CHAPTER 6

Pharmacological activity
PHARMACOLOGICAL ACTIVITIES AGAINST CANCER

Pharmacological studies have been done of six compounds isolated from three medicinal plants namely *Aralia cachemirica*, *Lavatera cachmiriana* and *Salvinia natans*. The activities of these six compounds against seven different cancer cell lines has been done in Regional Research Laboratory (CSIR), in Jammu. The six compounds were coded as (SN-1, SN-2, LK-1, LK-2, AC-1 and AC-3) were tested in-vitro for their cytotoxicity against seven different human cancer cell lines from different tissues viz lung:A-549, Liver:Hep-2, Ovary:ovcar-5, Neurolastoma: SK-N-MC, Colon: HT-29, Prostate: Pc-3, Cervix: SiHa and Lung:HOP-62 at two different concentration (10 and 30 μg/ml) using sulforhodamine B. Out of six compounds tested only two compounds showed >50% activity against all the seven cell lines and these two compounds can be considered cytotoxic against cancer cell lines. Rest of four compounds (SN-1, SN-2, AC-1 and AC-3) showed very little or no activity against all the cell lines studied and can be considered as inactive.

**Background of invention:**

Cancer or neoplasm is the malignant new growth anywhere and elsewhere in the body system. It is characterized by unregulated proliferation of cells and a growing public health problem whose estimated worldwide new incidence is about six million cases per year. In most of the countries, cancer is second only to heart disease as cause of death. It can arise in any organ of the body but some sites are prone to attack than others such as breast, throat, intestine, leukocytes etc. Each cancer is propagated from a single cell that cut at some stage, it becomes free from its territorial restraints, which form a family of cells that multiply without limits and appear in the form of tumors.

During the transition from normal cell to a tumor cell a profound and heritable change occurs which allows a tumor cell to determine its
own activities largely irrespective of the laws that govern so precisely the
growth of all normal cells in an organism. This newly acquired property,
which is known as autonomy, is the most important single characteristic
of tumor cells since without it there would be no tumors. Another
distinguishing characteristic of tumor cells is their lack of perfect form of
function. The differences that exist between cancer and normal cells are
that, compared to normal cells, cancer cells have a) low pH  b) greater free
radical character  c) tumor produced hormone peptides d) tumor
associated antigens e) lower calcium ion and higher potassium ion
concentration f) different potassium isotope ratios g) elevated amounts of
methylated nucleotides h) higher concentrations of plasma microproteins
and mucopolysacharides i) greater need of exogenous zinc and j) high
biowater content.

Many of the gross causes of cancer, such as dietary, environmental, occupational exposure to certain chemical substances or
forms of electromagnetic radiation, have been elucidated through
epidemiological studies. It is imperative, therefore, that they be identified
and eliminated from the environment in so far as that is possible in,
modern industrial societies.

In the annals of therapy, a quest to conquer, the impasse of cancer
has been always fascinated, by and large, all disciplines of scientific
community, especially natural product chemists. In 19th and 20th
century, lot of research work has been carried out to find out the driving
force behind this dreadful disease as well as large number of drugs have
been introduced to counter its menace. The plants have always
fascinated the scientists and as a result number of drugs for different
diseases including cancer have been isolated from the plants. It is
worthwhile at this juncture to look briefly at a few most powerful
chemotherapeutic agents, which have been of paramount importance to
the mankind and also to the researches who have been actively involved
in the synthesis and isolation of anticancer drugs.
There is a strong association between exposure of the skin to the ultraviolet light component of sunlight and the development of skin cancers, such as malignant melanoma and the non-melanoma skin cancers, mainly basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs). The incidence of these cancers has been rapidly increasing worldwide. In Britain, there were 4000 newly diagnosed cases of malignant melanoma in 1994, an 80% increase over the past ten years with sex. In the United States, approximately 34,100 new cases were expected, an increase of 45 per year. Queensland, Australia, has the highest incidence of melanoma in the world but early detection and widespread public health campaigns and the promotion of use of sunscreens and reduction of ultraviolet exposure have helped to reduce the number of deaths. BCCs currently affect one in 1,000 in the U.K. population, and the incidence has more than double in the last 20 years\(^1\). One million new cases of BCCs and SCCs are expected to be diagnosed in USA in 1997 compared to 600,000 in 1990 and 400,000 in 1980\(^2\). In Australia, there is no reason to suspect that a similarly increasing incidence would not also apply, despite extensive publicizing of the dangers of solar and UV radiation, with the Queensland population being at the greatest risk.

Over 90% of all skin cancers occur on areas of the skin that have been regularly exposed to sunlight or other ultraviolet radiation, with UVB responsible for burning the skin and associated with malignant melanomas, and UVA associated with premature skin aging and the development of BCCs and SCCs. Childhood sun exposure has been linked to the development of malignant melanoma in younger adults. Other risk factors include a genetic predisposition (fair complexion, many skin moles), chemical pollution, over-exposure to X-rays, and exposure to some drugs and pesticides. Depletion of the ozone layer of the stratosphere is considered to contribute to long term increase in skin cancer. When the tumour is past the stage amenable to surgery, the
most common treatment for melanoma or metastatic skin cancer of all type is chemotherapy, which has been largely unsuccessful. In theory, an ideal drug would be one that when applied topically to an exposed melanoma, BCCs or SCC, selectively necroicizes the tumour cells or induce them to undergo apoptosis, with out causing damage to the surrounding healthy skin cells. In practice, this has yet to be achieved. The drugs currently available are neither selective nor penetrative. However, anecdotal reports suggest that plant sap extracts are still being used by the general public for the treatment of sunspots or solar keratoses, with some success being claimed as a part of the screening program for anti-cancer activity carried out on 114,000 extracts from 35,000 terrestrial plant species carried out by United States Cancer Institute, a number of diterpenes were tested. A recent report describes selected cytotoxicity of a number of diterpene esters from the latex of *Euphorbia poisonii*, a highly toxic plant found in Northern Nigeria. One of these compounds has a selective cytotoxicity for the human kidney carcinoma cell line A-498 more than 10,000 times greater than that of adriamycin. It is in the treatment of cancers and in anti-infective areas that natural products have made their major impact as templates or direct treatment. In the cancer area, of 92 drugs commercially available prior to 1983 in the United States or approved worldwide between 1983 and 1994, approximately 62% ca be related to a natural product origin, ignoring those of biological origin. A common feature with many of the natural product- derived anti-cancer drugs now in clinical use is that the original natural product was too toxic. Chemists, therefore, had to make semi-synthetic compounds based on the natural product structures.

The well known anticancer drugs from plants are *Vinca* alkaloids, vinblastine and vincristine from *Catharanathus roseus* and podophyllotoxin from *podophyllum peltatum* which is not used but its two semi synthetic glycosides, Etoposide and Tenioposide are in clinical use. The later addition in the list of anticancer drugs from plants is
paclitaxel\textsuperscript{7}. The complex diterpene, Taxol\textsuperscript{8}, was isolated from the bark of \textit{Taxus brevifolia}. Paclitaxel along with several key precursors (baccatins) occurs in the leaves of various taxus species, such as Docetaxel\textsuperscript{9}, has provided a major, renewable natural source of this important class of drugs.

The plant genus \textit{lavatera} is known to have cathartic and anti-inflammatory activities\textsuperscript{10}. The isolated compounds from the plant genus \textit{lavatera} are dihydromalvalic acid from the seed oils\textsuperscript{11}, cyclopropene and dihydrostercolic acids\textsuperscript{12}, cycloopenoid from the seeds\textsuperscript{13}.

With the above background attention was focussed towards the identification and isolation of potent cytotoxic agent(s) from \textit{lavatera}. Since there is hardly any report regarding isolation and characterization of compounds from \textit{Lavatera cachmiriana} (family malvaceae).

Diterpenes have been isolated from a number of plants. Recent studies on the biological activities of diterpenes prove beyond doubt the efficacy of these phytochemicals as cytotoxic agents. Four diterpenes have been isolated and characterized from \textit{Lavatera cachmiriana}. The two diterpenes were found to be potent cytotoxic against \textit{in vitro} human cancer cell lines.

\textbf{In vitro cytotoxicity of Compound LX-1 against human cancer cell lines:}

The human cancer cell lines were obtained either from National center for cell science, Pune, India or National Cancer Institute, Frederick, MD, USA. Cells were grown in tissue culture flasks in complete growth medium (RPMI-1640 medium with 2mM glutamine, 100 µg/ml streptomycin, pH 7.4, sterilized by filtration and supplemented with 10% sterilized fetal calf serum and 100 units/ml penicillin before use) at 37\textdegree C in an atmosphere of 5% CO\textsubscript{2} and 90% relative humidity in a carbon dioxide incubator. The cells at subconfluent stage were harvested
from the flask by treatment with trypsin (0.05% trypsin in PBS containing 0.02% EDTA) and suspended in complete growth medium. Cells with cell viability of more than 97% by trypan blue exclusion technique were used for determination of cytotoxicity.

**Compound LK-1** was dissolved in DMSO (dimethyl sulfoxide) to obtain a stock solution of 20 mg/ml. The stock solution was serially diluted with complete growth medium containing 50 μg/ml of gentamycin to obtain three working test solutions of 200, 60 and 20 μg/ml.

The suspension of human cancer cell lines of required cell density in complete growth medium was prepared and cell suspension of each cell line was placed in 96-well tissue culture plate (100 μl per well) for control, **Compound LK-1** and positive control(s). The blank wells for each cell line and concentration of test material were also included that contained equivalent amount of complete growth medium only. The plates were incubated at 37°C in an atmosphere of 5% CO₂ and 90% relative humidity in a carbon dioxide incubator. The experiment was carried out in triplicate.

After 24-hours of incubation, the working solutions of **Compound LK-1** of different concentrations (100 μl) were added in the experimental wells and blanks whereas positive controls of required concentrations (100 μl) were added into wells for positive controls. The equivalent amount of complete growth medium was added to control set.

The plates were further incubated for 48-hours (at 37°C in an atmosphere of 5% CO₂ and 90% relative humidity in a carbon dioxide incubator) after addition of test material etc. and then the cell growth was stopped by gently layering of 50 μl of TCA (50% trichloroacetic acid) on top of the medium in all the wells. The plates were incubated at 4°C for one hour to fix the cells attached to the bottom of the wells. Liquids of all the wells were gently pipetted out and discarded. The plates were
washed five times with distilled water to remove TCA, growth medium, low molecular weight metabolites, serum proteins etc. Plates were air dried.

Cell growth was measured by staining with sulforhodamine B dye (SRB). The SRB solution (100 µl of 0.4% SRB in 1% acetic acid) was added to each well and the plates were incubated at room temperature for 30 minutes. The unbound SRB was quickly removed by washing the wells five times with 1% acetic acid and plates were air dried. Tris-buffer (100 µl of 0.01M, pH 10.4) was added to all the wells and plates were gently stirred for 5 minutes on a mechanical stirrer. The optical density was recorded on ELISA reader at 540 nm.

The cell growth in presence of test material was determined by subtracting mean OD value of respective blank from the mean OD value of experimental set. Like wise, cell growth in absence of test material (control set) and in presence of positive control was also determined. The per cent cell growth in presence of test material was determined considering the cell growth in absence of test material as 100% and in turn per cent inhibition was calculated.

In vitro cytotoxicity of Compound LK-1 was determined against human cervix (SiHa), CNS (SK-N-MC), colon (HT-29), lung (A-549 & HOP-62), liver (Hep-2), ovary (OVCAR-5) and prostate (PC-3) representing different organs. The results are summarized in Table 5. Compound LK-1 showed dose dependent inhibition of cell growth of all the human cancer cell lines studied except HOP-62. The inhibition varied from 66-85% at 10 & 30 µg/ml. It was most effective against human prostate cancer cell line PC-3 and least effective against cervix cell line SiHa.
In vitro cytotoxicity of Compound LK-2 against human cancer cell lines:

The human cancer cell lines were grown, harvested and cytotoxicity was determined exactly as mentioned in Compound LK-1 except that the test material used was Compound LK-2 that was dissolved in DMSO and three working test solutions were prepared of the same concentrations as in Compound LK-1.

In vitro cytotoxicity of Compound LK-2 was determined against the same human cancer cell lines as in Compound LK-1 representing different organs. The results are summarized in Table 5. Compound 2 showed dose dependent inhibition of cell growth of all the human cancer cell lines studied except HOP-62. The inhibition varied from 66-85% at 10 & 30 μg/ml. It was least effective against human cervix cancer cell line (SiHa) but was highly active against rest of the cell lines.
<table>
<thead>
<tr>
<th>Test material</th>
<th>Conc. (µg/ml)</th>
<th>Cell line &amp; Tissue (Growth inhibition, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-549</td>
<td>Hep-2</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>Liver</td>
</tr>
<tr>
<td>LK-1</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>72</td>
</tr>
<tr>
<td>LK-2</td>
<td>10</td>
<td>09</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>71</td>
</tr>
<tr>
<td>AC-1</td>
<td>10</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>7.6</td>
</tr>
<tr>
<td>AC-3</td>
<td>10</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>13.9</td>
</tr>
<tr>
<td>SN-1</td>
<td>10</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>13.2</td>
</tr>
<tr>
<td>SN-2</td>
<td>10</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>12.1</td>
</tr>
<tr>
<td>5FU</td>
<td>1x10⁻⁴M</td>
<td>-</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>1x10⁻⁸M</td>
<td>19</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>1x10⁻³M</td>
<td>98</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>1x10⁻³M</td>
<td>87</td>
</tr>
</tbody>
</table>
Graph 1: Inhibitory effects at 1, 10, 30 μg/ml (A) & 300 μg/ml (B) of compounds (LK-1 & LK-2) against human cancer cells
Graph 2: Inhibitory effects at 10 μg/ml (A), 30 μg/ml (B) of compounds (AC-1, AC-2, SN-1 & SN-2) against human cancer cells.
REFERENCES: