RESEARCH ARTICLE

Chemoprotective Effect of Eugenol against Single Dose Doxorubicin (DOX)-Induced Toxicopathological Alterations in Swiss Albino Mice with Special Reference to Cardiotoxicity

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Abstract

Doxorubicin (DOX), is utilized in the treatment of various canine and feline cancers. However, its therapeutic application is associated with marked adverse effects on heart as well as myelosuppression and potential for secondary malignancy. In order to reduce the side effects, potential of eugenol was investigated against Doxorubicin in Swiss albino mice. Toxicity was induced by a single intraperitoneal injection of doxorubicin (25 mg/kg, b.wt.) and Eugenol treatment (10 mg/kg/day, orally) was initiated 15 days before doxorubicin administration. Oral administration of eugenol markedly alleviated DOX-induced oxidative stress in cardiac tissues, as evidenced by reduced levels of lipid peroxidation, nitric oxide. Furthermore, eugenol enhanced the activity of antioxidant enzymes, including catalase and superoxide dismutase. In addition to attenuating oxidative stress, eugenol administration significantly reduced DOX-induced inflammation by downregulating the gene expression of pro-inflammatory mediators, namely, TNF alpha, iNOS and IL. Gross and histopathological examinations revealed substantial structural and functional impairment in the heart, brain, liver, spleen, bone marrow and kidney due to DOX intoxication. This study suggests the promising chemoprotective efficacy of eugenol against DOX-induced toxicopathological alterations, indicating its potential as an adjuvant in chemotherapy.

Key words: Acute cardiotoxicity, Doxorubicin, Eugenol, Mice, Nitric oxide.

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Introduction

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body (Li *et al.,* 2007). The rate of cancer among dogs and cats is similar to the rate of humans (Todorova *et al.,* 2016). Cancer is one of the leading causes of death in companion animals. Dogs can develop variety of cancers that include sarcomas of connective tissues and bones, lymphomas or leukemias of the circulatory system and carcinomas of epithelial cells and organs (Gardner *et al.,* 2016). Chemotherapy is one of the conventional treatment methods for cancer. The anticancer drugs which are used in chemotherapy act by killing rapidly dividing cells in a cytotoxic manner. However, these drugs are not selective only for cancer cells as they may also kill healthy cells. Several drugs are used as chemotherapeutic agents against various forms of human and canine cancers. These anticancer drugs also produce genotoxicity to normal cells which leads to secondary malignancies (Venkatesh *et al.,* 2007). Doxorubicin (DOX) is an anthracycline antibiotic first isolated from *Streptomyces peucetiusvarcaesius* (Jagtia and Venketesh, 2015) with broad-spectrum and potent antineoplastic activity, used either alone or in combination with other chemotherapeutic drugs. It is mainly used in therapy for a wide variety of solid tumours in humans, canines, and felines (Farag *et al.,* 2021). As anticancer agents are not selective in action, the sensitivity of normal tissues, such as bone marrow, limits the maximum tolerated doses.

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The most severe side effect of doxorubicin is cardiotoxicity, leading to life-threatening heart failure, although genotoxicity, hepatotoxicity, nephrotoxicity, reproductive toxicity, and gastrointestinal disturbances are also common sequelae of DOX chemotherapy (Farag *et al.,* 2021).

Combination therapy with phytochemicals is found to be beneficial in providing protection against chemotherapy

induced oxidative damage. To combat the adverse effects of these cytotoxic drugs, it is common to prescribe vitamins and dietary supplements, which contain substantial amounts of antioxidants, free radical-scavenging agents and general blocking agents. Such natural agents have the potential to diminish the physiological side effects (Venkatesh *et al.,* 2007). Eugenol ($C_{10}H_{12}O_2$; phenylpropanoid), is an aromatic compound belonging to the group of phenols commonly obtained from the natural essential oils of plants like clove (*Syzygium aromaticum*) oil (Ulanowska and Olas, 2021). Eugenol has demonstrated various antioxidant, analgesic, antimutagenic, anti-platelet, antiallergic, anti-swelling, and anti-inflammatory properties (Hajra *et al.,* 2018; Ulanowska and Olas, 2021). These facts motivated us to evaluate the protective role of eugenol against DOX induced toxicities through inhibition of oxidative stress, and inflammatory response pathway in Swiss albino mice.

Materials and Methods

Experimental Animals and Chemicals

Adult (5-6 weeks old) Swiss albino female mice (n=24, 25±2 g b.wt.), bred in disease free small animal house, LUVAS, Hisar (Haryana, India) were procured and used in this study. They were maintained at control temperature (23±2 °C) and humidity (55±10%) under alternating light and dark conditions (12 h/12 h). Animals were fed with standard feed prepared at Department of Animal Nutrition, LUVAS, Hisar and drinking water was provided *ad libitum*. All procedures for animal experimentation used were approved by the Institutional Animal Ethics Committee (TV3240-200623, Dated: 28.06.2023). Eugenol used in the study was purchased from Sigma Chemicals and was diluted in peanut oil for administration in mice. Doxorubicin hydrochloride (DOX) was obtained from Zydus Lifesciences Limited and was dissolved in normal saline for intraperitoneal administration in mice.

Experimental Design

After acclimatization of 7 days the mice were divided into four groups containing six mice (n=6) in each group. Vehicle control group (VC) mice were administered with single intraperitoneal (i.p.) injection of normal saline (vehicle of doxorubicin); DOX treated group (DOX) mice were intraperitoneally treated with Doxorubicin hydrochloride @ 25 mg/kg b.wt. (Single dose); DOX + Eugenol pretreated group (DOX+EUG) mice were intraperitoneally treated with Doxorubicin hydrochloride @ 25 mg/kg b.wt. (Single dose) and eugenol was given orally @ 10 mg/kg b.wt. daily by gavage (Fouad and Yacoubi, 2010) 15 days prior to Doxorubicin hydrochloride treatment and continued till the end of the experiment. The mice were sacrificed 24 h after DOX treatment.

Sample Collection

Before euthanasia, blood samples were collected from mice by retro-orbital venous plexus in sterile EDTA coated vials and processed for evaluation of haematological parameters using automatic haematological analyzer (MS4Se). Heart was collected for analysis of oxidative stress (LPO, Catalase, SOD and NO) and gene expression study of TNF-α, iNOS, IL-1. To study histopathological alterations heart, brain, liver, spleen, kidney and bone marrow tissues were collected and processed as per the standard protocol.

Estimation of Oxidative Stress Parameters in Cardiac Tissue

Quantitative estimations of Lipid Peroxidation (LPO) in terms of malondialdehyde (MDA) formed by using TBARS test, Superoxide Dismutase (SOD) activity, Catalase (CAT) activity, and Nitric Oxide (NO) activity in cardiac tissue homogenates were performed as per the methods described by Shafiq-Ur-Rehman (1984), Madesh and Balasubramanian (1998), Aebi (1984), and Titheradge (1998), and were expressed as nM of MDA formed per mL, units/mg of protein, mmol H_2O_2 utilized/min/mg protein, and µmol/g tissue homogenates, respectively.

Gene Expression Study of Pro-Inflammatory Genes TNF-α, iNOS, IL-1 by qPCR

Heart tissues of each animal were removed, homogenized with liquid N_2, placed in RLT buffer, and then frozen at -20°C. RNeasy Mini Kit (Qiagen, Heidelberg, Germany) was used for total RNA extraction from cardiac tissue and quantified with ND2000 spectrophotometer (NanoDrop Technologies). DNase I-treated RNA was reverse transcribed into cDNA using the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit. Previously published primers were used (Zhao *et al*., 2020; Zhu *et al.,* 2022) as given in Table 1. Quantitative real-time PCR (qPCR) was carried using SYBR Green dye and POWERUP® SYBR Green qPCR Master Mix (2X). The data obtained were subjected to comparative Ct method (ΔΔCt method) for the analysis of the expression levels of targeted gene and an endogenous control. The fold changes in expression, *i.e.,* RQ (Relative Quantification) values were calculated for temporal expression analysis of TNF-α, iNOS and IL-1 gene along with standard deviation (RQ ± SD). More than two fold increase or decrease in gene expression was considered as significant (according to global convention mice guideline).

Table 1: Primer sequences used for Real-time RT-PCR analysis

Type of Gene	Primer Sequences	Amplicon size
TNF-α	F5′ AAACCACCAAGTGGAGGAGC ACAAGGTACAACCCATCGGC	119
iNOS	GAAGAAAACCCCTTGTGCTG TCCAGGGATTCTGGAACATT	116
IL-1	AGCTTCCTTGTGCAAGTGTCT GACAGCCCAGGTCAAAGGTT	156
GAPDH	GGCATTGCTCTCAATGACAA TGTGAGGGAGATGCTCAGTG	200

Genotoxicity Studies

Micronuclei occurrences in bone marrow of all the groups were assayed as per method of Borroto *et al.* (2003). Evaluation of the activity of eugenol to reduce micronuclei induced by doxorubicin in Polychromatic erythrocytes (PCEs) and Normochromatic erythrocytes (NCEs) was carried out using formula:

Reduction in micronucleated cells (%) = [(micronucleated cells in doxorubicin treated mice) - (micronucleated cells in doxorubicin + eugenol treated mice) / (micronucleated cells in doxorubicin-treated mice - micronucleated cells in control)] X 100

DOX-induced possible DNA damage was evaluated by comet assay technique to evaluate the activity of eugenol to reduce DNA damage in terms of tail moment using formula:

Reduction in tail moment = [(tail moment in Doxorubicin treated mice) - (tail moment in Doxorubicin + eugenol treated mice)/ (tail moment in Doxorubicin treated mice - tail moment in control)] X 100

Gross and Histopathological Evaluation of Vital Organs

Gross lesions in heart, brain, liver, spleen, kidney and bone marrow were recorded immediately during post-mortem examination of the mice. Representative tissue samples were collected in 10 % buffered formalin and were processed for paraffin embedding technique. Tissue sections of 4-5 µm thickness were cut using an Histo Core MULTICUT semi-automated rotatory microtome, stained with haematoxylin and eosin stain and evaluated.

Statistical Analysis

All data were presented as mean ± SD. One way ANOVA followed by Tukey's Multiple Comparison Test using SPSS 16.0 version software was performed. Significant difference was indicated when the P value was < 0.05.

Results and Discussion

The effect of single dose of intraperitoneal administration of Doxorubicin caused significant reduction in Hb, PCV and TLC. Granulocyte percentage was more and lymphocyte percentage was reduced in doxorubicin treated mice. Improvement was observed in mice, supplemented with eugenol 15 days prior (Table 2). Doxorubicin reduced the proliferation of B and T lymphocytes and their clonogenicity resulting into decrease in the count (Zeiss *et al.,* 2019) as observed in the present study. Destroying the structure of the white pulp, caused the depletion of splenic lymphocytes, and an influx of monocytic macrophages into the spleen resulted in leucopenia and bone marrow suppression. Supplementation of eugenol 15 days prior reversed the toxic effect due to its antioxidant potential as well as immunomodulatory and immunostimulatory potential (Aprotosoaie *et al.,* 2019).

Table 2: Ameliorative effect of eugenol on Doxorubicin induced haematological parameters of mice (Mean ± SD, n=6)

Group	Hb (g/dL)	PCV (%)	TEC ($x10^6$/µL)	MCV (fl)	MCH (pg)	MCHC (%)	Platelets ($x10^3$/µL)	WBC ($x10^3$/µL)	Granulocytes (%)	Lymphocytes (%)
Control	13.30^{b}±0.10	45.40^{b}±1.10	8.10^{a}±0.20	61.20^{a}± 0.30	16.5^{a}± 0.10	26.50^{a}±0.50	352.70^{a}±40.90	10.20^{b}±0.20	11.30^{b}±0.60	87.50^{ab}±0.80
DOX	11.50^{a}± 0.38	39.13^{a}±0.36	7.52^{a}±0.24	61.92^{a}± 1.05	16.6^{a}± 0.20	26.77^{a}±0.45	258.00^{a}±31.57	5.86^{a}±0.36	16.50^{a}±2.72	82.17^{a}±3.07
DOX+EUG	12.52^{ab}±0.32	43.22^{ab}±2.67	7.08^{a}±0.45	61.65^{a}± 0.46	16.8^{a}± 0.69	27.45^{a}±1.04	339.67^{a}±33.67	8.38^{b}±1.02	12.67^{b} ±0.21	82.17^{a}±3.71
EUG	12.80^{b}± 0.17	46.57^{b}±0.77	7.30^{a}±0.13	60.51^{a}± 1.18	17.6^{a}± 0.23	26.90^{a}±0.24	339.67^{a}±15.76	10.38^{b}±0.39	11.50^{b}± 0.22	92.50^{b} ±1.77

Mean ± SDs with different superscripts (a,b) in the same column differ significantly ($p \leq 0.05$)

Control: Vehicle control **DOX:** Doxorubicin hydrochloride @ 25 mg/kg b.wt. i.p. **EUG:** Eugenol @ 10 mg/kg b.wt. (orally) daily. **DOX +EUG:** Doxorubicin hydrochloride @ 25 mg/kg b.wt. i.p. (Single dose) + eugenol @ 10 mg/kg b.wt. orally daily by gavage 15 days prior to Doxorubicin hydrochloride treatment

LPO and NO activity in heart were significantly ($p<0.05$) increased and antioxidant enzymes SOD and catalase were significantly reduced in mice intoxicated with Doxorubicin (Table 3) suggesting formation of free radicals is primary cause for toxicopathological alterations in heart as well as other organs as evidenced by histpathological alterations (Fouad and Yacoubi, 2010). Eugenol supplementation showed significant ($p<0.05$) decrease in LPO as well as NO activity and rise in levels of SOD and catalase indicating strong ameliorative effect due to anti-oxidative and cardio-protective effect of eugenol (Jagetia and Venkatesh, 2015).

Gene expression of pro-inflammatory markers TNF-α, IL-1 and iNOS was upregulated in heart of mice intoxicated with Doxorubicin (Table 4) suggesting cell injury and inflammation. This might be because DOX administration has increased the iNOS transcription and protein expression (Rawat *et al.,* 2021), as well as stimulation of signaling pathway for inflammatory mediators production, such as proinflammatory cytokines and chemokines. Eugenol supplementation to DOX-treated mice showed significant downregulation in mRNA expression of TNF-α, IL-1 and iNOS. Supplementation of eugenol to Doxorubicin treated

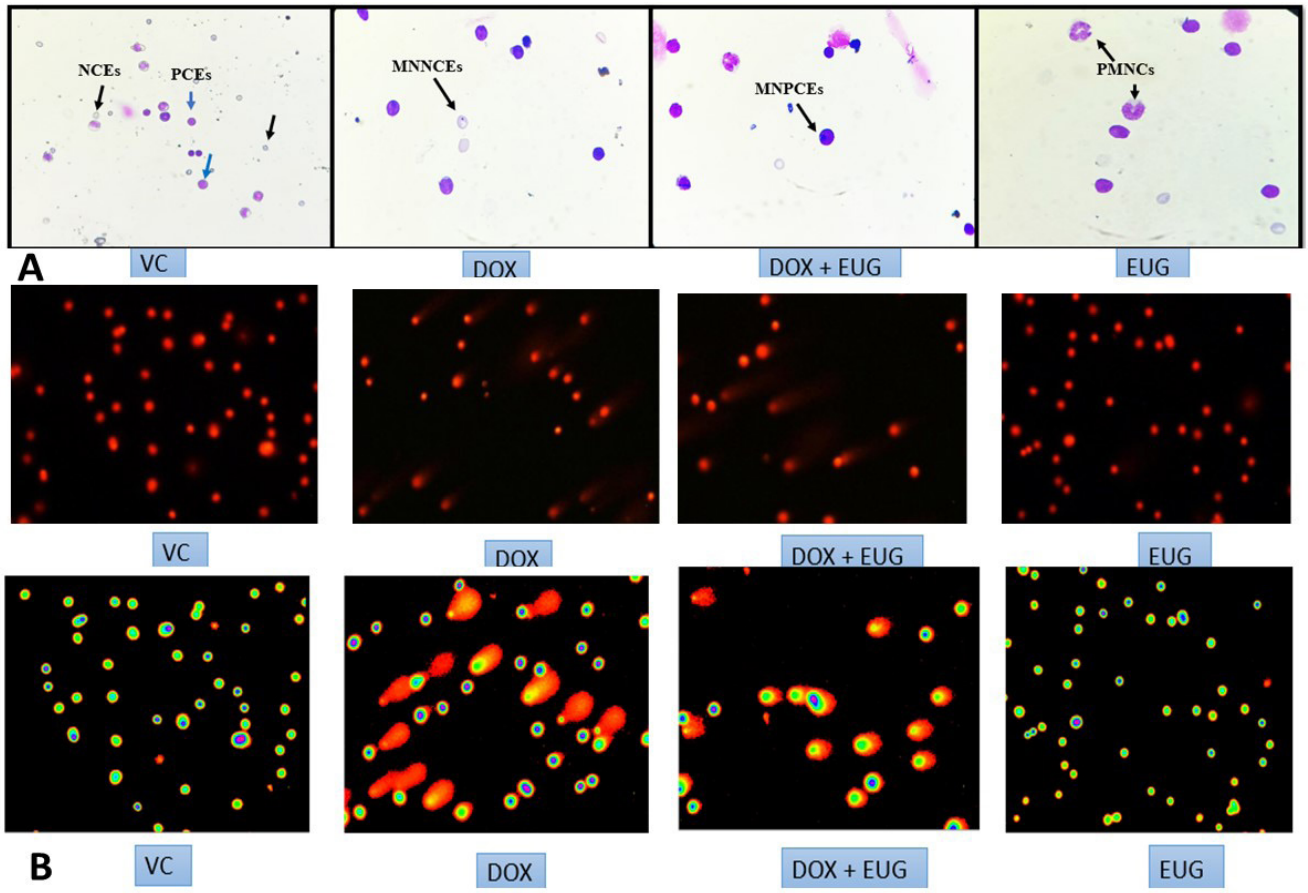

Fig. 1: Ameliorative effect of eugenol against genotoxic effect of Doxorubicin in bone marrow cells. **A:** formation of micronuclei; **B:** Formation of comets . VC: Vehicle Control, DOX: Doxorubicin 25 mg/kg B wti/p , DOX+ EUG: Doxorubicin hydrochloride + eugenol, EUG: Eugenol @ 10 mg/kg b.w. (orally) daily by gavage 15 days prior to DOX

Table 3: Ameliorative effect of eugenol on Doxorubicin induced oxidative stress in heart of mice (Mean ± SD, n=6)

Group	LPO activity (nM MDA/ gm) in heart tissue	NO activity (µmol/g tissue) in heart tissue	SOD activity (IU/mg protein) in heart tissue	Catalase activity (mM H_2O_2 utilized/min/mg protein) in heart tissue
Control	276.33^{a} ±54.67	1.00^{a} ± 0.10	345.83^{a} ± 31.84	245.00^{a} ± 54.5
DOX	802.00^{b} ±149.23*	4.80^{b} ± 0.20*	262.67^{b} ± 34.33	195.90^{b} ± 29.95
DOX+EUG	278.17^{a} ±36.64#	3.20^{ab} ± 1.80	355.83^{a} ± 41.94	253.09^{a} ± 41.01
EUG	232.65^{a} ±32.42	2.60^{a} ± 2.10	481.17^{a} ± 83.18	334.60^{a} ± 34.49

Mean ± SD with different superscripts (a,b) in the same column differ significantly ($p\leq0.05$)

mice showed downregulation of TNF-α, iNOS and IL1. This might be due to inhibition of ROS production and signaling pathway for inflammatory mediators production, such as proinflammatory cytokines and chemokines, *i.e.,* antioxidant and anti-inflammatory potential of eugenol (Magalhaes *et al.,* 2019). Doxorubicin has produced significant genotoxic changes in bone marrow cells in the present study.

Table 4: Ameliorative effect of eugenol on the altered mRNA expression of inflammatory cytokines genes TNF-α, iNOS, and IL-1 due to Doxorubicin in heart of mice (Mean ± SD, n=6)

Group	TNF-α	iNOS	IL-1
Control	1.00^{a} ± 0.03	1.00^{a}±0.61	1.00^{a}±0.31
DOX	4.55^{b}±0.24	21.70^{c}±0.33	1.42^{b}±0.24
DOX+EUG	0.86^{a}±0.06	15.07^{b}±1.2	0.42^{a}±0.08
EUG	0.77^{a}±0.15	1.77^{a}±0.61	0.41^{a}±0.36

Mean ± SD with different superscripts (a,b,c) in the same column differ significantly (p≤0.05)

The mean value of micronucleated polychromatic erythrocytes (MnPCEs) and normochromatic erythrocytes (MnNCEs) was increased in DOX-treated mice (Fig. 1A) which is due to numerical and structural chromosomal irregularities produced by Doxorubicin (Renu *et al.,* 2022). When eugenol was supplemented to doxorubicin treated mice, micronuclei formation was reduced (Table 5). Further, a single dose of doxorubicin exposure over a period of 24 h in mice has resulted into increased DNA damage leading to DNA migration out of nucleus into the tail of the comets in bone marrow cells. Doxorubicin administration significantly ($p<0.05$) increased the DNA damage resulting in long comet length (Table 5, Fig. 1B), tail DNA (%), average tail length (µm) and non-significant increase in tail moment (AU) formation in large number of cell population. Co-administration of eugenol in Doxorubicin treatment significantly ($p<0.05$) mitigated comet length compared to DOX-treated mice and prevented DOX-induced DNA damage in bone marrow cells. These results conclusively indicate that the compound eugenol possess potent geno-protective efficacy by inhibiting DOX-induced free radicals generation (Abdeldaim *et al.,* 2017). Doxorubicin produced significant gross (Fig. 2) and histopathological (Fig. 3) alterations in all vital organs such as severe congestion of coronary blood vessels and petechial to ecchymotic haemorrhages at the apex and auriculoventricular junction in heart, pale discoloration of liver with fragile consistency, congestion of meningeal blood vessels and swelling of brain along with severe red discoloration and petechial haemorrhages on kidney. Histopathological changes noticed in the heart were of severe category such as congestion of blood vessels and haemorrhages in mycocardium, necrosis of individual muscle fiber, haemorrhages and degeneration of fat cells as well as foci of haemorrhages in endocardium. Degeneration of hepatocytes in liver, perivascular cuffing and neuronal degeneration in cerebrum (Fig. 3), lymphocytic depletion in spleen, haemorrhages in bone marrow and decreased cellularity were extensive in DOX-treated mice (Kumar *et al.,* 2021).

The toxicopathological alterations were reversed to normal structure in eugenol treated mice suggesting ameliorating potential of eugenol as it is reported that Eugenol acts by trapping the reactive oxygen species, including superoxide anion and hydroxyl radicals as well as by inhibiting propagation of free radical chain reactions.

Table 5: Ameliorative effect of eugenol on Doxorubicin induced genotoxic changes-micronuclei formation and comet in bone marrow cells of mice (Mean ± SD, n=6)

Group	P/N ratio	% MnPCE	% MnNCE	% PCE	Reduction in MnNCE (%)
Control	1.30^{a} ± 0.00	0.00^{a} ± 0.00	0.00^{a} ± 0.00	55.7^{a} ±0.50	_
DOX	0.69^{a} ± 0.13	0.15^{a} ± 0.1	0.14^{b} ± 0.05*	38.90^{a} ± 5.43	_
DOX+EUG	2.41^{a} ± 1.39	0.00^{a} ± 0.00	0.05^{ab} ± 0.03	59.14^{a}±15.10	64.28
EUG	1.24^{a} ± 0.10	0.02^{a} ± 0.02	0.00^{a} ± 0.00	55.30^{a} ± 1.70	_
	Comet length (µm)	**Tail length (µm)**	**Tail DNA (%)**	**Tail moment (AU)**	**Reduction in tail moment**
Control	24.40^{a}± 0.40	1.27^{a} ± 0.27	4.57^{a} ± 1.14	0.69^{a} ± 0.17	-
DOX	31.75^{b}±1.89*	1.55^{b}± 0.21*	7.95^{b}± 1.14*	0.93^{a}± 0.19	-
DOX+EUG	24.52^{a}±0.67#	1.11^{ab}±0.15	7.61^{b}± 0.51	0.80^{a} ± 0.10	54.16%.
EUG	22.41^{a}±0.35	0.60^{a}± 0.26	3.70^{a}± 1.11	0.40^{a} ± 0.12	-

MnPCE: micronucleated polychromatic erythrocytes; MnNCE: micronucleated normochromatic erythrocytes; P/N: ratio of polychromatic erythrocytes to normochromatic erythrocytes; and PCE: polychromatic erythrocytes. $p<0.05$ significantly different from control, #$p<0.05$ significantly different from DOX

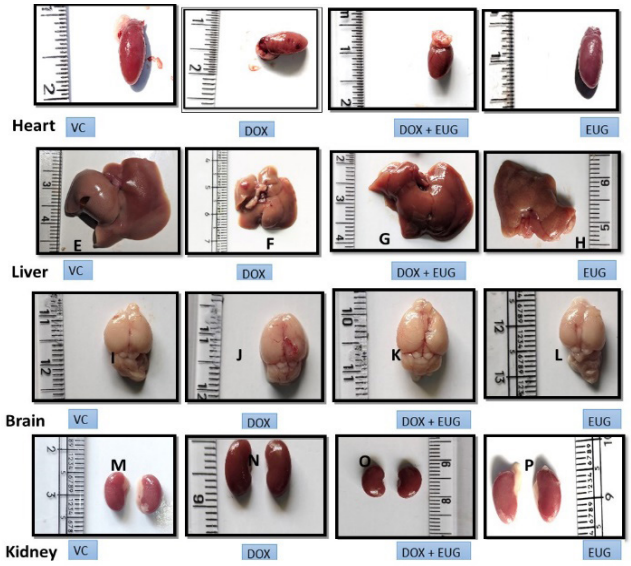

Fig. 2: Protective effect of eugenol against doxorubicin induced gross lesions in heart, liver, brain and kidney in mice. VC: Vehicle Control, DOX: Doxorubicin 25 mg/kg b.wt. i/p, DOX+ EUG: Doxorubicin hydrochloride + eugenol, EUG: Eugenol @ 10 mg/kg b.wt. (orally) daily by gavage 15 days prior to DOX.

Conclusion

Eugenol offers efficient protection against DOX-induced toxicopathological alterations in mice. The chemo-protective efficacy conferred by eugenol is probably due to inhibition of DOX induced free radicle formation by scavenging free radicles, inhibition of initiation and propagation steps leading to termination of reaction and delaying oxidation process. The efficacy shown by the pretreatment group might be due to compound providing some added protection to the target cells before exposure to the chemotherapeutic agent. Therefore, eugenol can serve as potential chemo-protective agent against DOX-induced cardiotoxicity and toxic effects observed on brain, liver, kidney, bone marrow and spleen.

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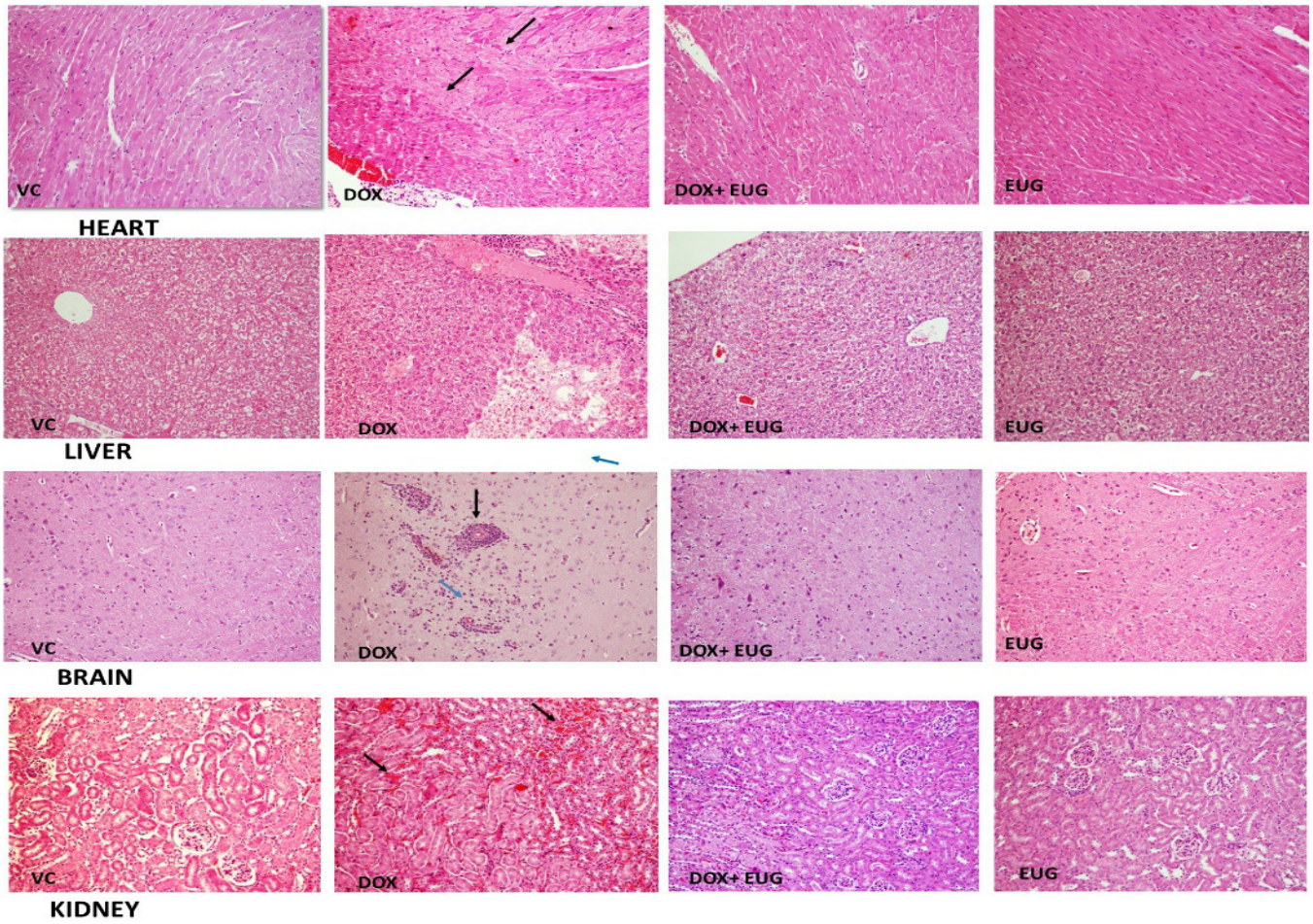

Fig. 3: Ameliorative effect of eugenol against doxorubicin induced histopathological changes in heart, liver, brain and kidney in mice VC: Vehicle Control, DOX: Doxorubicin 25 mg/kg b.wt. i/p, DOX + EUG: Doxorubicin hydrochloride + eugenol, EUG: Eugenol @ 10 mg/kg b.wt. (orally) daily by gavage 15 days prior to DOX.

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