CHAPTER 4

DISCUSSION

Natural plant products have been reported as antistress, growth promotion, appetite stimulation, tonic and immunostimulation, and to have aphrodisiac and antimicrobial properties in finfish and shrimp larviculture due to the active principles such as alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids, and essential oils (Citarasu, et al., 1998, 1999, 2001, 2002; Sivaram, et al., 2004). Herbs rich sources of immune-enhancing substances are used in many countries to promote health, increase the body's natural resistance to infection and in prevention and treatment of various diseases (Agarwal and Singh, 1999; Devasagayam and Sainis, 2002). Herbal immunostimulants can modulate the innate immune response and as an alternative its cost prohibitive or limited effectiveness to the antibiotics, chemicals or drugs currently being used to control fish and shellfish diseases (Jeney and Jeney, 2002; Hou and Chen, 2005; Fu et al., 2007; Yeh et al., 2009a, 2009b). Disease outbreaks in commercial fisheries may be controlled by the enhancement of innate immunity through the application of natural immunostimulants (Sivicki et al., 1994), but are still rejected by the scientific community due to a lack of standardization (Ponni, 2002).

Herbal products are cheaper source for therapeutics, have greater accuracy than chemotherapeutic agents, and offer a viable solution for all problems which aquaculture faces today. Several antimicrobial, anti-stress, immunostimulant and growth-promoting plant products significantly influence the fish or shrimp larviculture (Citarasu et al., 1998, 2002, 2003a, b). Plant phenolics, polysaccharides, proteoglycans, and flavonoids play a major role in preventing or controlling infectious microbes (Citarasu, 2010). Herbal compounds have the ability to inhibit the generation of oxygen anions and scavenge free radicals. Picrorhiza kurroa has been effectively used as an anti-
stress compound for shrimps (Citarasu et al., 1998) and Ocimum sanctum positively influenced the immunostimulatory effects, enhanced the antibody response and disease resistance in Oreochromis mossambicus against Aeromonas hydrophila infection (Logambal and Michael, 2000). Curcuma longa and Allium sativum enriched diet also increased the serum bactericidal activity and phagocytosis in rohu, Labeo rohita (Sahu et al., 2007). Superoxide anion production, serum lysozyme level and serum bactericidal level were enhanced in fingerling rohu, L. rohita fed with diet enriched with Achyranthus at different doses (Rao et al., 2006). Application of immunostimulants such as plant extracts significantly enhances the phagocytic activity in various fish (Gopalakannan and Arul, 2006).

Biochemical analysis of the glucose, Total cholesterol, H.D.L., triglycerides, total protein, and albumin of hemolymph, hepatopancreas, ovary, spermatheca, muscle and the gills shows interesting results. The V. harveyi, V. parahaemolyticus, V. alginolyticus treated group shows decrease in the protein content which might be due to the existence of proteolysis. The depletion in the protein content may also be due to the blocking of protein synthesis or protein denaturation or interruption in the amino acid synthesis (Jha, 1991) or due to the rapid utilization of tissue protein as the food utilization decrease when the animals are under stress conditions.

A similar report on the decrease in the protein content in the tissues of the prawn, Palaemon serratus and the crab, Uca annulipes during Cd exposure (Papathanassiou and King, 1986; Prasad, 1990, Reddy and Bhagyalakshmi, 1994) and Naphthalene exposure in the crab, S. serrata (Vijayavel and Balasubramanian, 2006) was reported.

In the M. tinctoria and R. communis ethanol extract treated group the protein has significantly increased in all tissues. The hemolymph shows higher levels of protein than the other tissues.
The protein is the major intake of energy source. It has been observed that the proximate composition of aquatic animals under various feeding regimes and the energy gain or depletion from the body is due to the changes in the amount of content of the cell is considered to be an important tool for the evaluation of physiological standards. The tissue protein is metabolized to produce glucose by the process of gluconeogenesis and it is utilized for the energy production under stress condition (Elumalai and Balasubramanian, 1999).

Decrease in serum protein concentration and the albumin/ globulin ratio in the blood indicates some liver dysfunction. When exposed to stressors, the gills become “leaky” to water and ions, often resulting in osmoregulatory imbalances (Mazeoud et al., 1977). Thus the decline in serum total protein, albumin and globulin may also be due to a high degree of haemodilution under the stress of pollution. The A/G ratio is an index used to track relative changes in the composition of serum or plasma (Jacobes, et al., 1990). A reduction in A/G ratio can indicate a shift from albumin production to globulin proteins in response to stress. Nayak et al., 2004, opined that reduction of total serum protein, albumin, globulin and A-G ratio is a strong indicator of an immunosuppressive effect of pesticide on the organism (Adekunle et al., 2007).

Enzyme regulates the addition of glucose to proteins by glycosylation, which is often essential for the body function. Globulins like gamma globulin are essential for maintaining a healthy immune system. Serum albumin and globulin values were always higher in the fish treated with different immunostimulants than those in the control (Choudhury et al., 2005). Increase in the serum protein, albumin and globulin levels is thought to be associated with a stronger innate immune response in fish (Wiegertjes et al., 1996). The biochemical and immunological parameters also alter the level of anti-protease activity, lysozyme activity; complement factors and serum bactericidal components, which could be correlated with the enhanced leucocytic response.
and the production of reactive oxygen species (Harikrishnan et al., 2003). As a first line of defense, various peptides such as anti-protease, lysozymes, antibodies, complement factors and other lytic factors are present in serum, where they prevent adherence and colonization of microorganisms (Alexandar and Ingram, 1992) leading to prevention of infection and disease.

The present study shows a significant decrease in glucose levels in the hemolymph, hepatopancreas, ovary, muscle and gills of the bacteria treated group.

Glucose is the main monosaccharide in crustacean hemolymph and is used for chitin, glycogen, ribose and pyruvate production (Chang and O'Connor, 1983). Stable glucose hemolymph levels are very important for the regular functioning of the nervous, muscular and reproductive systems. Glucose can be accumulated in the form of glycogen in the hepatopancreas and in other tissues, such as the muscles and the gills (Oliveira et al., 2003). Glucose concentrations were selected as a stress indicator over cortisol or adrenaline level rise in concentration almost immediately following stress. During stressful situation; higher blood glucose is maintained in fish normally through breakdown of glycogen from liver, mainly through glycogenolysis (Vijayan et al., 1997). The glucose level increased in the infected or stressed animals (Yoganandhan et al., 2003). It was also reported that the glucose level elevated in stress condition (David et al., 2005). Injection of *A. hydrophila* was a stress indicator, which was indirectly confirmed by the glucose level during that period and the entire metabolic pathway produces a burst of energy to prepare the fish for an emergency situation (Rottmann et al., 1992).

This shows that the decrease in the carbohydrate level was due to glycogenolysis, possibly by increasing the activity of glycogen phosphorylase to meet the energy demand under stress condition or the toxicant has an effect
on glycogenesis by inhibiting the activity of carbohydrate metabolism or also due to hypoxic condition in the tissues as a result of pollutant stress (Dhavale and Masurekar, 1986).

The decrease in the glucose level in the present study is may be due to the stress by *V. harveyi, V. parahaemolyticus, and V. alginolyticus* infection. The hepatopancreas shows a significant increase in the glucose level than the other tissues of the plant leaf ethanol extract treated group.

In the present study the *M. tinctoria* and *R. communis* ethanol extract treated group shows a significant increase in the lipid – Total cholesterol, H.D.L, levels when compared to the *Vibrio* spp. treated group. It has been reported that the decrease in the total lipid content during stress indicates that the endogenous lipid is the only metabolite left to supply the energy needs of the animal during stress (Vijayavel and Balasubramanian, 2006).

Cholesterol is an important steroid that occurs free or chemically bound to fatty acids in all cells and blood, and in crustaceans it serves as precursor of numerous compounds such as sex hormones, molting hormones, adrenal corticoids, bile acid and vitamin D (Sheen, 2000). Most animals can synthesis sterols from acetate, but crustaceans, like other arthropods, have been found to be incapable of synthesizing sterols *de novo* (Sheen et al., 1994). Dietary Cholesterol is therefore considered essential for good growth and survival in crustaceans (Sheen, 2000).

Cholesterol is an important metabolic precursor for ecdysone biosynthesis in crustaceans Y – organ uptake of cholesterol is greatly improved at the time the molting sequence is initiated (Watson and Spazani, 1982).

In the absence of an adipose tissue in crustaceans, the hepatopancreas seems to be the main site of lipid storage (García et al., 2002), although lipids can also be accumulated in the muscle tissue and in the female gonad (Komatsu
Crustacean egg yolk is composed of proteins, mainly a lipoprotein (lipovitellin), lipids, carbohydrate and carotenoids (Avarre et al., 2003). Lipids represent 18–41% of the dry weight of mature ovaries in various crustaceans. These ovarian lipids originate from ingested food, either directly or after storage in the hepatopancreas, and are transported via the hemolymph to the ovaries (Avarre et al., 2003). According to Oberdörster et al., (2000), lipovitellin is produced in large quantities during crustacean oocyte development. The site of synthesis may be the hepatopancreas or ovary, or both, depending on the species, and it is accumulated in the developing oocytes during primary vitellogenesis (Oberdörster et al., 2000; Tseng et al., 2001; Abdu et al., 2002). Plasma lipid transport in crustaceans, as in other animals, is carried out by the association with proteins, forming lipoproteins. The main lipoprotein in crustacean hemolymph seems to be HDL (high-density lipoprotein) although LDL (low-density lipoprotein) and VHDL (very high-density lipoprotein) may also be present (García et al., 2002; Avarre et al., 2003). García et al., (2002) observed two different forms of HDL in the hemolymph of the shrimp, Macrobrachium borellii, one that is found in males and females and another HDL, found exclusively in females, during the reproductive season. According to Walker et al. (2003), these lipoproteins are called highdensity lipoprotein I (LpI) and II (LpII), and LpI plays an important role in the transfer of lipids from the hepatopancreas to peripheral tissues and in crustacean immune recognition, while LpII, also called lipovitellin, is involved in vitellogenesis and is the major source of energy for the developing embryo (Avarre et al., 2003; Walker et al., 2003).

In the present study the M. tinctoria and R. communis ethanol extract treated group shows a significant decrease in the Triglycerides levels when compared to the Vibrio spp. treated group.

The increase in the triglyceride content in tissues suggests that these compounds may help in the synthesis of cellular compounds and detoxify the
toxic molecules to reduce toxic impact (Martin et al., 1981; Surendranath 1989). Triacylglycerides (TAGs) are the predominant form of storage of fatty acids and comprise the main energy reserve in all animals (Wolins et al., 2006). The ability to store and use this energy involves a regulated balance between TAG synthesis and hydrolysis. Invertebrates, such as insects accumulate TAG as lipid droplets within the cytoplasm of fat body cells, from which they are released when required and transported to target tissues to support anabolism. Storage of TAG during the larval stages of crustaceans is primarily used to support metamorphosis that includes non-feeding periods during molting and in insects to meet energy demands during migratory flight (Beenakkers et al., 1985; Pistillo et al., 1998). The content of TAG in the fat body is influenced by several factors, including development stage, nutritional state and sex (Beenakkers et al., 1985).

The whiteleg shrimp, *Litopenaeus vannamei* is widely used as a crustacean model. The main storage site of lipids is the digestive gland (Dall et al., 1990); the lipid depots are stored in R-cells (Heffington Bunt, 1968). Enzymes responsible for the hydrolysis of lipid droplets are intracellular lipases. These enzymes have also been reported in insects (Rajesh Patel et al., 2005), but not in crustaceans. Total, digestive and intracellular lipase activity has been reported in the same species with focus on the effect of oligotrophic and eutrophic pond water on digestive enzymes (Moss et al., 2001) and measuring the effect of probiotics on these enzymes (Yan-Bo, 2007). Currently, there are no studies on intracellular lipases in crustaceans. Indirect participation of these enzymes have been proposed in shrimp by measuring the changes of lipids of the digestive gland and hemolymph during fasting (Sánchez-Paz et al., 2007) and during ontogeny e.g. *Farfantepenaeus paulensis* (Lemos and Phan, 2001), showing that lipids are used as energy reserve during stressing conditions and during ontogeny.
An increase of enzyme activity in the extra cellular fluid or plasma is a sensitive indicator of even minor cellular damage since the levels of these enzymes within the cell exceed those in the extra cellular fluids by more than three orders of magnitude (Moss et al., 1986). Toxicants can also inhibit the activity or synthesis of enzymes (Goetz, 1980). The AST and ALT are liver specific enzymes and they are sensitive markers of hepatotoxicity and histopathological changes can be assessed within a shorter time (Balint et al., 1997).

In the present investigation, the marker enzymes such as ACP, ALP, AST, ALT and LDH show variable results within the tissues of *O.senex senex*. Phosphatases are phosphomono-esterase having pH specificity, which hydrolyze various phosphate esters and liberate phosphate from the substrate. The phosphate also plays a major role in molting physiology of many crustaceans (Vijayavel and Balasubramanian, 2006). ACP hydrolysates the phosphorous esters in acidic medium and autolysis process of the cell after its death. ALP is a brush border enzyme involved in carbohydrate metabolism, growth and differentiation.

The ACP activity is increased in the hepatopancreas, muscle, hemolymph, ovary, spermatheca and the gills of the *Vibrio* spp. treated group. The activity of ALP has significantly increased in the hepatopancreas, spermatheca, hemolymph, muscle, ovary and the gills of the *Vibrio* spp. treated group when compared with the *M. tinctoria* and *R. communis* ethanol extract group.

The increased activity of ACP and ALP suggests enhanced breakdown of phosphate to release energy due to the contamination of the crabs by the *Vibrio* spp. treatment and in the ALP is due to increased transphosphorylation. The decreased activity of ACP and ALP indicate disturbance in the structure and integrity of cell organelles (lysosome) and physiological process (hormonal
regulation) of organism, causing deleterious consequences (Joshi and Kumar, 2001). Restoration of Phosphatases enzyme activity by *Psidium guajava* leaf extract in *Pseudomonas aeruginosa* infected fresh water crab, *Oziotelphusa senex senex* has been reported. (Devakumar et al., 2011a).

Transaminases such as AST and ALT constitute a group of enzymes that catalyzes the interconversion of amino acids and α-ketoacids by transfer of amino groups to form α-glutamate and pyruvate in ALT and glutamate and oxaloacetate in AST (Moss et al., 1986).

The results of the present study shows a significant increase in the AST content in the hepatopancreas, spermatheca, ovary, gills, hemolymph and the muscle of the *Vibrio* spp. treated group. The ALT content shows a higher concentration in the hepatopancreas than the other tissues of the *Vibrio* spp. treated group when compared with the *M. tinctoria* and *R. communis* ethanol extract treated group. Increased concentration of AST and ALT in the present study is due to the increased proteolysis and transamination of amino acids.

Increased AST and ALT have also been reported by Devakumar et al., (2011b) *Pseudomonas aeruginosa* infected fresh water crab, *Oziotelphusa senex senex* treated with *Psidium guajava* leaf Extract. Christensen (1975) reported increased transaminase activities in the tissues of trout exposed to Cadmium chloride. Gould et al., (1976) also reported a similar kind of increase in transaminase in the rock crab, *Cancer irroratus* during Cd exposure. Increased AST and ALT have also been reported by Reddy and Bhagyalakshmi (1994) in the crab, *S. Serrata* exposed to cadmium. The lower activity of this enzyme is exhibited in the Endosulfan exposed prawn, *Macrobrachium malcolmsonii* (Bhavan and Geraldine, 2001) and in *S. serrata* exposed to Naphthalene (Vijayavel and Balasubramanian, 2006).
Several aminotransferases of crustaceans have been studied, including AST and ALT in lobster, *Homarus americanus* (Devereaux, 1986), Kynurenine aminotransferase in tiger prawn (Meunpol *et al*., 1998); and D-Alanine oxidase and D-Aspartate oxidase in several crustacean species (D’Aniello and Giuditta, 1980). For crustaceans, AST and ALT studies have been used only recently to study the effects of pesticide (Galindo-Reyes *et al*., 2000) and heavy metal pollution (Zhao *et al*., 1995; Li *et al*., 1998).

LDH shows an increased activity in all the tissues of the *M. tinctoria* and *R. communis* ethanol extract treated group. The decrease in the LDH content of the *V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus* treated group indicates the reduced mobilization of pyruvate into the citric acid cycle.

Several reports revealed that decreased LDH activity in the tissues under various toxic conditions (Tripathi and Shukla, 1990). Alteration in the LDH activity have been noted in the prawn, *Penaeus indicus* exposed to Phosphomidon (Jayapratha *et al*., 1991), in *M. malcolmsonii* exposed to heavy metal (Yamuna, 1997; Bhavan *et al*., 1997) and in *S. serrata* exposed to Naphthalene (Vijayavel and Balasubramanian, 2006).

The phosphatases and transaminases are considered as indices for the diagnosis of tissue damage. Once cells are damaged, these enzymes leak into the circulatory body fluid and it is generally accepted that an increase of these enzymes in the extra cellular fluid is indicative of even minor cell damage (Van der Oost *et al*., 2003). The measurement of phosphatases and transaminases activity in the circulating fluid has frequently been used as a diagnostic tool in water pollution studies (Palanivelu *et al*., 2005).

In the last decades, enzymes have been widely used as environmental biomarkers, including LDH, ChE, and GST. These enzymes play an important role in physiological functions determinant for the survival and performance of
the organisms, namely neurotransmission, energy production and detoxification respectively (Elumalai et al., 2007).

LDH is a cytoplasmic enzyme also having different forms, which catalyses the interconversion of pyruvate to lactate in glycolysis. Its activity in muscle or in whole body homogenates has been used as indicative of potential effects on energy production mechanisms induced by chemical stress (De Coen et al., 2001; Frasco and Guilhermino, 2002). An increase of LDH activity has been found in Daphnia magna exposed to mercury (De Coen et al., 2001) while an inhibition of enzymatic activity was found in animals exposed to Zinc (Diamantino et al., 2001). In fish exposed to an acid mine drainage impacted effluent, both LDH activity (in Gambusia holbrooki) and increased LDH (in P. reticulate) were observed (Castro et al., 2004).

LDH is widely used in ecotoxicity studies to diagnose cell, tissue and organ damage (Diamantino et al., 2001). Valarmathi and Azariah (2003) exposed Sesarma quadratum the estuarine crab to Copper and found increased LDH activity. LDH has been used as a biomarker for hypoxia in muscles and in situations of chemical stress, as in fish (Cohen et al., 2001; Gagnon and Holdway, 1999) isopods (Ribeiro et al., 1999) and Daphnia (Guilhermino et al., 1994; Diamantino et al., 2001) when organism requires additional energy.

In the present study the M. tinctoria and R. communis ethanol extract treated group shows a significant increase in the amylase, lipases activity when compared to the V. harveyi, V. parahaemolyticus, V. alginolyticus treated group.

Digestive enzymes were found (amylase, pectinase, alginase, lipases and proteases) in gastric juice, hepatopancreas and the intestine of juvenile crayfish of Cherax albidus. Generally, the optimum pH range for amylase, lipase and
protease activity in juvenile *Cherax albidus* was broadly congruent with that reported in other crustaceans (Figueiredo and Anderson, 2009).

Amylase activities in the gastric juice and hepatopancreas of *Cherax albidus* were comparable with the ones reported in *Cherax quadricarinatus* (Lopez-Lopez *et al*., 2005). Amylase activity is the highest among carbohydratases. Lipase activity values found in the present study is comparable to those reported by Lopez-Lopez, *et al*., (2005) in *P. monodon*, which has one of the highest values for amylase specific activity among crustaceans, is able to survive on an algal diet and is known to be the most herbivorous of penaeid (Primavera and Gacutan, 1989).

The crustacean hepatopancreas is involved in several vital physiological processes, such as secretion of digestive juice and absorption and storage of digested food (Miyawaki *et al*., 1984). A significant body of information exists on the effects of pollutants on various enzymes in aquatic organisms, but most studies have dealt with fishes. Mukherjee and Bhattacharya (1977) found that the amylase activity in the fresh-water teleosts, *Ophicephalus punctatus* and *Clarias batrachus*, exposed to industrial pollutants, including copper, decreases markedly. With respect to crustaceans, Sarojini *et al*., (1992) reported that tributyltin exposure depresses amylase, protease and lipase activity in the hepatopancreas of the prawn, *Caridina rajadhari*.

In the present study, lipase activity was significantly higher in the hepatopancreas and intestine than in gastric juices as reported in the spiny lobster (*Panulirus argus*). This is likely due to the fact that lipid digestion does not start in the gastric chamber as it does in the case of carbohydrate and protein hydrolysis in *Panulirus argus*.

Lipases play an important role in lipid metabolism and energy homeostasis because fatty acids, mostly stored as triacylglycerides (TAG), are
the major endogenous source of energy. In mammals, lysosomal and digestive lipases have been recognized by several authors (Roussel et al., 1999; Miled et al., 2000). Among digestive lipases, two major groups, gastric and pancreatic lipases, are of fundamental physiological interest because they are responsible for TAG hydrolysis (Miled et al., 2000). Studies of invertebrate lipid metabolism demonstrate lysosomal and digestive lipases. Food lipid sources have been studied in penaeid species (González-Félix and Pérez-Velazquez 2002). They demonstrate that shrimp have a limited ability to synthesize de novo, the n-6 and n-3 highly unsaturated fatty acids. Lipids found in fish oils and plant oils rich in linoleic and linolenic acids are better utilized by crustaceans and have better nutritional value (Lim et al., 1997; González-Félix and Pérez-Velazquez 2002). The enzymes responsible for TAG hydrolysis from dietary lipids to free fatty acids are digestive lipases.

Lipase variation and regulation mechanism can be obtained from the study of fasting, which takes place during molting (Dall et al., 1990). The midgut gland, besides its role in digestion, participates in molting as the major lipid storage organ, mainly storing triacylglycerides (TAG), which are the main source of energy during fasting or increased energy demands (Birnbaum, 2003). Variations in lipase activity in crustaceans have been studied during fasting in lobsters, such as Jasus edwardsii (Johnston et al., 2004); other studies on lipase activity showed that oligotrophic and eutrophic pond water induce changes in lipase activity in L. vannamei (Moss et al., 2001), as does the use of probiotics in feeds for shrimp farming (Yan-Bo, 2007), but these results are inconclusive since lipase activity is commonly confused with esterase activity when using unspecific substrates. Lipase present in the digestive tract has been studied in lobster, Panulirus argus (Perera et al., 2008), crab, Carcinus mediterraneus (Slim et al., 2007), and shrimp, such as M. borelli (González-Baró et al., 2000).
In the present study the *M. tinctoria* and *R. communis* ethanol extract treated group shows a significant decrease in the ammonia, urea, and uric acid levels when compared to the *Vibrio* spp. treated group.

In decapod crustaceans, nitrogen is excreted mainly as ammonia (60–70% of total nitrogen [total-N]) and amino acid (10%) as well as urea and uric acid (in small amounts). Ammonia is formed by the catabolism of metabolic amino acids, deamination of amides and deamination of adenylate following the purine nucleotide cycle (Claybrook, 1983; Regnault, 1987). Ammonia-N excretion is affected by intrinsic factors such as the molt cycle, nutritional status and size, and also by extrinsic factors including temperature, salinity and ambient ammonia (reviewed by Regnault, 1987). An inverse relationship between ammonia-N excretion and salinity has been observed in the shore crab, *Carcinus maenas* (Haberfield *et al*., 1975), Chinese crab, *Eriocheir sinensis* (Jeuniaux and Florkin, 1961), blue crab, *Callinectes sapidus* (Mangum *et al*., 1976), *M. japonicus* (Chen and Chen, 1996), tiger shrimp, *Penaeus monodon* (Chen *et al*., 1994) and mud crab, *Scylla serrata* (Chen and Chen, 1996). In summary, ammonia excretion increases when animals are hyper regulating and decreases when they are hyporegulating.

Urea is formed through the ornithine–urea cycle (OUC), hydrolysis of arginine and degradation of uric acids in the uricolytic pathway. Uric acid is formed through the degradation of nucleic acids (Claybrook, 1983; Regnault, 1987). Arginase, a final enzyme in the OUC, has been found in the gill, midgut and hepatopancreas of *C. maenas*, Atlantic rock crab, *Cancer irroratus* (Hanlon, 1975) and *M. japonicus* (Chen and Chen, 1997). *P. monodon*, which has an isoosmotic point of 26.5 ‰, significantly increased their hemolymph urea levels when placed in 45‰, indicating the shrimp has a genetically built-in capability to produce urea in hyperosmotic environments (Fang *et al*., 1992). Higher urea excretion and hemolymph urea have also been observed in *S. serrata* in higher levels (Chen and Chen, 1996). Increased Nitrogenous
wastes have also been reported by Devakumar et al., (2012) in the crab *O. senex senex* infection to *Aeromonas hydrophila*

Ammonia is the major end-product of protein catabolism excreted by a decapod; and by increasing ammonia concentration in the water, ammonia excretion is reduced; consequently, the ammonia level in the blood and tissues increases with serious effects on the physiology of the shrimp. The first reaction of penaeids is the reduction or cessation of feeding to reduce the production of metabolic ammonia (Colt and Armstrong, 1981), which could be a serious problem for shrimp aquaculture farming. An increase in ammonia excretion with increasing temperature has been observed in the shore crab, *Carcinus maenas* (Needham, 1957). In crustaceans, nitrogenous excretion is affected by both extrinsic factors, such as temperature and salinity, and intrinsic factors, such as molt cycle, nutritional level and neuroendocrine control (Regnault, 1987).

Lallier and Walsh (1991) reported the measurable activities of both uricase and xanthine dehydrogenase in *C. sapidus* and stone crab, *Menippe mercenaria*, which indicate production of uric acid and urea. The activities of uricase, allantoinase and allantoicase, which are the enzymes that catalyze uric acid to form urea in the uricolytic pathway, have been detected in freshwater rusty crayfish, *O. rusticus* (Sharma and Neveu, 1971).

Arginase, which cleaves arginine to produce urea, has been detected in the gill, midgut and hepatopancreas of *C. maenas* and *M. japonicus* (Hanlon, 1975; Chen and Chen, 1997). A higher concentration of hemolymph urea was observed in *P. monodon* adapted to 40 ‰ (Fang et al., 1992) and *S. serrata* adapted to 40 ‰ (Chen and Chen, 1996). Fang et al., (1992) suggested that in hyperosmotic environments, OUC is activated so that the shrimp will produce urea, a less toxic by-product as an osmolite.
Crustaceans require small amount of minerals, to support a number of key metabolic processes (Desilva and Anderson, 1995). For example, minerals can act as biocatalysts for enzymes, hormones and proteins or from components for hard-tissue matrices (exoskeleton), soft-tissues, and as cofactor or activator of enzymes. Minerals are also involved in maintenance of osmoregulation, pH balance and membrane potential (Davis and Lawrence, 1997).

Crabs have protein value along with minerals and vitamins. The cations such as Calcium, Potassium, and Sodium are also distributed in considerable quantities. Ionic concentrations play an important role in the osmo-ionic regulation of the organisms and some extent in the enzymatic activities of the organisms. The cations also influence in the normal physiological activities and nervous co-ordination and also in gamete production (Gross 1958; Lockwood 1969). The ions like Na⁺, K⁺, Ca++, and Mg++ are important in the composition of the tissues. The concentration of these ions vary in various tissues during reproductive, nutritive and moulting cycles like pre-moult, post-moult and inter-moult stages of the crab. They also play a vital role in the metabolism of the organism information on the distribution and concentration of cations in the aquatic organisms like fishes, prawns, shrimp and crabs (Sather, 1967).

The level of sodium, potassium, and chloride shows significant decrease in all tissues of the *V. harveyi, V. parahaemolyticus, V. alginolyticus* treated group. Administration of *M. tinctoria* and *R. communis* ethanol extract restored the electrolyte balance in all the tissues.

The level of magnesium shows significant decrease in all tissues of the *V. harveyi, V. parahaemolyticus, V. alginolyticus* treated group, whereas in the *M. tinctoria* and *R. communis* ethanol extract treated group a significant increase was seen in all the tissues.
The cations also undergo changes during the reproductive and moulting cycles. Sodium is the most abundant cation in sea water which is known to participate in several developmental processes (Lockwood, 1969). Sodium is the principal cation of extracellular fluids of most animals. In freshwater field crab, Paratelphusa hydrodromus the sodium level in gonad slightly increased as the size group advanced as in hepatopancreas and the muscle. Similar observation was noticed in the sodium concentration of hemolymph in the crab, Neptunus pelagicus and Scylla serrata. In these animals the sodium levels are possibly affected by increasing temperature of the medium. Seasonal variations in hemolymph composition with reference to age, sex and moult cycle of Oreconectus limosus (Andrews, 1967), Carcinus maenas (Greenway, 1976) has been reported. Shaw and Sutcliffe (1961) reported that Gammarus duebeni and G.pulex pulex active uptake of sodium is involved in the distribution of this ion between blood and medium. Sodium ion was partially reabsorbed from the urine, in the crab, Carcinus maenas. The cation distribution between blood and medium is mainly passive, hyper ion regulation in dilute sea water is affected largely by changes in the rates of uptake (Zander, 1980).

Potassium is another important cation, which also influences the osmionic regulation of marine animals (Dehnel and Carfoot, 1965; Skinner 1965; Sather 1967; Poat, 1967). Magnesium was found to be decreased in all the tissues of the V. harveyi, V. parahaemolyticus, V. alginolyticus treated group. There is a slight increase in the magnesium content of the M. tinctoria and R. communis ethanol extract treated group.

The Mg\(^{2+}\) ion is an essential requirement for the activation of certain enzymes, e.g. isocitric dehydrogenase (Vernberg and Vernberg, 1970) and muscle ATPase (Perry and Grey, 1956). Excess of this ion has been shown to exert a narcotizing effect on many marine invertebrates (Pantin, 1931) and Robertson (1960) has suggested that species such as Dromia sp., Lithodes maia, Maia squinado, and Hyas sp. may live in a semi-narcotized state.
Robertson (1953) proposed that the general activity of a species is inversely related to the normally-maintained magnesium concentration of its blood. This contention was supported by Gross (1964) who found that, amongst semiterrestrial species of crabs, the more terrestrial species had greater capabilities for concentrating magnesium in their urine than less terrestrial species.

Calcium and Magnesium ions are essential for normal growth, survival, and osmoregulatory function of crustaceans. The lack of adequate levels of aqueous $K^+$ could thus be potentially detrimental in terms of the ability to effectively osmoregulate, because enzyme activity can be directly related to $K^+$ concentration (Bursey and Lane, 1971). Magnesium also plays a role in the normal metabolism of lipids, proteins, and carbohydrates serving as a cofactor in a large number of enzymatic and metabolic reactions (Davis and Lawrence, 1997).

The level of calcium shows significant decrease in all tissues of the *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus* treated group. The *M. tinctoria* and *R. communis* ethanol extract treated group shows a significant increase in all the tissues.

In the present investigation the level of phosphorous shows an increase in all tissues of the *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus* treated group, whereas the phosphorous level significantly decreased in the *M. tinctoria* and *R. communis* ethanol extract treated group.

In decapod crustaceans wide variation of calcium concentration have been reported by many workers. Hormonal control and calcium level in the crab, *Scylla serrata* have been observed (Menon and Sivadass, 1963). Dall (1965) reported that the calcium ion concentration differs, with body size, calcium ion concentration varied in the exoskeleton and tissues during moulting and reproducing cycles of the shrimp.
Phosphorous is an important constituent of nucleic acids and cell membranes in all living species and directly involved in energy producing cellular reactions. Crustaceans have relatively high content of ash (15.9 %) in their body (Sze, 1973) and hence proper mineralization is important for normal growth. Minerals is obtained from feed in terrestrial animals. However, in aquatic organisms some of the mineral ions are extracted from the surrounding water (Gilles and Pequeex, 1983). Unlike calcium, phosphorous is generally found at low concentration in natural water (Boyd, 1981). Consequently absorption of significant amount of phosphorous from water is unlikely, making a dietary source essential for most aquatic species. Supplementary phosphorous in practical shrimp feeds improved the weight gain and feed conversion ratio (FCR) as reported in many penaeid shrimp species (Ahamad Ali, 1999; Ambasankar and Ahamad Ali, 2002).

The increased levels of calcium and phosphorous in the hepatopancreas and depressed levels in the exoskeleton of soft-shelled prawns may indicate impaired mobilization of these mineral elements from the former to the latter for use in shell formation and hardening. External sources of Ca are diet and the water medium. In *P. japonicus* fed with purified diet, it was shown that dietary Ca (0.251.5%) had little effect on growth and it was surmised that the shrimp fulfils its Ca requirement from seawater (Cheng, 1985). Cheng (1985) showed that the diet is a principal source of phosphorous in *P. japonicus*. The dietary phouphorous level that resulted in best growth in *P. japonicus* was 1.75% (Cheng, 1985). It also appeared that the level of phosphorous affects the rate at which Ca is retained in the body. This means that the animal can maintain Ca/P ratios within narrow limits.

Organo-calcium phosphate crystals may play a role as a Ca reservoir for the exoskeleton (Starch et al., 1982). The exact molecular basis for Ca and P utilization and the role of selected micronutrients in certain aspects of
exoskeleton biosynthesis in crustaceans is still poorly understood (Conklin, 1982).

Total Hemocyte count was increased in the *V. harveyi, V. parahaemolyticus, V. alginolyticus* treated group. The decrease in the Total Hemocyte count of the *M. tinctoria* and *R. communis* ethanol extract treated group was noticed in the present study.

The circulating Hemocyte number is a stress indicator (Le Moullac, and Haffner, 2000; Kumaran et al., 2013a, b) and Hemocyte counts may be a valuable tool in monitoring the health status of crustacean species (Jussila, *et al.*., 1997). Studies on the Hemocytes of crustaceans contribute to the accumulation of the basic knowledge on Hemocytes, especially with regard to the physiological condition of the animal.

Decapod’s circulating Hemocytes play extremely important roles not only by direct sequestration and killing of infectious agents but also by synthesis and exocytosis of a battery of bioactive molecules (Smith and Chisholm, 2001). Essentially, the Hemocytes execute inflammatory-type reactions such as phagocytosis, Hemocyte clumping, production of reactive oxygen metabolites and the release of microbicidal proteins.

In intensive culture the application of antibiotics, chemotherapeutants, and vaccines is quite expensive and leads to undesirable effects such as bioaccumulation, pollution, and antibiotic resistance that can be transferred to wild and human pathogenic microbes, thus posing a threat to human health and a host of socio-political and environmental problems as evidenced in countries like India forcing the intervention of judiciary imposing severe restrictions in shrimp farming.

As a conclusion the present study clearly suggests that plant extracts or their bye products contain several active compounds such as phenols,
polyphenols, alkaloids, quinones, terpenoids, lectins, and polypeptides that have been shown to be effective alternatives to traditional chemotherapies and vaccines. Several herbal immunostimulants administered at various concentrations through oral or injection enhances the innate and adaptive immune response in different freshwater and marine fish and shellfish against various bacterial, viral, and parasitic diseases. Hence herbal extracts have a potential application as an immunostimulant in aquaculture; further they are often locally available, inexpensive, act against a broad spectrum of pathogens, easily biodegradable and environment friendly. The preparation of herbal extract is simple and inexpensive. Hence phytotherapy will prove to be very effective in aquacultural operations. They can also be used as a potential supplementary additive to fish feed; however, appropriate field trials remain necessary before using herbs as immunostimulants in aquaculture farms.

Further in this present study, the role of *M. tinctoria* and *R. communis* by restoring the levels of marker enzymes and minerals proves to be excellent antibacterial agent against *Vibrio* spp. and immunostimulant in the crab, *O.senex senex*. 