Chapter 2
MATERIALS AND METHODS
2.1 MODELS FOR THE STUDY

2.1.1 Bacterial Model - *Salmonella typhimurium*

Test offered for the study is Ames *Salmonella*/microsome assay. Ames test is the simplest and rapid screen test used to identify chemical mutagens and carcinogens. The organism used is *Salmonella typhimurium*. The scientist Prof. B. N. Ames and his co-workers had genetically engineered these bacteria. The test is highly efficient in detecting carcinogens.

The test detects chemicals by means of their ability to damage DNA and is about 90% accurate in detecting the chemical status. Many carcinogens and non-carcinogens are tested by this method. The economy of the *Salmonella*/mammalian microsome assay suggests its usefulness as a tool in rapidly obtaining information about the potential mutagenic or carcinogenic properties of uncharacterized compound in complex mixture. In this test quantitative information is obtained. The test is quite valuable as bioassay in identifying and purifying complex biological mixtures (Maron & Ames, 1983).

The objective was to evaluate the test compound and/or its metabolites for the ability to induce reverse mutations at the histidine locus in the four strains of the *Salmonella typhimurium* in the presence and absence of an exogenous metabolic activation system (S9) containing mammalian microsomal enzymes. This assay design was based on OECD Guideline 471, updated and adopted in July, 21, 1997.

The sensitivity of the *Salmonella*/microsome test is due to several factors-

1) The tester strains contain, either base pair substitution (strains TA100, TA102) (Levin et al., 1982) or frame shift mutations
(strains TA97a, TA98) (Levin et al., 1982) in the histidine operon. The frame shift mutations are long strings of repetitive bases (-C-G-C-G-C-G-C- or C-C-C-C-) that are ‘HOT SPOTS’ for mutagenesis by certain classes of mutagens.

2) The tester strains contain a mutation that deletes the uvrB gene, which causes loss of the excision repair system. This greatly increases the sensitivity of the test for the detection of many mutagens.

Two of the test systems (TA100 & TA98) were developed by transferring a resistance transfer factor, pKM 101, to strains TA1535 and TA1538 respectively (Mc Cann et al., 1975; Walker and Dobson, 1979).

<table>
<thead>
<tr>
<th>Strain</th>
<th>His-mutation</th>
<th>LPs</th>
<th>Repair</th>
<th>PKM101</th>
<th>Nature of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA 97a</td>
<td>his O 1242</td>
<td>rfa</td>
<td>ΔuvrB</td>
<td>+</td>
<td>+ 4 near CCC</td>
</tr>
<tr>
<td>TA98</td>
<td>his D 3052</td>
<td>rfa</td>
<td>ΔuvrB</td>
<td>+</td>
<td>- 1 near CG</td>
</tr>
<tr>
<td>TA100</td>
<td>his G 46</td>
<td>rfa</td>
<td>ΔuvrB</td>
<td>+</td>
<td>AT→GC</td>
</tr>
<tr>
<td>TA102</td>
<td>PAQ 1 His G 428 / Δ his</td>
<td>rfa</td>
<td></td>
<td>+</td>
<td>GC→AT ochre</td>
</tr>
</tbody>
</table>

These Salmonella strains can be easily preserved on the master plates, and as per the requirements same can be procured (Maron & Ames, 1983.).

For the screening of Vitae Elixxir four different strains were used. These strains were received from BRUCE AMES LABORATORY, Molecular and Cell Biology, 401, Barker Hall, Berkeley, CA 94720-3202. By considering all these parameters Ames test was chosen as one of the evaluating system.
Table 2.2 Study design for Ames *Salmonella* / microsome assay

<table>
<thead>
<tr>
<th>Study</th>
<th>Name of the study</th>
<th>Strains used</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TA97a</td>
</tr>
<tr>
<td>I</td>
<td>Dose Range Finding Study (Spot Assay)</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>Plate Incorporation Assay (with and without S9)</td>
<td>✓</td>
</tr>
<tr>
<td>III</td>
<td>Preincubation Test (with and without S9)</td>
<td>✓</td>
</tr>
<tr>
<td>IV</td>
<td>Modulation Study (with and without S9)</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 2.1: Characterization of *Salmonella typhimurium* strains
2.1.2 Mammalian Model - Mouse

In selection of animal model, various complex factors were taken into consideration. For any biological study, selection of animal model is very important, as the social and ethical implications do not allow human subjects. The animal model should be such, that their biological systems more or less similar to human. The availability, number, life cycle, space required, food etc. should be easily manageable in the laboratory condition and economical. Different authorities worldwide suggested the use of mice, rats, and rabbits for laboratory experiments (Little et al., 1956; Crispens, 1975).

Ideally toxicity test should be conducted with an animal that will elicit compound related toxic response similar to those that occur in man. Animal that metabolizes the test compound identically to man and which has more or less same susceptible organ system/systems are to be used. Under such condition animal data may be extrapolated to man. For the selection of animal species, emphasis was given to metabolism, physiological and biochemical parameters similar to man and the most important criterion that the convenience, economical factors and existing database for the animal.

Mouse and rat is widely used for its small size, and easy handling. Both give many generations within small period and can be easily caged. It requires very less food so comparatively economical and suitable to maintain in the laboratories.

More than 50 million mice and rats are used annually in scientific research and testing areas such as toxicity aging, virology, histocompatibility, haemolytic anemia, congenital defects, neoplasia, genetics, radiobiology, amyloidosis, giardiasis, autoimmune disease, amebiasis, obesity, dwarfism, monoclonal gammopathys, diabetes mellitus, renal disease, behavior and drugs and cosmetics (optional) (Little, 1956).

Mouse satisfied many conditions of choice for this study. For the toxicity study of Vitae Elixxir mouse was used as animal model for all its parameters with equal number of sex, age groups, health condition, body weights and genetic make up for whole study.
The Swiss albino and Balb C strains of the mice were used in the study which were bred and reared in the laboratory. By referring the guidelines such as Gaitonde Commission (India) and OECD (Paris), the equal numbers of sexes were used for complete screening of Vitae Elixxir.

Housing was totally in controlled environment such as temperature, light, humidity, air cycles and number of animals/cage. The acute, subacute and mutagenicity studies were performed using Swiss albino mice. Antitumor activity was performed by using Balb C strain of albino mice. These animals were bred and reared at INTOX. Animals chosen for the studies were randomly assigned, grouped in 3 or 4 or 5 per sex (as per the studies) in polypropylene cages of 5”x6”x10” size with stainless steel top. Animals were identified by cage tag and body marking. Animals were caged with bedding of rice husk. Tap water was provided *ad libitum*. Palleted food of Nav Maharashtra Chakan Oil Mills, Pune was given to the animals with water *ad libitum*. Since the animals were from the same premises and laboratory it was not needed to quarantine/ acclimatization.

Mice were kept in air-conditioned room (22°C, ± 3). Humidity of the room was maintained in between 30 – 70%. Sequence of 12 hours light and 12 hours dark cycles were observed in the animal room.

<table>
<thead>
<tr>
<th>Study</th>
<th>Name Of The Study</th>
<th>Strain</th>
<th>Animal ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Dose range finding</td>
<td>Swiss albino</td>
<td>001 to 016</td>
</tr>
<tr>
<td>II</td>
<td>LD₅₀</td>
<td></td>
<td>017 to 066</td>
</tr>
<tr>
<td>III</td>
<td>Micronucleus test</td>
<td></td>
<td>067 to 096</td>
</tr>
<tr>
<td>IV</td>
<td>Chromosome aberration test</td>
<td></td>
<td>097 to 126</td>
</tr>
<tr>
<td>V</td>
<td>Subacute oral study (28 days)</td>
<td></td>
<td>127 to 150</td>
</tr>
<tr>
<td>VI</td>
<td>Evaluation of antitumor activity</td>
<td>Balb C</td>
<td>151 to 198</td>
</tr>
</tbody>
</table>

Table 2.3 Study design for mouse system
Mouse model- Swiss Albino

Mice cage & spares

Experimental room

Figure 2.2: Animal Model
2.2 TEST MATERIAL & CHEMICALS

2.2.1 TEST MATERIAL

Vitae Elixir


Suggested Use : First day stir 2 drops of extracts into any kind of juice or food, and then every day thereafter increase drops by 2 more additional drops until usage is 6 to 12 drops per day. Divide drops in equal amounts and take after each meal. Maintain this usage for several months or more, or as desired. Then reduce drops to 4 to 6 each day for one year or more. Do not take on an empty stomach. Drink lot of liquids after taking. Maintain traditional nutrition, avoid extreme diets. Shake well before use. Extract does not deteriorate, lose no potency or dose reasonable heat or cold affect it. It is compatible with any product you may be using.
Stains: Removed by lemon juice or bleach.
Manufacturing by U.S.A., Organic Resource Limited, Prince Building, Hong Kong.

**Ingredients:** Chlorophyllin,
Sanguinaria canadensis,
Impatiens pallida,
Hydrastis canadensis,
Ferula galbaniflua,
Hypericum perforatum,
Rubus villosus,
Fumaria officinalis,
Frasera caroliniensis,
Allicin, Garlic.

Figure 2.3: Vitae Elixir
2.2.2 **POSITIVE CONTROLS**

1. **Methyl Methane Sulfonate (MMS)**
   
   Chemical formula: $C_2H_6SO_3$  
   Molecular Weight: 110.13  
   Solubility: Water and DMSO  
   Concentration: 1.3 g/ml  
   Source: Sigma, U.S.A.

   Therapeutic use: Useful in cancer Chemotherapy (Fishbein, 1978).

   Mutagenicity: It is known to be mutagenic in mice in vivo (Heddle & Carrano, 1977) and in human lymphocytes in vitro (Shiraishi & Sandberg 1979a) and in Ames *Salmonella* / microsome assay (Ames et al., 1975).

2. **2- Amino Fluorine (97%) (2-AF)**
   
   Chemical Formula: $C_{13}H_{11}N$  
   Molecular weight: 181.23  
   Source: Lancaster Synthesis

   It is used as positive control in Ames *Salmonella* / microsome assay for the strains such as TA 97a, TA98 and TA100 with metabolic activation (Maron and Ames, 1983).

3. **1, 8- Dihydroxy anthraquinone (95%) (Danthon)**
   
   Chemical Formula : $C_{14}H_8O_4$  
   Molecular weight: 240.22  
   Source : Fluka Chemica

   It is used as Positive control for Ames *Salmonella* / microsome assay, for strain TA102 with metabolic activation (Levin et al., 1984).

4. **4-Nitroquinoline N - Oxide (NQNO)**
   
   Chemical formula: $C_9H_6N_2O_3$  
   Molecular Weight: 190.16  
   Source: Fluka Chemica

   It is used as positive control for Ames *Salmonella* / microsome assay, for the strains such as TA 97a and TA98 without metabolic activation (Maron and Ames, 1983).

   For Media, Reagents, Stains and Other chemicals refer chemical preparation.
2.3 METHODS

2.3.1 IN VITRO METHOD:
AMES SALMONELLA / MICROSOME REVERSE MUTATION ASSAY

The procedure proposed by Dorothy M. Maron and Bruce N. Ames (1983) was used for assessment of mutagenic and/or anti mutagenic potential of Vitae Elixir.

Media and stock solutions: -
The media and stock solutions recommended by Maron and Ames (1983) were used for the present study.
1. Nutrient broth
2. Minimal glucose agar
3. Top agar
4. L - Histidine – HCl
5. D - Biotin
6. L - Histidine – HCl + D - Biotin
7. Nutrient agar
8. Liver homogenate (S9 fraction)
9. S9 mix

Characters of the strains: -
The strains used were Salmonella typhimurium TA 97a, TA98, TA100 and TA102. These are standard bacterial tester strains with different types of
mutations in the histidine operon. All the strains, used in this study, except \textit{S. typhimurium} TA102 are having deletion of a gene coding for the DNA excision repair system. TA102 was constructed primarily for detecting mutagens that require an intact excision repair system. The tester strains, TA 97a, TA98, TA100 and TA102 contain the R – factor plasmid, pKM 101. In addition to this, TA102 also contains the multi-copy plasmid pAQ1 which carries his G 428 mutation and tetracycline resistance gene (Maron and Ames, 1983). All the strains have a lipopolysaccharide barrier on the cell wall (rfa). These two properties confer extra sensitivity to DNA damage and also greater permeability of large molecules into the cell. Plasmid pKM 101 is resistance transfer factor present in these strains, which confers resistance to ampicillin.

The strains used to study of Vitae Elixxir were TA97a, TA98, TA100 and TA102. The strains were tested for cell membrane permeability and antibiotic resistance for ampicillin and tetracycline before initiation of the study.

Sub cultures were grown overnight in the nutrient broth at 37°C and used for the study. These cultures attaining the cell count approximately 1 – 2 $\times 10^9$ cells per ml were used for experiment (Maron & Ames, 1983).

\textbf{Mammalian liver homogenate (S9) :-}

\textbf{Need for metabolic activation :-}

A variety of chemicals require for metabolic activation by enzymes to exhibit their mutagenic potential. It was realized that \textit{Salmonella} lacked the oxidative N-demythylation and N-hydroxylation process (Ames et al., 1973). These processes are carried out enzymetically in the livers by mixed function oxidizes. Hence, it was necessary to supplement the bacterial system with a post mitochondrial (S9) fraction from the homogenate of rodent liver. In general, these oxidizes are nonspecific and are readily induced by treatment with lipophilic substrates. Aroclor 1254 is a lipophilic substrate to induce a wide spectrum of enzymatic activity. The use of metabolic activation with S9 fraction plays important role in confirming the weak mutagenicity of the test article.

For general mutagenesis, liver homogenate (S9) from rat liver was used.
Wistar male rats ranging from 175 to 225 g and 6 – 8 weeks of age were used. All the parameters such as food, water and housing maintained as per the norms. These animals were injected intraperitoneally with polychlorinated biphenyl Aroclor 1254. First two digits of the Aroclor indicates the number of carbon atoms i.e. 12 and last two digits indicates the % of chlorine i.e. 54 (Composition: \( \text{C}_{12}\text{H}_6\text{Cl}_4, \text{C}_{12}\text{H}_4\text{Cl}_4 \)) (Menzie, 1980). The various Aroclors represents a series of technical mixtures.

\[
\begin{align*}
\text{Cl} & \quad X & \quad \text{Cl} & \quad Y \\
X + Y & = 4 \\
X + Y & = 5 \\
X + Y & = 6
\end{align*}
\]

Figure 2.4 : Chemical Structure Aroclor 1254

Stimulation of rat liver : -

Aroclor 1254, used for present study was received from Agharkar Institute, Pune. Mixed function oxidizes systems in the liver were stimulated following an intraperitoneal single injection of Aroclor 1254. Aroclor 1254 was diluted in corn oil. The concentration used for stimulation of liver enzymes was 500 mg/kg body weight of rat. The animals were fed for 5 days with normal diet. Food was removed 10 hours before sacrifice. The rats were killed by cervical dislocation and livers aseptically removed.
Inject single shot of Aroclor 1254 (500mg/kg body weight) intra-peritoneally to the male rats

\[\text{Sacrifice the rats by cervical dislocation on day 6 of administration of Aroclor 1254}\]

\[\text{Excise the livers; wash with chilled 0.15 M KCl to remove hemoglobin.}\]

\[\text{Mince the livers and homogenize with Potter – Elvehjer homogenizer (3 ml KCl: 1g liver)}\]

\[\text{Centrifuge the homogenate at 9000 g for 10 minutes at 4 °C}\]

\[\text{Collect the supernatant, (S9 fraction)}\]

\[\text{Distribute in 2ml aliquots and store at - 80°C.}\]

(All steps were carried out in sterile condition at 0 to 4°C)

Figure 2.5: Flow diagram of S9 preparation
Preparation of liver homogenate (S9) : -

The preparation of homogenate was carried out in sterile atmosphere. The glassware and solutions were maintained at 0 – 4°C. Excised livers were transferred in 0.15M KCl. Livers were washed thrice in 0.15M KCl to remove blood. Livers were weighed and transferred in fresh 0.15M KCl (3 ml KCl/g liver). Further these livers were minced with sterile scissors and homogenizer. The homogenate was centrifuged for 10 minutes at 9000 g and supernatant was stored in sterile 2 ml aliquots. The complete procedure of obtaining liver enzymes was carried out in chilled and sterile condition to avoid destruction of enzymes and contamination respectively. Sterility was checked and protein content was determined before preservation (Ames et al., 1975).

S9 characterization: -

Sterility of S9 fraction was tested by adding 0.5 ml of S9 in the top agar and overlaying it on minimal glucose agar plates. It was allowed to incubate at 37°C for 48 hours. Plates were examined for contamination to confirm sterility. S9 was randomly checked during the experimentation for its protein content and sterility.

The total protein of S9 homogenate was determined by Lowry’s method (Lowry et al., 1951). Protein content was 4.3g/100 ml.

Control groups : -

1. Negative Control: - To check spontaneous reversions of the strain only the respective culture and top agar supplemented with histidine was allow to incubate along with treatment group.

2. Solvent Control: - Solvent used during toxicity and/or mutagenicity testing is called vehicle. Mostly used vehicle in Ames test is either DMSO or water. Similar set of 3 plates of each strain was allowed to run along with treatment groups. It is further labeled as solvent control. Plates containing bacterial culture, DMSO in top agar supplemented with histidine to check the solvent effect.
3. **Positive Control :-** A set of positive controls were run parallel to the test control. The positive control groups used in Ames tests are strain specific. The *Salmonella* strain and respective positive controls are listed in the table below.

**Table 2.4 Positive controls for *Salmonella typhimurium* strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Positive Control With S9</th>
<th>Positive Control Without S9</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA97a</td>
<td>2AF</td>
<td>4NQNO</td>
</tr>
<tr>
<td>TA98</td>
<td>2AF</td>
<td>4NQNO</td>
</tr>
<tr>
<td>TA100</td>
<td>2AF</td>
<td>MMS</td>
</tr>
<tr>
<td>TA102</td>
<td>Danthron</td>
<td>MMS</td>
</tr>
</tbody>
</table>

**Dose Preparation :-**

Vitae Elixxir was dissolved in DMSO at a concentration of 5 mg/ml. 5000 µg/plate was the highest concentration selected for the preliminary cytotoxicity study (Spot test assay).

**SPOT TEST ASSAY :-**

Spot test for dose range finding study was performed. Normally dose range is performed either by filter paper disc method or well preparation by cork borer method. Normally for spot test assay TA98 and TA102 are used. Filter paper disc assay was employed in present study. Spot test assay tells about toxicity of the test substance. A set of minimal glucose agar plates were prepared prior to the experiment. Plates were kept in the incubator upside down for overnight to check for sterility.

Over-night cultures were prepared by transferring a colony from appropriate master plate to a flask containing 100 ml nutrient broth. The cultures were placed in a 37°C shaker – incubator at 120 rpm for overnight till the titer obtained 1–2X10^9 cells per ml. The cultures were harvested on checking the cell count (Friederich et al., 1982). 10 ml of L –Histidine - HCl + D – biotin (0.5mM) were added to 100ml of molten top agar. Into each 10 mm X 100mm sterile
culture tube, 2 ml of top agar was added. The tubes were held in a heating block at 45°C. In the tube containing top agar, 0.1ml *Salmonella* culture was added and mixed thoroughly. The contents of the tube were then poured on to the minimal glucose agar plate, allowed to spread and set evenly on the agar. Two sterile filter paper discs (Whatman No.1) of 5mm diameter were placed on each plate at least 20mm away from each other. On each disc 10µl of freshly prepared concentrations of the test substance were poured. Each disc was labeled with respective concentration and strain on the basal plate.

Plates were kept in fridge for an hour for diffusion of test chemical in top agar. Then the plates were incubated for 24 hours or longer at 37°C. The plates were scanned for a uniform background lawn of auxotrophs and for presence of zone of inhibition along with the colonies (revertants) well away from the disc.

![Figure 2.6 Ames Test – Dose range finding study](image)

**Observation (Spot Test Assay):**

Plates of spot test assay were observed after 24 hours of incubation for the cytotoxicity of the test article and compared with control plates. The zone of inhibition of bacterial growth near the filter paper disc was the criterion to find the dose level. Doses were selected on the basis of zone of inhibition.
**Dose levels :-**

Cytotoxicity of Vitae Elixxir was tested the concentration up to 5000 µg per disc. The 5000 µg per disc was found to be toxic, hence the following doses were selected for the assay; 1000, 500, 100, 50 and 10 µg per plate. The doses were freshly prepared for each experiment.

**PLATE INCORPORATION ASSAY: -**

Quantitative evaluation of Vitae Elixxir was performed by using plate incorporation assay. It was designed to establish relationship between the number of induced revertants and the doses of test substance used. Vitae Elixxir understudy was tested using four tester strains viz. TA 97a, TA98, TA100, TA102. Procedure for *Salmonella* microsome assay described by Maron and Ames (1983) was adopted in this study. The doses selected were 1000, 500, 100, 50 and 10 µg per plate since 5000 µg per disc found to be cytotoxic. A set of minimal glucose agar (MGA) plates was prepared in triplicates for selected doses.

**Without metabolic activation : -**

Micronutrients such as 10 ml of 0.5 mM L–Histidine–HCl + 0.5 mM Biotin were added to 100 ml of molten top agar. In each sterile culture tube 0.1ml *Salmonella* strain suspension, 0.1 ml of freshly prepared doses of Vitae Elixxir and respective vehicle and positive controls were added to the 2 ml top agar. These mixtures were vertexed and the tubes were held in a heating block at 45°C. Then the tubes were overlaid on to the harden surface of 25 ml MGA plates. After solidification of the top agar plates were inverted and kept in incubator at 37°C for 48 ± 6 hours. Each dose was listed in triplicates. The revertant colonies were counted after 48 hours. Pin point to pin head colonies on background lawn were considered during counting as revertants. The colonies were counted using colony counter.

**With metabolic activation – (Rat liver S9 fraction) : -**

Procedure mentioned for metabolic activation was employed as it is with
addition of 0.5 ml of S9 mix to the mixture of top agar. Each dose was listed in triplicates. Observations were made similarly like without metabolic activation.

**PREINCUBATION ASSAY (WITH AND WITH OUT S9) :-**

In this method, respective culture along with Vitae Elixxir was incubated for 30 minutes at room temperature along with and without S9 metabolic activation. Then the same mixture was poured in the plates along with top agar. This modification was first described by Yahagi & his colleagues in 1975.

After solidification of the top agar plates were inverted and kept in incubator at 37°C for 48 ± 6 hours. Each dose was listed in triplicates. Observations were made by counting revertant colonies as described above.

**ANTIMUTAGENICITY TEST :-**

**MODULATION STUDY (WITH AND WITH OUT S9)**

Vitae Elixxir was assessed by one step modification in the preincubation assay. In this assay *Salmonella* strains TA98 and TA100 were treated with Vitae Elixxir and respective positive mutagen with and without microsomal fraction. This mixture was preincubated for 30 minutes at 37°C (Wang & Russel, 1999; Premkumar et al., 2002; Saroj et al., 2002). Further procedure and observations were made as in the plate incorporation assay.

Procedure for *Salmonella* microsome assay described by Maron and Ames (1983) was adopted in this study.

After solidification of the top agar plates were inverted and kept in incubator at 37°C for 48 ± 6 hours. Each dose was listed in triplicates.

Observations were made by counting revertant colonies of the size of pinpoint to pin head on background lawn.
2.3.2 IN VIVO METHODS

A) ACUTE ORAL TEST IN MOUSE (LD$_{50}$)

This study was designed to determine the LD$_{50}$ of the ‘Vitae Elixxir’ or to establish a non-lethal dose of the Vitae Elixxir at limit dose i.e. 5 g/kg body weight of the animal and to study the toxic effects of the Vitae Elixxir with onset, severity.

Dose range was screened by conducting study in four (2M + 2F) mice per group at the doses of 625, 1250, 2500 and 5000 mg/kg body weight. Mice were administered with Vitae Elixxir as a single oral dose and were observed for the incidence of mortality and clinical signs for 7 days (Leclair & Willard, 1970).

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Dose (mg/kg)</th>
<th>Animal No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>1</td>
<td>625</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>1250</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>2500</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>5000</td>
<td>2</td>
</tr>
</tbody>
</table>

Main study :-

On the basis of results of dose range finding study, the main study was conducted. Mice were administered orally with Vitae Elixxir as a single dose and observed for the incidence of mortality and clinical signs for 14 days.
Table 2.6 Acute test : Main study : Allotment of animals

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Dose (mg/kg)</th>
<th>Animal No.</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>628</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>995</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>1577</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>2500</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

**Dose preparation :-**

Doses of the Vitae Elixir were prepared shortly before dose administration. 2500 mg of Vitae Elixir was weighed accurately and homogenized in the pestle mortar by adding distilled water gradually. The final volume is made 10 ml. A series of dilutions were prepared for the doses of 628, 995, 1577 and 2500 mg/kg and maintained the constant dosage volume rate of 10ml/kg body weight.

**Administration of test article :-**

The animals were dosed by oral gavage using 1ml graduated syringe with stainless steel intubation needle (16 G). The dosage volume administered to individual mouse was adjusted according to its most recently recorded body weight. The control group mice received the vehicle (distilled water) only, by oral gavage at the same dosage volume of 10ml/kg body weight.

**Observations**

Following observations were made during the course of treatment.

**Mortality**

On the day of dosing, all animals were observed for mortality at 30 min, 1, 2, 4 and 6 hours following dosing and thereafter they were observed daily once for 14 days.
Clinical signs

The treated animals were observed for signs of intoxication, at 30 min, 1, 2, 4 and 6 hours after treatment and thereafter once a day for 14 days. The appearance, progress and disappearance of these signs were recorded.

The animals were examined particularly for changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity such as lacrimation, piloerection, pupil size and unusual respiratory pattern. Changes, if any, in gait, posture and responses to handling as well as the presence of clonic or tonic movements, stereotypies or bizarre behaviour were also recorded.

Body Weights

The animals were weighed individually on the day of the treatment and weekly thereafter. Group mean body weights and individual group mean percent gain in body weight over basal values were calculated.

Necropsy

Necropsy was performed on all dead animals during the course of the test and sacrified at the termination of the test. Gross pathological changes were recorded.

LD_{50} Calculation

LD_{50} value with fiducial limits at 95% confidence level was calculated following method of Litchfield & Wilcoxon (1947).
Feeding syringe with canula

Animal ID by body marking

Oral gavage

Figure 2.7 Force feeding spares and animal handling
B) MICRONUCLEUS TEST IN MOUSE BONE MARROW

The protocol proposed by Schmid (1975) was used with few modifications. Total 30 mice of 25.4 ± 2.4g body weight, (15 males and 15 females) were assigned for the experiment. For each dose 6 animals were employed (3 males, 3 females) and equal numbers of animals were used for positive and vehicle control groups.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Dose (mg/kg)</th>
<th>Animal No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>1</td>
<td>0.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>40.0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>125.0</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>312.5</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>625.0</td>
<td>3</td>
</tr>
</tbody>
</table>

The treatment doses were selected from the data obtained from acute toxicity of the Vitae Elixxir. Doses were selected for in vivo mutagenicity tests (micronucleus test and chromosomal assay) based on LD$_{50}$ value (Unknown author, 1990).

<table>
<thead>
<tr>
<th>Dose</th>
<th>% of LD$_{50}$</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>--</td>
<td>0.0</td>
</tr>
<tr>
<td>Positive Control</td>
<td>--</td>
<td>40.0</td>
</tr>
<tr>
<td>Low</td>
<td>10 % of LD$_{50}$</td>
<td>125.0</td>
</tr>
<tr>
<td>Intermediate</td>
<td>25 % of LD$_{50}$</td>
<td>312.5</td>
</tr>
<tr>
<td>High</td>
<td>50 % of LD$_{50}$</td>
<td>625.0</td>
</tr>
</tbody>
</table>
**Dose preparation :-**

Doses of the Vitae Elixxir were freshly prepared before treatment on each day. 625 mg Vitae Elixxir was weighed accurately and homogenized in the pestle mortar by adding distilled water gradually. The final volume is made 10 ml and labeled as original stock. Original stock was used as high dose i.e. 625 mg/kg body weight. A series of dilutions were prepared using original stock for further doses (312.5 and 125.0 mg/kg body weight) and maintained the constant dosage volume rate of 10ml/kg body weight.

**Administration of test article:-**

All the animals were fasted for four hours prior to the administration of test article. Vitae Elixxir was administered orally at 0th hour and subsequently after 24 hour by oral gavage at approximately the same time both the days using a graduated syringe and a stainless steel intubation needle. The dosage volume administered to individual mouse was adjusted according to its most recently recorded body weight. The control group mice received the vehicle (distilled water), by oral gavage at the rate of 10ml/kg body weight. Positive controls were run in parallel and orally fed with Methyl Methane Sulphonate (MMS), at a dose of 40 mg/kg body weight.

**Dissection and handling of bone marrow: -**

The animals were sacrificed at 48th hours after the first treatment by cervical dislocation as per the procedure suggested by Kliesch et al. (1981). Both the femora were carefully removed. The femora were cleaned by tissue paper to remove adhering muscles and tissues. The proximal and distal end of each femur was cut to an extent just to expose the marrow cavity. A syringe along with 26-G needle, filled with 2 ml of goat serum was passed through the marrow cavity. The cells were directly aspirated in the clean and dry test tube. The tubes containing marrow cells were centrifuged at 1000 rpm. Supernatant was discarded and sediments of cells were resuspended by gentle tapping in 0.5 ml of goat serum. The slides were prepared by taking a drop of cell suspension on slide by blood smear
technique. A drop of this cell suspension was used to obtain a thin uniform smear of the cells. Three slides were prepared for each mouse. The slides were allowed to dry in a dust free area for overnight.

**Staining and screening of bone marrow smears:**

70% ethanol was used to fix the smear. Bone marrow smear were stained with undiluted May-Grünwald solution for 3 minutes followed by 2 more minutes in 1:1 diluted May-Gruenwald : Distilled water. Further the slides were stained with Giemsa stain diluted with distilled water (1:6). Slides were rinsed with distilled water and air dried. Stained dried slides were scored after coding of slides. Coded slides were scored. From each animal about 1000 polychromatic erythrocytes (PCEs) were scored for micronuclei if any and subsequent number of normochromatic erythrocytes (NCEs). The ratio of PCE to NCE was determined. This ratio was used as a parameter to judge the cytotoxicity of Vitae Elixxir. Micronucleated PCEs were used as an index for the clastogenic ability of Vitae Elixxir.

Student t test was employed for assessing statistical significance of the result. Increases in the frequency in micronuclei induction above the vehicle control frequency that are significant at P< 0.05 were considered to be indicative of an active clastogenic activity (Gad & Weil, 1982).
C) CHROMOSOMAL ABERRATION TEST IN MOUSE BONE MARROW

The animal number, dose groups of animals, selection of doses, dose preparation, dosing schedule, the route of administration of Vitae Elixxir and controls were same as in vivo micronucleus test in mouse bone marrow.

Total 30 mice of 24.6 ± 2.8g body weight, (15 males and 15 females) were assigned for the experiment. For each dose 6 animals were employed (3 males, 3 females) and equal numbers of animals were used for positive and vehicle control groups.

Table 2.9 Chromosomal aberration test : Allotment of animals

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Dose group</th>
<th>Dose (mg/kg)</th>
<th>Animal No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>0.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Positive Control</td>
<td>40.0</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Low</td>
<td>125.0</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Intermediate</td>
<td>312.5</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>High</td>
<td>625.0</td>
<td>3</td>
</tr>
</tbody>
</table>

The dosage volume administered to individual mouse was adjusted according to its most recently recorded body weight. The volume of Vitae Elixxir was kept 10 ml/kg. The vehicle control group mice received distilled water only, by oral gavage at the volume of 10ml/kg body weight. Positive controls were run in parallel with Methyl Methane Sulphonate (MMS), at concentration of 40 mg/kg body weight. 46 hours after the 2\textsuperscript{nd} treatment animals were subjected to the colchicine treatment.
Colchicine Treatment: -

Colchicine is a spindle poison, which arrests the metaphases after specific time interval. In mouse it takes 2 hours to arrest the metaphases. All animals were treated with intraperitoneal colchicine treatment to arrest the metaphases 2 hours prior to the necropsy i.e. 46 hours after administration of Vitae Elixxir. Colchicine was injected intraperitoneally with tuberculin syringe (Top, India) mounted with 26G needle. The concentration of colchicine was 4 mg/kg body weight at 46th hour of first oral administration of the Vitae Elixxir (EHC 51, 1985).

Dissection and handling of bone marrow: -

48 hours after the treatment i.e. 2 hours after colchicine treatment animals were sacrificed by cervical dislocation. Both the femora were removed, cleaned with the help of tissue paper. Both the ends of femora were cut to expose marrow cavity. Bone marrow was slowly aspirated in 2 ml of 2.2% pre-warmed sodium citrate (Das and Kar, 1980) using 26G needle. 2.2% sodium citrate acts as isotonic solution for the marrow cells. The suspension was centrifuged at 1000 rpm for 10 min. Supernatant was discarded and the cells were transferred to the 2 ml of 1.1% sodium citrate. The cells were allowed to mix by gentle tapping and suspension was incubated at room temperature for 15 minutes (Adler, 1984). 1.1% sodium citrate acts as hypotonic solution. Further the tubes were centrifuged at 1000 rpm for 5 min. The supernatant was discarded and chilled methanol: glacial acetic acid was added to it (methanol 3 parts, glacial acetic acid 1 part). This mixture act as fixative and fixes the cells. After two washing of the chilled fixative the cell suspension was kept overnight in chilled condition (at 4°C). On the following day, the suspension was centrifuged at 1000 rpm. The supernatant was discarded. The palette was resuspended in 0.5 ml of freshly prepared chilled fixative. From this cell suspension slides were prepared by air drop method keeping slide at 45º angle to the floor and dropping the cell suspension about 30-40cm away from slide with the help of fine pasture pipette. Slides used for preparation were absolutely clean, grease free and chilled in absolute alcohol (EHC 51, 1985).
Staining and screening of bone marrow smears :-

Air dried slides from dust free area further processed for Giemsa staining (6%). The stained coded slides were scored for chromosome abnormalities, if any, under binocular microscope by using 100 X oil immersion objective. 100 metaphases were scored per animal and stage reading was recorded for the same (Snell, 1956). The chromatid breaks, fragments and gaps were observed and recorded from the slides.

Chromatid gaps were counted separately, but not considered as chromosomal anomalies, therefore excluded from the statistical analysis.

Student ‘t’ test was employed for assessing statistical significance of the results. Increase in the frequency in aberration above the vehicle control frequency that are significant at $P< 0.05$ were considered to be indicative of an active clastogenic activity.
D) SUBACUTE ORAL STUDY IN MOUSE (28 DAYS)

Subacute (28 days) oral study was performed to assess the toxicological profile of Vitae Elixxir after repeated administration in mouse. It provides information on the possible health hazards likely to arise after repeated exposure over a relatively limited period of time.

The results of subacute study should provide information on target organs, the possibilities of cumulation if any and can provide an estimate of a no observed adverse effect level (NOAEL) of exposure which can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure. The housing is provided as mention in animal model.

Selection of dose levels :-

Dose levels for the subacute study were selected on the basis of acute oral toxicity of Vitae Elixxir. Based on LD\textsubscript{50} value (1250 mg/kg body weight) obtained from acute toxicity studies and severity of clinical signs, the selection of doses for subacute toxicity study was carried out. The dose selected for subacute toxicity study ranges from 20 to 200 mg/kg body weight. The doses were calculated with the difference of 0.5 log doses of 628 mg/kg dose of acute test which showed cyanosis and recovered after 4 hours without mortality. The doses calculated were 200, 63 and 20 mg/kg body weight as high, intermediate and low dose groups.

Table 2.10 Subacute study : Selection of doses

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Dose group</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Low</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Intermediate</td>
<td>63</td>
</tr>
<tr>
<td>5</td>
<td>High</td>
<td>200</td>
</tr>
</tbody>
</table>
Study design :-

Three Swiss albino mice per sex per group were administered Vitae Elixxir by oral gavage daily for 28 days at 10 ml/kg dose volume. Concurrent control group receiving the vehicle (distilled water) was also maintained. The doses of Vitae Elixxir were prepared according to LD\(_{50}\) values.

Table 2.11 Subacute : Allotment of animals

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Dose Group</th>
<th>Dose (mg/kg)</th>
<th>Animal No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>0</td>
<td>Male: 3</td>
</tr>
<tr>
<td>1</td>
<td>Low</td>
<td>20</td>
<td>Male: 3</td>
</tr>
<tr>
<td>2</td>
<td>Intermediate</td>
<td>63</td>
<td>Male: 3</td>
</tr>
<tr>
<td>3</td>
<td>High</td>
<td>200</td>
<td>Male: 3</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dose Preparation :-

200 mg Vitae Elixxir was weighed accurately and homogenized in the pestle mortar by adding distilled water gradually. The final volume is made 10 ml. A series of doses were prepared by the dilutions, for the doses of 20, 63 and 200 mg/kg body weight. Doses of the Vitae Elixxir were prepared freshly before dosing on each day.

Administration of test article :-

Animals were force fed by oral gavage at approximately the same time each day, using a graduated syringe and a stainless steel intubation needle (16G). The dosage volume administered to individual mouse was adjusted according to its most recently recorded body weight. The control group mice received the vehicle (distilled water) only, by oral gavage at the same dosage volume of 10ml/kg body weight. Treatment in this study was continued once a day, seven days a week, for a total period of 28 days.
Observation :-
Following observations were made during the course of treatment period.

Mortality
Throughout the study all animals were supervised twice a day to look for dead or moribund to allow necropsy and gross pathological examination to be carried out.

Clinical Signs
All signs of ill health, together with any behavioural changes or reaction to treatment were recorded for individual animals.

Body Weights
The weight of each mouse was recorded at the time of allocation of the animal to groups, i.e. on day zero prior to the day of commencement of treatment, twice in a week thereafter and at the time of necropsy.

Food Consumption
The quantity of food consumed by mice in each cage was recorded on the day of commencement of treatment and twice a week thereafter. Food intake per mouse was calculated using the amount of food offered to and left in each cage after 24 hours and the number of mice surviving in each cage.

Pathology
Urine analysis
Urine analysis was performed on all animals before termination of the treatment period. Urine samples were collected using a battery of specially designed stainless steel urine collection cages. Each mouse was housed in this cage. Urine samples were collected over a period of 4 hours. Food and water was not offered during this period.
Tests were performed using Multistix® (SG Multiple Reagent Diagnostic Strips manufactured by Bayer Diagnostics India Ltd., Baroda, India) and were
used as qualitative / semi-quantitative indicators to analyze concentration.

**Clinical Pathology**

Food was removed overnight from animals to be sampled for laboratory investigations. Samples of blood were withdrawn at termination of treatment under carbon dioxide anaesthesia, from the retro-orbital plexus of all mice using heparinized glass capillary. The samples were collected in tubes containing heparin (for clinical chemistry) and K$_2$-EDTA (for haematology) as an anticoagulant. Blood smears were also prepared for differential count and gross morphological observations of all the blood cells. Clinical chemistry samples were centrifuged at a speed of 5000 rpm for 5 minutes.

**Haematology**

Haematological parameters were studied at pre-treatment and post-treatment using 'Coulter AC.T diff’ Haematology Analyzer (Beckman Coulter, Inc., Miami, Florida, USA):

- Haemoglobin (Hb) (g/dl)
- Packed cell volume (PCV) (%)
- Total red cell count (Total RBC) (x10$^6$/mm$^3$)
- Total white cell count (Total WBC) (x10$^3$/mm$^3$)
- Platelet Count, Total (Platelets) (x10$^5$/mm$^3$)

Following absolute erythrocyte indices were calculated:

- Mean corpuscular volume (MCV) (fl)
- Mean corpuscular haemoglobin (MCH) (pg)
- Mean corpuscular haemoglobin concentration (MCHC) (g/dl)

Differential WBC counts: Were determined by microscopy of blood smear, stained with Leishman’s stain, counting 100 cells –

<table>
<thead>
<tr>
<th>Category</th>
<th>%</th>
<th>Category</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils (N)</td>
<td></td>
<td>Lymphocytes (L)</td>
<td></td>
</tr>
<tr>
<td>Eosinophils (E)</td>
<td></td>
<td>Monocytes (M)</td>
<td></td>
</tr>
</tbody>
</table>
Clinical Chemistry

Serum chemistry parameters, with their units of measurement as listed below, were analysed using the "Erba Smartlab Random Access Batch Analyser / Erba EC5 Plus Analyser" (Transasia Bio-Medicals Ltd., India) using standard methodology:

- Glucose (Gluc) (mg/dl)
- Blood Urea Nitrogen (BUN) (mg/dl)
- Total Protein (TP) (g/dl)
- Albumin (Albu) (g/dl)
- Serum Glutamate Pyruvate Transaminase (SGPT) (IU/L)
- Serum Glutamate Oxaloacetate Transaminase (SGOT) (IU/L)

Terminal Studies

Necropsy Examination

On completion of 28 days of treatment, all surviving mice were sacrificed by exsanguinations under carbon dioxide anesthesia and were subjected to complete necropsy. The necropsy performed at termination of the treatment was staggered. The tissues such as liver, spleen, kidney and testes/ovary from all animals, were preserved, in 10% neutral buffered formalin. In addition, samples of any macroscopically abnormal tissues were routinely preserved, along with samples of adjacent tissue where appropriate.

Organ Weights

Following organs, from all animals sacrificed at the scheduled, were dissected free of fat and weighed wet as soon as possible to avoid drying (Feron, 1969) and relative organ weights were calculated as percent body weights.

- Testes/ovaries,
- spleen,
- kidney,
- liver

Histopathological Examination

Tissues that were subjected to microscopic examination embedded in paraffin wax, sectioned at five micron and stained with haematoxylin and eosin (Elizabeth, 1956; Kiernan, 1990). A full histopathological examination was conducted on the specified list of tissues including all macroscopically abnormal
tissues of all animals from the control and the high dosage level groups, sacrificed at termination. Additional tissues, of animals from other groups, which exhibited gross pathological changes at necropsy, were also subjected to histopathological evaluation.

**Statistical Analysis**

The experimental results were expressed as mean±SD. Data were assessed by the method of analysis of ANOVA followed by Dunnett’s test. P<0.05 was considered as statistically significant.
Figure 2.8: Subacute test: Terminal Procedure (Necropsy)
E) **ANTITUMOR ACTIVITY** :-

Anticancer agents are generally toxic and more precisely mutagenic. They are selected on the basis of toxicity towards cancer cell lines and animal tumor models. It is the challenge to design the therapy with highly specific and efficient to the targeted tumor tissues. None of the drug fulfills the criteria. Cytotoxicity of the drug is limited to differentiate between normal and malignant cells.

**Study design**

Balb C mice were chosen for this study. The body weight was ranging from 20 to 24 g at the time of initiation of the study. All the individuals were of female sex since the compound was mainly meant for breast cancer and the females are more prone to this. All other parameters such as housing, caging, animal marking were same as subacute study. Animals were randomized to obtain six groups as follows.

**Table 2.12 Antitumor activity : Selection of doses***

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Dose Group</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>Water</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Tumor control</td>
<td>Tumor cells</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Drug control</td>
<td>Tumor cells + Cyclophosphamide</td>
<td>250</td>
</tr>
<tr>
<td>4</td>
<td>Low</td>
<td>Tumor cells + Vitae Elixxir</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>Intermediate</td>
<td>Tumor cells + Vitae Elixxir</td>
<td>63</td>
</tr>
<tr>
<td>6</td>
<td>High</td>
<td>Tumor cells + Vitae Elixxir</td>
<td>200</td>
</tr>
</tbody>
</table>

**Table 2.13 Antitumor activity : Allotment of animals***

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Dose Group</th>
<th>Treatment</th>
<th>Animal No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>Water</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Tumor control</td>
<td>Tumor cells</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Drug control</td>
<td>Tumor cells + Cyclophosphamide</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Low</td>
<td>Tumor cells + Vitae Elixxir</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>Intermediate</td>
<td>Tumor cells + Vitae Elixxir</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>High</td>
<td>Tumor cells + Vitae Elixxir</td>
<td>6</td>
</tr>
</tbody>
</table>
On day 16, blood was withdrawn by heparinizied capillary under anesthesia, from retro-orbital plexus of the mouse. The samples were collected in heparinized tubes.

**Preparation of tumor model**

The sarcoma 180 cell line no. S180 was issued from ATCC national facility, Pune university campus, Pune-07. This cell line derived from the sarcoma 180 ascitis of the Swiss Webster. The working culture was brought in the Minimal Essential Medium (MEM) with Earle’s Hanks’ Balanced Salts Solution (HBSS) and Fetal calf serum. The cell count was $2 \times 10^6$ viable cells/ml. This diluted culture was inoculated intraperitoneal (IP) region of the Balb C mice except plain control group.

**Administration of drugs**

Tumor was implanted in each animal through intraperitoneal injection of S180 cell at the count of $2 \times 10^6$ cells/ml. on $0^{th}$ day tumor was allow to grow in the peritoneal cavity for 5 days from the implantation. After day 6, animals were received Vitae Elixxir orally till day 28 at the concentration of 20, 63 and 200 mg/kg body weight. Drug control animals received cyclophosphamide as standard drug. Tumor and vehicle controls animals received distilled water at 10ml/kg body weight.

**Observation**

**Mortality**

Throughout the study all animals were supervised twice a day to look for dead or moribund to allow necropsy and gross pathological examination to be carried out.

**Body Weight**

The body weights of each animal were recorded during pre-treatment, on the day of commencement of the treatment and daily thereafter to monitor the development of the tumor.
Food consumption
The quantity of food consumed by mice in each cage was recorded on the day of commencement of treatment and twice a week thereafter.

Haematology
Haematological parameters were studied at pre-treatment and post-treatment using 'Coulter AC.T diff' Haematology Analyzer (Beckman Coulter, Inc., Miami, Florida, USA):

Haemoglobin (Hb) (g/dl)

Packed cell volume (PCV) (%)

Total red cell count (Total RBC) (x10⁶/mm³)

Total white cell count (Total WBC) (x10⁹/mm³)

Platelet Count, Total (Platelets) (x10⁵/mm³)

Following absolute erythrocyte indices were calculated:

Mean corpuscular volume (MCV) (fl)

Mean corpuscular haemoglobin (MCH) (pg)

Mean corpuscular haemoglobin concentration (MCHC) (g/dl)

Differential WBC counts: Were determined by microscopy of blood smear, stained with Leishman’s stain, counting 100 cells –

Neutrophils (N) %

Lymphocytes (L) %

Eosinophils (E) %

Monocytes (M) %

Assessment of the activity
The inhibitory rate (%) against growth of tumor was calculated by the formula (Chihara et al., 1970) –

Inhibitory rate (%) = [(A-B)/A] X 100

A - Avarage tumor weight of tumor control group

B - Avarage tumor weight of treatment group
Tumor growth response

The effect of Vitae Elixxir on tumor growth was estimated by evaluating % increase in life span (ILS) of Balb C mice (Ahluwalia et al., 1984).

Increase in Life Span (ILS) % = [(Mean survival time of treated group/ mean survival time of control group)-1] X 100

The mean survival time and spleen indices were calculated (Gothoskar and Ranadive, 1971; Vijayabaskaran et al, 2010).

Mean Survival Time (MST) = (Day of First Death + Day of Last Death)/2

Spleen Index (mg/g) = Spleen weight / Body weight (Zhao et al, 2002)

Statistical Analysis

The experimental results were expressed as mean±SD. Data were assessed by the method of analysis of ANOVA followed by Dunnett’s test. P<0.05 was considered as statistically significant.

Figure 2.9: Sarcoma 180 bearing Balb C mice