

CHANGES IN ENDOGENOUS GLUTATHIONE LEVEL ASSOCIATED WITH THE ANTITUMOR ACTIVITY OF THE STEM BARK EXTRACT OF *DILLENIA PENTAGYNA* AGAINST MURINE ASCITES DALTON'S LYMPHOMA

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Summary

D. pentagyna is a herbal plant which is most commonly used as traditional herbal medicine in cancer suspected cases by the Mizo tribe in Mizoram state, India. The objective of the present study was to evaluate the changes in the levels of glutathione and its related enzymes in tumor cells and other tissues of the host so as to explore their possible role in its antitumor activity. Treatment of ascites Dalton's lymphoma-bearing mice with methanol extract of *Dillenia pentagyna* at a dose of 20 mg/kg body weight showed antitumor potential. Plant extract treatment causes an increase in the level of glutathione in kidney, spleen and testes while a significant decrease was found in liver and Dalton's lymphoma (DL) cells. The assay of glutathione-s-transferase (GST) and glutathione reductase (GR) activities in Dalton's lymphoma cells showed that the treatment caused an increase in GST activity and a decrease in GR activity. It is proposed that the decreased level of GSH may partially be due to decreased GR activity in Dalton's lymphoma cells, and may contribute to the antitumor potential of *Dillenia pentagyna*. However, other contributory steps may also be involved in the antitumor activity of *D. pentagyna* and further investigation is needed in this direction.

Keywords: Antitumor potential; Dalton's lymphoma; *Dillenia pentagyna*; glutathione; glutathione reductase; glutathione-s-transferase.

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Introduction

Chemotherapy against cancers is commonly used either singly or in combination with surgery and/or radiotherapy. In chemotherapy, drugs like cisplatin, carboplatin, cyclophosphamide, bleomycin procarbazine, mitozantrone, ifosfamide, melphalan, 5-fluorouracil, gemcitabine etc. are some of the drugs used for the treatment of cancers. However, the efficacy of most of the cancer chemotherapeutic drugs is found to be limited due to the development of various side effects in the host and/or the development of acquired drug resistance by cancer cells. Various side effects like anaemia, neurotoxicity, nephrotoxicity, myelosuppression, dermatological reactions, hypersensitivity etc are commonly noticed in the host (1,2). In an attempt to diminish these side effects and better remedy against malignancies, many plant derivatives have been used with varying success (3) and a variety of plant extracts have been reported to have potential antitumor and/or anticarcinogenic activities (4-12). Earlier, we studied the possible antitumor activity of aqueous and methanol extracts of *Blumea lanceolaria* Linn. (Asteraceae), *Dillenia pentagyna* Roxb. (Dilleniaceae), *Ageratum conyzoides* Linn. (Asteraceae), *Taxus baccata* Linn. (Taxaceae) and *Potentilla fulgens* Wall. (Rosaceae), which have been used by the local people of Mizoram and Meghalaya states of India, as a traditional home medicine for the treatment of cancer suspected diseases. Among the five plants studied, methanol extract of stem bark of *Dillenia pentagyna* showed the highest antitumor activity (13). Therefore, this plant i.e., *D. pentagyna* was selected presently for further study.

D. pentagyna Roxb. (Dilleniaceae) is a deciduous tree, about 12-14 m in height; branches gradually ascending. Stem bark is greyish-white outside, green underneath the corky layer and light red inside. It is found in most places of Mizoram state and flowering is usually seen in the month of March and April. The local people commonly use the juice of stem bark extracted by boiling with water as a traditional home medicine for suspected gastric cancer and other diseases. Recently, we have reported that the extract treatment of this plant causes some significant changes in the level of sialic acid and lipid peroxidation in DL cells as well as other tissues.

Glutathione, an endogenous intracellular thiol-containing tripeptide (L- γ -glutamyl-L-cysteinyl-glycine), is an important cellular antioxidant and has been the focus of interest in cancer chemotherapy (14,15). Under normal physiological conditions mammalian cells maintain more than 98% of glutathione in the reduced form (GSH) at intracellular concentration of 0.5 to 10mM. It is involved in many cellular functions, i.e, bioreductive reactions, maintenance of enzyme activity, amino acid transport, protections against oxidative stress, detoxification of xenobiotics, and drug metabolism (16,17). On the other hand, reduction of GSH levels in tumor cells has been explored as a means of enhancing the cytotoxic effects of chemotherapeutic agents (18). Loss of GSH and oxidative damage has been suggested to constitute early, possibly signalling events in apoptotic cell death (19). Therefore, it is of interest to determine changes in cellular GSH levels and some GSH related enzymes as a parameter involved in the antitumor activity of the extract of *D. pentagyna* against murine ascites Dalton's lymphoma.

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 was prepared and dissolved as described previously (13) for the intraperitoneal treatment. For the treatment through

diet, the plant extract powder was dissolved in 50% alcohol (14, 35, 70 and 105 mg/100 ml) and mixed thoroughly with the feed powder in the ratio of 1:2 (volume:weight/ml:g) which was then dried in an oven at 35-40°C. Since the daily intake of feed per animal was about 7.5 g, the different doses of extract treatment come to 20, 50, 100 and 150 mg/kg body weight/day.

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. -caused an increase in the level of glutathione in kidney, spleen and testes while a significant decrease was found in liver and Dalton's lymphoma (DL) cells. The assay of glutathione-s-transferase (GST) and glutathione reductase (GR) activities in Dalton's lymphoma cells showed that the treatment caused an increase in GST activity and a decrease in GR activity. It is proposed that the decreased level of GSH may partially be due to decreased GR activity in Dalton's lymphoma cells 21 days. This study using these animals is as per animal ethical norms and has been cleared by institutional ethical committee.

Antitumor activity:

The antitumor activity of the extract of *D. Pentagyna* was evaluated using two routes of treatment of the tumor-bearing mice i.e. intraperitoneal (i.p.) and through diet. In the first mode of treatment, group of mice were administered i.p. with the methanol extract of *D. pentagyna* (20–200 mg/kg body wt.) beginning 1st day after tumor transplantation, once daily for 5 days. The control mice received the same volume of extract dissolving vehicle i.e. 0.05% NaOH. For the treatment through diet, the animals were fed with the diet supplemented with different concentrations of plant extract (70, 175, 350 and 525 mg/kg of diet) beginning 7 days before tumor inoculation till the end of the experiment. As the daily intake of diet per animal is about 6.8-7.5 g, the daily intake of plant extract from different diet with different concentration of extract, are approximately 20, 50, 100 and 150 mg/kg body weight respectively. Control mice were fed with the diet mixed with extract vehicle only. The deaths, if any, of the hosts were recorded daily and the survival pattern of the hosts was determined. The antitumor efficacy in different groups was expressed as the average percentage increase in life span (ILS%) which was calculated using the formula:

$$(T/C \times 100) - 100,$$

where, T is the mean survival days of treated mice and C is that of the control mice.

Glutathione and related enzymes:

As the i.p. treatment of the hosts showed better antitumor efficacy than the treatment through diet, only this treatment schedule with the dose 20 mg/kg body wt. showing highest antitumor activity was used in the determination of GSH and GSH related enzymes. The plant extract was injected (i.p.) on the 10th day post-tumor transplantation which is the logarithmic phase of tumor growth. After 24, 48, 72 and 96 h of treatment, animals were killed by cervical dislocation, and different tissues (liver, kidney, spleen, testes and DL cells) were collected for glutathione estimation following the method of Sedlak and Lindsay, 1968 (20). GSH determination was also done in the tissues of tumor-bearing mice collected on the 5th, 10th and 15th day of tumor growth, considered as initial, middle and late stage of tumor growth.

The assay of glutathione S-transferase (GST; EC 2.5.1.18) and glutathione reductase (GR; EC 1.6.4.2) activity in DL cells from different treatment groups of mice was done following the

method of Habig et al., 1974 (21) and Smith et al., 1988 (22) respectively. The GST and GR enzyme activity was calculated using the extinction coefficient ($E_{340} = 9.6 \text{ mM}^{-1}\text{cm}^{-1}$) and ($E_{412} = 13.6 \text{ mM}^{-1}\text{cm}^{-1}$) respectively. One unit of GST activity was defined as the amount of enzyme that catalyzed the conjugation of 1 μmol of 1-chloro-2,4-dinitrobenzene (CDNB) per minute, and the specific activity of the enzyme is reported per mg protein. One unit of GR activity was defined as the amount of enzyme that catalyzed the reduction of 1 μmol of NADPH per minute, and the specific activity of the enzyme is expressed as units per mg protein. The protein content in the respective tissue was determined by the method of Lowry et al., 1951 (23).

Statistical analysis

One-way analysis of variance (ANOVA) for significance of differences between groups, and Student's *t*-test for paired data were used for statistical analysis. $p < 0.05$ were considered as indicative of significance and values are expressed as mean \pm S.D.

Results

Antitumor activity:

The intraperitoneal injection of methanol extract of *D. pentagyna* dose dependently prolonged the survival time of Dalton's lymphoma-bearing mice. The maximum increase in the survival time of the hosts (ILS% \sim 71%) was observed at a dose of 20 mg/kg body wt. Inoculation of plant extract through diet did not show significant increase in the survival time of tumor-bearing mice except at a dose of 100 mg/kg body weight/day (Table 1).

Table 1. Antitumor activity of stem bark extract of *Dillenia pentagyna* against murine ascites Dalton's lymphoma.

i.p. Treatment			Treatment through diet		
Dose (mg/Kg body wt.)	Survival days	ILS (%)	Dose (mg/Kg body wt.)	Survival days	ILS (%)
Control ^a	20.0 \pm 0.49	-	Control ^b	20.0 \pm 2.16	-
200	13.5 \pm 0.70	-32	150	21.8 \pm 1.98	9
100	19.6 \pm 1.64	-2	100	24.6 \pm 1.71	23*
50	23.7 \pm 0.94	19	50	20.6 \pm 2.54	3
30	32.4 \pm 0.60	62*	20	19.6 \pm 1.83	-2
25	32.7 \pm 0.81	63*			
20	34.2 \pm 0.95	71*			
15	27.1 \pm 1.08	35*			
10	26.3 \pm 0.57	31*			

Results are mean \pm standard deviation (S.D). ^aControl animals received the same volume of the vehicle. ^bControl animals were fed with food pellets without the plant extract. *%ILS > 20 compared to respective control, n = 20.

Glutathione levels:

GSH levels decreased in liver, kidney, spleen and testes during tumor growth, and a significant decrease were observed in tumor-bearing mice on the 15th day of tumor growth (Fig. 1). In Dalton's lymphoma cells, a significant increase of total GSH ($5.96 \pm 0.28 \mu\text{moles/g wet wt.}$) was observed on the 10th day of tumor growth as compared to day 5 and decreased gradually thereafter (Fig. 1).

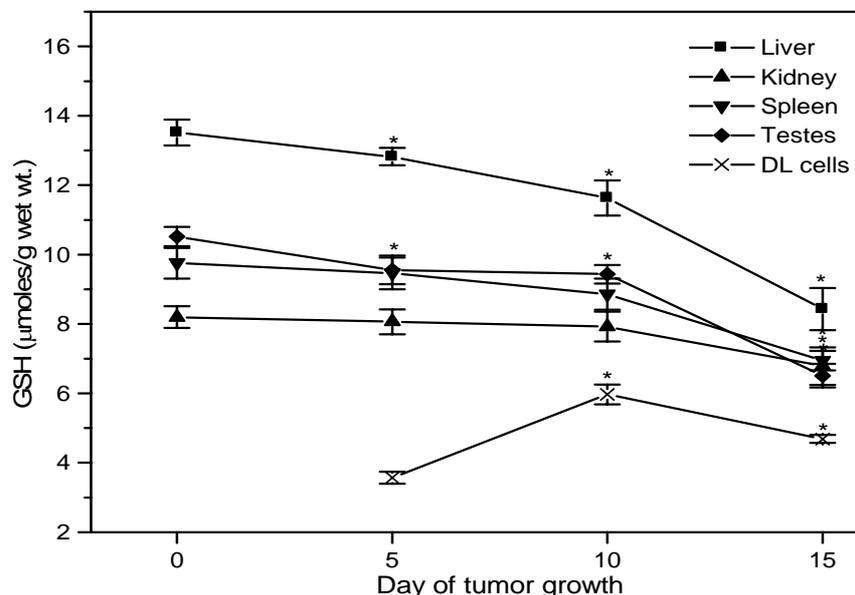


Figure 1. Changes in glutathione levels in different tissues of tumor-bearing mice at different stages of tumor growth. Results were expressed as mean \pm S.D. Student's *t*-test; as compared to the normal tissue counterpart except DL cells where comparison was done with the fifth day of tumor growth, $n = 5$, * $P < 0.05$.

In comparison to their respective control values, extract treatment of tumor-bearing mice for 24 to 96 h showed a significant ($P < 0.05$) decrease of total GSH level in liver and DL cells while an increased level was observed in other tissues (Table 2). In kidney and spleen, an increased level was observed during 48 to 72 h of treatment and a decreased thereafter. In testes, a significant increase was observed after 72 h of treatment.

Table 2. Changes in GSH level in different tissue of mice following *D. pentagyna* treatment.

Treatment	Hour	Liver	Kidney	Spleen	Testes	DL cells
Normal mice		13.52 ± 0.37	8.19 ± 0.95	9.75 ± 0.44	10.52 ± 0.28	--
Group I Tumor-bearing (control) ^a	24 h	11.51 ± 0.25	7.96 ± 0.26	8.75 ± 0.16	9.37 ± 0.09	5.82 ± 0.35
	48 h	10.62 ± 0.41	7.47 ± 0.58	7.73 ± 0.19	8.16 ± 0.01	5.73 ± 0.24
	72 h	9.31 ± 0.48	7.32 ± 0.5	7.21 ± 0.11	7.08 ± 0.11	5.31 ± 0.12
	96 h	8.56 ± 0.39	6.96 ± 0.24	7.04 ± 0.14	6.65 ± 0.22	4.68 ± 0.05
Consolidated (24-96 h) Control		10.00 ± 1.22	7.40 ± 1.01	7.68 ± 0.69	7.82 ± 1.12	5.39 ± 0.65
Group II Extract treated	24 h	$9.01 \pm 0.51^*$	8.12 ± 0.40	8.82 ± 0.18	9.30 ± 0.11	$3.13 \pm 0.20^*$
	48 h	$8.61 \pm 0.27^*$	$10.26 \pm 0.38^*$	$8.59 \pm 0.15^*$	8.09 ± 0.11	$4.52 \pm 0.17^*$
	72 h	$7.89 \pm 0.41^*$	$8.22 \pm 0.30^*$	$7.96 \pm 0.16^*$	$8.61 \pm 0.13^*$	$4.67 \pm 0.52^*$
	96 h	$7.01 \pm 0.25^*$	6.69 ± 0.28	7.11 ± 0.12	$9.03 \pm 0.22^*$	4.77 ± 0.31
Consolidated (24-96 h) Extract treated		$8.13 \pm 0.85^\dagger$	8.04 ± 1.81	8.12 ± 0.69	$8.76 \pm 0.56^\dagger$	$4.27 \pm 0.96^\dagger$

Results are mean \pm standard deviation (S.D., $n = 5$). ^aControl animals received the same volume of extract vehicle. * $P < 0.05$ compared to respective control (Student's *t*-test); $^\dagger P < 0.05$ compared to their respective consolidated control (one-way ANOVA).

Enzyme activities:

In order to determine the possible reasons for the change in glutathione status, we measured the activity of GST and GR in DL cells, the main enzymes involved in the glutathione redox cycle. In tumor-bearing mice GST activity of DL cells increases during tumor growth while GR activity decreases. Plant extract treatment causes significant increases in GST activity during 24 to 96 h of treatment and a significant decrease in GR activity was observed during 24 and 48 h of treatment (Figure 2).

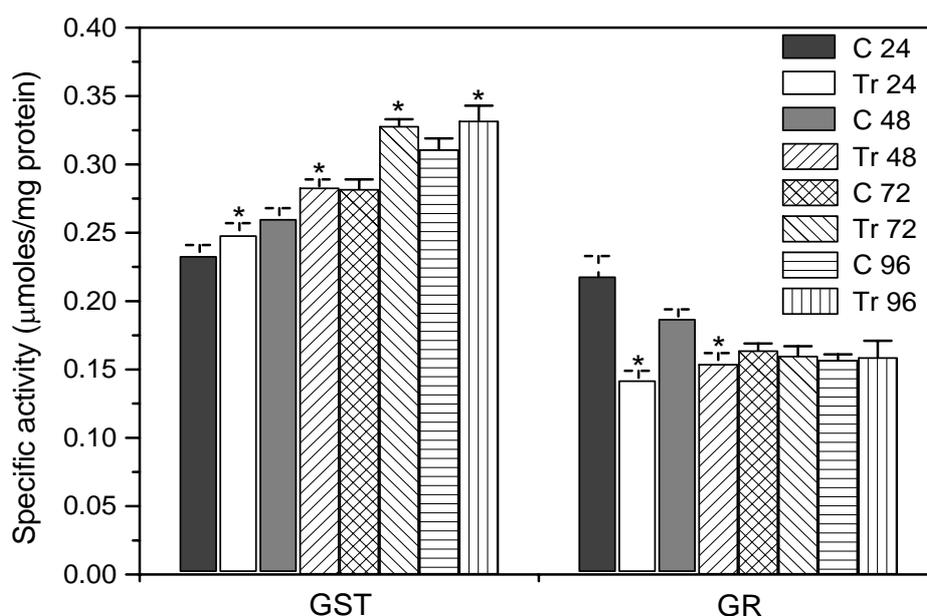


Figure 2. Histogram showing changes in glutathione-s-transferase and glutathione reductase activities in Dalton's lymphoma cells of control (untreated tumor-bearing mice) and treated mice. Results are expressed as mean \pm S.D. Student's *t*-test; as compared to the corresponding control values, $n = 5$, * $P < 0.05$.

Discussion

In the antitumor studies, ascites Dalton's lymphoma has been commonly used as an important murine experimental tumor model (24). Control group of mice, treated with the same volume (0.25 ml) of 0.05% NaOH, did not show significant differences in survivability from untreated tumor-bearing mice. Details on the doses of the extract and its effect on the survivability of the hosts in different experimental groups have been shown in Table 1.

During different stages of tumor growth, i.e. 5th, 10th and 15th day, GSH content of different tissues were found to be decreased, while DL cells showed maximum increase in GSH content on the 10th day of tumor growth and a slight decrease over the next 4-5 days (Fig. 1). In Ehrlich ascitic tumor cells maximum GSH concentrations was observed on the 7th day of tumor growth, followed by a decrease on the 14th day, which was correlated with a decrease in cell proliferation (25). The increase of GSH in tumor cells could be involved in facilitating the proliferation and metabolism of tumor cells in the host as it has been reported that GSH controls the onset of tumor cell proliferation by regulating protein kinase C activity and intracellular pH (26). As the GSH level in DL cells was observed to increase with tumor growth (Fig. 1), it became an investigating interest to find the changes in the GSH content of various tissues, if any, after the extract

treatment. Plant extract treatment significantly decreased the level of GSH in liver as well as DL cells (Table 2). On the other hand, an increase in the level of GSH was observed in kidney, spleen and testes (Table 2). However, the decrease in GSH level, particularly in DL cells by the extract treatment may be a noteworthy step in the antitumor activity of the extract of *D. pentagyna* against Dalton's lymphoma.

GSH is involved in the cellular defence against cytotoxic insults (27). It protects tissues from damage produced by oxidative stress, radiation and chemotherapy (25). Elevation of intracellular GSH levels has been suggested to be involved in the resistance of cancer cells to oxidative stress, radiotherapy and chemotherapy (28,16), while a depletion of GSH levels could increase the cytotoxicity of a variety of antitumor agents (14,15) which in turn could induce the apoptotic cell death also (19) and increase DL cell's susceptibility to oxidative stress. In an attempt to further explore the significance of GSH in the extract-mediated cytotoxicity of tumor cells, the activity of GST and GR was assayed in DL cells. It was noted that the GST activity increased while GR activity decreased in tumor cells with the tumor growth (Fig. 2). Glutathione S-transferases (GSTs) function as detoxification enzymes that catalyze the conjugation of electrophilic chemicals with glutathione in the cells (29). As the GSH levels and GST activity (Fig. 1 & 2) both increased in tumor cells, this may be involved as a step by tumor cells as a detoxification mechanism in host. Certain tumor cells can be sensitized to the action of chemotherapeutic drugs by co-administration of GST inhibitors (30,31), and over expression of GSTs can increase tumor cell resistance to such chemotherapeutic drugs (32,33). In present studies, the plant extract treatment showed an increase in GST activity in DL cells (Fig. 2). At present the details of the extract composition and actually signifying its interaction with GSH, if any, are not known. As the extract treatment showed definite antitumor activity and also causing decrease in GSH level, and an increase in GST activity in the tumor cells, it may indicate that the changes in GSH level in tumor cells may be playing major role than that of GST in the antitumor activity of the extract. Glutathione reductase mediated reduction of GSSG to GSH maintaining the GSH/GSSG ratio, which is of considerable importance for cell viability (34). GR also plays an essential role in the cellular defence against oxidative stress and a controlled decrease of the level of GR in human fibroblasts results in lowering the cell viability (35). When GR activity is impaired; the ability of the cell to reduce GSSG to GSH may be devastated, leading to GSSG accumulation within the cytosol. GR activity is noted to be decreased at 24-48 h (Fig. 2) which may affect the conversion of GSSG to GSH, thus, causing to decrease cellular GSH levels. The decrease in GR activity after extract treatment could be one of the other possible steps involved to decrease the GSH level, thus, affecting cellular antioxidant machinery, and resulting antitumor activity.

In conclusion, data of present work shows that *D. pentagyna* exhibits the highest antitumor activity at a dose of 20 mg/kg body wt. It is proposed that the decreased level of GSH and GR activity in DL cells may contribute to the antitumor potential of *D. pentagyna* against murine ascites Dalton's lymphoma. However, other contributory steps may also be involved in the antitumor activity of *D. pentagyna* and further investigation is needed in this direction.

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