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Research Article

ANTITUMOR ACTIVITY OF *CYNOGLOSSUM ZEYLANICUM* (VAHL EX HORNEM) THUNB. EX. LEHM. AGAINST DALTON ASCITES LYMPHOMA IN SWISS ALBINO MICE

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ABSTRACT: The present study aims to evaluate the antitumor activity of ethanol extract of whole plant of *Cynoglossum zeylanicum* on DAL model in Swiss Albino mice. Evaluation of the antitumor effect of ethanol extract of whole plant of *Cynoglossum zeylanicum* on tumor growth and host survival time was made by the study of the following parameters: tumor volume, viable and non viable cell count and life span of host. The results showed decrease in tumor volume and cell viability. Hematological studies revealed that, the Hb count decreased in DAL treated mice, whereas, it was induced by the drug treated animals and showed an increase in Hb near to normal levels. The results suggested that, the extracts of whole plant of *Cynoglossum zeylanicum* exhibited significant antitumor activity on DAL bearing mice.

Keywords: *Cynoglossum zeylanicum*, antitumor, lifespan, WBC.

INTRODUCTION

Cancer is a major public health burden in both developed and developing countries. It was estimated 12.7 million now cancer cases and 7.6million cancer deaths in 2008 (Ferlay *et al.,* 2010). The environmental, chemical, physical, metabolic and genetic factors play a direct and/or indirect role in the induction and deterioration of cancers. The limited success of clinical therapies includes radiation, chemotherapy, immunomodulation and surgery in treating cancer, as evident by the high morbidity and mortality rates, indicates that there is an imperative need of new cancer management (Dai and Mumper, 2010). Drug discovery from medicinal plants has played an important role in the treatment of cancer and, indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last half centuary have been applied towards combating cancer (Balunas and Kinghorn, 2005). The National Cancer Institute collected about 35,000 plant species from 20 countries and has screened around 1,14,000 extracts for anticancer activity. It was estimated that 14 cancer drugs of the top 35 cancer drugs in year 2000 based on worldwide sales were natural products and natural products derivatives (Shoeb, 2006). Thus, it is urgent to find more and more safe new compounds that kill cancer cells. Cynoglossum zeylanicum (Vahl Ex Hornem) Thunb. Ex. Lehm. belongs to Boraginaceae family. It is commonly known as “Jathakkai”. Decoction prepared from the whole plant is used to arrest vomiting by Badaga community in Nilgiri Biosphere Reserve, Tamil Nadu. Taking into consideration of the medicinal importance of Cynoglossum zeylanicum, the ethanol extract of whole plant of Cynoglossum zeylanicum, were analyzed for their anticancer activity.

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MATERIALS AND METHODS

Collection

The whole plants of *Cynoglossum zeylanicum* were collected in the month of February and March, 2012, from the Kothagiri, Nilgiri Biosphere Reserve, Western Ghats, Tamil Nadu. The plant specimen were identified with the help of local flora and authenticated in Botanical survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited in Ethnopharmacology Unit, Research Department of Botany, V.O. Chidambaram College, Tuticorin, Tamil Nadu.

Preparation of plant extract for anticancer activity

The whole plants of *Cynoglossum zeylanicum* were cut into small pieces, washed dried at room temperature; the dried leaves and bark were powdered in a Wiley mill. Hundred grams of powdered whole plant were packed in a Soxhlet apparatus and extracted with ethanol. The ethanol extracts were concentrated in a rotary evaporator. The concentrated ethanol extracts of whole plant were used for anticancer activity.

Animals

Healthy male adult Swiss Albino mice (20-25gm) were used for the study. The animals were housed in microlon boxes in a controlled environment (temperature 25±20c) and 12 hr dark/eight cycle) with standard laboratory diet (Sai Durga feeds and foods, Bangalore) and water *ad libitum*. The mice well segregated based on their gender and quarantined for 15 days before the commencement of the experiment. They were fed on healthy diet and maintained in hygienic environment in our animal house.

Tumor Cells

Dalton Ascites Lymphoma (DAL) cells were obtained from Division of Oncology Department of Biotechnology, Tamil Nadu, Veterinary and Animal Husbandry, Chennai, Tamil Nadu, India. The DAL cells were maintained *in vivo* in Swiss albino mice by weekly intra peritoneal (i. p) inoculation of 10^6cells / mouse after every ten days. DAL cells 9 days old were used for the screening of the anticancer activity.

Acute oral toxicity study

Acute oral toxicity was performed by following OECD guideline - 420 fixed dose procedure for ethanol extract of whole plant of *Cynoglossum zeylanicum* and it was found that dose increasing up to 2000 mg / kg body weight, shown no toxicity or mortality in experimental mice. The LD50 of ethanol extracts of whole plant of *Cynoglossum zeylanicum* as per OECD guidelines-420 is greater than 2000 mg/kg (Ecobichon ,1997; Turner 1965).

Antitumor activity

Healthy Swiss albino mice were divided in to six groups of six animals (n=6) each. The test samples were dissolved in isotonic saline (0.9% NaCl W/V) and used directly in the assay. DAL cells were collected from the donor mouse and were suspended in sterile isotonic saline. The viable DAL cells were counted (Trypan blue indicator) under the microscope and were adjusted at 1 X 10^6 cells/ ml. 0.1 ml of DAL cells per 10g body weight of the animals were injected (i. p) to each mouse of each group except normal saline group (Group I). This was taken as Day 0. Group I served as a normal saline control (1mL/kg, p.o) and group II served as DAL bearing control. On day 1, the ethanol extracts of *Cynoglossum zeylanicum* at a dose of 100 and 150mg/kg each of the Group III, IV were administrated orally and continued for 14 consecutive days respectively. Group V served as tumor induced animal administrated with vincristine (80mg/kg body weight) for 14 consecutive days. On day15, half of the animals (n=3) in each case were sacrificed and the remaining animals were kept to observe the life span study of the tumor hosts. The effect of ethanol extract of *Cynoglossum zeylanicum* on tumor growth and host's survival time were monitored by studying parameters like tumor volume, tumor cell count, viable tumor cell count, nonviable tumor cell count, mean survival time and increase in life span (Gothoskar and Ranadive 1975; Mazumder *et al*.,1997).

Tumor growth response

The effect of ethanol extracts of *Cynoglossum zeylanicum* on tumor growth and hosts survival time were examined by studying the following parameters such as tumor volume, tumor cell count, viable tumor cell count, non viable tumor cell count, median survival time and increase in life span.

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Determination of Tumor volume

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube. Packed cell volume was determined by centrifuging the ascitic fluid at 1000 rpm for 5min.

Determination of Tumor cell count

The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension as placed on the Neubauer counting chamber and the number of cells in the 64 small squares was counted.

Estimation of viable and non viable tumor cell count (Tryphan blue dye assay)

The cells were then stained with tryphan blue (0.4% normal saline) dye. The cells that did not take up the dye were viable and those that took the stain were non viable. These viable and non viable cells were counted.

Percentage increase of life span (% ILS)

Animals were inoculated (1 x 10^6 cells/ml) 0.1ml of DAL cells per 10g body weight of the animals was injected i.p) on day zero (day 0).A day of incubation was allowed for multiplication of the cells. Fourteen doses of the Test samples (100 mg/kg and 200 mg/kg, 0.1 ml/10g body weight) and control group was treated with same volume of Saline (0.9%sodium chloride solution) and compared with vincristine (80mg / kg body weight) were injected i.p from the first day up to the 9th day with 24 h intervals. The effect of ethanol extracts of whole plant of *Cynoglossum zeylanicum* tumor growth was monitored by recording the mortality, daily for a period of 9 days and percentage increase in life span (% ILS) was calculated from the following equation.

$$\textbf{Increase in life span} = \frac{T - C}{C} \times 100$$

Body Weight

Body weights of the experimental mice were recorded both in the treated and control group at the beginning of the experiment (zero day) and sequentially on every 5th day during the treatment period.

Hematological studies

At the end of the experimental period, all mice were sacrificed by cervical dislocation. Blood was collected from freely flowing tail vein and used for the estimation of Hemoglobin content(Hb), Red blood cell count (RBC) and White blood cell count (WBC). WBC differential count was carried out from Leishman stained blood smears (Dacie and Lewis, 1958).

Statistical analysis

The data were analyzed using student's t- test statistical methods. For the statistical tests, *p* values of less than 0.01 and 0.05 were taken as significant.

RESULTS

The acute toxicity study, ethanol extracts of *Cynoglossum zeylanicum* whole plant did not show any toxic effect up to the dose of 2000mg/kg body weight, according 100mg/kg and 150mg/kg body weight were taken as low and high dose of whole plant *Cynoglossum zeylanicum* for the experiment. The present investigation indicates that ethanol extracts of whole plant of *Cynoglossum zeylanicum* showed significant antitumor activity in DAL bearing mice. The administration of ethanol extract of whole plant of *Cynoglossum zeylanicum* to DAL bearing mice showed reduction in body weight, spleen, thymus, liver, kidney and lungs (Table 1). In the case of tumor growth response study, treatment with ethanol extract of whole plant of *Cynoglossum zeylanicum* showed significant ($p<0.01$) reduction in tumor volume (Table 2). Table 3 depicts the effect of ethanol extract of whole plant of *Cynoglossum zeylanicum* on life span, viable cell count and non viable cell count. It revealed that there was increase in mean survival time. Administration of ethanol extract of *Cynoglossum zeylanicum* appreciably decreases the viable cell count compared to DAL bearing mice. Non viable cell count was significantly higher with increase in dosage of extracts. Table 4 showed that hematological parameters of tumor bearing mice on day 15 were found to be significantly different as compared to the extracts treated groups. In tumor bearing mice, it was found that there was increase in WBC count, and decrease in Hb content and RBC count. In differential count of WBC, present of neurophils and monocytes increased while the lymphocyte count decreased in the DAL control group. Treatment with *Cynoglossum zeylanicum* whole plant at the dose 100mg/kg and 150mg/kg significantly ($p<0.05$ and $p<0.01$ respectively) increased the Hb and RBC count to normal levels.

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The total WBC count was found to be increased significantly in the DAL control group when compared to normal group. Administration of *Cynoglossum zeylanicum* whole plant extracts (100mg/kg and 150mg/kg) in DAL bearing mice significantly ($p<0.05$ and $p<0.01$) reduced the WBC count as compared with DAL control.

Table 1. Effect of *Cynoglossum zeylanicum* extract on relative organ weights of tumor induced (DAL) mice

Treatment	Relative Organ Weight (g/100g body wt.)					
	Body weight (g)	Spleen	Thymus	Liver	Kidney	Lungs
Normal control(Group I)(Saline)	17.54±0.31	0.36±0.021	0.17±0.012	2.24±0.81	0.93±0.06	0.42±0.024
Tumor induced control(Saline)	39.23±2.10**	0.67±0.043**	0.41±0.017**	4.17±0.51*	2.13±0.17**	0.74±0.014*
CZW extract(100 mg/kg)+ DAL	27.33±2.17*	0.49±0.013	0.30±0.017*	3.24±0.21ns	1.49±0.014*	0.57±0.016
CZW extract(150mg/kg)+ DAL	21.43±0.38a	0.54±0.024*	0.18±0.027a	2.36±0.54a	0.84±0.17a	0.40±0.014a
Vincristine (80 mg/kg)+ DAL	19.13±1.17aa	0.39±0.024a	0.24±0.013a	2.54±0.27ns	0.86±0.87ns	0.41±0.011a

Each Value is SEM of 6 animals * P < 0.05 ; ** P < 0.01 Significance between normal control vs tumor induced control , drug treated group and ;aP < 0.05 ;aa P < 0.01 tumor induced control vs drug treated group ns :Not significant

Table 2: Antitumor activity of *Cynoglossum zeylanicum* extract on solid tumor volume in tumor (DAL) induced mice

Treatment	Solid Tumor Volume			
	15th day	20th day	25th day	30th day
Normal control (Saline) (Group I)	-	-	-	-
Tumor (DAL) induced control(Saline) (GroupI I)	4.39±0.21	6.08±0.13	6.81±0.31	6.97±0.26
CZWextract(100 mg/kg)+ DAL (Group III)	4.03±0.26	3.65±0.21ns	2.42±0.11**	2.08±0.38*
CZW extract(150mg/kg)+DAL (Group IV)	2.55±0.27*	2.03±0.19**	1.93±0.15**	1.53±0.2**
Vincristine (80mg/kg)+ DAL(Group V)	2.64±0.17	2.40±0.18*	2.04±0.37**	1.88±0.18**

Each Value is SEM of 6 animals * P < 0.05 ; ** P < 0.01 Significance between tumor induced control vs drug treated group ; ns :Not significant

Table 3 .Antitumor activity of Cynoglossum zeylanicum extract on the survival time, life span, tumor volume and viable and non-viable cell count in tumor induced mice

Treatment	Mean Survival time (Days)	Increase of life span (%)	Packed cell volume	Viable cell count X 106 cells/ml	Non-viable tumor cells count X 106cells/ ml
Normal control (Saline) (Group I)	-	-	-	-	-
Tumor (DAL) induced control(Group II)(Saline)	18.45±0.24	-	3.64±0.019	15.23±0.84	0.91±0.017
CZW extract(100 mg/kg)+DAL(Group III)	29.15±0.63*	57.99	1.53±0.017*	6.21±0.16**	1.24±0.065ns
CZW extract(150mg/kg)+DAL (Group IV)	36.22±0.24**	96.31	0.93±0.019**	5.37±0.13**	2.24±0.055*
Vincristine (80mg/kg)+ DAL(Group V)	34.65±0.74**	87.30	0.82±0.026**	5.13±0.16**	2.14±0.011*

Each Value is SEM of 6 animals * P < 0.05; ** P < 0.01 Significance between tumor induced control vs drug treated group; ns : Not significant

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Table –4 Anticancer activity of *Cynoglossum zeylanicum* extract on hematological parameters in tumor (DAL) bearing mice

Parameter	Hb (gm%)	RBC (million/mm3)	WBC (103 cells/ mm3)	Differential count		
				Lymphocytes	Neutrophils	Eosinophil
Normal control(Group I)	10.54±0.16	4.19±0.33	8.31±0.73	52.21±0.16	40.16±0.74	7.63±0.14
Tumor (DAL) induced control(Group II) (Saline)	7.94±0.34*	3.84±0.21	14.16±0.36**	47.13±0.24	50.13±0.16	2.74±0.15*
CZW extract(100 mg/kg)+ DAL(Group III)	8.17±0.17ns	4.01±0.19	9.56±0.24	40.13±0.38	51.66±0.30	8.21±0.21a
CZW extract(150mg/kg)+ DAL(Group IV)	10.88±0.94*	4.24±0.14	8.14±0.12a	42.56±0.16	50.13±0.77	7.14±0.16a
Vincristine (80mg/kg)+ DAL(Group V)	11.68±1.23*	4.13±0.24	8.06±0.15a	48.14±0.54	47.28±1.24	4.58±0.11ns

Each Value is SEM of 6 animals * $P < 0.05$; ** $P < 0.01$ Significance between normal control vs tumor induced control, drug treated group and ;a $P < 0.05$; tumor induced control vs drug treated group ns:Not significant

DISCUSSION

The present study was carried out to investigate the antitumor potential of whole plant of *Cynoglossum zeylanicum* against DAL bearing mice. The ethanol extract treated animals at the doses of 100 and 150 mg/kg significantly inhibited the tumor volume, packed cell volume, tumor (viable) cell count and brought back the hematological parameters to more or less normal levels.

In DAL tumor bearing mice, a regular rapid increase in ascitic tumor volume was observed. Ascetic fluid is the direct nutritional source for tumor cells and a rapid increase in ascitic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells (Prasad and Giri, 1994). Treatment with ethanol extract of *Cynoglossum zeylanicum* inhibited the tumor volume, viable tumor cell count and increased the life span of the tumor bearing mice. The reliable criteria for judging the value of any anticancer drug are the prolongation of the life span of animals (Gupta *et al.,* 2004). It may be concluded that ethanol extract of *Cynoglossum zeylanicum* by decreasing the nutritional fluid volume and arresting the tumor growth increases the life span of DAL bearing mice. Thus, ethanol extract of *Cynoglossum zeylanicum* has antitumor activity against DAL bearing mice.

Usually, in cancer chemotherapy the major problems that are being encountered are of myelosuppression and anemia (Price and Greenfield, 1958; Hogland 1982; Rajeshwar et al., 2005). The anemia encountered in tumor bearing mice its mainly due to reduction in RBC or hemoglobin percentage, and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions (Rajeshwar *et al*., 2005; Sarada *et al*., 2012). Treatment with ethanol extract of *Cynoglossum zeylanicum* whole plant brought back the hemoglobin (Hb) content, RBC count, indicating the anticancer nature of the extracts and WBC count more or less to normal levels. This clearly indicates that *Cynoglossum zeylanicum* whole plant possess protective action on the hemopoietic system.

Plant derived compounds have played an important role in the development of several clinical useful anticancer agents (Newman and Cragg ,2003) Tetradecanoic acid , phytol, 9,12- octadecadienoic acid (Z,Z)- , oleic acid and squalene were reported in the ethanol extract of *Cynoglossum zeylanicum* whole plant by GC-MS analysis. These compounds may have the role in anticancer property (Anitha *et al.,* 2012). Further, the isolation of the compounds responsible for the activity has to the taken up which may result in a modern drug from these plants.

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