Aqueous *Morinda citrifolia* Leaves Extract Enhancing Glutathione Peroxidase Activity and α2-Macroglobulin Gene Expression on *Macrobrachium rosenbergii*

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**ABSTRACT**

*Morinda citrifolia*, known commercially as noni is often used for enhancing immunity, these plant-rich phenolic compound with antioxidant properties. In the present study, *Macrobrachium rosenbergii* were fed diets containing aqueous *M. citrifolia* leaves extract (AMLE) at 0.6, 4 and 6 g kg\(^{-1}\). Glutathione peroxidase (GPx) and α2-macroglobulin (α2-M) activity were conducted to measure an immune parameter, which was evaluated before and after 7, 21, 35, 49 and 63 days of feeding trial. The results showed that after 63 days of feeding treatment, significantly increased in GPx activity. Moreover, the gene expressions of α2-macroglobulin 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*.

**Keyword:** Morinda citrifolia, Macrobrachium rosenbergii, GPx, α2-M.

**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, *M. rosenbergii*, which is indigenous to South and Southeast Asia, has been imported into many other tropical and subtropical areas of the world and these species most favored for farming purposes (New, 2002).

Decreased production of freshwater prawn *M. rosenbergii* caused by disease outbreaks often occurred. In Taiwan, during the past few years, commercial prawn farming has been negatively impacted with yeasts in the cool season (Hsu and Liu, 1994) and bacteria in the hot season (Cheng and Chen, 1998), which have caused severe economic losses. Therefore, an increased immune system and disease resistance in the giant freshwater prawn must be considered, because as it was known that the prawn merely depends 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 essential for health. The significant advantages of medicinal plants in various treatments are their safety besides being less expensive, efficacy and availability through out the world. Recently, a growing interest has developed in using herbs in 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 tumor, and in other immuno-inflammatory disorders. Also, 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) were demonstrated that *M. citrifolia* leaf methanol extract against *Vibrio parahaemolyticus* in freshwater crab, *Oziotelphusa senex senex* and as well enhanced non-specific and specific defense mechanism of freshwater crab. However, mechanism of *M. citrifolia* leaves as an immunostimulant for giant freshwater prawn *M. rosenbergii* has not been studied in detail.

According to the previous research, it 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 freshwater prawn *M. rosenbergii*, which is considered to be the aquatic species contributing in aquaculture production in the world.

**MATERIALS AND METHODS**

**Plant Extraction, Diet Preparation, and Determination of Total Phenol by Folin-Reagent Method.**

*Morinda citrifolia* leaves, all of the 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°C for 12 hours. The dried leaves were ground into a powder with a grinder. A known weight of noni leaves powder used to extract in hot water. To determine *M. citrifolia* leaves powdered concentration, the methods have been a modification, noni leaves powder (1, 3 and 5 gram) boiled in 100 ml of distilled water at 100±4°C for 2 hours, respectively. The aqueous *Morinda citrifolia* leaves extract centrifuged at 3000 x g, 28°C for 15 minutes and discard the pellet. Then, the supernatant containing the noni leaves extract was lyophilized using freeze dryer to obtain 0.01, 0.07, and 0.10 mg.

Four diets containing a 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. Ingredients were ground up in Hammermill 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 spaghetti-like strings were dried in a drying cabinet using air blower at 50°C until the moisture levels were lower than 10%. After drying the mixture, the finished pellets were stored in a plastic bin at 4°C until being used. The experimental feeds were...
Prepared in the basal diets contained 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 (90 ml). Then, 10% Na$_2$CO$_3$ was prepared by dissolving Na$_2$CO$_3$ (10 g) in water (90 ml). Each crude sample (0.2 ml) was taken in the tube and added 10% Folin-Ciocalteu reagent (1.5 ml). Then, keep in a dark place for 3 minutes. Furthermore, added 10% Na$_2$CO$_3$ (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 the total phenolic compound in all plant extract expressed as milligram of Gallic acid equivalents (GAE) per gram dry weight of the plant.

**Table 1. Composition of The Basal Diet for Macrobium Rosenbergii**

| Ingredients            | 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           | 0.6 | 4   | 0.6 |

**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%      |
Experimental Design.

Giant freshwater 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 uropods which partial retraction of the epidermis could be distinguished (Cheng et al., 2005). During the acclimation period, prawns were fed with control diet twice daily at 08:00 and 17:00.

The experiment was carried out for 63 days with a replacement of 50% water weekly to maintain water quality. Fecal matter, molting and uneaten food was 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 tanks containing aerated fresh water 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 to 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 daily at 08:00 and 17:00. Measurement of the immune response glutathione peroxidase (GPx) activity and the gene expression α2-macroglobulin.

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 manufacturer'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 at 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\(^{-1}\).

Immune genes of *Macrobrachium rosenbergii*.

Total RNA was measured using Total RNA Isolation Reagent (Zymo Research, Quick-RNA\textsuperscript{TM} 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 real-time polymerase chain reaction (RT-PCR) assay in ABI PRISM 7900 Sequence Detection System (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). Specific primers of the 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\(^{ΔΔCt}\) 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 subtracted as the ΔΔCt value that allowed measurement of the change in expression of immune-related genes in the treatment compares than the control sample.

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ΔΔCt = (ΔCt \text{ of prawn fed diet containing aqueous } M. \textit{citrifolia} \text{ leaves extract at 0.6, 2 and 6 g kg}^{-1} \text{ for immune genes}) - (ΔCt \text{ of the control group}).
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Statistical Analysis.

One-way analysis of variance (ANOVA) was used to analyze the data. When ANOVA identified differences among groups, multiple comparisons (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.

RESULTS & DISCUSSION

Glutathione Peroxidase (GPx) Activity of Macrobrachium rosenbergii.

Glutathione peroxidase (GPx) activity 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. After 35 days of feeding treatment, no significant differences of glutathione peroxidase activities at all of the 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 (Figure 1).

![Figure 1](image_url)

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.
Immune gene expression (α2-macroglobulin) of *Macrobrachium rosenbergii*.

The gene expression of α2-macroglobulin in hemocytes of prawn was 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 (described in figure 2).

**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 hemocytes of prawn indicated that the fluctuation of O₂⁻ production in prawns related with the level of total hemocyte count (THC) and phagocytic activities. Decreased of O₂⁻ 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 hemocyte 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 a major 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 to 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. 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 leave extract-supplemented diet might be lead to the suppression immune responses and immune genes expression in prawns.

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REFERENCES
Arizo, M.A., R.A.G.Beroncal, W.A.K.T. Chua, J.J.E. Lim, K.C.S. Rogando and M. B.B. Maningas. 2016. Immune response of Macrobrachium rosenbergii immersed in aqueous extract of Gracilaria edulis challenged with white spot syndrome virus. AACL Bioflux. 9 (2): 215-220.
Balasundram, N., K. Sundram, S. Samman. 2006. Phenolic compounds in plants and agri-industrial by products: antioxidant activity, occurrence and potential uses. Food Chemistry. 99: 191-203.
Cheng, W. and J-C Chen. 1998. isolation and characterization of Enterococcus- like bacterium causing muscle necrosis and mortality in Macrobrachium rosenbergii in Taiwan. Disease of Aquatic Organisms. 34: 93-101.
Cheng, W., Su-Mei Chen, Feng-I Wang, P-I. Hsu, C-H Liu, J-C. Chen. 2003. Effects of temperature, pH, salinity and ammonia on the phagocytic activity and clearance efficiency of giant freshwater prawn Macrobrachium rosenbergii to Lactococcus garvieae. Aquaculture. 219: 111-121.
Govind, P., S. Madhuri, Mandloi A.K. 2012. Immunostimulant Effect of Medicinal Plants on Fish. International Research Journal of Pharmacy. 3 (3): 112-114.
Hossain, M.A., K. A. S. Al-Raqmi, Z. H. Al-Mijzy, A. M. Weli, Q. Al-Riyami. 2013. Study of total pheol, flavonoids contents and phytochemical screening of various leaves crude extracts of locally grown Thymus vulgaris. Asian Pacific Journal of Tropical Biomedicine. 3 (9): 705-710.
Hsu, J.P, Liu, C.I. 1994. Studies on yeast infection in cultured giant freshwater prawn (Macrobrachium rosenbergii). Fish Disease Research. 15:55-0.68.
Jayaraman, S.K., M.S. Manoharan, S. Illancheizan. 2008. antibacterial, antifungal and tumor cell suppression potential of Morinda citrifolia fruit
extracts. *International Journal of Integrative Biology.* 3 (1): 44-49.

Kumaran, T., Devakumar, D., Jayanthi, J. Ragunathan, M.G. 2013. Stimulated immune response by *Morinda tinctoria* methanol leaf extract in a *Vibrio parahaemolyticus* infected fresh water crab, *Oziotelphusa senex senex.* *International Journal Current Science.* 5: 145-152.

Liu, C.-H., S.-P. Yeh, P.-Y. Hsu, W. Cheng. 2007. Peroxinectin gene transcription of the giant freshwater prawn *Macrobrachium rosenbergii* under intrinsic, immunostimulant and chemotherapeutant influences. *Fish and Shellfish Immunology.* 22 (4): 408-417.

Liu, C.-H., C.-C. Chang, Y.-C. Chiu, W. Cheng, M.-S. Yeh. 2011. Identification and cloning of transglutaminase from giant freshwater prawn *Macrobrachium rosenbergii*, and its transcription during pathogen infection and moulting. *Fish and Shellfish Immunity.* 31: 871-880.

Livak, K. J., T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods.* 25: 402-408.

Masuda, M., K. Muraa, A. Fukuhama, S. Naruto, T. Fujita, A. Uwaya, F. Isami, H. Matsuda. 2009. Inhibitory effects of constituents of *Morinda citrifolia* seeds on elastase and tyrosinase. *Journal of Natural and Medicines.* 0.63 (3): 20.67-273.

Nayak, B.S., S. Sandiford, A. Maxwell. 2009. Evaluation of the wound-healing activity of ethanolic extract of *Morinda citrifolia* leaf. *Hindawi Publishing Corporation Scientific World Journal.* 0.6(3): 0.631-0.630.6.

Nayak, S., S. Mengi. 2010. Immunostimulant activity of noni (*Morinda citrifolia*) on T and B lymphocytes. *Pharmaceutical Biology.* 48 (7): 724-731.

New, M. B. 2002. Farming Freshwater Prawns, A Manual For The Culture Of The Giant River Prawn (*Macrobrachium rosenbergii*). FAO, Fisheries Technical Paper, 428 p.

Nworu, C.S., P.A. Akah, F.B.C. Okoye, C.J.Onwuakagba, U.O. Okorafor. C.O. Esimone. 2012. Supplementation with aqueous leaf extract of *Morinda lucida* enhances immunorestitution and upregulates the expression of cytokines and immunostimulatory markers. *Journal of Molecular and Cellular Immunology.* 41 (8): 799-819.

Pham. M.A., K.-J. Lee, B.-J. Lee, S.-J. Lim, S.-S. Kim, Y.-D.Lee, M.-S. Heo, K.-W. Lee. 2006. Effects of Dietary *Hizika fusiformis* on growth and immune response in juvenile olive flounder (*Paralichthys olivaceus*). *Asian-Australasian Journal of Animal Science.* 19: 1769-1775.

Rattanavichai, W., Winton Cheng. 2015. Dietary supplement of banana (*Musa acuminate*) peels hot-water extract to enhance the growth, anti-hypotermal stress, immunity and disease resistance of the giant freshwater prawn, *Macrobrachium rosenbergii.* *Fish and Shellfish Immunology.* 43: 415-420.

Rivera, A. Cedillo, L., Hernández, F., Castillo, V. Sánchez, A. Castañeda, D. 2012. Bioactive constituents in Ethanolic extract leaves and fruit juice of *Morinda citrifolia.* *Annals of Biological Research.* 3 (2): 1044-1049.

Usha, R., S. Sashidharan, M. Palaniswamy. 2010. Antimicrobial activity of rarely known species, *Morinda citrifoia* L.
Halim, A.M. et al.: Aqueous Morinda citrifolia Leaves Extract Enhancing Glutathione

Journal of Ethnobotanical Leaflets. 14: 300.6-311.

Wang, Wei-Na, B-S. Li, J-J, Liu, L. Shi, M.J. Alam, S-J. Su, J. Wu, L. Wang, An-Li Wang. 2012. The respiratory burst activity and expression of catalase in white shrimp, Litopenaus vannamei, during long-term exposure to pH stress. Exotoxicology. 21: 1609-1616.

Zin, Z.M., A.A., Hamid, A., Osman, N. Saari. 2000.6. Antioxidative activities of chromatographic fractions obtained from root, fruit and leaf of Mengkudu (Morinda citrifolia L.). Food Chemistry. 94: 10.69-178.