MATERIALS AND METHODS

It has been mentioned previously that the main objective of the work are—

1. To explore the role of *Moringa oleifera* (MO) leaf in behavioural pattern, brain electrical activity, cholinergic enzymes, antioxidants and monoamines of adult albino rats.

2. To establish the protective effect of MO leaf in Alzheimer’s disease by modulating the behavioural pattern, antioxidative enzyme activity, cholinergic enzymes and monoaminergic and EEG functions in different regions of brain which are involved in Alzheimer’s disease.

3. To establish the protective effect of MO leaf in hypobaric hypoxia induced memory loss by alteration of different enzymes and neurotransmitter that undergoes variation under chronic exposure to high altitude and thus leads to potent memory loss akin to Alzheimer’s disease.

Major emphasis has been given to demonstrate the role of MO in behavioural pattern, EEG study, antioxidant enzymes like SOD, CAT and LPO, cholinergic enzymes like cholineacetyltransferase and acetylcholinesterase, monoamines like norepinephrine, dopamine and serotonin, Histological studies of neuronal loss by cresyl violet staining, counting of dendritic branches of motor and sensory cortical neurons by golgi cox method and the Neuropathological hallmarks of AD, amyloid plaques and neurofibrillary tangles by the method of Bielchowsky were observed in experimental Alzheimer rat model and also in hypobaric hypoxia induced memory loss.
The methodology is described as follows:

1. Animal use and maintenance.

2. Animal treatment
   a) Collection and authentication of plant.
   b) Preparation of extract
   c) Drugs and Doses.
   d) Treatment of animal with standardized dose of plant extract.

3. Animal preparation
   a) Colchicine infused Alzheimer model.
   b) Chronic Hypobaric hypoxic exposure.
   c) Post operative care.

4. Analysis of behavioural pattern
   a) Radial Y arm maze.

5. Electroencephalographic studies.

6. Biochemical estimation
   a) Serotonin (5-HT), dopamine (DA) and norepinephrine (NE) estimation in discrete brain areas.
   b) Catalase (CAT), superoxide dismutase (SOD) and lipid peroxidase (LPO) in cerebral cortex.
   c) Acetylcholinesterase and Cholineacetyltransferase enzyme in hippocampus and cerebral cortex.

7. Histological studies
   a) Morphological study by Hematoxylin Eosin stain
   b) Neuronal Cell count by cresyl violet staining.
   c) Dendritic branches by golgi cox stain.
d) Hagamethanamine silver stain for amyloid plaques.
e) Bielchowsky stain for neurofibrillary tangles.

Collection of Plant Materials

The leaves of *Moringa oleifera* were procured from United Chemical & Allied Products, Calcutta and authenticated by Botanical Survey of India, Howrah. (Authentication No. CNH/I-I (20)/2004-Tech.II)

Preparation of Ethanolic extract of MO

Fresh, young, healthy leaves of MO were bought from local market and the identity of the plant was authenticated by Botanical Survey of India, Howrah and kept in the laboratory. The leaves were shade dried and ground with the help of an electrical grinder to get a free flowing powder. This powder was subjected to extraction with dehydrated alcohol at room temperature for 24 hours. The extract obtained was filtered through Whatman filter paper and vacuum dried at 40-50°C to get a blackish green semisolid mass, which was dissolved in saline (0.9% NaCl) solution for final use (Ganguly and Guha, 2006).

Experiment on Intact Animals

*Animals and Experimental Design:*

Holtzman strain adult rats of both sexes and weighing between 180-220g were used throughout the experiment. The rats were housed individually in a photoperiod cycle of 12h: 12h (light & dark), at room temperature (around 28°C) with standard laboratory diet. Drinking water was supplied *ad libitum.* Body weight of rats were recorded every day and maintained in the laboratory throughout the experimental period.
Plate 2: Radial Y arm maze
**Behavioural testing by radial Y arm maze training:**

**Habituation session:** During RAM training the animals were food deprived to about 80% of their *ad libitum* body weight and trained for 5 days to run on a radial arm maze. (Brown, wooden, 60x10 cm arms) extending from an octagonal central platform (Plate 2). The maze was kept in the centre of a dimly lit room (15 ft x 10ft) with many posters and objects hanging on the wall. The animals were placed in the center of the maze with all arms accessible and baited with chocolate chips. The rats were removed from the maze after visiting all the arms. Arms were rebaited only after the animal left the arm and the maze was cleaned with 50% alcohol solution between animals. Only animals reaching this criterion were trained on the memory tasks. Entry into an arm previously visited within any daily trial was scored as an error (Dwaine and Thomas, 1990; Jonathan and Etan, 1998).

Following habituation session, the animals were trained for 10 daily trials per day on RAM task.

**Animal Treatment:**

Adult albino rats were divided into control, sham control and experimental groups. Sham control rats were treated with saline (5 ml/kg, orally) and experimental rats were treated with MO extract (250 mg/kg, orally) once daily for 14 consecutive days between 9:00 to 11:00 a.m.

**Preparation of Alzheimer Animals:**

1. Colchicine induced Alzheimer Model.

2. Chronic hypobaric hypoxia induced memory loss.
Alzheimer's disease
Loss of tau and accumulation of hyperphosphorylated tau in somatodendritic compartments

Amyloid plaques
APP processing disrupted
Loss of memory

Colchicine
Disrupts Axonal microtubules

Mechanism of action of Colchicine

Colchicine
Disrupts Cholinergic neurons of MS/VLDB
Lateral Ventricle
Diffuse to SHC system
Cholinergic neurons in MS/VLDB that project to Hippocampus

15μg/5μL
Cholinergic neurons of MS/VLDB and septum/vertical limb of the diagonal band, SHC, Septo hippocampal cholinergic system, APP, Amyloid precursor protein, L.V. Lateral Ventricle

Granule cells, Pyramidal cells, Interneurons
Hippocampus

Memory

Abbreviations: MS/VLDB=Medial septum/vertical limb of the diagonal band, SHC=Septo hippocampal cholinergic system, APP=Amyloid precursor protein, L.V=Lateral Ventricle
late 3: Photomicrograph of coronal section (8 μ) of the rat brain showing the position of syringe in lateral ventricle for injection of colchicine.
**Animal Preparation for Surgery:**

Prior to surgery, all the animals were subjected to overnight fasting though drinking water was not withdrawn.

**Anaesthesia:**

Anaesthetic ether (Kabra Chemicals India Ltd.) was used.

**Preparation of experimental Alzheimer model by colchicine:**

Intraventricular infusion of colchicine was done stereotaxically by anesthetizing the animals with pentobarbitone (40 mg/kg B.W). The anesthetized animals were mounted on stereotaxic instrument. Head was fixed in such a position that lambda and bregma sutures were in the same horizontal plane. The scalp was incisioned in the midline and the pericranial muscles and fascia were retracted laterally. The overlying bone was drilled at the specific loci in the lateral ventricle following the co-ordinates of the stereotaxic atlas. (A.P- 0.6mm; Lateral- 2.6mm; Depth- 1.8mm) Colchicine was then slowly infused (0.125 μl/min) into the lateral ventricle (Plate 3) (*Bhattacharya et al.*, 2001).

**Post operative care:**

After surgery, all aseptic measures were taken for different periods and particular care was taken for feeding until they recovered from surgical stress. Antibiotic was given post operatively to all animals for 3 consecutive days by intramuscular route. After 3 days of surgery, experimental sessions started and continued routinely until sacrificed.
Exposure of animals to hypoxia

Chronic hypobaric hypoxic exposure leads to potent loss of memory in human. In animals a simulated hypobaric hypoxic condition was made in a hypoxic chamber. The experimental design and the principle for simulating the high altitude condition was the same as described earlier by Bhatia et al., 1969. It is a stainless steel rectangular box having a glass cover on the top fitted tightly with the help of grease. A pressurized tube, from the chamber is attached to a rotary high vacuum pump (Precivac Eng Co, Model VSP 85, Kolkata, h.p. 0.5, rpm 500). The airflow was maintained at 2 litres /min throughout the experimental period. The animals were exposed in the hypoxic chamber for 21 days (2 hrs Daily) where atmospheric pressure was reduced to 412.0 mm Hg. (11% O₂ balanced with N₂ and 5% CO₂). The animals were then exposed to high altitude stress at 412.0 mm Hg equivalent to 5400 meters above sea level for two hours each day up to 21 days. After completion of 2 hrs exposure the vacuum was gradually released and finally the animals were taken out and returned to their cages. The control animals were kept in similar conditions but without the hypoxic exposure (Plate 4).

Surgical procedures for EEG studies

For electroencephalographic recordings rats were anaesthetized with pentobarbitone sodium (40mg/kg, i.p) and was placed in a stereotaxic instrument and surgery was done by a midline incision at the back of the head. At the first stage, bipolar electrodes were implanted on the surface of the somatosensory cortex and fixed with dental cement. The electrical activity from the cerebral cortex was monitored frequently through an 8-channel EEG (Recorder & Medicare, India Ltd.).Recordings were taken approximately
Plate 4: Hypobaric hypoxia chamber
every 5min throughout the session before and after MO treatment (Ganguly and Guha, 2006).

Biochemical Estimation

1) Estimation of Serotonin (5-HT), Norepinephrine (NE) and Dopamine (DA)

Different regions of brain tissues e.g. Cerebral cortex, cerebellum, caudate nucleus, midbrain and hippocampus were collected in cold condition (4°C) and was homogenized in 10 ml acidified butanol. 4 ml homogenate was mixed with 10 ml of 10% heptane and 5 ml of 0.003 N HCl. It was then shaken for 5 mins and centrifuged at 2000 r.p.m. for 10 mins. 4.5 ml of acid layer was eluted and mixed with 200 mg alumina and 1 ml of 2M sodium acetate. The mixture was shaken for 5 mins and centrifuged at 2000 r.p.m. for 10 mins. The supernatant of this step was used for the estimation of serotonin (5-HT) and the precipitate was used for the estimation of norepinephrine (NE) and dopamine (DA). A Perkin-Elmer Spectrofluoro-meter measured the fluorescence of all the samples (Hazra and Guha, 2003).

2) Estimation of Antioxidant Enzymes:

Collection of samples: Cerebral cortex (CC) and Hippocampus (HC) was isolated and dissected in cold condition (4°C) for enzyme assays.

Estimation of SOD:

SOD was assayed according to the method of Mishra and Fridovich (1972) by comparing the adenochrome formation. The assay was based on the ability of SOD to inhibit the spontaneous oxidation of adrenaline to adenochrome. Results are expressed as units of SOD activity/mg protein or % inhibition of activity of
SOD compared to control. One unit of SOD induced approximately 50% inhibition of auto-oxidation of adrenaline.

**Estimation of CAT**

In the estimation of CAT activity, decomposition of H$_2$O$_2$ was measured. The assay was based on the ability of CAT to induce the disappearance of hydrogen peroxide (H$_2$O$_2$). One unit of CAT was defined as the amount of the enzyme required to decompose 1µM of H$_2$O$_2$ per min, at 25°C and pH 7.0. Results are expressed as units (U) of CAT activity/mg protein or % inhibition of activity of CAT compared to control (Cohen *et al.*, 1970).

**Estimation of LPO**

LPO was determined by estimating the accumulation of the peroxidative product, thiobarbituric acid reactive substances (TBARS), using a standard curve of 1, 1, 3, 3-tetramethoxypropane, and was expressed as nmol TBARS/g tissue (Bhattacharya *et al.*, 2001).

**Estimation of Protein**

Protein was estimated by the method of Lowry. 0.1 ml of hydrolyzed precipitate solution was mixed with 0.4 ml of distilled water. To this 5ml of alkaline reagent was added and waited for 10 minutes. 0.5ml of phenol reagent was given to this solution and after 10 minutes reading was taken in spectrophotometer at 600nm (Lowry *et al.*, 1951).

3) **Estimation of Cholinergic enzymes**

**Estimation of Acetylcholinesterase:**

Brain tissue homogenate was dissolved in Phosphate buffer and Ellman’s reagent was added followed by acetylthiocholine iodide as a substrate was
added to it and rate of reaction was noted by measuring absorbance in a spectrophotometer over a period of 2 min and change per min was recorded (Ellman et al., 1961).

**Estimation of Cholineacetyltransferase:**

Acetyl CoA and Choline chloride, the two major reactant of formation of acetylcholine were made to react in a medium containing Phosphate buffer, Neostigmine sulphate and EDTA at 37°C. This was followed by addition of homogenate and reaction was stopped and the precipitated protein with EDTA addition were removed. And the supernatant was made to react with 4,4'-Di thio pyridine to develop a colour which was measured spectrophotometrically (Haba et al., 1988).

**Histological studies:**

*Staining with Hematoxylin and Eosin for morphological observation of cells:*

The hematoxylin and eosin stain is probably the most widely used histological stain. Its popularity is based on its comparative simplicity and ability to demonstrate clearly an enormous number of different tissue structures. Hematoxylin can be prepared in numerous ways depending on its application. In our experiment, Harry’s hematoxylin has been prepared. The cut sections of 10-12 μ were taken and undergone various grades of alcohol in descending order (100% to 50%) for hydration and then taken to water. Then the sections were stained with Harry’s Hematoxylin and counterstaining was done by eosin. (Stevens and Wilson, 1990).
**Staining with Cresyl violet for cell counting in hippocampal areas:**

Nissl stain is used routinely in neurohistology not only to demonstrate nissl substances but also to show the cellular pattern. Nissl granules can be demonstrated by many dyes, e.g. neutral red, methylene blue, thionine, toluidine blue and also cresyl violet. In our experiments, the cellular pattern in different hippocampal regions was observed by cresyl violet staining. The sections were taken to water as earlier. 1% cresyl violet stain (Sigma Aldrich, USA) was prepared. The sections were stained with cresyl violet and rinsed with 90% alcohol to remove excess stain. *(Lowe, 1996).*

**Staining with Bielchowsky for Neurofibrillary tangles:**

There are several techniques for the demonstration of axons and cell processes in the CNS which rely on silver impregnation. Bielchowsky pioneered present day methods by use of a primary sensitizing silver solution and finally reduction in formalin. Neurons develop intracellular filamentous inclusions termed as “neurofibrillary tangles”. These may be detected with Bielchowsky’s silver stain and appear as skeins of filament around the nucleus, also extending into the axonal hillock of the cell body. *(Wallington, 1965).*

**Staining with Methenamine silver method for amyloid plaques:**

Amyloid is deposited in the cerebral cortex, derived from a fragment of a neuronal cell membrane protein, termed as amyloid precursor protein or APP. Deposits of amyloid become surrounded radially by dilated and distorted neuronal processes to form senile plaques. These structures can be demonstrated with silver techniques. Haga methanamine silver method is an excellent
screening stain for the presence of amyloid plaques. The sections were kept in silver solution for at 60°C and rinsed in distilled water. For stain fixation, the sections were placed in 5% sodium thiosulphate solution. Then the sections were rinsed in tap water. (Haga et al., 1989).

**Staining with Golgi-Cox method for neuronal degeneration:**

The block impregnation technique give tremendous insight into the three dimensional nature of the neuron and its processes. Following impregnation, of tissue blocks, thick sections are cut and mounted. The unexplained phenomenon which makes the method so useful is that only a few cells become impregnated giving a clear picture of neuronal architecture uncluttered by surrounding cell processes. In this method, the tissue slices are hardened by Potassium dichromate and mercuric chloride mixture (containing 5% mercuric chloride, 5% Potassium dichromate, 5% Potassium chromate and distilled water) for sixteen weeks. Then the blocks were processed into paraffin wax and were cut at 50 μm. After section cutting, the sections are blackened. (Pugh and Rossi, 1993).

*The detailed methodology has been described in respective chapters.*

**Drugs:**

a) Colchicine (SRL India Ltd).

b) Sodium Pentobarbitone (Neon Laboratories, India)

c) Sodium benzyl penicillin (Alembic Laboratories, India)
Statistical Analysis

The data were expressed as mean ± SEM. Statistical analysis of the differences between control, colchicine infused Alzheimer animals and MO treated Alzheimer animals were done by two way ANOVA and appropriate pair wise comparisons were performed by two tail-test in behavioral changes in RAM task (P≤ 0.05 was considered statistically significant) where as in other experiments statistical analysis was performed using one way analysis of variance (ANOVA) followed by multiple comparison t test. (P≤ 0.05 was considered statistically significant).