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Enhancement glutathione peroxidase activity and α2-macroglobulin gene expression of Macrobrachium rosenbergii Fed With Aqueous Morinda citrifolia Leaves Extract-Supplemented Diet

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ABSTRACT

Morinda citrifolia, known commercially as noni is often used for enhance immunity, these plant rich of phenolic compound with antioxidant properties. In the present study, Macrobrachium rosenbergii were fed with diets containing of aqueous M. citrifolia leaves extract (AMLE) at 0.6, 4 and 6 g kg-1. Glutathione peroxidase (GPx) activity was conducted to measure immune parameter, these parameter was evaluated before and after 7, 21, 35, 49 and 63 days of feeding trial. Immune gene expression of α2-macroglubulin (α2-M) was evaluated in this study. The results showed that after 63 days of feeding treatment, significantly increased in GPx activity. Gene expressions of α2-macroglubulin was significantly upregulated. These results recommend that administration of AMLE can be used as an immunostimulant and regulated immune response and immune gene expression in M. rosenbergii.

Keywords: *Morinda citrifolia*, *Macrobrachium rosenbergii*, GPx, a2-M

1. Introduction

The giant freshwater prawn *Macrobrachium rosenbergii* is an important freshwater farmed crustacean species in many countries because of its high commercial value. Although several species of freshwater prawns are currently being cultured, farming of the major commercial species *M. rosenbergii*, which is indigenous to South and Southeast Asia, parts of Oceania and some Pacific Islands, *M. rosenbergii* has been imported into many other tropical and subtropical areas of the world and these species most favoured for farming purposes (New, 2002).

Decreased production of freshwater prawn *M. rosenbergii* caused by disease outbreaks often occured. In Taiwan, during the past few years, commercial prawn farming has been negative impacted with yeasts in the cool season (Hsu and Liu, 1994) and bacteria in the hot season (Cheng and Chen, 1998), which have caused serious economic losses. Therefore an increased immune system and desease resistance in the giant freshwater prawn must be considered, because as it was known that the prawn merely depend on innate immunity.

Plants are the primary source of medicines. Medicinal plants are considered to be very rich sources of secondary metabolites and oils which are important for health. The important advantages of medicinal plants in various treatments are their safety besides being less expensive, efficacy and

animal feeds by both researchers and feed companies (Govind et al. 2012). Morinda citrifolia, known commercially as noni, grows widely throughout the Pacific and most significant sources of traditional medicines among Pacific Island societies. This small evergreen tree or shrub is native from Southeastern Asia (Indonesia) to Australia, and now has a pantropical distribution. Various parts of noni plant extracts have been reported to have many significant effect such as fruit extract of M. citrifolia as antibacterial, antifungal, tumor suppression (Jayaraman et al. 2008), antioxidative activities from root of M. citrifolia (Zin et al. 2000), inhibitor on elastase and tyrosinase from seeds of M. citrifolia (Masuda et al. 2009) and also originally, the leaves were applied directly to the skin to treat ulcerations and minor infections (Usha et al. 2010). Nworu et al. (2012) explained that Noni is a very popular for immune boosting reported to be beneficial in immunosuppression tumour, and in other immuno-inflammatory disorders. In addition, octanoic acid, cyclopropyl, hexanoic acid, n-decanoic acid, allantoin, sorbitol, mannitol, glycerin and γ-tocopherol have identified compounds for medicinal importance in M. citrifolia leaves extract (Rivera et al. 2012). Ethanolic extract from M. citrifolia capable of promoting wound-healing activity (Nayak et al. 2009). Nayak and Mengi (2010), also explained that M. citrifolia can be used for immunostimulant on T and B lymphocytes. Kumaran et al. (2013) was demonstrated that M. citrifolia leaf methanol extract against Vibrio parahaemolyticus in fresh water crab, Oziotelphusa senex senex and as well enhanced non specific and specific defense mechanism of fresh water crab. However, mechanism of M. citrifolia leaves as immunostimulant for giant freshwater prawn M. rosenbergii has not been studied in detail.

According to the previous research that explained the advantages of *Morinda citrifolia* leaves, this study is the first research conducted to evaluate the effects of aqueous *Morinda citrifolia* leaves extract on immunity and gene expression of giant fresh water prawn *M. rosenbergii,* which is considered to be the aquatic species contributing in aquaculture production in the world.

2. Materials and Methods

2.1. Plant extraction, diet preparation and determination of total phenol by Folin-reagent method

Table 1. Composition of the basal diet for *Macrobracium rosenbergii*

Ingridients	Composition (g kg^{-1})			
	Control (0)	0.6	4	6
Fish meal	370	370	370	370
Wheat flour	250	250	250	250
Soybean meal	245	245	245	245
Squid liver meal	70	70	70	70
Fish oil	37.08	37.08	37.08	37.08
Vitamin Pre-mix	10	10	10	10
Mineral Pre-mix	20	20	20	20
Choline chloride	2	2	2	2
Cholesterol	1.5	1.5	1.5	1.5
Noni Leaves Extract	0	0.0.6	4	0.6

Morinda citrifolia leaves, all of green leaves were collected from Noni Farm, Pingtung, Taiwan. Fresh leaves of *M. citrifolia* cleaned with tap water and rinsed with distilled water to remove contaminant and debris. The leaves were chopped into small pieces and dried in an oven at 60 ^{0}C for 12 hours. The dried leaves were ground into a powder with a grinder. A known weight of noni leaves powdered was used to extract in hot water. To determine *M. citrifolia* leaves powdered concentration followed by Arizo *et al*. (2010) methods that have been modification, noni leaves powder (1, 3 and 5 gram) boiled in 100 ml of distilled water at 100 ± 4 ^{0}C for 2 hours, respectively.

The aqueous *Morinda citrifolia* leaves extract centrifuged at 3000 x g, 28 ^{0}C for 15 minutes and discard the pellet. Then, supernatant containing the noni leaves extract was lyophilized using freeze dryer to obtain 0.01, 0.07 and 0.10 mg.

Four diets containing different concentration of aqueous *M. citrifolia* leaves extract were prepared as described in Table 1. For the experimental diets, aqueous *M. citrifolia* leaves extract added to the basal diets at 0, 0.6, 4, 6 g kg^{-1}, respectively. Ingridients were ground up in Hammer mill until passed through an 80-mesh screen. Experimental diets were prepared by mixing the dry ingredients with fish oil until a stiff dough resulted. Each diet was then passed through a mincer with a die and the resulting sphagetti-like strings were dried in a drying cabinet using air blower at 50 ^{0}C until the moisture levels were lower than 10%. After drying the mixture, the finished pellets were stored in plastic bin at 4^{0}C until being used. The experimental feeds were prepared in the basal diets contained of proximate composition (Table 2).

The total phenolic compound of aqueous *M. citrifolia* leaves extract were determined using a modified version of the Folin–Ciocalteu method by Hossain *et al.* 2013. Briefly, from each crude extract (1 g) were dissolved in 100 ml of different solvent (water, methanol and ethanol), respectively. A total of 10% Folin-Ciocalteu reagent was prepared by adding Folin-Ciocalteu reagent (10 ml) in water (90ml). Then, 10% Na_2CO_3 was prepared by dissolving Na_2CO_3 (10 g) in water (90 ml). Each crude sample (0.2 ml) was taken in tube and added 10% Folin-Ciocalteu reagent (1.5 ml). Then, keep in a dark place for 3 minutes. Furthermore, added 10% Na_2CO_3 (1.5 ml) and allowed in the dark place for 2 hours. The absorbance was measured for all solution by using UV-spectrophotometer at 750 nm. Quantification was done according to a standard curve with gallic acid. The concentration of total phenolic compound in all plant extract expressed as milligram of Gallic acid equivalents (GAE) per gram dry weight of plant.

Table 2. Proximate Composition of Basal Diet and Enriched with Aqueous *M. citrifolia* Leaves Extract at 0.6, 4 and 6 g kg^{-1}

Proximate Composition	Aqueous *M. citrifolia* leaves extract g kg^{-1}			
	Control (0)	0.6	4	6
Crude protein	46.52 ± 1.05%	44.58 ± 0.41%	45.08 ± 2.30%	43.38 ± 1.09%
Crude lipid	9.62 ± 0.14%	10.11 ± 0.35%	9.76 ± 0.18%	9.99 ± 0.20%
Ash	8.30 ± 0.21%	8.26 ± 0.21%	7.96 ± 0.17%	7.84 ± 0.09%
Moisture	5.09 ± 1.57%	5.44 ± 1.45%	5.26 ± 0.06%	5.19 ± 0.05%
Crude fiber	5.46%	6.71%	6.25%	7.01%
Carbohydrate	25.01%	24.90%	25.69%	26.59%

2.2. Experimental Design

Giant fresh water prawn, *Macrobrachium rosenbergii* were obtained from a commercial farm in Pingtung, Taiwan, and reared in National Pingtung University of Science and Technology, Department of Aquaculture, and acclimatized at room temperature for two weeks before experimentation. Only prawns in the intermoult stage were used in this experiment. The moult stage was determined by examination of uropoda which partial retraction of the epidermis could be distinguished (Cheng *et al.* 2005). During the acclimitation period, prawns were fed with control diet twice dialy at 8.00 hour and 17.00 hour.

The experiment was carried out for a period of 63 days with a replacement of 50% water weekly to maintain water quality. Fecal matter, molting and uneaten food were removed daily. This study evaluated growth performance, immune responses and immune related genes of *M.*

rosenbergii, 16 prawns were stocked in each tank in triplicate and four diet groups. Twelve tank containing of aerated freshwater were used for this study.

Feeding trial conducted for 63 days and immune parameters of prawns that are fed with aqueous *M. citrifolia* leaves extract containing diets determine at the beginning and after 7, 21, 35, 49 and 63 days of feeding treatment. Prawns were fed with aqueous *M. citrifolia* leaves extract at 0.6, 4 and 6 g kg^{-1} twice dialy at 8.00 hour and 17.00 hour. Measurement of the immune response glutathione peroxidase (GPx) activity and the gene expression α2-macroglobulin.

2.3. Glutathione Peroxidase (GPx) Activity

GPx activity was measured following the method by Cheng *et al.* (2005) using Ransel RS-505 kit (Randox, Crumlin, UK), following the manufacture's instructions. GPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized form of glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^{+}$. The decrease in absorbance was measured at 340 nm in room temperature and the rate of reaction was estimated from the absorbance readings at the first 1 min and 3 min after adding cumene hydroperoxide. Specific activity was expressed as GPx units/g protein.

2.4. Immune genes of Macrobrachium rosenbergii

Total RNA was measured using total RNA isolation Reagent (Zymo Research, Quick-RNA™ MiniPrep, R1054, USA) following the manufacturer's instructions. First-strand complementary cDNA synthesis in reverse transcription (RT) was measured according to the methods of Liu *et al.* (2007). Expression mRNA of immune genes including LGBP, peroxinectin, α-2 macroglobulin, proPO, transglutaminase, crustin and lysozyme were measured using an SYBR green I real-time RT polymerase chain reaction (PCR) assay in ABI PRISM 7900 Sequence Detection System (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). Specific primes of immune gene (α2-macroglobulin) and the β-actin primer were used for the quantitative RT-PCR (Table 3). Data processing and analysis were performed using Sequence Detection Software (SDS vers. 2.1, Applied Biosystems). The $2^{-\Delta\Delta Ct}$ method was chosen as the calculation method following Livak and Schmittgen, (2001). Differences in the Ct values of each gene and the corresponding internal control β-actin gene, called ΔCt. The value of ΔCt for treated sample was substracted as the ΔΔCt value that allowed measurement of the change in expression of immune-related genes in the treatment compared than control sample. ΔΔCt = (ΔCt of prawn fed diet containing aqueous *M. citrifolia* leaves extract at 0.6, 2 and 6 g kg^{-1} for immune genes) – (ΔCt of the control group).

Table 3. Primer sequences (Forward and Reverse) of gene target

Gene name	Gene Bank Number	Primer	Sequence (5'-3')
α-2 M	ABK60046	F	CTC GGC CAT CTT ATC CGT ATG
		R	GGG AGC GAA GTT GAG CAT GT
β-actin	Liu *et al*	F	CATCACCAACTGGGACGACATGGA
	(2011)	R	GAGCAACACGGAGTTCGTTGT

2.5. Statistical Analysis

One-way analysis of variance (ANOVA) was used to analyze the data. When ANOVA identified differences among groups, a multiple comparasion (Tukey's test) was conducted to examine

significant differences among treatments using SPSS Version 16.0 computer software. Data are presented as the mean ± SD. Statistical significant differences required that $p < 0.05$.

3. Results And Discussion

3.1. Glutahione Peroxidase (GPx) Activity of Macrobrachium rosenbergii

Glutathione peroxidase (GPx) activitiy of prawn significantly increased when fed aqueous *Morinda citrifolia* leaves extract-supplemented diet at 0.6 g kg^{-1} after 5 days by 244.96 %, compared than those of control group. However, GPx activity of prawn decreased significantly at 4 and 6 g kg^{-1} of aqueous *Morinda citrifolia* leaves extract-supplemented diet, compared to the other groups and control group.

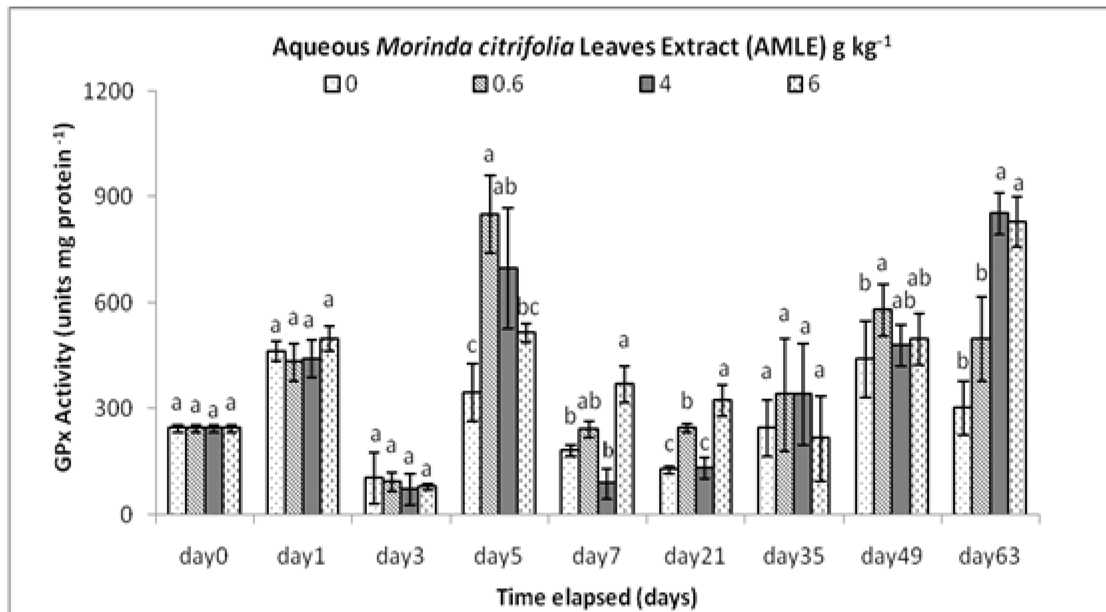

Figure 1. Glutathione peroxidase (GPx) activities of *Macrobrachium rosenbergii* fed with aqueous *Morinda citrifolia* leaves extract-supplemented diet at 0, 0.6, 4 and 6 g kg^{-1} for 63 days. Data (mean ± SEM) with different letters are significantly different ($p<0.05$) among treatment.

After 35 days of feeding treatment, no significant differences of glutathione peroxidase activities at all of supplemented diet. Likewise, after 63 days of feeding treatment, no significant differences were observed at 0.6 g kg^{-1} of aqueous *M. citrifolia* leaves extract, compared than those of control group. However, at 4 and 6 g kg^{-1} of aqueous *M. citrifolia* leaves extract-supplemented diet, the GPx activity of prawn significantly increased by 274.07 % and 282.14 % after 63 days of feeding treatment (Fig. 1).

3.2. Immune gene expression (a2-macroglobulin) of Macrobrachium resenbergii

The gene expression of α2-macroglobulin in haemocytes of prawn were significantly increased fed with aqueous M. citrifolia leaves extract-supplemented diet at 0.6, 4 and 6 g kg^{-1} after 3 days to 63 days of post feeding. After 3 days of feeding treatment gene expression of α2-macroglobulin higher at 0.6 g kg^{-1} of AMLE than another concentration and control group. The α2- macroglobulin still increased at the end of feeding treatment at 0.6 g kg^{-1} of aqueous M. citrifolia leaves extract.

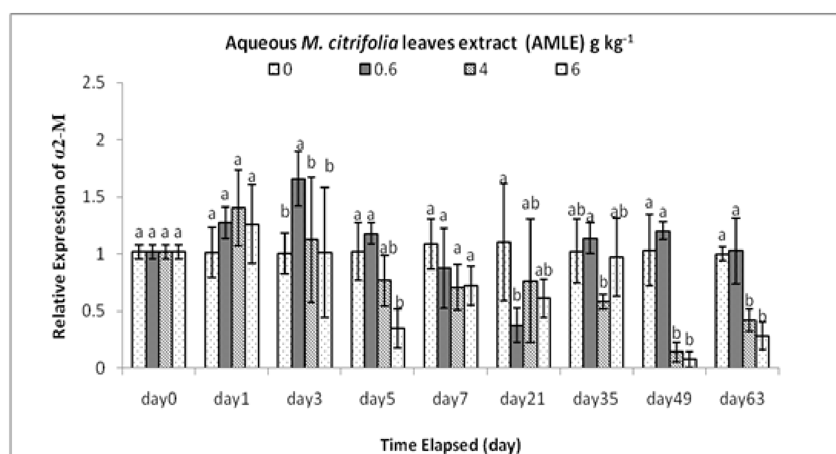

Figure 2. Relative expression of α2-macroglobulin in prawn Macrobrachium rosenbergii fed with aqueous Morinda citrifolia leaves extract-supplemented diet at 0, 0.6, 4 and 6 g kg^{-1} for 63 days. Data (mean ± SEM) with different letters are significantly different ($p<0.05$) among treatment.

Reactive oxygen species (ROS) in haemocytes of prawn indicated that the fluctuation of O_2^- production in prawns related with the level of total haemocyte count (THC) and phagocytic activities. Decreased of O_2^- production can be resulted from increased of superoxide dismutase activities (SOD) or glutathione peroxidase (GPx) activities of prawns. In these study, the GPx activity of prawn significantly increased during 63 days of feeding treatment, these results indicated that aqueous *M. citrifolia* leaves extract-supplemented diet exhibit to balancing the antioxidant system in haemocyte of prawn. In addition, the aqueous *M. citrifolia* leaves extract contained total phenol by 45.46 mg of gallic acid equivalent/g of extract. Phenolic compounds are secondary metabolites of plants and play an important role in growth, reproduction, prevent the pathogens intruders and also attributed to antioxidant activity (Balasundram *et al.* 2006). This result in agreement with Pham *et al*. (2006), higher content of polyphenols in *Hizika fusiformis*-supplemented diet could enhance the nonspecific immune response and improve the resistance of juvenile olive flounder to *Streptococcus iniae*.

The expression of α-2 macroglobulin significantly increased and revealed that aqueous *M. citrifolia* leaves extract-supplemented diet induce enhancement of gene expression of the prawn. This result indicated that aqueous *M. citrifolia* leaves extract leading to upregulated the gene expression, which later used for defense mechanism against pathogen intruders and enhances immune ability in prawn. Similarly with Rattanavichai *et al*. (2015) when used banana peel extract (BPE) at 6.0 g kg^{-1} increased the PO activity of giant freshwater prawn and those considered to be related with up-expression of proPO, LGBP and PE genes to increase resistance against the pathogen. Wang *et al*. (2008) revealed that LGBP of white shrimp significantly increased fed the diet containing beta glucan at 2 g kg^{-1} after 3 days of feeding trial. In addition, Liu *et al*. (2007) explained that the PE gene expression was significantly higher in prawns fed the 1.0 g kg^{-1} sodium alginate. Nevertheless, after 63 days of feeding treatments with aqueous *M. citrifolia* leaves extract at 4 and 6 g kg^{-1} the α-2 macroglobulin gene expression was decreased significantly. These facts suggest that excessive amounts of aqueous *M. citrifolia* leaves extract-supplemented diet might be lead to the suppression immune responses and immune genes expression in prawns.

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