Anticancer efficacy of methanolic extracts of some medicinal plants from Jammu region, Jammu & Kashmir, India

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Received 17 December 2014; revised 30 May 2015

The methanolic extracts from ten medicinal plants (Alstonia scholaris, Azadirachta indica, Calotropis gigantea, Emblica officinalis, Mentha citrate, Mentha piperita, Musa paradisiaca, Olea europaea, Punica granatum and Trachyspermum ammi) selected from Jammu region, Jammu & Kashmir, India, were evaluated against five human cancer cell lines from four different origins, viz., A-549 (lung), HCT-116 (colon), MCF-7 (breast), PC-3 (prostate) and T-47D (breast) at the concentration of 100 µg/mL using sulphorhodamine blue (SRB) assay. A. indica, O. europaea, M. piperita and M. paradisiaca showed in vitro cytotoxic effect against one or the other human cancer cell line. The methanolic extract of stem and leaves of C. gigantea showed significant cytotoxic activity against four human cancer cell lines from four different tissues. The methanolic extract showed 82-90% growth inhibition at 50 µg/mL and 73-78% growth inhibition at 10 µg/mL against lung, colon and prostate cancer cells. However, 1 µg/mL the extract displayed only 52% growth inhibition against lung (A-549) and prostate (PC-3) cancer cells. The results have shown that C. gigantea may have potential for development of anticancer agents, especially for lung, colon and prostate cancer cells.

Keywords: Alstonia scholaris, Azadirachta indica, Calotropis gigantea, Emblica officinalis, Mentha citrate, Mentha piperita, Musa paradisiaca, Olea europaea, Punica granatum, Trachyspermum ammi

Plants have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer1. The ethanolic extract of Alstonia scholaris (satparna) stem bark has shown anticancer activity against human sarcoma2 while ethanolic extract from the leaves exhibit potential in vitro cytotoxic effect against lung, oral, breast, neuroblastoma and colon cancer cells3. The leaves of Azadirachta indica (neem) and its constituents have been demonstrated to exhibit anti-carcinogenic properties4. The ethanolic and aqueous extracts from the leaves and stem bark of the plant showed remarkable in vitro cytotoxic effect against colon, liver, neuroblastoma and prostate cancer cells5. The extracts from Calotropis gigantea (milkweed), possess hepatoprotective, antibacterial, diarrheal, analgesic properties6-9 while roots of the plant are known for anticancer properties10. The aqueous extract of Emblica officinalis (amla) inhibites the growth of A549 (lung), HepG2 (liver), HeLa (cervical), MDA-MB-231 (breast), SK-OV3 (ovarian) and SW620 (colorectal) cells in vitro and the extract induce apoptosis in HeLa, A549, MDA-MB-231 and SK-OV3 cells11. An amla extract possesses antiproliferative activity in MCF7 and MDA-MB-231 breast cancer cell lines12.

Mentha (pudina) is a plant with worldwide distribution and cytotoxic activities of its several species have been reported13-15. Chloroform and ethylacetate extracts from leaf of Mentha piperita show significant dose and time dependent anticarcinogenic activity against HeLa, MCF-7, Jurkat, T24, HT-29 and MIAPaCa-2 cancer cell lines16. Methanolic extracts and essential oils from six Mentha species viz; Mentha piperita, Mentha spicata, Mentha pulegium, Mentha longifolia, Mentha aquatica and Mentha crispa have shown cytotoxicity against HeLa and HeP2 cancer cell lines17. Musa paradisiaca (banana) is commonly used in Ayurveda for the treatment of asthma, diabetes, hypertension18. In addition to its antidiabetic19, antioxidant20, and anti-ulcer21 potential, the plant possesses in vitro cytotoxic effect against lung, colon, leukemia and breast cancer cells22.

Olea europaea (olive) oil intake has been shown to induce significant levels of apoptosis in various cancer cells including breast, prostate and colon23.

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Antioxidants are present in olive/olive oil and consumption of antioxidants are believed to reduce the risk of mutagenesis and carcinogenesis\textsuperscript{24,25}. \textit{Punica granatum} (pomegranate) fruit extracts rich in ellagitannins have shown efficacy as antioxidant and anticancer agents especially against breast and colon cancer\textsuperscript{26-29}. Anticancer/antioxidant activities of standardized whole fruit, pulp and peel extract of Egyptian pomegranate has also been reported\textsuperscript{30}. In the present study, \textit{in vitro} anticancer potential of the above mentioned ten medicinal plants of Jammu region has been investigated against five human cancer cell lines (A-549, HCT-116, MCF-7, PC-3 and T-47D) from four different tissues (breast, colon, lung and prostate).

Materials and Methods

Chemicals

RPMI-1640 medium, Dulbecco’s minimum essential medium (DMEM), dimethyl sulfoxide (DMSO), EDTA, fetal calf serum (FCS), sulphorhodamine blue (SRB) dye, phosphate buffer saline (PBS), trypsin, gentamycin, penicillin and 5-fluorouracil were purchased from Sigma Chemical Co., USA. All other chemicals were of high purity and obtained locally with the brand Sigma-Aldrich Chemicals Pvt. Ltd. and S.D. Fine Chemicals Pvt. Ltd.

Plant materials and preparation of extracts

The plant/plant parts like stem, leaves, fruit, seeds \textit{etc}. were authenticated at site by Dr. S.K. Gupta, Professor, Division of Agroforestry, SKUAST-Jammu and then collected throughout the year from Herbal Garden, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu (SKUAST-Jammu), Jammu & Kashmir, India. The freshly plant material collected was chopped, shade-dried and ground into powdered form. The methanolic extract was prepared by percolating the dried ground plant material (100 g) with 95% methanol and then concentrating it to dryness under reduced pressure. Stock solutions of 20 mg/mL were prepared by dissolving 95% methanolic extract in DMSO. Stock solutions were prepared at least one day in advance and were not filtered and the microbial contamination was controlled by addition of 1% gentamycin in complete growth medium \textit{i.e}. used for dilution of stock solutions to make working test solutions of 200 μg/mL.

Cell lines and cultures

The human cancer cells: A-549, HCT-116, MCF-7, PC-3 and T-47D were obtained from National Centre for Cell Science, Pune, India and National Cancer Institute, Frederick, USA. These human cancer cells were further grown and maintained in RPMI-1640 medium and DMEM. The media was supplemented with FCS (10%), penicillin (100 units/mL), streptomycin (100 μg/mL) and glutamine (2 mM).

Preparation of positive controls

Positive controls like adriamycin, mitomycin-C and 5-fluorouracil were prepared in distilled water, while paclitaxel was prepared in DMSO. These were further diluted in gentamycin medium to obtain desired concentrations of $2 \times 10^{-5}$ M and $2 \times 10^{-6}$ M.

In vitro assay for cytotoxic activity

Extracts were subjected to \textit{in vitro} anticancer activity against various human cancer cell lines\textsuperscript{32}. In brief, the cells were grown in tissue culture flasks in growth medium at 37°C in an atmosphere of 5% CO\textsubscript{2} and 90% relative humidity in a CO\textsubscript{2} 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% viability (trypan blue exclusion) were used for determination of cytotoxicity. An aliquot of 100 μL of cells ($10^5$ 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\textsuperscript{33}. In brief, the cell growth was stopped by gently layering 50 μL of 50% (ice cold) trichloroacetic 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% 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 reported 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. Percent growth in presence of test material was calculated as 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 (a) % Growth in presence of control = 100/OD change in presence of control (b) % Growth in presence of test sample = % Growth in presence of control × OD change in presence of test sample, (c) % Inhibition by test sample = 100 − % Growth in presence of test sample and (d) The growth inhibition of 70% or above was considered active while testing extracts, but in testing of active ingredients at different molar concentrations, the growth inhibition of 50% or above was the criteria of activity.

### Results and Discussion

In vitro cytotoxic activity of methanolic extracts from ten prominent medicinal plants from Jammu region is summarized in Table 1. The methanolic extract from the stem and leaves of *Alstonia scholaris* exhibited significant cytotoxic effect (growth inhibition of 71%) against only one human cancer cell line of lung origin A-549. However, the methanolic stem-leaf extract from *Azadirachta indica* did not exhibit significant *in vitro* cytotoxicity against any of the human cancer cell lines as the growth inhibition was observed in the range of 10-61%, which is not considered significant.

The methanolic extract of the stem and leaves of *Calotropis gigantea* showed significant *in vitro* cytotoxic effect against four human cancer cell lines from four different tissues. Maximum growth inhibition 100% was observed against MCF-7 (breast). The extract also showed 95% growth inhibition against lung cancer cells (A-549), 92% against prostate cancer cells (PC-3) and 82% against colon cancer cells (HCT-116). However, the extract was found to be inactive against T-47D cells (breast). The leaves of *Emblica officinalis* extract suppressed the proliferation of three human cancer cell lines from breast and lung origin in the range of 77-99%.

### Table 1 — Growth inhibitory effect of methanolic extracts of ten medicinal plants from Jammu region with appropriate positive controls on human cancer cell lines

<table>
<thead>
<tr>
<th>Generic name of the plant</th>
<th>Part used</th>
<th>Conc. (µg/mL)</th>
<th>Human cancer cell lines from four different tissues</th>
<th>Lung</th>
<th>Colon</th>
<th>Breast</th>
<th>Prostate</th>
<th>Breast</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A-549</td>
<td>HCT-116</td>
<td>MCF-7</td>
<td>PC-3</td>
<td>T-47D</td>
</tr>
<tr>
<td><em>Alstonia scholaris</em></td>
<td>Stem-leaf</td>
<td>100</td>
<td></td>
<td>71</td>
<td>65</td>
<td>55</td>
<td>35</td>
<td>24</td>
</tr>
<tr>
<td><em>Azadirachta indica</em></td>
<td>Stem-leaf</td>
<td>100</td>
<td></td>
<td>61</td>
<td>15</td>
<td>35</td>
<td>24</td>
<td>10</td>
</tr>
<tr>
<td><em>Calotropis gigantea</em></td>
<td>Stem-leaf</td>
<td>100</td>
<td></td>
<td>95</td>
<td>82</td>
<td>100</td>
<td>92</td>
<td>67</td>
</tr>
<tr>
<td><em>Emblica officinalis</em></td>
<td>Leaves</td>
<td>100</td>
<td></td>
<td>77</td>
<td>62</td>
<td>99</td>
<td>37</td>
<td>85</td>
</tr>
<tr>
<td><em>Mentha citrata</em></td>
<td>Whole plant</td>
<td>100</td>
<td></td>
<td>74</td>
<td>55</td>
<td>68</td>
<td>57</td>
<td>24</td>
</tr>
<tr>
<td><em>Mentha piperita</em></td>
<td>Whole Plant</td>
<td>100</td>
<td></td>
<td>29</td>
<td>14</td>
<td>00</td>
<td>26</td>
<td>23</td>
</tr>
<tr>
<td><em>Musara paradisiaca</em></td>
<td>Stem-leaf</td>
<td>100</td>
<td></td>
<td>32</td>
<td>13</td>
<td>26</td>
<td>25</td>
<td>05</td>
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<tr>
<td><em>Olea europaea</em></td>
<td>Leaves</td>
<td>100</td>
<td></td>
<td>57</td>
<td>25</td>
<td>00</td>
<td>43</td>
<td>10</td>
</tr>
<tr>
<td><em>Punica granatum</em></td>
<td>Fruit</td>
<td>100</td>
<td></td>
<td>83</td>
<td>62</td>
<td>99</td>
<td>50</td>
<td>66</td>
</tr>
<tr>
<td><em>Trachyspermum annii</em></td>
<td>Seeds</td>
<td>100</td>
<td></td>
<td>82</td>
<td>42</td>
<td>78</td>
<td>99</td>
<td>92</td>
</tr>
</tbody>
</table>

Positive controls

<table>
<thead>
<tr>
<th>Conc. (Molar)</th>
<th>5-Flurouracil</th>
<th>Paclitaxel</th>
<th>Adriamycin</th>
<th>Mitomycin-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>2×10^-5</td>
<td>-</td>
<td>78</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1×10^-6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1×10^-6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>67</td>
</tr>
<tr>
<td>1×10^-6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Growth inhibition of 70% or above is indicated in bold.

The mark (-) indicates that particular human cancer cell line was not treated with that particular positive control.
inhibition again against breast cancer cells (T-47D) and 77% growth inhibition was observed on lung cancer cells (A-549).

The methanolic extract derived from the whole plant of Mentha citrata displayed in vitro anticancer efficacy (74%) against only A-549 cancer cell line (originated from lung tissue) and the methanolic extract from the whole plant of Mentha piperita did not exhibit cytotoxic effect against any of the human cancer cell line. The methanolic extract from the stem and leaves of Musa paradisiaca showed only growth inhibition of human cancer cell lines in the range from 5-32% which was considered inactive. The methanolic extract of the leaves of Olea europaea showed insignificant cytotoxic effect in the range of 0-57%.

The methanolic extract of the fruit part of Punica gurum showed significant activity against two human cancer cell lines from breast and lung origin. The growth inhibition of 99% and 83% was produced by this extract against MCF-7 and A-549 cancer cells respectively. The seeds of Trachyspermum ammi showed remarkable in vitro anticancer activity in the range of 78-99% against four human cancer cell lines. The extract showed maximum 99% growth inhibition against prostate cancer cells (PC-3), followed by 92% on breast cancer cells (T-47D), (A-549) 82% and 78% (MCF-7). Surprisingly, the extract exhibited only 42% growth inhibition (non-significant) against HCT-116, a human cancer cell line from colon origin.

In the present investigation, methanolic extracts from six out of ten selected medicinal plants showed significant activity against two human cancer cell lines from breast and lung origin. The growth inhibition of 99% and 83% was produced by this extract against MCF-7 and A-549 cancer cells respectively. The seeds of Trachyspermum ammi showed remarkable in vitro anticancer activity in the range of 78-99% against four human cancer cell lines. The extract showed maximum 99% growth inhibition against prostate cancer cells (PC-3), followed by 92% on breast cancer cells (T-47D), (A-549) 82% and 78% (MCF-7). Surprisingly, the extract exhibited only 42% growth inhibition (non-significant) against HCT-116, a human cancer cell line from colon origin.

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In the present investigation, methanolic extracts from six out of ten selected medicinal plants showed (cell line specific) in vitro cytotoxic activity with one or the other human cancer cell line. Most significant results, that is cytotoxic effect against human cancer cell lines was displayed by the methanolic stem and leaf extract from Calotropis gigantea. cytotoxic potential of the extract was much stronger than that shown by standard anticancer drugs (used as positive controls). The extract showed 100% growth inhibition in case of breast cancer cells (MCF-7). Further, the methanolic extract from Calotropis gigantea was evaluated at lower concentrations of 50 µg/mL, 10 µg/mL and 1 µg/mL against same human cancer cell lines and the result is summarised in Table 2. The extract showed 82-90% growth inhibition at 50 µg/mL and 73-78% growth inhibition at 10 µg/mL against lung, colon and prostate cancer cells. However, at 1 µg/mL the extract displayed 52% growth inhibition against lung (A-549) and prostate (PC-3) cancer cells.

Most of the drugs used in cancer chemotherapy exhibit cell toxicity and can induce genotoxic, carcinogenic and teratogenic effects in non-tumor cells. Therefore, there is a need for alternative drugs of natural origin that are less toxic, endowed with fewer side effects and more potent in their mechanism of action. Recently, we have reported that methanolic extract from the leaves of Nardostachys jatamansi (commonly known as muskroot) exhibits in vitro anticancer effect against five human cancer cell lines viz., NCI-H23, HeLa, SK-N-MC, SW-620 and COLO-205 in the range of 70-93%14. Similarly, the methanolic extract from the fruit part of ‘Kamala tree’ (Mallotus philippinensis) has displayed significant cytotoxic effect against fourteen human cancer cell lines: A-549, COLO-205, DU-145, HEP-2, HeLa, IMR-32, KB, MCF-7, NCI-H23, OVCAR-5, SiHa, SK-N-MC, SW-620 and ZR-75-15. Also, the methanolic extract from the stem-leaves of Calotropis procera has shown 70% growth inhibition of colon cancer cells (HCT-15)36 and the ethanolic extract from the seed part of Apium graveolens is also observed to be active against COLO-205, HeLa, KB, SK-N-MC37. The methanolic extract from the stem and leaves of Alstonia scholaris exhibited remarkable in vitro anticancer efficiency against leukemia and lung cancer cells38. Moreover, the methanolic extracts from the whole plants of four Mentha spp. viz, M. arvensis, M. longifolia, M. spicata and M. Viridis have shown

<p>| Table 2 — Growth inhibitory effect of methanolic extract of Calotropis gigantea at different concentrations on human cancer cell lines |</p>
<table>
<thead>
<tr>
<th>Generic name of the plant</th>
<th>Part used</th>
<th>Conc. (µg/mL)</th>
<th>Human cancer cell lines from four different tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calotropis gigantea</td>
<td>Stem-leaf</td>
<td>100</td>
<td>Lung (A-549) 95 82 100 92 67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>Colon (HCT-116) 90 85 60 82 40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>Breast (MCF-7) 78 78 49 73 26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>Prostate (PC-3) 78 78 49 73 26</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Breast (T-47D) 52 00 00 52 11</td>
</tr>
</tbody>
</table>

Growth inhibition of 70% or above is indicated in bold.
The active ingredients from this promising methanolic extract from stem and leaves of *Calotropis gigantea* can act as lead molecules, for the development of drugs especially for colon, lung and prostate carcinoma to provide a great service and promise to cancer patients. The overall results analysis of ant cancerous activity of selected medicinal plants reported can be arranged based on the growth cytotoxic effect as follows. *Calotropis gigantea* > *Trachyspermum ammi* > *Emblica officinalis* > *Punica granatum* > *Mentha citrate* > *Alstonia scholaris* > *Azadirachta indica* > *Olea europaea* > *Musa paradisiaca* > *Mentha piperita*.

**References**

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