CHAPTER 5

METHODOLOGY
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Certified sample of three species of wheat viz. *Triticum dicoccum*, *Triticum durum* and *Triticum aestivum* were acquired from Wheat Research Center, Gujarat Krushi University, Junagadh, Gujarat, India. These wheat species were grown in plastic tray as per procedure described by Wigmore 1985 as follow;

a. Adequate quantities of unpolished wheat-grain were soaked overnight in water in a plastic container.

b. On the next day, the soaked wheat-grain were spreaded on the surface of the soil filled in plastic tray. Care was taken so that the grains did not touch one another.

c. A thin layer of soil was sprinkled on the wheat grains.

d. The tray was covered with a newspaper to provide darkness, which helps the sprouting.

e. The tray was kept in a covered balcony.

f. Next day, the tray was uncovered to spray on some water and was covered again with the newspaper. This step was repeated every day until sprouting took place, after which the tray was left uncovered and watered everyday upto 8 days.

g. On 9th day, the wheat grass was harvested by cutting with a clean pair of scissors about $\frac{1}{2}$” above the surface of the soil.

h. The grass was cleaned and dried in a dark place for 4 days. It was powdered, passed through 40# and kept in airtight bottle. The wheat grass powder was used for phytochemical study.

i. Wheat grass tablets were purchased from market (Herbal Hills Wheat grass Tablets, Manufactured by Herbal Hills, Lonavala Industrial Estate, Maharashtra, India) for clinical study.
5.1 PHYTOCHEMICAL STUDY:

Phytochemical study was performed to evaluate the presence of Abscisic acid and laetrile in *Triticum dicoccum*, *Triticum durum* and *Triticum aestivum* (wheat grass species) using HPLC technique at Amneal Pharmaceuticals (India) Pvt. Ltd., Ahmedabad, India.

**Preparation of Extract for Determination of Abscisic Acid from Wheat grass by HPLC:** 100 gm of wheat grass sample (*Triticum aestivum*, *Triticum durum* and *Triticum dicoccum*) was taken into 1000 mL water. Each sample was sonicated for 25 minutes. 100 mL methylene dichloride was added in each sample and thereafter sample was extracted. 5 mL from each sample was taken and diluted up to 50 mL with methylene dichloride. The extract was subjected to HPLC injection.

**Preparation of Extract for Determination of Laetrile from Wheat grass by HPLC:** 150 gm of wheat grass sample (*Triticum aestivum*, *Triticum durum* or *Triticum dicoccum*) was taken into 200 mL volumetric flask. 20 mL of 0.0005M ammonia solution was added in to the sample. Each sample was sonicated up to 1 hour with shaking and diluted up to volume with water. 5 mL from the solution was pipetted out into 100 mL volumetric flask and diluted up to volume with water. The extract was subjected to HPLC injection.

The extracted sample and reference standard were used for the determination of Abscisic acid and Laetrile using HPLC technique.

**Preparation of Reference Standard:**
Abscisic acid: Weigh accurately about 50 mg Abscisic acid into 100 mL volumetric flask, add about 70 mL of Methylene chloride and sonicate to dissolve it and dilute up to the mark. Pipette 2 mL from above solution in to 100 mL volumetric flask and dilute up to the mark with Methylene chloride.
Laetrile: Weigh accurately about 50 mg Laetrile into 200 mL volumetric flask, add about 140 mL of water and sonicate to dissolve it and dilute up to the mark. Pipette 1 mL from above solution in to 100 mL volumetric flask and dilute up to the mark with Water.

5.2 ESTIMATION OF ANTI PROLIFERATIVE ACTIVITY BY MTT ASSAY (In-Vitro)

Traditionally, the determination of cell growth is done by counting viable cells after staining with a vital dye. Trypan blue staining is a simple way to evaluate cell membrane integrity (and thus assume cell proliferation or death), but the method is not sensitive and cannot be adapted for high throughput screening. Measuring the uptake of radioactive substance, usually tritium-labeled thymidine, is accurate, but it is time-consuming and involves handling of radioactive substance.

The MTT assay is colorimetric assays for measuring the activity of enzyme that reduces MTT to formazan dye, giving a purple color. A main application allows assessing the viability and the proliferation of cells (cell culture assays). It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cell viability and growth.

Yellow MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide) is reduced to purple formazan in the mitochondria of living cells. The absorbance of the colored solution can be quantified by measuring at a wavelength, usually between 500 and 600 nm, by a spectrophotometer. The absorption max is dependent on the solvent employed. This reduction takes place only when mitochondrial reductase enzyme is active, and therefore conversion can be directly related to the number of viable cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in
causing death of cells can be deduced through the production of a dose-response curve. Solution of MTT solubilized in tissue culture media or balanced salt solution, without phenol red, is yellowish in color. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solution. The crystals can be dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance.

The use of MTT method has limitations influenced by the physiological state of cells and variance in mitochondrial dehydrogenase activity in different cell types. The MTT method of cell determination is useful in the measurement of cell growth in response to mitogens, antigenic stimuli, growth factors and other cell growth promoting reagents, cytotoxicity studies, and in the derivation of cell growth curves (Freshney 2005).

In the present study, MTT assay was performed to evaluate the antiproliferative activity of wheat grass species. The reagents, media, glassware and equipments used for the MTT assay are listed in Table 2;
### Table 2: List of Reagents, Media, Glassware and Equipments

<table>
<thead>
<tr>
<th>REAGENTS</th>
<th>MEDIA</th>
<th>GLASS WARES AND PLASTIC WARES</th>
<th>EQUIPMENTS</th>
<th>CELL LINES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotic/Antimycotic solution, 100X (10000 U/mL Penicillin G, 10000µg/mL Streptomycin, 25 µg/mL Amphotericin B) (Hyclone)</td>
<td>DMEM (Dulbecco Modified Eagle’s medium, low glucose with glutamine) (US Biological)</td>
<td>Falcon tubes (15 mL, 50 mL), Cryotubes (2 mL), Cell scrapper</td>
<td>Biosafety cabinet class II (Esco, Singapore)</td>
<td>A549, U937, HL60 (Cancer cell line) and Vero (one normal cell line) was obtained from NCCS, Pune.</td>
</tr>
<tr>
<td>DMSO cell culture grade (MP Biomedicals)</td>
<td>FBS (Fetal Bovine Serum, South American origin, 500 mL) (Bioclot)</td>
<td>Micro tips (Volex)</td>
<td>Cytotoxic safety cabinet (Esco, Singapore)</td>
<td></td>
</tr>
<tr>
<td>HBSS –1X (Hank’s Balanced Salt solution) (Hyclone)</td>
<td>Fluid thioglycolate media (TGM)</td>
<td>Tissue culture flasks (25 cm² T Flask vented &amp; non-vented, 75 cm² T Flask vented, 150 cm² T Flask vented)</td>
<td>ELISA plate reader (Thermo, USA)</td>
<td></td>
</tr>
<tr>
<td>Penicillin and Streptomycin solution (MP Biomedicals)</td>
<td>RPMI1640 (with L-glutamine) (Hyclone)</td>
<td>96-well microtiter plate (Flat Bottom, U Bottom, V Bottom)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triton X100 (MP Biomedicals)</td>
<td>Sodium bicarbonate (MP Biomedicals)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Trypan blue (Hyclone)</td>
<td>Methotrexate</td>
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<tr>
<td>0.25% Trypsin 1X (Invitrogen)</td>
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<td></td>
</tr>
<tr>
<td>DPBS/modified 1X (Dulbecco’s phosphate buffer saline without Ca+ &amp; Mg+) (Hyclone)</td>
<td></td>
<td></td>
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</tbody>
</table>
Cell Lines:

**A549:** A549 cells are human alveolar basal epithelial cells. These are squamous in nature and responsible for the diffusion of substances, such as water and electrolytes, across the alveoli of lungs. They grow adherently, as a monolayer, in vivo. The squamous epithelial cells are positive for keratin, as is evidenced by immunoperoxidase staining. These cells are also able to synthesize lecithin and contain a high percentage of desaturated fatty acids, which are utilized by the cytidine-diphospho-choline pathway and important for the maintenance of membrane phospholipids in cells. (http://a549.com/)

**U937:** U937 cells are a model cell line used in biomedical research. They are used to study the behaviour and differentiation of monocytes. U937 cells mature and differentiate in response to a number of soluble stimuli, adopting the morphology and characteristics of mature macrophages. U937 cells are of the myeloid lineage and so secrete a large number of cytokines and chemokines either constitutively (e.g. IL-1 and GM-CSF) or in response to soluble stimuli. TNFα and recombinant GM-CSF independently promote IL-10 production in U937 cells. IFNγ increases the expression of chemokine receptors (CCR1, CCR3 and CCR5) as well as that of nuclear receptors for melatonin. IFNγ treatment enhances thrombin-induced IL-8 production and is required for the melatonin-induced production of IL-6. U937 can be grown in DMEM or 1640 medium plus 10% fetal bovine serum. Atromentin induces apoptosis in human leukemia U937 cells. (http://www.copewithcytokines.de/cope.cgi?key=U937)

**HL60:** The HL-60 (Human promyelocytic leukemia cells) cell line is a leukemic cell line that has been used for laboratory research on how certain kinds of blood cells are formed. HL-60 proliferates continuously in suspension culture in nutrient medium supplemented with fetal bovine serum, L-glutamine, HEPES and
antibiotic chemicals. The doubling time is about 36–48 hours. The cell line was derived from a 36-year-old woman with acute promyelocytic leukemia at the National Cancer Institute. HL-60 cells are predominantly a neutrophilic promyelocyte (precursor). Proliferation of HL-60 cells occurs through the transferrin and insulin receptors, which are expressed on cell surface. The requirement for insulin and transferrin is absolute, as HL-60 proliferation immediately ceases if either of these compounds is removed from the serum-free culture media. With this line, spontaneous differentiation to mature granulocytes can be induced by compounds such as dimethyl sulfoxide (DMSO), or retinoic acid. Other compounds like 1,25-dihydroxyvitamin D3, 12-O-tetradecanoylphorbol-13-acetate (TPA) and GM-CSF can induce HL-60 to differentiate to monocytic, macrophage-like and eosinophil phenotypes, respectively. The HL-60 cultured cell line provides a continuous source of human cells for studying the molecular events of myeloid differentiation and the effects of physiologic, pharmacologic, and virologic elements on this process. HL-60 cell model was used to study the effect of DNA topoisomerase (topo) IIα and IIβ on differentiation and apoptosis of cells and is especially useful in dielectrophoresis studies, which require an aqueous environment with suspended and round cells (Gallagher R 1979, Breitman T 1980, Sugimoto K 1998, Ratanchoo K 2002).

**Vero:** Vero cells are lineages of cells used in cell cultures. Vero cells are used for many purposes, including:

- Screening for the toxin of Escherichia coli, first named "Vero toxin" after this cell line, and later called "Shiga-like toxin" due to its similarity to Shiga toxin isolated from Shigella dysenteriae.
- As host cells for growing virus; for example, to measure replication in the presence or absence of a research pharmaceutical, the testing for the presence of rabies virus, or the growth of viral stocks for research purposes.
- As host cells for eukaryotic parasites, especially of the Trypanosomatids.
The Vero cell lineage is continuous and aneuploid. A continuous cell lineage can be replicated through many cycles of division and not become senescent. Aneuploidy is the characteristic of having an abnormal number of chromosomes. (http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Cell_Culture/Key_Resources/ECACC_Handbook/Cell_Culture_Techniques_5.html.)

5.2.1 CHARACTERIZATION OF CELL LINES AND CULTURE MEDIA:

Characterization is essential not only when deriving new lines, but also when a cell line is obtained from a cell bank or other laboratory. Cultures are examined under an inverted phase microscope before start of experiment and frequent assessments are made of the viability of the cell population throughout the experimental period.

Testing for Microbial Contamination: The two methods were used in present study to check for bacterial and fungal contamination. Detection was carried out using special media like fluid thioglycolate media (TGM) and tryptone soya broth (TSB) and direct observation using Gram’s stain. Contamination by bacteria, yeast or fungi was detected by an increase in turbidity of the medium or/and a decrease in pH (yellow in media containing phenol red as a pH indicator). Cells were inspected daily for presence or absence of microbial growth.

Protocol: (Freshney 2005)
1. Cell lines were cultured in the absence of antibiotic prior to testing using 25cm² non-vented T flask.
2. In case of adherent cell line, attached cells were brought into suspension using a cell scraper. Suspension cell lines were tested directly.
3. 1.5 mL test sample (Cells) were inoculated in two test tubes of each containing thioglycollate medium (TGM) and tryptone soya broth (TSB).
4. 0.1 mL of E. Coli, B. subtilis or 0.1 mL C. sporogenes was inoculated in to
the test tubes (duplicate) containing thioglycollate medium (TGM) and tryptone soya broth (TSB), These were considered as positive controls. Whereas two test tubes of each containing thioglycollate medium (TGM) and tryptone soya broth (TSB) un-inoculated as negative controls.

The broths were incubated as follows:
- For tryptone soya broth, one broth of each pair was incubated at 32°C for 4 days and the other at 22°C for 4 days.
- For thioglycollate medium, one broth of each pair was incubated at 32°C for 4 days and the other at 22°C for 4 days.
- For thioglycollate medium inoculated with C.sporogenes was incubated at 32°C for 4 days

Note: Test and Control broths were examined for turbidity after 4 days.

Criteria for a Validity of results: The control broth showed evidence of bacteria and fungi within 4 days of incubation and the negative control broths showed no evidence of bacteria and fungi.

Criteria for a Positive Result: Test broths containing bacteria or fungi showed turbidity.

Criteria for a Negative Result: Test broths were clear and showed no evidence of turbidity.
5.2.2 PREPARATION OF MEDIA:

Preparation of Dulbecco's Modified Eagle's Medium (DMEM): 10.7 gm of DMEM powder was added in 1 liter of distilled water and stirred continuously until clear solution formed. To this, NaHCO₃ was added to maintain pH 7.0-7.2. The solution was filtered using membrane filtration assembly. It was stored in reservoir bottle under room temperature.

Preparation of the Trypsin dilution: 5 mL of Trypsin solution was pipetted out in to 50 mL falcon tube containing 45 mL of phosphate buffered saline (PBS) using 10 mL pipette.

5.2.3 DETERMINATION OF CELL VIABILITY, DENSITY AND POPULATION DOUBLING TIME

Cell viability by Trypan Blue Dye Exclusion Method:
The viability of cells was determined by the trypan blue dye exclusion method. It takes advantage of the ability of healthy cells with uncompromised cytoplasmic membrane integrity to exclude dyes such as trypan blue (Freshney 2005).

Haemocytometer Cell Count:
1. Hemocytometer slide and cover slip were cleaned and wiped with 70% V/V alcohol. Then cover slip was placed on haemocytometer slide.
2. In 2 mL centrifuge tube, cell suspension (cells in culture media) was added. The two fold dilution was prepared by mixing aliquot of 0.1 mL cell suspension with 0.1 mL trypan blue.
3. 0.1 mL of cell suspension was placed in to chamber of haemocytometer.
4. By using a Lieca inverted microscope, numbers of cells were counted in 1mm² area with 10X objective.
5. Viable and non-viable cells were counted in both halves of the chamber.
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**Calculations:** Each large square of Haemocytometer, with cover-slip in place, represents a total volume of 0.1 mm$^3$ or $10^{-4}$ cm$^3$. Since 1 cm$^3$ is equivalent to approximately 1 mL, the total number of cells per mL will be determined using the following calculations;

1. Total number of viable cells = $A \times B \times C \times 10^4$
2. Total dead cell count = $A \times B \times D \times 10^4$

Where,

- $A =$ Volume of cell solution (mL)
- $B =$ Dilution factor in trypan blue
- $C =$ Mean number of unstained cells
- $D =$ Mean number of dead/stained cells

3. Total cell count = Viable cell count + dead cell count

$\% \text{ viability} = \left( \frac{\text{Viable cell count}}{\text{Total cell count}} \right) \times 100$


**Population doubling time (PDT)** (Das HK, 2007)

It is the time in hours, taken for cell number to double and is reciprocal of the multiplication rate. $(1/r)$

\[ N^H = \text{no. of cells harvested at the end of growth period that is } t_2 \]
\[ N^I = \text{no. of cells inoculated at time } t_1 = 0 \]
\[ n = 3.32 \left( \log N^H - \log N^I \right) \]

PDT = total time elapsed/no. of generations = $1/r$

**Multiplication rate (r):** The numbers of generation that occurs per unit time and is usually expressed as population doubling in 24 hours.

\[ r = 3.32 \left( \log N^H - \log N^I \right) / (t_2 - t_1). \]
5.2.4 SUBCULTURING /PASSAGING OF CELL LINES

Sub culturing of adherent cell lines: A549 or Vero both is adherent cell line which was sub cultured as follow (Freshney 2005):

All the reagents and cell lines were brought at room temperature before start of sub culturing which include Fetal Bovine Serum (FBS), Dulbecco’s Modified Eagle’s medium (DMEM), EDTA –Trypsin solution (Trypsin-EDTA made by diluting the stock 1/10 by adding PBS only) and antibiotics. The cell line was handled under cytotoxicity cabinet to prevent cross contamination of cell lines.

1. Cells were split when they were about 80-90% confluence.
2. Cells were washed with 0.1 mL cm² / flask (2.5 mL in case of 25cm² flask) DPBS- EDTA (1mM EDTA) solution. The monolayer adhered to flask was gently rinsed by rocking the flask back and forth.
3. After 5 minutes, aspirate offs the excess PBS-EDTA from the flask.
4. To the above flask, 0.1-0.2 mL/cm² trypsin was added until the entire monolayer was covered and incubated for 3-5 minutes at room temperature to detach the cells from monolayer.
5. Cells were dispersed into a single cell suspension by pipetting the cell solution up and down. These cells were added into media flask (DMEM + BSS) containing FBS (FBS inactivates the trypsin, which was why it had to be rinsed off with PBS-EDTA initially).
6. Cells were counted by haemocytometer and diluted to the appropriate concentration for seeding. Finally, the appropriate volume of cell suspension were added into a new flask containing medium along with 1% antibiotic solution and the flask was placed in 5 % CO₂ incubator at 37°C.
7. The splitting/passage was repeated every 3-4 days, so that they were not diluted too much or overgrown.
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**Sub-culturing of Suspension Cell Line** (Freshney 2005): HL60 and U937 are suspension cell lines hence the method of sub culturing was little different than adherent cell line.

1. Cultures were observed under an inverted microscope (Leica, DMIL) using 10X objective.
2. Yellow colors of media indicated that cells were overgrown and then centrifuge the culture flask at 150 gm for 5 minutes. After centrifugation (minispin centrifuge, eppendorf), flask was re-seeded at a slightly higher cell density and 10- 20% of conditioned medium (supernatant) was added to the fresh media.
3. Sample of the cells were taken from the cell suspension (100-200 μl). Cell density was calculated and the desired numbers of cells were re-seeded into freshly prepared flask without centrifugation just by diluting the cells.
4. Flask was placed in 5 % CO₂ incubator at 37⁰C.
5. This splitting/passage was repeated every 3-4 days, so that they were not diluted too much or overgrown.
5.2.5 PREPARATION OF COMPOUND DILUTION

- **Preparation of plant extraction:** Fresh wheat grass was crushed with water and the juice was filtered through filter paper. It was kept in stopped bottle.

- **Preparation of Stock solution of plant extraction:** Plant extract was stored as stock solution prepared in water at concentration of 100 µM.

- **Dilution of plant extracts:** 15 µl extract of 100µM test compound was added in to 135 µl of culture cell media as a result 150 µl of 10 µM concentration was obtained in 1st well of plate. Then, 1:3 serial dilution of test compound was done by pipetting out 50 µl compound mixed cell media culture to the next well. Same dilution was repeated 9 times way proceed further (Table 3), in order to get final dilution of test compound to 0.000508 µM (0.508 nM) in well -10. This led to 1:3 dilution of test compound in the next 9 wells.

**Table 3: Dilution of test compound used in the MTT assay (1:3)**

<table>
<thead>
<tr>
<th>Well no:</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compo und dilution (µl)</td>
<td>100</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>Taken from</td>
<td>CM 15 TC 35M</td>
<td>well 1</td>
<td>well 2</td>
<td>well 3</td>
<td>well 4</td>
<td>well 5</td>
<td>well 6</td>
<td>well 7</td>
<td>well 8</td>
<td>well 9</td>
</tr>
<tr>
<td>Final conc. (µM)</td>
<td>10 (µM)</td>
<td>3.33 (µM)</td>
<td>1.11 (µM)</td>
<td>0.37 (µM)</td>
<td>0.123 (µM)</td>
<td>0.041 (µM)</td>
<td>0.013 (µM)</td>
<td>0.0045 (µM)</td>
<td>0.0015 (µM)</td>
<td>0.0005 (µM)</td>
</tr>
</tbody>
</table>

Where, TC = Test compound; CM = Culture Media, M=Media (µl)

- **Reference substance:** Methotrexate is an anticancer (antimetabolites) drug used in the treatment of lymphoma (cancer of the lymph nodes) and certain forms of leukemia. It is also given to treat some forms of cancers of the uterus, breast, lung, head, neck, and ovary. Methotrexate inhibits folic acid reductase which is responsible for the conversion of folic acid to
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tetrahydrofolic acid. Molecular Weight of Methotrexate is 454.4. Stock solution of it was prepared by mixing 0.454 mg of drug with 10 mL of Dimethyl sulfoxide (DMSO). The concentration of Methotrexate in stock solution was kept at 100 μM.

- **Preparation of Abscisic acid solution:** Molecular weight of Abscisic acid is 264.32. Its stock solution was prepared by mixing 0.264 mg of drug with 10 mL of methylene dichloride. The concentration of Abscisic acid in stock solution was kept at 100 μM.

- **Preparation of Laetrile solution:** Molecular weight of Laetrile is 309.27. Its stock solution was prepared by mixing 0.309 mg of drug with 10 mL of Water. The concentration of Laetrile in stock solution was kept at 100 μM.
5.2.6 EXPERIMENTAL SETUP

**Cell Lines and Culture Medium:** Stock cells of Vero, U937, A549 and HL60 cell lines were cultured in Dulbecco’s Modified Eagle’s medium (DMEM), supplemented with 10% FBS (fetal bovine serum). Along with media cells were also supplemented with 5% HBSS, penicillin, streptomycin and amphotericin-B (MP Biomedicals), in a humidified atmosphere with 5% CO₂ at 37°C until confluence reached. The cells were dissociated with 0.2% trypsin, 0.02% EDTA in phosphate buffer saline solution. The stock cultures were grown initially in 25 cm² tissue culture flask, then in 75 cm² and finally in 150 cm² tissue culture flask and all cytotoxicity experiments were carried out in 96 well microtitre plates. 2 X 10⁴ cells/well were added in to each well of 96 well plates. The calculations were as follow.

**Calculation for number of cells in 96 well plate:** The no. of cells required for 100 wells = 96 well,

\[
\text{No. of cells/well} \times 100 = 2 \times 10^4 \times 100 = 2 \times 10^6 \text { cells/plate}
\]

Total volume of media for 100 wells

\[
\text{Total volume of media/well} \times 100 = 100 \mu l \times 100 = 10 \text{ mL}
\]

Therefore, total of 2 X 10⁶ cells were in 10 mL of medium, then aliquoted the required volume of cell suspension in to each wells.

**Design of experiment:** Cell lines in exponential growth phase were washed, trypsinized and re-suspended in complete culture media. Cells were plated at 2 x 10⁴ cells/well in 96 well microtitre plate and incubated for 24 hour during which a partial monolayer form. The cells were exposed to various concentrations of the test compounds (as indicated in plate assignment) and standard methotrexate.
Control wells were received only maintenance medium. The plates were incubated at 37°C in a humidified incubator with 5% CO₂ for a period of 24 hour. The morphological changes of drug treated cells were examined using an inverted microscope at different time intervals and compared with the cells serving as control. At the end of 24 h, cellular viability was determined by using MTT assay [MTT solution (5 mg/mL in phosphate buffered saline (PBS) pH 7.5), HCl, Propan-2-ol 96-well microtitre plate, ELISA reader].

5.2.7 SCREENING OF TEST COMPOUNDS BY MTT ASSAY;

- 2 X 10⁴ Cells were grown in microtitre plates in final volume of 100 µl culture medium per well. Cells were incubated for 24 hour at 37°C and at 5% CO₂.
- Various amounts of compound (final concentration e.g. 10µM - 0.0005µM, refer table 3) were added into micro plates (96 wells, flat bottom).
- After incubation period, 10 µl of the MTT labeling mixture was added.
- The plate was allowed to incubate for additional 4 hour at 37°C under CO₂ incubator.
- Add 100 µl solubilization solutions (MTT solvent - 60% DMSO and 40% Ethanol).
- The plate was read on a microplate reader using a wavelength of 550 nm (reference wavelength of 650 nm).
- After 24 hour, the cytotoxicity data was standardized by determining absorbance and calculating the corresponding concentrations. Linear regression analysis with 95% confidence limit and R² were used to define dose-response curves and to compute the concentration of chemical agents needed to reduce absorbance of the MTT by 50% (IC₅₀).
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- Percentage cell growth inhibition or percentage cytotoxicity was calculated by the formula:

\[
\% \text{ viability} = \frac{(AT-AB)}{(AC-AB)} \times 100, \text{ Where,}
\]

- \( AT = \) Absorbance of treated cells (drug)
- \( AB = \) Absorbance of blank (without cell)
- \( AC = \) Absorbance of control (untreated)

There by,

\[
\% \text{ cell growth inhibition} / \% \text{ cytotoxicity} = 100 - \% \text{ cell survival}
\]

- **Determination of IC\(_{50}\) Value:** According to the FDA, IC\(_{50}\) represents the concentration of a drug that is required for 50% inhibition in vitro. In the present study, IC\(_{50}\) is a concentration of drug at what 50% of cell population die.

  - For primary screening, a threshold of 50% cell growth inhibition as a cut off for compound toxicity against cell lines was used. IC\(_{50}\) was determined from plot of dose-response curves between log of compound concentration and percentage growth inhibition.

  - Graph was plotted by keeping log concentration of drug on X-axis and % cell growth inhibition or % cytotoxicity on Y-axis. IC\(_{50}\) was estimated as a concentration of drug at 50% position on Y-axis.

  - The relationship should be sigmoidal, log drug concentration on the x -axis and 'response/measurement' of the Y-axis.
5.3 ESTIMATION OF ANTICANCER ACTIVITY BY CLINICAL STUDY (In-Vivo):

The clinical study of wheat grass tablet on patients of leukemia cancer was carried out at Bharat Cancer Research Center, Surat. The necessary permission for conducting the clinical study was obtained from the concerned ethical committee. The objective was to study clinical efficacy of wheat grass tablets as a supportive treatment in leukemia patients who were subjected to chemotherapy. The clinical efficacy was assessed:

- By comparing Side Effect Index between 2 leukemic patients’ groups (Side Effect Index included headache, nausea, vomiting, bone pain, fever, skin rash, hair loss, mouth ulceration, anorexia, loss of weight and overall life quality as determined from patient and assessment by a physician).
- By comparing changes in laboratory parameters (Complete Blood Count, Alkaline Phosphatase, SGOT, SGPT and Blood Urea Nitrogen) between 2 leukemic patients’ group.

A total of thirty patients of clinically diagnosed with leukemia cancer, who were subjected to chemotherapy treatment, age ranged between 20-70 years, who meet all the inclusion criteria and none of the exclusion criteria, based on history and clinical examination were recruited in the present study during the screening visit (V1: Day 0). All the patients were signed the informed consent before participating into study. All thirty patients were divided into 2 groups;

- Group-I (ALONE): Including 15 patients and were kept on chemotherapy treatment alone.
- Group-II (WITH WHEAT GRASS): Including 15 patients and were kept on chemotherapy treatment and wheat grass tablets as a supportive treatment. The patients of second group were given wheat grass tablets with dosage regimen of 2 tablets (Wheat grass powder 500 mg), 3 times a day for 270 days (9 months).
Patients of both groups were instructed to visit the facility at every month as follow:

V1: Screening Visit 1: Day 0
V2: Visit 2: Day 30
V3: Visit 3: Day 60
V4: Visit 4: Day 90
V5: Visit 5: Day 120
V6: Visit 6: Day 150
V7: Visit 7: Day 180
V8: Visit 8: Day 210
V9: Visit 9: Day 240
V10: Visit 10: Day 270

On day 0, the blood samples of the suspected patients with leukemia cancer were taken to check the blood laboratory parameters. The patients were recruited into study after the assessment of eligibility criteria. Instructions were given by physician about the dose administration and subsequent visits. On every visit, blood samples of the patients were taken to check effects of wheat grass tablets on blood laboratory parameters. Patients were asked about experienced adverse events and improvement in lifestyle. The improvement in lifestyle was assessed by physical well-being, social/family well-being, emotional well-being and functional well-being. The clinical efficacy was assessed by comparing Side Effect Index (SEI) between and by comparing changes in laboratory parameters.
Side Effect Index (SEI) included headache, nausea, vomiting, bone pain, fever, skin rash, hair loss, mouth ulceration, anorexia, loss of weight and overall life quality as determined from patient and assessment by a physician.

The blood samples were analyzed for Complete Blood Count, Alkaline Phosphatase, SGOT, SGPT and Blood Urea Nitrogen (BUN) at laboratory.

**Statistical Analysis:** The results were presented as mean ± SEM. Statistical difference between the means of various groups was evaluated using student’s paired ‘t’ test. Data were considered as statistically significant at ‘P’ value of 5 % (P ≤ 0.05).