CHAPTER II

MATERIALS AND METHODS
Animals:

The present investigation has been carried out in a group of healthy, male albino rats of approximate same age group and body weight between 80-100 gms. The experimental animals were housed in individual cages provided with drinking water facilities.

Reagents and Chemicals:

Arecoline hydrobromide was obtained from Sigma chemical Co., St. Louise, M.O. All other reagents used in the study were of analar grade. The preparations of the reagents were described in each chapter and redistilled water was used for their preparations. Oven dried corning glass apparatuses were used in the various experiments.

Normal feeding regime:

All the experimental animals were provided a maintenance diet. The composition of mineral salt mixture and diet mixture has been shown in Table 7 and Table 8.
FIG. 10: Showing:
(A) Tender betelnut
(B) Ripe, unprocessed betelnut
(C) Fermented betelnut.
TABLE - 7: Showing the composition of mineral mixture.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium carbonate</td>
<td>381.400 gm.</td>
</tr>
<tr>
<td>Cobalt nitrate</td>
<td>0.230 gm.</td>
</tr>
<tr>
<td>Cupric sulphate</td>
<td>0.477 gm.</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>57.300 gm.</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>27.000 gm.</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>4.010 gm.</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>0.7904 gm.</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>389.000 gm.</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>139.300 gm.</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>0.548 gm.</td>
</tr>
</tbody>
</table>

TOTAL: 1000.00 gm.

The above salts were mixed properly and stored.

TABLE - 8: Showing the composition of basal diet mixture.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skimmed milk powder</td>
<td>25.64 gm.</td>
</tr>
<tr>
<td>Salt mixture</td>
<td>4.00 gm.</td>
</tr>
<tr>
<td>Refined ground nut oil</td>
<td>5.00 gm.</td>
</tr>
<tr>
<td>Multi-vitamin tablets</td>
<td>1.00 gm.</td>
</tr>
<tr>
<td>Sago powder</td>
<td>64.36 gm.</td>
</tr>
</tbody>
</table>

The above ingredients were mixed and pulverized into a powder form and stored.

Preparation of betelnut (BN) mixed diet:

Ripe fresh, betelnut of unprocessed variety (Fig.1C) were sliced and pulverized into a powder form with the help
of mixer cum grinder.

For the preparation of betelnut feed additive, 15 gms. of betelnut powder was mixed with normal basal diet in the ratio of 15 : 85 which was found to be the most effective after trial and error. The prepared experimental diet was used as oral diet for animals. Each animal was given 25 gms. of the prepared diet each day and the quantity of the given diet consumed by the experimental animal was measured daily.

Preparation of the arecoline solution:

Arecoline solution was prepared by dissolving the chemical in physiological saline at the concentration of 5 mg/ml. Fresh solution was used every time.

Arecoline was administered in two different doses namely 0.2 ml. (1.0 mg) and 0.4 ml. (2.0 mg) for 7 and 14 days to the different group of animals as recommended by Panigrahi and Rao (1982).

To evaluate the biochemical and histopathological changes in the hepatic cells due to oral feeding of betelnut and parental administration of arecoline solution, two set of experiments were conducted in male albino rats.

Oral feeding of betelnut mixed diet:

Experiment I: To study the effect of oral feeding of betelnut mixed diet, a total of 32 numbers of rats were taken. The animals were maintained on betelnut mixed diet for maximum twenty eight days and sacrificed at seven days interval from the day of commencement of the treatment.
The following groups were maintained:

**GROUP I**: Animals of this group received betelnut mixed diet for 7 days and were sacrificed on 8th day.

**GROUP II**: Animals of this group received betelnut mixed diet for 14 days and were sacrificed on 15th day.

**GROUP III**: Animals of this group received betelnut mixed diet for 21 days and were sacrificed on 22nd day.

**GROUP IV**: Animals of this group received betelnut mixed diet for 28 days and were sacrificed on 29th day.

**Intraperitoneal administration of arecoline solution**:

**Experiment - II**: To evaluate the changes due to arecoline alone, parental administration of arecoline solution on rat liver, a total of 32 numbers of healthy rats were selected.

The animals were divided into 4 groups:

**GROUP I**: Animals of this group received a single dose of 1.0 mg. of arecoline per day for 7 days and were sacrificed 24 hours later from the last injection.

**GROUP II**: Animals of this group received a single dose of 2.0 mg. of arecoline per day for 7 days and were sacrificed 24 hours later from the last injection.
GROUP III: Animals of this group received a single dose of 1.0 mg. of arecoline per day for 14 days and were sacrificed 24 hours later from the last injection.

GROUP IV: Animals of this group received a single dose of 2.0 mg. of arecoline per day for 14 days and were sacrificed 24 hours later from the last injection.

The arecoline solution prepared in the normal saline was injected intraperitoneally with all aseptic measure with the help of 22 gauge needle syringe.

Normal control group:

Along with the experimental groups ten numbers of animals were taken as a control for both the experiments. The animals belong to this group were normal, healthy and maintained with basal diet (Table 8) and provided with drinking water ad lib.

The liver in situ were collected after sacrificing the animals from each group. The animals were sacrificed after 24 hours interval without any arecoline injection or betelnut feed. The liver tissues obtained from animals of experiment I (Group I, Group II, Group III, Group IV) and also experiment II (Group I, Group II, Group III, Group IV) were processed for biochemical studies described in subsequent chapters from III to VI. Along with the biochemical
studies of liver tissue, a part of a tissue belong to all the
groups viz. Experiment I and Experiment II were processed
for histopathological studies.

**Method of evaluation:**

The different experimental values obtained were
evaluated by application of a number of statistical methods
to find out the statistical significance of the effect of
betelnut mixed diet and alkaloid arecoline on liver enzymes.

**Standard deviation (S.D.):**

The standard deviation of the experimental sets
were determined by the equation of:

\[
S.D. = \sqrt{\frac{\sum(X - \bar{X})^2}{n - 1}}
\]

Where \( X \) = Value of each observation in a set.
\( \bar{X} \) = Mean value of the set.
\( n \) = Number of observation.

**Standard error mean (SE\(_M\)):**

The standard error mean of the experimental set was
determined by the equation of:

\[
SE_M = \frac{S.D.}{\sqrt{n}}
\]

Where \( S.D. \) = Standard deviation of the set.
\( n \) = Number of observation.

**Degree of freedom (df):**

\[
df = n - 1
\]

Where \( n \) = Number of observation of the experiment.
**Significant test (t):**

The significance difference between two sets e.g. between control and a test group or between two test groups were compared by "student t test" for possibility of event.

\[
t = \frac{X_1 - X_2}{\sqrt{\frac{n_1 n_2}{n_1 + n_2}} \sqrt{\frac{(X_1 - \bar{X}_1)^2 + (X_2 - \bar{X}_2)^2}{n_1 + n_2 - 2}}}\]

Where S.D. = \[\sqrt{\frac{(X_1 - \bar{X}_1)^2 + (X_2 - \bar{X}_2)^2}{n_1 + n_2 - 2}}\]

- $\bar{X}_1$ = Mean value of the first set.
- $\bar{X}_2$ = Mean value of the second set.
- $X_1$ = One individual value of the first set.
- $X_2$ = One individual value of the second set.
- $n_1$ = Number of observations of the first set.
- $n_2$ = Number of observations of the second set.

$n_1 + n_2 - 2 = df$, degree of freedom.

**Correlation coefficient (r):**

The correlation coefficient (r) was determined by the following formula in a group between two different parameters.

\[
Correlation\ coefficient\ (r) = \frac{\sum [(X - \bar{X}) / SD_X] \times [(Y - \bar{Y}) / SD_Y]}{n - 1}
\]
Where $\bar{X}$ and $\bar{Y}$ = Mean of the 1st and second set.

$X$ and $Y$ = Individual value of 1st and second set.

$SD_X$ and $SD_Y$ = Standard deviation of 1st and second set.

$n$ = Total number of observations.

(Bancroft, 1967; Lapin, 1975; Bhattacharyya and Johnson, 1977).