CHAPTER 4

MATERIALS AND METHODS
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Three plants were selected for neurotoxicological study from the survey site. Then behavioral study, biochemical parameters and ultrastructural study in albino mice brain was done. Standard methods and literature were consulted for all the parameters performed.

4.1 Field study & Collected plant information:

Extensive field study was conducted from 2009 to 2010 in study area. Field data was collected for each plant. Information on toxic plants was collected from ethnic people. After this medicinal property of the particular plant were found in previous literature study.

4.2 Preparation of Extract:

Three plants were selected for neurotoxicological screening depending upon the field information and literature study. The plants are *Alstonia scholaris* R.Br. (stem bark), *Thevetia peruviana* Merr. (seed) and *Semecarpus anacardium* L.f. (leaf). The plants were identified by Botanical Survey of India, Shillong Branch. The fresh part of selected three plants was collected from the nearest forest belongs to Cachar district of Assam, India. The collected material was shade dried and grinded for extraction. Than the grinded material was dipped in distilled water (100 g in 1 L) for 24 h. The filtrate was oven dried at 40°C. The extract was weighed and dissolved in distilled water for desired concentration (Akindele and Adeyemi, 2006).
4.3 Animal Experiments:

The albino mice (25-30g) were collected from Pasteur institute, Shillong. The mice were acclimatized under laboratory conditions and food and water was provided ad libitum. The handling and experiments of animals were according to the rules and guidelines of "Assam University Animal Ethics Committee". After acclimatization, mice were treated with sub lethal dose for evaluation of neurotoxicity. The mice were divided into three groups, each containing six mice and were treated for seven days. The first one was considered as control group and treated with distilled water (i.p.) of 100mg/kg bw and 200 mg/ kg bw. Which were considered as low dose and high dose respectively.

Similarly two doses of *T. peruviana* ie., 50 and 100 mg/ kg bw was administered orally for seven days to mice. The third plant ie., *S. anacardium* was administered (i.p.) at two doses of 500 and 1000 mg/ kg bw to mice.

4.4 Behavioural study

For behavioural study, there were two another groups of mice taken to which standard drug has been treated. First group was treated by Fluoxetine (20 mg/kg, ip.) and considered for Force swim test. The second group was treated by Diazepam (1mg/kg, i.p.) and behavioral experiments were performed ie., Hole board test, Elevated plus maze test, Locomotor activity, and black & white test.
Force swim test: This was performed by Porsolt et al., 1977. The mice were forced to swim in glass vessel (22cm diameter, 40cm height) containing 20cm height fresh water at approximately 25°C. The depressant activity of the plant extract was considered when mice became immobile and lost its hope for escaping from glass vessel. The mice were judged to be immobile when neither hind limb was moving and slightly hunched forward. The duration of immobility was observed and mice were put for five minutes in glass vessel. Fluoxetine was used as standard drug.

Hole board test: The hole board was done by the method of (Masood et al., 2008). The hole-board apparatus consisted of a wooden box (60 x 60 x 35 cm) with four equidistant holes 4 cm in diameter in the floor. Each animal was placed in one corner of the hole board and was observed for 5 min for the number of head dips and total time spent head dipping. Diazepam was used as standard drug.

Locomotor activity: In locomotor activity test Rebai and Djebli, 2008 procedure used. Mice were tested in cages (45 x 25 cm) divided into 16 equal squares. The number of crossed squares was recorded for each mouse per time of 5 min for 20 min investigation. Diazepam was used as positive control drug.

Elevated plus maze test: The elevated plus maze test was done by the method of Onasanwo et al., 2010. This apparatus was made of wood and consisted of 2 open arms
and 2 closed with 25 cm walls. The maze was elevated 38.5 cm from the room floor. The
time spent in open arms was recorded for 5 mins. Diazepam was used as standard drug.

**Black and white test**: In black and white test Young, 1991 method was followed. The
apparatus consisted of wooden box with two compartments, one of which was
illuminated with a white light while the other remained dark. The time spent in
illuminated places was recorded for 5 mins. Diazepam was used as standard drug.

**4.5 Biochemical observations**:  
After behavioural study a portion of cerebral cortex and midbrain were separated from
brain and washed with saline. Than the tissue were homogenized and used for following
biochemical assay.

**Catalase (EC 1.11.1.6)**: This was assayed by the method of Aebi, 1974. Catalase is a
common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the
decomposition of hydrogen peroxide to water and oxygen. The brain tissue homogenate
was added to H₂O₂ and product obtained , in which disappearance of H₂O₂ was measured
at 240 nm. The unit of enzyme was measured as u/min/mg protien. It is very important
enzyme in protecting the cell from oxidative damage by reactive oxygen species.
**Lactate dehydrogenase (EC 1.1.1.27):** Portion of cerebral cortex and midbrain tissue were homogenized for performing lactate dehydrogenase assay (Wroblewski and Ladue, 1955). Lactate dehydrogenase catalyzes the inter conversion of pyruvate and lactate with concomitant interconversion of NADH and NAD⁺. Brain tissue homogenate was added to sodium pyruvate and NADH. Then LDH was determined by measuring the rate of oxidation of NADH. The optical density of the product was read at 340 nm. The unit of LDH activity was measured as u/min/mg protein.

**Lipid peroxidation:** Brain (cerebral cortex and mid brain) tissue were homogenized in 0.15 M KCl for LPO assay by the method of Wilbur et al., 1949. Lipid peroxidation was assayed by estimating the level of malondialdehyde (MDA). TCA and TBA were added to brain tissue homogenate supernatant. The optical density of the mixture was recorded as 530 nm. The unit of LPO was measured as nm MDA /mg protein.

**Protein:** Protein was measured by Lowry et al., 1951 for calculating specific activity of the enzymes.

**Serum Glutamate Oxaloacetate Transaminase (EC 2.6.1.1):** Blood samples were collected (immediately after decapitation) in plain tubes and used for evaluation of SGOT by Reitman and Frankel, (1957) method. SGOT is an enzyme that is normally affected when the liver is damaged. SGOT converts L- Aspartate and α ketoglutarate to oxaloacetate and glutamate. The oxaloacetate formed reacts with 2, 4, dinitrophenyl hydrazine to produce a hydrazone derivative. The unit of the activity is u/ml.
**Serum Glutamate Pyruvate Transaminase (EC 2.6.1.2):** Blood samples were collected (immediately after decapitation) in plain tubes and used for evaluation of SGPT by Reitman and Frankel (1957) method. SGPT is an enzyme that is normally affected when the liver is damaged. SGPT converts L-alanine and α-ketoglutarate to pyruvate and glutamate. The pyruvate formed reacts with 2, 4 dinitrophenyl hydrazine to produce a hydrazone derivative. The unit of the activity is u/ml.

**4.6 Ultrastructural observations:**

**Transmission Electron Microscopy:** The mice were sacrificed and a small portion of cerebral cortex of the treated groups was transferred to 2% glutaraldehyde and preserved in fixative for transmission electron microscopy. The TEM of the sample was done in SAIF, North Eastern Hill University, Shillong.

**4.7 Calcium Channel Blocker Experiment:**

After the above mentioned study was done, the experiments with CCB mainly nimodipine was done to evaluate if it is able to attenuate the neurotoxicity of the plant extracts. In this study the plant extracts at following doses was administered - *A. scholaris* (100mg/kg, ip), *T. peruviana* (50mg/kg, orally) and *S. anacardium* (500mg/kg, ip). In another set of three groups the above mentioned plant extracts was added at specific doses. However, half an hour before the administration of these plant extracts, nimodipine was administered at 4 mg/ kg i.p. (Yanpallēwar *et al.*, 2004) to study if neurotoxicity is attenuated by administering nimodipine. In this set of experiments two
separate groups were also added viz. control to which distilled water was administered and the other to which nimodipine was administered at 4 mg/kg ip.

4.8 Antimicrobial study:

Antimicrobial study was performed to see the effect of the plant extract against microbes. This was determined against 5 bacterial strains. Viz, were *Bacillus sp*, *Staphylococcus sp* (Gram positive) and *Pseudomonas sp*, *Escherichia coli*, *Klebsiella sp* (Gram negative). Disc diffusion method was used to determine the zone of inhibition (Vincent *et al.*, 1944). Disc of all the three plants ie. *Alstonia scholaris*, *Thevetia peruviana* and *Semecarpus anacardium* were tested against the bacteria. The nutrient agar was cooled up to 45°C and was inoculated with bacteria under aseptic condition. Whatman No. 2 filter paper disc of 6mm diameter containing 5 mg/disc of plant extract was placed over the inoculated plate. Than the plates were incubated at 37°C for 24 hours. After that the zone of inhibition was measured using a zone reader. The standard disc taken was Ampicillin. The inoculums were collected from the Microbiology Department of Silchar Medical College & Hospital.

4.9 Statistical Analysis: All the values are expressed as mean ±se. All the values were evaluated by ANOVA along with Students-Newman Keuls post hoc analysis and Turkeys multiple comparison test (Rebai and Djeblí, 2008). The significant level is 0.05.
Plate: 1. Plants used for the study in various experiment

(A) *Alstonia scholaris* R.Br.  (B) *Thevetia peruviana* Merr.  
(C) *Semecarpus anacardium* L.f.