Chapter-III

ANTIOXIDANT ACTIVITY OF AN ETHANOLIC EXTRACT OF THE
PLEUROTUS OSTREATUS IN DIFFERENT TISSUES OF AGED RATS

1. Introduction

Ageing is characterized by a progressive decline in biochemical and physiological functions of various tissues and organs in an individual. The causes of this decline are still unclear, but it has been proposed that increased oxidative stress, disturbances in energy metabolism, and a primary dysregulation of the immune system might play an important role (Prolla and Mattson, 2001). According to the free radical theory of ageing (Harman, 1956) and its modern version, the oxidative stress theory of ageing, the disruption of the delicate balance between generation of reactive oxygen species (ROS) and antioxidant scavenging systems with increasing age could lead to a shift to an oxidative cellular milieu. Ageing is now considered to be associated with an increase in oxidative damage to biomolecules (Ames et al., 1993; Goto et al., 2001) and also with enhanced levels of inflammation (Miller, 1996; Johnson et al., 1999; Cheng et al., 2002).

Oxidative damage to DNA, proteins and other macromolecules accumulates with age and has been postulated to constitute a major type of endogenous damage leading to ageing (Fraga et al., 1990). Proteins are particularly susceptible to oxidative damage because of their inherent structural flexibility and reactive amino acid residues (Requena et al., 2003; Stadtman and Levine, 2003). Oxidative damage to protein is reflected in the level of protein carbonyl (PCO). The reaction of free radicals, such as \( \text{OH}^* \) or \( \text{O}_2^- \) with the side chain of lysine, arginine, proline, threonine and glutamic acid...
residues of protein leads to the formation of carbonyl derivatives (Stadtman and Berlett, 1997). Carbonyl groups can also be introduced into proteins by glycation and glycoprotein reactions (Baynes, 1991). Therefore, modifications in protein carbonyl are good indicators of the presence of oxidized proteins that increase with advancing age (Sohal et al., 1994a; Liu et al., 1996).

Although almost all organisms are equipped with antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are often inadequate to completely prevent the damage (Simic, 1988). The increased oxidative damage observed during ageing might be due to the insufficiency of antioxidants (Reiter, 1995). In fact, there is an increased interest in the study of the beneficial effects of nutritional antioxidants on health in terms of delaying ageing and age-related diseases (Vaya and Aviram, 2001; Youdim et al., 2002; Galli et al., 2002; Klein and Ackerman, 2003; Joseph et al., 2005). Mushrooms have been part of the normal human diet for thousands of years. This chapter of the thesis under review describes the use of an extract of the mushroom *Pleurotus ostreatus* to treat chronic (age-related) oxidative stress in male Wistar rats.

2. Materials and Methods

2.1. Animal experiment

Male albino Wistar rats weighing approximately 80-110 g (4 months old) and 300-375g (24 months old), procured from the Central Animal House, Rajah Muthiah Medical College, Annamalai Nagar, India, were used for the experiments. The animals were acclimatized for 20 days prior to dosing, during which time they had free access to food and water *ad libitum*. Eighteen such acclimated rats were divided into 3 groups of
six each: Group I, normal young (4 months old) rats; Group II, normal aged (24 months old) untreated rats; Group III, normal aged rats treated with the extract of the mushroom *P. ostreatus* (200mg/kg bw administered intraperitoneally) for 21 days. On the 22nd day, rats were sacrificed by decapitation; the liver, kidneys, heart and brain were removed from each rat and stored at -80°C until further analysis.

2.2. Biochemical analyses

Preparation of tissue homogenates and the analysis of various biochemical parameters, including isozymic patterns of antioxidant and xanthine dehydrogenase (XDH) enzymes, in this phase of the study were performed by methods similar to those described in chapter II. The protein carbonyl content, an indicator of the oxidative damage to protein, was also evaluated in this chapter.

2.3. Determination of protein carbonyl content

The protein carbonyl content was determined by the most common and reliable method based on the reaction of carbonyl groups with 2, 4-dinitrophenylhydrazine to form a 2, 4-dinitrophenylhydrazone, described by Nakamura and Goto (1996). The protein in each liver and kidney tissue homogenate in two equal (100 μl) volumes was precipitated with 10% TCA. The precipitates were then treated with either 2N HCL alone (control) or 2N HCL containing 10 mM DNPH (test) at 15°C for 1h. After the completion of the reaction, the mixture was centrifuged at 3400×g for 10 min and the precipitates were then washed thrice with an ethanol:ethylacetate (1:1) mixture; the precipitates were finally dissolved in 8 M urea. The absorbance was measured at 360 nm and the protein carbonyl content was determined as nanomoles per mg protein using molar extinction coefficient of 1/22,000.
3. Results

3.1. Concentration of MDA

An increase in the mean MDA level, a measure of lipid peroxidation, was found in the liver, kidneys, heart and brain of aged untreated (Group II) rats relative to young (Group I) rats (Tables 6 & 6a). Treatment of aged (Group III) rats with the extract of *Pleurotus ostreatus* was found to lower the mean MDA concentration so that it approached that of normal young rats, presumably by limiting lipid peroxidation in the tissues of the major organs.

3.2. Concentration of protein carbonyl

A significant (p<0.001) increase in the level of protein carbonyl was observed in the liver, kidneys, heart and brain tissues of aged rats, when compared to the levels in young rats (Tables 6 & 6a). Treatment of aged rats with *P. ostreatus* extract Group III) resulted in protein carbonyl levels that were significantly (p<0.01) lower than those observed in aged untreated (Group II) rats.

3.3. Activity of XDH

A significant increase in the activities of XDH was observed in the liver (p<0.05), kidneys, heart (p<0.01) and brain (p<0.001) tissues of aged rats, when compared to the activity of XDH in the same tissues of young rats. In aged rats that had been treated with the extract of *P. ostreatus*, the activity of this enzyme was found to be significantly lower (p<0.05) in the kidneys, heart and brain than in the corresponding organs of aged- untreated rats; however, no significant decrease was observed in the liver tissues (Tables 6 & 6a).
Table 6. Effect of extract of mushroom (*Pleurotus ostreatus*) on malondialdehyde (MDA), protein carboxyls (PCO), xanthine dehydrogena (XDH), reduced glutathione (GSH) and Vitamins C and E in liver and kidney tissues of aged rats.

<table>
<thead>
<tr>
<th>Parameters tested</th>
<th>Tissues</th>
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<tbody>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td>Young rats (Group I)</td>
</tr>
<tr>
<td>MDA</td>
<td>0.932±0.2</td>
</tr>
<tr>
<td>PCO</td>
<td>5.11±0.02</td>
</tr>
<tr>
<td>XDH</td>
<td>7.57±1.15</td>
</tr>
<tr>
<td>GSH</td>
<td>10.31±0.35</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>2.8±0.15</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.65±0.15</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td>Young rats (Group I)</td>
</tr>
<tr>
<td>MDA</td>
<td>1.24±0.25</td>
</tr>
<tr>
<td>PCO</td>
<td>2.23±0.04</td>
</tr>
<tr>
<td>XDH</td>
<td>31.15±1.71</td>
</tr>
<tr>
<td>GSH</td>
<td>12.24±1.18</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>1.65±0.15</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.14±0.04</td>
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</tbody>
</table>

Values are expressed as mean ± SD of six rats in each group.
MDA- n moles of MDA produced / mg protein; PCO- n moles/mg protein; XDH- μ mole of NADP reduced/min/mg protein; GSH - μg of reduced glutathione / mg protein; Vitamins C and E - μg/mg protein
*p<0.05; ** p<0.01; ***p<0.001; NS- Not significant
Table 6a. Effect of extract of mushroom (*Pleurotus ostreatus*) on malondialdehyde (MDA), protein carbonyls (PCO), xanthine dehydrogenase (XDH), reduced glutathione (GSH) and Vitamins C and E in heart and brain tissues of aged rats.

<table>
<thead>
<tr>
<th>Parameters tested</th>
<th>Tissues</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Heart</td>
<td>Heart</td>
<td>Heart</td>
<td>Brain</td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>Young rats</td>
<td>Aged rats</td>
<td>Aged rats treated with mushroom extract</td>
<td>Young rats</td>
<td>Aged rats</td>
</tr>
<tr>
<td>MDA</td>
<td>1.29±0.35</td>
<td>1.48±0.3 NS</td>
<td>1.31±0.34 NS</td>
<td>1.07±0.49</td>
<td>1.33±0.26 NS</td>
</tr>
<tr>
<td>PCO</td>
<td>3.18±0.05</td>
<td>4.23±0.19 ***</td>
<td>3.58±0.71 **</td>
<td>4.62±0.08</td>
<td>5.80±0.27 ***</td>
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<tr>
<td>XDH</td>
<td>37.3±2.70</td>
<td>63.21±3.96 **</td>
<td>51.39±2.13 *</td>
<td>19.84±1.22</td>
<td>39.02±1.11 ***</td>
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<tr>
<td>GSH</td>
<td>12.10±0.39</td>
<td>8.40±0.49 *</td>
<td>10.11±0.53 *</td>
<td>13.08±3.60</td>
<td>10.58±2.82 *</td>
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<tr>
<td>Vitamin C</td>
<td>1.51±0.40</td>
<td>1.01±0.53 *</td>
<td>1.44±0.48 NS</td>
<td>1.37±0.23</td>
<td>1.13±0.13 NS</td>
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<tr>
<td>Vitamin E</td>
<td>1.18±0.02</td>
<td>0.986±0.07 *</td>
<td>1.11±0.068 NS</td>
<td>1.27±0.020</td>
<td>1.06±0.089 *</td>
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</tbody>
</table>

Values are expressed as mean ± SD of six rats in each group.
MDA- n moles of MDA produced / mg protein; PCO- n moles/mg protein; XDH- μ mole of NADP reduced/min/mg protein;
GSH - μg of reduced glutathione / mg protein; Vitamins C and E - μg/mg protein
*p<0.05; ** p<0.01; ***p<0.001; NS- Not significant
3.4. Concentration of GSH

In aged untreated rats (Group II), a significant decrease (relative to the levels in young rats) in the level of GSH was observed in the liver, kidneys, heart and brain tissues (Tables 6 & 6a). Administration of the extract of *P. ostreatus* to aged rats (Group III) elevated GSH to levels that approximated the levels in young rats.

3.5. Concentration of vitamins C and E

Significantly lower levels of vitamin E were observed in the liver, kidneys, heart and brain of aged untreated (Group II) rats, when compared to levels in normal young (Group I) rats (Tables 6 & 6a). Significantly lower concentrations of vitamin C were also noted in liver and heart (and lower concentrations in kidney and brain) of Group II rats, compared to levels in Group I rats. However, the levels of vitamins C and E in liver, kidneys, heart and brain of aged rats administered *P. ostreatus* extract (Group III) were found to be at near normal levels, that is the levels found in young (Group I) rats (Tables 6 & 6a).

3.6. Activities of antioxidant enzymes

Significantly lower activities of CAT, SOD, Gpx, GST, GR, G6PDH and Apx were observed in the liver, kidneys, heart and brain of aged untreated (Group II) rats when compared to the values in young (Group I) rats (Tables 7 & 7a). In aged rats that had been administered extract of *P. ostreatus* (Group III), a significant increase (compared to the activities in aged-untreated rats) was observed in the activities of some of these enzymes. However, no significant increase was observed in the activities of CAT and SOD in all tissues and Gpx in heart and brain tissue.
Table 7. Effect of extract of mushroom (*Pleurotus ostreatus*) on catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (Gpx), glutathione -S-transferase (GST), glutathione reductase (GR), acorbate peroxidase (Apx) and glucose-6-phosphate dehydrogenase (G6PDH) in liver and kidney tissues of aged rats.

<table>
<thead>
<tr>
<th>Parameters tested</th>
<th>Tissues</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young rats</td>
<td>Aged rats</td>
<td>Aged rats treated with mushroom extract</td>
</tr>
<tr>
<td>CAT</td>
<td>73.37±4.44</td>
<td>39.6±6.33*</td>
<td>57.16±3.88**</td>
</tr>
<tr>
<td>SOD</td>
<td>13.87±2.27</td>
<td>9.8±0.10*</td>
<td>12.69±0.34**</td>
</tr>
<tr>
<td>Gpx</td>
<td>62.36±2.88</td>
<td>42.63±0.90*</td>
<td>53.23±1.96*</td>
</tr>
<tr>
<td>GST</td>
<td>1.72±0.15</td>
<td>1.43±0.032**</td>
<td>1.59±0.03*</td>
</tr>
<tr>
<td>GR</td>
<td>0.88±0.002</td>
<td>0.67±0.008**</td>
<td>0.76±0.032*</td>
</tr>
<tr>
<td>Apx</td>
<td>3.08±0.001</td>
<td>2.57±0.034**</td>
<td>2.91±0.025*</td>
</tr>
<tr>
<td>G6PDH</td>
<td>1.41±0.002</td>
<td>1.19±0.042**</td>
<td>1.39±0.068*</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD of six rats in each group.
CAT- μmoles of H₂O₂ utilized /min/mg protein; SOD, Apx- Unit/mg protein; Gpx- μmoles of GSH oxidized/min/mg protein; GST- μ moles of CDNB Conjugated/min/mg protein; GR- μ moles of NADH oxidized /min/mg protein; G6PDH-Unit/min/mg protein.  **p<0.01;  *p<0.05;  NS- Not significant
Table 7a. Effect of extract of mushroom (Pleurotus ostreatus) on catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (Gpx), glutathione –S-transferase (GST), glutathione reductase (GR), acorbate peroxidase (Apx) and glucose-6- phosphate dehydrogenase (G6PDH) in heart and brain tissues of aged rats.

| Parameters tested | Tissues | | | |
|-------------------|---------|---------|---|---------|---------|---|
|                   | Heart   | Aged rats treated with mushroom extract | Brain | Aged rats treated with mushroom extract |
|                   | Young rats | Aged rats (Group I) | (Group II) | (Group III) | Young rats (Group I) | Aged rats (Group II) | (Group III) |
| CAT               | 47.8±4.09 | 39.98±2.21* | 41.22±4.96NS | 32.66±4.7 | 21.66±5.86* | 29.32±3.61NS |
| SOD               | 11.55±1.62 | 7.96±1.79* | 10.06±095NS | 21.81±2.56 | 17.08±1.92* | 18.92±2.59NS |
| Gpx               | 51.97±3.78 | 38.39±2.63* | 47.82±1.32NS | 34.76±2.30 | 20.77±1.22* | 31.33±2.76NS |
| GST               | 0.76±0.03 | 0.44±0.012** | 0.620±0.053* | 0.521±0.008 | 0.290±0.001** | 0.484±0.002* |
| GR                | 0.617±0.09 | 0.53±0.024** | 0.608±0.006* | 0.452±0.009 | 0.314±0.001** | 0.398±0.044* |
| Apx               | 2.41±0.05 | 1.17±0.021** | 2.08±0.34* | 1.59±0.012 | 0.96±0.029** | 1.56±0.03* |
| G6PDH             | 1.24±0.05 | 1.04±0.046** | 1.12±0.004* | 1.107±0.001 | 0.995±0.007** | 1.031±0.07NS |

Values are expressed as mean ± SD of six rats in each group.
CAT- μmoles of H2O2 utilized /min/mg protein; SOD, Apx- Unit/mg protein; Gpx- μmoles of GSH oxidized/min/mg protein; GST- n moles of CDNB Conjugated/min/mg protein; GR- μ moles of NADH oxidized /min/mg protein; G6PDH- Unit/min/mg protein.  **p<0.01;  *p<0.05; NS- Not significant
3.7. Isozyme pattern of enzymes in liver, kidneys, heart and brain

3.7.1. Isozymes of CAT

An analysis of the electrophoretic pattern of catalase isozymes in the liver, kidney, heart and brain tissues (Fig. 21a, b, c and d) revealed a single band, with variations in the staining intensity of the band among the three groups of rats; the samples of liver, kidneys, heart and brain of young (group I) rats exhibited a single band of high intensity (band areas 220.0, 223.0, 161.87 and 112.76, respectively), while those of group III rats (aged rats treated with *P. ostreatus* extract) exhibited a single band of almost equal intensity (band areas 212.37, 221.87, 159.91 and 109.87, respectively); however, homogenates of the liver, kidneys, heart and brain of group II (aged-untreated) rats revealed a single band of lower intensity (band areas 189.12, 180.24, 135.09 and 98.54, respectively) (Fig. 22).

3.7.2. Isozymes of SOD

Two isozymes of SOD were detected in the liver, kidneys, heart and brain of all three groups of rats (Fig. 23a, b, c and d). Isozyme SOD1 appeared as a band of essentially similar intensity of staining in the liver (band areas 153.75, 150.8 and 152.87), kidney (band areas 136.24, 129.27 and 134.89), heart (band areas 129.81, 127.24 and 128.47) and brain (band areas 166.87, 159.19 and 164.98) of rats of Groups I, II and III, respectively. The intensity of staining of isozyme SOD2 was essentially similar in rats of groups I and III with respect to the liver (band areas 146.15 and 144.73, respectively), kidney (band areas 114.62 and 104.75, respectively), heart (band areas 111.98 and 107.31, respectively) and brain (band areas 66.75 and 51.09, respectively); however, Group II rats exhibited a decreased staining intensity of SOD2.
Fig. 21. Electrophoretic pattern of CAT isozyme in (a) liver (b) kidney (c) heart and (d) brain tissues of rats

L1- young rats
L2- aged untreated rats
L3- aged rats treated with mushroom extract
Fig. 22. Densitometric pattern of catalase isozyme in liver, kidney, heart and brain tissues of rats.
Fig. 23. Electrophoretic pattern of SOD isozymes in (a) liver (b) kidney (c) heart and (d) brain tissues of rats

L1- young rats
L2- aged rats
L3- aged rats treated with mushroom extract
Fig. 24. Densitometric pattern of SOD isozyme in liver, kidney, heart and brain tissues of rats.

S1-SOD1; S2-SOD2
in liver (band area 129.63), kidney (band area 99.0), heart (band area 87.63) and brain (band area 45.29) (Fig. 24).

3.7.3. Isozymes of Gpx

a) Five isozymes of the Gpx enzyme were noted in the liver of all three groups of rats (Fig. 25a, b, c &d). The staining intensity of Gpx 1 was essentially similar in rats of groups I, II and III (band areas 175.39, 171.74 and 173.17, respectively); a similar phenomenon was noted for Gpx isozyme 3 (band areas 168.55, 164.57 and 166.98, respectively) and Gpx isozyme 5 (band areas 156.71, 151.65 and 153.45, respectively) in rats of groups I, II and III (Fig. 25a). However, the staining intensity of the Gpx 2 and 4 isozymes was less in group II (band areas 110.09 and 96.13, respectively) rats than that in Group I (band areas 124.88 and 117.60, respectively) and in Group III (band areas 118.24 and 101.18, respectively) rats. b) Four isozymes of the Gpx enzyme were noted in kidney tissues of all three groups of rats. The staining intensity of the Gpx1 (band areas 132.85, 129.47 and 130.05) and Gpx3 (band areas 121.96, 117.34 and 119.88) isozymes was essentially similar in kidney tissues of groups I, II and III rats, respectively (Fig. 25b); however, the staining intensity of Gpx 2 and 4 isozymes was less in kidney tissues of Group II rats (band areas 99.61 and 67.27, respectively) than that in Group I rats (band areas 128.38 and 98.98, respectively) and Group III rats (band areas 121.81 and 73.10, respectively). c) Four isozymes of the Gpx enzyme were detected in the heart of all three groups of rats. In rats of groups I, II and III, the staining intensity of the Gpx2 isozyme (band areas 101.62, 99.54 and 100.71, respectively) and Gpx3 isozyme (band areas 122.5, 119.89 and 120.1, respectively) was essentially similar (Fig. 25c). However, the staining intensity of the Gpx1 and 4 isozymes was less in the heart of group II rats (band areas 89.78 and 121.89,
Fig. 25. Electrophoretic pattern of Gpx isozymes in (a) liver (b) kidney (c) heart and (d) brain tissues of rats.

G1- Gpx1; G2-Gpx2; G3-Gpx3; G4-Gpx4; G5-Gpx5

L1- young rats
L2- aged rats
L3- aged rats treated with mushroom extract
Fig 26. Densitometric pattern of Gpx isozymes in (a) liver and kidney (b) heart and brain tissues of rats

G1- Gpx1, G2-Gpx2, G3-Gpx3, G4-Gpx4; G5-Gpx5
respectively) than that in the heart group I (band areas 99.82 and 130.76, respectively) and that in group III (band areas 97.90 and 129.48, respectively) rats. d) In the case of brain tissue, four isozymes of the Gpx enzyme (Gpx isozymes 1, 2, 3 and 4) were detected in rats of group I (band areas 139.0, 103.4, 62.0 and 141.7, respectively) and group III (band areas 127.31, 101.81, 59.14 and 135.55, respectively). However, only two isozymes, namely Gpx1 and Gpx 4, were noted in group II (band areas 100.91 and 68.57) rats (Fig. 25 d and 26).

3.7.4. Isozymes of GST

Four isozymes of the GST enzyme were detected in the liver and kidneys of all three groups of rats (Fig. 27a and b). a) In the liver of rats of groups I, II and III, the staining intensity of the GST1 isozyme (band areas 164.24, 162.31 and 163.10, respectively) was essentially similar. However, the staining intensity of the GST2, 3 and 4 isozymes in the liver was less (band areas 109.44, 99.31 and 87.98, respectively) in group II rats compared to that in group I (band 128.62, 116.41 and 101.62, respectively) and that in group III (band areas 125.17, 114.51 and 98.97, respectively) rats. b) Four isozymes of the GST enzyme were expressed in kidneys of all three groups of rats. In rats of groups I, II and III, the staining intensity of the GST2 (band areas 119.86, 117.20 and 118.78, respectively) and GST3 (band areas 118.94, 116.56 and 117.10, respectively) isozymes was essentially similar. However, the staining intensity of the GST1 and 4 isozymes was less (band areas 139.68 and 41.47, respectively) in group II rats compared to that in group I (band areas 145.13 and 56.27, respectively) and that in group III (band areas 144.56 and 54.27, respectively) rats. c) In heart tissue, five isozymes of the GST enzyme were detected in all three groups of rats (Fig. 27c). In rats of groups I, II and III, the staining intensity of GST1 (band areas
Fig. 27. Electrophoretic pattern of GST isozymes in (a) liver (b) kidney (c) heart and (d) brain tissues of rats

G1- GST1; G2-GST2; G3-GST3; G4- GST4; G5-GST5

L1- young rats
L2- aged rats
L3- aged rats treated with mushroom extract
Fig 28. Densitometric pattern of GST isozymes in (a) liver and kidney (b) heart and brain tissues of rats.

G1-GST1; G2-GST2, G3-GST3; G4-GST4; G5-GST5
39.17, 37.98 and 38.81, respectively), and GST3 (band areas 120.03, 118.97 and 119.72, respectively) isozymes was almost similar. However, the staining intensity of the GST2, 4 and 5 isozymes was less (band areas 27.69, 88.61 and 130.47, respectively) in group II rats than that in group I (band areas 32.31, 94.07 and 142.31, respectively) and that in group III (band areas 30.40, 91.24 and 140.07, respectively) rats. d) In the case of brain tissue, three isozymes of the GST enzymes (GST1, 2 and 3) were detected in all three groups of rats (27d). In rats of group II, the staining intensity of GST1 (band area 91.17), GST2 (band area 78.37) and GST3 (band area 80.20) was less compared to that in group I (band areas 98.6, 89.99 and 92.17, respectively) and that in group III (band areas 97.20, 87.96 and 90.80, respectively) rats (Fig. 28).

3.7.5. Isozymes of Apx

The Apx enzyme exhibited four isozymes in the liver and heart tissues and three isozymes in the kidney and brain tissues of all three groups of rats (Fig. 29 a, b, c and d). a) In liver, four isozymes of Apx enzyme were detected in all three groups of rats (Fig. 29a). In rats of groups I, II and III, the staining intensity of the Apx1 (band areas 117.87, 114.98 and 115.99, respectively) and Apx2 (band areas 74.13, 71.09 and 72.68, respectively), Apx3 (band areas 96.85, 93.17 and 95.98, respectively) isozymes was essentially similar. However, the staining intensity of the Apx4 isozyme was less (band area 29.86) in group II rats compared to that in group I (band area 37.65) and that in group III (band area 34.17) rats. b) Three isozymes of the Apx enzyme were observed in the kidneys in all three groups of rats (Fig. 29 b). In rats of groups I, II and III, the staining intensity of the Apx1 (band areas 101.71, 99.89 and 100.72, respectively), Apx2 (band areas 53.34, 51.20 and 52.39, respectively) and Apx3 (band areas 79.65, 77.75 and 78.17, respectively) isozymes was essentially similar. c) Four isozymes of
Fig. 29. Electrophoretic pattern of Apx isozymes in (a) liver (b) kidney (c) heart and (d) brain tissues of rats

A1- Apx1; A2- Apx2; A3- Apx3; A4- Apx4

L1- young rats
L2-aged rats
L3-aged rats treated with mushroom extract
Fig. 30. Densitometric pattern of Apx isozymes in (a) liver and kidney (b) heart and brain tissues of rats.

A1-Apx1; A2-Apx2; A3-Apx3; A4-Apx4
the Apx enzyme were detected in the heart tissue in all three groups of rats (Fig. 29 c). In rats of groups I, II and III, the staining intensity of Apx3 (band areas 96.63, 96.55 and 95.68, respectively), and Apx4 (band areas 21.55, 19.99 and 20.97, respectively) isozymes was almost similar. However, the staining intensity of the Apx1 and 2 isozymes was less (band areas 30.98 and 41.74, respectively) in group II rats than that in group I (band areas 49.03 and 50.93, respectively) and that in group III (band areas 45.45 and 48.32, respectively) rats. d) In the case of brain tissue, the staining intensity of Apx1 (band areas 96.04, 94.39 and 95.12) isozyme was essentially similar in rats of groups I, II and III respectively (Fig. 29 d). However, the staining intensity of the Apx2 and 3 isozymes was less (band areas 27.45 and 50.90, respectively) in group II rats compared to that in group I (band areas 46.24 and 59.54, respectively) and that in group III (band areas 36.81 and 58.17, respectively) rats (Fig. 30).

3.7.6. Isozymes of G6PDH

The G6PDH enzyme exhibited a single isozyme in the liver and three isozymes in the kidney, heart and brain tissues of all three groups of rats (Fig. 31a, b, c and d). a) The liver of groups I and III rats exhibited a single band of high intensity staining (band areas 219.10 and 211.85, respectively) compared to that in group II (band area 183.93) rats (Fig. 31a). b) Three isozymes of G6PDH were noted in the kidneys in all three groups of rats (Fig. 31b). In rats of group II, the staining intensity of the G6PDH1, 2 and 3 isozymes (band areas 41.55, 72.17 and 209.89, respectively) was found to be lower than that in group I (band areas 54.48, 83.68 and 217.89, respectively) and that in group III (band areas 49.22, 78.16 and 212.34, respectively) rats. In the heart and brain tissues, three isozymes of the G6PDH enzyme were observed in all three groups of rats. c) In the heart tissue of rats of group II, the staining intensity of G6PDH 1, 2 and 3
Fig. 31. Electrophoretic pattern of G6PDH isozymes in (a) liver (b) kidney (c) heart and (d) brain tissues of rats

G1- G6PDH1; G2- G6PDH2; G3-G6PDH3

L1- young rats
L2- aged rats
L3- aged rats treated with mushroom extract
Fig. 32. Densitometric pattern of G6PDH isozymes in (a) liver and kidney (b) heart and brain tissues of rats.

G1- G6PDH1; G2-G6PDH2; G3-G6PDH3;
isozymes was less (band areas 182.58, 214.01 and 207.65, respectively) than that in group I (band areas 205.79, 220.31 and 214.07, respectively) and that in group III (band areas 185.17, 219.96 and 211.85, respectively) rats (Fig. 31c). d) Three isozymes of G6PDH were detected in the brain tissue in all three groups of rats (Fig. 31d). In rats of group II, the staining intensity of G6PDH1, 2 and 3 isozymes was less (band areas 143.21, 181.63 and 225.46, respectively) than that in group I (band areas 203.84, 216.15 and 229.97, respectively) and that in group III (band areas 197.41, 213.74 and 227.83, respectively) rats (Fig. 32).

3.7.7. Isozymes of XDH

The XDH enzyme exhibited a single isozyme in the liver, but four isozymes in the kidney, heart and brain tissues of all three groups of rats (Fig. 33a, b, c and d). a) The liver of group I and group III rats exhibited a single band of low intensity (band areas 195.21 and 199.41, respectively) compared to that in group II (band area 204.82) rats (Fig. 33a). b) Four isozymes of XDH were detected in the kidneys in all three groups of rats (Fig. 33b). In rats of groups I, II and III, the staining intensity of the XDH3 (band areas 104.89, 114.60 and 109.08, respectively) and XDH4 (band areas 125.21, 127.49 and 126.91, respectively) isozymes was essentially similar. However, the staining intensity of the XDH1 and 2 isozymes was greater (band areas 219.37 and 98.81, respectively) in group II rats compared to that in group I (band areas 133.49 and 89.76, respectively) and that in group III (band areas 196.48 and 92.75, respectively) rats. c) In the heart tissue of rats of groups I, II and III, the staining intensity of the XDH2 (band areas 88.12, 90.68 and 89.62, respectively), XDH3 (band areas 130.90, 134.69 and 131.01, respectively) and XDH4 (band areas 136.72, 139.87 and 138.45, respectively) isozymes was essentially similar (Fig. 33c). However, the staining
Fig. 33. Electrophoretic pattern of XDH isozymes in (a) liver (b) kidney (c) heart and (d) brain tissues of rats

X1 - XDH1; X2 - XDH2; X3 - XDH3; X4 - XDH4

L1 - young rats
L2 - aged rats
L3 - aged rats treated with mushroom extract
Fig. 34. Densitometric pattern of XDH isozymes in (a) liver and kidney (b) heart and brain tissues of rats.

X1-XDH1; X2-XDH2; X3-XDH3; X4-XDH4
intensity of the XDH1 (band area 98.81) isozyme was greater in group II rats than that in group I (band area 89.76) and that in group III (band areas 92.75) rats. d) Four isozymes of XDH were noted in the brain tissue of all three groups of rats (Fig. 33d). In rats of groups I, II and III, the staining intensity of XDH1 (band areas 79.58, 82.67 and 80.17, respectively) and XDH2 (band areas 89.09, 90.41 and 89.98, respectively) isozymes was essentially similar. However, the staining intensity of the XDH3 and 4 isozymes was less in group I (band areas 45.71 and 99.90, respectively) and in group III (band areas 50.76 and 103.85, respectively) rats compared to that in group II (band areas 52.63 and 108.47, respectively) rats (Fig. 34).

4. Discussion

Ageing may be defined as a gradual, progressive change in an organism that increases the probability of death. The alterations associated with ageing compromise an organism’s ability to meet both internal and external challenges. A prime example of such alterations is the age-related damage to tissues due to the endogenous generation of free radicals. This condition is worsened by an age-related decline in the organism’s ability to counteract these changes, resulting in a phenomenon referred to as oxidative stress (Yu, 1994). A primary source of damage brought about by oxidative stress is lipid peroxidation, which is attributed to its high propagative nature and cytotoxicity from its metabolic byproducts.

4.1. Lipid peroxidation

Lipid peroxidation, a process induced by free radicals, leads to oxidative deterioration of polyunsaturated lipids. Under normal physiological conditions, only low levels of lipid peroxides occur in body tissues. The excessive generation of free
radicals leads to peroxidative changes that ultimately result in enhanced lipid peroxidation (Rikans and Hornbrook, 1997). Malondialdehyde (MDA), a secondary product of lipid peroxidation, is used as an indicator of tissue damage (Ohkawa et al., 1979). Ageing is reported to be associated with increased disruption of membrane lipids leading to subsequent formation of peroxide radicals (Niki et al., 1993). In the present investigation, such a disruption of membrane lipids possibly accounted for the observed increase in MDA levels in the organs of aged (Group II) rats when compared to young (Group I) ones. In addition, insufficient levels of antioxidants to scavenge peroxy radicals during ageing (Sohal et al., 1990 and Wei, 1998) could also have contributed to the elevated level of MDA observed in the aged rats. These data from the present investigation corroborate findings of earlier investigations (Arivazhagan and Panneerselvam, 2000; Balu et al., 2005; Augustyniak et al., 2005; Li et al., 2006). Antioxidants are necessary for preventing the formation of free radicals and they inhibit some of the deleterious actions of reactive oxygen species on lipids, DNA and proteins (Halliwell, 1996). Administration of natural extracts of grape seed (Balu et al., 2005) and of green tea (Augustyniak et al., 2005) has been reported to reduce the levels of MDA in the central nervous system and the liver of aged rats, respectively. So also, in the present study, the observed reduction in the MDA level in aged rats following administration of the mushroom extract suggests the putative antioxidant activity of Pleurotus ostreatus.

4.2. Protein carbonyls

Protein oxidation is an exothermic event in which peptides react with free radicals, resulting in the modification of several amino acids and aggregation and fragmentation of proteins. An evaluation of the carbonyl level is possibly the most
common method used for assessing the oxidative modification of proteins (Butterfield et al., 1998). Increased levels of this protein derivative have been documented in the lungs (Hatao et al., 2006), spinal cord and brain region (Balu et al., 2005) and the hippocampus of patients suffering from Alzheimer’s disease (Hensley et al., 1995). So also, in the present investigation, the levels of protein carbonyl were significantly (p<0.001) increased in the liver and kidneys of aged (Group III) rats when compared to young (Group I) rats. The increase in carbonyl derivatives, as a marker of protein oxidation with age was possibly due to the inability of the antioxidant defense system to cope with increases in H₂O₂ (Hatao et al., 2006). However, when aged rats were treated with mushroom extract (Group III) a significant (p<0.01) decrease was noted in the concentration of protein carbonyls in the liver and kidneys, relative to the levels in aged untreated rats; this effect was possibly due to the presence of antioxidant principles in the mushroom extract (data shown in chapter I) that functioned by direct scavenging of ROS. Balu et al. (2005) reported that the administration of grape seed extract could reduce protein carbonyl levels in the spinal cord and brain regions of aged rats.

4.3. Non-enzymatic antioxidants

The most important cellular non-enzymic antioxidant, and the scavenger of free radicals is reduced glutathione (GSH) (Griffith and Meister, 1985). GSH is a major, non-protein thiol in living organisms which performs a key role in co-ordinating the innate antioxidant defense mechanisms. It is involved in the maintenance of the normal structure and function of cells, probably by its redox and detoxification reactions (Gueeri, 1995). In the present study, the level of GSH was found to be lower in aged (Group II) rats than in young (Group I) rats. Similar decreases in the levels of reduced
glutathione in liver (Liu and Choi, 2000; Mosoni et al., 2004; Mallikarjuna et al., 2006) and brain (Arockiarani and Panneerselvam, 2001) of aged rats have been reported previously. Lowered GSH levels may result from increased utilization of GSH by antioxidant enzymes, such as Gpx, which scavenge H₂O₂ (Anand et al., 1996). This decrease may also be due to the enhanced oxidative damage caused by free radicals during ageing. Interestingly, in the present study, when mushroom extract was administered to aged rats (Group III) the GSH level was found to be very similar to that in young (Group I) rats. This again strongly suggests the antioxidant potential of the extract of this mushroom. An increase in the levels of GSH in aged rats treated with grape seed extract as a source of antioxidant has also been reported (Balu et al., 2005).

Circulating antioxidants such as vitamin C and vitamin E are non-enzymatic scavengers of free radicals. Vitamin E reacts with lipid peroxy radicals, acting as a chain terminator of lipid peroxidation, and protects the cellular structures from attack by free radicals (Arivaghagan et al., 2000), while vitamin C facilitates the maintenance of vitamin E levels at optimum concentrations. A decrease in the ascorbic acid level in leucocytes and in the blood of rats with increasing age has been reported by Jayachandran and Panneerselvam (1995). A decrease in the vitamin E level of aged human platelets has also been reported (Verical et al., 1992). In the present study, significantly lower (relative to young rats) levels of vitamin C and vitamin E were recorded in aged rats; however, in aged rats that were treated with mushroom extract, the levels of these vitamins were essentially similar to the levels in young rats. A similar increase in vitamins C and E levels in liver of aged rats treated with a potent antioxidant, green tea extract, has also been reported (Augustyniak et al., 2005).
4.4. Enzymatic antioxidants

Living tissues are endowed with inherent antioxidant defense mechanisms, which include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Gpx), the three primary enzymes involved in direct elimination of ROS, while glutathione reductase (GR), glutathione-S-transferase (GST), glucose-6-phosphate dehydrogenase (G6PDH), and ascorbate peroxidase (Apx) are secondary enzymes, which help in the detoxification of ROS by decreasing peroxide levels or by maintaining a steady supply of metabolic intermediates (e.g. glutathione, NADPH) necessary for optimal functioning of the primary antioxidant enzymes (Vendemiale et al., 1999; Singh et al., 2003). According to the free radical theory of ageing, one might expect the activity of antioxidant enzymes to be altered (Harman, 1988). A reduction in the activity of the primary enzymes is associated with the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of integrity and function of cell membranes (Reedy and Lokesh, 1992; Krishnakantha and Lokesh, 1993; Sheela and Angusti, 1995). A decrease in the activity of these enzymes during ageing has been well-documented (Barja de Quiroga et al., 1990; Matsuo et al., 1992; Kalaiselvi and Panneerselvam, 1998; Arivazhagan et al., 2000; Balu et al., 2005; Valls et al., 2005). A decline in the activities of the secondary antioxidant enzymes GST, GR and G6PDH in the tissues of aged rats has also been noted (Arivazhagan et al., 2000, 2001, 2002; Kumaran et al., 2004). So also, in the present study, significantly lower activities of these enzymes were observed in aged rats, when compared to young rats. Administration of the mushroom extract markedly elevated the levels of these antioxidant enzymes so that they approached the levels seen in young rats, indicating the antioxidant potential of the mushroom *Pleurotus ostreatus*. 

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Supplementation of the diet with fruits and vegetables is reported to be beneficial in reversing the deleterious effects of ageing on neuronal communication and behavior (Joseph et al., 2005). Augustyniak et al. (2005) reported that the administration of green tea extract increased the activities of CAT, SOD and Gpx in the liver of aged rats. Balu et al. (2005) reported that supplementation of the diet with grape seed extract could improve the antioxidant status in the central nervous system of aged rats. Administration of the antioxidant component DL-α-lipoic acid to aged rats was found to lead to an elevation in the activities of GR, GST and G6PDH (Arivazhagan et al., 2001 and 2002). Kumaran et al. (2004) reported a significant increase in the activities of GR and G6PDH in the skeletal muscle and heart of aged rats following treatment with DL-α-lipoic acid and L-carnitine. So also, in the present investigation, administration of P. ostreatus extract appears to have brought about a remarkable improvement in the activity of antioxidant enzymes in aged rats.

4.5. Isozymes of antioxidant enzymes

The utilization of multiple isozymes of enzymes is believed to be one of the primary control mechanisms that control cellular metabolism (Sang et al., 2005). However, there is a paucity of data on the regulation of expression of the isozymes of antioxidant enzymes during oxidative stress, an aspect that deserves study. In this thesis, an attempt has been made to evaluate the pattern of isozymes during ageing and following supplementation with mushroom extract. The present study on the electrophoretic patterns of antioxidant enzymes in the liver, kidneys, heart and brain revealed characteristic isozyme patterns. In general, the number of bands and the staining intensity of the isozymes of the antioxidant enzymes was less in aged untreated (Group II) rats than in the two other groups; tissue-specific variations were also noted.
Such a pattern of differential expression of enzyme isozymes has been noted in barley shoot and root exposed to saline stress (Sang et al., 2005). Modifications in the pattern of enzyme isozymes during stress have been attributed to some shift in gene expression (El-baky et al., 2003). Interestingly, in the present study, the decreased intensity of CAT and SOD2 isozymes and complete absence of Gpx 2 and 3 isozymes in brain of group II rats suggests that the brain tissue may be particularly vulnerable to oxidative stress. A possible reason for this could be that since the brain is rich in non-heme iron, it is catalytically involved in the production of oxygen free radicals (Subbarao and Richardson, 1990). Another possible explanation is that the presence of unsaturated fatty acids in high concentrations makes the brain tissue a good substrate for the occurrence of peroxidation reactions (Ogawa, 1994).

The variations in antioxidant enzyme isozyme profiles, during ageing observed in the present investigation may possibly have been due to alterations in gene expression. Since direct genomic action of melatonin has been well-documented (Becker –Andre et al., 1994; Wiesenberg et al., 1995; Steinhiller et al., 1995); supplementation with melatonin during ageing has been proposed as a means of increasing the activity of antioxidant defense system (ADS) genes with a view to promoting the synthesis of antioxidant enzymes (Srinivasan, 1999). In the present study, a similar phenomenon possibly occurred, resulting in the increased intensity of staining of antioxidant enzyme isozymes following supplementation with mushroom extract.
5. Conclusion

Administration of an extract of the oyster mushroom *Pleurotus ostreatus* appeared to protect the liver, kidneys, heart and brain of aged Wistar rats against oxidative stress by reducing the intensity of lipid peroxidation and protein carbonyls, and activity of XDH and by enhancing the activities of enzymatic and non-enzymatic antioxidants. The results of the present study suggest that an ethanolic extract of the mushroom, *P. ostreatus*, can be conveniently incorporated in the diet as a nutritional supplement, therein augmenting the body’s defenses against oxidative stress.