3: MATERIALS and METHODS:

3.1: Materials:

1. Rhizomes of *Curcuma caesia* Roxb.
2. *Salmonella typhimurium* strains (TA98 and TA 100)
3. Animals (SwissAlbino Mice)
4. Bone marrow
5. Liver
6. S9 mix
7. Kidney
8. Cancer Cell lines: MDAMB 231(Breast cancer cell lines) and Calu6 (Lung Cancer cell lines).

3.2: Chemicals and Reagents

1. ABTS (7mM)
2. BHT
3. Bismuth nitrate
4. BSA (Bovine Serum Albumin)
5. Chloroform
6. Concentrated Hydrochloric acid
7. Concentrated Sulphuric acid
8. Copper II (CuII) sulfate pentahydrate

9. Cyclophosphamide (CP)

10. Dilute HCl

11. Dimethyl Sulphoxide (DMSO)

12. DPPH (2, 2-Diphenyl-1-picryl hydrazyl)

. 13. DTNB (0.6mM)

14. D-Biotin (0.2mM)

15. EDTA (15mM)

16. Eosin

17. Ethanol

18. Ethyl Acetate

19. $FeCl_3$ solution

20. $FeCl_3$ (1%)

21. $FeCl_3$ (0.5%)

22. $FeCl_3$ (10mM)

23. Fetal bovine serum albumin (FBS)

24. Folin-Ciocalteu's reagent (10%)

25. Formalin (10%)

26. Formazan

27. Gallic acid

28. Geimsa stain

29. Glacial acetic acid

30. Glacial acetic anhydride

31. Glucose-6-phosphate (1M)

32. Gum acacia (2%)

33. GSSG

34. Haematoxylin
35. HCl (0.25N)
36. Iodine
37. Isopropylalcohol

38. L-histidine (0.2mM)

39. Lead acetate solution

40. Magnesium (turning chips)

41. MgCl2 (0.4M)

42. May Grunwald stain

43. Mercuricchloride
44. Methanol
45. MTT
46. Napthol
47. Nicotinamide Adenine Dinucleotide Hydrogen (NADH) (156µM)
48. NicotinamideAdenine Dinucleotide Phosphate Hydrogen (NADPH) (9.6mM)
49. Nicotinamide Adenine Dinucleotide Phosphate (NADP) (0.1M)
50. NBT (60µM)
51. Nutrient Broth
52. Petroleum Ether
53. Phenazine methosulphate(PMS)(468µM)
54. Phosphate buffer

55. Phosphate buffer saline (PH=7.4)

56. Phosphate buffer (0.2 M, pH 7.4)

57. Phosphate buffer (0.12M, PH=7.2)

58. Phosphate BufferSaline (1X)

59. Picric acid
60. Potassium Chloride (KCl) (1.65M)
61. KCl (0.2M)
62. Potassium Hydroxide (KOH)
63. Potassium ferricyanide (1%)
64. Potassium iodide
65. Potassium persulphate (2.45mM)
66. Potassium sodium tartarate

67. NaOH

68. $NaHCO_3$ (7.5%)

69. Sodium azide(10mM)

70. TBA

71. TCA (10%)

3.3: Plant Material Collection and Extraction:

3.3.1: Extraction:

Rhizomes of *Curcuma caesia* Roxb.were collected from Nambol, Bishnupur District, Manipur, in the month of November, 2012; were cut into pieces and sun dried. The dried rhizomes were coarsely powdered and subjected to hot continuous successive extraction in a Soxhlet apparatus with solvents in the increasing order of polarity using petroleum ether, ethyl acetate, ethanol, methanol and water under controlled temperature (50-60^{0}C). Extractives were concentrated below 40^{0}C under water bath. The five dried extracts were stored in the desiccator for further uses (Satinder and Karen, 2003).

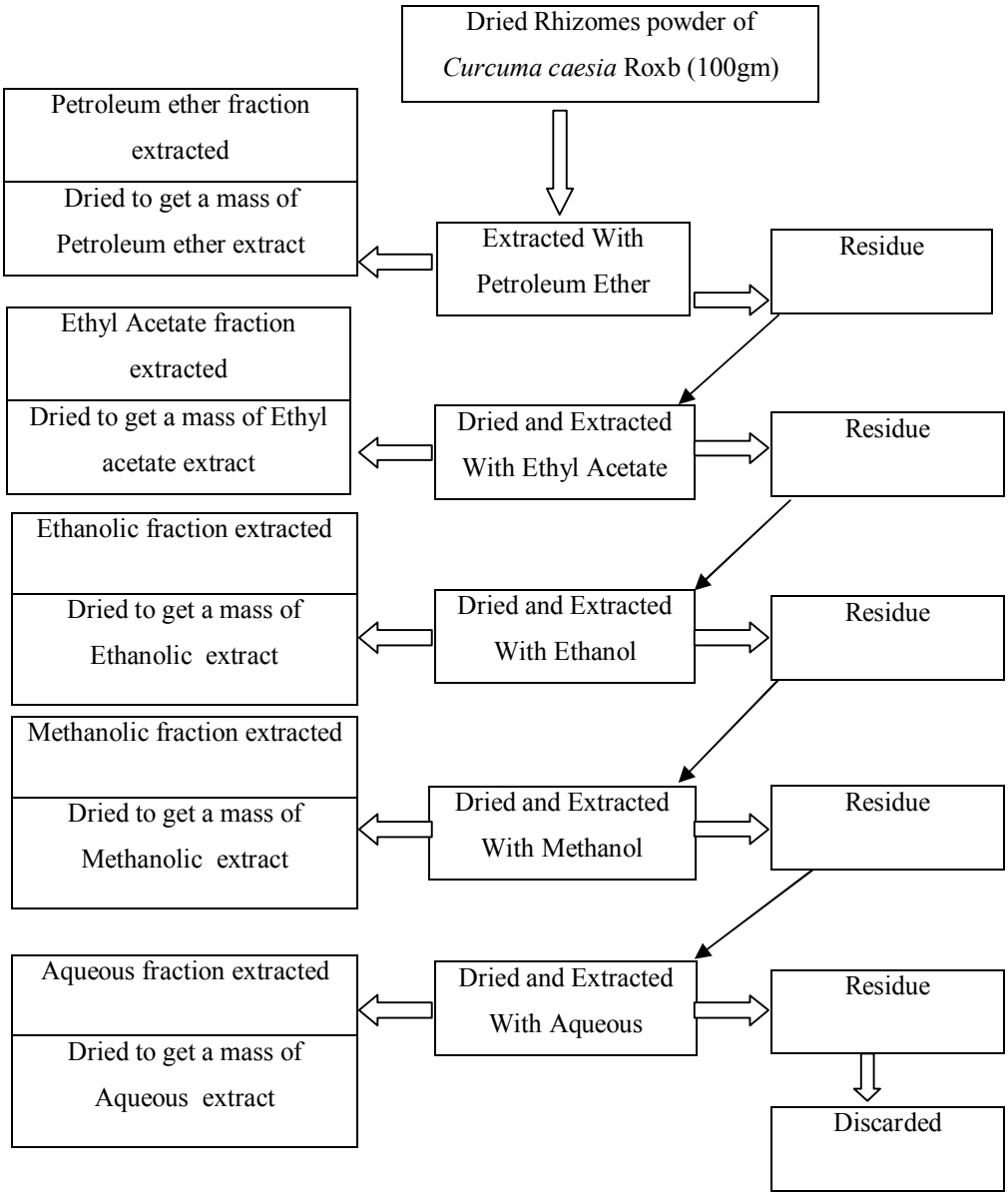


Fig. 4: Schematic Representation of Successive Extraction of the rhizomes of *Curcuma caesia* Roxb.

3.3.2: Percentage Yield:

The percentage yield of the extracts was calculated using the formula:

% yield = (weight of the extractives/weight of the crude extract) ×100.

3.4: Preliminary phytochemical screening:

Preliminary phytochemical screening was performed using the specified protocols for the qualitative analysis of alkaloids, carbohydrates, reducing sugars, flavanoids, terpenes, steroids and tannins. The screening test as follows:

3.4.1. Test for alkaloids (Khandelwal, 2006**):**

3.4.1.1: Dragendroff's test:

Dragendroff's reagent:

Solution A: 0.85g of basic bismuth nitrate was dissolved in glacial acetic acid followed by addition of 40 ml of water under heating.

Solution B: 8g of potassium iodide was dissolved in 30ml of water. Stock solutions weremixedin the proportion of 1:1(A+B= 1:1).

To the extract few drops of acetic acid was added, followed by the addition of few drops of Dragendroff's reagent and shaken well. Observance of orange red precipitate indicates the presence of alkaloids.

3.4.1.2: Mayer's test:

Mayer's reagent: 1.36gof mercuricchloride was mixed with 5g of potassium iodide and was dissolved in 100ml of distilled water.

To 2-3 ml of filtrate from each extractive, few drops of Mayer's reagent were added. Presence of cream colored precipitate indicates the presence of alkaloids.

3.4.1.3: Hager's test:

Hager's reagent: 1g of picric acid was dissolved in 100ml of distilled water and dissolved properly.

To a few ml of the filtrate from each extractive, 1 or 2 ml of Hager's reagent was added. Presence of prominent yellow precipitate indicates the presence of alkaloids.

3.4.1.4: Wagner's test:

Wagner's reagent: 1.27g of iodine was mixed with 2g of potassium iodide in 100ml of distilled water.

A fraction of the extract was treated with Wagner's reagent and observed for the formation of reddish brown precipitate for the presence of alkaloids.

3.4.2: Test for carbohydrates (Sofowara, 1993)**:**

Molisch's test (General test):

Molisch reagent: 15g of napthol was dissolved in 100ml of alcohol or chloroform.

Few drops of Molisch's reagent were added to each of the extractives which was followed by the addition of 1ml of the concentrated H_2SO_4 from the side of the test tube. The mixture was then allowed to stand for 2 minutes and then diluted with 5ml of distilled water. Formation of red and dull violet color at the interface of two layers was confirmed as positive test.

3.4.3: Test for steroids (Sofowara, 1993)**:**

Libermann-Burchard Reaction:

The few ml of the extract was dissolved in a few drops of chloroform which was then followed by the addition of 3ml of glacial acetic anhydride and 3ml of glacial acetic acid. The mixture was warmed and cooled under the tap water, followed by the addition of few drops of conc.H_2SO_4 along the sides of the test tube. The appearance of red green color indicates the presence of steroids.

3.4.4: Test for Flavanoids (Treas and Evans, 2002)**:**

3.4.4.1: Aqueous NaOH test: To a small fraction of each extractive few drops of 1N aqueous NaOH (10% NaOH dissolved in water) was added and observed for the formation of the yellow orange color. A change in color from yellow to colorless on the addition of dilute HCl indicates the presence of flavanoids.

3.4.4.2: Concentrated H_2SO_4 test: To a small fraction of each extractive, concentrated H_2SO_4 was added and observed the formation of orange color which is an indicative for the presence of flavanoids.

3.4.4.3: Schinoda's test: To a small fraction of the extract, a piece of magnesium (turning chips) was added and followed by the addition of concentrated HCl and then heated slightly. The formation of dark pink color indicates the positive result.

3.4.5: Detection of Phenolic compounds (Khandelwal, 2002)**:**

3.4.5.1: Ferric chloride test: A fraction of each extract was treated with 5% $FeCl_3$ solution and observed for the formation of deep blue color.

3.4.5.2: Lead acetate test: A fraction of each extract was treated with 10% lead acetate solution and observed for the formation of white precipitate.

3.4.6: Detection of Saponins (Treas and Evans, 2002)**:**

Foam Test: A fraction of each extract was vigorously shaken with water and boil and observed for the persistent foam.

3.4.7: Test for Soluble Starch (Vishnoi, 1979**):** Few quantity of the portion was boiled with 1ml of 5% KOH, cooled and acidified with concentrated H_2SO_4. Formation of yellow color was taken as the presence of starch.

3.4.8: Test for Reducing Sugars (Sofowora, 1993**):**

3.4.8.1: Fehling's Test for free reducing sugar:

Fehling'A: 69.28g of CuII sulfate pentahydrate (Cu_2SO_4. $5H_2O$) was dissolved in 1 liter of distilled water.

Fehling'B: 350g of Rochelle's salt, i.e (potassium sodium tartarate) and 100g of sodium hydroxide were dissolved in 1 litre of distilled water.

About 0.5g of each extract was dissolved in distilled water and filtered. The filtrate was heated with 0.5ml of each Fehling's solution A and B. Formation of red precipitate was an indication of the presence of free reducing sugars.

3.4.8.2: Fehlings test for combined reducing sugar:

About 0.5 g of each extract was hydrolyzed by boiling with 5ml of dilute HCl and resulting solution was neutralized with sodium hydroxide solution. To this few drops of Fehling's solution was added and then heated in a water bath for 2 minutes. Appearance of reddish brown precipitate of cuprous oxide indicates the presence of combined reducing sugar.

3.4.9: Test for Tannins (Treas and Evans, 2002)**:**

About0.5g of each extract was stirred with about 10ml of distilled water and then filtered. To the filtrate, few drops of 1% $FeCl_3$ solution was added. Observance of a blue black, green or blue precipitate indicates the presence of tannins.

3.4.10: Test for terpenoids (Sofowara, 1993**):**

A little of each portion of the extract was dissolved in ethanol and 1ml of acetic anhydride was added to each of the filtrate which was then followed by the addition of concentrated H_2SO_4. A change in color from pink to violet showed the presence of terpenoids.

3.5: Determination of total phenol contents in the plant extracts:

Principle:

Folin ciocalteau reagent (FC) is a heteropolyacid complex made up of orthophosphoric acid and molybdotungstic acid. The hydroxyl groups present in Gallic acid brings about the reduction of oxygen atoms present in tungstic acid or molybdate of FC reagent at the positions 1, 2 or 3 of oxygen atom. The reaction gives intense blue color which increases its intensity with the increase in concentration of Gallic acids and extracts exhibiting the absorbance at 765nm (Singleton *et al*., 1999).

3.5.1: Procedure:

The concentration of phenolics in plant extracts was determined using Folin Ciocalteau method (Singleton *et al*., 1999) with little modifications. The extracts in the concentration of 1 mg/ml were used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of each

extract solution, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% NaHCO3. Blank was prepared**,** containing 0.5 ml ethanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of $NaHCO_3$. The samples were thereafter incubated at 45°C for 45 min. The absorbance was determined using spectrophotometer at λ max = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of Gallic acid and the calibration line was constructed. Based on the measured absorbance, the concentration of phenolics was calculated from the calibration line; then the content of phenolics in each extract was expressed in terms of Gallic acid equivalent (mg of GAE/g d.wt of extract).

3.6: In Vitro Antioxidant Studies:

3.6.1: DPPH radical scavenging activity:

Principle:

DPPH is a stable free radical which gives purple color in methanol and shows maximum absorbance at 517nm. When 2, 2-Diphenyl-1-picryl hydrazyl radical (DPPH) reacts with the donors of hydrogen, it gets reduced to a corresponding 2, 2-Diphenyl-1-picryl hydrazine reducing the purple color to yellow color. The assay evaluates the addition of antioxidants to DPPH solution where the decrease in absorbance is measured (Espin *et al*., 2000; Huang *et al*., 2005).

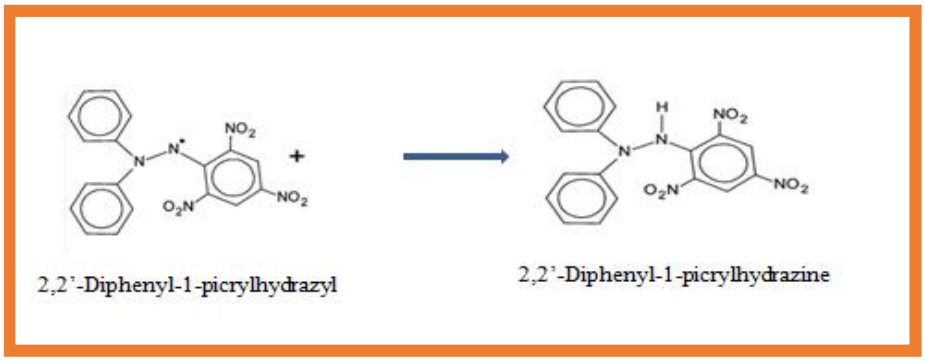


Fig: 5: Mechanism of action of rhizome extracts in DPPH Assay.

3.6.1.1: Procedure:

The quenching of free radical activity of different extracts was determined by spectrophotometric method against 2, 2-Diphenyl-1-picryl hydrazyl (DPPH) following Blois; 1958**.** 1ml of each extract of various concentrations (25-800µg/ml) were mixed with 1ml of DPPH (0.1mM) solution prepared in ethanol/methanol and incubated in dark for 20 minutes and absorbance values were recorded at 517nm. 1ml of ethanol and 1ml of ethanol/methanol solution of DPPH (0.2mM) was taken as control. Similarly 1ml of ethanolic solution of ascorbic acid (200µg/ml) was mixed with 1ml of DPPH ethanolic/methanolic solution and absorbance values were recorded. The radical scavenging activity was calculated using the following formula:

DPPH radical scavenging activity (%) = {[Ab-Aa] /Ab} x 100

Where Ab is the absorption of the control and Aa is the absorption of the extract/ascorbic sample.

3.6.2: Reducing Power Assay:

Principle:

The assay evaluates the reduction potential of the extracts involving the reduction of Ferricyanide ion $[Fe(CN)_6]^{3-}$ to ferrocyanide ion $[Fe(CN)_6]^{4-}$ by the electrons donated by polyphenols present in the plant extracts. Fe (III) ions combined with ferrocyanide ion $[Fe(CN)_6]^{4-}$ under acidic conditions resulting in the formation of ferricferrocyanide complex $Fe_4[Fe(CN)_6]_3$, a blue color solution (Graham, 1992). The intensity of blue color solution is measured at 700nm and its intensity increases with the increase in the concentration of extract.

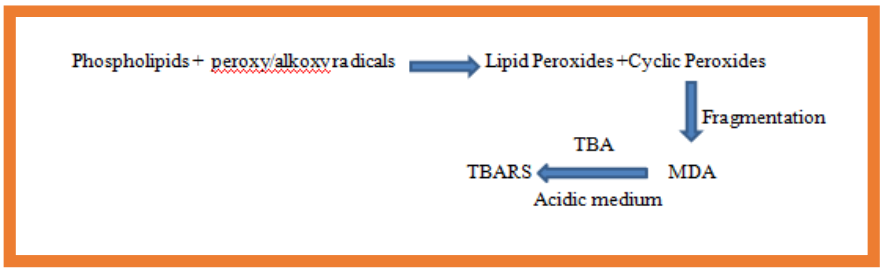


Fig: 6: Mechanism of action of Polyphenols in reducing power assay.

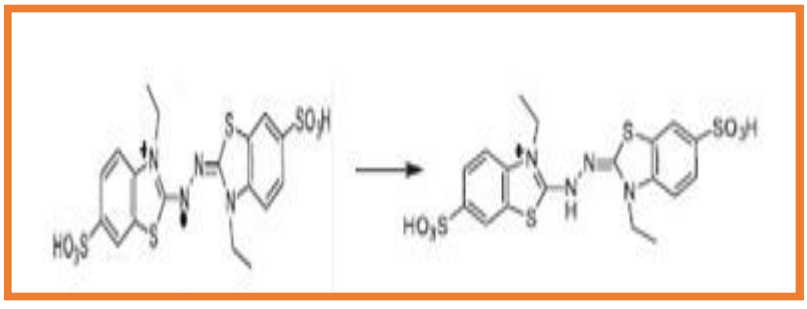
3.6.2.1: Procedure:

The ability to reduce ferric ions to ferrous ions by the antioxidants present in rhizomes of *Curcuma caesia* Roxb. was determined by the method of Oyaizu, 1986with little modification. From the different concentrations of each extract solutions (200µg- 1000 µg per ml), 1ml of each was mixed with 2.5 ml of 0.2 M phosphate buffer (P^H= 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was heated at 50 °C for 20 minutes and then cooled, followed by the addition of 2.5ml of 10% TCA and then centrifuged at 3000rpm for 10 minutes. 2.5ml of the supernatant was again mixed with 2.5ml of distilled water and 0.5ml of 0.5% $FeCl_3$ and the absorbance was recorded at 700nm against blank made without extract. Increase in the absorbance values shows the increasing reducing ability of the extracts. The entire test was performed in triplicate.

3.6.3: ABTS Radical Cation Decolourisation Assay:

Principle:

The basicprinciple behind this assay involves the generation of ABTS free radical cation and it is generated by mixing ABTS and potassium persulphate. The reaction mixture produce blue green color radical. The blue green color radical is converted into colorless ABTS in the presence of antioxidants from plant extracts. The absorbance of these colorless ABTS is measured at 734nm (Re *et al*., 1999).



$ABTS^+$ Antioxidants

Fig.7:Scavenging of ABTS radical cation by the antioxidants.

3.6.3.1: Procedure:

The assay was performed following Re *et al*., 1999, with slight modifications. ABTS radical cation was generated by the addition of

7mM ABTS and 2.45mM potassium persulphate. The reaction mixtures were allowed to stand for 12-16 hrs at 30^0C in the dark. After 16 hrs the reaction mixture was diluted with ethanol or phosphate buffer saline (PH=7.4). Following that 0.3ml of ABTS+ and 0.5 ml of extract solution (5-100µg/ml) were mixed and absorbance was read at 734nm without any incubation period against the sample blank prepared by mixing 0.3ml of methanol and 0.5ml of DMSO. Similarly control was also read in the same wavelength by adding together of 0.3ml of ABTS+, 0.5ml of DMSO and 1ml of methanol. With the same procedure measurement of Gallic acid standard were also recorded. Percentage of inhibition was calculated using the formula given below:

(%) inhibition = {[Ab-Aa] /Ab} x 100

Where Ab is the absorption of the control and Aa is the absorption of the extract sample.

3.6.4: Superoxide anion radical scavenging assay:

Principle:

It involves the generation of superoxide anion free radicals (O_2^-) in the presence of phenazine methosulphate (PMS) - NADH system. Superoxide anion free radicals reduce the NBT (Nitroblue tetrazolium) to diformazan which is indicated by the production of blue color and is measured spectrophotometrically at 560nm (Nishikimi *et al*., 1972). The non enzymatic generation of O_2^- can be reduced by the introduction of extracts having potential antioxidant activity to decrease the intensity of blue color.

3.6.4.1: Procedure:

It was performed following Nishikimi *et al*., 1972, with slight modifications. The reaction mixture contains 1ml of different concentrations of extract (20-200µg), 1ml of 156µM NADH, 1ml of 60µM NBT, and 1ml of 468µM phenazine methosulphate(PMS) in phosphate buffer(PH=8.3). The reaction mixture was incubated at 25^0C for 10 minutes and absorbance was taken against blank at 560nm. The

standard taken was Gallic acid. The inhibition mixture was calculated using the formula:

% inhibition= {[Ab-Aa] /Ab} x 100

where Ab is the absorption of the control and Aa is the absorption of the extract sample.

3.7: Antimutagenicity Assay:

Antimutagenic potential of rhizome extracts of *Curcuma caesia* Roxb was performed using Ames assay proposed by Maron and Ames 1983 with slight modifications. The present study employed *Salmonella typhimurium* tester strains TA 98 and TA 100 because they can detect more than 100 chemical mutagens.

Principle:

The principle behind Ames Salmonella assay/ microsomal mutagenicity assay is that it uses specially designed auxotrophic mutants of Salmonella strains to detect a wide variety of chemicals which is capable of inducing genetic damage that leads to mutations. The test evaluates the mutagenic properties of test compounds. The tester strains are incapable of synthesizing histidine (His-) amino acid required for their growth and hence they are unable to form colonies in its absence. The strains carry specific mutations (frameshift mutation and base pair deletion mutation) in their histidine operon, thus act as hot spot region for mutagens that cause DNA damage through different kinds of mechanisms. Those bacteria which are independent of histidine (His+) able to form colonies. However number of spontaneously induced revertant colonies per plate is relatively constant. The reversion of histidine independence bacterial colonies i.e. prototrophs (His+) serves as a marker for identification of mutation that occurs at the site of pre-existing mutation or near the genes of previously mutated genes, thus restore the gene function. When a mutagen is added to the plate, the number of bacterial colonies increases dose dependently. There are certain compounds which are non-mutagenic in mammalian systems but their derivatives which are formed

by the action of cytochrome P450 of liver microsomes are potent carcinogens. In order to make bacterial systems useful to check the mutagenicity of compounds, exogenous mammalian metabolic system (S9 liver mix from mice) is included in the assay (Tezs, 2008).

While testing the antimutagenic potential of plant extracts of *Curcuma caesia* Roxb, the assay was modified with the inclusion of plant extract along with the known mutagen. In the presence of extract, the number of revertant colonies decreases as compared to the colonies produced by positive control (mutagen alone).

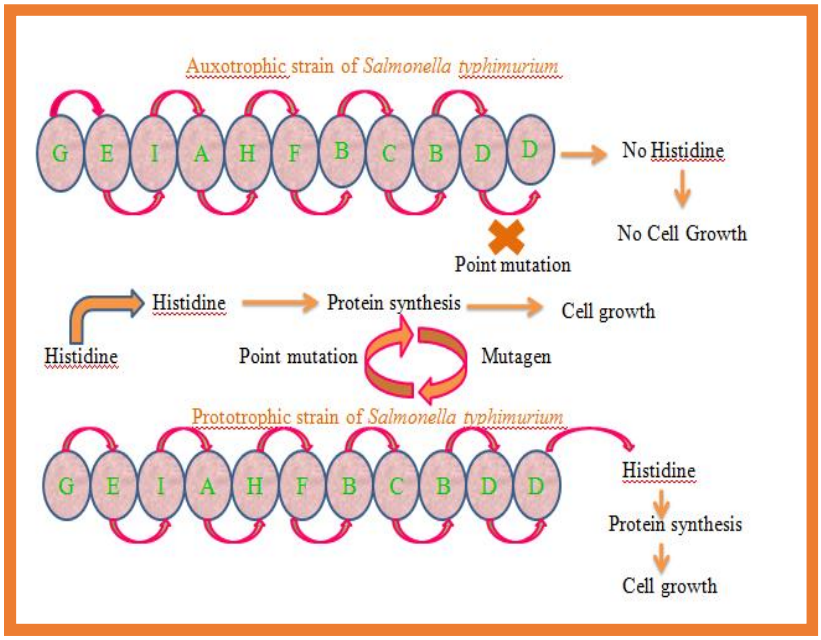


Fig.8: Schematic overview of Histidine biosynthesis in *Salmonella typhimurium* (Wycisk *et al.*, 2004).

3.7.1: Bacterial strains:

Salmonella typhimurium strains TA98 and TA100 which is histidine-requiring mutants, were kindly provided by IMTECH, Chandigarh, India and are maintained as described byMaron and Ames, 1983. The genotypes of the test strains were checked routinely for their histidine requirement, rfa mutations, UV sensitivity (uvrB mutation). They were stored at -80^{o}C for further use.

3.7.2: S9 preparation:

S9 is the mitochondrial enzyme mix required for metabolic activation of indirect acting mutagens like cyclophosphamide. The S9 mixture was prepared from male rat liver using the chemicals 1M Glucose-6-phosphate, 0.1M NADP, 0.2M Phosphate buffer, 0.4M $MgCl_2^+$ 1.65MKCl (Himedia- India) as described by Maron and Ames *et al* **.,**1983. S9 mix was prepared fresh for each assay.

3.7.3: Salmonella-microsome assay:

The bacterial strains were incubated in Nutrient Broth for 16 h at 37 ◦C in an orbital shaker to obtain a density of 2×10^9 colony forming units (CFU/mL).0.1ml of an overnight culture of bacteria and 0.5ml of sodium phosphate buffer (0.2 M, pH 7.4 for assay without S9) supplemented with 0.2mM L-histidine and 0.2mM D-biotin solution containing different concentrations of each extract. These were mixed using vortexer for 10 minutes. The resulting complete mixture was poured on minimal agar plates (minimal agar plates are prepared by mixing 50X Vogel Bonner salts, 40% glucose, sterile histidene, sterile ampicillin, and agar) prepared as described by Maron and Ames, 1983. The plates were incubated at 37^oC for 48 h and the revertant bacterial colonies of each plate were counted. Data were collected with a mean ± standard deviation of three experiments (n = 3).

3.7.4: Antimutagenicity testing:

For the experiment with S9 mix, in a tube containing 0.1ml of overnight grown bacterial cultures, 0.2mM Histidine-Biotin solution supplemented with each extract at different concentrations were added, mixed properly and incubated for 3minutes. After incubation 0.1ml of the CP (500µg/plate) and 0.5ml of S9 mix were added. The experiment was performed as mentioned above. Percentage inhibition was calculated using the formula (Lakshmi, 2006) as given below:

$$\%\text{Inhibition of mutagenicity} = [\{(R1 - SR) - (R2 - SR)\}/(R1 - SR)] \times 100$$

Where *R*1 is the number of revertants without extracts, but with CP, *R*2 the number of revertants with extracts plus mutagen (CP) and SR is the spontaneous revertants i.e without extracts and mutagen.

3.7.5: Statistical analysis:

The results are presented as the average and S.D (standard deviations) of three experiments with triplicate plates/dose/experiment. The regression analysis was carried out in Microsoft Excel 2007 between % inhibition of mutagenicity and concentrations of the plant extracts. Students't test was performed to compare the mean values of treatment with the positive control and positive control with the negative control using the following formula (two tailed analysis):

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{(n_1 - 1)s^2_1 + (n_2 - 1)s^2_2}{n_1 + n_2 - 2}\left[\frac{n_1 + n_2}{n_1 n_2}\right]}}$$

$\bar{X}_1$ is the mean of Group1., $\bar{X}_2$ is the mean of Group 2., n_1 is the number of mice in Group 1

n_2 is the number of mice in Group 2., s^2_1 is the variance for Group 1

s^2_2 is the variance for Group 2., Degree of Freedom is n+n-2.

3.8: Acute Toxicity Study:

The animals were procured from the Pasteur Institute, Shillong, India and were acclimatized for 15 days. For acute toxicity study female mice of about 20-25g weight were used and study was conducted for three rhizome extracts (ethyl acetate, ethanolic and methanolic extracts) in accordance with OECD guidelines 423, 2001. Animals were grouped as per the following schedule:

Table.3: Study of acute toxicity of rhizome extracts of *C.caesia* Roxb.

Groups	Rhizome extracts(mg/Kg; per oral)	Sighting study No. of mice	Main study
Group I	5	3	-
Group II	50	3	-
Group III	300	3	-
Group IV	2000	3	5
Group V	Control(Vehicle)	3	5

The study procedure was divided into two phases one for sight study and one for main study. Animals were made fasted for 18hrs by depriving of food; water was withdrawn 4 hrs before dosing and body weights were noted before dosing. For sighting study, animals received fixed doses such as 5, 50, 300, 2000 mg/kg b. wt. orally and the animals were observed continuously for the first 2hrs and then daily for 14 days and for main study animals received highest dose of extract i.e. 2000mg/kg. b.wt. The control group was given 2% gum acacia and 1ml/ 100g of volume was administered to all the animals.

3.9: Antigenotoxic study:

3.9.1: Experimental Design:

The study was conducted on 25-30g body weight male Swiss albino mice. They were maintained under controlled conditions of temperature and light (12hrs light: 12 hrs dark). They were provided standard mice feed. The study protocol was approved by the Institutional Ethical Committee (IEC/AUS/2-013-33, dt. 20/3/13 Assam University, Silchar, India).

The experimental animals were divided into eight groups, each containing five mice designated as follows:

Group 1: Negative Control: Each animal received distilled water.

Group 2: Positive Control: CP was administered intraperitoneally at a dose of 50 mg /kg b.wt.

Group 3: Animals received 100mg/kg.b.wt of each extract only intraperitonially.

Group 4: Animals received 250mg/kg.b.wt of each extract only intraperitonially.

Group 5: Animals received 500mg/kg.b.wt of each extract only intraperitonially.

Group 6: Pretreatment: Each extract was administered at a dose of 100mg/kg b. wt (i.p) followed by CP (i.p) treatment 2hrs later.

Group 7: Pretreatment: Each extract was administered at a dose of 250mg/kg b. wt (i.p) followed by CP (i.p) treatment 2hrs later.

Group 8: Pretreatment: Each extract was administered at a dose of 500mg/kg b. wt (i.p) followed by CP (i.p) treatment 2hrs later.

After 7 days of the experimental period, the animals were sacrificed and parameters described below were studied.

3.9.2: Micronucleus assay:

Principle:

The assay is a mutagenic test system that detects the chemicals responsible for the formation of small membrane bound DNA fragments i.e. micronuclei in the cytoplasm of interphase (metaphase/anaphase) cells. The assay detects those agents which modify the chromosome structure and segregation in such a way that those agents lead to the introduction of micronuclei in interphase cells. Micronuclei originates from whole chromosome (aneugenic agent leading to chromosome loss) or an acentric chromosome fragment separated from a chromosome after breakage (clastogenic event) which are not capableof migrating along with the rest of the chromosome during anaphase cell division. The formation of micronuclei after exposure to the test substance is harvested and analysis is done for the presence of micronuclei microscopically after staining the cells. A positive result is classified as concentration dependent increase in the number of micronuclei after exposure to the test substance (Fenech *et al*.,, 2000, 2003; Kirsch *et al*., 1997, 2001;

Pary, 1997). If a fraction of plant extract is able to scavenge the radicals produce by tested compounds then the number of micronuclei will be reduced as compare to positive control group.

3.9.2.1: Procedure:

Mice bone marrow micronucleus test was carried out according to Schmid, 1975. Bone marrow cells from both the femurs of each animal were flushed out with fetal bovine serum albumin (FBS) in a centrifuge tube. The cell suspensions were centrifuged at 10000 rpm for 10 minutes and supernatant was removed. The pellet was resuspended in FBS before being used for preparing slides. The air dried slides were stained with May Grunwald stain and Geimsa stain. Thousand polychromatic erythrocytes (PCEs) were scored for each group of animals to determine the frequency of micronucleated polychromatic erythrocytes (MnPCEs). All the slides were coded and scored by the same observer. The percentage reduction in the frequency of micronuclei was calculated using the formula given by Serpeloni, 2008.

Reduction (%) = (mean DI in A- mean DI in B) / (mean DI in A- mean DI in C)

A= group treated with Cyclophosphamide (CP)

B= group treated with CP plus each extract of the rhizome.

C= negative groups.

DI= damage index

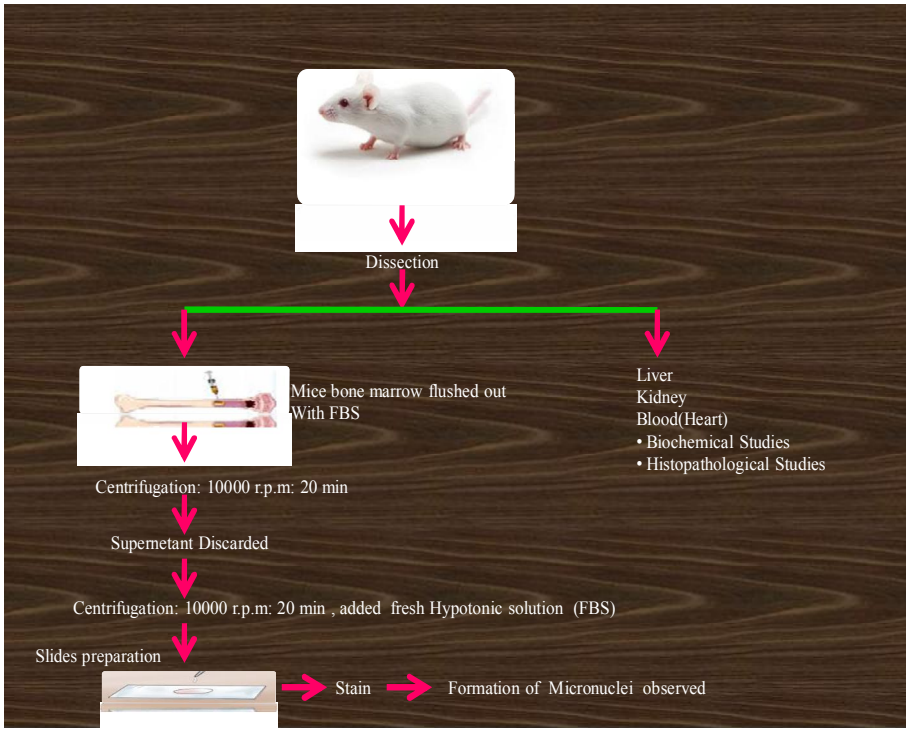


Fig.9: Schematic representation of mice micronucleus assay and biochemical analysis

3.10: Biochemical Analysis:

3.10.1: Serum Sample collection:

The blood sample was collected from the heart and kept it undisturbed for 2hrs. Serum was then removed by centrifugation at 10000g for 10 minutes and isolated serum sample was kept in -80^{0}Cfor further analysis of SGOT and SGPT. Besides, the removed kidney and liver were washed with phosphate buffer saline and blotted with filter paper and kept in deep freezer for further analysis.

3.10.2: Determination of serum SGOT and SGPT:

Principle for SGPT:

SGPT converts L-Alanine ans α-Ketoglutarate to Pyruvate and Glutamate. Pyruvate formed reacts with 2, 4-Dinitrophenyl hydrazine to produce a hydrazone derivative, which in an alkaline medium produces a brown colored complex whose intensity is measured. The reaction does not obey Beer's law and hence a calibration curve is plotted using a

Pyruvate standard. The activity of SGPT is read off from the calibration curve (Reitman and Frenkel, 1957).

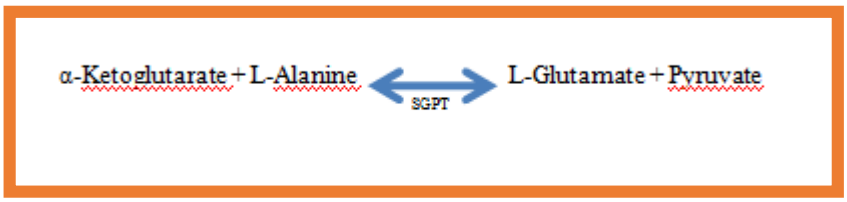


Fig.10: Reaction Catalyze by the SGPT.

Principle for SGOT:

SGOT catalyses the transfer of the amino group of L-aspartate to α-ketoglutarate resulting in the formation of oxaloacetate and L-glutamate. Oxaloacetate formed reacts with 2, 4-Dinitrophenyl hydrazine to form 2, 4-Dinitrophenyl hydrazone derivative which gives brown colored in alkaline medium whose intensity is measured. The reaction does not obey Beer's law and hence a calibration curve is plotted using a Pyruvate standard. The activity of SGPT is read off from the calibration curve (Reitman and Frenkel, 1957).

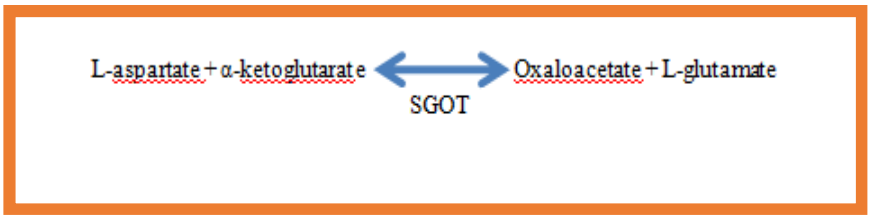


Fig.11: Reaction Catalyze by the SGOT.

3.10.2.1: Procedure:

The SGOT and SGPT levels were determined by a standard enzymatic method using commercial kits provided {SGOT (ASAT) and SGPT (ALT) kits} (Reitman and Frenkel, 1957).

3.10.3: Quantitative assay for lipid peroxidation:

Principle:

The principle behind lipid peroxidation assay is that it determines the ability of plant extracts to inhibit the process of lipid peroxidation induced by harmful chemicals. It allows the plant extracts to scavenge

the peroxyl and alkoxyl radicals which are responsible for the initiation and propagation of the process of lipid peroxidation. The lipids present in the liver microsomal fractions of mice easily undergo peroxidation in the presence of Fe (III) salt alone or along with reducing agents like ascorbate. The attack of peroxyl radicals or alkoxyl radicals formed degraded product i.e. malondialdehyde (MDA) and MDA forms color complex (pink) TBARS on reaction with TBA in acidic conditions. The TBARS so formed are measured spectrophotometrically at 532nm. If a fraction of plant extract is able to scavenge peroxyl radicals, the intensity of colored complex formed reduces as compared to positive control.

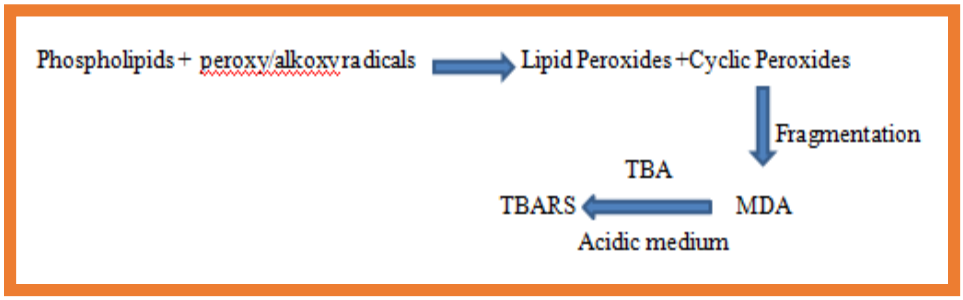


Fig: 12: Mechanism of peroxy/ alkoxyl radicals leading to the damage of lipids.

3.10.3.1: Procedure:

Lipid peroxidation was estimated in the liver and kidney microsomal fractions. The assay was performed following Halliwell and Gutteridge, 1989, with slight modifications. 0.2g of each sample was homogenized in 2ml of 0.2M KCl followed by centrifugation at 10000 rpm for 10 minutes in cooling centrifuge (Heraeus Biofuge Startos centrifuge). 0.5ml of the homogenate was mixed with 100µl of 10mM $FeCl_3$ and incubated at 37^0C for 30 minutes. After incubation 400 µl of TCA, 50 µl of BHT, 0.5ml of TBA and 50 µl of 0.25N HCl were added and heated at 100^0C for 60 minutes. The reaction mixture was cooled and then centrifuged. Absorbance was recorded against blank at 532nm.

3.10.4: Estimation of GSH level:

Principle:

The principle behind the GSH estimation is that it demonstrates the hepatic as well as nephritic level of GSH altered by toxin treatments. Glutathione is present in all type of living cells and tissues such as liver and kidney normally contain high level of reduced glutathione. GSH functions as intracellular redox buffer serving as reservoir of cystein and protects the thiol groups in protein from oxidation (Moron, 1979). The depletion of GSH has been reported to initiate hepatic and kidney necrosis and is because of the covalent bonding with the toxic metabolites (Yuan *et al*., 1991; Kim *et al*., 2012).

3.10.4.1: Procedure:

GSH level was estimated in the liver and kidney cytosols spectrophotometrically. 0.1g of each sample was homogenized in 2.5ml of 10% TCA followed by the centrifugation at 10000 r.p.m for 10 minutes. 0.1ml of the supernatant was mixed with 0.9ml of 0.2M phosphate buffer and 0.2ml of 0.6mM DTNB and the absorbance was read at 412nm against blank.The level of GSH was expressed as nmole of GSH/g tissue (Moron *et al*., 1979).

3.10.5: Estimation of Glutathione Reductase:

Principle:

Glutathione reductase is found in many tissues and it enables the cells to sustain adequate levels of cellular GSH. The enzyme catalyses the reduction of GSSG to reduced glutathione (GSH); GSH is the substrate for glutathione peroxidase that detoxifies the peroxides and glutathione S-transferases that invovle in the conjugation and elimination of xenobiotics from the organism. Reduced glutathione produced by glutathione reductase also acts as an antioxidant by reacting with free radicals and organic peroxides. The assay is based on the reduction of GSSG by NADPH in the presence of glutathione reductase and this activity of glutathione reductase can be measured by decrease in

absorbance caused by the oxidation of NADPH at 340nm. The enzyme is inhibited by divalent metal ions such as Zn^{2+} and Cd^{2+} in which it is prevented by the addition of EDTA to the extraction buffer (Dolphin *et al*., 1989; Worthington and Rosemeyer, 1965; Han and Han., 1994).

Glutathione reductase catalyses the following reaction:

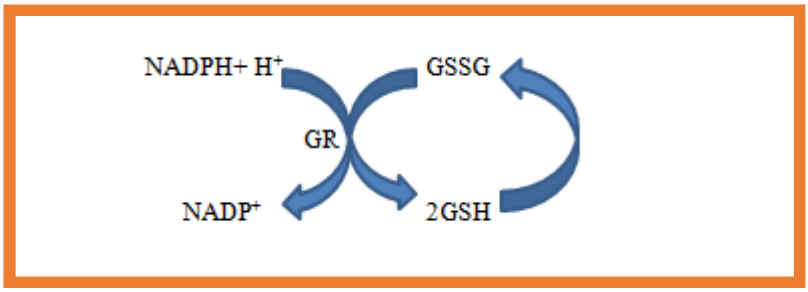


Fig: 13: Mechanism of Glutathione Reductase.

3.10.5.1: Procedure:

Glutathione reductase was assayed in both liver and kidney following David and Richard, 1983; and the absorbance were recorded at 340nm. 0.1g of tissue was homogenized in 1ml of phosphate buffer followed by the centrifugation at 10000 r.p.m. To 0.1ml of the enzyme source (supernatant), 1ml of (0.12M, PH=7.2) phosphate buffer, 0.1ml of 15mM EDTA, 0.1ml of 10mM sodium azide, 0.1ml of GSSG, 0.6ml of dH_2O were added and the volume was made up to 1ml with buffer. The reaction mixture was incubated for 3 minutes, followed by the addition of 0.3ml of NADPH (9.6mM). The absorbance was recorded at intervals of 15 seconds for 2-3 minutes. Enzyme activity was expressed as µmole of NADPH oxidized/minute/gram.

3.10.6: Estimation of protein concentration:

Principle:

The method is based on the formation of protein-copper complex and reduction of Folin Ciocalteau reagent by tyrosine and tryptophan residues of protein to form a color product. Copper ions react with peptide nitrogen and bring about the oxidation of aromatic amino acids such as tyrosine and tryptophan under alkaline condition and also reduces phosphomolybdic phosphotungstic acid to

heteropolymolybdenum, blue colour. The concentration of reduced folin reagent is measured by absorbance at 750nm. Cystein is also reactive to the FC reagent so cystein residues also probably contributes to the absorbance in Lowry Assay. Therefore the total protein concentration can be determine from the concentration of Trp and Tyr residues that reduces Foli-Ciocalteu reagent (Lowry *et al*., 1951; Everette *et al*., 2010).

3.10.6.1: Procedure:

Protein concentration was estimated following Lowry *et al*., 1951. 0.025g of each tissue was homogenized in 1ml of phosphate buffer saline. 0.5 ml of each homogenate was diluted with 6ml of PBS and from this 0.5ml of the diluted sample was used for the analysis. The reaction mixtures contain 0.5ml of the homogenate, 0.7ml of Lowry's solution. It was mixed through the vortex and incubated for 20 minutes. 0.1ml Folin Ciocalteau Reagent was added and mixed in the vortex. After 30 minutes of incubation absorbance was read at 750nm against the reagent blank. The amount of protein was estimated from the standard calibration curve obtained using BSA.

3.11: Statistical analysis:

The results regarding antigenotoxicity study and biochemical studies are presented in the form of mean±S.D and statistical significant differences are calculated in MS Excel using One Way Anova test.

3.12: Histopathological examination:

Liver and kidney were quickly removed after sacrificing the mice and were preserved in 10% formalin. The tissues were washed in running water for 12 hrs followed by dehydration with isopropylalcohol of increasing strength (70%, 80% and 90%) and the final dehydration was done with absolute alcohol with three changes for 12hours. The tissues were embedded in paraffin wax and longitudinally sectioned in microtome followed by haematoxylin and eosin staining and observed under microscope (Swarnlata *et al*., 2014).

3.13: Determination of cytotoxicity of the extracts of rhizome of *Curcuma caesia* Roxb:

Principle:

This is a colorimetric assay that is based on the conversion of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] into formazan crystals by living cells which determines mitochondrial activity. MTT enters the cells and passes into mitochondria where it is reduced to an insoluble purple colored product, formazan. Formazan releases when the cells are solubilised with an organic solvent (isopropanol/DMSO) and this released solubilised formazan is measured spectrophotometrically. For most population of cells, the mitochondrial activity is related to the number of viable cells, the assay is broadly used to measure the cytotoxic activity of drugs on cell lines (Meerloo *et al*., 2011).

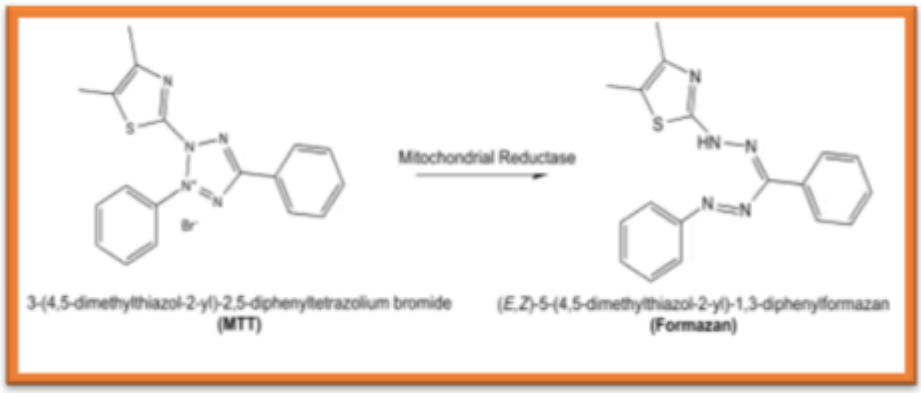


Fig: 14: Conversion of MTT into Formazan by mitochondrial enzymes.

3.13.1: Cell lines and culture preparation:

The cell lines used in the present study was MDAMB breast cancer cells and lung cancer cell lines Calu6 and they were cultured in Dulbecco's modified eagle's medium supplemented with 10% FBS and 1% Streptomycin and incubated at 37^0C, 5% CO_2 incubator for 24 hrs.

Fig : 15: Cancer Cells growing in T-25 Cm2 Flask.

3.13.2: Procedure:

The cytotoxicity of each extract was tested by the quantitative colorimetric MTT assay (Umthong *et al.*, 2011; Meerloo *et al.*, 2011) with slight modification. 70-80% confluent cell lines (breast cancer cell lines: MDAMB 231 and lung cancer cell lines: Calu6) were trypsinized and they were checked for viability of cells and centrifuged. 50000 cells were seeded into 96 well plates and incubated at 37^0C, 5% CO_2 incubator for 24 hrs. After incubation, each concentration of each extract (0.5-80µg/ml) was added, followed by the incubation at 37^0C, 5% CO_2 incubator for 24 hrs again. Later MTT solution [100µl/well (50µg/well) {5mg/10ml of MTT in 1X PBS}, the solution was then filtered through a 0.2µm filter and stored at 2-8^0C for further uses] was added and incubated for 3-4 hrs. It was followed by the addition of 100µl of DMSO to solubilise the formazan and absorbance was recorded at 590nm. The percentage inhibition was calculated comparing with the cell control using the following formula:

% inhibition= 100- (O.D of the treatment/O.D of the control) × 100.

Or, % inhibition= 100-(proliferation) × 100

3.14: GC-MS analysis:

Principle:

GC-MS is a technique used to separate volatile molecules present in the sample. The technique separates the chemical mixtures and identifies the components present at the molecular level through mass spectrophotometry. It is based on the principle that a mixture present in the plant sample will separate into individual substances when heated and the heated gases are carried through a column with an inert gas, helium. The separated substance emerges from the column opening and then flow into the Mass spectrophotometry and compounds present in it are identified.

3.14.1: Procedure:

The active principles of ethyl acetate extract of the rhizome of *Curcuma caesia* Roxb. with their retention time (RT), molecular formula (MF), molecular weight (MW) was examined by GC-MS. It was performed using GC/MS Clarus 500(Perkin Elmer) system consist of a gas chromatograph interfaced to a mass spectrophotometer (GC-MS) equipped with Elite-5MS (5% diphenyl/95% dimethyl polysiloxane) fused capillary column (30 meter × 0.25 mm). The injection volume employed was 1µl and the injector temperature was set at 250^0C. The oven temperature was set at 40^0C and mass spectra were taken and total separated peaks were observed. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in NIST library.

3.15: UV-Visible Analysis:

It was performed following Sahaya, *et al*., 2012 with little modifications. Each extract were diluted to 1: 5 with the same solvent and were centrifuge at 3000 r.p.m for 10 minutes and it was followed by filtration through Whatmann No.1 filter paper. Absorbance for each extract was recorded in the wavelength range of 200-800nm using UV

Visible Spectrophotometer (Spectrascan UV 2600, Thermo, USA) characteristic spectrum was plotted to determine the highest peak.