Chapter - 1

Material & Methods
AIMS AND OBJECTIVES

To study the *in vivo* effect of selected doses of Amphotericin-B on selected metabolic parameters in rat tissues.

**Plan of work**

1. To treat the albino rats with 0.25 mg/kg of Amp-B over four and 10 weeks and with 1.5 mg/kg of Amp-B over 4 and 10 weeks.
2. To study the impact of Amp-B on rat Brain, heart, liver, kidney based tissues major organic, constituents like total protein, carbohydrates, lipids, glycogen, glucose, free amino acid (FAA) and free fatty acids (F.F.A) Lactate & pyruvate.
3. To study the effect of Amp-B on major enzyme activity levels involved in protein metabolism i.e., proteases AST (Aspartate Amino Tranferase) and ALT (Alanine Amino Transferase)
4. To study the effect of Amp-B on rat tissue based major enzyme activity levels involved in carbohydrate metabolism like phosphorylase ‘a’ phosphorylase ‘ab’, aldolase, succinate dehydrogenase (SDH) lactate dehydrogenase (LDH) malate dehydrogenase (MDH), lipase and phospholipase activities.
5. To study the effect of Amp-B on key enzyme of energy metabolism like ATPases, Cytochrome-C-oxidase and ATP levels.

**Materials:**

Albino rats of the weight range 150 ± 10 gm were used for the present study. They were fed with commercial diet (Kamadhenu Agencies, Bangalore) and were kept under constant laboratory
conditions (20±5°C). Animals were divided into eight groups of seven each and maintained in separate cages.

Amphotericin-B (Amp-B) was a product from Bristol-Myeris Squib Company, I Squibb Drive, New Burns Wiek, New Jersey 08903, USA.

All chemicals used were purchased from Sigma Co (St-Louis) or SDH, or BDH India and they are all of technical grade ones.

Properties of Amphotericin-B

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Molecular formulae</td>
<td>C₄₇H₇₃NO₁₁</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>924.1</td>
</tr>
<tr>
<td>Colour</td>
<td>Yellow or Orange</td>
</tr>
<tr>
<td>Odour</td>
<td>Odour less</td>
</tr>
<tr>
<td>Physical State</td>
<td>Powder</td>
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The chemical structure of Amphotericin B is as shown below

**Amphotericin B**

*Fungizone (Squibb)*

Potency: not less than 750 μg of amphotericin B/mg.

**Treatment of Animal**

Amp-B in selected doses was injected through the tail vein. The II group of albino rats received 0.25 mg/kg weight of Amp-B over 4 weeks, the IV group received 0.25 mg/kg of Amp-B over 10 weeks (weekly doses). The I and III groups acted as 4 and 10 weeks control ones (received equal volumes of sterile water) simultaneously, the VI group of rats were administered with 1.5 mg/kg weight of Amp-B over 4 weeks (weekly doses) and the VIII group was treated with 1.5 mg/kg of weight of Amp-B over 10 weeks (weekly doses). The V and VII group of rats receiving sterile water acted as 4 and 10 weeks experimental ones.

**Isolation of tissues**

After treatment of rats, the control and experimental group of animals were anaesthetized with ether and were sacrificed. Major tissue like brain, heart, liver, kidney were isolated, quickly blotted on a filter paper, and were placed in liquid nitrogen and were stored at -80°C until used. Whenever needed blood was collected by cardiac puncture and
allowed to clot at room temperature, the clot was removed and samples were centrifuged at 2000 rpm for 10 minutes. The separated serum was kept for several hours in the refrigerator or several days in freezer and subsequently used for experimentation.

METHODS

Estimation of Total Proteins

The total protein content was estimated by the method Lowry et al., (1951) to express the activity of enzymes for mg protein.

1% (w/v) homogenate of the tissues were prepared in 0.25 M cold sucrose solution 2 ml of 10% trichloroacetic acid (TCA) was added to 0.5 ml of tissue homogenates and the samples were allowed to stand for 30 min at room temperature and again centrifuged at 1000 x g for 15 min. The sediment was dissolved in 1 ml of 1 N sodium hydroxide (NaOH) and 0.2 ml of this solution was added to 4 ml of alkaline copper reagent. After 10 min, 0.4 ml of Folin-phenol reagent (1:1) was added and the colour was read at 600 nm, in a spectrophotometer against a reagent blank. Bovine serum albumin was used as standard protein solution.

Estimation of Total Carbohydrates

Total carbohydrate content in both control and experimental rat tissues were estimated by the method of Carrol et al., (1956). The tissues were homogenized in 10% TCA and centrifuged at 1500 x g for 15 min. To 1 ml of TCA supernatant 4 ml of anthrone reagent was added and the colour was read against a reagent blank at 600 nm in a
spectrophotometer. From the optical density, the total carbohydrate content was calculated on comparison with the standard and the values were expressed as mg carbohydrate / gm wet wt of tissue.

**Estimation of Total Lipids**

Total lipid content in control and experimental tissues were estimated by the method of Folch et al., (1957). The tissues were homogenized in a mixture of chloroform methanol (2:1) and centrifuged at 3000 x g for 15 min. A small quantity of water was added to the supernatant and the contents were vigorously shaken. The aqueous layer was separated from biphasic solution.

A small aluminum foil boat was weighed and known volume of chloroform layer was added and evaporated at 50-60°C in vacuum drying oven. The container was weighed after complete evaporation of chloroform phase. The difference between initial and final weights gave the total lipid content. The lipid content was expressed as mg of total lipid / gm dry weight of tissue.

**Estimation of Free Amino Acids (FAA)**

Total free amino acids in tissues of both control and experimental tissues were estimated by the method of Moore and Stein (1954). 2% homogenate of tissues were prepared in 10% TCA and centrifuged at 1000 x g for 15 min. To 0.5 ml of supernatant 2 ml of ninhydrin reagent was added. The contents were kept in a boiling water bath for 5 min and cooled immediately. After cooling, the colour was read at 570 nm
in a spectrophotometer using a reagent blank. The amino acid content was expressed as m moles of tyrosine/gm wet wt of tissue.

**Estimation of Total Free Fatty Acids (FFA)**

Lipids were extracted with chloroform: methanol mixture (2:1) ratio and free fatty acids: present in the tissues were determined by the procedure of Bergmeyer (1974). The samples were treated with chloroform and copper reagent and the contents were thoroughly shaken for 20 min. Finally the chloroform phase was carefully separated with the help of a separating funnel. The chloroform phase so collected was taken and 0.2 ml of sodium diethyl dithiocarbomate was added to develop the colour. The colour was read at 440 nm against a reagent blank. Stearic acid was used as standard. The values of free fatty acids were expressed as m moles stearic acid/gm wet weight of tissue.

**Estimation of Glycogen Content**

Glycogen content in both control and experimental tissues were estimated by the method of Carrol et al., (1956) using anthrone reagent. 5% (w/v) homogenate of tissues were prepared in 10% trichloroacetic acid. The homogenates were centrifuged at 1500 x g for 10 min and the supernatant was taken for the estimation of glycogen. To known aliquots of supernatant, 5 ml of 95% ethanol was added and the contents were kept in refrigerator overnight for complete precipitation of glycogen. The contents were centrifuged for 15 min at 1500 x g and the residue was dissolved in 2 ml of distilled water. To this, 4 ml of anthrone reagent was added and kept for 15 min in a boiling water bath. After cooling, the colour was measured at 620 nm in a spectrophotometer.
against blank. The amount of glycogen is expressed as mg of glycogen/gm wet weight of tissue.

**Estimation of glucose content:**

The amount of glucose in blood serum was estimated by the method of Kemp and Mayers (1954). The blood is allowed to clot, and centrifuged to get the serum. 1ml of the serum was deproteinized with 5% trichloroacetic acid containing traces of silver sulphate and centrifuged at 1500 x g for 15 min. To 1.5 ml of the clear supernatant, 4.5 ml of concentrated sulphuric acid were added and mixed by vigorous shaking. The mixture was heated in boiling water bath for exactly 6 min and subsequently cooled in running tap water. The intensity of pink colour produced was measured in spectrophotometer at 520 nm and glucose concentration was read from standard curve in terms of mg of glucose / 100 ml of blood.

**Estimation of Pyruvic Acid:**

Pyruvate was estimated by the method of Friedman and Hangen (1942).

The tissues were homogenized in 10% trichloroacetic acid (w/v) and the homogenates were centrifuged at 2000 x g for 15 minutes. From the filtrate 0.5 ml was taken and made up to 2 ml with distilled water. To this 0.5 ml of dinitrophenyl hydrazine was added and the tubes were kept for 5 min at 25°C and 3 ml of sodium hydroxide was added. After 10 min, the colour was read at 540 nm in spectrophotometer against reagent. Standard graph was prepared by taking potassium pyruvate.
The pyruvate level was expressed as μ moles of pyruvate /gm fresh tissue.

**Estimation of Lactic Acid:**

Lactic acid in the tissues was estimated by the method of Barker and Summerson (1941) as modified by Huckabee (1961). The tissues were isolated and chilled immediately in freezing mixtures. After 2 to 3 hours of chilling, the tissues were quickly weighed in cold room, immediately homogenized in cold 10% trichloroacetic acid, and centrifuged at 3000 x g for 15 min. The supernatant of 0.5 ml was taken in a graduated centrifuge tube marked at 10 ml level. To each tube 1.0 ml of 20% copper sulphate solution was added the solution was made up to the mark with distilled water. Powder calcium hydroxide (1gm) was added and the tube was shaken vigorously until the contents were dispersed uniformly. The tubes were kept for an hour with intermittent shaking and then centrifuged. The supernatant of 1.0 ml was transferred in to a clean dry test tube and 0.05 ml of 4% copper sulphate, followed by 6.0 ml of sulphuric acid (AR) were added. The contents were mixed well by lateral shaking, kept in boiling water-bath for exactly six and half minutes, and cooled. When the contents were sufficiently cooled, 0.1 ml of p-hydroxy diphenyl was added directly into the solution, and then kept at laboratory temperature for 30 minutes. Later, the contents were placed in boiling water-bath for 90 seconds, followed by cooling, and the colour was read at 560 nm against a reagent blank in spectrophotometer. The lactic acid content was calculated as suggested by Baker and Summerson (1941) and expressed as mg lactic acid / gm fresh tissue.
Assay of Protease activity:

Protease activity was estimated by the method of Moore and Stein (1954).

5% homogenates of tissues were prepared in ice cold distilled water. The homogenates were centrifuged at 1000 xg for 15 min. The supernatant were employed for enzyme assay. The mixture of 2 ml contained 100 μ moles of phosphate buffer (pH 6.8) 12 mg of heat denatured haemoglobin as substrate and 0.5 ml of the homogenate supernatant. The contents were incubated at 37°C for 15 min. The reaction was stopped by adding 2 ml of 10% TCA. The incubated samples were treated with 2.0 ml of 10% TCA prior to the addition of the enzyme source. The contents of both incubated and unincubated samples were filtered and the free amino acid content was determined in the filtrates. To 0.2 ml ninhydrin reagent was added and heated in boiling water bath for 5 min and then cooled. The volume was made up to 10 ml with distilled water. The color absorbance was measured at 570 nm against a reagent blank in a spectrophotometer. All samples are corrected for zero time controls. The proteolytic activity is expressed as μ moles of tyrosine equivalents / mg of protein/hr.

Assay of Serum Transaminases

Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT)

In the calorimetric method these enzymes, otherwise called glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) catalyze the transfer of an α-amino groups from
specific amino acids to α-ketoglutaric acid to yield glutamic acid and oxaloacetic acid or pyruvic acid. The keto acids formed were then determined calorimetrically by the method of Reitman and Frankel (1957).

Determination of Aspartate Aminotransferase (AST) Activity
(L Aspartate: 2-Oxaloglutarate Aminotransferase: EC.2.6.1.1)

0.2 ml of serum was pipetted out into a clean test tube. To this 100 µ moles of L-aspartate, 100 µ moles of phosphate buffer (pH 7.5) and 2 µ moles of α-ketoglutaric acid were added. Then reaction was carried out at 37°C for 30 min. After incubation 0.5 ml of 2, 4-dinitrophenyl hydrazine (DNPH) was added to arrest the reaction. After keeping the tubes for 20 min at room temperature, 5 ml of NaOH was added and mixed thoroughly. The colour developed was read in a spectrophotometer at 505 nm against the reagent blank. Zero time controls were also maintained. The color intensity was proportional to the transaminase activity and was expressed as µ moles of pyruvate formed/mg protein/hr.

Measurement of Alanine Aminotransferase (ALAT) (L-Alanine: 2-Oxaloglutarate Aminotransferase: EC.2.6.1.2)

0.2 ml of serum was pipetted out into a clean test tube. To this 100 m moles of L-alanine, 100 m moles of phosphate buffer (pH 7.5) and 2 m moles of α-ketoglutaric acid were added. Then reaction was carried out at 37°C for 30 min. After incubation, 0.5 ml of 2, 4-dinitrophenyl hydrazine (DNPH) was added to arrest the reaction. After keeping the tubes for 20 MIN at room temperature, 5 ml of NaOH was added and mixed thoroughly. The colour developed was read at 505 nm.
against a reagent blank in a spectrophotometer. Zero time controls were also maintained. The colour intensity was proportional to the transaminase activity and was expressed as μ- moles of pyruvate formed/mg protein/hr.

**Determination of Glycogen Phosphorylase (L - 1, 4 - glucose: Orthophosphate glycosyl transferase: EC. 2.4.1.1)**

Phosphorylase activity in control and experimental samples were assayed by the method of Cori et al., (1955) in the direction of glycogen synthesis by determining the amount of inorganic phosphate formed from glucose-1-phosphate.

2% (w/v) homogenates of tissues were prepared in the medium containing 0.1 M sodium fluoride (NaF) and 0.03ml ethylene diamine tetra acetic acid (EDTA) (pH 6.0) as recommended by Guillory and Mommaerts (1962) to avoid interconversion of phosphorylase.

The enzyme was diluted (1:3) with cysteine (0:03M) β-glycerophosphate (0.015 M) buffer (pH 6.0) 0.1 ml of the diluted enzyme was added to 0.2 ml of 1% glycogen. For each sample, two tubes were maintained. The reaction was started by the addition of 0.2 mg of glucose-1-phosphate (0.06 M) to one of the tubes and 0.2 ml of mixture of 0.06 M glucose-1-phosphate and 0.004 M adenosine-5-monophosphate (AMP) to the other for estimating the active phosphorylase (a) and total phosphorylase (ab), respectively.
After 15 min incubation for total phosphorylase and 30 min for active phosphorylase (at 37°C) the reaction was stopped with 2.0 ml of 10% trichloroacetic acid (TCA).

The inorganic phosphate formed was estimated by the method of Fiske and Subba Row (1925). The above contents were centrifuged. To the filtrate 1.0 ml of ammonium molybdate solution and 0.4 ml of aminonaphthasulfonic acid (ANSA) were added. The colour was read at 660 nm against a blank prepared with 2.0 ml of trichloroacetic acid 1.0 ml of ammonium solution and 0.4 ml of ANSA.

Phosphorylase activity was expressed as m moles of inorganic phosphate formed mg/protein/hr.

Assay of Aldolase Activity

(Fructose-1,6-diphosphate D-glyceraldehyde 3-phosphatellyase:
EC.4.1.2.6)

The aldolase activity in control and experimental rat tissues were estimated by the method of Bruns and Bergmeyer (1963) where in the triose phosphates formed were estimated by using 2, 4-dinitrophenylhydrozine.

2% (w/v) homogenates of tissues were prepared in cold distilled water and centrifuged at 1000 x g for 5 min. The reaction mixture of 3 ml contained 1.75 ml of collidine hydrazine buffer (pH 7.4) 0.25 ml of fructose-1, 6-diphosphate (0.02 M, pH 7.4) 0.5 ml of distilled water and 0.5 ml of homogenate supernatant. The reaction mixture was incubated
for 15 min at 37°C and the reaction was arrested by adding 3.0 ml of 10% trichloroacetic acid. Then the contents were filtered and 1.0 ml of 0.75 N NaOH was added to 1.0 ml of the filtrate and allowed to stand for 10 min at room temperature. Then 1.0 ml of 2, 4-dinitrophenyl hydrazine was added and the contents were incubated at 37°C for 10 min. After incubation, 8 ml of 0.75 N NaOH was added. The reddish brown color developed was read at 540 nm in a spectrophotometer against a zero time control.

The aldolase activity was calculated according to Bruns (1954) and the values were expressed as m moles of fructose 1, 6-diphosphate cleaved / mg protein/hr.

Assay to Lactate dehydrogenase (LDH) Activity (L-Lactate: NAD-Oxidoreductase: EC.1.1.1.27)

Lactate dehydrogenase activity of control and experimental tissue samples were assayed by the method of Nachals et al., (1960) and as modified by Prameelamma and Swami (1975).

Five percent (5%) homogenates of tissue samples were prepared in 0.25 M ice cold sucrose solution and centrifuged at 2500 x g for 15 min. The supernatant was used as enzyme source. The reaction mixture in a final volume of 2 ml contained 40 μ moles of sodium lactate, 100 m moles of phosphate buffer (pH 7.4) and 4 m moles of 1-p-idophenyl-3-p-nitrophenyl tetrazolium chloride (INT).

The reaction mixture was incubated for 30 min. The reaction was stopped by the addition of 5 ml glacial acetic acid and the resultant
formazan was extracted into 5 ml of toluene at 5°C overnight. The intensity of the colour was read at 495 nm against toluene blank in a spectrophotometer. The protein content in the enzyme source was determined by the method of Lowry et al., (1951) using bovine serum albumin as the standard. The enzyme activity was expressed as m moles of formazan/mg protein/hr.

**Measurement of Malate dehydrogenase (MDH) Activity**

(L-Malate: NAD-oxidoreductase: EC.1.1.1.27)

The activity of malate dehydrogenase was measured by the method of Nachlas et al., (1960) and as modified by Prameelamma and Swami (1975).

Five percent (5%) homogenates of control and experimental tissues were prepared in 0.25 M ice cold sucrose solution and centrifuged for 15 min at 2000 x g the supernatant fraction was used for the enzyme assay. The reaction mixture in a total volumes of 2 ml consisted of 40 µ moles sodium malate, 100µ moles phosphate buffer (pH 7.4) 0.1µ moles of NAD and 4µ moles of 1-p-iodophenyl 3-p-nitrophenyl tetrazolium chloride (INT) and reaction was initiated by the addition of 0.2 ml of supernatant. The reaction mixture was incubated at 37°C for 30 min and the reaction was stopped by the addition of 5 ml of glacial acetic acid. The formazan formed was extracted into 5 ml of toluene at 5°C overnight. The optical density was measured at 495 nm in a spectrophotometer against toluene blank. The protein content in the enzyme source was estimated by the method of Lowry et al., (1951) using bovine serum albumin as the standard. The enzyme activity was expressed as m moles of formazan/mg protein/hr.
Assay of Succinate dehydrogenase (SDH) Activity  
(Succinate acceptor: Oxidoreductase: E.C.1.3.99.1)

The activity levels of SDH in control and experimental tissue samples were measured by the method of Nachlas et al., (1960) and as modified by Prameelamma and Swami (1975).

Five percent (5%) homogenates of the tissues were prepared in ice cold sucrose solution and centrifuged for 15 min at 2000 x g. The supernatant fraction was separated and used for the enzyme assay. The reaction mixture in a final volume of 2 ml contained 40μ moles of sodium succinate, 100 μ moles of phosphate buffer (pH 7.4) and 4μ moles of 1-p-iodophenyl-3-p-nitrophenyl tetrazolium chloride (INT). The reaction was initiated by adding 0.2 ml of supernatant. The reaction mixture was incubated at 37°C for 30 min and the reaction was stopped by the addition of 5 ml of glacial acetic acid. The formazan formed was extracted into 5 ml of toluene at 5°C in overnight. The intensity of the colour developed was read at 495 nm against toluene blank in a spectrophotometer. The protein content in the enzyme source was determined by the method of Lowry et al., (1951) using bovine serum albumin as the standard. The enzyme activity was expressed as p moles of formazan formed/mg protein/hr.

Estimation of lipase activities:

Lipase activity was estimated by the method of Bier (1957) 5% tissue homogenates were prepared in ice-cold distilled water and centrifuged at 1000 x g for 10 min. To 1 ml of the enzyme source 2 ml of M/15 phosphate buffer (pH 7.0) was added followed by 3 ml of
distilled water. Then the tubes were brought to $25^0C$ in a water bath and at a precise time interval of 1 minute. 0.5 ml of paranitrophenyl acetate (PNNA) of pH 7.0 was added and the yellow colour developed was read at 435 nm against a reagent blank in a spectrophotometer. The lipase specific activity was expressed as $\mu$ moles of paranitrophenol formed / mg protein / hour.

**Phospholipase activity:**

The method adopted for the enzyme assay is that Magee and Thomspson (1960). 5% homogenates were prepared in triethanol amine hydrochloride buffer (pH 7.5) and the homogenates were centrifuged. The following are used in the incubation mixture: 0.5 ml of supernatant, 1 mg of lecithin, 0.05 ml of calcium chloride (5 $\mu$ m). The samples are incubated for 30 min at 37 $^0$C in a metabolic shaking incubator with constant shaking. The activity are stopped by heat killing. Zero time controls are maintained side by side. Fattyacid liberated is determined as mentioned above. Values are expressed as $\mu$ moles of stearic acid / mg protein / hr.

**Assay of ATPases (ATP Phosphorylase: EC.3.6.1.3)**

Total ATPase activity was measured by the method of Fritz and Hamrich (1966) and as modified by Desaiah and Ho (1979).

**Total ATPases**

Total ATPase activity was determined by measuring the inorganic phosphate liberated from hydrolysis of ATP. The reaction mixture contained 135 m moles imidazole-HCl buffer (pH 7.5), 5m moles
MgCl₂, 0.05 m moles CaCl₂ m moles KCl, 5 m moles ATP and 30-50 mg of enzyme protein. The mixture was incubated at 37°C for 30 min and the reaction was stopped by the addition of 0.1 ml of 50% TCA. The inorganic phosphate liberated was estimated by the method of Fiske and Subba Row (1925). Enzyme activity was expressed as m moles of inorganic phosphate liberated/ mg protein / hr. Protein content was estimated by the method of Lowry et al., (1951) using bovine serum albumin (BSA) as standard.

**Determination of Cytochrome-C-Oxidase (Cyt-c-oxidase) Activity (Ferrocytochrome-c-oxygen oxidoreductase. EC: 1.9.3.1)**

In control and experimental tissues Cytochrome-C-Oxidase activity was measured by the procedure of Oda et al., (1958). 5% (w/v) homogenate of control and experimental tissues were prepared in 0.1 M cold phosphate buffer of pH 7.6 and centrifuged at 1000 x g for 15 min. The supernatant were used for the enzyme assay. The reaction mixture of 1.0 ml contained 0.2 ml of 0.2 M P-phenyline diamine, 0.2 ml of 0.2% neotetrazolium chloride, 0.2 ml of 10⁻⁴ cytochrome-c and 0.4 ml of supernatant as enzyme source. The contents were incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.4 ml of 1 N sulphuric acid. The yellowish brown precipitate is the oxidized product of P-phenyline diamine, which was extracted in 5.0 ml of 1:1 ether: acetone mixture. Its colour density was measured in a spectrophotometer at 520 nm against a blank. The ether: acetone (1:1) mixture served as blank. The enzyme activity was expressed in µg of formazan formed/mg protein/hr.
Measurement of ATP levels:

ATP levels in the culture medium after subsequent incubations was determined by using Bioluminescent somatic cell assay Kit (Sigma, St. Lou’s). To the vials containing 0.1 ml of ATP assay mix 0.1 ml of somatic cell ATP releasing reagent, 0.05 ml of distilled water plus 0.02 ml of cell samples were mixed and the light emitted (LSM) was measured immediately with a illuminometer. An internal standard (4 x 10^4 nm) in 0.05 ml was used to record the light emitted by the sample and standard (L_{SAM} + is). ATP in the medium was calculated by the formulae as given below:

\[
\frac{ATP_{is} \times L_{SAM}}{L_{(SAM + is)} - L_{SAM}}
\]

The ATP levels were expressed as nm/ml of Culture Medium.

Statistical Analysis

For each parameter, the mean of individual observations (for both control and experimental groups) were taken into consideration. Statistical significance of the data was analysed through two way ANOVA (Analysis of variance); Student New man Keuls test and regression analysis (Zar, 1984). P value < 0.001 was considered significant.