IN VITRO ANTICANCER STUDY OF *CLERODENDRON PANICULATUM*

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Summary

The plant *Clerodendron paniculatum* (Verbenaceae) is widely found in Udupi district of Karnataka, India. Plants of the genus Clerodendron were reported to possess potent anti-cancer, antioxidant and anti-inflammatory activities [1] [2] [3] [4] [5], but, no such reports are available on the plant *Clerodendron paniculatum*. Hence, the present study was undertaken to evaluate the possible anticancer effect of alcoholic extract and their fractions such as pet ether, chloroform, ethyl acetate, ethyl methyl ketone of the plant by *in vitro* methods like Trypan blue dye exclusion, MTT and SRB assays. Our studies revealed that alcoholic extract and their fractions showed moderate anticancer potential.

Keywords: *Clerodendron paniculatum,* anticancer, *in vitro* assay

Introduction

The burden of cancer is still increasing worldwide despite advances for diagnosis and treatment. Epidemiological studies have shown that many cancers may be avoidable. It is widely held that 80-90% of human cancers may be attributed to environmental and lifestyle factors such as tobacco, alcohol and dietary habits[6]. Cancer prevention includes primary, secondary and prevention methods. Primary prevention refers to avoiding cancer–causing substances in the environment or dietary elements associated with increased risk; dietary supplementation with putative protective agents. Secondary prevention aims at early detection and removal of benign tumours of oral, cervical and breast cancers[7]. According to the recent reports, over 10 million new cases of cancer occurred (approximately 5.3 million men and 4.7 million women) and over 6 million people died from cancer[8]. The most frequently affected organs are lung, breast, colon, rectum, stomach and liver.

Urbanization, industrialization, changes in lifestyles, population growth and ageing all have contributed for epidemiological transition in the country. The absolute number of new cancer cases is increasing rapidly, due to growth in size of the population, and increase in the proportion of elderly persons as a result of improved life expectancy following control of communicable diseases. In India, the life expectancy at birth has steadily risen from 45 years in 1971 to 62 years in 1991, indicating a shift in demographic profile[9]. It is estimated that life expectancy of Indian population will increase to 70 years by 2021-25. Such changes in the age structure would automatically alter the disease pattern associated with ageing and increase the burden of problems such as cancer, cardiovascular and other non-communicable diseases in the society.

India has a rich heritage of medicinal plants and natural products. The rich herbal resources offer a unique opportunity in our country to evaluate them specifically. The use of plant drugs is increasing in many of the developing countries because modern life saving drugs are beyond the reach of common man and have high toxic potential. Currently there is renewed interest in developing drugs from plants for cancer therapy. Many indigenous plants are being tested against a spectrum of experimental tumors with the perceived advantage of local availability, low cost and less side effects. The present study was on the plant *Clerodendron paniculatum* belongs to Verbenaceae family. Selection of the plant was based on the fact that other species of the genus Clerodendron viz. *Clerodendron serratum* and *Clerodendron viscosum* showed potent anticancer properties. In view of above facts, the present study has been undertaken on this plant to carry a preliminary investigation for their possible in vitro anticancer activity.

Materials and Methods

REQUIREMENTS

CO_2 incubator, inverted microscope, microplate reader, haemocytometer, tissue culture flasks, sterile petridishes, 96 well plates, cancer cell lines, cell culture media and micropipettes.

$HepG_2$, HeLa and MCF_7 originally procured from NCCS, Pune were sub cultured in Pharmaceutical Biotechnology lab of Manipal College of Pharmaceutical Sciences, Manipal. The cells were grown as monolayer culture in appropriate media supplemented with 10% NBCS (new born calf serum) and antibiotics in an incubator at 37º C along with 5% CO_2 and 95% air. The cells were maintained by routine sub culturing in 60 mm plastic tissue culture petriplates. EAC cell lines were procured from Amala Cancer Institute, Thrissur, Kerala.

CULTURE MEDIA

$HepG_2$ –DMEM, HeLa - DMEM, MCF_7 - MEM

Subculture of human cancer cell lines

The culture media from the flasks containing monolayer culture was carefully decanted and washed thoroughly with sterile phosphate buffered saline (PBS). To the flasks, 3ml of 0.1% trypsin-EDTA solution was added and incubated at 37º C for 1-2 min. The flasks were removed from the incubator and gently tapped to detach all the adhering cells. The cell detachment was confirmed by observing under an inverted microscope. Once the cells were completely detached from the flasks, an equal amount of media containing 10% NBCS was added and mixed well to

stop further enzymatic activity. Cell viability was calculated with a small sample of the suspension by Trypan blue dye exclusion test. From the stock cell suspension, 1 x 10^4 viable cells/ml suspended in media were seeded in 75 mm culture flasks and incubated until the plates attained 60-70% confluency.

CELL VIABILITY BY TRYPAN BLUE EXCLUSION TEST[10]

Determination of the percent viable cells:

Using a tissue paper, the surface of Neubauer's chamber and glass cover slip was wiped first with 70% alcohol followed by DDW (double distilled water). In an eppendorf tube, 90µl of the cell suspension was diluted with 10µl of Trypan blue dye (0.1%) (1:10 dilution ratio) and mixed well. The cover slip was placed on the centre of the grid and 10 µl of the cell-dye mixture was carefully loaded on the Neubauer's chamber (between the counting slide and the glass cover slip) without any air bubble.

The stained (dead) and unstained (viable) cells in four WBC chambers of the haemocytometer were counted separately under the light microscope. Total number of cells counted in 4 WBC chambers divided by four gave the average number of total cells in one quadrant. Total number of cells was calculated by multiplying the average number of cells per quadrant by the dilution factor. Multiply this number by 10^4 to calculate the number of cells.

Number of cells/ml = [(A+B+C+D)/4] x Dilution factor x 10^4.

To calculate the percentage viable cells, divide the number of unstained cells by the total number of cells and multiply by 100. The equation is as follows:

Percent viable cells = (Number of unstained cells/Total number of cells) x 100.

DETERMINATION OF MITOCHONDRIAL SYNTHESIS BY MICROCULTURE TETRAZOLIUM (MTT) ASSAY[11]

The monolayer cell culture was trypsinized and the cell count was adjusted to 5x 10^5 cells per ml using medium containing 10% new born calf serum. To each well of the 96 well microplate, 0.1 ml of the diluted cell suspension (approximately 50,000 cells) were added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once and 100µl of different drug concentrations was added to the cells in the microtitre plates. The plates were then incubated at 37^0C for three days in 5% carbon dioxide atmosphere, and microscopic examination was carried out and observations were recorded every 24 hours.

After 72 hours, the drug solutions in the wells were discarded and 50µl of MTT in HBSS (Hanks balanced salt solution) was added to each well. The plates were gently shaken and incubated for three hours at 37^0 C in 5% CO2 atmosphere. The supernatant was removed and 50µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage inhibition was calculated using the formula

$$\% \text{ Growth Inhibition} = 100 - \left[\frac{\text{Mean OD of Individual Test Group}}{\text{Mean OD of Control Group}} \right] \times 100$$

DETERMINATION OF TOTAL CELL PROTEIN CONTENT BY SULPHORHODAMINE B (SRB) ASSAY[12]

The monolayer cell culture was trypsinized and the cell count was adjusted to 5 x 10^5 cells per ml using medium containing 10% new born calf serum. To each well of the 96 well microplate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) were added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once and 100µl of different drug concentrations was added to the cells in the microtitre plates. The plates were then incubated at 37^0 C for three days in 5% carbon dioxide atmosphere, and microscopic examination was carried out and observations were recorded every 24 hours.

After 72 hours 25µl of 50% tri chloro acetic acid was added to the wells gently such that it forms a thin layer over the drug dilutions to form an overall concentration of 10%. The plates were incubated at 4^0 C for one hour. The plates were flicked and washed five times with tap water to remove traces of medium, drug and serum were then air dried. The air dried plates were stained with SRB for 30 minutes. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were then air dried. 100µl of 10mM Tris buffer was then added to the wells to solubilize the dye. The plates were shaken for 5 minutes.

The absorbance was measured using microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the formula

$$\% \text{ Growth Inhibition} = 100 - \left[\frac{\text{Mean OD of Individual Test Group}}{\text{Mean OD of Control Group}} \right] \times 100$$

Results

Trypan blue exclusion assay

Alcoholic extract and all the five fractions were tested by the trypan blue assay. Among them only the alcoholic extract and petroleum ether fraction were found to have considerable cytotoxicity. Petroleum ether fraction showed 100% cell death at a concentration of 50µg/100µl and the alcoholic extract showed 100% cell death at 100µg/100µl. Benzene fraction showed least cytotoxicity and hence this fraction was not taken for further studies. The alcoholic extract and the remaining fractions (pet ether, chloroform, ethyl acetate and ethyl methyl ketone) were taken up for further evaluation of their activities.

Table 1. Anti proliferative effects of *C. paniculatum* on growth of $HepG_2$ cell lines.

Drug	Concentration (µg/ml)	Mean absorbance		% Growth inhibition	
		MTT	SRB	MTT	SRB
Control	0	1.244	1.642	-	-
Cisplatin	12	0.404	0.384	67.52	78.80
Alc. extract	125	1.121	1.557	9.88	5.17
	250	1.078	1.204	13.34	26.67
	500	0.819	1.088	34.16	33.73
	1000	0.726	0.959	41.63	41.59
Pet ether	125	1.130	1.453	9.16	11.50
	250	1.099	1.195	11.65	27.22
	500	0.945	1.032	24.03	37.14
	1000	0.797	0.903	35.93	45.00
Chloroform	125	1.113	1.436	10.53	2.37
	250	1.041	1.398	16.31	14.85
	500	0.984	1.345	20.90	18.08
	1000	0.958	1.203	22.99	26.73
Ethyl acetate	125	1.196	1.563	3.85	4.81
	250	1.179	1.456	5.22	11.32
	500	1.135	1.321	8.76	19.54
	1000	1.071	1.139	13.90	30.63
Ethyl methyl ketone	125	1.220	1.397	1.92	14.90
	250	1.186	1.277	4.66	22.20
	500	1.162	1.190	6.59	27.52
	1000	0.973	1.113	21.78	32.20

Fig. 1

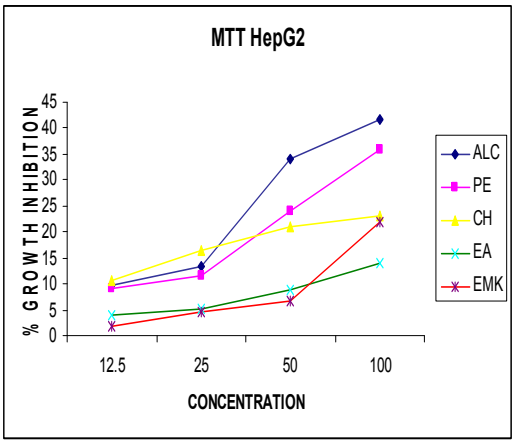
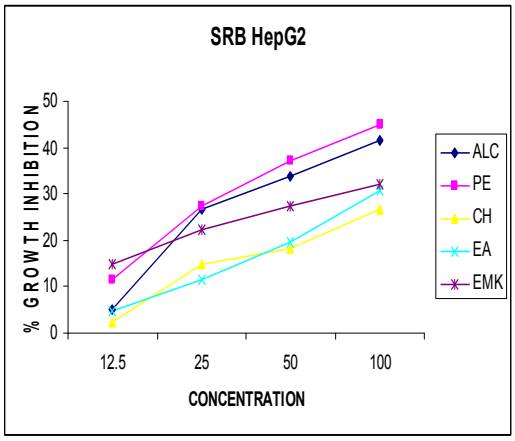


Fig 2



A dose depend decrease in cancer cell proliferation was observed after 48 hours of incubation with various drug concentration. Alcoholic extract and petroleum ether fraction showed maximum decrease in cell proliferation.

Table 2 Anti proliferative effects of *C. paniculatum* on growth of HeLa cell lines.

Drug	Concentration (µg/ml)	Mean absorbance		% Growth inhibition	
		MTT	SRB	MTT	SRB
Control	0	0.689	0.646	-	-
Cisplatin	12	0.195	0.197	71.69	69.50
Alc. extract	125	0.507	0.590	26.40	8.66
	250	0.423	0.523	38.60	19.04
	500	0.354	0.477	48.62	26.16
	1000	0.287	0.412	58.34	36.22
Pet ether	125	0.494	0.569	28.30	11.9
	250	0.436	0.546	36.71	15.47
	500	0.305	0.481	55.73	25.54
	1000	0.226	0.426	61.39	34.05
Chloroform	125	0.534	0.621	22.49	3.86
	250	0.484	0.583	29.75	9.75
	500	0.452	0.512	34.39	20.74
	1000	0.377	0.481	45.28	25.50
Ethyl acetate	125	0.512	0.569	25.68	11.9
	250	0.453	0.542	34.25	16.09
	500	0.436	0.507	36.71	21.51
	1000	0.422	0.473	38.75	26.78
Ethyl methyl ketone	125	0.528	0.567	23.36	12.22
	250	0.481	0.532	30.18	17.64
	500	0.430	0.513	37.59	20.58
	1000	0.391	0.479	43.25	25.85

Fig 3

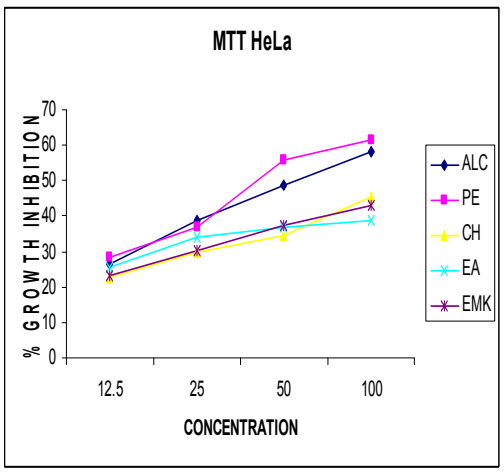
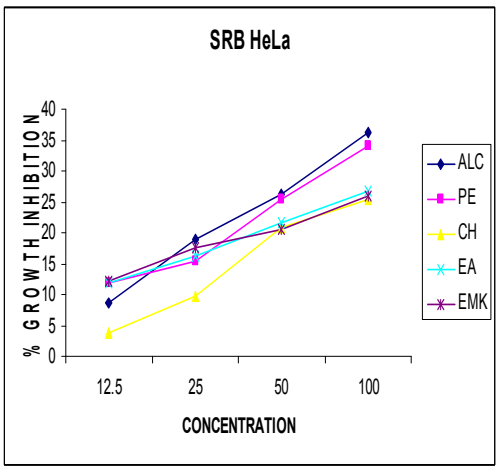


Fig 4



A dose depend decrease in cancer cell proliferation was observed after 48 hours of incubation with various drug concentration. Alcoholic extract and petroleum ether fraction showed maximum decrease in cell proliferation.

Table 3. Anti proliferative effects of *C. paniculatum* on growth of MCF_7 cell lines.

Drug	Concentration (µg/ml)	Mean absorbance		% Growth inhibition	
		MTT	SRB	MTT	SRB
Control	0	0.363	0.425	-	-
Cisplatin	12	.110	0.114	69.69	73.17
Alc. extract	125	0.302	0.331	16.80	22.11
	250	0.281	0.297	22.58	30.11
	500	0.231	0.270	36.36	36.47
	1000	0.203	0.264	44.07	37.88
Pet ether	125	0.284	0.347	21.76	18.35
	250	0.243	0.313	33.05	26.35
	500	0.225	0.282	38.01	33.64
	1000	0.216	0.226	40.49	46.82
Chloroform	125	0.315	0.378	13.22	11.05
	250	0.297	0.368	18.18	13.41
	500	0.282	0.338	22.31	20.47
	1000	0.277	0.310	23.69	27.05
Ethyl acetate	125	0.318	0.350	12.39	17.64
	250	0.295	0.331	18.73	22.11
	500	0.289	0.307	20.38	27.76
	1000	0.259	0.269	28.65	36.70
Ethyl methyl ketone	125	0.312	0.362	14.04	14.82
	250	0.298	0.330	17.90	22.35
	500	0.284	0.297	21.76	30.11
	1000	0.257	0.273	29.20	35.76

Fig 5

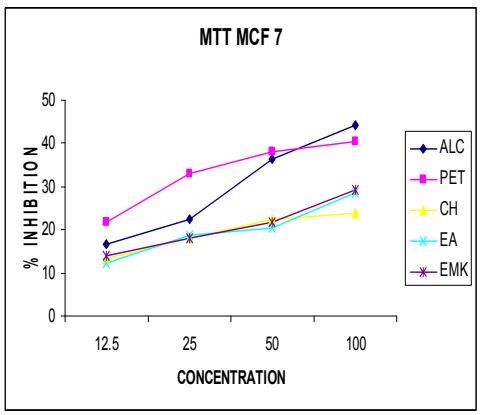
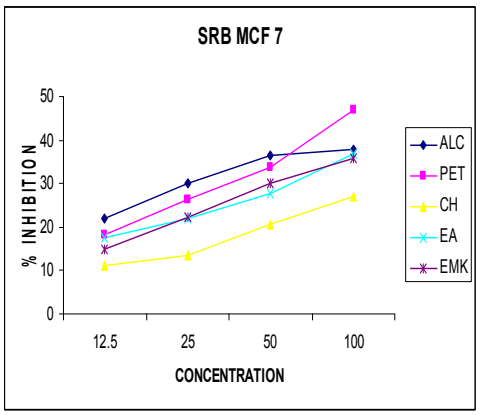


Fig 6



A dose depend decrease in cancer cell proliferation was observed after 48 hours of incubation with various drug concentration. Alcoholic extract and petroleum ether fraction showed maximum decrease in cell proliferation.

Discussion

The present study was undertaken to establish the anticancer activity of the *Clerodendron paniculatum* by in vitro methods. The Trypan blue assay was carried out on EAC cell lines. Both MTT and SRB methods were carried out using three different human cancer cell lines ($HepG_2$, HeLa and MCF_7). The alcoholic extract and petroleum fraction showed the maximum anticancer potential but it was not significant. The present study shows that the plant possess anticancer potential but not significant. The plant may show activity at higher concentrations. Further studies should be carried out.

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