



Chemoprotective Effect of Daphnetin against Benzene Induced Leukemia Cancer via Alteration of CYP2E1

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ABSTRACT

Leukemia is a malignant disease of blood forming tissue inducing the over-production of large number of immature blood cells that enter the peripheral blood. Leukemia considered as the 9th most common cancer in men and 12th in women. Available treatment for leukemia are chemotherapy, allogeneic cell transplantation and radiation therapy with side effects. Due to side effect linked with the treatment, medicinal herbs treatment having the more attraction to treat the leukemia. The current study was to scrutinize the anti-leukemic effect of daphnetin against benzene induced leukemia in rats and explores the underlying mechanism. Benzene was used for the induction of leukemia in experimental rats. The rats were divided into different groups and received the daphnetin (12.5, 25 and 50 mg/kg). The body weight, haematological parameters, deoxyribonucleic acid (DNA) fragmentation and cell cycle regulatory parameter were also estimated. Reverse transcription polymerase chain reaction (RT-PCR) was used for the estimation of messenger RNA (mRNA) expression of cytochrome P450 2E1 (CYP2E1). Daphnetin treated rats showed the up-regulation of body weight as compared to other groups. Moreover, daphnetin reduced the blasts in leukemic rats. It also altered the hemotological parameters such as red blood cell (RBC), white blood cell (WBC), lymphocytes, neutrophils, monocytes, eosinophils, monocytes and basophils, respectively. Daphnetin treated rats showed the increased level of p21 and p53 and reduced level of cyclins D1 and E. RT-PCR showed the up-regulated of mRNA expression of CYP2E1 of daphnetin treated group rats as compared to other groups. The current study showed the anti-leukemic effect of daphnetin and highlights the possibility of its use in leukemia to minimize the side effect of the usual therapy.

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Authors' Contribution

JL performed the experimental study. CS, QY and YL interpreted the biochemical data. SL and MT drafted the manuscript. All authors equally contributed in proof reading.

Key words

Daphnetin, Benzene, Haematological parameters, Antioxidant, CYP2E1, Gene expression.

INTRODUCTION

Cancer is the major health threat worldwide. Various types of the cancer spread worldwide and among all of the cancer's leukemia is the most dreadful disease. Leukemia is the most common form of blood cancer and it mostly affects the adults and infants (Ilson, 2008). Leukemia is group of disorder which affects the hemopoietic stem cell, categorized via uncontrolled proliferation and deposition of cancerous white cells in the peripheral blood and bone marrow (Armstrong *et al.*, 2002; Brunning, 2003). The hemopoietic neoplasm resultant from the mutation in the DNA and some few mutations in the DNA that activate or deactivate the tumor suppressor genes, thereby inducing cell death, division or differentiation. Few mutations occur due to predisposing factor including radiation or exposure

of carcinogenic substances such as benzene (Badham *et al.*, 2010; Snyder, 2012).

It is well known that benzene is commonly used for manufacturing of plastics and gasoline and is also found in the cigarette smoke. Human exposure to the benzene may be occupational (fuel and chemical industry) or environmental (exposure to cigarette smoke, gasoline and automobile exhaust) (Whysner *et al.*, 2004; Zhang *et al.*, 2007; Snyder, 2012). Benzene is soluble in the lipids and is commonly deposited in the tissues having high content of lipid. Around 50% of benzene absorbed may be excreted unchanged via exhalation and remaining may be

Abbreviations

RT-PCR, reverse transcription polymerase chain reaction; mRNA, messenger RNA; CYP2E1, expression of Cytochrome P450 2E1; DNA, deoxyribonucleic acid; RBC, red blood cell; WBC, white blood cell; RBC, reactive oxygen species; DMSO, dimethyl sulfoxide; Hb, hemoglobin; PCV, packed cell volume; BM, bone marrow; FBS, fetal bovine serum; GSH, glutathione; MDA, malonaldehyde; IL-1 β , interleukin-1 β ; IL-2, interleukin-2; IL-6, interleukin-6; IL-7, interleukin-7; IL-10, interleukin-10; OD, optical density; MCV, mean corpuscular hemoglobin; HCT, hematocrit; and MCHC, mean corpuscular hemoglobin concentration.

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metabolized into the liver tissue, primarily via CYP2E1 to form reactive metabolites including catechol, hydroquinone and benzene oxide (Lindsey *et al.*, 2005; Kawasaki *et al.*, 2009). Benzene is oxidized in liver via cytochrome P450 2E1 to generate benzene oxide, further it is converted into phenol enzymatically and non-enzymatically. Phenol itself undergoes into the hydroxylation reaction to yield hydroquinone or catechol. These metabolites can exert the toxicity in single or in combination to induce the synergistic effect via targeting the cellular targets leading to the enhanced toxicity (Snyder *et al.*, 1993; Doty *et al.*, 2007).

Benzene induces leukemia via inducing the reactive oxygen species (ROS) and may also bind with the cellular macromolecules. ROS acts as the signaling molecule, which affect the regulation of cell death, cell growth and gene expression; it also increases the lipid peroxidation (Korte *et al.*, 2000; Irons and Gross, 2002). During physiological conditions, specific scavenging and metabolizing systems control the ROS level generated via tissues and cells. Moreover, the imbalance between the generation and excretion of ROS via components of antioxidant defense system induces the oxidative stress (Korte *et al.*, 2000; Irons and Gross, 2002; Snyder, 2012).

Any natural or synthetic agent which inhibits or resists the progression or expansion of any disease or reverts the normal physiological condition is called chemo-preventive (Afzal *et al.*, 2017; Kumar *et al.*, 2017). Moreover, it is not easy to opt for an excellent chemo-preventive agent. Currently approved chemo-preventive agent such as raloxifene is being approved by Food and Drug Administration, United States, used as chemo-preventive agent in women breast cancer. Other, approved drug raloxifene is used for the treatment of osteoporosis, having high risk of breast cancer. For 5 years, postmenopausal women (nearly 19,000) received the raloxifer or tamoxifene. Previous study conducted against the male smoker (29,133 male) on α -tocopherol β -carotene and found the shocking result that 50 mg / kg α -tocopherol and 20 mg / kg β -carotene combination or alone showed the higher percentage of lung cancer between the treatment alone and also reduced mortality (32% to 41%) respectively. (Mukhopadhyay *et al.*, 2017). In the current study, we used the plant based phyto-constituent (daphnetin) and scrutinize against the benzene induced leukemia and explore the underlying mechanism.

MATERIALS AND METHODS

Chemicals

Daphnetin (98%), benzene and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). Biochemical kits were purchased from the

Nanjing Jiancheng Bioengineering Institute, China. All other the chemicals were purchased from the Sigma Aldrich (St. Louis, MO, USA).

Animals

Albino Wistar strain rats (weighing 150±20 gm) were used for the current experimental study. The rats were procured from the departmental animal house and kept in the polyethylene cages. Animals were acclimatized under the standard condition (temperature 25±2°C; 12/12 h light and dark cycle). The rats were fed with the standard rat chow and water *ad libitum*.

Induction of leukemia

Benzene (300 ppm) for 6 h/day, 5 days/week for 2 weeks in 1.3m³ inhalation chambers was used for induction the leukemia.

Experimental procedure

The rats were divided into 6 groups as follow: Group I: normal control (received PBS); Group II: Benzene only; Group III: Benzene only +Daphnetin (12.5 mg/kg); Group IV: Benzene only +Daphnetin (25 mg/kg) and Group V: Benzene only + Daphnetin (50 mg/kg), respectively.

The body weight, water and food intake of all group rats were estimated at regular interval (Kabeel *et al.*, 2018). At end of the study, blood sample of all group rats were collected via puncturing the heart and all group of rats scarified via cervical dislocation. The blood samples of all groups of rats were kept at 4°C and centrifuged for isolation of plasma for further biochemical and other parameters estimation (Mukhopadhyay *et al.*, 2017).

At the end of experimental study, rats were anesthetized with excess of anesthesia and blood was collected in an anticoagulation tube for estimation of RBC, WBC, hemoglobin (Hb), packed cell volume (PCV), platelets, mean corpuscular hemoglobin (MCV), hmatocrit (HCT) and mean corpuscular hemoglobin concentration (MCHC) (Saha *et al.* 2012). The rats were sacrificed by decapitation. Spleen, lung, liver and kidney were excised and weighed. The relative organ weight was calculated as the ratio between the body weight and organ weight. Bone marrow cells were flushed from one tibia using the 23-gauge needle to make smears. The bone marrow smears were stained with Wright-Giemsa and used for determination of differential blood counts (Eastmond *et al.*, 2001).

Collection of hematopoietic stem cells

Bone marrow (BM) cells were harvested from rats femur and tibia, flushed with stain buffer (fetal bovine serum (FBS)) (BD Pharmingen, San Jose, CA, USA), and

pipetted gently. The 70 μm cell strainer (BD Pharmingen, San Jose, CA, USA) was used to get a single cell suspension. To get the hematopoietic stem and multipotent cells (LSK cells), the BM cells were then stained with fluorescent-labeled antibody namely APC lineage cocktail, Sca-1 PE-CY7 and c-Kit PE-CY5 (BD Pharmingen, San Jose, CA, USA) and incubated at 4°C in the dark for 45 min. The cells were washed twice and re-suspended in 1 mL stain buffer. Then one drop of 7-AAD was added for 5 min. BD FACS Aria II Cell Sorter (BD Bioscience, San Jose, CA, USA) were used for flow cytometric analysis and cell sorting. The purity of isolated LSKs that was routinely obtained was >97%.

Antioxidant parameters

An antioxidant parameter such as glutathione (GSH) 8-hydroxy-2'-deoxyguanosine (8-OHdG) and malonaldehyde (MDA) was estimated by standard method with minor modification (Saha *et al.*, 2012).

Estimation of cytokines

The cytokines such as interleukin-1 β (IL-1 β), interleukin-2 (IL-2), interleukin-6 (IL-6) (Termo Fisher Scientific company, USA), interleukin-7 (IL-7) and interleukin-10 (IL-10) (Abcam, UK) were scrutinized by using the commercial ELISA kits following the manufacture's instruction.

Estimation of apoptotic marker

A commercial ELISA kit was used for the estimation of level of cleaved PARP. Briefly, for the preparation of samples, the samples were incubated with primary and secondary antibodies followed by color formed under the ELISA based experiments was performed as per the respective instruction provided with the kits and finally the optical density (OD) was estimated at 405 nm by using the ELISA plate reader.

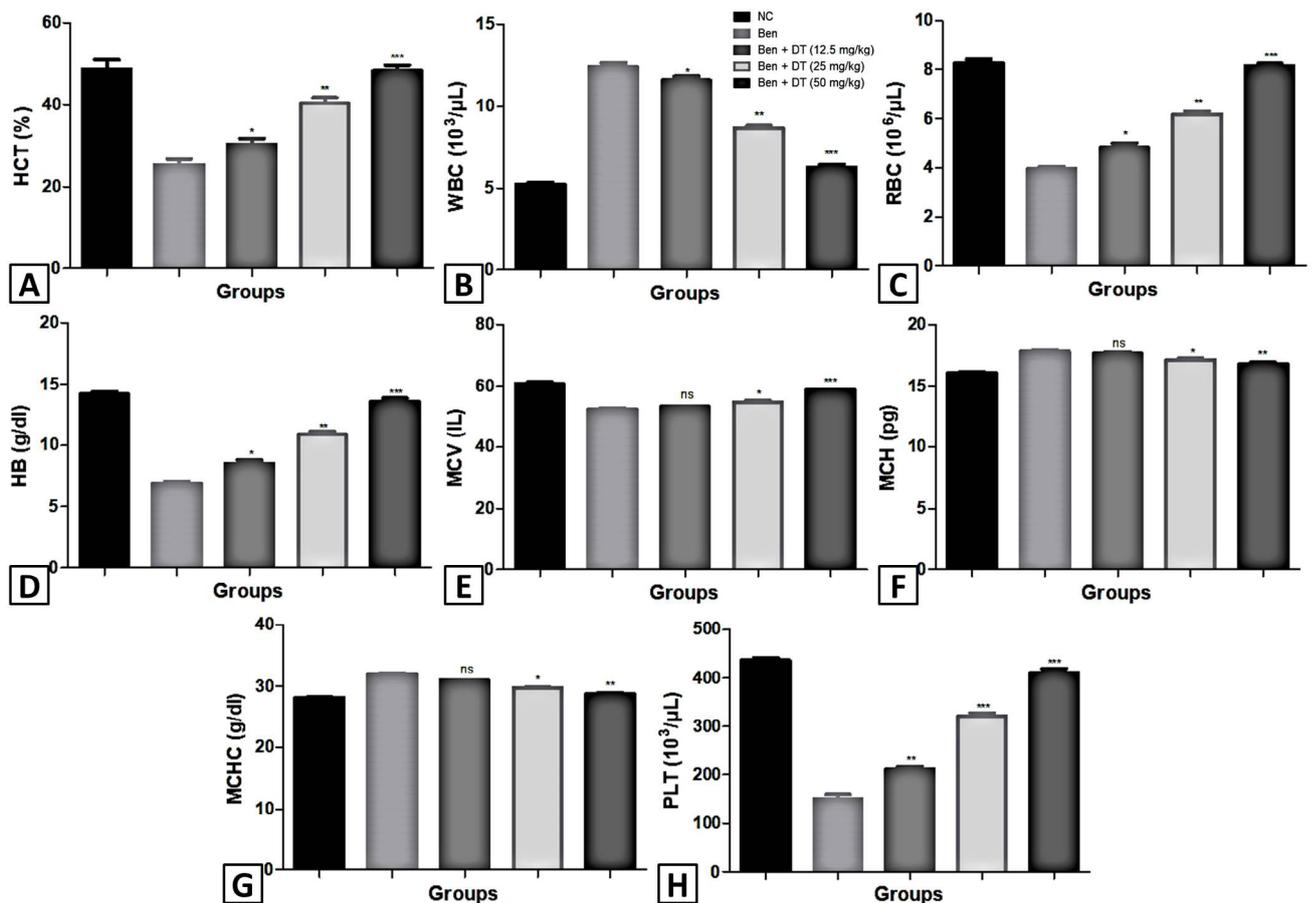


Fig. 1. Effect of daphnetin on various haematological parameters in benzene induced leukaemic rats. A, HCT; B, WBC; C, RBC; D, Hb; E, MCV; F, MCH; G, MCHC; H, PLT. The data are shown as mean \pm SEM. * P <0.05, ** P <0.01 and *** P <0.001 compared with the benzene induced leukaemia rats.

Table I: List of primer sequence.

S. No	Primer	Forwarded	Reverse
1	CYP2E1	5'-TGCCATCAAGGATAGGCAAG-3'	5'-AATGCTGCAAAATGGCACAC-3'
2	β -Actin	5'TGACGGGGT CAC CCA CAC TGT GCC CAT CTA 3'	3'CTAGAAGCATTTGCGGTG GAC GAT GGA GGG 5'

RT-PCR for CYP2E1

Reverse transcription of RNA to cDNA was performed following kit manufacture instructions (Promega, Madison, WI, USA) and stored at -20°C for further use. Briefly, for the preparation of cDNA sample, PCR buffer, cDNA, deoxynucleotide triphosphate, MgCl₂, AmpliTaq DNA polymerase and primers of CYP2E1 were added. The sequence of the primer is listed in Table I (Saha *et al.*, 2012).

Data analysis

Results are expressed as mean \pm SEM. Total variation present in a set of data was estimated by one-way analysis of variance (ANOVA) followed by Dunnet's test. P < 0.05 was considered significant.

RESULTS*Effect of daphnetin on various haematological parameters*

Figure 1 shows the effect of daphnetin on the benzene induced leukemia rats. Various haematological parameters such as HCT, MCV, MCH and MCHC in benzene induced leukemia group rats showed up-regulation in the level of HCT, MCV and down-regulation in the level of MCH, MCHC. Daphnetin significantly (P<0.001) decreased the level of HCT, MCV and increased level of MCH and MCHC at concentration dependent manner.

Figure 1 also shows the effect of daphnetin on RBC and WBC. Benzene induced leukemia rats showed increased level of WBC and decreased level of RBC and Hb. The concentration dependent daphnetin significantly (P<0.001) reduced the level of WBC and enhanced level of RBC and Hb.

Effect of daphnetin on differential leukocytes count

Figure 2 shows the effect of daphnetin on the differential leukocytes count in benzene induced leukemia rats. Normal control group rats showed the normal values of differential leukocytes count. Benzene induced group rats showed the reduced level of lymphocytes, neutrophils and increased level of blasts, monocytes, eosinophils and basophils. Dose dependent treatment of daphnetin significantly (P<0.001) boosted the level of lymphocytes, neutrophils and suppressed the level of blasts, monocytes,

eosinophils and basophils.

Effect on body weight

The body weights of normal control rats increased in normal manner. On the other hand, benzene induced leukemia rats showed reduced body weight compared to other rats group at end of the experimental study. Dose dependent treatment of daphnetin significantly (P<0.001) increased the body weight compared to the benzene induced leukemia rats group (Table II). Daphnetin treatment at 50 mg/kg increased the body weight almost near to normal in control rats.

Table II: showed the effect of daphnetin on the body weight of benzene induced leukaemia rats.

S. No	Group	Initial	Final
1	Normal Control	165.4 \pm 6.92	185.63 \pm 5.43
2	Ben Control	170.6 \pm 9.34	165.45 \pm 12.34
3	Ben + Daphnetin (12.5 mg/kg)	169.6 \pm 7.54	178.7 \pm 8.54***
4	Ben + Daphnetin (25 mg/kg)	171.3 \pm 6.83	183.45 \pm 9.34***
5	Ben + Daphnetin (50 mg/kg)	170.8 \pm 9.34	190.34 \pm 8.76***

The data are showed in mean \pm SEM. *P<0.05, *P<0.01 and ***P<0.001 compared with the benzene induced leukaemia rats.

Effect of daphnetin on antioxidant parameters

Figure 3 shows the effect of daphnetin on endogenous antioxidant parameters in leukaemic rats. In the normal control group rats, the level of endogenous antioxidant parameters remains normal at end of the experimental study. Benzene induced leukemia showed increased level of MDA and decreased level of GSH, 8-OhdG in the serum as compared with the normal control. Daphnetin significantly (P<0.001) reduced the level of MDA and increased the level of GSH, 8-OhdG (Fig. 3).

Benzene induced leukemia rats showed enhanced level of MDA and reduced level of GSH in the hepatic tissue as compared to normal control rats. Daphnetin significantly (P<0.001) suppressed the level of MDA and enhanced the level of GSH in a concentration dependent manner (Fig. 4).

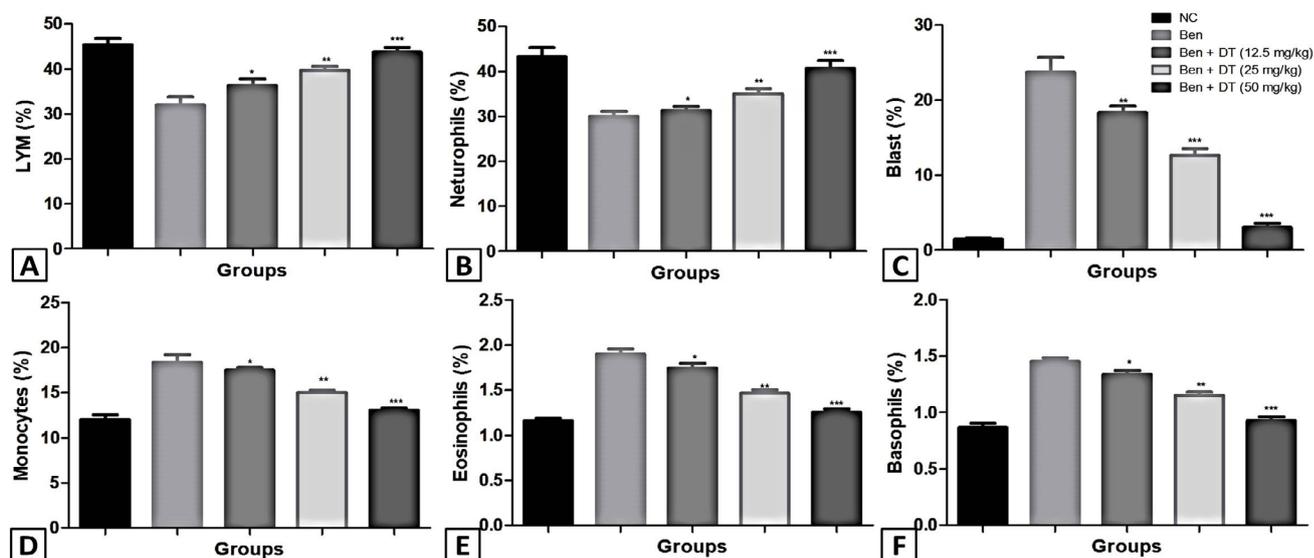


Fig. 2. Effect of daphnetin on differential of leukocytic count of benzene induced leukaemic rats. A, LYM; B, Neutrophils; C, Blast; D, Monocytes; E, Eosinophils; F, Basophils. The data are shown as mean±SEM. *P<0.05, **P<0.01 and ***P<0.001 compared with the benzene induced leukaemia rats.

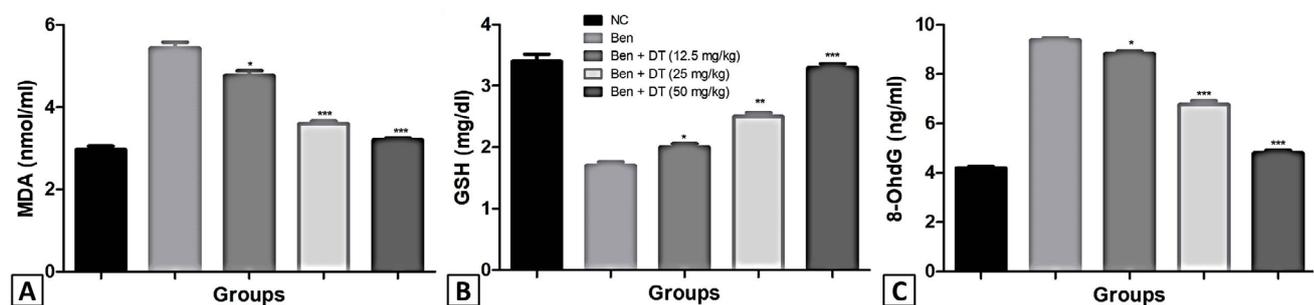


Fig. 3. Effect of daphnetin on antioxidant parameters in serum of benzene induced leukaemic rats. A, MDA; B, GSH; C, 8-OHdG. The data are shown as mean±SEM. *P<0.05, **P<0.01 and ***P<0.001 compared with the benzene induced leukaemia rats.

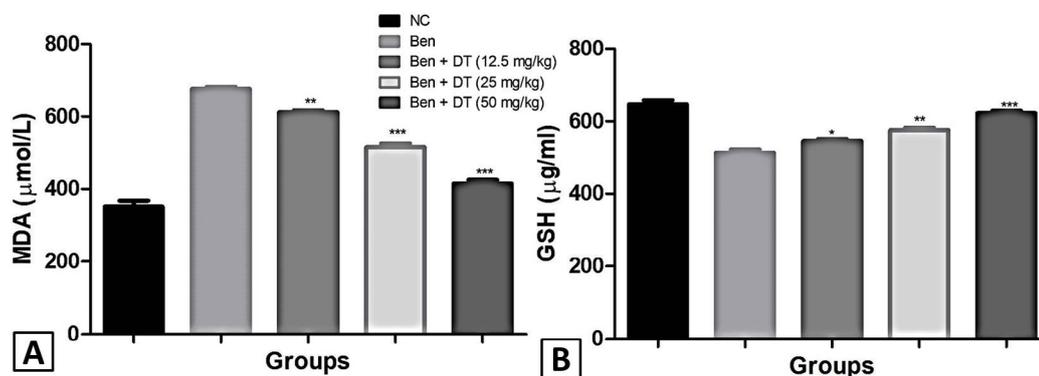


Fig. 4. Effect of daphnetin on antioxidant parameters in liver tissue of benzene induced leukaemic rats. A, MDA; B, GSH. The data are shown as mean±SEM. *P<0.05, **P<0.01 and ***P<0.001 compared with the benzene induced leukaemia rats.

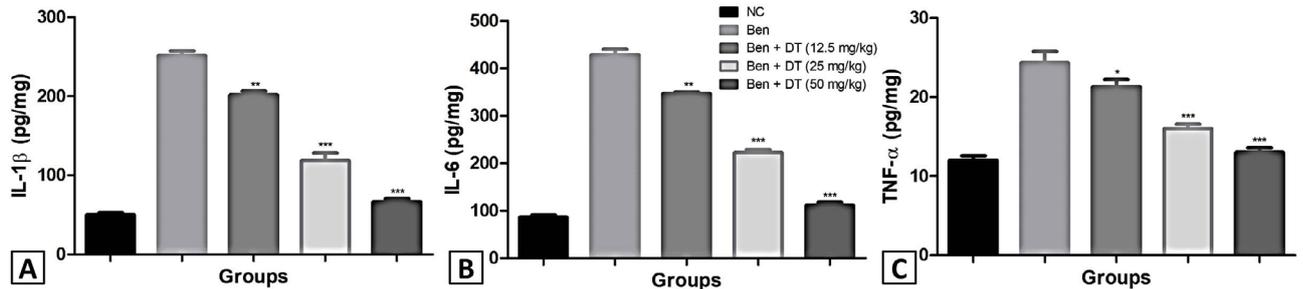


Fig. 5. Effect of daphnetin on proinflammatory cytokines of benzene induced leukaemic rats. A, IL-1 β ; B, IL-6; C, TNF- α . The data are shown as mean \pm SEM. * P <0.05, ** P <0.01 and *** P <0.001 compared with the benzene induced leukaemia rats.

Effect of daphnetin on pro-inflammatory cytokines

Figure 5 shows increased inflammatory reaction and increased level of inflammatory mediators and pro-inflammatory cytokines. Benzene induced control group rats showed the up-regulation in the level of pro-inflammatory cytokines *viz.*, IL-1 β , IL-6 and TNF- α in comparison with normal control. Daphnetin significantly (P <0.001) decreased the pro-inflammatory cytokines in concentration dependent manner compared to benzene control rats.

Effect of daphnetin on cytochrome P450 E1

Benzene induced leukemia showed increased level of cytochrome P450 E1. On the other hand, normal control group rats showed the normal level of cytochrome P450. Concentration dependent treatment of leukemia significantly (P <0.001) reduced the level of cytochrome P450 (Fig. 6).

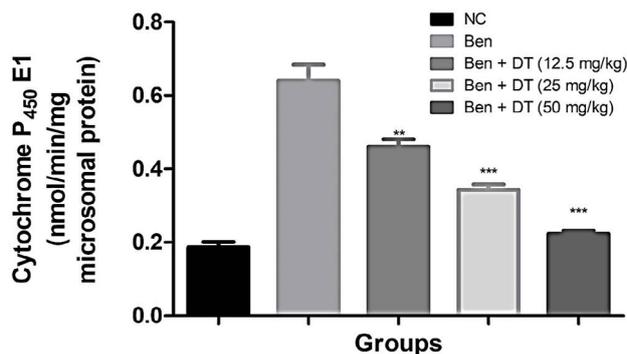


Fig. 6. Effect of daphnetin on cytochrome P450E1 of benzene induced leukaemic rats. The data are shown as mean \pm SEM. * P <0.05, ** P <0.01 and *** P <0.001 compared with the benzene induced leukaemia rats.

Effect of daphnetin on CyP2E1

The expression of CyP2E1 was upregulated after leukemia induction. Benzene induced group rats showed increased level of CyP2E1 as compared to normal control.

Dose dependent treatment of daphnetin significantly (P <0.001) reduced the level of CyP2E1 compared to benzene induced leukemia rats (Fig. 7).

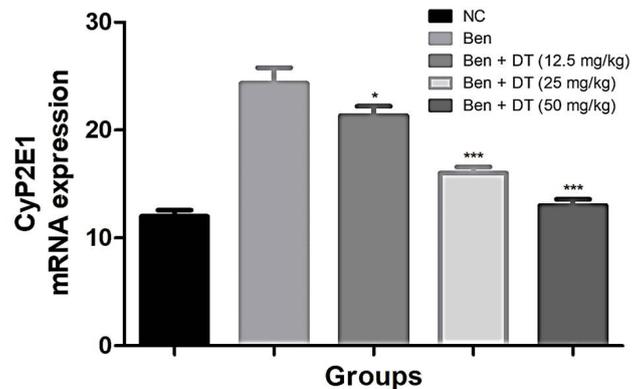


Fig. 7. Effect of daphnetin on the level of CyP2E1 mRNA of benzene induced leukaemic rats. The data are shown as mean \pm SEM. * P <0.05, ** P <0.01 and *** P <0.001 compared with the benzene induced leukaemia rats.

DISCUSSION

Although various investigation have been carried out to perform the benzene induced leukemogenicity and toxicity, but the underlying mechanism has still not been fully explained. In the current experimental study, we made attempts to scrutinize the anti-leukemic effect of daphnetin and explore the possible mechanism of action (Yoon *et al.*, 2003). Benzene is known to induce the leukemia along with myelogenous leukemia, hepatic leukemia of erythroblastic stem cells, thymic leukemia and lymphoblastic leukemia (Hirabayashi *et al.*, 2004).

Blood is a significant maker of pathological and physiological status in human and rodents and the parameters usually estimated are RBC, hemoglobin, WBC and total leukocytes counts (Natelson, 2007; Snyder, 2012). Benzene induced leukemia is categorized via

reduction of erythromyelopoiesis, resultant reduction of erythrocyte and leukocyte (especially lymphocyte count) level in the peripheral blood (Huff, 2007; Natelson, 2007). During the benzene induction, the rats showed the modulation of blood parameters and dose dependent treatment of daphnetin considerably restored the blood parameters (Huff, 2007). Moreover, after treatment with daphnetin, it was observed that it shows the protective effect of blood cell count. During benzene treatment rats showed the destruction of DNA and daphnetin showed the protective effect, may be due to repair of DNA.

Moreover, ROS produce the necrosis and cellular injury through various mechanism such as peroxidation of lipid, DNA and protein (Jang *et al.*, 1997). In the current experimental study, we have found the increased level of lipid peroxidation which was estimated in term of MDA in the benzene induced leukemia group rats as compared to normal control. The MDA level decreased in the serum as well as hepatic tissue and also reduced the endogenous antioxidant activity (Kumar *et al.*, 2013). In the current experimental study, we observed the increased level of MDA, which proved the boost in the lipid peroxidation. On the other hand, daphnetin decreased the lipid peroxidation due to interaction of daphnetin with toxin induced cellular injury and peroxidation in the experimental rats and confirm the antioxidant effect of daphnetin. Previous studies suggested that during the benzene induced leukaemia activate the apoptosis and caspase reactions (Jang *et al.*, 1997; Irons and Gross, 2002; Snyder, 2012). Daphnetin proved their beneficial role in alleviating the oxidative stress induced apoptosis via scavenging the ROS, increasing the redox status via chelating intralysosomal iron and preventing the rupturing of lysosomes. It is well documented that antioxidant altering enzymes and GSH play a significant role in the purification of ROS and various pollutants via either direct interaction with ROS or via decreasing the disulphide linkage in various proteins, thus protecting cells from the oxidative injury. Therefore, the reduced level of GSH was observed in the benzene induced group rats and dose dependent treatment of daphnetin significantly increased the level of GSH suggesting the antioxidant effect.

According to the Thomas *et al.* (2014), the mRNA expression of CYP2E1 was altered in the hepatic tissue during exposure to benzene. Other studies conducted by Gonzalez-Jasso *et al.* (2003) showed that dose dependent induction of benzene increased the mRNA expression of CYP2E1 and protein in both peripheral lymphocytes and liver tissue. Benzene increased the mRNA expression of CYP2E1 probably via post-transcriptional or transcriptional regulation. The mRNA expression of CYP2E1 considerably increased due to formation of toxin

metabolites of benzene like hydroquinone which may be responsible for the induction of leukemia (Neafsey *et al.*, 2009; Zhang *et al.*, 2011). It is well documented that CYP2E1 play a significant role in the expansion of oxidative stress and reactive oxygen species (ROS). ROS and oxidative stress induce the cell damage, and CYP2E1 is considered as the major contributor to benzene induced hepatic injury and oxidative stress (González-Jasso *et al.*, 2003; Thomas *et al.*, 2014). CYP2E1 also reduce the NADPH oxidase activation and also decrease the NADPH induced production of ROS. Benzene induced leukemia rats showed similar results that increased mRNA expression of CYP2E1 and dose dependent treatment of daphnetin considerably reduced the mRNA expression of CYP2E1 which suggest the hepatic protective effect. Daphnetin showed the hepatic protective effect due to reduced NADPH CYP2E1 reductase activity which is responsible for proper catalytic role of CYP2E1 (Cheung *et al.*, 2005; Neafsey *et al.*, 2009; Kanagal-Shamanna *et al.*, 2012).

CONCLUSION

On the basis of result, we can conclude that daphnetin ameliorate the benzene induced leukaemia in the experimental rats via alteration of differential RBC count and differential platelet count. This shows anti-leukemic effect of daphnetin via alleviating the endogenous antioxidants. Daphnetin also decreased the expression of CYP2E1 and hence suggests the anti-leukaemia effect.

Statement of conflict of interest

The authors declare no conflict of interest.

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