A monoterpenic antioxidant, linalool, mitigates benzene-induced oxidative toxicities on hematology and liver of male rats

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ABSTRACT
Benzene is a volatile chemical implicated in leukemia and hepatic injury through oxidative stress-mediated mechanism. Linalool is a naturally small molecular monoterpenic with proven antioxidant properties. This study was carried out to investigate the potential role of Linalool in ameliorating the oxidative stress-mediated hematologic and hepatic dysfunctions by Benzene in rats. Hepatic injury and hematological imbalance were induced in Wister rats by intravenous administration of 0.2 mL of benzene solution every other days for 3 consecutive weeks. Following benzene intoxication, 40 mg/kg linalool was post-administered each day orally for two weeks. Hematological alteration was assessed by comparing the hematological parameters of control and benzene-exposed animals with or without linalool supplementation. Plasmal activities of liver biomarker enzymes (ALT, AST, and ALP), total thiol and advanced oxidation protein products (AOPPs) along with hepatic activities of SOD, CAT, and hepatic contents of thiol-reduced glutathione (GSH), malondialdehyde, and percentage DNA fragment were measured. Linalool showed improvements in liver functions and restored altered hematology, decreased levels of AOPPs and oxidative stress and mitigated genotoxicity affected by benzene. In conclusion, linalool exerted its organ protective and myeloprotective effect through its influence on antioxidant defense system and reduction of oxidative stress.

Introduction
The redo-hematological influence of benzene have been well documented where it perturbed both the oxidative status and hemato logical parameters of living system. Benzene is a known leukemogen which caused plastic anemia, reduced hemoglobin content and resulted in leukocytosis, leukemic symptom that depicts leukemia in exposed organism especially in rat model and human being [1,2]. Human exposure to benzene has been associated with reduced activities of free radical scavenging enzymes such as superoxide dismutase and catalase and resulted to elevated malondialdehyde in the blood of occupationally exposed workers [3]. This may probably due to enzymatic bioactivation of benzene to reactive inter mediates that increased formation of reactive oxygen species (ROS) [4]. Animal experimental studies corroborated the evidence that exposure to benzene increases the risk of cancer in multiple organ systems including the hematopoietic system, liver, and stomach [5]. Hepatic injury by benzene has been
linked to oxidative stress through generation of reactive oxygen species during its metabolic biotransformation [6]. These compounds form covalent adducts with macromolecules such as proteins and nucleic acids in liver, kidney, spleen, and blood [7,8]. Oxidation of benzene in the liver by cytochrome P450 2E1 (CYP2E1) to benzene oxide and other reactive intermediates is an initial step in the bioactivation of benzene and is a prerequisite for cellular toxicity [7]. Benzene oxide can be hydrolyzed by microsomal epoxide hydrolase to benzene dihydrodiol that is then converted to a catechol or can undergo ring opening to produce trans–transmuconaldehyde or spontaneously rearrange to form phenol, which is then hydroxylated in the liver to form hydroquinone [9].

The link between liver organ and blood tissue has been established in which changes in the morphology and phenotype of hepatic epithelial cells during development correlate with hematopoietic activity [10,11]. Cells of the hepatocyte lineage interact with erythroblasts and produce stem cell factor and erythropoietin which are erythropoietic cytokines [11–13]. Moreover, derangement of hepatocyte function such as the synthesis of hemostatic factors has been reported in chronic liver disease [14]. The bone marrow of patients with liver cirrhosis was reported to exhibit ineffective hematopoiesis and dysplastic changes of karyocytes [15]. Therefore, toxicant that induces karyocytes may have influence on hematology or blood-related conditions such as anemia [16].

Linalool is a natural small molecule monoterpen with strong activity against different solid tumors such as renal cell adenocarcinoma cells, HepG2, and MCF-7 cells [17–19]. It exist as two scent racemic forms; 3 R-(-)-linalool (licareol) and 3S-(+)-linalool (coriandrol) as shown in Figure 1 which are main component of essential oil of Lavandula officinalis and Coriandrum sativum seeds, respectively, [20]. Pharmacologically, linalool possesses sedative, analgesic, anti-inflammatory, antioxidant, antimicrobial, and antitumor properties [20,21]. Linalool was reported to possess strong anti-leukemic activities and induce cell cycle arrest and/or apoptosis in leukemia [22–24]. Linalool has been found to offer hepatoprotective potential against carbon tetrachloride toxicity and exert regression of hepatocarcinogenesis induced by diethylnitrosamine and 2-acetylaminofluorine in rats [25,26]. In consonant with anti-leukemic and hepatoprotective potential of linalool, the present work, therefore, investigated the hepatoprotective effect of linalool in benzene-induced leukocytosis.
examining the biomarkers of oxidative stress in liver and blood Figure 2.

Materials and methods

Chemicals and reagents
Linalool is a product of Santa Cruz Biotechnology Inc Finnell Street Dallas, Texas, 1-chloro-2, 4-dinitrobenzene (CDNB), Glutathione (GSH), 5’,5’-dithio-bis-2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA), epinephrine, and hydrogen peroxide were purchased from Sigma® Chemical Company (London, UK). ALT and AST kits were obtained from Fortress Diagnostics Ltd., Antrim, UK. All other chemicals and reagent were of analytical grade and were obtained from British Drug House Poole UK.

Experimental animals
Twenty four adult male Wistar strain rats of weight range 100–120 g were used for this study. The animals were got from the Department of Veterinary Medicine University of Ibadan and acclimatized for 2 weeks in the animal house of the Department of Chemical Sciences, Ajayi Crowther University, Oyo. They were kept in research plastic cages and fed with standard commercial rat feed (Ladokun feeds Nigeria Ltd.) and clean tap water being supplied ad libitum. Anesthesia was not involved in this research and the protocol conformed to the guidelines of the National Institute of Health for laboratory animal care and use [27] with ethical approval code of Fns/Erc/2,019,002 from Faculty of Natural Sciences Ethical review committee of Ajayi Crowther University on January 8, 2019.

Drug treatments and animal grouping
After 2 weeks of adaptation period, intravenous injection of wister rats with 0.2 mL of a 1:10 diluted benzene solution (Chromasolv, in water/2-propanol [50/50] v/v) every 2 days for 3 consecutive weeks successively induced leukocytosis in the rats. Following induction, the animals were randomly assigned into four main experimental groups of 5 animals each. The doses of linalool was carefully chosen from literature review [28] and administered once daily by oral gavage using oral intubator as shown in Table 1

Collection of blood and liver samples
After 24 hours of final treatment, the blood samples were collected from each animal through retro orbitals plexus into lithium heparinized tubes centrifuged at 4000 rpm for 5 min bench centrifuge to obtain the plasma, and thereafter sacrificed. The bone marrow of femurs was collected for the assessment of bone marrow micronucleus frequency and livers were also excised, homogenized in 4 volumes of ice-cold 0.01 M potassium phosphate buffer (pH 7.4) and centrifuged for 15 min at 12,500 g in an Eppendorf (UK) refrigerated centrifuge at 4°C and the supernatants, termed as the post-mitochondrial fractions (PMF), were used for estimation of CAT, SOD, GST, lipid peroxidation estimated by quantitation of malondialdehyde (MDA) and GSH.

Table 1. Experimental design.

| Treatment groups       | Treatment (14 days)                                      |
|------------------------|----------------------------------------------------------|
| A. Control (CTRL)      | Distilled water                                          |
| B. Linalool (LN)       | 40 mg/kg linalool every day for 2 weeks                   |
| C. Leukocytosis rats (Leuk) | 0.2 ml of benzene mixture every two days for 3 weeks   |
| D. Leukocytosis rats + Linalool (Leuk + LN) | 0.2 ml of benzene mixture every two days for 3 weeks then + 40 mg/kg linalool every day for 2 weeks |
**Determination of plasma and liver protein content**

The Biuret method of Gornall et al., 1949 [29] was used to determine protein concentration in the plasma and liver homogenate after which the protein concentration in the samples was extrapolated from the standard bovine serum albumin (BSA) curve.

**Assay of biomarkers of hepatotoxicity**

Biomarkers of hepatotoxicity: activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed using Fortress Diagnostics kits based on the manufacturer’s procedure. Determination of plasma ALT and AST activities were based on the principle described by Reltman and Frankel in 1957 [30]. The activity ALP was determined in accordance with the principles of Tietz (1994) [31]. The p-nitrophenol formed by the hydrolysis of p-nitrophenyl phosphate confers a yellowish color to the reaction mixture and its intensity can be monitored at 405 nm to give a measure of enzyme activity.

**Assay for oxidative stress maker in the plasma**

Plasma AOPP was determined by the method described by Witko et al. (1996) [32] as modified by Zhang et al., 2014 [33]. Plasma total thiol was measured spectrophotometrically using DTNB (2, 2’-dinitro-5, 5’-dithiodibenzoic acid) [34].

**Assay for non-enzymatic antioxidants in the liver**

**Hepatic reduced glutathione level (GSH)**

The method of Jollow et al. (1974) [35] was used to evaluate the level of hepatic GSH. The chromophoric product resulting from the reaction between Ellman’s reagent, 5,5’-dithiobis -(2-nitrobenzoic acid) (DTNB) and reduced glutathione possesses a molar absorption at 412 nm.

**Assay of hepatic antioxidant enzymes**

The activity of hepatic GST was determined by the method described by Habig et al. (1974) [36]. Briefly, the assay mixture (3 mL) was made up of 150 µL of CDNB (3.37 mg/mL), 30 µL of reduced GSH (0.1 M), 2.79 mL phosphate buffer (0.1 M, pH 6.5) and 30 µL of liver PMF. The absorbance was measured at 340 nm against the blank after the reaction has stayed for 60 seconds.

The activity of superoxide dismutase (SOD) in hepatic tissue was determined by the procedure of Misra and Fridovich (1972) [37] where inhibition of auto-oxidation of epinephrine at alkaline medium pH 10.2 was being monitored. The procedural method outlined by Singha (1972) [38] was used to estimate the catalase activity in liver based on the reduction of dichromate in acetic acid to chromic acetate when heated in the presence of hydrogen peroxide (H₂O₂). The chromic acetate produced was measured spectrophotometrically at 570 nm and the amount of H₂O₂ remaining was extrapolated from the standard curve for H₂O₂. Catalase activity in the sample was expressed as micromole of H₂O₂ consumed per min per mg protein.

**Assay of hepatic level of lipid peroxidation**

The degree of lipid peroxidation (LPO) in the post mitochondrial fraction of liver organ was determined as described by the method of Varshney and Kale (1990) [39]. The method entailed the reaction between malondialdehyde and thiobarbituric acid to give a stable pink chromophore with maximum absorption at 532 nm. Lipid peroxidation in nmol/mg protein was computed as:

\[ MDA = \text{Abs} \times \text{Volume of Reaction Mixture} \times \text{Volume of Sample} \times \text{mgprotein} \]
Where $E_{5332}$ is the molar extinction coefficient for MDA = $1.56 \times 105$ M $\cdot$ 1 Cm$^{-1}$

**Percentage DNA Fragmentation Assay**

The method of Wu et al. (2005) [40] was used for DNA fragmentation assay where the endonuclease cleavage of the end product of apoptosis was assessed. DNA was extracted from the homogenate and treated with diphenylamine (DPA). The chromophore formed was measured on the spectrophotometer at 620 nm. Briefly, the livers were homogenized in 10 volumes of Tris-EDTA buffer (TE) pH 8.0 containing Triton-X100. Homogenates were centrifuged at 27,000 g for 20 min to separate the intact chromatin (pellet named A) from fragmented ones (supernatant named B). The pellet (A) was suspended in Tris-EDTA buffer (TE!) of pH 8.0 without Triton-X100. 0.5 mL aliquot of each sample (pellet and supernatant) was placed in separate test tubes and 1.5 ml of freshly prepared diphenylamine solution was added to each test tubes. Reaction mixture was incubated at 370° C for 20 h. Absorbance of mixture was then measured at 620 nm.

**Calculation**

Quantity of fragmented DNA was estimated by using the formula;

$$\text{% fragmented DNA} = \frac{B}{A + B} \times 100$$

**Statistical analysis**

Data are presented as the mean ± standard deviation (SD) of six replicates. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Duncan’s multiple comparisons between control and treated rats in all groups using SigmaPlot® statistical package (Systat Software Inc., San Jose, CA, USA). P-values less than 0.05 (P < 0.05) were considered statistically significant.

**Results**

**Influence of linalool on benzene-induced hematological changes**

The benzene-intoxicated leukocytosis group (Leuk) showed a reduction in packed cell volume (PCV), Hemoglobin and RBC content when compared with control values. Moreover, the WBC count in group of leukocytosis rats (leuk) was markedly increased, when the leukocyte counts were compared with control rats which reveal a leukocytosis condition, an indication of leukemia. However, the group treated with linalool alone and leukocytosed rats treated with linalool showed similar hematological parameters closed to that of control group which indicated that the extract did not have adverse hematological effect but has protective role against hematological influence by benzene exposure.

**Influence of linalool on benzene-induced changes on plasma liver function markers of leukocytosis rats**

Administration of benzene mixture significantly increased the plasma activities of aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline Phosphatase (ALP) in the plasma of leukocytosis rats by 54.2, 43, and 25.3%, respectively, when compared to the control group (Figure 3). However, post-treatment with linalool showed a significant decrease in the activity of AST, ALT, and ALP in the plasma by 31.9, 29.8, and 29.5%, respectively, when compared with the untreated leukocytosis group.

**Effect of linalool on benzene – induced oxidative stress in the plasma of leukocytosis rat**

Administration of Benzene mixture showed a significant elevation in the concentration of advanced oxidation protein products (AOPPs) present in the blood plasma by 28.8% with concomitant reduction in total thiol content by 33.51% when compared to the control group (Figure 4).
However, co-treatment with Linalool significantly (p < 0.05) attenuated the effect of Benzene toxicity by reducing the generation of plasma AOPP and caused an increase in thiol protein concentration in the plasma when compared to untreated leukocytosis animal group.

Influence of Linalool on Benzene-induced changes in liver biomarkers of oxidative stress in Leukocytosis rats

Hepatic levels of the non-enzymatic antioxidants reduced GSH was significantly reduced (P < .05) after induction of leukocytosis in rats. A similar reduction was also observed in the hepatic activities of enzymatic antioxidants: SOD and CAT (Figures 5A and 5B respectively). However, post-treatment with linalool significantly ameliorated the levels of GSH and the activities of hepatic SOD and CAT in the leukocytosis rats. MDA is
one of the products of lipid peroxidation resulted from oxidative stress. Compared to the control group, animal intoxicated with benzene has a significant ($P < .05$) increase in the hepatic LPO as observed by the MDA content in figure 5D. However, post-treatment with linalool, attenuated this increase in hepatic MDA levels.

**Figure 3.** Protective effects of Linalool on Benzene-induced changes in the plasma activities of aspartate amino transferase (A), alanine amino transferase (B) and alkaline phosphatase (C) in rats. Notes: Data represent the means ± SD for five rats in each group; * Significantly different from control group ($p < 0.05$). # Significantly different from Leuk only group ($p < 0.05$)

**Effect of linalool on benzene-induced hepatic percentage DNA fragmentation in leukocytosis rats**

The depletion in hepatic antioxidant molecules induced by intravenous administration of Benzene was accompanied by a significant increase in the hepatic level of percentage DNA
fragmentation as shown in figure 6. The level of Percentage DNA Fragmentation was significantly elevated in leukocytosis rats by 52.2% when compared with the control. Treatment with Linalool significantly attenuated this increase when compared with untreated Leuk group.

**Discussion**

Benzene is a volatile aromatic hydrocarbon that can alter the microanatomy and physiology of different organs including the liver in affecting its toxicity [41]. This toxic effect is probably mediated through the generation of reactive oxygen species intermediate. Linalool is monoterpene used for medicinal purposes due to its pharmacological activities, such as sedative, analgesic, anti-inflammatory, antioxidant, antimicrobial, and antitumor properties among others [20,21]. The research investigated the protective effects of linalool against the toxicological influence of benzene on hematology and the liver of rats through the analysis of biochemical indices, basic redox status markers and assessment of genomic instability.

The result from the assessment of hematological parameters are presented in. It shows the comparisons of various hematological parameters among groups that are post-treated with linalool after induction of leukocytosis. The untreated leukocytosis group (Leuk) showed a reduction in packed cell volume (PCV), Hemoglobin and RBC content when compared with control values. PCV is a measure of relative volume of RBCs to the total volume of blood which reflects the amount of circulating RBCs and the degree of anemia or polycythemia in living system. Moreover, the WBC count in group of leukocytosis rats (leuk) was markedly increased when the leukocyte counts were compared with control rats which reveal a leukocytosis condition as an indication of leukemia. However, the group treated with linalool alone and leukocytosis rats treated with linalool showed similar hematological parameters close to that of control group which indicated that the extract did not have adverse hematological effect but has protective role against hematological influence by benzene exposure.

![Figure 4. Effect of Linalool on plasma concentration of total thiol (A) and advanced oxidized protein products (B) in Benzene-induced leukocytosis in Wistar rats.](image)

Notes: Data represent the means ± SD for five rats in each group; * Significantly different from control group (p < 0.05). # Significantly different from Leuk only group (p < 0.05)
Hepatic necrosis resulted from increased lipid peroxidation and serum activities of liver-specific biomarker enzymes like AST and ALT has been reported in animals intoxicated with benzene [42]. Plasma ALT and AST are used as markers of liver injury as their significant elevation in the plasma is linked to their leakage from damaged hepatocytes [43–45]. ALP is associated with cell membrane and its relative increase in plasma is associated with

**Figure 5.** Effect of linalool on liver concentration of fragmented DNA in Benzene-induced leukocytosis in Wistar rats. Notes: Data represent the means ± SD for five rats in each group; * Significantly different from control group (p < 0.05). # Significantly different from Leuk only group (p < 0.05)
impairment of intrahepatic and extrahepatic bile flow and hepatobiliary injury [46,47]. The result of this research work shows that these marker enzymes were significantly increased in leukocytosis rats due to benzene exposure. However, administration of linalool buffered cellular integrity of liver cell as observed in significant reduction in the activities of these liver function enzymes in animals treated with monoterpene linalool when compared with untreated group. Therefore, linalool functions in alleviating the toxicity of benzene on liver damage.

Oxidative stress is now recognized to be a prominent feature of many acute and chronic diseases, and even cancer and leukemias [48--50]. Reduced glutathione (GSH), play a crucial role in cellular redox balance by acting as first line of defense against oxidation through scavenging of free radicals in cells [51]. Depletion of reduced glutathione which is a cellular non-protein thiol defenses system is linked to oxidation of critical sulfhydryl-containing proteins consequent to oxidative stress [52,53]. This oxidative effect usually alters the function of protein sulfhydryl groups that

**Figure 6.** Protective effects of Linalool on Benzene-induced changes in the plasma activities of aspartate amino transferase (A), alanine amino transferase (B) and alkaline phosphatase (C) in rats. Notes: Data represent the means ± SD for five rats in each group; * Significantly different from control group (p < 0.05). # Significantly different from Leuk only group (p < 0.05).
participate in transport of reducing equivalents and antioxidant defense as protein scavenge 50–75% of reactive species generated through thiol groups present on them [54,55]. The plasma levels of protein thiol in the body indicate antioxidant status and low levels of protein thiol correlated positively with the increased levels of lipid peroxides [56] and advanced oxidation protein products (AOPPs) [57]. In this present work, the exposure of rats to benzene resulted in decline in hepatic reduced glutathione and plasma total thiol with concomitant increase in plasma advanced oxidation protein products and liver lipid peroxidation relative to control. The generation of intracellular oxidized proteins is associated with increased production of ROS which results from a disruption in the balance between pro-oxidants and antioxidants [58]. It is produced in plasma when the plasma albumin is subjected to oxidation by various oxidants like reactive oxygen species and hypochlorous acid [59] and its elevated levels has been associated with some pathological conditions such as atherosclerosis, diabetes, nephropathies and cancer [60]. Reduced levels of protein thiols were earlier reported to correlate negatively with the levels of AOPPs [61] which is in consonant with result of this present work. However, administration of extract of Eulophia gracilis in this study significantly reduced the elevated generation of AOPP and MDA levels in leukocytosis rats group and effectively restored the total thiol status and GSH when compared to untreated group. This shows that linalool may improve antioxidant activity by preserving sulphydryl protein pools in plasma and GSH, non-protein cellular thiol.

The result of this study showed a significant increase in the level of MDA in leukocytosis animals intoxicated with benzene when compared with control group. Induction of lipid peroxidation is a crucial manifestation of many chemical substances that depicted the index of oxidative stress and tissue damage [62]. It is a phenomenon initiated by free radical attack on unsaturated fatty acid moiety of lipid that resulted into formation of complex series of compounds like MDA, reactive carbonyl compounds, and lipid peroxide [63–65]. Therefore, elevated levels of MDA are considered to be a pointer of enhanced lipid peroxidation that reflects tissue damages [66]. In normal physiological system, endogenous radical like superoxide radical is dismutated to hydrogen peroxide and dioxygen by SOD where hydrogen peroxide is later converted to water and molecular oxygen by catalase [67]. The enzymatic activities of CAT and SOD maintain redox balance in the cell and when overwhelmed by xenobiotic-generated free radicals may expose liver to oxidative stress [31,68]. In addition to these enzymes, glutathione-S-transferase is also involved in free radical scavenging activities in the cell and ensures detoxification process where it catalyzes conjugation of highly reactive electrophiles [69,70]. It transforms a wide range of compounds including xenobiotic and products of oxidative stress [71]. Therefore, the significant increase in lipid peroxidation observed in untreated leukocytosis rat group relative to control may be as a result of reduction in the activities of SOD, CAT, and GST by free radical intermediates of benzene which exposed the liver to oxidative stress. However, treatment with linalool in this study restored the cellular activities of CAT, SOD, and GST which resulted into corresponding significant decrease in hepatic MDA level, a product of lipid peroxidation.

The findings of other researchers have linked intoxication by chemical toxin to excessive lipid peroxidation, increased DNA damage and protein oxidation in rat liver [72,73]. In consonant with this, the results of the present study revealed that exposure to benzene induced DNA damage in liver of untreated leukocytosis rats as determined in the level of percentage DNA fragmentation spectrophotometrically using Diphenylamine (DPA) when compared to control group. Moreover, this work confirmed the higher
frequency of occurrence of polychromatic micronucleated cells in bone marrow cells of benzene intoxicated rats relative to the control rats. Therefore, our results support earlier report that benzene degrade DNA in liver tissue of rats by generating free radicals and increasing DNA fragmentation [74]. However, treatment with linalool significantly reduced the percentage of the DNA damage and ameliorated the occurrence of polychromatic micronucleated cells in bone marrow cells when compared to untreated leukocytosis animals.

**Conclusion**

The result from this research study showed that benzene altered hematology, induced liver damage and caused oxidative stress by depleting the antioxidant system. However, administration of linalool restored the benzene-induced hematological perturbation and improved biochemical indices of liver damage which may be as a result of its antioxidant capacity in stabilizing the redox status in blood and liver organ. It is, however, recommended that a detail molecular mechanism of hepatoprotective capacity of linalool should be investigated on specific liver diseased model.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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