Exploring \textit{in vitro} anti-proliferative efficacy of Jammu botanicals

\textbf{SUMMARY}

Cancer is becoming a big load on families and economies. Cancer cases related deaths on rise in J&K during past four years with the total of 11,815 cancer cases and 5,198 mortality cases have been reported in the state during the current year. Cancer research has, therefore, become a major area of scientific research supporting the foundations of modern biology to a great extent. Chemotherapy is a major treatment modality for cancer, but most of the drugs used in cancer chemotherapy exhibit cell toxicity and can induce genotoxic, carcinogenic and teratogenic effects in non tumor cells. Therefore, the research for alternative drugs of natural origin, which are less toxic, endowed with fewer side effects and more potent in their mechanism of action, is an important research line. In the present investigation, methanolic and aqueous extracts from two medicinal plants (\textit{Allium sativum} and \textit{Holarrhena antidysenterica}) selected from Jammu region were evaluated against eight human cancer cell lines from six different origins, viz., A-549 (lung), NCI-H322 (lung), HCT-116 (colon), COLO-205 (colon), MCF-7 (breast), PC-3 (prostate), THP-1 (leukemia) and U-87-MG (glioblastoma) at the concentration of 100 \textmu g/ml using sulphorhodamine blue (SRB) assay. Results revealed that methanolic extract from the stem-leaves of \textit{H. antidysenterica} displayed \textit{in vitro} cytotoxic effect against leukemia and colon cancer cells.

\textbf{Key Words} : Cancer cells, \textit{Allium sativum}, \textit{Holarrhena antidysenterica}, SRB assay


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\textbf{M}edicinal plants have played a key role in world health. Population rise, inadequate supply of drugs, prohibitive cost of treatment, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases, have led to increased emphasis on the use of plant material as a source of medicine for a wide variety of ailments. Out of total 250,000 plant species existing on earth approximately one thousand have anticancer potential. A large number of plant species have been screened through bioassays for the search of novel plant based anticancer drugs. The State of J&K has great botanical diversity and widespread use of traditional medicine practice known as ayurvedic medicine, however, only a relatively small number of these plants have been subjected to accepted scientific evaluation for their potential anticancer effects. Garlic is a relative of the onion and leek, and other related species containing the aromatic sulphur-based compounds,
which contribute to the characteristic odour and taste, as well as garlic’s beneficial healing effects (Linnaeus, 1957). Garlic has historically been used to treat leprosy, deafness, diarrhea, constipation, parasitic infections and relieve ear and stomach aches (Hahn, 1996). The use of garlic in the treatment of tumors dates back to 1550 BC when ancient Egyptians administered it orally and topically, but modern era begins in 1950s when its thiosulfinate extracts inhibited the growth of malignant cells and prevented growth of sarcoma 180 ascites tumor in vitro and in vivo (Weisberger and Pensky, 1958). *A. sativum* is widely used as herbal vegetable and has been suggested as an anticancer agent for several decades in epidemiological studies (Thomson and Ali, 2003). Fresh garlic aqueous extract possesses antiproliferative potential against neoplastic transitional cell carcinoma cells (Talebi et al., 2006). The plant displayed anti-tumor activity in sarcoma, mammary carcinoma, hepatoma, colon cancer, squamous cell carcinoma of skin and esophagus (Lamm and Riggs, 2001). Two main water soluble constituents of garlic, S-allylcysteine (SAC) and S-allylmercaptocysteine (SAMC), were able to suppress prostate cancer cell proliferation and proved their novel anticancer effect (Chu et al., 2006). *A. sativum* >24 kg/year had 60 per cent reduced risk of gastric/stomach cancer compared to those with low consumption <11.5 kg/year (You et al., 1989). In vivo and in vitro studies have shown that S-allylcysteine (SAC) and S-allylmercaptocysteine (SAMC) are not only able to suppress the skin, esophageal, stomach, colon, liver, lung and breast cancer growth in animal models (Thomson and Ali, 2003; Herman-Atosiewiez and Singh, 2004), but also directly inhibit proliferation of a variety of cancer cell lines derived from colon, lung, leukaemia, skin, breast and prostate cancer in vitro (Herman-Atosiewiez and Singh, 2004). The stem bark crude aqueous and alcoholic extracts of *H. antidysenterica* exhibit anti-bacterial activity against the known enteric pathogens (Ballal et al., 2001). Efficacy of aqueous extract of seed for the management of diabetes was also carried out in experimental model in rat (Ali et al., 2009; Wahab and Yousuf, 2004). This plant also showed in vitro antioxidant potential according to ferric thiocyanate assay method (Zahin et al., 2009). Bitter oleander (*H. antidysenterica*) has been considered as a popular remedy for the treatment of dysentery, diarrhea and intestinal worms (Kavitha et al., 2004) and derivative of 2, 6-diisopropylphenol (propofol) was prepared by coupling with 9-hydroxy-11-Z-octadecenoic acid (isolated from seed oil of *H. antidysenterica*) with the Cα,α-hydroxy function of 2, 6-diisopropylphenol and the analog was found cytotoxic against HeLa, MCF-7 and HL-60 cancer cells (Mohammad et al., 2010). In the present study, in vitro anticancer potential of garlic and bitter oleander has been investigated against eight human cancer cell lines from six different tissues.

**MATERIAL AND METHODS**

**Preparation of extracts :**

Bulb of garlic and stem-leaves of bitter oleander were collected in the month of July-August from Baish village of Udhampur, Jammu and Herbal Garden of SKUAST-Jammu, J&K, India respectively. Extraction of the plant material was carried out as per Jabar and Al-Mossawi, 2007 and Kandil et al., 1994.

**Cell lines and positive controls :**

The human cancer cells were obtained from National Centre for Cell Science, Pune, India and were further grown and maintained in RPMI-1640 medium. Positive controls like Adriamycin and 5-fluourouracil were prepared in distilled water, while paclitaxel was prepared in DMSO. These were further diluted in gentamycin medium to obtain desired concentrations.

**In vitro assay for cytotoxic activity :**

Extracts were subjected to in vitro anticancer activity against various human cancer cell lines (A-498, A-549, HCT-116, MCF-7, MDA-MB-435, OVCAR-5, PC-3, SF-295, T-47D) (Monks et al., 1991). In brief, the cells were grown in tissue culture flasks in growth medium at 37°C in an atmosphere of 5 per cent CO₂ and 90 per cent relative humidity in a CO₂ incubator (Hera Cell, Heraeus; Asheville, NCI, USA). The cells at sub-confluent stage were harvested from the flask by treatment with trypsin (0.05% trypsin in PBS containing 0.02% EDTA) and suspended in growth medium. Cells with more than 97 per cent viability (trypan blue exclusion) were used for determination of cytotoxicity. An aliquot of 100 μl of cells (10⁴ cells/ml) was transferred to a well of 96-well tissue culture plate. The cells were allowed to grow for 24 h. Extracts (100 μl/well) were then added to the wells and cells were further allowed to grow for another 48 h.

The anti-proliferative SRB assay which estimates cell number indirectly by staining total cellular protein with the dye SRB was performed to assess growth...
inhibition. The SRB staining method is simpler, faster and provides better linearity with cell number. It is less sensitive to environmental fluctuations and does not require a time sensitive measurement of initial reaction velocity (Skehan et al., 1990). The cell growth was stopped by gently layering 50 µl of 50 per cent (ice cold) trichloro acetic acid on the top of growth medium in all the wells. The plates were incubated at 4°C for 1 h to fix the cells attached to the bottom of the wells. Liquid of all the wells was then gently pipetted out and discarded. The plates were washed five-times with distilled water and air-dried. SRB 100 µl (0.4% in 1% acetic acid) was added to each well and the plates were incubated at room temperature for 30 min.

The unbound SRB was quickly removed by washing the cells five-times with 1 per cent acetic acid. Plates were air-dried, tris buffer (100 µl, 0.01 M, pH 10.5) was added to all the wells to solubilize the dye and then plates were gently stirred for 5 min on a mechanical stirrer. The optical density (OD) was recorded on ELISA reader at 540 nm. Suitable blanks (growth medium and DMSO) and positive controls (prepared in DMSO and distilled water) were also included. Each test was done in triplicate and the values recorded were mean values of three experiments.

The cell growth was determined by subtracting average absorbance value of respective blank from the average absorbance value of experimental set. Per cent growth in presence of test material was calculated as under:

- OD change in presence of control = Mean OD of control – Mean OD of blank
- OD change in presence of test sample = Mean OD of test sample – Mean OD of blank
- Per cent growth in presence of control = 100/OD change in presence of control
- Per cent growth in presence of test sample = Per cent growth in presence of control × OD change in presence of test sample
- Per cent inhibition by test sample = 100 – Per cent growth in presence of test sample

The growth inhibition of 70 per cent or above was considered active while testing extracts, but in testing of active ingredients at different molar concentrations, the growth inhibition of 50 per cent or above was the criteria of activity.

**RESULTS AND DISCUSSION**

The methanolic extract from the bulb part of *Allium sativum* did not exhibit significant cytotoxic effect (growth inhibition of 70% or above) against any of the human cancer cell line. The extract is considered inactive

### Table 1 : Growth inhibitory effect of *Allium sativum* along with positive controls against human cancer cell lines

<table>
<thead>
<tr>
<th>Plant part used</th>
<th>Extract</th>
<th>Conc. (µg/ml)</th>
<th>Lung</th>
<th>Colon</th>
<th>Colon</th>
<th>Breast</th>
<th>Lung</th>
<th>Prostate</th>
<th>Leukemia</th>
<th>Glioblastoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulb</td>
<td>Methanolic</td>
<td>100</td>
<td>08</td>
<td>42</td>
<td>37</td>
<td>56</td>
<td>41</td>
<td>00</td>
<td>27</td>
<td>04</td>
</tr>
<tr>
<td></td>
<td>Aqueous</td>
<td></td>
<td>00</td>
<td>00</td>
<td>51</td>
<td>26</td>
<td>19</td>
<td>19</td>
<td>05</td>
<td>15</td>
</tr>
<tr>
<td>Positive controls</td>
<td>Conc.(Molar)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>2×10^{-5}M</td>
<td>-</td>
<td>51</td>
<td>68</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>52</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>1×10^{-5}M</td>
<td>79</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>1×10^{-5}M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>59</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The mark (−) indicates that particular human cancer cell line was not treated with that particular positive control

### Table 2 : Growth inhibitory effect of *Holarrhena antidysenterica* along with positive controls against human cancer cell lines

<table>
<thead>
<tr>
<th>Plant part used</th>
<th>Extract</th>
<th>Conc. (µg/ml)</th>
<th>Lung</th>
<th>Colon</th>
<th>Colon</th>
<th>Breast</th>
<th>Lung</th>
<th>Prostate</th>
<th>Leukemia</th>
<th>Glioblastoma</th>
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<tr>
<td>Stem-</td>
<td>Methanolic</td>
<td>100</td>
<td>59</td>
<td>78</td>
<td>19</td>
<td>58</td>
<td>50</td>
<td>0</td>
<td>82</td>
<td>22</td>
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<tr>
<td>Leaf</td>
<td>Aqueous</td>
<td>100</td>
<td>63</td>
<td>64</td>
<td>60</td>
<td>0</td>
<td>27</td>
<td>0</td>
<td>19</td>
<td>8</td>
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<tr>
<td>Positive controls</td>
<td>Conc.(Molar)</td>
<td></td>
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<tr>
<td>5-Fluorouracil</td>
<td>2×10^{-5}M</td>
<td>-</td>
<td>51</td>
<td>68</td>
<td>-</td>
<td>-</td>
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<td>52</td>
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</tr>
<tr>
<td>Paclitaxel</td>
<td>1×10^{-5}M</td>
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<td>-</td>
<td>-</td>
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<td>Adriamycin</td>
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<td>60</td>
<td>-</td>
<td>59</td>
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</tr>
</tbody>
</table>

Growth inhibition of 70% or above has been indicated in bold numbers
The mark (−) indicates that particular human cancer cell line was not treated with that particular positive control
as the growth inhibition by this extract was observed in the range of 0-56 per cent. Similarly, aqueous extract from the bulb part of the same plant was found inactive as the growth inhibition by this extract was observed in the range of 0-51 per cent which is not considered active (Table 1). The methanolic stem-leaf extract from *H. antidysenterica* showed *in vitro* anticancer potential against two human cancer cell lines as 82 per cent growth inhibition was observed against THP-1 (a human cancer cell line from leukemia origin) and 78 per cent growth inhibition was observed against COLO-205 (a human cancer cell line from colon origin). However, the aqueous extract from the stem-leaf part of the same plant did not exhibit *in vitro* cytotoxicity against any of the human cancer cell line. The growth inhibition by this aqueous extract was observed in the range of 0-64 per cent, which is not considered significant (Table 2). Cancer is a deadly disease facing the humanity today and has emerged as an important health problem in the developed / developing countries and recognized as the important cause of morbidity, mortality, disability in India also. Cancer originates within a single cell and can be classified by the type of cell in which it originates or by the location of the cell. Presently, more than hundred types of cancer are known, the most commonly occurring ones are breast, colon, cervical, liver, lung, oral, ovary and prostate cancer. In recent years, cancer research has become a major area of scientific research supporting the foundations of modern biology to a great extent. Diverse biological disciplines such as cytogenetics, virology, cell biology, molecular genetics, epidemiology and biochemistry together with the clinical sciences have close links in their research of how cancer develops and to find remedies to stop the abnormal growth that is characteristic of cancerous cells. Despite the recent advances in surgery, endocrine therapy, radiotherapy and chemotherapy, it is considered that the management of cancer is still not upto the mark and we are in emergent need of drugs for the treatment of cancer having no side effects. Natural products or related substances or extracts of folk medicine accounted for 30 per cent of the top 35 worldwide natural product-based drugs sold and it has been estimated that > 50 per cent of all patients diagnosed with cancer explore complementary and alternative medicine – especially herbal medicine. Current evidence suggests that garlic, green tea, tomatoes and soy intake as part of the diet may be useful in preventing various cancers. A number of exciting researches suggest that vegetables, fruits, whole grains, herbs, nuts and seeds contain an abundance of polyphenolic compounds, terpenoids, sulphur compounds, pigments and other natural antioxidants, that have been associated with protection from or treatment of conditions such as cancer. Therefore, we can say that natural products have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer. In the search of potential anticancer agents from natural products, the present research work was carried out to examine the *in vitro* cytotoxic potential of Jammu botanicals against human cancer cell lines originated from different tissues.

To conclude, the present study demonstrated significant *in vitro* antitumor potential of *H. antidysenterica* and active ingredients from the promising methanolic extract of the plant can act as lead molecules for the development of anticancer drugs to provide a great service and promise to cancer patients.

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REFERENCES


