# CHAPTER 4

# MATERIALS AND METHODS

## MATERIALS

Ladu, Jilebi and Halwa were the three sweets items selected for the study of usage of synthetic food colourants in the sweets of Kozhikode District of Kerala. Kozhikode (formerly Calicut) is the most important part of Malabar, its very heart. The district is 38% urbanised and divided into 3 taluks (Vadakara, Koyilandi and Kozhikode), 12 blocks, 78 panchayaths and 117 villages.

This land is important for its tempting cuisine. The city has a strong mercantile streak to it, with the major hub of commerce being the *Mithai Theruvu*, a long street crammed with shops that sell everything from sarees to cosmetics, and house hotels to sweetmeat shops. The name *Mithai Theruvu* or *SM Street* comes from the sweet- *Kozhikode Halwa*, which was often called as Sweetmeat by European traders. Other important sweet items are Ladu and Jilebi (Kozhikode Dist official website, 2011).

### Halwa

Halwa refers to a dense sweet desert made from all purpose wheat flour. Ingredients used for Halwa are (1) All purpose wheat flour - 3 cups, (2) Sugar - 2 cups, (3) Coconut oil - 1 cup, (4) Crushed cardamom, (5) Water and (6) Food colour.

Flour is first mixed with water to form a thin batter. It is then covered and left for one day. Water from the top is removed, made up with water again and left for one more day. Flour will start fermenting by this time. Removed the water form top and filtered out any lumpy portions from the flour paste. The

coconut oil is heated to 350 degrees and kept aside. Sugar syrup is also kept ready by boiling sugar with a cup of water. The desired food colour is added to the sugar syrup. The sugar syrup is then mixed with the flour paste and cardamom seeds. The mixture is stirred well and to this hot oil is added. Stirring is continued on medium/high heat until the Halwa separates from oil and starts to stick together. Continued stirring for two more minutes and transferred to a greased pan. Cooled down and cut into desired size. Cooking in excess hot oil is a key step in getting the correct consistency and finishing it faster. The separated out oil can be reused (Food Buzz, 2011).

**Ladu**

Ladu is and Indian sweet made from flour, sugar and shortening, which is fried and then shaped into a ball. The ingredients used are a) Gram flour – 500 g, b) Raw rice flour – 2 tbsp, c) Sugar – 265 g, d) Ghee/oil, and e) Salt. A wide mouth mixing bowl is taken and mixed together the gram flour or besan, rice flour, a pinch of salt and ghee with required amount of water to make a thick batter. Oil is heated in a frying pan. The batter is spread on a boondi plate (a plate with lot of holes) kept above the oil so that only very small pieces of batter drop into the oil. Boondis are stirred until fully cooked and excess oil is drained off. Sugar syrup is made by boiling sugar and water in the ratio 1:1 for about 5 minutes. The fried boondis are dropped into the sugar syrup containing added colourant and mixed well. It is then cooled to room temperature and made into even balls by pressing lightly with hand (Pachakam, 2011).

**Jilebi**

Jilebi is a popular sweet dessert of India sub-continent. It is made by deep frying batter in a coil shape and then soaking in syrup. Ingredients are a) Maida flour – 2 cups, b) Sugar – 2 cups, c) Water – 2 cups, d) Baking powder – ½ tea spoon, e) Food colour and f) Ghee or vegetable oil for frying.

Flour is mixed with baking powder and water to make a batter of the consistency of a thick cream. Set aside for about 24 hours to ferment. Dissolved 2 cups of sugar in 2 cups of water and boiled till it becomes syrup of one thread consistency. A pinch of food colour is added to the syrup. Poured the batter through a coconut shell with a hole to form coils in heated oils under a steady stream. It is then deep fried until they are golden in colour and crisp all over, removed, drained and immersed in sugar syrup. The fried coils are kept for at least 4-5 minutes in sugar syrup so that they get soaked in the syrup. Finally taken out of the sugar syrup (Jilebi recipe, 2011).

**Figure 1 and 2** shows the picture of the sweets (Ladu, Jilebi and Halwa) in different shades available in the market.

In Kozhikode the sweets like Halwa, Ladu, Jilebi etc are sold mainly through sweet stalls, bakeries, supermarkets and also they are available in stalls in railway stations, bus stands cinema theaters and in shopping malls. There are around a total 600 bakeries in the district both in urban and rural areas. They belong to both organized and unorganized sector. The major organized bakeries in this area are Cochin bakery, K. R Bakery, Delecta and Ojin bakery. Most of the bakeries in the organized sector manufacture these items in their own

manufacturing facilities. On the other hand small bakeries depend mainly on the centralized manufacturing units who manufacture these items in bulk and supply at wholesale rates.

One thousand samples of the above sweets were collected from various outlets both in urban and rural areas of Kozhikode. The area of sample collection is shown in the attached map of Kerala (**Figure 3**). In the market, Ladu and Jilebi are available in yellow, red and orange shades, while Halwa is available in yellow, red, orange, brown, black, white and green shades. The details of the samples collected are given in **Table 7 and Figures 4 to 7**.

## METHODS

### 1. Isolation, Identification and Estimation of Synthetic Food Colours

The general scheme of identifying synthetic food colours present in foods normally involves a) Removal of fat from food materials, b) Extraction of the colour by dyeing wool, c) Separation of mixed colours if more than one colour is present by paper chromatography, d) Identification of the separated colours by co-chromatography with the standard dyes and e) Estimation of the colour by spectrophotometric technique.

**Reagents and Consumables:**

1. Pure white wool: Wool may contain fluorescing material which may appear as a fluorescent spot on the chromatogram. To overcome this interference, pure white knitting wool is boiled successively in dilute ammonia solution (1+4), and finally washed in water again.
2. Chromatographic paper, Whatman No. 1.

3. Standard permitted synthetic colours (0.2% solution in 0.1 N HCl)
4. Ammonia solution- 25%
5. Ethanol
6. Acetone
7. Petroleum Ether (40-60$^0$C).
8. Solvent system: Butanol: Water: Acetic acid (20:15:5).
9. Aluminium oxide (for column chromatography).
10. 0.1 N HCl.

All chemicals and consumables were purchased from M/s. Sigma Aldrich and M/s. E Merch India Ltd.

**a) Procedure for Wool Dyeing:**

About 10 g of samples were defatted with three changes of petroleum ether followed by extraction of colour with 50 ml of 2% ammonia in 70% ethanol. It was kept for one hour, centrifuged and the clear solution transferred into a dish and evaporated on a water bath. The residue was dissolved in 30 ml dilute acetic acid. To the solution 20 cm length of white woolen thread was introduced and boiled till the wool takes up the colour fairly completely. The wool was removed from the beaker, washed under running tap water and finally with distilled water. Acidic coal-tar dyes are stripped by dilute ammonia. The colour from the dyed wool was stripped by boiling it in a dilute ammonia solution (Natural colours may also dye the wool but the colours are not removed by ammonia). At present, all the permitted water-soluble coal-tar dyes are acidic and hence an indication of the presence of basic dye at this stage indicates that an unpermitted colour is present. The wool was removed, solution made faintly acidic and boiled with a fresh piece

of wool until the colour is removed. The dye from the woolen thread is again extracted with dilute ammonia, filtered through a cotton plug and evaporated on a hot water bath to a low bulk for spotting (Mahindru, 2000).

**b) Identification of Colours by Paper Chromatography:**

Colours were separated using ascending paper chromatographic technique. 20×20 cm Whatman No. 1 chromatographic paper was taken. A base line was drawn 2 cm from and parallel to the bottom of the paper. A few drops of the extracted and concentrated solution of the unknown synthetic colour were spotted on this line using a capillary tube. In addition to sample, a series of standard permitted dyes (0.2% solutions in 0.1 N HCl) of similar colours were also spotted. The spots were dried; paper curled into a cylinder and joined the two ends at two places with white thread. The paper was then placed in a chromatographic chamber containing about 300 mL of the solvent system Butanol: Water: Acetic acid (20:15:5). Allowed the chromatogram to develop until the solvent front has reached a height of 12 cm from the base line. Then the paper is removed and dried. The colourants were identified by comparing the $R_f$ values of sample colours with standard colour solutions. The $R_f$ is given by the following expression:

$$Rf = \frac{\text{Distance moved by the spot of the food colour}}{\text{Distance moved by the solvent front}}$$

The $R_f$ values depend on many factors like composition of the solvent used for the development phase, kind of paper, direction of paper fibers, manner of developing, length of paper used for development, distance of starting line from

the solvent, concentration of the solute, presence of other substances and temperature of development (Ranganna, 1987).

**c) Estimation of Added Colourants in Sweets:**

The concentration of the colourants in sweets samples was estimated by spectrophotometric technique as per ICMR protocol (ICMR, 1990).

The colour was first identified by paper chromatography separation technique as described above. To estimate quantitatively the added dyes in food, the dyes should be removed completely from the product. The procedure involved should avoid any severe heat treatment or the use of strong acids or alkalies, as this could alter its chemical composition. The wool-dyeing technique is undesirable, as repeated boiling is required to obtain complete recovery of the dyes. Hence the column chromatographic method was employed to purify the extracted colour solution using activated alumina.

**Column Chromatography:** A known weight of the sample (approximately 5 - 10 gm) was transferred into a glass stoppered separating funnel. The colour was extracted with 70% acetone. The acetone extract was shaken with petroleum ether (40-60$^{o}$C) in order to remove carotenoids and other natural pigments, if any. Extraction with petroleum ether was continued until petroleum ether extract is colourless. The acetone extract containing only coal-tar food colours were then passed through a column (2.1 × 45 cm) containing aluminium oxide acidified with 1% HCl. The absorbed colour was eluted with 1 % ammonia solution. The eluate was evaporated to dryness on a hot water bath, dissolved the residue in 0.1 N HCl, transferred quantitatively to a 100 ml volumetric flask and made up to the volume with 0.1N HCl. The Optical Density of the above solution was determined.

If the sample contained a mixture of colours, it was separated into individual colours by paper chromatography. The purified colour solution was made upto a known volume with water (5 mL). An aliquot (approximately 0.5 to 1.0 ml) of the purified dye was spotted on a Whatman No.1 filter paper as a band and the chromatogram developed using butanol : acetic acid: water (20:5:12) solvent system. The chromatographic paper was dried in air and the separated portions of the colour bands cut individually, eluted and made upto a known volume with 0.1 N HCl. A blank was prepared by cutting an equivalent strip from plain portions of the chromatogram and eluted with 0.1 N HCI. Individual concentration of each colourant was estimated spectrophotometrically and added all-together to get the total concentration in the sample.

**Spectrophotometric Determination:**

***a) Preparation of Standard Curve:***

**i) Stock solution:** 100 mg of each reference colour is accurately weighed and is dissolved in 0.1 N HCl in separate 100 ml volumetric flasks. The volume is made up with the 0.1 N HCl in each case.

**ii ) Working standard:** 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 ml of stock solution (i) of the reference colour is pipetted into series of clean and dry 100 ml volumetric flasks and diluted to volume with 0.1 N HCl .

Optical density of each of the reference colours was determined at the respective wavelength of maximum absorption ($\lambda_{max}$) using a Shimadzu UV 2450 spectrophotometer. Standard curve is obtained by plotting optical density against

concentration. The concentration of the unknown colourant is noted from the calibration curve and the amount of colourant present in the food product is calculated. The $\lambda_{max}$ of the permitted colourants is given in **table 8.**

**2. Determination of Genotoxicity of Food Colourants**

**Cytokinesis Block Micronuclei (CBMN) Assay:**

The cytokinesis-block micronuclei assay is a comprehensive system for measuring DNA damage, cytostasis and cytotoxicity. DNA damage events are scored specifically in once-divided binucleated (BN) cells and include micronuclei (MNi), a biomarker of chromosome breakage and/or whole chromosome loss. The assay is being applied successfully for biomonitoring of in vivo genotoxic exposure, in vitro genotoxicity testing and in diverse research fields. Because of its reliability and good reproducibility, the CBMN assay has become one of the standard cytogenetic tests for genetic toxicology testing in human and mammalian cells.

In the CBMN assay, once-divided cells are recognized by their BN appearance after blocking cytokinesis with cytochalasin-B (Cyt-B), an inhibitor of microfilament ring assembly required for the completion of cytokinesis. The development of the cytokinesis-block (CB) technique has transformed the human-lymphocyte micronucleus assay (MN) into a reliable and precise method for assessing chromosome damage. The CBMN methodology is simple and allows for rapid assessment of cells thus making it an economical procedure to implement on a large scale. The procedure can take up to 5 days to complete.

Prepare the culture medium in sterile tissue-culture-grade glass or plastic bottles. Smaller volumes of culture medium may be prepared depending on the number of cultures required. Culture medium can be stored for 1 week at $4^{o}$ C before use. It is not usually necessary to sterile filter the prepared culture medium if rigorous aseptic technique is followed; however, to be absolutely certain, medium may be sterile-filtered using a 0.22 mm filter.

**Protocol:**

Isolation and counting of leukocytes – Timing; approximately 3 h and all procedures until slide preparation must be carried out aseptically.

1. Collect fresh blood by venipuncture into vacutainer blood tubes with lithium heparin anticoagulant. Keep blood tubes at room temperature (i.e. 20–$22^{o}$ C). The volume taken is dependent on the number of cells needed. (Usually one can expect to collect up to 1x106 leukocytes per 1 ml blood).
2. Isolate lymphocytes on Ficoll (sigma) gradients as follows: Add 2ml of Ficoll to a 10ml centrifuge tube and carefully overlay 6ml of diluted blood sample.
3. Centrifuge at 1000 rpm for 10 min.
4. Remove the leukocyte layer located at the interface of Ficoll–Paque and diluted plasma into a fresh 10 ml tube, using a sterile, plugged, Pasteur pipette, taking care not to remove too much Ficoll–Paque.
5. Discard the supernatant and resuspend the cell pellet (slightly beige colour) in RPMI 1640 medium and centrifuge for 10 minutes.
6. Remove the supernatant and repeat step 5.

7. Culture the lymphocytes in sterile bottles using RPMI 1640 medium containing 15% foetal calf serum. Lymphocytes stimulate to divide with phytohaemagglutinin (PHA) and incubate for 72 hours at 37ºC.

   Critical Step -Cyt-B must be added exactly 44 h after PHA stimulation.

8. 44 hr after PHA stimulation, add cytochalasin-B to the cultures to give a final concentration of 4.5µg/ml.
9. Return cultures to the incubator and incubate the cultures for a further 28 h.
10. 28 hr after addition of cytochalasin –B, transfer the whole contents into a sterile centrifuge tube and centrifuge for 10 minutes; remove the supernatant, shack the pellet in a cyclomixer.
11. Add 10 ml of 0.075 M KCL solution to the cell button and keep at 37ºC for 10 minutes.
12. After this, add 2 drops of freshly prepared fixative (Methanol: Acetic acid) in the ratio 3:1.Again centrifuge at 1000 rpm for 10 minutes.
13. Remove the supernatant and mix the cell button in a cyclomixer and add 10 ml of freshly prepared fixative and centrifuge at 1000 rpm for 10 minutes.
14. Repeat this process until the supernatant becomes clear and the cell button becomes white.
15. From the cell button, prepare cell suspension.
16. Drop the cell suspension drop by drop on to pre cleaned, labelled, chilled slides

17. Air-dry the slides for 10 min, then fix in absolute methanol for 10 min and stain with May-Grunwald Giemsa for 10 minutes.
18. Code the slides before scoring and examine at 100X magnification. The number of MN in no less than 1000 binucleated cells should be scored and the distribution of MN among binucleated cells should be recorded.

**Criteria for Identifying Binucleated Cytokinesis Block Cells:** The chosen harvest time should maximize the proportion of BN cells and minimize the frequency of mononucleated and multinucleated (three or more nuclei) cells.

The cytokinesis blocked cells scored for micronucleus frequency have to satisfy the following criteria:

- Cells should have two nuclei of approximately equal size;
- The 2 nuclei may be attached by a fine nucleoplasmic bridge; and
- The 2 nuclei may overlap slightly or touch each other at the edges.
- Cells should not contain more than 6 micronuclei.
- The cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

**Criteria for Identifying Micronuclei:** Micronuclei are morphologically identical to, but smaller than normal nuclei. They also have the following characteristics.

- Diameter between 1/6 and 1/3 that of the main nuclei.
- They are non-refractile.

- They are not linked to the main nuclei via a nucleoplasmic bridge.
- Micronuclei may sometimes overlap the boundaries of the main nuclei.
- MNi usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

The microscopic appearance of Cytokinesis Block Binucleated Cells with and without micronuclei is shown in **Figure 8**.

**Preparation of Reagents:**

Phytohaemagglutinin (PHA-M: 5mg stock):-

Dissolve 5mg stock of PHA-M in 5ml of distilled water.

Hypotonic KCl solution (0.075M):-

Dissolve 5.6gm of KCl (99.9%), (MW-74.55) in 1000ml of distilled water.

Fixative [Methanol and Glacial Acetic acid in the ratio 3:1 (v/v)]:-

Mix three parts of absolute methanol (99.8%) (32.04g/mol) and one part of glacial acetic acid (99.7%) (MW-60.05). Methanol acts as a fixative and acetic acid as the scavenger. Better morphological fixation occurs at low temperatures.

Cytochalasin-B:- Dissolve 1mg Cytochalasin-B in 1ml of dimethyl sulphoxide and store in freezer.

Four parallel lymphocyte microcultures in duplicate were set up for each sample to evaluate the extent of genotoxicity produced by various colouring agents and their combinations as described by Fenech (2000). Culture A was kept as control and colouring agents and their combinations were added to culture B, C and D with varying concentration of 100, 200, and 500 µg/ml respectively at the time of culture initiation.

The results were subjected to statistical analysis. Multivariate analysis and Pairwise comparison were performed using Statistical Package for Social Sciences (SPSS).

**Table - 7**

**Sample Collection Data**

| Item | Colour | No. of Samples | |
|---|---|---|---|
| | | **Urban** | **Rural** |
| **Ladu** | Yellow | 50 | 50 |
| | Orange | 50 | 50 |
| | Red | 50 | 50 |
| | **Total** | **300** | |
| **Jilebi** | Yellow | 50 | 50 |
| | Orange | 50 | 50 |
| | Red | 50 | 50 |
| | **Total** | **300** | |
| **Halwa** | | | |
| | Yellow | 40 | 40 |
| | Red | 40 | 40 |
| | Orange | 40 | 40 |
| | Brown | 40 | 40 |
| | Green | 40 | 40 |
| | **Total** | **400** | |

**Table - 8**

**Absorption Maxima of Permitted Food Colours**

| Sl. No | Name of Colour | Absorption Maxima (nm) |
|---|---|---|
| 1 | Carmoisine | 516 |
| 2 | Ponceau 4R | 507 |
| 3 | Erythrosine | 527 |
| 4 | Fast Green FCF | 624 |
| 5 | Indigo Carmine | 609 |
| 6 | Brilliant blue FCF | 630 |
| 7 | Tartrazine | 427 |
| 8 | Sunset yellow FCF | 482 |

**Figure – 1**

## Ladu and Jilebi

**Figure – 2**

## Halwa

**Figure - 3**


KERALA
KASARGOD
KANNUR
WYNAD
Kalpatta
KOZHIKODE
MALAPPURAM
PALAKKAD
THRISSUR
IDUKKI
ERNAKULAM
KOTTAYAM
ALAPPUZHA
PATHANAMTHITTA
KOLLAM
THIRUVANANTHAPURAM

**Figure – 4**

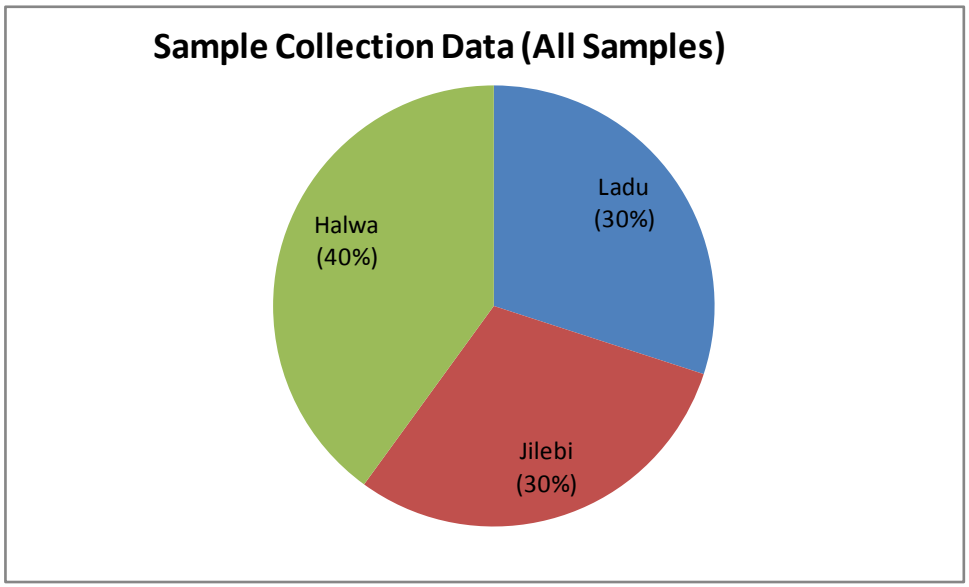



**Figure -5**

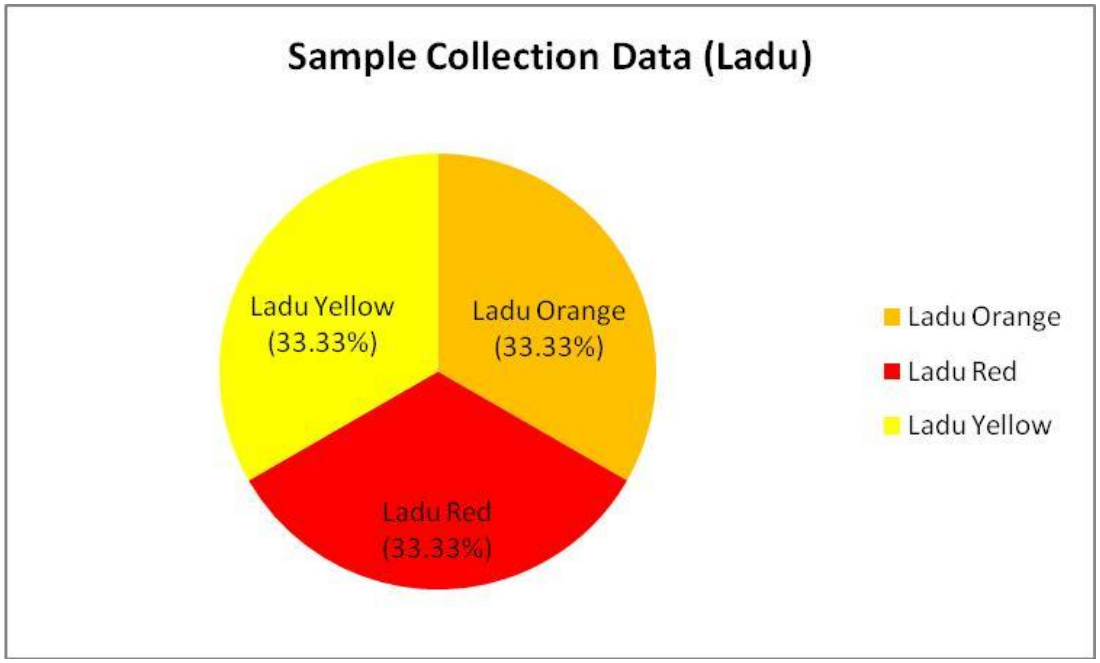


**Figure - 6**

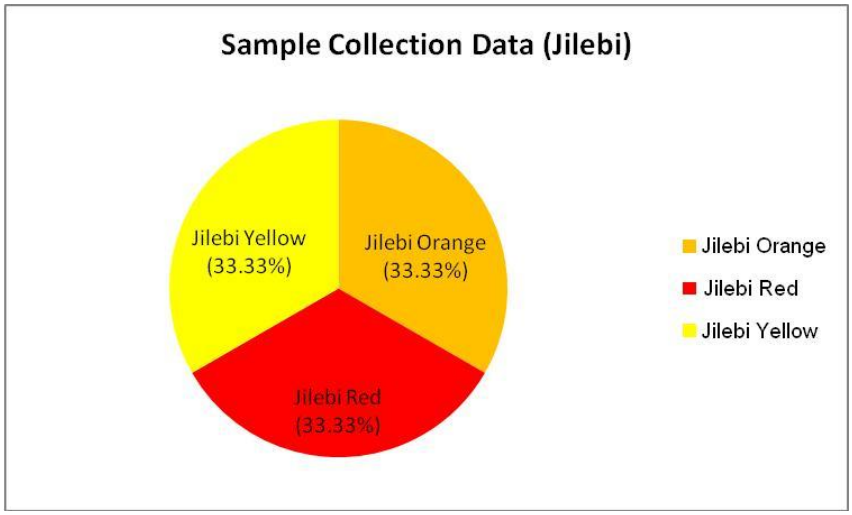



**Figure - 7**

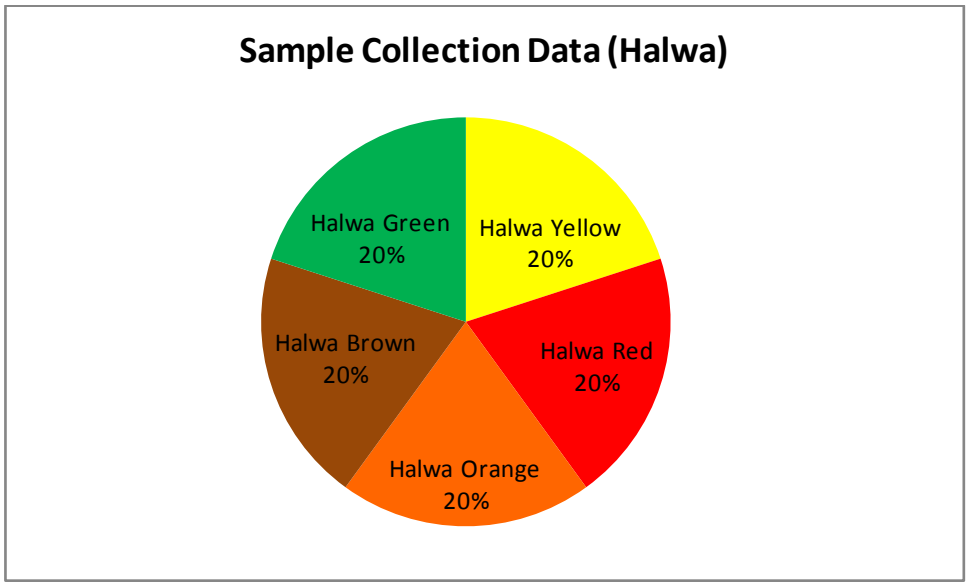
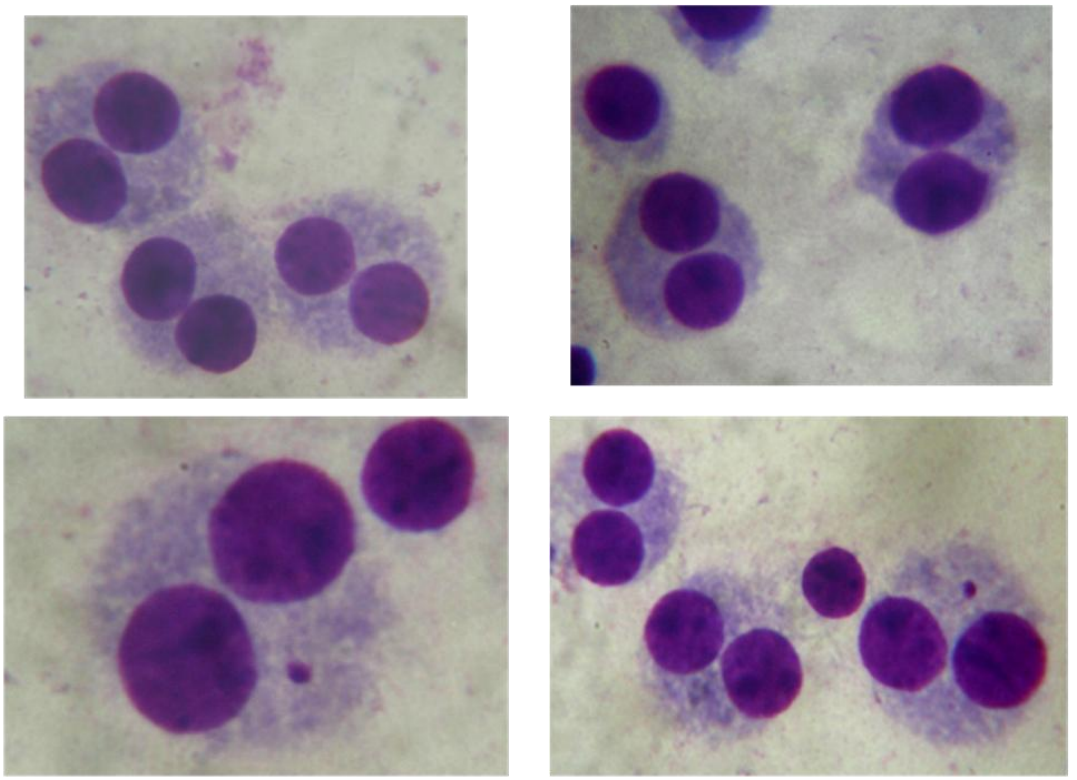


**Figure – 8**



Cytokinesis- block binucleated cells with and without micronuclei