CHAPTER 3

MATERIALS AND METHODS

3.1. Plant material

The fresh leaves of *Cassia alata* Linn. (CA). The plant specimen was authenticated by the Botanical Survey of India (BSI), shillong, India. The voucher number of identified plant sample is BSI/ERC/2014/Plant identification/618.

Plate 3: Herbarium of *Cassia alata* Linn.

3.2. Chemicals and reagents

1. 0.1% naphthylenediamine dihydrochloride,
2. 1% sulphanilamide,
3. 2,2-diphenyl-1-picrylhydrazil (DPPH)
4. Acarbose,
5. Acetate buffer,
6. Acetone
7. Agarose
8. Ammonia solution
9. Anthrone reagent
10. Ascorbic acid
11. Benedict's reagent
12. Benzene

13. Boric acid
14. BSA
15. Chloroform
16. Chloroform-isoamyl alcohol
17. Citrate buffer,
18. Copper Acetate
19. CTAB (Cetyl trimethylammonium bromide)
20. Dinitrophenol (DNPH),
21. Dinitrophenylhydrazine
22. Dinitrosalicylic acid (DNS),
23. DPPH
24. EDTA (Ethylene Diamine Tetra Aceticacid)
25. Eosin,
26. Ethanol
27. Ethyl acetate
28. Fehling's A & B solutions
29. Ferric Chloride
30. Ferrous sulphate,
31. Folin-Ciocalteu reagent,
32. Formalin,
33. Gallic acid
34. Gelatin
35. Glacial Acetic acid
36. Gum Acacia
37. Haematoxylin,
38. Hydrochloric acid (HCL)
39. Iodine
40. Isoamyl alcohol
41. Isopropanol
42. $K_3Fe(CN)_6$,
43. L-aspartate
44. Lead acetate solution
45. Methanol
46. Na_2CO_3

47. NaOH
48. Ninhydrin reagent
49. Nitric acid (HNO_3)
50. Orthophosphoric acid.
51. Para-Nitrophenyl-α-D-glucopyranoside (PNPG).
52. Petroleum ether
53. Phosphate buffer,
54. Picric acid
55. Potassium
56. Potassium Iodide
57. Potassium Mercuric Iodide
58. Potassium phosphate
59. PVP (polyvinylpyrolidone)
60. Pyridine
61. Sodium Chloride
62. Sodium Chloride (NaCl)
63. Sodium Nitropruside
64. Sodium phosphate buffer
65. Sodium potassium tartrate
66. Sodium pyruvate
67. Starch
68. Streptozotocin (STZ)
69. Sulphuric acid (H_2SO_4)
70. TPTZ
71. Tri-chloro Acetic acid (TCA)
72. α-Amylase
73. α-glucosidase
74. α-ketoglutarate
75. α-naphthol solution

3.3. Plant sample/extract preparation

The leaves of CA were collected during the month of November 2010 from Sangaiprou village, district of Imphal, Manipur, India. The collected leaves were washed, air dried under shade, powdered and stored for further use. The general steps followed for the preparation of extract is displayed in Fig.3.1.

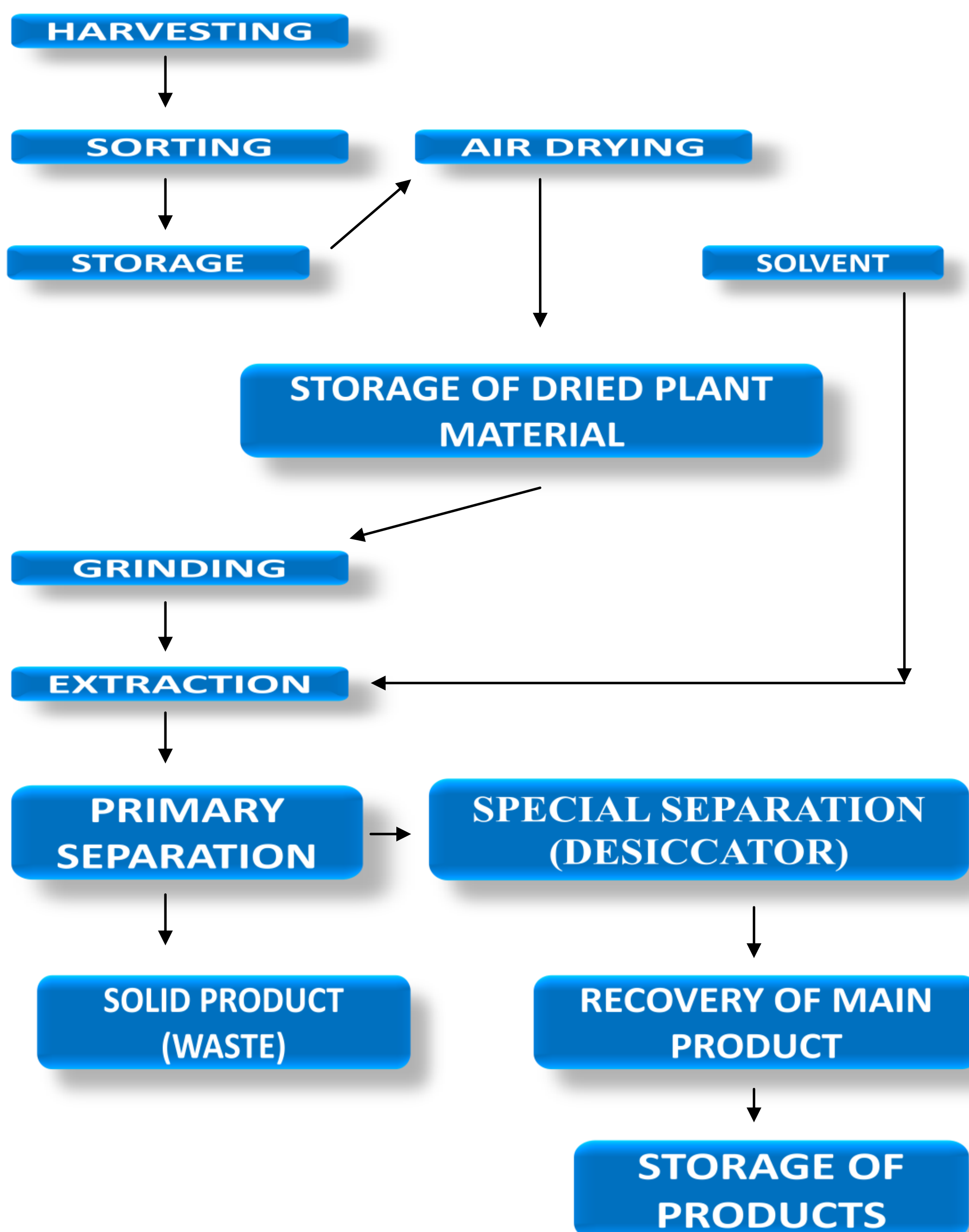

Fig.3.1: General scheme of extract preparation.

3.3.1. Successive solvent extraction of the plant leaves

Sequential extraction with solvents of increasing polarity was carried out using the method described by Bruneton with slight modification (Bruneton, 1999). Dried powdered leaves (30 gm) of the plant were subjected to successive solvent extraction with Soxhlet apparatus by using solvents of ascending polarity (petroleum ether, acetone, ethyl acetate, ethanol, methanol and water). Residue left after extraction with one solvent was dried and the same was used for the next solvent extraction (Fig.3.2). The extracts obtained were dried; weight and the percentage (%) yield of the plant extracts in different solvents used were calculated using the formula: Percentage yield = weight of final extract/weight of powdered leaves X 100.

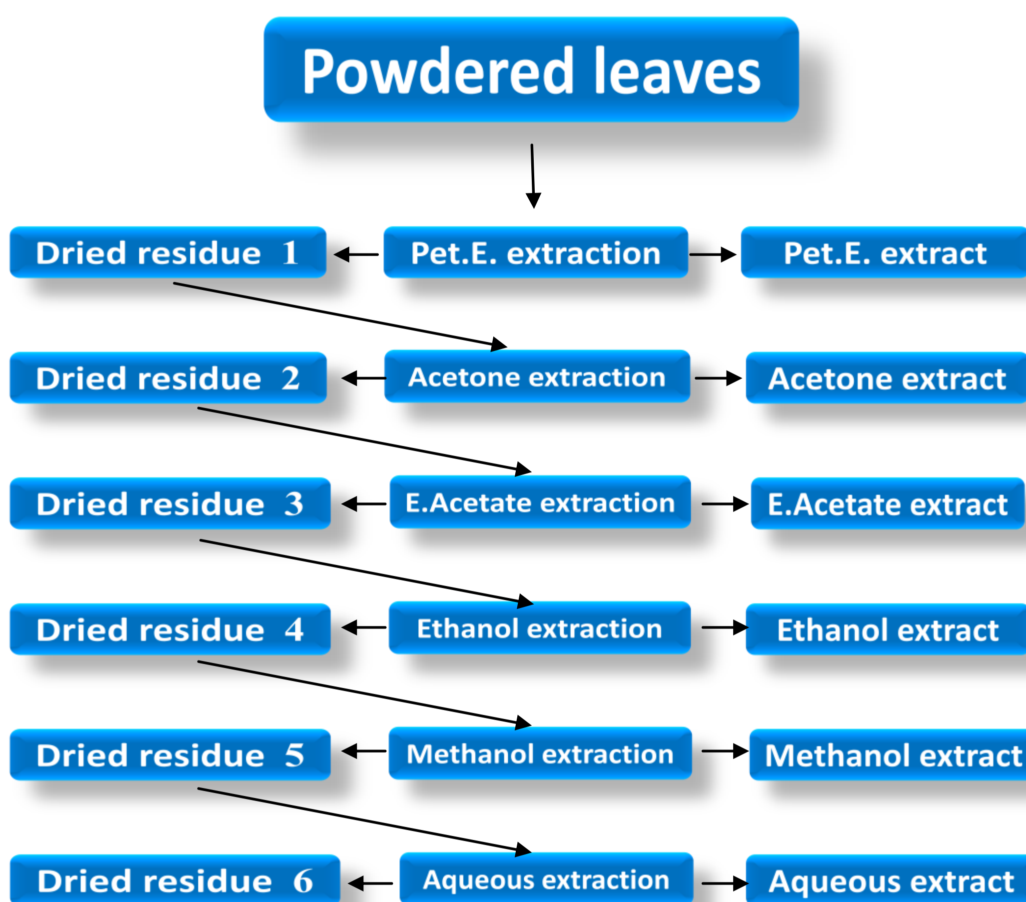

Fig.3.2: Successive solvent extraction of the plant leaves using solvents of ascending polarity.

3.4. Preliminary phytochemical screening

Phytochemical screening of ethanol, methanol and aqueous extract of *Cassia alata* Linn. was carried out base on standard protocols (Brain and Turner, 1975; Trease and Evans, 1996).

i) Detection of alkaloid: extracts were dissolved individually in dilute Hydrochloric acid and filtered.

   a) Mayer's test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of yellow coloured precipitate indicates the presence of alkaloids.
   b) Wagner's test: Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.
   c) Hager's test: Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.

ii) Detection of Carbohydrates: Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

   a) Molisch's test: Filtrates were treated with 2 drops of alchoholic α-naphthol solution in a test tube and concentrated sulphuric acid was added. Formation of the violet ring at the junction indicates the presence of Carbohydrates.
   b) Benedict's test: Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.
   c) Fehling's test: Filtrates were hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

iii) Detection of resins: Acetone H_2O test.

iv) Detection of Flavonoids:

   a) Alkaline reagent test: Extracts were treated with 4-5 drops of sodium hydroxide solution. Formation of intense yellow colour, which

becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

b) Lead acetate test: Extracts were treated with 4-5 drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

v) Detection of terpenoids: Salkowski test: To 0.5g each of the extract was added 2ml of chloroform. Concentrated H_2SO_4 (3ml) was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

vi) Detection of Anthraquinones: 0.5g of the extract was boiled with 10ml of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5ml of chloroform. The chloroform layer was pipette into another test tube and 1ml of dilute ammonia was added. The resulting solution was observed for colour changes.

vii) Detection of diterpenes: Copper acetate test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

viii) Detection of glycosides: Extracts were hydrolysed with dil. HCL, and then subjected to test for glycosides.

a) Borntrager's test: Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose pink colour in the ammonical layer indicates the presence of anthranol glycosides.

b) Legal's test: Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

ix) Detection of phenols: Ferric Chloride test - Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black indicates the presence of phenols.

x) Detection of Tannins: Gelatin test - To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

xi) Detection of proteins and aminoacids:

a) Xanthoprotein test: The extracts were treated with 4-5 drops of concentrated nitric acid. Formation of yellow colour indicates the presence of proteins.

b) Ninhydrin test: To the extract, 0.25% w/v Ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

xii) Detection of Cardiac Glycosides: Killer kilani test: To 0.5g of extract diluted to 5ml in water was added 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

3.5. In vitro antioxidant activity assessment

Antioxidant activity of CA extracts was determined by DPPH assay, Reducing Power Assay, FRAP and NRSA.

3.5.1. DPPH free radical scavenging assay

Antioxidant activity of the test extracts on the basis of scavenging activity of 2,2-diphenyl-1-picrylhydrazil (DPPH) free radical, was determined following the method described by Brand-Williams *et al.* (1995) with slight modifications. The absorbance of the samples was measured at 517nm on UV-vis spectrophotometer (Spectoscan UV 2600) after incubation for 30 min at room temperature. Percent inhibition was calculated as follows:

DPPH Scavenged (%) = (A control- A sample)/A control x 100

Where A control is the absorbance of the control and A sample is the absorbance of the extracts. Ascorbic acid was used as positive control. And IC_{50} for each sample was calculated from the linear regression curve.

4.5.2. Reducing power assay (RPA)

The reducing power assay was performed following the method suggested by Oyaizu (1986) with minor modifications. 0.5ml plant extract of different concentration was

mixed with 1ml phosphate buffer (0,2M, pH 6.6) and 1ml 1% $K_3Fe(CN)_6$, shaken well and incubated at 50˚C. 1 ml of TCA (10%) was added to stop the reaction after 20 min of incubation. It was centrifuged at 3000rpm for 10min. 1.5ml supernatant, 1.5ml ddH_2O and 0.1 ml $FeCl_3$ (0.1%) were mixed and incubated for 10min. The absorbance was read at 700 nm on UV-vis spectrophotometer. Ascorbic acid as positive control was also tested for the reducing power assay. Higher absorbance indicates higher reducing power.

3.5.3. Ferric reducing/ antioxidant power (FRAP) assay

The ferric reducing power of the extracts was determined according to the method described by Pulido *et al.* (2000) with slight modifications. To each test tube with 20µl of sample, 90µl ddH_22 and 900µl of a freshly prepared solution of FRAP reagent (mixture solutions: 2.5ml of 20mmol/L of TPTZ in 40mmol/L of HCl, 2.5mL of 20mmol/L of ferric chloride, 25mL of 0.3mol/L of acetate buffer, pH 3.6) are distributed. After incubation at 37˚C for 30 min the absorbance of the product (ferrous tripyridyl triazine complex) was read at 593 nm with UV-vis spectrophotometer. From the calibration curve (generated using known ferrous sulphate), the ferrous ions reduced by the extracts were calculated using a regression equation. The antioxidant activity was expressed in mmol FeII/mg of extract.

4.5.4. Nitric oxide radical scavenging assay (NRSA)

Nitric oxide radical scavenging activity was measured following the method of Marcocci *et al.* (1994) with few modifications. Different concentrations (10µg/ml, 15µg/ml, 20µg/ml and 25µg/ml) of sample extracts or standard (ascorbic acid) (500µg) were mixed with sodium nitroprusside (final concentration 5mmol/L) in phosphate buffer saline, pH 7.4 to the final volume of 1mL. After 150 min of incubation at 25˚C the reaction mixture was mixed with Griess reagent (1% sulphanilamide and 0.1% naphthylenediamine dihydrochloride in 5% orthophosphoric acid). The absorbance was measured at 540 nm with UV-vis spectrophotometer. The degree of nitric oxide radical scavenging activity (NRSA) was calculated as follows:

NRSA%=Ac-As/Ac x 100.

Where Ac is absorbance of the control;

As is the absorbance of the sample.

3.6. Quantification of Total Phenolic Content (TPC)

Total phenolic content of the extracts were determined by the Folin-Ciocalteu method (Slinkard and singleton, 1977). The extracts (100µl) were mixed with 0.2ml Folin-Ciocalteu reagent, 2ml of H_2O, and 1ml of 15% Na_2CO_3. The absorbance of blue coloured mixtures was recorded after 40 min at 725 nm against blank. The amount of TPC was calculated from the calibration curve of gallic acid standard solutions and expressed in mg gallic acid equivalent/gram of dry weight (extracts).

3.7. In vitro Antidiabetic activity assessment

3.7.1. α-Amylase inhibitory test:

The α-Amylase inhibitory test was determined spectrophotometrically according to the method of Bernfeld (1955), with minor modifications. The extracts or acarbose (500µl) and the α-amylase 500µl (2units/ml) were mixed and pre-incubated in 20mM sodium phosphate buffer (pH 6.7) for 5min at 37˚C. Then, 1ml of starch (0.2% w/v) dissolved in the buffer was added to the reaction mixture to make up to 2ml, and the whole was incubated for 5min at 37˚C. After the incubation, 1ml of dinitrosalicylic acid (DNS) colour reagent was added and placed in a boiling water bath for 5min. And then this mixture was cooled to room temperature and added another 6ml of ddH_2O. α-Amylase activity was determined by measuring the absorbance of the mixture at 540 nm. Percentage (%) inhibition was calculated according to the formula-

> Inhibition (%) = Ac-As/Ac x 100, where Ac is absorbance of the control; As is the absorbance of the sample.

3.7.2. α-glucosidase inhibitory test:

Inhibition of α-glucosidase activity was determined spectrophotometrically by following the method of Feng *et al.* (2011) with slight modifications. 120µl of sample (extract or acarbose) and 20µl α-glucosidase (1unit/ml) were pre-incubated in 0.1M potassium phosphate buffer (pH 6.8) at 37˚C for 15min. The reaction was initiated by the addition of 20µl of 5mM para-nitrophenyl-α-D-glucopyranoside (PNPG) in 0.1M potassium phosphate buffer to the mixture and was further incubated for 15min. The reaction was terminated by the addition of 80µl of 0.2 MNa_2CO_3 in 0.1M potassium phosphate buffer, and the absorbance of the mixture was recorded at 405 nm.

Percentage (%) inhibition was calculated according to the formula-

Inhibition (%) = Ac-As/Ac x 100, where Ac is absorbance of the control; As is the absorbance of the sample.

3.7.3. Insulin secretion test using MIN6 pancreatic β-cell line

Glucose stimulated insulin secretion (GSIS) from MIN6 was determined using a static incubation protocol. MIN6 were cultured in 96-well plates at density 50,000 cell/well until 80% confluent. On the day of experiment, growth medium was removed and the cells were washed twice with Krebs-Ringer bicarbonate (KRB) buffer (115mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 1.2mM KH2PO4, 1.3mM $CaCl_2$, 24mM $NaHCO_3$, 0.1%BSA, and 10mM Hepes, pH7.4), pH 7.5, containing 0.1%BSA (KRB-BSA), to remove serum. The cells were then preincubated for one hour in KRB-BSA supplemented with 1.1mM glucose at 37 C, 5% CO2. Incubation medium was removed, and the calls were washed in glucose free KRB. KRB-BSA containing 5.6nM glucose (untreated negative control), various concentrations of standard (positive control) and CAME (0.01-25mg/ml) were incubated for two hours. Gliclazide was used as a reference standard. For investigation of the effects of extracellular Ca^{2+} -free incubation on pancreatic insulin secretion, $CaCl_2$ was removed from KRB buffer preparations, so that cells were incubated in Ca^{2+} free KRB in the same procedure described above. For all experiments, incubation medium was collected and stored at -20ºC for a subsequent ELISA determination of the amount of secreted mouse insulin.

3.8. Experimental animals

Plate 4: Swiss albino mice.

Swiss albino mice (8 week of age) weighing 20-30g were obtained from Pasteur Institute Shillong, Meghalaya, India and were allowed to acclimatize to their surroundings for 2 weeks. Mice were housed in standard laboratory condition (temperature 20˚C to 24˚C, 45 to 65% humidity and 12 hour light/dark cycle). All mice received standard laboratory pellet diet and water *ad libitum*. Antidiabetic activity assessment was performed after the approval from Institutional Animal Ethical Committee (approval number: IEC/AUS/2013-031 dt-20/3/13) and in accordance with institutional ethical guidelines for the care of laboratory animals.

3.9. Toxicity test

The ethanol extract, methanol extract and aqueous extract of *Cassia alata* Linn. were analyzed for their acute toxicity profile with reference to behavioural aspects, in Swiss Albino mice. Acute oral toxicity test was performed as per Organization for Economic Co-operation and Development (OECD) guidelines 423. The test was performed using healthy young adult female Swiss albino mice, nulliparous, non-pregnant and weighing 22-30g. The mice were divided into 10 groups/sub-groups containing 3 mice each s follows –

Group 1: received CAEE

Sub-group 1: received 1000 mg/kg b.w. CAEE

Sub-group 2: received 2000 mg/kg b.w. CAEE

Sub-group 3: received 3000 mg/kg b.w. CAEE

Group 2: received CAME

Sub-group 1: received 1000 mg/kg b.w. CAME

Sub-group 2: received 2000 mg/kg b.w. CAME

Sub-group 3: received 3000 mg/kg b.w. CAME

Group 3: received CAAE

Sub-group 1: received 1000 mg/kg b.w. CAAE

Sub-group 2: received 2000 mg/kg b.w. CAAE

Sub-group 3: received 3000 mg/kg b.w. CAAE

Group 4: Control mice

The test substance was administered by gavage using specially designed mice oral needle following a period of 5hours fasting, animals were weighed and the test substance was administered orally at a dose of 1000, 2000, 3000 mg/kg body weight. The volume of the test substance administered was 1ml/kg body weight. The animals were observed individually after dosing, with special attention given during the first 4 hours and daily thereafter, for a period of 14 days.

3.10. Induction of diabetes in mice

To induce diabetes a reliable protocol for establishing diabetes in mice with multiple low-dose injections of STZ was followed (O'Brien *et al.,* 1986). Diabetes was induced by intraperitoneal injection of freshly prepared streptozotocin (STZ-40mg/kg body weight) in cold citrate buffer (0.1M, pH 4.5) to each pre-starved (for 12 hours before STZ injection) mouse for five consecutive days. To stabilize the blood glucose level, diabetic mice were kept under standard laboratory condition up to 7 days. Blood glucose was determined on day 7, and diabetic mice showing blood glucose > 200 mg/dl were selected to assess the antidiabetic activity of the extracts.

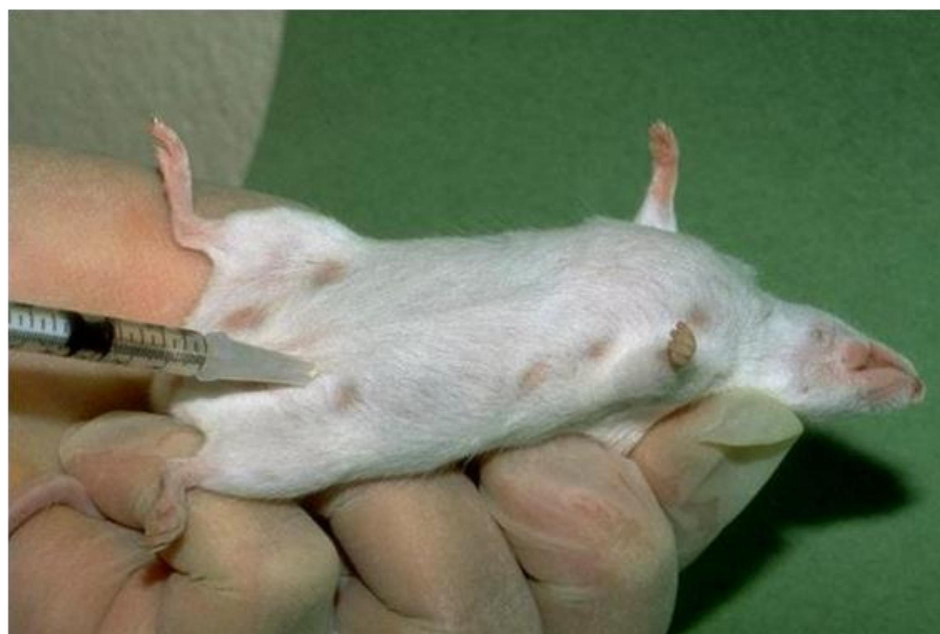

Plate 5: Intraperitoneal injection of STZ

3.11. Oral glucose tolerance test (OGTT)

Oral glucose tolerance test was carried out following the method described by Badole *et al.* 2006. All the mice were divided into two main groups, with 5 sub-groups, each sub-group containing 3 mice. The divided 2 main groups are –

Group 1: Normal mice group

Sub-group 1: normal control mice

Sub-group 2: received standard drug glibenclamide

Sub-group 3: received CAEE

Sub-group 4: received CAME

Sub-group 5: received CAAE

Group 2: Diabetic mice group

Sub-group 1: normal control mice

Sub-group 2: received standard drug glibenclamide

Sub-group 3: received CAEE

Sub-group 4: received CAME

Sub-group 5: received CAAE

Diabetes was induced to Group 2 by intra-peritoneal injection (IP) of STZ (40 mg STZ/kg body weight for five consecutive days). The blood glucose levels were measured from tail-vein blood of all groups by glucometer. Mice with blood glucose level above 200mg/dL were considered as diabetic and were recorded as 0min. Without delay, glucose solution (2gm/kg body weight) was administered to all groups orally. After 30min time glibenclamide (10mg/kg body weight) and extracts (200mg/kg body weight) were administered orally to respective groups. The blood glucose level was measured at 30mins, 60min, 90mins and 120mins after glucose administration.

3.12. In-vivo antidiabetic activity assessment.

3.12.1. Study Design for in vivo antidiabetic activity assessment

To assess the antidiabetic activity of extracts the following groups (six mice each) were made.

Group 1: normal control mice,

Group 2: diabetic control mice,

Group 3: diabetic mice received CAEE at 200 mg/kg dose,

Group 4: diabetic mice received CAEE at 400 mg/kg dose.

Group 5: diabetic mice received CAME at 200 mg/kg dose.

Group 6: diabetic mice received CAME at 400 mg/kg dose.

Group 7: diabetic mice received CAAE at 200 mg/kg dose.

Group 8: diabetic mice received CAAE at 400 mg/kg dose.

Group 9: diabetic mice received glibenclamide at 10 mg/kg dose.

Treatments were done with the freshly prepared samples (5ml/kg body wt of mice) (CAME, CAEE, CAAE and glibenclamide-standard antidiabetic drug), administered by mice oral needle to their respective group of mice up to 28 days. Blood glucose was monitored weekly by tail prick method using GlucoCard (Arkray GlucoCard 01 Mini Glucose Meter) and the change in blood glucose level was noted everyday during treatment. Percentage change in Blood Glucose Level (BGL) was determined using the formula –

Percentge (%) change in BGL = Initial BGL (before extract treatment) – Final BGL (after treatment)/ Initial BGL (before extract treatment) x 100

3.12.2. Measurement of biochemical parameters

Various biochemical parameters were determined from the serum obtained from whole blood sample. The whole blood sample was collected by the cardiac puncture method, in the centrifuge tubes and allowed to clot for 30min at room temperature. Blood sample were centrifuged at 3000rpm for 20min. Serum was separated as supernatant and stored at -20˚C until analysis. Enzymatic serum estimations were done spectrophotometrically using standard kits.

3.12.2.1. Estimation of serum triglyceride (TG)

Triglycerides were estimated by GPO/POP method using triglycerides kit (Span Diagnostic, India). Working reagent was prepared by adding enzymes (lipoprotein lipase, glycerol kinase, glycerol-3-phosphate oxidase, peroxidase, 4-aminoantipyrine and adenosine tri phosphate) to triglyceride reagent buffer (3,5 dichloro-2-hydroxybenzene sulphonate, pH 7.0) in a ratio of 15:1.1 and stored at 2-8˚C until use but not more than two weeks. Estimation of triglycerides was carried out by preparing 1ml each of blank (1ml working reagent), standard sample [1ml working reagent + 0.01ml standard (200mg/dL concentration)] and test sample (1ml of working reagent + 0.01 ml of blood serum). Blank, standard sample and test sample were mixed and incubated for 15 min and the absorbance of the standard and test was read against blank sample at 546 nm.

The serum triglyceride level was calculated by using the formula:

Triglycerides (mg/dL) = Absorbance of Test sample/Absorbance of Standard x Concentration of the standard (200mg/dL).

3.12.2.2. Estimation of serum total cholesterol (TC)

Total serum cholesterol was estimated by enzymatic method using diagnostic kit from Autopak, Siemens Ltd, Product code: 802. Working reagent was prepared by dissolving enzymes (cholesterol esterase, cholesterol oxidase, peroxidase,4-aminoantipyrine) into reagent 1A buffer (pipes buffer, phenol, sodium cholate, pH 37 6.95) and then allowed to stand for 5min at room temperature. The instrument was adjusted to zero with distilled water. For the estimation of serum cholesterol, 10μl each of sample and standard along with 1ml reaction reagent were pipette into a cuvette using a micropipette and incubated for 5 minutes at 37˚C and the absorbance was read at 500nm. The intensity of the colour developed was proportional to the concentration of cholesterol in the sample.

Calculation: Total Cholesterol (mg/dl) = (Abs. of sample/ Abs. of standard) X concentration of the standard (200 mg/dL)

3.12.2.3. Estimation of serum HDL-cholesterol

The serum HDL-cholesterol was estimated by PEG/CHOD-PAP method using commercial diagnostic kit. O.1ml of precipitating reagent was mixed with 0.1ml of sample/serum. Mixed well and incubated at R.T. for 5 min which was then centrifuged at 2500-3000 rpm to obtain a clear supernatant. Supernatant contain the HDL-cholesterol while HDL, VLDL and chylomicron fractions are precipitated. The aliquot thus obtained was used for the estimation of HDL-cholesterol. The blank (1ml working reagent + 0.05ml distilled water), standard (1ml working reagent + 0.05ml HDL standard) and test sample (1ml working reagent + 0.05ml aliquot) were prepared, mixed well and incubated at room temperature (25˚C) for 15 min. The absorbance of the Standard (Abs. S), and Test Sample (Abs. T) against Blank were measured at 505nm within 60 minutes.

Calculation: HDL Cholesterol in mg/dL = Abs. T/ Abs. S x 25 x 2.

(where, 2 is the dilution factor due to the deproteinization step)

3.12.2.4. Serum total protein was estimated by Biuret method (Reinhold, 1953).

For the quantitative determination of serum total protein, Lowry's method was followed. Complex-forming reagent was prepared immediately before use by mixing the stock solution A: 2% (w/v) Na_2CO_3 in distilled water, B: 1% (w/v) $CuSO_4 \cdot 5H_2O$ in distilled water and C: 2% (w/v) sodium potassium tartrate in distilled water in the proportion 100:1:1 by volume, respectively. To 0.1mL of sample or standard (1, 2, 3, 4, 5, 6, 7, and 8µg.ml of BSA), 0.1mL of 2N NaOH was added. Hydrolyze at 100°C for 10min in a boiling water bath. Cooled down to room temperature and added 1mL of freshly mixed complex-forming reagent. Incubated it at room temperature for 10min, and then added 0.1mL of Folin reagent. The whole mixture was vortex and allowed to stand at room temperature for 30–40 min. The absorbance of the test sample and standard was read at 750 nm. BSA standard curve was plotted as a function of initial protein concentration and use it to determine the unknown protein concentrations.

3.12.2.5. Estimation of Serum Glutamic Oxaloacetic Transaminase (SGOT)

Serum SGOT activity was determined according to the method of Reitman and Frankel (1957). SGOT substrate (2mM of α-ketoglutarate and 100mM L-aspartate in 100ml phosphate buffer, 0.1M, pH 7.4) was prepared. Sodium pyruvate is used as a standard. 5ml of substrate in Test, Control, Blank and standard was boiled for 5min at 37˚C. Then 0.1ml of serum was added to the test, 0.1ml sodium pyruvate to the standard and incubated at 37˚C for 60min. 0.5ml of DNPH was added to all the tubes and 0.1ml of serum to the control. The preparation was allowed to stand for 20min. Then sodium hydroxide was added to all the tubes and the absorbance was determined after 10mins at 520nm. SGOT level was estimated from the sodium pyruvate standard curve and is expressed as U/dL.

3.12.2.6. Estimation of Serum Glutamic Pyruvic Transaminase (SGPT)

Serum SGPT activity was determined according to the method of Reitman and Frankel (1957). 0.25ml of Buffered alanine α-Ketoglutarate (pH 7.4) was incubated at 37˚C for 5min. Then 0.05ml of serum was added, mixed and incubated at 37˚C for 30 min. 0.25ml of DNPH was added, mixed and allowed to stand for 20min. 2.5ml of diluted sodium hydroxide was added. The absorbance was determined after 10min at 520nm. Serum glutamate pyruvate transaminase (SGPT) was estimated from the sodium pyruvate standard curve and expressed as U/dL.

3.12.2.7. Estimation of liver glycogen content

Liver glycogen content was determined according to the method of Morales et al. (1975). Known weight of the liver & kidney tissues were subjected to alkali digestion with 30% KOH in boiling water bath for 20 minutes. 3.0ml of ethanol was added and tubes were kept in a freezer overnight. They were centifuged at 3000rpm for 40 minutes. The precipitate was dissolved in warm water, re-precipitated with ethanol ad centrifuged again. The final precipitate was dissolved in 3.0ml of distilled water and heated for 5 minutes in a boiling water bath. Liquots of the sample and standard (glucose) were mixed with 4.0ml of Anthrone reagent, heated in a boiling water bath for 20 minutes. The green colour developed was read at 600 nm using UV–VIS spectrophotometer. Abs (sample)/Abs (standard) x Concentration (standard). The glycogen content in the tissues is expressed as mg/g wet tissue.

3.12.3. Histological studies

For histological studies, the organs (pancreas, liver and kidney) were removed, washed with cold physiological saline and fixed in 10% formalin. Then dehydrated on treatment with a series of different concentrations of ethanol and embedded in paraffin. Paraffin sections (3-5µm) of liver, pancreas and kidney tissue were prepared using microtome, stained with haematoxylin and eosin to observe histopathological changes (Dunn, 1974). The specimens were observed with phase contrast microscope (Nikon Eclipse E200).

3.13. GC-MS analysis of the extract

The GC-MS analysis of CAME was performed using a Perkin–Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with a Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused a capillary column (Restek Rtx^{R} – 5, 30 meter X 0.25 mm ID x 1µm). For GC-MS detection, an electron ionization system was operated in electron impact mode with an ionization energy of 70 eV. Helium gas (99.99%) was used as a carrier gas at a constant flow rate of 1ml/min, and an injection volume of 1µl was employed (a split ratio of 10:1). The injector temperature was maintained at 280°C. The oven temperature was programmed from 40°C (maintained this temperature for 5 min), with an increase of 6°C/min to 280°C, ending with a 9min isothermal at 280°C. Total GC/MS run time was 60min. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The constituents

were identified by matching the spectra with those found in the NIST 05 library. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The mass-detector used in this analysis was Turbo-Mass Gold-Perkin-Elmer, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver-5.2.

3.14. Molecular characterization of the plant

For the identification of the plant at molecular level, DNA fingerprinting/genotyping of the plant was carried out by RAPD analysis using fluorescent (FAM) dyed primers. The samples collected for the analysis are – F1, *Cassia alata* Linn. (Place of collection: Imphal, plain area, which has moderate/humid subtropical climate). F2, *Cassia tora* Linn. (Place of collection: Imphal, plain area which has moderate/humid subtropical climate). F3, *Cassia alata* Linn. (Place of collection: Silchar, hilly area which has tropical climate).

3.14.1. Genomic DNA isolation

Fresh, young and tender leaves were collected and wiped with 70% ethanol for the extraction of DNA by the following protocol.

Reagents: 100mM Tris–HCl (pH 8), 1.4M Sodium Chloride (NaCl), 20mM-Ethylenediaminetetraacetic acid (EDTA) (pH 8), 2% (w/v) Cetyl trimethylammonium bromide (CTAB), Chloroform-isoamyl alcohol (24:1), Isopropanol, 70% ethanol, TE buffer (pH 8): 10mM Tris–HCl, 1mM EDTA, 0.5× Tris/Borate/EDTA (TBE) (10× stock contained 1M Tris, 0.8M boric acid, 0.5M EDTA) Agarose (molecular grade)

Protocol:

1. Preheated the extraction buffer containing 100mM Tris–HCl (pH 8), 1.4M NaCl, 20mM EDTA (pH 8), 2% (w/v) CTAB in water bath at 60°C for about 15 minutes.

2. 1g of plant tissue was submerged in 5ml of absolute alcohol for 5 minutes and allowed alcohol to evaporate.

3. The tissue was grinded in presence of 1% PVP (Polyvinylpyrrolidone) and pre-warmed extraction buffer by using a pre-chilled mortar and pestle (−40°C/−80°C) at room temperature.

4. The ground material was transferred into 2ml centrifuge tubes and incubated in water bath at 60°C for 1 hour.

5. The tubes are centrifuged at 10,000 rpm for 10 minutes at 4°C and collect the supernatant in 1.5ml centrifuge tube using wide bored tip.

6. To the supernatant equal volume of chloroform:isoamyl alcohol (24:1) was added and mix by inversion for 15 minutes.

7. The tubes were centrifuged at 10,000 rpm for 10 minutes at 4°C and collected the supernatant in 1.5ml centrifuge tube.

8. Again equal volume of chloroform: isoamyl alcohol (24:1) was added to the supernatant and mix by inversion for 15 minutes.

9. The tubes were centrifuged at 10,000 rpm for 10 minutes at 4°C and collected the supernatant.

10. Added twice the volume of chilled isopropanol to the supernatant to precipitate the DNA and incubate it at −20°C for 30 minutes.

11. The tubes were centrifuged at 10,000 rpm for 10 minutes at 4°C and collect the pellet.

12. The pellet was washed 2–3 times with 70% ethanol and air dried the pellet in room temperature.

13. Added 50–100µl of TE buffer to dissolve the DNA and stored at −20°C for further use.

For visualization of DNA, Samples were subjected to electrophoresis in 1×TBE buffer for 1 hour at 80V. 5 µl of the isolated genomic DNA was loaded on 0.8% agarose gel stained with ethidium bromide to check DNA quality. The gels were photographed under a Gel Documentation system.

3.14.2. RAPD fingerprinting

The isolated genomic DNA of the samples (F1, F2 and F3) were subjected to RAPD PCR and amplified. The primers used for the amplification are –

Primer 1: 5' – CCCHGCAMCTGMTCGCACHC – 3';

Primer 2: 5' – AGGHCTCGATAHCMGVY – 3';

Primer 3: 5' – MTGTAMGCTCCTGGGGATTCHC – 3'.

All the primers were Fluorescent (6fAM) labeled. Amplifications of genomic DNA were performed in 25µl reaction volumes containing 0.5µl *Taq* polymerase, 2.5µl *Taq*PolAssa buffer (10X), 1.0µl dNTPs (10mM), 2.0µl RAPD primer (100ng/ µl),

1.0µl of genomic DNA and 18µl of water. The cycle program included an initial 5min denaturation at 94°C, followed by 40 cycles of 1min at 94°C, 1min at 40°C and 2min at 72°C with a final extension at 72°C for 7min. After the amplification the fluorescent labeled PCR products were then analyzed on an automated DNA sequencer (ABI 3130 Genetic analyzer). RAPD fragments were separated electrophoretically on 1.5% agarose gels in 1X TBE buffer, stained with ethidium bromide. The binary output obtained after analyzing the band patterns was used to generate phylogenetic relationship between groups.

Scoring and data analysis: Fragment analysis was carried out for bands in the range of 35-500 bp. For diversity analysis, bands were scored as presence (1) or absence (0) to form a raw data matrix. A square symmetric matrix of similarity was then obtained with the Jaccard's similarity coefficient (Jac-card, 1908). The average similarity matrix was used to generate a tree for cluster analyses by UPGMA (unweighted pair group method with arithmetic mean) using the NTSys v 2.2 software.

3.15. Statistical Analysis

All data were expressed as mean ± S.D. Statistical analysis was performed by one-way analysis of variance (ANOVA) using Microsoft excels. The results were considered statistically significant if $p < 0.05$.

3.16. Graphical presentation of the methodology

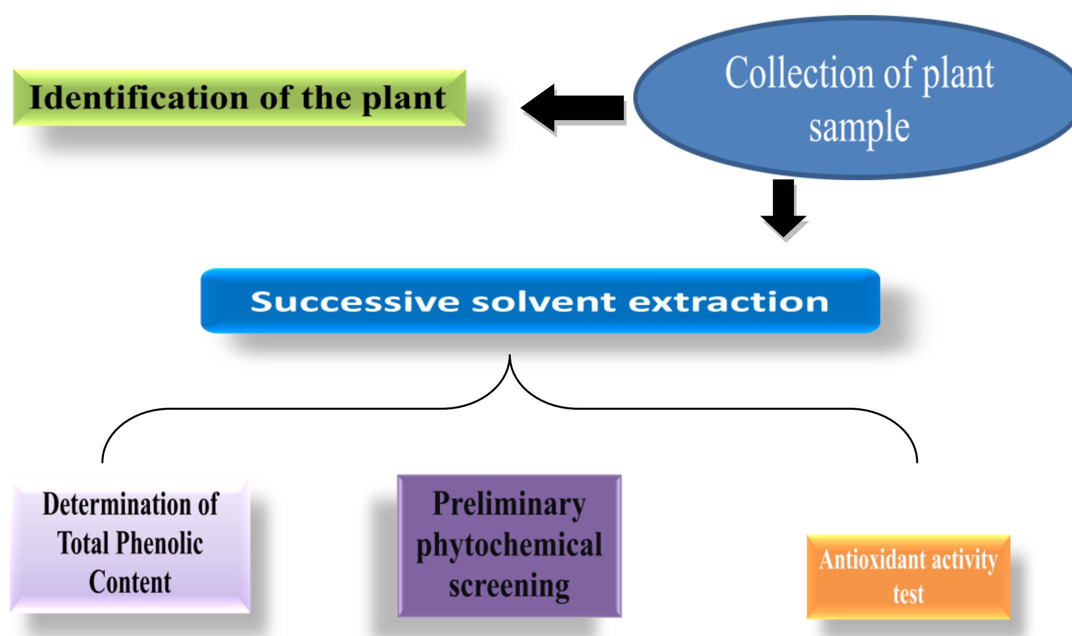

Fig.3.3: Graphical presentation of the methodology (phase 1)

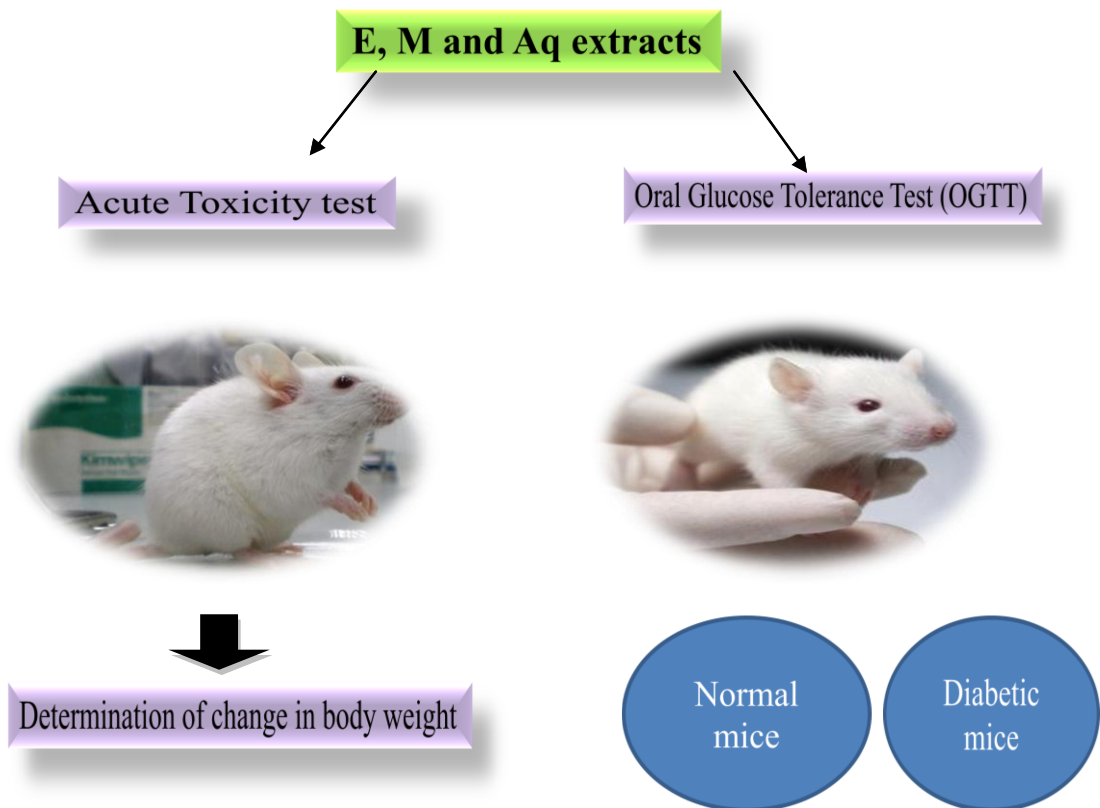

Fig.3.4: Graphical presentation of the methodology (phase 2)

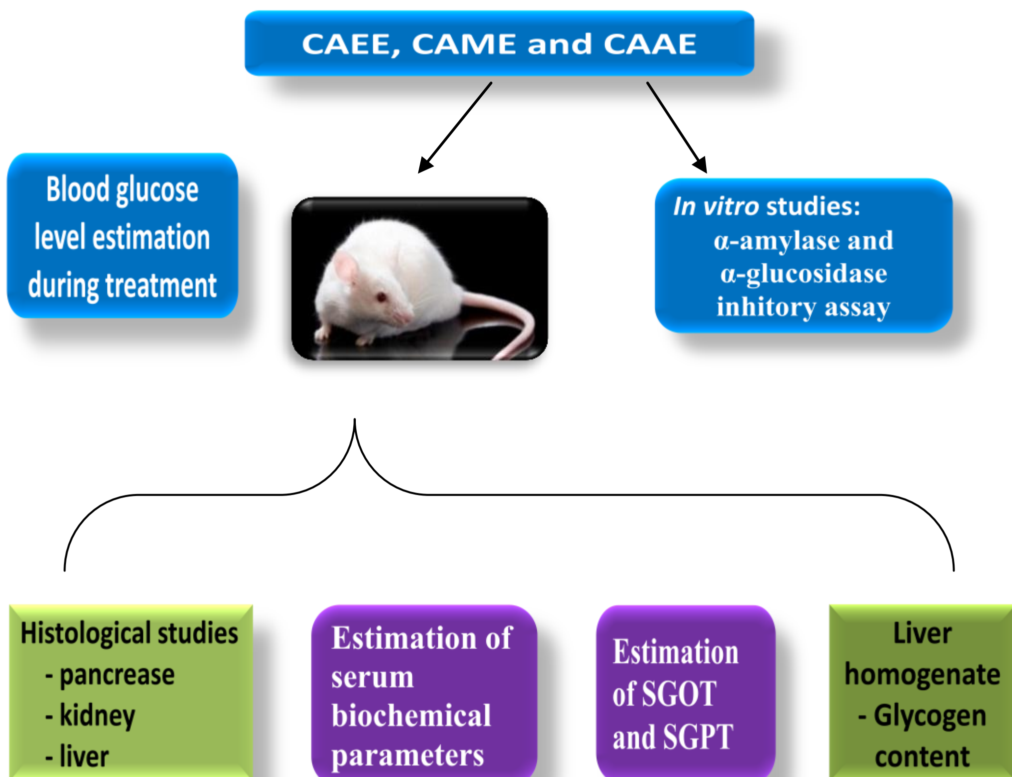

Fig.3.5: Graphical presentation of the methodology (phase 3)

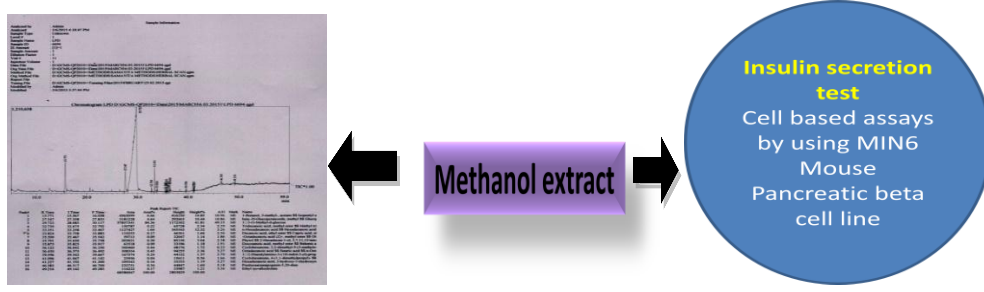

Fig.3.6: Graphical presentation of the methodology (phase 4)

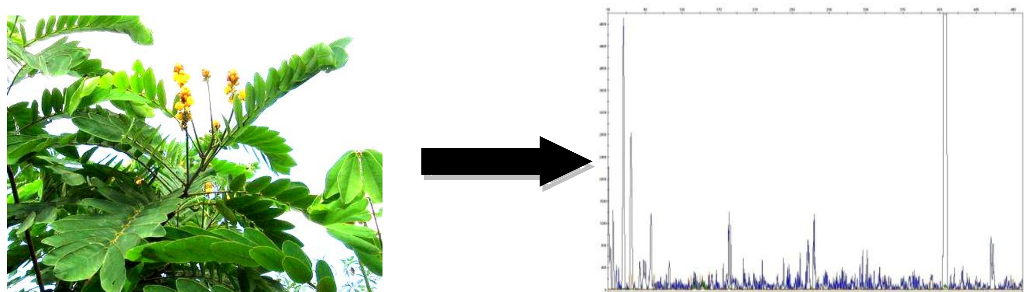

Fig.3.7: Graphical presentation of the methodology (phase 5)