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Antitumor activity of ethanolic extract of *phyllanthus longiflorus* Heyne Ex. Hook. F. against dalton's ascitic lymphoma in mice

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Abstract

The present study was planned to evaluate the effect of Ethanolic Extract of *phyllanthus longiflorus* against Dalton's Ascitic Lymphoma (DAL). Male Swiss albino mice (20-25gm) were procured from central animal house, and used throughout the study. Various technique for induction of cancer in animals, viz. chemically induced (using DMBA/croton oil. etc) virus induced, cell line induced (sarcoma–180, ULCA fibro sarcoma and Jensen sarcoma, mouse lung fibroblast cells L-929, Dalton's Lymphoma Ascites (DLA), Ehrlich Ascites Carcinoma (EAC) methods have been used in experimental studies of anticancer activity. The antitumor nature of ***phyllanthus longiflorus*** was evidenced by the significant reduction in percent increase in body weight of animal treated with ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/kg body weight when compared to DLA tumor bearing mice. Treatment with ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/kg significantly increases the Hb content, RBC, Platelets and significantly decreased the WBC count to about normal level. The inoculation of DLA cells caused significantly increase in the level of Total Cholesterol, Aspartate amino Transferase, Alanine, amino Transferase, Alkaline Phosphatase in the tumor control animals(G2), when compared to the normal group.

Keywords: Anti tumor activity, *phyllanthus longiflorus*, Dalton's Ascitic Lymphoma, Male Swiss albino mice, Hematological Parameters.

Introduction

Tumor is a mass of tissues which proliferate rapidly, spread throughout the body and may eventually cause death of the host (Mohan 2006). By 2050 over 20 million new cancer cases and over 17 million cancer deaths are probable to occur in the world (American cancer society 2006). Chemotherapy is an effective treatment against various types of cancer either singly or in combination with surgery and/or radiotherapy. However, chemotherapeutic effects of most of the drugs showed limited efficacy due to the development of various side effects. This fostered our attempts to evaluate some plant products against cancer as they are less likely to cause serious side effects. Many Indian spices are quoted to be useful in different types of cancer (Unnikrishnan & Kuttan 1990; Babu 1995).

The traditional systems of Siddha and Ayurvedic medicine use this plant alone or in combination with other medicinal plants for the treatment of various diseases. A vast literature collection fails to produce a scientific evidence to prove the anti tumor activity of *phyllanthus longiflorus*. Hence this study was planned to evaluate the effect of Ethanolic Extract of *phyllanthus longiflorus* against Dalton's Ascitic Lymphoma (DAL).

Materials and Methods

Plant material

The aerial part of *Phyllanthus longiflorus* Heyne Ex Hook.F. was collected from Courtallam hills (Tamil Nadu) at 1000m ht.

Preparation of plant extract for phytochemical screening and anti tumor activity

The leaves of the plant were dried under shade and then powdered with a mechanical grinder to obtain a coarse powder, which was then subjected to successive extraction in a soxhlet apparatus using ethanol. The extract was subjected to qualitative tests for the identification of various phytochemical constituents as per the standard procedure. The ethanolic extracts of the leaves were concentrated in a rotary evaporator. The concentrated ethanol extract were used for anti tumor activity.

Selection Grouping and Acclimatization of Laboratory Animal

Male Swiss albino mice (20-25gm) were procured from central animal house, and used throughout the study. They were housed in micro nylon boxes in a control environment (temp 25±2°C) and 12 h dark /light cycle with standard laboratory diet and water *ad libitum*. The study was conducted after obtaining institutional animal ethical committee clearance. As per the standard practice, the mice were 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 (Unnikrishnan & Kuttan 1990).

Induction of Tumor

Various technique for induction of cancer in animals, viz. chemically induced (using DMBA/croton oil. etc) (Agarwal 2009) virus induced, cell line induced (sarcoma–180, ULCA fibro sarcoma and Jensen sarcoma, mouse lung fibroblast cells L-929, Dalton's Lymphoma Ascites (DLA), Ehrlich Ascites Carcinoma (EAC) (Becerra *et al* 2006; David Apple man *et al* 1950; Chitra *et al* 2009) methods have been used in experimental studies of anticancer activity.

Induction of cancer using DLA cells

Dalton's Lymphoma ascites (DLA) cells were supplied by Amala cancer research center, Trissur, Kerala, India. The cells maintained *invivo* in Swiss albino mice by intraperitoneal transplantation. While transforming the tumor cells to the grouped animal the DLA cells were aspirated from peritoneal cavity of the mice using saline. The cell counts were done and further dilution were made so that total cell should be 1×10^6, this dilution was given intraperitonealy. Allowed the tumor to grow in the mice for a minimum of seven days before starting the treatments.

Treatment Protocol

Swiss Albino mice were divided into seven groups of six animals each. All the animals in Six groups were injected with Dalton's Lymphoma Ascites (DLA) cells (1×10^6 cells per/ml/ mouse) (Nagineni Sujana *et al* 2012) intraperitoneally, and the remaining one group is normal control.

Group 1 The normal control.

Group 2 The tumor control

Group 3 The positive control, was treated with injection fluorouracil at 20mg/kg body weight, i.p (Sathiyanarayanan *et al* 2006).

Group 4 Served as the treatment control, which was treated with Ethanolic Extract of *phyllanthus longiflorus* at a dose of 200 mg/kg body weight, in oral route.

Group 5 Served as the treatment control, which was treated with Ethanolic Extract of *phyllanthus longiflorus* at a dose of 400 mg/kg body weight, in oral route.

Treatment

In this study, drug treatment was given after 24 h of inoculation, once daily for 14 days. On day 14th day after the last dose, all mice from each group were sacrificed by euthanasia. Blood was withdrawn from each mouse by retro orbital puncture method and the following parameters were checked (Senthil *et al* 2007; Gupta et al 2004).

1. **Hematological parameters**
   - WBC count
   - RBC count
   - Hb content
   - Platelet count
   - Packed cell volume (PCV)
2. **Serum enzyme and lipid profile**
   - Total Cholesterol (TC)
   - Triglycerides (TGL)
   - Aspartate amino Transferase (AST)
   - Alanine amino Transferase (ALT)
   - Alkaline Phosphatase (ALP)
3. **Derived parameters**
   - Body weight; Life span (%)
   - Cancer Cell Count

Evaluation of Clinical Parameters

Cancer cell count

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 tryphan blue (0.1 mg/ml) and total numbers of the living cells were counted using heamocytometer (Spiridon 2006).

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

Hematological parameters

- WBC count
- RBC count
- Platelet count
- Hb
- PVC

i) WBC count

The WBC count was found to be increased in cancer control, when compared with normal and treated tumor-bearing mice. The WBC count were found to decrease significantly in animals treated with plant extract when compared with cancer control, indicating that the antitumor nature of the extract (Santhosh Kumar 2007).

ii) RBC and Hb

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

iii) Platelets

In Hodgkin lymphoma, increase in platelet count often reported in laboratory finding. Hence, this parameter in the study is investigated (Jacqueline 1998).

iv) Packed cell volume

In the case of anemia the packed cell volume decreases.

Serum Enzyme and Lipid Profile

The serum was analyzed for the following parameters

- Aspartate amino Transferase (AST)
- Alanine amino Transferase (ALT)
- Alkaline Phosphatase (ALP)
- Total Cholesterol (TC)
- Triglycerides (TGL)

1. Total Cholesterol and Triglycerides

Abnormal blood lipid profile has been associated with cancer. In Hodgkin lymphoma, high cholesterol level and low triglyceride level has been reported and investigated in this parameter study (Ronald 1995).

2. Liver Enzymes (AST, ALT, ALP)

Abnormal liver function seen in patient with Hodgkin lymphoma (Viroj wiwanikit 2001) that these liver enzyme levels markedly increase in tumor bearing mice. AST, ALT, ALP is an enzyme mainly derived from the liver, bones and in lesser amount from intestines, placenta, kidneys and leukocytes. An increase in AST, ALT and ALP levels in the serum are frequently associated with the variety of disease (Intyre & Rosalki 1991). AST, ALT and ALP comprise a group of enzyme that catalyzes the phosphate esters in an alkaline environment, generating an organic radical and inorganic phosphate.

Markedly elevated serum AST, ALT and ALP, hyperalkaline-phosphatasemia, is seen predominantly with more specific disorders; including malignant biliary cirrhosis, hepatic lymphoma and sarcoidosis (Jahan 2008).

Derived Parameters

1. Body weight:

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

2. Percentage increase in life span (ILS)

% ILS was calculated by the following formulae

$$\frac{\text{Life span of treated group}}{\text{Life span of control group}} - 1 \text{x} 100$$

All biochemical investigations were done by using OBAS MIRA PLUS-S Auto analyzer from Roche Switzerland.

Hematological test are carried out in COBAS MICROS OT 18 from Roche.

Newly added Hi-Tech instruments MAX MAT used for an auto analyzer for all biochemical investigations in blood sample.

Results

Effect on Hematological Parameters

As shown in (Table: 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 ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/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 extract. However, the standard 5-FU at the dose of 20 mg/kg body weight produced better result in all these parameters.

Table 1: Effect of *phyllanthus longiflorus* on Hematological Parameters

	Total WBC Cells /mlx10^3	**RBC Count Millon/cumm**	**Hb gm/dl**	**PCV in %**	**Platelets Lakhs/cumm**
G1	9.80 ±1.70	4.90±0.90	12.10 ±1.75	15.30±2.42	3.60±0.95
G2	14.65 ±3.15a**	2.35±0.40a**	7.20 ±0.90a**	30.70±3.35a**	1.45±0.80a**
G3	11.32 ±1.30b**	4.30±0.82b**	11.3 ±1.40b**	17.30±1.80b**	2.60±0.85b**
G4	12.74±2.20b**	2.95±0.52b**	9.42±0.62b**	23.30±2.30b**	1.95±0.45b**
G5	12.95 ±2.70b**	3.10±0.50b**	9.70±1.08b**	22.90±2.45b**	2.32 ±0.70b**

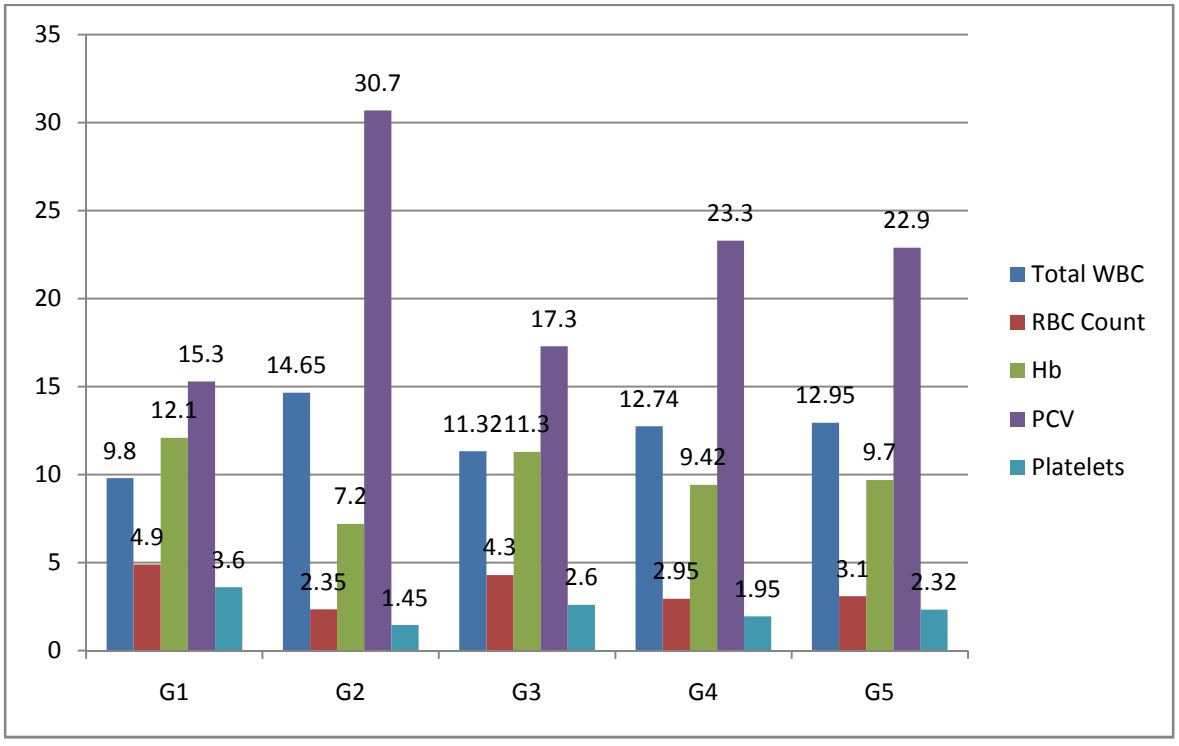
G1 – Normal Control, G2 – Cancer Control, G3 – Positive control, G4 – Treatment control (PS-1-200mg/kg), G5 – Treatment control (PS-1-400mg/kg),

All values are expressed as mean ± SEM (n=6).

a** – Values are significantly different from control (G1) P < 0.001

b** – Values are significantly different from cancer control (G2) P < 0.001

Figure 1: Effect of *phyllanthus longiflorus* on Hematological Parameters



Effect on Biochemical Parameters

The inoculation of DLA cells caused significantly increase in the level of Total Cholesterol, Aspartate amino Transferase, Alanine, amino Transferase, Alkaline Phosphatase in the tumor control animals (G2), when compared to the normal group. The treatment with ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/kg body weight reversed these changes towards the normal level (Table-2) All the values were found to be significant. The treatment with standard 5- FU also gave similar results.

Table 2: Effect of *phyllanthus longiflorus* on Serum Enzymes and Lipid Proteins

Treatment	Cholesterol (mg/dl)	TGL (mg /dl)	AST (U/L)	ALT (U/L)	ALP (U/L)
G1	105.20±5.70	127.30±2.60	39.50 ±1.30	34.30 ±1.50	130.50 ±3.30
G2	152.40±7.30a**	216.10±5.70a**	93.60±2.75a**	68.50±3.20a**	242.70±7.35a**
G3	118.20±4.50b**	159.50±3.60b**	53.35 ±1.90b**	42.30±2.10b**	163.55±4.25b**
G4	125.50±4.90b**	166.20±3.40b**	71.30 ±2.25b**	50.00±2.80b**	182.50±4.85b**
G5	125.88±4.98b**	163.70±2.96b**	68.30±1.96b**	48.52 ±2.45b**	183.45±4.30b**

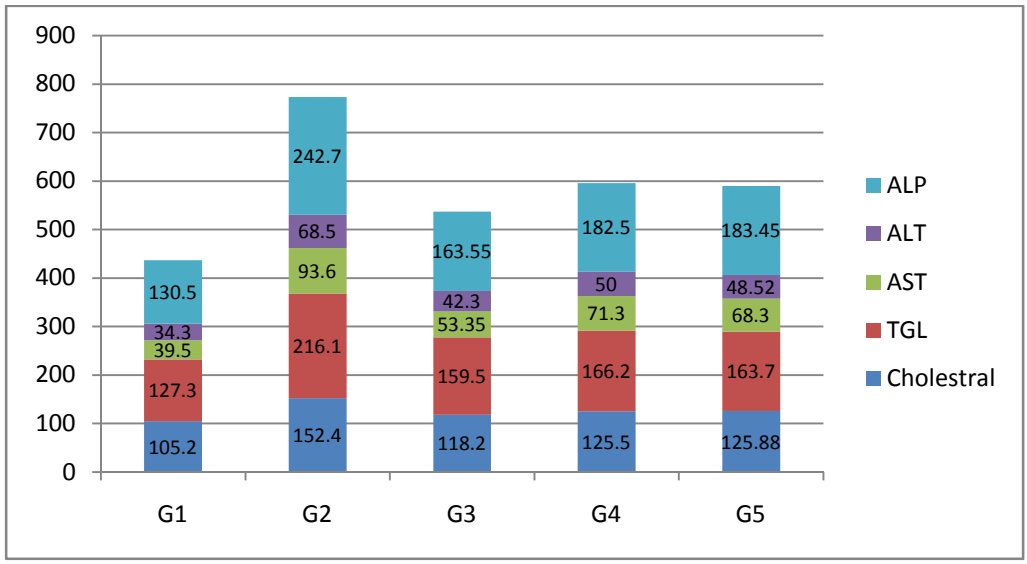
G1 – Normal Control, G2 – Cancer Control, G3 – Positive control, G4 – Treatment control (PS-1-200mg/kg), G5 – Treatment control (PS-1-400mg/kg)

All values are expressed as mean ± SEM (n=6).

a **– Values are significantly different from control (G1) $P < 0.001$

b** – Values are significantly different from cancer control (G2) $P < 0.001$

Figure 2: Effect of *phyllanthus longiflorus* on Serum Enzymes and Lipid Proteins



Effect on Tumor Growth

In the DLA tumor control group, the average life span of animal was found to be 50% where as ***phyllanthus longiflorus*** at a dose of 200 mg/kg and 400mg/kg body weight increase the life span to 70% and 72% respectively. These values were significant. However the average life span of 5- FU treatment was found to be 90%, indicating its potent antitumor nature. The antitumor nature of ***phyllanthus longiflorus*** was evidenced by the significant reduction in percent increase in body weight of animal treated with ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/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 both the extracts of treatment when compared to the DLA tumor control (Table: 3)

Table 3: Effect of *phyllanthus longiflorus* on the Life Span, Body Weight and Cancer Cell Count of Tumor Induced Mice

Treatment	Number of animals	% ILS Life span	Body weight in grams	Cancer cell count ml X 106
G1	6	>>30 days	2.30±0.50	-
G2	6	50%	7.90±0.90a**	2.68±0.40a**
G3	6	90%	3.85±0.23b**	1.35±0.36b**
G4	6	70%	4.16±0.70b**	1.97±0.58b**
G5	6	72%	4.55±0.75b**	1.62±0.37b**

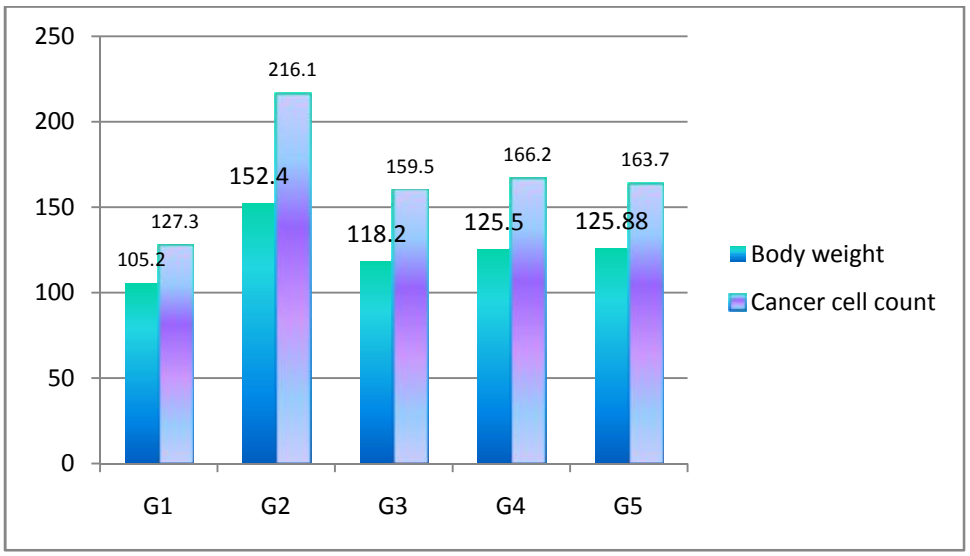
G1 – Normal Control, G2 – Cancer Control, G3 – Positive control, G4 – Treatment control (PS-1-200mg/kg), G5 – Treatment control (PS-1-400mg/kg)

All values are expressed as mean ± SEM (n=6).

a** – Values are significantly different from control (G1) at P < 0.001

b** – Values are significantly different from cancer control (G2) at P < 0.001

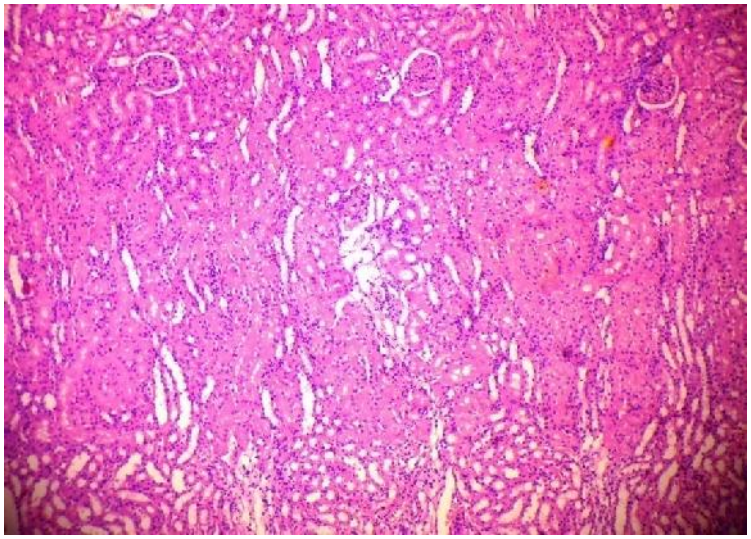
Figure 3: Effect of *phyllanthus longiflorus* on Body Weight and Cancer Cell Count of Tumor Induced Mice



HISTOPATHOLOGY STUDY

Figure No: 1

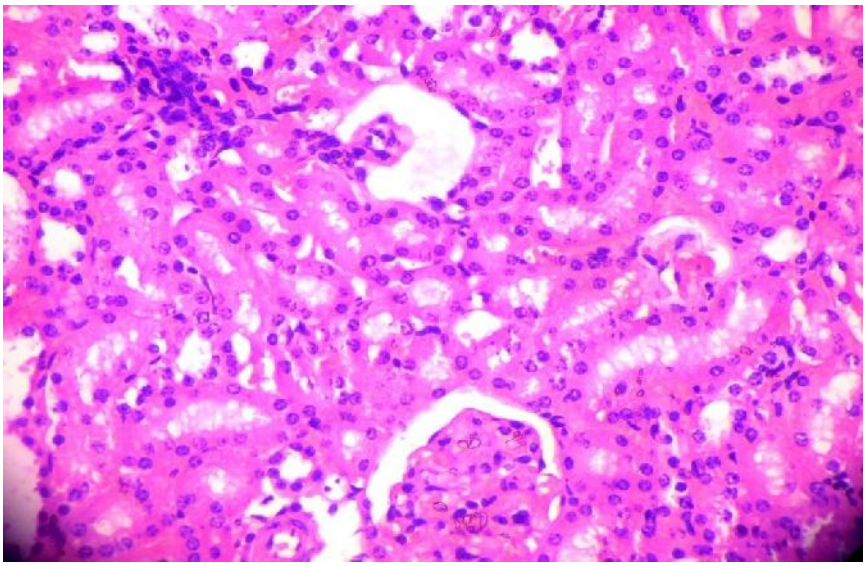
Normal Control Rat



Section of liver parenchyma with hepatocyte which appear normal, and central vein & portal tract are normal.

Figure No: 2

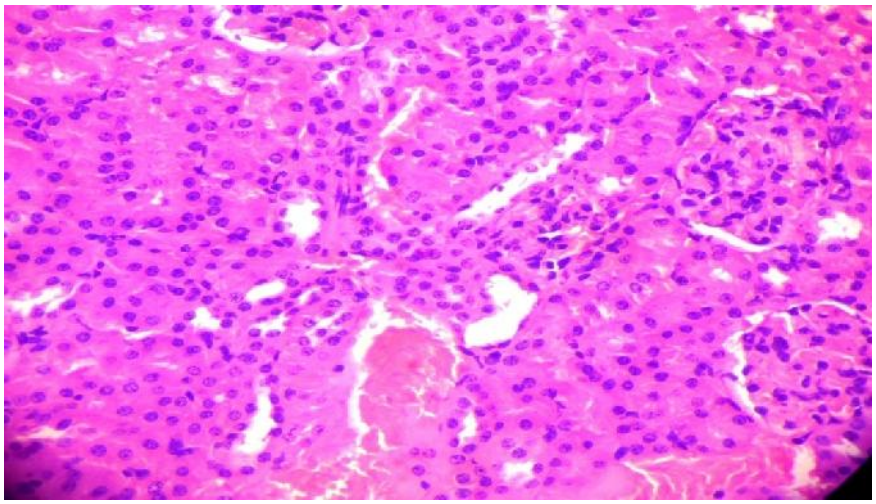
Toxic Control



Section of liver parenchyma with scattered focal area of necrosis of hepatocyte.

Figure No: 3

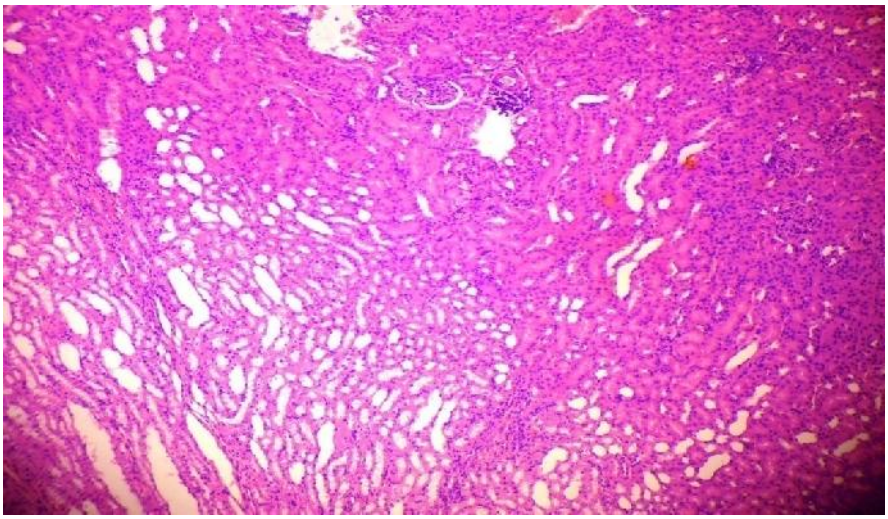
Positive Control



Section of liver parenchyma shows normal hepatic architecture

Figure No: 4

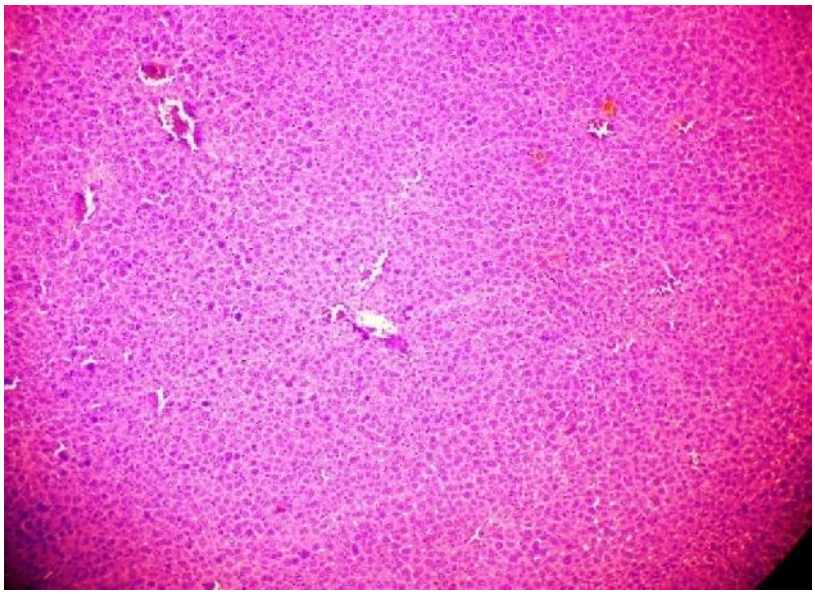
Treatment Control



Section of liver parenchyma shows normal hepatic architecture

Figure No: 5

Treatment Control



Section of liver parenchyma shows 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 Ethanobotanical grounds. A large number of plants possessing anticancer properties have been documented (Jasmine *et al* 2008; Abeeu & Abeeu 1979; Ramalingam Radha *et al* 2008; Dauod 2004; Rajkapoor 2004; Ashok Kumar Durairaj *et al* 2009).

Plants of ***phyllanthus longiflorus*** are traditionally used in the treatment of tumors. The present investigation was carried out to evaluate the antitumor activity of Ethanolic extracts of ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/kg in DLA tumor bearing mice. The ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/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 ascitic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells (Prasad & Giri 1994). Treatment with ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/kg 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 (Clarkson & Burchenal 1965). It may be concluded that ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/kg by decreasing the nutritional fluid volume and arresting the tumor growth increases the life span of DLA bearing mice. Thus ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/kg have antitumor activity against DLA bearing mice.

Usually, in cancer chemotherapy the major problems that are being encountered are myelo suppression and anemia (Price & Greenfield 1958; Hogland 1982). The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or Hb and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions (Fenninger & Mider 1954). Treatment with ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/kg brought back the (Hb) content; RBC and WBC count more or less to normal levels significantly. This clearly indicates that ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/kg possess protective action on the haemopoietic 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 serum of tumor inoculated animal indicated liver damage and loss of functional integrity of cell membrane. The significant reversal of these changes towards the normal by ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/kg 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 ***phyllanthus longiflorus*** at the dose of 200 mg/kg and 400mg/kg possesses significant antitumor and hepatoprotective effect of the extracts.

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