Nutritional Composition, Antioxidants and Antimicrobial Activities in Muscle Tissues of Mud Crab, *Scylla paramamosain*

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Abstract
Mud crab, Scylla paramamosain known as a green mud crab, has become a popular seafood due to its meat quality. In addition, this marine invertebrate has been found to possess peptides with different biological activities and potentials. The aim was, first, to determine the basic nutritional content and second, to screen for the antioxidants and antimicrobials activities in the tissue of mud crab, S. paramamosain. Percentages of carbohydrate, protein and fat in S. paramamosain were 2.32%, 12.53% and 0.23% respectively. The IC_{50} of methanol extract of tissue S. paramamosain in DPPH scavenging assay was found to be 1.79±0.0 mg/mL. Moreover, superoxide dismutase (SOD) activity was measured as percentage inhibition by SOD was found to be 88.7% ± 2.2, followed by catalase (CAT) activity which was 44.3 ± 2.9 µmol of hydrogen peroxide (H_2O_2) per minute respectively.

The total glutathione (GSH) in the tissues extract was 0.5 µg/mL. In antimicrobial assay, the methanolic extract of S. paramamosain revealed its potential as antimicrobial agents against three human clinical pathogens with minimal inhibitory concentration (MIC) values ranging between 7 to 62 µg/mL. Minimal bactericidal concentration (MBC) values were higher than 125 µg/mL. The study reported that muscle tissues of S. paramamosain are enriched with high protein content and possess antioxidant and antimicrobial activities. Therefore, further studies are needed to identify and isolate the bioactive compounds in the tissue extracts of S. paramamosain to add more values and potentials of mud crabs.

Keywords: Mud crab, Nutritional content, Antioxidants, Antimicrobial, Bioactive compounds.

Introduction

Mud crabs are much in demand due to highly delicate flavor and meat content, thus supporting the economy of fresh water-fishery industry around the world. Belonging to the family of *Portunidae*, this euryhaline organism has been characterised into 4 distinct species which are *Scylla paramamosain*, *Scylla serrata*, *Scylla transquebarica* and *Scylla olivacea*. Interestingly, *S. paramamosain* also known as a green mud crab is one of the popular species and widely distributed in mangrove area with high water salinity such as continental coast of South China Sea and Java Sea.[10]

Among the other species, *S. paramamosain* has triangular frontal lobe spines and easily identified by the dotted pattern on its propodus. From nutritional point of view, mud crabs have high protein, minerals and polyunsaturated fatty acids contents.[23] Apart from nutritional view, many studies have been conducted on the biological activities in mud crabs, specifically about antioxidant properties and antimicrobial peptides AMPs.[6,13,24] The presence of these defense molecules in marine invertebrate immune systems is vital to protect the cells against oxidative stress or invading bacterial.[8]

Marine derived-peptides have been studied by the researchers due to their numerous functional properties which can be applied in food industry and nutraceuticals.[11] Tissues and haemolymph of mud crabs are also reported to possess antioxidant properties and many AMPs enable to inhibit the growth and kill many pathogenic organisms.[1,28]

Generally, AMPs in marine invertebrates have low molecular sizes below 10kDa with many physiological functions.[9] Recently, researchers in China have successfully identified the latest type II cationic cysteine-rich AMP in mud crab *S. paramamosain.* In the same study, the AMP displayed a stronger antibacterial activity against Gram-negative bacteria.[26] Thus, marine-derived bioactive peptides can be recommended to be applied in pharmaceutical industry. In Malaysia, mud crabs especially female crabs are sold at a high value compared to male crabs due to their good taste and high meat content. Nutrient composition has been reported to be affected by the sex of animals and also body parts.[4]

The current study represents the primary conceive to investigate the basic nutritional content, antioxidants and antibacterial properties in the muscle tissues of *S. paramamosain* which are found in Borneo, Malaysia.

Material and Methods

Collection of *S. paramamosain*: Mud crabs, *S. paramamosain* were collected from the mangrove area located in Asajaya Village, Sarawak (1°31'55.0"N

110°39'52.5"E) with the help of field assistants. Species identification was done according to Keenan et al[10] as shown in figure 1. Mature *S. paramamosain* were collected, washed with tap water, fed with razor clams and transported to the laboratory for the purpose of analysis.

Figure 1: Dorsal view(top) of *S. paramamosain*

Preparation of tissue homogenates: Mud crabs were euthanized in cold water for 5 min to reduce consciousness. Then, mud crabs were sacrificed by spiking process within 15 seconds. The muscle tissues were dissected out immediately for homogenization[12]. 0.2 g of the muscle tissues were homogenized in 1.8 mL of 0.9% (w/v) sodium chloride solution using a Hercuvan digital hand-held tissue homogenizer. This step was done in tube containing ice to preserve the protein content.

The homogenates were centrifuged at 10000 rpm for 15 min at 4 °C using a refrigerated centrifuge. The supernatant was stored at a temperature of −20 °C. All proximate compositions and enzymatic and non-enzymatic antioxidant assays were completed within a duration of two weeks after the tissue homogenate was prepared.

Preparation of methanol extract of muscle tissues: Crab tissue was extracted using methanol as a solvent and conducted based on method of Laith et al.[13] Crab tissue was frozen with liquid nitrogen and immediately grinded into powder form. The powder sample was soaked in 1:10 w/v of methanol for 3 days and then filtered using a filter paper. Methanol extract was dried under a fume hood to ensure the solvent evaporated out and used for the determination of DPPH scavenging activity and antibacterial assays.

Proximate analysis: Protein, carbohydrate and fat contents were calculated according to the in-house method 0512 in reference to the method of Analysis for Nutritional Labelling, AOAC.[25]

Antioxidant analysis

DPPH radical scavenging activity: The ability of free radical scavenging activity in mud crab tissue extracts was evaluated by DPPH scavenging assay.[21] Briefly, 0.1mM solution of DPPH in methanol was prepared. 0.8mL of DPPH solution was added to 0.2 mL of all tissue extracts at different concentrations ranging from 0.0010 mg/mL to 3 mg/mL. The mixtures were shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance was measured at 517 nm using a JASCO UV-Vis spectrophotometer. Ascorbic acid was used as a standard. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. All the tests were performed in triplicate. The ability of scavenging the DPPH radical was calculated using the following formula:

$$\text{DPPH scavenging activity (\% inhibition)} = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in presence of all the extract samples and reference.

SOD activity: The determination of antioxidant enzyme, SOD, activity in tissues homogenate was conducted using Oxiselect™ Superoxide Dismutase Activity Assay Kit (STA-340) from Cell Biolabs Incorporation, USA. The experimental procedures were conducted according to the manufacturer protocols. Absorbance of samples was read at 450 nm using BioTek microplate reader. SOD activity was then calculated based on the percentage of inhibition rate of SOD whereby unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50% in 1 mL reaction solution.

CAT activity: The other enzymatic antioxidant, CAT activity in tissues homogenate was measured according to Sinha.[22] A reaction mixture containing 1ml of 0.01M phosphate buffer, pH 7.0, 0.5 mL of 0.2M hydrogen peroxide and 0.4 mL of distilled water was prepared in this study. Next, 0.5 mL of extract was added and the mixture was incubated at 25°C for 5 min in water bath. The reaction was ceased with the addition of 2 mL acid reagent (a mixture of dichromate and acetic acid in volume ratio of 1:3).

The control was prepared by adding the extract once the acid reagent was added. The absorbance of samples was recorded at 610 nm using JASCO UV-Vis spectrophotometer after the sample was heated for 10 min. High concentration of hydrogen peroxide produced indicates a lower CAT activity.

The results were expressed in µmoles of H_2O_2 consumed per minute per milligram protein.

Total Glutathione: Total glutathione in the sample was measured using a commercial Glutathione Assay Kit from Biovision Incorporated (K261-100). The sample preparation and procedures were conducted according to manufacturer's protocols. Absorbance of the sample was read at 412 nm using Biotek Microplate Reader. The total glutathione was presented in unit of µg/mL.

Antimicrobial analysis

Minimum inhibitory concentration (MIC): The MIC of *S. paramamosain* methanol extract was determined using broth micro-dilution method in 96-well plate. The broth microdilution method was conducted according to Wiegand et al[27] with slight modification. Methanol extract of *S. paramamosain* was tested against two-gram negative bacteria namely *E. coli and S. typhi* and one-gram positive bacteria, *S. aureus.* The bacteria were strike on Mueller Hinton Agar (MHA) plate and incubated at 37℃ for 18-24 hours to obtain single colonies. Few single colonies were then transferred to 5 mL of Mueller Hinton Broth (MHB) and turbidity of the culture was compared with 0.5 McFarland standard using spectrophotometer at a wavelength of 620 nm. Broth culture with similar absorbance value to 0.5 McFarland standards contained bacterial concentration of approximately 1×10^8 CFU/mL.

The methanol extracts were prepared at a concentration of stock of 1000 µg/mL. Then, two-fold dilutions were conducted with MHB in a 96-well plate. 100 µL of bacteria suspension were then added into the resultant serial dilution series and incubated with 150 rpm shaking at 37°C for 24 hours. After the incubation period, the plates were examined with naked eyes under adequate lighting for turbidity. The lowest concentration of the sample where no visible growth was detected is the MIC. The absorbance value of each well at 620nm was also determined using a Biotek Microplate Reader and the MIC_{50} were calculated. Tetracycline was used as a positive control in the experiment. The broth microdilution was conducted in triplicate for every sample.

Minimum bactericidal concentration (MBC): MBC test was undertaken to determine the minimum concentration of the extracted samples needed to kill the microorganisms or bactericidal. It was done according to Romainor et al.[18] It was done by sub-culturing broth dilutions that prevent the growth of bacterial organism (those concentrations at or above MIC). 10µL of the mixture from 96-well microtiter cell culture plates with no turbidity observed was put onto MHA and then incubated for 18-24 hours at 37℃. The MBC point was determined as the lowest concentration in serial dilution series that shows no colony growth after 24 hours of incubation at 37°C.

Data analysis: The data were analyzed and presented in mean ± standard deviation (SD) using the statistical software of SPSS (Statistical Package for the Social Sciences) version 24.0 for Windows.

Results and Discussion

Crabs are good source of protein to marine life as well as human. It is important to have the biochemical information of mud crabs since the demand is increasing as one of the fisheries products. The percentages of carbohydrate, protein and fat in *S. paramamosain* are presented in figure 2 respectively.

The muscle tissues of *S. paramamosain* were found to have 2.32% carbohydrate, 12.53% protein and 0.23% fat. These results elucidated that protein is the main composition among the three macromolecules in muscle tissues of *S paramamosain*.

The present results are in agreement with the previous reports. A study by Zaliha et al[30] reported that mud crab tissues contained 14.63% protein, 3.30% carbohydrate and 0.04% fat. Meanwhile, Sreelaksmi et al[23] in their study revealed that the protein content in the mud crabs ranged between 15.63% to 17.63% and crude fat content ranged between 0.53% to 1.54%. In contrast, Paul et al[17] reported a lower protein content than this study which was 11% in *S. serrata*. Sarower et al[20] have reported that female mud crabs of *S. serrat*a have higher protein and lipid contents compared to males *S. serrata* suggesting that nutrient content may differ between gender.

Additionally, a study by Zafar and Siddiqui[29] suggested that the protein values showed different pattern during pre-breeding and post-breeding period in male and female *S. serrata*. Apart of being important in gametogenesis of the crustacea, protein is fundamentally nutrient to human[3], therefore it is good to consume mud crabs as one of good sources of protein. Fat in mud crabs is vital in ovarian maturation of the female crabs and also acts as a source of energy as well as provides essential fatty acids and fat-soluble vitamins.[2]

Additionally, low fat content shows that mud crab acts as an excellent diet due to its balance nutrient content and delicacy seafood. However, various procedures during preparation, extraction methods or storage of mud crabs may affect the nutritional quality of the crabs.[5] On another note, antioxidant related enzymes are directly involved in crustaceans' innate immune reaction.[14] The antioxidant activities of the tissues of S. *paramamosain* measured by the DPPH, SOD and CAT activities as well as total glutathione are presented in table 1.

In this study, concentration of sample that causes 50% loss of DPPH activity, IC_{50}, was measured. The IC_{50} of methanol extract of tissue *S. paramamosain* in DPPH scavenging assay was found to be 1.79±0.0 mg/mL. The activity of SOD was measured in percentage inhibition by SOD for a duration of 15 min. Water-soluble formazan dye was formed upon reaction of SOD in samples.

Table 1
Antioxidants analysis in *S. paramamosain* tissue extract

Parameters*	
IC_{50} of DPPH scavenging activity (mg/mL)	1.79 ± 0.0
SOD (Percentage inhibition by SOD, %)	88.7±2.2
CAT (µmol of H_2O_2 per minute)	44.3±2.9
Total Glutathione (µg/mL)	0.5 ± 0.0

*All data in antioxidants analysis were expressed in mean ± SD of samples

In the present study, the percentage of SOD activity was found to be 88.7 ±2.2 %. The other antioxidant enzyme CAT activity was measured based on the concentration of H_2O_2 produced in the reaction. Blue precipitate formed in the reaction indicated that H_2O_2 in the reaction has reacted with dichromate. In this study, the CAT activity was found to be 44.3±2.9 µmolmin^{-1}mg^{-1}. SOD is vital in antioxidant defense systems which catalyzes the conversion of the superoxide anion (O^{2-}) into oxygen (O_2) and hydrogen peroxide (H_2O_2).

Catalase CAT plays role in enzymatic detoxification to degrade hydrogen peroxide, a powerful and harmful oxidizing agent to water and oxygen gas. Liu et al[14] have suggested that the increase of SOD dismutation rate is accompanied with the enhancement activity of CAT when the crabs are exposed to oxidative stress. Furthermore, in this study, the total glutathione in the muscle extract was found to be 0.5 µg/mL. Glutathione is an antioxidant that play roles in maintaining cell redox status when exposed to oxidative stress such as in hypoxia condition.[7]

Previous studies have suggested that the antioxidant enzymes in crabs are considerably affected by water salinity, seasonal changes, tissue and gender of the species.[16,19] Screening for antibacterial activity was performed using methanol extract of muscle tissue. Percentage inhibitions of *E. coli*, *S. aureus* and *S. typhi* being treated with methanol extract of tissue *S. paramamosain* and tetracycline were displayed in figure 3. MIC (µg/mL) and MBC (µg/mL) values of *S. paramamosain* methanol extract against three bacteria were displayed in table 2. This study revealed that the MIC values of methanolic extracts of *S. paramamosain* against three pathogens range from 7 µg/mL to 62 µg/mL. The most susceptible pathogen was the gram-positive bacteria; *S. typhi* with MIC_{50} value was 32 µg/mL.

On the same note, MICs values for the gram negative, *E. coli* and gram positive, *S. thypi* were ranging from 15 µg/mL to 62 µg/mL respectively. MIC_{50} values against *E. coli* and *S. aureus* was found to be 48 µg/mL and 43 µg/mL respectively. However, the MBCs for all pathogens tested were higher than 125 µg/mL whereby no growth of bacteria were detected above this concentration as shown in figure 4.

The results suggested that the methanol extract of muscle tissue *S. paramamosain* could be a bactericidal agent at higher concentrations and a bacteriostatic agent at lower concentrations. The present findings are supported by the works of Laith et al[13] who studied the antibacterial activity of the other mud crab species which was *S. transquebarica*. Their findings showed that the crab extract inhibited bacterial growth at lower concentrations of 0.78 µg/mL to 3.125 µg/mL.

In the same study, the crab extracts at concentrations of 6.25 µg/mL to 25 µg/mL resulted in microbial death. In other study, Meiyalagan and Arumugam[15] have suggested that the protein present in undiluted serum of *S. serrata* could inhibit the growth of *Bacillus sp*. and *E. coli* and contributed to the host defense system.

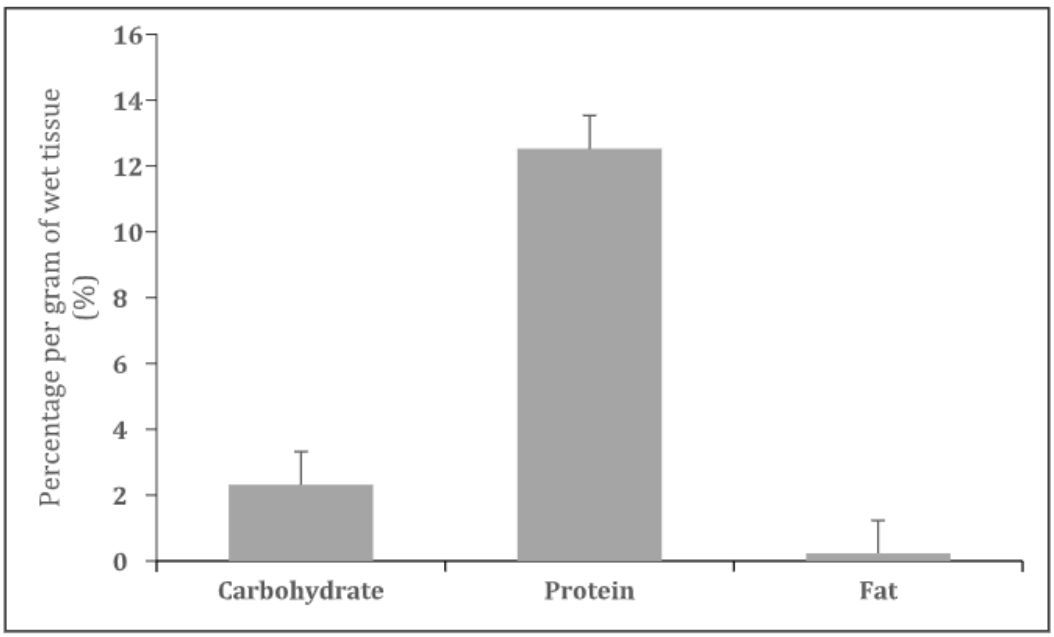

Figure 2: Basic nutrition content in *S. paramamosain*

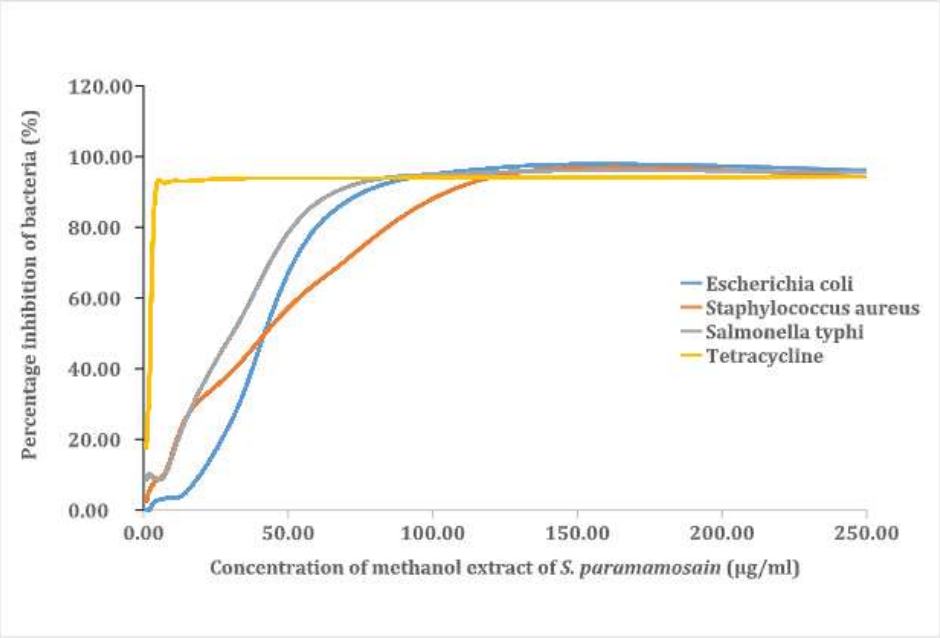

Figure 3: Percentage inhibition of *E. coli, S. aureus* and *S. typhi* being treated with methanol extract of tissue *S. paramamosain* and tetracycline

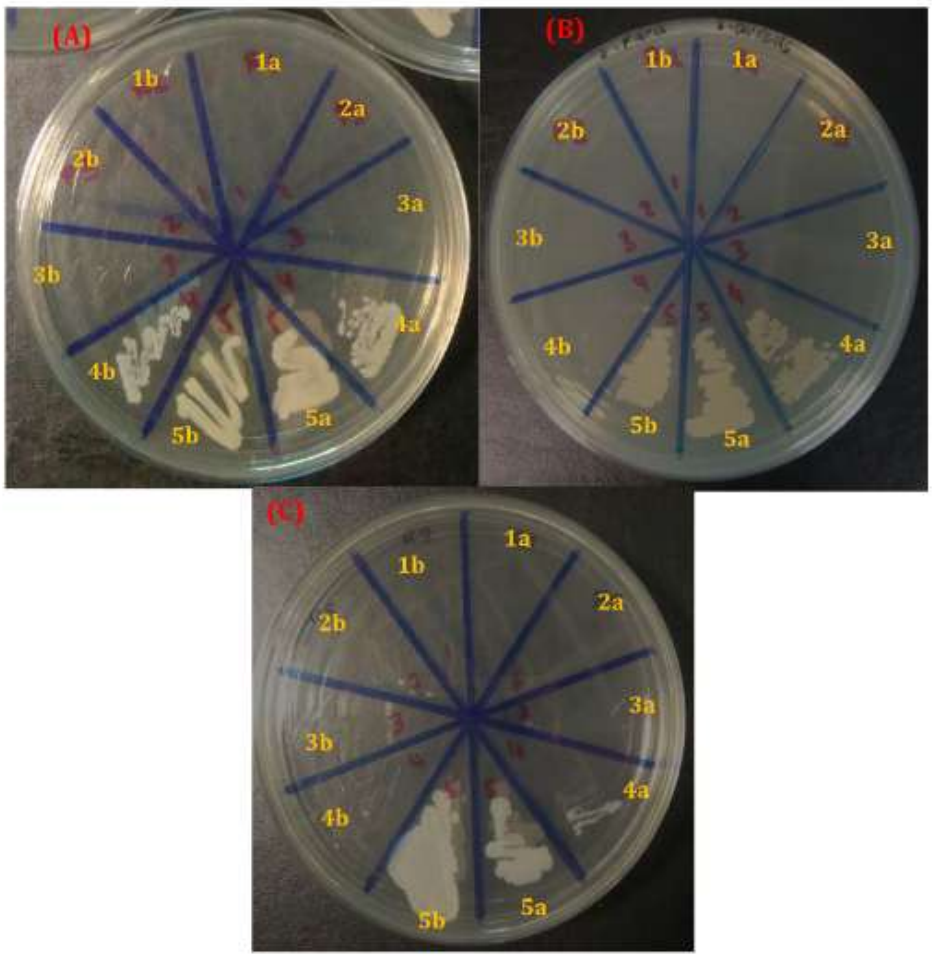

Figure 4: Antibacterial activity of mud crab, *S.paramamosain* against tested bacteria by minimum bactericidal concentration method. (A): *E. coli*, (B): *S. aureus* (C): *S. typhi* at different concentrations: (1a/1b) = 1000 µg/mL, (2a/2b) = 500 µg/mL, (3a/3b)= 250 µg/mL, (4a/4b)= 125 µg/mL, (5a/5b)= 62.5 µg/mL.
[ab]All concentrations were done in duplicate

Table 2
Antibacterial activity of methanol extract of tissue *S. paramamosain.*

Bacterium	Minimum inhibitory concentration (µg/mL)		Minimum bactericidal concentration (µg/mL)
	Range	MIC_{50} value	
Escherichia coli	15–62	48	>125
Staphylococcus aureus	7–62	43	>125
Salmonella typhi	15–62	32	>125

Conclusion

The present study revealed the information of basic nutrient content in edible mud crab, *S. paramamosain*. From the study, it can be concluded that mud crab has good nutritive value in human diet. In addition, the tissue extracts also possess the anti-oxidative and antibacterial properties which could become an important source of marine bioactive compounds. Therefore, further research on the isolation and purification of bioactive compounds from mud crab tissues is needed to discover the new potentials of this marine organism.

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