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Evaluation of Anticancer Activity of *Clerodendrum serratum*

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ABSTRACT

Clerodendrum serratum, is an important medicinal plant has already been in used as antibacterial, Antiasthmatic, antidiabetic, antihypertensive. In this study an attempt of the plant has been made to evaluate the anticancer activity of the methanolic extract of the plant, as stated in many hypotheses. The methanolic extract of leaves of *Clerodendrum serratum* were screened for their anticancer activity by *in vivo* the various parameters such as hematological studies and protein estimation, solid tumor volume, median survival time (MST), life span (%LS) and *in vitro* studies was carried out by Tetrazolium salt assay and Tryphan blue dye exclusion method. The relative cell survival progressively decreased in dose dependant manner. Cytotoxicity studies by Tryphan Blue exclusion method also confirmed the anticancer activity of *Clerodendrum serratum* (200µg/ml showed 79% of Cytotoxicity inhibition). Our studies point the possibility of developing *Clerodendrum serratum* as a novel potential agent in the area of cancer chemotherapy.

Keywords: *Clerodendrum serratum,* Anticancer Activity and Cytotoxicity.

1. INTRODUCTION

Cancer is a major public health burden in both developed and developing countries and it has become a leading cause of mortality next to heart attack, accounting for 23% of all deaths [1]. Cancer is the second leading cause of death in the United States [2], where one in four deaths is due to cancer. Plants have long been used in the treatment of cancer [3]. The National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity. *Clerodendrum serratum,* is an important medicinal plant distributed throughout India, it has many therapeutic uses in different traditional medicine of the world. Different parts of the plant are of different therapeutic values. The importance of this herb has made us to investigate its anti-hypertensive property. The leaf was shown to possess potent analgesic, antipyretic and anti-inflammatory properties of different extracts. The cytotoxic activities of the leaves have so far been undertaken [4]. In this study an attempt of the plant has been made to evaluate the anticancer activity of the methanolic extract of the leaves by *in vivo* and *in vitro* method

2. MATERIALS AND METHODS

2.1. Preparation of plant extracts:

Freshly collected leaves were washed, shade dried under room temperature. The dried plant material was made into a coarse powder. A weighed quantity of the powder (500g) was passed into sieve number 80 and subjected to hot solvent extraction in a soxhlet apparatus using methanol at a temperature range of 40-80^{0}c before and after every extraction the marc was completely dried and weighed [5]. The filtrate was evaporated to dryness at 40^{0}c under reduced pressure in a rotary vacuum evaporator.

2.2. Preliminary phytochemical analysis

Methanol extract of *Clerodendrum serratum* leaves were subjected to preliminary phytochemical analysis to test for presence or absence of various phyto-constituents [6].

2.3. Experimental animals

Adult male Swiss albino mice (25-30g) collected. The animal was maintained in well-ventilated room with 12:12 hour light /dark cycle provided *ad libitum* throughout the experimentation period.

2.4. Tumour cell line

Dalton's ascetic lymphoma (DAL) cells were obtained and were maintained by weekly intra-peritoneal inoculation of 10^6 cells/per mouse [7].

2.5. Effect of MECS on median survival time (MST)

Animals were inoculated with $2x10^5$ cells/mouse on day '0' and treatment with MECS started 24hr after inoculation, at a dose of 100mg/kg/day p.o (Group-1), 200mg/kg/day p.o (Group-2). The control group (Group-3) was treated with same volume of 0.9% sodium chloride. All treatments were carried out for 9 days. Median survival times for each group were noted. The anti-tumor efficacy of MECS was compared with that of 5Fluorouracil (20 mg/kg/day i.p. for 9 days) [7-9]. MST was noted with reference to control. Survival times of treated groups were compared with those of control groups using the following calculation.

Median Survival Time (MST) = Day of 1st death + Day of last death /2.

2.6. Effect of MECS on normal peritoneal cells

Three groups of normal mice (n=6) were used for the study. One group was treated with 100 mg/kg, p.o of MECS and second group received 200 mg/kg, p.o of MECS. The untreated third group was used as control. Peritoneal exudates cells were counted 24hr after treatment for each of the treated groups and compared with those of the untreated groups [10,11].

2.7. Percentage Increase Life Span (%ILS)

For DAL tumor growth was monitored by percent increase in life span which was considered to determine the antitumor efficacy and mortality was recorded for each group. The increase in life span was calculated by the formula [12].

%ILS = (MST of treated group) – (MST of Control group) X 100 /MST of Control group

2.8. Effect of MECS on hematological parameters

Comparison was made amongst groups (n=6) to detect the influence of MECS on the hematological status of DAL bearing mice after inoculation. The four groups comprised (1) tumor bearing mice treated with MECS (100mg/kg/day p.o for first 9 days) (2) tumour bearing mice treated with MECS (200mg/kg/day p.o for first 9 days) (3) tumour bearing control mice and (4) standard group receiving 5 Fluorouracil (20 mg/kg/day i.p. for 9 days). Blood was drawn from each mouse in the conventional way and the white blood cell count, red blood cell count, hemoglobin, protein and packed cellular volume were determined [13,14]. All the results were analysed by variance of analysis.

2.9. Effect of MECS on solid tumor

Mice (n = 6) were divided into 3 groups and tumor cells (1×10^6cells/mice) were injected into the right hind limb (thigh) of all the animals, intramuscularly. Group 1 animal was tumor control group, while groups 2 and 3 received MECS (100 and 200 mg/kg p.o respectively) for 5 alternative days [15]. Tumor mass was measured from the 11th day of tumor induction. The measurement was carried out every 5th day for a period of 30 days and the volume of tumor mass was calculated using the formula [16].

$$V = 4/3 \pi r_1^2 r_2$$

Where r_1 and r_2 are the radii of the tumor from two directions.

2.10. Cytotoxicity Assay

2.10. 1. Tryphan blue dye exclusion technique

The reactivity of Tryphan blue was based on the fact that the chromophore was negatively charged and does not interact with the cell unless the membrane was damaged because Tryphan Blue was a vital dye. Therefore, all the cells which exclude the dye are viable. Short term Cytotoxicity was assessed by incubating $1x10^6$ DAL cells in 1ml phosphate buffer saline with varying concentration of MECS. The cell viability was assessed by Tryphan blue dye exclusion method [17].

2.10. 2. MTT-Assay

MTT measures the metabolic activity of the viable cells. The method involves culturing the cells in a 96 well micro titer plate, and then incubates with MTT solution for 2hrs. During the period viable cells convert MTT into a water insoluble formazan dye and it can be colorimetrically detected at 595 nm. The absorbance directly correlates with the cell [18]. The cells were treated with different concentration of tested drug, control which contain only the medium and incubated for 24hrs. MTT solution was added to each well to make the final volume concentration and further incubated at 37^0c incubate for 3hours. The reaction resulted in the reduction of concentration MTT by the mitochondrial dehydrogenase of viable cells to a purple formazan product. The MTT-formazan product was dissolved in DMSO and estimated by measuring the absorbance at 570nm in an Elisa reader [19,20].

2.11. Statistical analysis

The values are recorded as Mean ± SEM. The data were analyzed by using ANOVA followed by Dunnett's test, the P values < 0.05 were considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1. Preliminary phytochemical analysis

Results of different chemical tests on the methanol extract of *Clerodendrum serratum* showed the presence of alkaloids, glycosides, flavonoids, tannins, saponins and Triterpenoids.

3.2. Median Survival Time (MST)

Any potential anticancer drug is expected to increase the mean survival time and thus increasing life expectancy. Mice transplanted with DAL (control) in our studies have MST of 23days, which was increased to 29 days and 36days by MECS of 100mg/kg and 200mg/kg respectively. These results are almost comparable to that of 5-FU, the standard drug for which the MST was 41 days.

3.3. Percentage increase life span (ILS %)

The increase in the life span of tumor bearing mice treated with MECS (200mg/kg p.o.) and 5-FU was found to be 57.43% and 78.20% respectively ($p > 0.01$) as compared to the control group.

Table -1: Percentage increase life span (ILS %)

Treatment	MST	Life span (%)
Tumor control	23.12±1.20	-
5-FU	41.20±1.36**	78.20
MECS 200mg/kg	29.16±1.72*	26.12
MECS 400mg/kg	36.40±1.32**	57.43

** $p < 0.001$ Vs control, * $p < 0.05$ Vs control.

3.4. Effect of MEL on normal peritoneal cells

The average number of peritoneal exudate cells per normal mouse was found to be $6.7 \pm 0.4 \times 10^6$. MECS (100 mg/kg) single treatment enhanced peritoneal cells to $8.3 \pm 0.5 \times 10^6$ while MECS (200 mg/kg) single treatment enhanced peritoneal cells to $9.1 \pm 1.7 \times 10^6$.

3.5. Hematological parameters

Hematological parameters of tumor bearing mice on day 14 were found to be significantly altered from test group. The total WBC count, protein and PCV were found to be increased with a reduction of the hemoglobin content of RBC. In a differential count of WBC, the percent of neutrophils increased while the lymphocyte count decreased (control). At the same time interval, MECS (100mg/kg, 200mg/kg p.o.) treatment could change those altered parameters to near normal.

3.6. Solid Tumor Volume

Estimation of solid tumor volume is a direct method of evaluation of anticancer activity. It is indeed a suitable method, which does not involve sacrificing the animal. In the study, the tumor mass was directly measured after implantation intramuscularly. The solid tumor volume increase by 6.6±0.1 ml. DAL bearing mice, treatment with MECS decreased significantly ($P < 0.01$, $p < 0.05$), the tumor volume to 4.1±0.07 ml on dose dependant manner at the end of 30 days.

Table -2: Hematological parameters of MECS

Treatment	Hb (g %)	RBC (Millions/mm^3)	WBC 10^6cells/mm^3	Protein (g %)	PCV (mm)	Differential count %		
						Lymphocytes	Neutrophils	Monocytes
DAL control	8.0±0.34	3.8±0.07	15.2±1.15	14.5±1.2	28.1±0.61	30.1±0.64	67.1±0.2	2.8±0.5
5-flu (20mg/kg)	14.1±0.43**	6.0±0.21**	7.2±0.2 **	8.3±0.56**	17.5±0.4 **	70.5±0.91 **	28.4±0.13**	1.0±0.8 **
MECS 100mg/kg	9.8±0.52 *	4.8±0.03**	11.1±1.6 **	11.5±0.4 **	21.3±0.17**	53.6±0.24 **	45.2±0.41**	1.2±0.2 **
MECS 200mg/kg	11.5±0.61**	5.5±0.51**	9.3±0.7 **	9.7±0.3 **	19.0±0.23**	68.1±0.12 **	30.9±0.72**	1.0±0.2 **

**$P < 0.01$; *$p < 0.05$ Vs control.

Table -3: Solid Tumor Volume

Treatment	Dose (mg/kg)	Solid tumor volume (ml)			
		15th day	20th day	25th day	30th day
Tumor control	Normal saline	3.5±0.06	4.1±0.03	5.7±0.06	6.6±0.1
Test-I	MECS 100mg/kg	3.0±0.05*	3.6±0.09**	4.07±0.03**	4.7±0.09**
Test-II	MECS 200mg/kg	2.6±0.12**	3.2±0.06**	3.6±0.09**	4.1±0.07**

3.7. Short Term Cytotoxicity studies

Short term Cytotoxicity studies by Tryphan Blue exclusion method is a very simple method which can be carried out within a short time of 3hrs. It is a precise method, which takes in to account the viable and also the dead cells in addition to estimation of IC_{50} concentration. The IC_{50} of MECS was found to be 350μg/ml against DAL.

The reliable criterion for judging the value of any anticancer drug is the prolongation of lifespan of the animal and decreasing the WBC from blood [21,22]. The above results demonstrated the antitumor effect of MECS against DAL in Swiss albino mice. A significant ($P > 0.01$) enhancement of MST and peritoneal cell count was observed.

To evaluate whether MECS treatment indirectly inhibited tumor cell growth, the effect of MECS treatment was examined on the peritoneal exudates cells of normal mice. Normally, each mouse contains about $5x10^6$ intraperitoneal cells, 50% of which are macrophages. MECS treatments were found to enhance peritoneal cell counts. These results demonstrated the indirect effect of MECS on DAL cells, probably mediated through enhancement and activation of macrophages or through some cytokine product inside the peritoneal cavity produced by MECS treatment.

Analysis of the hematological parameters showed minimum toxic effects in the mice which were treated with MECS. After 14 days of transplantation, MECS treated groups were able to reverse the changes in the hematological parameters consequent to tumor inoculation. All these data point to the possibility of developing methanolic extract of *Clerodendrum serratum* as a novel, potential agent in the area of cancer chemotherapy.

Preliminary phytochemical screening indicated the presence of alkaloids, triterpenoids and flavanoids in MECS. Flavanoids, which have been shown to possess antimutagenic [23] and anticarcinogenic activity [24]. Moreover, flavanoids have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis [25]. The cytotoxic and antitumor properties of the extract may be due to these compounds.

4. CONCLUSION

The anticancer effect of MECS is evident from increasing lifespan, reduction in solid tumor volume and also the reversal of altered hematological parameter near to normal. The extracts were found to be cytotoxic on HEp2 and HT29 Cell Lines of Human carcinoma.

The present study points to the potential anticancer activity of *Clerodendrum serratum*. A further study to characterize the active principles and elucidate the mechanism of the action of MECS has been carried out.

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