
Discussion
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CHAPTER IV

DISCUSSION

Disease outbreaks in aquatic organisms appear to be escalating worldwide and a growing number of bacterial infections have been associated with recreational and commercial uses of aquatic resources (Tamplin, 2001). The tremendous increase in disease onset has reflected in global disease management and currently has become a subject of active research in aquaculture (Lipp *et al*., 2002).

Aquaculture remains a growing, vibrant production sector for higher protein food. The decline of fish from natural aquatic resources and increasing demand for fish, crab, shrimp and other aquatic organisms by consumers are the two main factors for the expansion of aquaculture in recent days (Haniffa and Kavitha, 2012). Aqua culturists are encouraged towards intensive farming system to increase production and profit. As a well known fact, intensive fishing activities increases infectious diseases among aquaculture production unit. The pathogens causing infections are usually indigenous to the aquatic environment and been associated with disease outbreaks in aquatic animals and carries a higher risk of transmitting to human beings also (Paulo Martins da Costa *et al*., 2013). Hence the growth of intensive aquaculture production has parallel serious challenges to solve in preventing and treating aquatic diseases accordingly.

A large number of pathogens have been reported from aquatic environments that can affect wide range of aquatic animals. The principal pathogens acquired topically from fish or shellfish are *Aeromonas hydrophila, Pseudomonas aeruginosa , Edwardsiella tarda, Mycobacterium marinum, Streptococcus iniae, Vibrio vulnificus* and *V. damsel* (Haenen *et al*., 2013). These pathogens are also responsible in causing serious life threatening diseases in other aquatic species such as fishes, crabs, shrimps and lobsters.

Effective disease control is highly required within aquatic farming systems to stop the spread of infectious pathogens which may cause huge economic loss. Implementation of an effective health management system with well organized process, ideas and practices, hygienic measures and improved tools to resist disease onset can widely help in reducing and controlling the diseases at farm sites (Alexandra Adams and Kim Thompson, 2011).Various new methods to treat pathogenic disease attack in aquaculture systems was adapted in recent days, still more novel methods need to be identified which should be cost effective and more importantly capable of treating disease attack without generating serious side effects (Adams, 2009). The key to deal with any bacterial infection is early recognition and treatment. The environment and the condition of the aquatic animals are important in determining the rate and severity of the infection. On studying the bacterial infections in aquatic species it is more evident that safe and efficient treatment

methods are highly required to fight back the infections as bacterial pathogens are capable of gaining resistance if treated incompletely.

As discussed above, bacterial populations are the major reason for many diseases causing heavy mortalities in aqua farming. Usually the causative micro-organisms are naturally occurring saprophytes, which makes use of the organic and mineral matter in the aquatic environment to grow and multiply rapidly. It has been studied that the normal bacterial flora of fish reflects the bacterial population of the water body in which they live.

In the present study, major fish pathogens *Aeromonas hydrophila* and *Pseudomonas aeruginosa* were chosen for bacterial challenge study in an animal model following the fact that they are currently causing higher mortality rates in fishes, crabs and shrimp varieties. As per reviews it is known that aquaculture sectors are also highly affected by the disease attack of these bacterial pathogens and hence a detailed study on using these pathogens in aquatic animal model is highly recommended.

Hassan Malekinejad *et al*., (2012) have reported that *A.hydrophila* is the causative agent for numerous diseases in cold-blooded animals, including fish and reptiles and in warm-blooded animals such as mammals and birds. *A.hydrophila* is heterogeneous both biochemically and serologically and thus presenting the biggest hinder in developing effective commercial vaccine against *A. hydrophila*.

To prevent future disease occurence caused by *A. hydrophila,* a vaccine that could offer protection against multiple serotypes from various regions are urgently needed (Poobalanea *et al*., 2010). Harikrishnan and Balasundaram (2005) have reported that *A.hydrophila* is associated with various disease conditions in aquaculture sector worldwide. In both normal aquatic environment and in aquaculture setup, stress condition is produced by biotic or abiotic factor(s) which extends the adaptive responses of the individual beyond the normal range, such that its chances of survival are significantly reduced. In that way Sannasi Muthu Anbazahan *et al*., (2014) reported that pathogenicity of the bacteria appears to mainly affect stressed or compromised fish, and the infection is often secondary and hence stress conditions are the preliminary reasons for higher mortality range.

Similarly, *Pseudomonas spp.* are emerging fish pathogens responsible for high mortality and disease outbreaks in various aquatic species. *Pseudomonas* species are widely distributed in aquaculture farms and considered to be one of the primary causes of diseases in fishery sector. Austin and Austin (2007) have reported that *Pseudomonas fluorescens, P. angulliseptica, P. aeruginosa and P. putida* were identified in various species of fish as causative agents of *Pseudomonas septicaemia*, a life threatening aquatic disease.

John Thomas *et al* ., (2014) have reported that *Pseudomonas spp*. is the major causative agent for red spot diseases in different aquatic animals,

including European eel, Atlantic salmon, sea trout, whitefish, sea bream and rainbow trout .

Boopathi Mahalaxmi *et al*., (2013) have studied the distribution of microbial population associated with crabs from Ennore seacoast, India and have reported that the sewage effluent is highly contaminated with *Aeromonas spp.* and *Pseudomonas spp.* and can be the major reason for fish and crab mortality in that region. Sharmila Joseph *et al* ., (2014) have reported that increasing incidence of shell diseases in the freshwater crab, *Barytelphusa cunicularis* is caused by various range of bacterial infections predominantly by *Aeromonas spp.* and *Pseudomonas spp.*

In the present study, *Aeromonas hydrophila* and *Pseudomonas aeruginosa* cultures was screened by biochemical tests and each cultures were confirmed thoroughly by the individual biochemical test results and the bacterial cultures were studied for their mortality rates in experimental male and female crab groups and were utilised for further bacterial challenge study. Post infection period was monitored by incubating the animal for 96 hrs and the hemolymph was subjected for further assays to understand the changes in immunological and biochemical parameters.

Current disease control protocols against microbial infections are often difficult to administer. Due to increasing antibiotic resistance they have not only become ineffective but also they stay expensive and sometimes even

environmentally hazardous. Alternative approaches could be developed by focusing and identifying antimicrobial compounds derived from natural resources (Wright, 2014).

In the present study, fresh ethanolic extract of *S.molesta* leaf extracts were analysed for its pharmacognostic properties and used as an antagonist agent in treating bacterially challenged crabs. *Salvinia molesta,* a freshwater aquatic weed collected from Kanyakumari district ,Tamilnadu , India was studied for its medicinal efficacy in crustacean animal model *Oziotelphusa senex senex*. On analysing the previous studies on the same plant, Muraleedharan Nair Jalajakumari Mithraja *et al*., (2011) have reported the phytochemical properties of three *Pteridophytes* with eighteen extracts and reported that promising pharmacological activity was found in *Salvinia molesta* extracts collected from Kattakada, Trivandrum district, Kerala, India. Though there were studies on *S.molesta,* the ethanolic extract of *S.molesta* was not analysed previously and hence in the current study ethanolic extract was taken for further assays .

Choudhary *et al*., (2008) has reported the phenolic constituents of freshwater fern *S.molesta* D.S. Mitchell collected from Haliji Lake (Sindh, Pakistan), by a non-physiological assay and showed that compounds of *S.molesta* showed potent antioxidant radical scavenging activity. Hence in the present investigation the weed was taken as a study material to assay its antioxidant and immunomodulator role in animal models.

According to Chetan Savant (2014), Indian medicinal plants are a rich source of substances which are claimed to induce paraimmunity and non-specific immunomodulation process in a system. A large population of India uses plants for its healing, preventive, curative and therapeutic property together with immunomodulatory and immunostimulatory effects. Immunostimulation and immunosuppression are two different parameters which need to be tackled and balanced in order to regulate the normal immunological functioning of a system. In the recent past, scientific studies on plants used in ethno medicine have led to the discovery of many valuable drugs possessing potent immunomodulatory activities (Namrata Singh *et al*., 2016).

Numerous studies have proven that the herbal immunostimulants can promote both specific and non-specific immune responses, enhance the intestinal microflora, growth performance and disease resistance in fishes. The non-specific defense mechanisms of fishes include neutrophil activation, production of peroxidase and oxidative radicals, together with initiation of other inflammatory factors (Nagarajan Balachandran Dhayanithi *et al*., 2015).

One of the most promising methods of controlling diseases in aquaculture is strengthening the defence mechanisms of fishes/crabs/shrimps and other aquatic animals by prophylactic administration of synthetic or natural immunostimulants. Immunostimulants of traditional Chinese medicine (TCM) has been widely used to cure human and domestic animal diseases since

ancient times in China which is biodegradable, biocompatible, and also enhanced immune system in aquatic animals.

In the present study, ethanolic extract of *S.molesta* was administrated to bacterially challenged crab groups to study the changes in biochemical and immunological parameters as a new effort in learning the pharmaceutical aspects of the aquatic weed and its following ability in curing diseased conditions of crabs. As per previous studies, it is understood that using plant product as a supplement to improve immune properties of aquatic animals is a highly preferred scenario where potential results were obtained in animal studies.

In that way, Harikrishnan and Balasundaram, (2008) have reported the antagonistic activity of the medicinal herbs *Curcuma longa*, *Ocimum sanctum*, and *Azadirachta indica* against *A. hydrophila* in goldfish varieties. Similarly, Harikrishnan *et al.*, (2010) have reported the effect of triherbal extract-enriched diets to control *A.hydrophila* infection in carp. Chandhirasekar Devakumar and Arulvasu Chinnasamy (2015) have reported the role of natural immunostimulants on growth performance, haematological, biochemical parameters and disease resistance of Asian Sea bass *Lates calcarifer.* Devi *et al.,* (2016) have reported the antimicrobial, and antioxidant effect of partially purified extracts of Sea grass (*Cymodocea serrulata*), Turmeric (*Curcuma longa*), *Spirulina* (*Spirulina platensis*), and beet root (*Beta vulgaris*) in

bacterially challenged *Pseudochromis dilectus* under *invitro* and *invivo* conditions and had recorded the relevant hemato-biochemical changes.

Ahilan *et al*., (2010) observed that the addition of *Phyllanthus niruri* and *Aloe vera* (Aloe) as herbal additives can positively enhance the growth and immune performance of goldfish, *Carassius auratus* as well as its resistance to *A. hydrophila* infections.

From the above studies it was obvious that plant products are well known for their medicinal values in aquaculture sector and also have proven effective in treating fish and crab diseases. For these reasons, studying their pharmacological effects and discovering new anti-infectious agents are highly required to solve many unanswered queries in disease management. Since, the main concern of the general public is in finding new natural and therapeutically active agents; scientists all over the globe have started to screen plants in searching new therapeutic agents for their effective multi level treatment in both animals and human beings. From the above studies it was more evident that plant products show inevitable results in treating aquatic infections.

In the present study when five different solvent extracts were assayed for phyto constituents, all the solvent extracts showed viable results whereas ethanolic extract of *S.molesta* leaf extracts was found to possess higher range of significant secondary metabolites *viz*., tannins, saponins, flavonoids,

phenols, terpenoids, alkaloids, glycosides, cardiac glycosides, coumarins and steroids.

On quantification of the phytochemicals it was found that the levels of phenol were high in ethanolic leaf extract when compared to other solvent extracts. Suganyadevi *et al*., (2011) have reported that phenolic compounds contain enhanced antioxidative property and hence suppress the initiation or propagation of chain reactions. The antioxidant activity of phenolic compounds is mainly attributed to their redox properties, which allow them to act as reducing agents, hydrogen donors and quenchers of singlet oxygen and thus plays a major role in free radical scavenging. Purushothaman Rama *et al*., (2013) have reported that polyphenols have received considerable attention because of their physiological functions, including, antioxidant, anti-mutagenic and antitumour activities which have beneficial implications for improving health.

In the present study in different leaf extract of *S.molesta* ,the levels of saponins were found to be considerably high in ethanolic extracts, though closer values were found in acetone and aqueous extracts also. Being widely distributed amongst plants, saponins have long been regarded as phytochemical material to protect plant against pathogens. Therefore, it is no doubt that saponins function as potential as antimicrobial agent (Hassan *et al*., 2013). Mamta Saxena, (2013) has reported that saponins possess potent antimicrobial,

antioxidant, anticancer and detoxification activity and thus enhances the stimulation of the immune system.

Tannin levels was predominantly found high in ethanolic extract of *S. molesta* than other four solvents showing the better extraction properties of ethanol as a solvent. Naveen Prasad *et al*., (2008) has reported that tannins are significant antimicrobial agents and are water-soluble polyphenols and exist as precipitated proteins in many plant foods and found to prevent the development of microorganisms by precipitating microbial protein. The growth of many fungi, yeasts, bacteria, and viruses were inhibited by these tannins. Ilhami Gulcin *et al*., (2010) have reported that tannins are responsible for free radical scavenging and antioxidant activity in *invitro* conditions.

In the present study, the levels of flavonoids were significantly high in ethanolic leaf extract of *S.molesta* elucidating the importance of the extract as a potent medicinal agent. Flavonoids are found to be synthesized by plants in response to a microbial infection condition. Hence it should not be surprising that they have been found to be effective as antibacterial substances against a wide array of infectious agents (Jasmine *et al*., 2007). Tapas *et al*., (2008) have reported that flavonoids possess significant nutraceuticals, antimicrobial and anti-oxidant activity.

Similarly alkaloids were found to be high in ethanol extract of *S.molesta*. The levels in aqueous and acetone solvents were considerably

moderate when compared to ethanolic extract. Farida *et al*., (2014) have studied the presence of alkaloids in medicinal plants and their importance in antimicrobial activities on some pathogenic microbial strains and confirmed the role of alkaloid as a potent antagonist agent against bacterial pathogens.

It is a well known fact that phytotherapy is the oldest form of healthcare known to man-kind. Bioactive substance present in herbs is well-known for their antimicrobial and immunomodulatory properties. Globally, plant extracts are utilised by general population for their antibacterial, antifungal and antiviral properties. It is known that more than 400,000 species of tropical flowering plants have medicinal properties and this has made traditional medicine cheaper than modern medicine particularly in the developing countries (Venkatachalam Uthayakumar *et al*., 2014). Numerous reports are available supporting the studies performed using plant products to treat aquatic diseases and also was observed that treatment procedures are highly effective against various ranges of bacteria, fungal and viral infections. Dubber and Harder (2008) have studied the ability of some herbs and seaweeds to inhibit potential fish pathogens. From the previous studies on phytochemical analyses, it is more clear that bioactive constituents are highly responsible for antibacterial, antioxidant, immunomodulatory, anticancer and many other therapeutic activities.

Azwanida (2015) has reported that ethanolic extracts of plant species are more effective and showed maximum presence of phytochemicals. Similarly,

William Patrick Cruiz Buhian *et al* ., (2016) have reported the importance of ethanolic solvent as a potent extraction agent showing higher pharmacological activities and hence ethanolic extract was further utilised for assay of antibacterial activity and antioxidant assays.

When analysing the antibacterial property of a plant product, it is essential for a medicinal plant to show wide range of anti microbial activity against multiple pathogens so that the plants innate ability to act as an potent therapeutic agent can be justified. The antibacterial activity of any plant product is proven to be possible because of presence of range of phytoconstituents which exerts multiple roles in treatment of infectious diseases and various studies have proven the correlation between the role of phytochemicals and the antimicrobial activity of the plant extracts (Sanower Hossain *et al*., 2014).

As a rich source of phytochemical constituents, *S.molesta* ethanolic leaf extract was believed to possess wide antimicrobial properties. Supporting the data and facts, when the ethanolic extract was utilised for antimicrobial activity assay against six different pathogens, the results obtained in the present study revealed that the ethanolic extract of *S.molesta* is able to significantly resist all the test pathogens in varying levels, where the antibacterial activity against *P.aeruginosa and A.hydrophila* were considerably higher than the other selected pathogens.

The results also showed that increase in concentration of the extract increased the zone of inhibition. The range of inhibition by ethanolic extracts against pathogens were in the following order of higher to lower, *viz.*, *Aeromonas hydrophila, Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis.*

Similar studies were found to be performed by many researchers utilising the phytochemical properties of plants to treat fish diseases. Sharifah Raina Manaf and Hassan Mohd Daud (2016) have performed studies to determine the phytochemical constituents and the antibacterial activity of *V. trifolia, A. vera, S. crispus, P. pellucida, C. nutans* and *P. grandifolia* plants towards selected aquatic pathogenic bacteria and reported that the antimicrobial role of the plant substances are by virtue of its phytoconstituents. Turker *et al*., (2009) have reported that the phytochemicals present in alcoholic and aqueous extracts of *Nuphar lutea, Nymphaea alba, Stachys annua, Genista lydia, Vinca minor, Fragaria vesca, ilipendula ulmaria* and *Helichrysum plicatum,* a group of traditional herbs of Turkey are responsible for the antibacterial activity against *A. hydrophila, Yersinia ruckeri, Lactococcus garvieae, Str. agalactae* and *Enterococcus faecalis* bacteria isolated from fish.

The antibacterial activity of *S.molesta* ethanolic leaf extract against general gram positive and gram negative bacterial cultures paved way for analysing the aquatic weed, *S.molesta* further to quantify the antioxidant ranges and qualitatively analyse the active component(s) responsible for its activities.

In the present study, *S.molesta* leaf extract was assayed for antioxidant activity *via* antioxidant assays. DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples. It is a stable radical with a characteristic absorption at 517 nm and is used to study the radical scavenging effects of plant extracts. As antioxidants donate protons to this radical, the absorption decreases. Antioxidants, on interaction with DPPH, either transfer electron or hydrogen atom to DPPH, thus neutralizing free radical character. Sherikar and Mahanthesh (2015) have reported that DPPH radical scavenging assay was a more accurate assay in evaluating the free radical scavenging capacity.

The ethanolic extract of *S.molesta* when subjected to DPPH assay showed maximum free radical scavenging activity when compared with standard ascorbic acid levels thus elucidating the importance of *S.molesta* as a potent anti-oxidant agent. The profound pharmacological properties of aquatic weeds and its antioxidant capability in curing various ailments is documented by many researchers.

Bright and Kanagappan (2016) have reported the presence of significant levels of antioxidant in five different aquatic weeds of India and revealed the role of aquatic weeds in disease prevention and the credit has been attributed to antioxidant properties of their plant phytoconstituents. It is believed that higher intake of antioxidant rich food is associated with decreased risk of degenerative diseases particularly enhanced protection from broad range

infectious agents and cancer causative agents. Surendraraj *et al*., (2013) have reported the antioxidant potential of water hyacinth , *Eichornia crassipes* and recorded that *Eichornia crassipes* could be a potential natural antioxidant source for food, fish feed, and pharmaceutical applications.

Nanadini *et al*., (2014) have reported the potent antioxidant property of an invasive weed, *Chromolaena odorata.* Similarly, Joash Ban Lee Tan *et al*., (2014) have reported the antioxidant activity five plants from the *Commelinaceae* family *ie*., group of weeds. Narintorn Rattanata *et al*., (2014) have reported the antioxidant and antibacterial properties of selected Thai weed extracts and mentioned that the weed population is also an equally medicinally important plant product and can be investigated for it pharmacological properties and therapeutical ability.

Gas chromatography mass spectrometer is a unanimously accepted method for the analysis of volatile constituents of herbal medicines, due to their sensitivity, stability and high efficiency. Especially, the hyphenation with mass spectrometer provides reliable information for the qualitative analysis of the complex constituents. Recently, the analysts had turned gas chromatography as a powerful separation method and combined it with mass spectrometry to aid identification. It has been shown that *invitro* screening methods like GC MS could provide the needed preliminary observations necessary to analyse crude plant extracts and also to elucidate potentially useful compounds for further

chemical and pharmacological investigations (Bagavathi Perumal Ezhilan and Ramasamy Neelamegam, 2012).

The unknown organic compounds in a complex mixture can be determined by interpretation and also by matching the spectra with reference spectra (Elizabeth Thomas *et al*., 2013). The identification was based on the peak area, molecular weight, molecular formula and retention indices and also comparison of their mass spectra with those of standards and MS library. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) in the extracts were recorded for comparison with other analytical data.

In the present study, GC MS results of *S.molesta* confirmed the presence of four major components highly responsible for its antioxidant, antibacterial, antiinflammatory and immunostimulant properties. All the four compounds are profoundly known for their therapeutical properties. The four important compounds isolated from the purified *S.molesta* ethanolic extracts are; Apiol, Hexadecanoic aci d-methyl ester, Hexadecatrienoic acid methyl ester, Heptadecanoic acid,16-methyl-, methyl ester.

Hui Zhang *et al*., (2006) have reported the prevailing antioxidant capability of apiol isolated from parsley (*Petroselinum crispum*) and their efficacy as an anticancer agent. Similarly, Yilmaz *et al*., (2011) have reported the role of apiol in altering the immunological parameters of infected fish

varieties. Recent studies have also concluded the significant anti inflammatory role of apiol present in essential oil of *Trachydium roylei* (Yu-Tao Wang *et al*., 2016).

The pharmaceutical role of hexadecanoic acid is more evident from previous studies.Mustapha Abubakar and Runner Majinda (2016) have reported the antimicrobial efficacy of hexadecanoic acid isolated from *Albizia adianthifolia and Pterocarpus angolensis* and their potent antibacterial activity against gram positive and gram negative microorganisms. Aparna *et al.,* (2012) have assessed the anti inflammatory property of hexadecanoic acid and their role in medicinal oils in treating chronic diseases. More importantly significant antioxidant role of hexadecanoic acid is reported by Jayanta Kumar Patra *et al*., (2015) in an edible seaweed, *Laminaria japonica L.*

In the same way,the antibacterial role of hexadecatrienoic acid is studied in *Spirulina platensis* (Vinay Kumar *et al*., 2011). Whereas, Zaha Elagbae *et al.,* (2016) have reported the antioxidant property of heptadecanoic acid in *Annona muricata* L. Seeds. Fayaz *et al* ., (2005) had studied *Kappaphycus alvarezzi*, an edible seaweed from the west coast of India, and analyzed its chemical composition and found that an high percentage of heptadecanoic acid was responsible for its significant antioxidant activity.

In the present study GC MS analysis of ethanolic extract of *S.molesta* leaves revealed significantly important bioactive compounds and its synergistic role responsible for major pharmaceutical properties as discussed previously.

Further, to analyse the active compound in the crude fraction of ethanolic leaf extract of *S.molesta*, Thin layer chromatography technique was performed. Sasidharan *et al.*, (2011) have reported the efficacy of TLC methods in extraction and purification of bioactive compounds from plant products. Barma and Goswami (2013), have reported the identification of fatty acid methyl ester compound by TLC method and have reported that 0.59 cm is closer to R_f value of fatty acid methyl groups. In the present study the R_f value of TLC confirmed the presence of fatty acid as an active component. Thus the compound is elucidated as long chain fatty acid. Still, further analytical assays are required to elucidate the structural aspect of the compound and hence NMR and FTIR studies were performed.

In the present study, the NMR spectral analysis revealed the presence of long chain fatty acid. Based on the ^{1}H NMR and ^{13}C NMR studies the compound was further confirmed as a fatty acid methyl ester (FAME) compound. Numerous NMR spectral studies were performed to elucidate structure of bioactive components from plant source. Piotr Michel *et al.,* (2017) have studied the range of metabolites in *Gaultheria procumbens* plant by NMR spectral methods to assay the structural characterization of numerous long chain fatty acids. In the present study, the elucidated FAME compound from

ethanolic extract of *S.molesta* was known to possess numerous biological and pharmaceutical roles. A closely related compound *Methyl-4, 8-dimethylundecanate*, a novel fatty acid isolated from actinomycete *Micromonospora aurantiaca* was found to possess significant pharmaceutical role (Jeroen Dickschat *et al*., 2011). Agoramoorthy *et al*., (2007) have reported the antibacterial and antifungal activity of FAME compounds present in *Excoecaria agallocha* mangrove plant species. Ama *et al*., (2015) have reported the oxidative stress potentiality of fatty acid methyl ester compounds present in extracts of *Albizia chevalieri* on Juvenile African Catfish (*Clarias gariepinus*).Since the ethanolic crude extracts of *Salvinia molesta* was found to possess significant antibacterial and antioxidant role and they were further utilised for study in animal models to evaluate the *invivo* potentiality of the compound.

Studies on the immunity of crustaceans have focused on identifying defence mechanisms and biochemical pathways activated during an infection and cellular factors responsible for the destruction of pathogens, regulation, and damage repair. Crustaceans possess an open circulatory system, where nutrients, hormones, oxygen, and cells are distributed in the hemolymph. The hemocytes (circulating cells) could be functionally analogous to vertebrate leukocytes, because they are mainly involved in non-self matter recognition and elimination (Lorena Vazquez *et al*., 2009). Invertebrates, lacking adaptive immune systems, have developed defense systems that can respond against

antigens on the surface of potential pathogens. The defense mechanisms of crustaceans depend totally on the innate immune system of the animal that is activated when pathogen-associated molecular patterns are identified by soluble or by cell surface host proteins.

In invertebrates, the physical barriers are the first obstacle to detain pathogenic micro-organisms. When there is an infection and the micro-organisms invade the tissue, proteolytic pathways takes place immediately, allowing elimination or destruction of microbes invading the organism. The effector mechanism of invertebrate immune responses include, coagulation cascade which is the main agent helps to avoid the loss of hemolymph and stimulates oxidative metabolites and production of melanin by activating the ProPo system. Prophenoloxidase activation stimulates other important processes in the immune response, such as phagocytosis, encapsulation and nodule formation (Vargas and Yepiz, 2000).

Research in the field of crustacean immunity has revealed that they have the arsenal to defend themselves from disease causing agents. The increasing changes in the environment due to natural and more often man-made destructions causes an compromise in their immune system. Knowledge on the action of cellular and humoral factors of crustaceans, particularly crabs, fishes and shrimps, during pathogenic attacks and during the presence of various environmental conditions and contaminants is scanty (Laura *et al*., 2006). Research in the use of hemolymph factors as crustacean health markers is

encouraging, since it helps to study the effects of environmental deterrents and in turn, to control their effect on culture animals.

When we use higher vertebrate models like mice and rabbits many ethical issues may arise pertaining to experimental works. Therefore, use of alternative organism is required to replace vertebrate animals (Sonali and Shashikant, 2015). Invertebrate organisms are widely used as an alternative for laboratory animal study. Comparatively, invertebrates hold numerous benefits, such as a brief life cycle, small size and simple anatomy, so that a large number of invertebrates can be studied in a single experiment within a short period which is less time consuming and cost effective (Susan and Wilson, 2011).

In the present study, the freshwater crab, *Oziotelphusa senex senex* is used as the experimental model. Numerous researches have been carried out using *Oziotelphusa senex senex* owing to the abundant availability of the species and easy handling and less mortality. The immune strength of both male and female *Oziotelphusa senex senex* was assessed in the present study when treated with medicinally rich ethanolic leaf extract of *S.molesta*. The reason to choose both male and female groups is to find the efficacy of the *S.molesta* extracts among female groups comparing with male groups, since female crabs need to be studied for its mortality rates during hatchery periods and hence should be treated with a effective alternate medicinal tool against pathogen attack. In the present study, both the experimental groups when

bacterially challenged by *A.hydrophila and P.aeruginosa* showed varying positive responses in biochemical and immunological parameters.

Many immune protection studies were performed in crustaceans using medicinal plants, especially *O.senex senex* was treated and analysed for changes in immune parameters by administrating medicinal plant products by many researchers. Kumaran *et al*., (2013) has reported the immune response by *Morinda tinctoria* methanol leaf extract in a *Vibrio parahaemolyticus* infected *O.senex senex.* Devakumar *et al*., (2011) has reported the restoration of marker enzymes when treated with herbal extract in *Pseudomonas aeruginosa* infected *Oziotelphusa senex senex* groups. Dayananthan Devakumar *et al*., (2014) has reported the immunomodulation by *Psidium guajava* leaf extract in *A.hydrophila* infected fresh water crab, *O.senex senex* which is highly related to the present study.

Many aspects of the crustacean immune system are now comparatively well characterized and documented. Lot of immunology research has been conducted on crab, crayfish and lobster using synthetic drug components which may cause severe side effects in predominant usage and may lead to serious life threatening consequences. Hence more safer drug parameters needs to be implemented for safer treatments (Sritunyalucksana *et al*., 2001).

Biochemical parameters are the major suitable tools for assessing environmental influences and stress effects of anthropogenic origin on the

condition and health of aquatic invertebrates since there is a close association between the circulatory system of crustaceans and the external environment (Celi *et al*., 2015).The effect of external stressors and toxic substances on exposed animals could be manifested through clinical diagnosis of its physiology. The body components like protein, carbohydrate and lipid play a significant role in body construction and energy production. They are involved in major physiological events and the assessment can be considered as diagnostic tool to determine the physiological phases of organism (Jiyavudeen and Puvaneswari, 2016).

In the present investigation carbohydrate content decreased significantly on infection by both the bacterial cultures *viz*., *A.hydrophila* and *P.aeruginosa* whereas increased significantly in both male and female groups and attained closer physiological values mentioning the normalising role of *S.molesta* leaf extract in *O.senex senex.* Srivalli *et al*., (2013) have stated that the decreased 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. Thus the *S.molesta* leaf extracts might be clearing the toxicity in hemolymph of *O.senex senex* and hence retaining back the physiological levels.

In crabs, carbohydrates constitute only a minor percentage of total biochemical composition. Carbohydrates in fishery products contain no dietary

fiber but only glucides, the traces of glucose, fructose, sucrose and other mono and disaccharides. Harikrishnan *et al.,* (2003) have reported a study on hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *A.hydrophila* infection and had reported compatible results elucidating the positive changes in biochemical and immune parameters.

In the present study, total protein levels decreased significantly in both the bacterial infected groups and reached normal levels on treatment with ethanolic leaf extract of *S.molesta* at 96 hrs of treatment period. Srivastava *et al*., (2002) observed a declining trend in proteins of muscle and liver after exposure of *Channa punctatus* to zinc. Rama Srivastava and Neera Srivastava (2008) have reported that the decline in protein levels is due to proteolytic effect caused due to chronic infection.

In the present study, the total cholesterol levels decreased significantly on infection. Whereas, in both the treated groups there was a significant increase in cholesterol levels at 48 hrs period. The levels of cholesterol at 96 hrs of treatment were closer to control values illustrating the pharmacological role of ethanolic leaf extract of *S.molesta*. Maruthi and Subba Rao (2000) have reported that the decrease in cholesterol levels are due to the effect of toxicant or infection which may induce disruption of plasma membrane and lead to altered steroidogenesis. Muley *et al*., (2007) reported that the decrease in cholesterol levels is due to the utilization of lipid to meet the additional energy

requirement under stress conditions of the animal. Thus ethanolic leaf extracts of *S.molesta* were able to alter the pathological conditions of the animal and could able to restore the physiological cholesterol level.

In the present study, the levels of microprotein decreased significantly both *A.hydrophila and P.aeruginosa* infected groups, whereas increased sequentially in all treated groups. The levels of microprotein were restored significantly on treatment with *S.molesta* leaf extracts at 96 hrs of incubation period reporting the therapeutic property of the extract which could cease further proteolytic process. Devakumar *et al.*, (2015) have reported the total biochemical changes in *A.hydrophila* infected and *P. guajava* leaves treated *O.senex senex* groups. In his study the levels of microproteins, protein, glucose and cholesterol restored to physiological levels in *O.senex senex* on treatment with *P. guajava* leaf extract proving the potent activity of the leaf extract in maintaining the basal metabolism of the crab.

Total and differential haemocyte counts are the important immunological parameters required mandatorily to assess the primary physiological state of the animal. The THC and DHC are the total number of cells present in milliIiter of haemolymph and is an indicator of the number of cells available for defence reactions and depicts the immune strength of a system (Aladaileh *et al*., 2007).

The circulating haemocytes play extremely important roles not only by direct sequestration and killing of infectious agents but also by synthesis of a battery of bioactive protein molecules. Importantly, the haemocytes execute inflammatory-type reactions such as phagocytosis, haemocyte clumping, production of reactive oxygen intermediates and the release of microbicidal proteins (Lambert *et al*., 2003).

Parrinello *et al*., (2015) has reported that classification of hemolymph components is generally based on the presence or absence of certain components such as cytoplasmic granules, and three types of circulating hemocytes (differential hemocytes). They are usually classified as hyalinocytes (cells without evident granules), semigranulocytes (containing small granules) and granulocytes (with abundant cytoplasmic granules. Each cell type is more active during defence reactions, with the hyaline cells mainly involved in phagocytosis, the semigranular cells in encapsulation, and the granular cells in the storage and release of the prophenoloxidase (ProPo) system and cytotoxic factors (Giulianini *et al*., 2007). The proportion and percentage of hemocyte types in the hemolymph of varies species differs accordingly. Total hemocyte counts (THC) and differential hemocytes counts (DHC) have been reported as stress indicators (Lorenzon *et al*., 2008) and may be valuable tools for monitoring the health status of crustacean species.

In the present study, the total hemocyte count significantly increased in both *A.hydrophila and P.aeruginosa* infected groups and the ranges of THC

were high at 96 hrs depicting the immune response of the hemolymph system when experienced a pathogen attack in the system. Both male and female infected groups when treated at 96hrs of incubation period, the levels returned to physiological levels mentioning the potent role of *S.molesta* extract treatment. The hemocyte counts increased significantly in all infected groups and decreased in all the treated groups and thus *S.molesta* could immune modulate the system and agent can regulate the levels of hemocyte during post infection period.

Similar changes were found in differential hemocytes cell counts ,were there was significant increase of LGC, SGC and significant decrease of HC in both male and female *A.hydrophila and P.aeruginosa* infected groups, whereas in the treated groups all the obtained values were closer to the normal physiological values.

The prophenoloxidase system is a complement like enzyme cascade, responsible for the formation of melanin. In lower vertebrates and in invertebrates, this dark pigment is deposited in the presence of microorganisms. Prophenoloxidase, a zymogen, is converted to active phenol oxidase, the tenninal enzyme in the system. Quinones are by-products of this system and are capable of pathogen destruction. Phenoloxidase is also involved in non-self recognition as well as generation of opsonins and release of cell adhesion proteins (Perazzolo *et al*., 2002). Phenol oxidase enzyme is mainly present in the large granule haemocytes. Thus, they are the main performers in

encapsulation, which ultimately leads to the deposition of melanin. Phenoloxidase is sometimes seen diffused in the granular and electron dense cytosol of large granule haemocytes.

In the present study, prophenoloxidase levels decreased significantly during infection at 96 hrs of infection as a reflection of pathogenic attack. Whereas when treated with relevant concentrations of *S.molesta* leaf extract, the ProPo levels were significantly retained back to physiological levels.

Kumaran *et al.,* (2013) reported the change of immunity parameters on treatment with plant source and has recorded the relevant changes in immune parameters such as THC, DHC and ProPo. Bernard Mark Asirvatham and Sekhar (2015) reported the antibacterial and immunostimulant activity of *Psidium guajava* leaf extract against the *V. harveyi* infected freshwater crab, *O.senex senex* and had recorded the relevant positive changes in total hemocyte ,differential hemocyte counts in treated groups thus elucidating the role of hemocytes in performimg immune functions.

Non-specific ACP and ALP are important for regulation of various metabolic processes that occur by phosphorylation and dephosphorylation with kinases (Zhou *et al*., 2008). ACP, a significant enzyme for intracellular digestion of phagocytized antigens, has been used as a marker of macrophage activation in mammal (Meng *et al.*, 2003). In crustacean cells, phosphatase is the most important element of lysosomal enzymes, which performs the double

function of digestion and defence (Zhang *et al*., 2005). It is generally accepted that an increase of enzyme activity in the extracellular fluid or plasma is a sensitive indicator of even minor cellular damage, since the levels of these enzymes increase relative to those in the extracellular fluids by more than the normal levels (Van der Osst *et al*., 2003).

Acid phosphatase (ACP) is a lysosomal enzyme and cellular damage is usually accompanied by increase in the activity of this enzyme. Alkaline phosphatase (ALP) is a brush border enzyme and involves in transport and transphosphorylation reactions. These enzymes are used as a potential biomarker for a variety of different organisms due to its high sensitivity and less variability among species and often easier to measure as stress indices (Sanjib Saha *et al*., 2009)

They hydrolyze a large variety of organic phosphate esters to form alcohol and phosphate ions. Their probable function is the transfer of phosphate group from a donor substrate to an acceptor compound containing a hydroxyl group. Thus, these are involved in several processes such as dephosphorylation, degradation of mucopolysaccharides, hydrolysis of acylglycerol and hydrolization of peptides bearing free amino acid groups. Acid phosphatase is activated by acidic pH while alkaline phosphatase requires alkaline pH. Joshi and Kumar (2001) have studied the acid and alkaline phosphatases activity in different tissues of freshwater crab, *Paratelphusa masoniana* when exposed to

pesticides and have reported that there was noticeable changes in the levels of ACP and ALP during stress conditions.

In the present study, in both male and female group the levels of ACP and ALP was high on infection with bacterial agents, whereas the levels decreased and reached physiological values in all the treated groups when ethanolic leaf extract of *Salvinia molesta* was ingested. There was a significant decrease in levels of ACP and ALP at 96hrs were observed in treated group hemolymph of *O.senex senex*.

In crustacean immune defence research, a thorough understanding of haemolymph function is important, but has not been studied in detail; in particular, the capacity to overcome oxidative stress responses, such as reactive oxygen intermediates (ROS). When micro-organisms are engulfed by hemocytes, a bulk of antimicrobial substances is generated. These substances include highly reactive oxygen species, such as superoxide anion, hydrogen peroxide, hydroxide ions, and singlet oxygen. Though oxygen is an essential element for aerobic cells, it causes potential cytotoxic problems by generation of highly reactive oxygen species in the respiratory process. The effective and quick elimination of reactive oxygen species (ROS) is mandatory for the proper functioning and survival of organisms. This is performed by anti-oxidant defence mechanisms that can scavenge the superoxide anion (De la Fuente and Victor, 2000).

The term “antioxidant” refers to any compound, molecule or substance capable of stabilizing or deactivating free radicals before they attack cells. Humans already possess highly complex antioxidant systems (enzymic and nonenzymic), which can work synergistically, and also in combination with each other to protect the cells and organ systems of the body against free radical damage. The antioxidants can be endogenous or obtained exogenously as a part of a diet or as dietary supplements. Some dietary compounds that do not neutralize free radicals, but enhance endogenous activity may also be classified as antioxidants (Khalid Rahman, 2007).

To eliminate the excess of ROS produced during many physiological and pathological processes, numerous ROS defence enzymes or antioxidant system are required to protect the host from the toxic effects by the activated oxygen species (Hanschmann *et al*., 2013). Hai-Peng Liu *et al*., (2010) reported that there are prominent group of antioxidant enzymes available in crustacean immune system that can scavenge the infectious state of an animal system. These enzymes include Superoxide dismutase (SOD), Catalase, Glutathione peroxidise (GPx), Glutathione-S-Transferase (GST), Glutathione Reductase (GR), Reduced glutathione (GSH) and Lipid peroxidase (LPO). Previous studies showed that a balance between the activities and the intracellular levels of these enzymatic and non enzymatic antioxidants are necessary for healthy survival of the animal.

The enzymatic antioxidants SOD, CAT, GPX, GST and GR play an important role in the biological system to act against the oxidative stress (Ahmed, 2014). SOD is the first antioxidant enzyme which scavenges superoxide radicals and CAT is responsible for detoxification of H_2O_2 formed as a result of the reaction catalyzed by SOD. Whereas the levels of GPX, GST and GR are equally important in contributing the major percentile in improving the immune parameters of a system.

In the present study conducted, the hemolymph of both male and female crabs were assayed for above discussed major antioxidant systems and it was observed that there was significant relevant changes in the levels of SOD, CAT, GPX, GST, GR, GSH and LPO due to the high antioxidant potentiality of *S.molesta* mentioning the improvement in immune strength of the animal so that the system can fight against further infections.

Superoxide dismutase (SOD), are those antioxidant enzymes which can rapidly convert the very unstable superoxide into the more stable hydrogen peroxide (H_2O_2). Superoxides are produced from molecular oxygen due to oxidative enzymes of body as well as *via* non-enzymatic reaction such as auto oxidation by catecholamines. Superoxide dismutase catalyses the dismutation of the highly reactive superoxide anion to oxygen and hydrogen peroxide (Yulema Valero *et al*., 2015).

Paital and Chainy (2010) have reported the importance of SOD and its antioxidant defence in oxidative stress parameters in tissues of mud crab, *Scylla serrata*. Nathan and Cunningham-Bussel (2013), have reported that though SOD can scavenge free radicals, high levels of SOD may damage the host tissues beyond its repairing role. Since there must a homeostasis to be maintained to regulate the levels of SOD.

In the present study, the superoxide dismutase (SOD) levels reduced significantly in infected groups and the levels slowly increased with time and reached physiological levels in treated groups and sustained further. And hence the retained levels on treatment stayed closer to physiological levels, proving *S.molesta* extracts are capable of regulating the levels of SOD in the hemolymph of *O.senex senex*.

Catalase is a hemoprotein which catalases the reduction of hydrogen peroxides and protects tissues from highly reactive hydroxyl radicals (Indradevi *et al*., 2012). Lan Wang *et al*., (2011) have reported the changes in levels of CAT during oxidative stress conditions in the freshwater crab, *Sinopotamon henanense.* Atli *et al*., (2006) have studied the response of catalase activity in five tissues of freshwater fish, *Oreochromis niloticus* and reported that CAT may be considered as a sensitive bio indicator of the antioxidant defense system.

In the present study, the levels of catalase in the hemolymph of *Oziotelphusa senex senex* decreased significantly in both *A.hydrophila and P.aeruginosa* infected groups and on administration of ethanolic leaf extract of *S.molesta* to the infected crabs, the levels were restored close to 96 hrs of incubation.

The glutathione system consists of reduced glutathione, glutathione reductase, glutathione peroxidase and glutathione S-transferases. Glutathione peroxidase (GPX) is an enzyme that catalyzes and breakdown the hydrogen peroxide and organic hydroperoxides. GPX consists of a family of antioxidants that are involved in the reduction of hydroperoxides using glutathione as an electron donor.

In the present study, glutathione peroxidise levels significantly decreased in the hemolymph of infected male and female groups, whereas there was a significant increase of GPX in *S.molesta* treated groups. Jinxiang Wang *et al* ., (2013) have reported similar result in cadmium exposed freshwater crab, *Sinopotamon henanens* and mentioned that the "adaptive stage" and the "inhibitive stage" of GPX enzyme is due to the initial exposure time which efficiently attenuated the accumulation of H_2O_2 to maintain a normal cellular balance.

Glutathione S-transferase is another class of glutathione dependent antioxidant enzymes that exhibits high activity with lipid peroxidase. These

enzymes are found high in the liver, helps in detoxification metabolism (Sharma *et al*., 2004). It is involved in the detoxification and conjugation of xenobiotics and in protecting against peroxidative damage. Hayes et *al*., (2005) reported that GST's protect tissues from endogenous organic hydroperoxides produced during oxidative stress thus developing resistance against infectious agents. High levels of GSTs have consistently been observed in resistant insect strains and play a major role in insecticide resistance (Alias and Clark, 2007).

In the present study, GST levels decreased significantly in the hemolymph of *A.hydrophila and P.aeruginosa* infected male and female groups. Whereas there was a increase in GST levels in *S.molesta* treated groups illustrating the pharmacological role of the leaf extract.

Glutathione reductase (GR) is a vital enzyme that reduces glutathione disulfide (GSSG) to the sulfhydrl form (GSH) by the NADPH- dependent mechanism, an important cellular antioxidant system. Due to its significance, the enzymes has been purified from a number of animals, plants and microbial sources and studied in an effort to identify and explain its structure, kinetic mechanism and molecular properties (Linster and Van Schaftingen, 2007). Iskusnykh *et al*., (2013) have studied the expression of glutathione reductase levels in hepatitis rats and reported that increase in levels of GR is due to formation of a cascade mechanism which was formed to scavenge the free radicals along with lipid peroxidation system.

The levels of glutathione reductase significantly reduced in hemolymph of both male and female infected groups, whereas showed increased levels in *S.molesta treated* groups at different time intervals.

Lipid peroxidation mediated by free radicals is considered to play a major role in antioxidant mechanism in tissues. Increased LPO is the index of oxidative stress in the animals. In the present study both male and female infected crab groups showed significant increase in LPO levels whereas there was a decrease in LPO levels on treatment with *S.molesta* ethanolic extract. Natalia Kurhalyuk *et al*., (2010) have reported that lipid peroxidation system is the major contributor to the loss of cell function under oxidative stress conditions. The peroxidation process was preceded by a decrease in the cell antioxidant defense system followed by the production of lipid and protein oxidation products. Gopalakrishnan Singaram *et al.*, (2013) have reported the changes in serum LPO of *Scylla serrata* when been exposed to higher mercury levels. Correlative relationships between peroxidative parameters and antioxidant enzyme activities elucidating the importance of peroxidation process following the consumption of the antioxidant defense system. Usually levels of GSH will be depleted during scavenging of ROS. Such depletion of antioxidant defenses due to exposure to pathogens could result in greater susceptibility to lipid peroxidation. Hence levels of LPO play a vital role in normalising the immune parameters of a crab system. In the present study, the levels of GSH decreased significantly during infection period, whereas there

was an increase in GSH levels at treated groups indicating its coordination with the pharmacological role of *S.molesta* leaf extracts.

Nitric oxide is a free radical generated by endothelial cells, macrophages, and neurons etc., and involved in the regulation of various physiological processes (Smitha *et al*., 2013). Excess concentration is associated with several diseases. It reacts with oxygen to produce its stable product nitrate and nitrite through intermediates NO_2, N_2O_4 and N_3O_3. Singaram Gopalakrishnan *et al*., (2011) have reported the increased levels of nitric oxide in lipopoysaccharide challenged *Scylla paramamosain* crab indicating the response of immune system to infection.

In the present study, the levels of nitric oxide increased significantly in all the infected groups whereas the levels of nitric oxide decreased by 96 hrs of treatment by ethanolic leaf extract of *S.molesta.*

To conclude, the present study revealed that *S.molesta a* freshwater weed is available abundant in nature but still not explored in detail for its medicinal properties. The present study of the aquatic weed in animal models revealed that *Salvinia molesta* leaf extracts possess remarkable pharmacological and therapeutic values and can act as an potent pharmaceutical drug. The preliminary phytochemical analyses data supports the medicinal value of the weed and its potentiality to act as an medicinal agent. The aquatic weed also possesses significant antibacterial activity against

selected pathogens by virtue of its bioactive constituents. On quantification of the phytochemicals it was more evident that there were significant levels of phytoconstituents available in the ethanolic leaf extract of *Salvinia molesta.* The compounds identified by GCMS, NMR and FTIR studies also revealed the presence of therapeutically important compounds which are the major reason for antibacterial, antioxidant and immunomodulatory activity role of the *S.molesta* leaf extract. When the experimental animal *O.senex senex* were bacterially challenged by two major fish pathogens, *A.hydrophila and P.aeruginosa* and treated with relevant concentrations of ethanolic leaf extract of *S.molesta*, positive changes in biochemical and immunomodulation parameters was observed in both male and female infected groups indicating the significance of *S.molesta* as a therapeutic agent. Owing to the pharmacological significance of the plant extracts more clinical studies can be performed in large scale attributes to understand the role of *Salvinia molesta* leaf extract as a pharmaceutical and therapeutically potent drug source in treating wide range of infections in aquaculture sectors and other higher animals.