3.1. Introduction:

Cancer is the second leading cause of death worldwide (Amin et al., 2009). Cancer chemoprevention is a relatively new concept. The pioneering work to reduce cancer incidence by chemical intervention was initiated by the groups of Wattenberg and Sporn in the early 1960s and 1970s (Wattenberg, 1985; Sporn 1993) Later, scientists have embraced the concept of cancer chemoprevention as a distinct new discipline of oncology (Kelloff et al., 1994; Greenwald et al., 1995; Hong and Sporn, 1997; Stoner et al., 1997; Trignali, 2001).

Conventional cancer therapies cause serious side effects and, at best, merely extend the patient’s lifespan by a few years. Cancer control may therefore benefit from the potential that resides in alternative therapies. The demand to utilize alternative concepts or approaches to the treatment of cancer is therefore escalating. There is compelling evidence from epidemiological and experimental studies that highlight the importance of compounds derived from plants “phytochemicals” to reduce the risk of colon cancer and inhibit the development and spread of tumors in experimental animals. More than 25% of drugs used during the last 20 years are directly derived from plants, while the other 25% are chemically altered natural products. Still, only 5-15% of the approximately 250,000 higher plants have ever been investigated for bioactive compounds. The advantage of using such compounds for cancer treatment is their relatively non-toxic nature and availability in an ingestive form. An ideal phytochemical is one that possesses anti-tumor properties with minimal toxicity and has a defined mechanism of action. As compounds that target specific signaling pathways are identified, researchers can envisage novel therapeutic approaches as well as a better understanding of the pathways involved in disease (Amin et al., 2009).
Plants have a long history of use in the treatment of cancer and played an important role as a source of effective anti-cancer agents. Over 60% of currently available anticancer agents are derived in one way or another from natural sources, including plants, marine organisms and microorganisms (Newman et al., 2003; Cragg and Newman, 2005; Cragg et al., 2005). The search for anticancer agents from plant sources started in earnest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic phodophyllotoxins. As a result, United States National Cancer Institute (NCI) initiated an extensive plant collection program in 1960, focused mainly in temperate regions. This lead to the discovery of many novel chemotypes showing a range of cytotoxic activities (Cassaday and Douros, 1980), including the taxanes and camptothecins, but their development into clinically active agents spanned a period of some 30 years, from the early 1960s to the 1990s. This plant collection program was terminated in 1982, but the development of new screening technologies led to the revival of collections of plants and other organisms in 1986, with a focus on the tropical and sub-tropical regions of the world. It is interesting to note, however that no new plant-derived clinical anti-cancer agents have, as yet, reached the stage of general use, but a number of agents are in preclinical development (Cragg and Newman, 2005).

Oxidative stresses are related with various diseases and pathological conditions such as aging, atherosclerosis and cancer (Cadenas and Davies, 2000; Aviram, 2000; Kogurre et al., 2004). Antioxidants may prevent these chronic disease by several mechanisms such as enzymic degradation of free radicals, chelation of metals which stimulate the production of free radicals and scavenging the free radicals (Penckofer et al., 2002). Medicinal plants are considered to be the important source of antioxidant compounds, and recently there has been considerable interest in finding
the natural antioxidants from plant materials to replace synthetic ones (Mehdipour et al., 2006).

Phytotherapeutic products are many times mistakenly regarded as safe because they are natural (Gester, 1992). Nevertheless, those products contain bioactive principles with potential to cause adverse effects (Bent and Ko, 2004). In addition poor pharmacovigilance services of this area make it difficult to determine the frequency of adverse effects caused by the use of phytotherapeutic products (Eisnberg et al., 1998). Thus all the natural products used in the therapeutics must be submitted to efficiency and safety tests by the same methods used for new synthetic drugs (Talay and Talay, 2001; Fères et al., 2006).

In the present study, we report for the first time the cytotoxic, anti-tumor, anti oxidant as well as the acute toxicity studies of aerial parts of different extracts of T. involucrata.

3.2. Materials and Methods:

3.2.1. Cell lines and Chemicals:

BHK, Vero, MCF-7 and KB cell lines were obtained from the repositories of National Center for Cell Sciences (NCCS), Department of Biotechnology, Pune, India.

**Sigma Chemical Co, USA:** Cell proliferation ELISA-MTT (colorimetric) kit, 2,2-diphenyl-1-picryl hydrzyl(DPPH) and 22’-azinobis(3-ethylbenzo-thozoline-6-sulphonic acid) diammonium salt(ABTS), Tween -20.

terpenoid, HPBT.

**E-Merc, India:** propylene glycol, Ascorbic acid, Sulpanilamide, Hydrochloric acid, Calbiochem, USA: MTT: (3-[4, 5-Dimethylthiazol-2yl]-2, 5-diphenyltetrazoliumbromide).
3.2.2. Preparation of Compounds:

Compounds were freshly prepared by dissolving in DMSO to a concentration of 200mg/ml and further diluted with RPMI-1640/DMEM for the experiments. The control cultures were treated with equivalent concentrations of DMSO alone, which up to the highest dose (0.1%), had no significant effect on any of the parameters measured.

3.2.3. Cell Culture:

BHK, Vero and KB cells were maintained in RPMI-1640 medium. MCF-7 was maintained in DMEM medium. All these media were supplemented with 10% FCS, penicillin G100 IU/ml and streptomycin 100μg/ml. All cell lines were maintained and sub cultured in 75 cm² cell culture flasks and was incubated at 37°C in a humidified 5% CO₂ air atmosphere. Every third day the medium was removed and supplemented with fresh medium. For passaging 70% confluent, cells were rinsed with PBS and then incubated with 3 ml of trypsin-EDTA solution at 37°C for 5 min. After detachment from the flask, cells were harvested in 10 ml RPMI-1640/DMEM/10%FCS. The cells were then pelleted at 500 X g for 5 min. The supernatant was aspirated and the cells were resuspended in the respective media and
counted in hemocytometer using trypan blue. Approximately 10,000 cells / cm\(^2\) were seeded in new flask for further studies.

3.2.4. MTT Solution:

MTT [3-(4, 5-demethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide] was prepared as a 5 mg/ml stock in PBS and stored for not more than 2 weeks in dark at 4°C. Before use it was filtered through a 0.22 μm filter to remove any blue formazan product formed and diluted with RPMI-1640 to 1 mg/ml (Danizot and Lang, 1986).

3.2.5. MTT Assay:

Cells in exponential growth phase were cultured in half-area flat-bottomed microtitre trays (NUNC, USA) at the volume of 0.1 ml (1 X 10\(^6\) ml\(^{-1}\)). After allowing 1h for the cells to attach, plant extract in different concentrations in the range of 0.1-10 μM (100 μl) were added to the wells and incubated in 5% CO\(_2\) air atmosphere with high humidity for 48 h. To each well 50 μl of a 1 mg/ml solution of MTT in RPMI-1640/DMEM medium was added. The culture plate was gently shaken and incubated for further 4 h at 37 °C. At the end of incubation period the culture plate was centrifuged (800 X g, 5 min) and untransformed MTT was removed carefully by blotting the culture plate. Propanol (50 μl) was then added to each well. The plate was vigorously shaken to ensure that the blue formazan was completely dissolved (Danizot and Lang, 1986). The optical density of each well was measured with an automatic plate reader at a test wavelength of 570 nm and a reference wavelength of 650 nm. The inhibitory rate was calculated as follows:

\[
\text{Inhibitory rate } \% = \frac{(\text{Absorption}_{\text{control}} - \text{Absorption}_{\text{test}})}{(\text{Absorption}_{\text{control}})} \times 100
\]
IC$_{50}$ was defined as the concentration of plant extracts killing 50% of the cells. IC$_{50}$ was determined for all four-cell lines.

3.2.6. *In vivo* Anti-tumor Activity: Determination of Acute Toxicity and Therapeutic Doses:

3.2.6.1. Mice and Tumor Model:

Swiss albino mice of either sex (8-10 weeks old) weighing 20-25 g, were used for the experiment. The animals were maintained under proper environmental conditions i.e., temperature 25 ± 2°C and humidity 50 ± 5% with a 12 h light and dark period. They were housed in polypropylene shoebox type cages with stainless steel grill top, bedded with rice husk. The animals were provided with pelleted diet (Gold Mohur, Lipton, India) and water *ad libitum*. Ten animals were used in each control and treated group. Swiss albino mice of either sex were used for implanting Erlich ascitis tumor model.

3.2.6.2. Acute toxicity:

HPBT was dissolved in propylene glycol: water (1:4) and terpenoid was dissolved in 1%Twenn 20 in PBS. Acute toxicity was assessed on healthy Swiss albino mice of either sex (8-10 weeks old) weighing 20-25 g, in one ip, administration. The drug dosage was fixed according to OECD/OCDE guidelines no.420 (*Fig.1.*), the mice were observed continuously for 1 h for any gross behavioral changes and deaths intermittently for the next 6 h and then again 24h after dosing. LD$_{50}$ of both extracts was calculated according to OECD guidelines. The behavior parameters observed were convulsion, hyperactivity, sedation, grooming, loss of righting reflex, increased or decreased respiration, food water intake etc.
3.2.6.3. Short-term toxicity:

To determine short-term (14 days) toxicity, groups of 8 mice each, were administered, daily 60, 75 and 90mg/kg, ip., of HPBT for 14 days. Control animals were treated with vehicle in similar way. Body weight, food and water intake and general behavior were monitored. After the treatment period, mice were sacrificed, blood was collected and important internal organs were removed, weighed and observed for pathological changes. Mice, which received 150mg/kg extract, did not survive for more than 3 days. Therefore, for this dose, another group of 6 animals were given a single dose (150 mg/kg) and the animals were sacrificed 24 h after dosing, (along with the other 3 groups). Blood hematological parameters were determined (Jain et al., 1986). Serum glutamate pyruvate transaminase (SGPT) and glutamate oxaloacetate transaminase (SGOT) were determined by the method of Reitman and Frankel (1960), using commercial assay kits. Urea was estimated by enzymatic method (Tietz, 1987), using assay kit (Wipro Biomed, India). Calcium was estimated by o-cresolphthalain complex method (Tietz, 1987). Phosphorus was estimated by calorimetric method (Henry, 1974), using commercial reagents (Reagents Applications, Irk, San Diego, CA).

3.2.6.4 Experimental Chemotherapy:

The study protocol was approved by the ethics committee. Mice (6-8 week old) were challenged with Ehrlich Ascitic carcinoma (EAC) cells (10^6 cells/mouse; subcutaneously) on day zero. The treatment began the day after tumor inoculation, and test compounds (HPBT and terpenoid) and vehicle were administered ip using ten mice per test group. Routinely, the compounds were administered five times: on the day after tumour inoculation (D1), on day D3, D5, D7, D9 and D11 (Scheme D1, 3, 5,
7 and 9). The compounds were also administered once (D1, single dose). In all chemotherapy trials mice were checked daily, with any adverse clinical reactions noted and deaths recorded. Mice were weighed 2-4 times weekly during treatment and once weekly thereafter. Tumors were measured by calipers twice weekly and tumor volume (mm$^3$) were estimated as $= 0.5$ (Length $\times$ Width$^2$). Results are presented for experiments involving ten mice per experimental group.

3.2.6.5. Evaluation of Anti-tumor Activity: Life Span:

Mortality was noted every day and the median life span was calculated as:

$$\text{MLS} = \frac{\text{Dm} + (\text{Mm} - \text{number of mice dead before Dm})}{\text{Number of mice dead on Dm}}$$

The median mouse (Mm) separates into two identical groups (one group, including the mice that died before Mm, the other group including those who died after) and the median day (Dm) is the day Mm died. Mice surviving for at least 45 days were considered as cured and were included in the calculation of the median life span. Compounds efficiency was expressed by T/C as follows:

$$\text{T/C}\% = \left( \frac{\text{MLS of treated animals}}{\text{MLS of control animals}} \right) \times 100$$

Or by the increase in life span ILS: $\text{ILS}\% = 100 \times \frac{\text{T-C}}{\text{C}}$.

The therapeutic index (defined as the ratio of the dose that kills 10% of tumor-free mice to the dose that gives a 50% increase in life span in tumor-bearing mice) was determined for each experiment. Survival curves of treated and control groups were statistically compared using the Log-rank test.
3.2.6.6. Tumour Growth:

Treatment efficiency is assessed in terms of the compound’s effects on the tumour volumes of tumour bearing mice relative to the control vehicle-treated mice. Two evaluation criteria were used in parallel: (i) Specific tumour growth delay (SGD), calculated as follows: for EAC tumor model = \[\frac{T_d (drug-treated group)-T_d (vehicle treated group)}{T_d (vehicle-treated group)}\], with \(T_d\) being the tumour doubling time of drug - treated and control groups, defined as the time in days required for the tumour volume to double. (ii) Tumour regressions defined as partial (PR) if the tumour volume decreased to 50% or less of that at the start of treatment, without dropping below measurable size (Plowman et al., 1997).

3.2.7. In vitro Antioxidant activity:

3.2.7.1. DPPH radical scavenging method:

The assay was carried out in a 96 well microtitre plate. To 200 µl of DPPH (100 µM) solution, 10 µl of various concentrations of the HPBT or the standard (ascorbic acid) solution were added separately in wells of the microtitre plate. The plates were incubated at 37 °C for 30 min. Absorbance of each solution was measured at 517 nm using ELISA reader (Rao, 1996).

3.2.7.2. Preparation of extract and standard solutions:

13.5 mg of each of HPBT and the standard, ascorbic acid were weighed separately and dissolved in 2 ml of freshly distilled DMSO. These solutions were serially diluted with DMSO to obtain lower dilutions.
3.2.7.3. ABTS Solution:

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.

3.2.7.4. Scavenging of ABTS radical cation:

To 0.2 ml of various concentrations of the extracts or standards, 1.0 ml of distilled DMSO and 0.16 ml of ABTS solution were added to make the final volume to 1.36 ml. Absorbance was measured after 20 min at 734 nm (Pellegrini et al, 1999).

3.2.8. In vivo antioxidant activity:

Healthy mice (20 ± 2 g) were divided into seven groups comprising of six animals in each group. Group I served as control and received 0.2ml propylene glycol: water (1:4). Group II, III and IV received a single dose of HPBT at the dose of 50, 70, and 90mg/kg body weight respectively. Group V, VI and VI received HPBT at 50, 70 and 90mg/kg body weight respectively on alternative days for 8 days. All these treatments were given intraperitoneal. On day 9, the mice were anesthetized using diethyl ether and blood was collected from heart and kept at 37° C in the incubator for 30 min. Later, it was cold centrifuged at 2000rpm for 15 min to get clear supernatant serum, which was used for the in vivo antioxidant assays.

3.2.8.1. Estimation of Nitric – oxide by Griess – Reagent Method:

0.1 ml of Serum sample was taken in a test tube. The volume was made up to 1ml with distilled water. 1 ml of sol A (0.5 gm of Sulphanilamide in 100 ml of 20%,
v/v HCl) was added and incubated at room temperature for 5 min. 1 ml of soln. B [N - (1 - naphthyl) – ethylene diamine dihydrochloride Solution] was added and incubated for 10 min at room temperature. Optical density was measured at 550 nm. Sodium nitrate standard (69 µg/ml) was used to find out the amount of Nitric oxide in the Serum (Bodis and Haregewin, 1993).

3.2.8.2. Estimation of Total Antioxidant in serum by Phosphomolybdenum method:

A spectrophotometric method has been developed for the quantitative determination of antioxidant capacity. The assay is based in the reduction of MO (VI) to MO (V) by the sample analyze and the subsequent formation of a green phosphate / MO (V) complex at acidic pH. The method has been optimized and characterized with respect to linearity interval, repetitively and reproducibility and molar absorption coefficients for quantitation of several antioxidants, including vit E (Prieto et al., 1999).

To 0.1 ml of serum, 0.9 ml 99% ethanol was added. To this, 1 ml of total antioxidant (mixture of 0.6 M H2SO4, 28 mM sodium monophosphate and 4 mM ammonium molybdate) reagent was added and incubated in a thermal block at 95°C for 90 min. Optical density was measured at 695 nm after cooling the reaction mixture. α - Tocopherol {(Vit-E)(12 µg/ml)} was used as standard.

3.2.8.3. Estimation of Super oxide Dismutase by NitroBlue Tetrazolium (NBT) method:

2.5 ml of methionine (380 µg/1 ml) and 0.2 ml of Riboflavin were pipetted out into 4 different tubes. 0.2 ml buffer (pH 7.8) was added to standard and control. 0.1 ml
Table 1. *In vitro* cytotoxicity of different extracts of *T. involucrata*.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC₅₀(mg/ml)*</th>
<th>HE</th>
<th>TE</th>
<th>DCME</th>
<th>AE</th>
<th>ME</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB</td>
<td>3.21 ± 0.20</td>
<td>NT</td>
<td>36 ± 0.005</td>
<td>47 ± 3.6</td>
<td>1.76 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>5.7 ± 0.39</td>
<td>NT</td>
<td>89 ± 0.35</td>
<td>177 ± 0.87</td>
<td>2.58 ± 0.54</td>
<td></td>
</tr>
<tr>
<td>Vero</td>
<td>1.82 ± 0.12</td>
<td>NT</td>
<td>19 ± 0.21</td>
<td>56 ± 7.4</td>
<td>0.88 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>BHK</td>
<td>1.34 ± 0.03</td>
<td>NT</td>
<td>15 ± 0.51</td>
<td>31 ± 1.23</td>
<td>0.127 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

*Results are the means ± SD of three independent experiments.

HE, hexane extract; TE, toluene extract; DCME, dichloromethane extract; AE, acetone extract; ME, methanol extract; NT- Not Toxic.

Doses that reduce the cell growth by 50%, after 48 h *in vitro* as compared to controls.
3.3.2. Toxicity of HPBT:

When the mice were observed for behavioral changes after i.p. administration of a single dose of the HPBT, none of the mice in the dose group of 50, 70, or 90 mg/kg exhibited any abnormal behavioral responses. Mice which received 150 mg/kg and above showed toxic symptoms. These include inactiveness, slow movement, dizziness, erection of hairs and hypothermia. These symptoms appeared almost 1 h after the extract administration and slowly progressed to some extent. There was a dramatic reduction in food and water intake. The food intake in the control animals was approximately 15 g/mouse/24 h whereas that in the treated mice was less than 1 g/mouse/24 h. The mean water intake in the untreated control mouse was 6.4 ml/24 h whereas that in the treated mouse was almost 2 ml/24 h. The LD50 value for a single i.p. dose for male mice was 300 mg/kg and that for female mice was almost the same.

Administration of repeated daily doses for 15 days at a dose of 50 or 70 mg/kg did not influence body weight (data not given). The weights of liver, kidney, brain and spleen were also not altered by the treatment (data not shown), these organs did not exhibit any gross morphological changes.

When the drug was administered repeatedly at a dose level of 150 mg/kg (i.p.) most of the animals died after the second dose (on the third day). Therefore, this group of mice was sacrificed 24 h after a single dose, for haematological, serum biochemical and pathological studies.

The effect of repeated low doses (50, 70 or 90 mg/kg) or a single high dose (150 mg/kg) on the haematological parameters is shown in Table 2. The repeated low doses did not significantly influence haemoglobin level and number of RBCs and platelets in the blood. The packed cell volume of blood and mean

90
cell haemoglobin content were also unchanged in the treated animals. However, the mice, which received the high dose, showed alterations in the haematological parameters (Table 2). The cell volume, RBC number and haemoglobin content were increased in the blood, whereas the number of WBC and platelets was not increased. Further, low level of haemolysis was observed while separating the serum from the blood of mice, which received 150 mg/kg drug.

None of the biochemical parameters studied was altered in the repeated low doses treated mice. In contrast, administration of a single high dose (150mg/kg) dramatically increased the urea level in the blood. The SGOT and SGPT activities were also significantly increased. However, the serum calcium and phosphorous levels were unaltered (Table 2).
Table 2. Effect of HPBT on hematological and serum biochemical parameters in mice

<table>
<thead>
<tr>
<th>HPBT (mg/kg)</th>
<th>0(control)</th>
<th>60</th>
<th>75</th>
<th>90</th>
<th>150</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb(g%)</td>
<td>14.9±1.6</td>
<td>8.8±1.03</td>
<td>15.0±1.2</td>
<td>14.2±1.01</td>
<td>13.8±0.7</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>38.8±2.31</td>
<td>20.1±3.8</td>
<td>38.9±3.7</td>
<td>40.5±2.93</td>
<td>31.5±4.1</td>
</tr>
<tr>
<td>RBC (10⁶/mm³)</td>
<td>8.62±0.67</td>
<td>7.42±1.7</td>
<td>8.47±2.2</td>
<td>8.88±1.56</td>
<td>7.10±1.8</td>
</tr>
<tr>
<td>Total WBC (10⁹/mm³)</td>
<td>1.5±0.02</td>
<td>0.8±0.03</td>
<td>1.1±0.02</td>
<td>2.1±0.031</td>
<td>1.2±0.12</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>38.5±2.41</td>
<td>43.6±1.85</td>
<td>38.4±1.65</td>
<td>35.1±3.2</td>
<td>43.7±2.6</td>
</tr>
<tr>
<td>Platelets (10⁹/mm³)</td>
<td>454±4.3</td>
<td>304±2.89</td>
<td>391±3.65</td>
<td>246±3.3</td>
<td>470±3.13</td>
</tr>
<tr>
<td>SGOT (U/L)</td>
<td>538±2.01</td>
<td>274±4.2</td>
<td>403±1.01</td>
<td>699±1.1</td>
<td>1135±5.4</td>
</tr>
<tr>
<td>SGPT (U/L)</td>
<td>88±1.23</td>
<td>67±3.54</td>
<td>89±3.15</td>
<td>73±4.27</td>
<td>453±2.85</td>
</tr>
<tr>
<td>Serum Calcium (mg/dl)</td>
<td>7.7±0.3</td>
<td>6.6±1.7</td>
<td>10.4±1.45</td>
<td>2.5±1.08</td>
<td>8.3±1.69</td>
</tr>
<tr>
<td>Serum Phosphorous (mg/dl)</td>
<td>12.2±1.87</td>
<td>10.8±1.8</td>
<td>13.9±1.23</td>
<td>10.5±1.05</td>
<td>11.0±1.11</td>
</tr>
</tbody>
</table>

Repeated daily dose of the drug was administered (i.p) for 14 days in all groups except ISOmg/kg where a single dose was administered. Results are the means ± SEM of three independent experiments. n = 8 in each group. PCV, Packed Cell Volume; MCHC, mean cell hemoglobin concentration; SGOT, serum glutamate oxaloacetate transaminase; SGPT, serum glutamate pyruvate transaminase.
3.3.3. Anti-tumor activity of HPBT and Terpenoid:

The highest non-lethal dose (LD₀) and lethal dose (LD₁₀₀) were determined after a single injection to mice by ip route. Highest non-toxic doses (LD₀) and lethal doses (LD₁₀₀) were found to be 130 mg/kg and 500 mg/kg respectively. Antitumor activity against EAC was determined for 5 HPBT and terpenoid using single dose and intermittent treatments over two weeks, i.e., five drug administrations by the i.p., route on days, 1, 3, 5, 7, and 9 after EAC cells inoculation. The extracts and the vehicle were administered ip using 10 mice per test group. HPBT exhibited antitumor activity against EAC, with a significant increase in life span. The effect of HPBT on the survival of tumor bearing mice is shown in the Table 3a. There was a dose-effect relationship and the increase in life span. The ILS% for HPBT was 18.18, 25 and 104.54% for the single dose and 4.54, 52.27 and 75% for the multiple doses at the dosage of 60, 75, and 90 mg/kg body weight respectively. When the tumor bearing mice were given 100 mg/kg body weight as well as higher concentrations mice did not survive beyond a week. There was reduction in the tumor volume of mice treated with HPBT. The tumor volume of control animal on the 30th day of tumor injection was 5.75 ± 0.59 mm³, where as the compound treated group it was 6.57 ± 0.47, 5.02 ± 0.82, and 2.18 ± 0.98 mm³ for the single dose and 3.11 ± 0.60, 2.68 ± 0.43, and 2.23 ± 0.83 mm³ for the multiple dose respectively for the doses of HPBT mentioned earlier. Seven or eight out of ten mice were cured with these compounds. The highest therapeutic index was obtained with multiple schedules. The results obtained for these compounds are shown in Figure 3a-b. Even the tepenoid showed significant antitumor activity against EAC tumor model at the dose of 50, 100 and 150 mg/kg body weight. Tepenoid was non-toxic even when the concentration was increased up
to 2000mg/kg body weight. Antitumor activity of tepenoid was also dose dependant and single dose had a better activity than the multiple drug administrations (*Table 3b* and *Fig.4a-4b*). The highest ILS % was found to be 86.22% at 150mg/kg body weight. Single injection (D1) led to the 8.13, 32.97 and 86.22% activity while the multiple schedules caused a reduction of 7.65, 21.05 and 66.14% of the tumoural volume at the end of the treatment, showing an *in vivo* antitumor effect of the extracts tested.
Table 3a. Antitumour activity of *T. involucrata* HPBT given ip against the sc implanted EAC.

<table>
<thead>
<tr>
<th>Treatment Schedule</th>
<th>MTD * (mg/kg)</th>
<th>TI b</th>
<th>Dose (mg/kg)</th>
<th>SGD</th>
<th>ILS%</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>130</td>
<td>6.66</td>
<td>60</td>
<td>-0.04</td>
<td>18.18</td>
<td>8/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>75</td>
<td>0.04</td>
<td>25.00</td>
<td>7/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90</td>
<td>-0.23</td>
<td>104.54</td>
<td>5/10</td>
</tr>
<tr>
<td>D1,3,5,7,9</td>
<td>130</td>
<td>6.66</td>
<td>60</td>
<td>0.14</td>
<td>4.54</td>
<td>5/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>75</td>
<td>0.09</td>
<td>52.27</td>
<td>6/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90</td>
<td>0.19</td>
<td>75.00</td>
<td>7/10</td>
</tr>
</tbody>
</table>

Tumour cells are inoculated ip as described in Materials and Methods.

Drugs are then injected ip with in different schedules, and ILS% are then determined. * MTD – maximum tolerance dose.

b TI, therapeutic index.
Fig. 2. Measurement of tumor volume by calipers.
Figure 3a: *In vivo* antitumour activity of HPBT on Ehrlich's Ascitic Carcinoma. Tumors were generated by s.c. inoculation of EAC cells in Swiss albino mice. The compound was administered i.p., once (D1, Single dose) on the day after tumor inoculation. Average tumour volumes of treated (n = 10) and control (n = 10) are shown.
Figure 3b: *In vivo* antitumour activity of HPBT on Ehrlich's Ascitic Carcinoma. Tumors were generated by s.c., inoculation of EAC cells in Swiss albino mice. The compound was administered i.p., six times: on the day after tumor inoculation (D1), on day D3, D5, D7, D9 and D11. Average tumour volumes of treated (n = 10) and control (n = 10) are shown.
Table. 3b. Antitumour activity of *T.* *involucrata* terpenoid given ip against the sc implanted EAC.

<table>
<thead>
<tr>
<th>Treatment Schedule</th>
<th>MTD $^a$ (mg/kg)</th>
<th>TI $^b$</th>
<th>Dose (mg/kg)</th>
<th>SGD</th>
<th>ILS%</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>&gt; 2000</td>
<td>13.33</td>
<td>50</td>
<td>-0.38</td>
<td>8.13</td>
<td>8/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>0.28</td>
<td>32.97</td>
<td>6/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>150</td>
<td>0.04</td>
<td>86.22</td>
<td>9/10</td>
</tr>
<tr>
<td>D 1, 3, 5, 7, 9</td>
<td>&gt; 2000</td>
<td>13.33</td>
<td>50</td>
<td>0.28</td>
<td>7.65</td>
<td>7/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>-0.38</td>
<td>21.05</td>
<td>7/10</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>150</td>
<td>0.19</td>
<td>66.14</td>
<td>8/10</td>
</tr>
</tbody>
</table>

Tumour cells are inoculated ip as described in Materials and Methods.

Drugs are then injected ip with in different schedules, and ILS% are then determined. $^a$ MTD – maximum tolerance dose.

$^b$ TI, therapeutic index.
Figure 4a: *In vivo* antitumour activity of Terpenoid on Ehrlich’s Ascitic Carcinoma. Tumors were generated by s.c., inoculation of EAC cells in Swiss albino mice. The compound was administered i.p., once (D1, Single dose) on the day after tumor inoculation. Average tumour volumes of treated (n = 10) and control (n = 10) are shown.
Figure 4b: *In vivo* antitumour activity of Terpenoid on Ehrlich’s Ascitic Carcinoma. Tumors were generated by s.c., inoculation of EAC cells in Swiss albino mice. The compound was administered i.p., six times: on the day after tumor inoculation (D1), on day D3, D5, D7, D9 and D11. Average tumour volumes of treated (n = 10) and control (n = 10) are shown.
3.3.4. Antioxidant activity of HPBT:

The results on the free radical scavenging activity of HPBT shown in Table 4a. HPBT exhibited significant antioxidant activity in ABTS and DPPH assays and the IC₅₀ values found to be 0.85 ± 0.012 and 6.46 ± 0.371µg/ml respectively. Above-mentioned difference in values between the two methods is due to the action of antioxidants at different levels. The DPPH radical assay corresponds to the primary radical scavenging activity where as the ABTS corresponds to the ability of an antioxidant to inhibit the radical formation (Dixit et al., 2005).

HPBT has shown a potent in vitro antioxidant activity. Hence it was subjected for in vivo antioxidant screening. The administration of HPBT at 50mg/kg body weight for 7 days or a single dose caused a significant increase in the level of total antioxidant, Nitric Oxide and SOD level in the serum (Table 4b). The antioxidant activity showed reciprocal dose dependence. At 50mg/kg body weight the activity found to be highest where as a decline in the antioxidant capacity as the concentration was increased to 90mg/kg.
Table 4a. *In vitro* antioxidant activity of HPBT of *T. involucrata*.

<table>
<thead>
<tr>
<th>compound</th>
<th>Method</th>
<th>IC$_{50}$ value µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>ABTS</td>
<td>11.25 ± 0.49</td>
</tr>
<tr>
<td>HPBT</td>
<td>ABTS</td>
<td>0.85 ± 0.012</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>DPPH</td>
<td>2.69 ± 0.05</td>
</tr>
<tr>
<td>HPBT</td>
<td>DPPH</td>
<td>6.46 ± 0.371</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M Values are mean of six replicates.
3.4. Discussion:

Anticancer drugs have well known therapeutic limitations and this has stimulated the search for new agents with enhanced therapeutic efficacy. Considerable efforts have been directed towards medicinal plants, which have been reported to be effective in the treatment of human cancers. Therefore, search for new drugs is required for the treatment of cancers (Xingming et al., 2009). Although the terpenoid and HPBT have demonstrated significant in vitro and in vivo antineoplastic activities against the tumour cell lines, the mechanism of this effect has not been fully examined.

The cytotoxic effects of plant flavonoids are shown to be mediated through apoptosis. Considering the ability of these natural flavonoids to absorb proteins and metal ions, there is a possibility that they can elicit apoptosis signals through various receptors or proteins. Apart from this, they are excellent antioxidants and they thus prevent free radical attack on DNA by acting as scavengers of these free radicals. A number of flavonoids are topo-II poisons inhibiting topo I/II isomerases thus enhancing the DNA cleavage.

Another possible mechanism of reported for anticancer drugs is inhibition of DNA synthesis and thus prevention of cell division. Folic acid supplied from the diet is essential for the production of tetrahydrofolic acid (THF). The conversion of folic acid to THF is carried out by an enzyme folate reductase. Anticancer drugs compete with folic acid for this enzyme thus restricting the production of THF required for synthesis of DNA and consequently for cell replication. Cells, which do not have adequate production of THF eventually, die.
It should be noted that there are a number of reports that that the anticancer plant extracts retarded development of ascetic tumour growth and increased the life span of tumour growth and increased the ILS% (Xingming et al., 2009; Abdel-Khader et al., 2007; Rajkapoor et al., 2004; Babu et al., 2002; Latha and Panikkar, 1998). For example, Loranthus extract significantly inhibited ehrlichs Ascetic Carcinoma (EAC) growth in mice (Mary et al., 1994). Preparations from Solanum trilobatum also reduced the growth of EAC in mice (Mohanan and Devi, 1996). In the present study the HPBT and terpenoid significantly increased the life span of ascetic tumour bearing mice dose dependently. Moreover, the extracts significantly reduced the solid tumor development in the mice.

In our studies we found that HPBT posses antioxidant effect. Flavonoids have been shown to posses antimutagenic, antimalignant and antioxidant effects. It was reported that plant derived extracts containing antioxidant principles showed cytotoxicity towards tumor cells and antitumor activity in experimental animals (Babu et al., 2002; Ruby et al., 1995). Antitumor activity of these antioxidants is either through induction of apoptosis or by inhibition of neovascularisation (Putul et al., 2000). The implication of free radicals in different steps of carcinogenesis is well documented (Player, 1982; Frenkel, 1992). The free radical hypothesis supported the fact that the antioxidants can effectively inhibit carcinogenesis and the observed properties may be attributed to the antioxidant principles present in the extract.

The major toxic symptoms due to high dose of this drug appear to be loss of food and water intake, a dramatic increase in blood urea level and decreased level of water in the blood. The apparent increase in RBC and hemoglobin in the blood could result from a decrease in water in the blood. The raise in urea may suggest kidney malfunction or metabolic alterations or both.
Repeated daily administration of the drug (50, 70 or 90 mg/kg) for 14 days did not exhibit any significant conspicuous adverse toxicity, whereas even a single administration of 150 mg/kg of the drug showed severe toxic symptoms. This suggests that the drug is not much accumulated in the body and it is detoxified or eliminated from the body.

In the present study, free radical scavenging and in vivo antioxidant activity of HPBT of T. involucrata has been studied.

Antioxidant capacity is often used to characterize food or medicinal plants and their bioactive components. Reduction of chronic diseases, like DNA damage, mutagenesis, carcinogenesis, etc and inhibition of pathogenic bacterial growth, which are often associated with termination of free radical propagation in biological system by natural antioxidants in plants are closely related to their biofunctionalities (Covacci et al., 2001; Zhu et al., 2002; Jayaprakasha et al., 2002; Gülçin et al., 2006).

DPPH and ABTS radicals are widely used in the model system to investigate the scavenging activities of several natural compounds (Ansari et al., 2005). Antioxidant activities are very important due to deleterious role of free radicals in biological systems (Min, 1998; Gülçin et al., 2006). The results of radical scavenging activities shows that HPBT has significant in vitro antioxidant activity (P<0.05). In this study, Ascorbic acid was used as a accepted radical scavenger. Many flavonoids and other phenolic compounds are known to exhibit potent antioxidant activity (Lee et al., 2004; Senthil Kumar et al., 2007)

Even the HPBT shows a significant in vivo antioxidant activity as well as the reciprocal dose dependence. In vivo antioxidant activity was maximum at 50mg/kg in all the three methods ie, total antioxidant, Nitric Oxide and SOD where as the activity was reduced when the dose was increased to 70mg/kg further reduced when the
concentration was 90mg/kg. This property may be because of the components present in the extract. Extent of protectiveness provided by phenolics antioxidants related to their permeability into the biomembranes of cells. It has been shown that flavonoids auto oxidize readily to free radicals like hydroxy radicals and the semiquinone radicals. Such species are known to be toxic and are reported to bind irreversibly to various cell constituents by the formation of covalent binding with sulphhydral groups and/or other essential groups producing secondary free radicals. The formation of these secondary free radicals is responsible for the prooxidant activity of flavonoids. The latter depends on the concentration of the flavonoids and the free radical source. (Monks et al, 1992; Simms et al., 1990; Geetha et al., 2005).

The selection of proper dose for extension of biological effects of flavonoids to clinical use thus becomes a very important factor to be monitored and need to be stressed up on the scientists involved in the study of these agents. Cautious uses of excessive flavonoids intake and uncertainty regarding the conditions and the levels of flavonoids, which may pose a potential health hazard, have been mentioned in the literature (Silva et al., 2002; Sahu & gray, 1996; Geetha et al., 2005).

3.5. Conclusion:

In summary, this study shows the cytotoxic and antitumor potential of HPBT as well as terpenoid isolated from the aerial parts of the T. involucrata. HPBT showed a significant activity against the in vitro as well as in vivo tumor models. It was even active as antioxidant agent. The high activity of HPBT against EAC must be considered as a new class of anti-tumor agents.