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Selective *in vitro* cytotoxicity of *Hypericum hookerianum* towards cancer cell lines

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SUMMARY

The methanol extracts of the aerial parts, leaves and stem of *Hypericum hookerianum* were tested for *in vitro* cytotoxicity on selected normal and cancer cell lines and anti tumor activity using DLA cells. Cell viability and morphological changes were assessed. Among the three extracts tested, the stem extract of *Hypericum hookerianum* showed potent cytotoxicity against HEp-2 and RD cell lines. The CTC_{50} (concentration required to reduce viability by 50%) of this extract was found to be 2.02 μg/ml for RD cell line, 10.25 μg/ml for HEp-2 cell line and 100.06 μg/ml for Vero cell line. In the clonogenic assay, no colony formation was observed up to a concentration of 100 μg/ml. In the short term cytotoxicity studies using DLA cells, 50% viability was observed in the concentration range of 50-100 μg/ml for aerial parts, 100-200 μg/ml for stem and more than 200 mg/ml for leaf extracts of *Hypericum hookerianum*. In the long-term activity using HEp-2 cell line, no colony formation was observed over a concentration of 200 mg/ml for the stem extract. *Hypericum hookerianum* stem extract was fractionated into petroleum ether, chloroform, ethyl acetate and methanol soluble fractions. The petroleum ether and chloroform soluble fractions showed higher cytotoxic activity against HEp-2 cell line when compared to the other two fractions. The methanol stem extract of *Hypericum hookerianum* has the potential for further investigation in animal models to determine its anti-tumor activity and to identify its active principles.

Key words: *Hypericum hookerianum*; Cytotoxicity; Anti-tumor; Colony formation

INTRODUCTION

Plants of the genus *Hypericum* (Family: Hypericaceae) are herbs, shrubs or small trees, and are distributed chiefly in the temperate regions of the world. Almost all the plants of the genus *Hypericum* are widely used in folk medicine (Anonymous, 1962). Among these, the constituents of the leaf essential oil of *Hypericum perforatum* Linn. are known to possess cytotoxic properties (Weyerstahh, *et al.*, 1995). Recent investigation on *Hypericum mysorens* Wight & Arn. and *Hypericum patulum* Thunb. carried out in our laboratories has shown potent cytotoxic properties (Vijayan *et al.*, 2003). *Hypericum hookerianum* Wight & Arnott. also known as *Norysca hookerianum* Wight & Arnott. is another plant belonging to the genus *Hypericum*. *Hypericum hookerianum* is a round-topped shrub with weakly spreading, non-erect branches with golden yellow flowers. The plant is mostly found in Sikkim, Khari and Jaintia hills and in Nilgiris, India (Anonymous, 1962). Recently, wound-healing properties of its leaf and stem extracts have been reported (Mukherjee and Suresh, 2000). Except this, so far no other biological properties have been reported. The present paper describes the *in vitro* cytotoxic properties of the methanol extracts of the aerial parts, leaves and stem of *Hypericum hookerianum* against the malignant rhabdomyosarcoma (RD) cells, caucasian male larynx epithelium carcinoma (HEp-2) cells, Dalton's lymphoma ascites (DLA) cells and normal African green monkey (Vero) cells.

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MATERIALS AND METHODS

Plant material and extraction

Fresh materials (aerial parts, leaves and stem) of *Hypericum hookerianum* were collected from Ootacamund, a famous hill station in southern India belonging to the district Nilgiris of Tamilnadu, India. It was identified and authenticated by Botanical Survey of India, Southern circle, Coimbatore, where voucher specimens are preserved. The aerial parts, leaves and stem were dried in the shade and each was pulverized separately in a mechanical grinder, passed through a 40 mesh sieve and stored in a closed vessel for further use.

The powdered aerial parts, leaves and stem of *Hypericum hookerianum* were extracted with methanol using Soxhlet extraction apparatus. The methanolic extracts were then concentrated and dried under reduced pressure. The methanol free semisolids, thus obtained were used for the experiments. The yield was 7.75% w/w, 11.75% w/w and 9.65% w/w with respect to dried powdered material from the aerial parts (HHAM), leaves (HHLM) and stem (HHSM) extracts, respectively. The extracts were separately dissolved in dimethylsulfoxide (DMSO) and the volume was made up to 10 ml with DMEM/RPMI medium to obtain 1000 μg/ml concentrated stock solutions and were stored at -20°C till use.

Cell lines and culture medium

HEp-2, RD and Vero cell cultures used in the experiments were obtained from National Center for Cell Sciences, Pune and Pasteur Institute of India, Coonoor. Stock cells of HEp-2, RD and Vero cell lines were cultured in RPMI-1640 and DMEM supplemented with 10% sheep serum, penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.05% glucose, 0.02% EDTA in phosphate buffered saline). The stock cultures were grown in 110 ml flat bottles and all experiments were carried out in 96 well microtiter plates, where the cell population was adjusted to 10,000 cells per well. DLA cells were obtained from Amala Cancer Research Centre, Trissur, and were maintained in Swiss albino mice at the JSS College.

Cytotoxic assay

The cytotoxicity assay was carried out using 0.1-ml of cell suspension containing 10,000 cells was seeded in each well of a 96 well microtiter plate (Nunc and Tarsons). Fresh medium containing different concentration of the extracts was added 24 h after seeding. Control cells were incubated without the test extracts and with DMSO (solvent). The very little percentage of DMSO present in the wells (maximal 0.2%) did not affect the experimental results. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO_2 for a period of 3 days. Twelve wells were used for each concentration of the extracts. Morphological changes were examined using inverted microscope. The cells were observed at different time intervals during incubation period in the presence or absence of the compound. Cellular viability was determined by using the standard MTT (3-(4,5-dimethylthiozole-2yl)-2,5-diphenyl tetrazolium bromide) assay (Francis and Rita, 1986; Ke *et al.*, 1999), Trypan blue dye exclusion method (Moldeus *et al.*, 1978) and cell metabolic function by Protein estimation (Lowry *et al.*, 1951) from the treated culture of 4 wells of each concentration.

MTT assay

MTT assay is based on the reduction of the soluble MTT into a blue purple formazan product mainly by mitochondrial reductase activity inside living cells. The number of viable cells were found to be proportional to the extent of formazan production for the cell lines used in this study. After incubation, the solutions in four wells of each concentration were discarded and 50 μl of a solution of 2 mg/ml of MTT (Sigma Chemicals Co., St. Louis, MO, USA) in DMEM (without phenol red) was added and the cultures were incubated for an additional 3 h at 37°C. The supernatant was removed and the cells were treated with propanol (100 μl/well) and kept aside for 10 min at room temperature. The absorbance was read on a microtiter plate reader (Bio-Rad, Model 550) at a wavelength of 540 nm and the mean absorbance from four wells was recorded. Mean absorbance taken from cells grown in the absence of the extracts was taken as 100% cell survival (control). The percentage inhibition was calculated using the

following formula.

Growth Inhibition %

$$=100 - \left[\frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100\right]$$

The percentage inhibition was plotted against concentration and the CTC_{50} (concentration required to reduce viability by 50%) value for each cell line was calculated.

Dye exclusion method

The solutions in the other 8 wells of each concentration were discarded and cells were trypsinized with 100 µl of TPVG (Trypsin, PBS, Versene, Glucose Solution) for 3 min at 37°C. To stop the activity of trypsin, 100 µl of growth medium was added, the cells were flushed and cells from four wells, of each concentration wise, into Eppendorf tubes. The pooled cells from four wells of each concentration tested were then subjected for dye exclusion and protein estimation.

For testing viability using dye exclusion method, the pooled cells from four wells of each concentration were mixed with 0.4% trypan blue in the ratio of 1:1 and the number of stained, non-stained and total number of cells were counted using haemocytometer. The percentage Inhibition and CTC_{50} were calculated.

Protein content estimation

The pooled cells from the remaining four wells of each concentration tested were centrifuged at 10,000 rpm in a cooling centrifuge. The cell pellets were then treated with 0.5 ml of 11% trichloro acetic acid and the precipitated proteins were assayed by Lowry method. The % inhibition and CTC_{50} values were calculated.

Clonogenic assay

Cytotoxicity was assayed by determining the ability of HEp-2 cell to form colonies after the treatment with the extracts (Christopher *et al.*, 1985). The monolayer cell culture was trypsinized and the cell count adjusted to 5000 cells/ ml. To each well of a 24 well microtiter plate, 1 ml of the diluted cell suspension was added and incubated overnight at 37°C in 5% CO_2 atmosphere. The supernatant was discarded and the cells were exposed to different concentrations of the extracts for 2h. Afterwards, the supernatant was discarded and the cells were washed with fresh medium, followed by addition of 1 ml growth medium. Cell colonies were allowed to grow for 2 weeks at 37°C, 5% CO_2. The medium was then removed, the colonies were stained with 1% crystal violet (Sigma Chemical Co., St. Louis, MO, USA) in 70% ethanol and then counted manually. The tolerance limit was determined by the ability of a cell to form a colony containing more than 50 cells.

Short term cytotoxic activity

Short-term cytotoxic activity (Moldeus *et al.*, 1978) of the extracts was assayed by determining the percentage viability of DLA cells using Trypan blue dye exclusion technique. DLA cells were cultured in the peritoneal cavity of healthy albino mice weighing between 25 to 30 g by injecting a suspension of DLA cells (1×10^6 cells/ml) intraperitoneally. The cells were aspirated aseptically from the peritoneal cavity of the mice on the 15th day. The cells were washed with Hanks balanced salt solution (HBSS) and centrifuged for 10 to 15 min at 1500 rpm in the cooling centrifuge. The pellet was re-suspended with HBSS and the process was repeated for three times. Finally, the cells were suspended in known quantity of HBSS and the cell count was adjusted to 2×10^6 cells/ml and 0.1 ml of the diluted cell suspension was distributed into Eppendorf tubes and exposed to 0.1 ml each of the different concentrations of the extract and incubated at 37°C, 5% CO_2 for 3 h. After 3 h, Trypan blue dye exclusion test was performed to determine the percentage viability.

Long term anti-tumor activity

In the above studies, HHSM was found to be the most potent extract. Hence, it was subjected for long term anti-tumor test for cell survival. This assay was carried out by determining the ability of HEp-2 cells to form the colonies after the extract treatment by the method of Freshney (1987). A series of cultures were prepared in 25 cm^2 flasks, three flasks for each concentration and three for controls by seeding 2×10^5 cells in 4 ml of growth medium and incubated at 37°C for 48 h. Growth

medium was discarded and cultures were exposed to different concentrations of the extract and incubated further for 72 h. Morphological changes in cultures were observed under the microscope. At 72 h past incubation, the medium was discarded, cultures were trypsinized and seeded at required density (500 cells/ml) for cell growth, diluting all cultures by same amount as control and the seeded cultures were incubated for 2 weeks until colonies were formed. Colonies were fixed, stained and counted.

RESULTS

The methanol extracts of the aerial parts, leaves and stem of the *Hypericum hookerianum* have shown dose dependent destruction of monolayer and morphological changes. All the three extracts showed cytotoxic activity against HEp-2, RD and Vero cell. Potent cytotoxic activity was observed in the HHSM compared to other two extracts. The CTC_{50} of the HHSM was found to be 2.02 μg/ml for RD, 10.25 μg/ml for HEp-2 and 100.06 μg/ml for Vero cell lines. Among the three cell lines, RD and HEp-2 cells were found to be most susceptible (Table 1). In the clonogenic assay, no colony formation was observed over a concentration of 100 μg/ml for HHSM, 200 μg/ml for HHLM and 600 μg/ml for HHAM (Table 2).

Table 2. Cytotoxic effect of the methanolic extracts of aerial parts (HHAM), leaves (HHLM) and stem (HHSM) of *Hypericum hookerianum* on HEp-2 cells using Clonogenic assay

Treatment	Concentration (μg/ml)	No. of Colonies*
HHAM	700	0±0.00
	600	0±0.00
	500	12±1.1
	400	24±1.8
Control	----	52±3.2
HHLM	400	0±0.00
	300	0±0.00
	200	0±0.00
	100	25±1.62
Control	----	50±3.74
HHSM	300	0±0.00
	200	0±0.00
	100	0±0.00
	50	12±0.95
Control	----	55±4.02

*Average of three independent experiments±SE

In the short term cytotoxic studies using DLA cells, 50% viability was observed in the concentration range of 50-100 μg/ml for HHAM, 100-200 μg/ml for HHSM and more than 200 μg/ml for HHLM (Table 3). In the long-term survival activity using HEp-2 cell line, no colony formation was observed over a concentration of 200 μg/ml for HHSM (Table 4).

Table 1. Cytotoxic effect of methanolic extract of *Hypericum hookerianum* aerial parts, leaves and stem on HEp-2, RD and Vero Cell lines

Extract	Cell line	Cytotoxicity (CTC_{50}) μg/ml by*			Mean CTC_{50} μg/ml
		MTT assay	Dye exclusion Method	Protein content	
Aerial parts	HEp-2	161.29±11.65	220.13±18.11	293.12±19.05	224.84
	RD	99.42±4.87	65.67±2.88	195.29±10.66	120.12
	Vero	264.37±12.42	287.57±21.09	261.54±11.64	271.16
Leaves	HEp-2	81.22±5.15	210.01±15.56	120.10±7.22	137.11
	RD	155.62±6.76	160.34±12.34	91.21±3.87	135.72
	Vero	227.63±10.43	200.00±7.95	201.13±12.48	209.59
Stem	HEp-2	6.52±0.28	11.02±0.76	13.21±1.08	10.25
	RD	1.30±0.07	0.64±0.04	4.12±0.29	2.02
	Vero	105.43±5.87	95.32±4.25	99.42±7.51	100.06
Stem extract fractions					
Petroleum ether	HEp-2	71.33±4.06	80.09±3.55	70.14±5.44	73.85
Chloroform	HEp-2	98.23±5.28	95.71±5.12	92.13±5.98	95.36
Ethyl acetate	HEp-2	110.23±6.45	185.23±12.77	110.23±7.02	135.23
Methanol	HEp-2	495.21±19.76	490.92±22.90	485.16±24.78	490.43

*Average of six independent determinations±SE

Table 3. The cytotoxic effect of the methanolic extracts of aerial parts (HHAM), leaves (HHLM) and stem (HHSM) of *Hypericum hookerianum* on DLA cells after 3 h exposure

Treatment	Concentration (μg/ml)	Percentage viability*
Control	----	99.56±7.28
HHAM	200	00.70±0.05
	100	23.78±1.90
	50	96.49±8.22
	25	99.42±7.68
HHLM	200	92.66±6.45
	100	98.03±6.23
	50	98.77±7.21
	25	98.93±7.69
HHSM	200	03.33±0.02
	100	70.37±5.72
	50	92.30±5.95
	25	92.59±6.42

*Average of three independent experiments±SE

Table 4. The anti-tumor effect of methanolic extract of *Hypericum hookerianum* (stem, HHSM) on HEp-2 cells by long term survival method

Treatment	Concentration (μg/ml)	No. of Colonies*
Control	----	56±3.08
HHSM	200	0±0.00
	40	22±1.33
	8	38±2.74
	1.6	52±2.98

*Average of three independent experiments±SE

DISCUSSION

In the present study, the methanol extracts of aerial parts, leaves and stem of *Hypericum hookerianum* were evaluated for *in vitro* cytotoxicity and short term and long term antitumour activity. The HHSM has shown strong cytotoxic properties against all the cancerous cell lines. Maximum cytotoxicity was observed against the RD cell line. The HHSM has shown poor cytotoxicity against the normal Vero cells. This gives an indication that the HHSM is inhibiting the growth of cancerous cells at very low concentrations. The clonogenic assay also confirms the cytotoxic activity of the extracts. Based on these results obtained, HHSM was fractionated into petroleum ether, chloroform, ethyl acetate and methanol soluble fractions. These fractions were subjected for cytotoxic activity against HEp-2 cells. The petroleum ether soluble fraction showed the highest and the methanol soluble fraction showed the lowest cytotoxicity. But the individual cytotoxicities of these fractions were less than that observed with HHSM. This indicates that there might be a synergistic effect among the fractions (Table-1). Further experiments are needed to prove this. Preliminary phytochemical tests carried out with HHSM indicated the presence of triterpenoids, flavonoids, saponins, glycosides, tannins, amino acids and proteins. Several of these compounds have been reported to be cytotoxic and anti-tumor in nature (Elena *et al.*, 1999; Ke *et al.*, 1999; Lyubov *et al.*, 1999; Ross *et al.*, 1999). The observed cytotoxic properties of the HHSM may be due to the presence of any of these compounds. The cytotoxic activity of this extract was found to be better than that obtained in our earlier studies on *Hypericum mysorens* and *Hypericum patulum* (Vijayan *et al.*, 2003). Hence, the methanolic extract of *Hypericum hookerianum* has the potential for further investigations in animal models to determine its anti-tumor activity and to identify its active principles.

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