CARDIOPROTECTIVE EFFECT OF GREEN TEA EXTRACT ON DOXORUBICIN-INDUCED CARDIOTOXICITY IN RATS

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Abstract: The *in vivo* antioxidant properties of green tea extract (GTE) were investigated against doxorubicin (DOX) induced cardiotoxicity in rats. In this experiment, 48 Wistar albino rats (200–250 g) were divided into eight groups (n = 6). Control group received normal saline for 30 days. Cardiotoxicity was induced by DOX (20 mg/kg *i.p.*), once on 29th day of study and were treated with GTE (100, 200 and 400 mg/kg, *p.o.*) for 30 days. Aspartate aminotransferase (AST), creatinine kinase (CK), lactate dehydrogenase (LDH), lipid peroxidation (LPO), cytochrome P$_{450}$ (CYP), blood glutathione, tissue glutathione, enzymatic and non-enzymatic antioxidants were evaluated along with histopathological studies. DOX treated rats showed a significant increased levels of AST, CK, LDH, LPO and CYP, which were restored by oral administration of GTE at doses 100, 200 and 400 mg/kg for 30 days. Moreover, GTE administration significantly increased the activities of glutathione peroxidase (GPX), glutathione reductase (GR), glutathione s-transferase (GST), superoxide dismutase (SOD) and catalase (CAT), in heart, which were reduced by DOX treatment. In this study, we have found that oral administration of GTE prevented DOX-induced cardiotoxicity by accelerating heart antioxidant defense mechanisms and down regulating the LPO levels to the normal levels.

Keywords: green tea extract, doxorubicin, cardiotoxicity, antioxidants, lipid peroxides

Doxorubicin is a potent broad-spectrum chemotherapeutic agent that is highly effective in treating patients with acute lymphoblastic leukemia, Hodgkin's lymphoma, aggressive non-Hodgkin's lymphomas, breast carcinoma, ovarian carcinoma and many solid tumors (1). However, the clinical use of this drug has been seriously limited by undesirable side effects especially dose-dependent myocardial injury, leading to potentially lethal congestive heart failure (2).

Due to the great importance of DOX in chemotherapy for the treatment of many types of cancer, researchers have exerted great efforts to attenuate the side effects of DOX. In view of this several strategies have been followed for dosage optimization and use of analogues or combined therapy but no promising results have been found (3, 4). The use of several DOX analogues available clinically did not show stronger antitumor efficacy as compared to DOX (5). Antitumor action of DOX is mediated by a wide number of mechanisms but one of the activities, i.e., generation of the free radicals, is among the main causes of cardiotoxicity. This fact allows the researchers to develop strategies to reduce the toxic effects of DOX without interfering with its antitumor properties.

Herbal extracts have many properties like antioxidant, anti-allergic, anti-inflammatory, antiviral, anti-proliferative and anti-carcinogenicity (6). Natural antioxidants, which are capable of protecting the cells from oxidative injury, should be included in the potential antioxidant therapy. Therefore, there is a need for identifying alternative, natural and safer sources of antioxidants (7).

Green tea (*Camellia sinensis*) is one of the most popular beverages, approximately three billion

kilograms of tea is produced and consumed yearly throughout the world. Green tea is favored in Asian countries and initial research on the benefits of green tea showed that daily consumption of green tea is safe and has no adverse effects on human health (8). The major catechin present in green tea extract is epigallocatechin-3-gallate (EGCG), which attributes to its beneficial effects and also reduces the risk of a variety of diseases (9, 10). Thus, the present study was designed to investigate whether pre-treatment of GTE has any protective effect on lipid peroxidation, activities of enzymatic and non-enzymatic and histopathological examination of myocardium in DOX treated rats.

MATERIALS AND METHODS

Experimental animals

This study was conducted in Wistar albino rats (200–250 g), which were kept in the animal house of Faculty of Pharmacy, Jamia Hamdard, New Delhi, India for one week prior to starting the experimental protocol for proper acclimatization under controlled condition of illumination (12 h light/12 h darkness) and temperature 20–25ºC. They were housed under ideal laboratory conditions, maintained on standard pellet diet (Lipton rat feed, Ltd., Pune, India) and water *ad libitum* throughout the experimental period. The study was approved by the Institutional Animal Ethics Committee (IAEC), Jamia Hamdard, New Delhi, India.

Drugs and chemicals

Standardized powdered, aquous extract of green tea was a gift sample by Sanat Products Ltd., India. DOX was procured from Dabur Pharmaceuticals Ltd., New Delhi, India. Vitamin E was procured from Merck Ltd., India. LDH, CK and AST assay kit were purchased from Span Diagnostics Ltd., Surat, India. All other chemicals used during the study were of analytical grade.

Experimental design

In this experiment, a total of 48 Wistar albino rats were used. The rats were randomly divided into eight groups comprising of six animals in each group as follows:

Group I: Normal control, received normal saline (1 mL/kg *p.o.*) for 30 days.

Group II: Toxic control, received DOX (20 mg/kg *i.p.*) once on 29ᵗʰ day.

Group III: GTE control, received GTE (400 mg/kg *p.o.*) for 30 days.

Group IV: Vitamin E control, received vitamin E (100 mg/kg *p.o.*) for 30 days.

Group V: GTE treated-1, received GTE (100 mg/kg *p.o.*) for 30 days and DOX on 29ᵗʰ day.

Group VI: GTE treated-2, received GTE (200 mg/kg *p.o.*) for 30 days and DOX on 29ᵗʰ day.

Group VII: GTE treated-3, received GTE (400 mg/kg *p.o.*) for 30 days and DOX on 29ᵗʰ day.

Group VIII: Vitamin E treated, received vitamin E (100 mg/kg *p.o.*) for 30 days and DOX on 29ᵗʰ day.

On 31ˢᵗ day, blood samples were collected from rat tail vein for biochemical determinations. Later, the rats were sacrificed under the influence of anesthesia. The hearts were excised out immediately, rinsed in ice-cold normal saline and used for the following assays.

Post mitochondrial supernatant (PMS)

The heart were quickly removed and perfused immediately with ice-cold saline (0.85 %, w/v NaCl) and homogenized in chilled phosphate buffer (0.1 M, pH 7.4) that contained KCl (1.17%, w/v). The homogenate was centrifuged at $800 \times g$ for 5 min at 4ºC in a refrigerated centrifuge to separate the nuclear debris. The supernatant was centrifuged at $10,500 \times g$ for 20 min at 4ºC to obtain PMS, which was used as a source of enzymes. A portion of the PMS was further centrifuged in an ultracentrifuge (Beckman, L7-55) at $34,000 \times g$ for 60 min at 4ºC to isolate microsomal fraction, which was finally suspended in phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17 %, w/v).

Biochemical determinations

Aspartate aminotransferase (AST) was determined by the method of Reitman and Frankel (11). The activity of creatine kinase (CK) was determined by the method of Tsung (12), whereas the lactate dehydrogenase (LDH) activity was assayed by the method of Lum and Gambino (13). The microsomal cytochrome P_{450} content was determined according to the method of Omura and Sato (14). Lipid peroxidation was determined by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), a product formed due to the peroxidation of membrane lipids (15). Protein content in various samples was estimated by the method of Lowry et al. (16). Blood glutathione was assayed by the method of Beutler et al. (17). Tissue GSH content was determined by method of Sedlak et al. (18). GPX and GR activities were measured by the oxidation of NADPH (19), GST activity was assayed by the method of Haque et al. (20) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, SOD activity was measured according to the method of Marklund et al. (21), in which the

enzyme activity was expressed as units/mg protein and 1 unit of enzyme is defined as the enzyme activity that inhibits autoxidation of pyrogallol by 50% and CAT activity was determined by the method of Claiborne (22).

Histopathological examination of heart

The heart was isolated immediately after sacrificing the animal and washed with ice-cold normal saline, and fixed in 10% buffered neutral formalin solution. After fixation, the heart tissue was processed by embedding in paraffin. Then, the heart tissue was sectioned and stained with hematoxylin and eosin (H.E.) for histopathological examination.

Statistical analysis

Data were expressed as the mean ± standard error (SE). For a statistical analysis of the data, group means were compared by one-way analysis of variance (ANOVA) with *post-hoc* analysis. The Tukey-Kramer *post-hoc* test was applied to identify significance among groups; p < 0.05 was considered to be statistically significant.

RESULTS

Effect of GTE on serum levels of AST, CK and LDH level

The effect of GTE on serum levels AST, CK and LDH levels are summarized in Figures
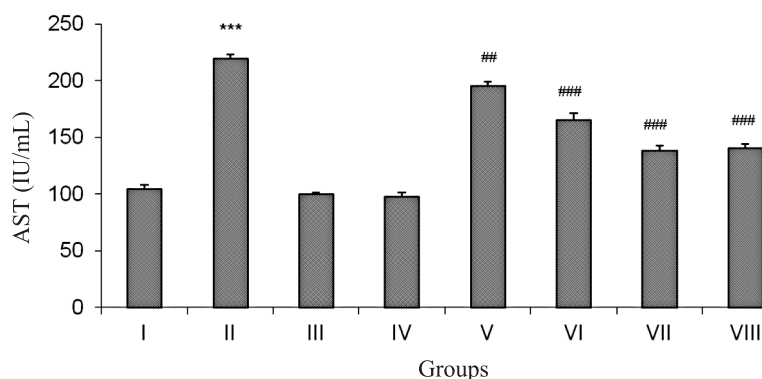

Figure 1. Effect of GTE on serum AST level in DOX treated rats. The data are expressed as the mean ± SEM; n = 6 in each group; ***p < 0.001 compared with the corresponding value for normal control rats (group I); ###p < 0.001, ##p < 0.01 compared with the corresponding value for DOX treated rats (group II)

Figure 2. Effect of GTE on serum CK level in DOX treated rats. The data are expressed as the mean ± SEM; n = 6 in each group; ***p < 0.001 compared with the corresponding value for normal control rats (group I); ###p < 0.001, ##p < 0.01 compared with the corresponding value for DOX treated rats (group II)
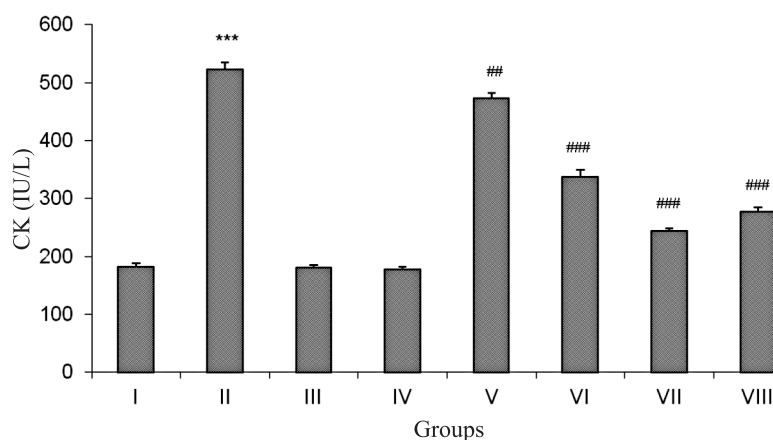
1–3, respectively. GTE treated groups V, VI and VII and vitamin E treated group VIII showed a significant (p < 0.001) decrease in the level of serum marker enzymes when compared with DOX alone treated rats (group II). No significant difference was observed in control groups (group III and IV) when compared to normal control rats (group I).

Effect of GTE on CYP contents

Figure 4 illustrates the effect of GTE on CYP contents in various groups. DOX alone treated rats showed a significant (p < 0.001) decrease in CYP contents when compared with normal control rats.

GTE treated groups V, VI, VII and vitamin E treated group VIII significantly (p < 0.001) increased the CYP contents when compared with group II rats. No significant difference was observed in control groups (group III and IV) when compared to group I rats.

Effect of GTE on LPO levels

Table 1 illustrates the effect on malondialdehyde (MDA), a secondary product of LPO, in various groups. DOX treatment resulted in a significant (p < 0.001) increase in MDA level in DOX alone treated rats when compared with normal control rats (group I). The level of MDA were significantly (p < 0.001) decreased in GTE treated groups V, VI and VII and vitamin E treat-
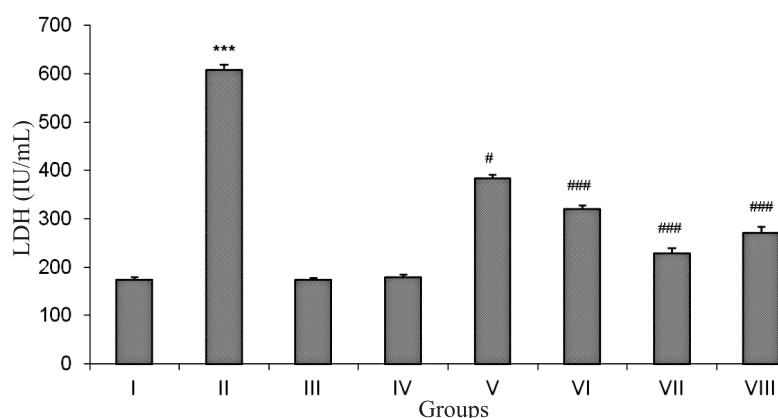

Figure 3. Effect of GTE on serum LDH level in DOX treated rats. The data are expressed as the mean ± SEM; n = 6 in each group; ***p < 0.001 compared with the corresponding value for normal control rats (group I); ###p < 0.001, ##p < 0.01 compared with the corresponding value for DOX treated rats (group II)

Figure 4. Effect of GTE on cytochrome P_{450} level in DOX treated rats. The data are expressed as the mean ± SEM; n = 6 in each group; ***p < 0.001 compared with the corresponding value for normal control rats (group I); ###p < 0.001, ##p < 0.01 compared with the corresponding value for DOX treated rats (group II)
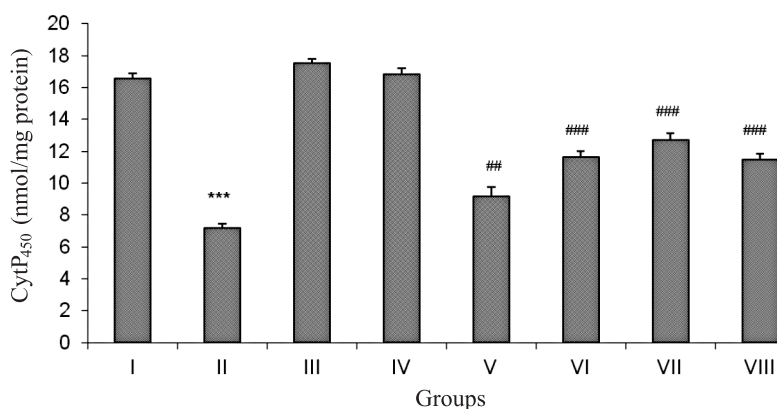
Table 1. Effect of GTE on LPO, blood GSH and tissue GSH levels in DOX treated rats.

Group	Treatment	TBARS (nmol of MDA/mg protein)	Blood GSH (mg %)	Tissue GSH (mmole/ mg protein)
I	Normal control	0.76 ± 0.013	1.71 ± 0.045	1.38 ± 0.049
II	DOX (20 mg/kg *i.p.*)	1.81 ± 0.050 ***	0.91 ± 0.031 ***	0.54 ± 0.044 ***
III	GTE (400 mg/kg *p.o.*)	0.73 ± 0.022	1.79 ± 0.035	1.49 ± 0.040
IV	Vit. E (100 mg/kg *p.o.*)	0.75 ± 0.017	1.77 ± 0.034	1.39 ± 0.029
V	GTE (100 mg/kg *p.o.*) + DOX	1.61 ± 0.021[##]	1.13 ± 0.055[##]	0.76 ± 0.044[#]
VI	GTE (200 mg/kg *p.o.*) + DOX	1.29 ± 0.033[###]	1.34 ± 0.048[###]	0.89 ± 0.049[###]
VII	GTE (400 mg/kg *p.o.*) + DOX	1.07 ± 0.046[###]	1.49 ± 0.024[###]	1.11 ± 0.038[###]
VIII	Vit. E (100 mg/kg *p.o.*) + DOX	1.23 ± 0.037[###]	1.41 ± 0.022[###]	0.96 ± 0.030[###]

The data are expressed as the mean ± S.E.; n = 6 in each group. ***$p < 0.001$ compared with the corresponding value for normal control rats (group I). ###$p < 0.001$, ##$p < 0.01$, #$p < 0.1$ compared with the corresponding value for DOX treated rats (group II).

ed group when compared with group II rats. No significant difference was observed in control groups (group III and IV) when compared to group I rats.

Effect of GTE on blood glutathione and tissue glutathione level

Table 1 also illustrates the activities of blood glutathione and tissue glutathione in various groups. DOX alone treated rats showed a significant (p < 0.001) decrease in blood and tissue glutathione levels. GTE treated groups V, VI, VII and vitamin E treated group VIII significantly (p < 0.001) increased the blood and tissue glutathione levels when compared with group II rats. No significant difference was observed in control groups (group III and IV) when compared to group I rats.

Effect of GTE on antioxidant enzymes (GPX, GR, GST, SOD & CAT) levels

Table 2 represents the effect on these antioxidant enzymes in various groups. Rats treated with DOX alone showed a significant (p < 0.001) decrease in the activity of these enzymatic antioxidants in heart as compared to the normal control rats. GTE treated groups V, VI, VII and vitamin E treated group VIII showed a significant (p < 0.001) increase in the activity of these enzymatic antioxidant when compared with DOX alone treated rats (group II). No significant difference was observed in control groups (group III and IV) when compared to group I rats.

Effect of GTE on histopathological changes in the heart

As shown in Figure 5, section of rat heart from normal control group showed normal myocardial fibres. There was no vacuolation, necrosis or inflammation found in the group I rats (Fig. 5A), but DOX alone treated rats (group II) showed a large and

irregularly shaped hypertrophic myocardial fibre with other fibres in the vicinity with small and large vacuoles (Fig. 5B). The histopathology of the heart was improved in GTE treated groups V, VI, VII and vitamin E treated group VIII and showed a normal shape, size and configuration of cardiac muscle fibres (Figs. 5C, 5D, 5E and 5F, respectively).

DISCUSSION

DOX-induced generation of reactive oxygen species (ROS) seems to be a leading cause of cardiomyopathy (9, 23). The diagnostic serum marker enzymes of cardiotoxicity are AST, CK, and LDH (24). It has been reported that the enzymes (AST, CK and LDH), that leak from the tissue damage, are the best marker of cardiotoxicity due to their tissue specificity and serum catalytic activity.

Our study also reveals an increase in the activities of these marker enzymes in DOX alone treated rats. Administration of DOX may leads to the damage of the myocardial cell membrane or it become permeable, that resulted in the leakage of AST, CK and LDH in the blood. This probably accounts for the increase in the level of these marker enzymes in the serum. Pretreatment with GTE (100, 200 and 400 mg/kg *p.o.*) restored the activities by reducing these marker enzymes level toward normal in serum. This may be due to the protective role of GTE on the myocardium, reducing the myocardial damage, thereby restricting the leakage of these enzymes in serum.

The target organelle of DOX induced cardiotoxicity is the mitochondria within which DOX accumulates (25, 26). Mitochondrial enzymes (e.g., NADH dehydrogenase) act on DOX in such a way that the quinone ring undergoes redox cycling between quinone and semiquinone states. During this

process, electrons are generated and captured by oxidizing agents, including oxygen, which then initiate a chain reaction leading to the generation of ROS (27). Cytochrome P_{450} reductase and xanthine oxidase also have been found to catalyze the reduction of anthraquinone to a semiquinone free radical (28). DOX has the ability to suppress cytochrome P_{450}, stimulating its own metabolism and thus could accelerate its elimination and increase the production of reactive toxic metabolites. Cytochrome P_{450} is not rapidly organized in the microsomal membrane and possesses lateral mobility, which largely depends on fluidity of the membrane (29). Exogenous supplementation of different doses of GTE to the DOX treated rats resulted in an increase in the cytochrome P_{450} contents. This may be attributed to the decreased activity of hemoxygenase, which in turn increased the cytochrome content. Moreover, reduction in the formation of lipid peroxides may likely to contribute to increment in detoxification process (30).
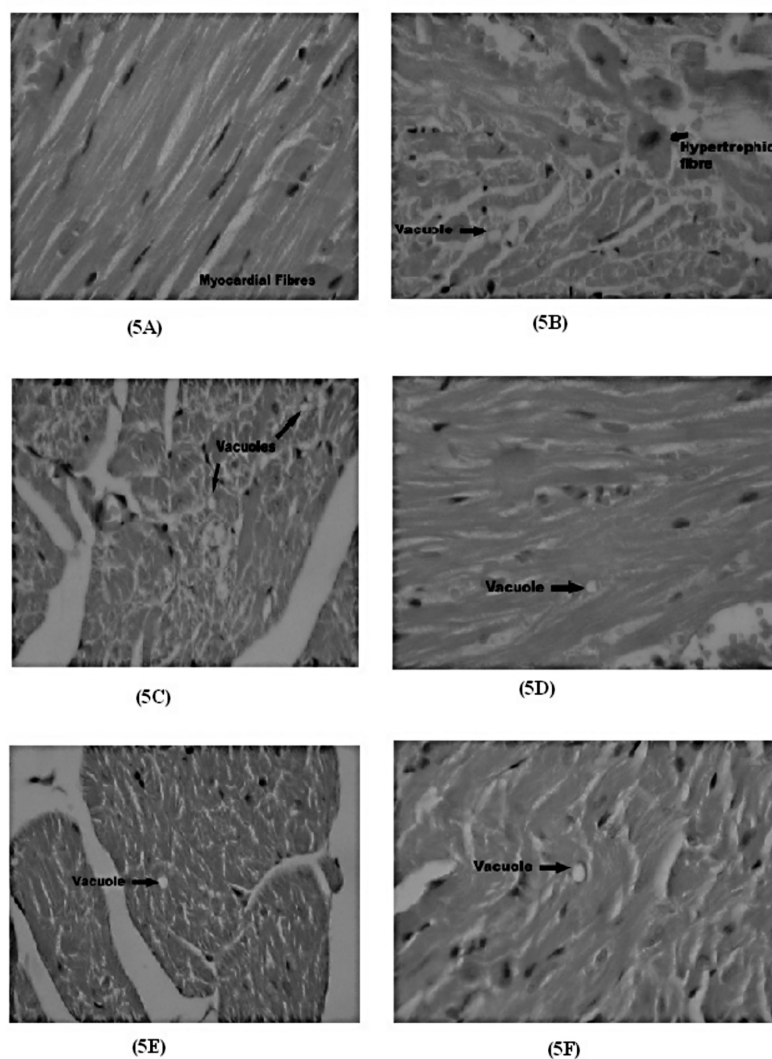

Figure 5. Hematoxylin and eosin-stained sections of rat heart, which were examined under high power (400×) of light microscope: (5A) Represents normal control rat showing normal myocardial fibers, no vacuolation, necrosis or inflammation. (5B) represents DOX alone treated rats, showing a large and irregularly shaped hypertrophic myocardial fiber with other fibers in the vicinity showing small and large vacuoles. (5C) represents GTE (100 mg/kg *p.o.*) + DOX treated rats, showing scattered vacuoles in the myocardial fibres. (5D) represents GTE (200 mg/kg *p.o.*) + DOX treated rats, showing cardiac muscle fibers of normal shape, size and configuration. A single myocardial fiber with an intracytoplasmic vacuole is seen in the photograph. (5E) represents GTE (400 mg/kg *p.o.*) + DOX treated rats, showing cardiac muscle fibers of normal shape, size and configuration. A single myocardial fiber with small intracytoplasmic vacuole is seen. (5F) represents Vit. E (100 mg/kg *p.o.*) + DOX treated rats, showing cardiac muscle fibers of normal shape, size and configuration. A single vacuole is seen in one of the myocardial fibers

Table 2. Effect of GTE on GPX, GR, GST, SOD and CAT in DOX treated rats.

Group	Treatment	GPX (nmol of NADPH oxidized/minute/mg protein)	GR (nmol of NADPH oxidized/minute/mg protein)	GST (nmol of CDNB conjugate/minute/mg protein)	SOD (U/mg protein)	CAT (nmol of H_2O_2 consumed/min/mg protein)
I	Normal control	143.56 ± 4.66	385.80 ± 5.75	140.10 + 4.07	9.61 + 0.26	28.40 + 0.47
II	DOX (20 mg/kg i.p.)	71.01 ± 3.54***	194.02 ± 5.0***	69.57 ± 2.71***	3.82 ± 0.14***	10.88 ± 0.43***
III	GTE (400 mg/kg p.o.)	152.40 ± 2.52	394.32 ± 6.92	148.38 ± 4.32	9.88 ± 0.10	30.95 ± 0.40
IV	Vit. E (100 mg/kg p.o.)	150.13 ± 4.55	393.52 ± 6.47	148.21 ± 4.25	9.56 ± 0.10	30.30 ± 0.69
V	GTE (100 mg/kg p.o) + DOX	89.33 ± 4.37#	238.11 ± 5.4##	92.61 ± 4.10##	4.76 ± 0.10##	13.55 ± 0.45#
VI	GTE (200 mg/kg p.o.) + DOX	101.26 ± 4.09###	301.05 ± 7.35###	108.19 ± 2.56###	6.46 ± 0.12###	17.27 ± 0.62###
VII	GTE (400 mg/kg p.o.) + DOX	117.35 ± 3.12###	340.33 ± 6.76###	121.02 ± 2.98###	7.92 ± 0.15###	23.34 ± 0.39###
VIII	Vit. E (100 mg/kg p.o.) + DOX	102.11 ± 3.26###	309.36 ± 7.98###	112.42 ± 2.84###	6.81 ± 0.084###	18.80 ± 0.47###

The data are expressed as the mean ± S.E.; n = 6 in each group. ***$p < 0.001$ compared with the corresponding value for normal control rats (group I). ###$p < 0.001$, ##$p < 0.01$, #$p < 0.1$ compared with the corresponding value for DOX treated rats (group II).

Oxidative stress is characterized by increased lipid peroxidation (LPO) and altered enzymatic and non-enzymatic antioxidant systems (31). In the present study, a significant increase in the level of LPO, in cardiac tissue of DOX alone treated rats was observed. Free radicals initiate LPO of biological membranes. GTE have been shown to neutralize reactive oxygen species, such as superoxide radical, singlet oxygen, hydroxyl radical, peroxyl radical, nitric oxide, nitrogen dioxide and peroxynitrite, thereby reducing the damage to lipid membranes, proteins and nucleic acids in cell-free systems. The assessment of the relative contribution of different pathways of free radical production elicited by DOX, is an important tool for toxicological mechanisms of these agents and for the implementation of adequate therapeutic approaches towards limiting their toxicological effects.

The glutathione antioxidant system plays a fundamental role in cellular defence against free radicals and other oxidant species (32). GSH plays a crucial role in both scavenging reactive oxygen species and the detoxification of the drugs. GSH with its –SH group functions as a catalyst for disulfide exchange reactions, and plays a major role in H_2O_2 detoxification. GSH depletion results in impaired cell defence and tissue injury. Following DOX administration, glutathione status get greatly impaired as indicated by a decrease in the GSH level in both blood and the heart. In this study, reduction in levels of MDA and elevation in GSH in GTE treated rats suggest that it scavenges free radicals, generated during oxidative stress (35).

In our study, the activity of all the antioxidant enzymes like: glutathione peroxidase, glutathione reductase, glutathione s-transferase, superoxide dismutase and catalase were significantly decreased in DOX alone treated rats. This result showed that DOX generates free radicals in heart and decreases its ability to detoxify ROS. However, GTE significantly increased the level of these antioxidant enzymes in DOX treated groups. The protection thus offered could be attributed to its antioxidant and ROS scavenging properties.

The histopathological changes of DOX induced cardiotoxicity, consist in order of increasing severity, swelling of sarcoplasmic reticulum, cytoplasmic vacuolization, myofibrillar degeneration, myocyte disruption and fibrosis (34–36). In our study, we have observed hypertropic fibre, disruption of myocyte structure, including damage to microtubules, vacuolization, dilation of sarcoplasmic reticulum, loss of myofibrils and alteration of mitochondrial functions such as decreased mito-

chondrial enzyme activities, which goes fine with the above finding. Rats pretreated with GTE showed cardiac muscle fibers of normal shape, size and configuration. Only a single vacuole is seen in one of the myocardial fibres. The protection exhibited by GTE as evident by the histopathological changes may be due to its antioxidant potential to counteract free radicals.

The overall protective effect of GTE is probably due to counter action of free radicals by its antioxidant nature hence its ability to restore normalcy in tissue under oxidative stress. However, the precise molecular mechanism by which GTE exerts its protective action against oxidative damage remains to be established. If this protective function is confirmed in cancer patients, GTE may have an important clinical significance as an adjuvant therapy with DOX.

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