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Phytochemical Investigation and Anti-Cancer Activity of *Vitex trifolia*

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Abstract

Aim: Traditional medicine has a long history of serving people all over the world. In recent years, the use of traditional medicine information in cancer research received considerable interest. Ethanolic Extract of *Vitex trifolia* has been used in traditional and folklore medicine for the treatment of cancer. The aim of the present study was to evaluate the effect of Ethanolic Extract of *Vitex trifolia* against trypan blue dye exclusion assay against Daltons ascites lymphoma cell lines.

Materials and methods: DAL cells were injected intraperitoneally (1×10^{6} cells/ml/mouse) to the mice. The Ethanolic Extract of *Vitex trifolia* at a dose of 400mg/kg body weight were administered orally for 14 consecutive days to the tumor bearing group of animals. Derived parameters, hematological parameters, packed cell volume and WBC, RBC count were measured and compared to the control group. 5-Flurouracil (20 mg/kg) was used as a standard drug.

Results: The dose of Ethanolic Extract of *Vitex trifolia* decreased average increase in body weight, reduced the packed cell volume (PCV) viable tumor cell count and increased the life span of DAL treated mice and brought back the hematological parameters near to normal values. All the values were found to be statistically significant with control group at $p<0.01$. These observations are suggestive of the protective effect of extracts in Dalton's Ascitic Lymphoma (DAL)

Conclusion: All these findings enable to conclude that the dose of Ethanolic Extract of *Vitex trifolia* possess a protective effect against DAL.

Keywords: Dalton's Ascitic Lymphoma, ethanol extract of *Vitex trifolia*, Trypan blue exclusion assay„ 5-Fluorouracil, Tumor volume, Lifespan.

INTRODUCTION

Cancer is the major cause of mortality in the world and it claims more than 6 million lives each year. Methods commonly used for the treatment of cancer, although possess some benefits, but still there is a significant need to improve current cancer therapies and search for novel compounds[1].Breast cancer is one of the most common causes of the cancer in females in the whole world[2]. It has been observed that breast cancer accounts for 23% of all newly occurring cancers in women worldwide and represents 13.7% of all cancer deaths due to the breast cancer in male and female. It is the most frequent cancer in both developed and developing regions, but the rate of human breast cancer is higher in developing countries in compared to developed nations.[3]

Vitex trifolia Linn. belonging to the family of Verbenaceae is commonly known as chaste tree(English), Nirnochi (Tamil) and jalanirgundi (Sanskrit). It is distributed throughout India in tropical and subtropical regions. Height of the plant 1-3.5 meter. Flowers are appearing insummer or late summer, and 6-12 inch long. Leaves are commonly used as poultice for rheumatic pains, inflammations, sprains, fever, anthelmintic, improve memory, favour the growth of hair, good for the eyes, leucoderma, bad taste in mouth and bronchitis[**4,5**]. Roots are used to treat febrifuge, painful inflammations, cough and fever. Flowers are used in treating fever and fruits in amenorrhoea[**6**]. This plant is known to possess various active constituents viz, essential oils[7], halimane type diterpenes, vitetrifolins[8] and several pharmacological properties have been studied viz, antipyretic[9], antibacterial[10], against asthma and allergic diseases[11]. In view of its diverse medicinal applications and in order to ensure complete quality of its supply in terms of adulteration and substitution prevailing on the crude drug markets of India, the present communication deals with a complete pharmacognostical evaluation of the leaves of *vitex trifolia*. The study include macroscopic and microscopic evaluation, fluorescence analysis of powder, physico-chemical values, preliminary phytochemical screening of different extractives **[12]**.

MATERIALS AND METHODS

Collection and identification of plants

Vitex trifolia plant materials were collected from Aritapatti village near Madurai district, in December and was identified by Dr.Stephen, Professor, American college, Madurai, Tamilnadu; a voucher specimen has been deposited at the herbarium unit of the Department of Pharmacognosy, Ultra College of pharmacy, Madurai, Tamilnadu, India.

Extraction and phytochemical screening of plant

The powdered plant materials (500g) was extracted with petroleum ether at 40-60○ C, by continuous hot percolation using soxhlet apparatus. The extraction was carried out by using solvent of increasing polarity starting from petroleum ether, ethanol and aqueous respectively. The extraction was carried out for 72 hours. The petroleum ether extract was filtered and concentrated to dry mass by using vacuum distillation. A dark greenish brown residue was obtained.

The marc left, after petroleum ether extraction was taken and then subsequently extracted with ethanol and aqueous for 72hours. Phyto chemical screening was performed using standard procedures [13-15].

Preliminary phytochemical investigation

The qualitative chemical test of various extracts of *Vitex trifolia* was carried out using standard procedure. Sterols, Terpenoids, saponins, Carbohydrates, Tannins, Alkaloids, glycosides, Flavanoids were present in all the extracts.(Table 1)

PHYTOCHEMICAL SCREENING OF *VITEX TRIFOLIA*

Table – 1

EXTRACT	PETROLEU	ETHANO	AQUEOU
Sterols	+	+	+
Terpenoids	-	+	+
Carbohydrate	-	-	-
Flavonoids	+	+	+
Proteins and	-	-	-
Alkaloids	+	+	+
Glycosides	+	+	-
Tannins	-	-	+
Saponin	+	+	-
Phenolic	-	-	-
Fixed oils	+	+	-
Gums and	-	-	-

***In-vitro* cytotoxicity assay**

Trypan Blue [16,17]

Trypan blue is a vital stain used to selectively colour dead tissues or cells blue. It is a diazo dye. Live cells or tissues with intact cell membranes are not coloured. Since cells are very selective in the compounds that pass through the membrane, in a viable cell Trypan blue is not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue colour under a microscope. Since live cells are excluded from staining, this staining method is also described as a Dye Exclusion Method.

Materials required

DLA (Daltons lymphoma ascites) bearing mice. The standard drug 5-Flurouracil Injection .Phosphate buffered saline (PBS) contains Nacl- 4gm, Na_2HPO_4- 0.72gm, KH_2PO_4- 0.1gm, KCl- 0.1gm and Distilled water- 500ml. The dye used is Trypan blue and the cell is counted by using Haemocytometer.

Animals

Adult male swiss albino mice weighing between 25-30 g were selected and acclimatized to the laboratory conditions for one week. They were fed with standard pellet diet obtained from Hindustan lever ltd. Bangalore, India, and water and libitum these mice were divided into four groups viz., Group IV each with twelve mice and cancer was induced in all the groups except Group I.

Induction of cancer

The DAL cells were procured from Amala cancer Institute, Amala Nagar, Thrissur. The cells were introduced into the peritoneal cavity of the mice for tumour development. The cells were than aspirated aseptically from the tumour developed mice during log phase (on the 15th day after tumour transplantation) using 18 gauge needle. The ascitic fluid was washed three times in PBS (Phosphate buffer Saline) or normal saline and the cell pellet was resuspended in PBS. The tumour cell count was done using tryphan blue dye exclusion method in a haemocytometer. The cell suspension was diluted to get 10^6 cells/ml. Cancer was induced in the test mice by I.P inoculation of 10^6 cells per mouse.

TREATMENT PROTOCOL

Group I :

In this group, the mice were not induced with cancer and not treated with solvent (sterile water). This group serve as normal control.

Group II :

This group was reserved as cancer control and treated with solvent (sterile water)

Group III :

This group received Ethanolic extract of *Vitex Trifolia* 400mg/kg and serve as test group.

Group IV :

This group received 5-fluro uracil equivalent to 20mg/kg and serve as standard group. After establishing the tumour in the peritoneal cavity treatment was given for 15 days.(2nd day to 15th day).

On the 16th day 6 animals from each group were sacrificed and the following parameters were studied.

1. Cancer cell count.
2. Packed cell volume of peritoneal ascites fluid.
3. Haematological parameters
   i) RBC count
   ii) WBC count
4. Body weight

The remaining 6 mice from all the groups were kept with normal diet to findout survival time.

1. Cancer cell count

The fluid (0.1ml) from the peritoneal cavity of each mouse was withdrawn by sterile syring and diluted with 0.8 ml of ice cold sterile normal saline or sterile PBS and 0.1 ml of tryphtan blue (0.1 mg/ml) and preserved in an ice bath of 10 min. The total number of the living cells were counted using haemocytometer.

2. Haematological Parameters[18,19]

i) RBC count

ii) WBC count

Blood were collected from all the animals to study the haematological changes associated with the anticancer study. Then the WBC and RBC count were done using haemocytometer.

3. Packed cell volume

After sacrificing the animal, peritoneal cavity was dissected tumour was transferred in the clean beaker, from that 1ml of fluid is taken in the PCV tube centrifuged at 3000 rpm for 30 minutes. From the packed cell volume the % tumour inhibition was calculated using the formula.

% Tumour inhibition = Test PCV
-------------x 100 = X
Control PCV
= 100-X

4. Body weight

All the mice were weighed, from the beginning to 16th day of the study. Body weight was noted every alternative days. Average gain in body weight on 16th day was determined and % decrease in body weight was calculated by the formula.

% decrease in Body Weight

Gain in body weight - Gain in body weight of
of caner control treated group
= --- x 100
Gain in body weight of cancer control

5) Survival Time[20,21]

After months of study the number of surviving animals were counted and the average survivel time was calculated and % increase in lifespan (%ILS) was calculated by the formula

MST of treated group-MST of control group
%ILS = -- X 100
MST of control group

Where MST =Mean Survival Time

STATISTICS

The results were expressed as mean and SEM .statistical analysis was carried out using student't' test.

ANTICANCER STUDIES

Table 2 Effect Of Ethanolic Extract Of *Vitex Trifolia* On Total WBC Count On Dal Tumour Model

Groups	Total WBC Cells / µL x	Groups Compared	T-Value	Level of Significant
G_1	4.82 ± 0.86	-	-	
G_2	9.44 ± 1.24	G_1 Vs G_2	3.14	P < 0.02 * *
G_3	4.52 ± 0.74	G_1 Vs G_3	1.05	P > 0.05
G_4	3.61 ± 0.76	G_1 Vs G_4	1.1	P > 0.05

Values are mean ± SEM of six animals
* → Significant
** → More Significant

Figure I Effect Of Ethanolic Extract Of *Vitex Trifolia* On Total WBC Count On Dal Tumour Model

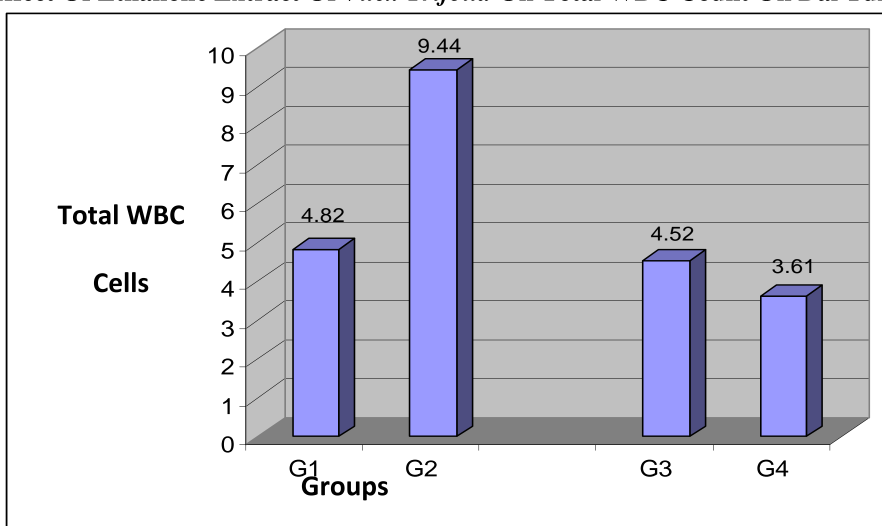


Table 3 Effect Of Ethanolic Extract Of *Vitex Trifolia* On Total RBC Count On Dal Tumour Model

Groups	Total RBC Cells / µL x	Groups Compared	T Value	Level of Significant
G_1	5.92 ± 0.86	-	-	
G_2	2.65 ± 0.44	G_1 Vs G_2	3.64	P < 0.02 * *
G_3	5.84 ± 0.64	G_1 Vs G_3	0.1	P > 0.05
G_4	4.36 ± 0.64	G_1 Vs G_4	2.16	P > 0.05

Values are mean ± SEM of six animals
* → Significant
** → More Significant

Figure II Effect Of Ethanolic Extract Of *Vitex Trifolia* On Total RBC Count On Dal Tumour Model

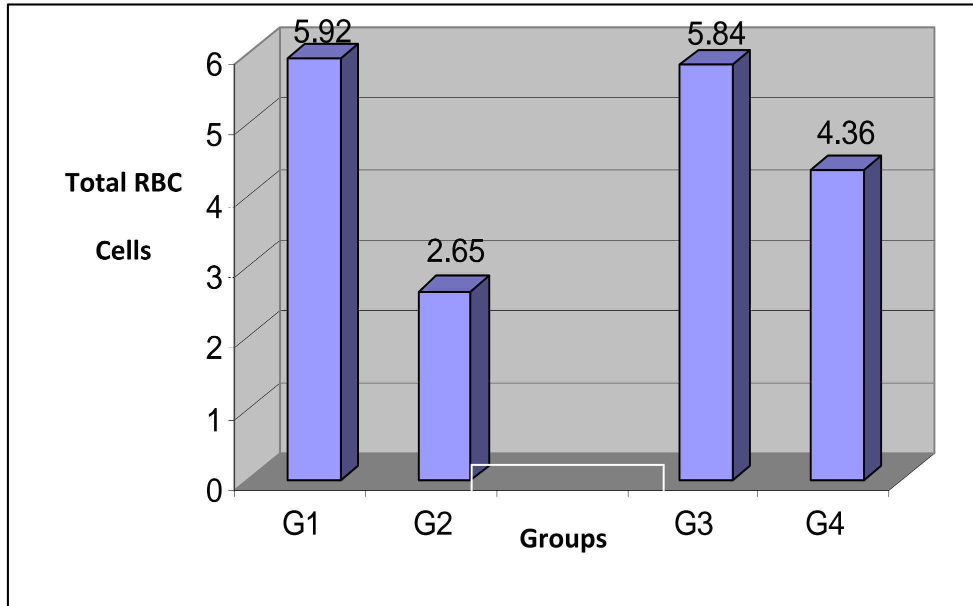


Table 4 Effect Of Ethanolic Extract Of *Vitex Trifolia* On The Survival On Dal Tumour Model

Groups	Mean Survival	Groups	T Value	Level of	Percentage Increase
G_1	>42	-	-	-	-
G_2	21.42 ± 1.62	-	-	-	-
G_3	36.84 ± 2.16	G_3 Vs G_2	5.72	P < 0.01* *	71.96
G_4	42.12 ± 2.84	G_4 Vs G_2	5.84	P < 0.01* *	98.72

Values are mean ± SEM of six animals
* → Significant
**→ More Significant

Figure III Effect Of Ethanolic Extract Of *Vitex Trifolia* On The Survival On Dal Tumour Model

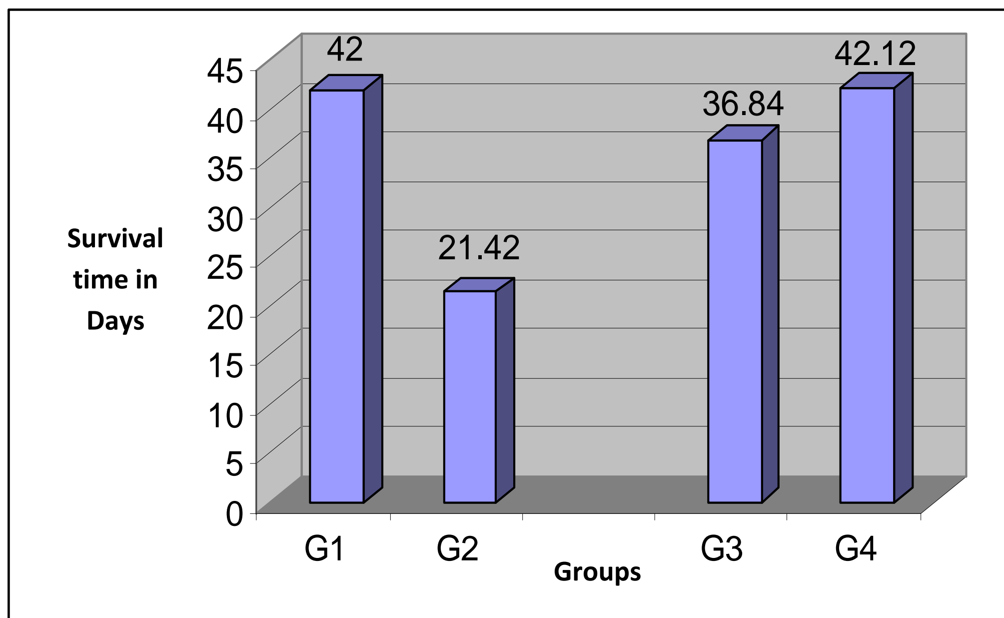


Table 5 Effect Of Ethanolic Extract Of *Vitex Trifolia* On Body Weight On Dal Tumour Model

Groups	Increase in Body	Groups	T Value	Level of Significant	Percentage
G_1	0.41 ± 0.02	-	-	-	-
G_2	11.12 ±0.44	-	-	-	-
G_3	3.23 ± 0.13	G_2 Vs G_3	18.78	P < 0.001* * *	70.95
G_4	1.89 ± 0.13	G_2 Vs G_4	54.64	P < 0.001* * *	83

Values are mean ± SEM of six animals
* → Significant
** → More Significant

Figure IV Effect Of Ethanolic Extract Of *Vitex Trifolia* On Body Weight On Dal Tumour Model

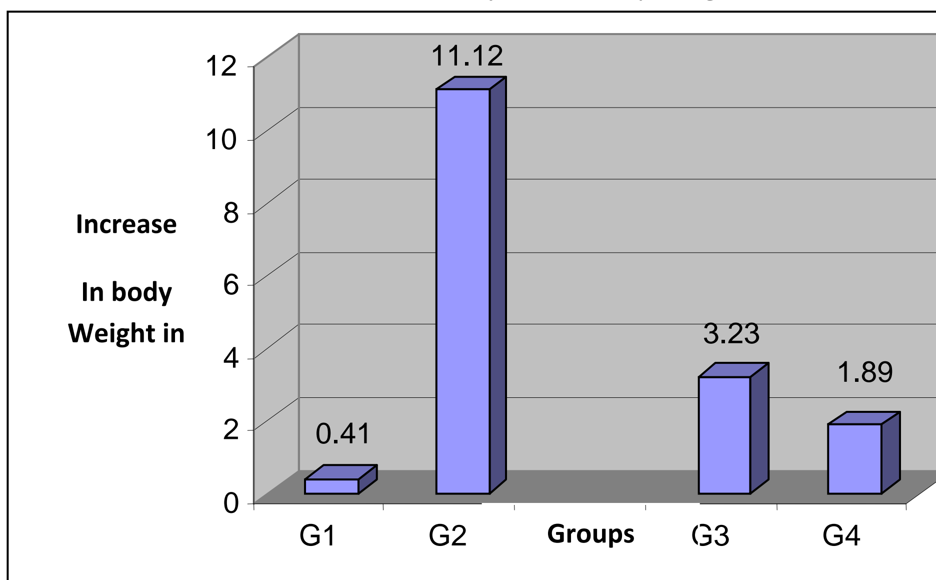


Table 6 Effect Of Ethanolic Extract Of *Vitex Trifolia* On Cancer Cell Count On Dal Tumour Model

Groups	Cancer Cell	Groups	T Value	Level of Significant	Percentage
G_1	-	-	-	-	-
G_2	1.52 ± 0.12	-	-	-	-
G_3	0.96 ± 0.08	G_2 Vs G_3	4.00	P < 0.01* * *	36.85
G_4	0.72 ± 0.06	G_2 Vs G_4	8.00	P < 0.001* * *	52.64

Values are mean ± SEM of six animals
* → Significant
** → More Significant

Figure V Effect Of Ethanolic Extract Of *Vitex Trifolia* On Cancer Cell Count On Dal Tumour Model

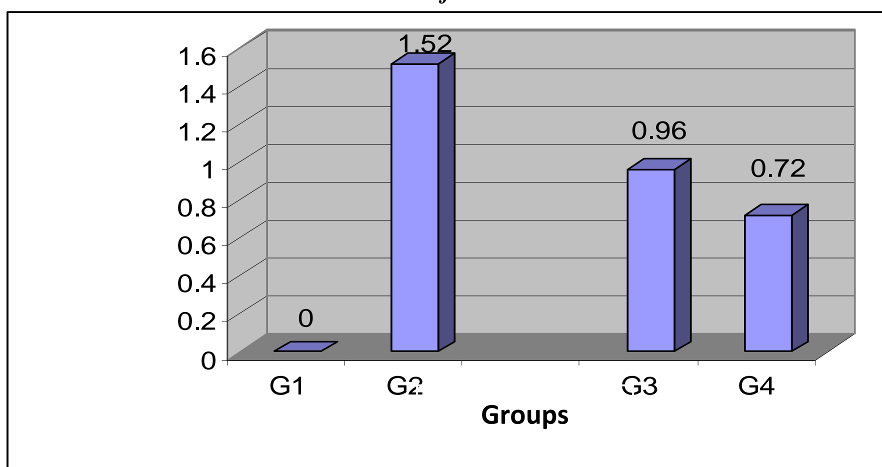
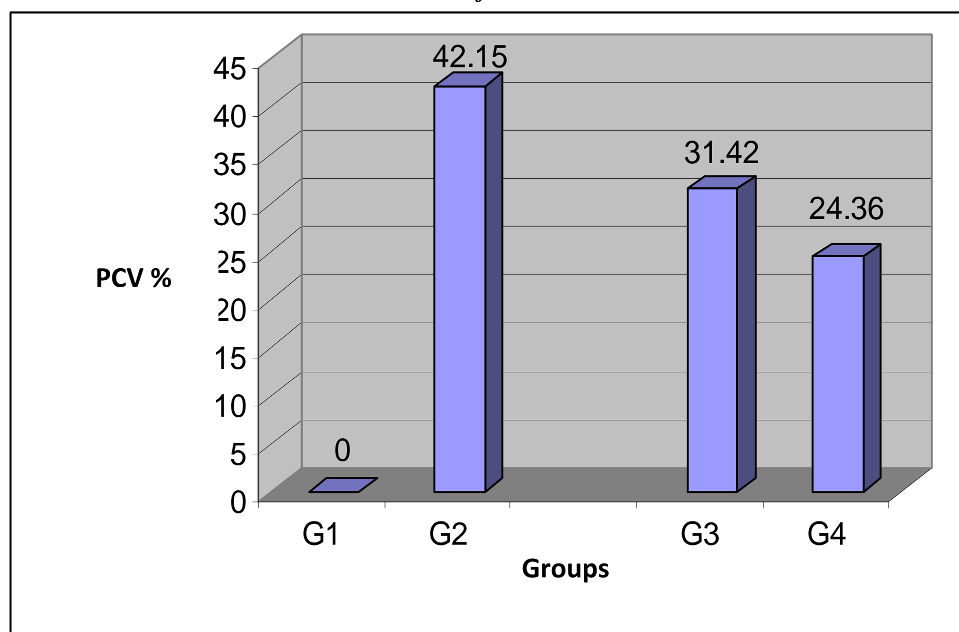


Table 7 Effect Of Ethanolic Extract Of *Vitex Trifolia* On Packed Cell Volume On Dal Tumour Model

Groups	Packed Cell	Groups	T Value	Level of Significant	Percentage
G_1	-	-	-	-	-
G_2	42.15 ± 2.44	-	-	-	-
G_3	31.42 ± 1.86	G_2 Vs G_3	3.52	P < 0.02* * *	25.46
G_4	24.36 ± 1.28	G_2 Vs G_4	7.9	P < 0.001* * *	42.24

Values are mean ± SEM of six animals
* → Significant
** → More Significant

Figure VI Effect Of Ethanolic Extract Of *Vitex Trifolia* On Packed Cell Volume On Dal Tumour Model



RESULTS

Effect on Tumor Growth

In the DLA tumor control group, the average life span of animal was found to be 42% where as ethanolic extract of *Vitex Trifolia* dose of 400 mg/kg body weight increase the life span to 71.96% (Table 4) respectively. These values were significant. However the average life span of 5- FU treatment was found to be 98.72%, indicating its potent antitumor nature. The antitumor nature of ethanolic extract of *Vitex Trifolia* was evidenced by the significant reduction in percent increase in body weight of animal treated with ethanolic extract of *Vitex Trifolia* at the dose of 400 mg/kg body weight when compared to DLA tumor bearing mice (Table 5). It was also supported by the significant reduction in packed cell volume and viable Tumor cell count in both the dose of treatment when compared to the DLA tumor control (Table 7)

Effect on Hematological Parameters

As shown in (Table 2,3) 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 *Vitex Trifolia* at the dose of 400 mg/kg significantly increases the Hb content, RBC 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.

DISCUSSION AND CONCLUSION

The present investigation was carried out to evaluate the antitumor activity of Ethanolic extracts of *Vitex trifolia* in DLA tumor bearing mice. The EECC treated animals at the doses of 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. World Health Organization investigation shows that 80% of world populations relies on traditional medicines [22]. Of these, at least 30% utilized medicinal plants from clinical indication E[23]. Available literatures on medicinal plants indicate that promising phytochemicals can be developed for many health problems [24-26]. Plant extracts as a traditional remedy are already being used to treat a variety of diseases, including cancer.[27-31] The utilization of medicinal plants is more common in underdeveloped countries[32], and experimental studies showed that the extracts of the various plants can also protect against breast cancer cells. 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 [33]. Treatment with EECC 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 [34].

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[35]. Treatment with both doses of EECC brought back the (Hb) content; RBC and WBC count more or less to normal levels significantly. This clearly indicates that EECC possess protective action on the haemopoietic system.

In conclusion *Vitex trifolia* can be a better candidate for isolation of cytotoxic and anticancer compounds, specially ethanol extract of *Vitex trifolia.* On the basis of present investigation this plant species can be further investigated for pharmaceutical applications and achievement of novel anticancer compounds.

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