EFFECT OF *DILLENIA PENTAGYNA* EXTRACT ON THE LEVEL OF SIALIC ACID AND LIPID PEROXIDATION IN DALTON'S LYMPHOMA-BEARING MICE

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Summary

Dillenia pentagyna (*D. pentagyna*) is a herbal plant which is most commonly used as traditional anticancer medicinal plant by the Mizo tribe in Mizoram state, India. The objective of the present study was to evaluate the level of sialic acid and lipid peroxidation in Dalton's lymphoma -bearing mice so as to explore their possible role in the antitumor activity of *D. pentagyna*. The host survivability experiment revealed that the extract treatment at the dose of 20 mg/kg body weight caused the highest percentage increase in life span (%ILS ~70) of tumor-bearing mice. The extract treatment of the host at the same dose significantly decreased ($p<0.05$) sialic acid content in liver, kidney, spleen and DL cells, and a significant increase in ascites supernatant. The treatment resulted in a decrease in lipid peroxidation in liver, kidney and testes whereas an increase was observed in the spleen. DL cells did not show significant changes in the level of lipid peroxidation. *D. pentagyna* extract mediated decrease in the lipid peroxidation in the tissues of tumor-bearing mice may indicate its possible protective function against tissue damage caused by oxidative stress in tumorous condition. Decrease in sialic acid content in the tissues of tumor-bearing mice and paricularly in the tumor cells after this plant extract treatment may also help in facilitating / increasing host survivability.

Keywords: Dalton's lymphoma; *Dillenia pentagyna*; antitumor potential; sialic acid; lipid peroxidation.

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Introduction

Sialic acid is usually found as terminal carbohydrate residue on the non-reducing end of oligosaccharide chains of glycoproteins and glycolipids in serum and tissue. A significant amount of sialic acid (98–99.5%) is bound to glycoproteins and a small portion to lipoproteins. In physiologic pH, the sialic acid is negatively charged, and this affects the physiological and chemical properties of glycoproteins significantly [1,2]. Lipid-bound sialic acid levels have been used as a tumor marker of various malignant neoplasms including bronchial, prostate, ovary, breast, and colon cancer, and malignant melanoma [3, 4, 5, 6, 7]. It was believed that changes in bound carbohydrates at the cell surface might result in persistent cell division, decreased intercellular adhesiveness, altered transport, altered/masked immunogenicity and other specialized functions accompanying malignant transformation [8]. The widely distributed sialic acid moieties of glycoprotein are reported to have damping, protective and regulatory functions at the cell surface [8]. Prasad [9] reported that the lectin mediated agglutination behavior of normal and malignant cells depends upon the changes in cell surface sialic acid moieties. Since sialic acid occupies a terminal position in carbohydrate chains of mammalian glycoproteins, it might be expected that it would play an important role as a receptor at the cell surface. Yet on masking of cell-surface antigens, it may be involved in nonspecific repulsion of cells or macromolecules by virtue of its negative charge [10]. Thus, the cell surface components could play vital role in the malignant transformation of a variety of cells. And the study of sialic acid in tumor cells treated with or without anticancer drugs may give some useful information to understand the chemotherapeutic mechanisms.

Lipid peroxidation, the oxidative breakdown of polyunsaturated fatty acids, is widely accepted as a general mechanism for cellular injury. It has been implicated in diverse pathological conditions, including atherosclerosis, aging and cancer, and in toxicity associated with certain solvents, drugs and metals [11]. Peroxidation of cell membrane lipids causes changes in cell structure and, in turn, the dysfunction of affected cells [12], and also to the production of toxic and reactive aldehyde metabolites called

free radicals. Reactive oxygen forms (ROF) or free radicals are highly reactive and responsible for oxidation damage of lipids. Of these free radicals, malondialdehyde (MDA) is the most important [13], and it has been extensively used as a marker of lipid peroxidation, mainly in processes associated with oxidative stress and vascular injury [14].

We have previously reported the antitumor activity of aqueous and methanol extract of stem bark of *Dillenia pentagyna* Roxb. (Dilleneaceae) [15]. The stem bark of this plant has been used by the local people of Mizoram state, India, as a traditional medicine for the treatment of cancer suspected diseases [16], and stem bark and fruit has also been used by other Indian ethnic communities as cure for blood dysentery, stomach pain and fistula [17]. However, the details of its effect in the tissues and tumor cells of tumor bearing mice while its antitumor activity in the host is largely unknown.

Therefore, in the present investigation, the effect of *Dillenia pentagyna* stem bark extract on the levels of sialic acid and lipid peroxidation were determined in various tissues of Dalton's lymphoma-bearing mice in order to find their possible role in the antitumor activity.

Materials and methods

Chemicals

Bovine serum albumin (BSA) was obtained from Sigma Chemicals Co. Ltd., U.S.A. All other chemicals of analytical grade were purchased from Sisco Research Laboratories Co. Mumbai, India.

Animals and tumor model

Inbred Swiss albino mice colony is being maintained under laboratory conditions keeping 5-6 animals in a propylene cage at 23-25^{o}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 intraperitoneal (i.p.) transplantation of 1 x 10^7 viable tumor cells per animal (in 0.25 ml phosphate buffered saline, pH 7.4). Tumor-transplanted mice usually survive for 19–21 days.

Plant material and preparation of test sample

The plant material, *D. pentagyna* was authenticated by Dr. P.B. Gurung, Department of Botany, North Eastern Hill University, Shillong, India and a voucher specimen (no. SBP 001) was deposited in the Department of Zoology, NEHU. The methanol extract of stem bark of *D. pentagyna* was prepared as described previously [15].

Survivability at different doses

Different dose of methanol extract of *D. pentagyna* (10, 15, 20, 25, 30, 50, 100 and 200 mg/kg body weight/day) was used for dose determination. For each dose of the plant extract, 15 animals were used. Tumor-bearing mice were treated with 0.25 ml of *D. pentagyna* extract (DPE) through intraperitoneal (i.p.) injection, daily for 5 days starting from day 1, and the hosts survival pattern were recorded. Tumor transplantation day was designated as day 0. Animals in the control group received the same volume of plant extract vehicle (0.25 ml of 0.05% NaOH). The antitumor efficacy of different doses of the extract was reported in percentage of average increase in life span (% ILS), and was calculated using the formula:

% ILS = (T/C x 100) – 100,

where, T and C are the mean survival days of treated and control groups of mice respectively.

Treatment

The dose of DPE (20 mg/kg body weight) showing the highest percentage increase in life span (% ILS ~ 70) was used in the biochemical studies. Animals were divided into 3 groups having normal (Group-I), untreated tumor-bearing control (Group-II) and DPE-treated (Group-III) mice. On the 10^{th} day of tumor transplantation when the tumor is in log/mid phase of tumor growth, group II and III animals received a single dose of extract vehicle (0.05% NaOH) and DPE (20 mg/kg body wt.) respectively. After 24, 48, 72 and 96 h of treatment, liver, kidney, spleen, testes and DL cells were collected

and used for determination of sialic acid and lipid peroxidation. Ascites supernatants (SN) were also used for sialic acid determination.

Sialic acid estimation

Sialic acid concentration in different tissues was determined following the method of Warren [18]. Briefly, 5% tissue homogenate prepared in 0.1N H_2SO_4 was incubated in a hot water bath (80^oC) for 1 hour, and the homogenate was centrifuged at 8000 rpm for 15 minutes. 0.2 ml of the supernatant was mixed with 0.1 ml of periodate solution (4.278% sodium metaperiodate in 58% orthophosphoric acid) and incubate at room temperature for 20 minutes. 1 ml of arsenite solution (10% sodium arsenite, 7.1% sodium sulphate and 0.01% potassium iodide in 0.1N H_2SO_4) and 3 ml of thiobarbituric acid (1.2% thiobarbituric acid and 14.2% sodium sulfate in water) were added and heated in a boiling water bath for 15 minutes, then, cooled in cold water. Equal volume (4.3 ml) of cyclohexanone was added, mixed thoroughly and centrifuged at 2000 rpm for 10 minutes. The upper clear supernatant showing pink colour was collected and absorbance was read at 532 and 549 nm wavelength against the blank in a spectrophotometer. The total sialic acid content was expressed as μmoles/gm tissue wet wt., and μmoles/ml for supernatants.

Lipid peroxidation assay

Lipid peroxidation in different tissues was measured following the method of Buege and Aust [19]. 10% tissue homogenate was prepared in 0.15 M KCl. To 1 ml of tissue homogenate, 2 ml of the trichloro acetic acid (15%) - thiobarbituric acid (0.375%) - HCl reagent (0.25 N HCL) was added and mixed thoroughly. The mixture was heated in a boiling water bath for 15 minutes, and then cooled at room temperature. The precipitate was removed by centrifugation at 1000xg at 4^oC for 10 minutes. The absorbance of the clear supernatant was read at 535nm. The malondialdehyde concentration was calculated using an extinction coefficient of 1.56 x 10^5 $M^{-1}cm^{-1}$ and expressed as nmoles/mg protein.

The protein content of the tissues was determined by the method of Lowry et al. [20] using bovine serum albumin as a standard.

Statistical analysis

Results were expressed as mean ± S.D. of number of experiments (n = 5). Significance was evaluated by Student's *t*-test. *p* values less than 0.05 were regarded as significant.

Results

Among eight different doses of DPE used, the maximum percentage increase in life span (% ILS ~ 70) of the hosts was observed at a dose of 20 mg/kg body wt./day (Figure 1). The percentage survivors with different doses has been shown in Figure 2, and hundred percent survivors was noted till 30 days under the treatment with 20 mg/kg body wt./day.

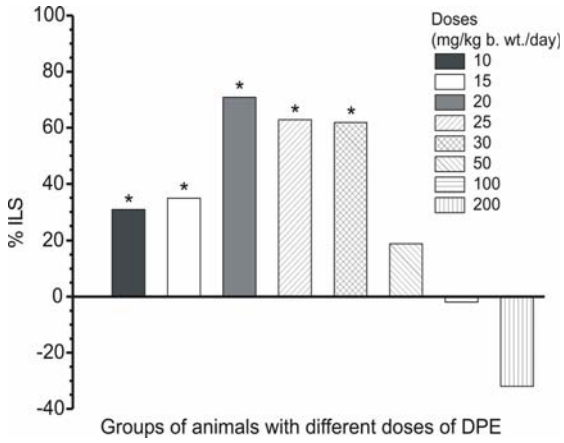

Figure 1. Graph showing percentage increase in life span (%ILS) of tumor-bearing mice treated with different doses of DPE. *Dose of DPE showing %ILS ≥ 20 were considered to possess antitumor potential.

In the normal mice, spleen showed the highest sialic acid content (1.36 µmoles/g tissue wet wt.) followed by kidney (1.09 µmoles/g tissue wet wt.), liver (0.86 µmoles/g tissue wet wt.) and testes (0.62 µmoles/g tissue wet wt.) (Table 1). During the tumor progression i.e. 5^{th}, 10^{th} and 15^{th} day, representing initial, middle and later stage of tumor growth, sialic acid content in liver, kidney, spleen, DL cells and SN increased except in testes where a decreased level was observed (Table 1).

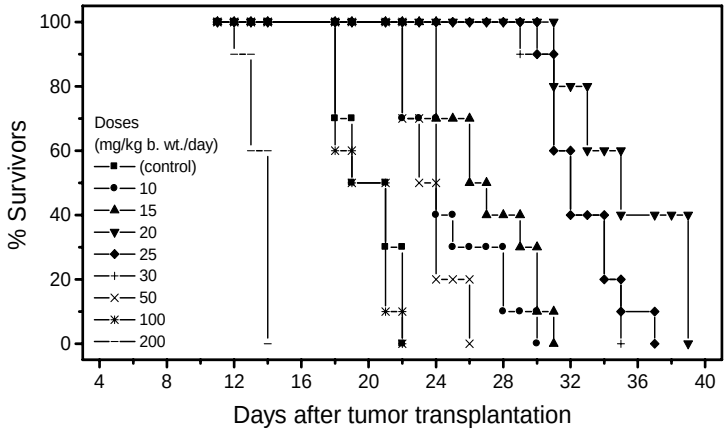

Figure 2. Comparative survival patterns of tumor-bearing mice treated with different doses of DPE. Control mice received extract vehicle alone (-■-). Doses of DPE, 10 (-●-), 15 (-▲-), 20 (-▼-), 25 (-♦-), 30 (-+-), 50 (-×-), 100 (-∗-) and 200mg/kg b. wt. (- -) were administered daily for 5 days beginning from day 1 of tumor transplantation.

Table 1. Changes in the sialic acid (µmoles/g tissue wet wt.) in various tissues of mice at different stages of tumor growth

Day of tumor growth	Liver	Kidney	Spleen	Testes	DL cells	SN[a]
Day 0[b]	0.86±0.04	1.09±0.03	1.36±0.04	0.62±0.04	-	-
Day 5	1.11±0.06*	1.14±0.03	1.51±0.02*	0.60±0.02	1.01±0.06	0.82±0.01
Day 10	1.37±0.06*	1.20±0.02*	1.64±0.01*	0.55±0.02*	1.27±0.11*	0.84±0.02
Day 15	1.75±0.02*	1.27±0.02*	1.79±0.03*	0.45±0.02*	1.34±0.08*	0.97±0.02*

Results are mean ± standard deviation (S.D). Student's t-test; as compared to the corresponding normal tissue, n = 5, *P ≤ 0.05. [a]Sialic acid content was expressed as µmoles/ml SN. [b]Normal mice.

Comparison with the respective corresponding controls showed that DPE treatment caused a significant decrease of sialic acid content in liver and kidney during 24 h to 96 h of treatment (Figure 3A and 3B), 48 h to 96 h of treatment in spleen and DL cells (Figure 3C and 3E) and a significant increase during 72 h to 96 h of treatment in ascites supernatant (SN) (Figure 3F). Testes did not show significant changes in the sialic acid content (Figure 3D).

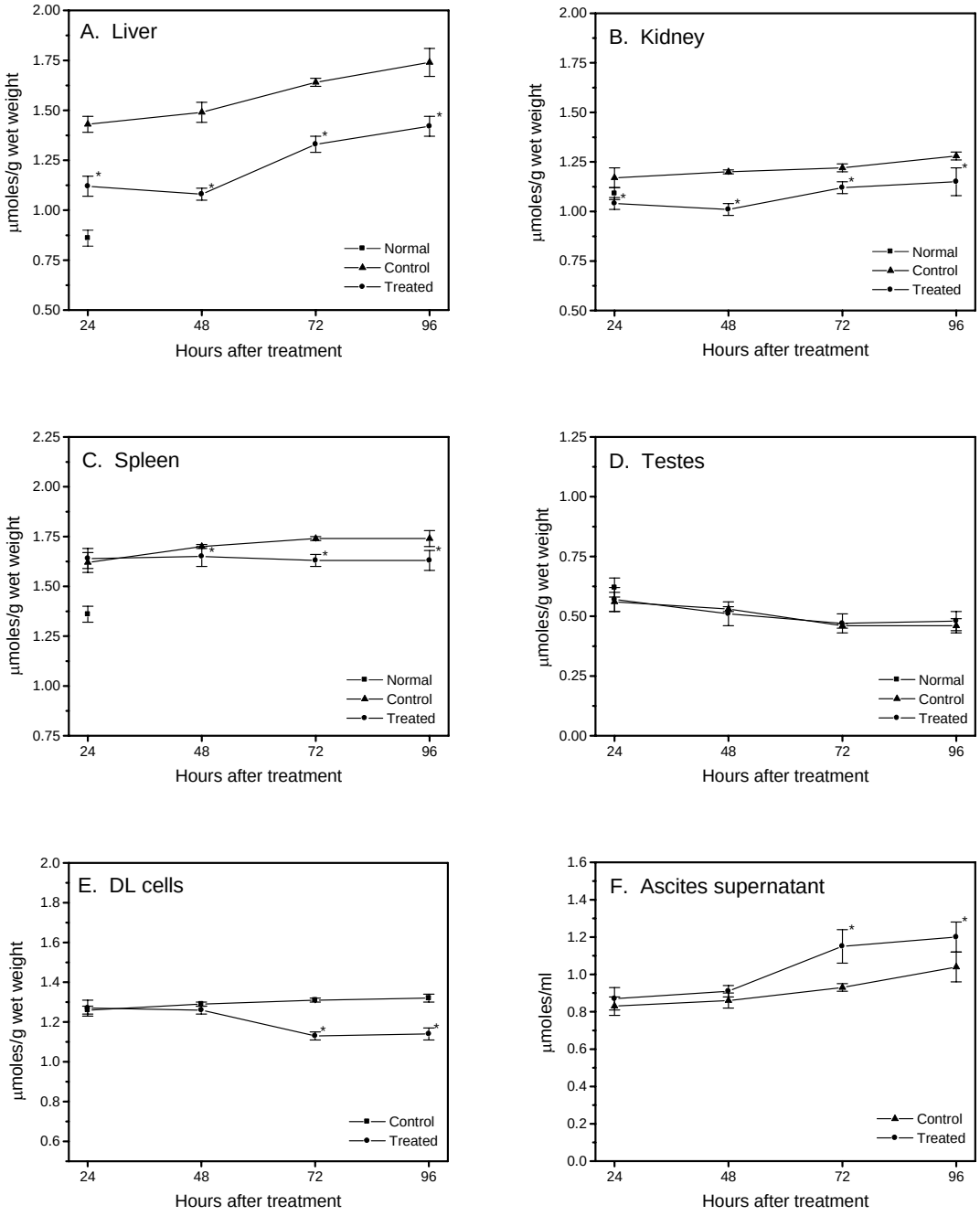

Figure 3. The pattern of changes in sialic acid content in the liver (A), kidney (B), spleen (C), testes (D), DL cells (E) and SN (F) of normal, control and treated mice. Treated mice received a single dose (20mg/kg. b. wt.) of DPE while control mice received extract vehicle alone. Results are mean ± standard deviation (S.D). Student's t-test; as compared to the corresponding controls, n = 5, *P ≤ 0.05.

In the normal mice, the highest level of lipid peroxidation was noted in spleen (0.252 nmoles/mg protein) followed by testes (0.170 nmoles/mg protein), kidney (0.160 nmoles/mg protein) and liver (0.122 nmoles/mg protein) (Table 2). As the tumor growth progresses, lipid peroxidation was observed to increase in liver, kidney and testes whereas a decreased level of lipid peroxidation was noted in spleen and DL cells (Table 2).

Table 2. The pattern of changes in lipid peroxidation (nmoles/mg protein) in the tissue of tumor-bearing mice during different stages of tumor progression.

Day of tumor growth	Liver	Kidney	Spleen	Testes	DL cells
Day 0[a]	0.122±0.016	0.160±0.010	0.252±0.023	0.170±0.018	--
Day 5	0.162±0.011*	0.184±0.017	0.207±0.016*	0.210±0.055	0.110±0.017
Day 10	0.167±0.011*	0.211±0.013*	0.121±0.015*	0.361±0.012*	0.104±0.012
Day 15	0.193±0.019*	0.245±0.016*	0.100±0.016*	0.403±0.018*	0.080±0.012*

Results are mean ± standard deviation (S.D.). Student's t-test; as compared to the corresponding normal tissue, n = 5, *P ≤ 0.05. [a]Normal mice.

A significant inhibition of lipid peroxidation by DPE was noted in liver, kidney and testes (Figure 4A, 4B and 4D). In spleen, lipid peroxidation increases significantly during 24 h to 96 h of treatment (Figure 4C) while not much change was observed in DL cells (Figure 4E).

Discussion

In the antitumor studies, ascites Dalton's lymphoma has been commonly used as an important murine experimental tumor model [21, 22]. The host survival data (Figure 1) indicate significant increase (% ILS ≥ 20) in survivability of tumor-bearing mice treated with DPE at a dose of 10, 15, 20, 25 and 30 mg/kg b. wt./day as compared to the control tumor bearing mice suggesting its antitumor potentials (Fig. 1 and 2) with the most potent antitumor activity (%ILS ~ 70) observed at the dose of 20 mg/kg body wt./day (Fig. 1)

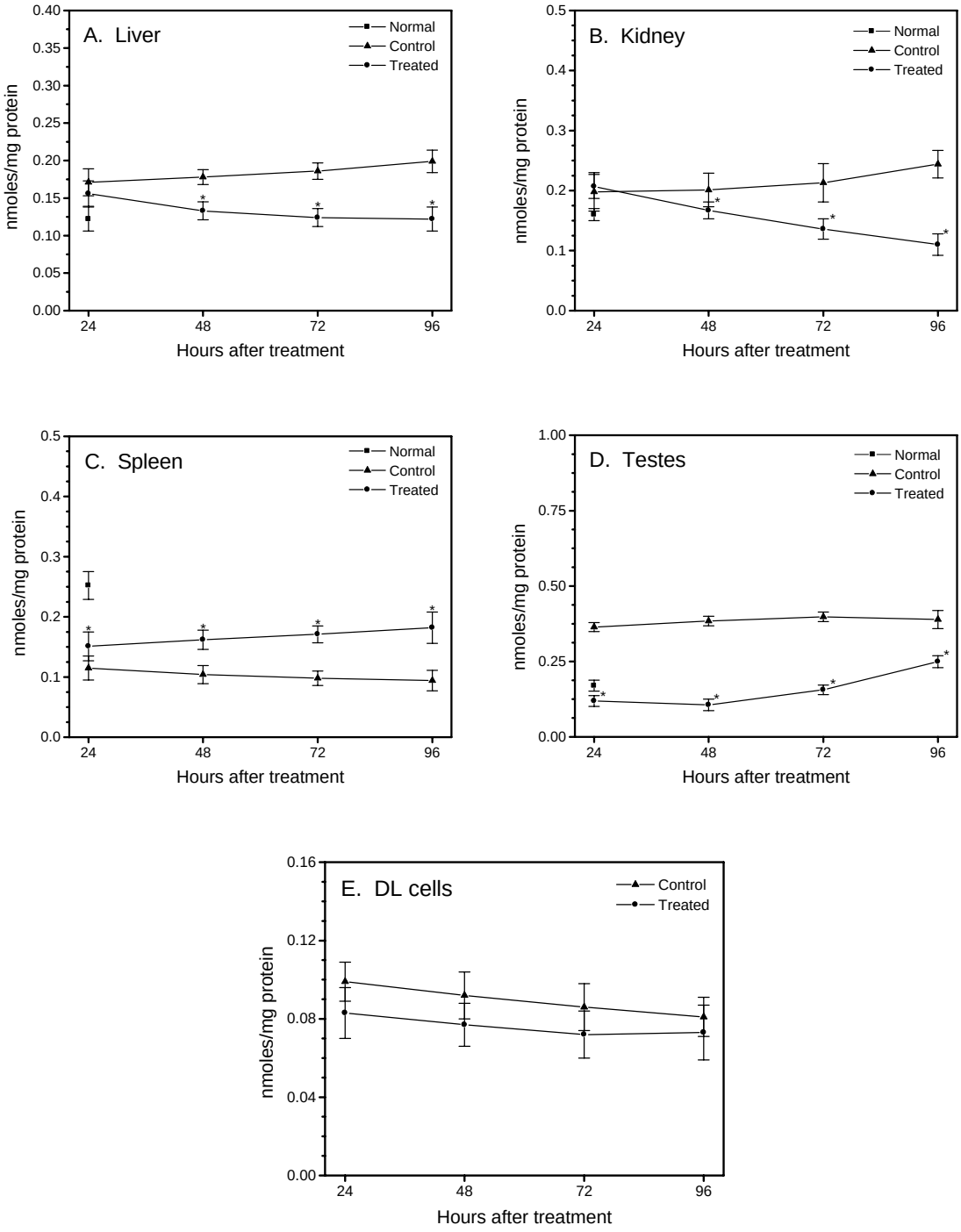

Figure 4. Graph showing the effect of a single dose (20mg/kg. b. wt.) of DPE in the level of lipid peroxidation in the liver (A), kidney (B), spleen (C), testes (D) and DL cells (E) of normal, control and treated mice. Control mice received extract vehicle alone. Results are mean ± standard deviation (S.D). Student's t-test; as compared to the corresponding controls, n = 5, *P ≤ 0.05.

Sialic acids consist of a family of acidic nine-carbon sugars that are typically located at the terminal positions on a variety of glycoconjugates. The largest structural variations of naturally occurring sialic acids are at carbon 5, which can be substituted with either an acetamido, hydroxyacetamido or hydroxyl moiety to form 5-*N*-acetylneuraminic acid (Neu5Ac), 5-*N*glycolylneuraminic acid (Neu5Gc) or deaminoneuraminic acid (Kdn), respectively [23]. It has been reported that sialic acid influences many properties of the cell surface such as the determination of the cell surface negativity and the losses of contact inhibition during malignancy and antigen masking agent. Cell surface glycoproteins and glycolipids are susceptible to such elevations as soon as a malignant growth starts to develop and metastasize, that they are referred to as 'tumor markers' [7]. The present findings showed an increase in sialic acid concentrations in all the tissues except testes (Figure 3A-F) with tumor growth in mice. The increase of sialic acid in DL cells with tumor growth may be due to enhanced activity of enzymes involved in sialic acid synthesis. Some reports have indicated a 3-5 times increased sialyl transferase activity in various virally transformed cells as compared to the corresponding normal cells, an event that may be associated with the increase in the amount of sialic acid in the transformed cells [24]. The elevated sialic acid levels in malignant cells have also been observed for murine Yoshida ascites sarcoma [25]. The influence of sialic acid on the oncogenecity of tumor cells may be based on a negative charge determining constituent on the cell surface, resulting in the loss of contact inhibition, an antigen-masking agent and a component of the cell surface involved in the adherence of tumor cells to the mesothelial membrane prior to their dissemination to form metastasis [10]. Furthermore, the observation of increased sialic acid content in the tissues of tumor bearing mice could be helpful for DL cells in the host since sialic acid has also been known to be important in the transport of proteins, amino acids and ions to cancer cells. As far as the effect of DPE on the quantitative changes in the sialic acid of DL cells and tissues is concerned, it was noted that DPE treatment of tumor-bearing mice for 24 h to 96 h caused a decrease of sialic acid in liver, kidney, spleen and DL cells (Figure 3A, 3B, 3C and 3E) while an increased level was observed in SN (Figure 3F). The decrease in sialic acid content in DL cells after DPE treatment may be associated with an increase in tumor cell immunogenicity thereby enhancing host's immune

response. The increase in the sialic acid content in the SN should be associated with the release of sialic acid moieties from DL cells after the treatment. Along with the sialic acid decrease in DL cells, sialic acid decrease in other tissues in tumor-bearing mice should also help to bring out restoration of the functional activity of the tissues closer to normalcy in the host, thereby facilitating survivability of the host.

Lipid peroxidation has been reported as a major contributor to the loss of cell function under oxidative stress situations. Oxidative stress leads to various types of damage at the molecular and cell level [26]. Numerous studies have shown that toxicity of superoxide and hydrogen peroxide is highly dependent on the presence of iron or copper and that the nature and extent of damage initiated by these species is related to the subcellular location of these metals [27]. All cellular components are susceptible to attack by reactive oxygen species (ROS), particularly by OH. Attack on proteins can lead to the modification of amino acids, oxidation of sulfhydryl groups leading to conformational changes, altered enzymatic activity, crosslinking, peptide bond cleavage as well as carbohydrate modification in glycoproteins, loss of metal in metalloproteins, altered antigenicity, and increased proteolytic susceptibility [28, 29]. ROS attack also causes DNA strand breaks and base modifications [29]. Lipid peroxidation can alter vital membrane protein structure and function. The presence of lipid hydro peroxides in a membrane disrupts its function by altering fluidity and allowing ions such as Ca++ to leak across the membrane, the consequences of which include activation of phospholysis, membrane blebbing and eventual membrane rupture. The antioxidants in such cases can act as stabilizers of homeostasis. Cancer cells can generate large amounts of hydrogen peroxides which may contribute to their ability to damage normal tissues [30]. Some chemical drug treatment is associated with induction of oxidative stress by generation of free radicals and reactive oxygen species [31, 32], and the potential role of dietary antioxidants, such as ascorbic acid, tocopherol, β-carotene etc. to reduce the activity of free radical-induced reactions has drawn increasing attention [33]. In the present studies treatment of tumor-bearing mice with DPE resulted in a significant decrease of lipid peroxidation in the tissues except spleen (Table 2; Fig. 4A - E). Therefore, these results may suggest that the increased level of lipid peroxidation noted in the tissues of tumor-

bearing mice was decreased by DPE, thereby minimizing tissue damage which might be occurring by oxidative processes.

It may be concluded that out of different doses of DPE used in the present study, 20 mg/kg. body wt./day exhibited the most effective antitumor potential against murine ascites Dalton's lymphoma. Along with the sialic acid decrease in DL cells, this plant extract-mediated decrease of sialic acid in the tissues of tumor-bearing mice should also help facilitating survivability of the hosts. DPE-mediated inhibition of lipid peroxidation in the tissues of tumor-bearing mice is also suggested to involve a reduction of tissue damage caused by oxidative processes, thereby increasing host survivability. Further, it needs to characterize the active component of DPE, toxicities and elucidate more insight on the mechanism of antitumor activity.

Acknowledgement

The financial assistance rendered by University Grants Commission (under DSA, UPE), Department of Science and Technology, New Delhi, and North-Eastern Hill University is gratefully acknowledged.

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