CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

Experimental animals:

The present study was carried out in pathogen free adult male white mice (C_3H/He strain) weighting between 18-22 gms, maintained under uniform laboratory conditions in the animal culture laboratory of the Department of Zoology, Gauhati University, India. The mice were provided with standard pellet diet (Hindustan Lever) or standard laboratory chow (Lipton India Ltd., Kolkata) and water were available ad libitum. The mice were randomly divided into three sets comprising of five different groups with six animals in each group, sacrificed on 10^{th}, 20^{th} and 35^{th} day from the onset of the experiment. Carboxymethyl cellulose (0.75% aqueous, Hi Media, India Ltd.) was used as vehicle for administration of BRH_2 to the mice. The vehicle used had same affect as distilled water on the biochemical parameters studied. Hence, only carboxymethyl cellulose was used in the control set of mice. The different animal groupings were:

I. Control Set:

Group A: Received only 0.2ml of 0.75% aqueous carboxymethyl cellulose.

II. Malignant Set :

Group B & C: Received 0.2 ml (i.e. 1 x 10^7 cells) ascite Dalton's lymphoma fluid intraperitoneally (i/p) sacrificed on the 10^{th} & 20^{th} day.

III. Anticancer drug treated Set:

Group D: Received BRH_2 (in 0.75% aqueous carboxymethyl cellulose) 100 mg/Kg bw on 1^{st}, 5^{th} & 9^{th} day i.p., sacrificed on the 20^{th} day.

Group E: Treated as in Group D sacrificed on the 35^{th} day.

Each group comprises of six animals.

A: PREPARARTION OF ANTICANCER DRUG BRH_2

The drug was prepared following the method of Patil et al. (1989). The details of the procedure for preparation of BRH_2 [*copper (II) complexes with 5 – phenylazo – 3 – methoxy salicylidene thiosemicarbazone*] and determination of dose is described in the chapter III.

B: DETERMINATION OF LACTIC DEHYDROGENASE (LDH) ACTIVITY

The LDH activity of Liver, Spleen, Kidney, and Bone marrow of white mice was measured following the method of King (1965).

Principle: The measurement of the enzyme activity was based on the principle of conversion of lactate to pyruvate in presence of NAD.

Lactic acid + NAD $\xrightarrow{LDH}$ Pyruvic acid + NADH + H^+

The pyruvic acid that formed will react with reduced NAD colour reagent to form the corresponding phenyl – hydrazone, which in alkaline medium, gives golden brown colour and absorbency was measured.

Reagents:

1. *Glycine buffer, 0.1M (Sorensen)*: 7.505 gms of glycine and 5.85 gms of sodium chloride were taken in 1 L volumetric flask and dissolved in double distilled water (all glass). The volume was made up to the mark with double distilled water.
2. *0.1 N Sodium hydroxide (NaOH)*
3. *0.4 N Sodium hydroxide (NaOH)*
4. *Buffer substrate*: 4 gms of lithium lactate or 5 ml of sodium lactate solution was dissolved in 125 ml of glycine buffer (Reagent – 1) and 75 ml of 0.1 N NaOH (Reagent – 2) solution in a glass stoppered bottle. The reagent was stored at 4^0C.

5. *Nicotinamide adenine dinucleotide (NAD) solution*: 10 mg of NAD was dissolved with 2 ml of double distilled water and stored at 4^0C. Fresh solution was prepared at regular interval.
6. *1 N Hydrochloric acid (HCl).*
7. *2,4 dinitrophenyl hydrazine (DNPH)*: 200 mg of 2,4 dinitrophenyl hydrazine was dissolved in hot 1 N HCl acid and made up to 1 L with this acid. The reagent was then transferred to a glass stoppered bottle and stored at 4^0C.
8. *Standard Sodium Pyruvate Solution*: 11 mg of sodium pyruvate was dissolved in 100 ml buffered substrate (reagent – 4). The reagent was then transferred into a glass-stoppered bottle and stored at 4^0C. This reagent contains 1µ mol of pyruvate/ml of buffered substrate.
9. *Reduced nicotinamide adenine dinucleotide (NADH) solution*: 71 mg of disodium salt of NADH was dissolved in 100 ml of buffered substrate (Reagent – 4) to obtain a solution containing 1 u mol of NADH/ ml of buffered substrate and stored at 4^0C.

Preparation of tissue homogenate:

Fresh tissues of liver, spleen, kidney and bone marrow were weighted and homogenized separately with ice-cold redistilled water in the proportion of 1:10 (1 – 2 gm tissue in 8 – 10 ml of water). The homogenized liquid was centrifuged at 2000 rpm for 20 minutes and the supernatant extracts were

siphoned off. 1 ml of tissue homogenate was taken in a centrifuge tube and kept in an ice bath.

1 ml of buffered substrate (reagent – 4) and 0.1 ml of tissue homogenate were taken in each of the two test tubes. 0.2 ml of double distilled water was then added to one of the two test tubes and was marked as blank (B). Both the tubes were then incubated at 37^0C for 15 minutes. 0.2 ml of NAD solution (reagent –3) was added to the other test tube marked as test (T) and mixed. Both the tubes (i.e. blank and test) were then placed again in an incubator. Exactly 15 minutes after addition of NAD solution (reagent – 5), 1 ml of DNPH (reagent – 7) was added to each tube, mixed and incubated for another 15 minutes. But the tubes were then taken out from the incubator and 10 ml of 0.4 N NaOH (reagent – 3) was added to each of the tube. Within 1 to 5 minutes of adding 0.4 N NaOH solution absorbance was measured in a spectrocolorimeter (Systronics; Model – 108) at 440nm. The result of the LDH activity was obtained by extraplotation from the standard calibration curve.

Preparation of standard calibration curve:

A standard calibration curve was set up by taking $NADH_2$, pyruvate solution, buffered substrate, NAD solution and double distilled (DD) water in different quantity in a series of test tubes as shown below in the ***table- 2.1***

Table. 2.1: Preparation of working standard solution for LDH activity.

IU /L	0	157	333	500	567	833	1000
ml of reduced NAD in substrate	0	0.05	0.1	0.15	0.2	0.25	0.3
ml of pyruvate solution	0	0.05	0.1	0.15	0.2	0.25	0.3
ml of buffered substrate	1.0	0.9	0.8	0.7	0.6	0.5	0.4
ml of NAD solution	0	0.2	0.2	0.2	0.2	0.2	0.2
ml of distilled water	0.3	0.1	0.1	0.1	0.1	0.1	0.1

Table. 2.2: Absorbance of standard solution.

Tube No	Replication					Mean
	1	2	3	4	5	
1	0.04	0.06	0.05	0.045	0.055	0.05
2	0.11	0.125	0.13	0.115	0.12	0.12
3	0.2	0.21	0.22	0.215	0.205	0.21
4	0.35	0.3	0.25	0.35	0.25	0.3
5	0.37	0.39	0.36	0.39	0.39	0.38
6	0.46	0.45	0.44	0.455	0.445	0.45

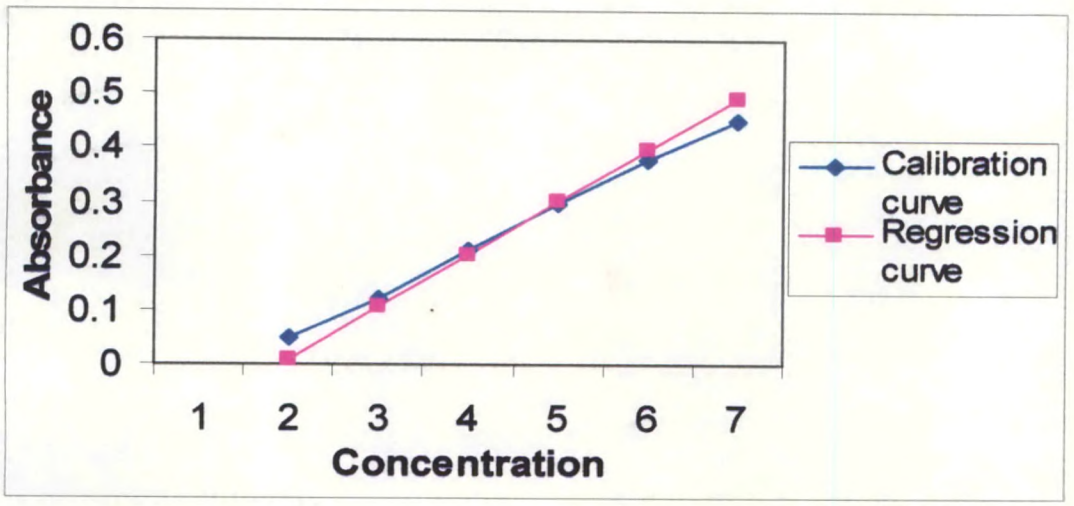

Fig. 2.1 Calibration Curve for LDH

When all the tubes were ready, they were incubated for 15 minutes at 37^0C. Exactly after 15 minutes of incubation, 1 ml of DNPH was added and were incubated for further 15 minutes at 37^0C. All the tubes were then taken out from the incubator and 10 ml of 0.4 NaOH was added and mixed. The absorbance was measured in Spectrocolorimeter at 440 nm.

Isolation of Isoenzyme of LDH:

LDH isoenzymes were separated on polyacrylamide gel electrophoresis (PAGE) following the procedure of Davis (1964). Electrophoresis was carried out in small glass tubes in a vertical dimension and polyacrylamide

gels (5.5% acrylamide gel cross linked with 0.14% bis – acrylamide) was made in the glass tubes.

Reagent:

I. **Solution A:** 48 ml of 1 N HCl was taken in a 100 ml volumetric flask. 36.6 gm of Tris and 0.23 ml of tetra methyl ethylene diamine (TEMED) was added into the flask and dissolved by adding DD water. The volume was finally made up to the mark with DD water.

II. **Solution B:** 28 gm of acrylamide and 0.735 gm of bis acrylamide were dissolved with DD water in a 100 ml volumetric flask and the volume was made up to the mark with DD water.

III. **Solution C:** 48 ml of 1 N HCl was taken in a 100 ml volumetric flask. Then 5.98 gm Tris and 0.46 ml TEMED were added to the flask and dissolved by adding DD water. The volume was made up to the mark with DD water.

IV. **Solution D:** 10 mg acrylamide and 2.5 gm of bisacrylmide were dissolved in DD water in a 100 ml volumetric flask and the volume was made up to the mark with DD water.

V. **Solution E:** 4 mg of riboflavin was added in DD water in 100 ml volumetric flask and the volume was made up to mark.

VI. **Solution F:** 40 gm of sucrose was dissolved with DD water in a 100 ml volumetric flask and the volume was made up to the mark with DD water.

VII. **Solution G:** 0.14 gm ammonium persulphate was dissolved with DD water in a volumetric flask and the volume was made up to the mark with DD water. This solution should freshly be prepared.

VIII. **Solution H (Electrode buffer)**: 0.6 gm of Tris and 2.88 gm of glycine were taken in a 1L. volumetric flask and dissolved in DD water and the volume was made up to the mark. pH was checked at 8.3.

IX. **Solution I (Staining mixture)**: The staining solution was prepared by adding 0.47 ml of NAD^+ solution (8 mM), 0.78 ml of Nitroblue tetrazolium (NBT) solution (4 mM), 0.2 ml of phenazine methosulphate (P.M.S.) solution (8 mM), 1.0 ml sodium lactate (5.35 M) and 2.5 ml of water to 5.0 ml of Tris – HCl buffer (0.05 M; pH 7.4).

Preparation of gel column:

Glass tubes of 0.5 cm diameter and 7.0 cm long were used for gel preparation. The tubes were thoroughly washed in chromic acid, detergent solution and finally ringed several times with DD water. The tubes were placed in a vertical plane by placing them in a rubber stopper.

The gel columns were prepared in segments, first the running gel and then spacer gel above it.

(a) **Running Gel Preparation:** In a electrophoretic glass tube, 1.275 ml of solution A, 2.55 ml of solution B and 1.275 ml of DD water were added and mixed thoroughly. The 5.1 ml of solution G was added and mixed properly. The above solution contains the acrylamide concentration of 5.5%.

The glass tubes were placed in a vertical plane by placing them with rubber stopper in one end. 1.7 ml of the above prepared gel solution was poured carefully into each tubes and 2 – 3 drops of DD water were added carefully above the gel solution with a syringe. The tubes were then placed under fluorescent light to polymerize (30 minutes). The water i.e. layered over it after polymerization was drained off by inverting the tubes.

(b) **Spacer Gel Preparation:** In a elctrophoretic glass tube, spacer gel solution was prepared by mixing of 0.45 ml of solution C, 0.9 ml of solution D; 0.45 ml of solution E and 1.8 ml of solution F.

After the running gel layer was polymerized the spacer gel solution was prepared as above and 0.1ml of the spacer gel solution was poured over the running gel with the help of a pipette, 2 – 3 drops of DD water was added above it and again photo polymerized under fluorescent light. After polymerization of

the gel, the water that accumulated above the gel was removed as mentioned before.

Then the gel tubes were placed carefully into the electrophoresis chamber and 0.1 ml of tissue extract (1 µg/ of supernatant for LDH) was added on the gel column of each tube, placed vertically in the hole of upper reservoir of the electrophoresis chamber. Then 2 drops of 40% sucrose solution poured over it. Finally, 0.01% Bromophenol blue was added at the top of it as marker. The blank space of the tube was filled with electrode buffer. Both the upper and lower chambers of the electrophoresis tank were filled with tris glycine buffer (solution H) and allowed to run the current at the rate of 2 mA (100 volt) for about 15 minutes. After 15 minutes the current was increased to 4 mA (at 4^0C).

Exactly after 90 minutes of electrophoresis at 4 mA the gels were removed from the gel tube by running a jet of water by the sides of the gel inside the tube (the amount of time is to travel the dye through the length of the gel tube). The gels were then incubated in the staining mixture (pH = 8.3, solution I) at 37^0C for 40 minutes in dark. The gels were washed in DD water and fixed and preserved in 50:40:10 of distilled water : methanol : glacial acetic acid (v/v) respectively. The analysis of gels was performed using

VDS – image master for scanning and the pixel intensity was calculated.

C: ESTIMATION OF ELEMENTS

The elements were estimated by Atomic Absorption Spectrophotometer following the Wet ashing method (oxidation procedure).

Wet Ashing Method: This method describes the determination of metals in tissue using a wet ashing (oxidation) procedure (Kahnke, 1966). Sample oxidation is accomplished by boiling formalinized tissue samples in water and a mixture of nitric aid and perchloric acid until a clear solution appears. A final dilution with deionized water was performed and the sample was ready for analysis.

Sample Preparation: 5 gm of formalinized tissue was taken in a 125 ml Erlenmeyer flask, added glass beads and 25 ml of deionized water. Then 10 ml of 1 : 2 mixture of concentrated nitric acid and perchloric acid was added and boiled until the solution appeared clear. The solution was then transferred to a 100 ml volumetric flask and diluted with deionized water.

Analysis: The concentration of elements were determined in the Parkin – Elmer atomic absorption spectrophotometer (M 2380) using the procedures

listed in the manual supplied with the instrument. Standards were prepared by diluting the stock standard solutions with deionized water. Deionized water was used as the blank solution.

D: SCANNING ELECTRON MICROSCOPY (SEM) OF CANCER CELLS (DALTON'S LYMPHOMA CELLS)

Specimen Preparation for SEM Study: The first step in the preparation of the specimens for SEM is the fixation. The commonly used fixatives for SEM of lymphoma cell is 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2 – 7.4), after washing in phosphate buffered saline (PBS).

Preparation of buffers:

1. *0.01 m sodium phosphate buffer:*

Sodium dihydrogen phosphate	-	0.3205 gm
Disodium hydrogen phosphate ($Na_2HPO_47H_2O$)	-	2.065 gm
$Na_2HPO_4.12H_2O$	-	2.76 gm

All glass DD water was used to make the volume 1000 ml.

2. *0.1 M Sodium cacodylate buffer (pH 7.2 – 7.4)*

Sodium cacodylate Na $(CH_3)_2AsO_2.3H_2O$	-	21.403 gm
N. Hydrochloric acid	-	6.9 ml

DD water was used to make the volume to 1000 ml.

Preparation of phosphate buffered saline (PBS):

8.775 gms of Sodium Chloride (0.15 M) was dissolved in 1000 ml of 0.01 M sodium phosphate buffer (pH 7.4).

Preparation of fixative:

2.5% glutaraldehyde in 0.1 M Sodium cacodylate buffer: 25 ml of 25% gluteraldehyde was taken and made the volume up to 125 ml with double distilled water. Added 125 ml of 0.1 M Sodium cacodylate buffer and stored at 4^0C.

Procedure for fixation:

The mice were killed by under anesthesia and all protocols were followed as per CPCSEA and ascites tumor was removed, centrifuged and washed once with PBS (0.15 M NaCl, 0.01 M Sodium phosphate buffer, pH 7.4). The cell pellet was diluted with PBS (1:3, weight : volume) gently. The cell suspensions were fixed in 2.5% glutaraldehyde prepared in 0.1 M Sodium cacodylate buffer for 10 minutes at 4^0C. Cell suspensions were washed in 0.1 M Sodium cacodylate buffer (buffer used for the preparation of the fixative). Tissue can be brought in the same buffer (0.1 M Sodium cacodylate buffer).

Drying and metal coating:

A drop of cell suspensions were taken on a clean cover slip and covered gently by another cover slip for uniform distribution of cell. Then both the cover slips were detached each other keeping sometime for air dried at room temperature (25^0C).

The dried cover slips were fixed on brass stub by electro conductive paint and are kept on the specimen stand of the ion sputter coater (JFC 1100, JEOL) and were coated with a thin layer of gold particles.

The coated specimens were then observed under SEM (JSM – 35 CF, JEOL) at an accelerating voltage at 15 KV and photographed on 125 ASA/22 DIN 112 mm black and white OROW film.

E. HISTOLOGICAL PREPARTAION FOR LIGHT MICROSCOPY

The tissues of the dissected parts of liver, spleen and kidney were washed in normal saline fixed in Carnoy's fixatives (6:4:1, Absolute alcohol : Chloroform : Glacial acetic acid) for 3 – 4 hrs. Now fixed tissues were washed in 100% alcohol and thereafter transferred to xylene. Then the tissues were treated with 1 : 1 xylene and paraffin for 30 minutes and thereafter tissues were immersed in pure paraffin with 3 changes (each for

45 minutes) and then impregnated in pure paraffin for block making. 5μ paraffin sections were cut and affixed on the slides. Now deparaffinised in xylene and brought to the water through downgrade of alcohol Routine Haematoxylene and Eosine staining was carried out and the sections were dehydrated through increasing concentrations of alcohols. Then, they were cleared in xylene and mounted in DPX and observed under the light microscope. Photographs were taken on Kodak 200 (Coloured) films.

F. STATISTICAL ANALYSIS

1. **Regression Line :** The regression line was prepared to test the accuracy of the methods of biochemical estimation by using the linear regression equation.

   $Y = a + bx$

2. **Analysis of variance (ANOVA):** The experimental data expressed as means ± SE were analyzed using Fisher's method of ANOVA (one way) and subjected to critical difference calculation (Raghuramulu et al., 1983) for evaluation of significance.

   The critical difference (CD) were calculated by using the following formula:

   $$CD = t_0 \sqrt{EMS \times (1/K_1 + 1/K_2)}$$

Where 't_0' is the tabulated value of 't' at the desired level and at the error degree of freedom. EMS is the error mean square and K_1, K_2, are the number of replications in the groups compared.

3. **Correlation analysis:** Correlation (Co) is used to describe the degree to which one variable is linearly related to another. The coefficient of correlation denoted by 'r' indicates the direction of relation between two variables (accordingly, the Co may be +Ve or – Ve). The Co were calculated by the formula suggested by Bailey (1994) as:

$$r = \Sigma xy \Big/ \sqrt{(\Sigma x^2)(\Sigma y^2)} \qquad \text{where, } x = X - \overline{X}$$

$$y = Y - \overline{Y}$$

Here, x and y are the deviation measured from their respective means.