**In vitro** cytotoxic activity of leaves extracts of *Holarrhena antidysenterica* against some human cancer cell lines

Vikas Sharma*, Shabir Hussain, Manish Bakshi, Neha Bhat and Ajit Kumar Saxena

Division of Biochemistry, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Main Campus Chatha, Jammu-180009, J&K, India

Cancer Pharmacology Division, Indian Institute of Integrative Medicine (IIIM) - CSIR, Canal Road, Jammu-180001, J&K, India

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**In vitro** cytotoxic potential of extracts (95% and 50% ethanolic extract and hot water extract at concentration of 100 µg/ml) from leaves of *Holarrhena antidysenterica* was evaluated against fourteen human cancer cell lines — A-549, COLO-205, DU-145, HeLa, HEP-2, IMR-32, KB, MCF-7, NCI-H23, OVCAR-5, SiHa, SK-N-MC, SW-620 and ZR-75-1 from nine different tissues (breast, colon, cervix, CNS, lung, liver, oral, ovary and prostate) using SRB assay. The 95% ethanolic extract displayed maximum anti-proliferative effect in the range of 73-92% against eight human cancer cell lines, while 50% ethanolic extract showed cytotoxic activity in the range of 70-94% against seven human cancer cell lines. However, the hot water extract did not show any activity. Among the fractions of 95% and 50% ethanolic extract, significant cytotoxic activity was found in the chloroform soluble fraction of 95% ethanolic extract at 100 µg/ml; it inhibited the growth in the range of 71-99% of seven human cancer cell lines from five different tissues viz., OVCAR-5 (ovary), HT-29 (colon), SK-N-MC (neuroblastoma), HEP-2 (liver), COLO-205 (colon), NIH-OVCAR-3 (ovary) and A-549 (lung). The cytotoxic activity of chloroform soluble fraction was found to be higher than 5-flurouracil, adriamycin, mitomycin-c and paclitaxel (anticancer drugs used as positive controls). Further **in vivo** studies and identification of active components from the chloroform fraction and their exact mechanism of action could be useful in designing new anticancer therapeutic agents.

**Keywords:** *Holarrhena antidysenterica*, Cytotoxic activity, Human cancer cells, SRB assay

*Holarrhena antidysenterica* (Linn.) Wall, popularly known as ‘bitter oleander’ and belonging to Apocynaceae family is a laticiferous deciduous shrub/small tree with white flowers, distributed throughout India in deciduous forests up to an altitude of 3500 ft. In traditional system of Indian medicine, the different parts of the plant have been considered as a popular remedy for the treatment of diarrhoea and intestinal worms.

*H. antidysenterica* is used by various Indian tribals in case of a number of ailments like anaemia, epilepsy, stomach pain, cholera, skin diseases and antiprotozoal activity of individual alkaloids of the plant, especially conessine has been extensively investigated. A hot decoction of *H. antidysenterica* has been used by Portuguese in bowel infection. The plant is used in the preparation of an ayurvedic formulation useful for the treatment of AIDS and has shown in vitro antioxidant activity. The leaves and roots of the plant are useful in treatment of scabies and the trunk-bark possesses amoebicidal property. Bark and seeds of the plant are used as a powerful antidysenteric, astringent, febrifuge, antihelmenthic, antibacterial agent and seeds possess antidiabetic activity.

The stem bark of the plant, commercially known as kurchi, has been used traditionally for the treatment of dysentry. In clinical study, stem bark powder has shown effectiveness against bleeding piles. The crude aqueous and alcoholic extracts of stem bark exhibit anti-bacterial activity against the known enteric pathogens. The stem bark contains a number of alkaloids; antibacterial activity of the stem bark is mainly associated with alkaloids; . The alkaloid holarrifine-24-ol isolated from the stem bark has shown good antibacterial (against ten pathogenic bacteria) and antifungal (against six phytopathogenic fungi) activity.

A derivative of propofol (2, 6-diisopropylphenol) prepared by coupling 9-hydroxy-11-Z-octadecenoic acid (isolated from seed oil of *H. antidysenterica*)
with Cl-α-hydroxy function of propofol has been found to show cytotoxic activity against HeLa, MCF-7 and HL-60 cancer cells. This derivative (3-8 µg/ml) is reported to decrease the metastatic potential of H71080, HOS, RPMI-7951 and HeLa cancer cells and has shown a beneficial effect on antitumor immunity in mice.

Cancer is the most common and fatal disease responsible for 2-3% deaths recorded worldwide annually. A total of 16,38,910 new cancer cases and 5,77,190 deaths from cancer were projected in USA in 2012. In India, around 5,55,000 people died of cancer in 2010. The phytochemical analysis of H. antidysenterica has shown the presence of many biologically active substances, but no systematic work has so far been reported on anticancer property of leaves of the plant. In the present study, we have evaluated in vitro cytotoxic activity of the leaves of H. antidysenterica against fourteen human cancer cell lines — A-549, COLO-205, DU-145, HeLa, HEP-2, IMR-32, KB, MCF-7, NCI-H23, OVCAR-5, SiHa, SK-N-MC, SW-620 and ZR-75-1 from nine different tissues (breast, colon, cervix, CNS, lung, liver, oral, ovary and prostate) using SRB assay.

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-flourouracil were purchased from Sigma Chemical Co., USA. All other chemicals were of high purity and obtained locally from M/s Ramesh Traders, Palace Road, Jammu (brand was Sigma-Aldrich Chemicals Pvt. Ltd. and S.D. Fine Chemicals Pvt. Ltd.)

Plant material and preparation of extracts
Holarrhena antidysenterica leaves were collected in the month of July from Herbal garden, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu (SKUAST-Jammu), J&K, India and were authenticated at site by Dr. M Saleem, Professor, Division of Agroforestry, SKUAST-Jammu, J&K, India. The freshly collected leaves were chopped, shade-dried and ground into powdered form, which was then extracted with different solvents at room temperature to obtain extracts for bioevaluation. The ethanolic extracts were prepared by percolating the dried ground leaf material (100 g) with 95% ethanol or 50% ethanol and then concentrating it to dryness under reduced pressure. The aqueous extract was obtained by boiling dried ground plant material (100 g) for 30 min in distilled water (300 ml) and freeze-dried.

Stock solutions of 20 mg/ml were prepared by dissolving 95% ethanolic extract in DMSO, 50% ethanolic extract in 50% DMSO and aqueous extract in sterile water. Stock solutions were prepared at least one day in advance and were not filtered, but the microbial contamination was controlled by addition of 1% gentamycin in complete growth medium i.e. used for dilution of stock solutions to make working test solutions of 200 µg/ml.

Preparation of fractions
The ethanolic extract was fractionated into four fractions. For hexane soluble fraction, dried ethanolic extract (10 g) was taken in a stoppered conical flask (250 ml) and vigorously shaken with hexane (100 ml). After standing for 30 min, supernatant was decanted. The procedure was repeated three-times and the combined hexane soluble portion was concentrated to dryness under reduced pressure. For chloroform soluble fraction, the residue left after removing the hexane soluble part was further macerated with chloroform (100 ml) four times. Combined chloroform soluble portion was then concentrated to dryness under reduced pressure. For n-butanol and water soluble fractions, the residue left after removing the hexane soluble and chloroform soluble part was suspended in water (200 ml) in a separating funnel and extracted four-times (each time with 100 ml) with n-butanol. The n-butanol and water soluble portions were collected and concentrated to dryness under reduced pressure. The fractions obtained were dissolved in DMSO to form stock solutions of 20 mg/ml. Similarly, 50% ethanolic extract was fractionated into three fractions (chloroform, n-butanol and water soluble) and the fractions obtained were dissolved in 50% DMSO to form stock solutions of 20 mg/ml.

Cell lines and cultures
The human cancer cells 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, 5-fluorouracil and mitomycin-C 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 $2 \times 10^{-4}$ M.

**In vitro assay for cytotoxic activity**

Extracts and fractions were subjected to *in vitro* anticancer activity against various human cancer cell lines. In brief, the cells were grown in tissue culture flasks in growth medium at 37°C in an atmosphere of 5% CO$_2$ and 90% relative humidity in a CO$_2$ incubator (Hera Cell; Heraeus; Asheville, NCI, USA). 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 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 and fractions were then added to the wells and cells were further allowed to grow for another 48 h.

The anti-proliferative SRB assay was performed to assess growth inhibition which estimates cell number indirectly by staining total cellular protein with the dye SRB. The SRB staining method is simpler, faster and provides better linearity with cell number. It is less sensitive to environmental fluctuations and do not require a time sensitive measurement of initial reaction velocity. 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 an hour 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 were 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 10.4) was added to all the wells to solubilize the dye and then plates were gently stirred for 5 min on a mechanical stirrer. The 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 under:

$$\text{OD Change in presence of control} = \text{Mean OD of control} - \text{Mean OD of blank}$$

$$\text{OD Change in presence of test sample} = \text{Mean OD of test sample} - \text{Mean OD of blank}$$

$$\% \text{ Growth in presence of control} = \frac{100}{\text{OD change in presence of control}}$$

$$\% \text{ Growth in presence of test sample} = \% \text{ Growth in presence of control} \times \text{OD change in presence of test sample}$$

$$\% \text{ Inhibition by test sample} = 100 - \% \text{ Growth in presence of test sample}$$

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**

The extracts (100 µg/ml) were evaluated against fourteen human cancer cell lines from nine different tissues and ethanolic extracts showed *in vitro* cytotoxic effect against human cancer cell lines. The 95% ethanolic extract exhibited *in vitro* anticancer potential against eight human cancer cell lines from seven different origins, *viz.*, A-549 (lung), COLO-205, SW-620 (colon), HEP-2 (liver), KB (oral), OVCAR-5 (ovary), SiHa (cervical) and SK-N-MC (neuroblastoma). Surprisingly, 50% ethanolic extract also showed remarkable activity against all the above-mentioned human cancer cell lines, except SiHa. The 95% and 50% ethanolic extracts show activity in the range of 73-92% and 70-94% respectively. The aqueous extract did not show any significant cytotoxic effect on human cancer cell lines; however, it showed 63% and 64% growth inhibition of A-549 and COLO-205, respectively (Table 1).

The fractions (100 µg/ml) obtained from the 95% and 50% ethanolic extracts were evaluated against ten
SHARMA et al.: IN VITRO CYTOTOXIC ACTIVITY OF LEAVES EXTRACTS OF H. ANTIDYSENTERICA

Out of a total of four fractions obtained from 95% ethanolic extract, significant anti-proliferative effect on human cancer cell lines was displayed by the chloroform soluble fraction. It exhibited highest cytotoxic effect (71-99%) against seven human cancer cell lines. The maximum growth inhibition (99%) was observed against OVCAR-5 (ovary), followed by HT-29 (98%), SK-N-MC (95%), HEP-2 (88%), COLO-205 (84%), NIH0VCAR-3 (82%) and A-549 (71%). The hexane-soluble fraction showed activity against four human cancer cell lines, namely HCT-15, HEP-2, OVCAR-5, SiHa and the growth inhibition ranged between 73-97%. However, n-butanol and water soluble fraction suppressed the proliferation of only HCT-15 (96% and 98% respectively) and SiHa (99% and 90%, respectively) as shown in Table 2.

Among the fractions obtained from 50% ethanolic extract, only chloroform soluble and n-butanol soluble fractions showed in vitro anticancer activity. The chloroform soluble fraction was active against five human cancer cell lines (A-549, COLO-205, HEP-2, NIH0VCAR-3, OVCAR-5) in the range of 76-98%, while n-butanol soluble fraction inhibited the growth of four human cancer cell lines (A-549, COLO-205, HEP-2, NIH0VCAR-3) in the range of 74-88%. The water soluble fraction was found inactive against all the ten human cancer cell lines (Table 3).

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. Earlier, the 95% ethanolic extract from the leaves of Nardostachys jatamansi, commonly known as muskroot has shown 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%, however, its chloroform fraction shows activity against only three cancer cell lines, namely SK-N-MC, SW-620 and HT-29. Similarly, the 95% ethanolic extract from the fruit part of ‘Kamala tree’ (Mallotus philippinensis) has displayed highest cytotoxic effect against fourteen human cancer cell lines and its chloroform fraction is found to suppress the proliferation of A-549, COLO-205, HT-29 HEP-2, HCT-15, NIH0VCAR-3, OVCAR-5, SiHa, SK-N-MC and T-47D. Further, purified compound (Rottlerin), isolated from the fruit part of
Table 2—Growth inhibitory effect of fractions (95% ethanolic leaves extract) from *H. antidysenterica* on human cancer cell lines along with positive controls

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Conc. (µg/ml)</th>
<th>Breast</th>
<th>Cervix</th>
<th>CNS</th>
<th>Colon</th>
<th>Lung</th>
<th>Liver</th>
<th>Ovary</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T-47D</td>
<td>SiHa</td>
<td>SK-N-MC</td>
<td>COLO-205</td>
<td>HCT-15</td>
<td>HT-29</td>
<td>A-549</td>
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<tr>
<td>Hexane soluble</td>
<td>100</td>
<td>34</td>
<td>86</td>
<td>60</td>
<td>64</td>
<td>97</td>
<td>0</td>
<td>64</td>
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<tr>
<td>Chloroform soluble</td>
<td>100</td>
<td>62</td>
<td>0</td>
<td>95</td>
<td>84</td>
<td>0</td>
<td>98</td>
<td>71</td>
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<tr>
<td>n-Butanol soluble</td>
<td>100</td>
<td>19</td>
<td>99</td>
<td>51</td>
<td>2</td>
<td>96</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Water soluble</td>
<td>100</td>
<td>12</td>
<td>90</td>
<td>0</td>
<td>0</td>
<td>98</td>
<td>0</td>
<td>8</td>
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Growth Inhibition (%)

Positive controls

<table>
<thead>
<tr>
<th>Conc. (Molar)</th>
<th>5-Fluorouracil</th>
<th>Paclitaxel</th>
<th>Adriamycin</th>
<th>Mitomycin-C</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2 × 10⁻⁵ M</td>
<td>1 × 10⁻⁶ M</td>
<td>1 × 10⁻⁶ M</td>
<td>1 × 10⁻⁶ M</td>
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<tr>
<td></td>
<td>64</td>
<td>66</td>
<td>66</td>
<td>66</td>
</tr>
</tbody>
</table>

(-) Indicates that particular human cancer cell line was not treated with that particular positive control

Table 3—Growth inhibitory effect of fractions (50% ethanolic leaves extract) from *H. antidysenterica* on human cancer cell lines along with positive controls

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Conc. (µg/ml)</th>
<th>Breast</th>
<th>Cervix</th>
<th>CNS</th>
<th>Colon</th>
<th>Lung</th>
<th>Liver</th>
<th>Ovary</th>
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<tr>
<td></td>
<td></td>
<td>T-47D</td>
<td>SiHa</td>
<td>SK-N-MC</td>
<td>COLO-205</td>
<td>HCT-15</td>
<td>HT-29</td>
<td>A-549</td>
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<tr>
<td>Chloroform soluble</td>
<td>100</td>
<td>66</td>
<td>16</td>
<td>63</td>
<td>85</td>
<td>68</td>
<td>50</td>
<td>78</td>
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<td>100</td>
<td>31</td>
<td>0</td>
<td>65</td>
<td>88</td>
<td>68</td>
<td>56</td>
<td>85</td>
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<tr>
<td>Water soluble</td>
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<td>5</td>
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Growth Inhibition (%)

Positive controls

<table>
<thead>
<tr>
<th>Conc. (Molar)</th>
<th>5-Fluorouracil</th>
<th>Paclitaxel</th>
<th>Adriamycin</th>
<th>Mitomycin-C</th>
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<tbody>
<tr>
<td></td>
<td>2 × 10⁻⁵ M</td>
<td>1 × 10⁻⁶ M</td>
<td>1 × 10⁻⁶ M</td>
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<tr>
<td></td>
<td>64</td>
<td>66</td>
<td>73</td>
<td>88</td>
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</table>

(-) Indicates that particular human cancer cell line was not treated with that particular positive control
M. philippinensis has shown significant activity against HT-29, HEP-2, HCT-15, HOP-62, OVCAR-5, SiHa, SK-N-SH and SW-620. The 95% ethanolic extract from the stem-leaves of Calotropis procera has also shown 70% growth inhibition of colon cancer cells (HCT-15) and its chloroform fraction exhibits 80% activity against HCT-15. Further, the 95% ethanolic extract from the seed part of Apium graveolens is observed to be active against COLO-205, HeLa, KB, SK-N-MC and its chloroform fraction inhibits the growth of A-549, COLO-205, HT-15, HEP-2, OVCAR-5, SNB-78, SK-OV-3 and SW-620 in the range of 84-99%

In conclusion, the present study showed significant in vitro antitumor potential of chloroform soluble fraction of leaves of H. antidysenterica; the cytotoxic activity was found to higher than 5-flurouracil, adriamycin, mitomycin-c and paclitaxel (anticancer drugs used as positive controls). Further studies are being carried out to characterize the bioactive constituents responsible for the anticancer activity from the chloroform soluble fraction.

References