8. ANTIOXIDANT ASSAY

8.1. INTRODUCTION

Marine invertebrates have pronounced pharmacological activities and other bioactive properties which are useful in the biomedical arena. Since marine natural products are becoming increasingly attractive due to their potential applications in the pharmaceutical industries, the identification of new sources of these materials is extremely important. Oxidative stress, the consequence of an imbalance of pro-oxidants and antioxidants in the organism, is rapidly gaining recognition as a key phenomenon in chronic diseases (Ines and Federico, 2000). As a consequence of this reactivity of ROS and their potential to damage cells and tissues, marine and other organisms balance the production of these radicals with a wide variety of cellular antioxidant defenses (Correia et al., 2003). Thus, antioxidants have gained more importance on account of their positive effects, as health promoters in the treatment of cardiovascular problems, atherosclerosis, many forms of cancer, ageing process, etc. Hence the quest for natural antioxidant compounds has initiated the search towards marine organisms which serves as a reservoir of unique molecules. A number of studies have demonstrated potential for antioxidant enzyme, free radical scavenger responses and oxidative damage in species of invertebrates (De Giulio et al., 1995).

The liver as a vital organ in the body is primarily responsible for the metabolism of carbohydrates, lipids, proteins and detoxifying xenobiotics and drugs. Thus the liver is prone to injury due to the chronic exposure to drugs, environmental toxicants and other xenobiotics (Amacher, 2002). The liver disorders are one of the serious health problems, throughout the world. More than 350 million people were affected with chronic hepatic infections worldwide and in India above 20,000 deaths were reported every
year due to liver disorders. Hepatocellular carcinoma is one of the most common tumors in the world with over 250,000 new cases each year (Salhab and Canelo, 2011). The antioxidant capacity of compounds has been related to the prevention of several diseases including cancer, coronary heart diseases, inflammatory disorders, neurological degeneration, and aging (Madhavi et al., 1996; Wollgast and Anklam 2000). The harmful effect of the free radicals can however, be blocked by synthetic antioxidants, but due to their adverse side effects, search for effective and natural antioxidants has become crucial. Hence, the search is still on to find a natural drug possessing and antioxidant properties (Choi et al., 2007; Adeolu et al., 2009). But there are no studies on antioxidant potential of D. dehaani crab through different experimental methods. Hence the present study was aimed to investigate the in vivo antioxidant potential D. dehaani hemolymph extract in NDEA induced rats.

8.2. MATERIALS AND METHODS

8.2.1. Drug and chemicals:

N-nitrosodiethylamine (NDEA) was purchased from Sigma-Aldrich Chemicals Co, 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), reduced glutathione (GSH), 2, 20-dipyridyl, xylenol orange, 2, 4-dinitrophenylhydrazine (DNPH), γ-glutamyl-p-nitro anilide, and 5, 50-dithiobis - 2-nitrobenzoic acid were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). The rest of the chemicals utilized were obtained from a local firm (India) and were of analytical grade.
8.2.2. Maintenance of animals, Tumor induction, Hemoplymph preparation, Serum preparation, Plasma preparation, Erythrocyte preparation:

Protocols are as same as that of second chapter in order to avoid the redundancy the methodology has not been repeated here.

8.2.3.1. Phase I Enzymes: Assay of Cytochrome P450:

The activity of cytochrome P450 was assayed by the method of Omura and Sato, (1964). This method is based on the formation of a pigment with an absorbance between 450-490 nm. According to this protocol, for 1 ml of buffer, 0.1 ml of sample was added followed by few mg of sodium dithionite. The CO gas was gently bubbled for approximately 1 min and the absorbance was read at 450 and 490 nm. The difference in absorption spectrum was used to calculate cytochrome P450 content using the extinction coefficient $91 \text{mM}^{-1}\text{cm}^{-1}$. Values are expressed as nmoles of cytochrome/mg protein.

8.2.3.2. Phase II Enzymes: Assay of Glutathione-S-Transferase:

Activity of GST was measured in tissue homogenate by following the increase in absorbance at 340nm using CDNB as substrate by the method of Habig et al., (1974). The reaction mixture contained 1 ml of phosphate buffer, 0.1 ml of CDNB, 0.1 ml of tissue homogenate and 0.7 ml of distilled water. The reaction mixture was incubated at 37°C for 5 min and then the reaction was started by the addition of 0.1 ml of 30 mM glutathione. The absorbance change was read at 340nm for 5 min. Reaction mixture without the enzyme was used as the blank. The activity of GST was expressed as µmoles of CDNB-GSH conjugate formed/min/mg protein.
8.2.3.3. Estimation of alpha-fetoprotein:

The solid phase is coated with monoclonal AFP-specific antibody labeled with a ruthenium complex. An aliquot of sample containing AFP gets conjugated with biotinylated monoclonal AFP-specific antibody and forms a sandwich complex when incubated. After incubation, streptavidin-coated micro particles are allowed to react with biotin of the sandwich complex. The reaction mixture is aspirated into the measuring cell where the micro particles are captured onto the surface of the electrode. Unbound substances are removed with ProCell. Application of a voltage to the electrode induces chemiluminescent emission which is measured by a photomultiplier. The results are determined via a calibration curve which is instrument specifically generated by 2-point calibration and a master curve provided via the reagent bar code. The values of AFP are expressed as mg/mL.

8.2.4. Determination of lipid peroxidation:

8.2.4.1. Estimation of thiobarbituric acid reactive substances:

The level of thiobarbituric acid reactive substances (TBARS) in plasma and tissues were estimated by the method of Niehius and Samuelsson (1968). In this method, malondialdehyde and other TBARS were measured by their reactivity with TBA in acidic condition to generate a pink colored chromophore, which was read at 535nm. The tissue homogenate was prepared in Tris-HCl buffer (pH 7.5). 1 ml of the tissue homogenate 0.5 ml of plasma was treated with 2 ml of TBA-TCA-HCl reagent and mixed thoroughly. The mixture was kept in a boiling water bath for 15 min. After cooling, the tubes were centrifuged for 10 min and the supernatant was taken for measurement. A series of standard solution were also treated in a similar manner. The absorbance of chromophore
was read at 535nm against the reagent blank. The values were expressed as mM/g – tissue or mM/dl - plasma

8.2.4.2. Estimation of Lipid Hydro peroxides:

The level of lipid hydro peroxides in plasma and tissues were estimated by the method of Jiang et al., (1992). In this method, oxidation of ferrous ions (Fe²⁺) under acidic conditions in the presence of xylenol orange leads to the formation of a chromophore with an absorbance maximum at 560nm. 1.8 ml of the Fox reagent was mixed with 0.2 ml of the tissue homogenate/0.2 ml of plasma. Then incubated for 30 min at room temperature and read at 560nm. The values were expressed as mM/g – tissue, 10⁻⁵ mM/dl – plasma.

8.2.4.3. Assay of Enzymic Antioxidants:
8.2.4.3.1. Estimation of Superoxide Dismutase:

The activity of superoxide dismutase (SOD) was determined by the method of Kakkar et al., (1984). Superoxide radicals react with NBT in the presence of NADH and produce formazan blue. SOD removes the superoxide radicals and inhibits the formation of formazan blue. The intensity of colour is inversely proportional to the activity of the enzyme. According to this procedure 0.5 ml of tissue homogenate was diluted to 1 ml with water. Then added 2.5 ml of ethanol and 1.5 ml of CHCl₃ (all the reagents were chilled). This mixture was shaken for 1 min at 4°C and then centrifuged. The enzyme activity in the supernatant was determined. The assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 ml of 186 μM PMS, 0.3 ml of 30 μM NBT, 0.2 ml of 780 μM NADH, appropriately diluted enzyme preparation and water in a total volume of 3 ml. Reaction was started by the addition of NADH. After incubation at 30°C for 90 sec the reaction was stopped by the addition of 1
ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml of n-butanol. The intensity of the chromogen in the butanol layer was measured at 560nm against butanol blank. A system devoid of enzyme served as control. One unit of the enzyme activity is defined as the enzyme reaction which gave 50% inhibition of NBT reduction in one minute under the assay conditions and expressed as specific activity in units/mg protein.

8.2.4.3.2. Estimation of Catalase:

The estimation of catalase (CAT) was determined by the method of Sinha (1972). Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate, when heated in the presence of H$_2$O$_2$. The chromic acetate formed was measured at 620nm. For this protocol 0.9 ml of phosphate buffer, 0.1 ml of tissue homogenate and 0.4 ml of H$_2$O$_2$ were added. 2 ml of dichromate acetic acid reagent was added after 1 min. The tubes were kept in boiling water bath for 10 min and the colour developed was read at 620nm. Standards in the range of 2-10 $\mu$mol were taken and proceeded as test with blank containing reagent alone. The activities were expressed as $\mu$moles of H$_2$O$_2$ consumed/min/mg protein.

8.2.4.3.3. Estimation of Glutathione Peroxidase:

The activity of glutathione peroxidase (GPx) was estimated by the method of Rotruck et al., (1973). A known amount of enzyme preparation was allowed to react with H$_2$O$_2$ in the presence of GSH for a specified time period. Then the remaining GSH was measured by the method of Ellman. According to this procedure 0.2 ml of Tris buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.5 ml of tissue homogenate were added. To this mixture, 0.2 ml of glutathione followed by 0.1 ml of H$_2$O$_2$ was added. The contents were mixed well and incubated at 37°C for 10 min along with a
tube containing all the reagents except sample. After 10 min the reaction was arrested by the addition of 0.5 ml of 10% TCA, centrifuged and the supernatant was assayed for glutathione by the method of Ellman (1959). The activities were expressed as μg of GSH consumed/min/mg protein.

8.2.4.4. Determination of Non-Enzymic Antioxidants:

8.2.4.4.1. Estimation of Ascorbic Acid (Vitamin C):

The level of ascorbic acid was estimated by the method of Omaye et al., (1979). The ascorbic acid was oxidized to copper to form dehydro ascorbic acid and diketoglutaric acid. These products when treated with DNPH form the derivative, bis-2, 4-dinitrophenyl hydrazone that undergoes rearrangement to form a product with absorption maxima at 520nm. Thiourea provides a mild reducing medium, which helps to prevent the interference from ascorbic acid chromogens. 0.5 ml of plasma/tissue homogenate was mixed thoroughly with 1.5 ml of 6% TCA and centrifuged for 20 min. To 0.5 ml of the supernatant, 0.5 ml of DNPH reagent was added and mixed well. The tubes were allowed to stand at room temperature for an additional 3 hrs and then placed in ice-cold water and added 2.5 ml of 85% H₂SO₄ and allowed to stand for 30 min. A set of standards containing 10-50 μg of ascorbic acid were taken and processed similarly along with a blank, containing 0.5 ml of 4% TCA. The colour developed was read at 530nm. The values were expressed as μM/mg - tissue, mg/dl - plasma.

8.2.4.4.2. Estimation of α-Tocopherol (Vitamin E):

α-tocopherol was estimated by the method of Desai (1984). This method involves the reduction of ferric ion to ferrous ion by α-tocopherol and the formation of a red coloured complex with 2, 2′-dipyridyl. Absorbance of the chromophore was measured at 520nm. To 0.1 ml of
plasma/lipid extract, 1.5 ml of ethanol and 2 ml of petroleum ether were added, mixed and centrifuged. The supernatant was evaporated to dryness at 80°C. To this 0.2 ml of 2, 2'-dipyridyl solution and 0.2 ml of ferric chloride solution was added. Mixed well and kept in dark for 5 min and added 2 ml of butanol. The intense red colour developed was read at 520nm. Standard α-tocopherol in the range of 10-100 μg were taken and treated similarly along with blank containing only the reagent. The values were expressed as μM/mg – tissue, mg/dl - plasma

8.2.4.5. **Estimation of liver marker enzymes:**

**8.2.4.5.1. Activities of serum aspartate and alanine transaminases:**

Oxaloacetate and pyruvate formed are coupled with 2, 4-DNPH to give the corresponding hydrazone, which gives brown colour in alkaline medium and this is measured calorimetrically by the method of Reitman and Frankel (1957). 0.5 ml of substrate was incubated for a few min at 37°C. Then 0.1 ml of serum was added and incubation was continued for an hour in the case of aspartate transaminase and 30 min for alanine transaminase. Adding 0.5 ml of DNPH solution arrested the reaction and the tubes were kept at room temperature for 20 min. 5 ml of 0.4 N sodium hydroxide was also added. A set of pyruvic acid standards was also treated in similar manner. The colour developed was read at 540 nm. Activity of AST and ALT were expressed in units/ml.

**8.2.4.5.2. Assay of alkaline phosphatase:**

Activity of alkaline phosphatase was assayed by the method of King and Armstrong (1951). An incubation mixture containing 150 μM of bicarbonate buffer and 100 μM of substrate in 2.9 ml was preincubated at 37°C for 10 min. 0.2 ml of serum was added to this and incubated at 37°C for 15 min. The reaction was arrested by the addition of 10 ml of Folin-
ciocalteau reagent. The suspension was centrifuged and 2ml of 10% sodium carbonate was added to the supernatant. The solution was incubated at 37°C for 10 min. Aliquots of standard phenol (2.5 - 10 μg) were also treated with Folin’s reagent and sodium carbonate. The blue colour developed was read at 640 nm. The enzyme activity was expressed in KA units.

8.2.4.5.3. Assay of lactate dehydrogenase:

The activity of lactate dehydrogenase (LDH) was assayed by spectrophotometrically method of Nieland (Varley et al., 1998) according to the standard procedures using commercially available automated Roche/Hitachi 912 kit. 1 ml of the buffered substrate and 0.1 ml of serum was added in a set of tubes and the tubes were incubated at 37° C for 15 min. After adding 0.2 ml of NAD solution incubated for another 15 min. Then the reaction was arrested by adding 1 ml of DNPH reagent and the tubes were incubated further period of 15 min at 37° C. 0.1 ml of serum was added to blank tubes after arresting the reaction with DNPH. 7 ml of NaOH solution was added and the colour developed was measured at 420 nm. Stable aliquots of the standard were also analyzed by the same procedure. The enzyme activity was expressed in IU/L.

8.2.4.5.4. Activity of Serum γ-Glutamyl Transferase:

The activity of γ-glutamyl transferase was estimated by the method of Rosalki et al., (1970). The p-nitroaniline liberated by the enzyme in the presence of substrate (L-γ-glutamyl-p-nitroanilide) produces a yellow colour, which was estimated spectrophotometrically at 410nm. 0.05 ml of serum was made up to 0.5 ml by the addition of L-γ-glutamyl-p-nitroanilide substrate and incubated at 37°C for 30 min. The standard tubes taken at a concentration ranging from 0.1 to 0.4μmole were also
incubated as above. The reaction was arrested by the addition of 2.5 ml of 10% acetic acid. Simultaneously, a control without serum was also subjected to the above treatment and incubation excepting that serum was added after arresting the reaction. The yellow colour developed after the addition of acetic acid was measured at 410nm against the blank using spectrophotometer. The activity of GGT was expressed as IU/L of serum.

8.2.4.5.5. Estimation of serum bilirubin:

The level of serum bilirubin was estimated by the method of Malloy and Evelyn (1937). Serum bilirubin was estimated by Van den Bergh reaction. It was based on the formation of purple colored azobilirubin when bilirubin reacts with diazotised sulphanilic acid. 0.2 ml of serum was diluted to 2 ml with distilled water in two tubes marked as test and blank. To the test, 0.5 ml of the diazo reagent and to the blank, 0.5 ml of 1.5% HCl was added. Finally to both tubes, 2.5 ml of methanol was added and the tubes were kept at room temperature for 30 min. The colour developed was read at 540nm. For a standard curve, one in five dilutions of stock standard in methanol was made to obtain a solution containing 2 mg/100 ml. The level of serum bilirubin was expressed as mg/dl.

Statistical analysis:

Data were analyzed by one way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) using a statistically software package (SPSS for Windows, V. 13.0, Chicago, USA). Results were presented as mean ± S.D. p-values < 0.05 were considered as statistically significant.
8.3. RESULTS

8.3.1. Cytochrome P450, GST level:

The status of phase I (cytochrome P450) and phase II detoxication enzymes (GST), in the liver of control and experimental animals in each group were shown in Fig. 17. The activity of GST was considerably decreased, whereas the status of cytochrome P450 was increased in liver of tumor-bearing animals (NDEA) as compared to control animals. Administration of hemolymph to NDEA treated animals significantly decreased phase I and elevated phase II enzyme activities compared to NDEA. Although treatment with hemolymph alone showed no significant difference in the activities of phase 1 enzymes, the activities of phase II enzymes in the liver were significantly increased compared to control.

8.3.3. AFP and CEA level:

Fig. 18 illustrated the changes in the levels of molecular markers of alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA) in control and experiment animals. In NDEA treated rats, the levels of alpha fetoprotein and carcinoembryonic antigen were significantly increased when compared to control. Administration of hemolymph significantly decreased the levels of alpha fetoprotein compared to control and also administration of hemolymph alone significantly decreased the levels of carcinoembryonic antigen compared to control.

8.3.4. Changes in the levels of lipid peroxidation in liver:

The changes in the levels of lipid peroxidation in the liver of control and experiment animals are described in Fig. 19. It describes the levels of TBARS and lipid hydroperoxides. In NDEA treated rats the level of TBARS and lipid hydroperoxides were significantly increased when compared to
control. Administration of hemolymph alone considerably decreased the levels of TBARS and LPO compared to control.

8.3.5. Changes in the levels of lipid peroxidation in Plasma:

Fig. 20 indicates the modifications in the levels of lipid peroxidation in the plasma of control and experiment animals. It describes the levels of TBARS and lipid hydroperoxides in the plasma. Level of TBARS and lipid hydroperoxides were significantly increased in NDEA treated rats when compared to control. Administration of hemolymph alone showed considerable decrease in the TBARS and LPO levels compared to control.

8.3.6. Enzymatic and non-enzymatic antioxidant status:

Variations in the levels of enzymatic and non-enzymatic antioxidant like superoxide dismutase, catalase, glutathione peroxidase, vitamin C, vitamin E and GSH of liver in control and experimental rats has shown in Fig 21. In the superoxide dismutase assay NDEA treated rats showed significant decrease in the enzymatic antioxidants activities. The hemolymph treated groups significantly increased the levels of enzymatic and non-enzymatic antioxidants in liver compared to NDEA treated rats. The rats provided only the hemolymph resulted with significant increase in the enzymatic and non-enzymatic antioxidants activity compared to control.

In the catalase activity the NDEA treated rats showed significant decrease. The hemolymph treated groups significantly improved the levels of catalase in liver compared to NDEA treated rats. The rats provided the hemolymph alone resulted with significant increase catalase activity comparatively to the control.
The NDEA treated rats showed significant decrease in the glutathione peroxidase activity. Administration of the hemolymph significantly improved the levels of glutathione peroxidase in liver compared to NDEA treated rats. The rats provided only with the hemolymph resulted with significant increase glutathione peroxidase activity comparatively to the control.

The assay of vit C and vit E showed significant decrease in the NDEA treated rats. Hemolymph treated group resulted significantly improved the levels of vit C and vit E in liver compared to NDEA treated rats. The rats provided with the hemolymph alone resulted with significant increase vit C and vit E activity comparatively to the control.

The GSH level significantly showed decrease in the NDEA treated rats. The rats treated with hemolymph showed significant improvement in the levels of GSH in liver comparatively to NDEA treated rats. The rats administrated only with the hemolymph resulted with significant increase GSH activity comparatively to the control.

### 8.3.7. Accomplishments of liver marker enzymes:

Fig. 23 shows the levels of serum hepatic marker enzymes in control and experimental rats. NDEA induced hepatocarcinogenesis caused abnormal liver function in all the tested rats. The activity of serum hepatospecific enzyme aspartate transaminase was significantly increased ($p < 0.05$) in NDEA treated animals when compared to control animals. However, hemolymph treatment to (NDEA+ hemolymph) leads to a reversal in the values towards normal control. No significant changes were observed in hemolymph alone treated rats compared to control.

In the level of alanine transaminase a notable increase ($p < 0.05$) has observed in NDEA treated rats when compare to control rats. However,
treatment with hemolymph leads to a reversal in the values towards normal control. There is no significant changes were observed in rats which treated only with hemolymph comparatively to control.

Alkaline phosphatase shown noteworthy increase ($p < 0.05$) in the rats which treated with NDEA compared to control rats. On the other hand, hemolymph treated rats leads to a reversal results towards normal control. Rats which treated only with hemolymph have not shown any significant changes comparatively to control.

Significant increase ($p < 0.05$) has been observed in the level of lactate dehydrogenase when treated with NDEA compared to control rats. But the rats treated with hemolymph displayed reversal results towards normal control. The hemolymph alone treated rats has not shown any significant changes comparatively to control.