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RESEARCH ARTICLE

COMPARISON OF STRESS ENZYMES LEVEL IN *PENAEUSSEMISULCATUS* SPECIES COLLECTED FROM FOUR ACCESSIONS

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ABSTRACT

Penaeussemisulcatus (grooved tiger prawn or green tiger prawn)accessions were collected from southern subcontinent of India and the anti-oxidant enzyme levels in their tissues were compared. The accessions were from Pondicherry, Pazhaverkadu, Royapuram and Thalankuppam. From the frozen tissue samples of these species, Lipid peroxidation, Catalase activity, Glutathione S Transferase, Reduced glutathione, Acetyl cholinesterase, and Superoxide dismutase enzyme levels were measured. All the enzyme levels were elevated in the species collected from Thalankuppam compared to other three species. This is possibly due to the associated environmental factors and is an indirect measure of pollution levels.

INTRODUCTION

Edible Crustaceans, such as Crab, Prawn, Lobster and Cray fish constitute one of the most important sources of human food of all the animals and plants found in the aquatic environment. These serve as a major source of protein and constitute the main part of the diet in many countries. The nutritive values of crustaceans depend upon their biochemical composition, such as Protein, Lipids, Amino acids, Carbohydrates, Vitamins, Fatty acids, and Minerals. There are about 150 species of freshwater prawns all over the world, of which only 40 species are found distributed in the rivers, reservoirs and estuaries of India. One among them is *Penaeussemisulcatus* (commonly known as grooved tiger prawn or green tiger prawn) is distributed throughout the Indo West Pacific and is fished commercially in many countries (De Grave, 2017). This species contribute an almost equal share to the fishery industry of India as shared by *Penaeusmonodon*. It is one of the most important marine crustacean species subjected to intense fishery exploitation and aquaculture practices worldwide (Hulata, 2001). This species has been identified as a candidate for mass-breeding programs in some countries (Seidman and Issar, 1988; Gürel Türkmen, 2001). Farming of this species has not been attempted yet, but it has potential for commercial culture in India. The study of availability of stress enzymes of wild stocks might provide useful information for selection of suitable stock for farming programs. Since anti-oxidant enzymes are involved in detoxifying and stress management, the level of these enzymes in a particular species may vary according to the environmental factor. Thus, to prove the variation in these enzymes in a prawn species collected from four different locations in Tamilnadu, this present study was carried out. In the current study, four accessions of *Penaeussemisulcatus* which were collected from Tamilnadu and Pondicherry regions of India were analyzed for its stress enzymes using biochemical analysis. Their levels will indicate the stress in animals which could be possibly due to the environmental impact posed on them. The stress enzyme levels can also be viewed as a measure of pollution in a particular place.

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MATERIALS AND METHODS

Sample collection and identification: The samples were specifically collected from the Coromandel Coast along the Tamil Nadu coast. Coromandel Coast, in the east coast of Tamil Nadu and Andhra Pradesh states, SE India, stretches more than 400 miles (644 km) from Point Calimere, opposite the northern tip of Sri Lanka to the delta of the Krishna River. Over most of its length, the Coromandel Coast consists of surf-beaten beaches, uninterrupted by natural harbors.A total of four shrimp samples were collected from four different sites, Pondicherry (11°.05 N, 79°.05 E), Royapuram (13°.10 N, 80°.29 E), Pazhaverkadu (13°.41 N, 80°.31 E), and Thalankuppam (13°.22 N, 80°.32 E) situated along

the Coromandel Coast of the Bay of Bengal, India. Fresh specimens were spot examined for specific morphological characters that define the green tiger prawns. Each catch sampled from different sites were investigated to ensure correct sampling and labeling. These species were identified by using taxonomic keys described by "Edible Penaeid Shrimps in India" in the Training Manual "GIS and Marine Biodiversity" edited by John Milton (2008) (Rajkumar *et al.,* 2015).

Lipid peroxidation assay: Lipid peroxidation products such as Malionaldehyde (MDA) are considered useful and reliable indicators of oxidative damage, due to the susceptibility of membranes to attack by reactive oxygen species (Wise and Naylor, 1987; Hodges *et al.,* 1999). For the measurement of MDA content, frozen tissue (4 g) was immersed in 20 mL of 0.1 M (pH 6.8) potassium phosphate buffer and then centrifuged at 10,000 g for 15 min. The supernatant was collected and used to determine MDA content according to Guidi *et al.* (2000) with minor modifications (Guidi *et al.,* 2000). A mixture of supernatant (2 mL) and 0.5% 2-thiobarbituric acid (2 mL, TBA, dissolved in 15% trichloroacetic acid) was heated at 100°C for 20 min. After rapid cooling, the mixture was centrifuged at 10,000 g for 10 min to clarify the solution. The absorbance of the supernatant was read at 450, 532 and 600 nm. MDA content was calculated as nmol g^{-1} fresh weight (FW) by using the following formula:

$$6.45\,(Absorbance_{532} - Absorbance_{600}) - 0.56 * Absorbance_{450}$$

Catalase activity: Catalase (CAT; EC 1.11.1.6) activity was determined by measuring the decrease of H_2O_2 concentration at 240 nm according to Koroliuk *et al.* (1988). The rate of decrease in H_2O_2 content is directly proportional to the CAT activity in the sample. To 1.2 ml of PBS, 0.2ml of serum was added, followed by 1 ml of H_2O_2 solution. The absorbance at 240 nm was constantly measured for 3 min at 30 Sec intervals. 1 ml of distilled water was used as blank and run simultaneously. The enzyme activity was expressed as µmoles of H_2O_2 decomposed/ min/mg protein.

GSH-S-transferase content: GST (EC 2.5.1.18) was determined according to the procedure described by Habig et al, 1974[10].The reaction mixture was prepared by mixing 2.7 ml sodium phosphate buffer (100 mM) pH 6.5, 0.1 ml GSH (75 mM), 0.1ml CDNB (30mM) and 0.1 ml of the sample. The absorbance was measured at 340 nm spectrophotometrically at the temperature of 25°C. The increase in absorbance was recorded at 60s intervals for a total of 3 minutes. The reaction solution without sample was used as blank. The specific activity of GST was expressed as µmol GSH-CDNB (1-chloro-2.4-dinitrobenzene) conjugate formed/min/mg protein using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.The enzymatic activity was calculated via the formula:

$$GST = \frac{\Delta\ O.\ D/min \times Vol.\ of\ assay \times Dilution\ factor}{9.6\ of\ Vol.\ of\ conjugate\ enzyme\ (PMS) \times Vol.\ of\ sample\ x\ Protein\ (mg)}$$

Where,
9.6= molar extinction coefficient of CDNB
Δ OD/min = (Δ OD_{340} standard/sample - Δ OD_{340} blank)

Reduced glutathione content: GSH in the tissue was determined according to the method of Moron *et al.* (1979). 0.5 ml of the tissue homogenate was mixed with 0.1 ml of 25 % TCA, kept on ice for few minutes. These were then subjected to centrifugation at 3000 g for few minutes to settle the precipitate. 0.1 ml of the supernatant was mixed with 2.4 ml of 0.1 M phosphate buffer (pH 7.4) and 0.5 ml of 10mM DTNB (prepared in 0.2 M buffer, pH 8). The yellow color obtained was measured after 10 min at 412 nm against a blank which contained 0.1 ml of 5% TCA in place of the supernatant. The GSH content was calculated as µmol DTNB conjugate formed/gram tissue using molar extinction coefficient of $13.6\times10^3\ M^{-1}\ cm^{-1}$with the help of Formula

$$GSH = \frac{\Delta OD / min \times Vol.\ of\ assay \times dilution\ factor}{13.6of\ mole\ GSH\ conjugate/gm\ tissue\ x\ Vol.\ of\ sample\ Protein\ (mg)}$$

Acetyl cholinesterase activity: Acetylcholinesteraseis an enzyme that degrades (through its hydrolytic activity) the neurotransmitter acetylcholine, producing choline and an acetate group. 100mg of tissue was weighed and homogenized in 1 ml of phosphate buffer (0.1 M pH 8). To 0.1 ml of homogenate, 2.9 ml of phosphate buffer (pH 8) was added followed by 100µl of DTNB reagent (0.01M) and mixed well. The absorbance was measured at 412 nm; when this had stopped increasing, the absorbance was set to zero. To this 20µl of the substrate (acetylthiocholine iodide 0.075M) was added. Changes in absorbance were recorded and the change in absorbance per min was calculated. The rates were calculated as follows

$$AChE = \frac{\Delta O.\ D / min \times Vol.\ of\ assay \times dilution\ factor}{13.6\ of\ mole\ conjugate/gm\ tissue\ x\ Vol.\ of\ sample\ Protein\ (mg)}$$

SOD activity: The method used for measuring the SOD activity in this study, was a slight modification of indirect inhibition assay developed by Giannopolitis and Ries (1977)[12]. Breifly, to detect SOD enzymatic activity, 10mg of the tissue was homogenized in 200 µL of extraction buffer (20 mMTr is HCl, pH 8.0, 1 mM EDTA (ethylenediamine tetra acetic acid), 1 mM PMSF, and 0.1 % TritonX-100). The supernatant was separated using centrifugation at 12000 rpm for 20 minutes, at 4°C. The supernatant was collected and heated for 5 min at 65˚C to obtain a new supernatant after centrifugation (crude extract), which was stored at 20˚C until use. Samples were maintained on ice at all times to avoid protein denaturation. 100 µl of the supernatant was added to the 2 ml reaction mixture consisting of0·1 mM EDTA, 13 M methionine, 0·75 mM NBT, and 20 M riboflavin in phosphate buffer 50 mM, pH 7·8. The reaction was started by adding riboflavin, which initiated the light-mediated reaction. Tubes containing the reactants were incubated under fluorescent light, at 22° C. After 25 min, the reaction was stopped by transferring the test tubes to darkness and absorbance of the mixtures at 560 nm was recorded using a Spectrophotometer. The percentage of inhibition is the basis on which the amount of activity is calculated as below

$$\%\ inhibition = \frac{Absorbance\ (reaction\ control) - absorbance\ (sample) \times 100}{Absorbance\ (reaction\ control)}$$

One unit of SOD was defined as the amount of enzyme that inhibits the rate of NBT reduction by 50%.

Then X% is equal to 1/50 x X= Y unit.

Table 1: LPO levels in *P. semisulcatus*collected from four locations. Its expressed in nmolg^{-1}

	Lipid peroxidation (nmol g^{-1} fresh weight (FW)			
Samples	**OD at 532**	**OD at 600**	**OD at 450**	**MDA content**
Pondicherry	0.849333333	0.454666667	0.368666667	2.339±0.05
Pazhaverkadu	0.924666667	0.497666667	0.394666667	2.53±0.04
Royapuram	1.256	0.687666667	0.487	3.39±0.07
Thalankuppam	1.269	0.623	0.478333333	3.89±0.02

Table 2: Catalase levels in *P. semisulcatus*collected from four locations. Its expressed in nmol/min

	Catalase Activity (nmol/min mg protein)			
Time (s)	**Pondicherry**	**Pazhaverkadu**	**Royapuram**	**Thalankuppam**
0	0.648	0.636	0.615	0.606
60	0.547	0.534	0.495	0.463
120	0.446	0.421	0.314	0.296
180	0.328	0.297	0.188	0.152
A240 (Initial-Final)/Min	0.1067	0.1129	0.142	0.1514
Catalase Activity	2.203±0.07	2.33±0.03	2.93±0.04	3.125±0.03

Table 3. GST levels in *P. semisulcatus*collected from four different locations. Its measured as nmol/min mg of total protein

	GST Activity (nmol/min mg protein)			
Time (s)	**Pondicherry**	**Pazhaverkadu**	**Royapuram**	**Thalankuppam**
0	0.073	0.074	0.105	0.116
60	0.158	0.161	0.229	0.28
120	0.246	0.252	0.441	0.495
180	0.334	0.364	0.657	0.688
A360 (Final-Initial)/Min	0.0869	0.0965	0.184	0.1906
GST Activity	7.81±0.107	8.70±0.230	16.9±0.248	17.53±0.218

Table 4. GSH levels in *P. semisulcatus*collected from four locations. It was expressed asnmol/min

	GSH (nmol/min mg protein)			
Time (s)	**Pondicherry**	**Pazhaverkadu**	**Royapuram**	**Thalankuppam**
0	0.5479	0.5486	0.5669	0.5674
60	0.597	0.601	0.684	0.705
120	0.656	0.673	0.793	0.814
180	0.7424	0.7537	0.8646	0.8875
A240 (Final-Initial)/Min	0.0648	0.0683	0.099	0.1067
GSH Activity	4.291±0.105	4.525±0.101	6.56±0.023	7.061±0.150

Table 5. AchE levels in *P. semisulcatus*collected from four locations. It was expressed in nmol ACTI /min/mg/protein

Sample	**0 s**	**60 s**	**120 s**	**180 s**	**Change in Absorbance per min**	**Average**	**Acetyl cholinesterase activity nmol ACTI /min/mg/protein**
Pondicherry	0.654	0.754	0.849	0.956	0.101	0.1	6.617±0.06
	0.653	0.755	0.851	0.951	0.099		
Pazhaverkadu	0.659	0.769	0.863	0.979	0.107	0.107	7.136±0.109
	0.662	0.762	0.874	0.989	0.109		
Royapuram	0.696	0.824	0.952	1.411	0.238	0.240	15.93±0.233
	0.691	0.833	0.959	1.421	0.243		
Thalankuppam	0.699	0.864	0.984	1.596	0.299	0.297	19.68±0.140
	0.701	0.861	0.991	1.589	0.296		

Table 6. SOD levels in *P. semisulcatus*collected from four locations. It was expressed as Units/mg

	SOD Activity (Units/mg)			
	Pondicherry	**Pazhaverkadu**	**Royapuram**	**Thalankuppam**
Absorbance (Blank)	0.483	0.483	0.483	0.483
Absorbance (Sample)	0.3241	0.3093	0.2476	0.2174
% inhibition of NBT reduction by SOD	32.93	36.006	48.765	55.02
50% inhibition =1 Unit of SOD	0.6586	0.72012	0.9753	1.1004
SOD units/mg	1.3172±0.05	1.44024±0.09	1.9506±0.08	2.2008±0.13

RESULTS

LPO, Catalase, GST, GSH, AchE and SOD play important roles in protection against oxidative stresses posed on animal tissues. The elevation in these enzymes is an indicator that the tissues were exposed to higher oxidative stress. In the present study, the samples collected from four different locations showed different levels of enzyme activities. All the enzyme levels were elevated in Thalankuppam samples compared to other three locations. It was found that the MDA content was higher in tissues of Royapuram and Thalankuppam compared to that of Pondicherry and Pazhaverkadu. The Table 1 and Figure 1 shows that LPO levels vary among the species collected in different locations. Similarly, catalase levels were measured from 0 to 18 seconds and were tabulated (Table 2 and Figure 2). The Thalankuppam species were showing highest catalase activity compared to other three.

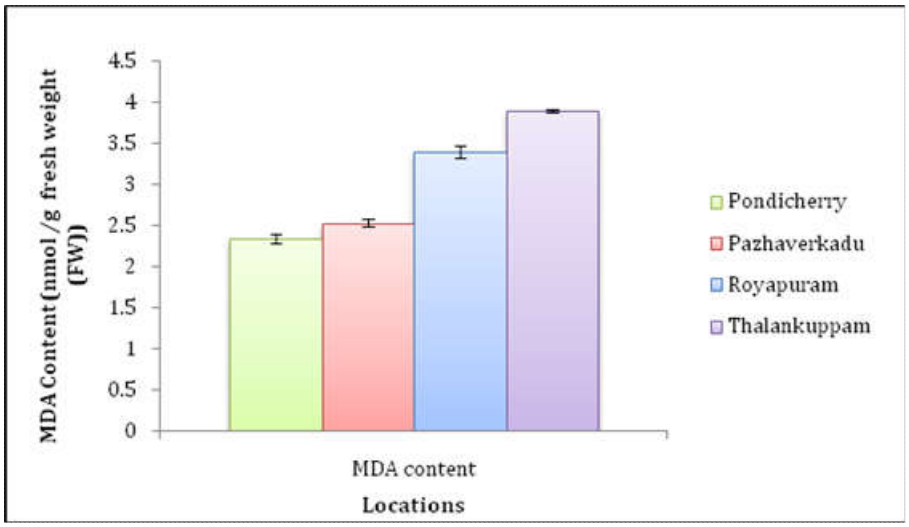

Figure 1. LPO levels in *P. semisulcatus* collected from four locations. The mean and standard deviation was calculated for triplicates and marked in the graph

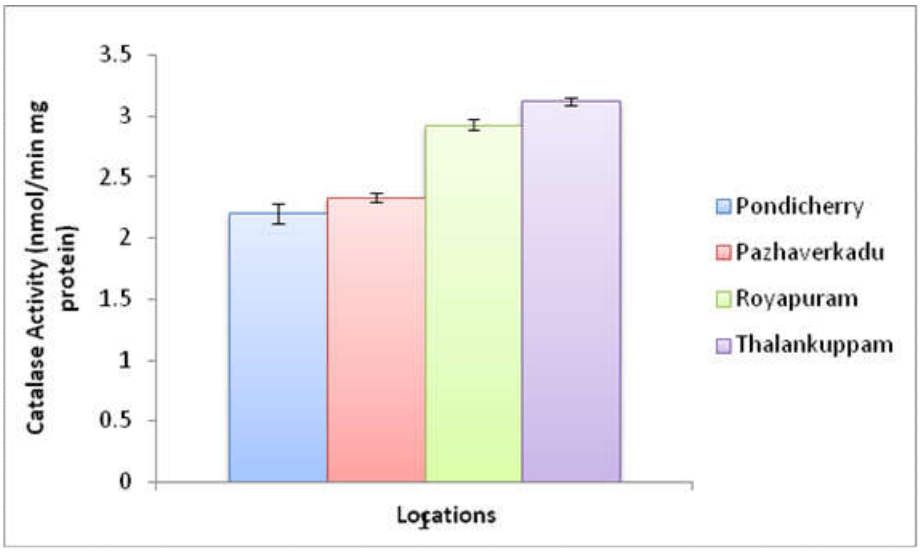

Figure 2. Catalase levels in *P. semisulcatus* collected from four locations. The mean and standard deviation was calculated for triplicates and marked in the graph

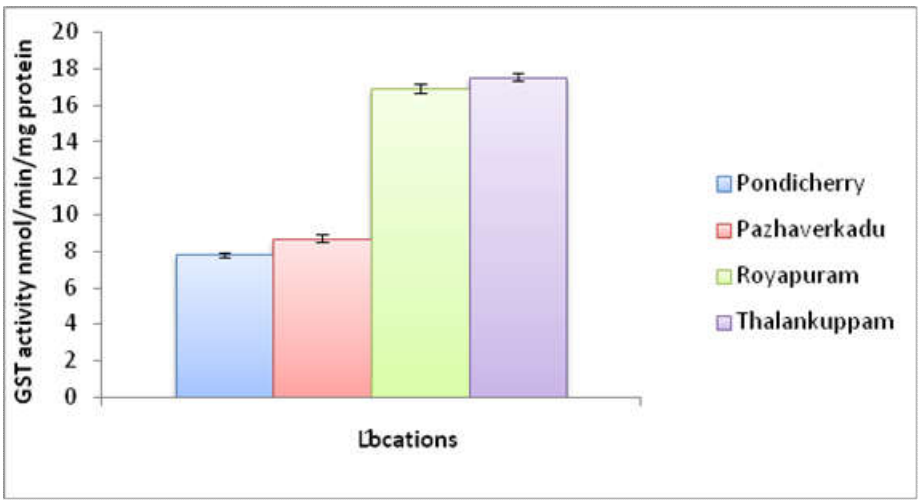

Figure 3. GST levels in *P. semisulcatus* collected from four locations. The mean and standard deviation was calculated for triplicates and marked in the graph

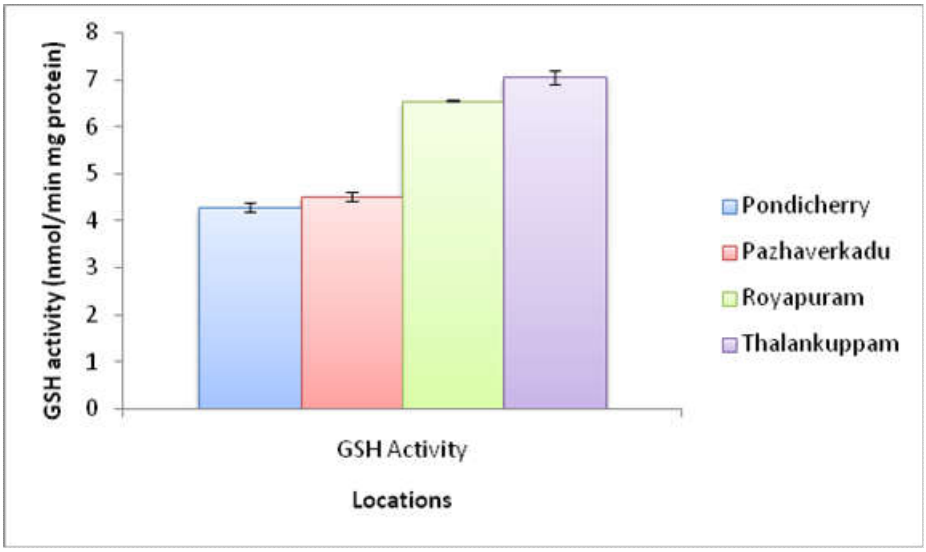

Figure 4 GSH levels in *P. semisulcatus* collected from four locations. The mean and standard deviation was calculated for triplicates and marked in the graph

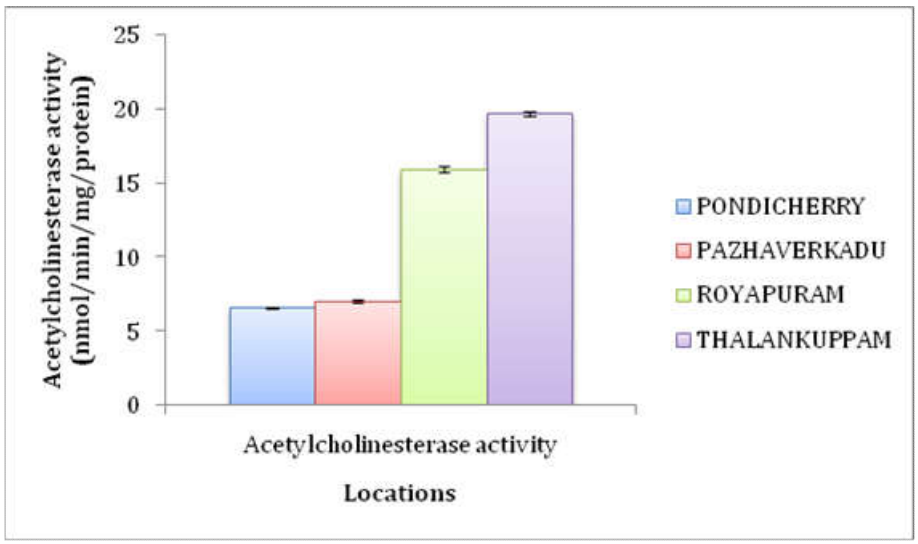

Figure 5. AChE levels in *P. semisulcatus* collected from four locations. The mean and standard deviation was calculated for triplicates and marked in the graph

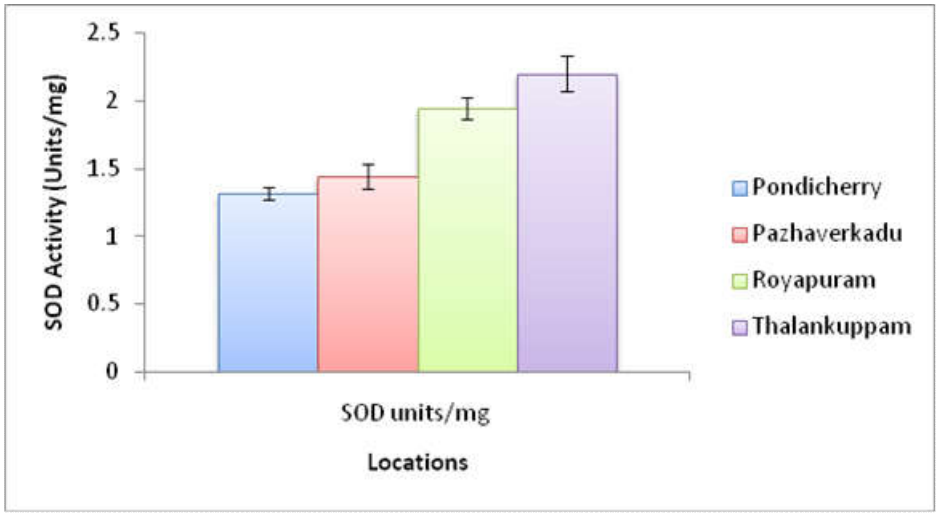

Figure 6. SOD levels in *P. semisulcatus* collected from four locations. The mean and standard deviation was calculated for triplicates and marked in the graph

The lowest among them was Pondicherry. As accordance with LPO and Catalase activity, lowest GST activity was found in Pondicherry samples.GST activity was highest in the samples collected from Thalankuppam followed by Royapuram (Table 3). As shown in the figure (Figure 3), the highest GSH activity was found in Thalankuppam and Royapuram species and the levels were as low as 4.21 in Pondicherry samples. Furthermore, AchE activity was compared among the four samples (Table 4 and Figure 4). It was 19.68 in Thalankuppam samples whereas in Pondicherry samples, it was found to be only 6.6. In case of SOD activity, though it was almost similar for all the samples, Thalankuppam samples showed a slight higher activity among other samples (Table 5 and Figure 5).

DISCUSSION

Aquatic organisms possess systems for generation and degradation of free radicals (Winston, 1991; Winston and Di Giulio, 1991; Valavanidis *et al.,* 2006; Lushchak, 2011; Dutta *et al.,* 2014) like other higher forms. Diverse protective systems must exist to enable adaptation to oxidative environments. Oxidative stress (OS) results when production of reactive oxidative species (ROS) exceeds the capacity of cellular antioxidant defenses to remove these toxic species (Lushchak, 2011; Dutta *et al.,* 2014; Limón-Pacheco and Gonsebatt, 2009). Similar studies were carried out in prawn species *Penaeusmonodon,* in which theoxidative stress response in hepatopancreas and gills of *P. monodon* to *V. parahaemolyticus* challenge were studied (Duan *et al.,* 2015). Many studies have investigated the effect of temperature on oxidative stress in aquatic animals (Vinagre *et al.,* 2012). In all these studies, oxidative stress has been used as biomarkers mostly as indicators of the effects of contamination in field studies. From the present study, it was clear that *P. semisulcatus* growing in the regions of Thalankuppam has higher stress enzyme levels compared to that of Pondicherry, Royapuram and Pazhaverkadu. Thus, among these four regions, contamination in the areas of Thalankuppam is higher which increases the physiological stress in the prawns.

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