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Anti-neoplastic Activity of *Anisochilus cornosus* (L.f) wall on HeLa cell line

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ABSTRACT

Anisochilus carnosus (L.f.) wall is an annual herb belonging to the family Lamiaceae which grows on rocks. It is used traditionally for the treatment of gastric ulcers, skin infections, cough, cold, digestion and eczema. Plant was collected and extracted with petroleum ether, ethanol and water separately and all the three obtained extracts were screened for in vitro anticancer activity against human cervical carcinoma cell lines (HeLa) by using MTT and SRB assays. Cytotoxic potential showed by Petroleum ether and ethanolic extracts with an IC_{50} of 5.88µg/ml and 78.73µg/ml respectively when compared with Doxorubicin with IC_{50} of1.86µg/ml in MTT assay. and IC50 of 17.93 µg/ml and 87.24 µg/ml respectively by SRB assay. Even though the cytotoxic activity shown by both petroleum ether and ethanolic extracts the possible mechanism for its cytotoxicity is not clear. Hence mechanistic evaluation of both plant extracts is needed in future research, so that we could come out with potent and selective anticancer agents derived from plant source.

Key words: Anisochilus, MTT assay, HeLa cell line, Anti-neoplastic

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INTRODUCTION

Cancer can be defined as ‘a group of diseases characterized by uncontrolled cell growth leading to invasion of nearby tissues and spread (metastasis) to other parts of the body. Cancer is the second most common cause of death, surpassed only by cardiovascular disease. According to the ACS report 2014, nearly 1 in every 4 deaths can be attributed to cancer with a possibility of 585,720 deaths due to cancer this year. Compounds of natural origin have provided new and potential leads for cancer chemotherapy in the past; many of them are drug of choice in cancer treatment. For instance, Taxol for breast cancer, Vinca alkaloids for leukemia, Podophyllum, etoposides and capotothecaetc., are some of the natural products in clinical use. Herbs these days are also being used as chemo-protectant against cytotoxicity caused by anticancer drugs. *Anisochilus carnosus* (L.f) wall is an annual herb belonging to the family Lamiaceae, commonly known as karpuravalli. It is an aromatic annual herb found on small rocks. It is native to tropical regions of Asia especially in India, Sri Lanka, Nepal, China, and Myanmar. In India it is widely distributed to high altitudes of Karnataka, Tamil Nadu, Maharashtra and Rajasthan [1]. It is used traditionally for the treatment of gastric ulcers, skin diseases, cold, cough, digestion and eczema [2-4]. Phyto-chemical review indicated *A. carnosus* to be enriched with phytochemicals such as flavonoids and essential oils [5]. Sesquiterpene hydrocarbons represent 39.7% of the oil and depending on type and concentration of the terpenes exhibit antitumor and cytotoxic effects on living cells or organism. The present study aimed to screen the different extracts of the plant for its anti-cancer activity against HeLa cell line.

MATERIALS AND METHODS

Plant source: The plant material was collected from Udyavar, Udupi. The plant was authenticated by Dr. Gopala Krishna Bhat, Taxonomist, and Dr. Richard Lobo, Pharmacognosist, Manipal College of Pharmaceutical sciences, Manipal, Karnataka. A voucher specimen (PP 573) has been deposited in the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal, India.

Chemicals: HeLa (Cervical carcinoma) cell line was obtained from NCCS Pune, India. DMEM (Dulbecco's Modified Eagles Medium), fetal bovine serum (FBS), SRB and MTT reagent were procured from Sigma Aldrich, USA. Tissue culture flasks and 96 well tissue culture plates were purchased from Tarson and Nunc, USA. Petroleum ether (60-80^{0}C), DMSO and absolute ethanol were purchased from NICE chemicals Ltd., Cochin and Ranbaxy Fine Chemicals Ltd. Doxorubicin was used as a standard and it was obtained from KMC hospital, Manipal.

Preparation of extracts: collected plant material was shade dried and powdered (500g). By using soxhlet extractor with petroleum ether and ethanol, solvent was removed by using vaccum rotavapour and dried extracts were obtained .Shade dried leaves (500g) were crushed and extracted by cold maceration at room temperature for 4 days by using chloroform: water (1:99) as a solvent. The filtrate obtained was concentrated to obtain crude aqueous extract.

Cytotoxicity assay: was performed by using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] and SRB (sulforhodamine B) assays.

MTT assay: HeLa (Cervical carcinoma) cells were harvested from 75 cm^2 tissue culture flasks and a stock cell suspension (1X10^5 cells/ml) was prepared. A 96-well plate was seeded with 0.1 ml of DMEM medium and supplemented by adding 10% FBS and allowed to attach for 24hrs. Just prior to the experiment, test compounds were dissolved in 0.1% DMSO. Cells were treated with 20 µl of test solutions from respective stocks (25, 50, 100 and 200µg/ ml) after 24hours of incubation and a fresh medium of 80 µl was added and incubated for 48 hrs. The medium containing 0.1% DMSO alone served as the control. Doxorubicin was used as standard. Drug containing media was removed after treatment and washed with 200µl of PBS. After adding100µl of MTT reagent, cells were incubated for 4hours at 37° C. After incubation, MTT reagent was removed by inverting the plate and formazan crystals were solubilized by adding 100µl of 100% DMSO. An ELISA plate reader at 540 nm was used to measure the optical density (O.D). By using the following formula cytotoxicity potential of the each extract was calculated [6-7].

$$\frac{\text{(Control-Blank)} - \text{(Test-Blank)}}{\text{(Control-Blank)}} \times 100$$

Experiment was done in triplicates; Results were expressed as Mean ± SEM values (proportional to cell survival) and graphs were plotted against the tested drug concentrations.

SRB assay: 100 µl of cell suspension was introduced into each well of 96-well tissue culture plate. Cells were treated with100 µl of various concentrations (25, 50, 100 and 200 µg/ml) of the test solution and incubated for 48hrs. The medium containing 0.1% DMSO only served as control and Doxorubicin was used as standard. After incubation, cells were fixed by treating with ice cold TCA for 1 hr at 40° C. Plates were washed and allowed for drying. Cells were subjected for staining at room temperature for 30min by adding 50 µl of SRB solution. 1% v/v acetic acid was added to remove unbound SRB and allowed to dry. 100 µl of 10 mMunbufferedTris Base (pH 10.5) was added to solubilize the bound SRB and the plate was kept on a shaker platform for 5 min. An ELISA plate reader at 570 nm was used to measure the optical density (O.D). The IC_{50} values were determined by plotting O.D values against the tested concentrations of the drug [8].

Percentage cytotoxicity of each extract was calculated by using this formula:

$$\frac{\text{(Control-Blank)} - \text{(Test-Blank)}}{\text{(Control-Blank)}} \times 100$$

Experiment was done in triplicates; Results were expressed as Mean ± SEM values (proportional to cell survival) were plotted against the tested drug concentrations.

Statistical analysis
Data will be analysed statistically by ANOVA followed by post-hoc Dunnett's test ($p<0.05$ will be considered significant)

RESULTS AND DISCUSSION

Extraction: The percentage yields (%w/w) of petroleum ether, ethanol and aqueous extracts were 23.8, 17 and 16.2 respectively. As the plant contains more amounts of oils like terpens, being a non-polar solvent petroleum ether extract yield was high.

MTT assay: all three extracts were screened for cytotoxic activity against human cervical cancer cell line (He La) and the results are shown in table 1and figure no:1 . Cytotoxicity was showed by both petroleum ether and ethanol extracts with an IC_{50} of 5.88 µg/ml and 78.73 µg/ml respectively when compared with Doxorubicin with IC_{50} of 1.86 µg/ml. In contrast, the aqueous extracts proved to be less cytotoxic with an IC_{50} of 313.60 µg/ml.

SRB assay: all three extracts were screened for cytotoxic activity against human cervical cancer cell line (He La) and results are shown in table 1 and figure no:2. *A. carnosus* petroleum ether and ethanol extracts showed cytotoxic effect with an IC_{50} of 17.93 µg/ml and 87.24 µg/ml respectively when compared with Doxorubicin as standard with IC_{50} of 1.89 µg/ml, whereas the aqueous extracts proved to be less cytotoxic with an IC_{50} of 303.56 µg/ml.In both assays, petroleum ether fraction showed potent cytotoxicity whereas ethanolic extract showed moderate cytotoxicity. Aqueous extract was less effective and showed cytotoxicity only at higher concentration.

In a previous study, it was reported that *A. carnosus* plant ethanolic and petroleum ether extracts showed substantial cytotoxicity against an invasive human breast cancer cell line (BT-549). In the study, luteolin, a dietary plant flavonoid with proven anti-cancer activity was detected in ethanol extract via HPLC fingerprinting [9]. Likewise, a recent study established that *A.carnosus* ethanol extract presented potent cytotoxicity against A-459, a human lung adenocarcinoma cell line in a dose dependent manner. Further, they proved that *A.carnosus* ethanol extract was also capable of inducing apoptotic cell death in A-549 cell line [10]. In general, numerous plants belonging to Lamiaceae have remarkable cytotoxic potential due to the presence of various chemopreventive phytochemicals such as terpenoids, flavonoids, royleanones etc [11].

Table 1: Results showing that % cell viability obtained by both MTT and SRB assays

Concentration of extracts µg/ml	% Viability							
	MTT assay				SRB assay			
	Aqueous extract	Pet. Ether extract	Ethanolic extract	Doxorubicin	Aqueous extract	Pet. Ether extract	Ethanolic extract	Doxorubicin
0	100±5.785	100±5.785	100±5.785	100±2.32	100±6.198	100±6.198	100±6.198	100±2.12
0.01	--	--	--	76.23±0.66	--	--	--	75.8±0.58
0.1	--	--	--	60.9±0.45	--	--	--	61.7±0.26
1	--	--	--	53.9±0.36	--	--	--	53.8±0.52
10	--	--	--	42.1±0.78	--	--	--	42.06±0.46
25	95.481±0.813	75.262±2.654	26.907±4.195	--	86.593±1.595	80.029±2.017	45.560±2.819	--
50	87.699±0.723	55.508±0.611	13.856±0.181	--	76.841±2.031	70.494±5.464	31.149±3.034	--
100	74.825±1.345	11.645±1.681	11.815±0.231	--	65.953±2.117	20.931±2.379	14.526±1.404	--
200	62.757±1.803	9.2696±0.159	3.3993±0.134	--	55.050±4.032	8.733±1.033	5.021±1.779	--
IC 50 value	**313.60**	**78.73**	**5.88**	**1.86**	**303.56**	**87.24**	**17.93**	**1.89**

Results were expressed as Mean ±SEM (n = 3) and A.carnosus extracts were tested at concentrations of 200, 100, 50, 25 µg/ml whereas Doxorubicin was tested at concentrations of 0.01, 0.1, 1 and 10 µg/ml.

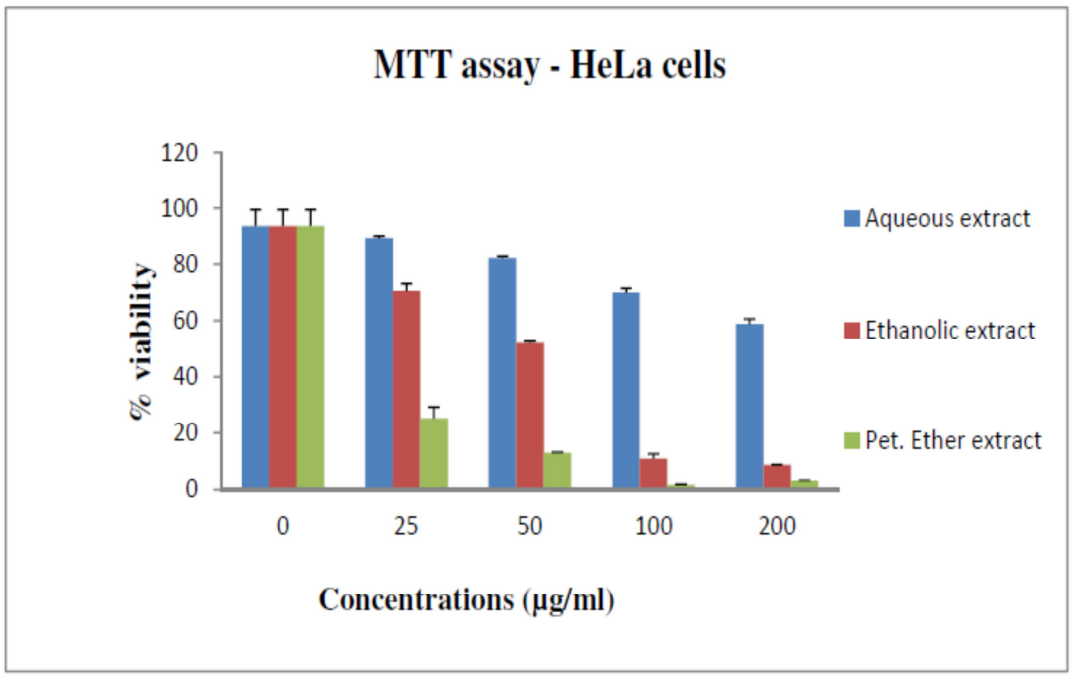

Figure 1: *In vitro* MTT assay on HeLa cells

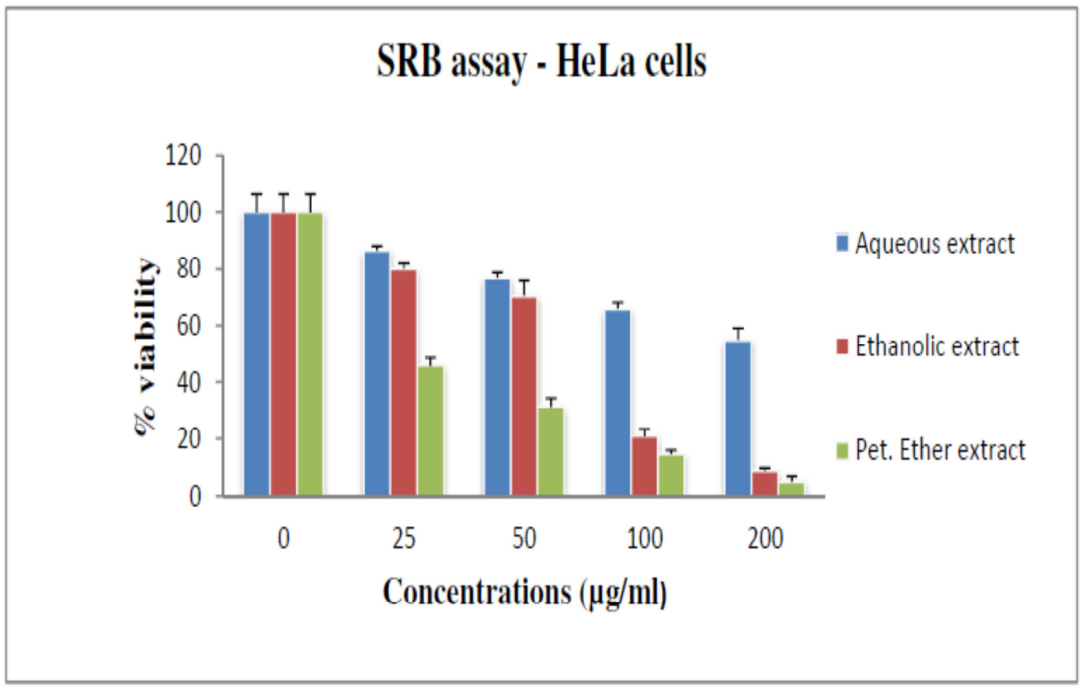

Figure 2: *In vitro* SRB assay on HeLa cells

CONCLUSION

Even though the cytotoxic activity showed by both petroleum ether and ethanolic extracts of the plant in both models, the possible mechanism for its cytotoxicity is very difficult to conclude at this point. Hence mechanistic

evaluation of both plant extracts is needed in future research, so that we could come out with potent and selective anticancer agents derived from plant source.

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