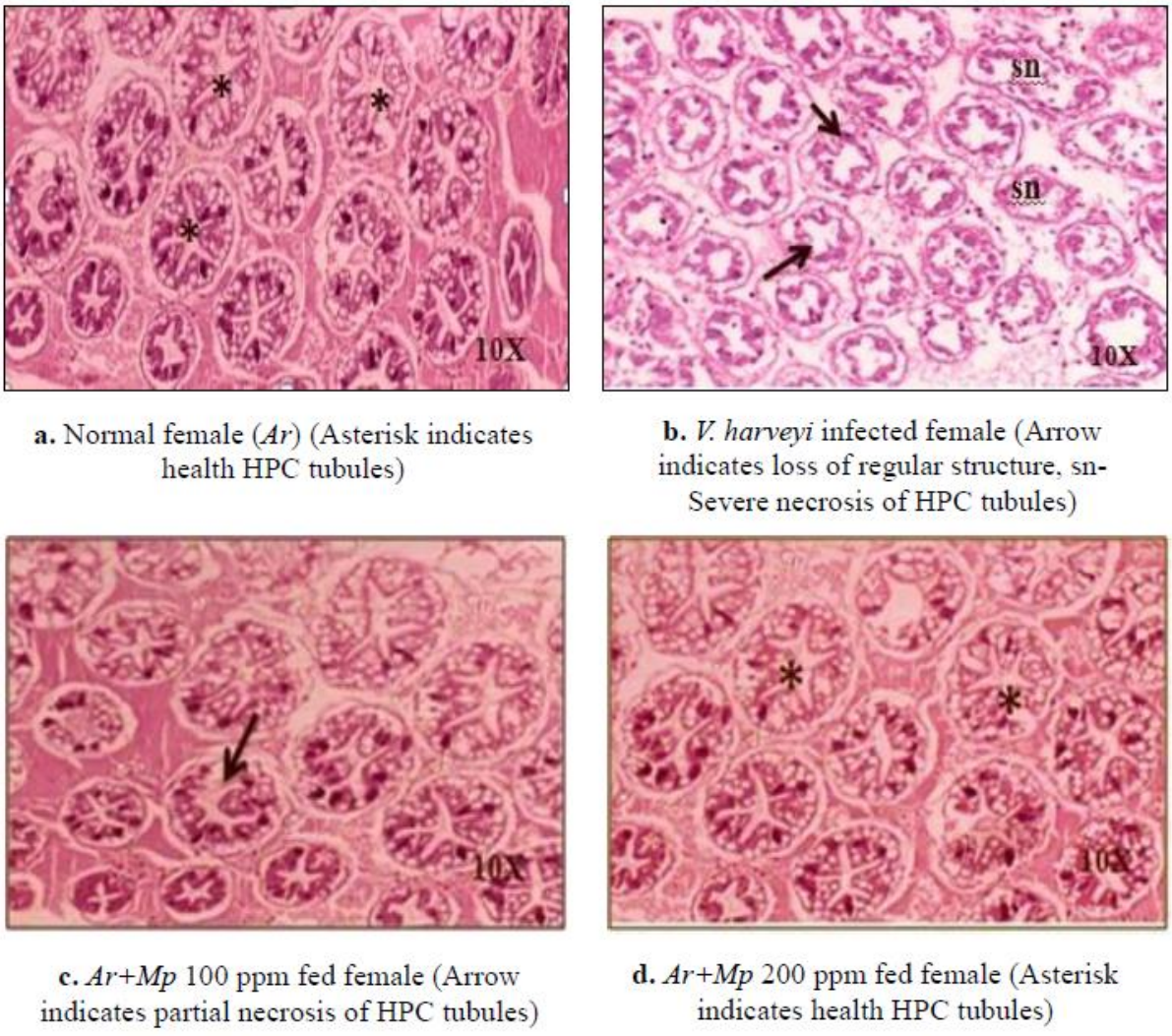
b. *V. harveyi* infected male (Arrow indicates loss of regular structure, nh - Necrosis of HPC tubules)

c. *Ar*+*Mp* 100 ppm fed male (Asterisk indicates health HPC tubules with some pigmentation)

d. *Ar*+*Mp* 200 ppm fed male (Asterisk indicates health HPC tubules)

Figure 3. HPC of Male shrimp, *L. vannamei*

The histo-pathological examination showed HPC tissue of control shrimps group had intact and healthy HPC tubules. The *V. harveyi* infected shrimp had severe necrosis in HPC tubules tissues of both male and female. *Ar* enriched with *Mp* 100 ppm diet fed groups show partial recovery in HPC tubules, whereas 200 ppm enriched diet fed groups returns to healthy HPC tubules (Image I - VIII). The result of histological study shows demorphies in their histo-architecture due to infection, whereas *Mp*-fed group restores to normal structure.



a. Normal female (*Ar*) (Asterisk indicates health HPC tubules)

b. *V. harveyi* infected female (Arrow indicates loss of regular structure, sn-Severe necrosis of HPC tubules)

c. *Ar+Mp* 100 ppm fed female (Arrow indicates partial necrosis of HPC tubules)

d. *Ar+Mp* 200 ppm fed female (Asterisk indicates health HPC tubules)

Figure 4. HPC of female shrimp, *L. vannamei*

4. DISCUSSION

Bacterial and viral infections are considered as the most devastating diseases affecting the shrimp industry around the world, even though recombinant DNA, protein vaccines, and RNA are available to control these.[21] Indeed, to overcome this problem, research on THmC, PO, SOD and RB activity of medicinal plant extract is being made world to treat such diseases

with terrestrial herbal extract, which are having ISt effect on shrimp, male and female *L. vannamei*.[22-24] The current investigation shows that the THmC, PO, SOD and RB activity of HmL using *Mp*-seed extract enriched with *Ar* in male and female *L. vannamei* infected by *V. harveyi*. The findings of the present examination were comparable with some of the other previous reports, the ISt effect of *Phillanthus niruri*-methanol extract on shrimp, P. monodon against WSSV was investigated, and quantification of WSSV infection through qRT-PCR analysis recorded that the control groups had 429×10^3 WSSV DNA copies, it experimental groups were considerable reduced from 4415 to 91.[24] Low level of THmC in HmL is caused by microbial infection because HmC are target for pathogen.[25] *Nodulisporium* sp. KT29 with induction treatment raised antibacterial activity with best treatment of NM, and *V. harveyi* infection resistance presented NM treatment of 20 mL/kg increase survival in *L. vannamei* shrimp reached 72.2%.[26] Continuously, PO role as humoral immune response in crustacean and it is shows as non-specific extracellular immune system mechanisms to prevent any infection caused by pathogenic bacteria. HmC takes part in PO mechanism by blocking harmful compound getting into the shrimp.[27,28] Recently, *Porpyridium aerugenum*-extract in shrimp feed was successful in increasing the number of HmC, and Pro-PO activity besides increasing the survival % of experimental shrimp.[29] Toll pathway and Imd pathway have been identified as the major signaling pathways in *L. vannamei* to participate in the process of immune responses.[30] Extracellular polysaccharide (EPS) from Porhyrydium enhances immune parameters in shrimp rapidly, and has the ability as an ISt or an immunomodulator.[31]

Supplementation on *Zingiber officinalis* and *Cyanodo dactylon* fed *M. rosenbergii* juvenile had increasing level of THmC, pahogocytic rate and pahogocytic index compared to the control.[32] Anion superoxide content and phagocytotic activity (PA) are directly proportional with the HmC number i.e. the increase in HmC counts will be followed by increase of anion superoxide content and PA.[33] The variations in the number of HmC are caused by the responses to infections, species, and environmental stress.[34,35] Further, the THmC pattern was directly proportional to the reduction of PA on the 18th and 21st day, and was due to the existence of phagocyte cells (hyaline cells).[36,37] Particularly, Yu-Ping-Feng polysaccharides (YPS3) group significantly increased the expressions of immune-related genes in the HmC and intestine, and *V. harveyi* challenge, the cumulative mortality in YPS groups was significantly lower than that of the control. Further, dietary YPS had significant effect on growth performance of shrimp.[38] THmC was gradual increased during ISt, which indicates infection was managed by immune system.[39] Particular HmC types percentage was increased due to rapid cellular differentiation or recruitment of cells from non-circulating compartments of the HmL, induced cellular proliferation in response to antigenic challenge.[40] On par with earlier reports, in this study also the alteration of THmC level among infected and *Mp* administered *L. vannamei* was witnessed. *Mp*-fed shrimps as significantly higher RB-activity than infected and control groups. *Gracilaria verrucosa*- extract using the THmC 1.8×10^6 cell/mL, phagocytic activity 65%, and survival rate increased up to 79.2%, and indicating that *G. verrucosa*-extract stimulated antibody responses.[41]

Many herbal compounds have been found to have non-specific immune stimulating properties in fish and shrimp specifically against WSSV, which screening of ISt plants having the ISt activity against the WSSV, and in vivo delivery of the plant active extracts in the Indian white shrimp *Fennerropenaeus indicus* against WSSV infection.[42] *G. verrucosa* derived from coarse sulphate polysaccharides researched being immune stimulators in shrimp. The polysaccharides isolated from seaweed had a molecular model that was recognized by shrimp

innate immune cells.[43,44] Commonly, total carbohydrate and glucose levels increases in infected or stressed animals toward off the infection or stress.[45] In the present study, *Mp*-fed groups has higher PO activity which indicates that this level of supplementation could stimulate HmC degranulation and activate proPO to become PO. SOD are one of the main antioxidant defense enzymes altered in response to oxidative stress, which converts the highly toxic superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2) by reduction. The oxidant formed (H_2O_2) is transformed into water and oxygen (O_2) by catalase (CAT) or GPx. Selenoprotein GPx enzyme removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSH).

SOD activity in HmL was significantly lowered in the WSSV-infected *P. monodon*, while *C. dactylon* extract treated (both invitro and invivo) shrimp significantly recovered when compared with control shrimps.[46] The *Mp*-fed group revealed an increased activity of SOD in *L. vannamei* and this may enhance the generation of other immunoproteins. An important component of antioxidant defense is composed of catalase (CAT) and GPx both convert H_2O_2 to water before hydroxyl radicals can be produced. CAT plays a relatively more vital role in detoxifying invertebrates compared to vertebrates.[47] Low PAs correlated with THmC, which also decreases, can be assumed that low HmC in the body of shrimp cause weakening of the non-specific immune system, which makes the shrimp unable to fight against bacterial infections.[48,49] GPx activity help convert the excess ROS so as to protect the host cells from oxidative damage. In crustaceans, in addition to CAT and GPx, peroxinectin a multifunctional protein containing biological activity of peroxidase enzyme, also plays a important role in the antioxidant defense by averting oxidative damage from H_2O_2.[50] This enzyme together with SOD, and catalase guard cells against damage caused by free radicals and hydroperoxides or lipoperoxides. GPx enhancement is considered to be associated with increased protection to reduce the harm from H_2O_2 after a pathogen invasion. In addition, plant sp increases immune response on the European Seabass.[51]

On comparing the above reports with this study results it is revealed that the increased levels of antioxidant in *L. vannamei* shrimps after exposing to *Mp*-seed extract might have increased their resistance against pathogen. Further the improvement of immunological status of the shrimps may be due to the ISt property of the *Mp*-extract. Particularly, the increase in endurance seen in the treatment with the addition of extracts thought to be associated with an increased immune response especially from the phagocytic activity which removes pathogens, viruses or invading microbes.[52]

Continuous, increase of immune response may have an impact on increasing endurance so that shrimp are more resistant to disease.[53] Histological analysis of *Mp*-fed on *L. vannamei* showed good healthy histological structures of HPC compared to infected shrimp. *Vibrio harveyi* is considered as the most frequently implicated in vibriosis to *L. vannamei* and cause major troubles in shrimp farming. Administration of *Ar* enriched feed with *Mp*-seed extract to the diseased *L. vannamei* has shown a protective effect against *V. harveyi* infection. Similarly, the melanisation of the body related to the decrease of total HmC will cause damage to the host.[54-56] Histopathology is a right tool for diagnosis and monitoring health, where the alteration at the tissues and cells due to the pathogen are interpreted to arrive at diagnosis. Pathology of HPC in typical of vibriosis showing loss of structure, severe necrosis, atrophy of tubule epithelial cells, vacuolation and sloughing and rounding of cells into the lumen.[57] Shrimp infected by vibriosis was characterized by the reddish carapace, melanosis of the skin, necrosis on the tail, lesions, and brownish HPC.[58-62]

5. CONCLUSION

As a concluding remark, the herbal *Mp*-seed extract has positive effect in shrimp antioxidant and ISt. The experimental study demonstrated that *Mp* enriched *Ar* in diet have a potential role and it is an effective antioxidant by regulating infectious stress, improves the immunity of shrimp against vibriosis. The present research highlighted the ISt role of *Mp* enriched *Ar* by increasing the resistance against *V. harveyi* infection. The study also suggested that the seed extract of *Mp* may be used as an alternate to synthetic antibiotic for vibriosis in aquaculture shrimp industry. Since, the synthetic antibiotic use is associated with human health and environmental problems, comprise develops pathogen resistance spread of antibiotic resistance to other organism. However, further study is warranted to isolate the bioactive principles and define the optimal dose and duration of administration in the field (shrimp farms).

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