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Anti Cancer Activity of Ethanolic Extract of Plumbago Zeylanica against Dalton's Ascitic Lymphoma in Mice

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Abstract
Aim: Traditional medicine serves people worldwide for a long time. In recent years, there has been significant interest in the use of traditional medicine information in cancer research. In traditional and common medicine for cancer treatments, Ethanolic Extract of *Plumbago zeylanica* (EEPZ) has been used to treat cancer. The aim of this study was to evaluate the effect on intraperitoneally injected Dalton Ascitic Lymphoma (DAL) cell lines in Swiss Albino mice, by ethanolic extract of *Plumbago zeylanica* (EEPZ) leaves. **Materials and Methods:** DAL cells ($1x10^6$ cells/ml/mouse) had injected into the mice intraperitoneally. The EEPZ was administered orally to the tumor-bearing group of animals at doses of 200 mg/kg and 400mg/kg body weight for 14 consecutive days. The measurement and comparison of derived parameters, hematological parameters, serum enzyme, and lipid parameters were carried out. The standard medicine was 5-Fluorouracil (20 mg/kg). **Result:** Both doses of EEPZ reduced average body weight gains, decreased viable tumor cell count for packed cell volume (PCV), and increased mice's lifetime for DAL treatment, with a reduction in blood flows, serum enzymes and lipid profile close to normal values. With the $p<0.01$ control group, all values were statistically important. The protective effect of Dalton Ascitic Lymphoma (DAL) extracts can be suggested for these observations. **Conclusion:** All these findings lead to the conclusion that both EEPZ doses have an Anti - DAL protective effect.

Keywords: Cancer, Plumbago zeylanica, EEPZ, DAL.

INTRODUCTION

Tumors are a mass of tissues that spread rapidly across the body and ultimately kill the hosts [1]. *Plumbago zeylanica L*, commonly known as chitrak or lead wort-white flowered is innate to South Asia. It is dispersed in tropical and subtropical countries of the world. Budding in deciduous woodland, savannahs, scrublands from sea level up to 2000 m altitude [2, 3]. In India it is sprinkled in central India to West Bengal, Maharashtra, and Uttar Pradesh to some parts of South India. The plant also enjoys regional names in different state Gujarati: Agni / vahini, Kannada:chitramula ,Malayalam: chitrakmula/ bilichitramula, Punjabi: Veellakeduveli, Bangali: chitra, Tamil: chita, Telugu: kodiveli/ chitramoolam,Hindi: chitraka/chitramol ,Sanskrit: chitra [4]. But commonly used name persisted to be chitraka [5,6]. Plumbago is from Plumbaginaceae family comprises of 10 genera and 280 species. The genus Plumbago take account of 3species that is Plumbago indica L. (P. rosea L.) P. capensis L., and P. zeylanica L.,in all these 3 species Plumbago zeylanica is most cultivated because of its high therapeutic uses. It is an oldest herb that was used in Ayurveda for several disorders over thousands of years. It grows wild in India and also refined commercially.
Chemotherapy provides efficient treatment either alone or in conjunction with surgery and/or radiotherapy for various types of cancer. The present study is aimed at to evaluate the ethanolic extract of *Plumbago zeylanica* against Dalton ascetic lymphoma.

MATERIALS AND METHODS

Preparation of Extract
The *Plumbago zeylanica.* Linn plant was purchased from the local market. The leaves were collected and they were washed to remove the surface contaminants in running water and desiccated between folds of soft tissue paper. Then the leaves were dried under shade and finely powdered. The powder was weighed to 50 gm and soaked overnight in petroleum ether to remove chlorophyll and the PET ether was drained. Then the remaining residue was refluxed using sox let apparatus using ethanol for 48 hrs. The extract was concentrated and used for analysis**.**

Selection Grouping and Acclimatization of Laboratory Animal [7]
Male Swiss albino mice (20-25 grams) were produced and used during the study from the experimental animal laboratory. They were housed in a controlled environment with a micro nylon box (temp 25±2°C) and an ad libitum water/dark/light cycle of 12 o'clock. The study was carried out following clearance of the institutional committee on animal ethics. The mice were separated by sex in accordance with standard practice and were quarantined 15 days before the experiment started. They have been fed on healthy food and kept in our animal house in a healthy environment.

Technique for Inducing Tumor
In experimental studies on the activity of cancers, various techniques have been employed in inducing induced cancer of animals, such as, chemically induced (use of DMBA/croton oil, etc.) [8] virus-induced; cell line induction of the disease; (sarcoma – 180, ULCA fibrosarcoma and Jensen sarcoma, mouse lung fibroblast cells L-929, Dalton's Lymphoma Ascites (DLA), Ehrlich Ascites Carcinoma

(EAC) [9-11] methods have been used in experimental studies of anticancer activity.
In the current study, the activity of the ethanol extract Plumbago zeylanica anti-cancer in mice was evaluated using cell lines induced by mice.

Induction of cancer using DLA cells
Amala Cancer Research Center, Thrissur, Kerala, India supplied Dalton's Lymphoma ascites (DLA) cells. In intraperitoneal transplantation, the cells were maintained in vivo in Switzerland. The DLA cells have been extracted from the peritoneal cavity of the mice using saline while transforming the tumor cells into the grouped animal. The cell counts were performed and further dilution was done so that the total cell was $1x10^6$, intraperitoneal dilution. Allow the tumor to grow in the mouse for at least 7 days before treatment.

Treatment Protocol
Mice of Swiss Albino were split into five groups, each with six. Intraperitoneally was injected into all of the animals in four groups with DLA cells ($1x10^6$ cells per mouse) [12] and the normal control group was in the remaining group.

- **Group 1** served as the normal control.
- **Group 2** served as tumor control. Group 1 and 2 receives a normal diet and Water.
- **Group 3** served as the positive control, was treated with injection 5-fluorouracil at 20mg/kg body weight, Intraperitoneally. [13**]**
- **Group 4** Served as a treatment control group and was administered ethanolic extract of *Plumbago zeylanica* at a dose of 200mg/kg orally.
- **Group 5** Served as a treatment control group and was administered ethanolic extract of *Plumbago zeylanica* at a dose of 400mg/kg intraperitoneally.

Treatment
In this study, medicinal products were treated once daily for 14 days after the 24 hours of inoculation. Euthanasia was sacrificed to every mouse of each group on Day 14, after the last dose. Retro-orbital plexus bleeding removed blood from every mouse and checked for the following parameters [14-17].

1. Hematological parameters
    a. WBC count
    b. RBC count
    c. Hb content
    d. Platelet count
    e. Packed cell volume
2. Serum enzyme and lipid profile
    a. Total Cholesterol (TC)
    b. Triglycerides (TG)
    c. Aspartate Amino Transferase (AST)
    d. Alanine Amino Transferase (ALT)
    e. Alkaline Phosphatase (ALP)
3. Derived parameter
    a. Body weight
    b. Life span (%)
    c. Cancer Cell Count

Evaluation of Clinical Parameters

Cancer cell count [18]
The fluid (0.1ml) from the peritoneal cavity of each mouse was withdrawn by sterile syringe and diluted with 0.8 ml of ice-cold Normal saline or sterile Phosphate Buffer Solution and 0.1 ml of trypan blue (0.1 mg/ml) and total numbers of the living cells were counted using hemocytometer.

$$\text{Cell count} = \frac{\text{No of cells Dilution}}{\text{Area} \times \text{Thickness of liquid film}}$$

1) Hematological parameters
i) WBC count
ii) RBC count
iii) Platelet count
iv) Hemoglobin
v) Packed Cell Volume

i) WBC count
The total WBC count was found to be increased in cancer control when compared with normal and treated tumor-bearing mice. [19]

ii) RBC and Hb
RBC and Hb content decreases with tumor-bearing mice when compared with Normal control mice.

iii) Platelets
In Hodgkin lymphoma, increased in platelet count often reported in laboratory finding. Hence, I investigated this parameter in the study. [20]

iv) Packed cell volume
In any case of anemia, the packed cell volume is decreased.

Serum Enzyme and Lipid Profile
The serum was analyzed for the following parameters
(a) Aspartate Amino Transferase (AST)
(b) Alanine Amino Transferase (ALT)
(c) Alkaline Phosphatase (ALP)
(d) Total Cholesterol (TC)
(e) Triglyceride (TG)

Total Cholesterol and Triglyceride (lipid profile)
Abnormal blood lipid profile has been associated with cancer. In Hodgkin lymphoma, high cholesterol level and low triglyceride level has been reported. Hence I investigated this parameter in the study. [21]

Liver Enzymes (AST, ALT, ALP)
Abnormal liver function is seen in a patient with Hodgkin lymphoma, that these liver enzyme levels markedly increase in tumor-bearing mice. ALP is an enzyme mainly derived from the liver, bones and in a lesser amount from intestines, placenta, kidneys, and leukocytes. An increase in ALP levels in the serum is frequently associated with a variety of disease. [22] ALP comprises a group of an enzyme that catalyzes the

phosphate esters in an alkaline environment, generating an organic radical and inorganic phosphate.
Markedly elevated serum ALP, hyperalakline-phosphatasemia, is seen predominantly with more specific disorders; including malignant biliary cirrhosis, hepatic lymphoma, and sarcoidosis. [23] Hence, I investigated this parameter in this study.

Derived Parameters

Body weight

All the mice were weighed, from the beginning to 15th day of the study. The average increase in body weight on the 15th day was determined.

Percentage increase in life span (ILS)

% ILS was calculated by the following formulae

The life span of the treated group

$$\%ILS = \frac{Life\ span\ of\ treated\ group}{Life\ span\ of\ control\ group} - 1x100$$

- All biochemical investigations were done by using COBAS MIRA PLUS-S Auto analyzer from Roche Switzerland.
- The hematological test is carried out in COBAS MICROS OT 18 from Roche.
- Newly added Hi-Tech instruments MAX MAT used for an autoanalyzer for all biochemistry investigations in a blood sample.

Table No. 1: Effect of *Ethanolic extract of Plumbago zeylanica* on Hematological parameters

Treatment	Total WBC Cells /mlx10^3	Rbc Count Mill/cumm	Hb Gm/dl	PCV %	Platelets Lakhs/cumm
G1	10.30 ±1.26	4.32±0.90	12.35 ±1.30	14.20±2.40	3.28±0.75
G2	15.20 ±2.58^{a**}	2.65±0.50^{a**}	6.75 ±0.90^{a**}	38.30±3.30^{a**}	1.72±0.55^{a**}
G3	12.25 ±1.25^{b**}	4.25±1.40^{b**}	11.97±1.50^{b**}	16.45±1.45^{b**}	2.99±0.92^{b**}
G4	13.70 ±1.40^{b**}	3.95±0.62^{b**}	12.02±1.35^{b**}	18.62±2.35^{b**}	3.12 ±0.66^{b**}
G5	12.90±2.01^{b**}	4.05±0.35^{b**}	12.19±1.65^{b**}	17.02±2.30^{b**}	3.19±0.75^{b**}

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 – Treatment control (200mg/kg) G5 – Treatment control (400mg/kg)
All values are expressed as mean ± SEM for 6 animals in each group.
**a – Values are significantly different from normal control (G_1) at $P < 0.01$
**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

Table No.2: Effect of ethanolic extract of *Plumbago zeylanica* on serum Enzymes and lipid proteins

Treatment	Cholesterol (mg/dl)	TGL (mg /dl)	AST (U/L)	ALT (U/L)	ALP (U/L)
G_1	110.05±3.45	134.80±2.50	38.45 ±1.62	33.30 ±1.40	130.30 ±2.35
G_2	147.90±4.25^{a**}	218.30±4.45^{a**}	78.4±2.70^{a**}	62.30±2.65^{a**}	262.35±4.30^{a**}
G_3	128.32±3.80^{b**}	168.10±2.62^{b**}	44.45 ±1.70^{b**}	34.50±1.65^{b**}	155.40±2.36^{b**}
G_4	120.25±2.60^{b**}	172.35±2.50^{b**}	50.40±1.95^{b**}	37.38 ±1.70^{b**}	174.35±2.50^{b**}
G_5	116.20±3.02^{b**}	169.60±2.70^{b**}	44.50 ±2.10^{b**}	36.22±1.95^{b**}	166.32±2.12^{b**}

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 – Treatment control (200mg/kg) G5 – Treatment control (400mg/kg)
All values are expressed as mean ± SEM for 6 animals in each group.
**a – Values are significantly different from control (G_1) at $P < 0.01$
**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

Table No.3: Effect of ethanolic extract of *Plumbago zeylanica* on the life span, body weight and cancer cell count of tumor-induced mice

Treatment	Number of animals	% ILS Life span	Increase in Bodyweight grams	Cancer cell count ml X 10^6
G_1	6	>>30 days	2.20±0.65	-
G_2	6	46%	$9.40\pm0.80^{a**}$	$2.70\pm0.38^{a**}$
G_3	6	92%	$5.65\pm0.40^{b**}$	$1.25\pm0.33^{b**}$
G_4	6	80%	$6.20\pm0.62^{b**}$	$1.60\pm0.43^{b**}$
G_5	6	84%	$5.75\pm0.78^{b**}$	$1.52\pm0.26^{b*}$

G_1 – Normal Control, G_2 – Cancer Control, G_3 – Positive control, G_4 – Treatment control (200mg/kg) G5 – Treatment control (400mg/kg)
All values are expressed as mean ± SEM for 6 animals in each group.
**a – Values are significantly different from control (G_1) at $P < 0.01$
**b – Values are significantly different from cancer control (G_2) at $P < 0.01$

RESULTS

Effect on Tumor Growth

In the DLA tumor control group, the average life span of the animal was found to be 46% whereas an ethanolic extract of *Plumbago zeylanica* at a dose of 200 and 400 mg/kg body weight increase the life span to 80%, and 84% respectively. These values were significant. However, the average life span of 5-FU treatment was found to be 92%, indicating its potent antitumor nature. The antitumor nature of ethanolic extract of *Plumbago zeylanica* at a dose of 200 and 400 mg/kg was evidenced by the significant reduction in percent increase in body weight of animal treated with ethanolic extract of *Plumbago zeylanica* at a dose of 200 and 400 mg/kg body weight when compared to DLA tumor-bearing mice.

It was also supported by the significant reduction in packed cell volume and viable Tumor cell count in ethanolic extract of *Plumbago zeylanica* at a dose of 200 and 400 mg/kg treatments when compared to the DLA tumor control. (Table No .2 & 3).

Effect on Hematological Parameters

As shown in (Table No.1) RBC, Hb, Platelets were decreased and WBC count was significantly increased in the DLA control group compared to the normal control group. Treatment with ethanolic extract of *Plumbago zeylanica* at a dose of 200 and 400 mg/kg significantly increases the Hb content, RBC, Platelets and significantly decreased the WBC count to about normal level. All these results suggest the anticancer nature of the ethanolic extract of *Plumbago zeylanica* at a dose of 200 and 400 mg/kg at a dose of 20 and 40 mg/kg. However, the standard 5FU at the dose of 20 mg/kg body weight produced a better result in all these parameters.

Effect on Biochemical Parameters

The inoculation of DLA cells caused a significant increase in the level of Total Cholesterol, Aspartate Amino Transferase, Alanine Amino Transferase, Alkaline Phosphatase in the tumor control animals (G_2) when compared to the normal group. The treatment with an ethanolic extract of *Plumbago zeylanica* at a dose of 200 and 400 mg/kg body weight reversed these changes towards the normal level. (Table No. 2) All the value was found to be significant. The treatment with standard 5-FU also gave similar results.

Histopathology

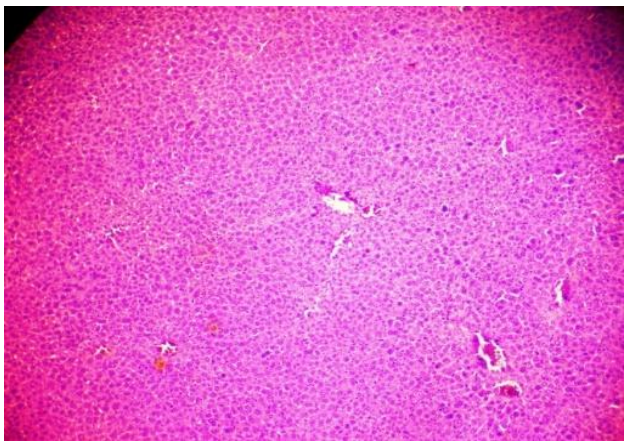

Fig. 1 Normal control

Section of liver parenchyma with hepatocyte which appears normal and central vein & portal tract is normal

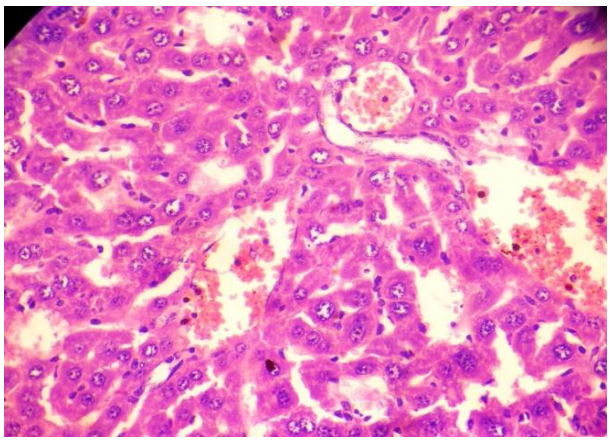

Fig. 2 Toxic control
Section of liver parenchyma with a scattered focal area of necrosis of hepatocyte.

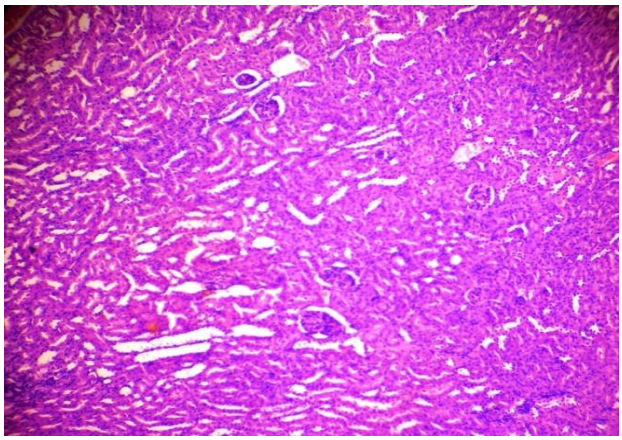

Fig. 3 Positive control (20mg /kg 5FU)
Section of liver parenchyma shows the normal hepatic architecture.

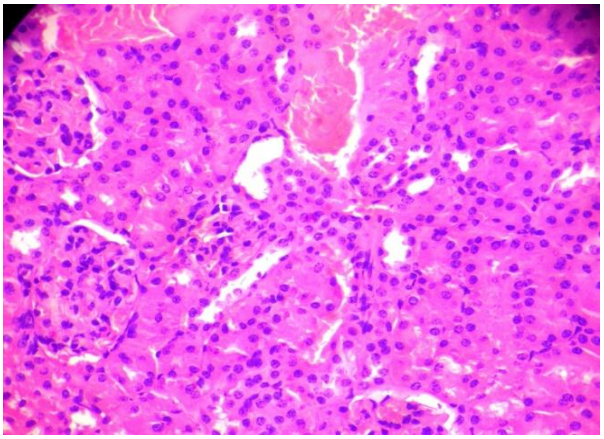

Fig. 4 Treatment control (EEPZ 200mg/kg)
Section of liver parenchyma shows the normal hepatic architecture.

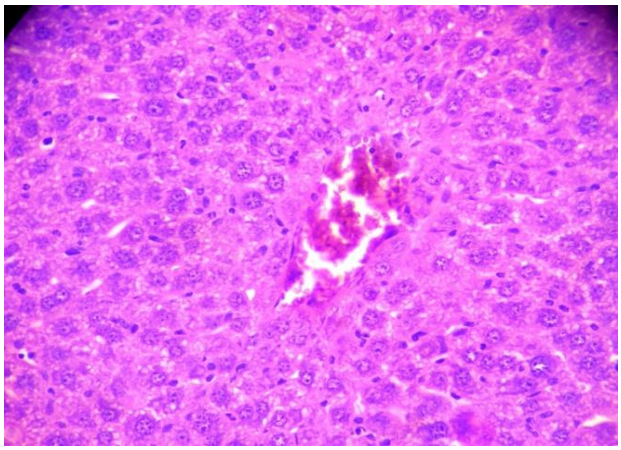

Fig. 5 Treatment control (EEPZ 400mg/kg)
Section of liver parenchyma shows the normal hepatic architecture

DISCUSSION AND CONCLUSION

The alternative system of medicines like Ayurvedic, Siddha, Unani and other tribal folklore medicines have significantly contributed to the health care of the population of India. Today these systems are not only complementary but also competitive in the treatment of various diseases. Plants have served as a good source of antitumor agents. Several studies have been conducted on herbs under a multitude of Ethnobotanical grounds. A large number of plants possessing anticancer properties have been documented [24-29].

Plants of *Plumbago zeylanica* were traditionally used in the treatment of tumors [30]. The present investigation was carried out to evaluate the antitumor activity of Ethanolic extracts of *Plumbago zeylanica* in DLA tumor-bearing mice. The EEPZ treated animals at the doses of 200 and 400 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 DLA tumor-bearing animals, a regular rapid increase in ascitic tumor volume was observed. Ascitic fluid is the direct nutritional source for tumor cells and a rapid increase in an ascitic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells [31]. Treatment with EEPZ 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 lifespan of animals [32]. It may be concluded that EEPZ by decreasing the nutritional fluid volume and arresting the tumor growth increases the life span of DLA bearing mice. Thus EEPZ has antitumor activity against DLA bearing mice.

Usually, in cancer chemotherapy, the major problems that are being encountered are myelosuppression and anemia [33, 34]. The anemia encountered in tumor-bearing mice is mainly due to a reduction in RBC or Hb and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions [35]. Treatment with both doses of EEPZ brought back the

(Hb) content; RBC and WBC count more or less to normal levels significantly. This clearly indicates that EEPZ possesses protective action on the hemopoietic system.

It was reported that the presence of tumor in the human body or in the experimental animals is known to affect many functions of the liver. The significantly elevated levels of total cholesterol, TGL, AST, ALT, ALP in the serum of tumor inoculated animal indicated liver damage and loss of functional integrity of the cell membrane. The significant reversal of these changes towards the normal by EEPZ treatments.

In the present study, the biochemical examination of DLA inoculated animals showed marked changes indicating the toxic effect of the tumor. The normalization of these effects observed in the serum treated with EEPZ possesses significant antitumor and hepatoprotective effect of the extracts.

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