Chemoprotective effect of eugenol on repeated dose doxorubicin, induced genotoxic lesions in mouse bone marrow

ABSTRACT

In present study, an attempt has been made to evaluate the possible in vivo chemoprotective potential of eugenol against doxorubicin induced genotoxic lesion in bone marrow. Swiss albino female mice were administeredEugenol (10 mg/kg/day, orally) 15 days before doxorubicin administration as well as concomitantly with doxorubicin. Genotoxic lesions were induced by a repeated dose intraperitoneal injection of doxorubicin (5mg/kg b.w.i.p.upto 9 days (alternate day). Micronuclei assay and COMET assay were carried out to determine genetic damage in bone marrow. Histopathological evaluation of bone marrow was also done to study lesions at cellular level. The study results demonstrated that eugenol significantly mitigated Doxorubicin induced micronuclei formation and DNA damage in the bone marrow niche. Histopathological observations revealed that Doxorubicn-intoxication resulted in massive structural impairment of bone marrow which was reduced by eugenol administration. Amelioration by eugenol is more significant in mice those received eugenol 15 days prior to Doxorubicin administration suggesting its chemoprotection. The present study suggests eugenol has the promising chemoprotectiveeffect against Doxorubin-induced genetic lesions in mice bone marrow.

Keywords: Bone marrow; Doxorubicin; Eugenol;Genotoxicity; Micronuclei

UNDER PEER REVIEW

1. Introduction

Cancers of various types are commonly diagnosed in companion animals, and are significant causes of death in humans and dogs[1.] A North American study, carried out with dogs, analyzed data from two decades of the Veterinary Medical Database; through a sampling of more than 74 thousand cases, the study concluded that neoplasia is the main cause of death in animals aged ~10 years[2.] Generally, approximately 50% of tumors are malignant, and the main sites of growth are the skin, mammary glands, soft tissues, genital tract, and oral cavity, consisting primarily of epithelial, mesenchymal, and lymphoid tumors[3, 4]. According to the oncological guidelines for dogs and cats published by the American Animal Hospital Association, there is an increase in the incidence of oncological cases that can be justified by the high life expectancy of small domestic animals as a result of improvements in nutritional management, disease control, vaccination, preventive veterinary medicine, and advances in clinical and diagnostic tests[5].Chemotherapy is one of the conventional treatment methods for cancer. The anticancer drugs used in chemotherapy act in nonselective manner by killing healthy cells along with cancer cells[6]. Several drugs are used as chemotherapeutic agents against various canine cancers such as doxorubicin, vincristine, vinblastin etc. These anticancer drugs also produce genotoxicity to normal cells that leads to formation of secondary malignancies[7.]

Doxorubicin (DOX) is an anthracycline antibiotic first isolated from *Streptomyces peucetius var caesius*[8] with broad-spectrum and potent anti neoplastic activity, used either alone or in combination with other chemotherapeutic drugs. It is used in therapy for a wide variety of solid tumours[9,10.]Although, Doxorubicin is widely used, it also produces adverse effects on body such as immunosuppression, leukopenia which will make animal prone for secondary infection and malignancies as well[11]. To combat this there is growing interest to find new chemical agent particulary of the origin of a plant that have been used by human being as well as pets and are known to have medicinal properties[7]. Phytochemicals plays an important role. Nowadays more emphasis is being given on the plant derived molecules. They have very good antioxidant properties. Antioxidants of plant origin such as curcumin, garlic, chlorella etc studied and reported to provide protection against DNA damage in rat and mouse model.

In present work, chemoprotective potential of natural compound eugenol has been evaluated against doxorubicin induced DNA damage. Eugenol (C10H12O2; phenylpropanoid), is an aromatic compound belonging to the group of phenols. It is commonly obtained from the natural essential oils of plants from the Lauraceae, Lamiaceae,Myristicaceae and Myrtaceaefamilies, and is the most important component of clove (*Syzygium aromaticum*) oil[12]. Eugenol showed versatile pharmacological actions in different types of cancer, it has genoprotective effects against oxidative and methylated DNA damage and can inhibit in vivo genotoxicity[13]. Eugenol has demonstrated various antioxidant, analgesic, antimutagenic, anti-platelet, antiallergic, anti-swelling, and anti-inflammatory properties[12]. These facts motivated us to evaluate the antigenotoxic effects eugenol on repeated dose doxorubicin, induced genotoxic lesions in mousse bone marrow.

2 MATERIALS AND METHODS

2.1. Experimental animals

Adult (5–6 weeks old) Swiss albino female mice (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 (HARYANA, India) and drinking water was provided ad libitum. All procedures for animal experimentation used were approved by the Institutional Animal Ethics Committee (CPCSEA Reg. No.-1669/GO/ReBiBt-S/Re-L/12/CPCSEA, India)].with proceeding/minutes of 28th meeting of IAEC circulated wide document number TV3240-200623, Dated: 28th June

2.2. Chemicals

Eugenol used in the experimental study was purchased from Sigma Chemicals and was diluted in peanut oil for administration in mice. Doxorubicin hydrochloride was obtained from Zydus Lifesciences Limited and was dissolved in normal saline for intraperitoneal administration in mice.

2.3. Experimental design

After acclimatization of 7 days the animals were divided into four groups containing six animals (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 @ 5mg/kg b.w.i.p.upto 9 days (alternate day); Only eugenol (EUG) treated group mice received eugenol at the dose of 10 mg/kg b.w. daily orally throughout the experimental

period; DOX+ eugenol concomitantly treated group (DOX+EUG con) mice were intraperitoneally treated with Doxorubicin hydrochloride @ 5mg/kg b.w.i.p.upto 9 days (alternate day) and Eugenol was treated orally at the dose @10mg /kg b.w. (1-10 days) by gavage. DOX+ eugenol pre-treated group (DOX+EUG pre) mice were intraperitoneally treated with Doxorubicin hydrochloride @ 5mg/kg b.w.i.p.upto 9 days (alternate day) and eugenol was given orally at the dose of 10 mg/kg b.w. daily by gavage 15 days prior to Doxorubicin hydrochloride treatment and continued till the end of the experiment.

All the mice were euthanized after end of experiment. Total dose of doxorubicin selected for present study was 25 mg/kg b.w.i.p. in mice. As per previous studies the most used dosage of doxorubicin is 60–75 mg/m^2 intravenously for two cycles in dogs which is equivalent to 20–25 mg/kg b.w. in mice[14]. DOX in this clinically relevant dose is previously reported for its cardiotoxicity in mice[14,15] and Eugenol dose at 10 mg/kg b.wt. orally by gavage in mice was selected on the basis of previous reports [16].

2.4. Sample collection and preparation

2.4.1. Preparation of bone marrow cell suspension

The bone marrow cells from femurs were collected in Dulbecco's phosphate-buffered saline (DPBS) for study of various genotoxicity parameters. After collection the bone marrow cellularity was determined and the samples were processed according to the methods described below.

2.5 Micronucleus assay

Micronuclei occurrence in all the groups were assayed by method as described[17,18]. The procedure is described briefly as follows:

The bone marrow cells suspension was subjected to spin at 1000 rpm for 10 min and cell pellet was resuspended in fetal bovine serum (FBS). A thin and uniform smear on a dry cleaned and non-greasy glass slide was prepared and air dried for 24 h. May-Grunwald's (MG) and Giemsa stains were used to stain the slides. The slides were stained with stock MG stain (0.3%) for 5 min and then with diluted MG stain (0.15%) for 2 min. Slides were rinsed twice in DW, counter stained with 20% Giemsa stain for 10 min followed by washing in DW and air drying. At lower magnification (40X) the slides with even spreading were selected and at high magnification (oil immersion objective 100X) analyzed for the presence of micronuclei.Polychromatic erythrocytes (PCEs) and Normochromatic erythrocytes (NCEs) were identified. To determine the frequency (%) of PCE to total erythrocytes (PCEs +NCEs) and PCE/NCE ratio of at least 4000 erythrocytes (i.e., PCE_S + NCE_S) from each animal were scored randomly [18].Evaluation of the activity of eugenol to reduce micronuclei induced by doxorubicin in PCEs and NCEs was carried out according to following formula [19].

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)] × 100

2.6. Measurement of extent of DNA damage by comet assay

DOX-induced possible DNA damage was evaluated by comet assay technique using alkaline single cell gel electrophoresis. The collected bone marrow cells were suspended in 1 ml chilled Dulbecco's PBS (DPBS) and centrifuged at 1200rpm for 4 min at 4^oC. Cell pellets were washed thrice with 1 ml of DPBS and were then re-suspended in 1ml DPBS. After assessing the viability using 4% (w/v) Trypan blue dye cells were counted and cell count was adjusted to 1.0x 10^6 cells/ml with DPBS. Embedding of cells in 1% and 0.5% low melting agarose (LMA) prepared in DPBS on base slide prepared by using 1 % normal melting agarose (NMA) was done and electrophoresis was conducted at 24 V (0.7 V/ cm) and a current of 300 mA using a power supply (Biorad Power Pac Basic) for 20 min on ice. The slides were stained with ethidium bromide in distilled water (20µg/ml; 80µl/slide) and scoring of slides was done by using a fluorescence microscope (Eclipse Make Nikon) at 200X magnification with green filter., Duplicate slides per treatment were made and randomly selected 150 cells (75 cells from each of the two replicate slides) were scored per treatment to get a reproducible data. An image-analysis system (TriTek Comet-Score Freeware v1.5 software) was used to take the photomicrograph of cells and to analyze various parameters of the comet.The comet parameters recorded were tail length (µm), tail DNA (%), tail moment, and comet length (µm). Evaluation of the activity of eugenol to reduce DNA damage in terms of tail moment induced by doxorubicin [Reduction (%)] was carried out by following 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

2.7. Histopathological evaluation of bone marrow

Femurs were collected from all groups of mice and washed in ice cold normal saline, soaked on blotting paper to remove the blood then fixed in 10% neutral buffered formalin for 24 h. Bones were decalcified in an 20% EDTA solution then processed for conventional paraffin-embedded histology with hematoxylin and eosin (H&E) staining.

2.8. Statistical analysis

All data (n=6 animals per group) were presented as mean ± SD. One way ANOVA followed by Tukey's Multiple Comparison Test using SPSS 16.0 version software was performed for comparisons among groups. Significant difference was indicated when the P value was< 0.05.

3. RESULTS AND DISCUSSION

Chemotherapy-induced bone marrow suppression and genotoxicity represents a fundamental challenge in canine oncology that could be regarded as an inevitable complication that could result in dose reduction or even discontinuation of the anti-neoplastic therapy[3]. Cancer survivors are prone to cope with unavoidable long-term chemotherapy-triggered nimmunosupression, secondary malignancies and bacterial infections that severely affect the quality of animal and could impose a great effect at socio-economic level [2]**.** It is very essential to reduce the toxic effects of doxorubicin. In this context nowadays, medicinal plants are playing an important role in current scenerio. Medicinal plants have been important sources of constituents with pharmacological activities. Phytochemicals present in plants have formed the basis of sophisticated traditional medicine systems as they are very useful in protecting the pet from the deleterious effects produced on the normal tissues[7]**.** In the present study, genetic lesions produced by doxorubicin were studied and chemoprotective effect of eugenol was evaluated against DNA damage in bone marrow in the form of the micronucleus assay , the comet assay and histopathological examination[13]. The genotoxic effects caused by the antineoplastic drug Doxorubin are associated with potential risk of inducing secondary tumors as well as secondary infections due to immuosupression[20.] Repeated dose adverse effects caused by Doxorubicin include bone marrow suppression. One of the major mechanisms responsible for these genotoxic lesion is the production of free radicals, leading to DNA damage and the formation of mutations in normal cells[16].The mean value of incidence of micronucleated polychromatic erythrocytes (MnPCEs) and normochromatic erythrocytes (MnNCEs) formation was increased (Table 1) in doxorubicin treated mice However administration of eugenol 15 days prior to doxorubicin treatment as well as concomitant treatment significantly inhibited MnNCEs formation. Eugenol had no adverse effect on animals. The mean value of percentage polychromatic erythrocytes was significantly(P<0.05) decreased and mean value of percentage normochromatic erythrocytes was significantly(P<0.05) increased in doxorubicin treated mice as compared to control (GR-1). Mice supplemented with eugenol concomitantly and given with 15 days prior showed significant increase in percentage polychromatic erythrocytes and significant decrease in percentage normochromatic erythrocytesin both groups. So, Doxorubicin administration significantly (P < 0.05) increased the frequency of micronuclei formation and decline in the polychromatic by normochromatic erythrocytes (PCE/NCE) ratio compared to vehicle control group (Table1). A high frequency of micronuclei formation in the bone marrow observed in present study indicates that the drug has the potential to damage DNA strands.

Table 1: Mitigation of micronuclei formation and P/N (Polychromatic erythrocytes/ Normochromatic erythrocytes) ratio in bone marrow cells by Eugenol in doxorubicin treated mice.

Group	P/N ratio	% MnPCE	% MnNCE	% PCE	% NCE	Reduction in MnNCE (%)
GR-1	1.26^{ab} ± 0.03	0.00^{a} ± 0.00	0.00^{a} ± 0.0	55.68^{b}± 0.49	44.35^{a} ± 0.49	–
GR-2	0.63^{a} ± 0.01	0.00^{a} ± 0.00	0.16^{b} ± 0.04*	38.48^{a} ± 0.54*	61.52^{b} ± 0.54*	–
GR-3	2.06^{ab} ± 0.23	0.01^{a} ± 0.01	0.06^{ab} ± 0.04	66.62^{b} ± $2.33^{\#}$	33.38^{a} ± $2.33^{\#}$	62.50
GR-4	1.99^{ab} ± 0.32	0.01^{a} ± 0.01	0.05^{ab} ± 0.03	64.54^{b} ± $3.73^{\#}$	35.46^{a} ± $3.73^{\#}$	68.75
GR-5	3.00^{b} ± 1.07	0.01^{a} ± 0.01	0.00^{a} ± 0.00	68.12^{b} ± 5.71	31.88^{a} ± 5.71	–

MnPCE: micronucleated polychromatic erythrocytes; MnNCE: micronucleatednormochromatic erythrocytes; P/N: ratio of polychromatic erythrocytes to normochromatic erythrocytes; and PCE: polychromatic erythrocytes.

Data were represented as mean ± SD, n=6.

* P < 0.05 significantly different from VC.
# P < 0.05 significantly different from DOX (one-way ANOVA followed by Tukey's post hoc test).

GR1: Vehicle control (normal saline) i.p, **GR2:**Doxorubicin hydrochloride @ 5mg/kg b.w.i.p.upto 9 days (alternate day). **GR-3**: Doxorubicin Hydrochloride@ 5mg/kg b.w.i.p.upto 9 days (alternate day) + Eugenol @10mg /kg b.w. (1-10 days) orally daily by gavage, **GR-4**: Eugenol @10mg/kg b.w. orally daily by gavage 15 days prior and till the end of experiment. Doxorubicin Hydrochloride @ 5mg/kg b.w.i.p.upto 9 days (alternate day, **GR-5:**Eugenol @ 10mg/kg b.w. orally daily by gavage throughout the experiment.

In contrast treatment with eugenol depleted the frequency of micronuclei formation and elevation in the PCE/NCE ratio, compared to only Dxorubicintreated mice.Percentage reduction in MnNCE in DOX group supplemented with eugenol concomitantly was 62.50% and DOX group supplemented with eugenol 15 days prior was 68.75%.[21]. Due to these effects, chromosome fragments do not take part in anaphase, and develop nuclear membrane around them and form micronuclei along with normal nuclei[21] . Eugenol has reduced the formation of micronuclei when administered 15 days prior as well as concomitantly however prior administration of eugenol has given very good results at genetic level[22]. This ameliorative effect of eugenol on micronuclei formation could be due to its strong antioxidant nature[27] since free radicals play important role in production of genetic damage due to cell injury[25]. Further The increase in micronuclei number is indicative of chromosomal irregularities. Another parameter, evaluated in present study i.ePCE/NCE ratio which is an indicator of the acceleration or inhibition of erythropoiesis.Reduction in polychromatic erythrocyte/ normochromatic erythrocyte (P/N) ratio indicates non-specific toxicity to bone marrow cells or it is myelotoxic as observed in present study. Conversely, oral administration of eugenol concomitantly and 15 days prior schedules caused an increase in the (P/N) ratio in the bone marrow of mice[23.] This demonstrates the eugenol can enhance the process of erythropoiesis that will further enhance the immunity of pet reducing immunosuppression[24].

DNA damage induction indicated by longer tail length, higher tail DNA percent, higher tail moment and higher comet length are considered as one of the important initial events in cellular toxicity. The tail DNA percentage indicates the amount of DNA migrated from the nucleus **[24].** Comet assay was carried out to examine Doxorubicin induced possible DNA damage bone marrow. In this purpose comet length,average tail length (µm),tail DNA (%) and tail moment (AU) were analyzed (Table 2; Fig. 1,2).

Table 2: Effect of compound eugenol against Doxorubicin induced DNA damage in bone marrow of different groups of mice.

Parameters	Comet length (µm)	Tail length (µm)	Tail DNA (%)	Tail moment (AU)
GR-1	24.4^{a} ± 0.36	1.27^{a} ± 0.27	4.57^{a} ± 1.14	0.69^{a} ± 0.17
GR-2	42.83^{d} ± 1.92*	1.32^{a} ± 0.09	7.60^{b} ± 0.10*	0.93^{a} ± 0.20
GR-3	34.82^{c} ± $1.88^{\#}$	0.66^{a} ± 0.17	3.35^{a} ± $0.42^{\#}$	0.33^{a} ± 0.08
GR-4	30.44^{bc} ± $0.46^{\#}$	0.96^{a} ± 0.17	4.36^{a} ± $0.60^{\#}$	0.58^{a} ± 0.13
GR-5	26.82^{ab} ± 0.94	1.00^{a} ± 0.17	4.49^{a} ± 0.67	0.65^{a} ± 0.11

Data were represented as mean ± SD, n=6.* P < 0.05 significantly different from VC.
# P < 0.05 significantly different from DOX (one-way ANOVA followed by Tukey's post hoc test).

GR1: Vehicle control (normal saline) i.p, **GR2:**Doxorubicin hydrochloride @ 5mg/kg b.w.i.p.upto 9 days (alternate day). **GR-3**: Doxorubicin Hydrochloride@ 5mg/kg b.w.i.p.upto 9 days (alternate day) + Eugenol @10mg /kg b.w. (1-10 days) orally daily by gavage, **GR-4**: Eugenol @10mg/kg b.w. orally daily by gavage 15 days prior and till the end of experiment. Doxorubicin Hydrochloride @ 5mg/kg b.w.i.p.upto 9 days (alternate day, **GR-5:**Eugenol @ 10mg/kg b.w. orally daily by gavage throughout the experiment.

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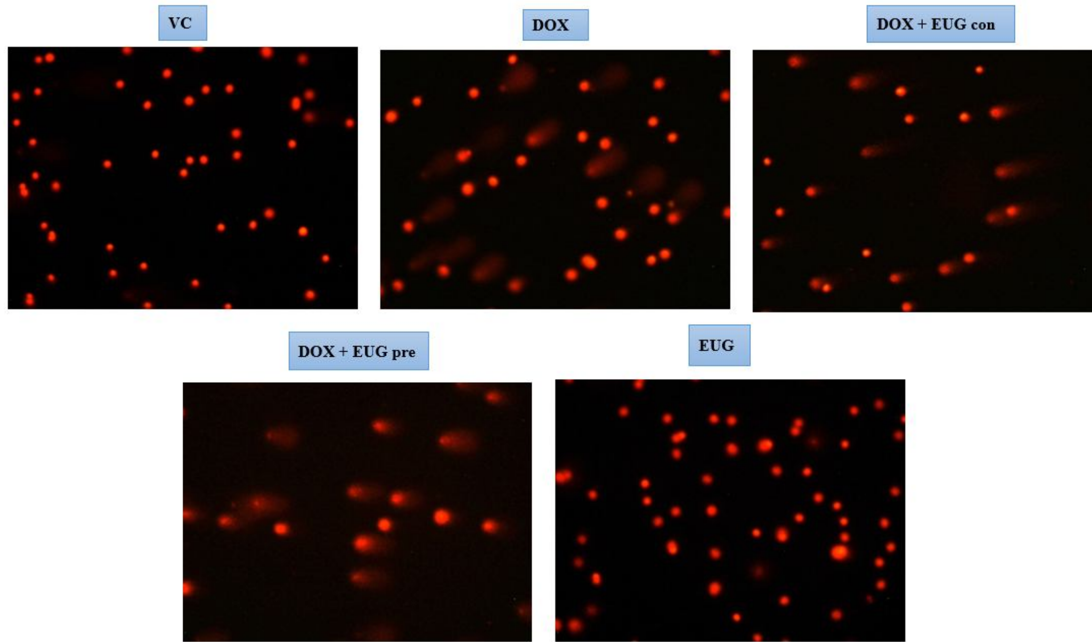

Fig. 1: Photographs of bone marrow cells at 200X magnification using a fluorescence microscope equipped with green filter. Compound eugenol attenuated DOX-induced DNA damage in bone marrow cells (× 200 magnifications). The vehicle control (VC) group showed intact DNA with no tail; only eugenol treated group (EUG) showed no DNA damage; only DOX-treated group (DOX) showed highly damaged DNA with scattered tail migration; concomitant treatment with eugenol (DOX+EUG Con) showed less migration of DNA with short comet tail; pretreatment with eugenol (DOX+EUG Pre) showed minimal migration of DNA with small tail

In vehicle control group, large round head and no tail was observed. In contrast, DOX-administration significantly ($P < 0.05$) increased the DNA damage, resulted in long comet length, tail DNA (%)and non-significant increase in average tail length (µm) and tail moment (AU) formation in large number of cell population. Result showed that repeated dose administration of DOX significantly ($P < 0.05$) increased the damage of cells in bone marrow compared to vehicle control group.

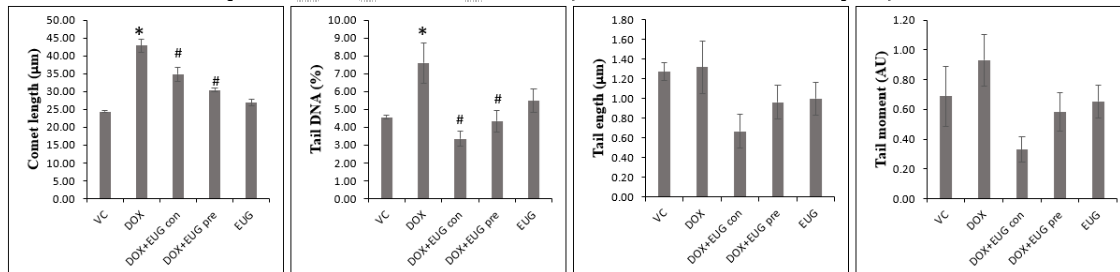

Fig. 2. Eugenol decreased bone marrow damage after DOX-administration. Data were represented as mean ± SD, n=6.*$P < 0.05$ significantly different from vehicle control (VC) group; #$P < 0.05$ significantly different from only DOX-treated group (one-way ANOVA followed by Tukey's post hoc test).

In contrast, co-administration of eugenol concomitantly and 15 days prior in DOX treatment group significantly ($P < 0.05$) mitigated comet length and tail DNA (%) and non-significantly mitigated average tail length (µm) and tail moment (AU) compared to DOX-treated mice and prevented DOX-induced DNA damage in bone marrow cells.

Doxorubicin has caused significant increase in COMET length and tail % suggesting strong genotoxic effect of drug on DNA. It might be due to drug's ability to bind DNA effectively by intercalation of the anthracycline portion; causing DNA damage via the production of free radicals from generation of

reactive oxygen species (ROS); stabilizing the topoisomerase II cleavage complex, which is critical for DNA function[23, 24]. It is also reported that doxorubicin can cause double as well as single-strand breaks induces incomplete excision repair processes[16]. However, oral administration of eugenol concomitantly and 15 days prior significantly ameliorated doxorubicin induced DNA damage in the present study. It is evidenced by minimization of comet length and tail DNA (%). These results conclusively indicate that the compound eugenol possess potent genoprotective efficacy as well as antioxidant activity by inhibiting DOX-induced DNA damage[25, 26].

Histopathological examination was also carried out to study lesions in bone marrow. Doxorubicin induced genetic lesions in mice bone marrow were further confirmed by bone marrow histology. Administration of Doxorubicin has caused increased in number of myeloid cells and severe hemorrhages along with congestion of blood vessels.However, experimental results showed that oral administration of eugenol resulted into reduction into lesions and almost normal histology of bone marrow (fig. 3)

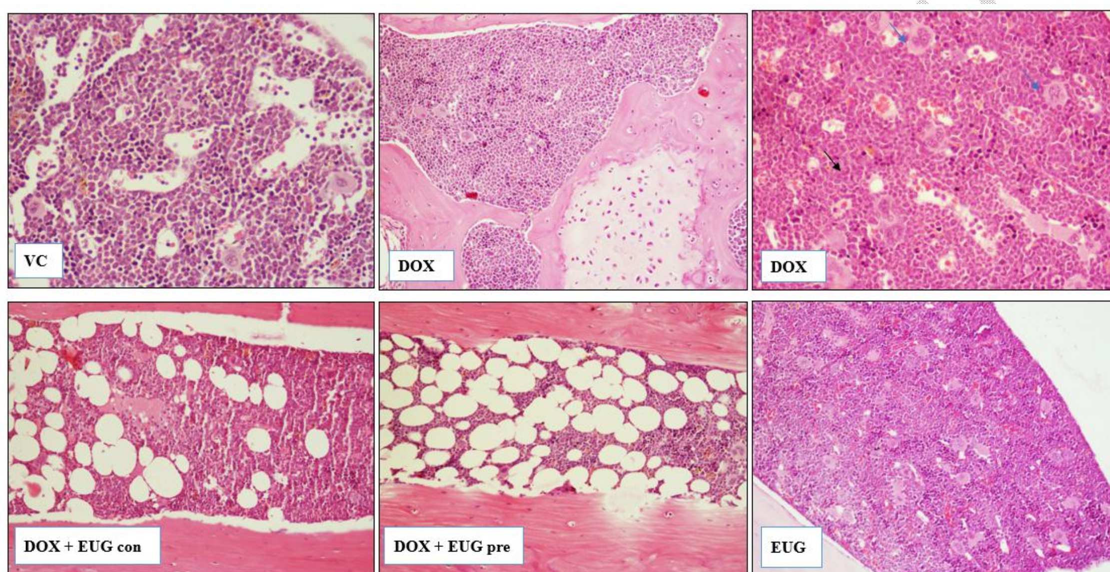

Figure 3. Photomicrographs of femur section of mice after stain with hematoxylin and eosin. Vehicle control (VC) group showed normal architecture of the femur (H&E×400); only eugenol treated group (EUG) also showed normal morphology of the femur (H&E×200); only DOX-treated group (DOX) showed increase in number of myeloid cells,severe congestion of blood vessels and haemorrhages (H&E×200), (H&E×400); concomitant treatment with EUG (DOX+EUG Con) showed decreased hyperplasia and hemorrhage in femur (H&E×200); pretreatment with EUG (DOX+EUG Pre) showed marked improvement in the myeloid hyperplasia and hemorrhages in femur (H&E×200).

Histopathological findings such as severe congestion of blood vessels containing lysed RBCs,diffuse hemorrhages, myeloid hyperplasia (increase cellularity) and increase in megakaryocytes in bone marrow of the mice in group intoxicated with repeated dose of DOX corroborated the genotoxic changes such as micronuclei formation and increased comet length. Genetic changes caused in bone marrow are also reflected in histopathological examination. Similar microscopic changes were also observed by other workers in mice and rats in DOX induced toxicity at different doses [7,27]. Mice supplemented with eugenol concomitantly and 15 days prior to doxorubicin treatment showed mild histological changes in bone marrow as compared to Doxorubicin administered mice.

4.CONCLUSION

Taken together, our results confirmed that eugenol pretreatment as well as concomitant treatment reduced the genetic lesions produced in bone marrow due to repeated doxorubin administration. is probably due to inhibition of DOX induced free radicle formation. The greater 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.

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