CHAPTER 5: ASSESSMENT OF EFFICACY AND TOXICITY OF CGE-1

5.1. Introduction

Decapod crustaceans are widely cultured in many countries around the world [data from Food and Agriculture Organization (FAO) of the United Nations]. Aquaculture production of these species has shown dramatic decrease in the contemporary years and the sustainability of crustacean farming is at present threatened by reduced production efficiency and susceptibility of the farmed crustaceans to diseases [1-4]. This has generated renewed concern to undertake the validation of novel growth enhancer to advance the growth rate of the species [5]. The exploration for cost effective, nutritionally adequate and practical feed sources has been directed towards the development of this alternative strategy. CGE-1 is designed to complement the necessity of novel approach to accelerate the growth and development, reduce the fattening period, accelerated weight gain, increase the degree of breeding and survival and develop the disease resistant capability.

Appropriate objectives, model organism and experimental design are essential to assess the activity and safety of novel way to enhance the growth in decapod crustaceans. Spiny lobsters including Panulirus homarus have received pronounced attention for aquaculture in India because of high foreign exchange earnings, demand for live lobsters in several countries and the decreased production of shrimps due to various diseases. Lobster production prospective is being gradually discovered throughout the world and
the introduction of cost effective and easy to use growth enhancers can assist in optimum utilization of the available lobster resources thus expanding aquaculture production. A drastically reduction in lobster production is observed in India due to overwhelming fishing pressure, over exploitation and antimicrobial resistance due to the use of antibiotics as growth promoters. Hence, the experiments were designed to evaluate the important differences between experimental and control groups in end points using spiny lobster, *Panulirus homarus* as model organism that replicate advantage of CGE-1 to decapod crustaceans. Such experiments deliver the gold standard to assess the efficacy and toxicity of novel growth enhancer. These experiments normally include various end points reflecting potential benefit of growth enhancer such as the molting frequency, weight gain and assessment of numerous other biochemical parameters such as total protein, carbohydrate and high density lipoproteins from various organs and hemolymph of *Panulirus homarus*.

Eyestalk ablation is widely used for rapid growth and breeding for induced maturation [6-13]. Hence, eyestalk ablation has been shown to result in shortening intermolt and advanced gonad development but it also causes a significant mortality in decapod crustaceans due to imbalance in various physiological functions [14-16]. In the present study, the effect of CGE-1 on crustacean growth and reproduction was evaluated using various experiments and compared with eyestalk ablation. The experiments were designed to substantiate that the CGE-1 has a superior replacement of several conventional methods such as bilateral/unilateral eyestalk ablation, use of antibiotics as feed additives, use of cholesterol rich diet etc. to stimulate growth and development in
decapod crustaceans. Since these conventional methods pose a risk towards human health, crustacean physiology and environment; this study also evaluates the plausible toxic effects of CGE-1 on *Panulirus homarus* by several biochemical and histopathological analysis and to demonstrate that this approach is superior to other conventional methods.

5.2. Materials and Methods

5.2.1. Analysis of Efficacy of CGE-1

5.2.1.1. Experimental Animals and Conditions

Male and female *Panulirus homarus* were purchased from local fisherman at Kanyakumari, Tamil Nadu, India and held in recirculating tanks at environmental conditions matching the condition of their natural habitat. The lobsters were fed with food pellets once in a day during night. Water temperature was held at 28 ± 2°C and salinity was maintained at 28‰ under a photoperiod of 12 h light: 12 h dark [17]. Water quality was retained by circulating the complete volume of water through a filter. *Panulirus homarus* ranging in weight from 55 to 101g were selected for the study and the length [total length, carapace length and width of each individual lobster was carefully measured using calipers.

To study the effects of CGE-1 on *Panulirus homarus*, 90 spiny lobsters were divided into nine groups of 10 [Table 5.1]. Molt staging was performed as described previously [18-21] and after acclimatization, the first group was served as controls injected with lobster saline [460mM NaCl, 13mM KCl, 13mM CaCl₂, 10 mM MgCl₂,
1.7mM Glucose, 10mM HEPES (pH 7.4)] [22, 23]. A premolt concentration (113.1ng/g of body weight) of 20-hydroxuedysone was injected into the intermolt Panulirus homarus through the sinus of the fifth walking leg. Bilateral eyestalk ablation was performed by cutting the eyestalk from their bases with the help of fine sterilized scissors. These groups i.e., bilateral eyestalk ablated and 20-hydroxyecdysone injected animals were used as positive controls during the experiment. Since there is no data available on the use and dosage of growth enhancers on crustaceans, the dosage were calculated by the computed effective concentration for Daphnia using Accelrys Discovery Studio 2.5. As per the prediction, the dosage were divided into three groups and injected with CGE-1 into the intermolt lobsters through the sinus of the fifth walking leg. The high dose group animals were injected with 254.5μg/g of CGE-1 while the medium and low dose group animals were injected with a concentration of 150μg/g and 53.6μg/g of CGE-1 respectively. For assessing the impact of CGE-1 on oral administration, the CGE-1 was orally administered in slurries prepared from anchovy fish pellets. The fish pellets were processed with seawater (2ml/g) to a smooth consistency. CGE-1 was prepared as suspension solution in carboxymethyl cellulose and added to three slurries in amounts that facilitated doses of 254.5μg/g, 150μg/g and 53.6μg/g to be administered in 1ml or less of slurry. The lobsters were administered with fresh slurries on each treatment day. All the animals were observed for physiological changes in hemolymph for three successive molting and subsequently the animals in all groups were sacrificed and various parameters were analyzed.
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<tr>
<th>S. No.</th>
<th>Name of the groups</th>
<th>No. of lobsters [Male + Female]</th>
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<tr>
<td>1.</td>
<td>Control</td>
<td>5+5</td>
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<tr>
<td>2.</td>
<td>Bilateral eyestalk ablation [EA]</td>
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<td>3.</td>
<td>20-hydroxyecdysone injected [EI]</td>
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<td>Low dose Injected [LDI]</td>
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<td>High Dose Oral [HDO]</td>
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Table 5.1: Design of experimental study to evaluate the effectiveness and toxicity of CGE-1.

5.2.1.2. Assessment of Growth

The carapace length, total length and body weight [24, 25] of every individual organism from different experimental groups were measured after every successive molting. The measurements of growth of molted lobsters were taken two days after ecdysis when the exoskeleton was sufficiently rigid to make sure accurate measurements. The carapace length (CL) of lobster was calculated as the distance between mid-dorsal [from the transverse ridge between the frontal spines] to the posterior margin of the cephalothorax.
5.2.1.3. Ecdysteroid Assay

The 20-hydroxyecdysone is synthesized and secreted from Y-organ in decapod crustaceans [31, 32]. Hence, the hormone level was quantified from Y-organs as well as hemolymph and correlated with the different molting stages [33]. For circulating ecdysteroid titer, hemolymph samples were extracted with 90% methanol and the supernatants were dried. The dry samples were then re-dissolved in water to their original volume [34]. The enzyme immunoassay (EIA) was performed as per the manufacturer instruction using specific antibodies raised against 20-hydroxyecdysone to quantitate the circulating ecdysteroid for both control and experimental lobsters at different time intervals throughout the molt cycle [35]. After three successive molting the Y-organs from all experimental lobsters were dissected in ice cold phosphate buffered saline and the ecdysteroid titer was quantified.

5.2.1.4. Biochemical Analysis of Hemolymph

The collection of hemolymph was performed at 0, 2, 4, 8, 12 and 24h post-treatments from CGE-1-injected and then subsequently on day 3, 5, 7, 14, 21, 28 or until the three consecutive molting completed. Lobsters were bled by piercing the third walking leg with a sterile needle [26, 27]. 100μl of hemolymph from each experimental animal were collected at each time interval and stored into an ice cold Eppendorf tube containing 1 mg of EDTA.
Hemolymph glucose level plays an important role as an indicator of fractional or complete failure of the physiological response and therefore glucose was quantified using GOD/POD method to monitor stress in all experimental groups [28]. Total protein quantification from hemolymph was used as an index for lobster health and vitality on the use of CGE-1 [29]. The protein titers in live lobsters are directly associated with the lobster food intake and hence, these levels were used as a nondestructive indicator physiological processes. Total protein was quantified using biuret assay during molt and between the experimental groups. Similarly, the high density lipoprotein (HDL) was quantified using direct enzymatic method [30].

5.2.1.5. Biochemical Analysis of Muscle and Hepatopancreas

Hepatopancreas and muscle were collected from each experimental lobsters and various biochemical analysis were performed. Total carbohydrate [36] and total protein [29] from both the organs were quantified to monitor the variation due to the CGE-1 treatment. Similarly, The HDL was extracted and quantified from hepatopancreas describes the correlation between reproduction and growth. HDL is required for the synthesis of vitellogenin, an egg yolk protein responsible for reproduction. HDL was also quantified from muscle to monitor the nutritional value of lobster from various experimental groups.

5.2.1.6. Analysis of Hepatosomatic Index (HSI)

Hepatosomatic index was calculated as the function of HDL absorption and vitellogenin production. In crustaceans, hepatopancreas plays a vital role in absorption
and storage of high density lipids derived from food and also serves as an organ for vitellogenesis. Vitellin is an egg yolk protein and hence, hepatosomatic index was used as quantitative method to verify reproduction as well as the gonadal development [37]. Hepatosomatic index was calculated using following formula:

\[
\text{Hepatosomatic index (HSI)} = \frac{H_w}{T_w} \times 100
\]

5.2.1.7. Molt Mineralization Index (MMI)

The mineralization of each lobster was determined in terms of gastrolith development in relation to the molt mineralization index (MMI). Molt mineralization index is defined as the ratio of gastrolith to the carapace length of the lobster. The deposition of calcium was observed by using X-ray radiograph analysis during premolt and post molt [38]. The X-ray radiography was adjusted to 60kV and exposure time 0.05s to avoid overexposure and underexposure.

5.2.1.8. Measurement of Cyclic Nucleotides

After three successive molting, the Y-organs from all the experimental lobsters were excised during intermolt. After collection, pair of Y-organs were transferred separately to micro centrifuge tubes and stored at −80°C until the assay was performed. Y-Organs were disrupted by homogenizer for several seconds in 0.1M HCl (200μl) in ice cold condition. The homogenate was transferred into centrifuge tubes and centrifuged (600×g, 20 min) at room temperature. cGMP quantification was performed
with the aliquots of the supernatant as per the protocol provided by the manufacturer (Cayman Chemical, Ann Arbor, MI). The data was represented as pmol/mg protein.

5.2.1.9. Analysis of Cuticle

After third successive molting, scanning electron microscopy (SEM), elemental analysis and FT-IR of cuticle was performed to perceive the plausible alterations in the network architecture during intermolt stage.

(a) Field Emission-Scanning Electron Microscopy [FE-SEM]

After the third consecutive molting, the mineralized cuticle were removed carefully from intermolt lobsters from all the experimental groups and cleaned with excess of tap water to remove cell debris. The dried samples were dipped in liquid nitrogen to enhance its brittleness and broken into small sections. These sections were then prepared for scanning electron microscopic (SEM) analysis [39, 40]. Sections were kept in vacuum chamber and coated with gold using Autofine Sputter coater [Jeol, Japan]. The gold sputtering was allowed for 1-3 minutes depending upon the size of the sample and analyzed using FE-SEM [model no. JSM-6701F], Jeol, Japan at an accelerating voltage of 10–15 kV with an upper detector (SEI-Secondary Electron Image) and a lower detector (LEI-Lower Electron Image).

An energy dispersive X-ray analyzer (EDX) was used in combination with FE-SEM to perform elemental examination of selected microscopic areas for the cuticle sample. The presence of two important elements calcium and phosphorous were
evaluated using FESEM-EDX which allows the qualitative and quantitative examination for three-dimensional deviations in elemental compositions of cuticle through elemental maps, line and spot scan.

(b) FT-IR Analysis

Amorphous CaCO$_3$, calcite and chitin are the three main component of lobster exoskeleton. Fourier transform infrared (FTIR) spectroscopic study of the exoskeleton after final molt was executed on a Perkin Elmer (Spectrum 100) equipped with a DTGS detector. For FT-IR analysis, the cuticles were carefully exuviated from the lobsters of each experimental group and washed with water several times to remove cell debris and external organic layer [39, 41]. The samples were dipped in liquid nitrogen to make them brittle and then ground into the fine powder by means of an agate mortar and pestle. The powdered cuticle samples were prepared in KBr pellets for FT-IR analysis and the finding were compared with the standard values published by [41].

5.2.1.10. Histological Evaluation of Hepatopancreas, Muscle, Ovary and Gills

After sacrificing the lobsters, all the organs were immediately dissected and fixed for eight hours in Bouin’s fluid. The fixed tissues were dehydrated using graded ethanol and cleared in xylol. The organs were then fixed in paraffin and 5μm sections were used for histological analysis and the sections were stained with hematoxylin and eosin (HE). The stained sections were washed and then analyzed using light microscopy [42, 43].
5.2.2. Statistical Analysis

One-way or two-way ANOVA were performed by using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). The level of significance was set at P≤0.001. All the assays were performed in triplicate.

5.3 Results and Discussion

5.3.1. Effect of CGE-1 on Weight Gain, Molt Frequency and Size Increment

In crustaceans, as molting progresses, the body weight, total length and carapace length are also relatively increases [44]. The calculated length-weight correlation therefore creates evidences for the overall health of crustaceans, efficacy of growth enhancer, discrepancy in growth and development according to sex and sexual maturity. Study of the length and weight of crustaceans after every successive molting has extensive use in describing the growth patterns during the experimental time. The intermolt stage is the longest period in crustaceans molt stages and hence, shortening of intermolt stage directly correlates with the higher efficiency of the growth enhancer.

As predicted from the in-silico drug designing, the efficacy of CGE-1 was directly related to the molting frequency in lobsters. The CGE-1 administration triggered substantial changes in molting frequency in a dose-dependent fashion. The most remarkable change was a gradual increase in the molt frequency in injected groups, which initiated around day 5 and the pattern was continued during the course of the experiment. The medium and high dose experimental groups were molted three times in
16 days as compared to control where the three molting were concluded in 43 days [Figure 5.1]. A similar fashion was observed in all the experiments.

Initially, the intermolt period presented a stable increase with size, but in high dose groups the intermolt period was considerably lesser as compared to the control lobsters after each consecutive molting. A higher mortality rate was observed in bilaterally eye-stalk ablated animals as compared to the CGE-1 administered.

Figure 5.1: CGE-1 treatment shortens molt cycle duration in *P. homarus*. CGE-1 assists an advanced molting frequency followed by eyestalk ablated and ecdysteroid injected experimental groups. Molt durations were measured in three consecutive molt cycles and results are expressed as mean number of days ± SEM. Asterisk (*) depicts significant decrease in intermolt stage in high dose lobsters as compared to
control. The duration of molting is described as first molting (■), Second molting (■), third molting (■) for all experimental groups.

In correlation with the molting frequency, a significant increase in total length, carapace length and carapace width was observed in the experimental group. A 3.3 mm increment in carapace length was observed as compared to 2.5mm in control lobsters. The 90% weight gain was observed in medium and high dose experimental group lobsters and it is incomparable with the control, eyestalk ablated and ecdysteroid injected lobsters where the weight gain ranged between 40-50% [Figure 5.2].

![Figure 5.2: The changes in mean weight gain (%) for the experimental groups of *P. homarus*. Both sets of treated groups gained significantly more weight than the control.](image-url)
respective sets of control groups (data represented as mean ± SEM where P<0.001 and n=10 for both ‘oral’ and ‘injected’ groups).

It is rather interesting to note that the CGE-1-induced size increment and weight gain was observed even at low and medium dose experimental groups that had only a little or no effects on the other parameters.

Furthermore, the pattern depicts that the increase was gradual during the progression of the CGE-1 administration at higher doses of CGE-1. In fact, our study revealed that the CGE-1-induced weight gain and molt increment was accompanied with the shortening of intermolt, as observed in all the treated groups.

It is also evident from the study that the bilateral eyestalk ablation and ecdysteroid injection has a minor effect on these parameters as compared to the CGE-1 dose groups. Additionally, a significantly higher mortality was observed in ablated animals most likely due to the imbalance in the various physiological processes since the X-organ/sinus gland complex is accountable for neuroendocrine regulation in decapod crustaceans. However, it is apparent from the study that such abnormalities in physiology are not induced in lobsters treated with various doses of CGE-1.

These results indicate that the negative response of molt inhibiting hormone was competitively inhibited by CGE-1. Also, a prominent increase in the molting frequency
of bilaterally ablated Panulirus homarus as compared to the saline treated lobsters has proved the presence of synthesis and secretion of MIH from the x-organ/sinus gland complex present in the eye-stalk of the crustaceans. Several reports suggested the presence of extra eye-stalk MIH secretory centers in various decapod crustaceans but as per our results from bilateral eye-stalk ablation studies the quantity is probably not sufficient as to affect moulting. The eyestalk ablation study in P. argus has suggested that the molting and gonadal development is not a simultaneous process as measured by weight gain, carapace length and gonadosomatic index; these observations leads to the hypothesis that MIH may play an important role in reproduction [45].

Later, it was substantiated that molt inhibiting hormone stimulated vitellogenesis in crustaceans. Since, the administration (injected/oral) does not affect the synthesis or secretion of MIH and only competitively inhibit the binding of MIH to the Y-organs, it is possible that both the processes i.e., molting and gonadal development can be work at the same time. The results from our studies has clearly demonstrated that CGE-1 is able to increase molt frequency, weight gain and carapace length regardless of the size/weight group, reproductive status and seasons, indicating the competitive inhibition of MIH binding to the Y-organs and an stimulating effect of MIH on vitellogenin production.

5.3.2 Ecdysteroid Quantification

As we have discussed earlier that the periodic molting is crucial for growth in crustaceans. In decapod crustaceans, the process of molting is triggered by 20-hydroxyecdysone synthesis and secreted by the Y-organs [46, 47]. A lower
hemolymphatic ecdysteroid titer is observed during the intermolt stage which surges to a threshold level during premolt stage and then drops to an undetectable amount after ecdysis [48-50]. These changes are directly linked to the inhibition and stimulation of ecdysteroid synthesis in the Y-organs. Since the molt inhibiting hormone suppresses ecdysteroidogenesis during considerably greater period of molt cycle, and molting triggers when MIH secretion diminishes. The hemolymphatic concentration of ecdysteroid determines the onset of molting and also the efficacy of a competitive inhibitor. As per long standing model which suggests that the level of ecdysteroid in hemolymph increases during premolt and reaches to its minimal level after ecdysis, our findings also established this hypothesis where hemolymphatic ecdysteroid titer was elevated during late premolt, and then dropped significantly during post molt.

During the experiment, 20-hydroxyecdysone was quantified initially for staging the molt cycle and an ecdysteroid level of 377ng/ml was observed. During experiment the ecdysteroid titer changes significantly in all the experimental groups at different molting stages. It was observed that the level was significantly higher in medium and high dose experimental groups (479.71ng/ml) as compared to saline control during premolt stage having a concentration of 377ng/ml [Figure 5.3].
Figure 5.3: Changes in hemolymph ecdysteroid concentration (20E) titer during different molting stages [Non detectable amount: Intermolt/Post molt, 50-470ng/ml: premolt stage]. Higher dose of CGE-1 causes a large, ephemeral increase in ecdysteroid concentration [479ng/ml] in the hemolymph as compared to controls [377ng/ml]. Values are represented as mean ± SEM, n=10. Lobsters were sampled at various time intervals and ecdysteroid was quantified with enzyme-immunoassay.
(EIA). Dotted line represents the significant increase in the ecdysteroid titer before ecdysis.

As anticipated, there was no significant quantity of ecdysteroid was estimated in Y-organs dissected from intermolt lobster after the third molt. These result also established that the binding of CGE-1 to the Y-organ cells is reversible and it does not cause a continuous uncontrolled growth in the organism. The ecdysteroid level were initially quantified from control animals at various molt stages and compared with the experimental animals [Figure 5.4].

![Figure 5.4: Stage dependent mean variation in 20-hydroxyecdysone titer in hemolymph of *P. homarus*. The lobsters were acclimatized and sampled at different time intervals and the molt staging was performed in accordance with the mean](image)

Figure 5.4: Stage dependent mean variation in 20-hydroxyecdysone titer in hemolymph of *P. homarus*. The lobsters were acclimatized and sampled at different time intervals and the molt staging was performed in accordance with the mean
ecdysteroid titer of male and female *P. homarus* for a successive molting. Results are expressed as mean ± SEM (P<0.001).

5.3.3 Variation in Hemolymph Glucose

The hemolymph glucose did not show a significant variation throughout the study on all experimental groups. As expected, the level was significantly higher after two hours of treatment. This is due to the stress caused by removal of organism from water for treatment and the injection or orally administration of compound or saline. The level got subsidized after 2nd hour of treatment, which suggests that the CGE-1 did not cause a prolonged stress to the lobsters [Figure 5.5]. Interestingly, the level of glucose remains low throughout the study on bilaterally eyestalk ablated animals due to the absence of crustacean hyperglycemic hormone (CHH) in the physiological system [Figure 5.6]. The CHH activates gluconeogenesis in the animals during the stress conditions which cause a sharp rise in the hemolymph glucose. Certain studies has shown previously that the level of glucose gradually reaches to normal level after several weeks of eye-stalk ablation, that suggests that the existence of the secretory centers other than X-organ/sinus gland complex in the nervous system of decapod crustaceans. Similar effect was observed in our experiments where the level of glucose progressively increases after the first week of eyestalk ablation. The changes in the hemolymph glucose can also be stage specific. As explained earlier, the level of glucose was found to be higher during the ecdysis as compared to the intermolt period. Since, the stress response is one of the most significant physiological processes in crustaceans and glucose estimation is among
conventional method of stress determination. The study has indicated that the CGE-1 did not cause a permanent stress to the animals as it was also reflected by the less mortality as compared to the bilateral eye-stalk ablated experimental group.

Figure 5.5: Time course activity of CGE-1, 20E and eyestalk ablation on hemolymph glucose titers in Panulirus homarus in relation to controls. Values are expressed as means ± SD. The level of hemolymph glucose in eyestalk ablated group was
significantly lower compared to eyestalk intact lobsters (p < 0.001). Asterisk represents maximum hemolymph glucose level was observed at 2h after initiating the experiment.

Figure 5.6: Mean changes in the hemolymph glucose titer during experiment. There was no significant difference was observed in the metabolic changes associated with CGE-1 administration but a lower glucose titer was detected in eyestalk ablated (EA) groups. The glucose was quantified at different time intervals as a function of stress caused during experiments. Asterisk represents the significant lower hemolymph glucose level during the course of the experiment. Mean ± SEM (P<0.001).
5.3.4 Variation in Hemolymph Protein Level

The results of hemolymph protein titer were in accordance with the previous studies where it has been seen that the level of hemolymph proteins varies according to the various molt stages. During the experiments, a higher protein concentration is observed during the premolt stage due to the presence of cuticular proteins secreted by hypodermal cells in all the experimental groups. These proteins also assist in the synthesis and hardening of new exoskeleton during postmolt. The glucose titer is a classical indicator of stress in crustaceans which may be caused as a result of traumatic physical and chemical environmental variations [Figure 5.7].
Figure 5.7: Total protein levels in the hemolymph of *Panulirus homarus* during the experiment. Hemolymph protein content was compared in different experimental groups at various molting stages in spiny lobster, *Panulirus homarus*. A comparable similar hemolymph protein concentration was observed between the control, EI, EA and CGE-1 injected/oral experimental groups during the intermolt stage. A substantial upsurge in hemolymph protein was observed just prior to ecdysis in all experimental groups due to the presence of cuticular proteins. Values represent mean ± SEM. (n=10 for each point, P<0.001).

### 5.3.5 Quantification of High Density Lipoproteins from Hemolymph

High density lipoprotein plays a vital role in the vitellogenin synthesis. Hence, an altered level signifies the problem in the reproduction. Our experiments have clearly demonstrated that the CGE-1 did not cause significant changes in the level of HDL
during the course of the study. The level was stable between 40-50mg/ml of hemolymph in all the experimental groups [Figure 5.8].

Figure 5.8: Time and molt related differences in quantity of high density lipoproteins (HDL) in hemolymph of *Panulirus homarus* during the experiment. No significant difference of hemolymph HDL was observed between the control, EI, EA and CGE-1 injected/oral experimental groups. The values given are the mean ± SEM (n = 10 and P<0.0001).
5.3.6 Biochemical Analysis of Muscle

Decapod crustaceans especially spiny lobsters are essential candidate species for mariculture and sea farming throughout the world due to their greater economic and nutritional values. Currently several conventional foods are available for lobster fattening such as clam meat, green mussel and sometimes even smaller crustaceans. Higher cost, their seasonal unavailability, requirement of deep freeze for storage, compromised water quality and also a risk of transmission of diseases are some of the disadvantages when these feedings are used. The reason to develop CGE-1 was to overcome these problems without compromising their nutritional values. Hence, the biochemical analysis of tail muscle was performed to determine the effect of CGE-1 on the nutritional value of Panulirus homarus.

In muscle, the total protein concentration was higher in medium (21.787mg/100g) and high dose group (21.878g/100g) lobsters as compared to control lobsters (19.669g/100g) [Figure 5.9]. A similar pattern was observed in ecdysteroid injected and eyestalk ablated lobsters where total protein concentration was higher than control but slightly lower than CGE-1 administered lobsters. There was no significant difference in the total carbohydrate was observed in all experimental groups. The total carbohydrate quantity was recorded 1.5, 1.6 and 1.6 g/100g in control, bilateral eyestalk ablated and high dose experimental group lobsters respectively [Figure 5.10]. Lobster muscle is also a great source of high density lipoproteins and it was observed that the concentration was marginally higher in CGE-1 administered experimental groups as compared to the controls [Figure 5.11].
Figure 5.9: Relative changes in total protein from muscle. The total protein quantification was used to evaluate the effect of CGE-1 upon the nutritional value of lobster, *P. homarus*. The treatment of CGE-1 led to an enhanced protein synthesis capacity as compared to control. Increased muscle protein content under CGE-1 administration suggests an affinity growth of tail muscle without causing damages in its structure. Results are expressed as the standard error of the mean where n=10 (P<0.001).
Figure 5.10: Total carbohydrate content from muscle. No significant difference of total carbohydrate was observed between the control, EI, EA and CGE-1 injected/oral experimental groups. Data represents as mean ± SEM [n=10, p<0.0001].
Figure 5.11: Variation in muscle HDL concentration after three molts in *P. homarus*. CGE-1, EI and EA caused a small, transient increase in high density lipoprotein concentration in the muscle (values are mean ± SEM, n=10). The dotted line represents the mean quantity of HDL from saline control group.
5.3.7 Biochemical Analysis of Hepatopancreas

Similarly, in case of hepatopancreas, there were no significant changes were observed in total protein [Figure 5.12] and carbohydrate [Figure 5.13] in medium (1.571g/100g) and high dose (1.60g/100g) as compared to control (1.521g/100g). But, the a higher level of high density lipoproteins was observed in treated lobsters as compared to controls probably due to the increased food intake and the result was correlated with the significant increased in the weight gain [Figure 5.14]. The results shown a higher level of high density lipoproteins (HDL) in the hepatopancreas of CGE-1 treated experimental groups especially in female lobsters and it was even higher than that of bilaterally eye-stalk ablated lobsters.

These results suggest that a higher fatty acid quantity could be linked with gonadal maturation. As discussed earlier phospholipid rich diet increases the efficacy of vital fatty acids due to more effective transport and improved lipid mobilization from the hepatopancreas to various other organs through hemolymph, causing a greater lipid deposition and improved energy accessibility for growth and development [51-53]. Similarly, the results from our study can be directly correlated with the higher level of HDL in hepatopancreas and a greater molting frequency. This has been appropriately replicated in the muscle and hepatopancreas of control and experimental animals.
Figure 5.12 Group based differences of hepatopancreas protein concentration (g/100g) in *P. homarus*. Muscle protein concentration differed significantly among the experimental groups (data represented as mean ± SEM, n=10, P<0.0001). Muscle protein concentration in HDI and EI was significantly higher than control. There was a significant positive correlation between muscle protein concentration and molting frequency indicating a strong relation between them.
Figure 5.13: Total carbohydrate content from hepatopancreas. A similar profile of total carbohydrate content was observed between the control, EI, EA and CGE-1 injected/oral experimental groups. Error bars indicate the standard error of the mean (n=10, P<0.0001).
Figure 5.14: Post experimental HDL quantification from hepatopancreas of *P. homarus*. There was a marked increase in the amount of lipoproteins when compared to control group HDL content. Such a difference was observed in both oral and injected groups. Results are expressed as the mean ± SEM (n=10, P<0.001).

5.3.8 Analysis of Hepatosomatic Index (HSI)

Gross indices are indicative of toxic effect of a compound and the hepatosomatic index (HSI) plays a vital role to demonstrate these effects on the health of crustaceans. The condition of the hepatopancreas and a ratio to the whole body, as evaluated through the hepatosomatic index (HSI) can deliver evidence on potential toxic impacts of CGE-1. HSI was used as an early screening biomarker to specify the effect of exposure of various concentrations CGE-1 that helps to understand the overall health of lobster population,
and it also serves as a good indicator of quality of the habitat and pollution levels [54-56]. It has been demonstrated that the toxicity of any compound leads to significantly lower values of HSI and a higher value reflects a better growth of the crustaceans [57, 58].

The lobster hepatopancreas is comprises of four key cell types, E-cells, F-cells, B-cells and R-cells. These cells play a vital role in the general functions of digestion, absorption, secretion, osmoregulation and detoxification. Hence, the evaluation of hepatosomatic index provides significant information about the intracellular digestion of CGE-1 and excretion of CGE-1 breakdown products. In the present study, a significantly higher hepatosomatic index was observed in medium and high dose experimental group lobsters (3.7) as compared to control (3.1), eyestalk ablated (3.3) and ecdysteroid injected (3.34) lobsters [Figure 5.15]. Hepatosomatic index reflects the size of hepatopancreas as compare to their body weight. It is very significant because in crustaceans the hepatopancreas plays a vital role not only in vitellogenin synthesis but it also helps in detoxification of the body. Hence, these results showed that the CGE-1 does not cause toxicity to the hepatopancreas.
Figure 5.15: Molt related variations of the hepatosomatic index over three consecutive molting cycles for specimens of *P. homarus*. The error bars represent the standard error of the mean for n=10 samples. The results of hepatosomatic index (HIS) were comparable with the controls for different experimental groups with lower P-value (P< 0.0001).

5.3.9 Effect of CGE-1 on Molt Mineralization Index (MMI)

In this study we followed the reverse dynamics of calcium reabsorption and its deposition in the form of gastrolith during premolt and the calcification of new cuticle during the postmolt by means of variations in their precise densities as detected in X-ray digital radiographs. The alterations in density precisely reveal the comparative mineral
content in each calcification site and, consequently, offer a precise method for evaluating the calcification state in the living crustacean. The accumulation of mineral and size of gastrolith in subsequent premolt period provides vital information about the absorption of mineral as compared to the size of the exoskeleton. The MMI index was calculated on the first premolt (initial) and compared with the MMI of the premolt stage of third successive molting (final).

It was observed that the molt mineralization index was higher in low (0.089), medium (0.089) and high (0.091) dose experimental groups as compared to the control (0.074), eyestalk ablated (0.079) and ecdysteroid injected (0.0781) groups [Figure 5.16].

Figure 5.16: Progression of gastrolith growth (expressed as MMI values) in the three consecutive molt cycles. The values were synchronized and are presented as mean ± SEM. The significant difference between the molt mineralization index between initial and final molts were represented as circles and squares respectively (p<0.0001).
During premolt stage, the cuticular proteins assist the reabsorption of amorphous calcium carbonate and stored in the body of lobster along with a very small amount of calcite. An increase in molt mineralization index correlated with the size increment of the lobster as this will help to form a new cuticle during post molt.

5.3.10 Quantification of cGMP in the Y-organs

Previous results have demonstrated the stage-specific changes in 20-hydroxyecdysone level in *Panulirus homarus* which consistent with the results that MIH mRNA abundance [59] and in MIH content in the sinus gland [50] inversely effect its production. It has been demonstrated in several decapod crustaceans that cGMP plays a vital role in the signal transduction in the Y-organs to inhibit the ecdysteroidogenesis. The signaling cascade activates when the MIH binds to its cognate receptor which consecutively inhibit the expression of phantom gene which encodes an enzyme critical for the synthesis of 20-hydroxecdysone from cholesterol.

The combined results from all these studies are in accordance with the hypothesis that variations in ecdysteroid secretion are governed by fluctuations in the MIH titer with a greater inhibition of ecdysteroidogenesis during intermolt period. Though, two current reports recommend that degree of ecdysteroid synthesis and secretion are also influenced by stage-specific variations in the receptiveness of the Y-organs to molt inhibiting hormone [60, 61]. As discussed earlier, the involvement of second messenger in synthesis and secretion of ecdysteroid by Y-organs have been explored in limited species [62, 63], and they demonstrated the role of cGMP on MIH based pathway [64, 65].
In studies reported here, the level of intracellular cGMP in the Y-organs was significantly higher during intermolt period of all experimental groups. The level of cGMP remains high throughout the intermolt period and the level becomes undetectable during late premolt which directly related the onset of ecdysis. To know whether the CGE-1 binding to its receptor is reversible or not, the cGMP quantification was performed. The level of cGMP during the intermolt in all CGE-1 administered experimental groups was practically similar to that of control lobsters [Figure 5.17].

Thus, ecdysteroid production by Y-organs was suppressed by cGMP and also CGE-1 does not cause an adverse effect on the molt cycle when the dosing discontinued. The collective outcomes from the above experiments back the conclusion that inhibition of ecdysteroid secretion by MIH is mediated by a cGMP second messenger and the binding of CGE-1 to the Y-organs is reversible and work in a dose dependent manner.
Figure 5.17: Effect of CGE-1 on the accumulation cGMP in Y-organs of *P. homarus*. Y-Organs were removed from lobster (in intermolt stage after recovering from the final molting) and the cGMP was determined by enzyme immunoassay (EIA). Results are expressed as mean ± SEM (n = 10, P< 0.001). A similar impact of CGE-1 was observed in the regulatory pathway of ecdysteroid synthesis in the Y-organs after the completion of experiments.
5.3.11 Effect of CGE-1 on Exoskeleton

The exoskeleton of crustaceans is a rigid structure which shields the soft body parts for defense from its predators. The deposition of calcium carbonate provides the desired rigidity to the crustacean exoskeleton after each molt. The present study evaluates the influence of CGE-1 on the exoskeleton physiology and biochemistry such as formation, various layers, presence of minerals and rigidity of the exoskeleton in relation to the molt cycles. We analyzed the cuticle on the basis of three main objectives which comprises (i) whether the CGE-1 causes an adverse effect on the reconstruction of chitin matrix after third consecutive molt as compared to the control lobsters (ii) how calcium carbonate is stacked into the chitin matrix and (iii) the presence of various minerals in the cuticle. The assessment of these areas of emphasis offers a basis on efficacy and toxicity of developed growth enhancer, and it also demonstrated that the drug can be potentially applied to other decapod crustaceans.

The exoskeletons of the spiny lobster, *Panulirus homarus* was analyzed with structural and chemical methods. The results suggested that the exoskeletons comprise of calcite, amorphous calcium carbonate, amorphous calcium phosphate (ACP), and chitin. The results from the study also revealed that the predominant mineral in the lobster cuticle is amorphous calcium carbonate embedded in the chitin matrix.

Scanning electron micrographs of the cuticle has shown that there were no significant changes in the formation of chitin matrix after three consecutive molt cycles
in experimental lobsters as compared to the control [Figure 5.18]. The matrix is mainly composed of α-chitin calcium carbonate [Figure 5.19] was observed in all the experimental groups.

The elemental analysis using FE-SEM for exoskeleton confirmed the presence of calcium and phosphorus in all the experimental groups and the occurrence was similar to that of control lobsters [Figure 5.20]. The FT-IR spectra provide crucial information about the presence of various functional groups and elements in the exoskeleton. In this study, the analysis was performed to analyze the presence of three main component including calcium carbonate (calcite and amorphous) and chitin. The probable changes in FT-IR spectra of the cuticle after the completion of three molt cycles in all experimental groups were recorded. The spectrum of amorphous calcium carbonate (868.12, 1072.58, 1420-1474cm\(^{-1}\)) and chitin absorption at 1650cm\(^{-1}\) was similar in all the experimental groups which demonstrate that the CGE-1 does not cause a toxic effect on the lobsters [Figure 5.21]. The results of SEM, FT-IR and elemental analysis were similar and it substantiated that CGE-1 does not interfere with the ion channel or the structure of cuticle.
Figure 5.18 Scanning Electron Microscope (SEM) micrograph of cross-sectional fracture surface of *P. homarus* chitin matrix after three successive molting. No significant difference was observed between control (a), eye-stalk ablated (b), ecdysteroid injected (c) and CGE-1 administered low (d), medium (e) and high dose groups (f). Similar outcomes were seen among CGE-1 oral and injected groups.
Figure 5.19: Scanning electron micrograph of calcium deposition in the cuticle of *P. homarus*. The cuticle was examined at various magnifications to observe the possible defects in calcium deposition during intermolt period after third molting. No significant differences were observed between the experimental groups even at high magnification as compared to control. Figure represents control (a), eye-stalk ablated (b), ecdysteroid injected (c) and CGE-1 administered low (d), medium (e) and high dose groups (f). Similar outcomes were seen among CGE-1 oral and injected groups. The calcium deposition was also analyzed at higher magnification (g, h, i) but no significant changes were observed.
Figure 5.20: Elemental analysis of fractured cuticle from *P. homarus* using scanning electron microscopy. Three major components of cuticle, carbon, calcium and phosphorus were analyzed from different locations in the cuticle. No significant difference was observed between control (a), eye-stalk ablated (b), ecdysteroid injected (c) and CGE-1 administered low (d), medium (e) and high dose group (f). Similar outcomes were seen among CGE-1 oral and injected groups.
Figure 5.21: Fourier transform infrared spectroscopy (FTIR) spectra of the cuticle during the intermolt in *P. homarus*. Solid vertical lines represent the main peaks in the vibrational spectrum of amorphous calcium carbonate (868.12, 1072.58, 1420-1474 cm\(^{-1}\)). The dashed vertical line represents the main chitin peak (1655.06 cm\(^{-1}\)). Similar pattern of spectrum for calcium carbonate and chitin was detected in all experimental groups. No significant difference was observed between control (a), eye-stalk ablated (b), ecdysteroid injected (c) and CGE-1 administered low (d), medium (e) and high dose group (f). Similar results were seen between CGE-1 oral and injected groups.
5.3.12 Effect of CGE-1 on Various Organs of *Panulirus homarus*

The aim of this study was to histologically evaluate various organs of *Panulirus homarus* in intermolt stage after three successive molting with the intention of providing significant information on the impact of CGE-1 in relation to the stress and cellular deformities and/or lesion.

(A) Effect of CGE-1 on Hepatopancreas

Hepatopancreas from all the experimental groups which includes both the sexes of *Panulirus homarus* were observed by means of histological techniques [Figure 5.22]. The tubules form the structure of hepatopancreas with limited intertubular space. Every tubule comprises of a rod-shaped layer of epithelial cells which are enclosed by myoepithelial cells and a basal lamina. The effect of CGE-1 was assessed in all the four major cell types of tubules i.e. E-cells, F-cells, B-cells and R-cells which provide complete regulation of digestion, absorption, secretion, osmoregulation and detoxification respectively. The results evidently indicated that CGE-1 does not cause cellular defects or lesion on the digestive cells, absorptive and secretory line of hepatopancreas.

(B) The effect of CGE-1 on Male and Female Reproductive System

The effect of CGE-1 on female reproductive system was evaluated in relation to the various ovarian development stages. The representative sections which illustrates germinative zone (CZ), Oocyte I (oI), Oocyte II (oII), Oocyte III (oIII) and Oocyte IV
(oIV) were analyzed for cellular damage and/or lesion [Figure 5.23]. The results indicated that CGE-1 did not cause any significant impairment of ovarian structure or function in female *Panulirus homarus*. Similarly, the cellular modifications due to CGE-1 on male reproductive system of *P. homarus* were examined histologically. In male reproductive system, simple squamous epithelium with spermatozoa (SZ) in the lumen covers the testicular lobule. After the third successive molting, the effect of CGE-1 was also carefully observed on the germinative zone (GZ), testicular cysts (TC) and proximal vas deferens of testes which is lined by a simple cylindrical epithelium (EP) with spermatozoids. No toxicity symptoms were observed even at higher doses of CGE-1 in the examined photomicrographs which indicate that the CGE-1 does not cause an adverse effect on gonadal development in male *Panulirus homarus* [Figure 5.24].

**(C) Effect of CGE-1 on Muscle**

In this study, as an outcome of the CGE-1 treatment of lobsters, no adverse effects were detected on the morphology of the gills [Figure 5.25] and tail muscle [Figure 5.26] of the experimental lobster groups. These observations could be a remarkable indicator for safety and non-toxicity of CGE-1 on decapod crustaceans. Histological sections of the gills and tail muscle derived from experimental lobsters treated with CGE-1 until three consecutive molts appeared normal when compared with the control group.
Figure 5.22: Cross sections (5μm) of *P. homarus* hepatopancreas. Light micrograph of cross sections of hepatopancreas tubules showing a lumen, uniform epithelium (E), and B-, F-, and R-cells. (F-cells are small and located between the R- and B-cells). No significant difference was observed between control (a), eye-stalk ablated (b), ecdysteroid injected (c) and CGE-1 administered low (d), medium (e) and high (f) dose groups. Similar outcomes were seen among CGE-1 oral and injected groups.
Figure 5.23: Effect of CGE-1 on gills of *P. homarus*. Light micrograph of a hematoxylin and eosin stained cross-section through the row of gill filament and lamellae. No significant difference was observed between control (a), eye-stalk ablated (b), ecdysteroid injected (c) and CGE-1 administered low (d), medium (e) and high (f) dose groups. Similar outcomes were seen among CGE-1 oral and injected groups.
Figure 5.24: Hematoxylin and eosin stained 5μm sagittal section trough the muscle of *P. homarus*. No structural changes in the fibrillar muscle were observed between the experimental groups. Figures represents control (a), eye-stalk ablated (b), ecdysteroid injected (c) and CGE-1 administered low (d), medium (e) and high (f) dose groups. Similar outcomes were seen among CGE-1 oral and injected groups.
Figure 5.25: Effect of CGE-1 on the ovary of female *P. homarus*. No significant changes were observed due to treatment on gonadal development, germinative zone (CZ), Oocyte I (oI), Oocyte II (oII), Oocyte III (oIII) and Oocyte IV (oIV). No significant difference was observed between control (a), eye-stalk ablated (b), ecdysteroid injected (c) and CGE-1 administered low (d), medium (e) and high (f) dose groups. Similar outcomes were seen among CGE-1 oral and injected groups.
Figure 5.26: Effect of CGE-1 on male reproductive system of *P. homarus*.

Photomicrographs of a cross section of male reproductive system stained with hematoxylin and eosin was observed from various experimental groups. Testicular lobule surrounded by a simple squamous epithelium with spermatozoa (SZ) in the
lumen. Testes showing the germinative zone (GZ) and testicular cysts (TC) and proximal vas deferens is lined by a simple cylindrical epithelium (EP) with spermatozoids. No significant difference was observed between control (a), eye-stalk ablated (b), ecdysteroid injected (c) and CGE-1 administered low (d), medium (e) and high (f) dose groups. Similar outcomes were seen among CGE-1 oral and injected groups.

5.4 Conclusion

The best growth enhancer, CGE-1 was validated with the intention of developing evidence-based tool for harmless and more effective approach to stimulate the growth in decapod crustaceans. Although there are various practices adopted including the bilateral eyestalk ablation or the use of antibiotics that influence molting, none of them has been identified for their ability to enhance molting without cause any harm to the animal or environment. Current evidence from structural analysis of MIH, although confirmed on spiny lobsters, suggests that the CGE-1 can not only be used on lobsters but also may have similar effects on different decapod crustacean species and facilitate the rapid growth. Among those treated with CGE-1, the high dose group (both oral and injected) exhibited a considerably better molting frequency in a specified period of time than other experimental groups. The high dose group results a 62% shortening of intermolt period as compared to the saline control after three consecutive molting. Assessment of contemporary methods with compared to the designed growth enhancer clearly indicated that there was a significant difference in hemolymph ecdysteroid titer and intermolt
period. Since, the eyestalk secretes several neurohormones, most of these methods produce various biological effects other than regulating the ecdysteroid production. In addition, CGE-1 did not exhibit any sign of toxicity, either histologically or biochemically, in animal model when compared with the control groups. Since, most of the species of decapod crustaceans are highly economically important and have a high export value, it offers convincing expectation in the enhancing the growth of decapod crustaceans in a comparatively shorter time span without compromising with the nutritional value.

5.5 References


