CHAPTER-III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant materials

Fresh leaves of *E.operculata* were collected from an area around Lilong Chajing of Manipur, India in the month of April, 2010. The plant was identified and authenticated as *Eugenia operculata* Roxb., by Botanical Survey of India, Shillong bearing No:BSI/ERC/2010/Plant identification/611 (Fig 3) and a voucher specimen was deposited at the Department of Biotechnology.

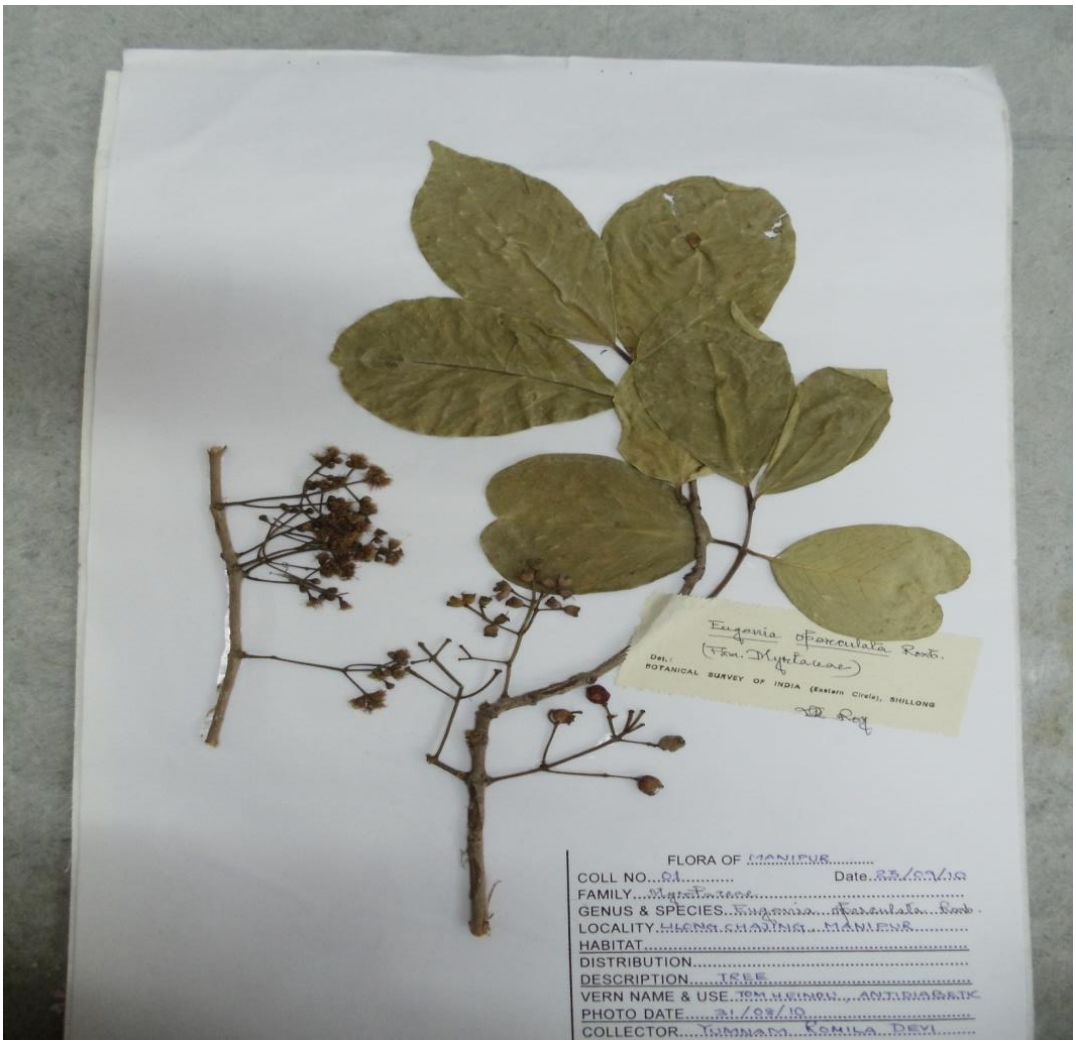

Plate 2: Herbarium of *E.operculata* Roxb.

3.1.2 Animal used

Albino mice (20-35 g) of either sex were employed in this study. The mice were bought from Pasteur Institute, Shillong and were maintained under standard laboratory conditions at 25 ± 2°C, relative humidity 50 ± 15% and normal photo period of 12hr light and 12hr dark cycle. The animal had free access to water and standard diet. The experimental protocol has been approved by the Institutional Ethics committee. (Number IEC/AUS/2, 013-032dt-20/3/13).

Plate 3: Swiss albino mice

3.1.3 Chemicals and reagents

1. 10% Formalin
2. 3, 5 Dichloro-2-hydroxybenzene sulphate
3. 4-Aminopyrine
4. Acarbose
5. Acetone
6. Adenosine tri phosphate
7. Alcohol
8. Alpha amylase
9. Alpha glucosidase

10. Alpha-naphthol
11. Aluminium chloride
12. Ammonia
13. Anthrone
14. Ascorbic acid
15. Benzene
16. Benzoic acid
17. Chloroform
18. Cholesterol esterase
19. Cholesterol oxidase
20. Citrate buffer
21. Conc Nitric acid
22. Copper acetate
23. Dinitrosalicylic acid
24. DMSO
25. DPPH
26. Dry glucose
27. Eosin
28. Epon
29. Ethanol
30. Ethyl acetate
31. Fehling solution
32. Ferric chloride
33. Ferric chloride
34. Folin-Ciocalteu reagent
35. Gallic acid
36. Gelatin
37. Glacial acetic acid
38. Glibenclamide
39. Glutaraldehyde
40. Glycerol kinase
41. Glycerol-3-phosphate oxidase
42. Gum acacia

43. Haematoxylin
44. Hexane
45. Hydrochloric acid
46. Iodine
47. Lead acetate solution
48. Lead citrate
49. Lipoprotein lipase
50. Magnesium ribbon
51. Methanol
52. Ninhydrin
53. Nitro blue tetrazolium
54. Osmium tetroxide
55. Peroxidase
56. Petroleum ether
57. Phenol
58. Phosphate buffer
59. Picric acid
60. Pipes buffer
61. P-nitrophenyl α-D-glucopyranoside
62. Potassium acetate
63. Potassium ferricyanide
64. Potassium iodide
65. Potassium mercuric iodide
66. Potato starch
67. Pyridine
68. Quercetin
69. Silica gel
70. Sodium carbonate
71. Sodium chloride
72. Sodium cholate
73. Sodium hydroxide
74. Sodium nitropruside
75. Sodium phosphate

76. Sodium potassium tartarate
77. Starch solution
78. Streptozotocin
79. Sulfuric acid
80. Thiourea
81. Trichloro acetic acid
82. Uranyl acetate
83. Xylene
84. Zinc dust

3.2 Methods

3.2.1 Sequential extraction of *E.operculata* leaves

Fresh leaves of *E.operculata* leaves were first rinsed with tap water and then with distilled water for several times. Then air dried at room temperature under shade, cut into small pieces, grounded and homogenized into a fine powder using electric grinder. The finely ground air-dried leaves (50gm×4) was sequentially extracted using soxhlet apparatus with solvents of increasing polarity (petroleum ether, ethyl acetate, methanol, ethanol and water). The marc obtained after every extraction was completely dried and weighed. The residue obtained after petroleum ether extraction was dried and then extracted with ethyl acetate to obtain ethyl acetate extract. The same processes were followed for methanol, ethanol and aqueous extract also. The extracts collected from each solvent were evaporated to dryness and then lyophilized for further use (Fig 3).

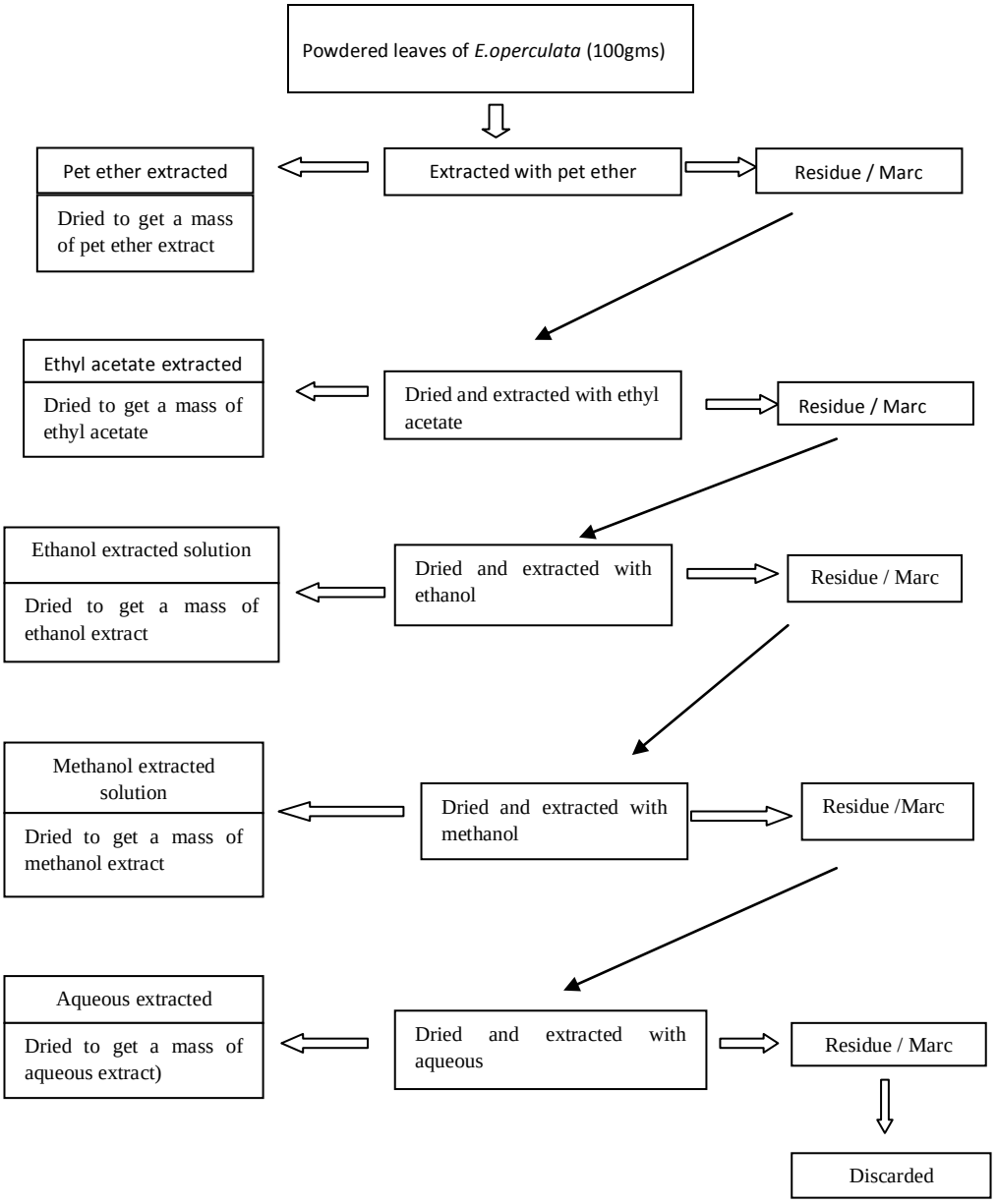

Fig 3: Schematic diagram of successive extraction.

3.2.2 Screening of *E.operculata* extract for in vitro antidiabetic activities

3.2.2.1 Alpha amylase inhibitory activity

This assay was conducted following the standard protocol (Giancarlo *et al*., 2006) with slight modification. The Starch solution (0.5% w/v) was obtained by boiling and stirring 0.25 g of potato starch in 50 ml of deionized water for 15 min. The enzyme solution (0.5 unit/ml) was prepared by mixing 0.001 g of α-amylase in 100 ml of 20 mM sodium phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride. The

extracts were dissolved in 2% DMSO to give concentrations from 10 to 100 mg/ml (10, 20, 40, 60, 80,100 µg /ml). The colour reagent was a solution containing 96 mM 3, 5-dinitrosalicylic acid (20ml), 1 M sodium potassium tartarate in 2 M sodium hydroxide (8 ml) and deionized water (12 ml). 1 ml of each plant extract of different concentrations and 1 ml enzyme solution were mixed in a tube and incubated at 25°C for 30 min. To 1 ml of this mixture 1 ml of starch solution was added and then the tubes were incubated at 25°C for 3 min. Then, 1 ml of the color reagent was added and the closed tubes were placed into an 85°C water bath. After 15 min, the reaction mixture was removed from the water bath and cooled thereafter, diluted with 9 ml distilled water and the absorbance value determined at 540 nm using uv-vis Spectrophotometer, Thermo Fisher, USA. Individual blanks were prepared for correcting the background absorbance. In this case, the color reagent solution was added prior to the addition of starch solution and then the tube placed into the water bath. The other procedures were carried out as above. Controls were conducted in an identical fashion replacing plant extract with 1 ml DMSO. Acarbose solution (at the concentrations of 10, 20, 40, 60, 80, 100 µg/ml) was used as positive control. The inhibition percentage of α-amylase was assessed by the following formula:

% of Inhibition = [O.D Control – O.D test / O.D control] X 100.

3.2.2.2 Alpha glucosidase inhibitory activity (Jayashri *et al*., 2009)

A volume of 50 µl of *E.operculata* (water, ethanol, methanol, ethyl acetate and petroleum ether) and 100 µl of 0.1 M phosphate buffer (pH 6.9) containing yeast α-glucosidase solution (1.0 U/ml) were incubated at 25°C for 10 min. After pre-incubation, 50 µl of 5mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each at 5 sec intervals. The reaction mixtures were incubated at 25°C for 5 min. After incubation, absorbance readings were recorded at 405 nm and compared to a control which had 50 µl of buffer solution in place of the extract. The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows:

% of Inhibition = [O.D Control – O.D test / O.D control] X 100.

3.2.3 In vivo antidiabetic effects of *E.operculata* extract

3.2.3.1 Induction of experimental diabetes

Diabetes mellitus was induced in overnight fasted albino mice by intraperitoneal injection (i.p) of freshly prepared streptozotocin dissolved in cold 0.01M citrate buffer, pH 4.5 at a dosage of 45mg/kg b.w daily for five consecutive days. The blood glucose concentration was measured every week from the day of STZ injection. Administration of multiple low doses of STZ produces significant hyperglycemia from 1st week. The animals were observed to be diabetic from 2nd week. After 2nd week of STZ injection animals with blood glucose concentrations increasing by more than 40% were considered diabetic and were included in this study (Arora *et al*., 2009 and Njomen *et al*., 2009).

3.2.3.2 Toxicity studies

Acute toxicity studies were carried out on mice. Extracts were administered at doses of 100, 300, 500, 1000 and 3000mg/kg b.w to five groups of mice each containing 6 animals. After administration of extracts the animals were observed for the first 3hrs for any toxic symptoms followed by observation at regular intervals for 24hrs up to 7 days. At the end of study the animals were also observed for general morphological behavior and mortality.

3.2.3.3 Acute effect of *E.operculata* extracts on blood glucose levels

Glucose tolerance tests were performed in experimental and control mice according to the method described by (Dimo *et al*., 2007 and Adiga *et al*., 2010) with slide modification in the mode of treatment. Briefly, animals were divided into two groups, normal and diabetic comprising of four subgroups. Two group of six normal mice and two group of six STZ induced diabetic mice were given *E.operculata* aqueous and ethanol extract at a dose of 250mg/kg b.w by gavages. Another group of normal and STZ induced diabetic mice were given standard drug glibenclamide at a dose of 5mg/kg b.w. A control group of normal mice and a control group of diabetic mice received gum acacia (1% w/v) at the same time. Grouped mice were subjected to overnight fasting from 8.00pm to 8.00am. After overnight fasting blood samples were taken from all groups at 8.01am for recording 0 min blood glucose level. One hour

later, after giving treatment glucose at the dose of 2.5mg/kg b.w were administrated to all groups. Blood glucose levels were then measured at 30, 60 and 120 minutes after glucose loading using glucometer. Blood glucose tolerance curves of experimental mice were plotted and compared with those of control groups.

	Normal mice	Diabetic mice
Group-1	Gum acacia (1% w/v; 0.5ml/kg b.w)	Gum acacia (1% w/v; 0.5ml/kg b.w)
Group-2	E.oAq (250mg/kg b.w)	E.oAq (250mg/kg b.w)
Group-3	E.oE (250mg/kg b.w)	E.oE (250mg/kg b.w)
Group-4	Glibenclamide (5mg/kg b.w)	Glibenclamide (5mg/kg b.w)

3.2.3.4 Long term antidiabetic effects of *E.operculata* extracts

Animals were divided into seven groups of six each. First group comprised of normal control and were given only gum acacia. Second to sixth group comprises of STZ induced diabetic mice. Second group received gum acacia and served as diabetic control. Third and fourth group were given *E.operculata* aqueous extract and fifth and sixth group were given *E.operculata* ethanol extract, dose of 250 and 500mg/kg b.w. Seventh group received the standard glibenclamide at the dose of 5mg/kg b.w. The treatment was continued for 21 days and fasting blood glucose level was monitored for every seven days after initiation of treatment.

Mice were sacrificed at the end of the experiment, on 21st day and the blood sample were collected to determine the effect of *E.operculata* extract on various biochemical parameters.

Group-1	Normal mice treated with vehicle alone (Gum acacia,1%w/v;1ml/kg b.w)
Group-2	Diabetic mice treated with vehicle alone (Gum acacia,1%w/v;1ml/kg b.w)
Group-3	Diabetic mice + Glibenclamide (5mg/kg b.w)
Group-4	Diabetic mice + Aqueous extract (250mg/kg b.w)
Group-5	Diabetic mice + Aqueous extract (500mg/kg b.w)
Group-6	Diabetic mice + Ethanol extract (250mg/kg b.w)
Group-7	Diabetic mice + Ethanol extract (500mg/kg b.w)

3.2.4 Effects of *E.operculata* extract on lipid profile

Blood sample were 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^{o}C until analysis.

3.2.4.1 Determination of triglyceride levels

Triglycerides were estimated by GPO/POP method using triglycerides kit from 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) into reagent buffer (3,5 dichloro-2-hydroxybenzene sulphonate, pH 7.0). And it was swirled to dissolve and allowed to stand for 10 min at room temperature. Serum triglycerides were hydrolyzed to glycerol and fatty acids by lipase enzyme. In the presence of ATP and glycerol-kinase, glycerol was converted into glycerol-3-phosphate and adenosine diphosphate (ADP). Glycerol-3-PO_4 oxidase dissociates glycerol-3-phosphate into dihydroxy-acetone phosphate and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide reacts with 4-aminoantipyrine to form a colored complex. The intensity of the color developed was proportional to the triglycerides concentration and was measured photometrically at 505 nm. The instrument was adjusted to zero with distilled water. For the estimation of triglycerides, 10µl each of sample and standard were mixed with 1ml of working reagent and incubated for 5 min at 37°C. The absorbance of the samples and standard of 200 mg/dl concentration was read out against the blank. The colour should be stable for 30 min. Triglyceride was estimated by using the following formula:

Triglyceride (mg/dl) =Absorbance of Samples/Absorbance of standard x Conc. of standard (mg/dl).

3.2.4.2 Estimation of total serum cholesterol

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

6.95) and then allowed to stand for 5min at room temperature. Serum cholesterol ester was hydrolyzed to cholesterol and fatty acid by cholesterol esterase. Cholesterol oxidase converted cholesterol into cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide reacts with 4-aminoantipyrine to form a coloured complex. The intensity of the colour developed was proportional to the concentration of cholesterol in the sample. 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^{o}C and the absorbance was read at 500nm.

3.2.4.3 Estimation of serum lipid

Phosphotungstate method was used to estimate the serum lipids like very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol level. The clear supernatant after removal of VLDL and LDL, containing HDL was used for determination of HDL-Cholesterol (HDLc). The VLDL and LDL from serum were precipitated by phosphotungstate in the presence of magnesium ions.

Serum/Plasma $^{\text{Phosphotungstate, mg}}$ HDL+ (LDL+VLDL+Chylomicrons)

VLDL-Cholesterol (VLDLc) and LDL-Cholesterol (LDLc) were respectively calculated by using Frederickson- Friedwald's formula as follows:

LDL Cholesterol = Total Cholesterol – Trigyceride/5- HDL Cholesterol.

VDDL Cholesterol= Triglyceride/5

For this 0.5 ml of serum was taken in a test tube and 0.5 ml of precipitation reagent was added. The mixture was shaken thoroughly and left to stand for 10 m at 25 to 30°c and then centrifuged for 20 m at 4000 rpm. Within 2 hrs after centrifugation, the clear supernatant was used for the determination of HDL-Cholesterol. The supernatant containing 0.05 ml was taken in a test tube and 1 ml reaction solution was added to it. In another test tube, 0.1 ml distilled water was taken and 1 ml reaction

solution was added. The mixtures were mixed thoroughly, incubated for 5 min at 37°c and measured for the absorbance of the sample against blank reagent at 510 nm.

3.2.5 Histological studies

3.2.5.1 Processing of tissue for light microscopy

At the end of the studies the animals were sacrificed, the pancreas of one animal from each group was excised and stored in 10% formalin after washing with normal saline. The tissue was washed, dehydrated with alcohol, cleared with xylene and paraffin blocks were made. Serial sections of 5 μm thickness were cut using a rotary microtome. The sections were then deparaffinised with xylene and hydrated in descending grades of alcohol. The slides were then transferred to haematoxylin for 10 min, followed by rinsing with water. These were examined and later counterstained with eosin, rinsed with water, dehydrated with ascending grades of alcohol, cleared with xylene and mounted.

3.2.5.2 Processing of tissue for Electron Microscopy (EM)

Tissue fragments, pancreas was cut into 1mm cubes and were prefixed in 2.7% glutaraldehyde solution in 0.1M PBS, pH 7.2, for 90 minutes at 4^0C. Then, samples were washed in 4 consecutive baths of 0.15 M PBS, 30 minutes each, and postfixed in 2% OsO_4 solution in 0.15 M PBS, for an hour, at 4^0C. Subsequently, the tissues were washed thoroughly in washing buffer to remove excess OsO_4. And then dehydrated gradually in ethyl alcohol and dealcoholized using propylene oxide. Infiltration was carried out with propylene oxide and spun's mixture at increasing concentrations at room temperature, using a low speed rotary shaker. Embedding was done in a flat embedding mould and ultrafine sections were obtained with a diamond knife at a Leica UC 6 ultra microtome. The sections, collected on a 200 Mesh electrolytic grids, were double contrasted with uranyl acetate and lead citrate and examined in a Jeol JSM 100 CX electron microscope.

3.2.6 Liver glycogen estimation (Nicholas *et al*., 1948)

The determination of glycogen in liver was done by solution of "anthrone reagent ". Purified anthrone (500 mg), thiourea (10 gm) and 1 liter of 72 % sulfuric

acid were placed in a flask. The mixture was heated up to 80-90°C. The flask was occasionally shaken to mix the contents. The mixture was cooled and stored in a refrigerator. Stock solution of standard was prepared by dissolving 100 mg of dry glucose in 100 ml of saturated benzoic acid solution. 5 ml stock solution was placed in a 100 ml volumetric flask and the volume was made up with saturated benzoic acid solution. Liver was blended by blender under trichloroacetic acid (TCA) and homogenized for 3 min. The homogenate was poured into a centrifuge tube. The supernatant fluid was centrifuged and decanted upon an acid-washed filter paper placed in a funnel and drained into a graduated cylinder. The residue was quantitatively transferred to the blender with TCA and homogenized again for 1 min. The mixture was centrifuged and the supernatant fluid was poured through the same filter. Two more extractions were made in the same manner. The desired volume was made up with 5 percent TCA and the solution was mixed thoroughly. 1 ml of the TCA filtrate was pipetted into a 15 ml Pyrex centrifuge tube. Duplicate samples of each unknown were analyzed to obtain the most reliable results. To each tube, 5 volumes of 95 percent ethanol were added with careful blowing. This was checked by noting the absence of an interface. The tubes were capped with clean rubber stoppers and allowed to stand overnight at room temperature. After precipitation was completed, the tubes were centrifuged at 3000 r.p.m. for 15 min. The clear liquid was gently decanted from the packed glycogen and the tubes were allowed to drain in an inverted position for 10 min. The glycogen was dissolved by adding 2 ml of distilled water, the water being added in a manner that was washed down the sides of the tube. Blank reagent was prepared by pipetting 2 ml of water into a clean centrifuge tube. A standard was prepared by pipetting 2 ml of standard glucose solution, containing 0.1 mg of glucose, into a similar tube. At this point 10 ml of anthrone reagent was delivered into the centre of the each tube with vigorous, but consistent, blowing to ensure good mixing. As each tube received anthrone reagent, it was tightly capped with an air condenser and placed in a cold tap water bath. After the temperature of all tubes had reached the temperature of cold water, they were immersed in a boiling water bath to a depth a little above the level of the liquid in the tubes for 15 min and then removed from water bath and cooled to room temperature. The tubes and stoppers were wiped dry and the contents of each tube were transferred to a calorimeter tube and the absorbance was read at 620 nm after adjusting the calorimeter with the blank reagent. Care was taken to avoid introduction

of lint or contaminating carbohydrate into the anthrone reaction. The calculation of glycogen content was done by using the following formula

DU/DS x 0.1 x Volume of Extract/100gm. of Tissue x 100 x 0.9 = mg. of glycogen per 100gm. of tissue.

3.2.7 Estimation of glycosylated hemoglobin

Glycated haemoglobin levels was measured by using diagnostic kit, Bio system. Into a test tube, 50µl of blood and 200µl of reagent were pipetted out. The contents in the test tube were mixed well and allowed to stand at room temperature for 10-15 min, and this formed the hemolysate. The column for the separation was prepared by removing the upper cap from the column first and then the lower cap, the rounded end of a pipette was used to push the upper disc down to the resin surface; care was taken to avoid compressing it. The column was allowed to drain completely.

50µl of the hemolysate was pipetted on the upper filter and the column was allowed to drain to waste. The sample residue left above the upper disc was removed by adding 200µl of reagent 2 and the column was allowed to drain to waste. Again 2ml of reagent 2 was added and the column was allowed to drain to waste. Next, the column was placed over a test tube and 4ml of reagent 3 was added to the column, the HbA1c was collected and the absorbance was read at 415nm against distilled water. In order to read the absorbance of the total hemoglobin 12ml of reagent 3 was mixed with 50µl of hemolysate and the absorbance was read at 415nm against distilled water.

HbA1c was calculated as: AHbA1c /3 x AHbtotal x 100 = % HbA1c

3.2.8 Antioxidant activity of *E.operculata* extracts

3.2.8.1 DPPH free radical scavenging activity

DPPH free radical scavenging assay was measured using DPPH free radical test, by employing the method of Wong *et al.* with slight modification. The different concentrations of each of the extracts were prepared in methanol and were added to 3ml of 0.1mM methanolic solution of DPPH. The tubes were shaken vigorously and allowed to stand for 30 min at room temperature in the dark. Changes in absorbance of samples were measured at 517 nm. A control reading was obtained using methanol

instead of the extract. Ascorbic acid served as the standard. Free radical scavenging activity was expressed as inhibition percentage and was calculated using the following formula,

$$\% \text{ Inhibition} = (A_0 - A_1)/A_0 X\ 100$$

Where,

A_0 is the absorbance of the control
A_1 is the absorbance of test samples.

All the tests were performed in triplicates and the results are reported as IC50, which is the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%.

3.2.8.2 Reducing power assay

A spectrophoptometric method was used for the measurement of reducing power. For this 2.5ml of each of the extracts was mixed with 2.5ml phosphate buffer (0.2M pH 6.6) and 2.5ml of 1% potassium ferricyanide (10mg/ml). The mixture was incubated at 50^0C for 20 min, then rapidly cooled, mixed with 2.5ml of 10% trichloroacetic acid and centrifuged at 6500rpm for 10min. An aliquot (2.5ml) of the supernatant was diluted with distilled water (2.5ml) and then ferric chloride (0.5ml, 0.1 %) was added and allowed to stand for 10min. The absorbance was read spectrophotometrically at 700nm. Ascorbic acid was used as standard for construction of the calibration curve and the reducing power was reported as ascorbic equivalent per 100gm of dry sample.

3.2.9 Phytochemical screening of *E.operculata* extracts

The ethanol and aqueous extract of *E.operculata* were screened for phytochemical constituents using standard procedures of analysis Brain and Turner 1975, Evans 1996 as given below.

3.2.9.1 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 a yellow colored 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 was confirmed by the formation of yellow coloured precipitate.

3.2.9.2 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 alcoholic α-naphthol solution in a test tube and concentrated H_2SO_4 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 dilute HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

3.2.9.3 Detection of resins

Acetone H_2O tests: Extracts were treated with acetone. Small amount of water was added and shaken. Appearance of turbidity indicates the presence of resins.

3.2.9.4 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.

c) Shinoda Test: To the alcoholic solution of extracts, a few fragments of magnesium ribbon and concentrated HCl was added. Appearance of magenta colour after few minutes indicates presence of flavonoids.

d) Zinc hydrochloric acid reduction Test: To the alcoholic solution of extracts, a pinch of Zinc dust and concentrated HCl was added. Appearance of magenta colour after few minutes indicates presence of flavonoids.

3.2.9.5 Detection of Terpenoids

Salkowski test: To 0.5 g each of the extract was added 2 ml of chloroform. Concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

3.2.9.6 Detection of Anthraquinones

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

3.2.9.7 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.

3.2.9.8 Detection of Glycosides

Extracts were hydrolysed with dilute 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 min. 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.

3.2.9.9 Detection of Phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

3.2.9.10 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.

32.9.11 Detection of Proteins and Aminoacids

a) Xanthoproteic 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.

3.2.9.12 Detection of Cardiac Glycosides

Killer Kilani Test: To 0.5 g of extract diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated H_2SO_4. 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.

3.2.10 Determination of total flavanoid and phenolic compounds

Total phenolic content of *E.operculata* extracts was measured by employing the method described by Skerget *et al*., 2005 involving Foin-Ciocalteu reagent as an oxidizing agent and gallic acid as a standard. To 0.5ml of extract solution (2mg/ml) in water, 2.5ml of Fiolin-Ciocalteu reagent (diluted 10 times with water) and 2.0ml of sodium carbonate (7.5%w/v) solution were added. After 20 minutes incubation at room temperature the absorbance was measured at 760nm using a UV-visible spectrophotometer. Total phenolic were quantified by calibration curve obtained from measuring the known concentration of gallic acid (0-100µg/ml). The phenolic contents of the sample were expressed as gm of GAE (gallic acid equivalent)/100gm of the dried extract.

Aluminum chloride colorimetric method was used for flavonoids determination (Chang *et al*., 2002). 0.5 mL of each plant extract was separately mixed with 1.5 mL of methanol, 0.1mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. The reaction mixture was allowed to stand at room temperature for 30 to 36 min and the absorbance of the reaction mixture was measured at 415 nm. The calibration curve was prepared by using quercetin at concentrations of 12.5 to 100 µg/ml in methanol.

3.3 Bioassay guided Isolation and characterization of antidiabetic compound(s) of *E.operculata* leaves extracts

3.3.1 Determination of TLC solvent system

Ethanolic extract of *E.operculata* was subjected to thin layer chromatographic studies, to find out the satisfactory developing or eluting solvent for column chromatography.

3.3.2 Preparation of TLC plates

Clean glass plates were coated with silica gel G60 to a thickness of 0.25 cm. The coated plates were air-dried and activated in an oven for 1hr at 110 ^{0}C. The plates were then cooled at room temperature. Ethanol extracts were dissolved in the respective solvent. The dissolved extracts were spotted on the plate. The plates were allowed to dry at room temperature. The spotted plates were developed in different solvent systems: - Petroleum ether: ethyl acetate, pet: acetone, pet: chloroform, pet: methanol, chloroform: ethyl acetate, chloroform: acetone, chloroform: methanol, ethyl acetate: acetone, ethyl acetate: methanol, ethyl acetate: ethanol and acetone: methanol. The solvent system was allowed to travel a pre-determined distance from the origin. The plates were removed from the chamber, dried and viewed under the UV viewer lamp before and after exposed to iodine vapour or concentrated H_2SO_4. In all cases, the plates were heated at 105º C for 10 minutes. The colours of spots developed and their Rf values were inspected in day light as well as long wave ultra-violate light (UV, 366 nm). The number of spots eluted was counted and the solvent system showing maximum spot were selected for column chromatography.

3.3.3 Fractionation of the crude extract

Concentrated ethanol crude extract was subjected to silica gel column chromatography for the isolation of bioactive principles. About 75gm of the extract was loaded on top of activated silica gel which was packed into a glass column (700mm x100mm).The column was eluted with chloroform: methanol mixtures of increasing polarity (0-100%). Total of 22 fractions measuring about 250ml each were collected and concentrated under desiccators. All the fractions were analysed by TLC (dimensions of the plates: 20cmx20cm) using different proportions of hexane and ethyl acetate as mobile phase. The plates were air dried and the spots on the plate were located using iodine vapour. Various fractions were pooled based on the Rf values. The fractions which were pooled were numbered from 1 to 6. All the pooled fractions were tested for antidiabetic activity by alpha amylase and glucosidase method using the same protocol mentioned above.

3.3.4 Characterization of the bioactive fraction

The fractions which show higher bioactivity were further subjected to column chromatographic as mentioned above for the identification of bioactive principles. And further characterized by spectroscopic technique.

3.4 NMR Spectroscopy

The isolated or fractionated compounds were characterized by analysis of their ^{1}H and ^{13}C NMR spectra by using Bruker advance II 400 operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C using DMSO-D_6 as a solvent and TMS as internal reference along with mass spectrometry and also by comparing with already reported spectral data.

3.5 Statistical analysis

The final results are expressed as Mean±SEM. The group mean were compared using ANOVA test and Post Hoc Tukey HSD was applied to compare the mean. Values were determined to be significant when p was less than 0.05 ($p<0.05$).