

**HEMATOLOGICAL CHANGES IN DALTON'S LYMPHOMA-BEARING MICE
AFTER TREATMENT WITH *DILLENIA PENTAGYNA* EXTRACT.**

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Summary

Dillenia pentagyna is a herbal medicinal plant the stem bark of which is used by the local people of Mizoram state, India, for the treatment of cancer suspected diseases and other stomach ailments. The objective of the present study was to determine the effect of *Dillenia pentagyna* extract treatment on some hematological parameters, and microscopical studies on the tumor-leukocyte interaction in Dalton's lymphoma-bearing mice in order to find their possible role in the antitumor activity of *Dillenia pentagyna*. *D. pentagyna* extract treatment at a dose of 20 mg/kg body wt/day caused a significant decline in the total ascites tumor volume and the body weight of tumor-bearing host after 12 and 8 day of treatment respectively, significant increase in red blood cell and white blood cell counts, packed cell volume and hemoglobin content in the host with the maximum increase after 96 h of treatment. The result of differential leukocyte counts shows maximum percentage increase with lymphocyte during 48 to 72 h of treatment. Leukocyte infiltration towards the tumor cells was also observed after plant extract treatment, and the plant extract treatment also caused the appearance of membrane vacuoles and plasma membrane disintegration leading to cell lysis. The observed increase in leukocyte infiltration towards tumor cells may be due to the increased production of leukocytes in the host, and this increased leukocyte infiltration may also play a role in reducing ascites tumor volume by causing tumor cell lysis, thereby increasing host survivability.

Keywords: Dalton's lymphoma; *Dillenia pentagyna*; Hematology; Luekocyte infiltration.

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Introduction

Our preliminary investigation through literature search and consultation of local herbal practitioners revealed that *D. pentagyna* Roxb. (Dilleniaceae) is one of the most commonly used anticancer medicinal plants in Mizoram state, India. The juice of stem bark of this plant prepared by boiling with water has been used by the local people (Mizo) of Mizoram as a traditional home medicine against cancer suspected diseases and other gastric problems. We have previously reported the antitumor activity of water and methanol extract of stem bark of this plant against murine ascites Dalton's lymphoma and the most potent antitumor activity was found with methanol extract at a dose of 20 mg/ kg body weight/day (1). We have also reported its inhibitory effect on the level of sialic acid and lipid peroxidation (2) as well as significant changes in the level of glutathione in the tissues of tumor-bearing mice (3). However, the effects of this plant extract on some hematological parameters in the tumor-bearing host have not been determined.

Alterations in the rate of erythrocyte production following tumor growth have been reported and anemia is a common hematologic finding in advanced neoplastic disease in human and experimental animals (4-7). Diminished erythropoiesis, shortened red cell life span, hemorrhage hemodilution have been considered either singly or in combination for the etiology of this phenomenon (8). In cancer therapy also low blood cell counts is one of the common side effects caused by radiation therapy and chemotherapy. One example is a dose dependent decrease on the level of RBC, WBC, Hb and PCV of tumor bearing mice after cisplatin treatment (9). Complications like myelosuppression and thrombocytopenia are usually associated during cancer chemotherapy (10).

Solid tumors, both primary lesions and metastases, are infiltrated by large numbers of tumor-associated leukocytes. These are a heterogeneous population of cells consisting of various subsets of T cells (helper, suppressor and cytotoxic), B cells, natural killer (NK) cells, and macrophages (11) and these subpopulations of tumor-associated leukocytes have been shown to be harmful to the host and beneficial to progressive tumor growth (12,13). In the present investigation, some hematological parameters were determined in Dalton's lymphoma-bearing mice in order to find their possible role in the antitumor activity of *Dillenia pentagyna* stem bark extract.

Materials and Methods

Plant material and preparation of test sample:

The stem bark of *D. pentagyna* was collected by G. Rosangkima from Kawlkulh village, Mizoram, in January 2005. The plant material was authenticated by Dr. P.B. Gurung, Herbarium specialist, Department of Botany, NEHU, Shillong (India), and a voucher specimen (no. SBP 001) was deposited in the department of Zoology, NEHU. The methanol extract of *Dillenia pentagyna* (DPE) was prepared and dissolved as described previously (1).

Animals and tumor model:

Inbred Swiss albino mice colony is being maintained under laboratory conditions keeping 5-6 animals in a propylene cage at 24-25°C. The animals were fed with commercially available food pellets and water *ad libitum*. Ascites Dalton's lymphoma tumor was maintained *in vivo* in 10-12 weeks old mice by serial (i.p.) transplantation of 1×10^7 viable tumor cells per animal (in 0.25 ml PBS, pH 7.4). Tumor-transplanted mice usually survive for 19 days.

Tumor growth pattern:

The antitumor activity of methanol extract of *D. Pentagyna* has been evaluated previously (3). Taking the day of tumor transplantation as day '0', the treatment was given with the methanol extract of *D. pentagyna* (20 mg/kg body wt) beginning 1st day after tumor transplantation, once daily for 5 days. The pattern of changes in body weight and tumor volume was monitored till the death of the animals.

Treatment:

In hematological and light microscopical studies, *Dillenia pentagyna* extract (20 mg/kg body weight) was administered to the tumor-bearing hosts on the 10th day post-tumor transplantation, i.e. the mid phase of tumor growth. The experimental mice were divided into 3 groups as follows;

Group 'I' - Normal mice, without tumor

Group 'II' - Tumor-bearing Control receiving extract vehicle only (0.25ml of 0.05% sodium hydroxide)

Group 'III' - Tumor-bearing mice treated with a single dose of *Dillenia pentagyna* extract (20 mg/kg body wt) on the 10th day of post-tumor transplantation.

Collection of blood samples:

The treatment schedule was carried out for 4 days i.e. 24, 48, 72 and 96 hours of time interval. Blood samples from different treatment groups of mice were collected from the tail vein into a sterilized tube containing heparin (15-20 IU per ml of blood) and used for hematological studies. All the hematological parameters were carried out following the method of Dacie and Lewis, 1975 (14).

Red blood cell counts:

Freshly collected blood was diluted (200 times) with RBC diluting fluid and mixed in a mechanical mixture. Counting was done in a Neubauer chamber using the 4 mm objective and 10x eyepiece, and the erythrocytes absolute values were calculated as follows:

Red-cell count per μl = $N \times 1 \times 200$ (dilution)/0.02

= $N \times 10000$

= $N \times 10^{10}/\text{L}$.

White blood cell counts:

Freshly collected blood was diluted (20 times) with white blood cells (WBC) diluting fluid (2% acetic acid) and the blood sample was mixed for about 1 minute and counted in a Neubauer chamber using the 16 mm objective and 10x eyepieces. The number of white blood cell was calculated as follows:

$$\begin{aligned}\text{White blood cell count per } \mu\text{l} &= N \times 10 \times 20 \text{ (dilution)} \\ &= N \times 200 \times 10^6/\text{L}.\end{aligned}$$

Packed cell volume (PCV) determination:

Blood was collected in a wintrobe tube and centrifuged for 30 min (2000xg, at 4°C). The height of the column of red cells was taken as the PCV (the volume occupied by the red cells expressed as a fraction of the total volume of the blood).

Hemoglobin estimation:

Blood was diluted (200 times) with cyanide-ferricyanide reagent (200 mg potassium ferricyanide and 50 mg potassium cyanide in 1 litre of distilled water) and allowed to stand for 10 min at room temperature. The absorbance was read at 540 nm in a Beckman DU-640 spectrophotometer. The hemoglobin content was calculated as follows:

$$\begin{aligned}\text{Concentration (g/dl)} &= \frac{A^{540} \times 64500 \times \text{dilution factor}}{44.0 \times d \times 1000 \times 10}\end{aligned}$$

where, A^{540} = Absorbance at 540 nm, 64500 = molecular weight of hemoglobin, dilution factor = 200, 44.0 = milimolar extinction coefficient, d = layer thickness in cm, 1000 = conversion factor for mg to g and 10 = conversion factor for g/litre to g/dl.

Differential leukocyte counts:

A drop of fresh blood was taken on a clean slide and a thin and uniform blood film was prepared with the help of another clean slide. The blood film was air dried for overnight, stained with Leishman's stain the following day and mounted in DPX. Counting was done under microscope in a narrow longitudinal strip of the blood film starting from one end of the film to the other end. The number of different types of white blood cells (neutrophils, basophils, monocytes, lymphocytes and eosinophils) were noted and expressed in percentage.

Light microscopical studies:

Animals in different treatment groups were sacrificed by cervical dislocation. The ascites tumor was collected and centrifuged at 1000 rpm for 5 min. at 4°C, washed in PBS (0.15M NaCl, 0.01 M sodium phosphate buffer, pH 7.4) . The cell pellet was resuspended in PBS (1:4) and a drop of the cell suspension was taken on a clean slide and a thin smear was prepared. The smear was air-dried, fixed in absolute methanol for 15 min. and stained the following day with Leishman's stain. The cells in different slides were studied and photographed.

Results

Tumor growth pattern:

Following tumor transplantation, the increase in the body weight and belly size with was noted from the 4th to 5th day onwards depicting an early sign of tumor development. Tumor-transplanted mice survived for 19 days. After treatment with *D. pentagyna* extract, the host's survivability was increased. With the progress of tumor, there was a regular increase in the body weight and ascites tumor volume of tumor-bearing control (group-II), which reached to about 35 gm and 9 ml by the 20th day of tumor growth respectively. After 8th day of treatment with *D. pentagyna* extract, a significant decrease in the body weight and ascites tumor volume (after 12th day) of tumor-bearing mice (group-III) was observed (Table 1; Figure 1 and 2).

Table 1: Changes in the body weight and tumor volume of tumor-bearing mice under different treatment conditions.

Days after transplantation	Changes in body weight (gm±S.D)		Changes in tumor volume (ml±S.D)	
	TB-control	DPE treated	TB-control	DPE treated
0	22.3±1.15	21.8±1.47	-	-
4	22.8±1.39	22.1±1.37	1.18±0.17	1.13±0.13
8	23.6±0.96	22.6±1.07*	3.43±0.19	3.33±0.16
12	25.3±1.25	23.5±1.17*	4.73±0.20	4.33±0.17*
16	30.8±1.13	27.4±2.11*	7.23±0.24	6.83±0.32*
20	34.5±1.08	30.0±1.56*	8.93±0.30	7.45±0.18*
24		32.1±3.51		7.70±0.32
28		33.0±2.21		8.28±0.24
32		33.9±2.37		8.40±0.14

Results were mean ± S.D. DPE = *D. pentagyna* extract (20 mg/kg body wt/day) was given for 5 consecutive days starting from day one of tumor growth. TB-control = tumor-bearing control were given extract vehicle alone. Student's t-test, n = 5, as compared to the corresponding control, *P ≤ 0.05.

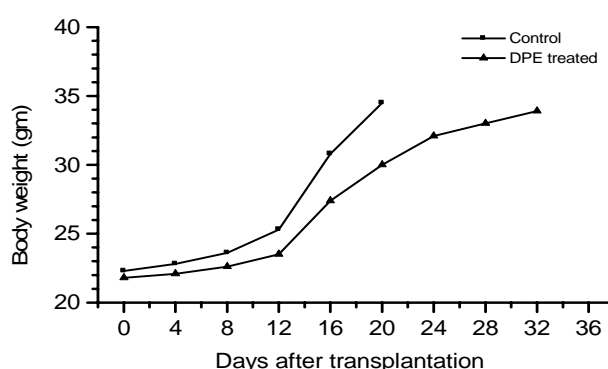


Figure 1. Figure showing the changes in the body weight of tumor-bearing control and after DPE treatment during tumor growth.

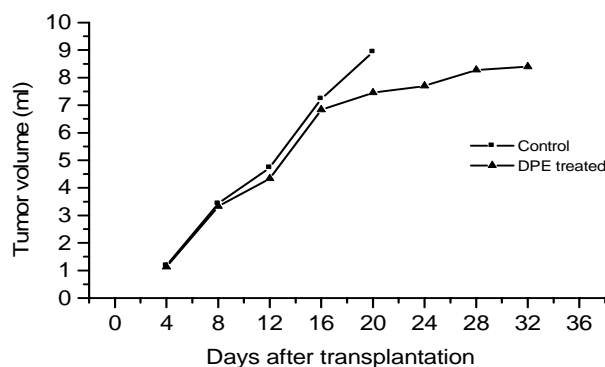


Figure 2. Figure showing the changes in the total ascites tumor volume in tumor-bearing control and after DPE treatment during tumor growth.

Red blood cell counts:

The red blood cells or erythrocytes (RBCs) count in normal mice was 7.57 ± 0.46 ($\times 10^{12}/l$). In tumor-bearing control, the number of erythrocytes decreases significantly showing 2.26 ± 0.22 ($\times 10^{12}/l$) on the 14th day of tumor growth. Treatment of tumor-bearing mice with the plant extract significantly increased the RBC counts during 48 to 96 h of treatment (Table 2) and maximum percentage increase in RBC counts was noted during 96 h of treatment (Figure 3).

Table 2: Changes in the hematological values of normal and tumor-bearing mice under different treatment conditions.

Treatment	RBC ($\times 10^{12}/l$)	WBC ($\times 10^9/l$)	PCV (%)	Hb (g/dl)
Normal	7.57 ± 0.46	04.95 ± 0.35	42.58 ± 3.32	14.36 ± 0.73
Control - 24 h	6.71 ± 0.67	10.41 ± 0.81	37.05 ± 3.91	10.13 ± 0.76
48 h	5.55 ± 0.58	11.82 ± 0.80	34.12 ± 3.65	09.49 ± 0.82
72 h	3.76 ± 0.37	12.42 ± 0.95	30.88 ± 3.37	09.07 ± 0.66
96 h	2.26 ± 0.22	13.36 ± 0.91	26.92 ± 2.53	08.10 ± 0.71
DPE Treated - 24 h	6.56 ± 0.54	$11.70 \pm 0.90^*$	38.45 ± 3.25	10.66 ± 0.60
48 h	$6.53 \pm 0.66^*$	$13.86 \pm 0.77^*$	36.61 ± 2.64	$12.58 \pm 0.90^*$
72 h	$6.52 \pm 0.61^*$	$15.16 \pm 1.23^*$	$35.89 \pm 2.69^*$	$12.50 \pm 1.29^*$
96 h	$6.45 \pm 0.70^*$	$16.70 \pm 1.12^*$	$35.49 \pm 2.29^*$	$12.13 \pm 1.24^*$

Results were mean \pm S.D. DPE = *D. pentagyna* extract, (20 mg/kg body wt.) was given on the 10th day of tumor growth. RBC = Red blood cells, WBC = White blood cells, PCV = Packed cell volume, Hb = Hemoglobin. Student's t-test, $n = 5$, as compared to the corresponding control, $*P \leq 0.05$.

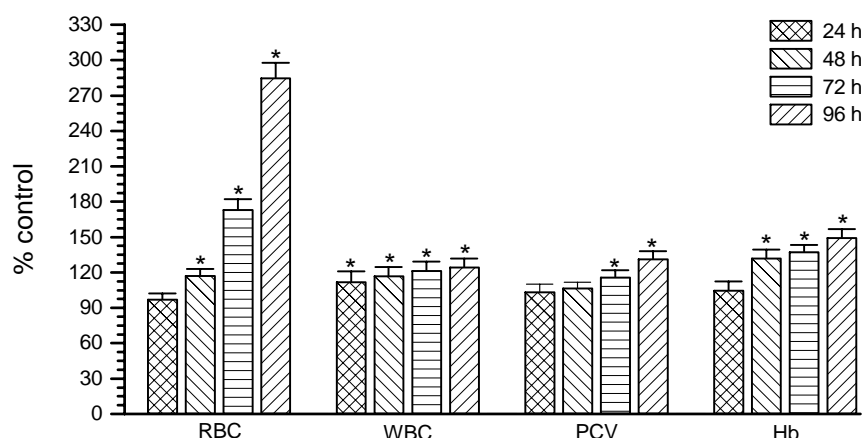


Figure 3. Histogram showing the percent changes in RBC count, WBC count, PCV and Hb concentration of tumor-bearing mice after DPE treatment (20 mg/kg b. wt.). Results are expressed as mean \pm S.D. Student's t-test, $n = 6$, as compared to the corresponding control, $*p \leq 0.05$.

White blood cell counts:

The number of white blood cells or leukocytes (WBCs) in normal mice was 01.03 ± 0.12 ($\times 10^9/l$). In tumor-bearing control, there was a significant increase in the number of leukocytes showing more than two folds increase on the 14th day of tumor growth. Treatment of tumor-bearing mice with the plant extract significantly increased the number of leukocytes during 48 to 96 h of treatment (Table 2; Figure 3).

Packed cell volume (PCV):

Packed cell volume in normal mice was observed to be 42.58 ± 3.32 (%), whereas, in tumor-bearing control, a significant decrease in the packed cell volume (PCV) was noted. As compared to the control, treatments with the plant extract significantly increased packed cell volume after 72 h of treatment (Table 2; Figure 3).

Hemoglobin concentration:

Hb content of normal mice was 14.36 ± 0.73 g/dl. As compared to normal mice, in tumor-bearing control, a significant decrease in Hb content was observed (8.1 ± 0.71 g/dl) on the 14th day of tumor growth. However, after the treatment (48 to 96 h) of tumor-bearing hosts with the plant extract, the Hb content increased significantly (Table 2; Figure 3).

Differential leukocyte counts:

As compared to normal mice an increase in the number of neutrophils, eosinophils, basophils and a decrease in lymphocytes and monocytes were observed in tumor-bearing control (Table 3). As compared to control, the extract treatment caused a significant increase in the number of lymphocytes and eosinophils during 48 to 96 h and 24 to 96 h of treatment respectively. In contrast, a significant decrease in the number of neutrophils during 48 to 96 h and basophils during 24 to 96 h of treatment with the extract was observed.

There was no significant change in the number of monocytes after treatment with the extract (Table 3; Figure 4).

Table 3: Differential leukocyte counts in the blood of normal and tumor-bearing mice under different treatment conditions.

Treatment	Lymphocytes	Monocytes	Neutrophils	Eosinophils	Basophils
Normal	63.95 ± 3.35	3.27 ± 0.35	30.36 ± 2.48	1.66 ± 0.16	0.80 ± 0.01
Control - 24 h	30.07 ± 2.12	2.31 ± 0.16	62.85 ± 3.61	3.65 ± 0.29	1.19 ± 0.07
48 h	26.84 ± 2.54	2.21 ± 0.20	65.84 ± 2.55	3.74 ± 0.21	1.18 ± 0.06
72 h	24.46 ± 2.26	2.07 ± 0.17	68.74 ± 2.35	3.90 ± 0.23	1.16 ± 0.08
96 h	20.83 ± 1.96	2.02 ± 0.18	72.49 ± 3.13	3.48 ± 0.19	1.15 ± 0.10
DPE treated – 24 h	31.23 ± 2.50	2.30 ± 0.18	62.53 ± 3.09	4.71 ± 0.35*	0.46 ± 0.04*
48 h	46.62 ± 2.76*	2.09 ± 0.07	46.47 ± 3.72*	4.30 ± 0.20*	0.32 ± 0.02*
72 h	44.31 ± 2.74*	2.06 ± 0.16	48.33 ± 2.59*	4.53 ± 0.32*	0.68 ± 0.04*
96 h	33.15 ± 2.32*	2.16 ± 0.19	59.89 ± 2.90*	4.20 ± 0.26*	0.72 ± 0.07*

Results were mean ± S.D. Normal = Blood from the mice without tumor or DPE treatment. Control = Blood from the tumor-bearing mice without DPE treatment. DPE = *D. pentagyna* extract, (20 mg/kg body wt.) was given on the 10th day of tumor growth. Student's t-test, n = 5, as compared to the corresponding control, *P ≤ 0.05.

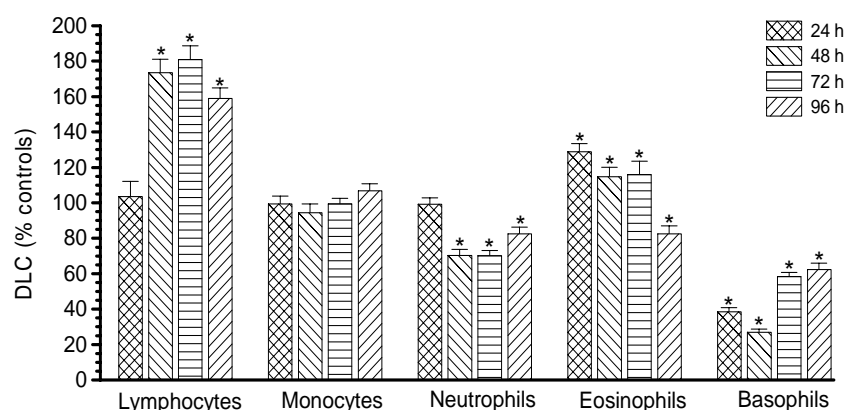


Figure 4. Histogram showing the percent changes in DLC of tumor-bearing mice after DPE treatment (20 mg/kg b. wt.). Results are expressed as mean ± S.D. Student's t-test, n = 6, as compared to the corresponding control, *p ≤ 0.05.

Light microscopy:

The light microscopical observation showed rounded shape of control tumor cells. They were surrounded by a very few leukocytes (Figure 5A). After DPE treatment, more leukocyte infiltration towards the tumor cell was noticed (Figure 5B and 5C). DPE treatment for 96 h resulted in the appearance of membrane vacuoles and gradual disintegration of plasma membrane leading to lysis of the tumor cells (Figure 5D, 5E).

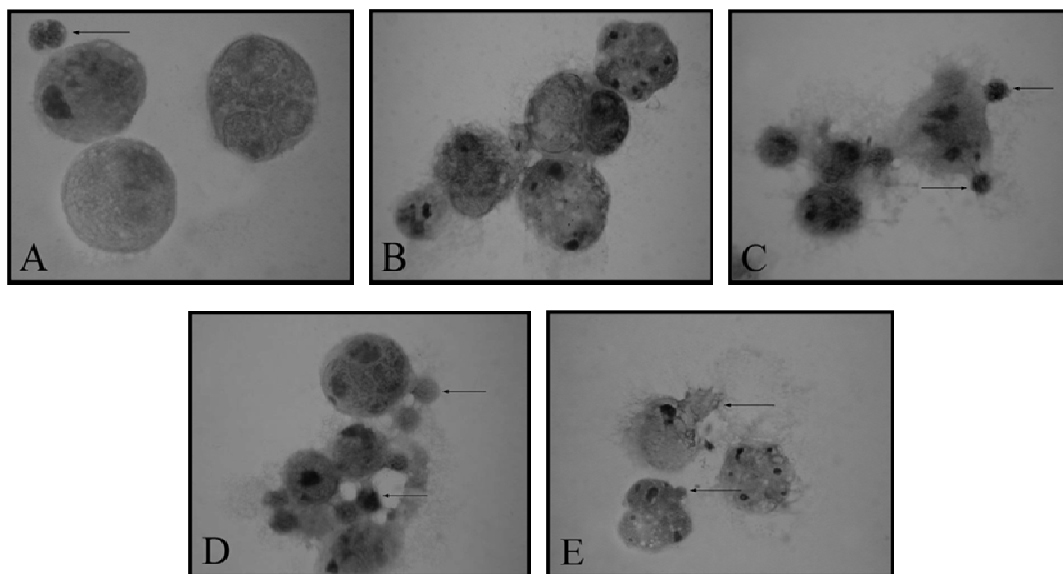


Figure 5. Light micrographs of tumor cells under different treatment conditions with DPE. Control tumor cells (A) are rounded in shape with very few surrounding leukocytes. 24 h of DPE treatment (B) showing infiltration of leukocytes towards the tumor cells. DPE treatment for 72 and 96 h (C,D) showing more infiltration of leukocytes towards the tumor cells and gradual disintegration of plasma membrane with membrane vacuoles (E).

Discussion

Dalton's lymphoma originated in the thymus gland of a DBA/2 mouse at the National Cancer Institute, Bethesda, M D, in 1947. Subsequently, an ascites form was developed by repeated i.p. transplantation of tumor (15). During tumor growth progression, an increasing volume of ascites tumor caused a steady increase in the body weight of the hosts with the maximum of 35 gm on the 20th day of tumor growth. In the present study, a significant decrease in the body weight of tumor-bearing mice and ascites tumor volume was noted after DPE treatment (Table 1). This result supported our previous report on the antitumor activity of DPE against murine ascites Dalton's lymphoma (1).

During tumor growth progression, red blood cell count, packed cell volume and hemoglobin concentration significantly decreased while white blood cells increased. After 14 days of tumor growth, red blood cell count reaches less than half of the normal value. Hemoglobin concentration also decreases to about half of the normal value, and white blood cells reaches more than double the normal value (Table 1). Anemia is a decrease in the number of red blood cells (RBCs). The extremely short survival and reduced number of erythrocytes in the host may be due to one or more secreted tumor product(s), and some tumor product which is directly responsible for the causation of anemia in the host have also been identified (16). An important part of the red blood cell is hemoglobin, the part that carries oxygen throughout your body. Therefore, when hemoglobin is low, oxygen levels are decreased and the body has to work harder in order to compensate.

In the present study, the observed increase in the level of red blood cells, packed cell volume and hemoglobin after plant extract treatment suggested that the plant extract may directly or indirectly involved in detoxification of some toxic product(s) of tumor cells, or it may also play a role in enhancing red cell production from the bone marrow. Since chemotherapy also often affected red blood cells leading to anemia in the host, *Dillenia pentagyna* extract may be helpful in developing suitable conditions in the host for anticancer drugs or in reducing the side effect(s) of some drugs in chemotherapy.

The present study also shows a significant increase in the total white blood cell count after the plant extract treatment. Although differential leukocyte counts after plant extract treatment showed decreased percentage of monocytes, neutrophils and basophils (Table 3; Figure 4), the overall increase in leukocyte counts revealed a significant increase in the level of all types of white blood cells. T lymphocytes play a key role in maintaining antitumor immunity. They provide an important opportunity for the immunotherapy of cancer (17,18). In adoptive immunotherapy, T lymphocytes with antitumor activity are transferred into a tumor-bearing host. The success of the therapy depends on the type of T cells transferred (19). T lymphocytes with antitumor activity can be categorized into tumor infiltrating lymphocytes and antigen-specific or tumor-specific cytotoxic T lymphocytes. Macrophages play several biological roles including antigen presentation, target cell cytotoxicity, removal of debris and tissue remodeling, regulation of inflammation, induction of immunity, thrombosis and various forms of endocytosis (20). In the setting of tumors, tumor-associated macrophages (TAMs) have a range of functions with the capacity to affect diverse aspects of neoplastic tissues including angiogenesis and modulation of tumor cell growth (enhancement and inhibition). Activated tumor-associated macrophages have been reported to induce neoplastic cell death (cytotoxicity, apoptosis) also (21). Thus, the observed increase in white blood cell counts may play a role in the antitumor activity of *Dillenia pentagyna* extract by enhancing antitumor immunity in the host.

Cell membrane/surface changes may influence the structural and functional properties of malignant cells (22,23). In solid tumors and few ascitic tumors, cell-cell contacts have also been observed (24-27). Cell associations in malignant cells regulate the pattern of growth and malignancy in tumors (28). In control ascites tumor very few leukocytes were seen among tumor cells which were round in shape (Figure 5A). The percentage ratio of leukocytes to tumor cells increased after treatment with DPE (Figure 5B and C). This increase in the number of leukocytes in tumor cell population after DPE treatment suggests the infiltration of many leukocytes towards tumor cells. The infiltration of lymphocytes and macrophages after potent antitumor drug, cisplatin treatment also has been noted in murine fibrosarcoma and Dalton's lymphoma (29). The plasma membrane disintegration observed after 96 h of DPE treatment could lead to the lysis of tumor cells. Singh and Sodhi, 1988 (30) reported that murine peritoneal macrophages treated with cisplatin *in vitro* showed increased binding to DL cells through distinct cytoplasmic extensions, which transfer the lysosomes from the cytoplasm of macrophages to the tumor cell cytoplasm. It has been suggested that lymphocytes kill tumor cells by the release of toxic factors that disrupt the cell membrane (31,32). The present study also revealed that the disintegration in the plasma membrane of tumor cells surrounded/connected by leukocytes (Figure 5E) could also be due to the release of some toxic factors by the leukocytes. *In vitro* cisplatin treatment of murine peritoneal exudates cells (macrophages) has shown the increased secretion of lysozyme, hydrogen peroxide, superoxide anions and interleukin-1 (33).

The formation of membrane vacuoles on the tumor cells (Figure 5E) following DPE treatment could be an indication of tumor cell lysis, eventually leading to cell death. Significant modifications of cell surface and disintegration of the plasma membrane of rat hepatoma cells have been reported after bacterially fermented mistletoe preparation (BFMP) treatment (34). Prasad and Sodhi, 1982 (35) reported that cisplatin treatment removes the cell surface sialic acid moieties and acid mucopolysaccharides which may in turn enhance the antigenicity of tumor cells. We have also previously reported the decreased level of sialic acid content in DL cells after *Dillenia pentagyna* extract treatment (2). In the present study also, this decreased sialic acid content of DL cells may have a role in enhancing DL cells antigenicity, thus facilitating immunological recognition.

It may be concluded that *Dillenia pentagyna* extract treatment caused a significant decrease in the ascites tumor volume. The observed increase in leukocyte infiltration towards tumor cells may be due to the increased production of leukocytes in the host, and this increased leukocyte infiltration may also play a role in reducing ascites tumor volume by causing tumor cell lysis, thereby increasing host survivability.

Acknowledgments

We acknowledge the financial support provided by the University Grants Commission and Department of Science and Technology, New Delhi under DRS and FIST programme respectively.

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