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# Effect of a Novel Compound as Dietary Supplement on Growth of Decapod Crustaceans

Sajal Shrivastava and S. Adline Princy*

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Several conventional methods have been used for many years to accelerate the growth in decapod crustaceans but they are suitable for aquaculture practices due to their harmful effects on the animals. The concern over water quality, environmental hazard, human health and food safety has led us to a search for alternative targets to design growth enhancers which can significantly improve the growth of crustaceans without harmful effects. Among such compounds has been CGE-1, an amine compound which have shown excellent molt inducing property. The lobsters treated with CGE-1 exhibited a higher weight gain and greater increment in the carapace length post-molt as compared to the controls even without inhibiting the synthesis and secretion of molt-inhibiting hormone (MIH). This is a significant finding considering the fact that MIH bind specifically to the membrane receptor of y-organs, hence CGE-1 might interferes with this interaction allowing an incessant production of 2-hydroxyedysone (20E). Here, we summarize our findings on the CGE-1 and its effects with particular focus on inclusive growth and vitellogenesis. Our observations and findings suggest that CGE-1 could be a useful growth enhancer that could be used either alone or as a dietary supplement for the rapid growth of decapod crustaceans.

## 1. Introduction

Antimicrobial agents has been used as an essential part of feed to improve and accelerate the livestock production for several years.[1] The use of antibiotics at very high concentration specifically in aquaculture has become the driving force for the development of drug resistance microorganisms. There are several incidences of infections caused by these multi-drug resistant (MDR) strains associated with the livestock and also on the increase of use of antibiotics in the therapy. These issues also concomitantly cause economic cost on the production of aquaculture related animals.[2,3] Therefore, the there is a strong need to better understand the regulatory pathway associated with the growth and development of production animals. Design and development of growth promoters or competitive inhibitors that can cause a rapid growth is a relatively rare occurrence. However, there is great interest in research on such growth promoting agents, not only because they might be remarkably effective to promote growth of various economically important species without causing any adverse effect, but also because they can be useful probes to understand the hormonal regulation of various physiological processes.

Among such economically important marine species, decapod crustaceans specifically plays a vital role in the aquaculture and fisheries sector. Hence, understating their physiology can provide significant insights for the development of novel growth enhancers. In decapod crustaceans, the regulation of growth is governed by molt inhibiting hormone, a neurohormone from crustacean hyperglycemic hormone (CHH) family, synthesize and secreted from the x-organ/sinus gland complex in the eyestalk. CHH family neurohormones form extremely interactive systems to accomplish the integrated function of various physiological processes for decapod crustaceans.[4,5] A critical role in the ecdysteroid production has a key neuropeptides, known as molt inhibiting hormones. Molt inhibiting hormone is a single-chain 113 amino acid proteohormone composed of a 35 residue signal peptide and a 78 residue mature MIH that play a key role in the regulation of growth and reproduction.[6,7] Its amino acid sequence exhibits homologies to other neurohormones including crustacean hyperglycemic hormone and vitellogenin inhibiting hormone and hence, designated as CHH family neuropeptides.[8] MIH is produced and secreted by X-organ/sinus gland complex, which acts on the endocrine system, in particular the Y-organs, suppressing the production of 20-hydroxyecdysone.[9] Therefore, eyestalk ablation leads to an upsurge in the hemolymph ecdysteroid titer and shortening of intermolt period, whereas the eye-stalk ablated animals injected with crude eyestalk extract significantly lowers the ecdysteroid level and delays molting.[10] Additionally, *in vitro* studies have also revealed that the recombinant MIH and crude eyestalk extracts inhibits 20-hydroxyecdysone production by Y-organs. MIH has dual regulation in crustacean physiology. Functional studies have shown that this peptide exerts a stimulatory effect on reproduction by stimulating the vitellogenesis in the hepatopancreas.[11] Ecdysteroid hormones profoundly affect growth, differentiation, and metabolism. Regulation of ecdysteroid synthesis, principally achieved by molt inhibiting hormone, is a protuberant factor of a complex network that govern the normal growth and proper function of the decapod crustaceans.[12,13] The three residues Asparagine (13), Serine (71) and Isoleucine (72) of MIH interact with the receptor in the Y-organs[14,15] and activated the signaling cascade leading to a significant increase cGMP activity and inhibition of the phantom gene expression.[16-18] From these observations it is hypothesized that a growth enhancer derived from the residues of MIH responsible for binding to the receptor can lead to a precocious molting and thus stimulates molting.

*Phone: +91 4362 264101, Fax: +91 4362 264120
E. Mail: adlineprinzy@biotech.sastra.edu
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Consequently, elucidation of the 20-hydroxyecdysone production in Y-organs and the development of inhibitors to reverse or modulate the effect of MIH are important issues. Toward these objectives, pharmacophore-based ligand design has been employed to develop specific inhibitors, which has attained a gradually noticeable position within the computer assisted drug discovery process during the past few years.[19,20] Our results from the study describes the first specific, highly potent growth promoter which also provides information on regulatory mechanisms that form the basis of the MIH specificity towards its transmembrane receptor on Y-organs.

## 2. Materials and Methods

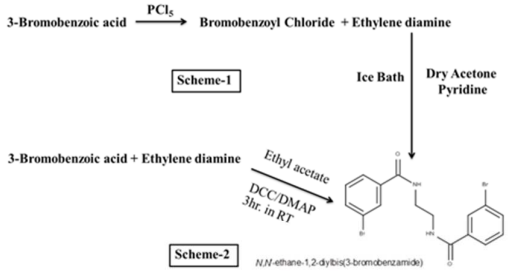
### 2.1. Design and synthesis of N,N'-ethane-1,2-diylbis (3-bromobenzamide)

**Pharmacophore feature generation and *in silico* screening**

The pharmacophore features, virtual screening and manual alterations were performed as described by earlier. [21] The HipHop Catalyst approach from Accelrys' Discovery Studio was used to generate common feature pharmacophores of binding site of the molt inhibiting hormone from five decapod crustaceans as described previously.[22] This approach utilizes the protocol which includes generation of three dimensional conformation model for each binding site, assigning them as active features and then use the common feature pharmacophores for drug screening. The pharmacophores features for two residues S71 and I72 were generated using the solution structure of molt inhibiting hormone from *Marsupenaeus japonicus* [PDB ID: 1J0T] and the 3-D structural libraries were virtually screened. The 3-D pharmacophores geometry serves as query in screening comprises of four features viz. hydrophobic groups, hydrogen-bond acceptor, hydrogen-bond donor, and ionizable groups. The screened hits from the compiled spatial arrangement of pharmacophores for two amino acids from MIH was then manually modified and then ranked according to their fit value. The best competitive inhibitor with highest fit value was then selected for further studies.

**Synthesis of crustacean growth enhancer, CGE-1**

The conventional dicyclohexyl carbodiimide (DCC) and Dicyclohexylcarbodiimide (DMAP) method which is used to synthesize amides was used to synthesize the CGE-1 (Scheme 1). The dicyclohexyl carbodiimide (DCC) was used as a coupling agent for the ester synthesis. The structure of CGE-1 was confirmed using $^{1}H$ and $^{13}C$-NMR.




**DCC**: N, N-Dicyclohexylcarbodiimide
**DMAP**: 4-Dimethylaminopyridine

**Scheme1. Route map for the synthesis of growth enhancer (CGE-1)**.

### 2.2. Analysis of efficacy of CGE-1

**Animals**

Male and female *Panulirus homarus* were purchased from local fisherman at Kanyakumari, Tamil Nadu, India. The lobsters were fed with food pellets once in a day during the night. Water temperature was held at 28 ± 2°C and salinity was maintained at 28%o under a photoperiod of 12 h light: 12 h dark. 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*, ninety spiny lobsters were divided into nine groups of ten. Molt staging was performed as described previously[23] and after acclimatization, the first group was served as controls injected with lobster saline [460mM NaCl, 13mM KCl, 13mM $CaCl_2$, 10 mM $MgCl_2$, 1.7mM Glucose, 10mM HEPES (pH 7.4)] and two of these groups were positive controls i.e., bilateral eyestalk ablated and 20-hydroxyecdysone injected animals. The three groups were injected with CGE-1 and remaining three groups were given CGE-1 orally in increasing increments as low, medium and high doses. 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. 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.

**Assessment of growth**

The carapace length, total length and body weight of every individual organism from different experimental groups were measured after every successive molting.[24] The measurements of growth of molted lobsters were taken two days after ecdysis when the exoskeleton was sufficiently rigid to make sure accurate measurements.

**Biochemical analysis of hemolymph**

The hemolymph was collected from third walking leg and assays were 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.

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 the GOD / POD method to monitor stress in all experimental groups.[25] Total protein quantification from hemolymph was used as an index for lobster health and vitality on the use of CGE-1.[26] 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.[27]

**Ecdysteroid assay**

The enzyme immunoassay (EIA) was performed as per the manufacturer's instruction (Cayman Chemical, Ann Arbor, MI) using specific antibodies raised against 20-hydroxyecdysone at different time intervals throughout the molt cycle.[28] 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.

**Biochemical analysis of muscle and hepatopancreas**
Total carbohydrate,[29] total protein[26] and HDL[27] from both the organs were quantified after three successive molts to monitor the variation due to the CGE-1 treatment.

**Analysis of hepatosomatic index**
Hepatosomatic index was calculated as the function of HDL absorption and vitellogenin production.[30] Hepatosomatic index was calculated by measuring weight of hepatopancreas to the total weight of the *P. homarus.*

**Molt mineralization index**
The molt mineralization index is defined as the ratio of gastrolith to the total weight of the lobster. The deposition of calcium was observed by using X-ray radiograph analysis (60kV, exposure time 0.05s) during premolt and post molt.[31]

**Measurement of cyclic nucleotide (cGMP)**
After three successive molting, the Y-organs from all the experimental lobsters were excised during intermolt. 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 were represented as pmol/mg protein.

**Analysis of cuticle**
After third successive molting, scanning electron microscopy and elemental analysis of cuticle was performed to perceive the plausible alterations in the network architecture during intermolt stage. Elemental analysis was performed to confirm the presence of calcium and phosphate in the exoskeleton. Similarly, Fourier transform infrared spectroscopy (FT-IR) spectra of the cuticle were recorded after the final molt. Calcium carbonate and chitin are the two main components of lobster exoskeleton. Hence, the peaks for both were analyzed using FTIR.[31]

**Histological evaluation of hepatopancreas, muscle, ovary and gills**
All the organs were dissected and fixed for eight hours in Bouin's fluid. 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.[32,33]

**Acute oral toxicity and cytotoxicity analysis**
The acute oral toxicity was performed as per the guidelines of the Organization for Economic Co-operation and Development (OECD). Acute oral toxicity was performed using 8-12 weeks old female Wistar rats to ensure the sensitivity of the test.

The cytotoxicity was performed using HepG-2 cell lines. Particulars of measuring cell growth inhibition using HepG-2 cells are described elsewhere. Briefly, cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and the $10^5$ cells/well were transferred in 96-well microtiter plate and incubated for 24h at 37°C with 5% $CO_2$. The medium was removed at the end of the incubation and cells were treated with different concentration of CGE-1 for 24 h in the settings stated above. The MTT solution was added and incubated for 20 min at room temperature. The percent cell viability was calculated by measuring optical density at 560nm.

## 3. Results and Discussion

### 3.1. Design and Synthesis of CGE-1

Pharmacophore based approaches were applied with the aim of design compounds having similar steric and conformational features of molt inhibiting hormone that would instead optimally bind to the receptor in the Y-organs. Ten distinct pharmacophore models were created and the significant interactions were considered by hydrophobic, hydrogen-bond donor or acceptor features. The best HypoGen model consisted of six pharmacophore features: one hydrogen bond acceptor, three hydrogen bond donor and two hydrophobic rings. The best pharmacophore model (Hypo1) was employed for virtual screening (3D database searching), including Lipinsiki's filter, to obtain a pool of more drug like molecules.[21] The compound screening was principally focused on providing the variety of comprehensive molecular data desired to facilitate the interaction of these compounds with y-organs. This includes spatial arrangements of atoms as compared to the selected amino acids from MIH, structure and pharmacological data about designed growth modulators. The designed molecules were ranked according to their fit values and N, N',di-(3-bromobenzoyl)diamine ethane (CGE-1) which exhibited the highest fit value of 5.6 was selected for further analysis. CGE-1 is unique, not only because of its structure but also in the level of fit value and depth of coverage of intrinsic features it achieves which is important to competitively inhibit the MIH-receptor interaction in the y-organs of decapod crustaceans. In addition to its extensive coverage to important features such as the presence of hydrophobic groups, hydrogen-bond acceptors and donors, CGE-1 demonstrated an excellent pharmacokinetic properties required for its usage as a growth promoter.

After analyzing the various properties of CGE-1, it was synthesized to assess its efficacy and toxicity *in-vivo.* As mentioned in the materials and methods section, two route map were established to synthesize CGE-1 but contrary to the acid chloride method for the synthesis of amides as depicted in the scheme-1, the scheme-2 for synthesis uses the mild environment and hence, avoids the use of toxic solvents for instance pyridine and strong reagents such as thionyl chloride. The compound was purified by column chromatography over silica gel followed by crystallization succeeded to obtain it as colorless crystals in good yield (65%). As shown in Fig. S1 and S2, the structure and composition of (1) are confirmed by $^1$H-NMR, $^{13}$C-NMR and further used for various biochemical and toxicological analysis.

### 3.2. Effect of CGE-1 on weight gain, molt frequency and increment

As predicted from the *in-slilico* drug designing, the efficacy of CGE-1 was directly related to the molting frequency of 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 (Fig. 1). In correlation with the molting frequency, a significant increase in total length, carapace length and carapace width were 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% (Fig. S3).

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 effect 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. Therefore, in the present study, all CGE-1 supplemented diets or injected doses resulted in a higher growth in *Panulirus homarus* than the control diets, signifying that the addition of CGE-1 improves the overall growth performance and diminishes the effects of stress factors which causes higher mortality in eye-stalk ablated animals during molting. Similar high mortality were observed by several researchers,[34-36] who studied the effect of unilateral and bilateral eyestalk ablation in decapod crustaceans. It is also evident from our study that the bilateral eyestalk ablation and ecdysteroid injection has a minor effect on these parameters as compared to the CGE-1 dose groups. 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.

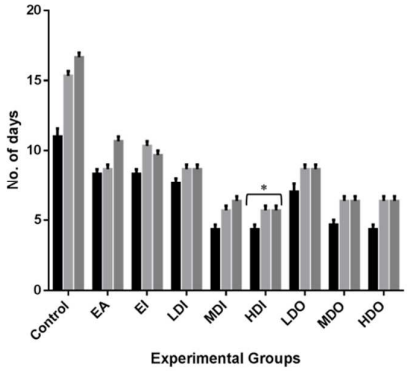



**Fig. 1. CGE-1 treatment shortens molt cycle duration in *P. homarus*.** Molt durations were measured in three consecutive molt cycles and results are expressed as mean number of days ± SEM. Asterisk (*) depicts significant decrease in intermolt stage. The duration of molting is described as first molting (■), Second molting (■), third molting (■) for all experimental groups. The group name represents: EA (eyestalk ablated), EI (Ecdysteroid injected), LDI (low dose injected), MDI (medium dose injected), HDI (high dose injected), LDO (low dose oral), MDO (medium dose oral), HDO (high dose oral).

### 3.3. Ecdysteroid quantification

Ecdysteroid was quantified initially for staging the molt cycle and an ecdysteroid level of 377ng/ml was observed. During the 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 controls during a premolt stage (Fig. S4). Individual animals treated with CGE-1 showed large, transient upsurge of 20E which increased in an extent with the commencement of premolt exhibiting a large premolt peak at this stage and gradually decreased in subsequent morphological stages. The level of 20E was remained significantly higher until ecdysis before reaching to the basal levels without causing any significant stress to the animals. There were no significant sex biased level of 20E was observed in the hemolymph of experimental animals and highly correlated at various molting stages. Also, the 20E quantitative values observed in the hemolymph with dose-supplemented diets were nearly equivalent to the injected group suggest that CGE-1 exhibit an enhanced absorption and distribution kinetics which permits a prompt interaction with the paired y-organs. As expected, there was no significant quantity of ecdysteroid was estimated in Y-organs dissected from intermolt lobster after the third molt. These results 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.

### 3.4. Variation in hemolymph glucose and protein

The hemolymph glucose did not show a significant variation throughout the study of all experimental groups. The results from this observation indicated that the injection cause a transitory hyperglycemia to the lobsters. The variation in the hemolymph glucose was occurred only during the few hours after the injection of CGE-1 and successively reached to the basal level rest of the time throughout the study. The glucose level was significantly higher after two hours of treatment and subsidized after two hour of treatment (Fig. S5). The changes were similar after every injection and this may be basically due to the time required for lobsters acclimatize to the CGE-1. It is evident from previous studies that the changes in the glucose level can cause due to the hypoxia, period of immersion and handling of animals. It became relevant to consider that during experiment the glucose levels and their period represent the acute stress in absolute terms and in all the cases there was no sustained hyperglycemia. Hence, considering these facts the stresses measured in *Panulirus homarus* as a function of hemolymph glucose level can be attributed predominantly by the handling, use of injection and blood sampling and hence, the CGE-1 in the hemolymph does not induce any stress to the animals. 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 (Fig. 2). During the experiments, a higher protein concentration is observed during the premolt stage due to the presence of presence of cuticular proteins secreted by hypodermal cells in all the experimental groups. These proteins also assist in the synthesis and hardening of the new exoskeleton during postmolt.

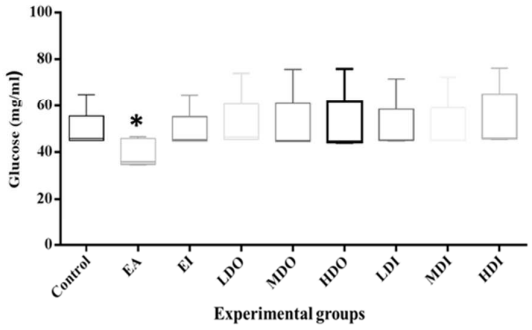



**Fig. 2. Mean changes in the hemolymph glucose titer during experiment.** The glucose was quantified at different time intervals as a function of stress caused during experiments. Asterisk represents the significant lower hemolymph glucose level during the course of the experiment. Mean ± SEM (P<0.001). The group name represents: EA (eyestalk ablated), EI (Ecdysteroid injected), LDI (low dose injected), MDI (medium dose injected), HDI (high

dose injected), LDO (low dose oral), MDO (medium dose oral), HDO (high dose oral).

### 3.5. Biochemical analysis of muscle and hepatopancreas

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) (Fig. S6). A similar pattern was observed in ecdysteroid injected and eyestalk ablated lobsters where total protein concentration was higher than the 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. The improved growth performance in CGE-1 treated animals is probably attributed to the interference of MIH binding to the y-organs resulted in higher synthesis and secretion of 20E. This 20E upsurge in the hemolymph may in turn explain the better growth and feed efficiency observed in the experimental animals. The variation in the biochemical parameters could be due to the different route of CGE-1 administration. It is also very interesting to observe that the increment in the molting-frequency due to CGE-1 treatment does not cause any adverse effect on the rates of protein synthesis.

In case of hepatopancreas, there were no significant changes were observed in total protein (Fig. S7) and also in case of carbohydrate where the medium (1.571g/100g) and high dose (1.60g/100g) groups are comparable with as compared to control (1.521g/100g). But, a higher level of HDL was observed in treating lobsters as compared to controls probably due to the increased food intake and the result was correlated with the significantly increased in the weight gain (Fig. S8).

### 3.6. Analysis of hepatosomatic and molt mineralization index

The different stages of molt cycle are the key factors influencing the crustacean condition,[37] the heaptosomatic index of each lobster were analyzed when all the lobsters reached the same molting stage. 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 (Fig. 3). A higher index suggest that the administration or diet supplemented with CGE-1improve the physiology by stimulating metabolism of biomolecules and energy storage in the hepatopanceas.[38] Furthermore, a significantly higher hepatosomatic index specifies the higher ability of treated animals to up-regulate the production of enzyme which enables absorption of lipids other biomolecules and efficacious utilization of their storage reserves during the intermolt stage. Moreover, 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.

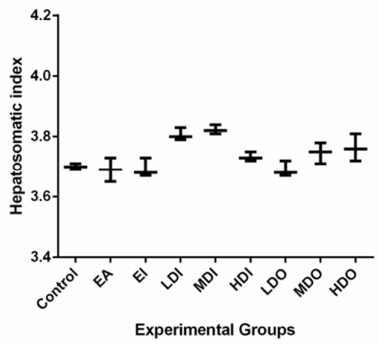



**Fig. 3. Molt related variations of the hepatosomatic index over three consecutive molting cycles for specimens of *P. homarus*.** The error bars represent the standard error of the mean for n=10 samples. No significant differences in the HSI for experimental groups were observed in relation to the treatment ($P < 0.0001$). The group name represents: EA (eyestalk ablated), EI (Ecdysteroid injected), LDI (low dose injected), MDI (medium dose injected), HDI (high dose injected), LDO (low dose oral), MDO (medium dose oral), HDO (high dose oral).

### 3.7. Effect of CGE-1 on cuticle and molt-mineralization index

The predominant mineral in the lobster cuticle is amorphous calcium carbonate embedded in the chitin matrix. It was observed that the molt mineralization index was higher in CGE-1 administered lobsters as compared to saline controls (Fig. 4). An increase in the molt mineralization index correlated with the size increment of the lobster as this will help to form a new cuticle during post molt.

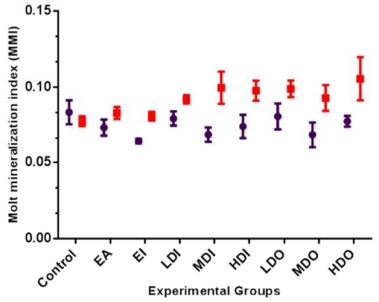



**Fig. 4. Progression of gastrolith growth (expressed as MMI values) in the three consecutive molt cycles.** The values were synchronized and are presented as mean ± SEM. Circles and squares represent initial and final molt mineralization index, respectively ($p<0.0001$). The group name represents: EA (eyestalk ablated), EI (Ecdysteroid injected), LDI (low dose injected), MDI (medium dose injected), HDI (high dose injected), LDO (low dose oral), MDO (medium dose oral), HDO (high dose oral).

Also, scanning electron micrographs of the cuticle has revealed that no significant changes in the deposition of calcium carbonate and chitin matrix were observed in all the experimental groups (Fig. 5). The elemental analysis for exoskeleton confirmed the presence of calcium and phosphorus in all the experimental groups. The probable changes in the FTIR 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 (Fig. 6). The *in vivo* experiment have shown that CGE-1 does not interferes with the shell hardening process.

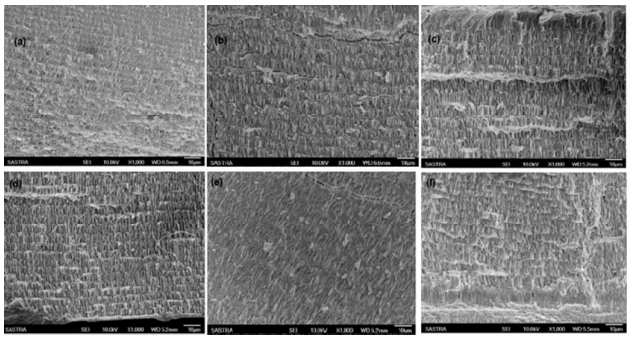


**Fig. 5. Scanning Electron Microscope (SEM) micrograph of the cross-sectional fracture surface of *P. homarus* chitin matrix after three successive molting.** No significant difference was observed between control (a), eye-stalk ablated (b), ecdysteroid injected (c) and CGE-1 administered low (d), medium (e) and high dose groups (f). Similar outcomes were seen among CGE-1 oral and injected groups.

### 3.8. Effect of CGE-1 hepatopancreas, muscle, ovary and gills

The photomicrographs of all experimental lobsters were evaluated for cellular integrity and lesion. There were no significant changes were observed in the organs from treated group lobsters as compared to the saline control. The results are in accordance with our various biochemical analysis where no abnormal variations were observed. The level of various proteins, lipids and glucose were shown a morphological stage dependent changes and these changes were relevant to the controls. Similarly, the biochemical analysis of these organs reflected a better growth and development and these factors were highly correlated with the histopathological changes in the various dose groups with different molting stages. The results also demonstrated that these are no concern over the effect of CGE-1 on gills due to their repeated contact through water or due to the CGE-1 supplemented diet. As we have already discussed about the role of MIH and 20E on the vitellogenesis and hence, reproduction the cellular integration has clearly indicated that the CGE-1 did not exhibited an inhibitory effect on the various stages of ovary development.

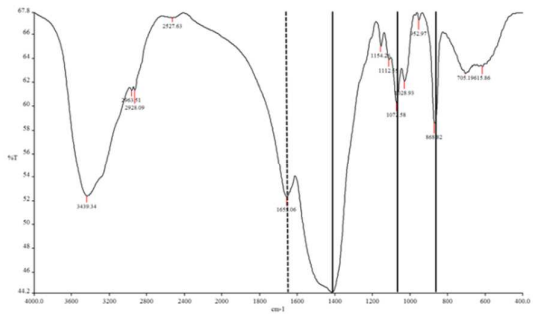


**Fig. 6**. **Fourier transform infrared spectroscopy (FT-IR) spectra of the cuticle during the intermolt in *P. homarus*.** Solid vertical lines represent the main peaks in the vibrational spectrum of amorphous calcium carbonate (868.12, 1072.58, 1420-1474 cm$^{-1}$). The dashed vertical line represents the main chitin peak (1655.06 cm$^{-1}$). Similar patterns of the spectrum for calcium carbonate and chitin was detected in all experimental groups.

### 3.9. CGE-1 does not cause acute oral toxicity and cytotoxicity

No sign of toxicity was observed during gross necropsy after 14$^{th}$ day for all drug groups. The food intake of drug animals was comparably similar to those of control animals. At a dose of 2000mg/kg of body weight no death was recorded during 14 days. These data indicate that the N, N',di-(3-bromobenzoyl)diamine ethane (CGE-1) has no toxicity and can be used to accelerate the growth in crustaceans. The effect of CGE-1 on the cytotoxicity to HepG-2 cells was evaluated. The results indicate that the CGE-1 will not cause any toxicity to the humans as the hepatic cells are able to metabolize the compound and ensure the excretion.

## 4. Conclusion

The criteria used to assess the effect of CGE-1 involves primarily of mortality, growth and reproduction. The other factors such as exoskeleton abnormalities, stress sensitivity and a comprehensive biochemical analysis of various organs were also evaluated. Using these factors, 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 the 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 93% 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 secrets several neurohormones, most of these methods have produced various biological effects other than regulating the ecdysteroid production. It is also very evident from the sequence analysis oand homology modeling MIH from various decapod crustaceans that they share high degree of similarity in the binding site, hence it is highly likely that the CGE-1 can also have similar effect on other decapod crustaceans. In that case, CGE-1 can be used as a universal growth enhancer to promote growth in several other economically important decapod crustaceans.

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