CHAPTER 5

ANTITUMOUR PROPERTY OF DIFFERENT FRACTIONS
OF CUTTLEFISH INK EXTRACT – *IN VIVO* STUDIES
5.1 INTRODUCTION

Major advances in the treatment of cancer have emerged from the recent revolution in clinical interventions. However, significant heterogeneity in the efficacy and toxicity of chemotherapeutic agents is consistently observed across the human population (Evans and Relling, 1999). Administration of a same dose of a given cancer drug to a population of patients results in a range of toxicity, from unaffected to lethal events (Sargent et al., 2001; Rothenberg et al., 2001). While many clinical variables have been associated with drug response (age, gender, diet, organ function and tumour biology), genetic differences in drug disposition and drug targets can have great impact on treatment outcome (Evans and Johnson, 2001; McLeod and Evans, 2001). The targets of any anticancer treatment are the rapidly dividing cells within a tumour mass. The strategies used today try to selectively eradicate these rapidly dividing ‘rogue’ cells without adversely affecting other organs and tissues of the body. However, drug cocktails and radiation exposures are toxic to living cells. As a consequence, other proliferating cells also become targets of therapy regimes due to the toxic nature of the treatment. These include follicular hair cells, which is manifested by hair loss and compromise of the body’s general defense mechanisms (Buchdunger et al., 1996; Kurzrock et al., 2003).

Attempts have been made in many parts of the world to isolate bioactive substances from the squid and cuttlefish ink. Takaya et al. (1994a) investigated the antitumour activity of peptidoglycan from the squid ink against Meth A fibrosarcoma in mice. The acetone delipidated squid ink was shown to have antitumour activity against Meth A fibrosarcoma in BALB/c mice (Sasaki et al., 1997). Whole peptidoglycan (cell
wall preparation) from *Bifidobacterium infantis* showed a significant suppression of tumour growth against syngenic Meth A fibrosarcoma in BALB/c mice (Sekine *et al*., 1985). The protein bound polysaccharides isolated from the mushrooms have been used as an immune therapy agent in the treatment of cancer in Asia for over 30 years (Wasser and Weis, 1999).

Investigations were carried out to evaluate the antitumour activity of peptidoglycan fractions from the ink of female cuttlefish, *Sepia pharaonis*. Studies were also carried out to evaluate the effect of peptidoglycan fraction on the level of antioxidant enzymes, antioxidants and lipid peroxidation products. The hepatotoxicity and neurotoxicity of the peptidoglycan was also investigated and the findings are reported in this chapter.

**5.2 MATERIALS AND METHODS**

**5.2.1. EXPERIMENTAL ANIMALS**

Inbred Swiss albino male mice of two months age were purchased from Veterinary College, Kerala Agricultural University, Mannuthy. The mice were obtained from the stock in-breed colony, which was maintained by mating brothers and sisters. They were housed in ventilated cages and maintained on dry feed pellets and water *ad libitum* for a week, prior to the experiments.

All animal experiments were carried out according to the guidelines prescribed by Animal Welfare Board and with the approval of Animal Ethic committee.

**5.2.2. TUMOUR CELL LINE AND THEIR MAINTENANCE**

Dalton’s Lymphoma ascites (DLA) tumour cell lines were provided by Amala Cancer Research Institute, Thrissur. The tumour cell line was maintained by serial
intraperitoneal (i.p.) transplantation in mice. Full grown tumour cell lines were aspirated from the mouse peritoneum, washed thrice with 0.9% HBSS (Hank’s Balanced Salt Solution) and suspended in HBSS. About $1 \times 10^6$ cells were injected intraperitoneally into a new healthy mouse.

5.2.3. PREPARATION OF DIFFERENT FRACTIONS FROM CUTTLEFISH INK

Crude preparation of cuttlefish ink was prepared as described in section 4.2.2.1

Fraction C of cuttlefish ink was prepared as described in section 4.2.2.2

Fraction $C_1$ of cuttlefish ink was prepared as described in section 4.2.2.3

Fraction $C_2$ of cuttlefish ink was prepared as described in section 4.2.2.3

Polysaccharide fraction of fraction $C_2$ of cuttlefish ink was prepared as described in section 4.2.2.8

5.2.4. ASSAY OF DIRECT CYTOTOXIC ACTIVITY IN VITRO

The *in vitro* cytotoxicity of fraction $C_2$ of cuttlefish ink was assayed using DLA cell lines. The ascitic tumour cells ($1 \times 10^6$ cells) were suspended in 0.5 ml of 10% FBS-RPMI containing 200 μg of sample and were incubated at $37^\circ$C for 8 h in moist air containing 5% CO₂. The viability of cells at 2, 4, 6 & 8 h were assayed by Trypan-blue exclusion method (Talwar, 1974).

5.2.5. ASSAY OF ANTITUMOUR ACTIVITY IN VIVO

Male BALB/c mice weighing 20 ± 5 g were used for the study. The animals were grouped into seven groups consisting of ten mice in each group. DLA cells ($1 \times 10^6$ cells/animal) in HBSS were transplanted as intraperitoneal injections to each mouse in all groups except group I. Samples were dissolved in 0.5 ml normal saline and were given as
intraperitoneal injections on the 2\textsuperscript{nd} & 4\textsuperscript{th} day after tumour transplantation. Mice receiving similar treatment with saline alone served as controls. The animals were fed on normal diet and water \textit{ad libitum}. The details of treatment given to each group is shown below.

Group 1 (Normal group) - Normal mice not bearing DLA

Group 2 (control group) - Each mice received intraperitoneal injections of $1 \times 10^6$ cells of DLA and 0.5 ml normal saline.

Group 3 (fraction C\textsubscript{2} treated group) - Each mice received intraperitoneal injections of $1 \times 10^6$ cells of DLA and fraction C\textsubscript{2} of cuttlefish ink (200 µg fraction C\textsubscript{2}/0.5 ml normal saline)

Group 4 (crude preparation treated group) - Each mice received intraperitoneal injections of $1 \times 10^6$ cells of DLA and crude preparation of cuttlefish ink (200 µg crude preparation/0.5 ml normal saline)

Group 5 (fraction C treated group) - Each mice received intraperitoneal injections of $1 \times 10^6$ cells of DLA and fraction C of cuttlefish ink (200 µg fraction C/0.5 ml normal saline)

Group 6 (fraction C\textsubscript{1} treated group) - Each mice received intraperitoneal injections of $1 \times 10^6$ cells of DLA and fraction C\textsubscript{1} of cuttlefish ink (200 µg fraction C\textsubscript{1}/0.5 ml normal saline)
normal saline) Group 7 (Polysaccharide treated group) - Each mice received intraperitoneal injections of 1 x 10^6 cells of DLA and polysaccharide of fraction C_2 of cuttlefish ink (200 μg polysaccharide from fraction C_2/0.5 ml normal saline)

The mice were fed on a normal diet for one month and observed for mortality. Length of survival of mice dying within 30 days of transplantation of tumour cells were studied. The mice surviving in group 1, 2 and 3 were used for biochemical studies.

5.2.6. PREPARATION OF SERUM AND TISSUE SAMPLES FOR THE BIOCHEMICAL STUDIES

a) PREPARATION OF TISSUE HOMOGENATES

The surviving mice were fasted overnight and the animals were sacrificed by cervical dislocation. The tissues namely liver, kidney, heart and brain were dissected out and rinsed thoroughly in ice cold saline to remove the blood. They were then gently blotted between two folds of blotting paper and desired amount of dried tissues were weighed and used for preparing tissue homogenates for different assays as detailed below.

b) PREPARATION OF SERUM

Blood was collected and serum was separated from blood cells by centrifugation at 2000 rpm for 30 min. The serum thus obtained was used to assay S-GPT, albumin and total protein.
5.2.7. METHODS USED FOR THE BIOCHEMICAL ANALYSES

5.2.7.1. ASSAY OF ANTIOXIDANT ENZYMES

Superoxide dismutase in tissues was determined using the method of Kakkar et al. (1984), catalase by the method of Machley and Chance (1954) and glutathione peroxidase by the method of Lawrence and Burk (1976). The detoxifying enzyme glutathione reductase was assayed using the method of Goldberg and Spooner (1983).

A) ASSAY OF SUPEROXIDE DISMUTASE (SOD; EC: 1.15.1.1)

REAGENTS

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium pyrophosphate buffer</td>
<td>0.052 M (pH 8.3)</td>
</tr>
<tr>
<td>Tris-HCl buffer</td>
<td>0.0025 M (pH 7.4)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.25 M</td>
</tr>
<tr>
<td>Phenazine methosulphate</td>
<td>186 μM</td>
</tr>
<tr>
<td>Nitroblue tetrazolium</td>
<td>300 μM</td>
</tr>
<tr>
<td>NADH</td>
<td>780 μM</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td></td>
</tr>
<tr>
<td>n-butanol</td>
<td></td>
</tr>
<tr>
<td>Ammonium Sulphate solution</td>
<td>90%</td>
</tr>
</tbody>
</table>

PROCEDURE

100 mg tissue was homogenized in 2 ml 0.25 M sucrose and differentially centrifuged at 10,000 rpm under 4°C to get cytosol fraction. Protein was precipitated from the supernatant with 90% ammonium sulphate and dialyzed overnight with 0.0025
M Tris-HCl buffer (pH 7.4). The supernatant was used as the enzyme source. The assay mixture contained 1.2 ml sodium pyrophosphate buffer (0.052 M, pH 8.3), 0.1 ml 186 μM phenazine methosulphate, 0.3 ml 300 μM nitro blue tetrozolium, 0.2 ml of diluted enzyme preparation, 0.2 ml 780 μM NADH. Incubated 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 shook with 4 ml of n-butanol. The mixture was allowed to stand for 10 min. and was centrifuged. The upper butanol layer was taken out. The colour intensity of chromogen in the butanol was measured at 560 nm against butanol blank. A system devoid of enzyme served as control. Protein estimation was carried out on the same enzyme source by the method of Lowry et al. (1951). One unit of enzyme has been defined as the enzyme concentration required to inhibit absorbance at 560 nm of chromogen production by 50% in one minute under the assay conditions and expressed as specific activity in milliunits/mg protein.

B) ASSAY OF CATALASE (CAT; EC:1.11.1.6)

REAGENTS

0.12 M phosphate buffer (pH 7.2)

H₂O₂ phosphate buffer - 0.16 ml H₂O₂ diluted to 100 ml with buffer

PROCEDURE

The tissue was homogenised in 2 ml phosphate buffer at 4°C and centrifuged at 5000 rpm. The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. 3 ml H₂O₂ phosphate buffer and 40 μl of the enzyme preparation were pipetted into the experimental cuvette. Read absorbance at 230 nm every 30
seconds for about 1-2 min. against control cuvette containing enzyme solution and H₂O₂ free phosphate buffer. Specific activity is expressed in terms of units / mg protein, where one unit has been defined as the velocity constant per second.

C) ASSAY OF GLUTATHIONE PEROXIDASE (GPx; EC: 1.11.1.9)

REAGENTS

0.25 M Sucrose solution

Sodium azide (Na N₃) - Stock 50 mg/10 ml distilled water

(Working Solution - 1 ml stock + 9 ml distilled water)

GSH (9 mg/ml distilled water)

0.12 M phosphate buffer (pH 7.2)

155 mmol/l EDTA buffer

9.6 mmol/l NADPH tetra sodium salt in 1% sodium bicarbonate

1% Sodium bicarbonate

PROCEDURE

The tissue was ground in 2 ml (0.25 M) sucrose solution. The assay system contained 0.3 ml NaN₃, 0.1 ml GSH, 1.5 ml phosphate buffer, 0.2 ml EDTA, 0.5 ml H₂O₂, 0.1 ml NADPH, 0.2 ml Enzyme, 0.1 ml H₂O₂. The reaction was followed at 340 nm for next 5 min. Values are expressed as difference in absorbance/min./mg protein.

D) ASSAY OF GLUTATHIONE REDUCTASE (GR; EC: 1.6.4.2)

REAGENTS

0.12 M phosphate buffer (pH 7.2)

155 mmol/l EDTA buffer
65.3 mmol/l GSSG

NADPH (9.6 mmol/l) tetra sodium salt in 1% Sodium bicarbonate

1% Sodium bicarbonate

**PROCEDURE**

Weighed sample of tissue was homogenised in a known volume of phosphate buffer. 2.6 ml of buffer, 0.1 ml EDTA and 0.1 ml GSSG acts as a reagent mixture to which was added 0.1 ml of the homogenised sample and waited for 5 min. Then 0.1ml of distilled water and 0.05 ml of NADPH were added, mixed thoroughly and read at 340nm for 5 min. against buffer blank. Control contained H₂O instead of GSSG. The enzyme activity is expressed as difference in absorbance / min. / mg protein.

**5.2.7.2. ESTIMATION OF ANTIOXIDANTS**

**A) ESTIMATION OF ASCORBIC ACID**

Ascorbic acid level in tissues was determined by using the method of Roe (1954).

**REAGENTS**

Trichloro acetic acid (TCA): 6%

Thiourea agent : 50% in alcohol

2, 4 dinitro phenyl hydrazine: 2% in 9 N H₂SO₄

Con. H₂SO₄ : 85%

Ascorbic Acid Standard

Stock : 100 mg ascorbic acid in 100 ml of 6% TCA

Activated charcoal
PROCEDURE

Immediately after sacrificing the animals, a weighed sample of tissue was homogenised in 5 ml ice cold 6% TCA in a pre-chilled mortar. The extract was shaken well in a test tube, added activated charcoal and allowed to stand for 15 min. The clear supernatant was filtered through whatman filter paper. To 4 ml of supernatant added a drop of thiourea reagent (50% in alcohol) and 1 ml of 2%, 2, 4 dinitro phenyl hydrazine in 9 N H\textsubscript{2}SO\textsubscript{4} and incubated for 3 h at 37\textdegree C in a water bath. At the end of the incubation, the test tubes were placed in an ice bath and added 4 ml of 85% conc. H\textsubscript{2}SO\textsubscript{4}. Kept for 30 min. in refrigerator. Centrifuged and OD measured at 540 nm in a spectrophotometer. The values are expressed as mg /100g tissue.

B) ESTIMATION OF GLUTATHIONE (GSH)

Glutathione level in tissue was determined using the method of Benk and Cheever (1974).

REAGENTS

1. Precipitating solution
   a) Glacial ortho / meta phosphoric acid - 1.67 gm
   b) Disodium / Dipotasium EDTA - 0.2 gm
   c) Sodium Chloride (NaCl) - 3 mg

   Dissolve one by one and make up to 100 ml with distilled water

2. 0.3 M phosphate solution

   \[ \text{KH}_2\text{PO}_4 \quad - \quad 3.55 \text{g} \]
   \[ \text{Na}_2\text{HPO}_4 \quad - \quad 7.268 \text{ g} \]
Dissolve in double distilled water and make up to 10 ml

3. 1% trisodium citrate solution

4. 0.04% DTNB (Ellman’s reagent) : 40 mg dissolved and made up to 100 ml
   with 1% sodium citrate solution

5. Glutathione Standard : 9 mg/ml of H₂O

**PROCEDURE**

500 mg tissue was homogenised in 5 ml precipitating solution. Allowed to stand for 5 min. at room temperature and filtered through course grade filter paper. To 0.2 ml homogenate solution added 5 ml of 0.3 M phosphate solution and 1 ml of 0.04% DTNB. Capped and inverted these tubes to mix and read the absorbance at 412 nm within 4 min.

5.2.7.3. **ESTIMATION OF LIPID PEROXIDATION PRODUCTS**

Malondialdehyde was estimated by the method of Niehaus and Samuelsson (1968) and conjugated diene by the method of Beuge and Aust (1978).

A) **ESTIMATION OF THIOBARBITURIC ACID REACTING SUBSTANCES (TBARS) / MALONDIALDEHYDE (MDA)**

**REAGENTS**

TCA - TBA – HCl solution

TCA - 15%

TBA - 0.375%

Prepare in 0.25 N HCl

**PROCEDURE**

Tissue was homogenised with 0.1M Tris-HCl (pH 7.5). 2 ml of TCA-TBA-HCl reagent (15% W/v TCA, 0.375% W/v 2-thiobarbituric acid in 0.25 N HCl) was added to
1 ml of the tissue homogenate and mixed thoroughly. The contents were heated in a boiling water bath for 15 min. After cooling, the flocculent precipitate was removed by centrifugation at 1000 x g for 10 min. The absorbance of the pink coloured supernatant was read at 535 nm against a blank that does not contain the sample. The concentration of MDA was calculated using extinction coefficient of 1.56 x 10^5 M^-1 cm^-1.

B) ESTIMATION OF CONJUGATED DIENES

REAGENTS

0.025 M Tris-HCl buffer (pH 7.5)

Chloroform: Methanol (2:1 v/v)

Cyclohexane AR

PROCEDURE

Weighed sample of tissue was homogenised in a known volume of the buffer. An aliquot of the homogenate was shaken with chloroform: methanol (2:1) and the lower layer was recovered. Then evaporated this layer to dryness and redissolved in a known volume of cyclohexane. Read the absorbance at 233 nm against cyclohexane as blank. The amount of conjugated diens is expressed in terms of mmol/100 g tissue.

5.2.7.4. ANALYSIS OF SERUM

5.2.7.4.1 DETERMINATION OF S-GPT, ALBUMIN & TOTAL PROTEIN

Blood was collected and serum was separated from blood cells by centrifugation at about 2000 rpm for 30 min. Parameters such as S-GPT, total protein and albumin in the serum were measured using kits provided by AGAPPE Diagnostics, Mumbai.
A) ASSAY OF S-GPT (SERUM GLUTAMATE PYRUVATE TRANSAMINASE)

ACTIVITY IN SERUM

PRINCIPLE

Kinetic determination of S-GPT activity is based on IFCC recommendation.

L Alanine + 2-oxal glutarate \( \xrightarrow{\text{GPT}} \) L-glutamate + Pyruvate.

Pyruvate + NADH + H\(^+\) \( \xrightarrow{\text{LDH}} \) L-Lactate + NAD\(^+\)

GPT \( \rightarrow \) Glutamate Pyruvate Transferase

LDH \( \rightarrow \) Lactate Dehydrogenase

REAGENT COMPOSITION

**REAGENT 1 (R1)**

Tris buffer (pH 7.5) \( \rightarrow \) 100 mmol/l

L alanine \( \rightarrow \) 500 mmol/l

LDH \( \rightarrow \) 1200 µmol/l

**REAGENT 2 (R\(_2\))**

2 Oxal glutarate – 15 mmol

NADH – 0.18 mmol/l

Working reagent: \( R_1 : R_2 = 4:1 \)

ASSAY PROCEDURE

Wavelength – 340 nm

No. of readings – 3

Interval – 60 Sec
Blank – distilled water

Sample 20 μl serum + 2 ml working reagent.

**CALCULATION**

\[ \text{OD/min} \times 1745 \]

GPT activity in U/L

**B) ESTIMATION OF TOTAL PROTEIN IN SERUM**

**PRINCIPLE**

Colorimetric determination of total protein based on the principle of Biuret (Copper Salt in Alkaline Medium). Protein in plasma or serum sample forms a blue colored complex when treated with cupric ions in alkaline solution. The intensity of blue colour is proportional to the protein concentration.

**REAGENTS**

Reagent:– Potassium Sodium Tartarte

Sodium Hydroxide

Copper sulphate

Surfactant

Standard : Protein 6g/dl

**ASSAY PROCEDURE**

Wave length – 550 nm

Temp: – 37° C

Blank – reagent

Sample volume – 20 μl
Reagent volume – 1 ml

**CALCULATION**

Total Protein Conc. (g/dl) = \( \frac{\text{Abs (Sample)}}{\text{Abs (Std.)}} \times 6 \text{g/dl} \)

**C) ESTIMATION OF ALBUMIN IN SERUM**

**PRINCIPLE**

The reaction between albumin in serum or EDTA plasma and the dye bromocresol green produces a change in colour that is proportional to the albumin concentration.

**REAGENT COMPOSITION**

1. Reagent: – Buffer pH 7.2
   - Bromocresol green
   - Preservative

2. Standard – Albumin 3 g/dl

**ASSAY PROCEDURE**

Mode of Reaction – End point

Wave length – 630 nm

Temp. – 37°C

Std. Concentration – 3 g/dl

Blank – Reagent

Reaction time – 1 min.

Sample volume – 10 µl
Reagent volume – 1ml

**CALCULATION**

\[
\text{Albumin (g/dl)} = \frac{\text{Abs. (Sample – Reag. Blank)}}{\text{Abs. (Standard – Reag. Blank)}} \times 3\text{g/dl}
\]

### 5.2.8. NEUROTRANSMITTER ASSAY

Brain neurotransmitters were assayed using HPLC as per the method of Varghese *et al.* (2001). A 10% homogenate of the hypothalamus and cerebral cortex was prepared in 0.4 N perchloric acid. The homogenate was centrifuged at 5000 x g for 5 min. at 40°C. The supernatant was filtered through 0.45 μm syringe top filters (millipore). The filtered sample was used for HPLC analysis using C-18 Reverse Phase Column and electrochemical detector.

### 5.2.9. STATISTICAL ANALYSIS

The data obtained were subjected to analysis of variance and the differences determined at 5% level of probability as described by Steel and Torrie (1980).

### 5.3. RESULTS

#### 5.3.1. PEPTIDOGLYCAN FROM CUTTLEFISH INK INCREASES LIFE SPAN OF DLA BEARING MICE

Antitumour activity exhibited by different fractions from cuttlefish ink is shown in Table 5.1. Among different fractions of the ink of cuttlefish, *Sepia pharaonis* the fraction C₂ has shown the highest activity (Table 5.1 and Fig.5.1). The C₂ fraction (peptidoglycan) increased the life span from 14 to 24 days (about 70% increase in life span) in mice bearing DLA. The antitumour activity exhibited by the polysaccharide part obtained from
Table 5.1: Antitumour activity exhibited by different fractions from the ink of cuttlefish, *Sepia pharaonis* against DLA in BALB/c mice

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Mean life span in days*</th>
<th>% increase in life span</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.6</td>
<td>-</td>
</tr>
<tr>
<td>Crude preparation</td>
<td>17.6</td>
<td>20.50</td>
</tr>
<tr>
<td>C</td>
<td>18.8</td>
<td>28.70</td>
</tr>
<tr>
<td>C₁</td>
<td>20.0</td>
<td>36.96</td>
</tr>
<tr>
<td>C₂</td>
<td>24.8</td>
<td>69.86</td>
</tr>
<tr>
<td>P S (polysaccharide)</td>
<td>24.52</td>
<td>65.75</td>
</tr>
</tbody>
</table>

* Mean life span of mice dying within 30 d of transplantation of tumour cells
Fig. 5.1. Antitumour activity of different fractions of cuttlefish ink, against DLA bearing mice.

A: crude preparation, B: fraction C, C: fraction C₁, D: fraction C₂, E: polysaccharide (PS)
fraction C₂ (66% increase in life span) did not significantly differ from that of C₂ fraction. The crude preparation of the cuttlefish ink has shown least activity (20% increase in lifespan).

5.3.2 *IN VITRO CYTOTOXICITY*

To ascertain whether the antitumour activity of fraction C₂ is due to the direct cytotoxic action of the fraction on the tumour cells, direct cytotoxic activity was studied. No direct cytotoxicity was elicited by the fraction C₂.

5.3.3. EFFECT OF DIFFERENT FRACTIONS ON ANTIOXIDENT ENZYMES

The antioxidant enzymes like SOD, catalase, glutathione peroxidase and glutathione reductase were estimated in heart, liver and kidney and the results are given in Table 5.2.

There was a significant decrease in superoxide dismutase, catalase and glutathione peroxidase and glutathione reductase activity in mice bearing DLA when compared with that of normal mice. These values were brought to almost normal levels when the mice were treated with the purified peptidoglycan (C₂ fraction).

5.3.4. EFFECT OF C₂ FRACTION ON ANTIOXIDANTS IN MICE BEARING DLA

Glutathione (GSH) and Ascorbic acid (Vitamin C) levels were studied in tissues of the experimental animals and the results are given in Table 5.3

Antioxidants like glutathione and ascorbic acid levels were found to be significantly reduced in DLA bearing mice when compared to normal mice. The mice treated with C₂ fraction, showed a significant increase in the above antioxidant levels similar to the normal mice, statistically.
Table 5.2: Effect of fraction C\textsubscript{2} from the ink of cuttlefish, *Sepia pharaonis* on the specific activity of SOD, Catalase, Glutathione Peroxidase and Glutathione reductase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tissue</th>
<th>Group</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>SOD</strong> (units x / mg protein)</td>
<td>Heart</td>
<td>3.92 ± 0.75\textsuperscript{a}</td>
<td>1.72 ± 0.31\textsuperscript{b}</td>
<td>3.14 ± 0.61\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>4.87 ± 1.15\textsuperscript{a}</td>
<td>1.79 ± 0.35\textsuperscript{b}</td>
<td>4.25 ± 0.82\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>4.27 ± 1.53\textsuperscript{a}</td>
<td>1.43 ± 0.21\textsuperscript{b}</td>
<td>6.98 ± 1.62\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td><strong>Catalase</strong> (x 10\textsuperscript{-3} units + / mg protein)</td>
<td>Heart</td>
<td>40.16 ± 4.16\textsuperscript{a}</td>
<td>19.92 ± 2.10\textsuperscript{b}</td>
<td>39.80 ± 4.01\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>51.20 ± 5.12\textsuperscript{a}</td>
<td>26.13 ± 2.64\textsuperscript{b}</td>
<td>49.17 ± 4.21\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>31.20 ± 3.20\textsuperscript{a}</td>
<td>15.31 ± 1.42\textsuperscript{b}</td>
<td>30.16 ± 3.22\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td><strong>Glutathione Peroxidase</strong> (units# / mg protein)</td>
<td>Heart</td>
<td>0.64 ± 0.05\textsuperscript{a}</td>
<td>0.40 ± 0.07\textsuperscript{b}</td>
<td>0.60 ± 0.06\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>0.95 ± 0.07\textsuperscript{a}</td>
<td>0.71 ± 0.08\textsuperscript{b}</td>
<td>0.92 ± 0.05\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>2.12 ± 0.38\textsuperscript{a}</td>
<td>1.87 ± 0.37\textsuperscript{a}</td>
<td>1.88 ± 0.41\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td><strong>Glutathione reductase</strong> (units# / mg protein)</td>
<td>Heart</td>
<td>0.49 ± 0.05\textsuperscript{a}</td>
<td>0.31 ± 0.03\textsuperscript{b}</td>
<td>0.47 ± 0.04\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>2.30 ± 0.03\textsuperscript{a}</td>
<td>1.20 ± 0.02\textsuperscript{b}</td>
<td>2.15 ± 0.03\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>2.22 ± 0.21\textsuperscript{a}</td>
<td>1.07 ± 0.02\textsuperscript{b}</td>
<td>2.10 ± 0.02\textsuperscript{a}</td>
<td></td>
</tr>
</tbody>
</table>

Group 1 – Normal mice not bearing DLA

Group 2- Mice bearing DLA

Group 3 Mice bearing DLA and treated with C\textsubscript{2} fraction

Values are average of six replicate samples ± SD

Values with different superscripts in the same row differ significantly (p< 0.05)

units\textsuperscript{x} - enzyme concentration required to inhibit absorbance at 560 nm of chromogen production by 50% in 1 min.

units\textsuperscript{+} - velocity constant / sec

units\textsuperscript{#} - One unit is defined as the difference in absorbance / minute
Table 5.3: Effect of fraction C\textsubscript{2} from the ink of cuttlefish, *Sepia pharaonis* on the levels of Ascorbic acid and Glutathione in tissues

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Tissue</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>Heart</td>
<td>12.5 ± 0.90\textsuperscript{a}</td>
<td>9.67 ± 0.60\textsuperscript{b}</td>
<td>11.46 ± 0.80\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>36.20 ± 2.50\textsuperscript{a}</td>
<td>27.90 ± 2.10\textsuperscript{b}</td>
<td>33.66 ± 2.80\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>28.80 ± 2.20\textsuperscript{a}</td>
<td>18.29 ± 0.90\textsuperscript{b}</td>
<td>27.57 ± 2.30\textsuperscript{a}</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Heart</td>
<td>0.32 ± 0.03\textsuperscript{a}</td>
<td>0.11 ± 0.01\textsuperscript{b}</td>
<td>0.29 ± 0.02\textsuperscript{a}</td>
</tr>
<tr>
<td>mg/ 100g tissue</td>
<td>Liver</td>
<td>0.33 ± 0.02\textsuperscript{a}</td>
<td>0.13 ± 0.02\textsuperscript{b}</td>
<td>0.30 ± 0.03\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.28 ± 0.02\textsuperscript{a}</td>
<td>0.09 ± 0.02\textsuperscript{b}</td>
<td>0.26 ± 0.02\textsuperscript{a}</td>
</tr>
</tbody>
</table>

Group 1 – Normal mice not bearing DLA

Group 2- Mice bearing DLA

Group 3 Mice bearing DLA and treated with fraction C\textsubscript{2}

Values are average of six replicate samples ± SD

Values with different superscripts in the same row differ significantly (p< 0.05)
5.3.5. EFFECT OF C₂ FRACTION ON LIPID PEROXIDATION PRODUCTS IN TISSUES

Lipid peroxidation products like malondialdehyde (MDA) and conjugated dienes (CD) in tissues of mice bearing DLA were studied and the results are given in Table 5.4. Malondialdehyde and conjugated dienes were found to be elevated significantly in DLA bearing mice when compared to normal mice. But these values were lowered to the normal values in mice treated with C₂ fraction.

5.3.6. EFFECT OF FRACTION C₂ ON SERUM TOTAL PROTEIN, ALBUMIN AND SGPT

Hepatotoxicity of fraction C₂ of cuttlefish ink was studied by evaluating the level of SGPT, total protein and albumin. Serum glutamate pyruvate transaminase (S-GPT) in serum was found to increase significantly in DLA bearing mice. But this value was decreased to the normal value in mice treated with the C₂ fraction. However, the level of total protein and albumin did not show any significant variation among the 3 groups (Table.5.5).

5.3.7. EFFECT OF C₂ FRACTION ON NEUROTRANSMITTERS

Effects of C₂ fraction on different neurotransmitters (5-HT, HIAA, DA, HVA, NE, and EPI) were studied. The results (Table 5.6) showed that the neurotransmitters in the two regions of the brain were significantly lower in the mice bearing DLA when compared to the normal mice. On the other hand, treatment with C₂ fraction resulted in the elevation of neurotransmitter levels, but they did not reach normal values in all cases (DA,NE,EPI). Some of the neurotransmitters did not show any variation (HIAA, 5-HT).
Table 5.4: Effect of fraction C₂ from the ink of cuttlefish, *Sepia pharaonis* on Lipid Peroxidation products in Tissues

<table>
<thead>
<tr>
<th>Product</th>
<th>Tissue</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
</table>
| **MDA mmol/ 100g tissue** | Heart   | 9.1 ± 1.10  
                      | Liver    | 22.2 ± 2.20  
                      | Kidney   | 26.40 ± 2.92a |
|                  |         | 16.0 ± 1.2b |
|                  |         | 41.6 ± 3.8b |
|                  |         | 49.6 ± 4.1b |
| **Conjugated dienes mmol / 100g tissue** | Heart  | 32.15 ± 3.00a |
                      | Liver    | 61.12 ± 5.20  
                      | Kidney   | 53.20 ± 5.10a  |
|                  |         | 39.12 ± 3.6b |
|                  |         | 76.74 ± 7.1b |
|                  |         | 68.56 ± 6.3b |

Group 1 – Normal mice not bearing DLA

Group 2- Mice bearing DLA

Group 3 Mice bearing DLA and treated with C₂ fraction

Values are average of six replicate samples ± SD

Values with different superscripts in the same row differ significantly (p< 0.05)
Table 5.5: Effect of fraction C₂ from the ink of cuttlefish, *Sepia pharaonis* on serum Total Protein, Albumin and SGPT

<table>
<thead>
<tr>
<th>Serum Parameters</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Protein (g/dl)</td>
<td>7.12 ± 0.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.91 ± 0.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.58 ± 0.71&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>2.70 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.75 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.56 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SGPT (u/l)</td>
<td>25.52 ± 2.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.15 ± 6.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.46 ± 2.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Group 1 – Normal mice not bearing DLA
Group 2- Mice bearing DLA
Group 3 Mice bearing DLA and treated with C₂ fraction

Values are average of six replicate samples ± SD
Values with different superscripts in the same row differ significantly (p< 0.05)
Table 5.6: Effect of fraction C\textsubscript{2} from the ink of cuttlefish, *Sepia pharaonis* on the levels of Neurotransmitters in the cerebral cortex and hypothalamus

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Brain region</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>5-HT</td>
<td>Cerebral cortex</td>
<td>0.83 ± 0.04\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Hypothalamus</td>
<td>0.42 ± 0.11\textsuperscript{a}</td>
</tr>
<tr>
<td>HIAA</td>
<td>Cerebral cortex</td>
<td>1.26 ± 0.18\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Hypothalamus</td>
<td>0.78 ± 0.10\textsuperscript{a}</td>
</tr>
<tr>
<td>DA</td>
<td>Cerebral cortex</td>
<td>1.65 ± 0.09\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Hypothalamus</td>
<td>2.29 ± 0.32\textsuperscript{a}</td>
</tr>
<tr>
<td>HVA</td>
<td>Cerebral cortex</td>
<td>0.43 ± 0.04\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Hypothalamus</td>
<td>4.47 ± 0.09\textsuperscript{a}</td>
</tr>
<tr>
<td>NE</td>
<td>Cerebral cortex</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Hypothalamus</td>
<td>1.59 ± 0.15\textsuperscript{a}</td>
</tr>
<tr>
<td>EPI</td>
<td>Cerebral cortex</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Hypothalamus</td>
<td>5.50 ± 0.28\textsuperscript{a}</td>
</tr>
</tbody>
</table>

Values are in nmoles/gm. wet weight of tissue

5-HT-5-Hydroxy Tryptamine, HIAA-Hydroxy Indole Acetic Acid, DA-Dopamine, HVA-Homovanillate, NE-Norepinephrin, EPI-Epinephrin.

Group 1 – Normal mice not bearing DLA
Group 2- Mice bearing DLA
Group 3 Mice bearing DLA and treated with C\textsubscript{2} fraction

Values are average of six replicate samples ± SD

Values with different superscripts in the same row differ significantly (p< 0.05)
5.4. DISCUSSION

The results of the present investigation revealed the antitumour activity of different fractions of female cuttlefish (*Sepia pharaonis*) ink in DLA bearing mice. The experimental results indicate that the C$_2$ fraction of cuttlefish ink possess significant antitumour activity compared to other fractions. The above fraction has showed 70% increase in lifespan of treated mice over the control group (mice bearing DLA). The results also showed that the antitumour activity is increased with increasing purification. As the purification of the fraction is increased the percentage of polysaccharide increased. The C$_2$ fraction is an uronic acid rich polysaccharide forming 85% of the peptidoglycan and the peptide part was 5% and the rest was pigment. The C$_2$ fraction had the least amount of protein and highest amount of uronic acid. The polysaccharide isolated from this fraction also showed similar antitumour activity. This may indicate that the antitumour property of fraction C$_2$ is vested mainly in the polysaccharide part.

Reports have shown that peptidoglycans from different biological sources have antitumour activity. The antitumour activity of peptidoglycan from squid (*Illex argentinus*) ink was first investigated against Meth A fibrosarcoma in mice (Takaya *et al.*, 1994a). Since the fraction had no direct cytotoxic effect on Meth A cells, they inferred that inhibition of tumour growth by this fraction might be due to stimulation of host mediated immune responses. Lu *et al.* (1994) working with cuttlefish ink and mice showed that the levels of specific antibody against Meth A sarcoma cell antigen in the serum from the mice treated with cuttlefish ink was significantly higher than that from control, indicating increased humoral immunity in treated mice. The antitumour activity of acetone delipidated squid ink was investigated by Sasaki *et al.* (1997) against Meth A
fibrosarcoma in BALB/c mice. The delipidated ink enhanced the phagocytic activity of macrophages but no direct cytotoxicity was observed for Meth A tumour cells. Hence the antitumour activity of delipidated squid ink was assigned to be due to augmented cellular immunity *in vivo*. In the present study also no direct toxicity was observed for the DLA cells by the C2 fraction. A polysaccharide grifolan NMF-5N, a beta – 1, 3- glucan obtained from mycelia of *Grifola frondosa*, exhibited antitumour activity against syngenic tumour mice (Suzuki *et al*., 1987). Whole peptidoglycan (cell wall preparation) from *Bifidobacterium infantis* showed a significant suppression of tumour growth against syngenic Meth A fibrosarcoma in BALB/c mice (Sekine *et al*., 1985). Seiki *et al.* (1982); Sekine *et al.* (1985) and Okamoto *et al.* (2001) also showed that peptidoglycan from the cell wall skeletons of bacteria exhibit antitumour activity *in vivo*. The protein bound polysaccharides isolated from the mushrooms have been used as an immune therapy agent in the treatment of cancer in Asia for over 30 years (Wasser and Weis, 1999). It was found that antitumour active fractions found in almost all mushrooms were polysaccharides or protein polysaccharide complex. These polysaccharides consisted mainly of 1,3 linked D-glucan and 1,4 linked mannosyl polymers (Jong and Donovick, 1989).

The fraction C2 which showed highest antitumour activity was studied for its effect on tissue antioxidant enzymes, antioxidants, lipid peroxidation products, S-GPT, serum proteins and neurotransmitter levels. It has been postulated that age dependent diseases like atherosclerosis, arthritis, neurodegenerative disorders and cancer involve oxygen free radicals (OFR) at least at some stage of their development (Halliwell *et al*., 1992; Ames *et al*., 1993). The antioxidant defense mechanism in our body protects the
cells from the attack of OFR. Studies on antioxidant system proved that in cell line induced tumour also, the disease promotion takes place through reactive oxygen species (Ruby et al., 1995; Anto et al., 1996). It was found that as the tumour progresses, the antioxidant enzymes like SOD, catalase, GPx, GR, GST and antioxidants like GSH and ascorbic acid were seriously affected by the OFR. Decreased activity of SOD, catalase and glutathione peroxidase in lymphoma condition may be due to either the direct inactivation of these enzymes by the superoxide radical (Thomas and Kamat, 1999) or by decreased aerobic metabolism in tumour cells (Greenstein, 1954). Treatment of the lymphomas (mice bearing DLA) with the fraction C$_2$ (peptidoglycan) of cuttlefish ink brought the SOD, catalase, glutathione peroxidase and glutathione reductase activities to near normal levels showing that the fraction may contain some factors that have the capacity to scavenge the toxicity of superoxides, there by protecting the cells from damage. This might have been the reason for the return to normal cellular functions.

Antioxidants such as GSH and ascorbic acid directly scavenge the free radicals. Prolonged oxidative stress can lead to the depletion of essential antioxidant system (Spencer et al., 1995). With the progression of tumour in mice, glutathione levels decreased significantly in testes and bone marrow cells while they increased in Dalton’s lymphoma cells and spleen (Khynriam and Prasad, 2003). Meister, (1994) observed a decrease in antioxidants in the liver and kidney in Dalton’s lymphoma and Ehrlich’s ascites lymphoma and attributed it to severe oxidative damage in these tissues. Antioxidants like glutathione and ascorbic acid levels were found to be significantly decreased in DLA bearing mice. The mice treated with C$_2$ fraction showed a significant increase in the above antioxidant levels and this may decrease the damage of cell due to
severe OFR attack. The decreased levels of antioxidant enzymes and antioxidants result in the accumulation of superoxides and other free radicals. These free radicals attack the lipids resulting in lipid peroxidation (Sies, 1985). Many observations support the notion that lipid peroxidation plays an important role in carcinogenesis (Vuillaume, 1987). Lipid peroxidation was found to be high in DLA bearing mice but treatment with the fraction C\textsubscript{2} (peptidoglycan) could control this increase. The increased level of lipid peroxidation might have been normalised by the fraction C\textsubscript{2} as it increased the levels of antioxidant enzymes and antioxidants.

Although chemotherapy is a widely accepted method for cancer treatment, chemotherapeutic agents show several toxic side effects. Activities of serum S-GPT, total protein and albumin levels are measured to evaluate the hepatotoxicity of the fraction C\textsubscript{2} of cuttlefish ink. DLA bearing mice showed a higher S-GPT activity. But this could be reversed by drug treatment. However, the level of total protein and albumin did not show any significant variation among the three groups. The results revealed that the peptidoglycan fraction had no hepatotoxicity on DLA bearing mice, on the other hand, the fraction showed hepato protective effect. The neurotransmitter levels of the DLA bearing mice were lower than those in normal mice. This may be due to low levels of ascorbic acid in DLA bearing mice. Vitamin C is a cofactor for several enzymes involved in the biosynthesis of neurotransmitters (Burri and Jaacob, 1997). But the neurotransmitter levels were increased on treatment with peptidoglycan probably due to an increase in vitamin C levels. This also showed that the C\textsubscript{2} fraction had no neurotoxicity, rather it protected the nerve cells.
The reactive oxygen species (ROS) play a major role in tumour progression by cellular damage. The present study indicated that the disease promotion might have taken place through ROS. As the tumour progresses, the antioxidant enzymes and the antioxidants are affected and this might be affecting the normal cell resulting in cell death. Treatment of the lymphoma with the peptidoglycan could control the changes in lipid peroxidation and antioxidant efficiency, resulting in the re-establishment of cellular metabolism and control of cellular damage. Thus the present experiment indicates that besides stimulating the immune response as cited by previous authors (Takaya et al., 1994a; Lu et al., 1994; Sasaki et al., 1997), the antitumour agent in the cuttlefish ink stimulates the antioxidant defence mechanism in experimental animals, thus arresting the tumour progression. The findings suggest the potential therapeutic use of the peptidoglycan (fraction C₂) as an anticancer agent in cancer chemotherapy.