3. EXPERIMENTAL
3. EXPERIMENTAL

3.1 Instruments and glasswares


Centrifuge: Remi centrifuge and R-8c Laboratory centrifuge, Remi Motors Ltd., Mumbai, India.

Elisa Reader: Bio-Rad Laboratories Inc, California, USA, model 550.

Fourier Transform Infrared: 1600 Series FT-IR, Perkin Elmer (India) Pvt. Ltd., Thane, India.


High Performance Liquid Chromatography: Shimadzu LC-2010A, liquid chromatography, Koyoto, Japan.

High Performance Thin Layer Chromatography: CAMAG, Switzerland.

HPTLC plates: silica gel 60 F$^{254}$ plates, E. Merk, Darmstadt. Germany.

HPTLC System: Linomate IV applicator and TLC Scanner 3, Camag, Muttenz, Switzerland.

HPTLC Syringe: Hamilton.

Twine through development chamber: Camag, Muttenz, Switzerland.

Homogenizer: Elvenjan homogenizer, Remi Motors Ltd., Mumbai.

Melting Point Apparatus: Lab India, Mumbai.

NMR: DDR X – 500 m/z – Bruker Daltonics, Germany.

Rotary Microtome: Leica RM 2135, Leica Microsystem GmbH, Nussloch, Germany.

Rotary Evaporator: Rotavapor R-205, Buchi Laboratory Equipments, Flawil, Switzerland.

Spectrophotometer: Shimadzu 160-A UV-VIS, Koyoto, Japan.
Experimental

**Muffle furnace:** Scientec, Genuine equipment manufacturers, Coimbatore.

### 3.2 Chemicals

3-(4, 5-dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), sulphorhodamine B (SRB), 2,2-diphenyl-1-picryl hydrazyl (DPPH) and 2,2’-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammmonium salt (ABTS) were obtained from Sigma Aldrich Co, St Louis, USA. Rutin and p–nitroso dimethyl aniline (p–NDA) were obtained from Acros Organics, New Jersy, USA. Naphthyl ethylene diamine dihydrochloride (NEDD) was obtained from Roch–Light Ltd., Suffolk, UK. Ascorbic acid, nitro blue tetrazolium (NBT), and butylated hydroxy anisole (BHA) were obtained from S.D. Fine Chem, Ltd., Biosar, India. 2-Deoxy–D-ribose was purchased from Hi–media Laboratories Ltd., Mumbai. 5–Flourouracil was purchased from Ranbaxy Laboratories Ltd., New Delhi, India. Cisplatin was obtained from Dabur India Ltd., New Delhi. Aspartate aminotransferase (ASAT), alanine aminotransaminase (ALAT) and alkaline phosphatase (ALP) diagnostic kits were obtained from Span Diagnostic Ltd., Surat, India. Aluminium backed high performance thin layer chromatography (HPTLC) plates coated with 0.2 mm layers of silica gel 60 F$_{254}$ plates and other diagnostic kits were obtained from E–Merck Ltd., Mumbai. All other chemicals used were of analytical grade.

### 3.3 Collection of plant and authentication

The stem and leaf of *Ipomoea leari* were collected from in and around of Nilgiris forests in the month of June 2009 and the plant materials were authenticated by Dr. Rajan, Botanist, Survey of Homeopathic Medicinal Plants and Collection Unit, Department of AYUSH, Ooty. A voucher specimen (No JSSU/OT/122) was deposited in the Department of Phytopharmacy and Phytomedicine, JSS College of Pharmacy, Ootacamund, India.
3.4 Pharmacognostical Studies

3.4.1 Evaluation of quality of raw materials

Quality of the dried leaves of the plant was evaluated by determining its ash values and extractive values. These were performed as per Indian Pharmacopoeia (IP) 2007.

Ash values

The ash values, namely total ash, acid-insoluble ash, water-soluble ash and sulphated ash were determined by using the shade dried parts of the selected plant materials powdering the same and passing it through sieve no. 40.

Determination of total ash

About 3 g of the powdered material was accurately weighed in a silica crucible which was previously ignited and weighed. The powdered drug was spread as a fine even layer at the bottom of the crucible. The crucible was incinerated at a temperature not exceeding 450°C until free from carbon. The crucible was cooled and weighed and the procedure was repeated to get a constant weight. The percentage of the total ash was calculated with reference to the air-dried drug.

Determination of acid-insoluble ash

The ash obtained as described in the total ash was boiled with 25 mL of 2 M HCl for five minutes. The insoluble ash was collected on an ashless filter paper and washed with hot water. The insoluble ash was transferred into preweighed silica crucible, ignited, cooled, weighed, and the procedure was repeated to get a constant weight. The percentage of acid-insoluble ash was determined with reference to the air-dried drug.
**Determination of water-soluble ash**

The ash obtained as described in the total ash was boiled in 25 mL of chloroform water for five minutes. The insoluble matter was collected in a Gooch crucible or ashless filter paper and washed with hot water. The insoluble ash was transferred into preweighed silica crucible, ignited for 15 minutes at a temperature not exceeding 450°C, cooled, weighed and the procedure was repeated to get a constant weight. The weight of the insoluble matter was subtracted from the weight of the total ash. The difference was considered as the water-soluble ash. The percentage of water-soluble ash was determined with reference to the air-dried drug.

**Determination of sulphated ash**

A silica crucible was heated to redness for about 10 minutes, allowed to cool in a desiccator and weighed. About 1 gm of the powdered drug was accurately weighed and taken in the crucible. The crucible was ignited gently first until the drug was thoroughly charred. The crucible was cooled and the residue was moistened with 1 mL of sulphuric acid, heated gently until no more white fumes were observed evolved and ignited at 800°C until all black particles disappeared. The crucible was allowed to cool; a few drops of sulphuric acid were added and again heated. The ignition was carried out as before, allowed to cool and weighed. The operation was repeated until two successive weighings did not differ by more than 0.5 mg. The percentage of sulphated ash was determined with reference to the air-dried drug.

**b. Extractive values**

**Water soluble extractives**

The macerated and air-dried coarse powder (5g) was mixed with 100 mL of chloroform water and kept in a closed flask for 24 hours, shaking frequently during the first 6 hours.
Experimental

and then allowed to stand for 18 hours. Thereafter, it was filtered rapidly, taking precautions against the loss of the solvent. About 25 mL of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105ºC and weighed. The percentage of water-soluble extractive was calculated with reference to the air-dried drug.

**Ethanol-soluble extractive**

The macerated and air-dried coarse powder (5g) was mixed with 100 mL of 95% ethyl alcohol in a closed flask and kept for 24 hours, shaking frequently during the first 6 hours and allowed to stand for 18 hours. Thereafter, it was filtered rapidly taking precautions against the loss of the solvent. About 25 mL of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105ºC and weighed. The percentage of ethanol-soluble extractive was calculated with reference to the air-dried drug.

### 3.4.2 Anatomical studies

Samples of different plant parts were cut; removed from the plant and fixed in FAA (Formalin, 5 mL + acetic acid, 5 mL + 70% ethyl alcohol, 90 mL). The specimens were dehydrated after 24 hours of fixing, with graded series of tertiary-butyl alcohol as per the schedule given by Sass (Sass J. E., 1940).

Paraffin wax (melting point, 58-60ºC) was added gradually until TBA solution attained super saturation to infiltrate the specimen. The specimens were then cast into paraffin blocks.

**Sectioning**

With the help of rotary microtome, the paraffin embedded specimens were sectioned. The thickness of the sections was 10-12 µm. Dewaxing of the sections was performed as per customary procedure (Plant micro technique, New York : Mc Graw Hill; 1940). The
sections were stained with toluidine blue as per the method given by O'Brien (O'Brien., 1965).

Wherever necessary, sections were also stained with safranin, fast-green and iodine in potassium iodide (for starch).

Paradermal sections of the leaf were cleared with 5% sodium hydroxide. Epidermal peelings were cleared by partial maceration employing Jeffrey’s maceration fluid (Sass et al., 1940).

The cleared paradermal sections and epidermal peelings were used in the preparation of glycerine mounted temporary slides. Powered materials of different parts were cleared with sodium hydroxide and mounted in glycerine medium after staining. Different cell components were studied and measured.

**Photomicrographs**

Microscopic descriptions of tissues were supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon Labphoto 2 microscopic unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light were employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures were indicated by the scale-bars (Easu K., 1964).

**3.5 Extraction**

*Ipomoea leari* plant material was air dried, powdered and successively extracted with n-Hexane (40-60°C), Chloroform, Ethyl acetate and Hydromethanolic solvants in a Soxhlet extractor for 18-20 h. The extracts were concentrated to dryness in a rotavapor under reduced pressure and controlled temperature (40-50°C). The nature and yields of the
extracts were noted. All the extracts were stored in a refrigerator at 4°C till further use. The successive n-Hexane (40-60 °C), Chloroform, Ethyl acetate and Hydromethanolic extracts of *Ipomoea leari* (leaf and stem) will henceforth be called ILH, ILC, ILE and ILHM, respectively.

3.6 Preliminary phytochemical screening of the extracts

All the extracts of the plant obtained were tested using the following qualitative chemical tests for the identification of various phytoconstituents (Harborne, 1984; Kokate 2002; Raaman N., 2006)

- **Tests for alkaloids:** Dragendorff’s test, Wagner’s test, Mayer’s test and Hager’s test.
- **Tests for steroids and sterols:** Libermann-Burchard test and Salkowaski test.
- **Tests for glycosides:** Legal test, Baljet test, Borntrager’s test and Keller-Kiliiani test.
- **Test for flavonoids:** Shinoda test.
- **Tests for tannins:** Lead acetate test and gelatin test.
- **Tests for triterpenoids:** Tin and thionyl chloride test.
- **Test for phenolics:** Ferric chloride test.
- **Test for carbohydrates:** Molish test.

3.7 Quantitative estimations

Total phenolic content and total flavonoid content estimation were performed for all the extracts. All the samples were tested using a solution of 1 mg/mL concentration.

3.7.1 Estimation of total phenol content (TP)

Total phenol content of the extracts was determined by using the Folin-Ciocalteu method (Sadasivam and Manikam, 1992). This test is based on the oxidation of phenolic groups.
with phosphomolybdic and phosphotungstic acids. After oxidation the green–blue complex formed was measured at 750 nm.

**Chemicals and Reagents used**

- Folin-Ciocalteu Reagent: Commercially available Folin-Ciocalteu reagent was diluted (1:10) with distilled water and used.
- Sodium Carbonate: 202.5 g of sodium carbonate was dissolved in 1mL of distilled water and used (0.7 M).

**Preparation of test and standard solutions**

Each extract (50 mg each) was dissolved in 50 mL of methanol. The solution was serially diluted with methanol to obtain lower dilutions. Gallic acid monohydrate (50 mg) was dissolved in 50 mL of distilled water. It was serially diluted with water to obtain lower dilutions.

**Procedure**

In a test tube, 200 μl of the extract (1 mg/ mL to 0.1 mg/ mL) was mixed with 1 mL of Folin-Ciocalteu reagent and 800 μl of sodium carbonate. After shaking, it was kept for 2 h for reaction. The absorbance was measured at 750 nm. Using gallic acid monohydrate, a standard curve was prepared when linearity was obtained in the range of 0.78-25 μg/mL. Using the standard curve the total phenol content was obtained. The total phenol content was expressed as gallic acid equivalent in mg/g of the extract.
3.7.2 Total flavonoid content (TF)

Aluminum chloride colorimetric method was used for the determination of total flavonoids (Chang et al., 2002).

**Preparation of sample solution**

Sample solution was prepared using distilled water, 50% methanol or methanol depending upon the solubility of the extract to get a solution of 1mg/mL concentration.

**Preparation of 10% aluminum chloride solution**

Accurately weighed 10 g of aluminum chloride was dissolved in distilled water and made up to 100 mL with distilled water.

**Preparation of 1M potassium acetate**

Accurately weighed 9.81 g of potassium acetate was dissolved in distilled water and made up to 100 mL with distilled water.

**Procedure**

The sample (1mL) was mixed with 3 mL of methanol, 0.2 mL of 10% aluminum chloride, 0.2 mL of 1 M potassium acetate and 5.6 mL of distilled water. The mixture was kept at room temperature for 30 minutes and the absorbance of the reaction mixture was measured at 415 nm with Perkin Elmer UV/Visible spectrophotometer. The calibration curve was prepared by preparing quercetin solutions of 20, 40, 60, 80 and 100 µg/mL concentrations in methanol. The concentration of flavonoids was found using the standard curve and the results was expressed as mg of quercetin equivalents (QE) per g dry weight (mg QE/g DW) of the sample. Results were averages of duplicate analysis.

3.8 Isolation and characterization of phytoconstituents

Isolation of phytoconstituents is a part of natural product chemistry. The biologically active ones can be incorporated as ingredients in the modern system of medicine. The
column chromatographic technique (adsorption chromatography) is widely used for the separation, isolation and purification of the natural products. The principle involved in this is the adsorption towards the adsorbent packed in the column. By changing the polarity of the mobile phase, separation can be achieved by column chromatography. Characterization of the isolated compounds can be carried out by different analytical techniques like UV, IR, NMR and Mass spectroscopy (MS). The ILC and ILE were subjected to column chromatographic separation to isolate the phytoconstituents.

### 3.8.1 Column chromatography of ILC

The ILC (5 g) was chromatographed over silica gel of 60-120 mesh, column length 100 cm and diameter 3 cm. Elution was carried out with solvents and the solvent mixtures of increasing polarities. Fractions were collected in 20 mL portions and monitored on TLC and the fractions showing similar spots were combined (Table 3.1).

**Table 3.1: Column chromatography of ILC**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Eluent</th>
<th>Residue on evaporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-17</td>
<td>Petroleum ether</td>
<td>No residue</td>
</tr>
<tr>
<td>18-28</td>
<td>Petroleum ether:Dichloromethane (95:5)</td>
<td>No residue</td>
</tr>
<tr>
<td>29-37</td>
<td>Petroleum ether: Dichloromethane (90:10)</td>
<td>No residue</td>
</tr>
<tr>
<td>38-50</td>
<td>Petroleum ether: Dichloromethane (80:20)</td>
<td>No residue</td>
</tr>
<tr>
<td>51-75</td>
<td>Petroleum ether: Dichloromethane (70:30)</td>
<td>No residue</td>
</tr>
<tr>
<td>76-91</td>
<td>Petroleum ether: Dichloromethane (60:40)</td>
<td>Light green residue</td>
</tr>
<tr>
<td>92-102</td>
<td>Petroleum ether: Dichloromethane (50:50)</td>
<td>Light green residue</td>
</tr>
<tr>
<td>103-117</td>
<td>Petroleum ether: Dichloromethane (40:60)</td>
<td>Light green residue</td>
</tr>
<tr>
<td>118-128</td>
<td>Petroleum ether: Dichloromethane (20:80)</td>
<td>no residue</td>
</tr>
</tbody>
</table>
The fractions 1-75 were mixed together and evaporated to minimal volume. Acetone was then added. Nothing settled down in the bottom of the container. Similar results were obtained with methanol also. The fractions 76-117 eluted in petroleum ether: dichloromethane (60:40, 50:50, 40:60) solvent system, showed two major spot along with minor impurities. Repeated recrystalisation in chloroform yielded a white powder. Its homogeneity was checked in petroleum ether: dichloromethane (40:60) solvent system (yield: 200 mg). The fractions eluted in dichloromethane and dichloromethane: ethyl acetate (100% and 80:20 and 60:40) solvent system almost showed similar number of spots. All the fractions were mixed together and rechromatographed. The remaining fractions were not worked out because of lower yields.

Rechromatography of white powder

The white powder (200 mg) obtained from fractions 76-117 was rechromatographed for further purification over silica gel of 100-200 mesh of column length 50 cm and diameter 1 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 10 mL portions and monitored on TLC (Table 3.2). The fractions 61–140 eluted in petroleum ether: dichloromethane solvent system (63:37-56:44), showed one major spot on TLC. The fractions 141-220 eluted in petroleum ether:
dichloromethane solvent system (55:45-48:52), showed two major spots on TLC. The fractions 221-330 eluted in petroleum ether: dichloromethane solvent system (47:53-37:63) showed one major spot on TLC. The fractions 61–140 were mixed together and evaporated to minimal volume. Repeated recrystallisation in chloroform yielded white crystals. It was designated as ILC-1 (yield: 58 mg) and the fractions 221-330 were mixed together and evaporated to minimal volume. Repeated recrystallisation in chloroform yielded white semi amorphous crystals. It was designated as ILC-2 (yield: 36 mg). These compounds were subjected to physical and spectral studies for characterization. The crystal structure and the cell parameters of the compound ILC-1 were also measured on a Bruker Axes SMART APEX II single crystal diffractmeter. A White Monocrystal (0.20x0.20x0.20mm\(^3\)) was selected and its X-ray diffraction intensity data were collected at 293 k on a three circle X-ray diffractometer fitted with Mo Kα radiation, \(\lambda=0.71069\). The \(\omega:2\theta\) scan technique was employed to measure these reflections. The reflections, 12339 reflections were unique. The data were corrected for the Lorentz and polarization effects and for absorption, employing an empirical correction. The crystal structure was solved by directed methods (SHELXS-97) and refined by a full matrix (SHELXL-97) least square procedure employing 9135 reflections that satisfied \(I > 2\sigma(I)\) criterion (Spek, A.L., 2009). The remaining fractions were not worked out as no residues were obtained.

**Table 3.2: Rechromatography of white powder**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Eluent</th>
<th>Residue on evaporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10</td>
<td>Petroleum ether</td>
<td>No residue</td>
</tr>
<tr>
<td>11-20</td>
<td>Petroleum ether:Dichloromethane(90:10)</td>
<td>No residue</td>
</tr>
<tr>
<td>21-30</td>
<td>Petroleum ether:Dichloromethane(80:20)</td>
<td>Light green</td>
</tr>
<tr>
<td>Temperature Interval</td>
<td>Solvent Mixture</td>
<td>Color/Residue</td>
</tr>
<tr>
<td>----------------------</td>
<td>-----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>31-40</td>
<td>Petroleum ether:Dichloromethane(70:30)</td>
<td>Light green</td>
</tr>
<tr>
<td>41-50</td>
<td>Petroleum ether:Dichloromethane(65:35)</td>
<td>Very light green</td>
</tr>
<tr>
<td>51-60</td>
<td>Petroleum ether:Dichloromethane (64:36)</td>
<td>Very light green</td>
</tr>
<tr>
<td>61-70</td>
<td>Petroleum ether: Dichloromethane (63:37)</td>
<td>White residue</td>
</tr>
<tr>
<td>71-80</td>
<td>Petroleum ether: Dichloromethane (62:38)</td>
<td>White residue</td>
</tr>
<tr>
<td>81-90</td>
<td>Petroleum ether: Dichloromethane (61:39)</td>
<td>White residue</td>
</tr>
<tr>
<td>91-100</td>
<td>Petroleum ether: Dichloromethane (60:40)</td>
<td>White residue</td>
</tr>
<tr>
<td>101-110</td>
<td>Petroleum ether: Dichloromethane (59:41)</td>
<td>White residue</td>
</tr>
<tr>
<td>111-120</td>
<td>Petroleum ether: Dichloromethane (58:42)</td>
<td>White residue</td>
</tr>
<tr>
<td>121-130</td>
<td>Petroleum ether: Dichloromethane (57:43)</td>
<td>White residue</td>
</tr>
<tr>
<td>131-140</td>
<td>Petroleum ether: Dichloromethane (56:44)</td>
<td>White residue</td>
</tr>
<tr>
<td>141-150</td>
<td>Petroleum ether: Dichloromethane (55:45)</td>
<td>White residue</td>
</tr>
<tr>
<td>151-160</td>
<td>Petroleum ether: Dichloromethane (54:46)</td>
<td>White residue</td>
</tr>
<tr>
<td>161-170</td>
<td>Petroleum ether: Dichloromethane (53:47)</td>
<td>White residue</td>
</tr>
<tr>
<td>171-180</td>
<td>Petroleum ether: Dichloromethane (52:48)</td>
<td>White residue</td>
</tr>
<tr>
<td>181-190</td>
<td>Petroleum ether: Dichloromethane (51:49)</td>
<td>White residue</td>
</tr>
<tr>
<td>191-200</td>
<td>Petroleum ether: Dichloromethane (50:50)</td>
<td>White residue</td>
</tr>
<tr>
<td>201-210</td>
<td>Petroleum ether: Dichloromethane (49:51)</td>
<td>White residue</td>
</tr>
<tr>
<td>211-220</td>
<td>Petroleum ether: Dichloromethane (48:52)</td>
<td>White residue</td>
</tr>
<tr>
<td>221-230</td>
<td>Petroleum ether: Dichloromethane (47:53)</td>
<td>White residue</td>
</tr>
<tr>
<td>231-240</td>
<td>Petroleum ether: Dichloromethane (46:54)</td>
<td>White residue</td>
</tr>
<tr>
<td>241-250</td>
<td>Petroleum ether: Dichloromethane (45:55)</td>
<td>White residue</td>
</tr>
<tr>
<td>251-260</td>
<td>Petroleum ether: Dichloromethane (44:56)</td>
<td>White residue</td>
</tr>
<tr>
<td>261-270</td>
<td>Petroleum ether: Dichloromethane (43:57)</td>
<td>White residue</td>
</tr>
<tr>
<td>Fraction</td>
<td>Solvent</td>
<td>Color</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>271-280</td>
<td>Petroleum ether: Dichloromethane (42:58)</td>
<td>White residue</td>
</tr>
<tr>
<td>281-290</td>
<td>Petroleum ether: Dichloromethane (41:59)</td>
<td>White residue</td>
</tr>
<tr>
<td>291-300</td>
<td>Petroleum ether: Dichloromethane (40:60)</td>
<td>White residue</td>
</tr>
<tr>
<td>301-310</td>
<td>Petroleum ether: Dichloromethane (39:61)</td>
<td>White residue</td>
</tr>
<tr>
<td>311-320</td>
<td>Petroleum ether: Dichloromethane (38:62)</td>
<td>White residue</td>
</tr>
<tr>
<td>331-340</td>
<td>Petroleum ether: Dichloromethane (36:64)</td>
<td>No residue</td>
</tr>
<tr>
<td>341-350</td>
<td>Petroleum ether: Dichloromethane (35:65)</td>
<td>No residue</td>
</tr>
<tr>
<td>351-360</td>
<td>Petroleum ether: Dichloromethane (34:46)</td>
<td>No residue</td>
</tr>
</tbody>
</table>

The ILC extract was concentrated and dissolved in a minimum amount of methanol and kept in room temperature for 24 h. A crystalline solid settled down to the bottom of the container. The crystalline solid was separated and washed with acetone. Repeated recrystallization of the combined crystalline solids with methanol yielded a colourless crystalline compound designated as ILC-3 (yield: 80 mg). The compound was subjected to physical and spectral studies for characterization.

### 3.8.2 Column chromatography of ILE

ILE (20 g) was chromatographed over silica gel of 60-120 mesh, the fractions were collected in 100 mL portions and monitored on TLC. The fractions showing similar spots were combined (Table 3.3).
Table 3.3: Column chromatography of ILE

<table>
<thead>
<tr>
<th>Fractions No.</th>
<th>Eluent</th>
<th>Residue on evaporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10</td>
<td>n-hexane</td>
<td>No residue</td>
</tr>
<tr>
<td>11-15</td>
<td>n-hexane: diethyl ether (99: 1)</td>
<td>No residue</td>
</tr>
<tr>
<td>16-20</td>
<td>n-hexane: diethyl ether (98: 2)</td>
<td>No residue</td>
</tr>
<tr>
<td>21-25</td>
<td>n-hexane: diethyl ether (97: 3)</td>
<td>No residue</td>
</tr>
<tr>
<td>26-32</td>
<td>n-hexane: diethyl ether (96: 4)</td>
<td>No residue</td>
</tr>
<tr>
<td>33-61</td>
<td>n-hexane: diethyl ether (95: 5)</td>
<td>No residue</td>
</tr>
<tr>
<td>62-103</td>
<td>n-hexane: diethyl ether (94: 6)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>104-106</td>
<td>n-hexane: diethyl ether (93: 7)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>107-116</td>
<td>n-hexane: diethyl ether (92: 8)</td>
<td>No residue</td>
</tr>
<tr>
<td>117-119</td>
<td>n-hexane: diethyl ether (91: 9)</td>
<td>No residue</td>
</tr>
<tr>
<td>120-121</td>
<td>n-hexane: diethyl ether (85: 15)</td>
<td>No residue</td>
</tr>
<tr>
<td>122-130</td>
<td>n-hexane: diethyl ether (80: 20)</td>
<td>Yellow residue</td>
</tr>
<tr>
<td>131-167</td>
<td>n-hexane: diethyl ether (75: 25)</td>
<td>Yellow residue</td>
</tr>
<tr>
<td>168-190</td>
<td>n-hexane: diethyl ether (70: 30)</td>
<td>Yellow residue</td>
</tr>
<tr>
<td>191-226</td>
<td>n-hexane: diethyl ether (65: 35)</td>
<td>Yellowish Brown residue</td>
</tr>
<tr>
<td>227-246</td>
<td>n-hexane: diethyl ether (60: 40)</td>
<td>Brown residue</td>
</tr>
<tr>
<td>247-253</td>
<td>n-hexane: diethyl ether (55: 45)</td>
<td>Dark brown residue</td>
</tr>
<tr>
<td>254-261</td>
<td>n-hexane: diethyl ether (50: 50)</td>
<td>Black brown residue</td>
</tr>
<tr>
<td>262-268</td>
<td>n-hexane: diethyl ether (40: 60)</td>
<td>Black brown residue</td>
</tr>
<tr>
<td>269-275</td>
<td>n-hexane: diethyl ether (20: 80)</td>
<td>Dark brown residue</td>
</tr>
<tr>
<td>276-280</td>
<td>diethyl ether</td>
<td>Dark brown residue</td>
</tr>
<tr>
<td>281-290</td>
<td>diethyl ether: Ethyl acetate (50:50)</td>
<td>Dark brown residue</td>
</tr>
</tbody>
</table>
The fractions 33-116 eluted with 10% methanol in ethyl acetate gave a yellowish brown semi solid and showed two major spots along with minor impurities. This was rechromatographed for further purification.

Rechromatography of fractions 33-116

The yellowish residue (40mg) obtained from fractions 33-116 was further chromatographed over silica gel 100-200 mesh of column length 50 cm and diameter 1 cm. Elution was carried out with solvents and solvent mixtures of increasing polarities. The fractions were collected in 10 mL portions and monitored on TLC (Table 3.4).

Table 3.4: Rechromatography of fractions 269-275

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Eluent</th>
<th>Residue on evaporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10</td>
<td>n-hexane</td>
<td>No residue</td>
</tr>
<tr>
<td>11-20</td>
<td>n-hexane: diethyl ether (98: 2)</td>
<td>No residue</td>
</tr>
<tr>
<td>21-30</td>
<td>n-hexane: diethyl ether (97: 3)</td>
<td>No residue</td>
</tr>
<tr>
<td>31-40</td>
<td>n-hexane: diethyl ether (96: 4)</td>
<td>No residue</td>
</tr>
<tr>
<td>41-50</td>
<td>n-hexane: diethyl ether (95: 5)</td>
<td>No residue</td>
</tr>
<tr>
<td>51-60</td>
<td>n-hexane: diethyl ether (94.9: 5.1)</td>
<td>Very Light yellow residue</td>
</tr>
<tr>
<td>61-70</td>
<td>n-hexane: diethyl ether (94.8: 5.2)</td>
<td>Very Light yellow residue</td>
</tr>
<tr>
<td>Fraction</td>
<td>Solvent System</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>71-80</td>
<td>n-hexane: diethyl ether (94.7: 5.3)</td>
<td>Very Light yellow residue</td>
</tr>
<tr>
<td>81-90</td>
<td>n-hexane: diethyl ether (94.6: 5.4)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>91-100</td>
<td>n-hexane: diethyl ether (94.4: 5.6)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>101-110</td>
<td>n-hexane: diethyl ether (94.2: 5.8)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>111-120</td>
<td>n-hexane: diethyl ether (94: 6)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>121-130</td>
<td>n-hexane: diethyl ether (93.9: 6.1)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>131-140</td>
<td>n-hexane: diethyl ether (93.8: 6.2)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>141-150</td>
<td>n-hexane: diethyl ether (93.7: 6.3)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>151-160</td>
<td>n-hexane: diethyl ether (93.6: 6.4)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>161-170</td>
<td>n-hexane: diethyl ether (93.5: 6.5)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>171-180</td>
<td>n-hexane: diethyl ether (93.4: 6.6)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>181-190</td>
<td>n-hexane: diethyl ether (93.3: 6.7)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>191-200</td>
<td>n-hexane: diethyl ether (93.2: 6.8)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>201-210</td>
<td>n-hexane: diethyl ether (93.1: 6.9)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>211-220</td>
<td>n-hexane: diethyl ether (93: 7)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>221-230</td>
<td>n-hexane: diethyl ether (92.8: 7.2)</td>
<td>Light yellow residue</td>
</tr>
<tr>
<td>231-240</td>
<td>n-hexane: diethyl ether (92.6: 7.4)</td>
<td>Very Light yellow residue</td>
</tr>
<tr>
<td>241-250</td>
<td>n-hexane: diethyl ether (92.4:7.6)</td>
<td>No residue</td>
</tr>
<tr>
<td>251-260</td>
<td>n-hexane: diethyl ether (92.2: 7.8)</td>
<td>No residue</td>
</tr>
</tbody>
</table>

The fractions 91-210 gave a dull yellow semi solid. This was dissolved in chloroform for further purification. An yellow amorphous powder separated. Its purity was checked in n-hexane: diethyl ether (1:1) solvent system. The compound, designated as ILE-1 (yield 25 mg), was subjected to physical and spectral studies for characterization.
3.9 High performance thin layer chromatographic (HPTLC) analyses

All the high performance thin layer chromatographic (HPTLC) studies were carried out using precoated HPTLC silica gel 60 F$_{254}$ plates (Merck) as a stationary phase. Samples were applied as an 8 mm band using Camag Linomat IV applicator. Application was done on the plate at a distance of 15 mm from bottom and 12 mm from the left margin with a 6 mm distance between the tracks at a constant application rate of 10 s/µl using nitrogen aspirator. Development was carried out in Camag twin through development chamber. The length of chromatogram run was maintained to 6 cm from the applied position. Subsequent to the development, TLC plates were dried in a current air with the help of a hair-dryer. The slit dimension setting of length 4 mm and width 0.30 mm, and a scanning rate of 20 mm/s and data resolution of 100 µm/step were used. Deuterium lamp, mercury lamp and tungsten lamp were used for scanning at 200-400 nm, 366 nm and 400-800 nm, respectively. Peaks were identified by densitometric scanning using Camag TLC scanner III and the results were obtained with the help of win CAT software version 1.4.3.

3.9.1 Fingerprint analyses

Preparation of sample solutions

Successive chloroform extract (ILC)

An accurately weighed quantity of the sample was extracted by heating at 40ºC for 5 minutes with 10 ml of chloroform. It was then filtered with the help of Whatman filter paper and the volume was adjusted to 10 ml with chloroform. The concentration of ILC solution was 25 mg/ml.
Fingerprint analyses

A fingerprint analysis of chloroform extract (ILC) was performed after developing suitable mobile phase. Mobile phase for fingerprint analyses was found by trial and error method.

Chromatography conditions for ILC extract

Mobile phase : Pet ether: ethyl acetate: Acetone (6.4: 1.18: 0.55)

Volume applied : 20 µl (ILC)

Detection wavelength before spray: 200 nm - 450 nm (with 50 nm interval)

Spray reagent : ortho phospho molibidic acid (Wagner et al., 1996)

Detection wavelength after spray: 450 nm

3.10 Pharmacological studies

3.10.1 In vitro studies

A. Antioxidant activity

The extracts and active fractions were tested for their in vitro antioxidant activity using standard methods. The in vitro methods are based on inhibition of free radicals. Samples are added to a free radical-generating system and the inhibition of the free radical activity is measured. This inhibition is related to antioxidant activity of the sample. Methods vary greatly as to the generated radical, the reproducibility of the generation process and the end point that is used for the determination.

Eventhough in vitro methods provide a useful indication of antioxidant activities, data obtained from in vitro methods are difficult to apply to biological systems and do not necessarily predict a similar in vivo antioxidant activity. All the methods developed have strengths and limitations and hence a single measurement of antioxidant capacity usually
is not sufficient. A number of different methods may be necessary to adequately assess in vitro antioxidant activity of a specific compound or a biological fluid. In the present study all the extracts were, therefore, tested for their in vitro antioxidant activity using several standard methods. The absorbance was measured spectrophotometrically against the corresponding blank solution. The percentage inhibition was calculated by using the formula,

\[
\text{Percentage inhibition} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100
\]

IC\textsubscript{50}, values, the concentration of the samples required to scavenge 50% of free radicals, were calculated.

**DPPH assay**

The 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical is reduced to its corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in colour and upon reaction with a hydrogen donor changes to yellow in colour. It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in absorbance is measured at 490 nm.

\[
\begin{align*}
\text{Purple DPPH} & \quad (2,2 \text{ diphenyl-1-picryl hydrazyl}) \\
& \quad \text{Yellow}
\end{align*}
\]
Experimental

Reagents

DPPH solution (100 μM): Accurately 22 mg of DPPH was weighed and dissolved in 100 mL of methanol. From this stock solution, 18 mL was diluted to 100 mL with methanol to obtain 100 μM DPPH solution.

Preparation of extract solution: Accurately 21 mg of each of the extract was weighed and dissolved in 1 mL of freshly distilled DMSO to obtain a solution of 21 mg/mL concentration. This solution was serially diluted to obtain lower concentrations.

Preparation of standard solutions: Accurately 10 mg ascorbic acid / rutin was weighed and dissolved in 0.95 mL of freshly distilled DMSO to obtain 10.5 mg/mL concentration. This solution was serially diluted with DMSO to get lower concentrations.

Procedure: The assay was carried out in a 96 well microtitre plate. To 200 μl of DPPH solution, 10 μl of the extract / standard solution was added to the wells of the microtitre plate. The plates was incubated at 37 °C for 30 min and the absorbance of the solution was measured at 490 nm (Hwang et al., 2001), using ELISA reader.

Scavenging of nitric oxide radical

Sodium nitroprusside in aqueous solution at physiological pH, spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of modified Griess Ilosvay reaction (Garrat, 1964). In the present investigation, Griess Ilosvay reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). Nitrite ions react with Griess reagent, which forms a purple azo dye. In presence of test components, likely to be scavengers, the amount of nitrite ions will decrease. The degree of decrease in the
formation of purple azo dye reflects the extent of scavenging. The absorbance of the chromophore formed is measured at 540 nm.

\[
\begin{align*}
\text{Sodium Nitroprusside} & \xrightarrow{\text{Aqueous soln}} \text{NO} \\
\text{NO} & \xrightarrow{\text{Dissolved O}_2/\text{Water}} \text{HNO}_3 + \text{HNO}_2 \\
\end{align*}
\]

\[
\begin{align*}
\text{HNO}_2 + \text{H}_2\text{N} & \xrightarrow{\text{SO}_3\text{H}} \quad \text{N}_2 \xrightarrow{\text{SO}_3\text{H}} \\
\text{Nitrous acid} & \quad \text{Sulfanilic acid} \quad \text{Diazonium salt} \\
\end{align*}
\]

\[
\begin{align*}
\text{1-Napthyl ethylene diamine dihydrochloride} & \xrightarrow{\text{Diazonium salt}} \quad \text{Azodye (Purple colored dye)} \\
\end{align*}
\]

**Reagents**

- Sodium nitroprusside solution (10 mM): Accurately 0.30 g of sodium nitroprusside was weighed and dissolved in distilled water and the volume was made up to 100 mL in a volumetric flask.
- Naphthyl ethylene diamine dihydrochloride (NEDD, 0.1%): Accurately 0.1 g of NEDD was weighed and dissolved in 60 mL of 50% glacial acetic acid by heating and the volume was made up to 100 mL with distilled water in a volumetric flask.
- Sulphanilic acid reagent (0.33% w/v): Accurately 0.33 g of sulphanilic acid was weighed and dissolved in 20% glacial acetic acid by heating and the volume was made up to 100 mL in a volumetric flask.

**Preparation of extract and standard solutions**: These solutions were prepared by similar procedure as in the case of DPPH.

**Procedure**: The reaction mixture (6 mL) containing sodium nitroprusside (10 mM, 4 mL), phosphate buffer saline (PBS, pH 7.4, 1 mL) and extract / standard (1 mL) in DMSO at various concentrations was incubated at 25 °C for 150 min. After incubation, 0.5 mL of the reaction mixture containing nitrite ion was removed, 1 mL of sulphanilic acid reagent was added to this, mixed well and allowed to stand for 5 min for completion of diazotisation. Then, 1 mL of NEDD was added, mixed and allowed to stand for 30 min in diffused light. A pink coloured chromophore was formed. The absorbance was measured at 540 nm.

**Scavenging of ABTS radical cation assay**

ABTS assay is relatively a recent one, which involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids. Its solubility in both the organic and aqueous media and the stability in a wide pH range raised the interest on the use of ABTS$^{*+}$ for the estimation of the antioxidant activity (Nenadis et al., 2004)
**Preparation of extract and standard solutions:** Accurately 13.5 mg of the extract ascorbic acid / rutin was weighed and dissolved in 2 mL of freshly distilled DMSO. This solution was serially diluted with DMSO to obtain lower dilutions.

**Procedure:** Accurately 54.8 mg of ABTS was weighed and dissolved in 50 mL of distilled water (2 mM). Potassium persulphate (17 mM, 0.3 mL) was then added. The reaction mixture was left to stand at room temperature overnight in dark before usage. To 0.2 mL of various concentrations of the extract / standard, 1.0 mL of distilled DMSO and 0.16 mL of ABTS solution was added to make the final volume to 1.36 mL. Absorbance was measured after 20 min at 734 nm (Re et al., 1999).

**Reducing power assay** (Ferreira et al., 2007)

Preparation of sample solution

Sample solutions were prepared using distilled water, 50% methanol or methanol depending upon their solubility to get a solution of 1 mg/ mL concentration.

Preparation of potassium ferricyanide solution (1%)

Accurately weighed 1 g of potassium ferricyanide was dissolved in distilled water and made up to 100 mL of distilled water.

Preparation of trichloroacetic acid solution (10%)

Accurately weighed 10 g of trichloroacetic acid was dissolved in distilled water and made up to 100 mL with distilled water.

Preparation of ferric chloride solution (0.1%)

Accurately weighed 100 mg of ferric chloride was dissolved in distilled water and made up to 100 mL with distilled water.

Procedure

The extract (1mL) / fraction / standard was added to 2.5 mL of 1% potassium ferricyanide and the mixture incubated for 20 minutes at 50°C. Aliquots of trichloroacetic
acid (2.5 mL, 10%) was added to the mixture and centrifuged at for 10 minutes. The upper layer (2.5 mL) was mixed with 2.5 mL of water and 0.5 mL of 0.1% ferric chloride solution. Absorbance was measured at 700 nm against the corresponding blank solutions.

**Scavenging of hydroxyl radical by p-NDA method**

Hydroxyl radical is measured by its inhibition of p-nitrosodimethyl aniline (p-NDA) bleaching (Elizabeth and Rao, 1989). Hydroxyl radical is generated through Fenton reaction. In this reaction, iron-EDTA complex reacts with hydrogen peroxide in presence of ascorbic acid to generate hydroxyl radical, which can bleach p-NDA specifically.

\[
\begin{align*}
\text{Fe}^{3+} + \text{EDTA} & \rightarrow \text{Fe}^{3+}\text{-EDTA complex} \\
\text{Fe}^{3+}\text{-EDTA complex} + \text{Ascorbate} & \rightarrow \text{Fe}^{2+}\text{-EDTA complex} + \text{Oxidized Ascorbate} \\
\text{Fe}^{2+}\text{-EDTA complex} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+}\text{-EDTA} + \text{OH}^- + \text{OH}^- \\
(\text{Fe}^{3+} + \text{O}_2^-) & \rightarrow \text{Fe}^{2+} + \text{O}_2 \\
\end{align*}
\]

Hydroxyl radical shows scavenging activity by inhibition of bleaching and the percentage of scavenging as absorbance is measured at 440 nm.

*Preparation of extract and standard solutions:* Accurately 30 mg of the extract / rutin was weighed and dissolved in 5 mL of freshly distilled DMSO. This solution was serially diluted with DMSO to obtain lower dilutions.

*Procedure:* Various concentrations of the extract / standard in distilled DMSO (0.5 mL) was added to a solution mixture containing ferric chloride (0.1 mM, 0.5 mL), EDTA (0.1 mM, 0.5 mL), ascorbic acid (0.1 mM, 0.5 mL), hydrogen peroxide (2 mM, 0.5 mL) and p-NDA (0.01 mM, 0.5 mL) in phosphate buffer (pH 7.4, 20 mM), to produce a final
volume of 3 mL. Sample blank was prepared by adding 0.5 mL sample and 2.5 mL of phosphate buffer. Absorbance was measured at 440 nm.

**Scavenging of hydrogen peroxide**

Hydrogen peroxide is generated *in vivo* by several oxidase enzymes. There is increasing evidence that hydrogen peroxide, either directly or indirectly via its reduction product hydroxyl radical (OH·) causes severe damage to biological systems. In this method, when a scavenger is incubated with hydrogen peroxide, the decay or loss of hydrogen peroxide can be measured spectrophotometrically at 230 nm (Jayaprakasha et al., 2004).

*Preparation of extract and standard solutions:* Accurately 30 mg of each of the extract / rutin was weighed and dissolved separately in 5 mL of methanol. These solutions were serially diluted with methanol to obtain lower dilutions.

*Procedure:* A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Various concentrations of 1 mL of the extract / standard in methanol was added to 2 mL of hydrogen peroxide solution in PBS. The absorbance was measured at 230 nm after 10 min.

**B. In vitro cytotoxicity studies**

Drug development programmes involve pre-clinical screening of a vast numbers of chemicals for their specific and non-specific cytotoxicity against many types of cells. Use of *in vitro* assay systems for the screening of potential anticancer agents has been a common practice almost since the beginning of cancer chemotherapy in 1946. The National Cancer Institute now routinely measures the growth inhibitory properties of every compound under test against a panel of 60 human tumor cell lines which are representative of major human tumor types. There are a number of advantages in *in vitro*
Experimental testing, using cell cultures which include analysis of species specificity, feasibility of using only small amounts of test substances, and facility to do mechanistic studies. A novel anticancer drug should possess cytotoxicity at a low concentration against cancerous cell lines and should be safe against normal cell lines even at higher concentrations (Masters, 2000). All the extracts and the isolated compound stigmasterol and β-sitosterol were, therefore, tested for cytotoxicity by MTT and SRB assays.

**Preparation of test solutions**

The extract was weighed, dissolved in distilled dimethyl sulphoxide (DMSO) and the volume was made up to 10 mL with MEM/DMEM, at pH 7.4 and supplemented with 2% inactivated FBS/NBCS (maintenance medium) to obtain a stock solution of 1 mg/ mL concentration, sterilized by filtration and stored at -20°C till use. Serial two fold dilution of the extract was prepared from the stock solution to obtain lower concentrations.

**Cell lines and culture medium**

Vero (normal African Green Monkey Kidney), L929, and HEp-2 (human laryngeal epithelial carcinoma) cell cultures were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of all cell lines were cultured in DMEM supplemented with 10% inactivated FBS/NBCS, penicillin (100 IU/ mL), streptomycin (100 µg/ mL) and amphotericin B (5 µg/ mL) in a 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 flat bottles and all experiments were carried out in either 96 or 6 well microtitre plates.
B.1. Determination of mitochondrial synthesis 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 cleavage of tetrazolium salt, 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to a blue formazan derivative by living cells is clearly a very effective principle on which the assay is based. The principle involved is the cleavage of MTT into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells is known to be proportional to the extent of formazan production by the cells used (Francis and Rita, 1986).

Procedure

The monolayer cell culture was trypsinized and the cell count adjusted to $1.0 \times 10^5$ cells/mL using MEM/DMEM medium containing 10% NBCS. To each well of the 96 well microtitre plate, 0.1 mL of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, and the monolayer washed once with medium. Different extracts (100μl each) were added to the cells in microtitre plates. The plates were then incubated at $37^\circ$ C for 3 days in 5% CO$_2$ atmosphere, microscopic examination was carried out and observations were noted every 24 h. After 72 h, the extract solutions in the wells were discarded and 50 μl of MTT in MEM – PR (Minimum essential medium without phenol red) was added to each well. The plates were gently shaken and incubated for 3 h at $37^\circ$ C in 5% CO$_2$ atmosphere. The supernatant was removed, 50 μl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate
reader at the wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and CTC\textsubscript{50} (concentration of drug or test extract needed to inhibit cell growth by 50%) values were generated from the dose-response curves for each cell line.

\[
\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}}\right) \times 100
\]

The pattern of all the cell lines as a group is used to rank the extracts/compounds as toxic or non-toxic.

**B. 2. Determination of total cell protein content by Sulforhodamine B (SRB) assay**

SRB is a bright pink aminoxanthine dye with two sulfonic groups. Under mild acidic conditions, SRB binds to protein basic amino acid residues in trichloro acetic acid (TCA) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of at least two orders of magnitude. Colour development in SRB assay is rapid, stable and visible. The developed colour can be measured over a broad range of visible wavelength in either a spectrophotometer or a 96 well plate reader. When TCA-fixed and SRB stained samples are air-dried, they can be stored indefinitely without deterioration (Philip et al., 1990).

**Procedure**

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/mL using the medium containing 10% new born calf serum. To each well of the 96 well microtitre plate, 0.1 mL of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 \(\mu\)l of different extracts were added to the cells in microtitre plates. The plates were then incubated at 37\(^\circ\) C for 3 days in 5%
CO₂ atmosphere, microscopic examination was carried out and the observations were recorded every 24 h. After 72 h, 25 µl of 50% trichloro acetic acid was added to the wells gently in such a way that it forms a thin layer over the extract to form an overall concentration of 10%. The plates were incubated at 4°C for 1 h. The plates were flicked and washed five times with water to remove traces of medium, extract and serum, and air-dried. They were stained with SRB for 30 min. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. Tris base (10 mM, 100 µl) was then added to the wells to solubilise the dye. The plates were shaken vigorously for 5 min. The absorbance was measured using microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the same formula used for MTT assay and the CTC₅₀ values were calculated.

C. In vitro short term toxicity studies

The short term toxicity studies were carried out against Dalton’s Lymphoma Ascities (DLA) and Ehrlich Ascitic Carcinoma (EAC) cells using standard procedures (Unnikrishnan and Ramadasan Kuttan., 1988). This test relies on a breakdown of membrane integrity determined by the uptake of a dye such as (Tryphan blue, erythorisin and nigrosin) to which the cell is normally impermeable.

Procedure

DLA and EAC cells were cultured in peritoneal cavity of mice by injecting intraperitoneally a suspension of DLA or EAC cells (1.0 x 10⁵ cells/mL). The peritoneal fluid containing cells were withdrawn from the peritoneal cavity of the mice between 12-15 days with the help of a sterile syringe. The cells were washed with HBSS and centrifuged for 10-15 minutes at 1,200 rpm. The procedure was repeated thrice. The cells
were suspended in known quantity of HBSS and the cell count was adjusted to $2 \times 10^6$ cells/mL. The diluted cell suspension was distributed into Eppendorf tubes (0.1 mL containing $2 \times 10^6$ cells). The cells were exposed to the extract / compound and incubated at $37^\circ$C for 3 h. After 3 h, equal quantity of the extract treated cells and trypan blue (0.4%) were mixed and left for a minute and loaded into a haemocytometer and viable and non-viable count was recorded within two minutes. (If kept longer, live cells also generate and take up colour). Viable cells do not take up colour, whereas dead cells take up colour.

The percentage growth inhibition was calculated and CTC$_{50}$ value was generated from the dose-response curves for each cell line.

\[
\text{% Growth Inhibition} = 100 - \left( \frac{\text{Total Cells} - \text{Dead Cells}}{\text{Total Cells}} \right) \times 100
\]

**D. In vitro long term toxicity studies by clonogenic assay**

Colony formation at low cell density, or plating efficiency, is the preferred method for analyzing cell proliferation and survival. This technique reveals differences in the growth rate within a population and distinguishes between alterations in the growth rate (colony size) and cell survival (colony number). When cells are plated out as a single-cell suspension at low densities (2-50 cells/cm$^2$), they grow as discrete colonies. The number of these colonies can be used to express the plating efficiency.

\[
\text{Plating efficiency} = \frac{\text{No. of colonies formed}}{\text{No. of cells seeded}} \times 100
\]

If it can be confirmed that each colony grew from a single cell, then this term becomes the cloning efficiency. Measurements of the plating efficiency are derived by counting the
number of colonies over a certain size (usually around 50 cells) growing from a low inoculum of cells.

While short-term tests are convenient and usually are quick and easy to perform, they reveal only cells that are dead (i.e., permeable to tryphan blue) at the time of the assay. Frequently, however, cells that have been subjected to toxic influences (e.g., irradiation, antineoplastic drugs) show an effect several hours, or even days, later. The nature of the tests required to measure viability in these cases is necessarily different, since by the time the measurement is made, the dead cells may have disappeared. Long-term tests are, therefore, used to demonstrate survival rather than short-term toxicity, which may be reversible. Survival implies the retention of regenerative capacity and is usually measured by plating efficiency (Freshney, 2000).

Long term survival studies against normal (Vero) and cancer (HEp-2) cell cultures were, therefore, carried out and regeneration capacity of the cells were estimated by standard clonogenic assay. Three non toxic concentration of each extract and stigmasterol was tested for their reduction in regenerative capacity, in comparison with untreated cell control.

**Procedure**

A series of cultures were prepared in 25 cm² flasks, three for each of three non toxic concentrations and three controls, cells were seeded at $5 \times 10^4$ cells/ mL in 4.5 mL of growth medium, and incubated at 37°C for 48 h, by which time the cultures would have progressed into the log phase. The supernatant medium was discarded, the monolayer washed once with medium and 4.5 mL of different selected test solutions were added to the bottles. The flasks were incubated for 72 h. The medium in the flask were removed and the cells trypsinized. The cell suspension was diluted from each bottle separately and the cell population was adjusted to $5 \times 10^3$ cells/ mL and seeded into Petri dishes or 6 well
Experimental plates. The cultures were incubated until colonies form. The cultures fixed in absolute methanol and stained for 10 min with 1% crystal violet. The dishes were washed in tap water and dried. The number of colonies was then counted (> 5 generations) and plating efficiency calculated.

3.10.2. In vivo Studies

A. Single dose acute oral toxicity studies

Acute oral toxicity was carried out as per OECD 423 guidelines using 6 Swiss albino mice. Limit test at a dose of 2000 mg/kg body weight was carried out using 3 mice per step. The extract was prepared at a concentration of 200 mg/mL in water and administered at a dose volume of 10 mL/kg body weight. All the animals were observed for clinical signs at 0, 1, 2 and 4 hours after drug administration and later once daily for a period of 14 days. Body weights of the animals were recorded on days 0, 7 and 14.

B. antioxidant studies

Liver is an important organ actively involved in the metabolism, detoxification and secretory functions in the body and its disorders are numerous with no effective remedies. Herbs are known to play a vital role in the management of various liver disorders. Ayurveda, the ancient system of Indian medicine, identified liver diseases and recommended a number of herbal remedies (Chatterjee, 2000). Carbon tetrachloride \((\text{CCl}_4)\) induced hepatic injury is the model used for hepatoprotective drug screening (Recknagel, 1983). The principle causes of carbon tetrachloride induced hepatic damage are free radical mediated lipid peroxidation leading to disruption of biomembrane, dysfunction of cells and tissues, decreased levels of antioxidant enzymes and generation of free radicals (Castro et al., 1974; Poli, 1993). The antioxidant activity involving inhibition of the generation of free radicals is important in providing protection against hepatic damage.
Several plant products have been reported for their hepatoprotective property by improving antioxidant status. Among them silymarin, a plant derived medicine obtained from the fruits of *Silybum marianum* is approved for use in liver cirrhosis and alcoholic liver diseases (Sallar et al., 2001). Among the *Ipomoea leari* plant extracts tested in the present investigations for *in vitro* antioxidant activity using different models, the Chloroform extract (ILC) exhibited potent antioxidant activity. Studies were, therefore, undertaken to evaluate its *in vivo* antioxidant properties.

**Animals**

Healthy Swiss Wistar rats of either sex, weighing 180-220 g, were procured from the animal house, J.S.S. College of Pharmacy, Ootacamund, India. The animal house was well ventilated and the animals had 12 ± 1 h day and night schedule. The animals were housed in large spacious hygienic cages during the course of the experimental period and the room temperature was maintained at 25 ± 1 °C. The animals were fed with standard rat feed and water *ad libitum*. The experiments were conducted as per the guidelines of CPCSEA, Chennai, India (Approval no. JSSCP/IAEC/Ph.D.,/PHYTO PHARM/02/2010-11).

**Preparation of extracts and standard**

ILC (100, 200 and 400 mg/ mL of) were prepared in 0.5% sodium carboxy methyl cellulose (CMC). Standard silymarin (100 mg/ mL) was prepared in 0.5% sodium CMC.

**Procedure**

Animals were divided into six groups comprising of six animals in each group. Group I served as normal and received 1 mL of 0.5% CMC. Group II served as CCl₄ treated control and received 1 mL of 0.5 % sodium CMC. Groups III received standard
Silymarin at 100 mg/kg body weight. Group IV, Group V and VI received ILC at 100, 200 and 400 mg/kg body weight, respectively. All these treatments were given orally for 7 days. On day 8, except for group I, all the other groups received 1 mL/kg body weight of CCl<sub>4</sub>, intraperitoneally.

**Isolation of blood serum**

On the ninth day, the rats were anesthetized using anesthetic ether and blood was collected from retro-orbital plexus. After collection, the blood was kept at 37 °C for 30 min. Later it was centrifuged at 2000 rpm for 15 min to separate serum, which was used for biochemical estimations.

**Preparation of liver and kidney homogenate**

All the animals were sacrificed by decapitation. The liver and kidney were removed, weighed and homogenized immediately with Elvenjan homogenizer fitted with teflon plunger, in ice chilled 10% KCl solution (10 mg/g of tissue). The suspension was centrifuged at 2000 rpm at 4°C for 10 min and the clear supernatant obtained was used for the biochemical estimations. The levels of antioxidant enzymes viz., catalase (CAT), superoxide dismutase (SOD) and lipid peroxidation (LPO) were measured spectrophotometrically in serum, liver and kidney samples.

**Estimation of antioxidant parameters**

**Estimation of catalase (CAT)**

Catalase (EC 1.11.1.6) is an enzyme present in the cells of plants, animals and aerobic (oxygen requiring) bacteria (Mates et al., 1999). Catalase is located in a cell organelle called the peroxisome. In animals, catalase is present in all major body organs. The role of catalase is to scavenge hydrogen peroxide and prevent oxidative damage to the cell. Catalase is a heme containing protein that can convert hydrogen peroxide to water and
oxygen in a two-step reaction cycle. In the first step, one molecule of hydrogen peroxide is converted to water. The catalytic cycle begins with the oxidation of the ferric heme by hydrogen peroxide to form the ferryl-oxo porphyrin/protein radical intermediate (compound 1). The catalase cycle is completed by the reduction of compound 1 to the ferric enzyme by hydrogen peroxide, resulting in the production of molecular oxygen (Li and Goodwin, 2004).

**Chemicals and reagents**

- *Hydrogen peroxide (7.5 mM):* 1.043 mL of 30% w/w H$_2$O$_2$ was made up to 100 mL with sodium chloride and EDTA solution (9 g of NaCl and 29.22 mg of EDTA dissolved in 1L distilled water).

- *Potassium phosphate buffer (65 mM, pH 7.8):* 2.2 g of potassium dihydrogen phosphate and 11.32 g of dipotassium hydrogen phosphate were dissolved in 250 mL and 1 L distilled water, respectively and mixed together. The pH was adjusted to 7.8 with KH$_2$PO$_4$.

- *Sucrose Solution:* 10.95 g of sucrose was dissolved in 100 mL of distilled water.

**Procedure**

2.25 mL of potassium phosphate buffer (65 mM, pH 7.8) and 100 μL of the tissue homogenate or sucrose (0.32 M) were incubated at 25°C for 30 min. H$_2$O$_2$ (0.65 mL, 75
mM) was added to initiate the reaction. The change in absorption at 240 nm was measured for 2-3 min, and dy/dx for 1 min for each assay was calculated and the results were expressed as CAT units / mg of tissue (Beers and Seizer, 1952).

Estimation of SOD

One of the most effective intracellular enzymatic antioxidants is superoxide dismutase (SOD) (EC 1.15.1.1). Superoxide dismutase is the antioxidant enzyme that catalyzes the dismutation of $O_2^\cdot -$ to $O_2$ and to the less reactive species $H_2O_2$. While this enzyme was isolated as early as 1939, it was only in 1969 that McCord and Fridovich proved the antioxidant activity of SOD (Mc Cord and Fridovich, 1969).

Superoxide dismutase exists in several isoforms, differing in the nature of active metal centre and amino acid constituency, as well as their number of subunits, cofactors and other features. In humans there are three forms of SOD: cytosolic Cu, Zn-SOD and mitochondrial Mn-SOD, and extracellular SOD (EC-SOD) (Landis and Tower, 2005). SOD destroys $O_2^\cdot -$ with remarkably high reaction rates, by successive oxidation and reduction of the transition metal ion at the active site in a “Ping-Pong” type mechanism (Mates et al., 1999).

Under physiological conditions, a balance exists between the level of reactive oxygen species (ROS) produced during normal cellular metabolism and the level of endogenous antioxidants, which serve to protect tissues from oxidative damage. Disruptions of this balance (either through increased production of ROS or decreased levels of antioxidants) produce a condition known as oxidative stress and leads to a variety of pathological conditions. To protect against oxidative damage, organisms have developed a variety of antioxidant defenses that include metal sequestering proteins, use of compounds such as vitamin C, E and specialized antioxidant enzymes. One family of antioxidant enzymes,
the superoxide dismutases (SOD), functions to remove damaging ROS from the cellular environment by catalyzing the dismutation of two superoxide radicals to hydrogen peroxide and oxygen (Misra and Fridovich, 1972).

\[
O_2^- + O_2^- \xrightarrow{SOD} O_2 + H_2O_2
\]

SOD measurement was, therefore, carried out for its ability to inhibit spontaneous oxidation of epinephrine to adrenochrome.

**Chemicals and reagents**

- **Sodium carbonate buffer (0.05 M, pH 10.2):** Sodium carbonate (5.3 g) and sodium bicarbonate (1.2 g) were dissolved separately in 1L of distilled water, which served as a stock solution. Buffer was prepared by mixing 64 mL of sodium carbonate and 70 mL of sodium bicarbonate solutions. The pH of the buffer was adjusted to 10.2 using the above stock solution accordingly.

- **Adrenaline (9 mM):** Adrenaline (0.038) was dissolved in distilled water and the final volume was made up to 10 mL with distilled water containing a drop of concentrated HCl (to bring pH down to 2). Adrenaline being sensitive, the vial was kept covered with aluminum foil at all times.

- **Sucrose (0.3199 M) solution:** Sucrose (10.96 g) was dissolved in distilled water and the volume was made up to 100 mL.

**Procedure**

Sodium carbonate buffer (2.8 mL, 0.05 mM) and 0.1 mL of tissue homogenate or sucrose (blank) was incubated at 30°C for 45 min. The absorbance was then adjusted to zero to sample. The reaction was then initiated by adding 10 μl of adrenaline solution (9 mM). The change in absorbance was recorded at 480 nm for 8-12 min. The temperature was
maintained at 30 ºC throughout the assay. Similarly, SOD calibration curve was prepared by taking 10 unit/ mL as standard solution. One unit of SOD produced approximately 50% inhibition of auto-oxidation of adrenaline. The results were expressed as unit (U) of SOD activity/mg of tissue (Saggu et al., 1989).

Estimation of thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation is commonly regarded as a deleterious process (Sevanian and Ursini, 2000), leading to structural modification of complex lipid protein assemblies, such as biomembranes and lipoproteins, and is usually associated with cellular malfunction. During lipid peroxidation, a polar oxygen moiety is introduced into the hydrophobic tails of unsaturated fatty acids. This process is of dual consequence: the presence of hydroperoxy group disturbs the hydrophobic lipid/lipid and lipid/protein interactions, which leads to structural alterations of biomembranes and lipoproteins; hydroperoxy lipids are sources for the formation of free radicals. When free radicals are generated, they can attack polyunsaturated fatty acids in cell membrane leading to a chain of chemical reactions called lipid peroxidation. As the fatty acid is broken down, the hydrocarbon gases and malonodialdehyde (MDA) are formed. The most common method used to assess MDA is the thiobarbituric acid (TBARS) assay (Halliwell and Chirico, 1993).

Chemicals and reagents

- **Thiobarbituric acid solution**: Thiobarbituric acid (0.8 g) was dissolved in distilled water and the volume was made up to 100 mL. The pH was adjusted to 7.4 with 1 N NaOH / 0.1 N HCl solution.

- **Acetic acid solution**: Acetic acid (20 mL) was dissolved in distilled water and the volume was made up to 100 mL with distilled water. The pH was adjusted to 3.5 with 1 N NaOH/0.1 N HCl solution.
Experimental

- **Sodium lauryl sulfate solution:** Sodium lauryl sulfate (8.1 g) was dissolved in distilled water and the volume was made up to 100 mL with distilled water.

- **Mixture of n-butanol and pyridine (15:1 v/v):** n-Butanol (15 mL) and 1 mL of pyridine were mixed together.

**Procedure**

To 1 mL of tissue homogenate, 0.2 mL of sodium lauryl sulfate solution (8.1%), 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of thiobarbituric acid solution (0.8% w/v, pH 7.4) were added. This incubation mixture was made up to 5.0 mL with double distilled water and then heated in boiling water bath for 30 min. After cooling, the red chromogen obtained was extracted with 5 mL of the mixture of n-butanol and pyridine (15:1 v/v) and centrifuged at 4000 rpm for 10 min. The organic layer was taken and its absorbance was measured at 532 nm. 1, 1, 3, 3-Tetraethoxy propane (TEP) was used as the external standard. The results were expressed as nM of MDA/mg of wet tissue or mL of serum using molar extension co-efficient of the chromophore (1.56 x 10^5 M^-1 cm^-1) (Tiwari, 2001). Similarly, the calibration curve was prepared for TEP and the results were expressed as nM of MDA/mg protein (Ohkawa et al., 1979).

\[
\text{nM of MDA/mg of tissue} = \frac{\text{OD x Volume of homogenate x 100 x 10}^3}{1.56 x 10^5 x \text{Volume of extract taken}}
\]

**C. anticancer studies**

In the *in vitro* antioxidant studies, ILC showed potent activity. In *in vitro* cytotoxicity studies, eventhough ILC, its fractions ILCF28, ILE and its fraction ILEF-1A showed moderate toxicity, it exhibited specific toxicity against cancer cell cultures. It was observed that among these extracts and fractions tested ILC, ILCF28 and ILE showed
good activity. These extracts and fraction were, therefore, selected for *in vivo* anticancer activity in different tumor models

**Selection and maintenance of animals**

Healthy adult Swiss albino mice weighing 25-30 g were obtained from the animal house of J.S.S. College of Pharmacy, Ootacamund, India, and were maintained under standard environmental conditions (22-28°C, 60-70% relative humidity 12 h dark/light cycle) and fed with standard rat feed (Amrut Rat Feed, Nav Maharasthra Chakan Oil Mill Ltd., Pune, India) and water *ad libitum*. The animals were housed in large spacious hygienic cages during the course of the experimental period. The experiments were conducted as per the guidelines of CPCSEA, Chennai, India. (Approval no: JSSCP/IAEC/Ph.D., / Phyto / 02/2010-11)

**Preparation of extracts and standard**

ILC, ILCF28, ILE and standard 5-Flurouracil (5-FU) were suspended in distilled water using sodium carboxy methyl cellulose (0.3%).

**C.1 Effect of ILC, ILCF-28 and ILE on mice bearing Dalton’s Lymphoma Ascities (DLA) cells.**

DLA (Dalton’s Lymphoma Ascites) cells were procured from Amala Cancer Institute, Amala nagar, Trissur, Kerala, India. The cells were maintained *in vivo* in Swiss albino mice intraperitoneally. Swiss Albino mice were divided into nine groups with six animals in each group. All the animals were injected with DLA cells (1 x 10^6 cells) intraperitoneally except the normal group. This was taken as day zero. Group I served as normal control and group II as tumor control. These two groups received sodium CMC
suspension (0.3%) administered orally. Group III served as a positive control and was treated with standard 5-Flurouracil (5-FU) at 20 mg/kg p.o. Group IV and V were treated with ILC at a dose of 100 and 400 mg/kg p.o., respectively. Group VI and VII were treated with ILCF-28 at a dose of 25 and 100 mg/kg p.o., respectively. Group VIII and IX were treated with ILE at 100 and 400 mg/kg p.o., respectively. All these treatments were given 24 h after the DLA cells were inoculated, once daily for 10 days. On day 11, blood was collected from the animals by retro-orbital puncture under mild anesthesia and was subjected to hematological parameters such as RBC, total WBC, differential count and hemoglobin estimation.

C.2 Effect of ILC, ILCF-28 and ILE on mice bearing Ehrlich Ascitic Carcinoma (EAC) cells.

Ehrlich tumor is a rapidly growing carcinoma with very aggressive behavior. It is able to grow in almost all mice strains (Chen and Watkins, 1970). The Ehrlich ascitic tumor, derived from a spontaneous murine mammary adenocarcinoma, is maintained in the ascitic form by passaging in Swiss mice, by i.p. route (Dagli et al., 1992). Swiss Albino mice were divided into nine groups with six animals in each group. All the groups were injected with EAC cells (2 x 10^6 cells) intraperitoneally except the normal group. This was taken as day zero. Group I served as normal control and group II as tumor control. These two groups received sodium CMC suspension (0.3%) administered orally. Group III served as a positive control and was treated with standard 5-Flurouracil (5-FU) at 20 mg/Kg p.o. Group IV and V were treated with ILC at a dose of 100 and 400 mg/kg p.o., respectively. Group VI and VII were treated with ILCF-28 at a dose of 25 and 100 mg/kg p.o., respectively. Group VIII and IX were treated with ILE at 100 and 400 mg/kg p.o., respectively. All these treatments were given 24 h after the DLA cells were inoculated,
once daily for 10 days. On day 11, blood was collected from the animals by retro-orbital puncture under mild anesthesia and was subjected to hematological parameters such as RBC, total WBC, differential count and hemoglobin estimation.

The following antitumour parameters were also calculated for both DLA and EAC experimental models;

**Average life span:** Average life span of the animals of all the groups were determined and noted.

**Percentage increase the life span (% ILS):** The effect of ILC, ILCF-28 and ILE on tumor growth was monitored by recording the mortality, daily for a period of 6 weeks and the percentage increase in life span (%ILS) calculated.

\[
\% \text{ ILS} = \left( \frac{\text{Mean survival of treated group}}{\text{Mean survival of control group}} - 1 \right) \times 100
\]

**Body weight analysis:** Body weights of the experimental mice were recorded both in the treated and control groups at the beginning of the experiment (day 0) and sequentially on every day during the treatment period. Percent increase in body weight was calculated on day 15 of the experiment using following formula;

\[
\% \text{ Increase in body weight} = \left( \frac{\text{Body wt. of animal on 15 day}}{\text{Body wt. of animal on 0 day}} \right) \times 100
\]

**c.3. Effect of ILC on mice bearing solid tumor**

Among the two extracts and one fraction tested for *in vivo* antitumour activity using DLA and EAC cells, fraction ILCF28 showed maximum activity followed by ILC and ILE. It was of interest, therefore, to study the anticancer activity of ILC and its fraction ILCF28 on DLA induced solid tumor model. DLA cells (1.5 x 10^6 cells/mouse) were injected
Experimental subcutaneously to the right hind limb of Swiss Albino mice. Swiss Albino mice were divided into six groups of 6 animals in each group. Group I served as tumor control and received sodium CMC suspension (0.3%). Group II, was treated with standard cisplatin at a dose of 2 mg/kg, Group III and IV were treated with ILCF28 at 25 and 50 mg/kg, Group V and VI received ILC at 50 and 100 mg/kg. All the treatments were given by i.p. 24 h after the DLA cells inoculation and the treatment continued once daily for 14 days. Initial diameter of the tumor on the right hind limb was noted using vernier calipers. From the 7th day onwards, tumor diameter was measured every third day up to 34 days. The tumor volume was calculated using the formula $V=\frac{4}{3}\pi r_1^2r_2$, where, $r_1$ and $r_2$ are the radii of tumors at two different planes.

3.11 Statistical Analysis: The significance of all the in vivo data was analyzed by One-Way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. Value of $(p < 0.05)$ was considered as statistically significant.