IN VIVO ANTICANCER ACTIVITY OF METHANOL EXTRACT OF THE WHOLE MEDICINAL PLANT *Enicostemma littorale* IN SWISS ALBINO MICE

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Summary

Cancer can affect people at all ages with the risk for most types increasing with age [1]. It caused about 13% of all human deaths in 2007[2] (7.6 million)[3]. Cancers are primarily an environmental disease with 90-95% of cases due to lifestyle and environmental factors and 5-10% due to genetics [4]. Common environmental factors leading to cancer death include: tobacco (25-30%), diet and obesity (30-35%), infections (15-20%), radiation, stress, lack of physical activity, environmental pollutants [4]. These environmental factors cause abnormalities in the genetic material of cells[5]. Many management options for cancer exist including: chemotherapy, radiation therapy, surgery, immunotherapy, monoclonal antibody therapy and other methods. In spite of advance development of synthetic anticancer drugs in recent years, some of the drugs of plant origin have still retained their importance. Plants used in folklore medicine continue to be an important source of discovery and development of novel therapeutic agents.

Keywords: Anticancer activity; *Enicostemma littorale*; Ehrlich ascetic carcinoma (EAC), Flavonoids

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Introduction

Enicostemma littorale (Family- *Gentianaceae*) also called as *Chota chirayata* in Hindi, *Mamejovo* in Gujrati, *Nagajivha* in Bengal and *Vellarugu* in Tamil has been used traditionally for many diseases. The plant is used in folk medicine for the treatment of diabetes mellitus, rheumatism, peptic ulcers, hernia, swelling, itching and insect poisoning [1]. It inhibited carrageen-induced edema and its anti-inflammatory activity is comparable to that of hydrocortisone [2]. Ethnomedical studies of North Gujrat (India) also revealed the use of hot aqueous extract of *E. littorale* by the tribal inhabitants for the treatment of diabetes, fever, stomach pain, dyspepsia and malaria [3]. The root extract showed antimalarial activity both *in vitro* and *in vivo* [4]. This herb is also known for its hypoglycemic, antioxidant and hypolipidemic potential in newly-diagnosed non-insulin-dependent diabetes mellitus (NIDDM) patients [5]. Anticancer activity of this plant has been evaluated for Dalton's ascetic lymphoma [6]. Swertimarin, alkaloids, steroids, triterpenoids, saponins, flavonoids, xanthones, and phenolic acid were isolated from this plant [7]. Swertiamarin is a major phytoconstituent present in *Enicostemma littorale* [6,7,8]. Hence, this study was aimed to evaluate the anticancer activity of the methanolic extract of *E. littorale* in Swiss Albino Mice.

Materials and Methods

Plant materials: *E. littorale* was collected from local market of Gujrat after proper identification, though it is a well known plant. The whole plants were shade dried and then powdered.

Preparation of extract: The powder was treated with petroleum ether for defatting as well as to remove chlorophyll then it was air dried. The dried powder was packed into a Soxhlet apparatus and subjected to hot continuous percolation using methanol (95% v/v) as solvent. The liquid extract was concentrated in vacuum rotary evaporator to get semidry mass. The solvent was completely removed by using lyophilizer and stored at 4° C until further use. For experimentation, the extract was dissolved in double distilled water just before intraperitonial administration.

Animals: Swiss albino mice (20-28 gm) of either sex were fed with standard pellet diet of and water ad- libitum. The mice were acclimatized in laboratory condition for 10 days before commencement of experiment.

EAC cell Transplantation in mice: EAC cells were obtained from Chittaranjan National Cancer Institute (CNCI), Kolkata, India. Ehrlich's Ascitic Carcinoma (EAC) was maintained from EAC cell bearing Swiss Albino mice. Ascitic fluid was collected from tumor bearing mice at day 9. The fluid was diluted with ice cold normal saline (0.9%) and the tumor cell number was adjusted to $2x10^6$ tumor cells / ml. Each animal was received 0.1 ml of tumor cell suspension containing 2 x 10^6 cells / ml intraperitoneally [9].

Dose calculation: The crude extract was evaluated for their acute toxicity (LD_{50}) in Swiss albino mice aged six to eight weeks and weighing 20-30 gm. Intraperitoneal administration of methanol extracts of *E. littorale* in mice, at doses from 100-1000 mg/kg did not cause mortality of mice within 24 hrs. Gross physical and behavioral observation of the experimental mice also revealed no visible signs of acute toxicity like lacrimation, hair erection, and reduction in their motor and feeding activities. They were physically active. The LD_{50} of methanol extract of *E. littorale* is 2000 mg/kg in i.p. route. So the 1/8th and 1/4th dose of LD_{50} was selected as the lower and higher dose in case of anticancer study.

Treatment Schedule: Swiss albino mice were divided into 5 groups (in each group, n = 10). All the groups (Table 1) were injected with EAC cells (0.1 ml of 2X 10^6 cells /mouse) intraperitoneally except the normal group. This was taken as day zero. Normal group was served as normal saline (5 ml/kg, p. o.) and Group II served as EAC control. On day 1 the methanol extract of *E. littorale* at a dose of 250 and 500 mg/kg body weight (Group-III & IV) were administered intraperitoneally and continued for 9 consecutive days. Standard drug Vincristine (30 mg/kg) administered in group V. On day 10, five mice of each group were sacrificed 24 h after the last dose and the rest were kept with food and water ad-libitum to check the mean survival time (MST) of the tumor hosts [9,10]. The effect of methanol extract on tumor growth and host's survival time were examined by studying the following parameters- body weight, tumor volume, tumor cell count, viable and nonviable tumor cell count, mean survival time (MST) and increase in life span.

Table 1: Treatment schedule of various groups

Group	Parameters	Doses
Group I	Normal	Normal saline 5 ml / kg
Group II	EAC control	2 X 10^6 cells /mouse
Group III	EAC + *E. littorale*	250 mg/kg body wt. i.p.
Group IV	EAC + *E. littorale*	500 mg/kg body wt. i.p.
Group V	EAC + Vincristine	30 mg/kg body wt. i.p.

Observation of tumor growth response: Anticancer effect of *E. littorale* was assessed by observing the change with respect of Ascitic tumor volume, packed cell volume, viable and non viable tumor cell count, mean survival time (MST) and percentage increase in life span (%ILS) [10, 11, 12].

Determination of tumor cell volume and packed cell volume: The mice were dissected for collecting ascetic fluid from peritoneal cavity. The transplantable murrain tumor was collected with the help sterile syringe and kept in graduated centrifuge tube. The tumor cell volume was measured in that tube and later on packed cell volume was determined by centrifuging at 1000 rpm for 5 min [10, 11, 12].

Determination of viable and non viable cell count: For viable and non viable cell counting, the ascitic cell were stained by the tryphan blue (0.4% in normal saline), dye exclusion test and count was determined in a neubauer counting chamber. The cells that did not take up the dye were viable and those that took the stain were non viable[10,11, 12].

Cell count= (No. of cells × Dilution) / (Area × Thickness of liquid film)

Mean survival time and percentage increased in life span: The effect of *E. littorale* on tumor growth was observed by MST and % ILS. MST of each group containing 5 mice were monitored by recording the mortality daily for 6 weeks and % ILS was calculated by using following equation[10, 11, 12].

MST = (Day of first death + Day of last death)/2

$$\% \text{ ILS} = \left\{ (\text{MST of treated group} / \text{ MST of control group}) - 1 \right\} \times 100$$

Hematological studies: Blood was collected from each mice by intra-cardial puncture with anticoagulant (EDTA) and white blood cells(WBC), red blood cells (RBC); Hemoglobin and differential count were determined[13, 14].

Data analysis: Results of the study were expressed as mean ± standard error of mean (M ± SEM). Statistical significance (*p*) was determined by one-way ANOVA (repeated measure of analysis of variance) between the treated groups and the EAC control followed by Dunnett's post hoc test of significance.

Results and Discussion

E. littorale increases mean survival time and ILS% of test animals when administered at different doses (250 mg/kg, 500 mg/kg) were shown in table 2. Administration of methanolic extract of *E. littorale* reduces the tumor volume, packed cell volume and also decreases viable tumor cell count in a dose dependant manner when compared to EAC control mice (Table 3).

The mean survival time was significantly increase (25.5, 31.0, and 36.0) days in *E. littorale* (250 mg/kg, 500 mg/kg) and standard drug Vincristine (30 mg/kg) respectively when compared with EAC control 18.5 days. The hematological parameters are shown in Fig. 1. The hemoglobin contents in the EAC control mice (8.88±0.30) was significantly decreased when compared with normal mice (13.27±0.42) whereas in the *E. littorale* treated doses, hemoglobin content were higher than EAC control. Significant changes in RBC count were also observed in *E. littorale* (500 mg/kg) treated mice. The *E. littorale* treated mice significantly reduced the WBC count as compared to that of control mice. In the differential count the percentage of Lymphocytes, Granulocyte and Monocyte was also significant as compared to EAC control.

Table2: Effect of methanol Extract of *E. littorale* on mean survival time (MST), percentage increase life span (%ILS) in EAC bearing mice.

Sl No.	**Experimental Groups**	**Mean survival time (MST) In days**	**% increase in life span**
1	Normal control (normal Saline 5ml/kg b. wt.)	-	-
2	EAC control	18.5	00
3	EAC + E. l. (250 mg/kg)	25.5	37.84
4	EAC + E. l. (500 mg/kg)	31.0	67.57
5	EAC + Vincristine(50 mg/kg)	36.0	94.59

Table3: Effect of methanol Extract of *E. littorale* on tumor volume, packed cell volume, viable and non viable tumor cell count in EAC bearing mice.

Parameter	EAC control	*E. littorale* 250 mg/kg	*E. littorale* 500 mg/kg	Vincristine 30 mg/kg
Body weight	25.4±1.07	22.8±1.07	21.6±0.51	23.8±1.24
Tumor volume (ml)	4.08±0.22	3.46±0.32	2.86±0.21	1.8±0.38**
Packed cell volume (ml)	2.76±0.29	2.02±0.34	2.86±0.21**	0.76±0.23**
Viable tumor cell count X 10^7 cells/ml	10.14±0.56	4.69±0.24**	2.36±0.14**	1.19±0.10**
Non viable tumor cell count X 10^7 cells/ml	0.56±0.11	2.02±0.34**	3.08±0.22**	3.94±0.22**

Values are mean ± SEM, (n=5), Experimental group compared with EAC control, **P < 0.01

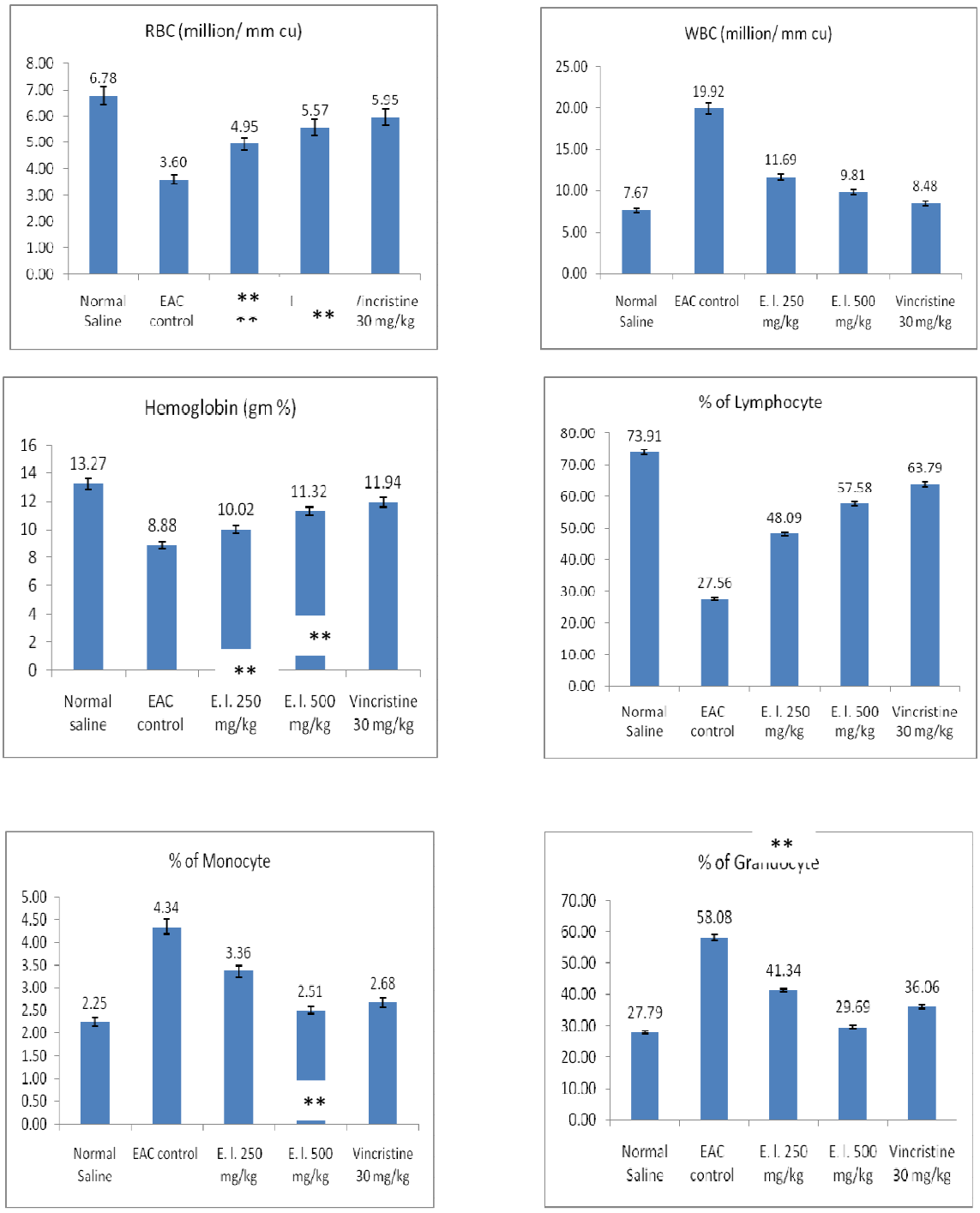

Fig. 1: Effect of the methanol extract of whole plant *Enicostemma littorale* on hematological parameters of EAC treated mice compared with normal, EAC control and standard drug treated mice. Statistical significance (*p*) calculated by one-way ANOVA between the treated groups and the EAC control followed by Dunnett's post hoc test of significance. Values are represent the mean ± S.E.M (n=5 mice per groups) and **p<0.01, when all treated groups are compared with EAC control group.

The study shows that methanolic extract of *E. littorale* were significantly increased the life span as well as decreased WBC compared to EAC control mice which are matching the criteria for any anticancer drug[15, 16]. The extract also reduced EAC cell volume and increased mean survival time compared to EAC control mice which revealed the delay on cell proliferation. The major drawback of anticancer medicine is anemia because of reduction of erythrocyte (RBC) as well as hemoglobin concentration and leucocytes [17]. The methanolic extracts of *E. littorale* were significant increase the RBC and hemoglobin level when compared to EAC control mice. This study show that extracts of *E. littorale* decreased viable cell count and increased level of non-viable cell count which proved it has direct relationship with cancer cells. Because these tumor cells are absorbed the anticancer drug by direct absorption in peritoneal cavity and this anticancer agent (extract of *E. littorale)* degraded the cells by direct cytotoxic mechanism. As earlier mentioned that phytoconstituents of *E. littorale* are flavonoids and alkaloids. Flavonoids induce so-called Phase II enzymes that help to eliminate mutagens and carcinogens, and therefore may be of value in cancer prevention. Flavonoids could also induce mechanisms that may kill cancer cells and inhibit tumor invasion [18, 19, 20]. Further more, flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation [21] and angiogenesis [22]. UCLA (University of California, Los Angeles) cancer researchers have observed that study participants who ate foods containing certain flavonoids, such as catechins found in strawberries and green and black teas; kaempferol from brussel sprouts and apples; and quercetin from beans, onions and apples, may have reduced risk of obtaining lung cancer [23].

Conclusion

Based on the above study results revealed that methanolic extract of *E. littorale* is moderately active for the treatment of cancer. Further pharmacological research using other cancer cells is necessary in order to established whether this plant can be used as a potential source for new anticancer medicine.

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