MATERIALS AND METHODS

a) Preparation of EEMC

Material used: Bitter melon (BM) fruits, Ethanol, Magnetic stirrer (REMI) with beads, Whatmann’s Filter paper no. 1, freeze dryer (Alpha 2-4 LD Plus from Christ, GmbH), Rotovapor R-215 (Buchi, Switzerland), Folin-Ciocalteu (F-C) Reagent (Merck Specialities Pvt. Ltd.), Gallic Acid Standard (GA) (Sisco Research Laboratories Pvt. Ltd.), Vortex mixture, UV/VIS Spectrophotometer (Eppendorf India Ltd).

![Diagram of preparation process]

**Procedure:** MC fruit was purchased from a Co-operative horticulture outlet and then washed in distilled water, weighed and paste was prepared of whole fruit using a regular household mixer without adding any additional water. The paste was then lyophilized using freeze dryer (Alpha 2-4 LD Plus from Christ, GmbH) and the powder obtained was stored at -80°C in air tight plastic container. Different percentage (50%, 70%, and 100%) of EEMC was obtained using 50 gm of this lyophilized powder.

50 grams lyophilized powder of MC was mixed with 50% ethanol till the whole powder was completely covered with the solvent, in stoppered container and kept in 4°C overnight and were subjected to maceration using magnetic stirrer the next day. After 3 hours of magnetic stirring, the solvent mixture was centrifuged and the supernatant was collected in a brown bottle. The pellet remaining at the bottom of centrifuge tube, i.e. marc was again collected into another stoppered container and again subjected for extraction using magnetic stirrer. This extraction procedure was done for 3 times with 50% solvent. Finally the complete
solvent mixture was filtered using Whatmann’s filter paper number-1 and stored in – 20º C. Sequential and gradient extraction was done using 50% ethanol followed with 70% ethanol & finally 100% absolute ethanol and preserved in –20º C in a stoppered brown bottle until used for further analysis.

The obtained different percentage solutions of EEMC were then concentrated using Rotovapor R-215 (Buchi, Switzerland). Solvent from 50% extract was evaporated by adjusting the temperature of water bath at 45ºC and pressure adjusted to 50 torr. Temperature was decreased as there was an increase in ethanol concentration from 50% to 100% due to decrease in boiling point. 70% extract was concentrated at 40ºC & 100% ethanolic extract was concentrated at 30ºC. Following low temperature holding mode, 50% extract was concentrated for 2 hours, 70% extract was concentrated for less than 2 hours and 100% extract was efficiently concentrated in just 1 hour, which was later filtered using Whatmann’s filter paper number-1 and stored in brown bottles at -20 ºC covered with aluminium foil. This concentrated different percentage EEMC were divided into two fractions. One of the fractions were subjected for biochemical analysis while the other fraction was again subjected to lyophilisation using freeze dryer (Alpha 2-4 LD Plus from Christ, GmbH) which was done by dehydrating all the 50%, 70% & 100% concentrated extracts completely at reduced pressure after being frozen at -80ºC. Once completely dehydrated, the concentrated lyophilized powder extracts were preserved at -80ºC wrapped with aluminium foil in air-tight container, until being used for further analysis.

For further analysis EEMC stock was prepared by dissolving the lyophilized powder in Phosphate buffered saline (PBS) (HiMedia Laboratories).

b) Biochemical studies:
Different percentages of EEMC were subjected to various biochemical analyses to determine the total phenolic content, reducing sugar, antioxidant activity, anti-inflammatory activity and flavonoids. All the analyses were done in triplicate and the reagents for each analysis were prepared freshly.

Estimation of Total Phenol Content (TPC) in EEMC using Folin-Ciocalteu (F-C) Method [161].
Gallic acid (Sisco Research Laboratories Pvt.Ltd.) was used as standard. Working stock standard range was fixed at 5ug- 60µg/µl. 50%, 70% & 100% EEMC were taken in volume of 100µl, 200µl and 300µl and made up to 1ml with absolute ethanol. To this, 1ml of F-C reagent and 0.8ml of 4% -NaHCO_3 was added and incubated along with standards for 30 minutes in dark at room temperature. Finally absorbance maxima was recorded at 760 nm using UV-Visible Spectrophotometer (Eppendrof India Ltd). Average of three trials was taken. Standard deviation (SD), Standard error (SE) was derived using Graph Pad Prism-5. TPC of 50%, 70% & 100% ethanolic extracts were expressed in terms of Gallic Acid Equivalents (GAE) from the standard calibration curve and Percentage Total Phenol (%TP) were obtained by back calculating for dried powdered plant materials and expressed as Percentage gram weight (% w/w).

Estimation of Total Flavanoid Content (TFC) in EEMC by Aluminum Chloride Colorimetric Method [162].
Quercetin (Sigma-Aldrich Co.LLC) was used as standard to make the calibration curve. Working stock standard range was fixed from 5µg/ml to 500µg/ml. 25µl of 50%, 70% and100% EEMC and standards were taken. To this 75µl 95% ethanol, 5µl aluminum chloride and sodium acetate and 140µl distilled water was added and incubated for 30 minutes at room temperature. Finally absorbance maxima was recorded at 415nm in a multimode plate reader (PerkinElmer). Average of three trials was taken. SD, SE was derived using Graph Pad Prism-5. Total content of flavonoid in the EEMC was expressed as quercetin equivalents (mg of QE/g sample).
**Estimation of reducing sugar in EEMC using 3, 5 - Dinitrosalicylic acid (DNS) Method.**

D-Glucose (Sisco Research Laboratories Pvt.Ltd.) was used as standard. Working standard range was fixed from 0.1 to 1mg/ml in distilled water. 50%, 70% & 100% EEMC were taken in volume of 100µl, 200µl and 300µl and made up to 1ml with distilled water. One ml of DNS working reagent was added to both standard and extract and incubated in boiling water bath for 10 minutes and then cooled. Finally 4.0 ml of distilled water was added. Mixed well and absorbance maxima was recorded at 540 nm and reducing sugar in different percentage of extracts was expressed in terms of D-Glucose concentration from the standard calibration curve. Average of three trials was taken. SD, SE was derived using Graph Pad Prism-5. The Reducing sugar level was expressed as D-Glucose concentration (mg/ml) and Percentage Reducing Sugar (%RS) was obtained by back calculating for dried powdered plant materials and expressed as Percentage Gram weight (% w/w).

**Identification of Reducing Sugar in EEMC by Paper Chromatography**

Paper chromatography was done using Butanol: Acetic acid: Distilled Water in the ratio 12:3:5 as mobile phase and 1.66 % of Phthalic acid in n-Butanol: Distilled water: Aniline in the ratio 95:4:1 as Visualizing agent. Rf (Relative fraction) for standards including extract were determined by the equation: Rf = Distance travelled by the substance/Distance travelled by the solvent front. Identification of sugars present in 50% EEMC was compared with the Rf values of standards in the same run and was confirmed by a cascade of colorimetric reactions.

**Quantitative estimation of Glucose present in the EEMC by Glucose Oxidase and Peroxidase (GOD-POD) Method**

D-Glucose was used as standard with working stock standard ranging from 50-350 mg/dl. 20µl of working stock standard, 20µl of distilled water and 20µl of 50% EEMC was taken for standard, blank and test respectively and 2ml of GOD-POD reagent was added. All preparations were vortexed well and incubated at room temperature for 30 minutes. Pink colour developed and absorbance was recorded at 505nm. Glucose concentration present in 50% EEMC was derived from standard calibration curve and expressed as concentration of glucose in mg/dl. Average of three trials was taken. SD, SE was derived using Graph Pad Prism-5.

**Preparation of stock of different percentage of EEMC**

Further biochemical analyses were carried out using the stock prepared for different percentage of EEMC based on TPC. Based on the total phenolic content the stock of 50%, 70% and 100% EEMC was prepared in the concentration of 80µg/ml, which was further serially diluted up to 1.25 µg/ml in ethanol.

**Estimation of Antioxidant Activity of EEMC using Ferric Reducing Antioxidant Power (FRAP) Method [163]**

FeSO₄ (Sisco Research Laboratories Pvt.Ltd.) was used as standard. Working stock standard ranged from 200µM - 1600µM. 150µl of working stock standard and 50%, 70% & 100% EEMC were taken in different concentrations from 40µg/ml to 1.25µg/ml and 2800µl of FRAP reagent was added to all samples and standards and incubated for 30 minutes in dark at room temperature. Finally maximum absorbance recorded at 593nm. Average of three trials was taken. SD, SE was derived using Graph Pad Prism-5. The antioxidant capacity based on the ability to reduce ferric ions of extracts to ferrous form was calculated from the linear calibration curve and expressed as mmol of FeSO₄ equivalents (FRAP units).

**Estimation of Free Radical Scavenging Activity of EEMC using 2,2-diphenyl-1-picrylhydrazyl (DPPH) Method [164]**

Ascorbic acid (Sisco Research Laboratories Pvt.Ltd.) was used as standard. The working stock standard ranged from 5µg/ml - 100µg/ml. 200µl of working stock standard were taken
from each of the different concentrations and an equal amount of 50%, 70% & 100% EEMC were taken in different concentrations from 40µg/ml to 1.25µg/ml. 1400µl of DPPH reagent was added to all samples and standards and incubated for 30 minutes in dark at room temperature. The purple colour changes to yellow as there is an increase in the concentration of standards. After incubation, the absorbance maxima of the solution mixtures were taken against blank at 536 nm. Average of three trials was taken. SD, SE was derived using Graph Pad Prism-5. The free radical scavenging activity of each fraction was determined from the linear calibration curve by comparing its absorbance with that of a blank solution. The radical scavenging activity was expressed as the percentage inhibition (I%) and calculated as per the equation:

\[ I(\%) = \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100, \]

Where A blank is the absorbance of the blank reagent with no testing compound, and A sample is the absorbance of the test sample with all reagents. The IC-50 value was calculated from the plot of inhibition (%) against the concentration of the extract.

**Estimation of Antioxidant Activity of EEMC using Reducing Power Assay [165]**
Ascorbic acid was used as standard. The working stock standard ranged from 5µg/ml - 100µg/ml. Assay was performed in 96well microplate reader. 30µl of 50%, 70% & 100% EEMC and standards were taken and 83µl of phosphate buffer and potassium ferricyanide was added and incubated for 20minutes at 50°C in a shaker incubator. To this 83µl of trichloroacetic acid was added and incubated for 5minute at room temperature. Now from this, 83µL of upper layer of solution was transferred to another 96 well microplate reader. To this 83µl of distilled water and 8.3µl of ferric chloride was added. The absorbance was measured at 700nm in a multimode plate reader (PerkinElmer). Higher absorbance of the reaction mixture indicates greater reducing power. Average of three trials was taken. SD, SE was derived using Graph Pad Prism-5

**Estimation of Anti-inflammatory Activity of EEMC using Human Red Blood Corpuscle (HRBC) Membrane Stabilization Assay (Hypotonicity Induced) [166]**
Preparation of suspension (10% v/v) of HRBC: 5ml of whole blood sample was collected in a EDTA centrifuge tube from healthy human volunteer who has not taken any alcohol, NSAID and abstinence from smoking for 2 weeks prior to the experiment. Blood samples were centrifuged at 3000 rpm at room temperature for 15 min. The supernatant (plasma and leucocytes) was carefully removed while the packed red blood cell was washed with fresh normal saline (0.85% NaCl). The process of washing and centrifugation was repeated four times until the supernatant turns clear. Then, Human erythrocytes suspension (10% v/v) was prepared by aspirating 2.5ml of pellet and mixing with Phosphate Buffer Isosaline to the final volume of 25ml.

Assay of Membrane stabilizing activity: Diclofenac solution (1mg/10ml) was used as positive control. The assay mixtures consisted of 2 ml of Hyposaline (0.25% NaCl), 0.8 ml of 0.15 M sodium phosphate buffer, pH 7.4, 0.5 ml of 10% (v/v) HRBC suspension, 0.2 ml of compound solution in Hyposaline (0.25% NaCl) (standard and extracts) and final reaction mixtures were made up to 4.5 ml with Isosaline. The reaction mixtures were incubated at 37°C for 30 minutes and centrifuged at 3000 rpm for 20 minutes. The absorbance of the supernatant solution was measured spectrophotometrically at 560 nm. Each experiment was carried out in triplicates and was averaged. The percentage inhibition of haemolysis or membrane stabilization was calculated using the following equation:

\[ \% \text{Inhibition of haemolysis} = \left( \frac{A_1 - A_2}{A_1} \right) \times 100 \]

Where: \( A_1 \) = Absorbance of hypotonic buffered saline and \( A_2 \) = Absorbance of test sample in hyposaline and were compared with the % inhibition of positive control.
c) Cell Line Studies

**Materials required**

Cell Culture: 3T3-L1 pre-adipocytes cell lines from National Centre for Cell Science, Pune, Dulbecco’s modified Eagle’s Media (DMEM) media (Gibco® by Life Technologies), Fetal Bovine Serum (FBS)- 10%(Gibco® by Life Technologies), Glutamine- 1%(Gibco® by Life Technologies), Penicillin-Streptomycin-1% (Gibco® by Life Technologies), Phosphate buffered saline (PBS) (HI Media Laboratories), Trypsin-EDTA- 0.25% (Gibco® by Life Technologies), CO2 Incubator- 5% (Heal Force Bio-Meditech Holdings Group), Inverted microscope (Magnus Analytics), Biosafety cabinet level -2 (Esco Technologies, Inc. USA), Adherent and treated cell culture flasks, 96 wells microtitre plate, Sulforhodamine-B (SRB)-0.4% (Sigma-Aldrich St. Louis, USA ), microtitre plate reader (Bio- Rad Laboratories, Inc.)

**Adipogenesis Assay** (Cayman chemicals item no 10006908) (Cayman Chemical Company, Ann Arbor, MI)

- Cell-Based Assay IBMX Solution (1,000X) 10008978
- Adipogenesis Assay Insulin Solution (1,000X) 10008979
- Adipogenesis Assay Dexamethasone Solution (1,000X) 10008980
- Lipid Droplets Assay Fixative (10X) 10008981
- Lipid Droplets Assay Wash Solution 600044
- Lipid Droplets Assay Oil Red O Solution 600045
- Lipid Droplets Assay Dye Extraction Solution 600046
- Cells treated with EEMC 96 wells microtitre plate
- Multimode plate reader (PerkinElmer)

**Adipolysis Assay** (Cayman chemicals item no 10009381) (Cayman Chemical Company, AnnArbor, MI)

- Adipolysis Assay IBMX Solution (1,000X) 10009948
- Adipolysis Assay Insulin Solution (1,000X) 10009949
- Adipolysis Assay Dexamethasone Solution (1,000X) 10009950
- Adipolysis Assay Isoproterenol Solution10009951
- Glycerol Standard Solution 10009952
- Free Glycerol Assay Reagent (10X) 10009953
- Cells treated with EEMC 96 wells microtitre plate
- Multimode plate reader (PerkinElmer)

3T3-L1 Pre-adipocytes Cell Lines were procured from NCCS Pune, India and were maintained in laboratory in DMEM media supplemented with 20% FBS, 1% Glutamine and 1% Penicillin-Streptomycin in adherent and treated tissue culture flasks and allowed to grow till 80% confluency. Based on the total phenol content of 50 % lyophilized powder working stock standard was prepared by serially diluting the stock to get concentrations range from 100µg/ml to 0.195µg/ml (2X).

Sulforhodamine-B (SRB) assay was carried out to evaluate the cytotoxic effect of 50% EEMC on the pre-adipocyte cells. The pre-adipocyte cells were grown according to the manufacturer’s instructions using Adipogenesis Assay Kit, item no-10006908(Cayman chemical). Ten thousand cells were seeded in 96 well plates and maintained in 100µL DMEM media supplemented with 20% FBS, 1% Glutamine and 1% Penicillin-Streptomycin and incubated at 37°C with 5% CO₂, till it reached confluence. The wells in the 96 well plate were divided into 5 categories consisting of media blank (without cells), non-differentiated (without treatment with induction or insulin media), control (treated with induction and insulin media), during differentiation group (treated with induction and insulin media along with EEMC upto 7th day), after differentiation group (treated with induction and insulin media upto 7th day and treated with EEMC on the 7th day).
Two days post confluence (day 0), the media was replaced with 100 µl of induction medium to control, EEMC during and after differentiation group and 100µL of 50% EEMC in the concentrations ranging from 50µg/ml to 0.09µg/ml (1X) was added to EEMC during differentiation group and incubated for 72 hours at 37°C in humidified atmosphere containing 5% CO₂. After that (day 3), induction medium was replaced with insulin medium and 100µL of 50% EEMC in the concentrations ranging from 50µg/ml to 0.09µg/ml (1X) was added again to EEMC during differentiation group. This step was done for every two days till day 7. On day 7 medium was again replaced to fresh insulin medium and 100µL EEMC in the concentrations ranging from 50µg/ml to 0.09µg/ml (1X) were added to both EEMC during and after differentiation group and incubated for 48 hours at 37°C in humidified atmosphere containing 5% CO₂. On day 9, the reaction was stopped and cytotoxicity was evaluated by Sulforhodamine-B (SRB) assay.

**Sulforhodamine-B (SRB) assay**
Cultures fixed with Trichloroacetic-acid (TCA) were stained with 0.4% Sulforhodamine-B dissolved in 1% acetic acid. SRB dyes bind with membrane protein of live cells. Unbound dye was removed by washing with 1% acetic acid. Finally membrane protein bound dye was extracted with 10mM unbuffered Tris base [tris (hydroxymethyl) aminomethane] and optical density was measured at 564nm using multimode reader (Enspire from PerkinElmer). Since the colour intensity of SRB dye treated wells is directly related to the number of viable cells, the cytotoxic effect of the compound can be evaluated. Decreased in absorbance signifies the increase in cytotoxic effect indicating decrease in the number of viable cells. From the SRB assay, it was found that concentration of EECM ranging from 3.725µg/ml to 0.09µg/ml had no cytotoxicity on 3T3-L1 pre-adipocytes. Based on this the concentration ranging from 4µg/ml to 0.25µg/ml (2X) of 50% EEMC was used for both adipogenesis and adipolysis assay.

**Adipogenesis Assay**
Cells were grown in the similar manner as mentioned for cytotoxicity assay from day 0 to day 9. On day 9, adipogenesis was evaluated by Oil Red O Staining according to manufacturers protocol (Cayman chemical, Adipogenesis Assay kit no 10006908). The staining procedure was done in a room temperature. Most of the medium was removed from the wells. To each of the well 75µl of diluted Lipid Droplets Assay Fixative was added and incubated for 15 minutes. Then the wells were washed with 100µl of wash solution two times for five minutes each. Once the wells are completely dry, 75µl of Oil Red O Working Solution was added to all well sincluding the background wells containing nocells and incubated for 20 minutes. After incubation period, Oil Red O Solution was removed andcells were washed with distilled water several times until the water contained no visible pink color. Next the wells were washed with 100µl of wash solution two times for five minutes each and allowed to dry. Once the wells were dry completely 100µl of dyeextraction solution was added to each well and allowed to stand for 30 min and the absorbance was read at 520nm with a multimode reader (Enspire from PerkinElmer).

**Adipolysis Assay**
Cells were grown in the similar manner as mentioned for cytotoxicity assay from day 0 to day 9. On day 9, adipolysis was evaluated according to manufacturer’s protocol (Cayman chemical, Adipolysis Assay kit no 10009381) using glycerol standard. 25µl of glycerol standards whose concentration ranged from 7.8µg/ml to 125µg/ml were added to a new 96 well plate. 25µl of the cell culture supernatants were collected and added to the corresponding well of the new 96 well plate. To this 100µl of diluted Free Glycerol Assay Reagent was added per well, incubated for 15 minutes at room temperature and absorbance was read at 540nm. The standard curve was plotted as a function of glycerol concentration and to determine the equation of the line. The glycerol concentration was determined using the formula.
Glycerol concentration (µg/ml) = \[ \frac{A_{540}-(y\text{-intercept})}{\text{slope} \times \text{Sample\text{Dilution}}} \]

**Statistical analysis:**
All statistical analysis was performed using GraphPad Prism, Prism 5 for Windows, version 5.01. Data were expressed as mean values ± SEM. A one-way ANOVA model was used to compare means between the groups. Each sample was analyzed thrice. *Post hoc* pairwise multiple comparisons were evaluated using the Bonferroni post-test, after ANOVA. Results were considered significant at \( p < 0.05 \).

d) Treatment of pre-adipocyte cells for western blot analysis

**Materials required**

1. **For SDS PAGE**:
   Acrylamide-30%,
   1.5M Tris HCl (pH 8.8): 18.5 g of tris base in 50 ml of distilled water adjust the pH to 8.8 using 1N HCl and then make up the volume to 100ml using distilled water.

   0.5M tris HCl (pH 6.8): 6g of tris base in 50ml od distilled water, adjust the pH to 6.8 using 1N HCl and make up the volume to 100ml using distilled water.

   **Table 8: Composition of Separating gel [1 GEL (10ml) 10%]**

<table>
<thead>
<tr>
<th>Tris HCl 1.5M</th>
<th>2.5 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled water</td>
<td>5.97 ml</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>30 % acrylamide</td>
<td>3.32 ml</td>
</tr>
<tr>
<td>10 % APS</td>
<td>50 microlitre</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 microlitre</td>
</tr>
<tr>
<td>10 ml</td>
<td></td>
</tr>
</tbody>
</table>

   **Table 9: Composition of Stacking gel (4 % 10 ml)**

<table>
<thead>
<tr>
<th>Distilled water</th>
<th>6.1 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5M Tris HCl, pH 6.8</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>100 microlitre</td>
</tr>
<tr>
<td>30 % acrylamide</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>10 % APS</td>
<td>50 microlitre</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 microlitre</td>
</tr>
<tr>
<td>10 ml</td>
<td></td>
</tr>
</tbody>
</table>

2. **1X running buffer (2000 ml)**
   - 25mM Tris Base ( 3.03 gram x 2 = 6.06 gram )
   - 192mM Glycine ( 14.4 gram x 2 = 28.8 gram )
   - 0.1% SDS ( 1 g x 2 = 2gram )
   - Add distilled water to 2000ml
   - pH 8.3

3. **Transfer buffer ( 2000 ml )**
   - 25mM tris Base ( 6.06 gram )
   - 192mM Glycine ( 28.8 gram )
   - 20% Methanol ( 400ml )
4. **TBST**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM Tris Base</td>
<td>1.21 gram</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>4.38 gram</td>
</tr>
<tr>
<td>50 mM KCl</td>
<td>1.865 gram</td>
</tr>
<tr>
<td>0.2% Tween 20</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Adjust pH to 7.6. Add distilled water to 500ml.

5. **Blocking buffer**
- TBST + Bovine Serum Albumin (BSA) (0.2 gram for 10ml of TBST)

6. **Stripping buffer**
- 6.25ml – 1M Tris pH 6.7
- 2 grams SDS
- Make up to 100ml with distilled water
- 700 microlitres of Beta Mercaptoethanol

(For cell lysate collection):

**RIPA buffer: (1X)**
- 20mM Tris HCl pH 7.5 (0.315 gram)
- 150mM NaCl (0.876 gram)
- 1mM Na₂EDTA (0.0372 gram)
- 1mM EGTA (0.038 gram)
- (1 % Triton – X 100) 1% NP – 40 (1gram)
- 1 % Sodium deoxy cholate (1 gram)
- 25.5mM sodium pyrophosphate
- 1mM Beta glyophosphate
- 1mM Na₂VO₄ (sodium orthovanadate)
- 1microgram / ml leupeptin

**Primary Rabbit mAb antibodies Cell Signaling Technology**
PPAR gamma (Catalogue No. #2443) and secondary antibody Anti-rabbit IgG HRP-linked (Catalogue No. #7074) Primary antibody SREBP 1 from Sigma-Aldrich (Catalogue No. SAB4502850)

The method was followed according to manufacturer's protocol (Cayman chemical, Adipogenesis Assay kit no 10006908). Cell count was done using Neubær’s chamber, one and a half million cells per flask were seeded in 8 flasks and maintained in 5 ml DMEM media supplemented with 20% Fetal Bovine Serum (FBS), 1% Glutamine and 1% Penicillin-Streptomycin and incubated at 37°C and 5% CO₂, till it reached confluence.

**Table 10: Flasks treated with different concentrations of EEMC**

<table>
<thead>
<tr>
<th>Flask</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>Undifferentiated preadipocytes</td>
</tr>
<tr>
<td>2nd</td>
<td>Positive Control</td>
</tr>
<tr>
<td>3rd</td>
<td>During differentiation 0.5 µg/ml</td>
</tr>
<tr>
<td>4th</td>
<td>During differentiation 1 µg/ml</td>
</tr>
<tr>
<td>5th</td>
<td>During differentiation 2 µg/ml</td>
</tr>
<tr>
<td>6th</td>
<td>After differentiation 0.5 µg/ml</td>
</tr>
<tr>
<td>7th</td>
<td>After differentiation 1 µg/ml</td>
</tr>
<tr>
<td>8th</td>
<td>After differentiation 2 µg/ml</td>
</tr>
</tbody>
</table>
Further process was similar to that mentioned for cytotoxicity assay from day 0 to day 9. On the day 7, cell lysate of during differentiation 3 flasks (3rd, 4th, 5th) were collected, stored in -80°C. On day 9, cell lysate of flask 1, flask 2, after differentiated flasks (6th, 7th, 8th) were collected and stored in -80°C.

**Cell Lysate Procedure:**
The floating and adherent cells were collected by scraping using cell scraper and centrifuged at 3500rpm, 5min, 16°C, discard supernatant. The pellet was washed with PBS, 100microlitre of lysis buffer was added to the pellet in each of the eppendorf tubes and mixed well by pipetting in and out and tapping for uniform mixture. Incubated for 30minutes in ice, and centrifuged at 14000rpm at 4°C. Supernatant was collected and estimation of protein concentration was done using BCA method.

**Western Blotting**
The required glass plates were taken for PAGE, and assembled them by using scotch cellophane tape. Leakage was checked using distilled water. Once there was no leakage, the separating gel was prepared and the gel was poured up-to 3/4th of the plate. Air bubbles were removed by adding isopropanol on top of the separating gel. The gel was allowed to polymerise (20 minutes) and the isopropanol was removed by holding the tissue paper at one corner and inverting the gel. With distilled water, it was washed twice. The stacking gel reagents were now mixed and poured over the separating gel until the edge of the plate and then the comb was placed.

Once the gel was polymerized, the comb was removed carefully and distilled water was added to remove the air bubbles and then drained them off. The gel was placed in the unit such that the wells faced the cathode and the running buffer was added. The concentration of the proteins was calculated and loaded 43.7 micrograms of sample and the protein marker mixed with the sample loading buffer was added to 1st well. The concentration of sample loading buffer was adjusted to 1x. After loading the samples, the upper reservoir tank was filled with running buffer. The anode and the cathode were connected to the power outlet and set for 50V and allowed for the run till dye enters into separating gel then increased the voltage to 100V. The dye was tracked and when it reached the lower edge of the plate, the run was ensured to be completed and the power was turned off (2hours).

**Transfer of the bands from the gel to the membrane:**
The transfer apparatus was set; the sponge and the filter papers were soaked in the transfer buffer. The required amount of PVDF membrane was taken and activated them in methanol for 30 seconds. The sponges and filter papers were placed, above the black plate, followed by the gel and then the membrane and again the filter paper followed by sponge and the red plate. The membrane and the gel were placed in the transfer unit such that the gel faces the cathode. The 1X transfer buffer was added and allowed for the transfer to occur for 2 - 4 hours at 100 Volts at 4°C. After the transfer, carefully the membrane was removed and rinsed once with distilled water and then added the Blocking buffer and left it overnight at 4°C. The gel was stained to ensure the transfer.

On the next day, once the membrane was at room temperature, primary antibody PPARγ was added to the blocking buffer in a dilution of 1:1000 (10microlitre of Ab in 10ml of blocking buffer) and incubated for 2 hours at room temperature. The blocking buffer was removed with Ab after the incubation and stored up to 1 week. This was later washed four times with fresh TBST, with each wash lasting for ten minutes and finally with distilled water for 5 minutes. The secondary antibody (anti-rabbit) was added to the fresh blocking buffer diluted 1:4000 times (2.5microlitres of Ab in 10ml of TBST) and the membrane was incubated in it for 1 hour at room temperature. This was washed with fresh TBST, with each wash lasting for 10 minutes and rinsed with distilled water. ECL – Enhanced
chemiluminescence (1:1 diluted) 2ml was added to the membrane and kept in dark for 5 minutes. The membrane was placed in the gel doc and the bands were captured.

**Stripping of the membrane**

After the capture of bands, the membrane was taken out, washed with distilled water, stripped by adding stripping buffer and incubated at 50°C for 30 minutes in shaker incubator at 60rpm. After removing the stripping buffer, the membrane was washed with fresh TBST thrice for 10minutes. The membrane was blocked with blocking buffer over night at 4°C.

Next day, the primary antibody SREBP1 was added and the above steps were followed to develop the bands. 3 trials were done, and the bands were obtained for PPARγ and SREBP1 respectively.
**Statistical analysis:**
Western blot images were analysed using ImageJ software. Further analysis was performed using GraphPad Prism, Prism 5 for Windows, version 5.01. Each sample was analyzed in triplicates.

![Refrigerated centrifuge](image12.png)

**Figure 12: Refrigerated centrifuge**

![Running Unit for WB](image13.png)

**Figure 13: Running Unit for WB**
Figure 14: pH meter

Figure 15: PAGE unit
Figure 16: Block heater

Figure 17: Gel documentation unit
Figure 18: Working of PAGE

Figure 19: Transferring unit for western blot
e) Real Time PCR Analysis

Cells were grown in T25 flasks in similar manner as explained for western blot analysis. After incubation both the floating as well as adherent cells were collected by trypsinization in dry sterile nuclease free vials and centrifuged at 3500 rpm to collect the cell pellet. Pelleted cells were washed with PBS to remove traces of media which may interfere with the RNA extraction procedure. RNA extraction was done using spin column technology from QIAgen (QIAamp RNA extraction kit). After RNA extraction, quantification was done using nanodrop compatible spectrophotometer (Eppendorf). Further, RNA was subjected for electrophoretic separation in denatured gel called Bleach gel electrophoresis. Unused RNA sample was aliquoted and stored at -80°C for further use in mRNA expression study. All the consumables were made RNase free by using RNaseZap solution which digest RNase enzyme so that RNA quality and quantity is preserved. All the Buffers were prepared in 0.1% diethylpyrocarbonate (DEPC) treated water. Now total RNA was converted into complementary.

Selection of primers for the gene of interest:

Based on previous literature, the following primers for PPARγ1, PPARγ2 and SREBP1 was selected. Housekeeping gene i.e. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also included for each sample group for normalization. These gene primers were cross verified using NCBI-Primer-BLAST search (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The Product sizes of these Primers were found as mentioned below.

Table 11: List of primers used for real time PCR

<table>
<thead>
<tr>
<th>S.No</th>
<th>Primers</th>
<th>Length</th>
<th>Pur</th>
<th>T_m</th>
<th>GC%</th>
<th>Sequence 5’-3’</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAPDH-F</td>
<td>21</td>
<td>DST</td>
<td>64.3</td>
<td>52.3</td>
<td>AAGGTGAAGGTCGGAGTCAAC</td>
<td>227</td>
</tr>
<tr>
<td>2</td>
<td>GAPDH-R</td>
<td>20</td>
<td>DST</td>
<td>64.1</td>
<td>45</td>
<td>TGGAAAGATGGTAGGGATT</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PPARγ1-F</td>
<td>20</td>
<td>DST</td>
<td>54.7</td>
<td>45</td>
<td>GAGTGTGACGACAAAGATTG</td>
<td>121</td>
</tr>
<tr>
<td>4</td>
<td>PPARγ1-R</td>
<td>20</td>
<td>DST</td>
<td>62.5</td>
<td>60</td>
<td>GGTGGGCCAGAGATGGCATCT</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>PPARγ2-F</td>
<td>21</td>
<td>DST</td>
<td>57.8</td>
<td>47.6</td>
<td>TCTGGGAGATTCCTCTGTGA</td>
<td>112</td>
</tr>
<tr>
<td>6</td>
<td>PPARγ2-R</td>
<td>20</td>
<td>DST</td>
<td>62.5</td>
<td>60</td>
<td>GGTGGGCCAGAGATGGCATCT</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>SREBP1-F</td>
<td>24</td>
<td>DST</td>
<td>65.4</td>
<td>50</td>
<td>TTGCGCAAGGCCCATCGACTACATT</td>
<td>235</td>
</tr>
<tr>
<td>8</td>
<td>SREBP1-R</td>
<td>24</td>
<td>DST</td>
<td>66.9</td>
<td>58.3</td>
<td>ACAAGGGGCTGCTTGAAAGGTG</td>
<td></td>
</tr>
</tbody>
</table>

Real Time – quantitative-Polymerase Chain Reaction (RT-qPCR):

Quantitative RT-PCR was done to study the expression of PPARγ and SREBP1 during and after differentiation. qPCR was done in Rotor-Gene Q-5PLEX HRM – QIAGEN. Fluorescent dye 2x syber green (QuantiTect SYBR® Green RT-PCR Kit) was used as a PCR mix in 1x along with appropriate concentration of cDNA and primers. 50 ng of cDNA and 0.5µM concentration of each primer was used to the final reaction volume of 25µl.qPCR cycling condition was programmed as below.
Table 12: RT-qPCR cycling conditions

<table>
<thead>
<tr>
<th>S.No</th>
<th>PCR steps</th>
<th>Time</th>
<th>Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PCR initial activation</td>
<td>5 mint</td>
<td>95°C</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>10 secs</td>
<td>95°C</td>
</tr>
<tr>
<td>3</td>
<td>Annealing</td>
<td>15 secs</td>
<td>60°C</td>
</tr>
<tr>
<td>4</td>
<td>Extension</td>
<td>20 secs</td>
<td>60°C</td>
</tr>
<tr>
<td>5</td>
<td>Melting Profile :</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ramp :</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acquire melt A.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Number of cycles</td>
<td></td>
<td>35 cycles</td>
</tr>
</tbody>
</table>

The relative quantification of marker gene RNA expression was calculated using the comparative threshold cycle (Ct) method: the value of target gene was normalized with an endogenous control (GAPDH) from each of the sample groups. Change in expression was compared with that of vehicle control and final analysis was done based on % fold change considering baseline as 1 (100%) for vehicle control. Down-regulation i.e. less than 1 indicates suppression of genes on treatment and more than 1 indicates up-regulation of genes.

Fold change is expressed as $2^{\Delta\Delta Ct}$ where $\Delta Ct = \text{Ct of the target gene} - \text{Ct of the endogenous control gene}$, and $\Delta\Delta Ct = \text{Ct of the target gene samples (treated)} - \text{Ct of the target gene samples (untreated/vehicle control)}$.

Figure 20: Conventional PCR
f) Animal Studies
The study was conducted at the Department of Biochemistry, JSS Medical College, Mysore and had the approval from Institutional Animal Ethics Committee with registration number of 261/PO/ReBi/2000/CPCSEA renewed on 14-08-2015 from Government of India.

Materials used

Drugs:
1. Pioglitazone: pioglitazone tablets- Pioz 15 mg, was purchased, powerdered, weighed and prepared by dissolving it in gum acacia. The solution was prepared freshly every day. 0.20 mg dissolved in 1 ml of gum acacia was used for the study.
2. Fresh bitter melon juice (BMJ): bitter melon fruits were purchased from a shop washed in water, weighed and juice was prepared from the whole fruit freshly in a regular household juicer. Dose of BMJ was 6 ml/kg body weight.

Chemicals:
1. Streptozotocin (STZ):streptozotocin(SISCO Research Laboratories) dissolved in sodium citrate buffer is used in this study.
   a. It produces a colourless solution in buffer. It should be stored between 0-50°C.
   b. It should be freshly made in to solution for inducing in rats.
   c. 500mg of STZ is dissolved in 25ml of Sodium citrate buffer.
2. Sodium citrate buffer: used for preparation of STZ. 1.05 g citric acid and 1.48 g sodium citrate in distilled water gives 0.1 M citrate buffer at pH 4.567.
3. Gum acacia: This is used as a suspending agent for the oral administration of the standard compound, concentration being 2 %. 2gm of Gum acacia is dissolved in 100ml of water. Acacia is the dried gummy exudates obtained from stem and branches of Acacia arabica or other species of acacia. It is an inert substance used as an emulsifying agent for the preparation of the emulsion. It has no effect on blood glucose levels.
4. Phosphate buffer saline: is used for preserving specimen. Sodium chloride-8 gm, potassium chloride-0.2 gm, disodium hydrogen phosphate-1.44 gm, dihydrogen
potassium phosphate- 0.24 gm dissolved in 1000 ml of distilled water. pH adjusted to 7.0.

Equipments:
1. MOUTH GAG: - to facilitate the introduction of oral feeding tube into the stomach of the rat.
2. ORAL FEEDING TUBE: - 18 Gauge syringe with polythene tubing (small) is used for the oral administration of the standard and test compounds.
3. TUBERCULIN SYRINGE (1ml):- Used for injecting STZ into the peritoneal cavity.

Animals used were adult healthy albino rats of Wistar strain, weighing between 170-250 gms of either sex. The rats were inbred in the central animal house of the JSS Medical College, Mysore, under suitable conditions of housing, temperature, ventilation and nutrition. The animals were fed with commercial laboratory food and water ad libitum. They were maintained at a temperature of 24-27°C with relative humidity of 30-70 % with 12 hr light dark cycle. Following overnight fasting, 18 rats were intraperitoneally injected with freshly prepared streptozotocin (dissolved in sodium citrate buffer) under aseptic precaution in a dose of 55 mg/kg body weight [167] 3 days before the experiment. Blood glucose level was recorded daily morning at around 9.00 am for 3 days. All animals developed stable hyperglycaemia after 3 days. Only those animals with blood glucose level more than 250mg/dl were selected for the study [167].

Animals were randomly divided into four groups, six animals in each group.

Group 1: NORMAL CONTROL: consisted of normal rats which were given 0.5 ml gum acacia.

Group 2: DIABETIC CONTROL: The diabetic rats [STZ 55mg/kg BW, i,p] were treated with 0.5 ml gum acacia.

Group 3: STANDARD GROUP: The diabetic rats [STZ 55mg/kg BW, i,p] were treated with pioglitazone suspended in 0.5 ml of gum acacia, in a dose of 45 mg/kg body weight.

Group 4: TEST GROUP: The diabetic rats [STZ 55mg/kg BW, i,p] were treated with fresh BMJ in a dose of 6 ml/kg body weight.

Drug administration:
Normal control: consists of normal rats which were given Gum acacia 2%, 0.5ml for 30 days through oral route

Diabetic control: The diabetic rats [STZ 55mg/kg BW, i,p] were treated with Gum acacia 2%, 0.5ml orally daily for 30 days.

Standard group: The diabetic rats [STZ 55mg/kg BW, i,p] were treated with pioglitazone suspended in 0.5 ml of gum acacia, in a dose of 45 mg/kg body weight orally for 30 days.

Test groups: The diabetic rats [STZ 55mg/kg BW, i,p] were treated with fresh BMJ in a dose of 6 ml/kg body weight orally for 30 days.

The animals are given the drugs and suspensions with the help of feeding tube once daily for 30 days.

Bitter melon fruits were purchased from a local shop washed with water, weighed and juice was freshly prepared from the whole fruit in a regular household juicer. Dose of BMJ was 6 ml/kg body weight [119].
**Estimation of body weight:**
Body weights (BW) of the individual rats were measured on the respective days before blood sugar estimation.
Blood was collected from 18 hr fasted rats after the last dose administration by tail bleeding and blood glucose was estimated by Accu chek- active glucometer. Blood glucose was estimated on 0, 7, 14, 21 & 28th day.

**Capillary blood glucose:**
The Glucometer used in this study is ACCU CHEK ACTIVE from Roche Diagnostics for measuring the blood glucose level of the capillary blood. The glucometer was first standardized by comparing the blood glucose levels with biochemical methods for few samples and later used.

**Method of using glucometer:**
Before using the instrument, 2 rechargeable cells are inserted in the space provided at the back. Then code chip is introduced into the slot provided in the meter. Afterwards the check strip was introduced to check the glucometer. After matching the code number with that of the glucose strips provided, one glucose strip was taken.
Blood was collected by rat tail bleeding method [17]. The animal was placed in a suitable restrainer and the tip of the tail was cut by a scissor under aseptic precautions. Blood drop which was formed was used for blood glucose estimation, a full drop of blood was applied to the orange test pad so that the target area is completely and evenly covered with blood and no part of orange mesh is visible. Within 5 seconds, the blood sugar level is displayed in the glucometer. After blood collection pressure over the site with help of cotton and spirit was applied to secure homeostasis.

**Chemical principle of the procedure:**
Dextrostix is based on the action of the enzyme glucose oxidase, which is specific for glucose and which catalyses the oxidation of glucose in blood by oxygen in the atmosphere, producing gluconic acid and hydrogen peroxide. Peroxides then catalyze the reactions of hydrogen peroxide with a chromogen system producing oxidized chromogens, which have characteristic colours.

After 30 days the rats were sacrificed by cervical dislocation and blood was collected by cardiac puncture in plain vacutainer for estimation of total cholesterol and triglycerides. Adipose tissue was dissected, washed in ice-cold phosphate buffer saline, weighed and stored in RNA later for western blot and gene expression analysis of PPAR-γ and SREBP.

**Estimation of total cholesterol and triglycerides [168]:**
**Total Cholesterol:** CHOP-PAP method (Agappe diagnostics)

**Principle:**
The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase, the absorbance of which is read at 510 nm (505-530 nm). The intensity of the colour is directly proportional to the concentration of total cholesterol in the serum.

\[
\text{Cholesterol ester} + \text{H}_2\text{O} \xrightarrow{\text{Cholesterol Esterase}} \text{cholesterol} + \text{fatty acids}
\]

\[
\text{Cholesterol} + \text{O}_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{cholestene} - 3 \text{ one} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + \text{phenol} + 4\text{-aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + 4\text{H}_2\text{O}
\]
**Triglycerides: by GPO-PAP method (Agappe diagnostics)**

**Principle:**
The triglycerides are determined after enzymatic hydrolysis with lipases. The indicator is quinoneimine formed from hydrogen peroxide, 4 aminophenazone and 4 chlorophenol under the catalytic influence of peroxidize, the absorbance of which is read at 510 nm (505-530 nm). The intensity of the red colour is directly proportional to the concentration of triglyceride in the serum.

\[
\text{Triglycerides} + H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty acids}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol Kinase}} \text{Glycerol 3 phosphate} + \text{ADP}
\]

\[
\text{Glycerol 3 phosphate} + O_2 \xrightarrow{\text{Glycerol 3 phosphate}} \text{Dihydroxy acetone phosphate} + H_2O_2
\]

\[
2H_2O_2 + 4 \text{ aminophenazone} + 4 \text{ chlorophenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4 H_2O
\]

Body weights (BW) of the individual rats were measured on the respective days before blood sugar estimation. Blood was collected from overnight fasted rats after the last dose administration by tail bleeding and blood glucose was estimated by glucose oxidase method on 0, 7, 14, 21 and 28th day. After 28 days, the rats were sacrificed by cervical dislocation and blood was collected from abdominal vena cava in plain vacutainer for estimation of triglycerides by Glycerol 3 phosphate oxidase phenol aminophenazone method and cholesterol by Cholesterol oxidase phenol aminophenazone method. Adipose tissue was dissected out and washed in ice-cold phosphate buffer saline (PBS). The tissue was minced to small pieces and homogenized in 5-10 mL of PBS with a glass homogenizer on ice. RNA extraction and real-time PCR was performed as described earlier.

**Statistical analysis:**
Collected data was expressed as mean ± SD. A one way ANOVA model was used to compare means between groups. All the grouped data were analyzed using SPSS version 18 for windows. The differences between means were interpreted statistically significant at p <0.05.
Figure 22: Intraperitoneal injection of streptozotocin in albino rat

Figure 23: Oral feeding with oral feeding tube
Figure 24: Rat tail cutting method

Figure 25: Estimation of blood glucose using glucometer
Figure 26: Dissection of rat