PAPER • **OPEN ACCESS**

Effect of *Chromolaena odorata* as bioactive compound in artificial diet on survival rate of tiger prawn *Penaeus monodon*

To cite this article: Harlina *et al* 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **253** 012015

Effect of *Chromolaena odorata* as bioactive compound in artificial diet on survival rate of tiger prawn *Penaeus monodon*

Harlina[1], Rosmiati[2], S Jafar[1], Sukmawati[3], Nurhidayah[2] and Kamaruddin[2]

[1] Faculty of Fisheries and Marine Science, Universitas Muslim Indonesia, Makassar, Indonesia
[2] Research Institute for Coastal Aquaculture and Fisheries, Maros, Indonesia
[3] Faculty of Economics, Universitas Muslim Indonesia, Makassar, Indonesia

Email: harlina.harlina@umi.ac.id

Abstract. The leaves of *Chromolaena odorata* can be used as alternative bioactive compounds to address high mortality in shrimp due to outbreaks of *Vibrio* sp. during both larval rearing and grow-out. The study aimed to determine the effect of *Chromolaena odorata* leaves on the survival and growth performance of tiger prawn *Penaeus monodon* larvae. Four different formulated diets with different concentrations of *Chromolaena odorata* leaves (1250 mg/kg, 1500 mg/kg, 1750 mg/kg) and a control (0 mg/kg) were fed to tiger prawns infected with *Vibrio harveyi*. Data were collected on total hemocyte count (THC), phenoloxidase (PO) and survival rate. One way ANOVA was applied for statistical analysis. The results show that *Chromolaena odorata* leaves were able to increase the immune response of tiger prawns infected with *Vibrio harveyi*. This was indicated by the increase in total haemocyte count (THC), phenoloxidase (PO) and survival rate with the values of 0.05 x 106 cells /mL, 0.05 and 84 %, respectively, for the formulated diet treatment with 1500 mg/kg feed. The increase in tiger prawn immune response led to increased resilience against *Vibrio harveyi*. As a result, survival rates of treated tiger prawns were higher than the control.

1. Introduction

The tiger prawn, *Penaeus monodon* Fabricius, is an important fishery commodity in Indonesia. Tiger prawn production reached 353% in 2010 [1]. However, the successful production of tiger prawn has been hampered by disease outbreaks leading to high mortality and financial loss [2].

Vibrio bacteria cause diseases which are a major issue in aquaculture. These bacteria can be found in almost all cultured marine organisms, such as crustaceans, molluscs, and fish [3]. *Vibrio harveyi* is a pathogen to post-larvae (PL-14) at a density of 107 CFU/mL [4]. Chemical and antibiotic products are often applied for disease outbreak prevention and control. However, the use of chemicals and antibiotics has led to bacteria resistance, antibiotic residues, and environmental pollution. Due to the side effects of chemical and antibiotic residues on human health, tiger prawns exported from Indonesia have received negative attention for the last few years. Therefore, the use of environmentally friendly compounds for the prevention and control of disease outbreaks is highly recommended.

The leaves of *Chromolaena odorata,* locally known as *kopasanda*, contain bioactive compounds which can be used as natural antibacterial agents. These compounds can increase the growth and survival rate as well as disease resistance. Moreover, they can modulate immune system parameters (Total Haemocyte Count (THC) and Differential Haemocyte Count (DHC). *Kopasanda* can function as an immune-stimulant which is able to prevent *Vibrio* spp. infection by increasing phagocytosis activity. This is known as a non-specific defence mechanism against disease outbreaks.

Kopasanda leaves contain natural antibacterial compounds such as flavonoids, alkaloids, steroids, saponin, and tannins [5]. Based on the antibacterial bioassay, *kopasanda* leaf extract has a strong antibacterial activity with an inhibition zone diameter of 22 mm. This suggests that the *kopasanda* leaves extract could be a future source of natural bioactive compounds which can significantly reduce *vibrio harveyi* population abundance [5]. Kopasanda leaves do not have a toxic effect on tiger shrimp post larvae [5], and histopathological damage to the hepatopancreas was not found [6]. These findings indicate that bioactive compounds from *kopasanda* leaves are safe to be used as bactericides in the prevention of *Vibrio* outbreaks.

The application of these natural antibacterial compounds through the immersion method has been attempted. However, the method did not prove to be very effective due to the high antibacterial compound dosage used and was uneconomical. Therefore, this study aimed to determine the effects of *kopasanda* leaf extract incorporated in feed formulation on the survival rate and growth of the tiger prawn *P. monodon*.

2. Materials and Methods

2.1. Chromolaena odorata leaf preparation

Chromolaena odorata

Samples were collected from the Marana Experimental Pond Station of the Research Institute for Coastal Aquaculture and Fisheries, Maros, South Sulawesi. The samples were dried by using herb drying methods at temperatures below 40 ^{o}C overnight. The dried leaves were then crushed into powder using a blender. The powdered leaves were filtered with a soft filter and weight to obtain the concentrations of 1250, 1500 and 1750 mg/kg for making artificial feed.

2.2. Preparation of Artificial Diet

The nutritional compositions of different diets are showed in Table 1. For each treatment, all the materials in the feed formulation were mixed with hot water to form a dough. The dough was printed using a mini-pelletizer. Pellets were dried using an oven at 40°C for 12 h. The pellets were ground into powder form before being fed to the larvae

Table 1. The composition of the different formulated feeds treatments

Materials	**Conc. Unit**	**Treatments**			
		A	B	C	D
Fish flour	%	40	40	40	40
Shrimp head flour	%	10	10	10	10
Copra cake	%	9	9	9	9
Corn flour	%	12	12	12	12
Soybean flour	%	17	17	17	17
Wheat flour	%	10	10	10	10
Vitamins and minerals	%	2	2	2	2
Kopasanda leaves	(mg/kg feed)	1250	1500	1750	0
Total	%	100	100	100	100
Crude protein	%	38.21	38.21	38.21	38.21
Crude lipid	%	9.27	9.27	9.27	9.27
Crude fibre	%	4.52	4.52	4.52	4.52
Ash content	%	12.48	12.48	12.48	12.48
NFE	%	32.86	32.86	32.86	32.86
Total Energy	(Kcal/kg feed)	1646	1646	1646	1646

2.3. Experimental Animals

Four days old post larvae (PL-4) of the black tiger prawn were collected from a commercial hatchery in Barru Regency, South Sulawesi, Indonesia. Larvae were acclimatized in the plastic bag at the Marana Experimental Pond Station of Research Institute for Coastal Aquaculture and Fisheries, South

Sulawesi for an hour. The larvae were reared in 1 x 0.8 x 1 m units called *happa*, with rearing water depth of 0.5 m, salinity of 28 ppt and post larvae density of 200 post larvae/m^3 for 21 days. The experimental animals were fed with diet as a treatment (Table 1) throughout the rearing period. Feed was given at 4 % of body weight four times a day (06:00 am, 10:00 am, 02:00 pm, and 06:00 pm).

2.4. Experimental Design

A completely randomized design was applied to four different formulated diets: 1250 mg/kg, 1500 mg/kg, 1750 mg/kg, and 0 mg/kg (control) of *kopasanda* leaves with three replicates for each treatment (4 x 3 = 12 experimental units).

2.5. Challenge Test

The challenge test was performed on larvae at PL-25. This test was carried out by challenging the tiger shrimp post larvae with a *V. harveyi* strain (MR 275 Rif) at a density of 5 x 10^6 CFU/mL. Conical glass containers with a volume of three litres were filled with 2 L of filtered and chlorinated seawater at 30 ppt salinity and continuously aerated during the experimental period. Thirty reared post larvae (PL-25) were selected at random and transferred into each of the prepared conical glass containers. The post larvae were then adapted for 2-3 hours before being challenged with *V. harveyi*.

2.6. Parameters for Evaluating Immunostimulant Efficacy

To determine the immune response of challenged post larvae, the following three parameters were measured in order to describe the immune system activity in shrimp post larvae.

2.6.1. Total Haemocyte Count (THC). The total haemocyte count (THC) was carried out according to [7]. The challenged post larvae from each treatment (n=5) were placed in 1.5 mL Effendorf glasses filled with 400 µl of anticoagulant (Natrium citrate 3.8%). The post larvae were then ground using a plastic pestle and homogenized through shaking by hand. Haemolymph was extracted using a pipette and placed on a hemocytometer (Improved Neubauer type). The total Haemocyte Count was carried out under a binocular light microscope at 40 x 100 times magnification [8]. Total Haemocyte Count was calculated using the following equation:

$$N = \left(\frac{n1 + n2 + n3 + n4 + n5}{5}\right) x\ 25\ x\ 10^4$$

where: N = Total Haemocyte Count (cell/mL)
n1, n2, n3, n4, n5 = the number of haemocytes in haemocytometer cells

2.7. *Differential Haemocyte Count (DHC).* The differential haemocyte count (DHC) was obtained based on the method in [9]. Haemolymph was dropped onto a glass slide, smeared, allowed to air dry, and fixed in absolute methanol for 5-10 minutes. The slide was then stained with 4 % Giemsa for 15-20 minutes and subsequently washed with distilled water. Differential haemocyte counts were obtained by grouping haemocyte cells into three types: granular, semigranular and hyaline, under a microscope (400 x magnification). The total percentage of each haemocyte cell type was calculated using the following equation:

$$\text{DGC (\%)} = \frac{\text{The number of haemocyte cell type}}{\text{Total haemocyte}} \text{ x } 100\%$$

2.7.1. Prophenoloxidase Activity Assay

Prophenoloxidase activity (ProPO) was measured according to the methods as described in [10]. ProPO was observed by using a spectrophotometer Genquant Merck with an absorbance of 490 nm. ProPO was measured by the formation of dopachrome from *L-dihydroxyphenilalanine* (L-DOPA) on a Multi-scan ELISA reader. Haemolymph was centrifuged at 700 rpm, at a temperature of 4 ^oC, for 20 minutes. The supernatant was removed and pellet was washed with 1 ml *cacodylate-citrate buffer* (0.01 M *sodium cacodylate*, 0.45 M *Sodium chloride,* 0.10 M *Trisodium citrate*; pH 7) and re-centrifuged under the same conditions. The pellet yielded was dissolved in *cacodylate buffer* (0.01 M

Sodium cacodylate; 0.45 M Sodium chloride; 0.01 M Calcium chloride; 0,26 M Magnesium Chloride; pH 7) and homogenized. The solution was then divided in two parts of 100 µl each. One part was incubated for 10 minutes at 25° C with 50 mL trypsin (1 mg.mL^{-1} cacodylate buffer*)* as elysator, and then with added 50 µl L-DOPA for 5 minutes, followed by the addition of 800 µL cacodylate buffer. Another aliquot of around 100 µL of the cell suspension was added to 50 µL cacodylate buffer (trypsin substitute) and 50 µL LDOPA, and was used as the control for background ProPO activity. Optical density of ProPO activity was measured with a spectrophotometer at the absorbance wavelength of 490 nm and expressed by the formation of dopachrome in 50 µL haemolymph.

2.8. Survival Rate

Survival Rate of post larvae was calculated using the equation according to [11] as follows:

$$SR = \frac{Nt}{No} \times 100\ \%$$

Note: SR = Survival Rate
Nt = Final number of post larvae
No = Initial number of post larvae

2.9. Data analysis

One way ANOVA was applied for statistical analysis in SPSS Version 21. The significance of the observed differences was tested using a Tukey post-hoc test at the 95% confidence level (P<0.05).

3. Results and Discussion

3.1. Effect of Immunostimulants on ProPhenoloxidase (proPO) Activity

ProPhenoloxidase (proPO) plays an active role in the process of foreign particle or pathogen destruction. The enzyme is activated by ProPhenoloxidase gen. ProPO activates phenol oxidase, catalysing the reactions which produce quinine which leads to the production of melanin [12]. The observed ProPO activity in post larvae infected by *V. harveyi* are illustrated in Figure 1.

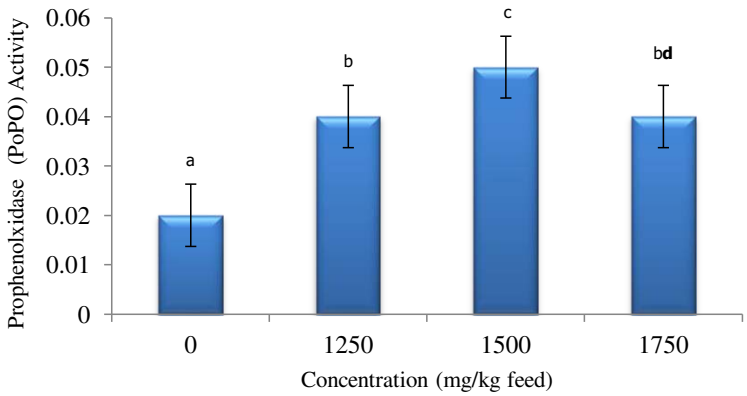

Figure 1. Prophenolxidase (proPO) activity in post larvae infected with *V. harveyi* fed with artificial feed containing *C. odorata* leaf bioactive compounds at different concentrations

The ProPO activity was higher in shrimp fed artificial diets containing *C. odorata* leaf bioactive compounds at all three concentrations tested than in the control (without *C. odorata* bioactive compounds). The highest ProPO activity was obtained under treatment C with a ProPO activity of 0.05, followed by treatments B, D and A (control). There was significant difference at the 95% confidence level (P>0.05) between treatment C and all other treatments, however treatment B and D were not significantly different at this level.

Bioactive compounds from *C. odorata* leaves can thus activate ProPO, as indicated by the higher ProPO activity of post larvae infected by *V. harveyi* and fed with artificial diet containing the bioactive

compounds. Granular, and semi-granular cell types known as haemocytes play an active role in ProPO activity, while hyaline cells have a function in phagocyte activity [13].

3.2. Effect of Immune stimulant on Total Haemocyte Count (THC)

The primary body protection mechanism in crustaceans is the hard carapace, which is followed by haemolymph activity. Haemocytes are found in the haemolymph, and play an important role in the immune response of shrimp against disease infection [13]. Total haemocyte cell count can be considered as an indicator of shrimp health.

Total Haemocyte Count (THC) in the post larvae challenged with *Vibrio harveyi* was higher than in the control larvae, as shown in Figure 2. The highest mean THC was found in the 1500 mg/kg feed treatment (0.05 x 10^6cells/mL), followed by the 1250 and 1750 mg/kg treatments, with equal means (0.04 x 10^6cells/m). The control (no addition of *kopasanda* leaf bioactive compounds) had the lowest mean THC (0.02 x 10^6cells/mL). These results indicate that the addition of *kopasanda* leaf extract to larval shrimp feed at 1500 mg/kg has potential as an immunostimulant treatment.

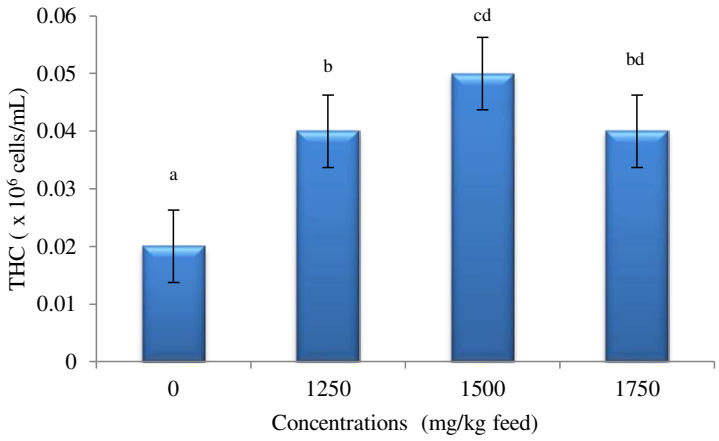

Figure 2. Total Haemocyte Count (THC) of post larvae challenged by *V. harveyi* with *kopasanda* compound as a feed additive at different concentrations

Haemocytes are the main component of the invertebrate protection system circulated in the haemolymph. They function as an automatic response to pathogens or injured tissue [14]. Haemocyte abundance can affect the ability of the host to react against foreign material and respond to infection [15].

Kopasanda (*C. Odorata* L) contains bioactive compounds such as phenolic compounds, steroids, flavonoids, and alkaloids [16], which can function as antibacterial agents [17, 6], anti-diabetes and ant cataract treatments [18] and anti-inflammatory agents [19]. In addition, *kopasanda* can promote wound healing, while the phenolic acid and flavonoids can be used as antioxidants [20]. Furthermore, it is reported that bioactive compounds from can increase the immunity of white mice by increasing phagocytic activity, lysosyme production, and reactive oxygen species (ROS) [21].

3.3. Effect of Immune-stimulant On Survival Rate

The survival rates of post larvae fed with artificial diet with and without *C. odorata* leaf bioactive compounds at 6 days post challenge are shown in Figure 3.

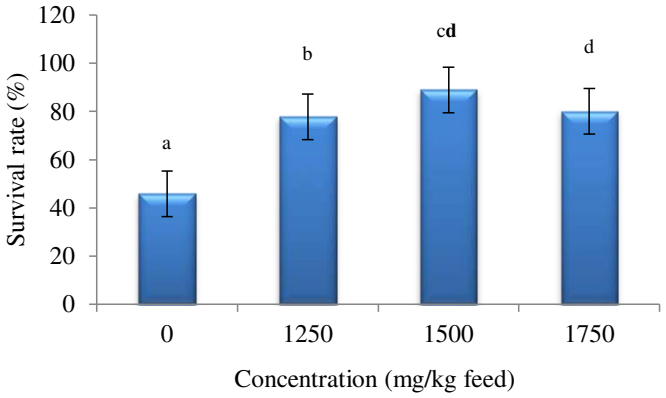

Figure 3. The percentage of survival rate of post larvae fed with artificial diet of supplemented with *C. odorata* leaves in different concentrations and challenged with *Vibrio harveyi*

The survival rate of post-larvae fed with all supplemented treatments was higher than the control at 6 days after being challenged with *V. harveyi*. The 1500 mg/kg treatment gave the highest mean percentage survival rate (85.56 %), while the followed by the 1750 mg/kg treatment (80.00 %), 1250 mg/kg treatment (77.78%) and 0 mg/kg control (45.78%). These results suggest that bioactive compounds from C. odorata positively affected the immune response of the post-larvae.

The post-larvae challenged with *V. harveyi* and fed with *C. odorata* bioactive compounds at a rate of 1500 mg/kg treatment showed a significantly different (higher) survival rate at the 95% confidence level ($p>0.05$) compared to the 1250 mg/kg, 1750 mg/kg and 0 mg/kg treatments. Meanwhile, the difference in survival rate of post-larvae challenged by *V. harveyi* under the 1250 mg/kg and 1750 mg/kg treatments was not significant at this level, but both treatments were significantly different from the control.

The *C. odorata* leaf compounds can increase the immune response of post larvae to the pathogen *V, harveyi*, this is likely due to positive interactions among post larvae immune system parameters acting against, leading to low shrimp mortality. The immune system of crustaceans, including shrimp, is dominated by haemocytes in the haemolymph, with the introduction of foreign material generally causing an increase of haemolymph production by the shrimp as defence mechanism. In the haemolymph, hyaline and semi-granular cells are responsible for phagocytic activity. The phenoloxidase activity and antibacterial activity by antimicrobial peptides (AMPs) such as penaidins, crustin and anti-lipopolysaccharide factors (ALFs) all actively attack pathogens [22].

4. Conclusions

The supplementation of tiger prawn diet with *kopasanda* increased the immune response of tiger prawn larvae infected with *Vibrio harveyi,* with the best results obtained from the formulated diet supplemented at a rate of 1500 mg/kg. The increased immune response was indicated by the increase in three parameters under this treatment, with the following mean values: total haemocyte count (THC) (0.05×10^6 cells/mL), phenoloxidase (PO) (0.05) and survival rate (89 %)

Acknowledgments

The authors would like to acknowledge support from the Directorate of Research and Community Service Fund, Kemenristek-Dikti under research grant contract: DIPA.042.06.1.401516/2017. We would like also to express our gratitude to the Rector of Universitas Muslim Indonesia, the chairman of the Research and Human Resource Development and his staff. The authors also express their gratitude to the head of Research Institute for Coastal Aquaculture and Fisheries Extension, Maros Regency, South Sulawesi for the use of laboratory facilities for analysing immunostimulant parameters.

References

[1] Subyakto 2011 *Current conditions of aquaculture fish resources to support fisheries industrialization* (Malang: Directorate General of Aquaculture Presented at a Public Lecture in the Framework of Quality and Safety of FPIK UB Fisheries Product November 19, 2011)

[2] Mariyono, Wahyudi A and Sutomo 2002 Controls on *Vibrio* outbreak through bacteria population at laboratory *Buletin Teknik Pertanian* **7** 25-27
[3] Isnansetyo A T, Setyowati E P and Anshory H H 2009 *In vitro* antibacterial activity of methanol extract of a sponge*, Geodia* sp. against oxytetracyclin-resistent *Vibrio harveyi* and its toxicity *J. Biol. Sci.* **9** 224-230
[4] Kadriah I A K 2012 *Analysis of morphological diversity, physiology, genetic and phagocyte test Vibrio sp* (Bogor: Thesis, Sekolah Pascasarjana, Institut Pertanian Bogor)
[5] Harlina, Prajitno A, Suprayitno E and Nursyam H 2013 The identification of chemical compound and antibacterial activity test of kopasanda (*Chromolaena odorata* L.) leaf extract against Vibriosis-causing *Vibrio harveyi* (MR 275 Rif) on tiger shrimp *Aquat. Sci. Tech.* **1** 15-29
[6] Harlina H, Prajitno A, Suprayitno E, Nursyam H and Rosmiati 2015 Potential study of kopasanda *(Chromolaena odorata* L.) leaves as antibacterial against *Vibrio harveyi,* disease causative agent of tiger prawn (*Penaeus monodon* Fabricius) post larvae *J. Aquat. Res. Dev.* **6** 1-5
[7] Chen T T 2000 Aquaculture biotechnology and fish disease *In: International Symposium on Marine Biotechnology* (Jakarta: Center for Coastal and Marine Resources Studies)
[8] McCarthy D H, Stevenson J P and Roberts M S 1973 Some blood parameters of the rainbow trout (*Salmo gairdneri Richardson*) The Kamloops variety *J. Fish. Biol.* **5** 1-8
[9] Martin G G and Graves L B 1985 Fine structure and classification of shrimp haemocytes *J. Morphol*. **185** 339-348
[10] Liu C H and Chen J C 2004 Effect of ammonia on the immune response of white shrimp *Litopenaeus vannamei* and susceptibility to *Vibrio alginolyticus Fish. Shellfish. Immunol.* **16** 321 – 334
[11] Effendi I 1997 *Biologi Perikanan* (Yogyakarta: Yayasan Pustaka Nusantara) van Den Braak K 2002 *Haemocytic Defence in Black Tiger Shrimp (Penaeus monodon)* (Netherland: PhD thesis, Wageningen University)
[12] Kakoolaki S, Soltani M, Ebrahimzadeh M H A, Sharifpour I *et al* 2011 The effect of different salinities on mortality and histopathological changes of SPF imported *Litopenaeus vannamei*, experimentally exposed to white spot virus and a new differential hemocyte staining method *Iran. J. Fish. Sci.* **10** 447–460
[13] Battison A, Cawthorn R and Horney B 2003 Classification of *Homarus americanus* haemocytes and the use of differential hemocyte counts in lobsters infected with *Aerococcus viridans* var. *homari* (Gaffkaemia) *J. Invertebr. Pathol.* **84** 177-197
[14] Johansson M W, Keyser P, Sritunyalucksana K and Soderhall K 2000 Crustacean haemocytes and haematopoiesis *Aquaculture* **191** 45-92
[15] Harlina H, Prajitno A, Suprayitno E and Nursyam H 2013 The identification of chemical compound and antibacterial activity test of kopasanda (*Chromolaena odorata* L.) leaf extract against Vibriosis-causing *Vibrio harveyi* (MR 275 Rif) on Tiger Prawn *Aquat. Sci. Tech.* **1** 15-29
[16] Zige D V, Ohimain E I and Nodu M B 2013 Antibacterial activity of ethanol, crude and water extract of *Chromolaena Odorata* leaves on *S typhi* and *E coli Greener J. Microbiol. Anti M* **1** 2354-2284
[17] Onkaramurthy M, Veerapur V P, Thippeswamy B S, Reddy T M, Rayappa H and Badami S 2013 Anti-diabetic and anti-cataract effects of *Chromolaena odorata* Linn. in streptozotocin-induced diabetic rats *J. Ethnopharmacol.* **145** 363–372
[18] Hanh T T H, Hang D T T, Van Minh C and Dat N T 2011 Anti-inflammatory effects of fatty acids isolated from *Chromolaena odorata Asian. Pac. J. Trop. Biomed.* **4** 760–763
[19] Phan T T, Wang L, See P, Grayer R J, Chan S Y and Lee S T 2001 Phenolic compounds of *Chromolaena odorata* protect cultured skin cells from oxidative damage: implication for cutaneous wound healing *Biol. Pharm. Bull.* **24** 1373-1379
[20] Nudo L P and Catap E S 2012 Anti-immunosuppressive effects of *Chromolaena odorata* (Lf.) King & Robinson (Asteraceae) leaf extract in cyclophosphamide-injected Balb/C mice *Philipp. J. Sci.* **141** 35–43

[21] Tassanakajon A and Somboonwiwat K 2011 *Antimicrobial peptides from the black tiger shrimp Penaeus monodon – A review* (Malaysia: Diseases in Asian Aquaculture VII. Fish Health Section, Asian Fisheries Society, Selangor, Malaysia)