ANTIBACTERIAL ACTIVITY OF *MUCUNA PRURIENS* SEED EXTRACT ON *VIBRIO HARVEYI* INFECTED *LITOPENAEUS VANNAMEI*

Thesis submitted to Pondicherry University in partial fulfilment of requirements for the award of the Degree of

DOCTOR OF PHILOSOPHY

in

ZOOLOGY

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CERTIFICATE

This is to certify that the thesis entitled **"ANTIBACTERIAL ACTIVITY OF *MUCUNA PRURIENS* SEED EXTRACT ON *VIBRIO HARVEYI* INFECTED *LITOPENAEUS VANNAMEI*"** submitted to Pondicherry University in partial fulfilment of requirements for the award of the Degree of **DOCTOR OF PHILOSOPHY in ZOOLOGY** by **Mr. G.SHANMUGAVEL** is the record of original research work carried out by him independently under my guidance and supervision. I also certify that this has not formed the basis of the award of any Degree / Diploma / Associateship / Fellowship or any other similar title to the candidate.

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DECLARATION

I hereby declare that the thesis entitled **"ANTIBACTERIAL ACTIVITY OF *MUCUNA PRURIENS* SEED EXTRACT ON *VIBRIO HARVEYI* INFECTED *LITOPENAEUS VANNAMEI*"** submitted to Pondicherry University in partial fulfillment of requirements for the award of the Degree of **DOCTOR OF PHILOSOPHY in ZOOLOGY** is my original research work and it has not been previously submitted either in part or whole to this or any other university for the award of any Degree/Diploma.

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ACKNOWLEDGEMENT

It gives me a great pleasure to express my deep sense of gratitude, indebtedness and sincere thanks to ***Dr. G. Krishnamoorthy,*** *Associate Professor & Head, Department of Zoology, Tagore Arts College, Puducherry, for suggesting the problem and able guidance, constant encouragement and help throughout the course of my research work.*

I wish to acknowledge my sincere thanks to ***Dr. Thamizharasi Tamizhmani,*** *Director,* ***Dr. V. Anandan, Dr. O.P. Shyma, and Dr. R. Seetha****, Formerly Directors, Kanchi Mamunivar Centre for Post Graduate Studies, Puducherry, for allowing me to carry out research work in this institution and their kind encouragement.*

I am very grateful to ***Dr. B. Kumaran,*** *(Associate Professor in Zoology) Principal, Indira Gandhi Government Arts and Science College, and* ***Dr. P. Latha,*** *Assistant Professor in Biochemistry and Molecular Biology, Pondicherry University, Doctoral Committee members for their deep interest, guidance and encouragement throughout this research.*

I extend my hearty gratitude to ***Dr. C. Sakunthula, Dr. S. Rajalakshmi, Dr. K. Sankaraiyah, Dr. V. Ramalingam,*** *Department of Zoology, Kanchi Mamunivar Centre for Post Graduate Studies, Puducherry, for their kind encouragement.*

I extend my sincere gratitude and thanks to ***Mr. K. Kaliyaperumal****, Rank Marine Hatchery, Chettikuppam, Villupuram District, Tamil Nadu for the encouragement and valuable suggestion and providing the laboratory facilities to carry out this research.*

I wish to express my sincere thanks to ***Dr. K. Kumaravel,*** *Postdoctoral fellow, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China for the timely help and encouragement.*

*I extend my sincere thanks to **Refsyn Biociences Pvt. Ltd.** Puducherry, for their help in the laboratory.*

I extend my sincere thanks to my PhD scholars colleagues for their support and encouragement throughout my research.

*I wish to thank **my family members** and **well wishers** for showering with love and care throughout my life.*

Finally, I would like to thank everybody who was important to the successful realization of thesis, as well as expressing my apology that I could not mention personally one by one.

(**G. SHANMUGAVEL**)

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List of Abbreviations

AHPND	Acute Hepatopancreatic Necrosis Disease
AMP	Antimicrobial Peptides
BLAST	Basic Local Alignment Search Tool
CAT	Catalase
CGA	Chlorogenic Acid
ECP	Extracellular Products
FAO	Food and Agriculture Organization
FCR	Feed Conversion Ratio
FOS	Fructooligosaccharides
FTIR	Fourier Transform Infrared Spectroscopy
G6PDH	Glucose-6-phosphate Dehydrogenase
GCMS	Gas Chromatograph Mass Spectroscopy
GOAL	Global Aquaculture Alliance
GPx	Glutathione Peroxidase
GSH	Reduced Glutathione
GSI	Gonadosomatic Index
H_2O_2	Hydrogen Peroxide
HFA	Herbal Feed Additive
HPT	Hematopoietic Tissue
IHHNV	Infectious Hypodermal and Hematopoietic Necrosis Virus
IMO	Isomaltooligosaccharides
LDH	Lactate Dehydrogenase

L-DOPA	L-dihydroxyphenylalanine
LPG	Lipopolysaccharides
LSS	Loose Shell Syndrome
MBV	Monodon baculovirus
MDH	Malate Dehydrogenase
MIC	Minimum Inhibition Concentration
Mn SOD	Manganese dependent Superoxide Dismutase
MOS	Mannooligosaccharides
MPEDA	Marine Products Export Development Authority
MRSM	Male Reproductive System Melanization
MT	Metric Ton
MTCC	Microbial Type Culture Collection
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NCBI	National Center for Biotechnology information
NOS	Nitric Oxide Synthetase
OD	Optical Density
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PG	Peptidoglycans
PI	Phagocytic Index
PL	Post Larvae
PO	Phenoloxidase
PR	Phagocytic rate
ProPO	Prophenoloxidase
PRPs	Pattern Recognition Proteins

PRRs	Pattern Recognition Receptors
RB	Respiratory Burst
RBC	Red Blood Corpuscles or Erythrocytes
RNIs	Reactive Nitrogen Intermediates
ROIs	Reactive Oxygen Intermediates
ROS	Reactive Oxygen Species
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SGR	Specific Growth Rate
SOD	Superoxide Dismutase
TCBS	Thiosulfate Citrate Bile salts Sucrose
THC	Total Haemocyte Count
TLRs	Toll-like receptors
TOS	Transgalactooligosaccharides
TSA	Trypticase Soy Agar
TSV	Taura syndrome virus
VHA	Vibrio Harveyi Agar
WBC	White Blood Corpuscles or Leukocytes
WGD	White Gut Disease
WHO	World Health Organization
WSSV	White Spot Syndrome Virus
XOS	Xylo-oligosaccharides
YHV	Yellow Head Virus
YRV	Yellow Head Rhabdovirus
16S rDNA	16S Ribosomal Deoxyribonucleic Acid
16S rRNA	16S Ribosomal Ribonucleic Acid

1. Introduction and literature review

1. INTRODUCTION AND LITERATURE REVIEW

1.1. Aquaculture

Aquaculture is defined as the farming of aquatic organism including fish, crustaceans, molluscs and aquatic plants. It is one of the fastest growing food farming systems at global level with tremendous potential for further development. The industry is growing faster than any other food production sector, due to the combination of strong increasing demand for seafood products and depleted wild aquatic animal stocks in the world's aquatic source. Aquaculture is not only increasing the production of high values species but also provide an affordable protein source to developing country. Aquaculture provides excellent economic growth opportunities by providing jobs from manual labour to skilled labour, income for millions of people and the potential foreign revenue investments. “Aquaculture offers sustainable development by meets the needs of present without compromising the ability of future generations to meet their own needs” It delivers the three dimensional benefits of ecological, economic and social sustainability.

Aquaculture is broadly classified into fresh water aquaculture and marine water aquaculture (Mariculture). Aquaculture has been responsible for introducing exotic animals, plants with latest technological innovation and adaptation to meet changing requirements. Use of nutrient inputs is an important aspect of aquacultural practices in the form of fertilizers, feeds or both. Aquaculture currently provides a critically important source of food for much of the world’s population. Globally many varieties of species are cultured based on local preferences and suitability of growing conditions.

Today’s universal reality of seafood market is that demand greatly exceeds the supply from wild fisheries. Seafood’s proven the role in health benefits include improving cardiac health, decreasing the risk of stroke and increasing cognitive function. Sea foods are rich in omega-3 fatty acids, it has ability to protect against diabetes, dementia, obesity, depression, and menopause. Thus, Nutritionists are also encourages the consumption of sea food is necessary to good health.

Aquaculture sectors are engaging in important public-private sector partnerships between farmer and seafood buyers of both importers and exporters. The stable supply of seafood products to the restaurant and food processing industries which promote sector reform for farm-raised aquaculture products. India is the second largest aquaculture producer and its contribution is nearly 5% of the global production. The country is endowed with a long coastline of 8129 km, 1.2 million hectares of potential brackish water area and 0.5 million sq.km of continental shelf with diverse ecosystems, offering huge scope for development and diversification of coastal aquaculture.

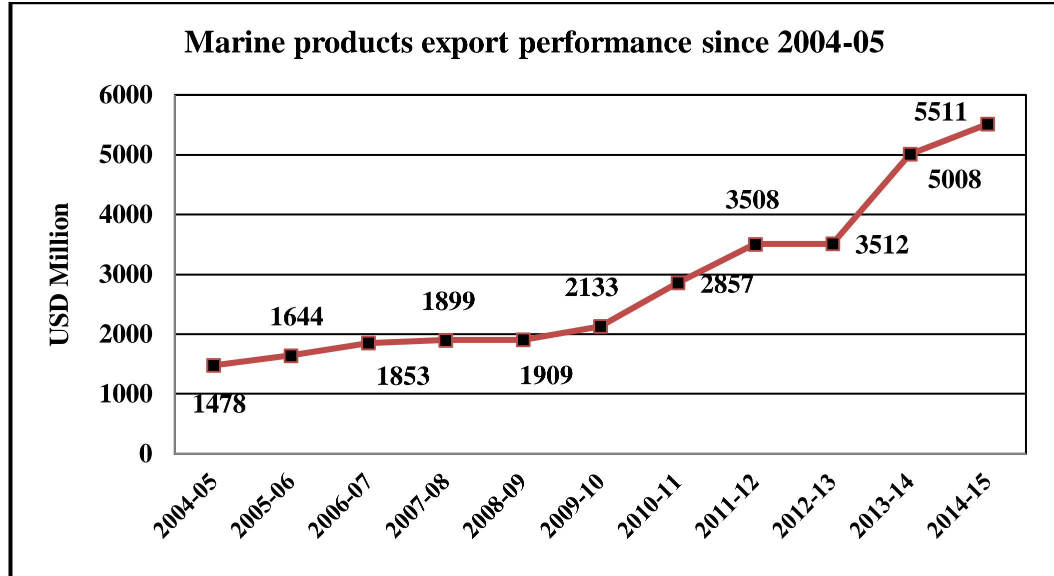
1.2. Shrimp culture:

Shrimp culture is one of the rapidly growing sectors within the aquaculture industries. It is regarded as the best economic activity in terms of returns to investment in areas of production, processing and exports is referred as a high pay-off activity (Krishnan *et al.,* 2000). Shrimp culture expanded significantly during the 1980s and now represents a multibillion dollar industry. Many factors responsible for faster expansion of shrimp aquaculture mainly high profits, high demand in international market, foreign currency earning and employment of poor coastal people are significant positive aspects. Due to limitations and fluctuations in supplies from capture fisheries of shrimps enhances the demand of farmed shrimps. Shrimp exports drive substantial foreign exchange to developing countries and may contribute to regional and national economic growth. Shrimp culture also generates improved incomes for farm workers.

1.3. Species of shrimp culture:

In India, shrimp farming is an age-old practice mainly cultivated at brackish water area. In the country 56 species of shrimp are available but only four species viz., *Penaeus monodon*, *Penaeus indicus*, *Penaeus semisulcatus* and *Penaeus merguiensis* have commercial importance. The recent trend shows replacement of *P.monodon* by *Litopenaeus vannamei* culture considerable increase in shrimp farming due to better growth rate and survive at extreme salinities. Presently more than 67% of farmed shrimp production comes from *L. vannamei* shrimp.

Marine Products Export Development Authority (MPEDA) reported that India marine product export show sizable good growth rate in consistent manner from the financial year 2004-05 to 2014-15.



During 2014-15, India marine product export reached USD 5511.11 million with growth rate of 10.05%. Among this shrimp contribution was 3709.76 million in which cultured shrimp is 76.45%. The export of *L. vannamei* has shown enormous growth to 2,22,176 MT from 1,75,071 MT and USD 2372.80 million from 1994.27 million compared to 2013-14. A total of 42.77% *L. vannamei* was exported to USA followed by 20.36% to South East Asian countries, 17.45% to European Union, 4.55% to Middle East and 4.18% to Japan.

1.4. Shrimp Disease:

Infectious disease is one of the limiting factors in shrimp farming. Besides many other factors also influence shrimp health status such as the age of shrimp, management conditions, biotic and abiotic stress. Shrimp can be threatened by protozoan, fungal, bacterial and viral pathogens, among these the viral and bacterial diseases cause major troubles in shrimp farming (Lightner, 1996). Global Aquaculture Alliance (GOAL) shrimp survey in 2013 revealed that impact of disease is foremost important challenge followed by feed cost and international market prices in shrimp industries. Survey also

revealed that 60% of losses were attributed to viruses and 20% to bacteria besides by 20% of water parameters, predators etc. Thus, the majority of our effort on disease control (80%) should clearly focused on viral and bacterial pathogens.

The first report on shrimp epidemic disease was for *Monodon baculovirus* (MBV) in Taiwan in the mid-1980's. This was followed by outbreaks caused by infectious hypodermal and hematopoietic necrosis virus (IHHNV) in America (Lightner *et al*., 1983), yellow head virus (YHV) in Thailand (Chantanachookin *et al*., 1993) and Taura syndrome virus (TSV) in the America (Hasson *et al*., 1995). While shrimp industries were still struggling with MBV, IHHNV, YHV and TSV outbreaks, the arrival of white spot syndrome virus (WSSV) was made a bigger disaster. WSSV first appearance at China in 1992, then it spread rapidly around Asia (Flegel and Alday-Sanz, 1998) eventually to the America.

The necrotizing hepatopancreatitis was first reported in shrimp crop on a farm in the state of Texas (United States of America) in 1985, the outbreaks have subsequently occurred in Mexico, Brazil, Peru, Ecuador, Venezuela, Panama and Costa Rica causing significant mortality. This disease is also called as granulomatous hepatopancreatitis or Texas necrotizing hepatopancreatitis. This type of infection has been reported in species of *Litopenaeus vannamei, Litopenaeus stylirostris, Litopenaeus setiferus, Farfantepenaeus aztecas* and *Farfantepenaeus californiensis* (Vincent and Lotz, 2007).

The shrimp infected with necrotizing hepatopancreatitis exhibit various symptoms such as darkening of gills, reduction in food ingestion, lethargy, an empty intestine, a flaccid body, an expansion of the chromatophores around the swimmerets leading to a darkened appearance, and marked atrophy of the hepatopancreas (Lightner, 1996). Physico-chemical changes like temperature, salinity and hypoxia which affect the immune response of shrimp and its susceptibility to pathogen bacteria. Li and Chen (2008) reported that water with low and high pH level, as well as those with low dissolved oxygen reduces the total haemocyte count (THC) and phenoloxidase (PO) activity in shrimp.

Bacteria cause disease like bacterial erosion, Zoea II syndrome, "white ball" and systemic vibriosis. Bacterial erosion of the shell is seen in all penaeid shrimp, juveniles and adults alike. It manifest with the appearance of black or brown stain in area that has been eroded through the action of chitinolytic bacteria, such as *Vibrio* spp., *Aeromonas* spp., *Flavobacterium* spp. and *Spirillum* spp. This disease is self-limiting and usually disappears when the shrimp molt. If the shrimp left untreated, it becomes serious and lead to a systemic infection. Zoea II Syndrome causes high mortality rates in the shrimp at juvenile stage. In 1993, this infection was detected for the first time in farms of *Litopenaeus vannamei* in Ecuador, Mexico and the United States (Morales, 2004). "White ball" disease causes appearances of small balls, emerge from desquamated hepatopancreatic cells or to hypertrophied, rounded hepatocytes that visible as spherical formations. It is suspected that these balls are created by toxins produced mainly by Vibrio spp. are *Vibrio harveyi* and *Vibrio alginolyticus* (Gómez-Gil *et al.,* 2001; Vandenberghe *et al.,* 1999).

1.6. Vibriosis:

Vibrios are ubiquitous in marine and estuarine environments as well as aquaculture farms worldwide. In aquatic environments, *Vibrios* are normal part of the bacterial flora and formerly considered to be more opportunistic pathogens (Raissy *et al.,* 2011). *Vibrio* species are not exclusive to one particular host because it is reported in both aquatic and terrestrial animals. *Vibrios* are the common genera associated with crustaceans are common inhabitants of the aquatic environment including shrimp farming ponds (Vijayan *et al.,* 2006). Most of the *Vibrio* species are also pathogenic to humans which are usually responsible for causing alimentary infections in countries with warm coastal waters, where as fish and shrimp are consumed raw or lightly cooked (Messelhäusser *et al.,* 2010). Several species within the *Vibrio* genus have a high tolerance for different salinity levels (Wright *et al.,* 1996), thus it associated with wide range of marine organism. In India, shrimp farms noted that *Vibrio* species accounted for 38–81% of the bacterial biota (Otta *et al.,* 1999; Vaseeharan and Ramasamy, 2003). Jaysree *et al* (2006) reported the tail necrosis, loose shell syndrome (LSS) and white gut

disease (WGD) caused by Vibrio spp. in *P. monodon* of Andhra Pradesh coastal culture ponds.

Vibriosis is one of the major bacterial diseases responsible for mass mortality of cultured shrimp worldwide (Chen *et al.,* 2000). The symptoms of vibriosis are classified based on the site of infections i.e. oral and enteric vibriosis, appendage and cuticular vibriosis, localised vibriosis of wounds, shell disease, systemic vibriosis and septic hepatopancreatitis (Lightner, 1996). Outbreaks of vibriosis may occur when the environmental factors trigger the rapid multiplication of bacteria already tolerated at low levels within shrimp blood (Sizemore and Davis, 1985), or by bacterial penetration of host barriers. Vibriosis infected organism shows lethargy signs, tissue and appendage necrosis, slow growth, slow metamorphosis, body malformation, bioluminescence, muscle opacity and melanization (Aguirre-Guzmán *et al.,* 2004). Vibriosis infected shrimp post larvae (PL) exhibits reduced motility, reduced phototaxis and empty guts (Chen, 1992). In adult shrimps suffering vibriosis may appear hypoxic, shows body reddening to brown gills, reduce feeding and may be observed swimming lethargically at the edges and surface of ponds (Anderson *et al.,* 1998). Vibriosis in penaeid shrimps is mainly caused by *V. harveyi* (Prayitno and Latchford, 1995) besides *Vibrio anguillarum* (Lightner, 1996), *V.alginolyticus* (Selvin and Lipton, 2003), *V. parahaemolyticus* (Alapide-Tendencia and Dureza, 1997). *V. harveyi* was considered as the most frequently implicated in vibriosis, is known to cause severe infections in penaeid livestock (Austin and Zhang, 2006). Although vibriosis is common bacterial disease among marine organisms, this disease normally occurs during the warm summer months when the water salinities and organic loads are high (Caipang and Aguana, 2011). Global warming leads to climate change which inducing water temperature increases is suggested to promote proliferation of *Vibrio*, particularly in temperate aquatic regions and its footsteps *Vibrio*-associated diseases (Harvell *et al.,* 2002, Baker-Austin *et al.,* 2013).

1.7. *Vibrio harveyi:*

Vibrio harveyi is a species of Gram-negative bioluminescent marine bacterium (Fig. 1 & 2). Rod-shaped, motile (via polar flagella), facultatively anaerobic, halophilic and competent for both fermentative and respiratory metabolism. It grows at 4°C - 35°C.

Free-swimming in tropical marine waters which occurring either as a free-living form or in association with intestinal microbiota of marine animals (Makemson and Hermosa, 1999), including prawns, Gorgonan corals, oysters, lobsters, the common snook, barramundi, turbot, milkfish and seahorses. It is also responsible for luminous vibriosis, a disease that affects commercially farmed penaeid prawns. *V. harveyi* emit blue-green

Figure 1. Colonies of the bioluminescent *Vibrio harveyi* glow in the dark petri dish

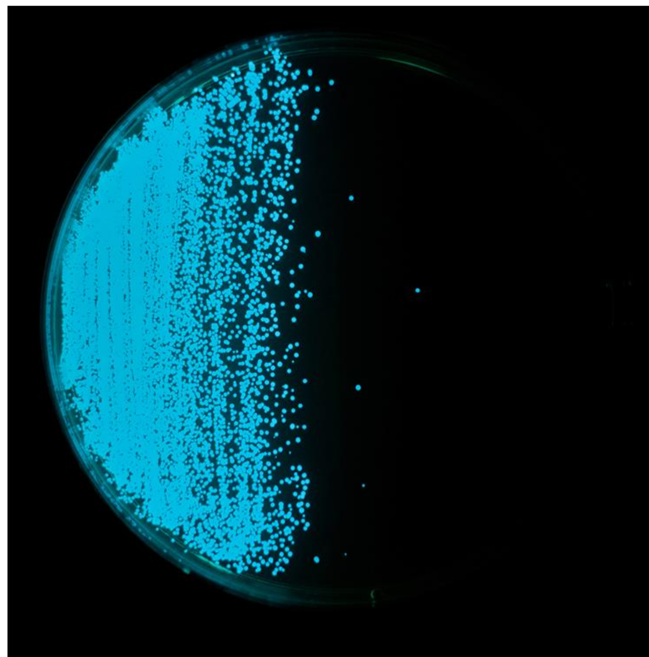
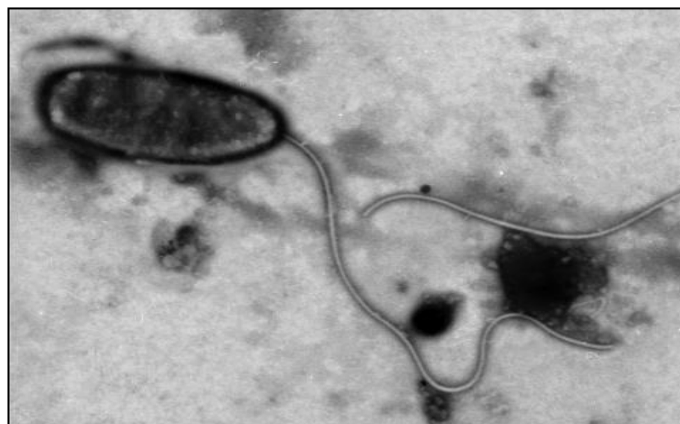


Figure 2. Electron micrograph of *Vibrio harveyi* strain



bioluminescence catalysed by the enzyme luciferase (Baumann and Schubert, 1984). It is thought to be the cause of the milky seas effect, in which, during the night, a uniform blue-green glow is emitted from the seawater. Raungpan *et al.,* (1995) also found that when luminous bacteria exceeded 10^4cells/ml in intensive cultured shrimp ponds caused serious health problems to the shrimp. *V. harveyi* is first isolated from a dead amphipod. *V. harveyi* was originally named as *Achromobacter harveyi* after E.N. Harvey, a pioneer in the systematics of bioluminescent bacteria (Johnson and Shunk, 1936). Subsequently at various stages, it has been named as *Lucibacterium harveyi* and *Beneckea harveyi* arriving at its current taxonomic position as *V. harveyi* (Farmer *et al.,* 2005).

Kingdom	:	Bacteria
Phylum	:	Proteobacteria
Class	:	Gammaproteobacteria
Order	:	Vibrionales
Family	:	Vibrionaceae
Genus	:	***Vibrio***
Species	:	***harveyi***

Vibrio harveyi is considered as a virulent pathogenic bacterium causing diseases in both invertebrates and vertebrates. *V. harveyi* are parts of the natural microflora of wild and cultured shrimps (Sinderman, 1990) and become infectious pathogens when natural defence mechanisms are suppressed (Brock and Lightner, 1990). It causes luminescent disease which leads to mass mortality in the shrimp hatcheries (de la Pena *et al*., 1993).

V. harveyi luminescent strains have been reported to cause major losses in the shrimp larviculture in the Philippine (Lavilla- Pitogo *et al.*, 1990). As a pathogen, *V. harveyi* manifests itself as luminous vibriosis in hatchery reared and commercially farmed penaeid shrimps resulting in severe economic losses to shrimp industry in Asia (Karunasagar *et al.* 1994; Conejero and Hedreyda 2003). *Vibrio harveyi* was found to cause disease in other organisms such as phyllosoma larvae of *Jasus verreauxi* [Rock lobster] (Diggles *et al.*, 2000).

V. harveyi is a predominant pathogen of cultured penaeid shrimp but high mortalities is usually occur in post larvae (PL) and juveniles shrimp. *V. harveyi* were found at all stages of shrimp in both diseased and healthy animal. Clinical sign of *V. harveyi* infection is examined by luminescence exhibits by shrimp which is readily visible at dark light (Ruby *et al.,* 1980), and the infected PL exhibit reduced motility and reduced phototaxis (Chen, 1992). A large number of shrimp hatcheries are located along the coastline of our country involved in shrimp seed production often suffer setbacks due to luminescent bacterial disease and suffer enormous economic losses (Jayasree *et al.,* 2006; Vinoda *et al.,* 2006).

1.8. Present scenario of disease control:

1.8.1. Antibiotics:

Antibiotics are widely used in shrimp farming to treat infectious diseases during the production cycle in both larval and growth phases. Aquaculture farmers rely on a wide range of antibiotics to prevent (prophylactic use) and treat (therapeutic use) bacterial infections in shrimps (Cabello *et al.,* 2013). The most frequently used antibiotics to control bacterial diseases are enrofloxacin, florfenicol, sarafloxacin and oxytetracycline (Roque *et al.,* 2001; Soto-Rodríguez *et al.,* 2006). Some other antibiotics are also used to control the disease i.e. tetracyclines, quinolones, ciprofloxacin, norfloxacin, oxolinic acid, perfloxacin, sulfamethazine, gentamicin, and tiamulin (Holmstrom *et al.,* 2003). Use of antibiotics is associated with environmental and human health problems which comprise pathogens resistance, spread of antibiotic resistance to other organism, disease persistence in the aquatic environment and also effects on the

biogeochemical composition. The antibiotic residues accumulates in the edible tissues of shrimp might alter human intestinal flora cause allergy problems and food poisoning (Ma *et al.,* 2006). Every year, more and more pathogenic Vibrio species have been reported to develop increasing levels of resistance toward most of the clinically used antibiotics (Letchumanan *et al.,* 2015).

In 2002, FAO reported that antibiotics uses poses considerable warning to the aquatic microorganisms prone to acquire antibiotic resistance and antibiotic residue accumulation from lower animals food chain to higher one including humans. These concerns prompted the ban on use of such therapeutics in Europe, USA (Patterson and Burkholder, 2003) paving the way in searching for new avenues and alternatives to replace antibiotic use against disease outbreak. Alternative strategies such as use of vaccine, dietary supplement of probiotics, prebiotics and immunostimulant may help to reduce the susceptibility of shrimp to diseases.

1.8.2. Shrimp vaccines:

Vaccination of shrimp provides protection against specific pathogens by improve the immunity to a particular disease or group of diseases which can be prepared with formalin, ethanol, sodium sulphate, ammonium sulphite, fatty acids and heating to inactivate the pathogen (Hossain *et al,* 2009). It can be administered either by orally along with feed or by injection (Li *et al*., 2005). Oral vaccination is considered as the most practical way to immunize cultured shrimps. Several commercial vaccines are available for teleosts and shrimp based on the adaptive immunity or the presence of specific immune memory (Sommerset *et al.,* 2005; Powell *et al.,* 2011). Shrimp are vaccinated against WSSV using inactivated virus (Singh *et al.,* 2005), DNA vaccines (Li *et al.,* 2010) and dsRNAs or siRNAs vaccines (Mavichak *et al.,* 2011), viral envelope proteins (Fu *et al.,* 2011), recombinant viral protein (Kono *et al.,* 2014). Vaccines available today are narrow specificity may not be applied to all bacterial and viral disease.

1.8.3. Prebiotics:

Prebiotics are a non-digestible food ingredient added to feed that beneficially affects the host by selectively stimulating the growth and/or activating the metabolism of one or a limited number of bacteria in the intestinal tract and thus improves resistance against infection (Gibson and Roberfroid, 1995). Prebiotics increase the microbial community of beneficial bacteria such as *Lactobacillus* spp. and *Bifidabacterium* spp. (Manning and Gibson, 2004). Several food are used as prebiotic, which includes resistant inulin and oligofructose, transgalactooligosaccharides (TOS), lactulose, isomaltooligosaccharides (IMO), lactosucrose, xylo-oligosaccharides (XOS), soyabean oligosaccharides, Mannooligosaccharides (MOS) and glucooligosaccharides. Ringo *et al.* (1998) studied the effect of nutrients such as linoleic acid, linolenic acid and soluble carbohydrates on the aerobic/facultative anaerobic intestinal microbiota of Arctic char *Salvelinus alpines.* Short chain Fructooligosaccharides (FOS) supplementation at different concentrations for 6 week trial, enhanced hemocyte respiratory burst of Pacific white shrimp *L. vannamei* cultured in a recirculation system (Li *et al*., 2007). Mahious *et al*., (2006) found that significantly changed gastrointestinal microflora in Turbot larvae by increasing Bacillus species to 14 % and decreasing *Vibrio* species with dietary supplementation of 2% inulin. Inluin reduce the WSSV prevalence in shrimp with low viral load by increasing the PO activity of *L.vannamei* (Partida-Arangure *et al*., 2013). Zhang *et al*., (2012) conducted 8 week feeding trial on *L. vannamei* with dietary supplementation of MOS enhance the growth and increase the resistance against NH_3 stress. In 30 days feeding trail, white leg shrimp fed with dietary supplementation of Copra-MOS can significantly improves growth, feed conversion, modulating intestinal microflora and enhance the resistance against *Vibrios*, *E. coli* and *Salmonella* (Cuong *et al*., 2013). Prebiotics serving as an energy and growth source for the beneficial bacteria, and unhelpful bacteria. This lead to imbalance in digestive flora, which experiences symptoms of food intolerance, indigestion and absorption in shrimp.

1.8.4. Probiotics:

Probiotics can be defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002). Shrimp farmers use probiotics to improve the growth leading to higher production, save their standing

crop and quality of pond environment. Probiotics act more than one way of mechanisms such as (a) the production of inhibitory compounds such as bacteriocins, competition for adhesion sites with opportunistic or pathogen microorganisms (b) competition for nutrients with other bacteria (c) an improvement of the immune status by increasing the production of immunoglobulins, acid phosphatase, antimicrobial peptides, improvement of cellular activities, etc. Probiotics were found to stimulate the feed conversion efficiency, augment weight in shrimp culture (Saenz de Rodriguez *et al.,* 2009). In highly stocked intensive aquaculture ponds, Probiotics are also being used as biological control agents (Morya *et al.,* 2013). A wide range of gram-positive (*Bacillus, Lactococcus, Micrococcus, Carnobacterium, Enterococcus, Lactobacillus, Streptococcus, Weissella*), gram-negative bacteria (*Aeromonas, Alteromonas, Photorhodobacterium, Pseudomonas* and *Vibrio*), yeast (*Debaryomyces, Phaffia* and *Saccharomyces*) and microalgae (*Tetraselmis*) has been evaluated as probiotics (Gibson *et al.*, 1998; Austin *et al.*, 1992; Tovar *et al.*, 2002). Commercially available preparation mainly contains *Lactobacillus, Saccharomyces cerevisiae* (Abidi 2003), nitrifying bacteria, *Streptococci, Roseobacter* (Wang *et al*. 2008; He *et al*. 2009) and *Bacillus* sp. (Ran *et al*. 2012).

Vibrio harveyi infected *P. monodon* shows an increased survival rate by dietary administration of Artemia encapsulated *Bacillus* spp. S11 (Rengipat *et al.,* 1998). *Bacillus subtilis* BT23 is exposed at 10^6 cfu ml^{-1} in *P. monodon* for 5 days (long-term treatment) or for 1hr (short-term treatment), and thereafter challenged with *V. harveyi*, showed a decrease in their cumulative mortality rate in both groups (Vaseeharan *et al.,* 2003). *P. monodon* fed on the *Bacillus* probiotics (BS11) shows significant growth and resistance to Vibrio (Rengpipat *et al*., 2003). Cumulative mortality was decreased in *L. vannamei* fed a supplemented diet with 10^{10} cfu kg^{-1} of *Lactobacillus plantarum* after injection with *V. alginolyticus* (Chiu *et al.,* 2007). Thompson *et al*., (2010) reported that *L. vannamei* fed supplemented diet with probiotic *Vibrio gazogenes* 3×10^5 cfu/shrimp, reduces mortality after infection with *Vibrio* spp. including *V. harveyi, V. anguillarum* and *V. alginolyticus*. Cruz *et al* (2012) states some of the aquaculture products are consumed raw or half cooked, has the residual probiotics which cause systemic infection to the consumer. Probiotics can be used in advance as prevention tools. They can prevent the disease rather than treatment of the disease. In the process of application of

probiotics, no other chemical or drug should be used for treating other diseases like fungal and protozoan caused by those other than bacteria. These probiotics can easily be destroyed by any other chemical or drug which generally interferes with the establishment of useful microbes.

1.9. Shrimp immune system:

The defense mechanism of shrimp is less developed than that in finfish and other vertebrates. More specifically, shrimp have no adaptive memory. Thus, they do not have the ability of producing immunoglobulins, so that they apparently depend only on innate defense systems (Roch, 1999). The study of shrimp immune system could be important in designing the strategies against pathogen infection. For shrimp, the innate immune responses play a major role in combatting invading pathogens and prevent them against diseases. Shrimp possess an innate immune system that consists of cellular and humoral reactions, which work in jointly coordination for the detection or elimination of all foreign organisms potentially hazardous for the host (Jiravanichpaisal *et al.*, 2006). Haemocytes play an important role in the cellular immune response. Cellular reactions are mediated by hemocytes include phagocytosis, encapsulation, cytotoxicity, nodule formation, cell adhesion, and hemolymph clotting mechanism. Humoral reactions involve the prophenoloxidase-activating cascade and immune-related proteins such as lectins, lysozymes, lysosomal hydrolytic enzymes and antimicrobial peptides.

1.9.1. Physical barriers:

Physical barriers are the first line of defense on shrimp. It consists of a rigid exoskeleton, which protects from injury and microbial attacks. The exoskeleton is composed of calcium carbonate, proteins and carbohydrates which contribute to different physiological processes associated with the immune response (Mylonakis and Aballay, 2005). Distribution of hemocyanin and catalytic phenol oxidation over the exocuticle and endocuticle of crustaceans offers immune response against microbes (Adachi *et al.*, 2005).

1.9.2. Cell mediated immune defense

Crustaceans have an open circulatory system with blue-green hemolymph, which circulates through the hemocele and irrigates the crustacean tissues. Hemocytes and humoral components are transported by the hemolymph favouring their encounter with foreign bodies (Rendon and Balcazar, 2003). Hematopoiesis is the source for mature effectors cells for the innate immune system, which show roles on host defense and homeostasis. The hematopoietic tissue (HPT) in crustaceans is an extensive network of packed lobules located at the dorsal and dorsolateral sides of the stomach, close to the antennal artery and at the base of the maxillipedes (Van de Braak *et al*., 2002). Hemocytes are produced within the walls of these tubules and released into the vessel lumens (Soderhäll *et al*., 2003).

Penaeid shrimp hemocytes have the same biological properties and functions with vertebrate macrophages, granulocytes and natural killer cells (Vande Braak, 2002). These cells participate in phagocytosis, encapsulation, nodule formation, wound repair, clotting, and prophenoloxidase activation. They also help the production of adhesion molecules, agglutinins and antimicrobial peptides (AMP) (Bachere *et al*., 2000). Hemocytes also have inhibitory enzymes needed for regulating the proteolytic cascade, preventing its over stimulation and the resultant tissue damage, while producing cytotoxic molecules such as lysozyme, phosphatase, esterase, phospholipase, peroxidase, protease, etc. (Johansson *et al*., 2000).

There are three classes of hemocytes, hyalinocytes, granulocytes and semi-granulocytes. Hyalinocytes (5- 15% of circulating hemocytes or CE) are small non-refractive cells, with a small nucleus relative to their cytoplasm, which have few or no cytoplasmic granules. Hyalinocytes have no phagocytic activity and easily adhere to glass surfaces, like fish and mammals macrophages. The primary role of these cells is related to clotting and phagocytosis. Granulocytes (10-20% of CE) have the smallest nucleus and a high number of cytoplasmic granules (0.8 µm width). Granulocytes display phagocytic activity and store the enzyme prophenoloxidase (proPO). These cells may be stimulated by β-1,3-glucans, peptidoglycans (PG) and lipopolysaccharides (LPS) to provoke exocytosis and enzyme release. Their function is encapsulation, initiating the proPO

cascade and phagocytosis (Zhang *et al*., 2006). Semi-granulocytes (75% CE) have a large numbers of small granules (0.4 μm width) similar to vertebrate granulocytes. These cells posses β-1,3-glucans receptors and their principal function involves phagocytosis, encapsulation and clotting (Martin and Graves, 2005).

1.9.2.1. Phagocytosis

Phagocytosis involves the internalization of foreign material. This is the main cellular defense mechanism in invertebrates, and is carried out by the semi- and granulocytes; it consists of chemotaxis, adherence, ingestion, pathogen destruction and exocytosis (Kondo *et al*., 1998). Phagocytic cells destroy the internalized organisms by two routes, an aerobic process which uses NADPH or NADH as an electron donor, and reduces an oxygen electron to form the superoxide ion. This radical in turn changes to hydrogen peroxide (H_2O_2) spontaneously or by the action of the superoxide dismutase (SOD), producing a new oxygen molecule. In penaeid hemocytes, the activation of the aerobic process has been demonstrated by the use of bacteria *(Vibrio parahaemolyticus and V. vulnificus)* and surface microbial antigens (β-1,3 glucan, PG, LPS, and zymosan), both increase the phagocytic capacity of hemocytes to destroy pathogens (Campa-Cordova *et al*., 2002). The anaerobic process is attributes to the action of diverse microbicidal enzymes, such as lysozyme and low molecular weight AMP (Nappi and Ottaviani, 2000).

1.9.2.2. Encapsulation and nodule formation

Semi granulocytes are responsible for the recognition of the invading agents and their encapsulation with proteins (76 kD) that work as an opsonins associated to the proPO activation system. These proteins act as a degranulation and adhesion factor for semi and granulocytes, and as an encapsulation promoter (Vargas- Albores and Yepiz-Plascencia, 1998). The hemolytical nodules, detected in gill and hepatopancreas, are formed by numerous hemocytes acting synergistically to trap microorganisms or big antigens that cannot be removed by phagocytosis. These nodules undergo the subsequent

activation of the proPO system, melanisation and destruction of microbes (Van de Braak, 2002).

1.9.2.3. Antioxidant system

Antioxidant factors protect the shrimp from the cytotoxic effects caused by the cellular metabolism and oxidative stress generated by the disequilibrium of the Reactive Oxygen Intermediates (ROIs). Downs *et al*., (2001) reported an increase in the levels of the antioxidant enzymes and immune system molecules and play an important role of the antioxidant enzymes as the immune response modulators.

ROIs and Reactive Nitrogen Intermediates (RNIs) are generated in phagocytic vacuoles. These molecules are capable of crossing the cell barrier and damaging the neighbouring cells. The antioxidant defense strategies have been developed including enzymatic substance (catalase, glutathione peroxidase and SOD) and nonenzymatic components (ascorbate, β-carotene, flavonoids, α-tocopherol and vitamin E), to neutralize the ROIs or repair the molecular damage in the cell (Nathan and Shiloh, 2000). SODs are one of the main defense mechanisms against oxidative stress caused by pollution, infections, hypoxia, hyperoxia, temperature and immunostimulants (Neves *et al*., 2000).

1.9.2.4. Oxyradical scavenging capacity

The production of oxidative compounds with antimicrobial effects has been studied in hemocytes from invertebrates (Buggé *et al*., 2007). This cellular response is rapid and transient, and is produced during microbe phagocytosis. These compounds include superoxide anions (O_2^-), hydroxyl radical (OH^-), H_2O_2, ROIs, and RNIs such as nitric oxide and peroxynitrite (Roch, 1999). Their production is mediated by the enzymes NADPH oxidase and nitric oxide synthetase (NOS). Other enzymes involved in ROI production are xanthine oxidase and glucose oxidase (Nappi and Ottaviani, 2000). The RNI are nitric oxide derivatives, which are synthesized from L-arginin by NOS.

1.9.2.5. Prophenoloxydase system

Granulocytes are responsible for the synthesis, storage and secretion of the Prophenoloxydase (proPO) system, which is activated by fungal β-glucans,

peptidoglycans (PG) and lipopolysaccharides (LPS). These molecules induce the granulocyte secretion of inactive proPO granules and their transformation (cascade reaction) to proPO enzyme. This oxidizes phenols into quinones, which may help to kill pathogens and are used for melanin production (Hellio *et al.*, 2007). In addition, the proteins interacting in the proPO cascade process are associated with cell recognition and hemocyte communication. Peroxynectin is a proPO system associated factor that creates cellular adhesion and acts as a peroxidase. This molecule is synthesized and stored by the granulocytes and activated upon cell secretion. Hemocyte's transmembrane receptors are responsible for the peroxynectin cell adhesion, hemocyte dispersion, phagocytosis, encapsulation, nodule formation and agglutination which resulted in peroxide activation and the invading agent destruction (Soderhäll *et al.*, 2003).

1.9.2.6. Melanization

Melanization plays an important role in the invertebrate defense mechanisms wherein a thick acellular capsule of melanin is generated around foreign objects (Barillas-Mury, 2007). Melanin, a product of the proPO system, is a dark brown pigment with antibacterial properties that inhibits antigens (Holmblad and Soderhäll, 1999). Although microbicidal properties have attributed to melanin and the other agents such as O_2^- and hydroxyl radicals which are generated during quinones formation (Hellio *et al.*, 2007).

1.9.2.7. Clotting protein cascade

Coagulation is used to prevent the loss of hemolymph through cuts and wounds in the exoskeleton, and to immobilization of invading pathogens (Meng-Yi *et al.*, 2005). Three types of hemolymph clotting systems (cascade) are known in crustaceans. Type A consists of rapid hemocyte agglutination without plasma coagulation; type B consists of cellular aggregation with limited plasma coagulation; and type C is limited cellular aggregation and lysis followed by plasma coagulation. Type C hemolymph coagulation is present in shrimp and other decapods (Yeh *et al.*, 1999). In crustaceans, the coagulation process is regulated by clotting proteins (coagulogens) and compartmentalized cellular factors within circulating cells. Clotting proteins in plasma are converted to covalently joined polymers by a Ca^{++} dependent transglutaminase secreted by the hemocytes (Wang

et al., 2001a). The cellular clotting proteins can be activated by LPS or β-1,3-glucan, and are related to the proPO activation system (Roux *et al*., 2002).

1.9.3. Humoral immune defense

Lectins are non-enzymatic proteins or glycoproteins that act in opsonization, agglutination, phagocytosis and pathogen encapsulation. Invertebrate lectins are considered primitive recognition molecules capable of detecting carbohydrates (Nappi and Ottaviani, 2000) which promote proPO system activation (Wang *et al*., 2001b). The pattern recognition proteins (PRPs) are lectins that detected the molecules like LPS, PG, bacterial lipoteichoic acid, fungal β-1,3-glucans and viral RNA (Song and Huang, 2000; Lee and Soderhäll, 2002), and which favour the activation of specific defense mechanisms by the host. The biological functions of PRPs are the initiation of a protein cascade and/or defense mechanisms' signalization routes and elimination of blood system invaders. When PRPs detect the antigens, the hemocytes are migrating to their location by chemotaxis, generating an inflammatory response. The crustacean open circulatory system favours this phenomenon, resulting in a fast and efficient defense mechanism against pathogens. Examples of PRPs present in crustacean plasma are β-1,3-glucan binding protein which induces degranulation and proPO system activation, and LPS binding protein, which helps in bacterial agglutination and removal by phagocytosis (Sritunyalucksana *et al*., 2002).

The innate immune system identifies pathogens through PRP and their corresponding pattern recognition receptors (PRR), which also are proteins. Toll-like receptors (TLRs) are an evolutionarily ancient family of PRRs presented in animals ranging from cnidarians to mammals, which can detect all kinds of pathogens (Robalino *et al*., 2004). TLRs are activated by bacterial and virus infection and have been reported in *Fenneropenaeus chinensis* and *Litopenaeus vannamei* (Li-Shi *et al*., 2007; Changjian *et al*., 2008). In mammals, TLRs on specialized antigen-presenting cells function as signal transducers by the way of nuclear factor kB, leading to the production of pro-inflammatory cytokines and the expression of costimulatory molecules on the cell surface (Inamori *et al*., 2004).

1.10. Immunostimulant activity of medical plants

An immunostimulant is a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens (Bricknell and Dalmo, 2005). The solubility and structure of the immunostimulant influences the biological activities of immunostimulators (Bohn and BeMiller, 1995). They may be crude preparations such as whole, dead microbial cells (e.g. yeasts or bacteria), semi-purified products from plants and microbes or pure chemicals (Raa, 1996). Immunostimulants also have ability to increase resistance to viral, bacterial and fungal infection (Anderson, 1992). Dietary additives of plant products have immunostimulant effect that enhance the innate (non-specific) defense mechanisms and increase resistance to specific pathogens (Sakai, 1999). Immunostimulants containing diets are used in aquaculture in order to increase resistance to stress and diseases of cultured shrimps by alerting the immune system.

In shrimp culture, medicinal plant especially herbs used as an immunostimulants to improve the quality and sustainability of aquaculture production. Hence, a growing interest has emerged in using medicinal plants that has attracted a lot of attention globally and has become the subject of active scientific investigation in many countries such as Egypt (Aly and Mohamed, 2010; Mehrim and Salem, 2013), Japan (Takaoka *et al.,* 2011), India (Sivaram *et al.,* 2004), Indonesia (Caruso *et al.,* 2013), Iran (Mousavi *et al.,* 2011), Korea (Harikrishnan *et al.,* 2011), Mexico (Auro de Ocampo and Jimenez, 1993), Nigeria (Okeke *et al.,* 2001) and Thailand (Direkbusarakom *et al.,* 1996a), because they are easy to prepare, cheap and are effective without any environmental and hazardous problems (Citarasu, 2010). Herbal products, stressol-I and stressol-II enriched *Artemia* nauplii fed to *P. indicus* postlarvae (PL 10–20) successfully increased the growth and efficiencies and also reduced osmotic stress (Chitra, 1995). Direkbusarakom *et al.,* (1996b) conduct a virucidal effect on polyvinylpyrolidone from *Clinacanthus nutans* extract in *Penaeus monodon* the result indicates that the extract is mixed with pellet at 1g/kg is more effectively control Yellow Head Rhabdovirus (YRV) infection in shrimp. *Picrorhiza kurroa* has been effectively used as an anti-stress compound for shrimps (Citarasu *et al.,* 1998). The methonolic extracts of three ayurvedic herbals viz. *Solanum*

trilobatum, Andrographis paniculata, and Psoralea corylifolia showed the protection of Penaeus spp. against nine pathogens such as *Bacillus subtilis, Proteus vulgaris, Salmonella typhi, K. pneumoniae, Pseudomonas aeruginosa, P. fluorescens, Vibrio* spp.*, S. aureus* and *A. hydrophila* (Citarasu, 2000). Artemia enriched with methanolic extracts of *Solanum trilobatum, Andrographis paniculata* and *Psoralea corylifolia* increased the survival and specific growth rate (SGR) and reduced bacterial load in a black tiger prawn culture system (Citarasu *et al*., 2003).

Administration of plant extract (*Psidium guajava*) supplemented diets showing higher efficacy than the antibiotic oxytetracycline to eliminate *Vibrio* infection in Black tiger shrimp *P.monodon* (Direkbusarakom, 2004). The herbal and seaweed diet enriched with Artemia boosted the survival and SGR of *Penaeus indicus* juveniles and reduced the bacterial load in muscle and hepatopancreas tissues (Immanuel *et al.,* 2004). *Vibrio harveyi* infected *Epinephelus tauvina* fed with methanolic extracts of *Ocimum sanctum, Withania somnifera* and *Myristica fragrans* significantly improved the immune parameters such as phagocytic activity, serum bactericidal activity, albumin– globulin (A/G) ratio and leukocrit (Sivaram *et al.,* 2004). Shrimp fed with butanolic extract of *W. somnifera* through *Artemia* enriched diet successfully controlled *V. parahaemolyticus* and *V. damsela* infection (Praseetha, 2005). Shrimp fed with methanolic extract of *Cynodon dactylon, Aegle marmelos, Tinospora cordifolia, P. kurooa* and *Eclipta alba* containing diet protected against white spot syndrome virus (WSSV) infection and better performance of hematological, biochemical, and immunological parameters (Citarasu *et al.,* 2006).

Post larvae of *Penaeus monodon* had significantly higher weight and SGR when fed with herbal appetizer, *Zingiber officinalis* enriched *Artemia* (Venkatramalingam *et al*., 2007). Indian traditional medicinal plants extracts such as *Aegle marmelos, C. dactylon, Lantana camara, Momordica charantia* and *Phyllanthus amarus* have antiviral activity against WSSV in shrimp (Balasubramanian *et al.,* 2007). Oral administration of *cyanodon dactylon* along with pellet feed act as a promoter of the shrimp immune system against WSSV infection (Balasubramanian *et al.,* 2008). Hsieh *et al.,* (2008) reported that an intraperitoneal injection of *Toona sinensis* extract in *L. vannamei* increase the

resistance against *V. alginolyticus*. *L. vannamei* injected with the hot-water extract of *Gracilaria tenuistipitata* (Hou and Chen*,* 2005), *Sargassum duplicatum* (Yeh *et al.,* 2006), *Gelidium amansii* (Fu *et al.,* 2007) and *Sargassum cristaefolium* (Chang *et al.,* 2013) acquires immunity against Vibrio infection. Diets with five herbal extracts decreased the Vibrio load in black tiger prawn PL after bath challenging *V. harveyi* (Velmurugan *et al.,* 2010). The growth rate of *Macrobrachium rosenbergii* fed 0.1% anthraquinone extract diet for 6 or 8weeks was higher than those without anthraquinone extract (Liu *et al.,* 2010). Immanuel *et al.,* (2010) reported that brown seaweeds such as *Sargassum duplicatum* and *Sargassum wightii* can be used as alternatives to antibiotics to control WSSV disease in black tiger prawns. Also a freshly squeezed garlic (*Allium sativum*) extract diet was used to alternate the use of antibiotics in control black gill disease in *Fenneropenaeus indicus* (Vaseeharan *et al.,* 2011). Dietary supplementation of *Gracilaria tenuistipitata* and *Hericium erinaceum* enhances the immune response and resistance against Vibrio infection in the Pacific white shrimp *L. vannamei* (Sirirustananun *et al.,* 2011; Yeh *et al.,* 2011). Immersion of *Sargassum hemiphyllum* var. chinense powder in *L. vannamei* showed increased immunity and resistance against *V. alginolyticus* and WSSV infection (Huynh *et al.,* 2011). Yogeeswaran *et al*., (2012) studied the role of herbal immunostimulants from the extract of *Acalypha indica, Cynodon dactylon, Picrorrhiza kurrooa, W. somnifera* and *Z. officinalis* along with formulated diets fed to shrimp for 60 days after vaccination, successfully protected them from WSSV. Zingerone supplement with feed to *L.vannamei* increased growth by stimulate appetite, immunity and disease resistance against *V. alginolyticus* (Chang *et al.,* 2012). Administration of *Zataria multiflora* essence to possibly control fungus contamination in cultured shrimp, *Litopenaeus vannemei* by eliminates *Candida albicans* and *Fusarium solani* in abiotic condition (Sharif Rohani *et al*., 2013). *L. vannamei* injected with the water extract of *Gynura bicolor* maintain physiological homeostasis and enhance immunity against *V. alginolyticus* infection (Hsieh *et al.,* 2013).

Herbs such as *Alteranthera sessilis, Eclipta alba and Cissus quadrangularis* acted as appetizers and enhanced the activities of digestive enzymes (protease, amylase and lipase) of freshwater prawns (Radhakrishnan *et al.,* 2014). In 28 day trail conducted by Raju *et al.,* (2014), *Indigofera aspalathoides* extract was supplemented with basal diet

fed with *Macrobrachium rosenbergii* in laboratory conditions was strongly induced in the defence system in fresh water shrimp which protect the bacterial infection of *V. harveyi*. The survival rate of *L. vannamei* that received *Petalonia binghamiae* extract at 6-10 µg/g was significantly higher than that of control shrimp (Chen *et al.,* 2014). Yang *et al.,* (2014) studied dietary administration of β-glucan from mushroom extract in Pacific white shrimps effectively enhance immune responses by significantly increased ratio of total haemocyte count and semi-granular cells. Chaweepack *et al.,* (2015) reported that the galangal (*Alpinia galanga* Linn.) extract has antimicrobial properties against white feces syndrome and Acute Hepatopancreatic Necrosis Disease (AHPND). Wang *et al.,* (2015) suggested that chlorogenic acid (CGA) possessed dual-modulatory effects on antioxidant capacity of *L. vannamei* and could be a potential feed additive that can enhance shrimp resistance against environmental stresses. *Cyperus rotundus* extracts effectively suppressed WSSV multiplication and improve the immune system in *F. indicus* against WSSV infection (Citarasu *et al.,* 2015).

1.11. *Mucuna pruriens:*

Mucuna pruriens is commonly known as Cowhage, Kiwanch or Konch (Hindi), Velvet bean or Cowitch (English), Atmagupta or Kapikacchu (Sanskrit), Alkushi (Bengali), Khaajkuiri (Marathi), Poonaikkaali (Tamil).

1.11.1. Botanical classification

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Fabales
Family	:	Fabaceae
Tribe	:	Phaseoleae
Genus	:	*Mucuna*

Species : *pruriens*

1.11.2. Geographical Distribution

The velvet bean *M. pruriens* is widespread in tropical and sub-tropical regions of the world. This plant is widely distributed in bushes and hedges at damp places, ravines and scrub jungles throughout the plains of South East Asia in Bangladesh, India, Sri Lanka and Malaysia. It is cultivated for its pods as vegetable and young leaves as fodder in Asia, America, Africa, and the Pacific Islands (Lampariello *et al,* 2012).

Figure 3. *Mucuna pruriens* seed pod

Figure 4. *Mucuna*

***pruriens* seeds**

1.11.3. Ethnobotanical Description:

The plant is an annual, leguminous, climbing shrub with long vines that can reach over 15 meter in length. It has long, slender branches with alternate, lanceolate leaves on hairy petioles. The leaves are trifoliate; the leaflets broadly ovate, elliptic or rhomboid ovate and unequal at the base. The flowers are large, white, growing in clusters of two or three, with a bluish- purple, butterfly – shaped corolla. The seed pods are about 10 cm long and are covered in loose orange hairs that cause a severe itching if they come in contact with skin (Fig. 3).

The pods or legume contains four to six seeds. The seeds are shiny black or brown colour (Fig. 4). The mature seed contain protein, carbohydrates, fat, fiber and minerals such as calcium, phosphorous, potassium, vitamin A, thiamin, riboflavin, amino acids. They are also rich in novel alkaloids, saponins and sterols. The seeds of all mucuna species contain a high concentration of L-dopa. Concentration of serotonin also have been found in the pod, leaf and fruit. The stinging hairs of the seed pods contain the phytochemical mucunain, which is responsible for causing skin irritation and itch.

Several countries used *M. pruriens* for various purposes in traditional medicine due to the presence of medicinally active phytochemicals. It is one of the most popular medicinal plants of India, widely utilized in the multiple pharmacological and is constituent of more than 200 indigenous drug formulations (Kavitha and Thangamani, 2014). Mucuna leaves are useful to treat ulcers, inflammation, cephalagia and general debility. Pods are used as vegetables; pod hairs (Trichomes) are antihelmintic. Hairs

mixed with honey used as vermifuge. Ointment prepared with hairs act as a local stimulant and mild vesicant (Sastry and Kawathekar, 1990). The seed are considered astringent, aphrodisiac, nervine tonic and nutritive. It is recommended for the nervous system, facial, paralysis, hemiplegra delirium. The roots are considered useful to relieve constipation, nephropathy, dysmenorrhoea, amenorrhorea, elephantiasis, dropsy, neuropathy, ulcers and heliminthiasis (Lindley, 1985; Shastry, 1995; Upadhyay, 2000). *M. pruriens* seed had been evaluated as a potent medicinal in terms of anti cholestrolemic, antiparkinson, antidiabetic, aphrodisiac, antivenom and antimicrobial agent (Natarajan *et al*, 2012).

1.11.4. Antioxidant effect

The antioxidant activity was evaluated for Hydroxyl radical scavenging activity, Nitric oxide radical scavenging activity with reference to Ascorbate and total phenol content respectively. *M. pruriens* seed has an antilipid peroxidation property which is mediated through the removal of super oxides and hydroxyl radicals (Tripathi and Upadhyay, 2002). Experiment on *in vitro* lipid peroxidation of *M. pruriens* methanolic seeds extract revealed the inhibition of ascorbate/FeSO4 induced peroxidation (Rajeshwar *et al.,* 2005).

An IC50 value was found that ethyl acetate extract of *Mucuna pruriens* is more effective in hydroxyl radical scavenging activity than that of methanolic and petroleum ether extract. The methanolic extract of *Mucuna pruriens* was found to more effective in the nitric oxide radical scavenging activity. Kottai Muthu *et al.,* (2010) found that ethylacetate and methanolic extract of whole *M. pruriens* plant contains large amounts of phenolic compounds, exhibits high anti-oxidant and free radical scavenging activities. These in vitro assays indicate that this plant extract is a significant source of natural anti-oxidant, which may be useful in preventing various oxidative stresses.

1.11.6. Antimicrobial activity:

Methanolic extract of *M. pruriens* showed broadspectrum antimicrobial activity against various microorganisms species like *Staphylococcus aureus, Bacillus pumillus, Escherichia coli* and *Vibrae cholera* (Rajeshwar *et al.,* 2005). Crude methanolic extracts

of *M. pruriens* leaves have been shown to have mild activity against some bacteria, probably due to the presence of phenols and tannins (Ogundare and Olorunfemi, 2007). The methanol extract of *M. pruriens* leaves had significant in vitro antimicrobial activity against *S. aureus, E. coli, B. subtilis, Proteus mirabilis* and *P. aeruginosa* comparable to that of the control streptomycin (Salau and Odeleye, 2007). Kumar *et al.* (2009) reported that *M. pruriens* was more effective against *Escherichia coli*, *Sigella dysenteriae, Salmonella typhi* and less effective against *Bacillus subtilis* due to the presence of the active chemical ingredients in the seeds.

Mucuna pruriens is also used for antimicrobial properties for extracting plant metabolites against plant pathogenic bacteria and fungi. The methanolic extract of leaves showed high antibacterial activity against *Erwinia carotovora*, *Pseudomonas syringae*, *Pseudomonas marginalis*, *Pseudomonas acruginosa*, *Xanthomonas campestris* and high anti fungal activity against *Curvularia lunata*, *Fusarium oxysporum*, *Pencillium expansum*, *Rhizoctonia solani*, *Tiarosporella phaseolina*, *Ustilago pomaydis* (Rayavarapu and Kaladhar, 2011). Aerial part of *M. pruriens* extract exhibited significant *in vitro* bacterial activity against *Staphylococcus saprophyticus, Staphylococcus aureus, Streptococcus epidermidis, Shigella sonnie, Shigella flexneri, Salmonella typhi and Vibrio cholera* (Bala *et al.,* 2011). The extracts of *M. pruriens* root and seed possess significant inhibitory effect against *Staphylococcus aureus, Klebsiella pneumoniae, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi*. Antibacterial activity was comparable with that of the standard antibacterial agent chloramphenicol and tetracycline against the tested organisms (Murugan and Mohan, 2011).

Saidu Garba *et al.,* (2012) reported that the ethanol extracts of both the leaves and stems of *M. pruriens* are more active antimicrobial activities against pathogens. The ethanol extract of *M. pruriens* leaves was found to be highly active against *Candida albicans* and *A. flavus*. Similarly the ethanol extracts of the *M. pruriens* stem was active against *Bacillus subtilis, streptococci* and *E. coli*. Marimuthu and Gurumoorthi (2013) evaluated two different germplasm of *M. pruriens*, black coloured germplasm was registered for higher levels of antifungal activity than white coloured germplasm.

Methanolic and ethanol solvents extracts of seed seemed to be brought better antifungal activity against *Aspergillus niger, Aspergillus flavus, aspergillus carneus* and *candida albigans* comparable with Fluconazole as standards. *M. pruriens* seed extract shows significant antibacterial activity against *P. vulgaris, E. coli, B. subtilis, S. aureus, K. pneumonia, E. aerogens, E. faecalis* and *Y. enterocolitia* which is mainly due to the presence of essential oil, flavonoids and triterpenoids and other natural polyphenolic compounds of free hydroxyl groups (Marimuthu *et al.*, 2013).

The methanolic extract of *M. pruriens* seed and leaves were more effectively inhibits the tested pathogens such as *Staphylococcus aureus, Salmonella typhi, Shigella dysenteriae, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa* (Pujari and Gandhi, 2013). The chloroform and benzene extract of *M. pruriens* seeds shown antimicrobial activity against both gram positive and negative compared to standard antibiotic solution of Amoxicilline (Vikran *et al.*, 2013). Stanley *et al.,* (2014) reported that *M. pruriens* seed extract have inhibitory effect on both gram positive (*S.aureus*) and gram negative (*E.coli*) but no inhibtion on the fungi (*C.albicans*).

1.11.7. Application of *Mucuna pruriens* in aquaculture:

Mucuna pruriens enriched Artemia supplementation have their swift positive influence over the reproductive performance and biochemical parameters in the spawners as well as offspring quality in the tiger shrimp *P. monodon* during the successive spawning (Babu *et al.,* 2008). Ojha *et al.,* (2014) reported that *M. pruriens* seed extract supplemented diet has significant role in improving growth, metabolism and immunity defence mechanism of *Labeo rohita* fingerlings. Fishes were fed with a diet significantly improved net weight, specific growth rate (SGR), food conversion ratio and also increase in digestive enzymes like protease, amylase, lipase supported the results of increase in growth at treatment level 0.06g/100g. The metabolic enzymes like Lactate dehydrogenase (LDH), Malate dehydrogenase (MDH) and *glucose-6-phosphate dehydrogenase* (G6PDH) levels were decreased, whereas the enzymes of protein metabolism were found increased in herbal extract supplemented groups. Further, the haematological parameters such as packed cell volume (PCV), haemoglobin concentration (Hb) and erythrocytes (RBC) were not significantly different in *M. pruriens* supplemented diets as compared to

control. A significant proliferation of the leukocytes (WBC) and improvement in respiratory burst activity.

2. Aim of the present research

2. AIM OF THE PRESENT RESEARCH

Aquaculture is not only increasing the production of high values species but also provide an affordable protein source to developing country. Aquaculture provides excellent economic growth opportunities by providing jobs from manual labour to skilled labour and income for millions of people and the potential foreign revenue investments. Shrimp culture is one of the rapidly growing sectors within the aquaculture industries. It is regarded as the best economic activity in terms of returns to investment in areas of production, processing and exports is referred as a high pay-off activity. The recent trend shows replacement of *Penaeus monodon* by *Litopenaeus vannamei* culture considerable increase in shrimp farming due to better growth rate and survived at extreme salinities.

Disease outbreaks is a significant constrains to the development of the aquaculture sector. Infectious disease is one of the limiting factors in shrimp farming. Shrimp can be threatened by protozoan, fungal, bacterial and viral pathogens, among these the viral and bacterial diseases cause major troubles in shrimp farming. Vibriosis is one of the major bacterial diseases responsible for mass mortality of cultured shrimp worldwide. *Vibrio harveyi* was considered as the most frequently implicated in vibriosis, is known to cause severe infections in penaeid livestock.

Isolation and identification of the causative agent is the initial step towards understanding the nature of a disease in any environment. Accurate and definitive identification of disease causing pathogen is indeed to develop appropriate prophylactic measures in any aquaculture setting.

Traditionally, antibiotics have been used in attempts to control bacterial disease in aquaculture. Aquaculture farmers rely on a wide range of antibiotics to prevent (prophylactic use) and treat (therapeutic use) bacterial infections in shrimps. Use of antibiotics is associated with environmental and human health problems which comprise pathogens resistance, spread of antibiotic resistance to other organism, disease persistence in the aquatic environment and also effects on the biogeochemical composition. The antibiotic residues accumulates in the edible tissues of shrimp might alter human intestinal flora which cause allergy problems and food poisoning.

In recent years, an increasing incidence of multiple resistances in pathogenic microorganisms to the synthetic antibiotics largely due to indiscriminate use of antimicrobial drugs commonly employed in the treatment of infectious diseases. In many developed countries use of antibiotic in aquaculture has been prohibited.

This has forced to search for new antimicrobial substances from alternative sources like the medicinal plants. Medicinal plants used as remedies for infectious diseases and offer new sources of biologically active chemical compounds as antimicrobial agents. Medicinal plants have been used as traditional medicines for centuries in India. The screening of plant extracts for antimicrobial activity has shown that plants represent a potential source of novel antibiotic prototypes.

Mucuna pruriens is one of the most popular medicinal plants of India, widely utilized in the multiple pharmacological and is constituent of more than 200 indigenous drug formulations. 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 phytochemicals to the environment. Additionally, they are free from toxic accumulation of the chemicals and moreover safe to environment and human during extensive application.

So, the present work necessitate to evaluate the antimicrobial, phytochemical and nutraceutical properties of *Mucuna pruriens* seed extract. It offers new sources of biologically active chemical compounds and its potential against pathogenic microorganism. This research was aimed to evaluate the impact of alternative treatment

(*Mucuna pruriens* seed extract) to control the disease caused by *V. harveyi* among cultivable *L. vannamei* shrimps.

3. Description of the study animal

3. DESCRIPTION OF THE STUDY ANIMAL

Litopenaeus vannamei also known as Pacific white shrimp is native to the Eastern Pacific coast of Mexico. The species grows to a size of 23 cm, and likes muddy bottoms from the depth of the shoreline down to about 72 meters (Holthuis, 1980). Females can reach a total length of 230 mm and males 187 mm (Jory and Cabrera, 2003). Females are commonly faster growing and larger than males.

3.1. Taxonomical classification:

Litopenaeus vannamei is belong to the phylum Arthropoda, whose members are characterised by a chitinous exoskeleton, a segmented body and jointed, paired appendages. There are thousands of terrestrial species in this phylum, and a large, predominately aquatic, the Crustacea. *L. vannamei* in the Order of Decapoda (with 10 walking legs).

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Subclass: Eumalacostraca

Order: Decapoda

Family: Penaeidae

Genus: *Litopenaeus*

Species: *vannamei*

Figure 5. Photograph of *Litopenaeus vannamei*

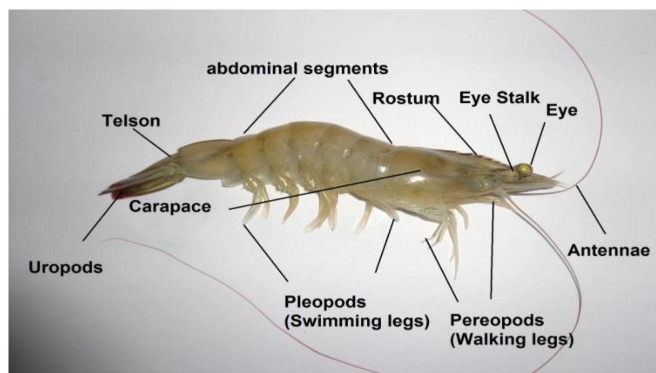
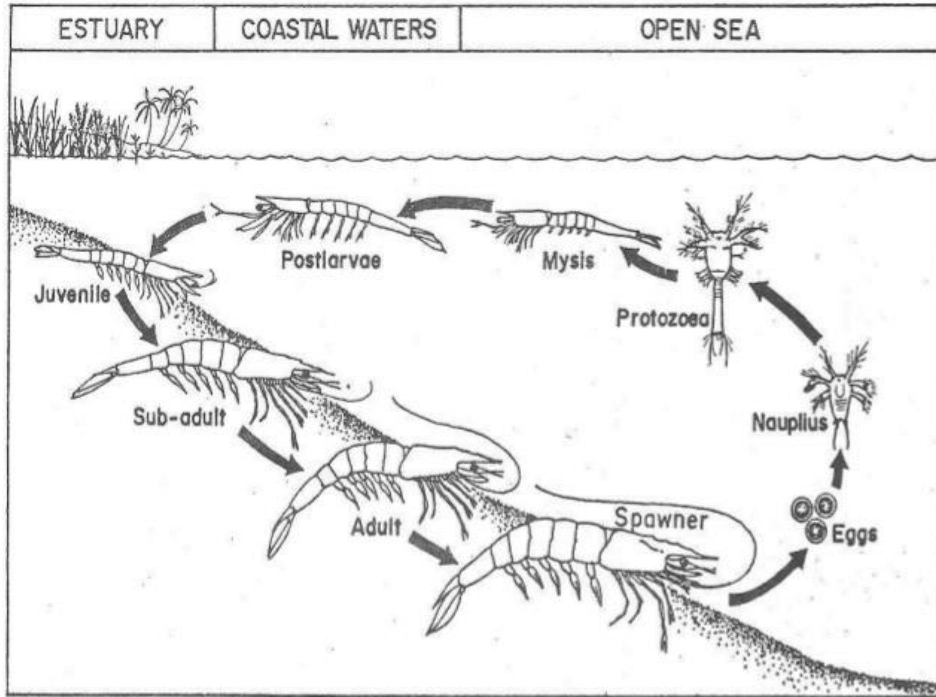


Figure 6. Life cycle of *Litopenaeus vannamei*



3.2. Morphology:

Litopenaeus vannamei is a laterally compressed, cylindrical body with bilateral symmetry (Fig. 5). The body of shrimp is composed of 19 segments, five make up the head, 8 are located in the thorax and 6 in the abdomen. The external anatomy of *L. vannamei* is characterised by a cephalothorax, abdomen and tail. Cephalothorax is fusion of head and thorax region which is characteristic by hard rostrum with 7-10 dorsal and 2-4 ventral teeth. The head region has a pair of antennule, antenna and stalked compound eye performs sensory functions. Internal region of head has a pair of mandibles for biting, cutting, holding food and maxillae 1st & 2nd for tasting and manipulating food. Most of the internal organs like heart, gills, hepatopancreas, lymphoid organ and stomach are present in cephalothorax region. The appendages of the cephalothorax are modified into different forms, maxillipeds are the first three pairs of appendages, for food handling and the remaining five pairs are the pereiopods (walking legs). Abdomen is composed by muscle which has a gut and reproductive organs. In mature male petasma is semi-open and symmetrical. Spermatophores complex consists of sperm mass encapsulated by sheath. Mature female has open thelycum. Five pairs of pleopods (swimming legs) are found on the abdomen. The last segment has one pair of uropods. The telson is the

posterior part of shrimp surrounded by the uropods, which together form the tail fan and is used to escape (Ruppert & Barnes 1994).

3.3. Life cycle:

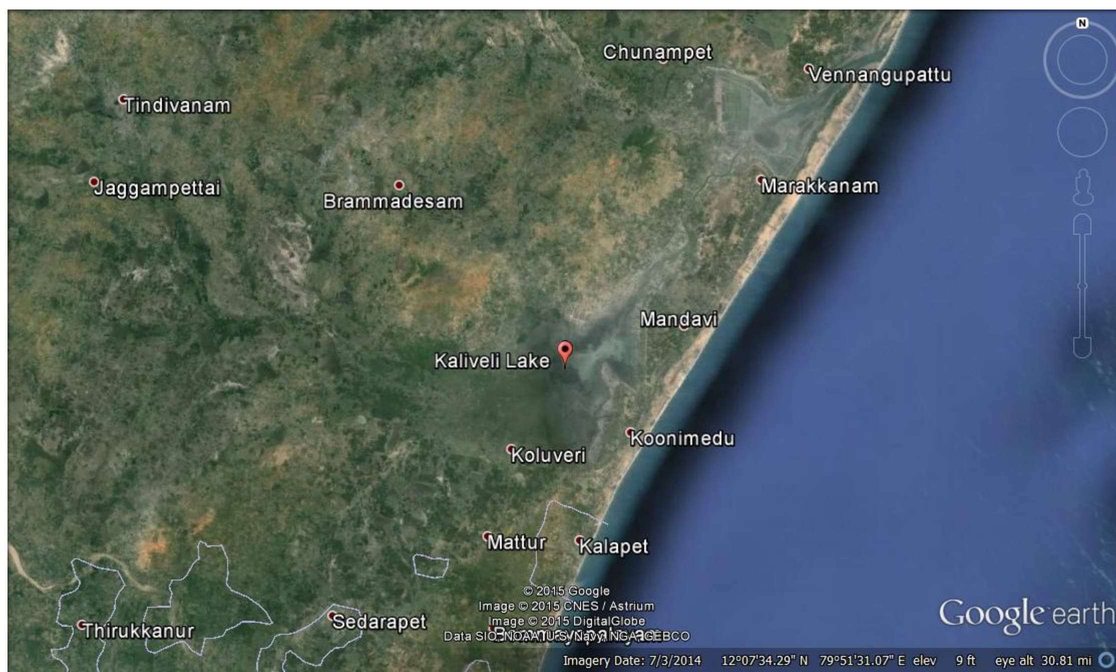
Adults *L. vannamei* live and spawn in the open ocean, while postlarvae migrate inshore to spend their juvenile, adolescent and sub-adult stages in coastal estuaries, lagoons or mangrove areas (Fig. 6). Generally, mating and spawning are take place during night. The maximum number of eggs spawned at a time is around 1,000,000 per female. Eggs hatch within 16 h after fertilisation. The larval stages comprise nauplius (6 stages in 2 days), protozoea (3 stages in 5 days), mysis (3 stages in 4-5 days) and megalopa (6-35 days). The megalopa and early juvenile are called postlarvae. Transition from juvenile to sub-adult takes 135-255 days and subsequently completion of sexual maturity occurs within 10 months (Bailey-Brock and Moss, 1992).

3.4. Culture advantage of *L. vannamei*:

L. vannamei is an important culture species, because it can be well adapted to a wide range of environmental conditions (Jory and Cabrera, 2003). It has a relatively rapid growth rate (more than 3g week $^{-1}$), can be grown at high densities (up to 400 m^2 in re-circulating systems), has a wide tolerance to salinity (0.5 to 45 ppt) and temperature (15–30° C) (Denisse Re *et al.,* 2012). It has the reputation of being a tolerant species, able to adapt to fluctuations in salinity, pH, and dissolved oxygen levels (Rosenberry, 1999). Recommended protein levels for feed are low, 30 percent compared to 45 percent protein feed for *Penaeus monodon*. In addition, *L. vannamei* are easier to reproduce than *P. monodon*. The uniform growth rate of this species is also an advantage in marketing.

4. Material and methods

Figure 7. Site of sample collected at Kaliveli lake



4. MATERIAL AND METHODS

4.1. Animal collection

Infected *L. vannamei* moribund shrimp were collected for isolation and identification of disease causing agent during disease outbreak season from culture pond near Kaliveli Lake (12° 7' 11.02' N, 79° 51' 27.66' E) located 25 km north of Pondicherry, south east coast of India (Fig. 7). The samples were transported to the laboratory in live condition in aerated polythene bags. Collected shrimp were inspected to macroscopic signs of disease. The *Vibrio harveyi* infected *L. vannamei* was identified by exposing the shrimp to the fluorescent light. Healthy adult *L. vannamei* shrimp of both male and female were also collected during harvesting period from culture pond at same location for immunological studies.

4.2. Animal acclimatization:

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 litre in 500-litre tanks containing 150 litre 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 sedimentation tank and biofilter. Approximately 20% of the water was changed per week and the sedimentation tank was siphoned once every 3–4 days. The water was taken from the sea through a sand filter, a 25 mm cartridge filter and an ultra violet sterilizer. Shrimps were fed twice daily with commercial shrimp pellets CP-Aquaculture, India.

4.3.1. Determination of LD_{50} of *L. Vannamei* in response to *V. harveyi*

L. Vannamei weighing 30 to 35g in total body weight were held in tanks (1000L) supplied with aerated 35 ppt seawater at 28-32 °C. The lethal dose 50 % end point (LD_{50}) tests, with batches of 10 animals per dose, were conducted by injection of *V.harveyi* (isolated from infected shrimp) at the ventral sinus of the cephalothorax with 24 hrs bacterial suspension (10^4 -10^8 CFU g^{-1}) into the shrimps (Trevors and Lusty, 1985). Sterile phosphate buffer saline (PBS) was injected into other group of shrimps as parallel control. The LD_{50} values were calculated using the method of Reed & Muench (1938). Mortalities were recorded daily for 11 days post challenge.

4.3.2. Experimental animals

Adult healthy spawners of *L.vannamei* (body weight of 30 ± 5g) of 15 (n=15) individuals per group were used in the present study. Shrimps were transferred to the individual spawning tank of 250 L capacity. The parameters such as salinity, dissolved oxygen, water temperature and pH were maintained at 35 ppt, > 5.5 mg L^{-1}, 32±0.5°C and 7.5±0.3, 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.

4.4.1. Collection of plant material

The seeds of *Mucuna pruriens* were collected from the local Ayurvedic shop in Pondicherry and the identity was confirmed by traditional and experts of plant biologist.

4.4.2. Preparation of plant extract

M. Pruriens seeds were made to shade dried for a month time to minimize its moisture content. Shade dried seeds were pulverized into fine powder using electric blender. 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.

The extraction was carried out by the method of Uhegbu *et al.* (2005). Finely powdered *M. Pruriens* seeds were subjected for alcoholic extraction with methanol and ethanol (Analytical grade) (Merck, Inc). 100 g powder of *M. Pruriens* seed was soaked in 1000 ml of methanol and ethanol sequentially, stirred for about 6 minutes and left overnight. Thereafter, the solution was filtered using filter paper (Whatman No. 1) and the extracts were evaporated to dryness under reduced pressure and temperature (below 40 C) in rotary vacuum evaporator. The concentrated *M. Pruriens* seed extract was dried and stored at 4ºC until use and this dry extract was subjected for antibacterial assay

4.4.3. Enrichment of *Artemia* for experiment diets

The *Artemia* enrichment with herbal extracts procedure was followed as described by Babu *et al.,* (2008). The *Artemia franciscana* nauplii (Tuticorin, India) were acclimatized in normal seawater for 5 hrs. The dried methanolic seed herbal extract of *Mucuna pruriens* were emulsified with oil (Super-Selco, *Artemia* systems, SA, Baasrode, Belgium) and enriched at 100 ppm and 200 ppm concentration in enrichment tank with *Artemia* biomass at the density of 5,000 *Artemia* /L seawater. After 4 hrs of enrichment, the enriched *Artemia* were rinsed in seawater of 32±2ºC temperature and 30 ppt salinity followed by fresh water dip and frozen into 1 cm^3 blocks. For the normal and infected control, the *Artemia* was enriched without any herbal extracts.

4.5. Experimental design

Eight groups of adult healthy spawners of *L. vannamei* were stocked in spawning tank of 250 litre capacity for 3 weeks. The shrimp were fed twice per day at 8.00 and 18.00hrs at 10% of the body weight. Uneaten feed and waste matters were removed before feeding on every experimental study. During the feeding time, the water exchange was stopped for one hr to avoid the loss of feed. The groups are as follows:

Group I. Normal male *L.vannamei*

Group II. *V.harveyi* infected male *L.vannamei*

Group III. *V.harveyi* infected male *L.vannamei* fed with *M.pruriens* (100 ppm) enriched *Artemia*

Group IV. *V.harveyi* infected male *L.vannamei* fed with *M.pruriens* (200 ppm) enriched *Artemia*

Group V. Normal female *L.vannamei*

Group VI. *V.harveyi* infected female *L.vannamei*

Group VII. *V.harveyi* infected female *L.vannamei* fed with *M.pruriens* (100 ppm) enriched *Artemia*

Group VIII. *V.harveyi* infected female *L.vannamei* fed with *M.pruriens* (200 ppm) enriched *Artemia*

4.6. Isolation of *Vibrio harveyi*:

The *Vibrio harveyi* agar (VHA) medium was used for the isolation of *V. harveyi* (Harris *et al.,* 1996). The hepatopancreas of infected shrimps were excised and washed extensively with 0.9% saline water. Hepatopancreas were weighed and the known amount of tissue sample was homogenized with Tris buffer (pH.7.0) for a minutes. Homogenized tissue sample was then centrifuged at 7000 rpm for 5 minutes and the clear supernatant was aspirated in to clean centrifuge tube. Clear supernatant was made into serial dilution (10^{-1} from 10^{-9}) using sterile water in the aseptic condition. From the diluted sample, 0.1 ml of sample was taken from 10^{-1} to 10^{-9} and spread over on specialized *Vibrio harveyi* selective agar medium separately and one plate maintained as a control without sample. This specialized agar inhibits the growth of other Vibrio species. The plates were incubated at 37ºC for 24 to 48 hrs to observe the bacterial colonies growth.

4.7. Identification of *Vibrio harveyi*

4.7.1. Phenotypic identification by biochemical and morphology characterisation:

The isolated clones from the culture medium were identified and confirmed by the following physiological and biochemical tests.

4.7.1.1. Gram stain

Gram stain technique was performed based on Manual of clinical Microbiology (Murray *et al.,* 2003).

Principle

The gram stain is used to differentiate between gram-positive and gram-negative bacteria. Cellular morphology can also be determined. Gram-positive and gram-negative bacteria are both stained by crystal violet. The addition of iodine forms a complex within the cell wall. Addition of a decolorizer removes the stain from gram-negative organisms due to their increased lipid content. These cells are stained pink with the counter stain safranin.

Reagents and Material (Store at room temperature)

1. Crystal Violet Stain
2. Gram Iodine (Combine Gram Iodine Concentrate to Gram Iodine Diluent)
3. Decolorizer Solution
4. Methanol
5. Safranin
6. Slides
7. Inoculating loop
8. Microscope with Immersion Objective

Procedure

1. Spread single loop of culture from the VHA medium to a microscope slide. Spread the culture over 1/3 to 1/2 to the total area of the slide.

2. Allow the smear to air dry. This may take up to 1 hr depending on the temperature and humidity of the room.

3. Cover the entire bacterial smear with 3 or 4 drops of methanol to fix the smear and allow to air dry. Again this may take up to an hr.

4. Cover the bacterial smear with crystal violet stain and allow to stand 1 minute. Gently wash the stain off with cool tap water and drain water from slide.

5. Cover the smear with grams iodine and allow to stand 1 minute. Gently wash the iodine off with water and drain the water from the slide.

6. Rinse the bacterial smear with decolorizer solution for 10 seconds; decolorization is complete when the solution runs clear from the slide. Gently rinse with water and drain the slide.

7. Cover the bacterial smear with safranin stain, and allow to stand for 1 minute, then gently wash the stain from the slide.

8. Blot the slide dry with absorbent paper and examine the slide under oil immersion lens.

Gram-positive → **Violet colour**

Gram-negative → **Red or pink colour**

4.7.1.2. Glucose fermentation by Methyl red test

Principle

Methyl red test was conducted to detect the organisms, which produced acid during the fermentation of glucose (Harrigan, 1998).

Reagents and Materials

1. Glucose phosphate broth
2. Methyl red indicator

Procedure

1. The ingredients were dissolved in distilled water and adjusted the pH to 7.5.

2. The medium was distributed in 5mL amounts in each test tube. Then these test tubes were sterilized for 20 minutes at 115°C.

3. The medium was inoculated with the specific/test organisms and incubated for 48-72 hrs at 37°C using 5 drops of methyl red indicator (0.1g of methyl red in 300 mL of 95% ethanol, made up to 500 mL in distilled water).

4. The results were recorded with change in color for the acid production.

Negative → Yellow colour

Positive → Red colour

4.7.1.3. Oxidase test

Principle

This test determines whether the organism produces an enzyme called cytochrome oxidase and therefore can use oxygen for energy production. The test uses filter paper impregnated with a reagent such as N,N dimethyl-p-phenylalnine, which is also a redox indicator. If the organism produces oxidase it will oxidize the reagent giving a purple color (Alsina and Blanch, 1994; Holt *et al.*, 1994).

$$\textbf{Phenylenediamine} \xrightarrow{\text{Oxidase enzyme}} \textbf{Deep purple color}$$

Reagents and Materials

1. Redox indicator (N,N dimethyl-p-phenylalnine)

2. Filter paper

3. Peti-dish

Procedure

1. In a clean peti-dish, a piece of filter paper and add a few drops of the oxidase reagent on it.

2. Using a clean slide, take some bacterial colonies and rub them on the wet filter paper.

3. If a purple color appears, the organism is oxdiase positive. If there is no change in color, the organism is oxidase negative.

4.7.1.4. Catalase

Principal

Hydrogen peroxide is used (H_2O_2) to determine if bacteria produce the enzyme catalase (Murray *et al.,* 2003).

Reagents and Materials

1. Three percent hydrogen peroxide is obtained from a commercial drug store.

2. Pipette

3. Slides

Procedure

1. The catalase test is best performed by flooding the growth of the bacteria (usually on an agar slant but blood free agar plates can be used) in question with 1.0 ml of 3% hydrogen peroxide and observing for effervescence (bubbling) which indicates a positive test.

2. The bacteria must be grown on blood free medium.

3. Modifications of the catalase test may be performed by very carefully removing a colony from a blood agar plate with a plastic needle or wooden applicator stick and transferring the colony to a glass slide.

4. A drop of 3% hydrogen peroxide is added to the colony on the slide and observed for effervescence.

5. Immediate formation of gas bubbles indicates the liberation of oxygen was considered as positive reaction. The absence of bubbles is considered as negative reaction.

4.7.1.5. Motility

Principle

The ability of bacteria to move through a semisolid media is useful in differentiating bacteria (Murray *et al.,* 2003).

Reagents and Materials

1. Motility test medium (Remel, number 061408)

2. Inoculating needle

Procedure

1. The medium is inoculated with an inoculating needle, not a loop. Apply a colony to the end of the needle from the agar plate.

2. The needle is inserted into the center of the medium in the tube for about 1 inch.

3. The inoculated tube is incubated at 30°C in ambient air and incubated until good growth is observed, in most cases 24 to 48 hrs is sufficient.

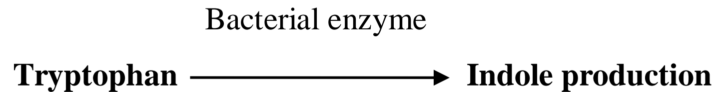
4. Strains that are motile will show growth outward to the edge of the tube and downward toward the bottom of the tube. Negative strains will only show growth in the line of the stab.

4.7.1.6. Indole formation

Principle

This test detects the ability of the organism to split indole from the amino acid, tryptophan by an enzyme called tryptophanase. Indole is then detected by the

kovac reagent giving a red color or a red colored ring if performed in a test tube (Alsina and Blanch, 1994; Holt *et al*., 1994).



Reagents and Materials

1. Liquid broth containing peptone

2. Kovac reagent

3. Inoculating loop

Procedure

1. To perform the test, we inoculate the organism into a liquid broth containing peptone.

2. Inoculation is done using a loop by taking a colony of the bacterium. The loop is then rubbed on the of the broth tube.

3. The tube is capped and incubated at 37˚C for 24 hrs.

4. After incubation, the kovac reagent is added. If a red ring appears, the organism is indole positive. If there isn't a red ring, the organism is indole negative.

4.7.1.7. H2S formation

Principle

This test determines whether the bacteria reduced sulfur-containing compounds to sulfides during the process of metabolism. If sulfide is released, it combines with iron compounds to produce FeS, a black precipitate. Triple Sugar Iron Agar (TSIA) is utilized for this test (Alsina and Blanch, 1994; Holt *et al*., 1994).

Reagent and materials

1. Triple sugar iron agar

2. Inoculated tube

Procedure

1. An isolate is transferred aseptically to a sterile TSIA slant.

2. The inoculated tube is incubated at 35-37ºC for 24 hrs and the results are determined.

3. Present in TSIA is an iron compound. The iron ions (Fe2+) have a high affinity (strong attraction) for *sulfide ions*.

4. The result is that H2S combines with the iron to make FeS, a black compound.

Positive → agar turns to black color

Negative → agar remains same color

4.7.1.8. Gelatin liquefaction

Principle

When proteolytic organisms are grown media containing gelatin, it is liquefied and loses the gelling property even when cooled to 4°C (Alsina and Blanch, 1994; Holt *et al*., 1994).

Reagent and materials

1. Nutrient gelatin medium

2. Inoculating loop

3. Test tube

Procedure

1. The molten medium was poured into tubes and autoclaved at 121°C for 15 minutes.

2. A 24 h old culture was inoculated and incubated at 28 ± 1°C for 3-5 days.

3. Un-inoculated tubes were maintained as control.

4. After the incubation period the tubes were kept at 4°C for 6 hrs.

5. The tubes were inverted to observe for liquefaction of gelatin while the control remains solid.

4.7.1.9. Arginine dihydrolase

Principle

Certain bacteria contain the enzymes to hydrolyze arginine. This hydrolysis results in an alkaline change in the media results in a color change in the media. This test can be used for differentiation of bacteria (Murray *et al.,* 2003).

Reagents and Materials

1. Moeller's decarboxylase broth containing arginine.

2. Pipette

Procedure

1. Add 1-3 drops of culture suspension to the tube of Moeller's decarboxylase medium containing arginine

2. Immediately overlay with sterile mineral oil (about 1 to 2 ml).

3. The medium is incubated at 35°C for up to 7 days in ambient air.

4. A positive reaction is recorded with the broth turns a deep purple color indicating an alkaline reaction, NH_3 is released. The development of a yellow color or no change in color of the broth indicates a negative reaction.

4.7.1.10. Amylase production

The production of amylase was observed by hydrolysis of the starch (Murray *et al.*, 2003).

Principle

Some bacteria are able to hydrolyze starch on starch supplemented agar. When iodine is added to starch, it turns dark bluish-black. If starch has been hydrolyzed, then it is not available to react with the iodine and the area around the bacterial growth is clear. This test can be used to differentiate some bacteria.

Reagents and Materials

1. Two percent starch agar

2. Gram's iodine

Procedure

1. Inoculate a starch agar plate with a heavy single streak of a fresh culture or run a drop of broth culture across the plate.

2. Incubate the plate in CO_2 at 35ºC for 48 hrs. Some strains may require longer incubation for sufficient growth.

3. After incubation, flood the plate with Gram's iodine.

4. A clear zone surrounding the growth is positive test that the strain hydrolyzed starch. A deep purple to black or bluish color of the agar indicates that starch has not been hydrolyzed and thus a negative test. For negative tests the deep color develops in the agar right up to the growth.

4.7.1.11. Colony colour on TCBS

Principle

Thiosulfate citrate bile Salts Sucrose agar (TCBS agar) is highly selective for the isolation and identify of Vibrios. Inhibition of gram-positive bacteria is achevied by the incorporation of oxgall, a naturally occurring substrate containing a mixture of bile salts and sodium cholate, a pure bile salts. Sodium thiosulfate serves as a sulfur source and in combination with ferric citrate, alkaline pH of the medium enhances the recovery of

V.cholerae. Thymol blue and bromthymol blue are included as indicators of pH changes (Alsina and Blanch, 1994; Holt *et al*., 1994).

Reagents and materials

1. TCBS agar

Procedure

1. Agar supplemented with 2.5% NaCl for 24 hrs at 28°C, colony colour and growth were recorded.

> ***V. harveyi*** → grayish-green to bluish-green colonies which show luminescence in dark
>
> ***V. cholerae*** → large yellow colonies
>
> ***V. parahaemolyticus*** → Blue to green centers in colonies
>
> ***V. alginolyticus*** → large yellow mucoidal colonies

4.7.1.12. β Blood hemolysis

Principle

The ability of bacterial colonies to induce hemolysis, when grown on blood agar Beta blood hemolysis is a true lysis of red blood corpuscles. (Murray *et al.,* 2003).

Reagents

1. Trypticase Soy Agar (TSA) plates containing 5% horse blood.

Procedure

1. Streak culture for isolation on TSA plate with 5% horse blood.

2. Incubate plate at 35°C in CO_2 for 24 hrs.

3. Complete clearing around the colony indicates the bacteria have positive growth.

4.7.1.13. Sensitivity to vibriostat O/129

Principle

The sensitivity of Vibrio to the Vibrio static agent 0/129 (2,4-diamino-6,7-di-isopropyl-pteridine phosphate). It was found to be useful in the differentiation of Vibrio from gram-negative bacteria, which are characteristically resistant to O/129. Even among the genus Vibrio, different species show different sensitivity to O/129 (Alsina and Blanch, 1994; Holt *et al.*, 1994).

Materials and method

1. ZoBell's agar plates

2. Filter paper (Whatman No. 1)

Procedure

1. Antibiotic assay filter paper disc of 6mm diameter (Whatman No.1) were prepared aseptically to contain l50 µg disc^{-1} of the vibriostatic agent O/129 (Fumiss *et al.,*. 1978).

2. The discs were stored at 4°C and used as required.

3. ZoBell's agar plates were swabbed with a suspension of the test bacterial organism to obtain a confluent growth and the discs were placed on it appropriately spaced.

4. Bacterial isolates sensitive to the pteridine compound developed a clearing zone around the disc.

4.7.1.14. Oxidation and fermentation

Principle

Some organisms metabolize glucose oxidatively and others ferment glucose fermentatively when the hydrogen acceptor is not oxygen. Such organism can be

differentiated based on the Oxidation fermentation test using Marine Oxidation Fermentation medium (Alsina and Blanch, 1994; Holt *et al*., 1994).

Reagent and materials

1. Marine Oxidation Fermentation medium (Himedia, Bangalore)

2. Test tubes

Procedure

1. Twenty two grams of the medium and l5 g agar were added to 1000 ml distilled water and sterilized by autoclaving at 121°C for l5 min.

2. To the above basal medium, l% glucose was added and 4 ml aliquots were transferred into sterile tubes which were then autoclaved for 10 min at 110°C.

3. The tubes were laid out to slants and after drying, the cultures were inoculated by stab and streaked on the slant.

4. The tubes were incubated at 28 ± 1°C and results were recorded as follows:

Oxidation	→	yellow colouration in the slant alone
Fermentative	→	yellow coloration throughout the tube
Alkaline reaction	→	no colour change in the tube

4.7.1.15. Phenylalanine deaminase

Principle

This test determines whether the microbe produces the enzyme phenylalanine deaminase which is needed for it to use the amino acid phenylalanine as a carbon and energy source for growth (Alsina and Blanch, 1994; Holt *et al*., 1994).

Reagent and materials

1. Phenylalanine agar

2. Ferric chloride (10%)

3. 0.1 N HCL

4. Test tube

Procedure

1. An inoculum from a pure culture is transferred aseptically to a sterile tube of phenylalanine agar to streak the slant.

2. The butt of the tube need not be stabbed. The inoculated tube is incubated at 35-37°C for 24 hrs.

3. After incubation five drops of 10% ferric chloride and five drops of 0.1N HCL are added and the tube is gently shaken.

4. A positive result is indicated if a green color develops within five minutes.

4.8. Genotypic identification using 16S rDNA:

The isolates identified based on phenotypic characterization, it was further confirmed of their identity based on sequence analyses of 16S rDNA. Hence the isolated *V.harveyi* was subjected for 16S rDNA sequencing and conformed with other findings.

4.8.1.Extraction of genomic DNA:

The genomic DNA was extracted from bacterial cell suspension culture in Luria-Bertani broth. A sample of 2 ml of bacterial cell suspension was centrifuged at 15,000 g for 10 min at 4°C. The pellet was collected and re-suspended in 500µL of TNE buffer (10mM Tris-Cl, 0.15 mM NaCl, 1 mM EDTA, pH 8.0) and centrifuged again at 15,000 g for 10 min at 4°C. Subsequently, the pellets were re-suspended in 500 µL buffer for cell lysis (0.05 mM Tris-HCl, pH 8.0, 0.05 mM EDTA, 0.1 mM NaCl, 2%, SDS, 0.2 % PVP and 0.1% mercaptoethanol) (Lee *et al.*, 2003) and 10µL of proteinase K (20mg/ml) was added and incubated initially for 1 hr at 37°C and then for 2 hr at 55°C. The extraction

was further carried out by phenol-chloroform method (Sambrook and Russell, 2001). The sample was deproteinated by adding equal volume of phenol (Tris- equilibrated, pH 8.0), chloroform and isoamyl alcohol (25:24:1) mixture. The aqueous layers and phenol were separated by centrifugation at 15,000g for 15 min at 4°C. The aqueous phase was pipetted out carefully into a fresh tube and the process was repeated once again. Following this, an equal volume of chloroform and isoamyl alcohol mixture (24:1) was added, mixed by gentle inversion and centrifuged at 15,000 g for 15 min at 4°C. The aqueous phase was separated and transferred to a fresh tube. Then, the DNA was precipitated by incubation at -20°C overnight after adding equal volume of ice cold absolute ethanol. The precipitated DNA was collected by centrifugation at 15,000 g for 15 min at 4°C and the pellet washed with 70% chilled ethanol. Centrifugation was repeated once again then the supernatant decanted and the tubes left open until the pellet got dried. The DNA pellet was dissolved in 100µL MilliQ (Millipore) grade water. Isolation of DNA was carried out using electrophoresis in 1% agarose gel. The isolated DNA was quantified spectrophotometrically (Abs_{260}) and the purity assessed by calculating the ratio of absorbance at 260 nm and 280 nm (Abs_{260}/Abs_{280}).

$$\text{Concentration of DNA } (\mu g\ \mu L^{-1}) = Abs_{260} \times 50 \times \text{dilution factor}$$

4.8.2. PCR amplification, cloning and sequencing of 16S rRNA gene:

Amplification of 16S rRNA gene was performed according to Reddy *et al.* (2000) using universal primers 16 S1 (GAG TTT GAT CCT GGC TCA) and 16 S2 (ACG GCT ACC TTG TTA CGA CTT) in DNA thermal cycler (Eppendorf, Germany). The reaction mixture (final volume 25 µL) contained 2.5 µL 10 X buffer, 1 µL 10 pmol each of oligonucleotide primer, 1.5 µL DNA template, 2.5 µL 2.5 mM each deoxynucleoside triphosphate, 1 µL Taq polymerase, and the remaining volume make up with sterile Milli Q water. The amplification profile consisted of initial denaturation for 5 min at 95°C followed by 34 cycles of denaturation for 20 sec at 94°C, annealing for 30 sec at 58°C and extension for 2 min at 68°C followed by a final extension for 10 min at 68°C. The PCR product was separated on 1 % agarose gel prepared in 1x TAE buffer and stained with ethidium bromide.

Gel purification was done using GenEluteTM Gel Extraction kit (Sigma, USA). For purifying the PCR products, the agarose gel that contained DNA fragments of appropriate size was excised and taken in a 1.5 mL tube, weighed and added 3 gel volumes (~450 µL) of gel solubilization solution and incubated for 10 min at 60 °C with repeated vortexing in every 2 min. After incubation, added 1 gel volume (~150 µL) of 100% isopropanol, mixed gently until it became homogenous. This solubilized gel solution was loaded into the binding column that was pretreated with column preparation solution, centrifuged at 12,000 x g for 1 min. Added 700 µL wash solution and centrifuged at 12,000 x g for 1 min, repeat the centrifugation, and residual wash solution was removed. The binding column was transferred to a fresh collection tube (2mL MCT) and added 50 µL of preheated (at 65°C) 10 mM Tris-HCl (pH 9.0), centrifuged at 12,000 x g for 1 min, stored at -20°C. The concentration of DNA was measured spectrophotometrically at 260/280 nm in a UV-VIS spectrometer (1800, Shimadzu, Japan). The purified PCR product of 16S rRNA gene was sequenced using the dideoxynucleotide chain termination method (Musa *et al.,* 2008) on a DNA sequencer (Model 373A, Applied Bios 2008).

The obtained nucleotide sequence was compared with known sequences in NCBI BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find out the percentage of identity.

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4.9. Antibacterial effect of *Mucuna pruriens*

The *Mucuna pruriens* seed extract was subjected for antibacterial assay. The extraction procedure has been described in the 4.4.2. section.

4.9.1. Bacterial strains used

1. *Staphylococcus aureus MTCC.* 9542
2. *Salmonella typhi MTCC.3224*
3. *Salmonella paratyphi MTCC 3220*
4. *Klebsiella oxytoca MTCC 3030*
5. *Klebsiella pneumoniae MTCC 7407*
6. *Pseudomonas aeruginosa MTCC 6458*

7. *Escherichia coli MTCC1698*
8. *Proteus mirabilis MTCC 9493*
9. *Vibrio cholerae MTCC 3906*
10. *Vibrio harveyi MTCC 7954*

These pathogen strains were obtained from the department of Medical Microbiology (Raja Muthiah Medical College Hospital) Annamalai University, Annamalai Nagar.

4.9.2. Bacterial medium and its preparation:

4.9.2.1. Composition of Muller Hinton Agar

Beef infusion	30g
Casein acid hydrolysate	17.5g
Starch	1.5g
Agar	17g

38g of Muller hinton Agar were suspended in 1000ml of distilled water and the pH was adjusted to 7.3± 0.2. The medium was boiled to dissolve completely and sterilized by autoclaving at 15 Lbs (121^0C) for 15 minutes.

4.9.2.2. Composition of Nutrient broth

Beef infusion	30g
Casein acid hydrolysate	17.5g
Soluble starch	1.5g

36g of Nutrient broth powder suspended in 1000ml of distilled water and the pH was adjusted to 7.2± 0.2. The medium was boiled to dissolve completely and sterilized by autoclaving at 15 Lbs (121^0C) for15 minutes.

4.9.2.3. Preparation of bacterial culture

Nutrient broth medium was prepared and sterilized in autoclave at 15 Lbs pressure. Ten bacterial species were inoculated in the nutrient broth and incubated at 28 $\pm 2^0$C for 24 hrs. Muller hinton agar medium was also prepared, autoclaved and transferred aseptically into sterile Petri dishes. On this, 24hrs old bacterial broth cultures were inoculated by using a sterile cotton swab.

4.9.3. Antibacterial activity by disc diffusion method

Antibacterial activity spectrum of the plant seed extract was studied in both gram positive bacteria and gram-negative bacteria with positive control agent erythromycin by Disc diffusion technique (Bauer *et al.,* 1996). 6 mm discs impregnated with 25 µl in ethanol and methanol extracts of *M. Pruriens* and positive control contained of a standard antibiotic disc were subjected for antibiogram assay. Erythromycin was used as standard antibiogram. After incubation at room temperature (37°C) for 24 hrs antibacterial activities was expressed in terms of diameter of zone of inhibition which was measured in mm using a scale and recorded.

4.9.4. Minimum inhibition concentration

A serial micro-dilution assay was used to quantify the minimum inhibitory concentration (MIC) values of *M. Pruriens* seed extract against the bacterial pathogen which exhibited maximum zone of inhibition in antibacterial assay. The MIC was assessed using tetrazolium violet reduction as an indicator of growth (Eloff, 1998; NCCLS, 2000). MIC of plant extract was determined by the method of broth dilution. Lysogeny broth was used to prepare bacterial aliquots. Aliquots of 10 µl of LB media was placed into 96 well microtitre plate before the plant extract solution was added to the tubes to the final concentration of 100 mg ml^{-1}. Next, the concentration of the plant extract was adjusted ranging from 10-250 µg ml^{-1} by adding different volumes of the LB media. All above reagents were autoclaved at 121^0C and cooling before the test bacteria suspension was added into to the inoculum size of 10^6 CFU ml^{-1} and then incubated at 37^0C for 24 h. Another diluted solution without adding any bacterium was prepared as control sample. The turbidity of all samples was determined at 540 nm in an ELISA

reader (Model: Cybershop microplate reader). The determination was based on the same turbidity of the two subjects of the same concentration, i.e., the minimum inhibitory concentration at which no bacteria grew in the culture media was defined as MIC.

4.10. Phytochemical analysis of *Mucuna pruriens* seed extract

M. pruriens seed methanolic extract was subjected for phytochemical validation by following methods.

4.10.1. Fourier transform-infrared spectroscopy (FT-IR) analysis

The qualitative analysis of the active principles of *Mucuna pruriens* seed extract was done by FTIR method, described by Kemp (1991).

Procedure

1. KBr discs were prepared by grinding the seed extract (5 mg) with dry potassium bromide (KBr).
2. The mixture was thoroughly mixed in a mortar and pressed at pressure of 6 bars within 2 min to form a KBr thin disc.
3. Then the disc was placed in a sample cup of a diffuse reflectance accessory.
4. The frequency of the spectra set to analysis was between 4000 - 400 cm^{-1} wave number and the vibration spectrum was recorded as graphical chart.
5. The IR spectrum was obtained using FTIR analysis (Shimadzu, Japan).

4.10.2. Gas Chormatograph-Mass Spectroscopy (GC-MS)

GC-MS analysis of methanolic extract of *M. pruriens* seed was performed using a Perkin-Elmer Clarus 680 Gas Chromatograph equipped and coupled to a Mass spectrometry - Perkin Elmer Clarus 600 (Software - Turbomass version 5.4.2) spectrometer with an Elite – 5(5 % diphenyl 95 % dimethyl polysiloxane), 30.0 mm X 0.25 mm X 250µm of capillary column. The instrument was set at an initial temperature of 60°C, and maintained this temperature for 2 min. At the end of this period the oven temperature was rise up to 300°C, at the rate of an increase of 10°C/min, and maintained for 6 min. Injection port temperature was ensured at 250°C and Helium flow rate at 1

ml/min. The ionization voltage was 70 eV. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 50-600 (*m/z*). The ion source temperature was maintained at 240°C and Interface temperature was at 240°C. The solvent delay time was of 2 min. Using computer searches on a NIST-2008 MS data library and comparing the unknown component mass spectrum obtained through GC-MS, compounds present in the samples were identified.

4.11. Nutraceuticals profile of *M. pruriens* seed powder

4.11.1. Proximate of *M. pruriens* seed by biochemical method

Moisture content – drying in oven at 105ºC, until a constant weight was obtained

Ash content - ashing at 600ºC for about 2 hrs

Protein - Kjeldhal method (Humphries, 1956)

Fat - Soxhlet extraction method (AOAC, 1990a)

Crude fibre - gravimetric method (AOAC, 1990b)

Carbohydrate - calculated based on Müller and Tobin (1980)

$$\text{Carbohydrates (\%)} = 100 - [\text{crude protein (\%)} + \text{crude lipid (\%)} + \text{crude fiber (\%)} + \text{ash (\%)}]$$

Energy value - calculated according to Osborne and Voogt (1978)

$$\text{Energy value (kcal/100g)} = [\text{crude protein (\%)} \times 4] + [\text{crude lipid (\%)} \times 9] + (\text{crude carbohydrates (\%)} \times 4]$$

4.11.2. Protein fractionation of *M. pruriens* seed using SDS PAGE

SDS-PAGE gel electrophoresis (Laemmli, 1970) was carried out to fractionate and analyze the protein content in seed.

4.11.2.1. Gel electrophoresis

The *Mucuna pruriens* seed powder were treated with denaturizing 4 sample buffer containing 62.5 mM Tris (pH 8.5), 20% (v/v) glycerol, 4% SDS (w/v), and 3% (w/v) DTT for 5 min at 85–95ºC in Protein Lobind Eppendorf cups. Serial dilutions of the proteins were labeled and loaded onto the gel lanes. Electrophoresis was carried out on self-cast polyacrylamide mini-gels (1 mm thick) using a discontinuous buffer system. The separation gel (pH 8.8) contained 12% polyacrylamide. The stacking gel (pH 6.8) contained 4% polyacrylamide. Both gels have an acrylamide/bis ratio of 37.5: 1. The running buffer contained 25 mM Tris (pH 8.6), 192 mM glycine and 0.1% SDS (w/v) in water. All solutions were freshly prepared prior to use. SDS-PAGE was carried out on a vertical polyacrylamide gel system at a current of 15 mA until the protein bands reach the interface of the separating gel. Separation was performed at 100 V for two hrs.

4.11.2.2. Coomassie brilliant blue (CBB) poststaining and destaining protocol

2.5 g of Coomassie blue R250 was dissolved in 1000 mL of 50% (v/v) methanol, 10% (v/v) acetic acid and 40% (v/v) water with stirring as needed. The solution was filtered to remove any insoluble material. The final concentration of Coomassie blue R250 was 0.25% (w/v). After electrophoresis, the apparatus was disassembled and the gel was immersed into CBB solution. The gel was stained at room temperature overnight with gentle agitation. The Coomassie stain was removed by aspiration after staining. The gel was then immersed into the destaining solution composed of 50% (v/v) methanol, 10% (v/v) acetic acid and 40% (v/v) water which allowed the gel to destain with gentle agitation. The destaining step was repeated several times with removal of destaining solution at each change by aspiration. Destaining was continued until the protein bands were seen clearly without any background staining of the gel.

4.11.2.3. Detection of proteins

Molecular weight is calibrated using UV-Tech Gel Documentation.

4.12. Growth performance

Growth performance parameters, total body weight gain, specific growth rate and survival rate (%) were evaluated. Shrimp were counted and weighted accurately in digital electronic balance at the beginning and the end of experimental.

Body weight increase (BWI) = Wt – W0 (Tacon, 1990)

Specific growth rate (SGR) = (ln Wt – ln W0) × 100 t -1 (Hevroy *et al.,* 2005)

$$\text{Survival Rate (SR)} = \frac{\text{No. of live shrimp}}{\text{No. of shrimp introduced}} \times 100$$ (Tekinay and Davis, 2001)

Where, Wt and W0 = Final and initial weights (g) respectively,

t = the experimental period in day.

4.13. Assessment of Female reproduction

The gonadosomatic index (GSI) was calculated as the weight of gonads to body weight as based on the methodology of De Vlaming *et al*., (1982). The GSI were calculated using the formula: GSI = Weight of gonad/weight of body

Berried female shrimps ovary from all the experimental groups were dissected out, weighed and cleaned with 0.9% normal saline. Oocytes were separated from one another and the ovarian membrane through a washing process (Bagenal and Braum 1978). The oocytes were flushed out of the ovarian membrane and into a 0.01 mm mesh sieve, which was held beneath the ovary. Oocytes collected in the sieve were again rinsed with fully flowing tap water to help separate them from one another. After draining the water, oocytes were transferred to containers where they were preserved in 2% neutrally buffered formalin. This formalin concentration was chosen because it was the lowest possible concentration that would ensure proper oocyte preservation and minimizing changes in oocyte size and appearance. Separated oocytes were preserved in 4% formalin for enumeration. Oocytes count and diameter was determined using the iterative method described in Sokal and Rohlf (1981). An ocular micrometer in a dissecting microscope was used to measure oocyte diameters to the nearest 0.038mm (1 micrometer unit at a

total magnification of 24x). Measurements were taken along the median axis of the oocyte, parallel to the horizontal micrometer gradations (Macer 1974, DeMartini and Fountain 1981).

4.14. Male fecundity assessment

Spermatophore quality was measured by spermatophore weight, sperm count, melanization of spermatophores and spermatophore absence. Spermatophores from each male were extruded using Electro-ejaculation technique at 7.82 volts (Soundarapandian *et al.*, 2013). This spermatophore was homogenized in 2 mL of calcium-free saline solution. Sperm counts were done by counting cells present in the resulting sperm–saline solution using hemacytometer under a light microscope according to the method described by Leung-Trujillo and Lawrence (1987). Melanization and spermatophore absence percentages were checked by visual examination of the coxae of the fifth pereiopod pair and the extruded spermatophore.

4.15. Evaluation of Immune parameters

Immune parameters were estimated in haemolymph of shrimp using following method:

4.15.1. Collection of haemolymph

Haemolymph 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 (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, 0.12 M glucose, pH 7.55, osmolality adjusted with glucose to 780 mOsm kg^{-1}). Haemolymph was collected from randomly selected shrimps in each group for total hemocyte count (THC), Phenoloxidase (PO) assay, Respiratory burst activity and superoxide dismutase.

4.15.2. Total hemocyte count.

A 100 µL haemolymph was mixed gently with anticoagulant and transferred to a sterile 1.5 mL eppendorf tubes. Hemolymph was fixed with an equal volume of 10% buffered formalin in using the following ratio: 1 part haemolymph, 3 parts anticoagulant,

5 parts formalin. A 20 µL aliquot was stained using 1.2% Rose Bengal in 50% ethanol and allowed to stain for 20 min. Total hemocyte was counted using a Neubauer haemocytometer under a compound microscope at 40x magnification. Obtained values were expressed as THC mL^{-1} hemolymph (Joseph and Phillip, 2007) using the following formula:

Total hemocyte count (THC) = $(A \times dcf)/(B \times (4 \times 10^{-6})$

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

Where:

A = total number of cell counted;

B = total number of squares counted;

Vh = volume of hemolymph;

Vac = volume of anticoagulant.

4.15.3. Phenoloxidase activity (E.C. 1. 14. 18. 1, PO):

Phenoloxidase activity was assayed according to the method of Ashida and Soderhall (1984).

Principle

Phenoloxidase oxidizes the L-DOPA (L-dihydroxyphenylalanine) into brown coloured dopachromes. The formation of dopachromes can be determined by reading at 490 nm in spectrophotometer.

Reagents

1. L-DOPA

    0.01M L-Dopa in 0.05M Tris- Hcl buffer at pH – 7.5

2. Cacodylate citrate buffer

0.01M sodium cacodylate, 0.45M sodium chloride, 0.10M trisodium citrate, pH-7.0

3. Cacodylate buffer

0.01M sodium cacodylate, 0.45M sodium chloride, 0.01M calcium chloride, 0.26 M magnesium chloride, pH 7.0

Procedure

The haemolymph 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, resuspended 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 phenoloxidase activity for all test groups was expressed as dopachrome formation in 50 µl of hemolymph.

4.15.4. Respiratory burst activity (E.C. 1. 15. 1. 1, O_2^-):

Respiratory burst activity was assayed according to the method of Song and Hsieh, (1994).

Principle

Nitroblue tetrazolium (NBT) assay is commonly used to measure the oxidative burst. NBT is used as an electron acceptor and, upon reduction by superoxide anion it changes from a yellow solute to a blue formazan precipitate.The formazan is then solubilized in dimethylsulphoxide (DMSO) and potassium hydroxide (KOH), and the

optical density of the solubilized formazan is subsequently measured on an ELISA reader. The colorimetric assay allows precise quantification of formazan.

Procedure

Haemolymph samples 100µl was collected in an eppendorf tube and centrifuged at 1600 rpm for 5min in a refrigerated centrifuge. After centrifugation the supernatant was discarded and 100µl of laminarin (2mg/ ml in HBSS) was added to the haemocytes and allowed to react for 30min at 37ºC. Then laminarin was discarded and the haemocytes were washed 3 times with 100µl HBSS, and then stained with 100µl NBT solution (0.3%) for 30min at room temperature. The staining reaction was terminated by removing NBT solution and adding absolute methanol. After three washings with 70% methanol, the haemocytes were air dried and finally 120 µl of 2M KOH and 140 µl of DMSO was added to dissolve the formazan. The optical density of the dissolved formazan was read at 630nm in a spectrophotometer. Respiratory bursts were expressed as NBT-reduction in 50 µl of haemolymph.

4.15.5. Superoxide dismutase activity (E.C. 1. 15. 1. 1, SOD):

The enzyme activity was assayed according to the method of Marklund and Marklund (1974).

Principle

The degree of inhibition of auto-oxidation of pyrogallol, at an alkaline pH, by superoxide dismutase was used as measure of the enzyme activity.

Reagents

1. Tris-HCl buffer (pH 3.2; 0.1 M, W/V) containing 2mM EDTA (W/V)

    1.21 g of Tris was dissolved in 100ml of distilled water and the pH was adjusted to 8.2 with HCl, then 74.4 mg of EDTA was added.

2. Tris-HCl buffer (pH 7.5; 0.05 M)

    605.7 mg of Tris was dissolved in 100 ml of distilled water pH was adjusted to 7.5 with HCl.

3. Absolute ethanol (AR grade)

4. Chloroform (AR grade)

5. Pyrogallol stock solution

25.2 mg pyrogallol was dissolved in 1 ml of 0.05 M Tris. HCl Buffer, pH 7.5 in an aluminum foil wrapped and stoppered test tube.

6. Pyrogallol working solution

At the time of assay, 0.5 ml of the above stock solution was diluted to 0.5 ml with 0.05M Tris. HCl buffer to give a 2mM working solution and used immediately.

Procedure

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 of 2 mM pyrogallal a final volume of 4ml. 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.

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

The SOD activity was expressed as Units/mg protein.

4.16. Validation of Antioxidant enzyme

Antioxidant enzyme assay were evaluated spectophotometrically (UV-VIS spectrometer-1800, Shimadzu, Japan) in tissue homogenates by the following methods:

4.16.1. Superoxide dismutase (E.C. 1. 15. 1. 1, SOD)

The superoxide dismutase was assayed according to the method of Marklund and Marklund (1974) has been described above.

4.16.2. Catalase (EC 1.11.1.6, CAT)

The activity of catalase was assayed by the method of Sinha (1972).

Principle

Dichromate in acetic acid is reduced to chromic acetate, when heated in the presence of hydrogen peroxide (H_2O_2) with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus formed was measured at 570nm.

Reagents

1. Sodium phosphate buffer (pH 7.0; 0.01M, W/V):
   Solution A: 156 mg of sodium dihydrogen phosphate was dissolved in 100 ml of distilledwater.
   Solution B: 178 mg of disodium hydrogen phosphate was dissolved in 100 ml of distilled water 39 ml of solution A + 61 ml of solution and then pH was adjusted to 7.0.
2. Hydrogen peroxide (H_2O_2) (0.2 M, V/V):
   1 ml of H_2O_2 was made up to 45 ml with distilled water.
3. Dichromate – acetic reagent.

This reagent was prepared by mixing a 5% solution of potassium dichromate with glacial acetic acid in the ratio 1:3 (V/V). This solution was further diluted to 1:5 with water, before use.

Procedure

The assay mixture contained 0.5 ml of 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 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 dichromate-acetic acid reagent. The tubes were then heated for 10 minutes, allowed to cool, and the green colour developed was read at 570 nm. The activity of catalase was expressed as μ moles of H_2O_2 consumed/min/mg protein.

4.16.3. Glutathione peroxidase (E.C. 1.11.1.9, GPx)

The activity of glutathione peroxidase was determined by the method Rotruck *et al.* (1973).

Principle

Glutathione peroxidase converts reduced glutathione (GSH) into oxidized form using hydrogen peroxide during its reaction. The amount of GSH utilized is estimated by measuring it in the assay mixture before and after the enzyme activity. GSH reacts with DTNB to give yellow colour, which was then measured at 412 nm.

Reagents

1. Sodium Phosphate Buffer: (pH 7.0; 0.4 M, W/V):
   Solution A: 6.24 g of sodium dihydrogen phosphate was dissolved in 100 ml of distilled water.
   Solution B: 7.11 g of disodium hydrogen phosphate was dissolved in 100 ml of distilled water.
   39 ml of solution A + 61 ml of solution B were mixed and pH was adjusted to 7.0.

2. Sodium azide (10mM, W/V):

   65 mg of sodium azide was dissolved in 100 ml of distilled water.

3. Reduced glutathione (4mM, W/V):

   12.3 mg of GSH was dissolved in 10ml of distilled water.

4. Hydrogen peroxide (2.5mM, V/V).

5. 10% TCA (W/V).

6. Disodium hydrogen Phosphate solution (0.3 W/V):

   5.34g of disodium hydrogen phosphate was dissolved in 100ml of distilled water.

7. Dithio-bis-nitrobenzoic acid (DTNB) reagent

   40 mg of DTNB was dissolved in 100 ml of 1% sodium citrate solution.

8. Standard Oxidized glutathione·

   20 mg of oxidized glutathione was dissolved in 100 ml of distilled water.

Procedure

0.5 ml of buffer, 0.1 ml of sodium azide, 0.2ml or reduced glutathione, 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 glutathione content, the supernatant was removed after centrifugation (1500 rpm for 8 minutes) and to this 4.0 ml of disodium hydrogen phosphate (0.3M) solution and 1ml of the DTNB reagent were added. The colour developed was read at 412 nm against a suitable reagent blank containing only phosphate solution and DTNB reagent. The enzyme activity was expressed as μ moles of GSH Consumed/min/mg protein.

4.16.4. Glutathione reductase (E.C. 1.6.4.2, GSH)

The enzyme assay was carried out by the method of Staal *et al.* (1969).

Reagents

1. Sodium phosphate buffer (0.3 M, pH 6.0):
   12.3 ml of 0.3 M disodium hydrogen phosphate solution and 87.7 ml of 0.3 M monosodium hydrogen phosphate solution are mixed and the volume made up to 200 ml with distilled water.

2. Ethylene Di-amine Tetra Acetic Acid (EDTA) (25mM W/V):
   930 mg of EDTA, tri sodium salt was dissolved in 100 ml of distilled water.

3. Glutathione (oxidized) (12.5 mM, W/V):
   765.79 mg oxidized glutathione was dissolved in 100 ml of distilled water, just before use.

4. Nicotinamide Adenine Di-nucleotide Phosphate (reduced form) (NADPH) (3 mM, W/V): 25 mg of NADPH was dissolved in 25 ml of distilled water before use.

Procedure

The assay mixture containing 0.2 ml of tissue extract, 1.5 ml of sodium phosphate 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 30seconds interval in a spectrophotometer against a reagent blank. GR activity was expressed as µ moles of NADPH oxidized/min/mg protein.

4.17. Analysis of Proximate composition

The proximate composition of shrimp muscles, such as protein, carbohydrate, lipid and moisture were assayed by spectrophotometrically (UV-VIS spectrometer-1800, Shimadzu, Japan).

4.17.1. Estimation of Total Protein

Total protein content was determined by the method of Lowry *et al* (1951).

Principle

Protein forms a complex with copper ions in alkaline solution and this copper protein complex reacts with Folin Ciocalteau reagent to give a blue colour which is due to the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of the colour developed was read at 720 nm.

Reagents

1. 0.1 N Sodium hydroxide : 0.4 g of sodium hydroxide in 100 ml of distilled water.

2. Reagent A : 2% sodium carbonate in 0.1 N sodium hydroxide (2 g of sodium carbonate in 100 ml of 0.1 N sodium hydroxide).

3. Reagent B : 0.5 % copper sulphate in 1.35% sodium potassium tartarate.

4. Reagent C- Alkaline Copper reagent : This was prepared just before use by mixing 50 ml of reagent A with 1 ml of reagent B.

5. Folin Ciocalteau reagent (1 N)

6. Protein standard : 12.5 mg of Bovine Serum Albumin (BSA) was dissolved in 50 ml of 0.1 N sodium hydroxide. Different concentrations ranging from 25 µg to 200 µg were used to prepare the standard graph.

Procedure

Aliquots of extracts were taken and made up to a final volume of 1 ml with distilled water. 5 ml of alkaline copper reagent was added, mixed and allowed to stand at room temperature for 10 minutes. Then 0.5 ml of Folin Ciocalteau reagent was added to this and shaken well. The blue colour developed was read against reagent blank at 720 nm after 20 minutes in a spectrophotometer. The amount of protein present in the aliquot of the sample was calculated by referring to the standard curve obtained. The total protein content is expressed as mg/100 mg of tissue (wet weight).

4.17.2. Estimation of Carbohydrate

Carbohydrate content was estimated by the method proposed by Hassid and Abraham (1957).

Principle

The tissues were digested with 30% KOH and glycogen was precipitated with ethanol. The precipitate was further treated with Anthrone reagent and the glucose content was determined colorimetrically.

Reagent

1. 30% (W/V) KOH: 30g of Potassium hydroxide was dissolved in 100ml of distilled water.

2. 95% ethanol (V/V): 95ml of alcohol was made up to100 ml with distilled water.

3. 95% Sulphuric acid (V/V): 95ml of concentrated Sulphuric acid was made upto 100ml with distilled water.

4. 0.2% anthrone reagent: This reagent was prepared by dissolving 0.2 g of anthrone in 100 ml of 95% sulfuric acid and just before use and stored in the refrigerator.

5. A standard glucose solution containing 100µg/of glucose per ml.

Procedure

A known weight of tissue was taken in a tube and 1ml of KOH was added. Digestion of the tissues was done by keeping the tube in a water bath for 20 minutes. It was cooled and 1.25ml of 95% ethanol was added. After a through mixing, the contents were boiled in water bath. It was cooled and then subjected to centrifugation for 15 minutes at 3000g. The precipitate was carefully taken by decanting the supernatant and allowing the tube to drain on a filter paper. To the precipitate 1ml of distilled water was added and redissolved in 1.25ml of 95% ethanol.

The supernatant was decanted off and the tube was drained on filter paper as before. The precipitate was then dissolved in 5ml of distilled water and placed in ice bath. To this 10ml of anthrone reagent was added and boiled in a hot water bath. Glass marbles were kept on the tubes while boiling to avoid the overflow of reagents. A green colour was developed which was then analyzed in a spectrophotometer at 620nm after cooling the tube. Similar procedure was employed for preparing "blank" and standard solutions. 5 ml of water and 5 ml of the standard solution containing 100 µg/l of glucose treated with 10ml of anthrone reagent served as blank and standard respectively. The Carbohydrate content of the tissue was expressed as gram %.

4.17.3. Estimation of Lipid

Lipid content was determined by the method of Folch *et al.,* (1957)

Principle

The quantitative determination of lipid by sulphophosphovanillin method depends on the reaction of lipids extracted from the sample using chloroform – methanol, with sulphuric acid, phosphoric acid and vanillin to give a red complex.

Reagents

1. Chloroform methanol (2:1): This reagent was prepared by mixing 200 ml of chloroform and 100 ml of methanol.
2. Sodium chloride (0.9%): 900 mg of NaCl was dissolved in 100 ml distilled water.
3. Sulphophosphovanillin reagent: 800 ml of Orthophosphoric acid was added to 200 ml of distilled water. To this, 2 g of vanillin powder was added and mixed well.
4. Standard: 10 mg of olive oil was dissolved in 10 ml chloroform methanol mixture (2:1) and different dilutions from this stock solution served as the standard.
5. Blank: Vanillin reagent was used as a blank solution.

Procedure

A known amount of tissue sample was taken and homogenized well with 4 ml of chloroform methanol mixture. After mixing well, 0.2 ml of 0.9% sodium chloride was

added and the mixture was kept undisturbed overnight. The lower layer of lipid was collected carefully and dried in a vaccum dessicator. The dried lipid content was dissolved in concentrated sulphuric acid (0.5 ml) and kept in a boiling water bath for 10 min. From the lipid sample, 0.2 ml was taken in a test tube and 5 ml of sulphophosphovanillin reagent was added, shaken well and kept undisturbed for 30 minutes. The intensity of red colour was measured at 520 nm in a spectrophotometer.

Calculation

$$\text{Lipid present in the sample (\%)} = \frac{\dfrac{\text{OD of the sample}}{\text{OD of the standard}} \times \text{Conc. of the standard (mg)}}{\text{Weight of the sample (mg)}} \times 100$$

4.17.4. Estimation of Moisture (APHA, 2005)

Principle

The wet tissue was allowed to dry by kept in desiccator. The difference between the wet weight of the tissue and its dry weight give the amount of water present in the fresh tissue.

Procedure

Known amount of wet tissue sample was taken individually on previously weighed concave glass and they were kept in a desiccator, maintaining 0.5% relative humidity. Dry the tissues in the desiccator till they reached a constant weight.

Calculation

$$\text{Moisture} = \frac{\text{Wet weight (g)} - \text{Dry weight (g)}}{\text{Wet weight (g)}} \times 100$$

4.18. Histology – Light microscopical study

Shrimp hepatopancreas and gill tissue were collected after completion of 21 days (experimental diet) fed for histological studies. The classic paraffin sectioning and Haematoxylin-Eosin staining techniques as follows.

Reagents

Davidson's Fixative Solution:

Davidson's fixative's solution was prepared by mixing the following chemicals.

1. Alcohol (95%)	: 300 ml
2. Formaldehyde (37%)	: 200 ml
3. Glacial acetic acid	: 100 ml
4. Distilled water	: 300 ml

Preparation of Eosin and Haematoxylin:

Eosin:

Eosin	: 1 g
Alcohol 95%	: 25 ml
Glacial acetic acid	: 75 ml

Ehrlich's Haematoxylin:

Haematoxylin	: 1 g
Absolute alcohol	: 10 ml
Potassium alum	: 20 g
Mercuric oxide	: 0.5 g
Distilled water	: 200 ml
Glacial acetic acid	: 8 ml

The solution was mixed thoroughly and exposed to light for six weeks to ripen the solution.

Fixation

Shrimp hepatopancreas and gill tissues were fixed in the Davidson's fixative for two consecutive changes of 1 hr and 1½ hrs as soon as they were excised. After 48 hrs, the tissue was taken out and washed in running tap water for a day to remove excess acid.

Dehydration

For dehydration, Isopropyl alcohol was used. The tissue was passed through the following series of alcohol: 85%, 95% and 100% (one and half an hr in each). Care was taken to have optimum dehydration.

Clearing

Xylol was used as the clearing agent for 1½ hrs with two to three changes.

Infiltration (or) **Impregnation**

The tissue was taken out of Xylol and kept in molten paraffin wax bath in metal pots. The temperature of the molten wax was maintained at about 58°C. The tissue was given 3 changes in the molten wax at 30 minutes intervals.

Embedding

The paraffin wax heated up to the optimum melting point (56°C-58°C) and maintained at the temperature till embedding. A clean glass plate was smeared with Glycerine and 'L' shaped models were placed on it to form rectangular cavity. The molten paraffin was poured slowly and the air bubbles were removed by using a hot needle. The tissue was placed in the paraffin and oriented with the surface to be sectioned. Then, the tissue was pressed gently towards the glass plates to make the tissues settle uniformly with metal pressing rod and allowed the wax to settle and solidify at room temperature. The paraffin block was kept in cold water for cooling.

Section cutting

Section cutting was done with rotary microtome. To produce uniform section, the microtome knife was adjusted to the proper angle in the knife holder with only the cutting edge coming in contact with the paraffin block and sections of 5µ thickness were cut.

Flattening and mounting of section

The sections were spread on a warm water bath, after they were detached from the knife with the help of hairbrush. Dust – free clean slides were coated with egg albumin over the whole surface. Required number of sections were spread on the clean slide and kept at the room temperature.

Staining

The sections were stained as follows:

- Deparaffinization treatment with Xylol two times, each for 5 minutes.
- Hydration through descending grades of Isopropyl alcohol (2 minutes) 90% alcohol, 70% alcohol and 50% (1 minute in each).
- Thorough washing in tap water and blowing for 10minutes.
- Rinsing with distilled water thoroughly.
- Staining with Haematoxylin and Eosin.
- Dehydration again with ascending grades of alcohol 85% alcohol (2 minutes), 95% alcohol and 100% (absolute) alcohol (2 minutes).
- Clearing up Xylol for three times, each at 3 minutes of interval.

Mounting

On the stained slide, the DPX mountant was applied uniformly and micro glass cover slide was placed gently, avoiding air bubbles. The slides were observed under microscope and microphotography was performed.

4.19. Statistical analysis

The resulting data obtained from the experiment was analysed 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.

5. Results

5. RESULTS

Vibrio harveyi is one of the most important pathogens, capable of causing vibriosis and devastating the diverse ranges of marine invertebrates. *V. harveyi* infected shrimp characterstic of body opaqueness, necrosis, lethargy and body malformations.

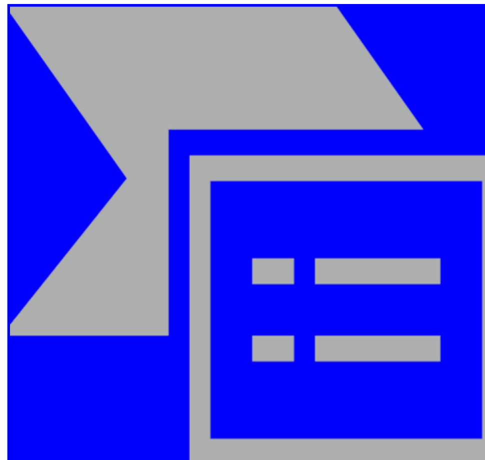
5.1. Isolation of *Vibrio harveyi* in VHA

Vibrio harveyi is gram negative, nonspore-forming, straight rod bacterium and classified as chemoorganotroph. Vibrio harveyi agar (VHA) was used for isolation and enumeration of *V. harveyi*. This medium is able to differentiate *V. harveyi* from 15 other vibrio species and has been shown to inhibit the growth of other *Pseudomonas* spp. and *Flavobacterium* spp. The colonies of *V. harveyi* on VHA were green in color with dark center (Fig. 8).

5.2. Characterization of *Vibrio harveyi*

Biochemical and physiological characteristics of the *V. harveyi* was done systematically (Table 1). All isolates were gram negative under light microscopic (Labomed 3100 microscopy, USA) observation after gram stain. All isolates were able to ferment the glucose and they are positive to glucose fermentation. All bacterial isolates were showed result for oxidase, catalase and motility test but unable to produce hydrogen sulfide. The production of amylase was also observed by the formation of clear zone around the colony (Fig. 9).

The isolates were able to degrade tryptophan and produce indole as final product, sensitive to vibriostat 0/129 (150 μg/disk) and produced green colonies on thiosulfate citrate bile salt agar (TCBS agar). The isolates were observed to be luminous in darkroom after 24 hr of growth. Isolated colonies were failed to utilize the L-arginine, phenylalanine and gelatinase with negative result. Positive result for both oxidative and fermentative test of isolates because they utilizes starch and lipid. Colonies were able to haemolyse horse's blood which resulted in breakdown of horse blood agar plate around the bacterial steak known as β-hemolysis (Fig.10)



G

Figure 8. ***Vibrio harveyi*** **colony in VHA (G – Dark green colonies)**

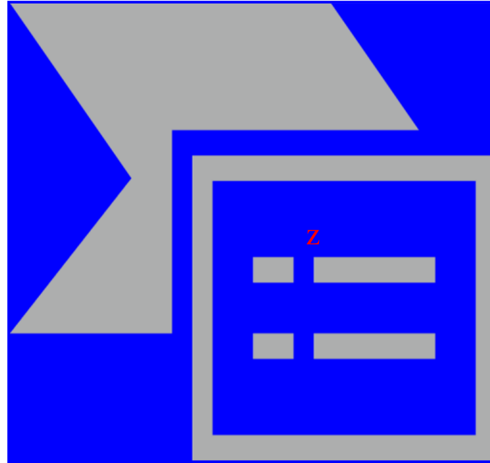


Figure 9. Starch hydrolysis by ***Vibrio harveyi***
on right streak with zone clearance (z)

Figure 10. β blood hemolysis (H) by ***Vibrio harveyi***

Table1. Biochemical and physiological characteristics of *Vibrio harveyi* isolates from *Litopenaeus vannamei*

Characteristics	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
Gram stain	-/R	-/R	-/R	-/R	-/R	-/R	-/R	-/R	-/R	-/R
Glucose fermentation	*	*	*	*	*	*	*	*	*	*
Motility	*	*	*	*	*	*	*	*	*	*
Indole formation	*	*	*	*	*	*	*	*	*	*
Oxidase	*	*	*	*	*	*	*	*	*	*
Catalase	*	*	*	*	*	*	*	*	*	*
H_2S formation	-	-	-	-	-	-	-	-	-	-
Luminescence	*	*	*	*	*	*	*	*	*	*
Gelatin liquefaction	-	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-
Amylase	*	*	*	*	*	*	*	*	*	*
Colony colour on TCBS	Gr	Gr	Gr	Gr	Gr	Gr	Gr	Gr	Gr	Gr
β Blood hemolysis	*	*	*	*	*	*	*	*	*	*
Sensitivity to vibriostat 0/129	*	*	*	*	*	*	*	*	*	*
Oxidase and fermentation	*/*	*/*	*/*	*/*	*/*	*/*	*/*	*/*	*/*	*/*
Phenylalanine deaminase	-	-	-	-	-	-	-	-	-	-
Identification	Vi	Vi	Vi	Vi	Vi	Vi	Vi	Vi	Vi	Vi

Vi = *Vibrio harveyi*, * = Positive, - = Negative, V = Colony, R = Short rod, Gr = Green

5.3. Nucleotide sequencing analysis for Molecular identification of *Vibrio harveyi*:

Molecular identification of the isolated *Vibrio harveyi* strains was carried out based on 16S rDNA sequence analysis. The sequence of the 1398 bp 16S rDNA PCR amplicon from isolate was compared with known sequences in the NCBI BLAST

accession numbers JQ920474.1. The nucleotide sequence analysis of the 16S rDNA fragments from this bacteria revealed high percentage of identity (>99) to 16S rDNA nucleotide sequences belonging to *V. harveyi*, they were also showed similarity (nearly 90%) to other *Vibrio* species. Therefore, the isolated were identified as *V. harveyi* based on their morphological, cultural, physiological, biochemical characteristics and 16S rDNA sequence analyses.

5.4. Antibacterial activity of *Mucuna pruriens*:

The *Mucuna pruriens* seed principles were extracted separately in ethanol and methanol, to study the antibacterial effect on the bacterial species. *M. Pruriens* methanolic extract exhibited the significant antibacterial activity against series of bacterial pathogens than ethanolic extract (Fig. 11). These extracts are more effective against *Staphylococcus aureus, Salmonella paratyphi, Escherichia coli, Proteus mirabilis, Vibrio cholerae, Vibrio harveyi.* Among the various strains maximum antibacterial effect was recorded against *Vibrio harveyi* with 16.8 mm diameter of zone of inhibition with methanol extract of *M. Pruriens. V. cholera* recorded its second far most antibacterial sensitiveness to *M. Pruriens* with 9.4mm followed by *E. coli* with 4.1 mm, *S. aureus* bacteria with 3.4 mm. The least antibiogram was recorded against *S. typhii, K. pneumonia* and *P. aeruginosa*.

5.5. MIC of *Mucuna pruriens*:

In the MIC assay *M. pruriens* methanol extract was screened against sensitive bacterial pathogens of *E. coli*, *S. aureus*, *V. cholerae*, and *V. harveyi* bacteria (Fig. 12). Differential concentration (10µg - 250 µg) of *M. pruriens* extract was screened against four bacteria in using microtitre plate. The bacterial growth inhibition was assessed using optical density measurement at 540nm. The results are promising enough that extract exhibits exhibits the maximum activity of MIC with 25 µg/ml concentration against *V. harveyi,* followed by *V. cholerae* at 75-80 µg/ml, *S. aureus* and *E. coli* on 175 µg/ml..

Nucleotide ▼

Display Settings: GenBank

Vibrio harveyi strain MCCB 155 16S ribosomal RNA gene, partial sequence

GenBank: JQ920474.1

FASTA Graphics PopSet

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  AUTHORS   Surekhamol,I.S., Deepa,G.D., Somnath Pai,S., Sreelakshmi,B.,
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  TITLE     Isolation and characterization of broad spectrum bacteriophages
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  JOURNAL   Lett. Appl. Microbiol. 58 (3), 197-204 (2014)
   PUBMED   24131105
REFERENCE   2  (bases 1 to 1398)
  AUTHORS   Surekha Mol,I.S., Somnath Pai,S., Bright Singh,I.S. and Deepa,G.D.
  TITLE     Morphological molecular and ecological properties of lytic Vibrio
            harveyi phages for the management of Vibrio harveyi in aquaculture
  JOURNAL   Unpublished
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  TITLE     Direct Submission
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      121 ccgcataatg cctacgggcc aaagaggggg accttcgggc ctctcgcgtc aggatatgcc
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      301 cagtggggaa tattgcacaa tgggcgcaag cctgatgcag ccatgccgcg tgtgtgaaga
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     1081 aatgtcggga actccaggga gactgccggt gataaaccgg aggaaggtgg ggacgacgtc
     1141 aagtcatcat ggcccttacg agtagggcta cacacgtgct acaatggcgc atacagaggg
     1201 cggccaactt gcgagagtga gcgaatccca aaaagtgcgt cgtagtccgg atcggagtct
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Figure 11. Disc diffusion technique plates

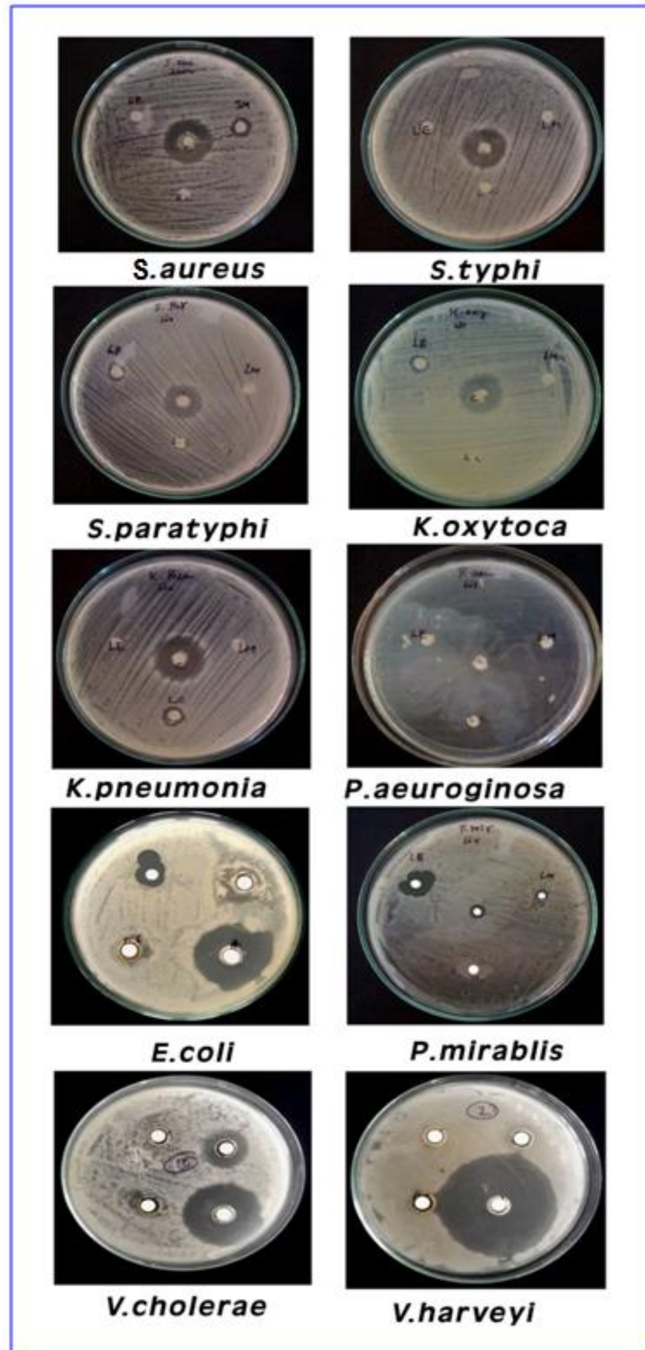
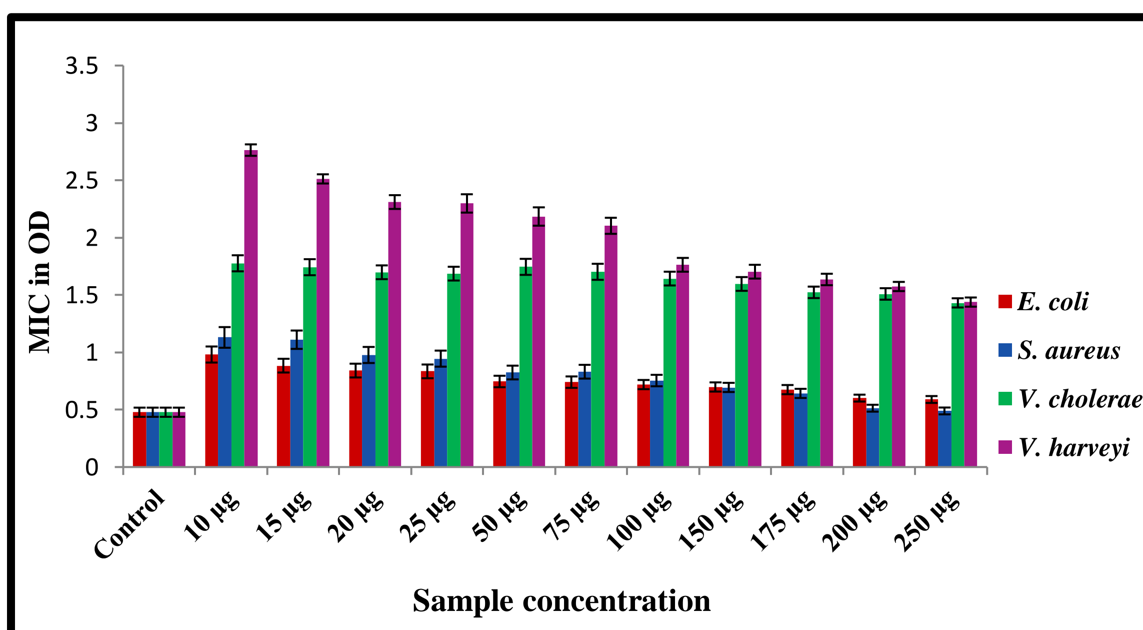


Table 2. Antibacterial activity of *M. pruriens* seed extract

Sl. No	Name of the Bacteria	Activity of Agent Erythromycin (C)	Zone of inhibition (mm in diameter)	
			Ethanol	Methanol
1	*Staphylococcus aureus MTCC* 9542	5.6	0.3	3.4
2	*Salmonella typhi MTCC 3224*	5.9	0.1	0.1
3	*Salmonella paratyphi MTCC 3220*	5.6	.05	0.1
4	*Klebsiella oxytoca MTCC 3030*	5.7	0.7	0.1
5	*Klebsiella pneumoniae MTCC 7407*	5.1	0.1	0.1
6	*Pseudomonas aeruginosa MTCC 6458*	0	0.1	0.1
7	*Escherichia coli MTCC1698*	4..9	3.6	4.1
8	*Proteus mirabilis MTCC 9493*	2.3	3.2	0.2
9	*Vibrio cholerae MTCC 3906*	0	3.9	9.4
10	*Vibrio harveyi MTCC 7954*	0	1.7	16.8

Figure 12. MIC assay of *M. pruriens* seed extract against selective pathogen



5.6. Phytochemical analysis of *M. pruriens* seed:

Phytochemical analysis of *M. pruriens* seed methanolic extract was done through FT-IR, and GC-MS analysis.

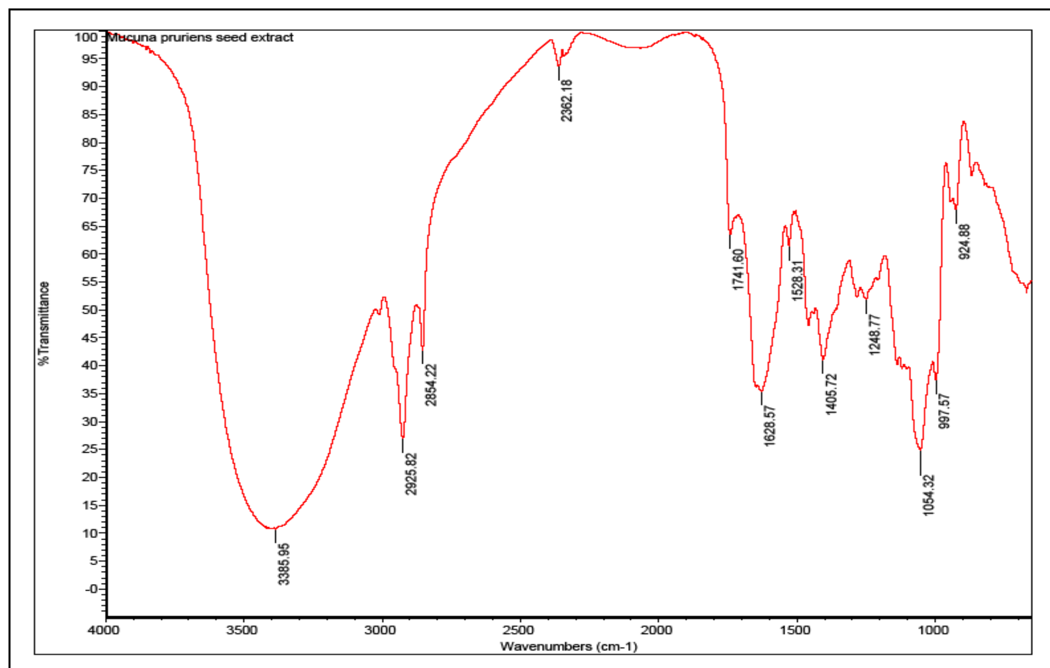
5.6.1. FTIR of *Mucuna pruriens*:

The FTIR spectrum was used to identify the functional group of the active components based on the peak value in the region of infrared radiation. The Infra-red spectroscopic (IR) analysis of the legume seed, in a band width ranging from 400 to 4000 cm-1, revealed the presence of different functional groups (Fig. 13). The peaks showed that the MP extract have the compounds like alcohol, amines, amides, phosphine, flurides, bromides, iodide, aliphatic and aromatic nitro compounds (Table 4). Further, it indicates the possible chemical bond and compound type as follows, O-H, N-H stretching for alcohol (3385.95cm-1), N-H stretching and for amine salt (2925.82,2854.22, 2362.18 cm-1), C=O stretching for amide (1741.60, 1628.57 cm-1), Asymmetric stretching for aliphatic and aromatic nitro compounds (1528.31cm-1), P-CH3 bending for phosphine (1405.72 cm-1), C-F, C-Br stretching for fluride and aryl bromide (1248.77 cm-1), C-Br, C-I stretching for bromide and iodide (1054.32) and P-H stretching for phosphine (997.57, 924.88 cm-1) was observed.

5.6.2. GCMS of *Mucuna pruriens*:

The GC-MS Chromatogram of the methanolic seed extract of *Mucuna pruriens* (Fig. 14) showed seventeen peaks indicating the presence of eleven significant compounds. The chemical compounds identified in the Methanolic extract of the seed of *Mucuna pruriens* are presented in Table. 5. The qualitative analysis identified the various phytochemicals compounds. Interpretation of mass spectrum GC-MS was conducted using the database of NIST data Library. Ethyl 9, 12-Hexa decadienoate (53.36%) was found as the major compoments followed by N Hexadecanoic Acid (17.72%), Methyl 11,14-Octadecadienoate (7.30%), (IS,15S)-Bicyclo [13.10] Hexadecan-2-one (7.12%),

Figure 13. FT-IR Spectroscopic analysis *Mucuna prurien* seed extract



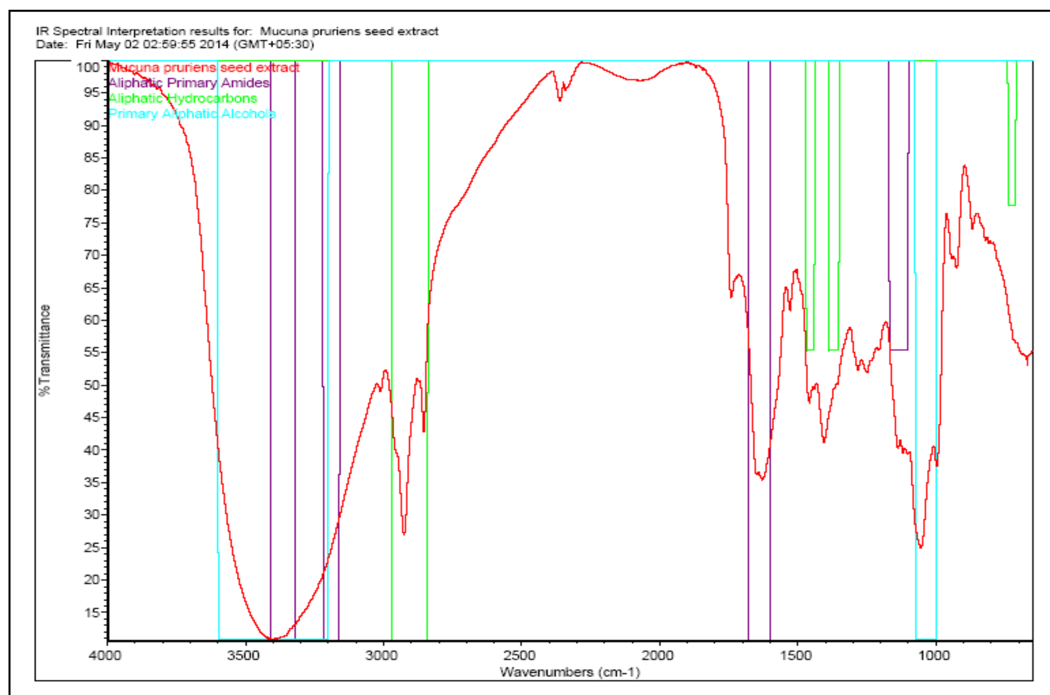


Table 4. FT-IR of *Mucuna pruriens* seed methanolic extract

Wavelength (cm^{-1})	Functional group	Type of bond	Compound type
3385.95	Alcohol and hydroxyl compound	O-H , N-H	Alcohol and Amines
2925.82	Saturated Aliphatic (alkane)	N-H	Amines salts
2854.22	Saturated Aliphatic (alkane)	N-H	Amines salts
2362.18	Alcohol and hydroxyl compound	N-H , O-H	Amines salts and Amides
1741.60	Oleifinic (alkene)	C=O , N-H	Amides
1628.57	Nitrogen Oxy compound	C=O , N-H	Amides
1528.31	Alcohol and hydroxyl compound	C=C	Aliphatic, Aromatic nitro compound
1405.72	Sulfur Oxy compound	$P-CH_3$	Phosphine
1248.77	Aliphatic organohalogen	C-F , C-Br	Flurides and Aryl bromides
1054.32	Oleifinic (alkene)	C-Br , C-I	Bromides and Iodides
997.57	Oleifinic (alkene)	P-H	Phosphine
924.88	Oleifinic (alkene)	P-H	Phosphine

Figure 14. GC-MS chromatogram of *M. pruriens* seed extract

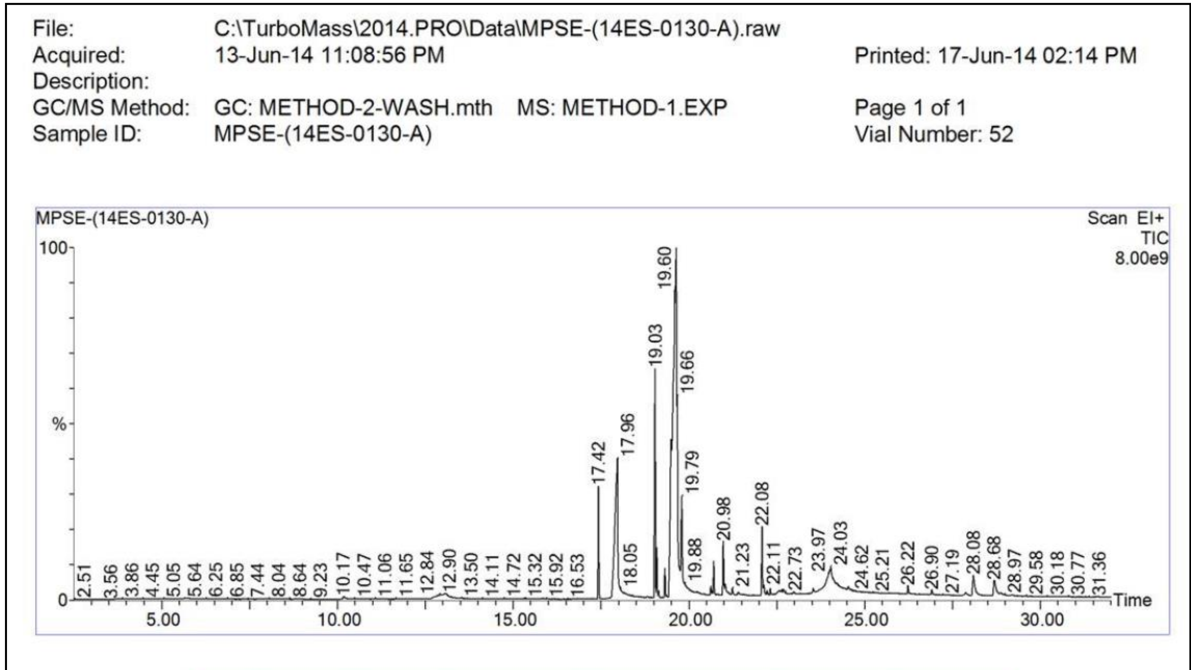


Table 5. GC-MS of *M. pruriens* seed methanolic extract

Sl. No.	Retention Time	Scan	Height	Area	Area %	Normal %	Compound Name	Molecular weight	Compound Structure
1	17.424	2984	2,563,727,616	63,264,888.0	3.426	6.39	Methyl 11-Methyl-Dodecanoate	228	$C_{14}H_{28}O_2$
2	17.965	3092	3,141,783,552	327,299,168.0	17.722	33.04	N-Hexadecanoic Acid	256	$C_{16}H_{32}O_2$
3	19.035	3306	5,135,240,192	134,861,568.0	7.302	13.61	Methyl 11, 14 – Octa decadienoate	294	$C_{19}H_{34}O_2$
4	19.085	3316	1,045,162,816	21,777,148.0	1.179	2.20	I-Propyl 9, 12 – Octa decenadienoate	322	$C_{21}H_{38}O_2$
5	19.635	3426	7,408,022,528	990,626,496.0	53.638	100.00	Ethyl 9,12 – Hexa decadienoate	280	$C_{18}H_{32}O_2$
6	19.790	3457	1,798,003,328	53,368,720.0	2.890	5.39	Pentadecanoic Acid, 15 – Bromo	320	$C_{15}H_{29}O_{Br}$
7	20.976	3694	1,144,097,920	30,004,310.0	1.625	3.03	Cyclobutanone, 2 - Tetradecyl	266	$C_{18}H_{34}O$
8	22.076	3914	1,493,184,256	37,368,788.0	2.023	3.77	Cyclobutane carboxylic Acid, 2 – Dimethyl amino ethyl ether	171	$C_{19}H_{17}O_2N$
9	24.027	4304	541,452,032	131,624,584.0	7.127	13.29	(1S, 15S) – Bicyclo [13.1.0] Hexadecan – 2 - one	236	$C_{16}H_{28}O$
10	28.084	5115	460,407,552	32,403,420.0	1.755	3.27	Stigmasterol	412	$C_{29}H_{48}O$
11	28.679	5234	340,732,992	24,265,312.0	1.314	2.45	Gamma – Sitosterol	414	$C_{29}H_{50}O$

Methyl 11-Methyl-Dedecanoate (3.42%), Pentade canoic acid, 15-Bromo (2.89%), Cyclobutane carboxylic Acid, 2-Dimethyl amino ethylether (2.02%), Stigmasterol (1.75%), cyclobutanone, 2-Terradecyl (1.62%), Gamma-Sitosterol (1.31%) and I-Propyl 9,12-Octa decenadienoate (1.17%). The analysis showed the presence of alkaloid, terpenoid and other secondary metabolites most of them are of pharmacological importance in terms of antioxidant and antimicrobial.

5.7. Nutraceuticals profile of *M. pruriens* seed powder

5.7.1. Proximate composition of *M. pruriens* seed by biochemical method

Nutraceutical analysis indicates the nutrients present in the seed of which carbohydrate content is relatively high with 54.1%, moisture with 9.8%, protein with 12.1%, fat with 6.9%. The total energy value was confined to be 327 Kcal/100g (Table. 6).

5.7.2. Protein fractionation of *M. pruriens* seed using SDS PAGE

Mucuna pruriens seed was subjected for SDS PAGE native gel electrophoresis for tracing out the plant seed protein. The results showed the presence of proteins in the range nearly 59 kDa and 95 kDa (Fig. 15).

5.8. Infection evaluation of *V. harveyi* against *L. Vannamei*

The LD_{50} value of *V. harveyi* against *L. vannamei* was 4.03 x 10^6 CFU g^{-1} (Table 7). No mortality was observed in the control injected with sterile PBS.

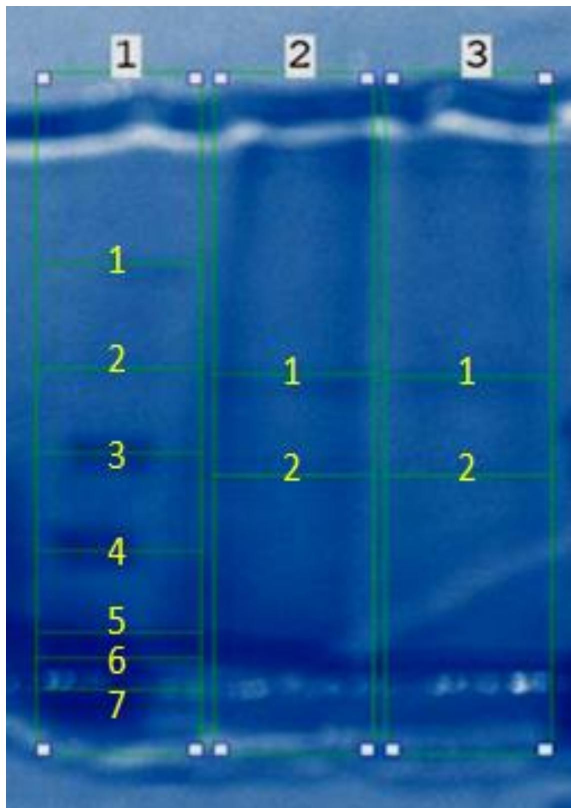
5.9. Growth performance

Growth performances of all experimental groups in male and female *L. vannamei* was recorded. After completion of the experiment final weight gain was recorded and final weight gain%, specific growth rate and survival were calculated. The result shows that shrimps fed with normal and experimental fed diet had no significant differences in average body weight (Fig. 16 & 17). However, the *V. harveyi* infected shrimps shows lowest weight gain of 1.36 ± 0.088 g in male and 1.53 ± 0.076 g in female (Fig. 18 & 19). Specific growth rate was low in infected shrimps compared with *M. pruriens* fed groups (Fig. 20 & 21). No mortality was observed in normal and experimental groups whereas the infected groups shows survival rate of 87% in male and 80% in female (Fig. 22).

Table 6. Proximate composition of *M.pruriens* seed extract

Parameters	Results (%) w/w
Moisture	**9.8**
Ash	**3.8**
Crude Fibre	**13.3**
Carbohydrate	**54.1**
Protein	**12.1**
Fat	**6.9**
Energy Value (kcal)	**327 Kcal/100 g**

Figure 15. SDS PAGE of *M.pruriens* seed extract



M.W. Values	Lane 1	Lane 2	Lane 3
Band 1	205.000	95.089	94.319
Band 2	97.400	59.701	59.701
Band 3	66.000		
Band 4	43.000		
Band 5	29.000		
Band 6	20.100		
Band 7	14.300		

Table 7. LD_{50} values of *V. harveyi* against *L. Vannamei*

Sl. No.	Dose (CFU g^{-1} body weight)	Mortality (%)	LD_{50} value (CFU g^{-1} body weight)
1	5.0×10^{4}	16.2	4.03×10^{6}
2	5.0×10^{5}	27.6	
3	5.0×10^{6}	46.6	
4	5.0×10^{7}	93.8	
5	5.0×10^{8}	98.4	

Figure 16. Body weight of male *L. vannamei*

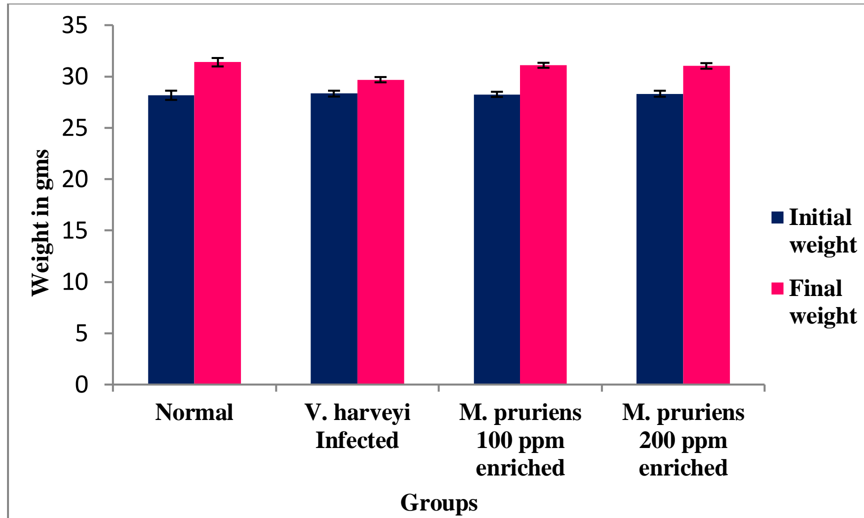


Figure17. Body weight of female *L. vannamei*

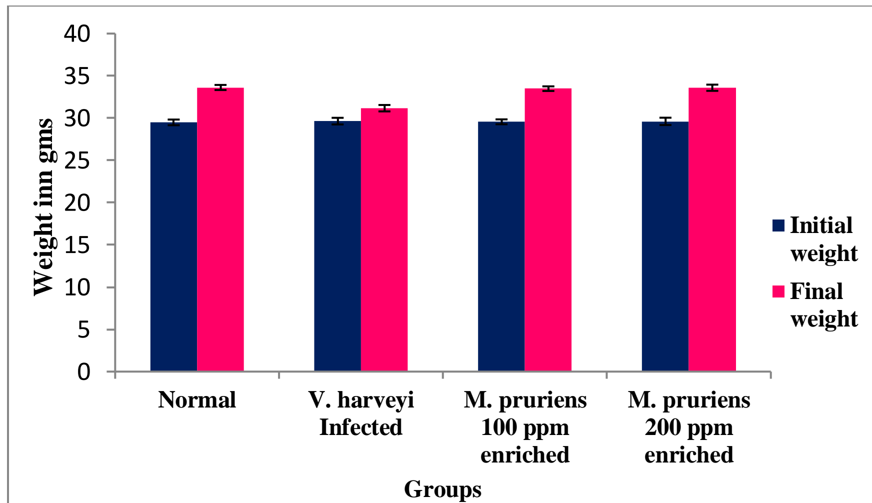


Figure 18. Body weight increase of male *L. vannamei*

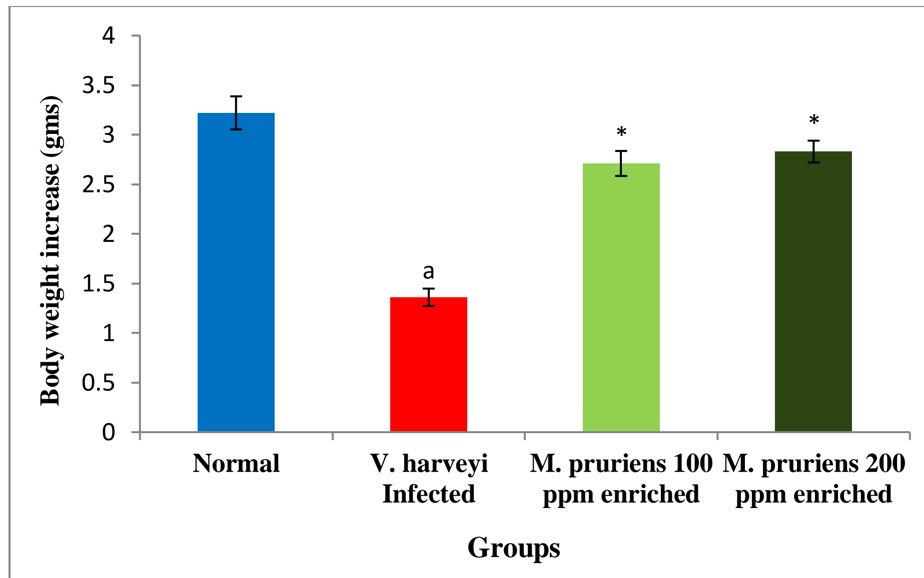
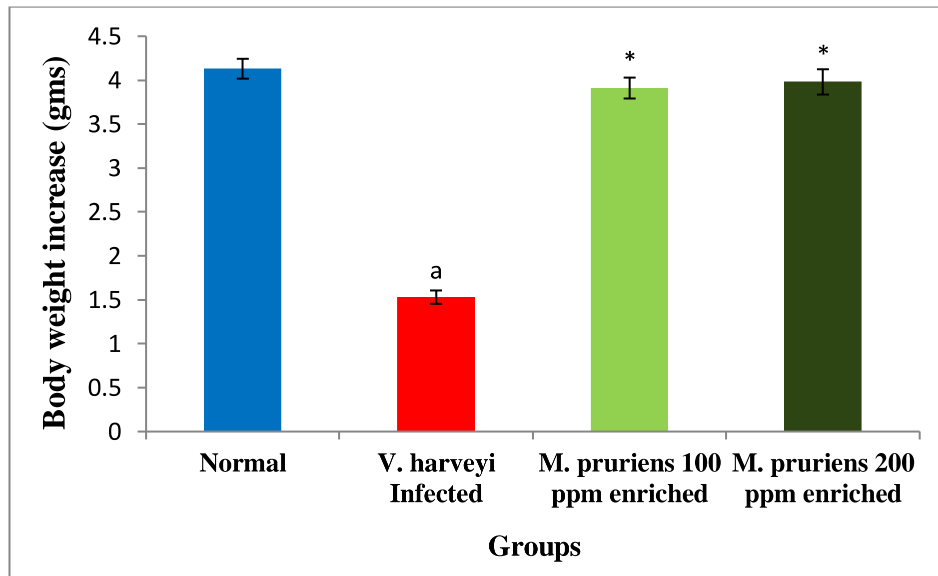


Figure 19. Body weight increase of female *L. vannamei*



a P > 0.05 Normal Vs Infected groups

*P > 0.05 Infected Vs *M. pruriens* fed groups

Figure 20. Specific growth rate of male *L. vannamei*

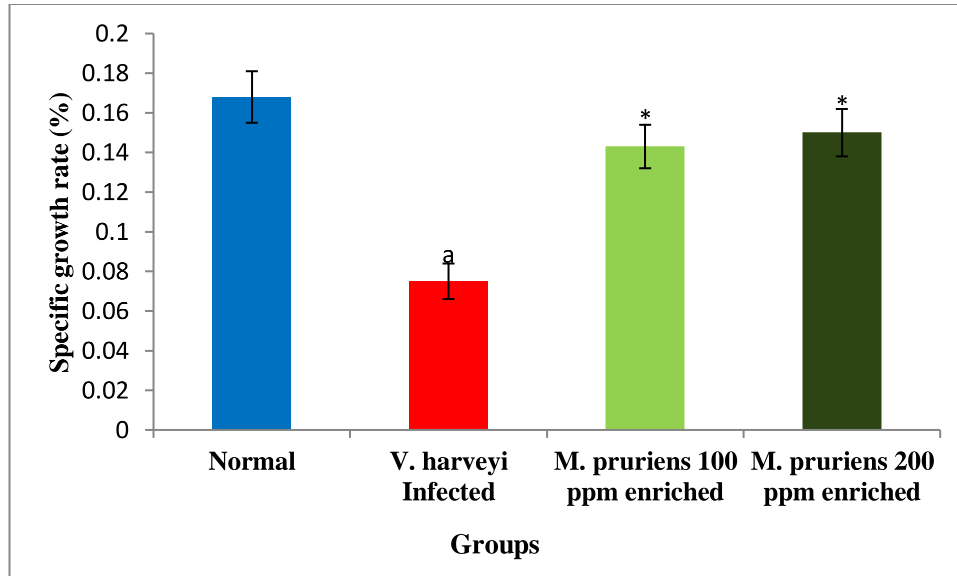
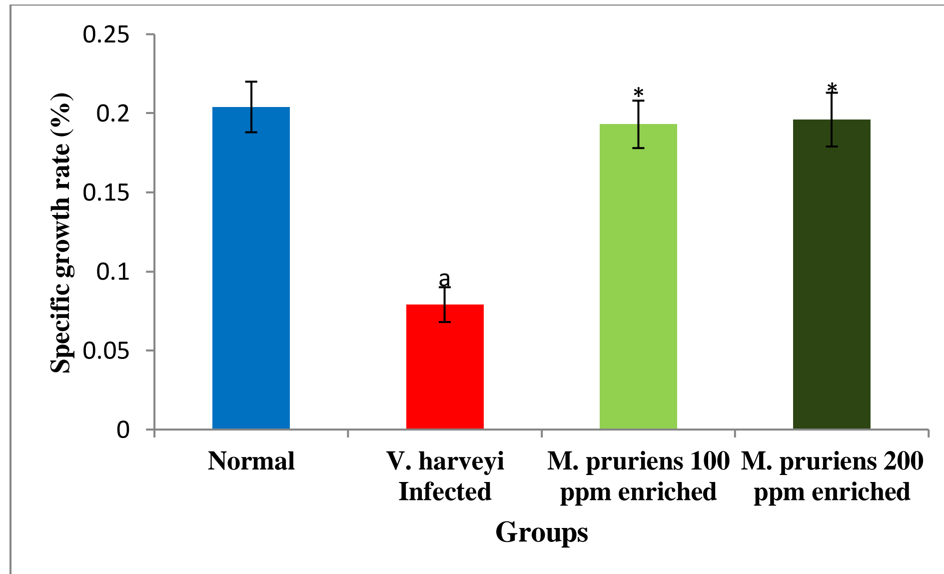


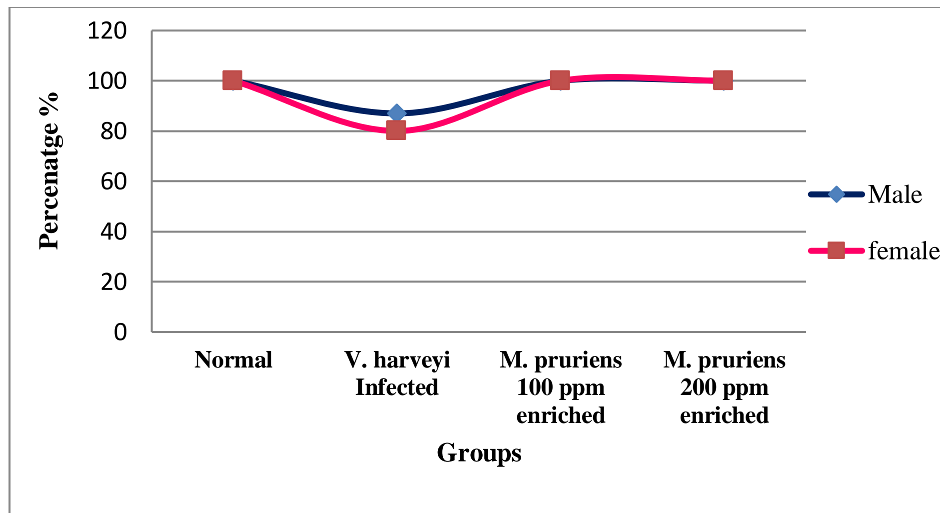
Figure 21. Specific growth rate of female *L. vannamei*



a P > 0.05 Normal Vs Infected groups

*P > 0.05 Infected Vs *M. pruriens* fed groups

Figure 22. Survival rate of male and female *L. vannamei*



5.10. Female fecundity assessment:

The female fecundity was assessed by gonadosomatic index (GSI), egg count and size as follows.

5.10.1. Female GSI:

Ovaries with GSI index values up to 5.1% was recorded in *M. pruriens* seed 100 ppm enriched Artemia fed group where as 5.2% of GSI index value was observed in 200 ppm. *M. pruriens* seed enriched Artemia fed group shows a significant match with GSI values of control group. *V. harveyi* infected female group showed a decreasing trend in its GSI value (Fig.23).

5.10.2. Egg count and size measurement:

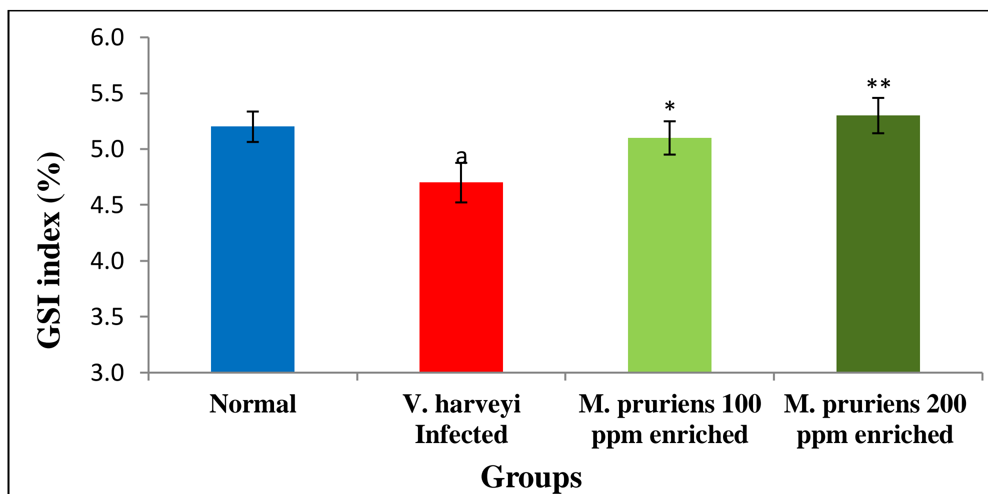
Vibrio harveyi infected groups exhibited profound decrease in egg count with reduction in egg size. The herbal extract of *M. pruriens* seed at 100 ppm and 200 ppm effectively returned the fecundity egg count and its size in fed group (Fig.24 & 25).

5.11. Male fecundity assessment:

The spermatophore quality data of males *L. vannamei* are summarized as follows. Spermatophore weight and sperm count were drastically lowered in *V. harveyi* infected groups. However, in all *M. pruriens* seed extract fed group, the means of spermatophore weight and sperm count were significantly increased at the end of the experimental period. Initially, none of the shrimp shows melanization or spermatophore absence effect in control and *M. pruriens* fed group (Fig.26 - 29). Highest percentages of melanization and spermatophore absence were observed in shrimp of *V. harveyi* infected groups. Above all the final survival rate of male *L. vannamei* was reduced by one fourth time in *V. harveyi* infected individuals and *M. pruriens* seed fed groups no mortality.

The results are promising enough that normal male and female *L.vannamei* shrimps shows usual pattern of reproductive index whereas the *V. harveyi* infected shrimps showed drastic fecundity decline. The herbal extract of *M. pruiens* fed groups shows returns of fecundity in both sexes of *L.vannamei*.

Figure 23. GSI index of female *L. vannamei*



a P > 0.05	Normal Vs Infected groups
*P > 0.05	Infected Vs *M. pruriens* fed groups
** P>0.01	Infected Vs *M. pruriens* fed groups

Figure 24. Egg count of female *L. vannamei*

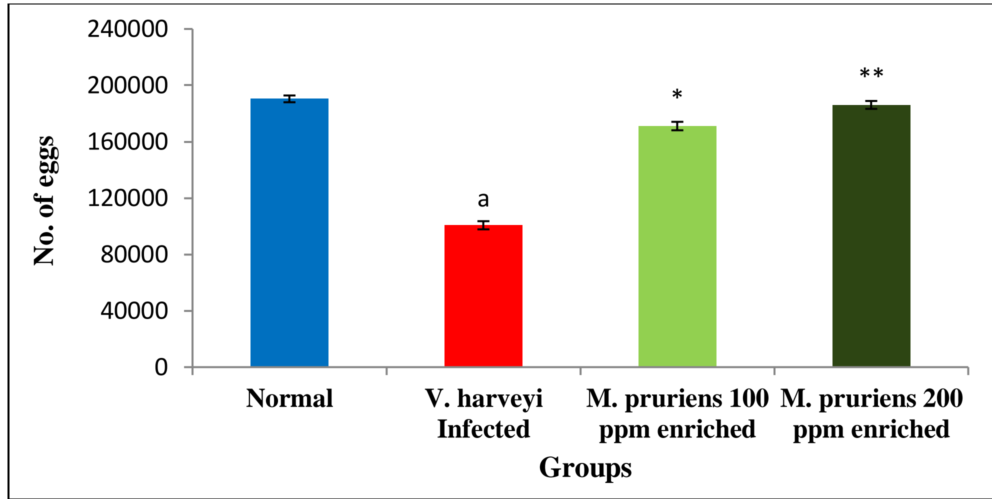
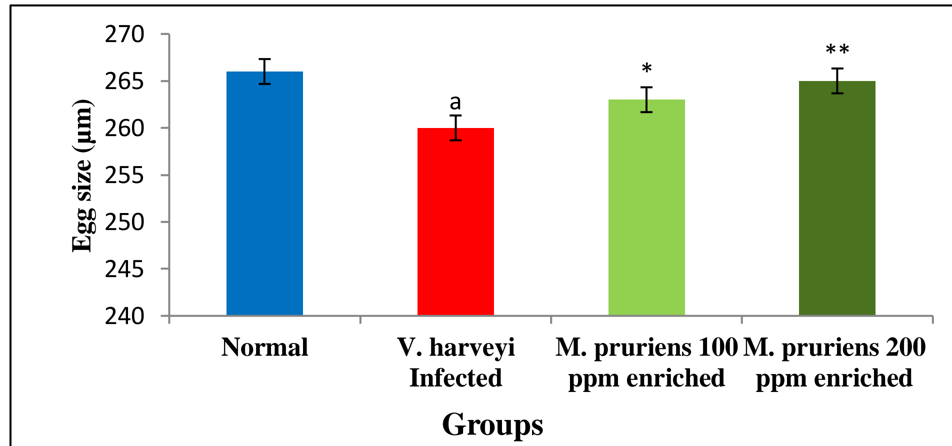


Figure 25. Egg diameter of female *L. vannamei*



a P > 0.05	Normal Vs Infected groups
*P > 0.05	Infected Vs *M. pruriens* fed groups
** P>0.01	Infected Vs *M. pruriens* fed groups

Figure 26. Spermatophore weight of male *L. vannamei*

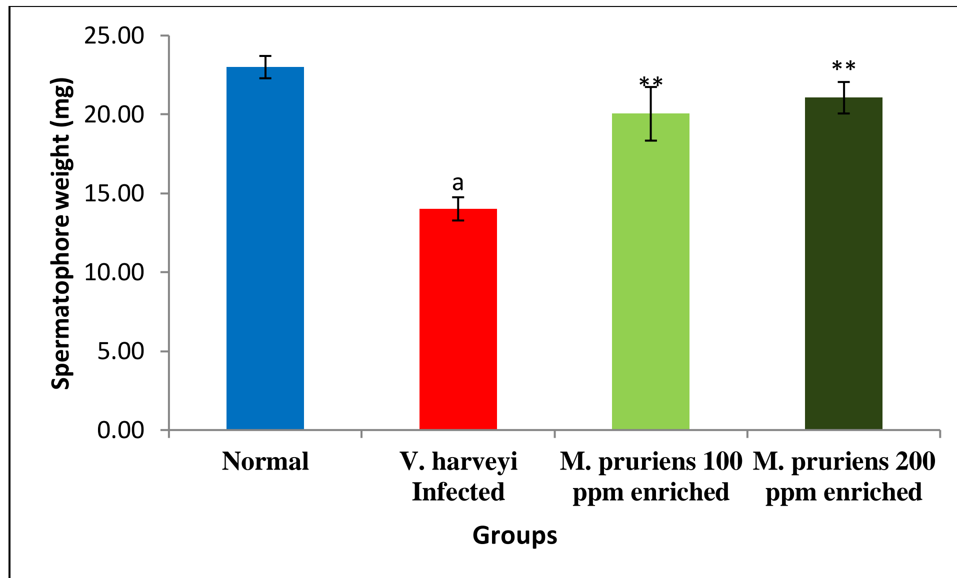
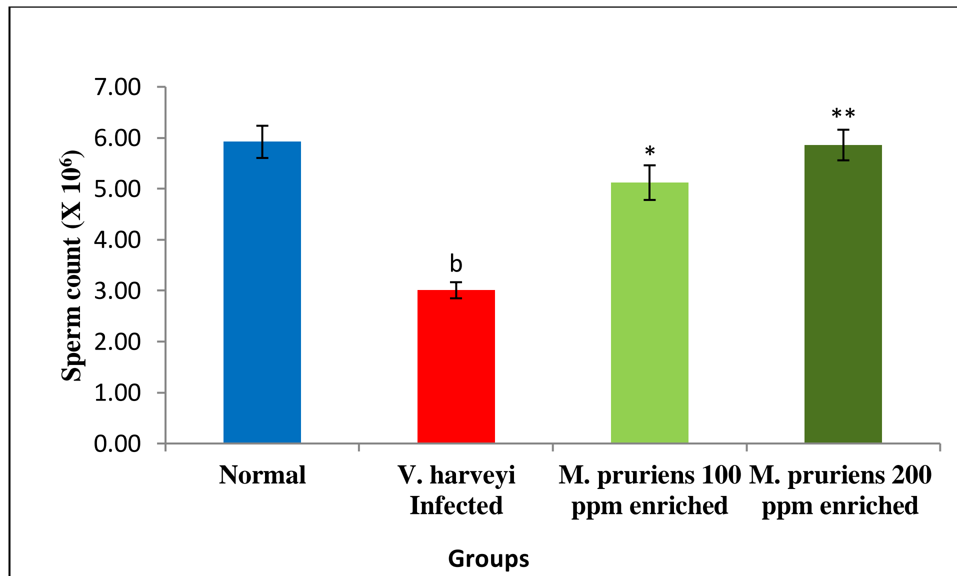


Figure 27. Sperm count of male *L. vannamei*



a $P > 0.05$	Normal Vs Infected groups
b $P > 0.05$	Normal Vs Infected groups
*$P > 0.05$	Infected Vs *M. pruriens* fed groups
** $P>0.01$	Infected Vs *M. pruriens* fed groups

Figure 28. Melanization of male *L. vannamei*

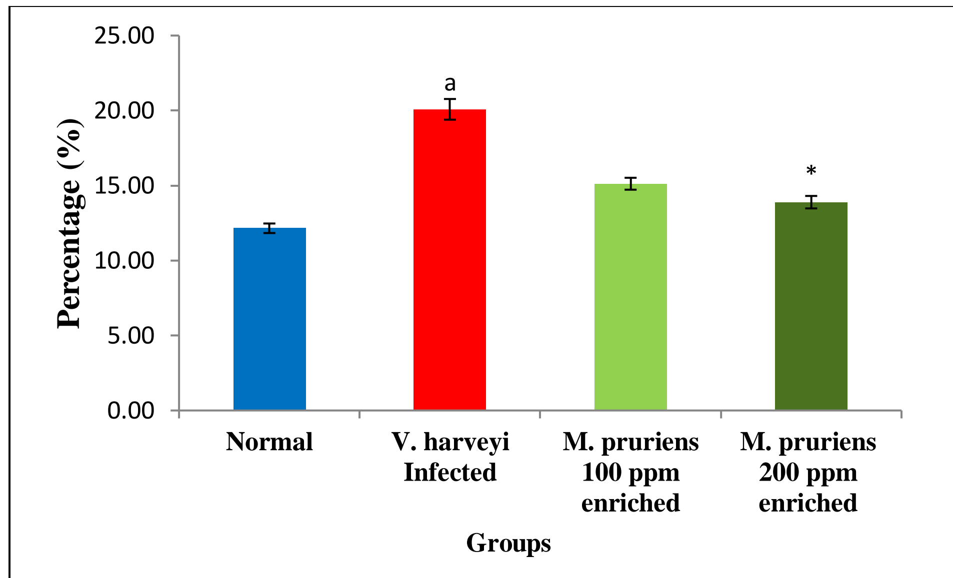
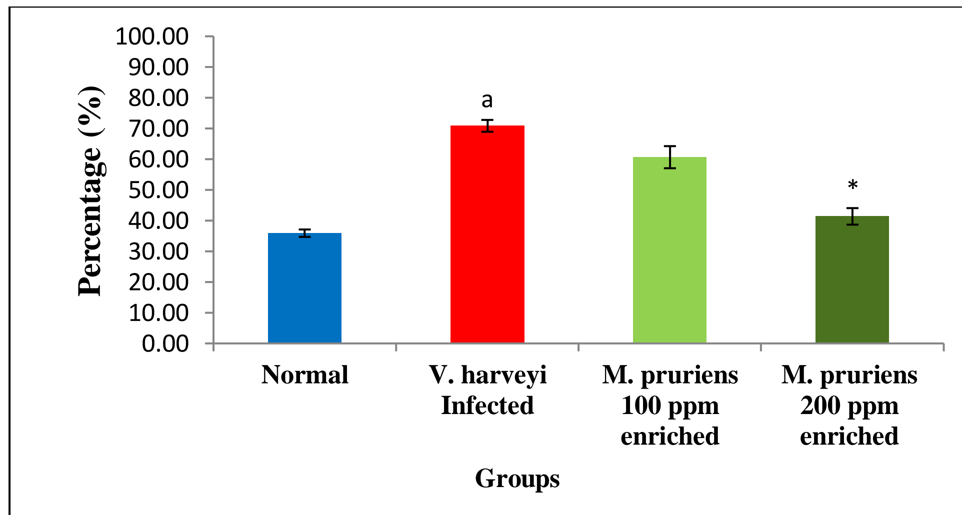


Figure 29. Spermatophore absence of male *L. vannamei*



a P > 0.05 Normal Vs Infected groups

*P > 0.05 Infected Vs *M. pruriens* fed groups

5.12. Evaluation of Immune parameters

The immunity of the shrimp was also evaluated by examine Total hemocyte count (THC), Phenoloxidase (PO), Superoxide dismutase (SOD) and Respiratory burst (RB) activity of haemolymph in male and female *L. vannamei*.

5.12.1. Total hemocyte count (THC)

The THC of control diet fed *L. vannamei* of both male and female was 13.40 ± 0.330 x 10^6 cell/ml and 13.01 ± 0.341 x 10^6 cell/ml (Fig.30 & 31). Artemia enriched with *M. pruriens* seed extract at 100 ppm and 200 ppm fed male shrimp shows THC level was 13.23 ± 0.493 x 10^6 cell/ml and 13.62 ± 0.316 x 10^6 cell/ml respectively. THC of female shrimp fed with experimental diet was 12.89 ± 0.472 x 10^6 cell/ml and 13.21 ± 0.328 x 10^6 cell/ml. The THC of *V. harveyi* infected male and female was 11.20 ± 0.458 x 10^6 cell/ml and 11.05 ± 0.435 x 10^6 cell/ml, which is significantly lower than control and *M. pruriens* fed groups. At the end of experiment *M. pruriens* enriched Artemia fed groups of both sexes shows significant increase in THC whereas infected groups shows lesser THC than normal control.

5.12.2. Phenoloxidase activity (PO)

The trend noticed for the phenoloxidase activity of control, infected and experimental diet fed male and female *L. vannamei* are shown in Fig. 32 & 33. Male shrimps fed on *M. pruriens* seed extract enriched Artemia at 100 ppm and 200 ppm diet displayed the PO activities of 0.114 ± 0.008 OD and 0.125 ± 0.006 OD, respectively and in control group, the value recorded was 0.124 ± 0.007 OD. However, infected shrimps displayed the minimum PO activity of 0.086 ± 0.004 OD. The experimental diet fed to female *L. vannamei* with PO activities of 0.123 ± 0.007 OD (100 ppm) and 0.139 ± 0.004 OD (200 ppm) which relatively matched with normal control group value of 0.135 ± 0.006 OD. Interestingly, the *V. harveyi* infected shrimp has low PO activity of 0.090 ± 0.003 OD. The results inferrred that the PO activity was high in MP 200 pm enriched Artemia groups when compared to infected shrimps of *L. vannamei*.

5.12.3. Respiratory burst (RB)

Respiratory burst activity in the haemolymph of control, infected and experimental diets fed male and female *L. vannamei* are shown in Fig. 34 & 35. Male shrimps fed on normal diet displayed RB activity of 0.063 ± 0.002 OD. The high RB activity of 0.064 ± 0.002 OD was registered by *M. pruriens* seed extract 200 ppm enriched Artemia fed diet, Whereas, in the *V. harveyi* infected shrimp has lesser RB activity of 0.046 ± 0.002 OD. In female shrimps fed with experimental diet displayed high RB activity of 0.074 ± 0.003 OD in *M. pruriens* seed extract at 200 ppm enriched Artemia fed groups when compared to *V. harveyi* infected shrimp 0.052 ± 0.002 OD. The results indicate that RB activities of 200 ppm MP enriched group were significantly higher than that of control shrimp. RB activity decreased significantly in *V. harveyi* infected shrimp of both sexes.

5.12.4. Superoxide dismutase (SOD)

SOD activity in the haemolymph of control, infected and experimental diets fed male and female *L. vannamei* are shown in Fig. 36 & 37. Shrimps fed on normal diet displayed SOD activity of 0.461 ± 0.011 OD in male and 0.445 ± 0.013 OD in female. SOD activity in *M. pruriens* 100 ppm and 200 ppm enriched diet fed male and female *L. vannamei* shows 0.419 ± 0.026; 0.485 ± 0.016 and 0.407 ± 0.028; 0.491 ± 0.012 respectively. SOD activity decreased significantly in infected shrimp has 0.282 ± 0.008 in male and 0.263 ± 0.010 in female. The SOD level of 200 ppm *M. pruriens* enriched fed group was 1.7 fold higher than the infected group, whereas in 100 ppm fed groups reaches to normal control level. *M. pruriens* fed groups shows significant increase in immune parameter whereas infected groups shows lesser than normal control.

Figure 30. Total Haemocyte Count of male *L. vannamei*

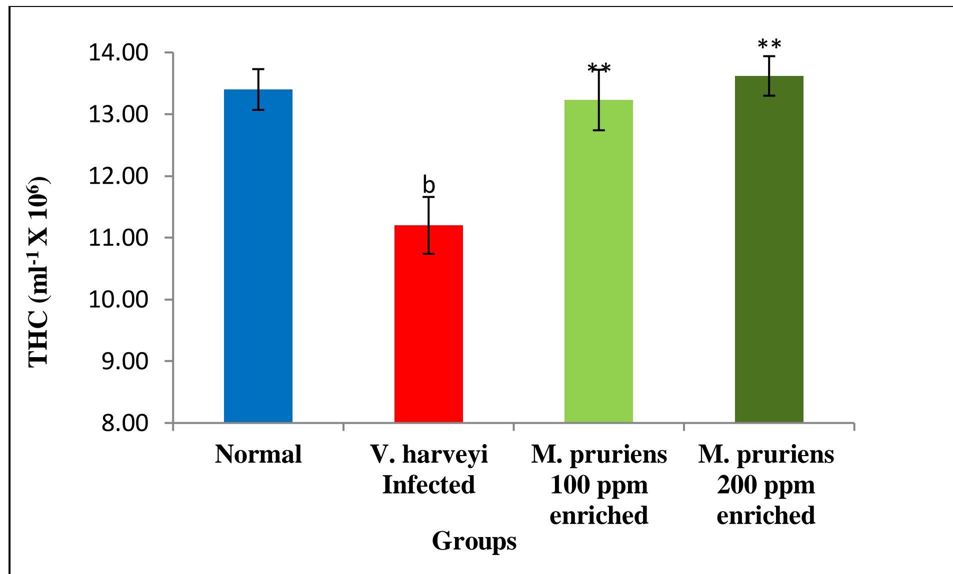
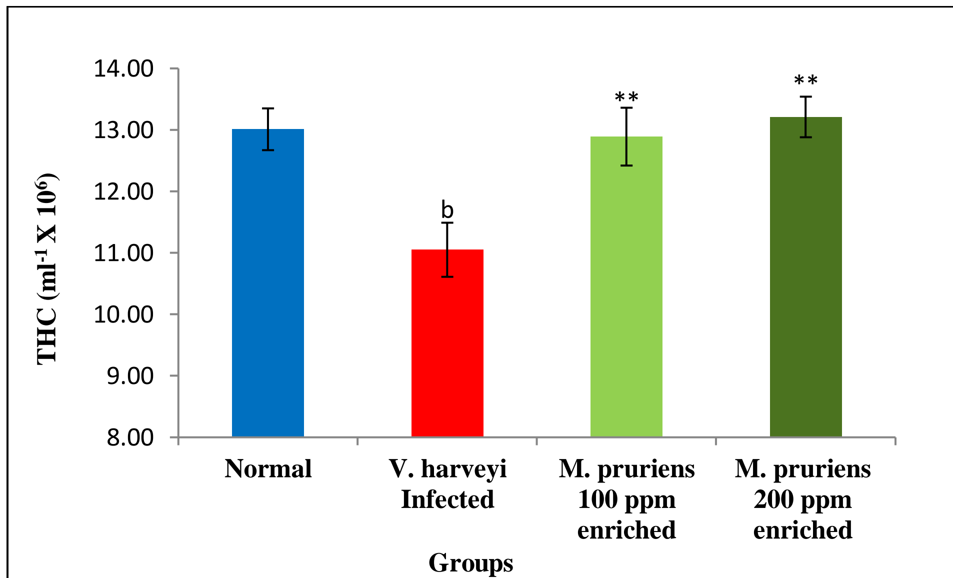


Figure 31. Total Haemocyte Count of female *L. vannamei*



b P > 0.01	Normal Vs Infected groups
** P>0.01	Infected Vs *M. pruriens* fed groups

Figure 32. Phenoloxidase activity of male *L. vannamei*

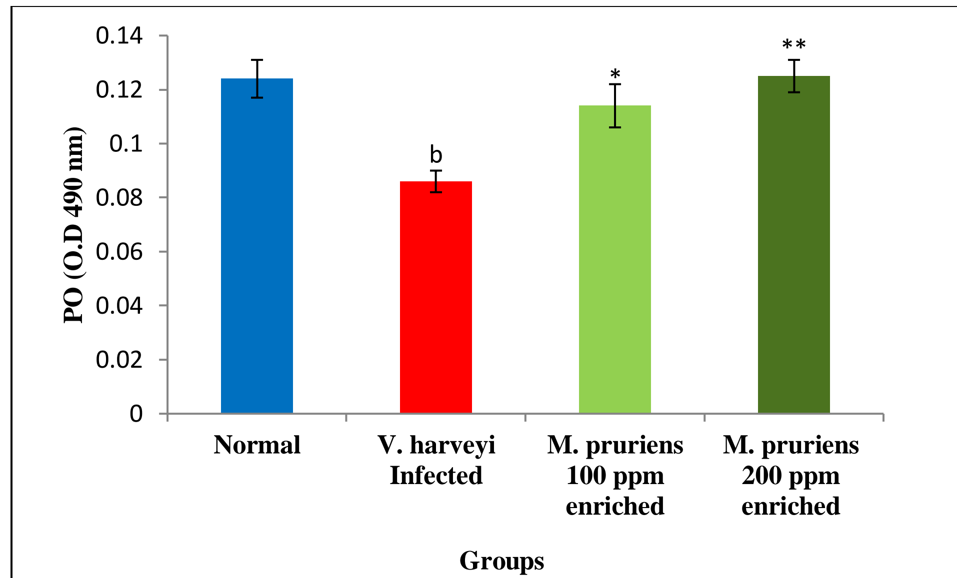
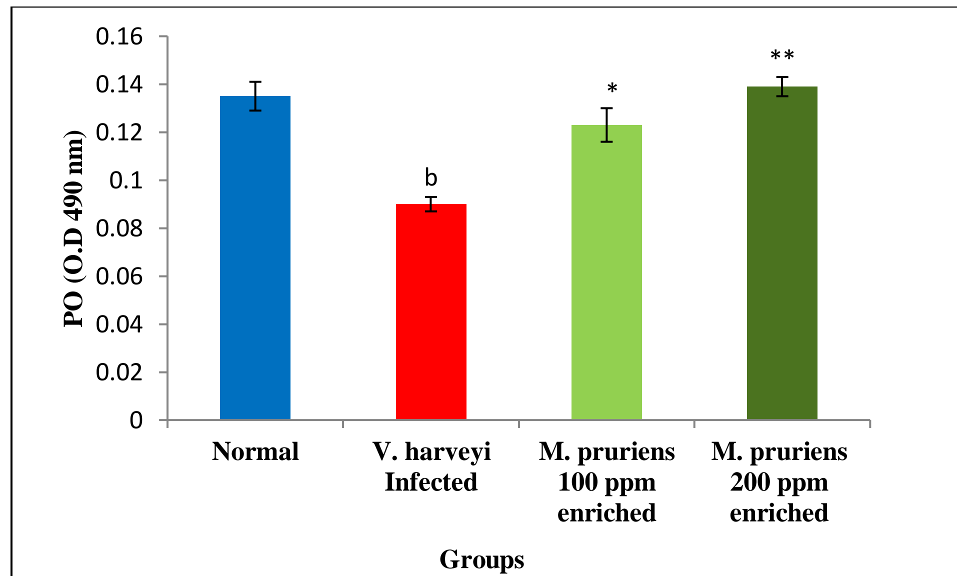


Figure 33. Phenoloxidase activity of female *L. vannamei*



b P > 0.01	Normal Vs Infected groups
* P>0.05	Infected Vs *M. pruriens* fed groups
** P>0.01	Infected Vs *M. pruriens* fed groups

Figure 34. Respiratory burst activity of male *L. vannamei*

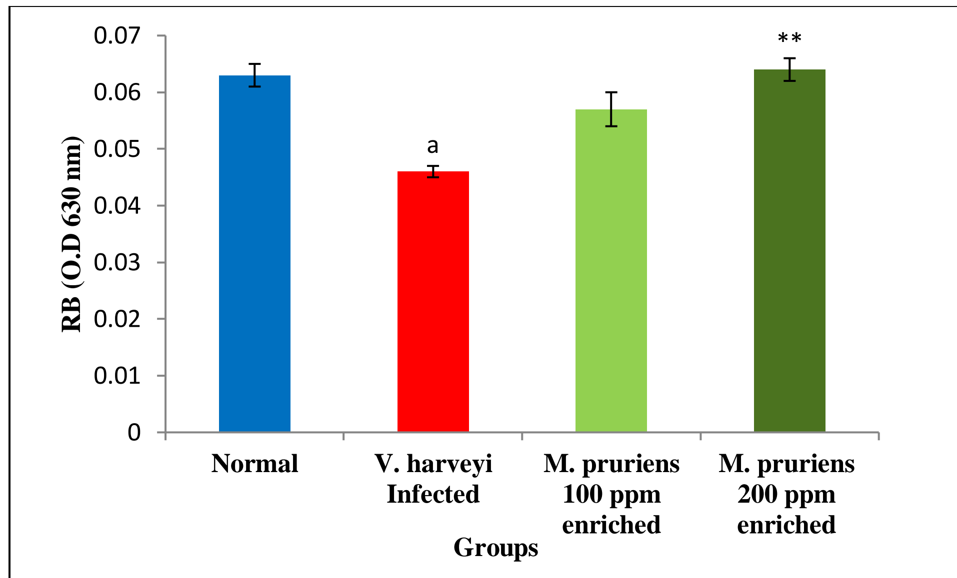
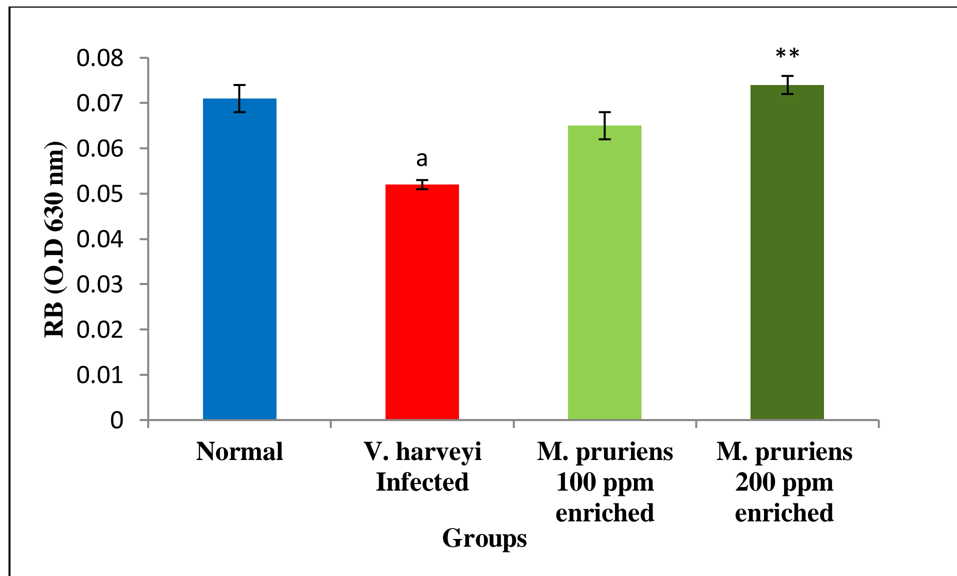


Figure 35. Respiratory burst activity of female *L. vannamei*



a P > 0.05 Normal Vs Infected groups

** P>0.01 Infected Vs *M. pruriens* fed groups

Figure 36. SOD activity of male *L. vannamei* (haemolymph)

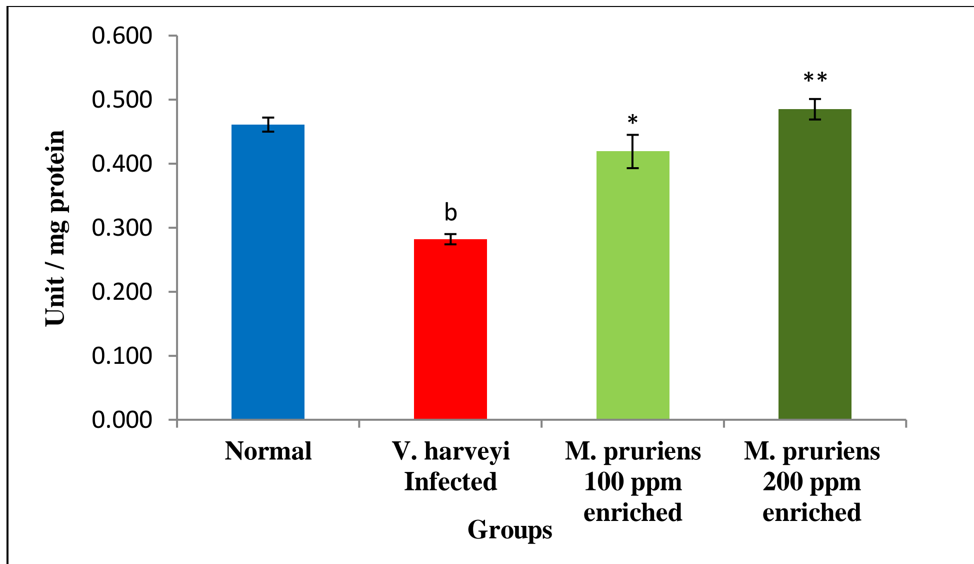
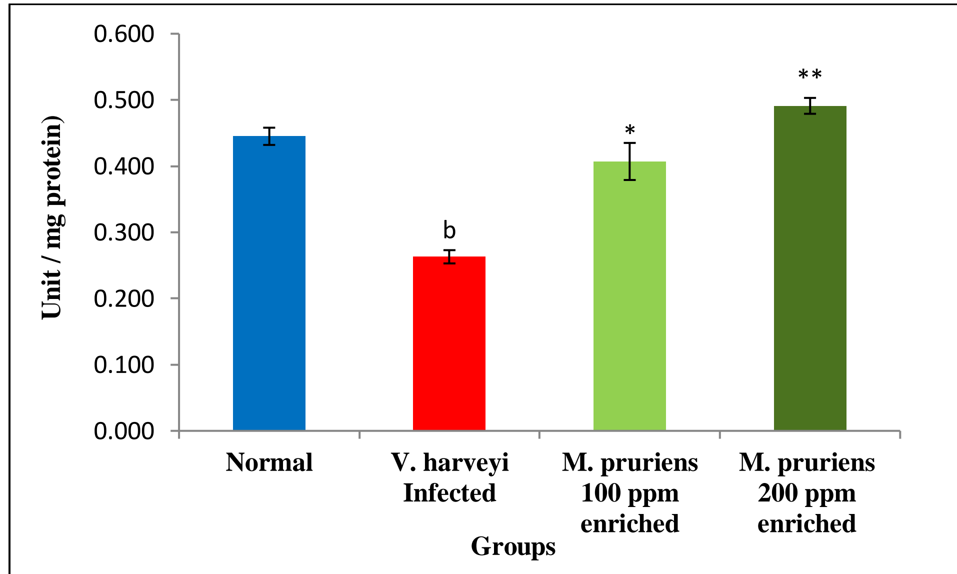


Figure 37. SOD activity of female *L. vannamei* (haemolymph)



b P > 0.01	Normal Vs Infected groups
* P>0.05	Infected Vs *M. pruriens* fed groups
** P>0.01	Infected Vs *M. pruriens* fed groups

5.13. Validation of antioxidants enzyme

Shrimps muscle tissues of each group are utilized for the estimation of antioxidant enzyme in male and female *L. vannamei*.

5.13.1. Superoxide dismutase level

The SOD level of normal male and female shrimps was (8.49 ± 0.212 U/ml) and (5.59 ± 0.325 U/ml) and there was decrease in SOD level in *V. harveyi* infected shrimps in the range of (4.5 ± 0.317 U/ml) and (2.19 ± 0.205 U/ml) respectively (Fig. 38 & 39). *M. pruriens* fed group recovered the enzyme to a significant state.

5.13.2. Catalase level

CAT enzyme level was totally low (0.52 ± 0.032 U/ml) and (0.32 ± 0.034 U/ml) in infected shrimps when compared to normal control which is observed as (1.01 ± 0.067 U/ml) and (0.71 ± 0.037 U/ml) (Fig. 40 & 41). CAT enzyme level reaches normal in *M. pruriens* fed group.

5.13.3. Glutathione peroxidase level

GPx level of normal male and female was (135.09 ± 6.03 U/ml) and (113.39 ± 4.94 U/ml) and which is low in infected individuals in the range of 98.49± 5.75 U/ml, 70.79± 4.66 U/ml (Fig. 42 & 43). *M. pruriens* fed group exerted recovery level of GPx.

5.13.4. Reduced glutathione level

GSH level in infected shrimps was (32.50 ± 1.90 U/ml) and (26.49 ± 2.02 U/ml) which considerably lower than that of normal individuals as (56.83 ± 4.39 U/ml) and (42.49 ± 2.28 U/ml). *M. pruriens* fed group adjusted the GSH level to normal (Fig. 44 & 45).

Significantly ($P < 0.05$) increased SOD, CAT, GPx and GSH activities were observed in shrimp fed with MP enriched Artemia diets compared to *V.harveyi* infected. Higher level of antioxidant status indicates that shrimps acquired resistance to *Vibrio harveyi* infection.

Figure 38. SOD activity of male *L. vannamei* (muscle)

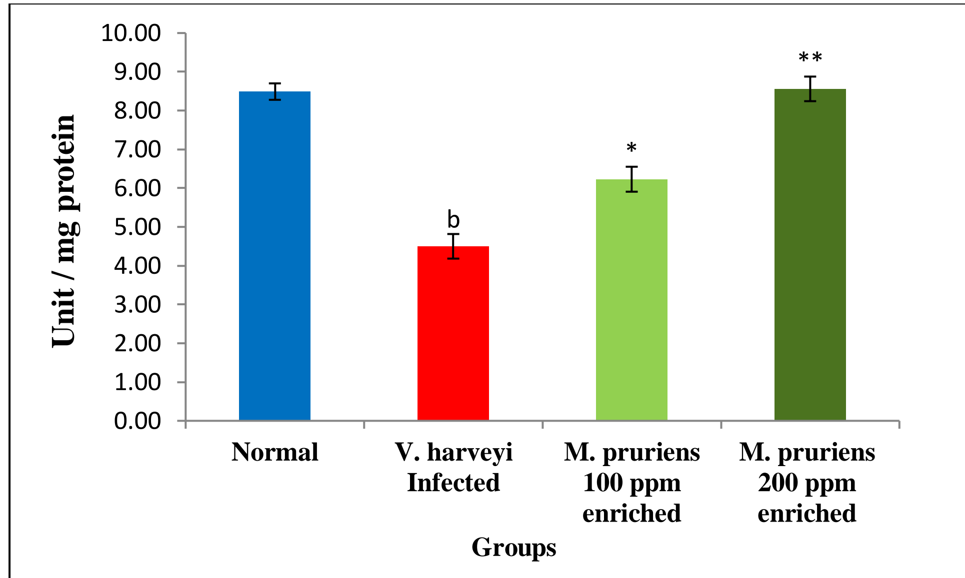
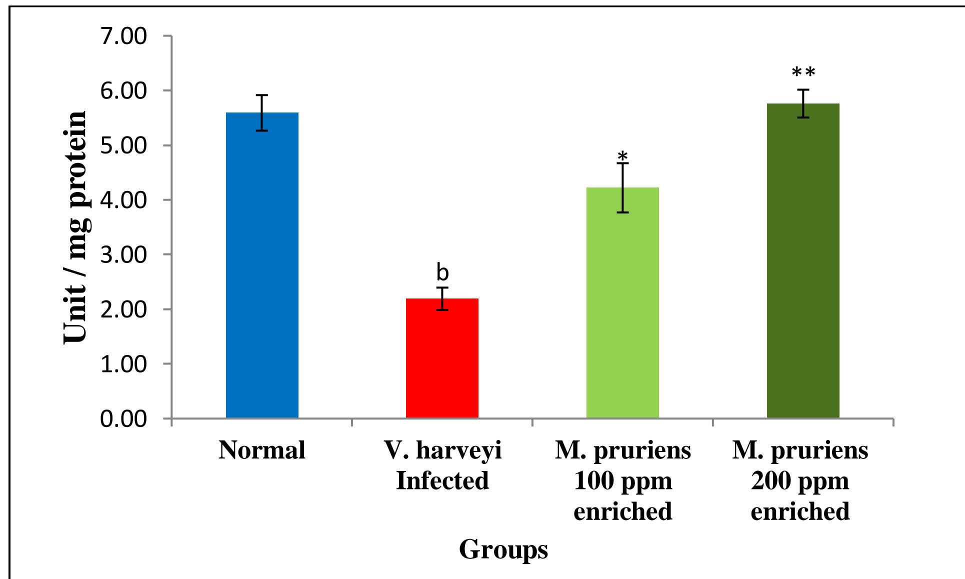


Figure 39. SOD activity of female *L. vannamei* (muscle)



b P > 0.01 Normal Vs Infected groups

* P>0.05 Infected Vs *M. pruriens* fed groups

** P>0.01 Infected Vs *M. pruriens* fed groups

Figure 40. Catalase activity of male *L. vannamei*

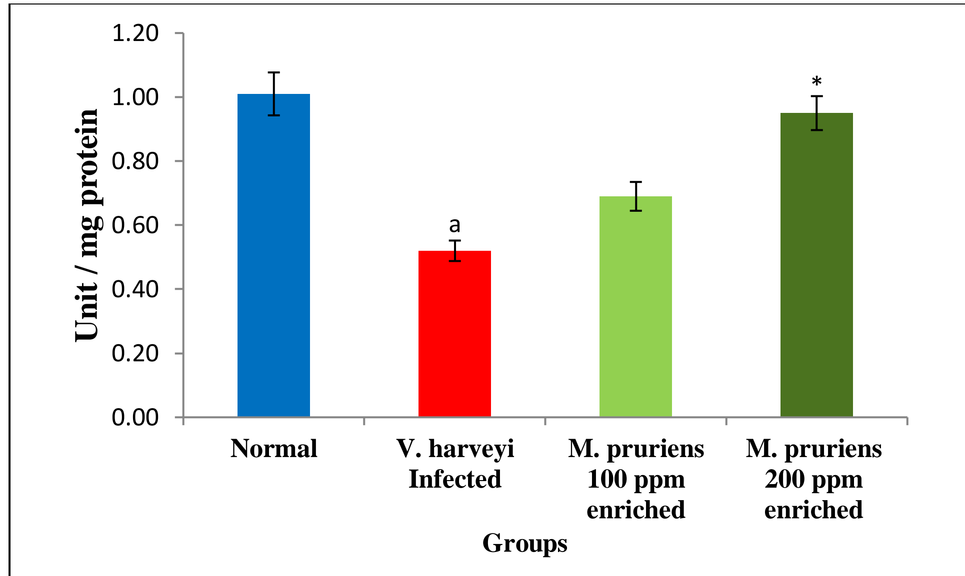
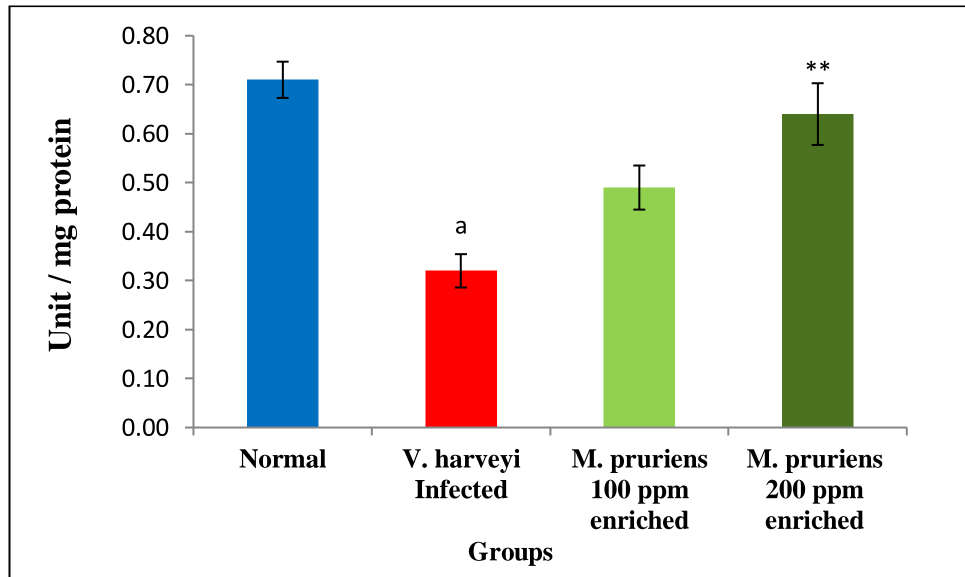


Figure 41. Catalase activity of female *L. vannamei*



a P > 0.05	Normal Vs Infected groups
* P>0.05	Infected Vs *M. pruriens* fed groups
** P>0.01	Infected Vs *M. pruriens* fed groups

Figure 42. GPx activity of male *L. vannamei*

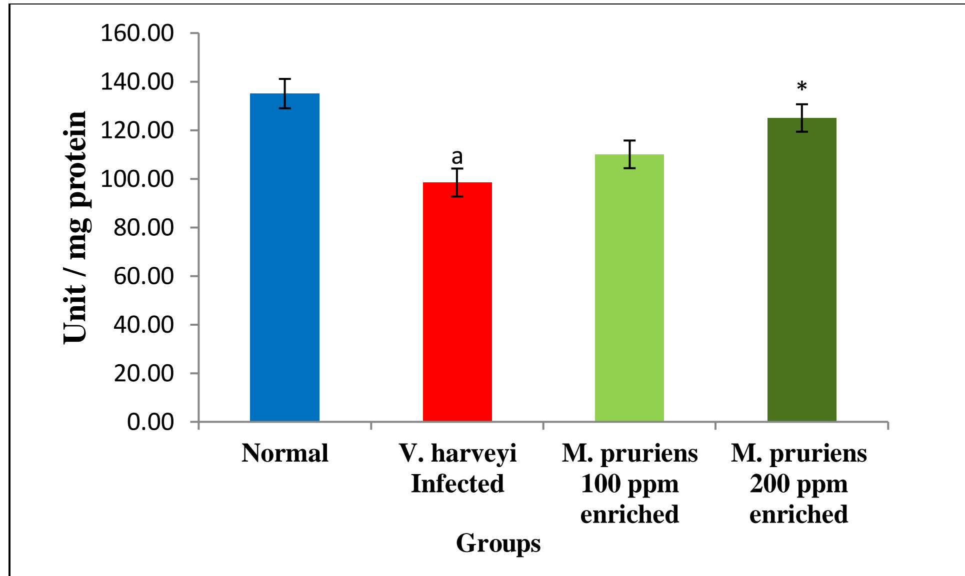
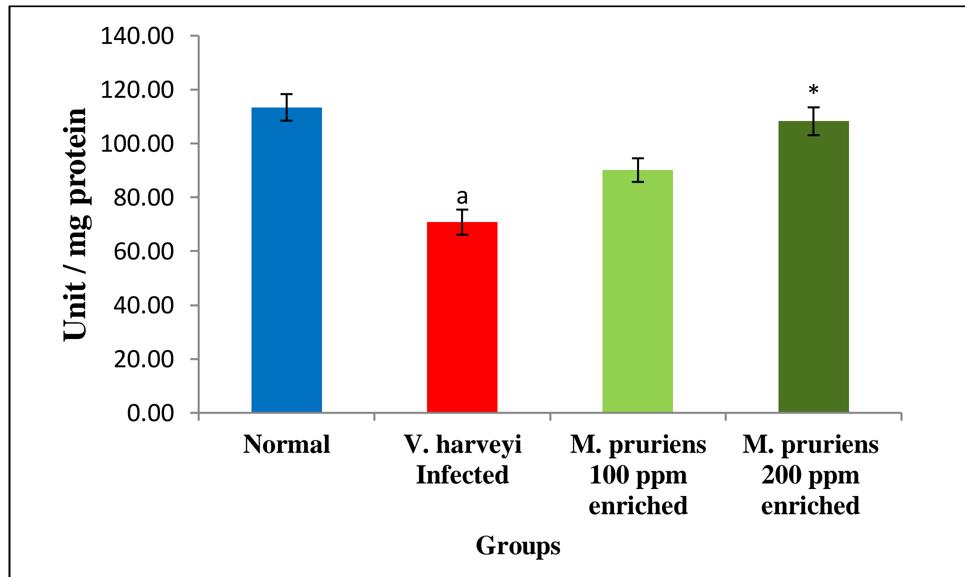


Figure 43. GPx activity of female *L. vannamei*



a $P > 0.05$ Normal Vs Infected groups

* $P>0.05$ Infected Vs *M. pruriens* fed groups

Figure 44. GSH activity of male *L. vannamei*

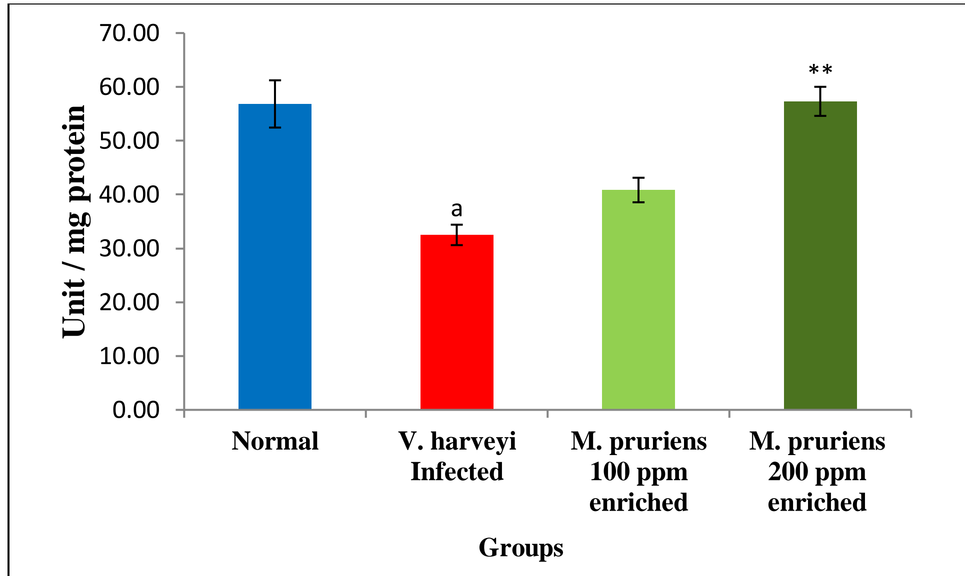
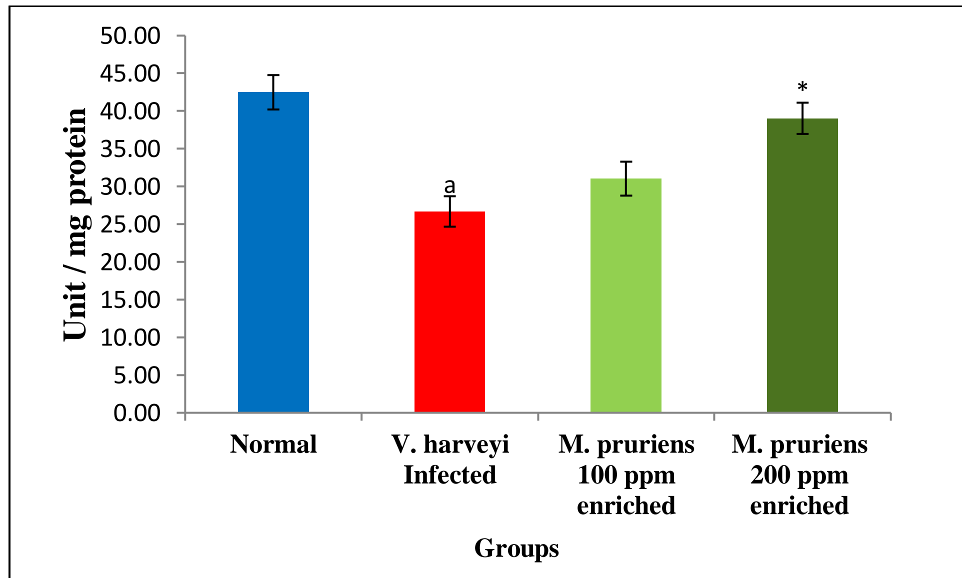


Figure 45. GSH activity of female *L. vannamei*



a P > 0.01	Normal Vs Infected groups
* P>0.05	Infected Vs *M. pruriens* fed groups
** P>0.01	Infected Vs *M. pruriens* fed groups

5.14. Analysis of proximate composition

The proximate composition of moisture, carbohydrate, protein and lipid content in male and female *L. vannamei* muscle tissue were estimated.

5.14.1. Protein content

At the end of the experiment, protein content of normal *L. vannamei* is 21.60% in male and 22.24% in female. Animal protein storage was moderately affected by bacterial infection (*V. harveyi*) where the protein percentage lies of 20.95% in male and 21.43% in female (Fig. 46 & 47).

5.14.2. Carbohydrate content

Carbohydrate percentage is also show similar alteration like protein in *V. harveyi* infected shrimp groups in the range of 3.96% in male and 4.12% in female (Fig.48 & 49).

5.14.3. Lipid content

Lipid content of infected *L. vannamei* slightly varies (2.03% in male and 2.94% in female) compared to normal and experimental groups (Fig. 50 & 51).

5.14.4. Moisture content

Moisture content in the infected shrimp was remain same in all groups (Fig.52 & 53)

The results of the present study revealed that the nutritive content of the shrimp was partially altered by the *V. harveyi* infection.

Figure 46. Total protein of male *L. vannamei*

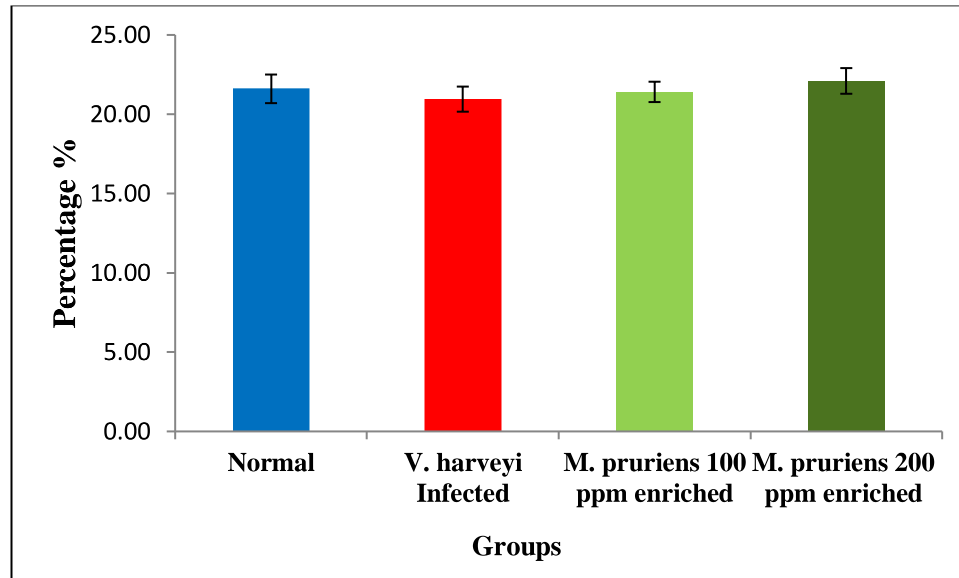


Figure 47. Total protein of female *L. vannamei*

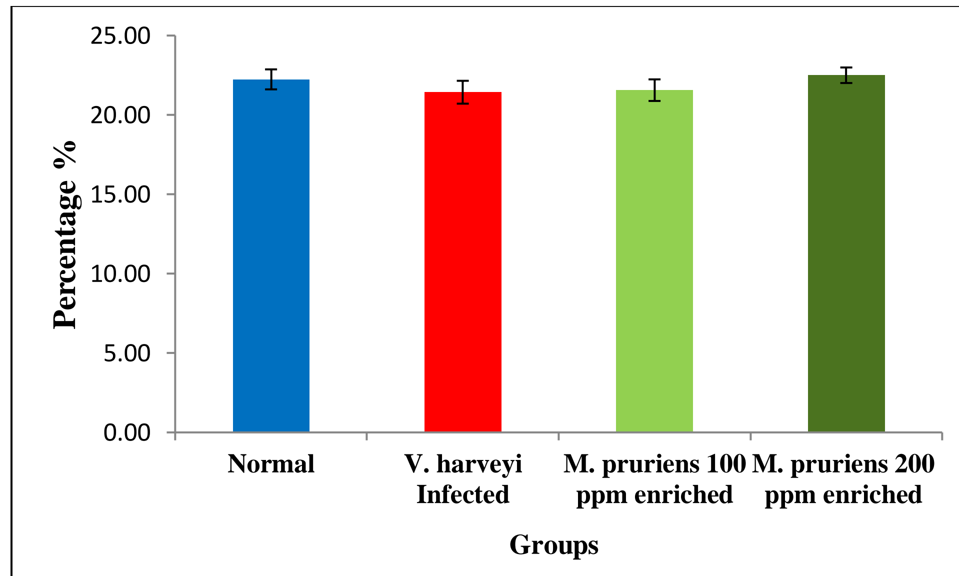


Figure 48. Carbohydrate of male *L. vannamei*

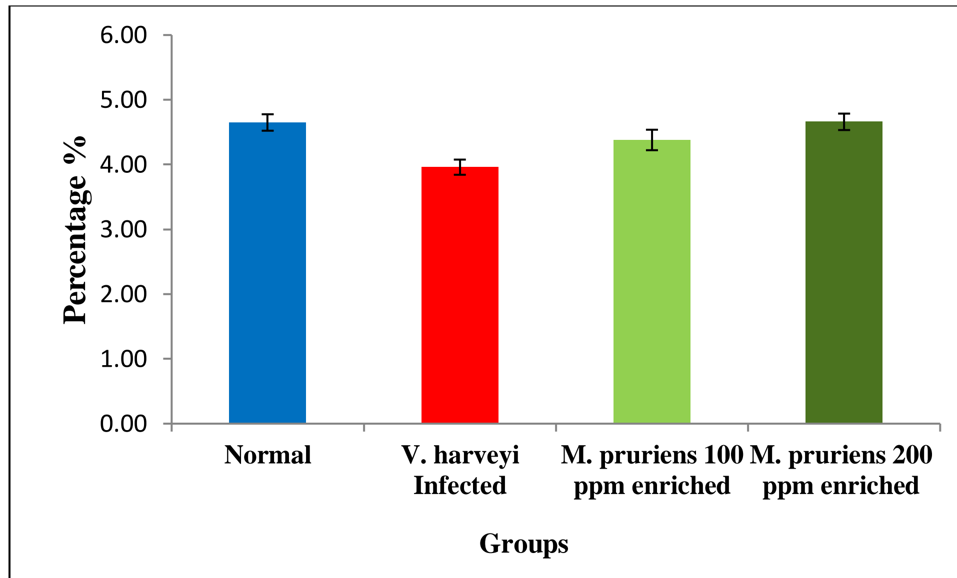


Figure 49. Carbohydrate of female *L. vannamei*

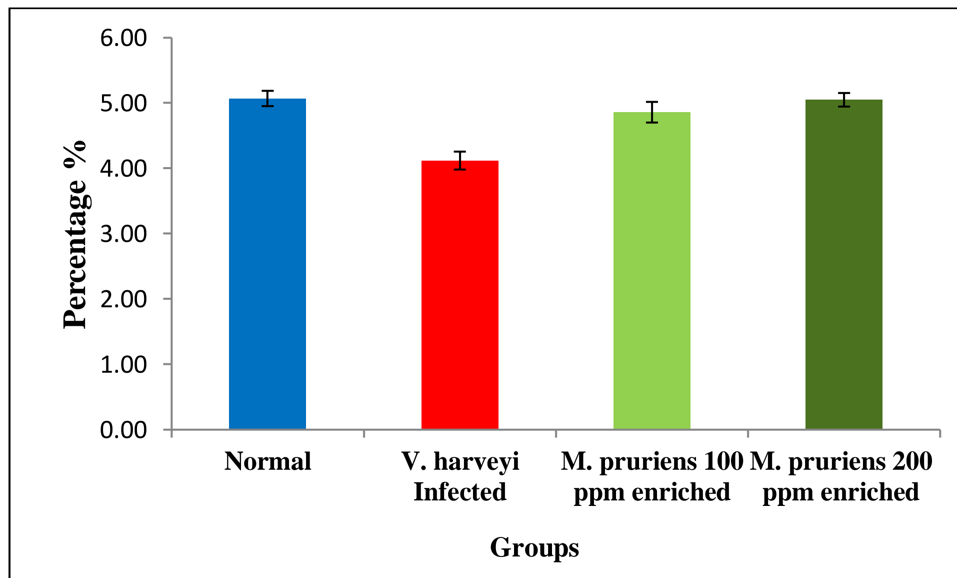


Figure 50. Total lipid of male *L. vannamei*

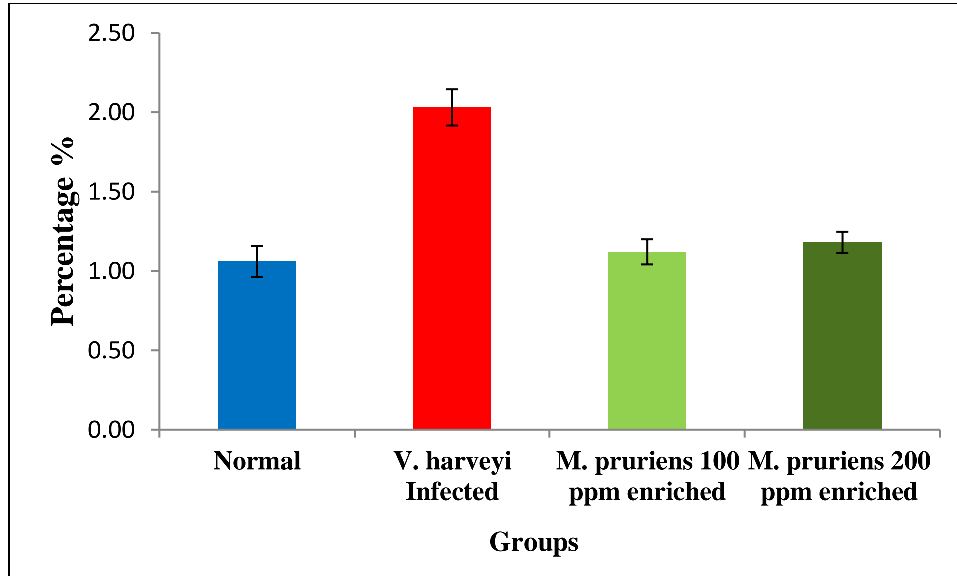


Figure 51. Total lipid of female *L. vannamei*

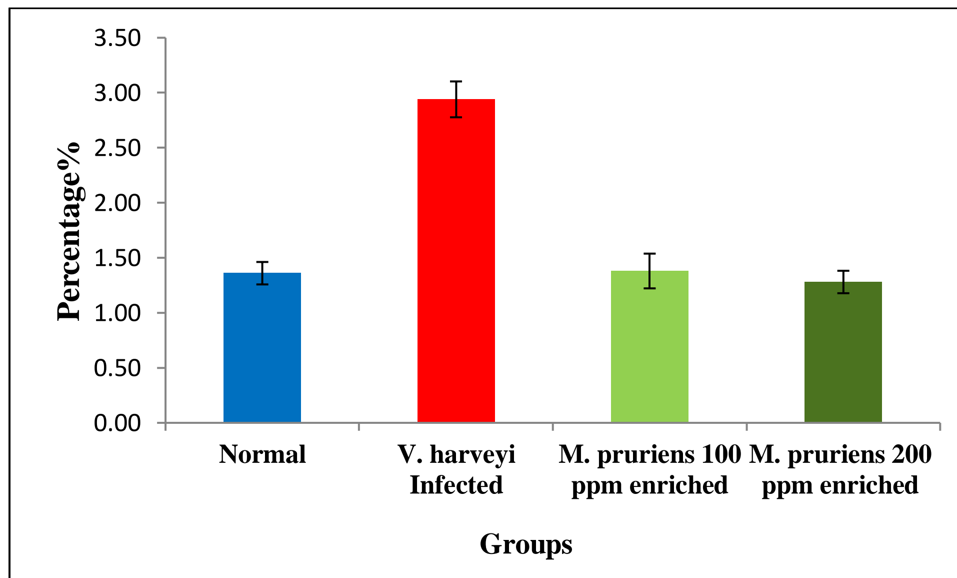


Figure 52. Total moisture of male *L. vannamei*

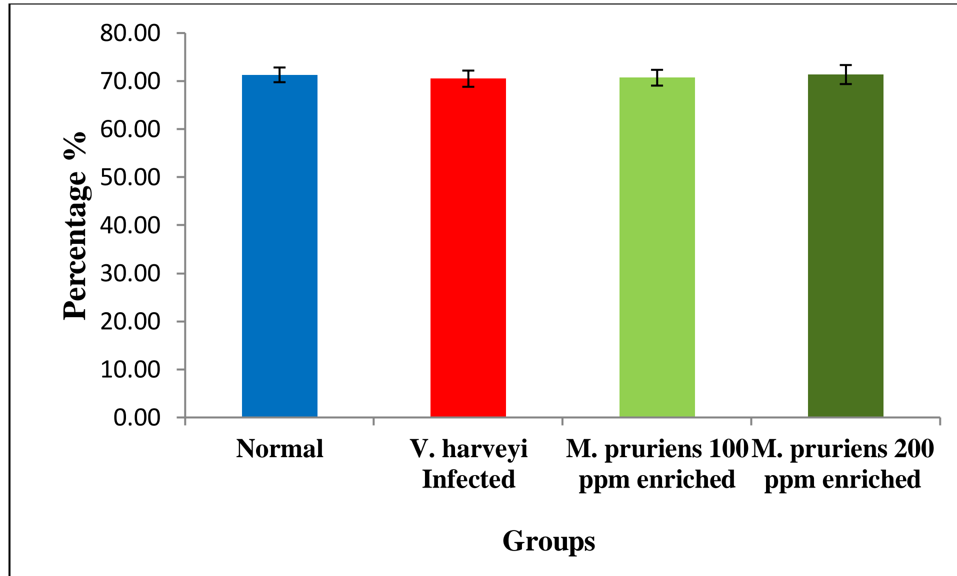
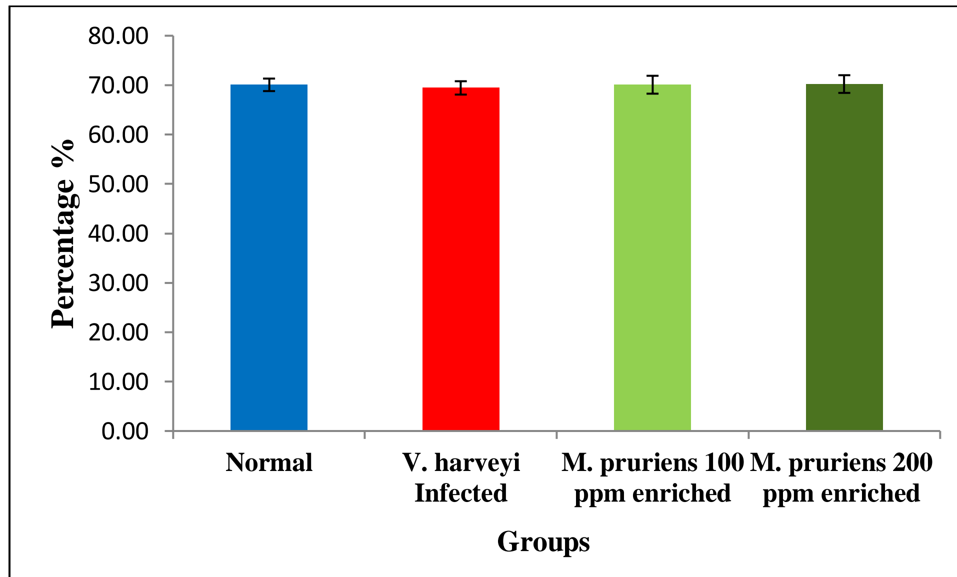


Figure 53. Total moisture of female *L. vannamei*



5.15. Histology

The histopathological examination showed hepatopancreas tissues of control shrimps group had intact and healthy hepatopancreatic tubules. The *V. harveyi* infected shrimp had severe necrosis in hepatopancreatic tubules of both male and female. Artemia enriched with *M. pruriens* 100 ppm diet fed groups show partial recovery in hepatopancreatic tubules, whereas 200 ppm enriched diet fed groups returns to healthy hepatopancreatic tubules (Plate I - VIII). In histopathology sections, gills showed healthy gill lamellae in normal control and experimentally diet fed group but some hemocytic infiltration was observed in *V. harveyi* infected shrimp (Plate IX - XVI). The result of histological study show demorphies in their historchitecture due to infection, where as *M. pruriens* fed group restores to normal structure.

PLATE I

Hepatopancreas – Section from normal male

Asterisk indicates health hepatopancreatic tubules

PLATE II

Hepatopancreas – Section from infected male

Black arrow indicates loss of regular structure
Necrosis of hepatopancreatic tubules (nh)

PLATE III

Hepatopancreas – Section from MP 100 ppm enriched male

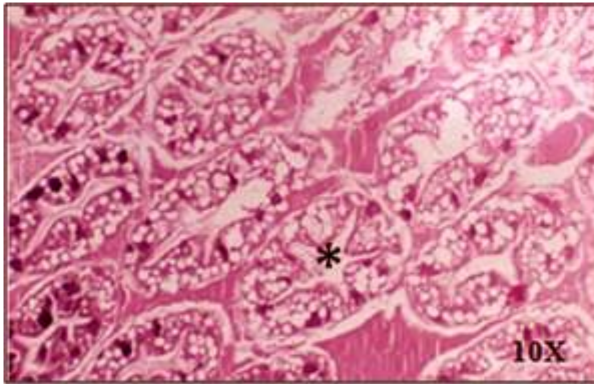
Normal hepatopancreatic tubules with some pigmentation (p)

PLATE IV

Hepatopancreas – Section from MP 200 ppm enriched male

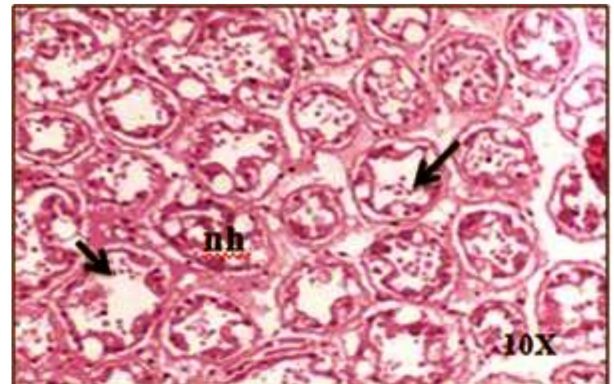
Asterisk indicates health hepatopancreatic tubules

PLATE I



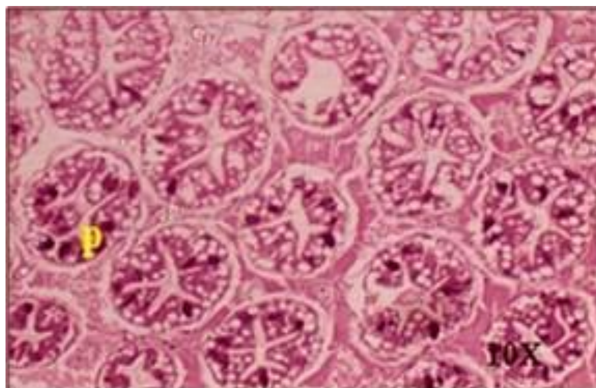
Magnification X10
Stained with Haematoxylin and Eosin

PLATE II



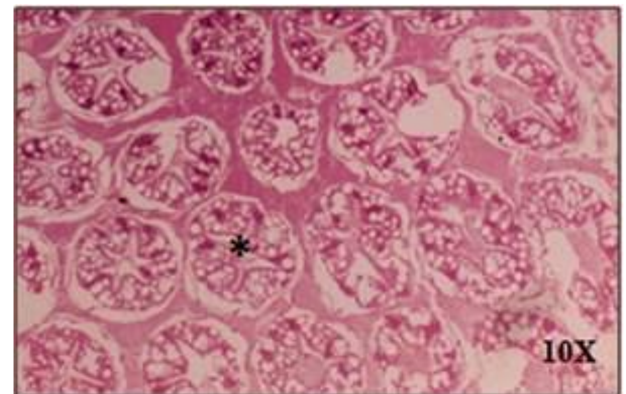
Magnification X10
Stained with Haematoxylin and Eosin

PLATE III



Magnification X10
Stained with Haematoxylin and Eosin

PLATE IV



Magnification X10
Stained with Haematoxylin and Eosin

PLATE V

Hepatopancreas – Section from normal female

Asterisk indicates health hepatopancreatic tubules

PLATE VI

Hepatopancreas – Section from infected female

Black arrow indicates loss of regular structure
Severe necrosis of hepatopancreatic tubules (sn)

PLATE VII

Hepatopancreas – Section from MP 100 ppm enriched female

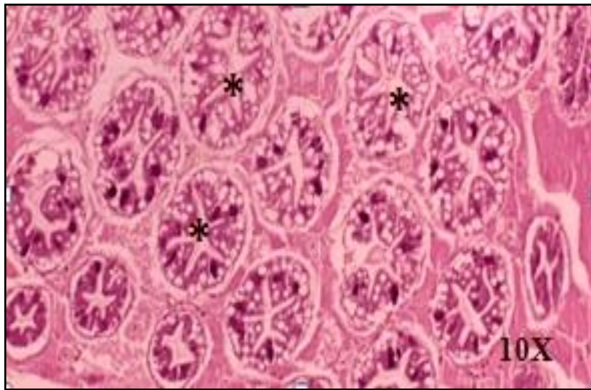
Black arrow indicates partial necrosis of hepatopancreatic tubules

PLATE VIII

Hepatopancreas – Section from MP 200 ppm enriched female

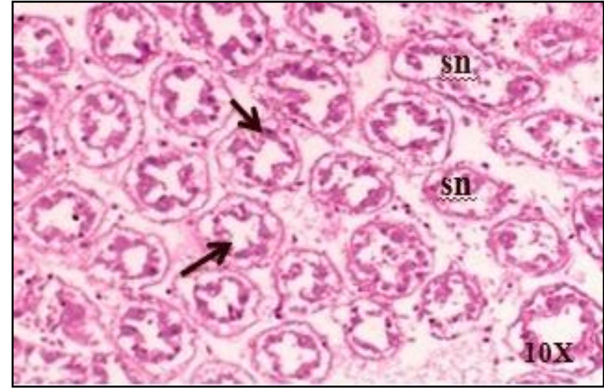
Asterisk indicates health hepatopancreatic tubules

PLATE V



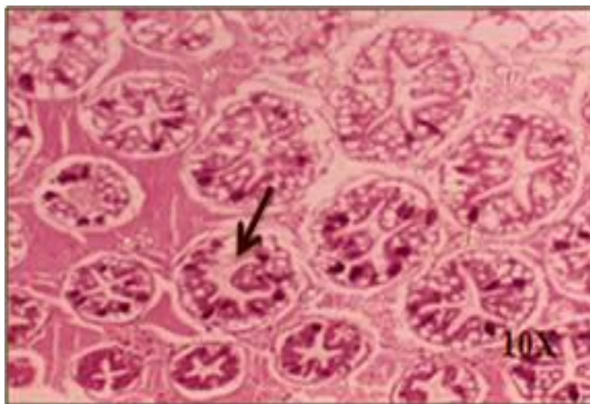
Magnification X10
Stained with Haematoxylin and Eosin

PLATE VI



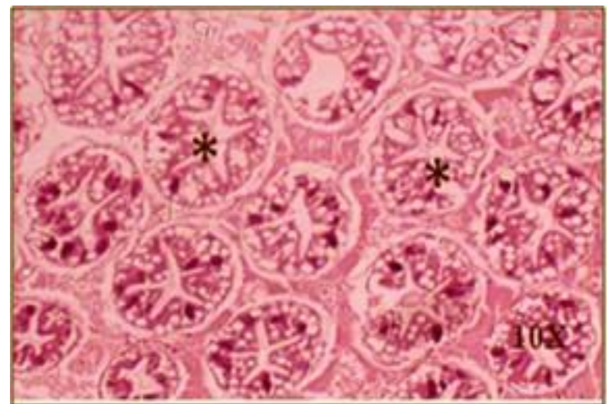
Magnification X10
Stained with Haematoxylin and Eosin

PLATE VII



Magnification X10
Stained with Haematoxylin and Eosin

PLATE VIII



Magnification X10
Stained with Haematoxylin and Eosin

PLATE IX

Gill – Section from normal male

Asterisk indicates lamellae are intact and healthy

PLATE X

Gill – Section from infected male

Black arrow indicates haemolytic infiltration

PLATE XI

Gill – Section from MP 100 ppm enriched male

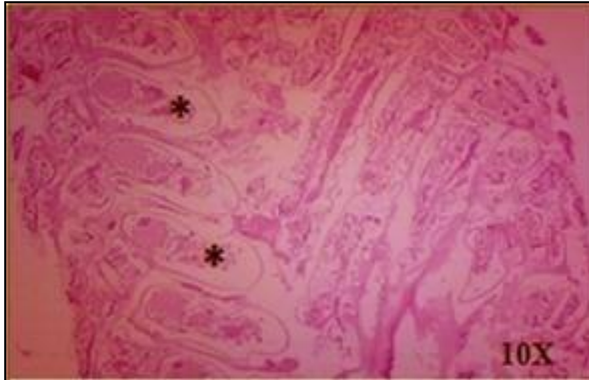
Black arrow indicates lamellae are intact and healthy

PLATE XII

Gill – Section from MP 200 ppm enriched male

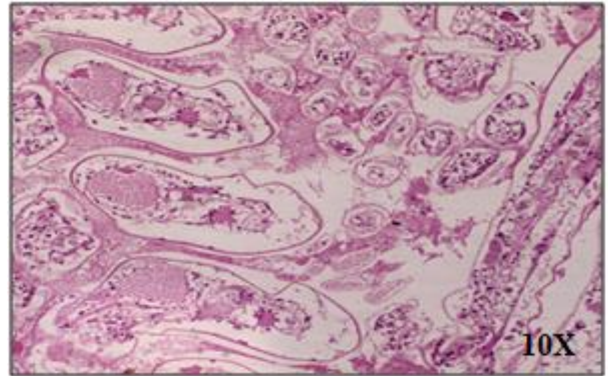
Black arrow indicates lamellae are intact and healthy

PLATE IX



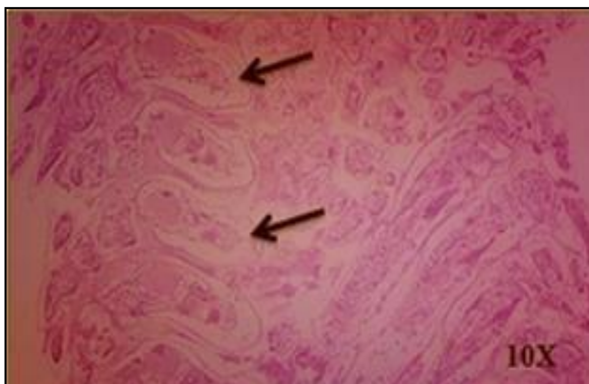
Magnification X10
Stained with Haematoxylin and Eosin

PLATE X



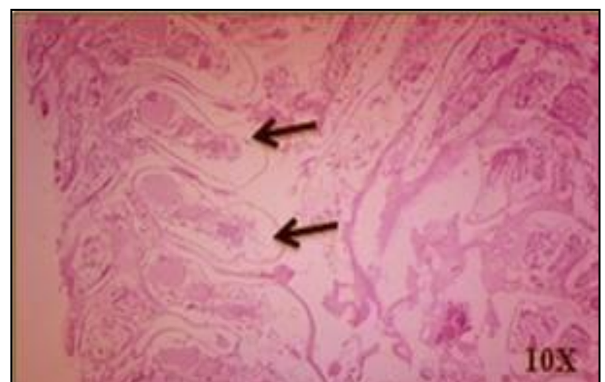
Magnification X10
Stained with Haematoxylin and Eosin

PLATE XI



Magnification X10
Stained with Haematoxylin and Eosin

PLATE XII



Magnification X10
Stained with Haematoxylin and Eosin

PLATE XIII

Gill – Section from normal female

Black arrow indicates lamellae are intact and healthy

PLATE XIV

Gill – Section from infected female

Black arrow indicates haemolytic infiltration

PLATE XV

Gill – Section from MP 100 ppm enriched female

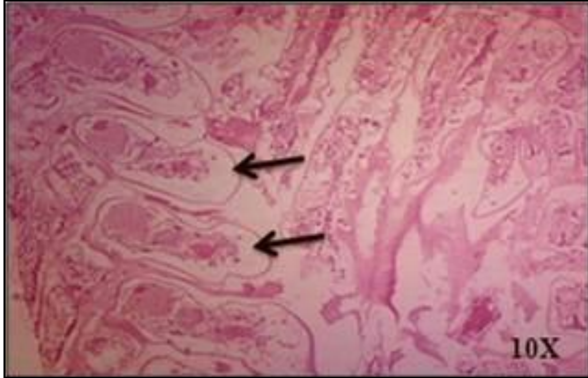
Black arrow indicates lamellae are intact and healthy

PLATE XVI

Gill – Section from MP 200 ppm enriched female

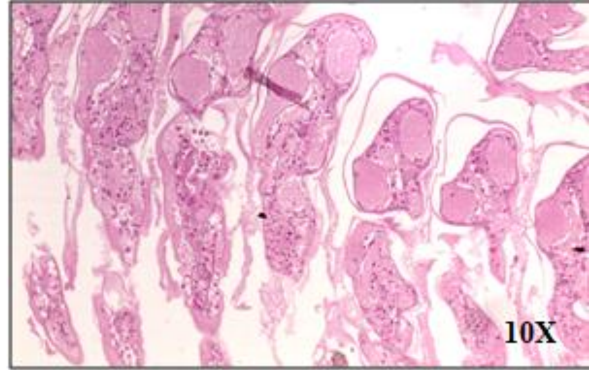
Black arrow indicates lamellae are intact and healthy

PLATE XIII



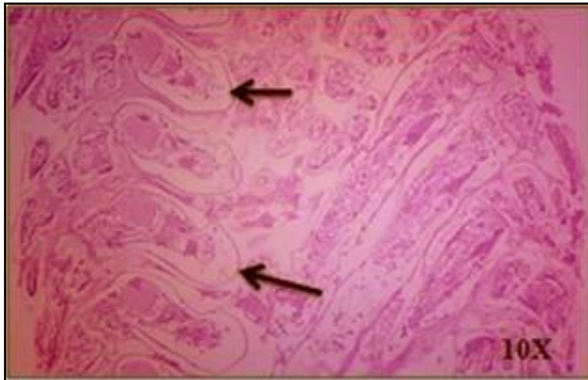
Magnification X10
Stained with Haematoxylin and Eosin

PLATE XIV



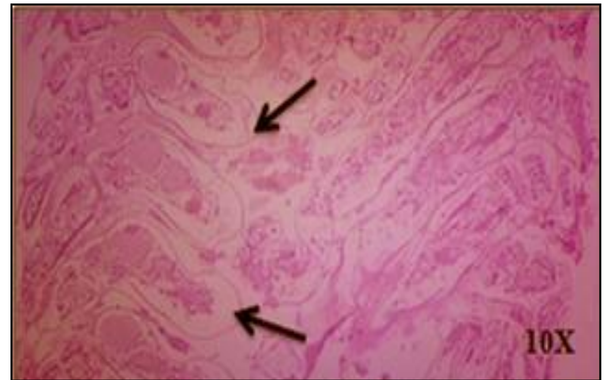
Magnification X10
Stained with Haematoxylin and Eosin

PLATE XV



Magnification X10
Stained with Haematoxylin and Eosin

PLATE XVI



Magnification X10
Stained with Haematoxylin and Eosin

6. Discussion

6. DISCUSSION

Vibrio harveyi has been implicated as the causative agent for vibriosis in shrimps. Often acting as an opportunistic pathogen, when stress or injury to shrimps with low resistance under poor environmental condition and rendering them susceptible to viral as well as bacterial infection (Liu, 1990). VHA is a unique medium for *V.harveyi* culture and isolation. *V. harveyi* are capable of utilizing the ornithine / cellobiose from the media and produce a green colour with dark center. Isolates with pathogenic potential were isolated according to their ability to lyse starch (Zamora-Rodriguez, 2003), positive hydrophobicity (Khuntia *et al.,* 2008), and production of extracellular enzymes such as proteases and lipases (Farzanfar, 2006).

Isolated clones shows luminous, Gram negative and possess short rod cell morphology; these morphological appearances were similar to isolated *V. harveyi* from *P. monodon* in Philippines described by Leano *et al.* (1998). Phenotypes of bacterium are related to its genotypic properties (Hernández and Olmos, 2003). However, various environment factors are also contribute to characterize phenotypes of a bacterium. *V. harveyi* isolates were positive to both oxidative and fermentative tests. Baumann and Schubert (1984) explained that most of the *V. harveyi* strains were positive to both oxidase and fermentative tests. All bacterial clones were green color on TCBS indicating the ability to ferment sucrose. *V. harveyi* isolated from shrimp were observed to be both green and yellow colony color on TCBS agar (Ruangsri *et al.,* 2004). Bacteria were positive for oxidase, catalase, and motility tests and sensitive to vibriostat indicating confirmed group to the Vibrionaceae. *V. harveyi* isolated from semi intensive penaeid shrimp hatcheries were also positive for motility, utilization of glucose, starch and tryptophan (Abraham and Palaniappan, 2004). Isolated clones were unable to obtain energy through arginine hydrolysis based on the negative result for the arginine hydrolysis test. It could not utilize citrate and unable to produce hydrogen sulfide which show negative result. Isolated bacteria unable to utilize phenylalanine thus it shows negative result. Result from the characterization study, it is evident that the bacterial species (*V.harveyi*) obtains energy specially from glucose, starch and tryptophan and not

from arginine, citrate and phenylalanine. So, the *V.harveyi* species is a unique organism can grow only the medium rich in above energy releasing molecule.

Another important finding in the study was that all isolates showed β haemolytic activity against horse erythrocytes. β haemolytic activity refers to the ability to lyse the whole cell of erythrocytes (Pollack *et al.,* 2002). Zhang and Austin (2000) described those bacterial hemolysins especially in Vibrios could be one of important pathogenic factors due to the fact that they could cause hemorrhagic septicemia and diarrhea in their host. Hemolytic bacteria are capable to synthesize exotoxins that cause lysis of blood erythrocytes of various animals.

The 16S and the 23S ribosomal RNAs are essential to the viability of bacterial cells, hence the genes coding for them are highly conserved. However, these genes also contain short variable sequences useful for characterization and discrimination of microbial populations at the level of family and in many cases, even at the level of genus and species. The combination of conserved and variable sites makes these molecules ideal taxonomic markers to identify *vibrio spp.* by PCR amplification and gene sequencing (Cano-Gomez *et al.,* 2009). Khamesipour *et al*., (2014) suggested that molecular-based detection techniques including PCR can be useful for a fast and accurate determination of the pathogens at the early phases of infection. PCR technique was employed using 16S rRNA gene sequence information is widely used for molecular identification of bacteria species (Dewhirst *et al.,* 2015). Its phenotypic characterization study by different biochemical tests indicates that the isolates were *V.harveyi*. Apart from this, the clear cut genotypic identification through 16S rDNA gene sequencing revealed that the isolated bacterial colony is *V.harveyi* strain with more than 99% similarity with *V.harveyi* strain MCCB 155 16S ribosomal RNA gene. These finding add new evidences to the wide distribution of this species, which has been frequently reported as one of the most abundant *Vibrio* species in marine environments, especially in south east coast of Tamil Nadu, India (Abraham and Palaniappan, 2004). In support of this study, Chrisolite *et al.,* (2008) observed the occurrence of *V. harveyi* infection in all stages of *L. vannamei* life cycle among the shrimp farms of Pondicherry coastal region. Therefore, the use of

PCR method to identify the *V. harveyi* in shrimp will facilitate disease identification and improve the cultivation process for successful harvest.

Plant cells contain sequestered glycosides and these glycosides may have antimicrobial activity against the invading pathogens. The glycosidases are able to hydrolyse the glycosides to yield more active glycones in the case of phenolic compounds, these may be oxidized to highly reactive antimicrobial quinines and free radicals (Dean and Kuc, 1987). Phytochemical screening of bioactive plants extracts has revealed the presence of alkaloids, carbohydrates, lactones, proteins, tannins, flavanoids, sterols, terpenes, glycosides, and saponins, of these, flavonoids and tannins have been linked to antibacterial activity and antidiarrheal activity (Ahmad *et al.,* 2006). Phytochemicals such as saponins, terpenoids, flavonoids, tannins, steroids and alkaloids also have anti-inflammatory effects (Lui, 2003).

Basic alkaloids and alkaloid salts were also present in most of the plants tested and may be responsible for the good antibacterial activity demonstrated by these plant extracts. Alkaloids have been reported to be responsible for the antibacterial activity in some plants (Doughari, 2006). Previous studies have also shown that alkaloids possess antimicrobial properties (Osbom, 2003). Studies have demonstrated that alkaloids have pharmacological effects and could be associated with inhibition of nucleic acid, protein, and membrane phosipholipids biosynthesis (Shelton, 1991).

Herb extracts have great anti-bacterial activity against both Gram positive and Gram negative bacteria. They can even be used to treat specific diseases caused by virus, parasites and fungi. Several compounds extracted from medicinal plants inhibited the growth of Gram-positive and Gram-negative organisms (Harikrishnan *et al*., 2009). The methonolic extracts of three ayurvedic herbals viz. *Solanum trilobatum*, *Andrographis paniculata*, and *Psoralea corylifolia* showed the protection of Penaeus sp. against nine pathogens such as *Bacillus subtilis, Proteus vulgaris, Salmonella typhi, K. pneumoniae, Pseudomonas aeruginosa, P. fluorescens,* Vibrio sp., *S. aureus* and *A. hydrophila* (Citarasu, 2000). Butanolic extract of *Withania somnifera* through Artemia enriched diet successfully controlled *Vibrio parahaemolyticus* and *V. damsela* infection in prawns

(Praseetha, 2005). *Penaeus indicus* juveniles fed with seaweed extracts were protected from *Vibrio parahaemolyticus* (Immanuel *et al.,* 2004).

In the present study alcoholic extract of *M. pruriens* seed in ethanol and methanol was screened for its antibacterial effect against multi drug resistant bacterial pathogens. Out of these two extracts methanol extract exhibited a significant antibacterial effect against *V. harveyi* bacteria and *Vibrio cholera*. Rajeshawar *et al* (2005) reported that the methanol extract of *Mucuna pruriens* had significant *in vitro* lipid peroxidation and antimicrobial activity. The antibiotic sensitiveness of *M. pruriens* may be due to the immense phytochemicals present in the form of alkaloids and terpenoids. It is reported that *M. pruriens* consists of gallic acid and Bufotenine as its major phytoconstituents (Kumar and Saha, 2013). These two alkaloids and tannin derivative is a very good antioxidant and antimicrobial in nature. Tannic acid, which is a mixture of Gallic acid esters of glucose can be used as a topical preparation for cold sores (Heinrich *et al*., 2004). Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, and cell envelope transport proteins, they also complex with polysaccharide (Brownlee *et al.,* 1990). This probably explains the reason as to why the plants containing these tannins showed good antibacterial activity.

Stanley *et al.,* (2014) have reported that *Mucuna pruriens* seed was effective antimicrobial agent to treat bacterial infection, apart from their roles as food additives and supplements. It can also be utilized as effective and cheap source of antimicrobial agent. Pujari and Gandhi (2012) were corroborated that *Mucuna pruriens* methanol extract were found to be more effective in inhibiting the pathogens compared to ethanol and acetone extracts.

In the Minimum inhibitory concentration (MIC) assay the specific bacterial strains of vibrio species was screened against methanolic extract of *M. pruriens* extract. In the MIC assay *V. harveyi* exhibited the significant IC50 values in lower concentration of 25µg/ml concentration next to that of *V. cholerae* species. From this study it is well evident that *M. pruriens* methanolic seed extract exhibited significant antibiotic effect against *Vibrio sp* than any other bacterial strain.

Previous studies have reported that flavonoids from the medicinal plant extract are water soluble antioxidants and free radical scavengers which are capable of preventing oxidative cell damage and have strong anticancer activity (Okwu, 2004). The sterols posses antibacterial and antibiotic activity and have been shown to act as inhibitors of tumor promotion in vivo (Yasukawa *et al.,* 1991). The presence of these sterols has been reported to account for the exertion of antimicrobial activity by plants containing them (Pretorius and Watt, 2001). The present study also reveals the sterols from *M. pruriens* seed extract may contribute to the good antibacterial activity against different species of bacteria.

In the present study active methanol extract of *M. pruriens* were subjected for GC-MS analysis and it is found that the extracts contain secondary actives metabolites in terms of polyphenolic, alkaloid and terpenoid derivatives. Many of these polyphenolic compounds from plant origin were seemed to have potential antioxidant properties. Polyphenol's affects bacterial growth through cytotoxic mechanisms.

In the infectivity studies, the LD_{50} value of *V. harveyi* was 4.03 x 10^6 CFU g^{-1} shrimp. The LD_{50} value of *V. harveyi* isolated from diseased shrimp was 10^6 CFU/shrimp as reported by Jiravanichpaisal *et al* (1994). According to Alapide-Tendencia and Dureza (1997), the symptoms in *P. monodon* such as red disease syndrome could be reproduced by *V. harveyi* injection with 10^7 CFU/shrimp.

In this study, the herbal *M. pruriens* was selected on their proven antibacterial activity as well as the nonhazardous nature of the phytochemicals to the environment (Citarasu *et al*., 2002). Application of the phytochemicals in aquaculture to overcome the drawbacks in the usage of chemical therapeutics is relatively new venture and the potential of the herbals with multifunctional active principles are promising (Sambhu and Jayaprakas, 2001; Sivaram *et al*., 2004).

No variation in body weight between normal and experimentally fed shrimps of both sexes was observed in this study. Similarly, Yune (2007) found unaltered body weight among herbal administered *L.vannamei.* No significant difference in the growth of *L. vannamei* after 2% *Houttuvnia cordata* (herb) supplementation with shrimp feed

compared to control groups. Xiao-hui (2009) observed no significant difference in the Feed Conversion Ratio (FCR) of *L. vannamei* when fed with diet supplemented with dietary Chinese herbal mixture of *Astragalus*, *Isatis* root, *Honeysuckle* and *Gypsum* in the concentrations of 0.5, 0.9, 1.3, 1.7 and 2.1%, respectively for 56 days. Peraza-Gomez *et al*. (2009) and Medina-Beltran *et al*. (2012) also found that feeding *Litpenaeus vannamei* powder of the herbs *Echinacea purpure* and *Uncaria tomentoosa* did not affect the survival and the growth of the shrimp.

Herbal diets enriched with Artemia boosted the survival rate of *Penaeus indicus* (Immanuel *et al*., 2004). A mixture of six herbs and plant materials enhanced or impaired enzyme activity and improved or diminished digestibility of nutrients, and stimulated the diet to pass quickly through the digestive tract of whiteleg prawns (Lin *et al.,* 2006). Papaya leaf meal contains an enzyme, papain, which increased protein digestion and weight gain of black tiger prawn PL (Penaflorida, 1995). The antimicrobial and anti-stress effects of herbal products significantly increased the survival rate of black tiger prawns larvae (Citarasu *et al.,* 2002).

In this study at the end of the experimental period, MP fed groups shows significant increase in survival rate compared to infected and normal control. Earlier study also reported the importance and acceleration of *P.monodon* survival rate by different herbal extract. The methanolic herbal extracts of *S. trilobatum, A. paniculata* and *P. corylifolia* were helped to increase the survival rate by reduced the bacterial load in the shrimp, *P. monodon* post larvae. Particularly, the *A. paniculata* extract performed well in the control of bacterial load in the various bacterial pathogens inoculated *P. monodon* post larval culture media (Citarasu *et al.,* 2003).

The difference in the maximum reproductive output among crustacean species seems to be primarily due to differences in female body size; however, other biotic or abiotic factors, such as egg size, latitudinal and seasonal variation (Boddeke, 1982), and habitat adaptation (Mantelatto and Fransozo, 1997), may also influence reproductive output. Large egg size at higher latitudes is usually associated with a more advanced larval stage at hatching and an increased development time (Hines, 1982). Egg size is an important diverse life history characteristic of species. In particular, reproductive patterns

and life history traits can be determined by the mode of energy allocation to either single embryos or brood output (Clarke, 1993).

A significant finding in the present study was that the female fecundity in terms of Gonad somatic index (GSI) was tentatively reduced in *V. harveyi* infected groups. On other hand *M. pruriens* herbal treated group exerted a positive influence on GSI index as compared to that of normal control group. The total number of egg count and egg size in diameter was much affected in *V.harveyi* infected groups. All these parameters were reversed to normal in *M.pruriens* treated group at the concentration of 200 ppm level. Similarly the positive role of herbal extract on crustaceans reproductive performance were also reported earlier. The crude combination of *Withania somnifera* (WS) and *M. pruriens* with other herbals has significantly influenced the offspring quality of the spent spawners (Babu and Marian, 2001).The methanolic extracts of the herb proved its significant influence over the various production parameters in shrimp hatchery industry than in its crude powder (Babu, 1999).The herbal extracts of both WS and *M. pruriens* have their swift positive influence over the reproductive performance and biochemical parameters in the spawners as well as offspring quality in the tiger shrimp *P. monodon* during the successive spawning (Babu *et al.*,, 2008).

The loss of spermatophore weight at the end of the experimental period has also been reported by Nakayama *et al*. (2008) in *F. paulensis*. Some studies have attributed the loss of spermatophore quality to stress, degeneration of the digestive tract and long time in captivity or nutrition due loss nutrient stores when wild animals are brought into captivity (Leung-Trujillo and Lawrence, 1987). Alfaro *et al*. (1993) reported that spermatophore and reproductive tract melanization may be the outcome of two different syndromes. Male Reproductive System Melanization (MRSM) is an infectious syndrome caused by microorganisms such as opportunistic bacteria, *Vibrio alginolyticus*, *Pseudomonas putrefaciens* and others. Male Reproductive Tract Degenerative Syndrome (MRTDS) is a stress believed to associated with the effects of captivity such as high temperatures (Pascual *et al*., 2003), unbalanced diet (Goimier *et al*., 2006) or simply lack of spermatophore ejaculation (Parnes *et al*., 2006). Whereas in this study, herbal extract

treatment of *M.pruriens* at dose dependant concentration reflected a normal reproductive pattern in terms of spermatophore quality and its biological nature.

Spermatophore absence to natural degeneration process (Alfaro and Lozano, 1993) and the fact that spermatophore degeneration is associated with the molt cycle (Heitzmann *et al*., 1993). Parnes *et al*. (2006) showed that spermatophores periodically disappeared from the terminal ampoules of males during 24 h premolt and then new spermatophores appeared after the exuviations. Spermatophore renewal is completed every two or three weeks (Pascual *et al*., 1998). However, this period may be decreased by improper captivity conditions like nutritional factors (Ceballos- Vázquez *et al*., 2004). At the end of the experiment, spermatophore reduction was only reported in *V.harveyi* infected groups. Nevertheless, the water quality for all treatments was considered ideal for the species, with temperatures between 24.5 °C to 29 °C and salinity of 33 to 35 ppt. Thus, bacterial infection of *V.harveyi* probably was important factor for aggrieved the spermatophore degeneration reported in the results of the present study.

Haemocytes play an important role in removing foreign particles such as bacteria from haemolymph by phagocytosis (Ratner and Vinson, 1983). In crustaceans, THC is a stress indicator, but varies non-specifically according to the environment as well as chemical and physico-chemical stress. Molting, development of an organ, reproductive status, nutritional condition, and occurrence of infection and even season have shown to influence hemocyte abundance (Cheng and Chen, 2001). The status of THC as a potential indicator of immune status in shrimp (Lorenzon *et al.,* 1999). Van de Braak (2002) observes that a decrease of THC is caused by pathogenic infection because hemocytes are target for pathogen. A decrease of THC may be due to cell lysis or increased movement of cells from haemolymph to tissues (Pipe and Cole, 1995). The decreasing of THC is also presumably due to the altered physiological conditions of the shrimp, as enhanced susceptibility to bacterial infection has also been found in oysters (Ford *et al*., 1993). Citarasu *et al.* (2006) have observed a significant decrease in THC on the onset of an infection against WSSV in *P. monodon.*

El-Desouky *et al*., (2012) demonstrated that supplementation on *Zingiber officinalis* and *Cyanodon dactylon* fed *M. rosenbergii* juvenile had increasing level of THC,

Pahogocytic index (PI) and Pahogocytic rate (PR) compared to the control. Phagocytotic activity and anion superoxide content are directly proportional with the number of hemocytes i.e. the increase in hemocyte counts will be followed by increase of phagocytotic activity and anion superoxide content (Nindarwi *et al*., 2013). When immunostimulation happens, gradual increases in THC are observed which indicates the immune system copes with infection (Declarador *et al.,* 2014). Aladaileh *et al*., (2007) predicted that the increased percentages of particular haemocyte types were due to induced cellular proliferation, recruitment of cells from non-circulating compartments of the haemolymph, or rapid cellular differentiation in response to antigenic challenge. On par with earlier reports, in this study also the alteration of THC level among infected and *M. pruriens* extract administered *L.vannamei* was witnessed.

Respiratory burst activity of shrimps in all *M. pruriens* fed groups was significantly higher than infected and control groups. The increase in respiratory burst capacity (Sung *et al.,* 1994) and antioxidant levels in stimulated haemocytes is considered to be a response to changes in the lipid composition of cell membranes, and to enhance the production of cell-activating factors (cytokines or chaperonins) that may improve the phagocytic capability of haemocytes (Itami *et al.,* 1998). Therefore, increased oxidant and antioxidant levels in immune cells against pathogens is expected after exposing shrimp to immunostimulants.

The granules in the granular hemocytes consist of precursors to prophenoloxidase (Supamattaya *et al.,* 2005). The activation of the prophenoloxidase cascade is exerted by extremely low quantities of microbial cell wall components such as, lipopolysaccharides, beta-1,3 glucans of peptidoglycans (Sritunyalucksana *et al.,* 2000). In the present study, the increased PO activity in shrimps *M. pruriens* fed groups which indicated that this level of supplementation could stimulate hemocytic degranulation and activate proPO to become PO.

Murugan (2005) reported a positive effect of Herbal Feed Additive (HFA) on PO activity with *P. monodon*. Citarasu *et al.,* (2006) recorded higher value of proPO when *P. monodon* was fed diet incorporated with methanolic extract of five different herbal medicinal plants like *Cyanodon dactylon*, *Aegle marmelos*, *Tinospora cordifolia*,

Picrorhiza kurooa and *Eclipata alba*. Dietary supplementation of sodium alginate extracted from the brown algae, *Macrocystis pyrifera* and *Lessonia nigrescens* and the hot-water extract of *Gracilaria tenuistipitata*, has shown to increase the PO activity and O^{-2} levels, and thereby the *L. vannamei* immunity also enhanced against *V. alginolyticus* (Cheng *et al.,* 2004). Similarly, the white shrimp, *L. vannamei*, injected with the hot-water extract of *Sargassum duplicatum* increased its immunity against to Vibrio infection (Yeh *et al.,* 2006). *L. vannamei* injected with rutin extracted of *T. sinensis* enhanced its resistance against *V.alginolyticus* through increased PO activity and O_2^- levels in *L. vannamei* (Hsieh *et al.,* 2008). Balasubrsmanian *et al.,* (2008) found that *in vivo* and *in vitro* administration of 2% of *Cyanodon dactylon* extract enhances the PO activity in the shrimp *P. monodon*. Xiao-hui (2009) obtained significant higher PO activity in *L.vannamei* serum when fed with diet containing 2.07% Chinese herbal mixture of *Astragalus*, *Isatis* root, *Honeysuckle* and *Gypsum*. PO activity was found to be significantly enhanced when fed with *L. vannamei* diet containing *Cinnamomum kanehira* twing hot-water extract compared to control (Yeh *et al.*, 2009).

Superoxide dismutases (SOD) are one of the main antioxidant defence enzymes altered in response to oxidative stress, which converts the highly toxic superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2) by reduction (Fridovich, 1995). The oxidant formed (H_2O_2) is transformed into water and oxygen (O_2) by catalase (CAT) or glutathione peroxidase (GPX). Selenoprotein GPX enzyme removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSH). Downs *et al.* (2001) reported increased levels of Mn–SOD, glutathione, heat shock proteins and ubiquitin in grass shrimp *P. pugio* after heat stress, specifically in response to increased protein synthesis and denaturation (Ellis, 1996), indicating that mitochondria were experiencing and responding to oxidative stress. Fridovich (1995) reported that increased levels of Mn–SOD provided tolerance to factors that induce oxidative stress, and moreover that Mn–SOD is a specific indicator of mitochondria experiencing oxidative stress. The superoxide anion is the first product released from the respiratory burst, and can be scavenged by SOD. Balasubramanian *et al.,* (2008) reported that the activity of SOD was significantly lowered in the WSSV-infected hemolymph of *P. monodon*, whereas *C. dactylon* extract-treated (both in vivo and in vitro) shrimp significantly recovered when

compared with control animals. The *M.pruriens* fed group revealed an increased SOD activity in *L. vannamei* and this may promote the generation of other immunoproteins.

A critical 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 important role in detoxifying invertebrates compared to vertebrates (Livingston *et al.*, 1992). GPx activity help convert the excess ROS so as to protect the host cells from oxidative damage. In crustaceans, in addition to GPx and CAT, peroxinectin, a multifunctional protein containing biological activity of peroxidase, also plays a critical role in the antioxidant defense by preventing oxidative damage from H_2O_2 (Liu *et al.,* 2004). GPx assumes an important role in detoxifying lipids and hydrogen peroxide, which rapidly form during phagocytosis or physiological metabolism, with the concomitant oxidation of glutathione (Mills, 1957). This enzyme together with SOD and catalase protect cells against damage caused by free radicals and hydroperoxides or lipoperoxides. The enhancement of GPx is considered to be associated with increasing protection to diminish the harm from H_2O_2 after the invasion by a pathogen.

On comparing the above reports with the present study results it is suggested that the increased antioxidant levels in *L.vannamei* shrimps after exposing to *M.pruriens* seed extract might have increased their resistance against pathogen. Further the improvement of immunological status of the shrimps may be due to the immunostimulant property of the *M. pruriens* extract.

A supplementation of *Ocimum sanctum* and *Withania somnifera* improved the immune system and reduced mortality in greasy grouper juveniles during *Vibrio harveyi* infections (Sivaram *et al.,* 2004). Herbal extracts enhanced the immune responses of grouper *Epinephalus tauvina* against the pathogen *V. harveyi* (Punitha *et al.,* 2008). Diet with five herbal extracts decreased the Vibrio load in black tiger prawn PL after bath challenging *V. harveyi* (Velmurugan *et al.,* 2010). Guava eliminated luminous bacteria from black tiger prawns more effectively than oxytetracycline (Direkbusarakom, 2004). Brown seaweeds (*Sargassum duplicatum* and *Sargassum wightii*) can be used as alternatives to antibiotics to control WSSV disease in black tiger prawns (Immanuel *et al.,* 2010). Also a freshly squeezed garlic (*Allium sativum*) extract diet was used to

alternate the use of antibiotics in control black gill disease in *Fenneropenaeus indicus* (Vaseeharan *et al.,* 2011).

The moderate variation of protein, carbohydrate and lipid content of shrimp muscle may be due to the infectious stress caused by *V. harveyi*. Whereas the *M. pruriens* seed extract supplementation overcome the stress effect of infectious agent and restore the nutritional value.

Histopathology is a suitable tool for monitoring and diagnosis health, where the changes at the cells and tissues due to the pathogen are interpreted to arrive at diagnosis. Pathology of hepatopancreas in typical of vibriosis showing severe necrosis, loss of structure, atrophy of tubule epithelial cells, vacuolation and rounding and sloughing of cells into the lumen (Ambipillai *et al.,* 2003). The observed pathological changes in the haepatopancreas and gills of *L. vannamei* in this study probably due to bacterial toxins. Extracellular products (ECP) have been considered as a virulent factor of *V. harveyi*. Furthermore, Liu, *et al.* (1996) studied the pathogenicity of strains in diseased *penaeus monodon* and explained that virulence is determined with both live bacteria and ECP. Proteases, phospholipase, haemolysins and other toxins may have important roles in the pathogenecity of *V. harveyi* (Austin and Zhang 2006). Histological analysis of *M. pruriens* fed on *L. vannamei* showed good healthy histological structures of hepatopancreas and gill compared to infected shrimp.

Vibrio harveyi was considered as the most frequently implicated in vibriosis to *L. vannamei* and cause major troubles in shrimp farming. Administration of Artemia enriched feed with *M. pruriens* seed extract to the infected *L. vannamei* has shown a protective effect against *V. harveyi* infection. The *M. pruriens* seed extract exhibited a significant antibacterial effect against *V. harveyi*. The antibacterial effect of *M. pruriens* may be due to immense phytochemicals present in the form of alkaloids and terpenoids. *M. pruriens* seed extract phytoconstituents not only possessing antibacterial and also very good antioxidant in nature. The enhancement of antioxidant enzyme activities were also recorded in the present study among the infected shrimp after *M. pruriens* seed extract supplementation. Further, the phytochemicals present in the *M. pruriens* extract auguments the immunological parameters of infected shrimps. Restoration of THC, PO

levels in infected shrimps by *M. pruriens* extract indicates their immunostimulant activity. Fecundity evaluation of infected and *M. pruriens* fed shrimps clearly shows the positive influence of *M. pruriens* on GSI. Reversal of normal reproductive pattern by *M. pruriens* extract in infected shrimps may be beneficial to shrimp hatchery industry. So, the *M. pruriens* seed was effective antimicrobial agent to control bacterial infection apart from their antioxidant and immunostimulant action.

The antibacterial effect of *M. pruriens* extract against *V. harveyi* might be attributed directly or indirectly by the enhancement of antioxidant system and modulation of immune system. The *L. vannamei* fed with the *M. pruriens* supplementation might have developed high resistance against the bacterial infection, which resulted in normal fecundity. So, the selected herbal extract of *M. pruriens* in the present study has proven antioxidant, immunostimulant, antibacterial and gonadal stimulant effect on *L. vannamei* shrimp.

The present study suggests that the herbal extract may be utilized as a effective and cheap source of antibacterial agent to control the bacterial infection in the shrimp farm. However, further investigation is warranted to isolate the bioactive principles and define the optimal dose and duration of administration in the field (shrimp farms).

7. Summary and conclusion

7. SUMMARY AND CONCLUSION

The antibacterial effect of *Mucuna pruirens* seed extract was assessed on *Vibrio harveyi* infected *L. vannamei* in laboratory conditions. *Vibrio harveyi* bacteria were isolated successfully from naturally infected *L. vannamei* shrimps by VHA selective media. The isolated *V.harveyi* strain was identified by 16S rDNA sequencing (Molecular-based detection technique) and these data were confirmed with NCBI BLAST data match. The isolated *V. harveyi* were used to infect the normal *L. vannamei* shrimp in laboratory for the experimental purpose.

The antioxidant system plays a vital role in preventing oxidative damages of tissue by free radicals generated by invading pathogens. Drastic reduction of antioxidant enzymes like SOD, catalase, GPx among the *V. harveyi* infected *L. vannamei* muscle tissue compared to control shrimps. The reduced antioxidant system among the infected shrimps might be the cause for lethargy and sluggishness of the shrimp. The antioxidant enzyme activities were brought back to normal in the infected shrimps received the *M. pruriens* enriched feed. *M. pruriens* extract containing principles might have enhanced either the synthesis or specific activities of the antioxidant enzymes in these shrimps.

The levels of immunological parameters like Total hemocyte count (THC), Phenoloxidase (PO), Respiratory burst (RB) are the potential indicator of immune status in shrimps. The observed significant decrease of THC, PO, RB in *L. vannamei* might be due to the altered physiological condition caused by onset of *V. harveyi* of shrimp after infection. Restoration of THC, PO, RB in infected shrimps might be due the positive influence of phytochemicals on these immunological status. These principles of *M. pruriens* would have acted as a immunostimulant in these infected shrimps and alleviated the bacterial load. The immunostimulant from naturally occurring compound modulates the immune system by increasing the host resistance against diseases caused by pathogens.

Reproductive potential of male and female infected *L. vannamei* shrimps were evaluated compared with normal control shrimps. Reduced total count and retarded motility of spermatozoa was observed in male infected shrimps. Similarly, smaller size

with low count of egg also seen among the female with vibriosis. The herbal extract of *M.pruriens* seed fed group with vibriosis replenished the fecundity in both sexes of shrimp. So, the phytochemicals present in the *M. pruriens* seed may enhance the reproductive potentials of the shrimps by overcoming the stress caused by bacterial infection.

As a concluding remark *Mucuna pruriens* seed herbal extract has positive effect in shrimp antioxidant and nutritional physiology. The experimental study demonstrated that *M. pruriens* enriched Artemia in diet have a potential role and it is an effective antioxidant by regulating infectious stress, improves the immunity, survival rate, nutritious value and enhanced fecundity rate of shrimp against vibriosis. The present research highlighted the immunostimulatory role of *M. pruriens* enriched Artemia by increasing the resistance against *V. harveyi* infection. The study also suggested that *Mucuna pruriens* herbal extract may be used as an alternate to synthetic antibiotic for vibriosis in aquaculture shrimp industry. Since, the synthetic antibiotic use is associated with environmental and human health problems, comprise develops pathogen resistance spread of antibiotic resistance to other organism. Further investigations are needed to define the optimal doses and duration of administration as well as to isolate, characterize and quantify the bioactive compounds contained in *Mucuna pruriens* extracts.

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