GREEN TEA EXTRACT PROTECTS DOXORUBICIN INDUCED TESTICULAR DAMAGE IN RATS

Leena J Patil[1]*, Balaraman R[2].

[1]*Dayananda Sagar College of Pharmacy, Shavige Malleshwara Hills, Kumarswamy Layout,Bangalore-560 078, Karnataka.
[2]Pharmacy Department, Faculty of Technology and Engineering, The M.S. University of Baroda, Baroda-390 001, Gujarat, INDIA.

Summary

Green tea extract was administered to rats (100 mg/kg/day p.o. for 28 days) along with doxorubicin (3mg/kg i.p. on day 1, 7, 14, 21, 28). Treatment with doxorubicin alone caused decrease in body weight, sperm count, serum testosterone and also reduction in the levels of antioxidant enzymes such as in superoxide dismutase, catalase and reduced glutathione, membrane bound enzymes like $Na^{+}K^{+}$ATPase, Ca^{2+}ATPase, Mg^{2+}ATPase and increase in serum levels of lactate dehydrogenase, creatine phosphokinase, glutamic oxaloacetate transaminase and lipid peroxidation. However, the combined treatment of green tea extract with doxorubicin restored the body weight, sperm count, testosterone level, lipid peroxidation, histopathological changes and serum markers of toxicity, with significant increase in levels of antioxidant enzyme and membrane bound enzymes in testes, indicating protection afforded by green tea extract administration. These findings indicate that green tea extract might be having protective effect against doxorubicin induced testicular toxicity.

Keywords: Green tea extract, Doxorubicin, Antioxidant, Testicular toxicity, Sperm count

Correspondence Address: Dr. Leena J. Patil,
Dayananda Sagar College of Pharmacy,
Shavige Malleshwara Hills, Kumarswamy Layout,
Bangalore-560 078, Karnataka
E mail: leenapatil27@yahoo.com
Telephone: 08026665460

1. Introduction

Doxorubicin, an anthracycline antibiotic, is a widely used anticancer agent. Inspite of its high antitumor efficacy, the use of doxorubicin in clinical chemotherapy is limited due to diverse toxicities, including testicular toxicity (1-5). Although a number of potential toxic mechanisms have been identified following exposure to doxorubicin, the major pathogenic mechanism appears to involve the generation of toxic reactive oxygen species(6). Doxorubicin induced toxicity has been alleviated by administering various natural and artificial compounds (7-11). Polyphenols are plant metabolites occurring widely in plant food and possess outstanding antioxidant and free radical scavenging properties (12, 13) .Green tea is an excellent source of polyphenol antioxidants, particularly of group known as green tea catechins (GTCs)(14). Green tea reduces iron-induced lipid peroxidation in brain homogenates as well as in cultured C_6 astrocytes and lung cells(15,16). In addition, green tea has also been shown to reduce the formation of the spin-adducts of hydroxyl radicals and hydroxyl radical to induced DNA strand breakage *in vitro* (17). Green tea has been found to have inhibitory effects on the chemical-induced lung tumorigenesis(18). There is also considerable epidemiological evidence suggesting that the consumption of green tea lowers the risk of heart disease as well as several types of cancer incidences as a result of these antioxidant mechanisms (19). Damage to the testicular germinal epithelium is a potential side effect of cancer therapy, and is of particular concern in case of men of reproductive age having tumors with high cure rates (20). Therefore, the aim of present study was to evaluate the protective effect of green tea extract (GTE) on doxorubicin induced testicular damage by using serologic, histopathologic, and biochemical analyses.

2. Material and methods

2.1. Chemicals

Standardized powdered, ethyl acetate extract of green tea leaves (C*amellia sinensis*) was gift sample from Cherain Chemicals, Baroda, India with total polyphenolic content 35%. Doxorubicin injection was gift sample from Serum Institute of India Ltd., Pune. Super oxide dismutase, malondialdehyde, catalase standared were purchased from Sigma Aldrich; USA. Reduced glutathione, 5, 5'Dithiobis (-2 nitrobenzoic acid) thiobarbituric acid from Hi Media; India. All other chemicals were of analytical grade.

2.2. Animals

Adult male rats (Wistar strain) weighing between 200 and 250 g were used for the study. The animals were fed ad libitum with standard pellet diet and had free access to water. All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of M. S. University, Baroda and are in accordance with guidelines as per "Guide for the care and use of laboratory animals" published by NIH publication (NO 85-23 revised 1996) and with permission from

Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

2.3. Experimental Protocol

2.3.1 Chemical analysis of green tea extract

TLC fingerprint profile of the extract was established using HPTLC. For development of TLC fingerprint, 500 mg of powdered green tea extract was extracted with (3x25 ml) of methanol. Extracts were pooled, filtered and concentrated to 25 ml. Suitably diluted stock solution of methanolic extract with gallic acid standard solution and catechin were spotted on a pre-coated Silica gel G60 F254 TLC plate (E.Merck) using CAMAG Linomat IV Automatic Sample Spotter and the plate was developed in the solvent system of Toluene: Ethyl acetate: Formic acid (6: 6: 1). The plate was dried at room temperature and scanned using CAMAG TLC Scanner 3 at UV 254 nm and R_f values, and peak area of the resolved bands were recorded. Relative percentage area of each band was calculated from peak areas. The TLC plate was developed by spraying with 5% methanolic ferric chloride solution for the detection of phenolic compounds.

2.3.2. Groups and Treatment Schedule

Powdered green tea extract was reconstituted in distilled water. Doxorubicin injection was dissolved in sterile water for injection. The animals were divided into four groups each consisting of six rats and received following treatment

Group I (Control): received distilled water (3ml/kg /day p.o. for 28 days) and sterile water for injection (1ml/kg, i.p.) on day 1, 7, 14, 21, 28.
Group II (DOX): Doxorubicin injection (3 mg/kg i.p.) on day 1, 7, 14, 21, 28.
Group III (DOX + GTE): Green tea extract (100mg/kg /day p.o. for 28 days) and doxorubicin injection (3 mg/kg i.p.) on day 1, 7,14,21,28.
Group IV (GTE): Green tea extract (100mg/kg /day p.o.) for 28 days.
After 48 hours of the last injection of either doxorubicin or vehicle, blood was collected for serological analyses. Epididymis was removed, cleared off the adhering tissues and weighed. The epididymal sperm count was done immediately. The testes was excised under euthanasia in chilled Tris buffer (10 mM pH 7.4) for measurement of tissue markers of oxidative stress the other one was collected for histopathology.

Epididymal sperm count: Epididymal sperm was collected by slicing the epididymis in 5 mL phosphate buffered saline (pH 7.2). An aliquot of the epididymal sperm suspension was used for spermatozoa count using Neubauer hemocytometer(21,22).
Serological analyses: Serum levels of lactate dehydrogenase (LDH) and serum creatine kinase (CK) were determined by using standard kits of Reckon Diagnostic Ltd, India while glutamic oxaloacetate transaminase (SGOT) was estimated by using standard kit of Span Diagnostic Pvt Ltd, India. Testosterone level was estimated by direct chemiluminescent assay (ADVIA CENTAUR).

Biochemical analyses: The excised testes was then weighed and homogenized in chilled Tris buffer (10 mM, pH 7.4) at a concentration of 10% (w/v). The homogenates were centrifuged at 10,000×*g* at 0° C for 20 min using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the assays of malondialdehyde content as indicator of lipid peroxidation (LP) (23)**,** endogenous antioxidant enzymes, superoxide dismutase (SOD)(24), catalase (CAT) (25) and reduced glutathione (GSH)(26). The sediment after centrifugation of tissue homogenate was resuspended in ice-cold Tris buffer (10 mM, pH 7.4) to get a final concentration of 10% and was used for the estimation of different membrane bound enzymes such as Na^+K^+ATPase(27), Ca^{2+}ATPase (28) and Mg^{2+}ATPase(29)and Total proteins(30).

Histopathologic examination: For histotological evaluation, the testes were fixed in 10% formalin, dehydrated and embedded in paraffin. Tissues were then sectioned at 4 μm, stained with haematoxylin and eosin (H&E) and examined for histopathological evidence under Olympus BX40 Photomicroscope.

2.4. Statistical analysis

Results of all the above estimations have been indicated in terms of mean ± S.E.M. Difference between the groups was statistically determined by analysis of variance (ANOVA) followed by Tukey –Kramer multiple Comparisons test with the level of significance set at $P \leq 0.05$.

3. Results

3.1. Chemical Analysis*:* The fingerprint chromatograms are shown in Fig. 1. Details of the fingerprint analysis are given in Table 1.

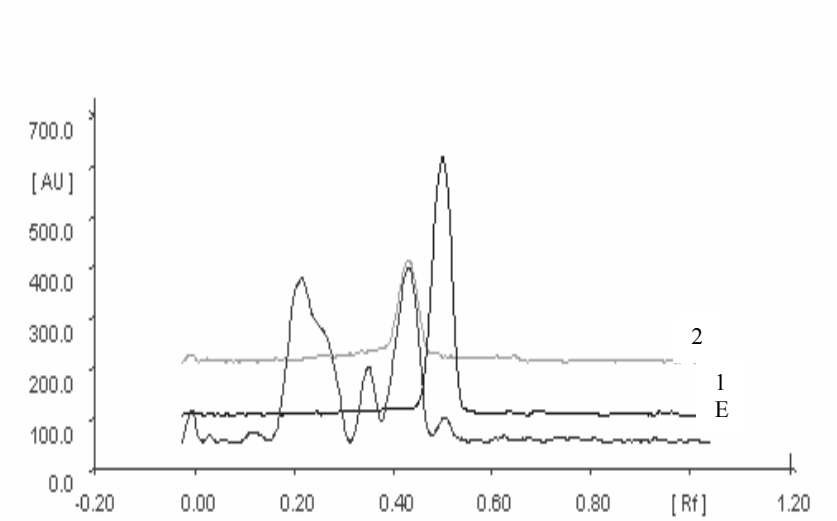


Table 1: Details of fingerprint chromatograms of green tea extract after scanning at 254 nm

Extract	Solvent system	No. of spots
Methanolic extract	Toluene: Ethyl acetate : Formic acid (6 : 6 : 1).	8
Rf values	0.03, 0.12, 0.22, 0.35, 0.43, 0.50, 0.63, 0.68	
Relative %	3.30, 1.84, 33.03, 15.11, 35.09, 4.99, 1.27, 1.05	

3.2. Body weight, testes weight and sperm count: All animals survived the experimental period. Administration of doxorubicin alone significantly reduced body and testes weight as compared to control animals. Administration of green tea extract along with doxorubicin restored body and testes weight to normal. Administration of doxorubicin alone significantly decreased sperm count while it was significantly increased with green tea extract coadministration (Table 2).

Table 2: Effect of doxorubicin alone and along with green tea extract on body weight, testes weight and sperm count

Groups	Final body weight (BW, g)	Absolute testes weight (g)	Relative testes weight (per BW, %)	Sperm Count (x 10^6/mg epididymis)
Control (Group I)	227.5± 4.23	2 ± 0.085	0.87±0.041	15.7±0.47
DOX (Group II)	204.2±4.16*	1.5±0.081**	0.73±0.024*	11.38±0.91**
DOX+GTE (Group III)	208±7.06^{NS}	1.86±0.076*	0.9±0.049*	14.65±0.94*
GTE (Group IV)	216.6±4.04	1.93±0.088	0.88 ±0.023	16.1±0.93
P value	P=0.0201	P=0.0017	P=0.012	P=0.0016
F value	4.10	7.24	4.70	7.35

Values are expressed as mean ±SEM. Group II was compared with Group I. Group III was compared with Group II. *P<0.05, **P <0.01, ***P <0.001, **NS** = Non significant

3.3 Serological analyses: Administration of doxorubicin alone significantly increased serum level of CK, LDH and GOT and decreased the level of testosterone as compared to control rats. The administration of green tea extract along with doxorubicin significantly restored serum marker levels towards the control value (Table 3).

Table 3: Effect of doxorubicin alone and along with green tea extract on serum levels of LDH, CK-NAC, SGOT and Testosterone

Groups	LDH (U/L)	CK-NAC (U/L)	SGOT (U/ml)	Testosterone (ng/ml)
Control (Group I)	169.83 ± 4.62	231.16±12.68	32.33 ± 2.0	0.80±0.045
DOX (Group II)	610.33 ±77.66 ***	511.5±17.69 ***	102.05±5.86***	0.63±0.061NS
DOX+GTE (Group III)	307.16±18.04 ***	261.66±17.77***	41.94± 2.35***	0.81±0.021*
GTE (Group IV)	177.5 ± 6.02	240 ±16.32	31.33±2.4	0.86±0.049
P Value	P <0.0001	P <0.0001	P <0.0001	P=0.010
F Value	26.45	68.19	91.68	4.91

Values are expressed as mean ±SEM. Group II was compared with Group I. Group III was compared with Group II. *P<0.05, **P <0.01, ***P <0.001, **NS** = Non significant

3.3 Biochemical analyses: Administration of doxorubicin alone significantly increases LP while there was a significant decrease in GSH, SOD and CAT levels as compared to control rats. Administration of green tea extract along with doxorubicin significantly restored GSH, SOD, CAT and LP levels towards control value (Table 4). Administration of doxorubicin alone significantly decreased the levels of membrane bound enzymes like Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase as compared to control. Administration of green tea extract along with doxorubicin significantly restored membrane bound enzyme levels towards control value (Table 5).

Table 4: Effect of doxorubicin alone and along with green tea extract on biomarkers of the oxidative stress

Groups	LP (nmoles of MDA/ mg protein)	GSH (µg of GSH/ mg protein)	SOD (Units/ mg protein)	CAT (µmoles of H_2O_2 consumed/min/ mg protein)
Control (Group I)	1.22 ± 0.043	4.15±0.32	4.92 ± 0.97	7.29±0.77
DOX (Group II)	1.82 ± 0.10***	2.63±0.15***	2.55 ± 0.15*	3.90±0.27***
DOX+GTE (Group III)	1.39± 0.059**	3.98± 0.065**	4.58 ± 0.17*	6.03±0.44*
GTE (Group IV)	1.13 ± 0.084	3.99±0.25	4.75 ± 0.14	7.10±0.23
P Value	P <0.0001	P=0.00027	P=0.011	P=0.00024
F Value	15.05	10.20	4.79	10.39

Values are expressed as mean ±SEM. Group II was compared with Group I. Group III was compared with Group II. *P<0.05, **P <0.01, ***P <0.001, **NS** = Non significant

Table 5: Effect of doxorubicin alone and along with green tea extract on membrane bound enzymes

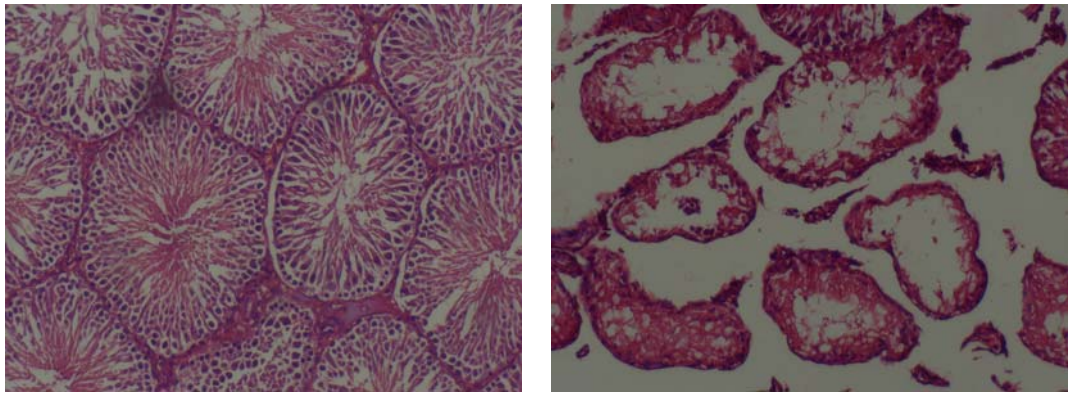
Groups	Na^+K^+ATPase (µmoles of inorganic phosphrous liberated /min /mg protein)	Ca^{2+}ATPase (µmoles of inorganic phosphrous liberated /min/ mg protein)	Mg^{2+}ATPase (µmoles ofinorganic phosphrous liberated / min/ Mg protein)
Control (Group I)	8.50±0.34	4.24±0.57	6.56±0.40
DOX (Group II)	5.01±0.30***	3.08±0.22NS	4.01±0.26***
DOX+GTE (Group III)	6.76±0.34**	3.71±0.30NS	5.25±0.22*
GTE (Group IV)	8.09±0.16	4.34±0.54	6.24±0.30
P Value	P <0.0001	P=0.19	P <0.0001
F Value	27.4	1.71	14.12

Values are expressed as mean ±SEM. Group II was compared with Group I. Group III was compared with Group II. *P<0.05, **P <0.01, ***P <0.001, **NS** = Non significant

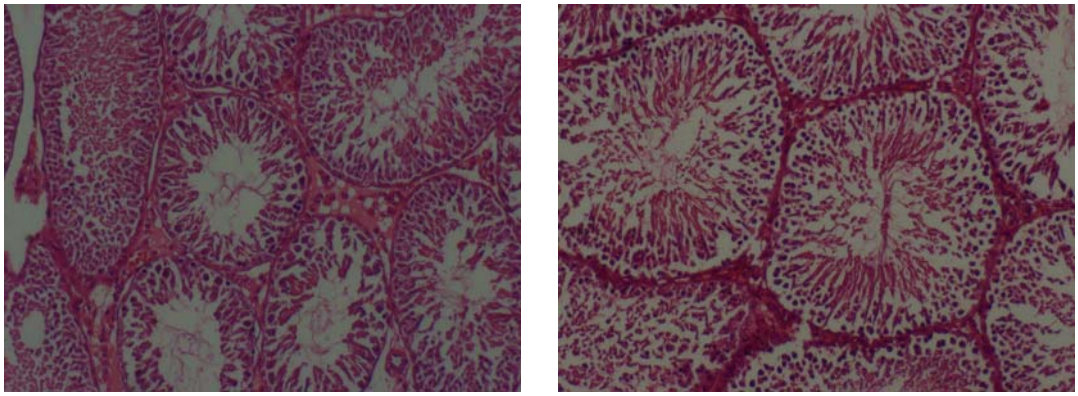
3.4. Histopathologic findings: Doxorubicin causes vacuolization and fibrinoid debris in the seminiferous tubules. Shrunken seminiferous tubules showed loss of germ cell. Widening of the interstitial space and severe vacuolization were also observed in interstitial tissues. Administration of green tea extract along with doxorubicin restored these changes towards normalcy (Fig.2).

Fig.2: Cross sections of testes in rats treated with doxorubicin and along with green tea extract.

A: Control **B:DOX**



C: DOX+GTE **D:GTE**



A: Control, **B**: DOX alone, **C**: DOX + GTE, **D**: GTE alone
Testes from control (A) and GTE treated rats (D) shows normal feature of seminiferous epithelium and interstitial tissue. However, testes from a doxorubicin treated rats (B) reveals markedly shrunken and empty seminiferous tubules. Administration of GTE along with doxorubicin (C) restored these changes towards normalcy.

4. Discussion

Many drugs used for cancer chemotherapy are known to produce toxic side effects in multiple organ systems including the testes. A strategy to diminish the side effects of anticancer drugs with preservation of their chemotherapeutic efficacy is necessary. Doxorubicin is known to disturb spermatogenesis in a dose-dependent manner in animal studies (3). With a low dose of doxorubicin, a significant but temporary reduction in spermatogenesis occurred (5). Ward et al. (1988) also reported that doxorubicin induced reductions in testicular sperm count (31). In the present study, male rats were treated with a total of 15 mg/kg of doxorubicin for 5 weeks. The results indicated above that doxorubicin induce pathological changes in serum and biochemical markers indicative of toxicity and increases the free radical production. These results were consistent with earlier studies (3-5). Elevated serum levels of LDH, CK and GOT suggest that doxorubicin may induce generalized toxicity in rats.

Further results also led to belief that administration of green tea extract improved the biochemical marker levels indicating decrease in oxidative stress as evident by increased levels of GSH, SOD and CAT with decreased production of LP. These protective effects were also supported by the restoration of sperm count, histopathologic study and serum markers of toxicity. $Na^{+}K^{+}$ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase are the membrane bound enzymes and the levels of $Na^{+}K^{+}$ ATPase, Ca^{++} ATPase and Mg^{++} ATPase were reduced in testes of doxorubicin treated rats. Since these membranes bound enzymes are thiol group containing enzymes(32), that are lipid dependant and hence the restoration of the activities of ATPase enzymes suggest the ability of green tea extract to protect the thiol group from oxidative damage through inhibition of lipid peroxidation.

In conclusion, green tea extract was found to be a potential candidate as an additive to chemotherapeutic agents that are toxic to testes. Green tea extract could be an effective regimen to enhance therapeutic efficacy and to reduce toxic side effects of doxorubicin in clinical chemotherapy.

Acknowledgement

The authors are thankful to Mr. V. R. Sarode, Serum Institute of India Ltd., Pune for providing gift sample of doxorubicin and Dr. Pasle, Baroda clinical laboratory, India for assisting in histopathology study.

References

1. Herman EH, Ferrans VJ. Animal models of anthracycline cardiotoxicity: basic mechanisms and cardioprotective activity. Prog Pediatr Cardiol.1998; 8:49–58.

2. Au WW, Hsu TC. The genotoxic effects of adriamycin in somatic and germinal cells of the mouse. Mutat Res. 1980; 79:351–61.

3. Lui RC, Laregina MC, Herbold DR, Johnson FE. Testicular cytotoxicity of intravenous doxorubicin in rats. J Urol.1986;136: 940–3.

4. Sawada T, Tamada H, Mori J. Secretion of testosterone and epidermal growth factor in mice with oligozoospermia caused by doxorubicin hydrochloride. Andrologia. 1994; 26:151–3.

5. Imahie H, Adachi T, Nakagawa Y, Nagasaki T, Yamamura T, Hori M. Effects of adriamycin, an anticancer drug showing testicular toxicity, on fertility in male rats. J Toxicol Sci. 1995;20:183–93.

6. Goodman J, Hochstein P.Generation of free radicals and lipid peroxidation by redox cycling of adriamycin and daunomycin. Biochem Biophys Res Commun. 1977; 77: 797–803.

7. Pritsos CA, Sokoloff M, Gustafson DL. PZ-51 (Ebselen) in vivo protection against adriamycin-induced mouse cardiac and hepatic lipid peroxidation and toxicity. Biochem Pharmacol. 1992; 44:839–41.

8. Shinozawa S, Gomita Y, Araki Y. Protective effects of various drugs on adriamycin (doxorubicin)-induced toxicity and microsomal lipid peroxidation in mice and rats. Biol Pharm Bull. 1993; 16:1114–7.

9. Mansour MA, El-Kashef HA, Al-Shabanah OA. Effect of captopril on doxorubicin-induced nephrotoxicity in normal rats. Pharmacol Res .1999; 39: 233 –7.

10. Shimpo K, Nagatsu T, Yamada K. Ascorbic acid and adriamycin toxicity. Am J Clin Nutr. 1991;54:1298–301.

11.Manabe F, Takeshima H, Akaza H. Protecting spermatogenesis from damage induced by doxorubicin using the luteinizing hormonereleasing hormone agonist leuprorelin. Cancer.1997; 79:1014–21.

12. Harbone JB. General procedures and measurement of total phenolics. In: Harbone JB, ed. Methods in Plant Biochemistry. New York: Academic Press, Vol. 1. 1989:p1–28.

13. Scott BC, Butler J, Halliwell B, Aruoma OI. Evaluation of the antioxidant action of ferulic acid and catechin. Free Radic Res Commun. 1993;19: 241–253.

14. Zhu QY, Zhang A, Tsang D, Huang Y, Chen ZY. Stability of green tea catechins. J Agric Food Chem. 1997; 45: 4624 – 4628.

15. Lin AM, Chyi BY, Wu LY, Hwang LS, Ho LT. The antioxida-tive property of green tea against iron-induced oxidative stress in rat brain. Chin. J. Physiol. 1998;41:189 –194.

16. Mazzio EA, Harris N, Soliman KF. Food constituents attenuate monoamine oxidase activity and peroxide levels in C6 astrocyte cells. Planta Med. 1998; 64: 603–606.

17. Hiramoto K,Ojima N,Sako K, Kikugawa K. Effect of plant phenolics on the formation of the spin-adduct of hydroxyl radical and the DNA strand breaking by hydroxyl radical. Biol. Pharm. Bull. 1996; 19: 558–563.

18. Xu Y, Ho CT, Amin SG, Han C, Chung FL. Inhibition of tobacco-specific nitrosamine-induced lung tumorigenesis in A/ J mice by green tea and its major polyphenol as antioxidants. Cancer Res. 1992;52: 3875–3879.

19. Ahmad N, Mukhtar H. Green tea polyphenols and cancer: biologic mechanisms and practical implications. Nutr. Rev.1999;57: 78–83.

20. Kobayashi H, Urashima M, Hoshi Y. Testicular morphological changes in children with acute lymphoblastic leukemia following chemotherapy. Acta Paediatr Jpn. 1996; 38:640–3.

21. Narayana K, D'Souza UJ, Rao KP. Effect of ribavirin on epididymal sperm count in rat. Indian J Physiol Pharmacol.2002; 46: 97-101.

22. Linder RE, Strader LF, McElroy WK. Measurement of epididymal sperm motility as a test variable in the rat. Bull Environ Contam Toxicol.1986; 36: 317-24.

23. Slater TF, Sawyer BC. The stimulatory effects of carbon tetrachloride and other halogenoalkanes or peroxidative reactions in liver fractions in vitro. Biochem J. 1971; 123: 805–814.

24. Misra HP, Fridovich I.The role of superoxide anion in the autooxidation of epinephrine and a simple assay of SOD. J Biol.Chem. 1972; 247: 3170-3175.

25. Colowick SP, Kaplan NO, Packer L. Methods in Enzymology, Academic Press, London Vol. 105.1984; pp. 121–125.

26. Moron MS, Depierre JW, Mannervik BLevels of glutathione, glutathione reductase and glutathione *S*-transferase activities in rat lung and liver. Biochimica et Biophysica ACTA. 1979; 582: 67–78.

27. Bonting SL .Presence of Enzyme System in Mammalian Tissues. Membrane and Ion Transport. Wiley, London.1970 pp.257– 263.

28.Hjerten S, Pan H. Purification and characterization of two forms of a low affinity Ca^{2+}ATPase from erythrocyte membranes. Biochimica et Biophysica ACTA. 1983; 728: 281–288.

29.Ohnishi T, Suzuki T, Suzuki Y, Ozawa K. A comparative study of plasma membrane Mg2+ATPase activities in normal, regenerating and malignant cells. Biochimica et Biophysica ACTA.1982; 684 :67–74..

30. Lowry OH, Rosenbrough NJ, Farr AC, Randell RJ. Protein measurement with folin-phenol reagent. J Biol Chem.1975; 193: 265–275.

31.Ward JA, Bardin CW, Knight M, Robinson J, Gunsalus G, Morris ID. Delayed effects of doxorubicin on spermatogenesis and endocrine function in rats. Reprod Toxicol.1988; 2:117–26.

32. Gubdjorson S, Halligrimson J, Skuldottir G. Properties of transport adenosine Triphosphatase. In: Peter H.,Gresham G A., Paoetti R., Arterial pollution. NewYork, Plenum Publishing Corp., 1983: pp.101.