3.1 Experimental Animal:

Swiss albino mice (*Mus musculus*) 10-12 weeks old and weighing between 22-28 g of both sexes purchased from the Pasteur Institute, Shillong (Meghalaya, India) were used in this study. The present investigation was conducted with due permission and proper guidelines from the Institutional Animal Ethics Committee. Many of the toxicological experiments were conducted using mice as a test model. In addition, mice were a popular experimental organism with geneticists because of the following reasons.

- They have fairly large litters; 8-10 is common for some strains.
- They are small for a mammal and easily housed.
- They have a short generation time.
- They are easy to work with in general.

As mammals, mice were considered a perfect model; their body plan, physiology, molecular mechanisms and genome share many features with human. It is found that 99% of mouse genes turn out to be analogues with human. In addition the advantage of using mouse as a model is that it posses proliferating cells both in somatic (bone marrow) and germinal (testes) tissues,
which makes the genotoxicity and tumorigenicity studies more convenient. Traditional breeding methods have resulted in the generation of many highly inbred lines of mice which have been very useful in studies of the immune system as well as other areas. Although other animals, plants and bacteria have been extremely useful in many ways, no other single experimental animal offers such a wide variety of uses to science and medicine as a mouse.

Figure 8:
Photograph of Swiss albino mice (A): normal front view; (B): normal side view; (C): tumor bearing front view; (D): tumor bearing side view.

Mice can tolerate the experimental stress conditions and they serve as a sensitive indicator of clastogenic, mutagenic and carcinogenic effects. Hence, mice act as a valuable model in order to understand the function and operation
of the genetic machinery in humans. Swiss albino mice of both sexes of closely inbred colony were used for genotoxic (Chromosome aberration assay, Micronucleus assay, Sperm head abnormality assay and Total sperm count) and tumorigenic studies.

**Maintenance of Animals:**

Animals were caged in solid-walled plastic cages with stainless steel mesh lids. They were housed in a group of two/three in small cages and four-six in large cages. Sexes were kept separate and the animals were maintained in closely inbred colony under conventional laboratory conditions at room temperature 26.0 ± 5.0 °C and in 12 hour light/dark cycle. Mice were provided with standard food pallet and water *ad libitum*. Cages were cleaned twice weekly. The experiments were conducted as per Assam University Institutional Animal Ethical Committee guidelines.

Three animals were selected for every experiment of genotoxic studies and five animals were selected for every experiment of tumorigenic studies. Since mice were selected from closely inbred colony there would be less probability of genetic divergence among the animals. Hence, a very small size of animals (3 and 5 animals per genotoxic and tumorigenic experiments respectively) would suffice the purpose as genetical response of the animals towards the chemical would not vary much from each other.
3.2 Experimental Chemicals:

3.2.1 Laboratory reagents:

Glacial acetic acid, Methanol, Sodium chloride, Potassium chloride, Vitamin C (L-Ascorbic acid) was purchased from Qualigens. Colchicine and P-DAB (Dimethyl Yellow) were purchased from Sigma chemicals Co. (St Louis, MO). Giemsa's stain, Eosin Y stain, Dichloromethane, Tricarboxylic acid (TCA), Thiobarbituric acid (TBA), Ammonium Chloride (NH₄Cl) were procured from Himedia Laboratories Pvt. Ltd., Mumbai, India. Mitomycin C (MMC) was obtained from Cadila Pharmaceuticals, India. Dimethyl sulphoxide (DMSO) was purchased from S.D. Fine-chem Limited, Mumbai, India.

All other chemicals were purchased from Himedia and Qualigens. All the reagents used for treatment, buffer were freshly prepared in distilled water before administration into the test system and prior to experimentation respectively.

3.2.2 Alkaloid, dose and treatment

Arecoline

Arecoline hydrobromide (1, 2, 5, 6-tetrahydro-1-methyl-3-pyridine carboxylic acid methyl ester hydrobromide), the major alkaloid of areca nut was selected for genotoxic studies and lipid peroxidation studies. Arecoline have the chemical formula C₈H₁₄BrNO₂, CAS No. 300-08-3, molecular weight of 236.11 g/Mol. Arecoline was purchased from Sigma-Aldrich (St. Louis, MO). The chemical structure is provided in Figure 9 in the next page.
3.2 Experimental chemicals

<table>
<thead>
<tr>
<th>Areca</th>
<th>Chemical Name</th>
<th>Chemical Formula</th>
<th>Molecular Weight</th>
<th>Chemical Structure</th>
<th>CAS No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>nut</td>
<td>Arecoline hydrobromide (1,2,5,6-tetrahydro-1-methyl-3-pyridinecarboxylic acid methyl ester hydrobromide)</td>
<td>C$<em>8$H$</em>{14}$BrNO$_2$</td>
<td>236.11</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>300-08-3</td>
</tr>
</tbody>
</table>

Figure 9:
Chemical structure of areca nut alkaloid Arecoline.

Three acute dose 20, 50 and 80 mg/kg body weight (bw) of arecoline were given orally by gavage (p.o) to Swiss albino mice. The oral LD$_{50}$ value for male mice was 371 mg/kg whereas it was 309 mg/kg bw for female mice (Selvan et al., 1989). Double distilled water was used as a solvent and it was freshly prepared, prior to the administration to the animals. The Table 6, below represents the alkaloid, arecoline dose, their route of administration and exposure time. The animals tolerated the dose of metabolite without any sign of toxicity.

MMC was obtained from Cadila Pharmaceuticals, India. MMC, 2 mg/kg bw was used as a positive control for the study of genotoxic effect in mice bone marrow cells and sperm cells from the cauda epididymis of testis. It was administered intra-peritoneally (i.p). The control animals received an equal volume of distilled water.

Vitamin C was purchased from Qualigens, India. The antioxidant, Vitamin C was selected for studying the protective role and oxidative stress. The
3.2 Experimental chemicals

Table 6

Table showing areca nut alkaloid Arecoline, Mitomycin C and Vitamin C dose, route of administration and exposure period

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Dose</th>
<th>Route</th>
<th>Exposure Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arecoline</td>
<td>(ARC1) 20 mg/kg bw</td>
<td>p.o.</td>
<td>24 hour</td>
</tr>
<tr>
<td></td>
<td>(ARC2) 50 mg/kg bw</td>
<td></td>
<td>48 hour</td>
</tr>
<tr>
<td></td>
<td>(ARC3) 80 mg/kg bw</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>(MMC) 2 mg/kg bw</td>
<td>i.p.</td>
<td>24 hour</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48 hour</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>(Vit. C) 500 mg/kg bw</td>
<td>p.o.</td>
<td>5 days</td>
</tr>
</tbody>
</table>

chemical formula and structure were provided in Figure 10. The antioxidant Vitamin C solution was prepared freshly in double distilled water, prior to the administration to the animals. The Table 6, above represents the antioxidant, Vitamin C dose, their route of administration and duration of treatment. The dose was selected on the basis of available literature and Vitamin C was always given orally for consecutive five days prior to the test chemical/extract treatment. The animals tolerated the dose of 500 mg/ kg bw of antioxidant without any sign of toxicity.

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Chemical Formula</th>
<th>Molecular Weight</th>
<th>Chemical Structure</th>
<th>CAS No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anticancer drug</td>
<td>Mitomycin C</td>
<td>C_{15}H_{18}N_{4}O_{5}</td>
<td>334.3</td>
<td><img src="image" alt="Mitomycin C" /></td>
</tr>
<tr>
<td>Antioxidant (Vitamin C)</td>
<td>Ascorbic acid</td>
<td>C_{6}H_{8}O_{6}</td>
<td>176.13</td>
<td><img src="image" alt="Vitamin C" /></td>
</tr>
</tbody>
</table>

Figure 10:
Chemical structure of Mitomycin C and Vitamin C.
### 3.2 Experimental chemicals

#### 3.2.3 Metabolites, dose and treatment:

<table>
<thead>
<tr>
<th>Arecoline Metabolites</th>
<th>Chemical Name</th>
<th>Chemical Formula</th>
<th>Molecular Weight</th>
<th>Chemical Structure</th>
<th>CAS No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Arecaidine hydrochloride (1-methyl-1,2,5,6-tetrahydropyridine-3-carboxylic acid hydrochloride)</td>
<td>C$<em>7$H$</em>{11}$NO$_2$.HCl</td>
<td>177.6</td>
<td><img src="image" alt="Arecaidine Structure" /></td>
<td>6018-28-6</td>
</tr>
<tr>
<td>2.</td>
<td>Arecoline N-oxide (((R,S))-1-Methyl-1,2,5,6-tetrahydropyridine-3-carboxylic Acid 1-Oxide Methyl Ester)</td>
<td>C$<em>8$H$</em>{13}$NO$_3$</td>
<td>172.097</td>
<td><img src="image" alt="Arecoline N-oxide Structure" /></td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>N-methyl nipecotic acid (1-methyl-3-piperidine carboxylic acid)</td>
<td>C$<em>7$H$</em>{13}$NO$_2$</td>
<td>144.102</td>
<td><img src="image" alt="N-Methyl Nipecotic Acid Structure" /></td>
<td>-</td>
</tr>
</tbody>
</table>

**Figure 11:**
Chemical structure of metabolites of Arecoline: Arecaidine, Arecoline N-oxide and N-methyl Nipecotic Acid.

**Arecaidine:**

Arecaidine hydrochloride (1-methyl-1, 2, 5, 6-tetrahydropyridine-3-carboxylic acid hydrochloride), the major metabolite of arecoline was selected for genotoxic studies and lipid peroxidation studies. Arecaidine have the chemical formula C$_7$H$_{11}$NO$_2$.HCl, CAS No. 6018-28-6, molecular weight of 177.6. Arecaidine was purchased from Sigma-Aldrich (St. Louis, MO). The chemical structure is provided in Figure 11, above.
Three acute dose 20, 50 and 80 mg/kg bw of arecaidine were administered intra-peritoneally (i.p) to Swiss albino mice. Double distilled water was used as a solvent and it was freshly prepared, prior to the administration to the animals. The Table 7, below represents the metabolite, arecaidine dose, their route of administration and exposure time. The animals tolerated the dose of metabolite without any sign of toxicity. The chemical dose, their dilution and mode of administration was selected and based on literature survey and toxicity studies.

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Dose</th>
<th>Route</th>
<th>Exposure Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arecaidine</td>
<td>(ARK1) 20 mg/kg bw</td>
<td>(ARK2) 50 mg/kg bw</td>
<td>(ARK3) 80 mg/kg bw</td>
</tr>
<tr>
<td>Arecoline N-oxide</td>
<td>(ARC-NO1) 20 mg/kg bw</td>
<td>(ARC-NO2) 50 mg/kg bw</td>
<td>(ARC-NO3) 80 mg/kg bw</td>
</tr>
<tr>
<td>N-methyl Nipecotic acid</td>
<td>(NA1) 20 mg/kg bw</td>
<td>(NA2) 50 mg/kg bw</td>
<td>(NA3) 80 mg/kg bw</td>
</tr>
</tbody>
</table>

**Arecoline N-oxide**

Arecoline N-oxide ((R, S)-1-Methyl-1, 2, 5, 6-tetrahydropyridine-3-carboxylic Acid 1-Oxide Methyl Ester), the metabolite of arecoline was selected for genotoxic studies and lipid peroxidation studies. Arecoline N-oxide has the chemical formula C₈H₁₃NO₃, molecular weight of 172.097 and it was
3.2 Experimental chemicals

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synthesized in NCI-NIH, USA. The chemical structure is provided in Figure 11, above.

Three acute dose 20, 50 and 80 mg/kg bw of arecoline N-oxide were administered intraperitoneally (i.p) to Swiss albino mice. Double distilled water was used as a solvent and it was freshly prepared, prior to the administration to the animals. The Table 7, above represents the metabolite, arecoline N-oxide dose, their route of administration and exposure time. The animals tolerated the dose of metabolite without any sign of toxicity. The chemical dose, their dilution and mode of administration was selected based on literature survey and toxicity studies.

**N-methyl Nipecotic acid**

N-methyl nipecotic acid (1-methyl-3-piperidine carboxylic acid), the metabolite of arecoline was selected for genotoxic studies and lipid peroxidation studies. N-methyl nipecotic acid has the chemical formula C$_7$H$_{13}$NO$_2$, molecular weight of 144.102 and it was synthesized in NCI-NIH, USA. The chemical structure is provided in Figure 11, above.

Three acute dose 20, 50 and 80 mg/kg bw of N-methyl nipecotic acid were administered intraperitoneally (i.p) to Swiss albino mice. Double distilled water was used as a solvent and it was freshly prepared, prior to the administration to the animals. The Table 7, above represents the metabolite, N-methyl nipecotic acid dose, their route of administration and exposure time. The animals tolerated the dose of metabolite without any sign of toxicity. The
3.2 Experimental chemicals

chemical dose, their dilution and mode of administration was selected based on literature survey and toxicity studies.

3.2.4. Extracts, dose and treatment

Table 8
Table showing areca nut extract, sadagura extract and areca nut with sadagura extract dose, route of administration and exposure period.

<table>
<thead>
<tr>
<th>Extract Name</th>
<th>Dose</th>
<th>Route</th>
<th>Scoring Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Areca nut extract</td>
<td>LOW</td>
<td>MIDDLE</td>
<td>HIGH</td>
</tr>
<tr>
<td>(AN1)</td>
<td>20 mg/kg bw</td>
<td>x 5 days</td>
<td>80 mg/kg bw x 5 days</td>
</tr>
<tr>
<td>(AN2)</td>
<td>50 mg/kg bw</td>
<td>x 5 days</td>
<td>80 mg/kg bw x 5 days</td>
</tr>
<tr>
<td>(AN3)</td>
<td>80 mg/kg bw</td>
<td>x 5 days</td>
<td>80 mg/kg bw x 5 days</td>
</tr>
<tr>
<td>Sadagura extract</td>
<td>LOW</td>
<td>MIDDLE</td>
<td>HIGH</td>
</tr>
<tr>
<td>(SG1)</td>
<td>20 mg/kg bw</td>
<td>x 5 days</td>
<td>80 mg/kg bw x 5 days</td>
</tr>
<tr>
<td>(SG2)</td>
<td>50 mg/kg bw</td>
<td>x 5 days</td>
<td>80 mg/kg bw x 5 days</td>
</tr>
<tr>
<td>(SG3)</td>
<td>80 mg/kg bw</td>
<td>x 5 days</td>
<td>80 mg/kg bw x 5 days</td>
</tr>
<tr>
<td>Areca nut with sadagura extract</td>
<td>LOW</td>
<td>MIDDLE</td>
<td>HIGH</td>
</tr>
<tr>
<td>(AN+SG1)</td>
<td>20 mg/kg bw</td>
<td>x 5 days</td>
<td>80 mg/kg bw x 5 days</td>
</tr>
<tr>
<td>(AN+SG2)</td>
<td>50 mg/kg bw</td>
<td>x 5 days</td>
<td>80 mg/kg bw x 5 days</td>
</tr>
<tr>
<td>(AN+SG3)</td>
<td>80 mg/kg bw</td>
<td>x 5 days</td>
<td>80 mg/kg bw x 5 days</td>
</tr>
</tbody>
</table>

Areca nut extract:

For the preparation of areca nut extract (AN), ripe, dry areca nuts were obtained from the local market of Irongmara, Silchar, India. After removal of the fibrous coats, the nuts were cutted into thin slices, oven dried and powdered by using mixer-grinder. Preparation of extracts was following the protocol of Bhide et al., 1991 with minor modifications. Briefly, powdered areca nut was mixed with dichloromethane in (1:10 ratio) and kept in shaker at room temperature for 72 hour. The mixture were then filtered and dried. The residue
was dissolved in DMSO at a concentration of 60 mg/ml and stored at -20°C for further use.

Three acute doses 20, 50 and 80 mg/kg bw of AN was given orally by gavage (p.o) to Swiss albino mice for five consecutive days and scoring was done after 24 hour and 48 hour of last treatment day, for genotoxicity and lipid peroxidation studies. Single dose of 80 mg/kg bw of AN was given orally by gavage (p.o) to Swiss albino mice for fourteen consecutive days and host survival and other parameters were monitored up to 90 days.

DMSO was used as a solvent and the extract solution was freshly prepared prior to the administration to the animals. The control animals received an equal volume of DMSO given orally. The Table 8, above represents the AN dose, their route of administration and exposure time. The animals tolerated the dose of extract without any sign of toxicity. The extract dose, their dilution and mode of administration was selected based on literature survey and toxicity studies.

**Sadagura extract**

Sadagura extract (SG) was prepared following local formulation with dried tobacco leaves, fenugreek seed and aniseed mixed in 2:4:1 ratio and all the ingredients were purchased from local market of Irongmara, Silchar, India. Then, the mixture was powdered. Now, the powdered mixture is locally called ‘Sadagura’. Preparation of extracts was following the protocol of Bhide et al., 1991 with minor modifications. In order to prepare the extract, sadagura was
mixed with dichloromethane in (1:10 ratio) and kept in shaker at room temperature for 72 hour. The mixture were then filtered and dried. The residue was dissolved in DMSO at a concentration of 60 mg/ml and stored at -20°C for further use.

**Preparation of Sadagura**

![Tobacco leaf (locally called Sada)](image1)

![Aniseed (locally called Mouri)](image2)

![Fenugreek (locally called Kali jira)](image3)

**Figure 12:**
Photograph showing the components of smokeless tobacco preparation, *sadagura*.

Three acute doses 20, 50 and 80 mg/kg bw of SG was given orally by gavage (p.o) to Swiss albino mice for five consecutive days and scoring was done after 24 hour and 48 hour of last treatment day, for genotoxicity and lipid peroxidation studies. Single dose of 80 mg/kg bw of *sadagura* extract was given orally by gavage (p.o) to Swiss albino mice for fourteen consecutive days and host survival and other parameters were monitored up to 90 days.
DMSO was used as a solvent and the extract solution was freshly prepared prior to the administration to the animals. The control animals received an equal volume of DMSO given orally. The Table 8, above represents the SG dose, their route of administration and exposure time. The animals tolerated the dose of extract without any sign of toxicity. The extract dose, their dilution and mode of administration was selected based on trial and error studies.

**Areca nut with sadagura extract**

*Areca nut with Sadagura* (AN+SG) extract was prepared in 1:1 ratio of areca nut powdered and *sadagura*, which was prepared by mixing dried tobacco leaves, fenugreek seed and aniseed mixed in 2:4:1 ratio and all the ingredients were purchased from local market of Irongmara, Silchar, India. Preparation of extracts was following the protocol of Bhide et al., 1991 with minor modifications. The powdered areca nut and *sadagura* were mixed with dichloromethane in (1:10 ratio) and kept in shaker at room temperature for 72 hour. The mixture were then filtered and dried. The residue was dissolved in DMSO at a concentration of 60 mg/ ml and stored at -20°C for further use.

Three acute doses 20, 50 and 80 mg/kg bw of AN+SG was given orally by gavage (p.o) to Swiss albino mice for five consecutive days and scoring was done after 24 hour and 48 hour of last treatment day, for genotoxicity and lipid peroxidation studies. Single dose of 80 mg/kg bw of areca nut with *sadagura* extract was given orally by gavage (p.o) to
Swiss albino mice for fourteen consecutive days and host survival and other parameters were monitored up to 90 days.

DMSO was used as a solvent and the extract solution was freshly prepared prior to the administration to the animals. The control animals received an equal volume of DMSO given orally. The Table 8, above represents the AN+SG dose, their route of administration and exposure time. The animals tolerated the dose of extract without any sign of toxicity. The extract dose, their dilution and mode of administration was selected based on trial and error studies.

3.3 Experimental Protocols:

Cytogenetic assays are essential tool of immense importance for measuring the genotoxic potential of drugs and other chemicals. The occurrence of chromosomal abnormalities in somatic cells may increase the chance of developing neoplasia while in germinal cells any such abnormality may give rise to a higher frequency of birth defects, heritable chromosome rearrangement, genetic diseases in offsprings' and spontaneous abortions. Considering the importance of such assays, four cytogenetical endpoints were selected for studying toxicity studies based on earlier established protocols with minor modifications. The four important endpoints considered in this study are Chromosomal aberration assay (CA), Micronucleus assay (MN), Sperm head abnormality assay (SHA) and Total sperm count per epididymis (TSC).
3.3.1 Chromosome aberration assay:

Chromosome aberration can be defined as the change of the number or modification of the structure of chromosomes. It can be detected both by cytological and genetical methods. It is a very important parameter for genotoxicity studies. It constitutes a set of efficient, reliable and economical criteria to measure genetic toxicity. Hence, Chromosomal aberration assay was developed to study such structural and numerical abnormalities and this technique provides reliable and sensitive information regarding genetical alterations induced by various chemicals and mutagens. By this technique 100 well spread metaphase chromosome plates are observed under microscope and structural changes in chromosome like breaks, gaps, exchanges, Robertsonian translocation, sister chromatid union and many additional types of abnormalities like pulverized, sticky chromosomes (Figure 16); numerical changes in chromosomes like polyploidy (2n and 3n) could be detected.

Protocol:

The Chromosomal aberration (CA) assay was conducted following the protocols given by Preston et al., 1987 and Krishna and Hayashi, 2000 with minor modifications. Mice were injected i.p. with colchicine (2 mg/kg bw) 1 hour 30 minutes prior to sacrifice and 24 hour and 48 hour of the last treatment. Animals were killed by cervical dislocation and bone marrow cells were collected from both the femora by flushing in Potassium chloride (KCl) (0.56% pre warmed at 37°C) and cells were incubated for 18-20 minutes at 37°C and fixed in 1:3 aceto-methanol (1 acetic acid: 3 methanol) for minimum 30 minutes in cold (refrigerator). Centrifugation for 5 minutes was repeated
twice with fresh aceto-methanol. The material, resuspended in a small volume of the fixative, the slides were prepared by dropping the sample on to chilled slides from a height of 2 feet and run over the frame, flame-dried (Flame drying method). The prepared slides were stained in the following day (after 24 hour of slide preparation) in 5% Giemsa stain. One hundred well spread metaphase plates were examined per animal for Chromosomal aberrations and Chromosome Abnormalities were classified, analyzed and scored as indicated in Table 10. Gaps were recorded separately but not included for statistical analysis because of their controversial genetic significance (WHO, 1985).

**Mitotic Index (MI):**

Mitotic index is the percent ratio of number of dividing cells to the total number of cells observed. The MI was significantly lower than that from the solvent control indicating a decrease in the number or rate of dividing cells, suggesting cytotoxicity.

\[
\text{Mitotic index (MI)} = \frac{\text{Dividing Cells}}{\text{Total number of cells studied}} \times 100
\]

**3.3.2 Micronucleus (MN) assay**

Micronucleus study is another very important and useful bio-marker for genotoxicity studies (Heddle et al., 1983). Micronucleus are small, extranuclear bodies that are formed in mitotic cells from chromosome fragments (acentric fragments) or whole chromosome itself lagging behind in the
cytoplasm during anaphase of cell division and are not integrated into daughter nuclei. Thus, micronucleus contains either chromosome fragments or whole chromosomes. Micronuclei harboring chromosome fragments may result either from direct DNA breakage, or from replication on a damaged DNA template, or inhibition of DNA synthesis. Micronuclei harboring whole chromosomes are primarily formed from failure of the mitotic spindle, kinetochore, or other parts of the mitotic apparatus or by damage to chromosome sub-structures, alterations in cellular physiology, and mechanical disruption (Oshimura and Barrett, 1986; Albertini et al., 2000). Thus, an increased frequency of micronucleated cells is a biomarker of genotoxic effects that can reflect exposure to agents with clastogenic (chromosome breaking; DNA as target), or aneugenic (aneuploidogenic; effect on chromosome number; mostly non-DNA target) mode of action of chemicals or other mutagens. The micronucleus study is conducted in the early and late maturing stages of RBC.

The RBC maturation takes place in the bone marrow of the animals. The first cell that can be identified as belonging to the red blood cell series is the proerythroblast (Figure 13). Large numbers of pro-erythroblasts are formed from the colony forming unit erythrocytes (CFU-E) stem cells. Once the proerythroblast is formed it divides multiple times, undergoes series of differentiations and maturations, eventually forming many mature red blood cells. The first generations of cells which develop from the proerythroblasts are basophilic in nature since they stain with basic dyes. The cells at that stage contain very little hemoglobin. The succeeding generations of cells becomes
filled with hemoglobin, the nucleus condenses to a small size and the final remnant extruded from the cells. Hence, the changes which are evident and observed in the erythrocytes while maturation are reduction in the cell size, increase in the cytoplasmic matrix, change in the staining reaction of the cytoplasm from basophilic to acidophilic (due to decrease in the amount of RNA and DNA), reduction in the size of the nucleus and finally disappearance of the nucleus with condensed chromatin material and the gradual acquisition of hemoglobin (Gyton and Hall, 2003). Different stages of a developing red blood cell are represented below in Figure 13.

Mature RBC does not contain DNA but if chromosomal aberrations occur in the mature RBC, fragments of chromosomes may be retained in the new mature RBC. In anaphase, chromosomal fragments from the damaged chromosomes or chromosomes from abnormal segregation lag behind when the centric elements move to the spindle poles. After telophase, these lagging fragments or chromosomes may be included in the daughter nuclei. During the development, the main nuclei are expelled from the mature erythrocytes while the secondary nuclei from the fragments or lagging chromosomes are retained as "micronuclei" (Figure 13 and 14).

The polychromatic erythrocytes (PCEs) are the early maturing stage of RBC taking light blue stain due to the presence of residual nucleic acids (Figure 17) whereas normochromatic erythrocytes (NCEs) are the older maturing stage of RBC and stain light reddish to transparent (Figure 17) due to the absence of nucleic acids in it (Li and Heflich, 1991). Total 2000 polychromatic erythrocytes (PCEs) and its corresponding number of
normochromatic erythrocytes (NCEs) were analyzed separately per mice for the presence of MN.

**Figure 13:**
Pictorial depiction of micronucleus formation. (1) Metaphase, (2) Anaphase, (3) Telophase, (4) The resultant micronucleus at interphase of cell cycle and (5) Mature cell with micronucleus after the extrudation of the main nucleus.

The ratio of PCE/NCE was scored to evaluate the cytotoxic effect of the chemicals tested. A PCE/NCE ratio was significantly lower than that from the solvent control indicating a decrease in the number or rate of erythrocytes maturation, revealing cytotoxicity (Li and Heflich, 1991).
3.3 Experimental protocols

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Figure 14:
Pictorial representation of the micronucleus formation (A) anaphase, (B) anaphase showing acentric fragment of chromosome or whole chromosome lagging behind during cell division, (C) interphase cell with micronucleus form from acentric fragment of chromosome (Clastogenic) and (D) interphase cell with micronucleus form from the whole chromosome lagging behind during cell division (Aneugenic).

Protocol:
The animals were sacrificed by cervical dislocation, after the appropriate time of treatment. Preparation of bone marrow smears and staining was done following the method of Schmid (1976) with minor modifications. The bone marrow cells were flushed out and collected in 0.9% NaCl pre-warmed at 37°C. Bone marrow smear was prepared, air-dried and fixed in methanol for 10 minutes. Staining was done in 5% Giemsa stain after 24 hour of slide preparation. The slides were mounted with cover slip using DPX mountant for microscopic study.
3.3.3 Sperm head abnormality (SHA) assay:

Study of germ cells for genotoxicity is one of the most important endpoint as such study indicates the possibility of transfer of faulty genetic information from one generation to next through abnormal gamete cells. Sperm abnormality study is of great importance since it is possible to detect the toxicity of any chemical on germ cells. Wyrobek and Bruce (1978) had stated that chemicals which can affect the somatic cells can also affect germinal cells in a similar manner. Further it was said that different stages of developing sperms react differently to the same chemical. Hence sperm head abnormality studies was undertaken after 35 days of chemical exposure order to observe the effects of the various chemicals on the primordial germ cells since spermatogenesis in mice takes about five weeks (35 days) to complete (develop and grow into mature sperms) according to Bruce and Heddle (1979).

Protocol:

The animals were sacrificed by cervical dislocation after 35 days of exposure. Both the cauda epididymis were dissected out and placed in 1 ml of 0.9% normal saline (NaCl). The sperms were released by mechanical disruption and washing of the epididymis. The suspension was sieved through two layers of muslin cloth to remove the tissue debris. A drop of the suspension was taken on a clean glass slide and a smear was made, air dried, and fixed in absolute methanol for 10 minutes. The slides were stained in the following day in 0.1% eosin Y for 10 minutes. Total 1000 sperms per animal
were analyzed and scored and different types of abnormalities were scored (Wyrobek and Bruce, 1978).

When the slides were observed under microscope, two tailed sperms were also seen but only the head morphology was examined for the present study. Different types of sperm head abnormalities taken into consideration used in the present study are as follows:

Normal sperm, amorphous, beaked, hooked, hookless, altered head, triangular, banana, pin-headed, giant, dwarf, double-headed (Figure 18), and some new, additional types such as mushroom, pea-nut, bent at cephalocaudal junction, bent at projecting filament. Abnormalities were categorized using the criteria as close to those described by Wyrobek and Bruce (1975).

3.3.4 Total sperm count:

Total sperm count was done by using Haemocytometer as described by Narayan et al., 2002 and Vega et al., 1988. Total sperm count analysis was also an important parameter indicating the toxic effects of test substances/chemicals on the spermatogenesis. This assay is used as a short-term bioassay to investigate the mutagenic or cytotoxic effects of agents responsible for significant human exposure. In addition, epidemiological evidences indicate that semen quantity in humans has declined progressively over the past 50 years with a general increase in the incidence of male reproductive pathogenesis including testicular cancer (Carlson et al., 1992; Auger et al., 1995).
Protocol:

The mice were killed by cervical dislocation. Testes were dissected out and epididymis was carefully separated and minced in 1 ml of normal saline (0.9% NaCl) to obtain a suspension (Narayana et al., 2002). The suspension was filtered through a nylon mesh. The total sperm count was performed in the filtrate as per the standard method in Neubauer's chamber (Vega et al., 1988; Narayana et al., 2002). An aliquot from the suspension (up to 0.5) was taken in leukocyte haemocytometer and diluted with normal saline up to mark 11. The suspension was well mixed and 1 or 2 drops was putted into the Neubauer’s counting chamber. The total sperm count in 8 squares (except the central erythrocyte area) of 1 mm² each was determined and multiplied by 5x10⁴ to express the number of spermatozoa/epididymis (Narayana et al., 2005).

3.3.5 Lipid peroxidation assay:

Lipid peroxidation has been established as a major mechanism of cellular injury in many biological systems of plant and animal origin. The mechanism involves a process whereby unsaturated lipids are oxidized to form additional radical species as well as toxic by-products that can be harmful to the host system. Polyunsaturated lipids are especially susceptible to this type of damage when in an oxidizing environment and they can react to form lipid peroxides (Figure 70). Lipid peroxides are themselves unstable, and undergo additional decomposition to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acids peroxides further react to form malondialdehyde (MDA).
MDA can be found in most biological samples including foodstuffs, serum, plasma, tissues and urine, as a result of lipid peroxidation, and has become one of the most widely reported analytes for the purpose of estimating oxidative stress effects on lipids. Extensive toxicological investigations have now established that increase in lipid peroxidation, actually denotes cytotoxicity and cellular dysfunction.

**Determination of malondialdehyde:**

The thiobarbituric acid (TBA) test is probably the single most widely used single assay for the measurement of lipid peroxidation. The sample under test is treated with TBA at low pH, and a pink chromogen is measured. In the TBA reaction, one molecule of MDA reacts with two molecules of TBA with the production of a pink pigment with an absorption maximum at 535 nm (Figure 15).

![Chemical structures](image.png)

**Figure 15:**
The reaction of thiobarbituric acid with malondialdehyde to produce a coloured product with absorbance at 535.

**Protocol:**

After sacrifice of mice, liver was removed, cleaned and weight in digital balance. Liver tissue was crush by using Pestle and Mortar, which was kept in
a plastic container containing ice-cubes. Tissue homogenate was prepared in ice-cold normal saline and centrifuged for 10 minutes at 3000 rpm. The supernatant was used for estimation of the biochemical attribute i.e., lipid peroxidation for assessment of induction of oxidative stress in liver. LPO was done by the method of Shafiq-U-Rahman (1984) and expressed as nmol malondialdehyde/g wet tissue. The step by step procedure is given as follows:

1. 1ml of sample (homogenate containing 0.8-0.9 mg of protein) was incubated at 37 ± 0.5°C for 2 hour.
2. To each sample, 1ml of 10% w/v trichloroacetic acid (TCA) was added.
3. After thorough mixing, the mixture was centrifuged at 2000 rpm for 10 minutes.
4. To 1ml of supernatant, an equal volume of 0.67% thiobarbituric acid (TBA) was added, kept in boiling water bath for 10 minutes.
5. After cooling, it was diluted with 1ml of distilled water (DW).
6. The absorbance was read at 535 nm.

Calculations:

The malonaldehyde concentration (MDA) of the sample was calculated by using extinction coefficient of 1.56x10^5 M^-1 cm^-1. The concentration of MDA expressed in μmolL^-1 was calculated by using Beer-Lambert law,

\[ A = \varepsilon c l \]

Where, \( A \) = Absorbance; \( \varepsilon \) = Extinction coefficient; \( c \) = concentration of MDA; \( l \) = path-length (1 cm standard Curvette).

Since the conversion of μmolL^-1 to n mol/g involves dividing and multiplying by 1000, these steps essentially cancel out, so that μmolL^-1 = nmolml^-1. Therefore, the original MDA concentration in μmolL^-1 can be simply
converted to the final concentration of n mol/g wet weight by the following calculation:

\[
\text{MDA n mol/g wet weight} = \left( \frac{\text{MDA} \, \mu\text{molL}^{-1} \times \text{volume normal saline ml}}{\text{g wet weight}} \right)
\]

3.3.6. Survival time of Dalton’s Lymphoma (DLA) bearing mice:

The tumor Dalton’s lymphoma was originated in the thymus gland of DBA/2 mouse at the National Cancer Institute, Bethesda MD, USA in 1947 and subsequently on ascites form was developed by repeated intraperitoneal (i.p) transplantation of the tumor (Goldie and Felix, 1951). In media probably this tumor cell line was first procured by Cancer Research Institute, Mumbai. The ascites tumor used in the present study was collected from North- East Hill University (NEHU), Shillong (Meghalaya), India in the year 2010.

Maintenance of DLA cell lines:

The DLA cells were propagated in the peritoneal cavity of the mice by injecting \(10^6\) cells. The cells were aspirated aseptically from developed tumor mice, during the log phase on the 15\(^{th}\) day of tumor transplantation using insulin syringe by withdrawing the fluid from peritoneal cavity. The ascitic fluid was washed three times in PBS (phosphate buffer saline) and the cell pellet was re-suspended in PBS. Tumor cell count was done using a hemocytometer. The cell suspension was diluted to get \(2 \times 10^6/0.2\) ml.

Studies were carried out using Swiss Albino mice. They were obtained from Pasteur Institute, Shillong and kept in close inbred colony in the animal
house of Assam University, Silchar. The mice were grouped and housed in polycyclic cages and maintained under Std. Laboratory conditions. They were allowed free access to Std. dry pellet diet and water *ad libitum*. All procedures were carried out in strict accordance with the guidelines prescribed by the committee for the purpose of control and supervision on experimentation on animals and was approved by the Institutional Ethical Committee.

**In vivo cancer activity:**

The enhancement or increases of tumor progression of extracts were determined in Dalton’s Lymphoma Ascites cells in murine test system. Male Swiss Albino mice were divided into 9 groups (n = 6 in each group). The experiment continued for 90 days. Treatment was given as per the table given below and mean survival days as well packed cell volume (PCV) were calculated. In all respective groups tumor cell line was transplanted on 14th day after single dose (80 mg/kg/day) of extract treatment for two weeks. The groups receiving tumor transplantation were injected with DLA cells (0.2 ml, 2x10⁶ cells/mice) intraperitoneally (i.p). Other groups received only extract treatment. Dimethyl yellow (p-DAB) (0.06% in Distilled Water p.o) (IARC, 2004) was used as positive control for the experiment which is a known carcinogen and induces tumorigenesis. The result has been discussed in Table 46 and Figure 65.
Table 9
Table showing areca nut extract, sadagūra extract and areca nut with sadagūra extract, Dalton's lymphoma ascites treatment mode and exposure period.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Groups</th>
<th>Extracts (for 14 days)</th>
<th>DLA (on 14th day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>DLA Control</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>AN</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>SG</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>AN+SG</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>AN+DLA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>SG+DLA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>(AN+SG)+DLA</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

3.3.7 Analysis of body weight of DLA bearing mice:

The enhancement or increases of tumor progression of extracts were determined in Dalton's Lymphoma Ascites cells in murine test system. Male Swiss Albino mice were divided into 9 groups (n = 6 in each group). In all respective groups tumor cell line was transplanted on 14th day after single dose (80 mg/kg/day) of extract treatment for two weeks. The groups receiving tumor transplantation were injected with DLA cells (0.2 ml, 2x10^6 cells/mice) intraperitoneally (i.p). Other groups received only extract treatment. Dimethyl yellow (p-DAB) (0.06% in Distilled Water p.o) (IARC, 2004) was used as positive control for the experiment which is a known carcinogen and induces tumorigenesis. The result has been discussed in Table 47 and Figure 66, 67.
All mice were weighed on the day of tumor inoculation and there after on daily basis until survival of animals.

3.3.8 Packed cell volume (PCV) of DLA bearing mice:

The enhancement or increases of tumor progression of extracts were determined in Dalton's Lymphoma Ascites cells in murine test system. Male Swiss Albino mice were divided into 9 groups (n = 6 in each group). In all respective groups tumor cell line was transplanted on 14th day after single dose (80 mg/kg/day) of extract treatment for two weeks. The groups receiving tumor transplantation were injected with DLA cells (0.2 ml, 2x10^6 cells/mice) intraperitoneally (i.p). Other groups received only extract treatment. Dimethyl yellow (p-DAB) (0.06% in Distilled Water p.o) (IARC, 2004) was used as positive control for the experiment which is a known carcinogen and induces tumorigenesis. The result has been discussed in Table 48 and Figure 68. After thirty-five days of tumor transplantation, tumor growth parameters were determined through measurement of packed cell volume.

**Determination of tumor volume:**

The mice were dissected and the ascetic fluid was collected from the peritoneal cavity. The volume was measured in a graduated centrifuge tube and packed cell volume determined by centrifuging at 1000 g for 5 minutes.
3.4 Statistical Analysis:

All the data were represented as mean ± S.E (for Chromosome aberration) and mean ± S.D (for Micronucleus, Sperm head abnormality and Total sperm count). For lipid peroxidation and tumor studies all the data were represented as mean ± S.E. The corresponding graphs of the tables were represented by histograms and line diagrams. The graphs were the diagrammatic representation of interdependence of two variables. In the present work the variables considered were the different chemicals (of different concentration) administered in the experiments and the other variable included the genetic changes (chromosomal aberrations or micronucleus formation or sperm head abnormality), biochemical changes (lipid peroxidation) and tumorigenic changes (survival time, body weight and packed cell volume) brought about by those chemicals.

ANOVA was used to determine the significance of genetic parameters. Since there is no significant heterogeneity of variance, one way analysis of variance was used to test whether there was evidence of any difference between mean of groups. Pair wise comparison of significance between the different groups was determined by using Tukey’s test. ANOVA values were calculated using GraphPad Prism, version 4.03 (GraphPad Inc., San Diego, CA, USA). A p-value of < 0.05 was considered to be significant.
3.5 Photomicrographs:
Figure 16:
Photomicrographs of murine bone marrow metaphase spreads showing different types of chromosomal aberrations induced by areca nut alkaloid Arecoline and its metabolites, Arecaidine, Arecoline N-oxide and N-methyl nipecotic acid and extracts of areca nut and sadagura alone or in combination under various treatment conditions. A, B, C: normal complement of chromosomes; D, F, G: chromatid breaks; E: chromatid gaps; H: isochromatid gap; I: terminal exchange; J: chromosome exchange; K, L: ring chromosome; M, N: Robertsonian translocation or centric fusion; O: PCS (Precocious centromeric separation); P: C-mitosis; Q: Pulverized and R: sticky chromosome.
Figure 17:
Photomicrographs of murine bone marrow cells i.e., polychromatic erythrocytes and normochromatic erythrocytes, with micronucleus induced by areca nut alkaloid Arecoline and its metabolites Arecaidine, Arecoline N-oxide and N-methyl nipecotic acid and extracts of areca nut and sadagura alone or in combination under various treatment conditions.
Figure 18:
Photomicrographs of murine sperms showing abnormal head morphology induced by areca nut alkaloid Arecoline and its metabolites Arecaidine, Arecoline N-oxide and N-methyl nipecotic acid and extracts of areca nut and sadagura alone or in combination under various treatment conditions as described in the text. A: Showing normal head morphology; B, C: Amorphous type of heads; D: Triangular type of head; E, F: Pin-head type of heads; G: Hook-type head; H: Beak-type head; I: Double headed; J: Banana type head; K: Bent at projecting filament; L: Bent at cephalocaudal junction.