Chapter – VI

3.8 IN VIVO ANTIOXIDANT ACTIVITY OF PURIFIED PEPTIDES ISOLATED FROM P. NIGER AND L. LUNARIS

3.8.1 Introduction

Ethanol is often used in pharmaco-toxicological research and testing as a solvent for water-insoluble substances [364]. However, in man as well as in animals, ethanol exposure has been associated with enormous production of free radicals [365]. α-Hydroxylethyl radicals are detected in microsomal incubations [366] and in vivo after ethanol treatment [367, 368], indicating that free radicals can be derived directly from ethanol. Moreover, hydrogen peroxide and products of lipid peroxidation increase in the liver after ethanol administration [369].

Ethanol is metabolized primarily in the liver by the multiple isoenzymes of alcohol dehydrogenase [370, 371] and the microsomal ethanol-oxidizing system [372], as well as by other ethanol-metabolizing enzymes [373]. Ethanol toxicity in various somatic tissues of adult animals, and particularly the liver, can, in part, be attributed to an increased production of reactive oxygen species (ROS) [374]. In hepatocytes, these potentially damaging ROS are generated, as a consequence of the metabolism of ethanol to acetaldehyde, mainly through the intervention of the ethanol inducible cytochrome P450 [375]. Various other mechanisms in these cells also generate ROS and free radicals from alcohol
Xanthine oxidase, among other enzymes, can oxidize the acetaldehyde generated as a consequence of alcohol metabolism and, in so doing, can further promote the levels of ROS [263].

Organisms have developed a variety of defenses, including enzymes and non enzymatic antioxidants to protect themselves from ROS. The major endogenous antioxidant enzyme includes superoxide dismutase, catalase, selenium-dependent glutathione peroxidase, and glutathione reductase. The major non enzymatic antioxidants include the reduced form of glutathione (GSH), vitamin C (ascorbic acid), and vitamin E (α-tocopherol) [376]. Recently, investigators and consumers are interested in seeking natural antioxidant components in the diet such as fruits, seeds, probiotics and marine fish, which may help to reduce oxidative damage. In this in vivo study, rats were induced to oxidative stress by ethanol and in parallel other group was treated with marine fish derived antioxidant peptide. Enzyme activities of SOD, GST and CAT in the blood were detected.

3.8.2 Materials and methods

Coomassie Brilliant Blue G-250, ethanol, phosphoric acid, NaOH, EDTA, bovine serum albumin (BSA), hydrogen peroxide, pyrogallol, reduced glutathione, 1-Chloro-2,4-dinitrobenzenewere purchased from Himedia Pvt., Ltd., India
3.8.2.1 Experimental animals

Adult male albino Wistar rats weighing 150–180g were obtained from Animal House, SRM Medical College Hospital & Research Centre, SRM University were used in this study. The animals were housed in polypropylene cages (47x34x18 cm) lined with husk, it was renewed every 24 h. The animals were fed on a standard pellet diet and water *ad libitum* throughout the experiment. The standard pellet diet comprised 21% protein, 5% lipid, 4% crude fiber, 8% ash, 1% calcium, 0.6% phosphorus, 3.4% glucose, 2% vitamins and 55% carbohydrate. It provides metabolisable energy of 3,600 kcal. The experimental animals were maintained in a controlled environment (12:12 h light/dark cycle) and temperature (24±2 ºC). The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and the experimental protocol was approved by the Ethical Committee for Animal Experimentation of SRM University. The animals were acclimatized for one week before starting the experiments.

3.8.2.2 Experimental design

The rats were divided into 6 groups of 3 rats in each group as follows:
Group I: Normal control; Group II: 20% ethanol (w/v); Group III: 20% ethanol + purified peptide (100μg/kg) from *P. niger* viscera; Group IV: 20% ethanol + purified peptide (100μg/kg) from *P. niger* muscle; Group V: 20% ethanol + purified peptide (100μg/kg) from *L. lunaris* muscle; Group VI: 20% ethanol + purified peptide (100μg/kg) from *L. lunaris* skin. Samples were orally
administrated to rats using a feeding tube daily for a period of 15 days. Normal control group received water alone.

3.8.2.3 Collection of *in vivo* samples

Food intake, water intake and body weight of the experimental animals were monitored regularly. Blood was collected from a fine catheter inserted into the retro orbital vein. After the blood was withdrawn the area was wiped to avoid the blood clotting. The anesthetized rats were fixed on an experimental desk, and then liver, kidney and heart were harvested and preserved in formaldehyde for histopathology analysis.

3.8.2.4 Preparation of erythrocyte lysate

Blood was collected from EDTA coated tubes, and centrifuged at 2500 x g at 4 °C for 10 minutes. Supernatant along with the upper layer of the red blood cell pellet which contains the buffy coat was discarded. And the erythrocytes were suspended in 4 volumes of ice-cold water and mix thoroughly for 5 min to allow lysis. The prepared erythrocyte lysate was stored in -70 °C.

3.8.2.5 Estimation of protein

Protein content in the erythrocyte lysate was determined by the method of Bradford [377].

**Procedure**

Sample (0.1 ml) was taken and dissolved with equal volume of 0.1 M NaOH and vortexed for 1 min. Then added 5 ml of Bradford’s reagent and
allowed to stand at room temperature for 5 min. After incubation, the blue color developed was read at 595 nm. A standard curve was obtained using BSA.

### 3.8.2.6 Assay of catalase

The activity of catalase was assayed in the erythrocyte lysate by the method of Aebi [378].

**Procedure**

To 50μl of the lysate, 2 ml of phosphate buffer and 1 ml of hydrogen peroxide were added. Catalase activity was measured at 240nm for 1 min using spectrophotometer. One unit of enzyme activity is defined as one millimoles of H₂O₂ degraded per minute.

### 3.8.2.7 Assay of Superoxide dismutase

Superoxide dismutase (SOD) activity in the erythrocyte lysate was determined according to the procedure of McCord and Fridovich [379].

**Procedure**

The assay mixture contained 50 μl of the lysate, 1.5 ml of Tris-HCl buffer, 0.3 ml EDTA, 0.2 ml of pyrogallol and distilled water to makeup a total volume of 3 ml. An increase in absorbance was recorded at 420 nm for 1 min by spectrophotometer. One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance/min at 420 nm.
3.8.2.8 Assay of Glutathione S-transferase

Glutathione S-transferase (GST) activity was assayed in the erythrocyte lysate by the method of Habig et al. [380].

Procedure

The reaction mixture contained an aliquot of sample, 2 ml potassium phosphate buffer, 0.3 ml of reduced glutathione and 0.2 ml 1-Chloro-2,4-dinitrobenzene, which was used as substrate and incubated for 1 min. After incubation the GST activity was read spectrophotometrically at 340 nm.

3.8.2.9 Histological observation

Histology of all the organs was conducted by the method of Galigher and Kozloff [381].

Procedure

After sacrificing the rats by cervical dislocation, tissues (liver, heart and kidney) were collected, washed in normal saline and fixed by using fixative (30% formaldehyde) for 24 h and dehydrated in a graded acetone series [(50–70–90–100–100–100)% (v/v); 10 min/step]. The samples were infused in with alcohol for complete dehydration. Then tissues were cleaned and embedded in paraffin (melting point 58-60°C), were cut into ultra-thin sections (3-5μm) by ultramicrotome and stained with the haematoxylin-eosin dye and finally, observed under a photomicroscope and morphological changes such as cell necrosis, degeneration of hepatocytes, congestion and cell infiltration were observed.
3.8.2.10 Statistical analysis

All the assays were conducted with three replicates and data were expressed as mean ± standard error of mean (SEM). The statistical analysis was performed using statistical package for the social science (SPSS) 10.0 software (SPSS Inc. Chicago, IL, USA). The significant difference was determined with 95% confidence interval (P<0.05).

3.8.3. Results

To establish ethanol as the source of the oxidative stress in Wistar rats, 20% ethanol (w/v) was used in the animal study. Over a period of 15 days rats were maintained in totally controlled experimental observations. Ethanol has not affected the social behavior of grouped rats and seemed more active for couple of minutes after the administration. Their physical health was not affected compared to the control group but, the enzymatic studies done from their blood appears to be different as shown in the table (6).

In comparison with normal group the activity of CAT (293.2±6.3), SOD (31.2±0.6) and GST (5.2±0.3) the negative control group (196.4±5.7, 15.1±0.4 & 1.3±0.5) showed very least activity. This might be the due to the oxidative stress and over load of free radicals inside the Wistar rats. But after supplementation of antioxidant peptides derived from two marine fishes P. niger and L. lunaris in the groups III – VI the activity of enzymes was quite improved.

Peptide isolated from L. lunaris muscle (283.65±8.6) and P. niger viscera (253.35±10.5) has shown better recovery of catalase (CAT) activity than the ethanol group followed by P. niger muscle (224.88±4.3) and L. lunaris skin (206.99±9.3). Whereas GST activity was better improved by L.
*lunaris* muscle and skin peptide (4.03±0.3 & 4.23±0.2) than that of *P. niger* peptides (3.72±1.3 & 2.77±0.2) which has a significant (P <0.05) effect on the tested animals.

On the other hand, SOD activity of administration all the four peptides for their respective groups showed a significant recovery (P <0.05), which is double the high than that of ethanol group and more or less to the normal control. The similar pattern of damage in ethanol group and recovery after peptides supplementation was observed in the histopathology sections of liver, kidney and heart of all the experimented rat groups as shown in figure (20).

**Table 6 The catalase, GST and SOD activities in the erythrocyte lysate of ethanol induced oxidative stress rats supplemented with peptides isolated from *P. niger and L. lunaris***

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Groups</th>
<th>Conc.</th>
<th>Protein*</th>
<th>Catalase *</th>
<th>GST *</th>
<th>SOD *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td></td>
<td>318.7±1.6</td>
<td>293.2±6.3</td>
<td>5.2±0.3</td>
<td>31.2±0.6</td>
</tr>
<tr>
<td>2</td>
<td>Ethanol 20 % w/v</td>
<td>301.6±2.5</td>
<td>196.4±5.7</td>
<td>1.3±0.5</td>
<td>15.1±0.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>BP Viscera</td>
<td>100 μg/Kg</td>
<td>278.9±3.9</td>
<td>253.35±10.5</td>
<td>3.72±1.3</td>
<td>25.9±0.7</td>
</tr>
<tr>
<td>4</td>
<td>BP Muscle</td>
<td>100 μg/Kg</td>
<td>289.04±1.9</td>
<td>224.88±4.3</td>
<td>2.77±0.2</td>
<td>27.68±0.8</td>
</tr>
<tr>
<td>5</td>
<td>PF Muscle</td>
<td>100 μg/Kg</td>
<td>320±2.8</td>
<td>283.65±8.6</td>
<td>4.03±0.3</td>
<td>28.42±0.7</td>
</tr>
<tr>
<td>6</td>
<td>PF Skin</td>
<td>100 μg/Kg</td>
<td>308±6.8</td>
<td>206.99±9.3</td>
<td>4.23±0.2</td>
<td>29.13±0.2</td>
</tr>
</tbody>
</table>

* U/min/mg of Protein

Values represent the mean ± SEM (standard error of mean) of triplicate assays in three animals in each group, P<0.05 when compared with control group.
(a) Liver  (b) Kidney  (c) Heart

(d) Liver  (e) Kidney  (f) Heart

(g) Liver  (h) Kidney  (i) Heart
Figure 20 Histopathology sections of rats after completion of treatment with ethanol and supplementation of peptide. Normal group (a-c), negative group (d-f), *P. niger* viscera peptide (g-i), *P. niger* muscle peptide (j-l), *L. lunaris* muscle peptide (m-o), *L. lunaris* skin peptide (p-r).
3.8.4 Discussion

Ethanol is one of the most commonly used in the experimental study of oxidative stress [262,382]. It was found that chronic administration of ethanol produced enormous free radical generation in rats. It is well documented that ethanol is bio-transformed under the action of cytochrome P450 in the microsomal compartment, by conversion of xanthine dehydrogenase into xanthine oxidase in cytosol and increases one electron reduction in mitochondria [383,384]. Leading to the generation of superoxide anion radical \( \text{O}_2^{•−} \) and hydrogen peroxide \( \text{H}_2\text{O}_2 \), and in the presence of metals, produces powerful oxidants such as the hydroxyl radical [264]. This effect on the redox status of the liver can cause activation of Kupffer cells and subsequently, hepatic stellate cells, and thus contributing to the generation of alcoholic liver disease (ALD) [266].

These free radicals bind covalently to the macromolecules and induce peroxidative degradation of the membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to the polyribosomal disaggregation, depression of protein synthesis, depletion of glutathione, cell membrane break down and death [265]. These indicate that ethanol induced oxidative stress pilot the reduction of antioxidant potential enzymes and damage the structural integrity of the liver cell architecture, which is confirmed by our blood parameter and histopathological studies of negative control group.
On the other hand, rat fed with purified peptide isolated from *P. niger* viscera, *P. niger* muscle, *L. lunaris* muscle and *L. lunaris* skin were successful in controlling the oxidative damage caused by ethanol exposure at *in vivo* level. This is possible because, most of the physiological and functional properties of proteins are attributed to peptides, making them biologically active. Bioactive peptides generally contain 3–20 amino acids [385]. The activity of these peptides is dependent on their length composition and sequence of amino acids.

Peptide studied in the current study comes within the range of bioactive peptides and proved to scavenge free radicals at *in vitro* experiments.

In free radical scavenging mechanism superoxide dismutase plays a major role in converting superoxide anions into H$_2$O$_2$ [268]. In mild exposure ethanol feeding should cause an up-regulation of the SOD enzyme at mRNA level, which seems to be a protective mechanism [386]. And the over expression of Mn-SOD prevents the ethanol-induced oxidative stress in the rat models [387]. On the contrary, with repeated ethanol administrations the increased level in Mn-SOD is gradually diminished. Thus adaptive response of Mn-SOD is blunted, leading to increased toxicity in prolonged ethanol exposure [386]. This might be the reason for 50% plummeting of SOD level in negative control group (15.1±0.4) compared to the normal group (31.2±0.6).

But, a significant recovery was observed after supplementing with the fish peptides to all the four groups (III - VI) as shown in table (6). As the peptide used in this study was known for its reducing properties (Fig. 12) it would have helped in scavenging the free radicals generated due to the ethanol metabolism.
Catalase is an enzymatic antioxidant widely distributed in all animal tissues [248]. Catalase decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals [135]. Glutathione (GSH) is one of the abundant tripeptide non-enzymatic biological antioxidants present in the liver [388]. It acts as a substrate for the H$_2$O$_2$ removing enzyme glutathione peroxidase and for dehydroascorbate reductase [255]. Therefore reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide [389], which was exactly happened in the negative control group. Administration of fish peptides successfully restored the activities of catalase in ethanol intoxicated rats. Our results revealed that these four peptides prevented excessive free radicals accumulation and protected the animals from ethanol induced oxidative stress. Similar, recovery of *in vivo* antioxidant enzymes was reported when the animal models were supplemented with antioxidants isolated from douchi, *A. polystachya* and *B. animalis* [267].

In summary, the results of this study demonstrate that peptides purified from *P. niger* and *L. lunaris* protein hydrolysates has a potent antioxidant action on ethanol induced oxidative damage in rats. These results show that the antioxidant effects of fish peptides may be due to its reducing power ability which indeed acquired from their amino acid composition and sequence. And improving the structural integrity of the tissue comes in combination with its ability to scavenge free radicals.