Material & Methods
MATERIALS AND METHODS

3.1 ANIMALS

Female Sprague Dawley rats weighing 150-200 g were obtained from Animal House of Panjab University. Animals were housed in polypinn cages on rice husk, and maintained in an ambient temperature, humidity and day/night photoperiod. They were maintained under good hygienic conditions and received pelleted rodent diet (Ashirwaad Industries, Tirpari; Punjab) and water ad libitum. All of the animal procedures as reported here strictly followed the protocols as laid down by the Ethics Committee on the Use of Experimental Animals Panjab University and in general according to the NIH guidelines (Rule No 2: revised in 1985).

3.2 DOSE STANDARDIZATION

3.2.1 Aluminium Neurotoxicity

Before starting with the neuroprotective studies using natural and synthetic antioxidants, the animal groups were started for establishing the appropriate aluminium dose. Three different treatment groups were run to which aluminium chloride was administered orally at three different doses of 40, 100 and 150 mg/kg of body wt. daily for a period of 8 weeks. The body weight of the animals was recorded at different time intervals (1st, 10th, 20th, 30th and 40th days) and the results are presented in the figure below.

![Fig 3.2.1: Effect of three different doses i.e. 40mg/kg, 100mg/kg and 150mg/kg of body wt of aluminium on body weight taken at different time intervals. Values are mean ± SD determinations 'p<0.05, 'p<0.01, 'p<0.001: treatment vs control'. Comparison of aluminium treated with respect to control.](image-url)
Various behavioural, biochemical and neurochemical studies were done in three different regions of brain i.e. cerebral cortex, mid brain and cerebellum.

We observed 60% of mortality rate in animals receiving dose of 150mg/kg body wt. No evidence for mortality was however seen in animals receiving 40 & 100 mg/kg of body wt dose. Though some neurological defects were evident in animals receiving 100mg/kg of dose which includes forward head tilt, hemiplegic gait, loss of appetite, splaying of the extremities and paralysis no such symptoms were observed in 40 mg/kg group animals. Further, the behavioural alterations like short-term memory loss and motor dysfunction were more prominent in

With these observations 100mg/kg of body wt dose was selected for further studies.
Role of Antioxidants in Al Induced Neurotoxicity

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animals receiving dose of 100mg/kg of body wt as shown in Fig 3.2 Significant alterations in oxidative stress parameters and biogenic amines (de the help of HPLC) were observed in 100mg/kg of body wt group in all ti regions of brain i.e. cerebral cortex, mid brain and cerebellum.

### 3.2.2 Dose Standardization of Curcumin

Three different treatment groups were run to which curcumin administered i.p. at three different doses of 50, 100 and 250 mg/kg of body wt. on alternate days for a period of 8 weeks along with aluminium at the dose of 100mg/kg of body wt daily for a period of 8 weeks. Control group was also run in which animals were given vehicle (oil) for curcumin. Various behavioural, biochemical and neurochemical studies were done in three different regions of brain i.e. cerebral cortex, mid brain and cerebellum (Fig 3.2.3-3.2.4).

![Effect of three different doses of curcumin on locomotor activity (B) short-term memory of aluminium rats. Values are mean ± SD of 6-7 determinations. *p<0.05, †p<0.001; treatment vs control group. Comparison of aluminium with respect to control.](image-url)
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Cerebral cortex
Mid brain
Cerebellum

□ Control ■ Aluminum * Al+ Cu (50mg/kg)
* Al+ Cu (250mg/kg) * Al+Cu (500mg/kg)

pmol H2O2 decomposed/min/mg protein

pmol of GSH/g tissue

48
Fig 3.2.4: Effect of three different doses of curcumin on (A) GSH content (B) GSH content (C) catalase activity (D) lipid peroxidation of aluminium treated rats. Value mean ± SD of 6-7 determinations "p<0.05, "p<0.01, "p<0.001; treatment vs control.

With these observations 50mg/kg of body wt dose was selected for further studies.

3.3 DEVELOPMENT OF THE EXPERIMENTAL MODEL
ALUMINIUM NEUROTOXICITY BY USING BIOCHEMICAL MARKERS

Biochemical markers are used to ascertain the degree of neuronal damage following biochemical markers were studies to assess Al neurotoxicity.

3.3.1 Acetylcholinesterase Activity

Estimation of acetylcholinesterase activity in serum was done by the method of Ellman et al.

Preparation of Serum: To prepare the serum, the animals were anaesthetized using mild ether anesthesia and the blood samples were drawn from animals of treated and normal control groups by puncturing the ocular vein, (retro orbital plexus), using fine sterilized heparinized capillaries. Thereafter, serum was separated by centrifugation at 3000×g for 20 min. and stored at −20°C until analysis.

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Materials & Methods

Cerebral cortex
Mid brain

Control
Aluminum
Al+Cur (250mg/kg)
Al+Cur (500mg/kg)

Fig 3.2.4: Effect of three different doses of curcumin on (A) GSH content (B) GSH content (C) catalase activity (D) lipid peroxidation of aluminium treated rats. Value mean ± SD of 6-7 determinations "p<0.05, "p<0.01, "p<0.001; treatment vs control.

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Acetylcholinesterase Activity: In this estimation, the substrate used was acetyl thiocholine, an ester of thiocholine and acetic acid. The mercaptan formed as a result of hydrolysis of ester, reacts with an oxidizing agent 5,5-dithio-bis2-nitrobenzoate that has maximum absorbance at 412 nm. To 3.0 ml phosphates buffer, 0.1 ml of acetyl thiocholine iodide was added followed by 0.10 ml of sample and change in absorbance was measured immediately at 412 nm for two minutes. Enzyme activity was calculated using molar extinction coefficient of DTNB at 412 nm, which is 13.6 nm$^{-1}$cm$^{	ext{-1}}$. The results were expressed as mmoles acetylcholine hydrolyzed /min./liter serum.

3.3.2 Cytochrome Oxidase Assay
Cytochrome oxidase was assayed in the platelets by the method of Sottocasa et al (1967) and the activity of cytochrome oxidase was measured spectrophotometrically by following the oxidation of reduced cytochrome c at 550 nm.

\[
\text{Cytochrome C (Fe}^{2+}) + \text{O}_2 \rightarrow \text{Cytochrome C (Fe}^{3+}) + \text{O}_2
\]

Preparation of Platelets: For platelet preparation, 1.0 ml of blood was drawn by puncturing the ocular vein, (retro-orbital plexus), using fine sterilized capillaries and collected in tubes containing ACD buffer (93 mM trisodium citrate, 213 mM citric acid and 11 mM dextrose, pH 5.0). Blood was washed 3 times with 2 ml ACD and centrifuged at 2000 × g each time for 10 min. The supernatant, that is platelet-rich plasma (PRP) was collected, and re-centrifuged at 12000 ×g for 20 min. The pellet containing platelets were then suspended in 75 mM sodium phosphate buffer (pH 7.5).

Cytochrome Oxidase Assay: The assay mixture (1.0 ml) contained 0.010 ml sample, 0.930 ml buffer and 0.060 ml reduced cytochrome c. The reaction was started by the addition of the requisite amount of platelets. The reaction was then followed spectrophotometrically for three min at 550 nm. Enzyme activity was calculated on the basis of molar extinction co-efficient of cytochrome c (29.5 M$^{-1}$cm$^{-1}$) and the results were expressed as mmol of cytochrome c oxidized/min./mg protein.

3.3.3 Glucose 6-Phosphate Dehydrogenase
Glucose-6-phosphate dehydrogenase (G-6-PD) was assayed in the hemolyzate according to the method of Kornberg and Horecker (1955). The increase in...
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Absorbance at 340 nm caused by the reduction of NADP⁺ in the presence of glucose phosphate catalyzed by G-6-PD was measured spectrophotometrically.

Preparation of Hemolyzate: Blood (0.2 ml) was drawn from ocular (retro-orbital plexus) and was washed 3 times with 2 ml of normal saline. It was centrifuged at 3000 rpm for 10 min. The washed cells were then suspended in 0.1 M saturated digitonin solution (= 200 mg/L) and refrigerated for 15 min to lyse red blood cells and was again centrifuged for 10 min, the supernatant thus obtained was regarded as hemolyzate.

Glucose 6-Phosphate Dehydrogenase Activity: Reaction mixture (3 ml) contained 0.3 mM of NADP and requisite amount of hemolyzate in 50 mM triethanolamine buffer (pH 7.6) the reaction was started by the addition of 0.5 mM G-6-P. The change in absorbance at 340 nm was recorded for 2 min. The enzyme activity was calculated on the basis of the molar extinction coefficient of NADP (6.23 M⁻¹ cm⁻¹) and the results were expressed in terms of EU/g Hb, where 1 EU is 1 umole of product formed/min.

3.4 EXPERIMENTAL DESIGN

3.4.1 Natural Antioxidant (Fig 3.4.1)

![Figure 3.4.1: Treatment design followed to evaluate the neuroprotective role of curcumin in aluminium induced neurotoxicity.](image-url)
Aluminium chloride was dissolved in normal drinking water and was administered through oral gavages, whereas curcumin was given i.p. in oil. All the treatments were carried out for a total period of two months.

3.4.2 Synthetic Antioxidant (Fig. 3.4.2)

Rats were segregated into four treatment groups with 10 animals each.

![Figure 3.4.2: Treatment design followed to evaluate the neuroprotective role of U74500A against aluminium induced neurotoxicity.](image)

3.4.3 Body Weight Changes

A weekly record of body weights of animals belonging to various treatment groups and their respective control animals was kept throughout the study. The animals were daily observed for their general behaviour and physical activity and an account of their daily intake of water and diet was also maintained throughout the study period.

3.5 ATOMIC ABSORPTION SPECTROSCOPY (AAS)

The spectroscopy was used for the estimation of concentration of aluminium in different regions of rat brain samples. Estimation of aluminium concentrations in the brain samples of control and different treatment groups were carried using atomic absorption spectrophotometer.

![Figure 3.5.1: Single Beam Atomic Absorption Spectrometer](image)
Following procedure was adopted for digestion of brain samples for the estimation of elements. To the known weights of cerebral cortex, mid brain and cerebellum, 2.5 ml of perchloric acid/ nitric acid mixture (1:4 in volume) was added. Digestion in sand bath was continued for 44 h till a white ash or residue was formed. The residue so obtained was dissolved in 2.5 ml of 10 mM nitric acid. The sample, in liquid form, was placed in the sample holder of atomic absorption spectrophotometer. Working of the atomic absorption spectrophotometer as shown in Fig 3.5.1 included the suckling of the samples from the nebulizer. After this, the sample in molecular form is dissociated into atomic form and this atomic form of the sample was present in the flame.

The flame constituted a mixture of compressed air and acetylene. The light was passed through the sample. Atoms absorb this light giving the absorbance, which depends upon the concentration of the metal ions in the sample. The total concentration in sample was calculated in $\mu g/gm$ tissue or directly expressed in ppm.

### 3.6 HISTOPATHOLOGICAL STUDIES

Peritoneum was opened and perfusion with chilled normal saline followed by formalin was carried out. Small sections of cerebrum and cerebellum from each of the normal control and treated animals were taken, washed with ice-cold 0.9% NaCl and fixed in the buffered formalin for about 24 to 48 h. After the fixation, tissues were processed carefully for embedding in paraffin wax (58-60°C) after subjecting them to different ascending grades of alcohols according to the standard technique (Pearse, 1968).

Paraffin sections with 5-7 microns thickness were cut, dewaxed in xylene, downgraded (hydrated) in decreasing grades of alcohol and brought to water. They were stained haematoxylin for approx. 20 sec, rinsed in ammonia water till the appearance of blue colour, again washed with water, treated with acid water (if over stained), upgraded (dehydrated) in alcohol till 70%, stained with 1% alcoholic eosin for 30 sec, differentiated with 90 % alcohol, washed with absolute alcohol and were cleared in xylene and finally mounted in DPX. Gallocyanin stain was used to stain nissle substance and solochrome was used to stain myelin (Humanson, 1961).
3.7 NEUROBEHAVIORAL STUDIES

3.7.1 Passive Avoidance Test

This test was used to assess short-term memory. In this experiment, the animals were exposed to light stimulus; avoiding this, the animals entered the maze fitted with electric grid, centrally located in a shock free zone. The apparatus for this test consisted of two open chambers and one close chamber. The three chambers were interconnected. The animals were placed in the open chamber and light from the table lamp (100V) was made to fall on the rat. The time taken by the rat to enter the closed chamber from the open lighted chamber was noted down and was termed as acquisition trial time. The shutter was then closed and the electric shock of 60 V was given for 5-7 sec. Then the animal was taken out through the second open chamber. After 24 h, the procedure was repeated again and the animals were again put in the lighted chamber and the time was noted down. The time taken by the animal to enter the closed chamber on the second day was termed as retention trial time. For retention trial time, the animal was allowed to stay in the open lighted chamber maximum for 300 sec.

3.7.2 Active Avoidance Test

Cognitive behaviour was assessed by the number of times, the animal escapes, in the 10 test trial series. The apparatus for this test consisted of two chambers separated by a partition. One chamber was lit. The animals were put in the lit compartment. After 10 sec, the buzzer was set on and after another 10 sec, an electric shock at 60 V was given. If the animal jumped to the other compartment as soon as the buzzer was set on, it means that the animal has avoided the test. However, if the animal jumps to the other compartment after the electric shock or tries to hide by some other means, this is termed as escapism. A total of 10 trials were given to every animal in a single day. To qualify, the animal has to jump at least 8 times out of 10 to avoid the test.

3.7.3 Elevated Plus Maze

The elevated plus maze was used to evaluate spatial long-term memory by the procedure of Reddy and Kulkarni (1998). Briefly the apparatus consisted of two open arms and two closed arms. The arms extended from a central platform, and the maze is elevated to a height of 50 cm from the floor. On the first day, each animal was
placed at the end of an open arm. Transfer latency (TL), the time taken by the rat to move into one of the enclosed arm was recorded on the first day. If the animal did not enter an enclosed arm within 90 sec, it was gently pushed into one enclosed arm and the TL latency was assigned as 90 sec. The rat was allowed to explore the maze for 20 s and then returned to the home cage. The rat was again placed on the maze on next day (24 h later) and TL was recorded (Itoh et al, 1994). Percent retention was calculated by the formula -
Transfer Latency (Day1) - Transfer Latency (Day2)/Transfer Latency (Day2) x 100

3.7.4 Morris Water Maze

The spatial memory was evaluated by the Morris water maze as described by Morris (1984). The experimental apparatus consisted of a circular water tank (120 cm in diameter, 45 cm high) filled with water whose temperature was maintained at 21-23°C. An invisible platform (15 cm in diameter, 35 cm high) was placed 1.5 cm below the surface of water. The pool was located in a test room and many clues external to the maze were visible from the pool (e.g., pictures, lamps, etc.), which could be used by the rats for spatial orientation. The position of the clues was kept constant throughout the task.

3.7.5 Muscle Activity

Muscle performance was evaluated using rota-rod (Techno) test. All the animals were given two initial training trials of 300 sec, approximately 10 min apart, to maintain posture on the rota-rod (3 cm in diameter and rotating at a constant 20 revolutions per minute). After the initial training trials, a baseline trial of 120 sec was conducted. The time each animal remained on the rota-rod was recorded. The animals not falling off the rota-rod were given a maximum score of 120 sec. (Dunham and Miya, 1957).

3.7.6 Locomotor Activity

Total locomotor activity (ambulation and rearing) was measured using a computerized Actophotometer (IMCORP, India). An array of 16 infrared emitter/detector pairs measured animal activity along a single axis of motion. The digital data was displayed on the front panel meter as ambulatory movements. Rats were allowed to acclimatize to the observation chamber for a period of 2 min. The
activity was monitored continuously for a total duration of 5 min. Total locomotor activity was expressed as mean of sum of total ambulatory photo beam counts and total rearing photo beam counts per 5 mins per animal (Bishnoi et al, 2006).

3.8 ANTIOXIDANT ENZYME SYSTEM

3.8.1 Collection of Brain Sample

At the end of behaviour studies, the animals were sacrificed by decapitation after anesthetization by diethyl ether. Brains were immediately removed, placed in ice-cold isotonic saline and dissected into three regions (viz cerebral cortex, mid brain and cerebellum). A small portion from mid brain and cerebellum were fixed in formalin (10%) for histological examination at the light microscopic level and a small portion used for neurotransmitters and atomic absorptions spectroscopic studies. The remaining samples from both the regions were used to prepare the tissue homogenates. A 10% (w/v) homogenate was prepared in ice-cold 10 mM PBS (phosphate-buffered saline, 0.15 M NaCl), pH 7.4. The homogenates were centrifuged at 2000 × g for 10 min at 4°C to remove the cell debris and nuclear pellet. The supernatants so obtained were again homogenized at 10,000 × g for 30 min. at 4°C to obtain post-mitochondrial supernatant (PMS) and crude mitochondrial pellet. In the present study, PMS were used to carry out the following estimations.

3.8.2 Protein Estimation

Protein contents in various samples were estimated by the method of Lowry et al (1951). The method is based on colour reactions of amino acids namely tryptophan and tyrosine with the folin phenol reagent. To 10μl of sample from each treatment group, 3ml of 50:1 mixture of 2% sodium carbonate in 0.1N NaOH and 0.5% CuSO4 /1% Na-K tartrate in distilled water was added. The tubes were incubated for 10min at room temperature. Then 300μl of 1N Folin's phenol reagent was added to each tube, mixed and again incubated for 30min at room temperature. The optical density was then measured at 620nm on spectrophotometer (UV-160A, Shimadzu). Bovine serum albumin (BSA) was used as standard (10-100μg).
3.8.3 Lipid Peroxidation

Lipid peroxide formation was assayed by method of Wills (1966). Since malondialdehyde is a degradation product of peroxidized lipids, the development of pink colour with the absorption characteristics (Absorption maximum at 532nm) as a TBA-MDA chromophore has been taken as an index of lipid peroxidation. 0.5 ml of tissue homogenates (10% w/v) from both the regions was diluted to 1.0 ml with ice cold TCA and after thorough mixing, the reaction mixtures were centrifuged at 800 x g for 10 min. To 0.5 ml of supernatants, 0.5 ml of TBA was added and the color was developed by boiling at 100°C for 10 min. Samples were cooled and the absorbance was read at 532 nm. The amount of MDA formed was calculated on the basis of molar extinction coefficient of MDA-TBA chromophore \(1.56 \times 10^5 \text{ M}^{-1} \text{cm}^{-1}\) and the results were expressed as n moles of MDA/min./mg protein.

3.8.4 Catalase

Catalase was estimated by the method of Luck (1971). The enzyme catalyses the decomposition of hydrogen peroxide \(\text{H}_2\text{O}_2\), which is measured by decrease in absorbance at 240nm.

\[ 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]

The reaction mixture consisted of 50mM phosphate buffer (pH 7) -2.5ml and 0.75 M \(\text{H}_2\text{O}_2\) - 50μl. The reaction was started by adding 100μl of enzyme (PMF) to the above reaction mixture and the rate of change in absorbance was recorded at 240nm for 2min. The enzyme activity was expressed as the rate of decrease in absorbance using the molar extinction coefficient of \(\text{H}_2\text{O}_2\) \(0.0394 \text{ mM}^{-1}\text{cm}^{-1}\). Results were expressed as μmoles of \(\text{H}_2\text{O}_2\) decomposed/min/mg protein.

3.8.5 Superoxide Dismutase

Activity of superoxide dismutase (SOD) was estimated by the method of Kono (1978). The reaction is designed to observe the inhibition of the rate of oxidation of nitroblue tetrazolium (NBT), which was recorded using hydroxylamine hydrochloride as the electron donor. The reaction mixture consisted of: Solution A (3.8mg EDTA and 0.53 g \(\text{Na}_2\text{CO}_3\) in 100ml distilled water, pH 10) -1.3ml, NBT (8 mg/100ml of Sol A) - 0.5ml, 0.6% Triton X in Solution A - 0.1ml, 2mM
hydroxylamine hydrochloride (pH 6) - 0.1ml. Reaction was initiated by the addition of 0.1ml of hydroxylamine hydrochloride to the reaction mixture and the rate of NBT reduction in the absence of the enzyme source was recorded for about one min. Following this, small aliquots of supernatants were added to the test cuvette as well as reference cuvette, which did not contain hydroxylamine hydrochloride. Finally, percentage inhibition in the rate of NBT reduction was noted. One unit of enzyme was expressed as inverse of the amount of protein (mg) required inhibiting the reduction rate of NBT by 50%.

3.8.6 Reactive Oxygen Species

Determination of reactive oxygen species was performed by the modified method of Driver et al (2000). This method is based on the principle that the acetate group of dichlorofluorescein diacetate (DCFH-DA) will be cleaved by esterases activated by various free radicals to form a fluorescent product, dichlorofluorescein (DCF) whose intensity can be measured at 530 nm. Homogenates from all the three regions of brain were prepared in ice-cold Locke’s buffer. The homogenates were allowed to warm at 21°C for 5 min. The reaction mixture containing DCFH-DA and 5 mg tissue/ml was incubated for 15 min at room temperature (21°C). After another 15 min of incubation, the conversion of DCFH to the fluorescent product dichlorofluorescein (DCF) was measured using fluorescence spectrophotometer with excitation at 485 nm and emission at 530nm. Background fluorescence (conversion of DCFH to DCF in the absence of homogenate) was corrected by inclusion of parallel blanks. The relative difference in fluorescence intensity was taken as the measure of amount of ROS in different treatment groups.

3.8.7 Reduced Glutathione

Reduced glutathione content was estimated according to the method of Ellman (1959). In this method, 5,5-dithiobis2-nitrobenzoic acid (DTNB) is reduced by –SH groups to form 2-nitro-5-mercaptobenzoic acid per mole of SH. The nitromercaptobenzoic acid anion released has an intense yellow color and can be used to measure –SH groups at 412 nm. 0.1 ml of TCA was added to 0.5 ml (cerebral cortex and mid brain) and 0.250 ml (cerebellum) homogenates. After mixing the contents, the precipitated proteins were separated by centrifugation at 2000 xg for 15 minutes. 0.5 ml of supernatant so obtained was diluted in a test tube to 1.0 ml with
sodium phosphate buffer. To this was added 2.0 ml of freshly prepared DTNB. Optical density of the yellow colored complex formed by the reaction of GSH and DTNB (Ellman’s reaction) was measured on a double beam spectrophotometer at 412 nm against a reference, which contained 0.5ml of TCA instead of the sample. For each set of assays, a standard curve for GSH was prepared. The GSH contents were expressed in terms of \( \mu \text{mol of GSH/g tissue} \).

3.8.8 Total Glutathione Content

The total glutathione content was measured by the method of Zahler and Cleland (1968). The method is based on the reduction of glutathione with dithioerythritol and determination of the resulting monothiols with DTNB in the presence of arsenite. The disulfide in 0.2 ml sample mixed with 0.1 ml dithioerythritol and the reduction is allowed to proceed for 20 mins or for the time for the disulfide to get reduced. After reduction, 0.2 ml of tris and 1.5 ml of sodium arsenite and water to give 2.9 ml is added and solution is mixed and allowed to stand for 2 min. 0.1 ml of DTNB prepared in sodium acetate is then added and the absorbance is recorded for 3 min. The absorbance resulting from the monothiols is determined by extrapolating the linear plot of the curve at the time of addition of DTNB and subtracting of the blank value from a sample containing no disulfide.

3.8.9 Oxidized Glutathione

Subtracting the value of glutathione reduced from total glutathione levels quantitated oxidized glutathione.

3.8.10 Glutathione Reductase

Glutathione reductase activity was measured according to the method described by Carlberg and Mannervik (1985). GR is a flavoprotein that catalyzes the NADPH-dependent reduction of glutathione disulphide (GSSG) to reduced glutathione (GSH).

\[
\text{NADPH} + H^+ + \text{GSSG} \rightarrow \text{NADP}^+ + 2\text{GSH}
\]

The oxidation of NADPH is followed spectrophotometrically at 340nm. To 3ml cuvette, 2.6ml of phosphate buffer, 0.15ml of NADPH and 0.15ml of GSSG were added. The reaction was initiated by the addition of 0.2 ml of brain sample to the cuvette and the decrease in absorbance at 340 nm was followed for 2 min. A unit of
GR activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH/min/mg protein using 6.22x10⁻³ as the molar absorption of NADPH.

3.8.11 Glutathione-s-Transferase

Glutathione-s-transferase was determined by the method of Habig et al. (1974). It catalyzes the formation of conjugate of glutathione and 1-chloro-2,4-dinitrobenzene, which was read at 340nm. 0.1ml of CDNB as substrate was added to 2.75ml of phosphate buffer. To which was added, 0.1ml of GSH and 0.1 ml of homogenates were added to make up the volume of approx 3ml. Glutathione conjugate of CDNB absorbs maximum at 340nm and the enzyme kinetics was observed for up to five minutes with an interval of one minute each. The blanks in all the cases constituted non-enzymatic reaction of GSH with the respective substrate. The activity was expressed as μmol conjugate formed/min/mg protein using the extinction coefficient of 9.6 mM cm⁻¹.

3.8.12 Glutathione Peroxidase

Total glutathione peroxidase activity was estimated in different regions of brain by the method of Flohe and Gunzler (1984) using H₂O₂ as substrate. The reaction was started by adding 20μl of PMF to 880μl of the above mixture (except H₂O₂) followed by incubation for 5min at room temperature. Later 100μl of H₂O₂ was added. Absorbance was recorded at 340nm (UV-160A, Shimadzu) for 3min. Enzyme activity was calculated from the slope of these lines and expressed as μmoles NADPH oxidized/min/mg protein.

3.8.13 Nitric Oxide Synthase (NOS)

Nitric oxide synthase activity was determined in terms of nitric oxide (NO) production. The estimation was carried out by the method of Radassi et al. (1994). NOS convert L-arginine to L-citrulline and NO, which then reacts with oxygen to yield nitrite. Nitrite, thus formed reacts with the Griess reagent to form a purple azo dye, which can be read at 540nm. To 0.100 ml of tissue homogenates, 0.100 ml of Griess reagent was added into the wells of ELISA plate. The ELISA plate was then incubated in dark at 37°C for 30 min. The pink colour so obtained was read at 540nm.
on ELISA plate reader. The amount of nitrite produced was determined by a standard curve prepared by using sodium nitrite. Results were expressed as \( \text{nM of nitrite/g tissue} \).

### 3.8.14 L-Citrulline

Estimation of L-citrulline was carried out by the method of Boyde and Rahmatullah (1980). Determination of L-citrulline in proteins and peptides utilizes the reaction of ureido groups with doacetylmonoxime to give pink colour after acid hydrolysis of proteins. To 0.1 ml of homogenates, 0.05 ml of ZnSO\(_4\) solution was added and mixed well to precipitate proteins. The tubes were then subjected to centrifugation at 10,000 \( \times \) g for 10 min. 0.1 ml of supernatants were taken and 0.4 ml of 0.1N HCl was added followed by an addition of 1.5ml of reagent 3 (2:1 ratio of 85% \( \text{H}_2\text{PO}_4\) solution and 0.5% (W/V) diacetylmonooxime containing 0.01% (w/v) thiosemicarbazide). After thorough mixing, the tubes were put in boiling water bath for 10 min. The tubes were then cooled at room temperature and the absorbance was measured against a reagent blank at 530 nm. The amount of citrulline produced was determined by a standard curve prepared by using citrulline. Results were expressed as \( \mu \text{M of citrulline/g tissue} \).

### 3.9 GLYCOLYTIC ENZYMES

#### 3.9.1 Succinic Dehydrogenase (SDH)

The SDH enzyme activity was measured by following the protocol of Kun and Abood (1949). The enzyme acts upon tetrazolium salt to form an acetone soluble red formazon in the tissue homogenates, which gives an absorbance at 420 nm. The reaction mixture comprised of 0.5ml TTC, 0.5ml buffer and 0.1 ml of enzyme source. The reaction was terminated by the addition of 1.5ml of acetone and finally the optical density of the supernatant was measured at 420 nm. The enzyme activity was expressed as \( \mu \text{g of TTC reduced/min./g tissue} \).

#### 3.9.2 Lactate Dehydrogenase (LDH)

The activity of Lactate dehydrogenase was determined by the method of Schatz and Segal (1969) using \( \alpha \)-ketoglutarate as substrate. Add 0.8 ml of buffer, 0.1 ml of NADH and 0.100 ml of \( \alpha \)-ketoglutarate along with 0.5 ml of sample. Thereafter,
the change in absorbance was monitored at 340nm. The activity of the enzyme expressed in terms of μ moles NADH/g tissue.

3.9.3 Hexokinase

The method of Crane and Sols (1965) was followed to estimate the specific activity of hexokinase. The reaction mixture contained 0.2ml of buffer, 0.2n substrate, 0.1 ml of sample and 0.10 ml of glucose. The final reaction volume was made to 1.0ml with distilled water. Control tubes were also run simultaneously with each test samples. Each control tube contained all the above except that 1.0ml of water was added prior to the incubation of enzyme with the substrate. The reaction mixture of test and control tubes were then incubated for 30 min at 37°C. In the control tubes, the incubation was terminated by the addition of 1.0ml of chilled 10% TCA. The contents were then centrifuged at 3000 rpm and the inorganic phosphorous released was estimated by the method of Fiske and Subbarow (1925). To 1.0 ml sample, 0.15 ml of ammonium molybdate and 0.72 ml of water were added. The contents were vortexed, followed by the addition of 0.05 ml of ANSA reagent. Samples were then incubated for 10 min at room temperature and optical density read at 660 nm. A standard curve (2-20 μg) for Pi was plotted and the amount of the sample was calculated from the standard curve. The activity of the enzyme expressed in nmoles of Pi liberated/min./g tissue.

3.9.4 Glucose-6-Phosphatase

The glucose-6-phosphatase enzyme activity was estimated by following procedure of Swanson (1955). The reaction mixture contained 0.4ml of buffer, 0.1 ml of glucose-6-phosphate and 0.1 ml of homogenate. Control tubes were also run simultaneously with each test samples. Each control tube contained all the above except that 1.0ml of TCA was added prior to the incubation of enzyme with substrate. The tubes were incubated for 60 min at 37°C, which were then followed by the addition of 1.0 ml of chilled TCA in test and standard tubes. The contents were then centrifuged at 3000 rpm and the inorganic phosphorous released was estimated by the method of Fiske and Subbarow (1925). A standard curve (2-20 μg) for Pi was plotted and the amount of Pi in the sample was calculated from the standard curve. The activity of glucose-6-phosphatase was expressed as μmoles Pi liberated/min./g tissue.
3.9.5 Glycogen Content

Glycogen was extracted and estimated by the method of Seifert et al (1950). An appropriate amount of tissue sample was digested in 3-5ml of 30% KOH by heating at 100°C for 20min. The glycogen in the digested sample was precipitated by the addition of saturated Na₂SO₄ followed by 95% alcohol to precipitate glycogen. The procedure was repeated twice. The glycogen content was estimated by adding the appropriate amount of the glycogen extract, distilled water and 0.2% anthrone in concentrated H₂SO₄ was kept in a boiling water bath for 10min. thereafter, the tubes were cooled and the colour thus developed, read at 625nm. The results were expressed in terms of μg glycogen/gm tissue weight.

3.9.6 Glucogen Phosphorylase

Glycogen phosphorylase was assayed as per the method of Niemeyer et al (1961). The method involves the measurement of inorganic phosphorus liberated following the phosphorylytic breakdown of glycogen by the action of the enzyme. The reaction mixture (1.0ml) containing 76mM citrate buffer (pH 6.0), 75 mM NaF, 25 mM glucose-1-phosphate, 2.5mM AMP, 1% glycogen and the required amount of homogenate was incubated at 37°C for 60min. the reaction was stopped by the addition of 1.0ml of chilled 10% TCA and the contents spun at 3000xg for 10min. the resultant supernatant was estimated for Pi and the results expressed as nmol Pi liberated/min/mg protein.

3.10 MITOCHONDRIAL DYSFUNCTION

3.10.1 Isolation of Rat Brain Mitochondria

Rat brain mitochondria were isolated by the method of Berman and Hastings (1999) by differential centrifugation. The brain from one adult rat is homogenized in 10ml of homogenizing buffer containing 225mM mannitol, 75mM sucrose, 5mM HEPES, 1mM EGTA, 1mg/ml BSA, pH-7.4. The homogenate is brought to 30ml with the same buffer and centrifuged at 2000g for 30min at 4°C. The pellet is discarded. The supernatant is divided into two tubes and centrifuged at 12,000g for 10min. the pellet containing the mixture of synaptosomes and mitochondria is suspended in 10ml of homogenization buffer containing 0.02% digitonin to lyse the synaptosomes.
followed by centrifugation at 12,000g for 10min to pellet down both extrasympotosomal mitochondria. The mitochondria pellet is washed twice in the same buffer without EGTA, BSA and digitonin. For some experiments like complex I and complex IV assay mitochondria are suspended in 50mM phosphate buffer, pH 7.4 and kept frozen at −20°C in aliquots and used within three days. In other experiments freshly isolated mitochondria are resuspended in isotonic buffer A (145mM KCl, 50mM sucrose, 5mM NaCl, 1mM EGTA, 1mM MgCl2 10mM phosphate buffer, pH-7.4 and used immediately.

3.10.2 Complex I Activity (NADH dehydrogenase)

Mitochondrial complex I activity was estimated by the method of King and Howard (1967). This assay used ferricyanide as the final acceptor and hence does not represent the activity of the total complex I. This method measures NADH oxidation by monitoring the absorption decreases at 340nm. The assay is carried out at 30° C and monitored at 340nm in a final volume of 1ml. All assay components are added to both sample and reference cuvette. An appropriate volume is added to the reference cuvette to allow for the mitochondrial sample to be added to the sample cuvette. The reaction was initiated by the addition of mitochondrial suspension (10-30μg protein) to the sample cuvette and the rate of oxidation of NADH was measured by the decrease in absorbance at 340nm. Assay is left to run for 3mins. The enzyme activity is then calculated by relating the rate of absorbance change to the mitochondria protein in cuvette. The extinction coefficient for NADH at 340nm is 6.22mM⁻¹cm⁻¹

Complex I activity = ΔOD/min X dilution factor / 6.22 X protein (mg/ml)

3.10.3 Complex II (Succinic Dehydrogenase)

Succinic dehydrogenase was assayed as per the method of King (1967). The method involves oxidation of succinate by an artificial electron acceptor, potassium ferricyanide which is measured spectrophotometrically at 420nm.

\[
\text{Succinate} + 2\text{Fe(CN)}_6^{3-} \rightarrow \text{Fumarate} + 2\text{Fe(CN)}_6^{2-} + 2\text{H}^+
\]

The reaction mixture (3.0ml) contained 0.2M sodium phosphate buffer, pH 7.8, 1% BSA, 0.6M succinic acid and 0.03M potassium ferricyanide. The reaction was initiated by addition of requisite amount of sample and absorbance change was
followed at 420nm for 2min. the enzyme activity was expressed as nmol succinate oxidized/min/mg protein.

3.10.4 MTT Assay

The MTT-assay is based on the reduction of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-H-tetrazolium bromide (MTT) by hydrogenase activity in functionally intact mitochondria. The hydrogenase catalyzes the conversion of the yellow MTT reagent to blue formazan crystals. 100µg mitochondrial sample was incubated with 20µl MTT (5mg/ml) for 30 min at 37°C. The samples were quenched with dimethylsulfoxide and MTT formazan was spectrophotometrically quantified in a microplate reader (Bio-Rad, Milano, Italy).

3.10.5 Complex IV Activity (Cytochrome Oxidase)

The enzyme activity was estimated by the method of King (1967). This assay measures the oxidation of reduced cytochrome c at 550nm by cytochrome oxidase. Reduced cytochrome c is prepared by reducing commercially available oxidized cytochrome c solution (10mg/ml) by adding few crystals of ascorbic acid followed by the removal of excess ascorbic acid by a passage through Biogel P-6 column equilibrated with 10mM phosphate buffer. Placing a sample in the spectrophotometer at 550nm and adding a few crystals of ascorbate check reduction of cytochrome c. If fully reduced, the addition of ascorbate will not result in a further decrease in absorbance. Exact concentration of reduced cytochrome c is assessed as follows:

50µl of reduced cytochrome c and 950 µl of phosphate buffer are placed into 1ml cuvette and take absorbance at 550nm. Then add 10µl ferricyanide. Note the difference in absorbance. Calculate the cytochrome c concentration using the extinction coefficient of cytochrome c at 550nm of 19.2mM⁻¹cm⁻¹ accounts for 20-fold dilution of the stock solution in the assay cuvette. The volume of reduced cytochrome c to be used in the assay is calculated to ensure that in each assay, the final concentration of cytochrome c is 50 µM. Reduced cytochrome c (50 µM) in 10mM phosphate buffer pH 7.4 is added in the sample cuvette and the reaction initiated in the sample cuvette by the addition of mitochondrial suspension (10-30 µg protein). The rate of decrease of absorbance at 550nm was measured at room temperature. The enzyme activity is then calculated by the following formula:

Complex IV activity = K x 50 x dilution factor/ protein mg/ml
3.10.6 Oxidative Phosphorylation (ATP Synthesis)

Oxidative phosphorylation was measured using a glucose/hexokinase system as described by Griffiths et al. (1977). Phosphorylation is determined by the disappearance of inorganic phosphorus. The reaction mixture (1.1 ml.) contained 0.25 M sucrose, 10 mM Tris HCl, 22 mM glucose, 5 mM KH$_2$PO$_4$, 2 mM NAD, 2 mM EDTA, 20 units of hexokinase (EC units, 1 μmol substrate/min), 2 mM MgCl$_2$, 20 mM succinate and an appropriate amount of mitochondrial preparation incubated at 30°C for 20 min. The reaction was terminated by addition of 1% trichloroacetic acid followed by centrifugation at 3000×g for 10 min. The supernatant was assayed for phosphorus and the results were expressed as nmol Pi uptake per mg of protein.

3.10.7 Mitochondrial ATPase (ATP Hydrolysis)

Mitochondrial ATPase was assayed according to the method of Houghton (1974). The method involves measurement of inorganic phosphate liberated following catalytic hydrolysis of ATP to ADP. The reaction mixture containing ATPase buffer (5 mM ATP, 2 mM MgCl$_2$, 50 mM Tris HCl, pH 8.4) and an appropriate amount of mitochondrial sample was incubated for 5 min at 30°C. The reaction was terminated by addition of 10% TCA followed by centrifugation at 3000×g for 10 min. The supernatant was assayed for phosphorus and the results were expressed as nmol Pi liberated per min per mg of protein.

3.10.8 ATP Levels

ATP levels were measured using a commercially available kit (BioMerieux, USA). The method involves conversion of 3-phosphoglycerate to 1,3-diphosphoglycerate with subsequent hydrolysis of ATP by the action of phosphoglycerate phosphokinase. 1,3-diphosphoglycerate is further converted to glyceraldehyde-3-phosphate with concomitant oxidation of NADH to NAD$^+$.
monitored spectrophotometrically at 340nm. Mitochondrial sample was deproteinized by adding 12% TCA solution and centrifuged at 3000 rpm for 5 min to obtain a clear supernatant. 1.0 ml. PGA buffered solution, 1.5 ml water and appropriate amount of supernatant was added to the NADH vial. The entire contents were decanted into a cuvette and initial absorbance was recorded using water as reference at 340 nm followed by addition of 0.04 ml GAPD/PGK enzyme mixture and again recorded absorbance at 340 nm until a minimum absorbance reading was reached. The results were calculated using molar extinction coefficient of NADH (0.22 x 10^3 M^-1cm^-1) and expressed as μmol ATP/mg protein.

3.10.9 Mitochondrial Cytochrome Levels

Cytochrome levels were estimated according to the method of Williams (1964). The method involves quantitative estimation of four cytochromes a, b, c1 and c simultaneously in a single mitochondrial sample. The differential spectrum of the mitochondrial sample (reduced vs oxidized) is recorded in the presence of deoxycholate. Knowing the contributions of absorbance of each cytochrome to the major maxima and minima of the other cytochromes, a set of four simultaneous equations with four unknowns are solved.

Equation 1-

\[
\begin{align*}
    b_{15} &= a_{15}/21.0 \\
    C_{25} &= (a_{25} - 6.51 b_{15})/15.6 \\
    D_{35} &= (a_{35} + 1.16 b_{15} - 1.48 C_{25})/13.8 \\
    E_{45} &= (a_{45} + 0.22 b_{15} + 0.482 C_{25} - 0.076 d_{35})/12.0
\end{align*}
\]

Where

- \( A_{15} \) = difference in absorbance from 550 nm to 535 nm which represents cytochrome c
- \( A_{25} \) = difference in absorbance from 554 to 540 nm which represents cytochrome c1
- \( A_{35} \) = difference in absorbance from 563 to 577 nm which corresponds to cytochrome b
- \( A_{45} \) = difference in absorbance from 605 to 630 nm which corresponds to cytochrome a
then Equation 2.
\[ X_4 = \frac{e_{45}}{d_{45}} = \text{Cytochrome a concentration} \]
\[ X_3 = d_{35} + 0.0263 X_4 = \text{cytochrome b concentration}. \]
\[ X_2 = C_{25} - 0.0484 X_4 - 0.225 X_3 = \text{cytochrome c}_1 \text{ concentration}. \]
\[ X_1 = b_{15} - 0.03 X_4 + 0.149 X_3 - 0.491 X_2 = \text{cytochrome c concentration}. \]

To each of two cuvettes were added mitochondrial suspension, 0.1 M sodium phosphate (pH 7.4) and 20% sodium deoxycholate. To cuvette 1 were added 0.05 M potassium ferricyanide and water to make reaction volume 1.0 ml. To cuvette 2 were added 0.05 M sodium ascorbate and water to make reaction volume 1.0 ml. Few grains of sodium hydrosulphite were added to cuvette 2 and mixed by repeated inversion. The spectrum of cuvette 2 Vs 1 was recorded from 500 to 630 nm. The difference in absorbance at the following pairs of wavelengths was recorded: 550-535 (cytochrome c), 554-540 (cytochrome c1), 563-577 (cytochrome b), and 605-630 nm (cytochrome a). The results were expressed as nmol/mg protein.

3.11 NEUROTRANSMITTERS AND RELATED ENZYMES

3.11.1 Monoamine Oxidase

The enzyme activity was estimated by the method of Mc Ewen and Cohen (1963). Benzaldehyde production from the oxidative deamination of benzylamine allows a convenient spectrophotometric assay of enzyme. The assay tube consisted of 10 μl of samples, 0.1 ml of buffer and 0.1 ml of benzylamine. The tubes were incubated in a metabolic shaker bath at 37 °C for 3 hrs. After incubation, 0.1 ml of 60% perchloric acid was added followed by the addition of 1.0 ml of cyclohexane. The contents in the tubes were emulsified with glass stirring rod and the tubes were kept at room temp for 15 mins. The tubes were emulsified again with rod and were centrifuged at 2000 × g for 30 mins. The absorbance of the cyclohexane extract was then measured at 242 nm. Control tubes were also run in parallel similarly, except that the substrate benzyl amine was added at the end of incubation. The results were expressed as μmoles benzaldehyde formed/min/mg protein.
3.11.2 Acetylcholinesterase Activity

The enzyme activity was estimated in tissue homogenates by following the protocol of Ellman et al. (1961), and the same has already been described for its assessment in serum and the enzyme activity was expressed as \( \mu \text{moles acetylcholine hydrolyzed/min/mg protein} \).

3.11.3 Estimation of Biogenic Amines

The concentrations of biogenic amines (serotonin and dopamine) were estimated by using the method of Cox and Perhach (1973). 200 mg of (cerebral cortex and mid brain) and 100 mg of cerebellum tissues were homogenized in 2.8 ml of acidified butanol. The homogenates were centrifuged for 5 min at 800 g. 2.5 ml of the supernatant fluid was transferred to a 13 ml glass stopper centrifuge tube containing 2.5 ml of distilled water and 5.0 ml of heptane. The tubes were shaken and centrifuged at 800 \( \times \)g for 5 min. 2.5 ml of the aqueous phase was then transferred to a screw capped test tube containing 200 mg of alumina, 1.0 ml of sodium acetate and the tubes were gently shaken for another 10 min and centrifuged for 5 min at 800 \( \times \)g. From the aqueous phase, 3.0 ml was transferred to a clean screw capped test tube and refrigerated for subsequent determination of serotonin. The remaining aqueous phase was aspirated from the alumina. Alumina was again washed by shaking with 2.0 ml of distilled water for 5 min and re-centrifuged at 800 \( \times \)g for 5 min. To the aqueous phase was discarded and 2.0 ml of acetic acid was added. The tubes were again gently shaken and centrifuged. Further, 1.0 ml of the aqueous phase was taken and to it 0.1 ml EDTA reagent was added and then mixed. 0.1 ml iodine reagent was then added and mixed for another 2 min followed by the addition of 0.2 ml of alkaline sulfite. Further, after 2 min, 0.2 ml of 5 N acetic acid was added, mixed and heated at 100°C for 5 min. The tubes were cooled, reheated for another 5 min, and the fluorescence was read at 320 nm and 370 nm respectively for the estimation of dopamine. To extract of serotonin, 3.0 g of NaCl, 1.0 ml of 0.35 M borate buffer (pH 11.0) and 6.0 ml of n-butanol solutions were added to the aqueous phase kept in the previous step for serotonin estimation. The tubes were shaken for 10 min and centrifuged for 5 min at 800 \( \times \)g; 5.0 ml of the organic phase was transferred to a centrifuge tube containing 0.5 ml of 0.1 N HCl and 6.0 ml of heptane. The tubes were then shaken for 5 min and re-centrifuged at 800 \( \times \)g for 5 min; 0.4 ml of the aqueous phase was then transferred to a test tube and 0.68 ml ninhydrin was added. The tube contents were mixed and
then heated in boiling water bath for 10 min. after cooling, fluorescence was read with the activation and emission wavelengths set at 360 nm and 460 nm, respectively. Internal standards were prepared by adding known amounts of each standard to a homogenate pool and running in parallel with the tissue samples. The pool homogenates without the standards served as a blank for the standards. The results were expressed as microgram per gram of wet tissue weight.

3.12 GENE AND PROTEIN EXPRESSION STUDIES.

3.12.1 mRNA Expression Studies

mRNA expression analysis by RT-PCR was performed using QIAGEN one step RT-PCR kit (Germany). Various genes associated with apoptosis like caspase 3, caspase 9, and Bcl-2 were analyzed. Gene expression of certain inflammatory genes like NF-kB, TNF-α was also analyzed. HSP-70 gene expression was also done in both neurons and glial cells. Mouse β-actin was used as an internal control. For these studies, total RNA isolation was carried out from the mid brain region.

i) **Total RNA Isolation:** Total RNA was isolated from the mid brain region using TRI-REAGENT (Mol. Res. Center, Inc, Ohio, USA). TRI-REAGENT combines phenol and guanidine thiocyanate in a monophase solution to facilitate the immediate and most effective inhibition of RNAase activity. This helps in the isolation of complete spectrum of RNA molecules. To obtain RNA, following procedure was performed:

**Homogenization:** 50mg of brain from different treatment groups was homogenized in 0.5ml TRI-REAGENT in 1.5ml polystyrene microfuge tubes using hand homogenizer.

**Phase Separation:** The samples were kept at room temperature for 5min to permit the complete dissociation of nucleoprotein complexes. Then 0.1ml chloroform was added and mixed vigorously for about 15 seconds. The homogenates were then kept at room temperature for 10min followed by centrifugation at 12,000g for 15min at 4°C. Following centrifugation three distinct layers, a lower phenol chloroform phase, interphase and an upper colourless aqueous phase were seen. The upper phase that is roughly 60% the volume of TRI-REAGENT contains RNA.
**RNA Precipitation:** The aqueous phase was transferred to fresh tubes and then 250 µl isopropanol was added to precipitate the RNA. The samples were kept at room temperature for 10 min and then centrifuged at 12,000 g for 10 min at 4°C. RNA precipitate was seen as a small white pellet on the side of the tube.

**RNA Wash:** The pellet was washed with 0.5 ml 75% ice-cold ethanol by centrifugation at 7500 g at 5 min at 4°C.

**RNA Solubilisation:** After removing the ethanol, the RNA pellet was briefly air-dried (not completely), and then dissolved in 10 µl of diethyl pyrocarbonate (DEPC) treated water.

### ii) Estimation of Purity and Concentration of RNA

**Purity:** Purity of RNA was checked by determining the ratio of absorbance values at 260 nm and 280 nm. The final ratio for all the RNA preparations was approximately 1.6, which indicates a pure preparation.

**Concentration:** Concentration of RNA was estimated by measuring the absorbance at 260 nm in spectrophotometer (UV-160A, Shimadzu) using $A_{260}=1\equiv40\mu g/ml$.

### iii) Agarose Gel Electrophoresis of RNA: Integrity and size distribution (quality) of isolated total RNA was checked by denaturing agarose-gel electrophoresis.

**Procedure:** 1.2% agarose gel (40 ml) was prepared in DEPC treated water in an Erlenmeyer flask using microwave oven, allowed to cool to 60°C and then 5 ml of 10X MOPS and 1.5 ml of formaldehyde were added and mixed well. The gel was poured into horizontal gel electrophoresis chamber with comb and allowed to stand at room temperature for 30 min to polymerize. Samples were prepared by mixing the following in microfuge tubes: RNA - 4.5 µl (5 µg), 10X MOPS - 1.0 µl, Formaldehyde - 3.5 µl, Formamide - 8.0 µl, Ethidium bromide (1 mg/ml) - 1.0 µl. The samples were incubated at 65°C for 15 min and then chilled on ice. To all the samples, 1.5 µl of 10X RNA loading buffer was added and mixed well. 1X MOPS was used as running buffer and electrophoresis was carried out at 70 V. Finally, the bands were viewed and photographed using Gel Doc (BioRad, UK).

Two distinct bands for 28s and 18s rRNA were observed and the intensity of the 28s rRNA band was almost double that of the 18s rRNA. RT-PCR products of
expected size i.e. 287bp were obtained for β-actin. The mRNA expression for gene was normalized with respect to β-actin mRNA, for which a uniform expression was obtained in all the treatment groups (Figure 3.12.1).

iv) Primer designing and synthesis: Optimal primer pairs were designed using the software “Gene Runner” or their sequence were obtained from literature and were got synthesized from Sigma-Aldrich (USA). Lengths of the primers chosen were ~20bp.

v) Reverse Transcriptase Polymerase Chain Reaction (RT-PCR): One RT-PCR kit was used (QIAGEN Inc, Germany) in which cDNA synthesis and are carried out sequentially in the same tube (Labware Scientific Inc., USA).

Procedure: RT-PCR was performed according to the manufacturer’s instructions. 3µg RNA was used for each reaction. Firstly, a master mix was prepared as follows: 5X QIAGEN RT-PCR buffer - 10µl, dNTP mix - 2µl, QIAGEN onx RT-PCR enzyme mix - 2µl, RNase inhibitor - 1µl.

The master mix was mixed thoroughly and 15µl of it was added to each PCR tube. To this 5µl of each sense and antisense gene specific primers (from 10µM stock) were added. Then 3µg template RNA was added and final volume was adjusted to 50µl with RNAase free water provided in the kit. All the reactions were carried out on ice. The PCR tubes were gently vortexed and centrifuged in order to settle all the components at the bottom. The PCR tubes were placed in a thermal cycler (Techmne Inc., UK), which was programmed as shown in Tab 3.12.1.

vi) Agarose Gel Electrophoresis for PCR Products:

30ml of 1.5% agarose was prepared in 1X TAE buffer in an Erlenmeyer in a microwave oven. After cooling to approximately 60°C, ethidium bromide was added to a final concentration of 0.5µg/ml. The gel was poured in to horizontal tray with comb and left undisturbed for 30min to allow it to polymerize. 3µl of

![Figure 3.12.1: Electrophoresis of total RNA through agarose gel containing formaldehyde. Lane I: Control, Lane II: Aluminum, Lane III: Al + Curcumin, Lane IV: Curcumin](image-url)
PCR product was mixed with 1μl DNA gel loading buffer and the sample loaded in separate wells. 1X TAE was used as the running buffer. Electrophoresis carried out at 70V. The bands were visualized and photographed on Gel Doc (UK).

Table No. 3.12.1: Thermal cycler conditions

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcription</td>
<td>50°C</td>
<td>50min</td>
</tr>
<tr>
<td>Initial PCR activation</td>
<td>95°C</td>
<td>15 min</td>
</tr>
<tr>
<td>3 step cycle</td>
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<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>variable*</td>
<td>45 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>No of cycles 35**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>10 min</td>
</tr>
<tr>
<td>Final Hold</td>
<td>10°C</td>
<td>10 min</td>
</tr>
</tbody>
</table>

**In initial experiments, RT-PCR was performed by using different number of cycles-20, 25, 35. It was found that the PCR products were progressively amplified till 35 cycles; 35 amplification cycles were performed.**

The PCR products were analyzed on agarose gel electrophoresis.

vii) **Densitometric Analysis of Bands:** Densitometric analysis of bands was carried out by using the Image J software (NIH).

3.12.2 **Enzyme Linked Immunosorbent Assay (ELISA):**

ELISA was carried out to quantitate the levels of caspase-3, caspase-8, and cytochrome C in various treatment groups under study. Titration of concentration of antigens and antibodies standardized the assay. 10% (w/v) homogenates were prepared in 50mM Tris-HCl (pH 7.4) under ice-cold conditions. The homogenates were then centrifuged at 10,000rpm for 30 min. The supernatant (post mitochondrial fraction, PMF) thus obtained was quantitated for protein by Lowry method and collected for ELISA. 2.5μg protein was loaded in ELISA strip wells in 100μl carbonate buffer and kept overnight at 4°C, in dark. Flicked the plate to remove the unbound antigen and the wells were washed with 1% BSA in 0.1M PBS for 1hr at 37°C. The plate was then flicked thrice with PBS containing 0.05% Tween-20. Wells were then incubated with primary antibody diluted in PBS containing 0.05% Tween-20 and 1% BSA.
for 2hr at 37°C. Plate was again washed thrice in the same manner and incubated with peroxidase labeled anti-rabbit IgG diluted in PBS containing 0.05% Tween-20 and 1% BSA (1:500) and kept for 2hr at 37°C. The plate was again washed thrice as above and one last washing was given with distilled water since Tween acts as an inhibitor of the substrate i.e. ABTS [2,2-azino-di-(3-ethylbenzthiazolinsulphonic acid)]. The substrate was then added to each well and the plate was kept in dark for 30min after which the color developed was read at 405nm.

3.13 APOPTOTIC STUDIES

3.13.1 DNA Fragmentation

DNA Isolation: Freshly excised tissue was grounded to powder form using pestle and mortar. The powdered tissue was added to approximately 10 volumes of extraction buffer, which consisted have Tris-Cl; 10mM (pH 8.0), EDTA; 0.1M (pH 8.0), SDS 1%. The solution was then incubated for 1 hr at 37°C. proteinase K was added to lyse the cells which was kept for 3hrs at 50°C. The solution was swirled periodically. The solution was allowed to cool to room temperature and an equal volume of phenol: chloroform in the ratio of 1:1 was added. Mix the two phases slowly by inversion for 10 min. Two phases were separated by centrifugation at 10,000 ×g for 15 min at room temperature. Viscous aqueous phase was transferred to a clean centrifuge tube and repeated the extraction twice with phenol: chloroform. To the aqueous phase, equal volume of 0.06 M sodium acetate was added. Two volumes of ice-cold isopropanol was then added and kept at -70°C for 1 h. The above solution was centrifuged at 10,000 ×g for 20 min to isolate DNA. The pellet obtained was washed with 70% ethanol and dissolved in 20 μl of TE buffer (pH 8.0)

Estimation of Purity and Concentration of DNA: This was checked by determining the ratio of absorbance values at 260nm and 280nm. The final ratio for all the DNA preparations was greater than 1.75, which indicates a pure preparation.

Concentration: Concentration of DNA was estimated by measuring the absorbance at 260nm in spectrophotometer (UV-160A, Shimazdu) using A_{260}=1=40μg/ml.

Agarose gel electrophoresis: 30 ml of 1.8% agarose was prepared in 1X TAE buffer in a flask in microwave oven. The gel was cooled to 60°C and ethidium bromide was added to a final concentration of 0.5 μg/ml and mixed thoroughly. The gel was poured into the horizontal gel electrophoresis chamber with comb and was
allowed to stand at room temperature for 30 min. 6μg of the DNA was mixed with 1.2 μl DNA gel loading buffer and the samples were loaded in separate wells. 1X TAE was used as the running buffer. Electrophoresis was carried out at 70V. The bands were visualized using ultraviolet transilluminator (BioRad systems, USA) and photographed on Gel Doc (BioRad, UK).

3.13.2 Comet Assay

Comet assay or single cell gel electrophoresis (SCGE) is a rapid, sensitive and relative simple method for detecting DNA damage at the level of individual cells (genotoxic changes). The assay works upon the principle that strand breakage of the supercoiled duplex DNA leads to the reduction of the size of the large molecule and these strands can be stretched out by electrophoresis.

Comet assay was performed according to Singh et al (1988). Briefly, 130 mg of brain tissue was homogenized in 1 × PBS using tip homogenizer. The homogenate (80 μl) was then mixed with 100 μl of 1% low melting temperature agarose at 37°C and then placed on a slide pre-coated with thin layer of 1% regular melting agarose. It was immediately covered with cover slips and the slides were kept at 4°C for 45 min to allow solidification of the agarose. After removing the cover slips, the cells were lysed in a lysing solution for 1 h. After washing in re-distilled water the slides were placed in a horizontal gel electrophoresis chamber. The chamber was filled with cold alkaline buffer and slides were kept at 4°C for 20 min to allow the DNA to unwind. Electrophoresis was performed for 30 min (25 V/cm, 300 mA). After electrophoresis, the slides were washed three times with neutralization buffer for 5 min. All preparative steps were conducted in dark to prevent additional DNA damage. The slides were stained with ethidium bromide for 10 min and analyzed with a fluorescence microscope at 450 × for comet assay using 550-560 nm excitations and 590 nm barrier filters.

3.14 STATISTICAL ANALYSIS

For analyzing the data, one way analysis of variance (ANOVA) followed by Newman Keul’s test was performed using the statistical software package “SPSS v 11 for windows”. The post-hoc comparison of means from different treatment groups were made by the method of least significant difference (LSD). Results corresponding to a p value of 0.05 or less were considered statistically significant.