Chapter 5

In-vitro Anti-adipogenic studies of the selected plant extracts *Boswellia ovalifoliolata, Commiphora caudata, Saccharum spontaneum* and *Garcinia mangostana.*

5.1 *In-vitro* cytotoxicity of the selected plant extracts in 3T3-L1 cells

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5.3 Results and Discussion
Chapter 5

*In-vitro* Anti-adipogenic studies of the selected plant extracts *Boswellia ovalifoliolata*, *Commiphora caudata*, *Saccharum spontaneum* and *Garcinia mangostana*.

5.1 *In-vitro* cytotoxicity of the selected plant extracts in 3T3-L1 cells

For testing the *in-vitro* cytotoxicity of the selected plant extracts, an MTT assay was performed to assess the cell viability with concentrations of test extracts of the selected plants ranging from 31.25 to 1000 μg/mL.

**Materials and Methods**

**Chemicals:**

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco’s Modified Eagle’s Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

**Cell lines and culture medium**

3T3L1 cell line was cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 μg/mL) and amphotericin B (5 μg/mL) in an humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).
Preparation of test solutions

For cytotoxicity studies, the test sample provided in liquid form (1.5 mg/mL) was made up with DMEM supplemented with 2 % inactivated FBS to obtain a stock solution of 750 mg/mL concentration and sterilized by filtration and centrifuged. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT Assay

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used [189].

Procedure:

1. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/mL using DMEM containing 10% FBS.
2. To each well of the 96 well microtitre plate, 0.1 mL of the diluted cell suspension (approximately 10,000 cells) was added.
3. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µL of different test conc. of test drugs were added on to the partial monolayer in microtitre plates.
4. The plates were then incubated at 37°C for 3 days in 5% CO₂, and microscopic examination was carried out and observations were noted every 24 h interval.

5. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO₂ atmosphere.

6. The supernatant was removed and 100 µL of propanol was added and the plates were gently shaken to solubilize the formed formazan.

7. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC₅₀) values is generated from the dose-response curves for each cell line.

\[
\text{% Growth Inhibition} = 100 - \left( \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \right) \times 100
\]
**Table 5.1: Cytotoxic properties of selected plant extracts on 3T3-L1 cells**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Extract</th>
<th>Test Conc.µg/mL)</th>
<th>% Cytotoxicity</th>
<th>CTC&lt;sub&gt;50&lt;/sub&gt; (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EBOG</td>
<td>1000 500 250 125 62.5 31.25</td>
<td>81.75±0.77 78.86±0.56 74.81±0.49 70.76±0.43 23.09±0.56 18.39±0.58</td>
<td>100±0.31</td>
</tr>
<tr>
<td>2</td>
<td>ECCB</td>
<td>1000 500 250 125 62.5 31.25</td>
<td>81.89±0.43 77.42±0.66 73.79±0.49 54.19±0.56 22.35±0.64 16.01±0.45</td>
<td>120±0.10</td>
</tr>
<tr>
<td>3</td>
<td>EBOL</td>
<td>1000 500 250 125 62.5 31.25</td>
<td>76.72±0.56 73.74±0.37 72.02±0.08 23.74±0.48 16.11±0.43 12.90±0.53</td>
<td>190±0.12</td>
</tr>
<tr>
<td>4</td>
<td>ECCL</td>
<td>1000 500 250 125 62.5 31.25</td>
<td>80.59±0.28 25.47±0.56 24.35±0.29 17.55±0.29 15.50±0.74 12.76±0.49</td>
<td>720±0.18</td>
</tr>
<tr>
<td>5</td>
<td>ESSW</td>
<td>1000 500 250 125 62.5 31.25</td>
<td>84.26±0.53 80.26±0.45 50.28±0.37 47.77±0.42 23.28±0.29 18.11±0.56</td>
<td>246.67±5.77</td>
</tr>
<tr>
<td>6</td>
<td>EGMP</td>
<td>1000 500 250 125 62.5 31.25</td>
<td>91.39±0.21 83.28±0.45 46.91±0.75 37.49±0.98 28.69±0.49 21.24±0.34</td>
<td>287.4±0.57</td>
</tr>
</tbody>
</table>

**EBOL:** Ethanolic extract of *B.ovalifoliolata* leaf  
**ECCL:** Ethanolic extract of *C.caudata* leaf.  
**ESSW:** Ethanolic extract of *S.spontaneum* whole plant  
**EBOG:** Ethanolic extract of *B.ovalifoliolata* gum  
**ECCB:** Ethanolic extract of *C.caudata* bark  
**EGMP:** Ethanolic extract of *G.mangostana* pericarp
Figure 5.1: Graphical representation of cytotoxic effect of selected plant extracts in 3T3-L1 cells

Figure 5.2: Cytotoxic effect of selected plant extracts in 3T3-L1 cells

EBOL: Ethanolic extract of B.ovalifoliolata leaf
ECCL: Ethanolic extract of C.caudata leaf,
ESSW: Ethanolic extract of S.spontaneum whole plant
EBOG: Ethanolic extract of B.ovalifoliolata gum
ECCB: Ethanolic extract of C.caudata bark
EGMP: Ethanolic extract of G.mangostana pericarp
5.2 *In-vitro* anti-adipogenic studies of selected plant extracts (*B. ovalifoliolata, C. caudata, S. spontaneum and G. mangostana*) in 3T3-L1 cells

The test concentrations for anti-adipogenic studies of the selected plant extracts were determined based on the results obtained in cytotoxicity studies. The anti-adipogenic studies were carried out on 3T3-L1 cells and the effect of plant extracts on inhibition of fat droplet formation was determined by quantification of Oil Red O staining method.

**Materials and methods**

**Chemicals**

Fetal Bovine serum (FBS), phosphate buffered Saline (PBS), Dulbecco’s modified Eagle’s medium (DMEM) and trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, glucose, insulin, dexamethasone, oil red stain and antibiotics from Hi-Media Laboratories Ltd., Mumbai. dimethyl sulfoxide (DMSO) and propanol from E.Merck Ltd., Mumbai, India.

**Equipments**

The following equipments were used for the Antiadipogenic studies

1. Automated Microplate reader - Biotek, USA
2. CO₂ Incubator - Nuaire, USA
3. Inverted Tissue culture Microscope- Lyzer, India

**Cell lines and culture medium**

3T3-L1 cell line was cultured in DMEM supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL) and amphotericin B (5 µg/mL) in an humidified atmosphere of 5% CO₂ at 37°C until
confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm$^2$ culture flasks and all experiments were carried out in 30 mm gelatin coated petri plates (Tarsons India Pvt. Ltd., Kolkata, India).

**Preparation of test solutions**

For adipogenesis studies, the test sample extracts was weighed and made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1000 mcg/mL concentration and sterilized by filtration and centrifuged.

**Culture and differentiation**

**Day 0:** 3T3-L1 preadipocytes (NCCS, India) were cultured in 4.5 g/L glucose-DMEM with 10% calf serum, antibiotic solution in 30 mm plastic Petri dishes until they reached 100% confluence.

**Day 1:** For differentiation, 2-day post-confluent cells were incubated for 48 h in DMEM with 10% FBS, antibiotics, and a differentiation cocktail termed MDI, which contained 0.5mM isobutylmethylxanthine, 1µM dexamethasone, and 100 nm insulin along with extracts.

**Day 3:** After 48 hours, cells were maintained with DMEM with 10% FBS, Insulin, antibiotics, and the test samples for 6 days.

**Day 9:** After 6 days, the cells were maintained with DMEM with 10% FBS along with test samples for 48 hours.

**Oil Red O Staining**

3T3-L1 adipocytes were washed with phosphate-buffered saline (PBS) and fixed with 10% formalin for 30 min. After washing twice with distilled water, cells were
stained for at least 1 h at room temperature in freshly diluted Oil Red O containing 0.5% Oil Red O in isopropanol. Finally, the dye retained in the 3T3-L1 cells was eluted with isopropanol and quantified by measuring the optical absorbance at 500 nm [90-91].

Table 5.2: Anti-adipogenesis activity of selected plant extracts in 3T3L1 cells

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the Extract</th>
<th>Test Concn. (µg/mL)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EBOG</td>
<td>100</td>
<td>46.1±0.026</td>
</tr>
<tr>
<td>2</td>
<td>ECCB</td>
<td>100</td>
<td>4.8±0.009</td>
</tr>
<tr>
<td>3</td>
<td>EBOL</td>
<td>150</td>
<td>8.3±0.014</td>
</tr>
<tr>
<td>4</td>
<td>ECCL</td>
<td>700</td>
<td>51.5±0.019</td>
</tr>
<tr>
<td>5</td>
<td>ESSW</td>
<td>250</td>
<td>26.0±0.079</td>
</tr>
<tr>
<td>6</td>
<td>EGMP</td>
<td>250</td>
<td>56.3±0.014</td>
</tr>
</tbody>
</table>

EBOL: Ethanolic extract of *B.ovalifoliat* leaf  
ECCL: Ethanolic extract of *C.caudata* leaf  
ESSW: Ethanolic extract of *S.spontaneum* whole plant  
EBOG: Ethanolic extract of *B.ovalifoliat* gum  
ECCB: Ethanolic extract of *C.caudata* bark  
EGMP: Ethanolic extract of *G.mangostana* pericarp

Figure 5.3: Anti adipogenesis activity of selected plant extracts in 3T3-L1 cells
5.3 Results and Discussion

To examine the effect of plant extracts on cell viability of 3T3-L1 preadipocytes, an MTT assay was performed, which assess cell viability by measuring mitochondrial activity in 3T3-L1 cells, treated with different concentrations of test extracts of the selected plants *B. ovalifoliolata, C. caudata, S. spontaneum* and *G. mangostana*. The alcoholic extracts of the selected plants were tested at concentrations of 31.25 to 1000 µg/mL. The extracts showed cytotoxicity with CTC$_{50}$ ranging from 100±0.31 to 720±0.18 µg/mL against 3T3L1 cell line. The CTC$_{50}$ values of the selected plant extracts were found to be in the order EBOG (100) > ECCB (120) > EBOL (190) > ESSW (246) >
EGMP (287) > ECCL (720). The control sample methanol showed poor toxicity at tested concentrations. Of all the extracts tested, ECCL was found to be less toxic and EBOG was more toxic than the other extracts tested.

One of the screening methods used in the discovery of anti-hyperlipidemic drugs is by measuring Oil Red O staining as indicators of lipid accumulation. The 3T3-L1 adipocytes were cultured and differentiated in a Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum for 6 to 8 days in the absence and presence of plant extracts. The effects of plant extracts on fat droplet formation in 3T3-L1 cells, and inhibition through the quantification of Oil Red O staining were presented in Fig. 5.4. Adipogenesis was substantially inhibited by the test extracts and among the six extracts screened for anti adipogenic activity, test extracts ECCL, EBOG, ESSW and EGMP significantly reduced the lipid accumulation with 51.5, 46.1, 26 and 56.3 (%) over control at 100, 700, 250 and 250µg/mL respectively through the quantification method of Oil Red O staining. Other two extracts namely ECCB (4.8) and EBOL (8.3)) did not show significant inhibition of fat accumulation at the tested concentration. These observations demonstrate that among the six extracts tested for anti-adipogenesis activity in-vitro, four extracts could efficiently suppress adipogenesis in 3T3-L1 preadipocytes and these extracts may have anti-hyperlipidemic anti-atherogenic effects. Hence the study was further continued to screen the selected plant extracts for anti-hyperlipidemic and anti-atherosclerotic activities in-vivo in atherogenic diet induced hyperlipidemic rats.