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Role of green tea extract against doxorubicin-induced cardiotoxicity in rats

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ABSTRACT

Tea consumption leads to a significant increase in the antioxidant capacity of blood. This study was aimed to evaluate the role of green tea extract (GTE) in Doxorubicin (DOX) induced myocardial infarction (MI). In this study 64 albino Wistar rats weighing between 120-150g were distributed into eight groups comprising of eight animals in each group. Animals were sacrificed on 31st day and estimation of Lactate dehydrogenase (LDH), and Creatinine phosphokinase (CPK) were estimated in blood and Thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), and Superoxide dismutase (SOD) were estimated in heart tissue. Histopathological studies were performed for the heart tissue of all the groups. Group II showed a significant increase in activity of marker enzymes such as CPK, LDH in blood, and a decrease in GSH and SOD with the increase in TBARS, in the heart tissue. Group V-VIII however showed reversal of this effect, as evident by the significant increase in the activities of marker enzymes, improvement of the antioxidant status by decrease in lipid peroxidative products and increase in the antioxidant enzymes and non-enzymatic antioxidant. Group III and IV however showed no significant effects. Among the three doses of GTE, 200 mg/kg was found to be more pronounced and was similar to that of Vit. E (100mg/kg) taken as a standard drug. The result of our study thus shows the preventive role of GTE in DOX- induced MI in rats.

KEYWORDS:

Green tea extract, Doxorubicin, Myocardial infarction, Antioxidants, Lipid peroxidation.

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INTRODUCTION

Cardiovascular diseases (CVDs) are responsible for 16.7 million or 29% of total global death per year according to World Health Report 2003. One of the CVDs, namely coronary (or ischemic) heart disease, is the dominating cause of disability and death in all industrialized nations. In India there is a great increase in the incidence of myocardial infarction (MI)[1]. The mortality rate with the acute infarction is approx 30% with more than half of these deaths occurring before the patient reaches hospital. Although the mortality rate after admission for MI has declined by about 30% over two decades, approximately 1 out of every 25 patient who survives the initial hospitalization, dies in the first year after MI[1].The reactive oxygen species produced due to ischemia/reperfusion injury causes damage to myocardial cells. There is increased production of lipid peroxidation and a transient inhibition of protective enzymes such as superoxide dismutase (SOD) in both MI and unstable angina. Therapeutic intervention that could diminish free radical production may be one of the treatments of MI[2]. Currently there is an increasing realization that herbs can influence the course of heart disease and its treatment. Recently, several plants of Indian origin have been found to possess anti-oxidant properties and beneficial effects in pathological conditions like atherosclerosis, ischemia, cancer, and liver dysfunction[3]. This study thus attempts to evaluate the cardioprotective effect of Green Tea (*Camellia sinensis*) Extract (GTE) with reference to Vit. E on Doxorubicin induced cardiomyopathy in rats. Green Tea is an important medicinal plant having strong anti-oxidant properties[4], however, its cardioprotective activity against Doxorubicin induced MI has not yet been studied. Green tea contains high levels of polyphenols. Polyphenols from green tea are efficient free radical and singlet oxygen scavengers that inhibit lipid peroxidation in *in vitro* systems, in experimental animals, and in humans[5]. Green tea has been receiving much attention because it contains useful compounds like polysaccharides, flavonoids, vitamin B-complex, vitamin C and fluoride in its natural state. The tea leaf contains as a main component (over 30% of the dry weight) a powerful anti-oxidant, the polyphenol epigallocatechin gallate (EGCG). The leaf also contains the enzyme polyphenol oxidase. Immediate heating of the harvested leaves inactivate the enzyme; the result is green tea, containing mainly EGCG. Green tea mainly contains polyphenols such as catechins, flavonols, flavandiols and phenolic acids[4,6,7].

We have taken Vit. E as a standard drug against Doxorubicin induced myocardial infarction in rats. Vit. E is the collective name of tocols and tocotrienol derivatives that represent α-tocopherol activity.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Male Wistar rats weighing (150-200 g) were used for this study. They were kept in the animal house (Singhania University, Jhunjhunu, Rajasthan, India) for one week for proper acclimatization before starting the experiment under controlled condition of illumination (12 h light and dark cycles) and temperature 20-25 ^{0}C. 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 Institutional Animal Ethics Committee, Singhania University, Jhunjhunu, Rajasthan, approved the experimental study (Letter No. SU/IAEC/PHD/PH.COLOGY/07/2008-10).

DRUGS AND CHEMICALS

Doxorubicin was purchased from Sigma Aldrich, USA. Green tea extract (GTE) was obtained from Sanat Products Ltd., India. Vitamin E was obtained from Himedia, India. All the diagnostic kits were procured from Span diagnostics, Surat and Reckon diagnostics, Baroda, India. All the other chemicals used were of analytical grade.

EXPERIMENTAL DESIGN

In this experiment, a total of 64 Wistar Albino rats were used. The rats were divided into eight groups comprising of eight animals in each. Groups were distributed as follows:

Group I: Normal control rats, received saline (1ml/kg, p.o) for 30 days. Group II: Toxic control rats, received Doxorubicin (85 mg/kg, s.c) twice at an interval of 24 hrs starting on 29th day. Group III: GTE control rats, received green tea extract (200 mg/kg/day, p.o.) for 30 days. Group IV: Vitamin E control rats, received Vit. E (100 mg/kg/day, p.o.) for 30 days. Group V: GTE treated-1 rats, received green tea extract (50 mg/kg/day, p.o.) for 30 days + DOX (20 mg/kg i.p.) once on 29th day. Group VI: GTE treated-2 rats, received green tea extract (GTE 100 mg/kg/day, p.o.) for 30 days + DOX (20 mg/kg i.p.) once on 29th day. Group VII: GTE treated-3 rats, received green tea extract (GTE 200 mg/kg/day, p.o. for 30 days + DOX (20 mg/kg i.p.) once on 29th day. Group VIII: Vit. E treated rats, received (Vit. E 100 mg/kg/day, p.o. for 30 days + DOX (20 mg/kg i.p.) once on 29th day.

Doxorubicin in normal saline was administered intraperitoneally (i.p) on 29th day of pretreatment with GTE and estimated after 48 hrs of first dose of DOX. On 31st day blood samples were collected for

biochemical estimations. Later the animals were sacrificed and hearts were removed, cleaned and washed with ice-cold saline for biochemical estimations.

BIOCHEMICAL ESTIMATIONS

LACTATE DEHYDROGENASE (LDH) ESTIMATION

The activity of lactate dehydrogenase was assayed by the method of Lum and Gambino (1974). LDH catalyses the conversion of lactate to pyruvate and the amount of pyruvate formed are measured at 340 nm.

CREATININE PHOSPHOKINASE (CPK) ESTIMATION

CPK was estimated by the method of Rosalki (1967), in this reaction creatine kinase (CK) catalyses the formation of ATP from creatine phosphate and ADP. Glucose is converted to Glucose-6-phosphate by hexokinase using ATP as source of phosphate moiety. Glucose-6-phosphate is oxidized by Glucose-6-phosphate dehydrogenase to 6-phosphogluconate reducing NADH to NADPH. The reaction after the lag phase is monitored by the increase in absorbance at 340 nm and is directly proportional to the creatine kinase activity (i.e. the formation of NADPH is in equimolar amount as that of formation of creatine).

POST-MITOCHONDRIAL SUPERNATANT PREPARATION (PMS)

Heart was removed quickly, perfused immediately with ice cold normal saline and homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing potassium chloride (1.17%) using a Potter Elvehjem homogenizer. The homogenate was centrifuged at 800 × g for 5 min at 4^{o}C in a refrigerated centrifuge to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500 × g for 20 min at 4^{o}C to get the PMS which was used to assay GSH, TBARS, SOD and CAT activity.

DETERMINATION OF GSH

GSH content was estimated by method of Sedlak and Lindsay (1968). The tissues were homogenized in 0.02M EDTA. Aliquots of 5 ml of the homogenates were mixed in test tube with 4 ml of cold distilled water and 1 ml of 50% TCA. The tubes were shaken for 10 minutes using vortex mixer and the centrifuged at 1200 × g for 15 minutes. Following centrifugation 2 ml of supernatant was mixed with 4 ml of 0.4 M Tris buffer (pH–8.9). The whole solution was mixed and 0.1 ml of 0.01 M DTNB {5, 5'-Dithiobis (2-nitrobenzoic acid)} was added to it. The absorbance was read within 5 minutes of

addition of DTNB at 412 nm using UV-spectrophotometer (Shimadzu, UV-1601, Japan) against a reagent blank with no homogenate.

DETERMINATION OF LPO

LPO was estimated by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), a product formed due to the peroxidation of membrane lipids (Ohkawa et al., 1979). Tissues were homogenized in chilled phosphate buffer (0.1M, pH 7.4) that contained KCl (1.17% w/v), using motor driven Teflon pestle. Aliquot of 1 ml of the suspension medium was taken from the supernatant obtained after the centrifugation of tissue homogenate (10% w/v) at 10,500 × g. 0.5 ml of 30% TCA followed by 0.5 ml of 0.8% TBA was then added to it. The tubes were kept in shaking water bath for 30 minutes at 80^{o}C. After 30 minutes of incubation tubes were taken out and kept in ice cold water for 10 minutes. These were then centrifuged at 800 × g for 15 minutes. The absorbance of supernatant was read at 540 nm at room temperature against appropriate blank. The concentration of MDA was measured from the standard calibration curve prepared by using tetraethoxypropane. Protein was estimated by the method of Lowry et al. (1951). Lipid peroxidation was expressed as n moles of MDA per milligram of protein.

DETERMINATION OF SOD ACTIVITY

SOD activity was measured according to the method of Marklund (1974). The enzyme activity was expressed as units mg^{-1} protein and one unit of enzyme is defined as the enzyme activity that inhibits autoxidation of pyrogallol by 50%.

STATISTICAL ANALYSIS

Data were expressed as the mean ± standard error (S.E) of the means. For a statistical analysis of the data, group means were compared by one-way analysis of variance (ANOVA) with post hoc analysis. The Tukey-Karmer post hoc test was applied to identify significance among groups. $P<0.05$ was considered to be statistically significant. Graph Pad software, Inc. (version 3.06) was used for statistical analysis.

RESULTS

EFFECT OF GREEN TEA EXTRACT ON DOXORUBICIN-INDUCED LDH ACTIVITY

The LDH activity in Group II rats were found to be significantly high when compared with Group I rats ($P<0.001$). Group V ($P<0.01$) and Group VI, VII, VIII ($P<0.001$) showed a significant decrease in

LDH activity when compared with group II. Group VI showed a significant decrease in LDH activity when compared with group V (P<0.001). Group VII showed a significant decrease in LDH activity when compared with Group VI (P<0.001). Other groups (group III and IV) however showed no significant change when compared with Group I.

EFFECT OF GREEN TEA EXTRACT ON DOXORUBICIN-INDUCED CPK ACTIVITY

The CPK activity in Group II rats were found to be significantly high when compared with Group I (P<0.001). Group V (P<0.05) and Groups VI, VII, VIII (P<0.001) showed a significant decrease in CPK activity when compared with Group II. Group VI showed a significant decrease in CPK activity when compared with Group V (P<0.001). Group VII showed a significant decrease in CPK activity when compared with Group VI (P<0.001). The test drug treated Groups (III and IV) showed the same activity as found in Group I.

Table 1- Effect of green tea extract (GTE) on blood lactate dehydrogenase (LDH) and creatinine phosphokinase (CPK) in normal and doxorubicin (DOX) induced myocardial infarction in rats

Group	Treatment	LDH (IU/L)	CPK (IU/L)
I	Normal control	422.63±19.90	142.29± 5.81
II	Doxorubicin (20 mg/kg i.p.)	986.27±25.21[a]	297.68±7.97[a]
III	GTE (200mg/kg/day, p.o.), per se	414.58± 18.70	134.66±4.97
IV	Vit. E (100mg/kg/day, p.o.), per se	421.08 ±17.54	139.85±5.37
V	GTE (50mg/kg/day, p.o.) + Doxorubicin (20 mg/kg i.p.)	954.42± 20.83 **	292.13±6.53 *
VI	GTE (100mg/kg/day, p.o.) + Doxorubicin (20 mg/kg i.p.)	895.63 ±23.33*** ##	275.63±7.25*** ##
VII	GTE (200mg/kg/day, p.o.) + Doxorubicin (20 mg/kg i.p.)	611.45± 24.36*** ## b	221.49±4.54*** ## b
VIII	VitaminE (100mg/kg/day, p.o.) + Doxorubicin (20 mg/kg i.p.)	629.57± 22.95***	230.22±5.17***

a : (p<0.001) when compared to Group I. * : (p<0.05), ** : (p<0.01), *** : (p<0.001) when compared to Group II.

b : (p<0.001) when compared to group VI. # : (p<0.01), ## : (p<0.001) when compared to Group V.

1. Each group contains eight animals. 2. Data shown in mean ± SD. 3. Data is analyzed with ANOVA followed by LSD.

EFFECT OF GREEN TEA EXTRACT ON DOXORUBICIN-INDUCED TISSUE GSH LEVELS

The tissue GSH levels of Group II showed a significant decrease when compared with Group I (P<0.001). Group V (P<0.01) and Group VI, VII, VII (P<0.001) showed a significant increase in tissue GSH levels when compared with Group II. Group VI showed a significant increase in tissue GSH levels when compared with Group V (P<0.001). Group VII showed a significant increase in tissue GSH levels when compared with Group VI (P<0.001). Other groups showed the same level as that of Group I.

Table 2- Effect of green tea extract (GTE) on heart glutathione (GSH), thiobarbituric acid reactive substances (TBARS) and superoxide dismutase (SOD) in normal and doxorubicin (DOX) induced myocardial infarction in rats

Group	Treatment	TISSUE GSH (ig/g wet wt. tissue)	TBARS (çmoles MDA/mg protein)	SOD (U/mg protein)
I	Normal control	578.37 ± 8.97	2.10 ± 0.07	1.93 ± 0.07
II	Doxorubicin (20 mg/kg i.p.)	331.13 ± 10.88 [a]	4.41 ± 0.11 [a]	0.98 ± 0.05 [a]
III	GTE (200mg/kg/day, p.o.), per se	2.18 ± 0.05	2.02 ± 0.06	2.01 ± 0.10
IV	Vit E (100mg/kg/day, p.o.), per se	580.36 ± 12.61	2.08 ± 0.07	1.97 ± 0.11
V	GTE (50mg/kg/day, p.o.) + Doxorubicin (20 mg/kg i.p.)	366.36 ± 8.12 *** #	4.24 ± 0.06 **	1.17 ± 0.04 ***
VI	GTE (100mg/kg/day, p.o.) + Doxorubicin (20 mg/kg i.p.)	636.36 ± 8.12 ***	4.24 ± 0.06 **	1.17 ± 0.04 ***
VII	GTE (200mg/kg/day, p.o.) + Doxorubicin (20 mg/kg i.p.)	477.38 ± 11.35 *** ## b	2.81 ± 0.08 *** ## b	1.53 ± 0.06 *** ## b
VIII	Vit. E (100mg/kg/day, p.o.) + Doxorubicin (20 mg/kg i.p.)	434.52 ± 7.64 ***	3.12 ± 0.10 ***	1.52 ± 0.07 ***

a : (p<0.001) when compared to Group I. *: (p<0.05), ** : (p<0.01), *** : (p<0.001) when compared to Group II.

b : (p<0.001) when compared to group VI. #: (p<0.01), ##: (p<0.001) when compared to Group V.

1. Each group contains eight animals. 2. Data shown in mean ± SD. 3. Data is analyzed with ANOVA followed by LSD.

EFFECT OF GREEN TEA EXTRACT ON DOXORUBICIN-INDUCED TISSUE TBARS LEVELS

The TBARS concentration of Group II showed a significant increase when compared with Group I (P<0.001). Group V (P<0.05), Group VI (P<0.01) and Group VII (P<0.001) showed a significant decrease in TBARS concentration when compared with Group II. Group VI did not show any significant decrease in TBARS concentration when compared with Group V, but Group VII showed a significant decrease when compared with Group VI (P<0.001). The test drug treated groups however showed the same level as that of Group I.

EFFECT OF GREEN TEA EXTRACT ON DOXORUBICIN-INDUCED TISSUE SOD ACTIVITY

The SOD activity showed a significant decrease in Group II when compared with Group I (P<0.001). Group V (P<0.01) and Group VI, VII, VIII (P<0.001) showed a significant increase in SOD activity when compared with Group II. Group VI did not show any significant increase in SOD activity when compared with Group V, however, Group VII showed a significant increase in SOD activity when compared with Group VI(P<0.001).Per se group showed the same levels when compared with Group I.

EFFECT OF GREEN TEA EXTRACT ON DOXORUBICIN-INDUCED HISTOLOGICAL CHANGES ON HEART

The histopathological observations are depicted in Figures 1-8. Figure 1 shows Group I or physiological saline treated section revealing normal myocardium while Figure 2 i.e. Doxorubicin treated heart section depicts interstitial polymorphonuclear infiltrate, edema and focal myocardial necrosis. Figure 3 reveals Group III i.e. GTE 200 mg/kg treated heart section showing morphologically normal myocardium which acts as control. Figure 4 i.e. Vit. E 100mg/kg treated section shows morphologically normal myocardium. Figure 5 which is GTE 50 mg/kg + Doxorubicin treated section shows myocardial necrosis, disruption of architecture, interstitial edema and inflammatory cell infiltrate revealing very less efficacy at this dose level. Figure 6 or Group VI (GTE 100 mg/kg + Doxorubicin) heart section depicts mild interstitial edema and moderate amount of inflammatory cell infiltrate mild degeneration of myocardium. However, figure 7 shows Group VII i.e. GTE 200mg/kg + Doxorubicin treated section reveals myocardium of nearly normal appearance with very mild inflammatory cell infiltrate and absence of necrosis. Also, figure 8 i.e. Vit. E 100mg/kg + Doxorubicin

treated one shows presence of moderate amount of interstitial inflammatory cell infiltrate, focal necrosis and edema.

Figure 1

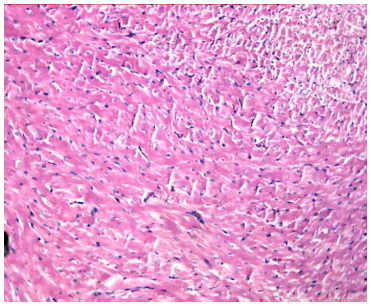

Figure 2

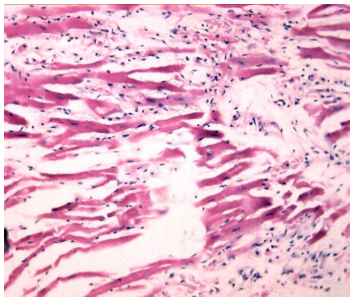

Figure 3

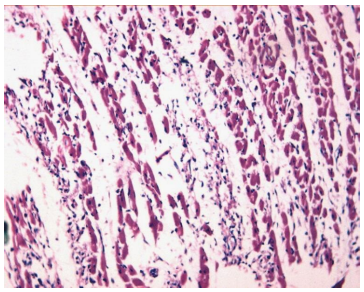

Figure 4

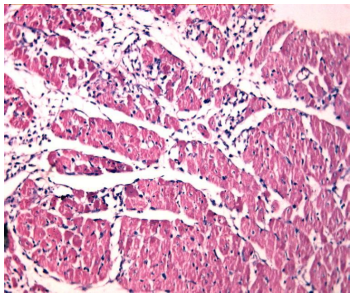

Figure 5

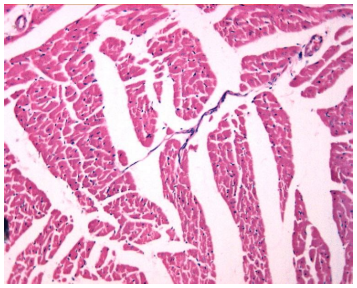

Figure 6

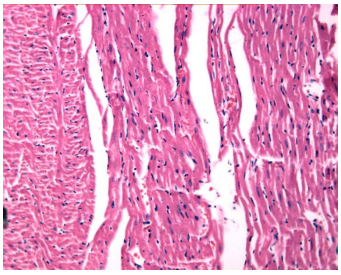

Figure 7

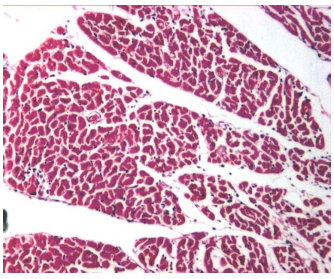

Figure 8

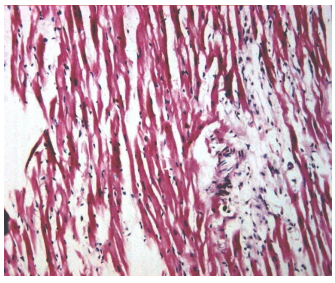

Figure 1-8: Histopathological observations on heart tissue

DISCUSSION

In a variety of *in vitro* and *in vivo* studies, green tea polyphenols were found to scavenge NO, H_2O_2, $^{\cdot}OH^-$ and O_2^- and reduce oxygen free radical damage[4,8-11]. It has been proposed that catechin polyphenols reacts with peroxy radicals involving termination of radical chain reaction[12,13]. Tea flavonoids are potent antioxidant that is absorbed from the gut after consumption. The fact that, catechins are rapidly and extensively metabolized during absorption in the small intestine, colon and liver, emphasizes the importance of demonstrating their antioxidant activity *in vivo*[14-16]. In animal model of atherosclerosis, green tea administration has resulted in modest improvement in the resistance of lipoproteins to *ex vivo* oxidation[15]. One mechanism that might explain a beneficial effect of tea on the cardiovascular system is that it improves the vascular endothelium function[17]. Doxorubicin induces myocardial necrosis, which is maximal in the subendothelial region of the left ventricle and interventricular septum. These changes resemble the subendothelial necrosis produced by myocardial necrosis produced in human[18]. Amongst the various mechanisms proposed to explain Doxorubicin induced cardiac damage, generation of highly cytotoxic free radicals through auto-oxidation of catecholamine has been implicated as one of the most causative factor. This free radical mediated peroxidation of membrane phospholipids and consequent changes in membrane permeability as well as intracellular Ca^{2+} overload is the primary target responsible for cardiotoxicity induced by Doxorubicin[19-21]. An increase in the activities of marker enzymes (CPK, LDH) in the serum on treatment with Doxorubicin could be due to the leakage of enzymes from heart as a result of necrosis and the amount of enzymes appearing in serum is in proportion to the number of necrotic cells[22,23].

In this study, Doxorubicin administration is found to reduce the GSH content of heart. It also reduces the antioxidant enzyme (SOD) activity in cardiac tissue, which is well in accordance with the earlier finding[24]. The cytotoxic free radicals generated by Doxorubicin, cause the loss of membrane integrity by initiating the lipid peroxidation of membrane bound polyunsaturated fatty acids[20]. In this case we also have observed an increase in the levels of TBARS in the heart tissue after Doxorubicin administration. The result of histopathology further confirms the Doxorubicin induced myocardial damage. The DOX treated group (Group II, Fig 2) demonstrated interstitial polymorphonuclear infiltrate, edema and focal myocardial necrosis as compared to normal control group (Group I, Fig 1). The treatment with GTE 50 mg/kg (Group V, Fig 5) did not demonstrate the reversal of myonecrosis, edema and inflammatory cell infiltration, which have been seen with DOX, treated group (Group II). However, the treatment with GTE 100 mg/kg (Group VI, Fig 6) and GTE 200 mg/kg (Group VII, Fig 7) groups did demonstrate the reversal of the conditions, which were seen with DOX, treated group

(Group II). Furthermore, between these two doses GTE 200 mg/kg treated group (Group VII) have shown better restoration or preservation of myocardium. Vitamin E 100mg/kg treated group (Group VIII, Fig 8) have only demonstrated mild reversal of myonecrosis, edema and inflammatory cell infiltration as seen with DOX treated group (Group II). The only test drugs treated groups, i.e., Group III (Fig 3) and Group IV (Fig 4), however, have shown the same myocardial status as that of the control. Thus, the histopathological results show myocardial damage by Doxorubicin and its prevention by GTE.

Chronic oral administration of GTE in all the doses prevented Doxorubicin induced myocardial injury at different levels. Myocardial activity of antioxidant enzyme shown to be preserved in all the three doses but the activity was more significant in dose 200 mg/kg when compared to 50 mg/kg groups. GTE at doses 100 and 200 mg/kg were shown to increase the myocardial GSH levels, hence GTE at these doses showed significant protection in terms of preservation of endogenous antioxidant system. The increase in endogenous antioxidant activity leads to decrease in lipid peroxidation of membrane phospholipids which is shown in this study by reduced myocardial TBARS concentration in all the three doses of GTE treated groups. GTE at a dose of 200 mg/kg showed significant inhibitory effect on lipid peroxidation when compared to groups treated with the dose 50 and 100 mg/kg. A significant reduction in the activities of marker enzymes LDH, CPK) in serum is indicative of the fact that green tea has cardioprotective action and maintains membrane integrity of myocytes. In the present study, chronic oral administration of Vit. E, taken as reference drug, attenuated the increase in TBARS level in myocardium upon Doxorubicin administration. α- tocopherol, being a lipid soluble chain breaking antioxidant reacts with O_2^- and lipid peroxy radicals, thereby inhibiting lipid peroxidation. Vitamin E increased the myocardial antioxidant enzyme and GSH levels in myocardium. The significant reduction in activities of marker enzymes (LDH, CPK) in serum in Vitamin E treated group suggests that it has cardioprotective action and maintain the membrane integrity of myocytes. The cardioprotective effect of Vit. E is well known in Doxorubicin induced myocardial infarction and the results obtained in this study are consistent with the earlier findings[25].

In summary, the results of this study based on biochemical and histopathological observations, indicate the protective role of GTE against Doxorubicin induced MI. Although the protection is seen with all the three doses, i.e., 50 mg/kg, 100 mg/kg and 200 mg/kg body weight, the best protective effect is indicated by 200 mg/kg dose.

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