CHAPTER 3
MATERIALS AND METHODS
Experiment I (A & B) and Experiment II were carried out separately with specific objectives as mentioned earlier. The materials and methodology for each experiment have been described below.

EXPERIMENT I (A): SKIN PAPILLOMAGENESIS STUDY:

To study the modulatory influence of Eclipta alba on 7,12-dimethylbenz(a)anthracene (DMBA) induced skin papillomagenesis in female Swiss Albino mice.

Chemicals:

The carcinogen (initiator), 7,12-dimethylbenz(a)anthracene (DMBA), and the promoter i.e., croton oil were procured from Sigma Chemicals Co., St. Louis, U.S.A. Acetone was procured from a local firm of India and was of highest purity grade.

Test animals:

Randomly bred female Swiss Albino mice, 6-8 weeks old were used for the experiment. Permission was obtained from the Institutional Animal Committee of Gauhati University to pursue the study. They were provided with standard pellet diet and tap water ad libitum. The animals were housed under uniform husbandry conditions having natural photoperiod, humidity and
ambient temperature. The hairs on the dorsal interscapular region of the mice were clipped off before the commencement of the experiment and kept under observation for three days. After three days, mice showing resting phase of hair growth cycle were selected for the study.

**Test material/ modulator:**

The herb, *Eclipta alba* was collected locally from various parts of Guwahati, Assam, India after proper identification by a competent botanist from the Department of Botany, Gauhati University, Guwahati, Assam (Plate I). The plant was washed and dried in shade without direct exposure to sunrays. It was then grounded and 50 gram of the material thus obtained was subjected to soxhlet extraction using 80% of hydro- alcoholic solvent (80% ethanol: 20 % distilled water). After that the alcohol was allowed to evaporate and the residue obtained was stored at 4°C until further use.

**Dose of the modulator used:**

The modulator was diluted appropriately in acetone to obtain a dose level of 5mg/Kg body weight / day so that the dose of the modulator remain at par the dose of initiator concentration.
Plate - I: Plant used in the study
(a) Flower of Eclipta alba
(b) Fruit of Eclipta alba
(c) Part of the Plant
(d) Plant in natural environment
Experimental design:

Mice showing no hair growths on the dorsal skin were segregated into four groups each containing ten (n=10) animals i.e. a total of 40 animals.

The chemopreventive action of the extract of *Eclipta alba* on DMBA induced mouse skin papillomagenesis have been studied by the method of Berenblum and Shubik (1947). Papillomas appearing on the shaven area of the skin were recorded at weekly intervals. Only those papillomas were considered for final analysis that persisted for two weeks or more. Animals were sacrificed 15 weeks after the commencement of the experiment. The following parameters were observed to study the influence of *E. alba* on mouse skin carcinogenesis:

1. Tumor incidence (percentage of papilloma bearing mice)
2. Tumor yield (average number of papillomas per mouse)
3. Tumor burden (papillomas per papilloma bearing mouse)
4. Cumulative number of papillomas
5. Percent inhibition of tumor multiplicity

Statistical analysis:

The significant level of difference between control and experimental values were statistically evaluated by student’s t test and considered significant at 5% significance level (P<0.05).
Table 6: Experimental design for evaluation of the modulatory influence of *Eclipta alba* on DMBA induced skin papillomagenesis in mice

<table>
<thead>
<tr>
<th>GROUP</th>
<th>NUMBER OF ANIMALS</th>
<th>TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10</td>
<td>Mice treated topically with DMBA (50μg/μl of acetone), followed by repeated application of croton oil (1% in acetone; three times a week) till the end of the experiment.</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>Mice treated with DMBA and croton oil as in group I + topical application of <em>Eclipta alba</em> extract (5mg/Kg body weight /day) for 14 days (i.e., 7 days before and 7 days after the application of DMBA)</td>
</tr>
<tr>
<td>III</td>
<td>10</td>
<td>Mice received a topical treatment of <em>Eclipta alba</em> extract (5mg/Kg body weight/day) for 91 days (i.e., starting from the time of croton oil treatment). DMBA and croton oil was given as in group I.</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>Mice received a topical treatment of <em>Eclipta alba</em> extract (5mg/Kg body weight/day) for 105 days (i.e., 7 days before the treatment of DMBA and continued till the end of the experiment). DMBA and croton oil was given as in group I.</td>
</tr>
</tbody>
</table>
EXPERIMENT I (B): Histopathological study

To study the effect of *Eclipta alba* extract on the dorsal skin of the control and treated mice in comparison to the normal skin tissue.

The animals from experiment I (A) were sacrificed at the end of the experiment (after 15 weeks of treatment) and were considered for histopathological study.

Chemicals:

The following chemicals were used for the histopathological study. Absolute alcohol, 90% alcohol, 70% alcohol, 50% alcohol, 30% alcohol, xylene, haematoxylin, eosin, paraffin, 10% formalin, Mayer’s albumen and DPX.

Preparation of skin for histology:

Affected skin and skin with papillomas were fixed in 10% formalin and dehydrated with graded alcohols starting from 30% alcohol to absolute alcohol. Then the tissues were embedded in paraffin after clearing in xylene. Serial microtome sections (4 μ) were stained in haematoxylin and eosin (Kehar and Wahi, 1967).
EXPERIMENT II: ENZYME ASSAYS.

To study the modulatory influence of *Eclipta alba* on Hepatic Phase I enzymes i.e., Cytochrome P$_{450}$ (Cyt P$_{450}$) and Cytochrome b$_{5}$ (Cyt b$_{5}$), Phase II enzymes i.e., Glutathione S-transferase (GST), Glutathione content (GSH), antioxidant enzyme profiles i.e., Superoxide Dismutase (SOD), Catalase (CAT) and Lipid Peroxidation (LPO) in Swiss albino mice.

**Chemicals:**

The following chemicals were used for the enzyme assay. 5,5-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), pyrogallol, triton-X-100, ethylene diamine tetra acetic acid (EDTA), thiobarbituric acid (TBA), reduced nicotinamide adenine dinucleotide (NADH) were obtained from Sigma chemicals Co. (St. Louis, U.S.A.). The rest of the chemicals were obtained from local firms (India) and are of highest purity grade.

**Test animals:**

Random bred, 6-8 weeks old female Swiss albino mice were selected for the study and maintained under hygienic conditions. They were provided with standard pellet diet and tap water ad libitum. The animals were housed under uniform husbandry conditions having natural photoperiod, humidity and ambient temperature.
Test material/modulator:

The herb, *Eclipta alba* was collected locally from various parts of Guwahati, Assam, India after proper identification by a competent botanist from the Department of Botany, Gauhati University, Guwahati, Assam. The plant was washed and dried in shade without direct exposure to sunrays. It was then grounded and 50 gram of the material thus obtained was subjected to soxhlet extraction using 80% of hydro-alcoholic solvent (80% ethanol: 20% distilled water). After that the alcohol was allowed to evaporate and the residue obtained was stored at 4°C until further use.

Experimental design:

A total of 40 animals were taken and randomly assorted into four groups containing ten animals in each group.

Body weights of the mice were recorded initially, at weekly intervals and at the end of the experiment. Twelve hours before the termination of the experiment diet was withheld from the animals.
Table 7: Experimental design for evaluation of the enzyme assays

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>NUMBER OF ANIMALS</th>
<th>TREATMENT</th>
<th>DOSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (NEGATIVE CONTROL)</td>
<td>10</td>
<td>Only vehicle; distilled water</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td><em>Eclipta alba</em> extract</td>
<td>125mg/Kg body weight/day</td>
</tr>
<tr>
<td>III</td>
<td>10</td>
<td><em>Eclipta alba</em> extract</td>
<td>250mg/Kg body weight/day</td>
</tr>
<tr>
<td>IV (POSITIVE CONTROL)</td>
<td>10</td>
<td>Butylated Hydroxy Anisole(BHA)</td>
<td>0.75% in diet</td>
</tr>
</tbody>
</table>
PREPARATION OF HOMOGENATES, CYTOSOL AND MICROSONAL FRACTIONS:

The animals were sacrificed by cervical dislocation and the gall bladder was carefully removed. Then the entire liver was perfused immediately with 0.9% ice cold NaCl solution and thereafter carefully dissected out and rinsed in chilled 0.15 M Tris KCl buffer. Acid microsomal fraction was prepared by the method of Fry and Bridges (1975). The liver was blotted dry and 500mg of liver was weighed quickly and homogenized in 2 ml of ice cold 1.15 % (w/v) KCl (by 3 up and down stroke at maximum speed). The homogenate was then centrifuged at 10,000g for 20 minutes at 4 ºC. The supernatant was taken and the pH was adjusted to 5.4 with acetate buffer (pH 4.0). It was then centrifuged at 10,000g for 10 minutes at 4 ºC. The supernatant was discarded and the refrigerated pellet after washing in 1.15% KCl; glycerol (4:1 v/v) was recentrifuged at 10,000g for 10 minutes at 4 ºC. The thin lipid layer over the pellet was removed and the supernatant was discarded carefully so that no loose pellet may fall down from the tube. The pellet that was obtained after centrifugation represented the microsomal fraction. The pellet was then suspended in 1ml of 50mM Tris-HCl buffer (pH-7.4) by one complete stroke in the hand held homogenizer to obtain the acid microsomal fraction, which was used for the assay of LPO.
The remaining portion of the liver was weighed quickly and homogenized in ice cold 0.15M Tris-KCl buffer (pH-7.4) to yield 10 % (w/v) homogenate at 4°C. An aliquot of this homogenate (0.5ml) was used for assaying acid soluble sulphhydryl group (-SH) while the remaining homogenate was centrifuged at 10,000g for 20 minutes at 4°C. The supernatant (cytosol fraction), after discarding any floating lipid layer and appropriate dilution, were used for the assay of GST and antioxidant enzymes.

ASSAY METHODS:

DETERMINATION OF SGOT AND SGPT:

Serum glutamate oxaloacetate transaminase (SGOT) and Serum glutamate pyruvate transaminase (SGPT) were determined following the method described in the kit. Activities of these two enzymes were expressed as U/L.

DETERMINATION OF MICROSOMAL Cyt b5 CONTENT:

Principle:

Cyt b5 was estimated from its redox spectrum of NADH reduced versus oxidized cytochrome that peaks at 424 nm.
Procedure:

Assays were performed according to the method of Omura and Sato (1964). The microsomal pellet obtained was re-suspended in 1 ml of homogenizing buffer. From this 500 ml sample was taken and mixed in a test tube containing 4.3 ml of 50 mM Tris HCl buffer (pH 7.4). The contents of the tube was thoroughly mixed, divided equally into two spectrophotometer cuvettes and were scanned in double beam spectrophotometer between 400-500 nm per baseline correction. To the cuvette containing test sample 100 ml of 2mM NADH and to the reference cuvette equal amount of Tris HCl (pH 7.4) buffer was added. Reaction was allowed to proceed for 2 minutes. The redox spectrum between 400nm-500nm was recorded. Cyt b₅ was determined as the absorption difference between 424nm-409nm. The cyt b₅ content is expressed as nano moles / mg protein using a molar extinction coefficient of 185 mM⁻¹cm⁻¹.

DETERMINATION OF MICROSOMAL Cyt P450 CONTENT:

Principle:

Reduced Cyt P₄₅₀ combines with carbon monoxide to give a characteristic absorption maximum at 450nm.
**Procedure:**

Assays were performed according to the method of Omura and Sato (1964). For estimation of Cyt P$_{450}$ the sample used for determination of Cyt b$_5$ content was not discarded but were mixed in a test tube properly. A pinch of sodium dithionate was added to this 5 ml sample to reduce haem proteins. The contents of the tube were equally divided between two spectrophotometrically matched cuvettes and were scanned for baseline correction between 400-500nm. Carbon monoxide produced by mixing formic acid and sulphuric acid in a specially designed apparatus was gently bubbled for 20 seconds and cuvettes were scanned between 400-500nm. Cyt P$_{450}$ was determined as absorption difference between 450-490nm. Cyt P$_{450}$ is expressed as nano moles/ mg protein using a molar extinction coefficient of 91mM$^{-1}$cm$^{-1}$.

**DETERMINATION OF CYTOSOLIC GST ACTIVITY:**

**Principle:**

The activity of GST was determined by the rate of formation of CDNB-GSH conjugate. The conjugate absorbs at 340 nm.

RX + GSH$_{GST}$$\rightarrow$ HX + R-SG
Procedure:

The cytosolic GST activity was determined spectrophotometrically at 37°C by the method of Habig et al (1974). The reaction mixture (1ml) contained 334μl of 100mM phosphate buffer (pH-6.5), 33 μl of 30mM CDNB (in 95% ethanol), 33 μl of 30mM of reduced glutathione and 590μl distilled water. After preincubating the reaction mixture for two minutes, the reaction was started by adding 10 μl of diluted cytosol and the absorbance was followed for 3 minutes at 340 nm using Cintra-5 UV- VIS double beam spectrophotometer of GBC, Australia make. The specific activity of glutathione S-transferase is expressed as moles of GSH-CDNB conjugate formed/min/mg protein using extinction co-efficient of 9.6mM⁻¹ cm⁻¹.

DETERMINATION OF ACID SOLUBLE SULPHHYDRL GROUP (GSH):

PRINCIPLE:

The acid soluble sulphhydryl group (non protein thiols of which more than 90% are reduced glutathione) forms a yellow coloured complex with DTNB that absorbs at 412 nm.

Reduced glutathione + DTNB → 5-thio-2-nitro benzoate

(Yellow)
PROCEDURE:

The GSH content of the tissue homogenate was estimated by the method as described by Moron et al (1979). 500 µl of homogenate was immediately precipitated by 100 µl of 25% trichloroacetic acid and the tissue debris was removed after centrifugation in a microfuge at 10,000g for 20 minutes at 4°C. Free –SH groups were assayed in a total volume of 3ml by adding 2 ml of 0.6 mM DTNB prepared in 0.2 M Sodium phosphate buffer (pH-8.0) and 900 µl of sodium phosphate buffer (pH-8.0) to 100 µl of the supernatant and the absorbance was read at 412 nm using a Cintra UV-VIS double beam spectrophotometer of GBC, Australia make. Sulphydryl content is expressed as nanomole GSH/gm tissue.

DETERMINATION OF CYTOSOLIC SOD ACTIVITY:

Principle:

The specific activity of SOD has been measured by means of inhibition of Pyragallol auto oxidation.

Procedure:

SOD activity was assayed by the method of Marklund (1974), which involves inhibition of Pyragallol auto oxidation by the enzyme at pH 8.0. A single unit of enzyme is defined as the quantity of SOD required to produce
50% inhibition of auto oxidation. The reaction mixture (1 ml, vol.) contained 10 μl of suitably diluted cytosol in 25% triton-X 100, 500 μl of 0.1M sodium phosphate buffer (pH 8.0), 33 μl of 0.111% EDTA, 60 μl of 8.1 mM Pyragallol and 397 μl distilled water. One unit of enzyme activity has been expressed as μ mole /mg protein measured at 420nm using a Cintra-5 UV-VIS double beam spectrophotometer of GBC, Australia make.

**DETERMINATION OF CYTOSOLIC CAT ACTIVITY:**

**PRINCIPLE:**

The rate of decomposition of H₂O₂ by catalase was employed for determination of CAT activity. In the UV range, H₂O₂ shows a continual increase in absorption with decreasing wavelength. The decomposition rate was measured by the decrease in absorbance at 240nm. The difference in absorbance per unit time is a measure of catalase activity.

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

**Procedure:**

Activity of CAT was estimated at 240nm in a Cintra-5 UV-VIS double beam spectrophotometer (GBC, Australia) as described by Aeibi (1984). The reaction mixture (1ml, vol.) contained 20 μl of suitably diluted cytosol in triton X-100 and ethanol, 500 μl of 0.1M sodium phosphate buffer (pH-7.0)
and 380 μl distilled water. The reaction was started by adding 100 μl of 2mM H$_2$O$_2$. The specific activity of CAT has been expressed as μ moles of H$_2$O$_2$ consumed/ min / mg protein.

**DETERMINATION OF LPO ACTIVITY:**

**PRINCIPLE:**

The level of LPO was estimated by measuring the formation of malondialdehyde using TBA reaction. The development of colour with the same absorption characteristics (absorption maxima at 531.8 nm) as TBA-MDA chromophore has been taken as an index of LPO. The decreased activity of LPO is expressed as nanomoles malondialdehyde formed/mg protein.

**PROCEDURE:**

LPO in microsomes prepared from liver, was estimated spectrophotometrically using Cintra-5 UV-VIS double beam spectrophotometer of GBC, Australia make by thiobarbituric acid reactive substances (TBARS) method as described by Varshney and Kale (1990) and is expressed in terms of malondialdehyde (MDA) formed /mg protein. In brief, 0.3 ml of microsomal sample was mixed with 1.7ml of 0.15M Tris KCl buffer (pH-7.4) to which 0.5ml of 30% TCA was added. Then 0.5ml of 52mM TBA was mixed and placed in a water bath for 45 minutes at 80°C cooled in
ice and centrifuged at room temperature for 10 minutes at 4000rpm. The absorbance of the clear supernatant was measured against reference blank of distilled water at 531.8nm.

DETERMINATION OF CYTOSOLIC AND MICROSOMAL PROTEIN:

PRINCIPLE:

The total protein content of various fractions was estimated by following the method of Lowry et al., 1951. The blue colour developed due to the reactions of the samples with Folin phenol reagent was read at 750nm using Bovine Serum Albumin (BSA) as standard.

PROCEDURE:

Firstly, two reagents were prepared –2% sodium carbonate in 0.1N sodium hydroxide solution (Reagent A) and 0.5% copper sulphate in 1% sodium – potassium tartarate (Reagent B). Alkaline copper solution (Reagent C) was prepared by adding 50ml of reagent A to 1ml of reagent B just before use. The Folin phenol reagent was diluted by adding 1 volume of Folin phenol reagent with 2 volume of water just before use to give a 1:2 dilution. 1% BSA solution was used as the standard. 0.1 ml of the liver homogenate was taken in test tube and was again added with 0.9ml of double distilled water. The same
volume of standard was taken and made up to 1ml with double distilled water. The blank contained 1ml of distilled water. To all the test tubes 5ml of the reagent C was added and allowed to stand for 10 minutes followed by addition of 0.5 ml of Folin phenol reagent. The solution in all the three tubes were mixed well and incubated for 30 minutes in a dark place at room temperature. After 30 minutes a blue colour developed and the absorption maxima were measured using a Cintra-5 UV-VIS double beam spectrophotometer, GBC, Australia make.

**STATISTICAL ANALYSIS:**

All the results are expressed as mean ± S.D of 8-10 animals and the statistical significance of differences between the groups was determined by ANOVA (Analysis of Variance).