4. Materials and Methods

4.1. MATERIALS

4.1.1. Plants chosen

<table>
<thead>
<tr>
<th>Plant</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aloe</td>
<td>Bharathidasan University Campus, Tiruchirappalli.</td>
</tr>
<tr>
<td>Chickpea</td>
<td>National Pulses Research Centre, Pudukottai.</td>
</tr>
<tr>
<td>Soybean</td>
<td>National Pulses Research Centre, Pudukottai.</td>
</tr>
</tbody>
</table>

4.1.1.1. Source

- Aloe (Leaf gel) - Bharathidasan University Campus, Tiruchirappalli.
- Chickpea (Seed) - National Pulses Research Centre, Pudukottai.
- Soybean (Seed) - National Pulses Research Centre, Pudukottai.

4.1.1.2. Process plant material

- Aloe gel and seeds of chickpea & soybean
  - Surface sterilization – Sodium hypochlorite
  - Gel collection – Manually with knife
  - Soaking – Water
  - Homogenising – Buffer

4.1.1.3. Prepare plant extract

- Mortar and pestle, buffers, juice extractor (Philips HL1631)

4.1.1.4. Purify plant extract

- Ammonium sulphate, Phosphate buffered saline (PBS), Dialysis membrane.

4.1.1.5. Quantify protein

Analytical reagents

Prepared by mixing 2 ml of (b) with 100 ml of (a)

- (a) 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solution (0.4 gm in 100 ml distilled water.)
- (b) 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37% sodium potassium tartarate solution.

- Folin - Ciocalteau reagent

4.1.1.6. Effect of plant extract on cell growth (*in-vitro*)

- Cell chosen: Human Peripheral Blood Mononuclear Cells (hPBMC)
- Source: Human volunteers
- Cell lines: Jurkat and Raji (T & B cancer cell lines)
An attempt to use Soybean protein as an alternate for Fetal Bovine Serum in animal cell culture

Source: National Centre for Cell Sciences (NCCS), Pune, India
Media: RPMI 1640
Serum: Fetal Bovine Serum
Chemicals: Histopaque, Tryphan blue, Phytohemagglutinin (PHA), Lipopolysaccharide (LPS), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Sulforhodamine B (SRB)

4.1.2. Select plant based alternate for Fetal Bovine Serum (FBS)

Results of protein concentration
Effect on cell culture – In-vitro studies

4.1.3. Prepare Soy Protein Concentrate (SPC)

Processing of seed: Cleaning, cracking and dehulling
Defatting: Soxhlet apparatus
Reagent: Hexane & Ethanol

4.1.4. Prepare Soy Protein Isolate (SPI)

Stationary phase: Sephadex G75 (Sigma – G75120)
Mobile phase: Tris–HCl buffer (0.05M, pH 7.4)
Column: Glass column with packing material

4.1.5. Separate of Soy Protein Fraction (SPF)

Buffer: Tris–HCl (pH 8) and Tris–HCl (pH 6.5)
Cut off membrane: Centrifugal filter device (Ultracel YM-100–42424)
Separation: Centrifugation (Sigma)

4.1.6. Characterize SPI

Tank buffer (pH 8.3), Gel loading buffer (pH 6.8), lower Tris (1.5M, pH 8.8), upper Tris (0.5M, pH 6.8) and chemicals needed for SDS PAGE.

4.1.7. Analysis Soy Protein Concentrate (Quality)

4.1.7.2. Protein – Lowery’s reagent, Standard – Bovine serum albumin.
4.1.7.3. Total free amino acids– Ninhydrine, Standard – Leucine.
4.1.8. Media formulation with SPI / SPF for suspension cell culture

Media: RPMI 1640 (HiMedia AT162)
Serum: Fetal Bovine Serum (HiMedia RM9952)
Supplement: Soy Protein Isolate / Soy Protein Fraction

4.1.9. Effect of the formulated media on Normal cell & Established cell lines

As in 4.1.1.6

Acid phosphatase assay Kit (M/s Sigma-Aldrich, CS0740)
Neutral red assay

4.1.10. Isolate & Identify Mesenchymal Stem Cells (MSCs) from Adipose Tissue (AT)

4.1.10.1. Sample

Adipose Tissue – From abdomen / hernia.

4.1.10.2. Source

Tissue sample – Kilpauk Medical College and Hospital, Chennai
Tamil Nadu, India.

4.1.10.3. Collection

Collection – by surgeons.

Transport Media – Phosphate buffered saline + 10000U Penicillin,
10mg Streptomycin, 25µg Amphotericin B per ml

4.1.10.4. Isolation

Cell dissociation: Collagenase I (1mg/ml, Boehler Mannheim)
Buffer: Phosphate Buffer Saline (pH 7.4)
Antibiotic solution: Penicillin, Streptomycin and Amphotericin B

4.1.10.5. Identification

In tissue

Paraffin wax, Xylene, Bouin’s fluid, Haematoxylin, Eosin, Alcohol and Microtome.

Antibodies for Immunohistochemistry and Immunofluorescence

Primary antibody
Purified anti-mouse/rat CD29; Cat.No.102201 (Biolegend, San Diego) and Purified anti-mouse/human CD44; Cat.No.103001 (Biolegend, San Diego)

Secondary antibody
Rabbit anti-mouse IgG FITC; Cat.No. 51056 FIC5 (Bangalore Genei)
HRP conjugate – Horseradish peroxidase (Bangalore Genei)
Molecular diagnosis

RNA isolation Kit : 5 Prime, USA

Primer for Molecular identification of MSCs

CD90
Forward primer : 5’-CTGGCCATCAGCATCGCT-3’
Reverse primer : 5’-TATTCTCATGGGCGGCAGTCC-3’

Alpha Smooth Muscle Actin
Forward primer : 5’-GATCTGGCTGGCCGAGATC-3’
Reverse primer : 5’-ATGTCCCGGACAATTCACG-3’

4.1.10.6. Culture & Maintenance

Media : DMEM (HiMedia AT007)
Serum : Fetal Bovine Serum (HiMedia RM9952)

4.1.11. Media formulation with SPI for adherent cell culture

Media : DMEM (HiMedia AT162)
Serum : Fetal Bovine Serum (HiMedia RM9952)
Supplement : Soy Protein Isolate.

4.1.12. Effect of the formulated media on Adherent cells

Cells chosen : Vero, HeLa and Adipose tissue derived Mesenchymal stem cells
Chemicals : TPVG Solution, Tryphan blue, Phytohemagglutinin (PHA), Lipopolysaccharide (LPS), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Sulforhodamine B (SRB)
Serum : Fetal Bovine serum
Supplement : Soy Hydrolysate and Serum Supplement x3
4.2. METHODS

4.2.1. Plants Chosen

Based on literature for their protein content and immunomodulation property three plants were chosen for this study. They are

Aloe – *Aloe vera*
Chickpea – *Cicer arietinum* (Kabuli)
Soybean – *Glycine max* (ADT 1)

4.2.1.1. Source

Aloe was collected from Bharathidasan University Campus, Tiruchirappalli, Tamil Nadu, India. The leaf gel was alone taken for the study.

Chickpea and soybean were obtained from National Pulses Research Centre (Unit of TNAU), Vamban, Pudukottai, Tamil Nadu, India.

4.2.1.2. Process plant material – (Gegengeimer, 1990)

**Aloe**

Undamaged and disease free leaves of Aloe were cut 2 cms away from the rosette and were washed in tap water, rinsed in 0.1% sodium hypochlorite solution for 15 sec. and again it was rinsed thoroughly with clean deionized water. Then the leaves were patted thoroughly to dry and the fresh leaves were used for gel extraction.

**Chickpea and Soybean**

Infection free dry seeds & beans were surface sterilized by soaking in 0.1% sodium hypochlorite solution for 30 sec. and rinsed thoroughly with deionized (distilled quality) water. Seeds were then soaked in sterile homogenization buffer for overnight at room temperature.

4.2.1.3. Prepare plant product extract

4.2.1.3.1. Aloe – (Ramachandra and Rao, 2008)

The *Aloe vera* leaf gel alone was collected by traditional hand-filleting method. The lower 1 inch of the leaf base (the white part attached to the large rosette stem of the plant), the tapering point (2-4 inch) of the leaf top and the short, sharp spines located along the leaf margins were removed by a sharp knife, then the knife was introduced into the mucilage layer below the green
rind avoiding the vascular bundles and the top rind was removed. The bottom rind was similarly removed and the rind parts, to which a significant amount of mucilage remains attached, were discarded. The gel was removed from the leaf without disrupting the latex portion (area between the rid and inner gel). The gel was then ground to a liquid and the pulp was discarded.

4.2.1.3.2. Chickpea & Soybean – (Bednar and Hadcock, 1988)

The seeds and beans were soaked at a ratio of 1 liter buffer / kg initial dry weight of the seeds / beans overnight. The soaked seeds and bean were made as homogenate in a mortar & pestle. The debris in the homogenate was removed in a commercial juice extractor (Philips HL1631 Juice Extractor). It was subjected for centrifugation and the supernatant was taken for further studies.

4.2.1.4. Purify plant protein
4.2.1.4.1. Precipitation of protein – (Englard and Seifter, 1990)

The proteins from the filtered extracts were precipitated in steps by successive addition of solid ammonium sulphate and the precipitate was removed at each step by centrifugation. Protein fractions containing 20%, 40%, 60% and 80% of ammonium sulphate were precipitated.

4.2.1.4.2. Desalting of proteins by Dialysis – (Pohl, 1990)

The precipitates were dissolved in known volume of distilled water and were kept for dialysis in dialysis membrane immersed in large volume of PBS (pH 7.4). The bags were kept at 4°C with constant stirring. The buffer was changed several times. After which the dialysis membranes were removed from the buffers and the sample was collected for further use.

4.2.1.5. Quantitative analysis of protein

Protein content of the plant material was estimated by Lowry et al., (1951). The phenolic group of tyrosine and tryptophan residues (amino acid) in a protein will produce a blue colour complex, with maximum absorption in the region of 660nm wave length, with Folin-ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of colour
depends on the amount of these aromatic amino acids present and will thus vary for different proteins. Most protein estimation techniques use Bovine Serum Albumin (BSA) universally as a standard protein, because of its low cost, high purity and ready availability. The method is sensitive down to about 10μg/ml and is probably the most widely used protein assay despite its being only a relative method, subject to interference from Tris buffer, EDTA, non-ionic and cationic detergents, carbohydrate, lipids and some salts. The incubation time is very critical for a reproducible assay. The reaction is also dependent on pH and a working range of pH 9 to 10.5 is essential.

A series of standard Bovine Serum Albumin ranging from 20μg-100μg was taken in a series of test tubes. The volume was made up to 1ml with distilled water. A blank of 1ml distilled water was taken in a different test tube. The required amount of sample was taken and made up to 1ml (0.02ml, 0.05ml, 0.07ml, 0.1ml). 2.5ml of analytical reagent was added to all the tubes. The test tubes were incubated for 10 minutes at room temperature. Then 0.2 ml of Folin Ciocalteau reagent was added to each tube and was incubated for 30 minutes. The colour developed in the solution was read at 660nm using spectrophotometer.

4.2.1.6. Effect of plant extract on cell growth (in-vitro) on

4.2.1.6.1. Human Peripheral Blood Mononuclear Cells (hPBMC)

4.2.1.6.1.1. Separation of hPBMC – (Böyum, 1968)

Principle

Human PBMC was separated from heparinised human blood based on density gradient centrifugation by Böyum, (1968)

Procedure

Human peripheral blood mononuclear cells were isolated by the technique described by Böyum, (1968). 10ml of venous blood was drawn from a donor and was defibrinated in a sterile 100ml conical flask containing 8 to 10 sterile glass beads of 2-3mm diameter by gently swirling the flasks for 10 minutes. Blood was then diluted with equal volume of RPMI 1640 media and carefully layered over histopaque (Sigma chemicals, U.S.A) in the ratio of two parts of diluted blood: one part of histopaque. After centrifugation at 200g for
20 minutes, at 25°C, the whole mononuclear cell layer seen at the interface was carefully transferred to a tube containing 5ml of RPMI 1640 medium. The cells were thoroughly mixed with the medium and washed by centrifugation at 300g for 10 minutes. The procedure of washing was repeated twice. The final suspension was made in 1 to 5ml RPMI 1640 medium with 10% fetal bovine serum (FBS). One drop of cell suspension (approximately 20µl) and one drop of 0.4% Tryphan blue solution (Sigma chemicals, U.S.A) were mixed and fed into a haemocytometer. Live and dead cells were counted under phase contrast objective. The cell concentration was adjusted to the desired number of viable mononuclear cells/ml in RPMI 1640 medium. Care was taken to obtain a cell suspension with 95% to 98% mononuclear cells with less than 5% to 7% contamination of erythrocytes, granulocytes, platelets and dead cells.

\[
\text{Viable cell count} = \frac{\text{No. of cells counted}}{\text{Area counted}} \times \text{depth factor} \times \text{dilution factor} \times 10^3
\]

4.2.1.6.1.2. Tryphan Blue Dye Exclusion Method – (Darlington, 2007)

A known concentration of the plant extracts were taken and reconstituted with known volume of PBS pH 7.2, which was the stock concentration of the extract. This was centrifuged and the supernatant was membrane filtered and used. From the stock various concentrations of the extracts were prepared and subjected for *in-vitro* analysis on hPBMC. Constant cell number was maintained. The assay was performed in 96 well tissue culture plates, using various negative controls like plain media, complete media, vehicle, cell and extracts. Human PBMC alone were also kept as control. A known positive control - phytohemagglutinin (PHA), a known immunopotentiator and Lipopolysaccharide (LPS) a toxin was also kept. After the addition of cell, media and extract, the cultures were incubated in an incubator (TC2323, Shell lab, U.S.A) with 95% air, 5% CO₂ and humidified atmosphere at 37°C for 24 hours.

After the incubation period a suitable volume of a cell suspension (20-200µl) was taken in an appropriate tube and an equal volume of 0.4% Tryphan blue was added and gently mixed. It was allowed to stand for 5 minutes at
room temperature. 10µl of stained cells were placed in a haemocytometer and the number of viable (unstained) and dead (stained) cells were counted. The average number of unstained cells in each quadrant was calculated and multiplied by $2 \times 10^4$ to find cells / ml. The percentage of viable cells is the number of viable cells divided by the number of dead and viable cells. Morphology of the cells was also assessed by microscopic method to determine the cytotoxic and cytopathic nature of the cells grown with the plant extracts.

**Calculation**

\[
\text{Percentage of viable cells} = \frac{\text{No. of cells counted} - \text{No. of dead cells}}{\text{No. of cells counted}} \times 100
\]

4.2.1.6.1.3. **Sulforhodamine B assay (SRB) – (Vichai and Kirtikara, 2006)**

- Molecular formula: $C_{27}H_{30}N_2O_7S_2$
- Molecular weight: 558.66 g/mole
- Colour: Red
- Solubility: water soluble

**Principle**

Sulforhodamine B is a fluorescent dye which uses spanning from Laser Induced Fluorescence (LIF) to the quantification of cellular proteins of cultured cells. The red, solid, water soluble dye is primarily used as a polar tracer.

The dye absorbs 515nm light and emits 586nm light. It does not exhibit pH dependent absorption or fluorescence over the range of 3 to 10.

**Method**

A known concentration of the plant extract was taken and reconstituted with known volume of PBS pH 7.2, which was the stock concentration of the extract. This was centrifuged and the supernatant was membrane filtered and used. From the stock various concentrations of the extracts were prepared and subjected for the *in-vitro* analysis on human PBMC. Constant cell number was maintained. The assay was performed in a 96 well tissue culture plate (Greiner, U.S.A), using various negative controls like plain media, complete media, vehicle, cell and extract. A known positive control like a known
immunomodulator-phytohemagglutinin (PHA) and a known cytotoxic compound - Lipopolysaccharide (LPS) were also maintained. After the addition of cell, media and extract, the cultures were incubated in an incubator (TC2323, Shel lab, U.S.A) with 95% air, 5% CO\textsubscript{2} and humidified atmosphere at 37ºC for 72 hours.

After incubation TCA was added to fix the cells with the substratum. The fixed cells were stained with SRB (stains only cellular proteins). The SRB stained proteins were solubilized using trizma base. The intensity of the colour developed was read using ELISA reader (iMark\textsuperscript{TM}, BioRad) at 515nm. Intensity of the colour developed is proportional to the number of viable cells.

**Calculation**

\[
\text{% of control cell growth} = \frac{\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{day 0}}}{\text{mean OD}_{\text{neg. control}} - \text{mean OD}_{\text{day 0}}} \times 100
\]

4.2.1.6.1.4. **MTT assay** – (Mosmann, 1983)

- **Molecular formula**: C\textsubscript{18}H\textsubscript{16}Br\textsubscript{5}S
- **Molecular weight**: 414.32
- **Colour**: Yellow
- **Solubility**: water soluble

**Principle**

Yellow MTT [3-(4, 5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide], a tetrazole is reduced to purple formazan in the mitochondria of living cells. A solubilisation solution (usually dimethyl sulfoxide or a solution of the detergent sodium dodecyl sulphate in dil. hydrochloric acid) is added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this coloured solution can be quantified by measuring at certain wavelength (between 500 and 600nm) by a spectrophotometer.

This reduction takes place only when mitochondrial dehydrogenase enzyme is active and therefore conversion is directly related to the number of viable cells. When the amount of formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agents in causing death of cells can be deduced, through the production of a dose-response curve. For each cell type a linear
relationship between cell number and absorbance is established, enabling accurate, straightforward quantification of changes in proliferation.

**Procedure**

A known concentration of the plant extracts were taken and reconstituted with known volume of PBS pH 7.2, which was the stock concentration of the extract. This was centrifuged and the supernatant was membrane filtered and used. From the stock various concentrations of the extracts were prepared and subjected for the *in-vitro* analysis on human PBMC. Constant cell number was maintained. The assay was performed in a 96 well tissue culture plate (Greiner, U.S.A) using various negative controls like plain media, complete media, vehicle, cell and extract. A known positive control like a known immunomodulator-phytohemagglutinin (PHA) and a known cytotoxic compound - Lipopolysaccharide (LPS) were also maintained. After the addition of cell, media and extract, the cultures were incubated in an incubator (TC2323, Shel lab, U.S.A) with 95% air, 5% CO₂ and humidified atmosphere at 37°C for 72 hours.

The assay was monitored after 72 hours based on spectrophotometer method by using an ELISA reader. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma catalogue no. M2128) was dissolved in PBS at 5 mg/ml and filtered to sterilize and remove a small amount of insoluble residue present in it. MTT solution (20µl per 200µl medium) was added to all wells of an assay, and plates were incubated at 37°C for 4 hours. After incubation period, the MTT was reduced by mitochondrial dehydrogenase as a result of which the colour was changed. Acid-isopropanol (100µl of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a iMark™ Microplate absorbance reader (BioRad), using a test wavelength of 570 nm, a reference wavelength of 630 nm. The data was analysed by plotting cell number versus absorbance in a graph. The rate of tetrazolium reduction is directly proportional to the rate of cell proliferation.
4.2.1.6.2. On Established cell lines

4.2.1.6.2.1. Cell lines chosen – Purpose based

Jurkat (T Cell Cancer) & Raji (B Cell Cancer) were the cancer cell lines taken to check the effect of plant extracts over established cell lines.

4.2.1.6.2.2. Source

The chosen cell lines were obtained from National Centre for Cell Sciences (NCCS) Pune, India and were maintained in RPMI 1640 with 10% FBS, 95% air, 5% CO₂, 99% humidity & 37°C temperature.

4.2.1.6.2.3. Tryphan Blue Dye Exclusion Method – (Darlington, 2007)

Effect of the plant extracts on established cell lines was done based on the method given in 4.2.1.6.1.2 using Jurkat and Raji cell lines.

4.2.1.6.2.4. Sulforhodamine B assay (SRB) – (Vichai and Kirtikara, 2006)

Effect of the plant extracts on established cell lines was done based on the method given in 4.2.1.6.1.3 using Jurkat and Raji cell lines.

4.2.1.6.2.5. MTT assay – (Mosmann, 1983)

Effect of the plant extracts on established cell lines was done based on the method given in 4.2.1.6.1.4 using Jurkat and Raji cell lines.

4.2.2. Characterization and comparison of soy protein with fetal bovine serum

4.2.2.1. SDS PAGE – (Sambrook and Russell, 2001)

The soy protein extracted and FBS (HiMedia RM9952) were analysed by SDS Polyacrylamide Gel Electrophoresis and Coomassie blue staining. 10% SDS PAGE was used for this purpose. The bands separated were stained with Coomassie blue and were identified to look for the soy storage proteins and the subgroups of serum proteins. They were compared and analysed.

4.2.3. Select plant based alternate for FBS

Based on the results obtained for protein quality and the effect of the extract on hPBMС soybean was chosen for further study.
4.2.4. Prepare soy protein concentrate

4.2.4.1. Defat soy flour – (Becker, 1978)

Soybean seeds of good calibre were taken, cleaned, cracked and dehulled. The cracked cotyledon and hypocotyledon were collected. Defatted soybean samples were made with 200ml of hexane at 70°C for 6 to 8 hours in a soxhlet extractor using a heating mantle. The condensation rate for the solvent was set at about 2 to 6 drops per second, depending upon the extraction period. An extraction period of 8 hours at a rate of 150 drops per min. was maintained finally. Later the sample in the thimble was cooled, weighed and used as fat free sample. This fat free soybean was ground into fine flour and the grits were removed though mechanical sieve.

4.2.4.2. Soy protein concentrate – (Alibhai et al., 2006)

The defatted soy flour was treated with aqueous alcohol (70% ethanol) to remove non protein constituents. After treatment the flour was dried as powder at room temperature. This dried powder with proteins and polysaccharides constitutes the soy protein concentrate and it was coded as SPC.

4.2.5. Prepare soy protein isolate (SPI)

SPC was suspended in Tris-HCl buffer (0.05M, pH 7.4). The suspension was passed through 0.4µM membrane filter to remove the insoluble materials.

4.2.5.1. Gel column chromatography – (Osterman, 1985)

The filtrate obtained was subjected to column chromatography using Tris-HCl buffer (0.05M, pH 7.4) as mobile phase and Sephadex G-75 (50 x 2) as stationary phase. The flow rate was 30ml/hour. Fractions of 4ml were collected. The quantity of protein in the fraction was detected by UV method. Fractions showing peaks at 280nm were pooled and taken for further studies and were dialysed against distilled water. The dialysed protein fractions were lyophilised. This lyophilised protein was named as soy protein isolate and it was coded as SPI.
4.2.6. Separate soy protein fraction (SPF) – (Bittencourt et al., 2007)

SPI was found to contain fractions of non-homogeneous mixtures of proteins. Based on the Svedberg coefficient it is mainly of two types namely 7S fraction and 11S fraction. 7S fraction contains 35% of soluble proteins and it is a mixture of some enzymes like hemagglutinins, lipoxygenase & globulins. The role of lipoxygenase is lipid oxidation and hemagglutinin is aggregation of cells in-vitro. 11S fractions contain 31% to 52% of soluble protein. It contains amino acids like leucine, isoleucine, phenylalanine and glycine. These proteins may play a vital role in cell proliferation. Hence 7S globulins and 11S globulins were isolated from SPI or SPC and were named as SPF7 and SPF11.

The 7S fraction was purified from the soy protein isolate, according to the procedure described by Bittencourt et al., (2007). The 7S fraction was extracted with 20 parts (m/v) of a solution containing 0.03M Tris-HCl buffer (pH 6.2), 0.001 M dithioerytritol, kept under stirring, at 9°C for 1 h; then, the preparation was centrifuged at 35,000g at 4°C for 20 min. The pellet was discarded and pH of the supernatant was adjusted to 4.8 with 1N HCl and centrifuged at 35,000g for 20 min, at 4°C. After the elimination of the supernatant, the pellet was diluted in 10 parts 0.03M Tris-HCl buffer (pH 6.2) and pH adjusted to 7.6 with 1N NaOH. This suspension was kept under agitation at 9°C until the pellet dissolved. The pH was adjusted again to 6.2 and the solution was centrifuged at 35,000g for 20 min, at 4°C and the supernatant was filtered on 100kDa membranes (Ultracel YM-100). The retained sample in the membrane was dissolved, concentrated under vacuum.

The 11S fraction was purified from the soy protein isolate, according to the procedure described by Bittencourt et al., (2007). It was initially extracted with 20 parts 0.03M Tris- HCl, pH 8.0, containing 0.01M 2-mercaptoethanol. This sample was allowed to rest for 1 hour at room temperature and then was centrifuged at 22,000g for 20 min at 20°C. The first pellet was discarded and the supernatant was considered as the crude extract. The pH was then adjusted to 6.4 with 2 N HCl and the sample was centrifuged at 22,000g for 20 min. at 4°C. The pH of the supernatant was adjusted to pH 4.8 and centrifuged again.
To eliminate the possible presence of the soy 2S fraction, whose MW is identical to the basic subunit of the 11S fraction, the supernatant was filtered on a 100kDa membrane (Ultracel YM-100) and the sample retained on the membrane was dissolved and concentrated under vacuum.

4.2.7. Characterize SPI

4.2.7.1. SDS PAGE – (Sambrook and Russell, 2001)

The soy protein extracted and fractionated by the above methods was analysed by SDS Polyacrylamide Gel Electrophoresis and Coomassie blue staining. The SPI and SPF were separated on 12% SDS PAGE and the bands separated were stained with Coomassie blue for identification of the soy storage proteins and its fractions.

4.2.8. Analyse SPI – Quality

4.2.8.1. Carbohydrate – (Hedge and Hofreiter, 1962)

Carbohydrates are the important components of storage and structural materials in the plants. They exist as free sugars and polysaccharides. The basic units of carbohydrates are the monosaccharide which cannot be split by hydrolysis into more simple sugars. The carbohydrate content can be measured by hydrolysing the polysaccharide into simple sugars by acid hydrolysis and by estimating the resultant monosaccharide subsequently.

Principle

Carbohydrates are first hydrolysed into simple sugars using dilute HCl. In hot acidic medium glucose is dehydrated to hydroxyl methyl furfural. This compound with anthrone forms a green coloured product with an absorption maximum at 630nm.

Materials

Anthrone reagent

200mg of anthrone was dissolved in 100 ml of ice cold 95% H$_2$SO$_4$. 
Standard glucose - Stock solution:

1mg of glucose was dissolved in 1ml of distilled water.

Working solution

10ml of the stock was taken and diluted with 90ml of distilled water.

Procedure

A series of 0.1ml standard glucose solution containing 10μg to 100μg was taken in different test tubes individually and 0.1ml of water was taken as blank in a separate tube. Known volume of test material containing glucose was taken in a test tube. The volume was made up to 1ml with distilled water. 4ml of anthrone reagent was added to all the tubes. After that the tubes were kept in an ice bucket to react. It was kept in a boiling water bath for 8 minutes and blank was prepared by mixing water and anthrone reagent. It was then cooled and the optical density was read at 630nm in spectrophotometer. A standard graph was drawn by plotting concentration of the standard on the x axis versus absorbance (O.D) on the y axis. From the standard graph the amount of carbohydrate present in the sample was calculated by extrapolating the test OD value in the standard graph. The amount of total carbohydrate present in the sample was calculated by the formula given below.

Calculation

\[
\text{Carbohydrate present in 100 mg of sample} = \frac{\text{mg of glucose}}{\text{Volume of test sample}}
\]

4.2.8.2. Protein – (Lowry et al., 1951)

Protein concentration was analysed as per the procedure given in 4.2.1.5.

4.2.8.3. Total free amino acid – (Sadasivam and Manickam, 1996)

Principle

Ninhydrin, a powerful oxidizing agent, decarboxylates the α-amino acids and yields an intensely coloured bluish purple product which is colorimetrically measured at 570nm.
Ninhydrin + alpha-amino acid $\rightarrow$ hydridantin + decarboxylated amino acid + CO$_2$ + NH$_4$

Hydridantin + Ninhydrin + ammonia $\rightarrow$ purple coloured product + H$_2$O

**Materials**

*Ninhydrine*: 0.8g of stannous chloride was dissolved in 500 ml of 0.2M citrate buffer (pH 5.0). This solution was added to 5gm of ninhydrin in 125ml of methoxyethanol.

*Solvent Diluent*: Equal volume of water and n-propanol was mixed well.

*Standard stock*: 10mg of leucine was dissolved in 10ml of distilled water.

*Working standard*: 10ml of the stock solution was made up to 100ml with distilled water.

**Sample extraction**

500mg of soy protein concentrate was taken and it was ground with small quantity of acid-washed sand. To this homogenate 5ml of 80% ethanol was added. It was centrifuged and the supernatant was saved. The extraction procedure was repeated twice and the supernatant was pooled. Then the volume was reduced and the extract was used for the quantitative estimation of total free amino acid.

**Procedure**

0.1ml of the extract was added to 1ml of ninhydrin solution and was made up to 2ml with distilled water. Then the tubes were boiled in water bath for 20 minutes. To this 5ml of diluents was added and mixed well. After 15 minutes the intensity of the purple colour developed was read against blank at 570nm. The colour was stable for 1 hour. Blank was prepared as above by taking 0.1ml of 80% ethanol instead of extract. Different concentration of stock solution of the standard amino acid was taken for standard graph. A standard graph was drawn for the standard amino acid and the unknown was calculated by extrapolating the OD value.
4.2.9.  Media formulation with SPI and SPF

Various batches of cell culture media were formulated and prepared with different concentrations of soy protein prepared i.e. SPI, SPF7 and SPF11 as given in Table 4.1. The newly formulated media were coded as SPIAM – Soy Protein Isolate Alternate media, SPISM – Soy Protein Isolate Supplement media, SPIRM – Soy Protein Isolate Replacement media, SPF7RM – Soy Protein Fraction 7S Replacement media, SPF11RM – Soy Protein Fraction 11S Replacement media. The concentration of SPI used in cell culture media was based on the equivalence of the protein concentration of FBS used in routine cell culture media.

4.2.9.1.  Major Media with SPI

Media formulated using SPI as supplement were grouped under major media.

4.2.9.1.1.  Alternate media (SPIAM)

A batch of media formulated with the basal medium and various concentration of SPI alone i.e. without FBS.

4.2.9.1.2.  Supplement media (SPISM)

A batch of media was formulated with basal medium, constant concentration of FBS supplemented with variable concentration of SPI.

4.2.9.1.3.  Replacement media (SPIRM)

A batch of media was formulated with basal medium, concentration of FBS was reduced step by step and that was compensated by step by step addition of SPI.

4.2.9.2.  Minor Media with SPF

Based on the results it was found that replacement media showed better effect in cell culture. In order to find which portion of the SPI fraction potentiates the cell growth, a media was formulated using fractions (SPF7 and SPF11) of SPI as additive and were grouped under minor media.
An attempt to use Soybean protein as an alternate for Fetal Bovine Serum in animal cell culture

Table 4.1. Composition of cell culture media formulated, prepared and used in suspension cell culture media

<table>
<thead>
<tr>
<th>Name</th>
<th>Type of Media</th>
<th>Basal Media</th>
<th>Supplement</th>
<th>FBS % (v/v)</th>
<th>SPI / SPF7 / SPF11 (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPIAM-1</td>
<td>RPMI + SPI</td>
<td>RPMI 1640</td>
<td>SPI</td>
<td>-</td>
<td>1</td>
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<tr>
<td>SPIAM-2</td>
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<td>SPIAM-4</td>
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<td></td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>SPIISM-1</td>
<td>RPMI + FBS + SPI</td>
<td>RPMI 1640</td>
<td>SPI</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>SPIISM-2</td>
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<td></td>
<td></td>
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<td>SPIIRM-1</td>
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<td>RPMI + FBS + SPF7</td>
<td>RPMI 1640</td>
<td>SPF7</td>
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<tr>
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</tr>
<tr>
<td>SPS11RM-1</td>
<td>RPMI + FBS + SPF11</td>
<td>RPMI 1640</td>
<td>SPF11</td>
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<td>1</td>
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<tr>
<td>SPS11RM-2</td>
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</tr>
</tbody>
</table>

SPIAM – Soy Protein Isolate Alternate Media
SPISM – Soy Protein Isolate Supplement Media
SPIRM – Soy Protein Isolate Replacement Media
SPF7RM – Soy Protein Fraction 7S Replacement Media
SPF11RM – Soy Protein Fraction 11S Replacement Media
4.2.9.2.1. Replacement media (SPF7RM)

A batch of media was formulated with basal medium, constant concentration of FBS supplemented with variable concentration of SPF7.

4.2.9.2.2. Replacement media (SPF11RM)

A batch of media was formulated with basal medium, constant concentration of FBS supplemented with variable concentration of SPF11.

4.2.10. Effect of Formulated media on hPBMC

4.2.10.1. Acid phosphatase assay (APA) (M/s Sigma-Aldrich, CS0740)

Effect of newly formulated media was analysed as per the procedure given in 4.2.1.6.1. Acid phosphatase (AP) is one of the acid hydrolases that normally reside in lysosomes. It is a classical marker for the identification of lysosomes in subcellular fractions. This assay provides a convenient method for detecting total acid phosphatase activity in a whole cell. This assay utilizes para-nitrophenyl phosphate (pNPP) as a chromogenic substrate for the enzyme. The intra-cellular AP in viable cell dephosphorylates pNPP and the phenolic OH-group is deprotonated under alkaline conditions resulting in p-nitrophenolate that yields an intense yellow colour which can be measured at 405 nm. This absorbance is directly proportional to the cell number ranging between $10^3$-$10^5$ cells.

Procedure

Sets of 96 well plates with cell density of $3 \times 10^3$ cells per well were prepared in formulated medias and incubated at appropriate conditions. For every 24 hours one set of plate was taken for AP estimation until the incubation time comes to 96 hours. For adherent cells the culture media was removed and cells were washed with PBS (pH 7.2). The cells were taken for AP analysis. Whereas for nonadherent cells, culture media along with the cells were
centrifuged for 10 mins at 800g and the pelleted cells were alone taken for AP analysis.

The content of the APA kit were brought to room temperature. The reaction components were added to the 96 well plates containing the cells as per the kit protocol and incubated for 10 min. at 37ºC. The reaction was stopped by the addition of 50µl of 0.5N NaOH. Absorbance was then read at 405 nm (iMark™, BioRad).

**Calculation**

Absorbance of p-nitrophenol at 405 nm is directly proportional to the cell number in the range of $10^3$ to $10^5$ cells (Yang et al., 1996).

4.2.10.1.6. **Neutral red assay - (Repetto et al., 2008)**

- **Molecular formula**: $C_{15}H_{17}ClN_4$
- **Molecular weight**: 288.78
- **Colour**: Red (pH 6.8) to Yellow (pH 8)
- **Solubility**: water soluble

This assay is based on the ability of the viable cells to incorporate and bind the supravital dye neutral red (Sigma, N4638). The weakly cationic dye penetrates cell membrane by non-ionic passive diffusion and concentrates in the lysosomes, where it binds by electrostatic hydrostatic bonds to anionic and / or phosphate group of the lysosomal matrix. The dye was then extracted from the viable cell by acidified ethanol solution and the absorbance of the solubilized dye was quantified at 540nm.

At physiological pH, the dye presents a net charge near to zero, enabling it to penetrate the cell membrane. Inside the lysosome, there is a proton gradient to maintain a pH lower than that of cytoplasm. Thus the dye becomes charged and is retained inside the lysosome.

**Procedure**

Sets of 96 well plates with cell density of $2 \times 10^6$ cells per well were prepared in formulated medias and incubated at appropriate conditions. For every 24 hours one set of plate was taken for neutral red uptake assay until the
incubation time comes to 96 hours. For adherent cells the culture media was removed and cells were washed with PBS (pH 7.2). The cells were taken for neutral red uptake. Whereas for nonadherent cells, culture media along with the cells were centrifuged for 10 mins at 600g and the pelleted cells were alone taken for analysis. To each well 100µl of neutral red medium (Neutral red medium was prepared one day before use by adding neutral red at a concentration of 40µg/ml in the formulated medias) was added and incubated for 2 hours at appropriate condition.

The neutral red medium was removed for adherent cells and the wells were washed with 150µl phosphate buffered saline (pH 7.2). For nonadherent cells, culture media along with the cells were centrifuged at 600g for 10 mins and the pelleted cells were washed with PBS. After which 150 µl of neutral red destaining solution (10ml water + 10ml ethanol + 0.2ml glacial acetic acid) was added per well and the plates were shaken for atleast 10 mins to extract the neutral red from the cells. Absorbance was then read at 540 nm (iMark™, BioRad).

4.2.10.1.7. BrdU (Bromodeoxyuridine) assay (M/S Chemicom, 2750)

This is an alternative method to [$^{3}$H]-thymidine uptake, i.e. BrdU, an analogue of thymidine is used in the place of [$^{3}$H]-thymidine and is incorporated into newly synthesised DNA strands of actively proliferating cells. Following partial denaturation of double stranded DNA, BrdU is detected immunologically allowing the assessment of the population of cells, which are actively synthesising DNA.

**Procedure**

Cells were plated at 3x10³ cells/well on a 96-well culture plate in the formulated medium. Cells were then incubated at 37°C, 5% CO₂, and 90% humidity for 48 hours. The cells were then assayed for their ability to incorporate BrdU according to the manufacturer's instructions.
Calculation

The change in colour from blue to deep yellow were read using microtiter plate reader (iMark™, BioRad) at 450nm with a reference wavelength 540nm.

4.2.10.1.8. Glucose analysis (M/S Enzychrome™, EBGL100)

Glucose is the major carbon source in the cultures.

Therefore, the monitoring and control of glucose in cultures has been one of the most essential elements of the measurement of cell proliferation. Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The formed H$_2$O$_2$ reacts under catalysis of peroxidase with phenol and 4-amino-antipyrine to form quinoneimine. The intensity of the colour is proportional to the glucose level present in the culture media.

Procedure

Cells were plated at 3x10$^3$ cells/well on a 96-well culture plate in the formulated medium. Cells were then incubated at 37°C, 5% CO$_2$, and 90% humidity for 96 hour. For every 24 hours 20µl of the culture supernatant was taken to determine the glucose concentration according to the manufacturer's instructions.

Calculation

The changes in colour were read using microtiter plate reader (iMark™, BioRad) at 570nm and concentration of glucose was determined from the standard curve.

\[
\text{Concentration of Glucose} = \frac{\text{OD sample} - \text{OD blank} \ (\mu M)}{\text{Slope}}
\]

4.2.10.1.9. Lactate analysis (M/S Enzychrome™, ECLC100)

Lactate is generated as an end product of glucose metabolism by lactate dehydrogenase enzyme. Measurement of lactate level in culture media
will therefore be an indicator for the balance between tissue oxygen demand and utilization. Lactate is measured based on lactate dehydrogenase catalysed oxidation of lactate, in which the formed NADH reduces MTT formazan reagent. The intensity of the product is directly proportional to the lactate concentration in the spent media.

**Procedure**

Cells were plated at $3 \times 10^3$ cells/well on a 96-well culture plate in the formulated medium. Cells were then incubated at 37°C, 5% CO2, and 90% humidity for 96 hour. For every 24 hours 20µl of the culture supernatant was taken to determine the lactate concentration according to the manufacturer's instructions.

**Calculation**

The changes in colour were read using microtiter plate reader (iMark™, BioRad) at 565nm and concentration of lactate was determined from the standard curve.

$$\text{Concentration of lactate} = \frac{\text{OD sample} - \text{OD blank} \ (\text{mM})}{\text{Slope}}$$

4.2.1.10. Glutamine analysis (M/S EnzyChrome™, EGLN100)

Glutamine supports the growth of cells that have high energy demand and synthesize large amount of proteins and nucleic acid. It is an alternate energy source for rapidly growing cells and cells that use glucose inefficiently. Glutamine analysis is based on the hydrolysis of glutamine to glutamate and colorimetric determination of the product.

**Procedure**

Cells were plated at $3 \times 10^3$ cells/well on a 96-well culture plate in the formulated medium. Cells were then incubated at 37°C, 5% CO2, and 90% humidity for 96 hour. For every 24 hours 20µl of the culture supernatant was taken to determine the glutamine concentration according to the manufacturer's instructions.
Calculation

The change in color was read using microtiter plate reader (iMark™, BioRad) at 565nm and concentration of glutamine was determined from the standard concentration.

\[
\text{Concentration of glutamine} = \frac{\text{OD sample} - \text{OD blank (mM)}}{\text{Slope}}
\]

4.2.10.1.11. Ammonia analysis (M/S EnzyChrome™, ENH3-100)

Ammonia is an inorganic source of nitrogen that exists primarily as a positively charged ion NH\(_4^+\), at physiological pH. In cell culture media, the catabolism of glutamine to glutamate and ammonia is mediated by mitochondrial enzyme called glutaminase. Ammonia produced in \textit{in-vivo} is not metabolized to urea and it accumulates in the culture media as ammonia ion. Ammonia is measured as NH\(_3\) and NH\(_4^+\). In this assay NADH is converted to NAD\(^+\) in the presence of NH\(_3\), ketoglutarate and glutamate dehydrogenase.

Procedure

Cells were plated at 3x10\(^3\) cells/well on a 96-well culture plate in the formulated medium. Cells were then incubated at 37°C, 5% CO\(_2\), and 90% humidiity for 96 hour. For every 24 hours 20µl of the culture supernatant was taken to determine the ammonia concentration according to the manufacturer's instructions.

Calculation

The change in color was read using microtiter plate reader (iMark™, BioRad) at 340nm and concentration of ammonia was determined from the standard concentration.

\[
\text{Concentration of ammonia} = \frac{\text{OD sample} - \text{OD blank (µM)}}{\text{Slope}}
\]

4.2.10.2. Established cell lines

Effect of newly formulated media was analysed as per the procedure given in 4.2.1.6.2 and 4.2.10.1.5 to 4.2.10.1.11.
4.2.11. Isolate and Identify Human Mesenchymal Stem Cells from Adipose tissue

4.2.11.1. Sample

Human Adipose tissue, an important source of easily available MSCs.

4.2.11.2. Source

The adipose tissues were collected from patients of age between 20 – 40 years attending Kilpauk Medical College Hospital, Chennai.

4.2.11.3. Collection – During surgery (abdominal/hernia)

The tissue was collected by the surgeons during surgery (abdomen/hernia). The isolation protocols and usage of the tissue were approved by the institutional review board of the hospital. Informed consent (oral and written) was obtained from the patients. The tissue samples were brought to the laboratory via transport media and were immediately processed.

4.2.11.4. Isolate hMSCs from Adipose tissue – (Zuk et al., 2001)

The adipose tissue was processed within 4 hours of sample collection to retain the viability of the cells. The tissue was placed in a sterile Petri dish, and was minced into small (4–5 mm) pieces with surgical scissors and scalpel. Attached skin and connective tissues were removed. Approximately 1-2 gms of adipose tissue was taken in a 15ml falcon tube together with 3-4 ml of type I collagenase solution [(1mg/ml), Boehiner Mannheim, Germany]. The adipose tissue-collagenase suspension was incubated at 37ºC in a water bath for 30 to 60 min. After incubation period, 4ml of Human mesenchymal stem cell growth medium supplemented with 20% fetal bovine serum was added to the adipose tissue digest to neutralize the collagenase. Then the content was centrifuged at 4000g for 10 min. to pellet the MSC-rich dense stromal vascular fraction. After centrifugation the adipocytes and fat appeared as a yellow oily layer at the top of the tube. This supernatant containing the oily layer and collagenase solution was decanted with the help of a transfer pipette. Care was taken not to pour off or disturb the pellet containing the cells.

The pellet was re-suspended in 2 ml of 160mM NH₄Cl and incubated at room temperature (RT) for 10 min. to lyse the red blood cells. The samples were transferred to new centrifuge tubes and centrifuged at 300g for 10 min. to
pellet the MSC-rich dense cell fraction. The supernatant was discarded and the pelleted cells were re-suspended in hMSC complete growth medium and with penicillin (100U/ml), streptomycin (100µg/ml) and amphotericin B. The cell suspension was filtered through a 100µm cell strainer and the cell debris were removed.

4.2.11.5. Identify Mesenchymal Stem Cell and their markers from Adipose tissue

4.2.11.5.1. Histology

To identify the stromal region of adipose tissue H & E stain was performed. The stromal vascular region has been reported to be rich in MSCs.

4.2.11.5.1.1. Haematoxylin & Eosin Staining – (Presnell and Schreibman, 1997)

Collected tissue samples were rinsed with an equal volume of sterile PBS with antibiotics to remove excess blood. This was repeated several times to remove entire blood from the tissue. It was cut into two equal pieces. One piece was placed in buffered formalin and used for normal histology followed by immunohistochemistry and immunofluorescence. Another half was used for MSC isolation followed by identification.

The adipose tissue was fixed in Bouin’s fluid. Fixation was carried out at room temperature for 24 hours, after which the tissues were transferred to 70% alcohol. Several changes of 70% alcohol were given until the yellow colour disappeared from the tissues. The tissues were then dehydrated by passing through ascending grades of alcohol (80% to 100%) and cleared by xylene, infiltrated with molten paraffin and finally embedded in paraffin wax (58°C). 3 to 5μm thick sections were taken making use of microtome (Leica 2035).

The section thus obtained, was stained in Harris haematoxylin and eosin, dehydrated using alcohol, cleared in xylene and mounted using DPX. The stained slides were observed in a research microscope (Carl Zeiss). Images were captured through a CCD camera in a computer and processing using Carl Zeiss Axion vision software.
4.2.11.5.1.2. Immunohistochemistry – (Sambrook and Russell, 2001)

This is an enzyme immunoassay used to locate and visualize a surface receptor (proteinaceous) in a section using specific antibodies.

Procedure

3-5µm paraffin embedded tissue sections were deparaffinised with xylene for 10-15 min. each. The slides were then rehydrated with graded ethanol series (100%, 90%, 70%, 50% and 30%) for 10 min. each and then brought to double distilled water and PBS for 10 min. each. The tissue sections were incubated in blocking buffer and were kept inside the moist chamber for 1h to block non-specific endogenous peroxidase activity. The slides were then treated with anti-CD29 and anti-CD44 (Biolegend, San Diego, CA) for 16-18hrs at 4°C.

After overnight incubation with primary antibodies (anti-CD29 & anti-CD44), the slides with tissue sections were washed with PBS twice for 5 min. and then incubated with secondary antibody (Rabbit Anti mouse IgG) conjugated with HRP (1:400 dilution) at room temperature followed by washing twice with PBS for 5 min. each. Finally, the tissue sections were treated with the substate solution 3, 3'-Diaminobenzidene (DAB), for 5-10 min. and the reaction was stopped by washing with PBS. The sections were then dehydrated in a series of graded ethanol (30%, 50%, 70%, 90% and 100%), cleared in xylene and mounted in DPX mount using cover slips. The slides were viewed under light microscope and photographed (Carl Zeiss, Germany).

4.2.11.5.1.3. Immunofluorescence – (Robertson et al., 2008)

Immunofluorescence is a technique for localization and visualizing an antigen in a section of a tissue by using a specific antibody for the target proteins. The sections are dewaxed, treated with a target retrieval solution, blocked with a protein based blocking solution and then incubated with a primary and corresponding secondary antibody FITC conjugates. Fluorescein isothiocyanate (FITC) enables the localization and identification of cellular antigens by means of an antigen antibody reaction. This technique involves the use of monoclonal
antibody to specific antigen, mostly proteins in the cells of the section spread on the microscopic slides.

The tissue was looked for CD29 & CD44 antigens. CD29 (Integrin) is a cell surface protein present in stromal cells and is a specific surface marker for mesenchymal stem cell. CD44 is a type of adhesion molecule used to identify specific type of MSCs.

**Procedure**

The 3-5µm paraffin embedded tissue sections were deparaffinised with xylene for 10-15 min. each. The slides were then rehydrated in graded ethanol series (100%, 90%, 70%, 50% and 30%) for 10 min. each and then brought to double distilled water and PBS for 10 min. each. The tissue sections were incubated in blocking buffer and were kept inside the moist chamber for 1 hour to block non-specific endogenous peroxidase activity. The slides were then treated with anti-CD29 and anti-CD44 (Biolegend, San Diego,CA) (1:200 dilutions) for 16-18hrs at 4°C.

After overnight incubation with primary antibodies (anti-CD29 & anti-CD44), the slides with tissue section were washed with PBS twice for 5 min. and then incubated with secondary antibody (Rabbit Anti mouse IgG) conjugated with FITC (1:400 dilution) at room temperature followed by washing twice with PBS for 5 min. each. The stained sections were viewed under fluorescent microscope (Carl Zeiss, Germany) and the results were photographed.

4.2.11.5.2.1 **RT-PCR – (Traktuev et al., 2008)**

RT PCR is used to amplify specific piece of RNA molecule. Amplification via reverse transcription polymerase chain reaction is highly sensitive as it can detect a very low no of RNA molecule. ADSCs were screened to look for the presence of the gene for CD90 (Thy-1, Extra cellular matrix protein) as it is one among the positive markers of human MSCs and α–SMA (alpha smooth muscle actin) as a marker for fibroblast formation.
Two days old cultured adipose derived stromal cells from adipose tissue derived stromal vascular fraction were taken and the total cellular RNA was isolated using single step RNA isolation kit (5Prime, USA). Then the isolated RNA was subjected to reverse transcription reaction with kit (5Prime, USA). The gene coding for CD90 and α–SMA was screened. CD90 gene is a cell adhesion molecule that is important for cell – cell interaction and is expressed more during the developmental stage of MSCs. α–SMA is used as a marker of fibroblast formation.

Subsequent PCR reactions were carried out according to standard procedures in 50 µl of reaction mix with 10µl of cDNA, 1.5 U of Taq polymerase (Boehringer Mannheim, Germany) and 10pmole of the following primers

- **CD90**: sense: 5’-CTGGCCATCAGCATCGCT-3’
  antisense: 5’-TATTCTCATGGCGGAGTCG-3’

- **α–SMA**: sense: 5’-GATCTGGCTGGCCGAGATC-3’
  antisense: 5’-ATGTCCCGGACAATCTCACG-3’

Amplification was done for 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 60 seconds. Amplified fragments were separated on 1% agarose gel and photographed after ethidium bromide staining.

4.2.11.6. **Culture and maintenance of hMSC – in-vitro –** (Zuk et al., 2002)

After enzyme dissociation of adipose tissue cell suspension containing 2 X 10⁵ stromal vascular fraction cells were seeded on average into Falcon 25cm² culture flasks containing 8ml of growth medium and incubated at 37°C in a humidified incubator with 5% CO₂ for 4 hours. After the incubation period, the flasks were washed twice with PBS to remove nonadherent cells. The adherent cells were removed with 0.25% trypsin/EDTA solution (HiMedia) and centrifuged at 400g for 10 mins. The resulting pellet was washed well with plain media and was reconstituted with known volume of complete growth media.

The viability of the cells was counted by dye exclusion method. The cell suspension containing 90% and above viable cells were taken for further studies.
4.2.12. Media formulation with SPI for adherent cell culture

Various batches of cell culture media were prepared as given in Table 4.2

4.2.13. Effect of formulated media on human adipose tissue derived MSCs

Based on the results obtained for SPIRM-3 in suspension cell culture the SPI concentration was used in the media formulation for adherent cells with DMEM as basal media.


Effect of newly formulated media was analysed as per the procedure given in 4.2.1.6.1.2

4.2.13.2. Sulforhodamine B assay (SRB) – (Vichai and Kirtikara, 2006)

Effect of newly formulated media was analysed as per the procedure given in 4.2.1.6.1.3

4.2.13.3. MTT assay – (Mosmann, 1983)

Effect of newly formulated media was analysed as per the procedure given in 4.2.1.6.1.4
Table 4.2. Composition of cell culture media prepared and used in adherent cell culture media

<table>
<thead>
<tr>
<th>Name</th>
<th>Type of Media</th>
<th>Basal Media</th>
<th>Supplement</th>
<th>FBS % (v/v)</th>
<th>SPI (mg/ml)</th>
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<tr>
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<td>DMEM</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
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<td>A1</td>
<td>DMEM + FBS + SPI</td>
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<td>SPI</td>
<td>5</td>
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<td>Soy Hydrolysate UF</td>
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<tr>
<td>A3</td>
<td>DMEM + FBS + SR</td>
<td>DMEM</td>
<td>Commercial Serum Replacement x3</td>
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<td>-</td>
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