Original Research Article

Myeloprotective and haematinic influence of *Harungana madagascariensis* in Benzene-initiated onco-hematologic process in Wistar rats.

ABSTRACT

Background: *Harungana madagascariensis* is a medicinal plant that is traditionally utilized for the treatment of anaemia. Benzene is an industrial solvent that constitutes an occupational hazards due to its adverse effect on hematology leading to onco-hematology. Therefore, the efficacy of *Harungana madagascariensis* against benzene-induced pre-leukaemic condition was investigated in rat model.

Methodology: Hematological disturbance leading to pre-leukemic conditions was induced in Wistar rats by intraperitoneal administration of 400mg/kg of benzene in prapan-2-ol:water, 1:1 v/v solvent every other day for 28 days. Following benzene intoxication, 200 mg/ kg HM was administered orally for 14 days. Hematological parameters and blood cell morphology were compared between baseline control and benzene-intoxicated rats with or without HM extract treatment.

Results: BZ-induced hematologically disturbed rats exhibited anaemic symptoms marked by reduction in hemoglobin level, red blood cells, and packed cell volume with morphologic blast cell appearance, polychromasia, hypersegmented neutrophil, anisocytosis and poikilocytosis. Moreover, there was an altered redox status depicted by a significant reduction in plasma level of total sulfhydryl content with concomitant increase in advanced oxidation protein products (AOPPs) that were accompanied by increased frequency of micronucleated polychromatic erythrocytes and hypercellularity in the bone marrow of benzene intoxicated rats. However, treatment with *Harungana madagascariensis* restored blood hematology and alleviated the blood cell morphological alteration induced by benzene. It also improve plasma redox status, reduced the frequency of micronucleus and improve the architecture of bone marrow cellularity in treated intoxicated rats.

Conclusion:*Harungana madagascariensis* protected against benzene-induced hematological alterations leading to pre-leukemic conditions in Wistar rats.

Conclusion: Non-invasive independent predictors for screening esophageal varices may decrease medical as well as financial burden, hence improving the management of cirrhotic patients. These predictors, however, need further work to validate reliability.

Keywords: [Harungana madagascariensis, onco-hematology, benzene, redox status }

1. INTRODUCTION

The decadence in health experienced by human beings is more of exposure to toxic chemicals, xenobiotics and pathogens or their toxin in the environment than spontaneous phenomenon of aging. Human exposure to various chemicals and toxins in the environment posed negative implication on their health status [1, 2, 3]. The resultant injuries due to exposure to environmental toxins and chemicals like aflatoxin B1 and arsenic compounds have been reported [4,5,6,7]. Benzene is a primary industrial chemical used for the manufacturing of plastics, resin and dyes. However, it constitutes an environmental hazard to humans when exposed to it through automobile repair, shipping, oil and other

industrial activities like in rubber production [8]. Exposure to relatively low levels of benzene is accompanied by hematotoxicity [9]. Some studies indicated that occupational exposure to benzene through proximity to automobile traffic and factories resulted in hematotoxic effect and increased the risk of acute myeloid leukemia and ~~haematological~~hematological malignancies [10,11]. There is alarming increase in the mortality of individual due to development of leukaemia in the whole world. Leukemia is the most common malignancy among the people under the age of 20 years with frequency of occurrence greater in males than females [12,13]. Worse still, leukemia and hematological disorder such as myelodysplasia coupled with vital organ toxicities are common observations in cancer treatment when synthetic chemotherapy and radiotherapy are employed [14,15,16]. Hence, there is need for remedies that possess minimal side effects for the treatment of cancer and protection against leukemia induction factors such as benzene.

Harungana madagascariensis is a medicinal plant that is native to Madagascar and tropical region and it belongs to *Hypericacae* family [17]. Previous studies on *Harungana madagascariensis* established its anticancer, antioxidant and antisickling activities [18, 19, 20, 21]. Its traditional utility is employed in the treatment of malaria, bleeding, dysentery and piles [22,23]. It is also used in the treatment of anaemia as it forms part offormulation for restoring hemoglobin level and pack cell volume [24, 19]. Therefore, due to its importance in treatment of blood related diseases, this study investigated the effect of *Harungana madagascariensis* on benzene-induced haematotoxicity in wistar rats.

Comment [HA1]: What r relation between malaria and leukemia !!

2. MATERIAL AND METHODS

2.1. Preparation of methanol extract of *Harungana madagascariensis*

Harungana madagascariensis stem barks were obtained from botanical garden, University of Ibadan and identified. It was air-dried at room temperature and pulverized into powder using electric blender. 600g of powdered *Harungana madagascariensis* stem bark was extracted by cold maceration using 3200mL of aqueous methanol (80%). The extract was concentrated by rotary evaporator, evaporated to dryness and stored at 4°C in air-tight bottle. The extraction yield was 23.817**%** (W/W).

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2.2. Experimental animals

Twenty four adult male Wistar strain rats of weight range 90-100g were used for this study. The animals were obtained from McTemmy Animal f~~F~~arm and acclimatized for seven days in the Departmental animal house of Chemical Sciences department, Ajayi Crowther University, Oyo, Nigeria. The designed work was conducted with the approval of the Faculty of Natural Sciences Ethical review of Ajayi Crowther University, Oyo with approval code: Fns/Erc/2019003 and the protocol conformed to the guidelines of the National Research Council for laboratory animal care and use [25].

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2.3. Animal treatments ~~and groupings~~

Twenty-four male Wistar strain albino rats were used for this study. Hematological disturbance was induced in 12 wistar rats by intraperitoneal injection of 400mg/kg body weight (BW) of benzene, every 2 days for 3 weeks. The animals were grouped and treated as follows: Group 1 (Control) served as control animals that were administered with water only. Group 2 are hematologically disturbed rats (HDR) that were administered with 400mg/kg body weight of benzene mixture (benzene: propanol: distilled water; 2:1:1) intraperitoneally every two days for 28 days. Group 3 (HDR + HM) was HDR rats post-treated with 400mg/kg body weight of methanol extract of *Harungana madagascariensis* every day for 14 days. Group 4 (HM) were normal baseline rats that were given 400mg/kg body weight (BW) methanol extract of *Harungana madagascariensis* every day for 14 days.

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2.4. Collection of blood and bone marrow

After 24 hours of final treatment blood samples were collected from each animal through retro orbitals plexus into lithium heparinized tubes for biochemical assays and ethylene diaminetetraacetic acid (EDTA) bottle for hematological parameters using the automated blood analyzer (SYSMEX KX21) and blood morphology. Thereafter, there animals were sacrificed and femur bones were excised to obtain bone marrow for micronucleus assay and hematoxylin and eosin staining for histopathological examination of the bone marrow cells.

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2.5. Assay for oxidative stress markers in the plasma

Plasma AOPP was determined by the method described by Witko et al. [26] with slight modification. Briefly, plasma (100µl) was added to 400µl of phosphate buffer saline (PBS) solution and 25µl 1.16M potassium iodide was then added followed 2min later by 50µl of acetic acid. The absorbance of the reaction mixture was immediately read at 340nm

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against a blank containing 500µl of PBS, 25µl of 1.16M potassium iodide, and 50µl of acetic acid. Plasma total thiol was measured spectrophotometrically using DTNB (2, 2'-dinitro-5, 5'-dithiodibenzoic acid) [27].

2.6. Micronucleus assay

Clastogenicity in pre-leukemic rats were evaluated in the bone marrow of the rats employing the micronucleus assay techniques as described by Heddle and Salmone, [28] with modification by Heddle, *et al.* [29]. Briefly, Bone marrow from femurs of rats was used for preparation of slides using standard procedure Matter and Schmid [30].

2.7. 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 comparison 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.

3. RESULTS

Table 1: Effect of *Harungana madagascariensis* on hematological parameters of benzene-initiated pre-leukemic rats.

GROUPS	White blood cell (10^9/L)	Neutrophils (%)	Red blood cell (10^{12}/L)	Haemoglobin (g/L)	Packed cell volume (%)
Control	6.275 ±1.892	5.1± 3.125	7.552± 0.446	15.05± 2.275	46.25± 4.588
HDR	8.3 ± 2.325	2.7± 0.687	5.967± 0.638	10.9± 1.211	43.55± 3.838
HDR +HM	6.75 ± 1.580	8.05± 3.924	6.542± 0.847	12.525± 1.391	42.55± 3.530
HM	6.15 ± 0.635	5.575± 4.438	7.79± 1.280	14.975± 2.189	48.427± 4.680

3.1. *Influence of Harungana madagascariensis on hematological parameters of benzene-initiated pre-leukemic rats.*

Comment [HA3]: ?

Table 1 showed the influence of *Harungana madagascariensis* on hematological parameters of benzene-initiated pre-leukemic rats. Hematologically disturbed rats (HDR) showed an increase in white blood cell (WBC) count when compared with control animal values. The red blood cell (RBC) and hemoglobin (HGB) content in Benzene-induced hematologically disturbed rats (HDR) were found to be decreased, when compared with the control values.

Comment [HA4]: Just add note about meaning of HDR, HM

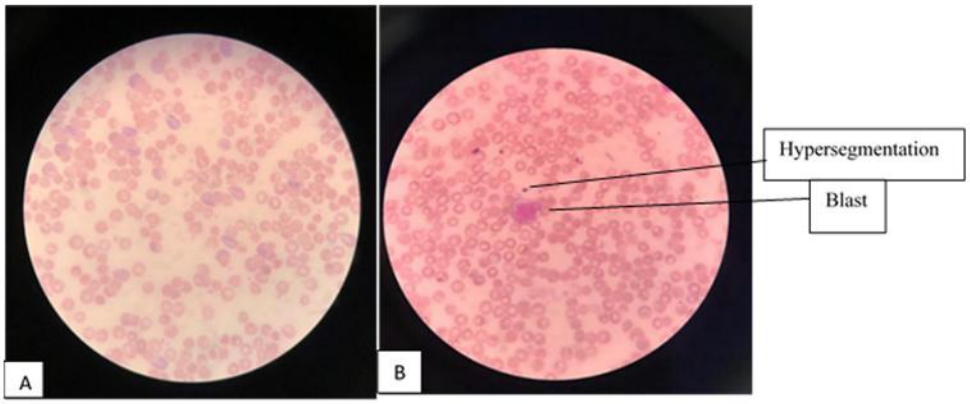

Figure 1: Blood morphology of representative of Control group rat (A) and hematologically disturbed rats (HDR) (B)

3.2. *Blood morphology of representative of Control group rat (A) and hematologically disturbed rats (B)*

Comment [HA5]: ?

Figure 1 showed the blood morphology of representative of Control group rat (A) and hematologically disturbed rats (B). Blood morphology of the control group animals showed the presence of normal blood cells as shown in Figure 1. However, the blood morphology of the hematologically disturbed rats (HDR) showed the presence of hypersegmented neutrophils, blast, anisocytosis and poikilocytosis.

TABLE 2: Influence of *Harungana madagascariensis* on blood cell morphology of benzene-initiated pre-leukemic rats.

Groups	Anisocytosis	Poikilocytosis	Microcyte	Macrocyte	Polychromasia	Hypersegmented neutrophils	Blast(%)	Nucleated red cell
Control	-	-	-	-	-	-	-	-
HDR	**+++**	**+++**	**+++**	**++**	**+**	**+**	10%	**+ +**
HDR+HM	**+**	**+**	**++**	-	-	-	2%	-
HM	-	-	-	-	-	-	1%	-

INDICATIONS
(-): absent
+ = occasional cells in every field
++ = few cells in each field
+++ = cells in 25% of each field

3.3. Influence of Harungana madagascariensis on blood cell morphology of benzene-initiated pre-leukemic rats.
Table 2 showed the influence of *Harungana madagascariensis* on blood cell morphology of benzene-initiated pre-leukemic rats. Hematologically disturbed rats (HDR) depicted the morphological derangement in blood cells as it showed the presence of irregular sized and shaped red blood cells (anisocytosis and poikilocytosis respectively), microcytes, macrocytes, immature red blood cells (polychromasia), hypersegmented neutrophils, blasts and nucleated red blood cells when compared to the control group in Table 2. Post-treatment with methanol extract of *Harungana madagascariensis* attenuated the effect of benzene toxicity by reducing the frequency of irregular sized and shaped red blood cells, microcytes, macrocytes, immature red blood cells (polychromasia), hypersegmented neutrophils, blasts and nucleated red blood cells when compared with the benzene-induced hematologically disturbed rats (HDR group).

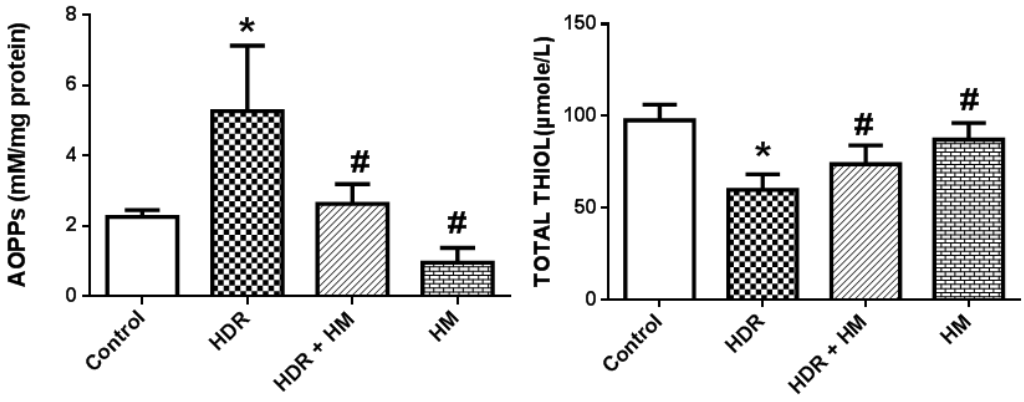

Figure 2: Effect of methanol extract of *Harungana madagascariensis* on plasma concentration of advanced oxidation protein products (AOPPs) and total thiol level on Benzene-initiated pre-leukemic rats.
Data are expressed as mean ±S.D for six rats in each group
* represent value significantly different from the control $p<0.05$
# represent value significantly different from hematologically disturbed rats (HDR)

3.4. Effect of methanol extract of Harungana madagascariensis on plasma concentration of advanced oxidation protein products (AOPPs) and total thiol level on Benzene-initiated pre-leukemic rats

Figure 2 showed the effect of methanol extract of *Harungana madagascariensis* on plasma concentration of advanced oxidation protein products (AOPPs) and total thiol level on Benzene-initiated pre-leukemic rats. Administration of benzene showed a significant elevation in the plasma concentration of advanced oxidation protein products (AOPP) in HDR group by133.21% when compared to the control group. Post-treatment with methanol extract of *Harungana madagascariensis* significantly attenuated the effect of benzene toxicity by reducing the concentration of plasma AOPP in treated animals by 50.01% when compared with the Benzene-induced hematologically disturbed rats (HDR) group that were treated with the extract.

Also, Benzene-exposed rats showed a significant decrease in the plasma concentration of total thiol by 38.81% when compared to the control group. Post-treatment with methanol extract of *Harungana madagascariensis* significantly increased the concentration of total thiol by 23.35% in the plasma when compared with the Benzene-induced hematologically disturbed rats (HDR) group.

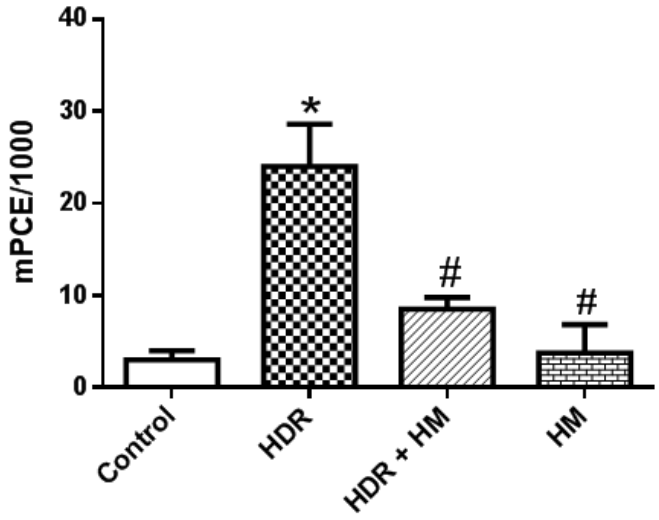

Figure 3: Effect of methanol extract of *Harungana madagascariensis* on bone marrow concentration of micronucleated polychromatic erythrocyte on Benzene-initiated pre-leukemic rats.

Data are expressed as mean ±S.D for six rats in each group

* represent value significantly different from the control $p<0.05$.
# represent value significantly different from hematologically disturbed rats (HDR) group

3.5. Effect of methanol extract of Harungana madagascariensis on bone marrow concentration of micronucleated polychromatic erythrocyte on Benzene-initiated pre-leukemic rats.

Comment [HA6]: ?

Figure 3 depicts the effect of methanol extract of *Harungana madagascariensis* on bone marrow concentration of micronucleated polychromatic erythrocyte on Benzene-initiated pre-leukemic rats. Administration of benzene showed a significant elevation in the frequency of micronucleated polychromatic erythrocyte present in the bone marrow by 700% when compared to the control group. Post-treatment with methanol extract of *Harungana madagascariensis* significantly attenuated the effect of benzene toxicity by reducing the bone marrow micronucleated polychromatic erythrocyte concentration by 64.58% in the bone marrow when compared with the Benzene-induced hematologically disturbed rats (HDR) group.

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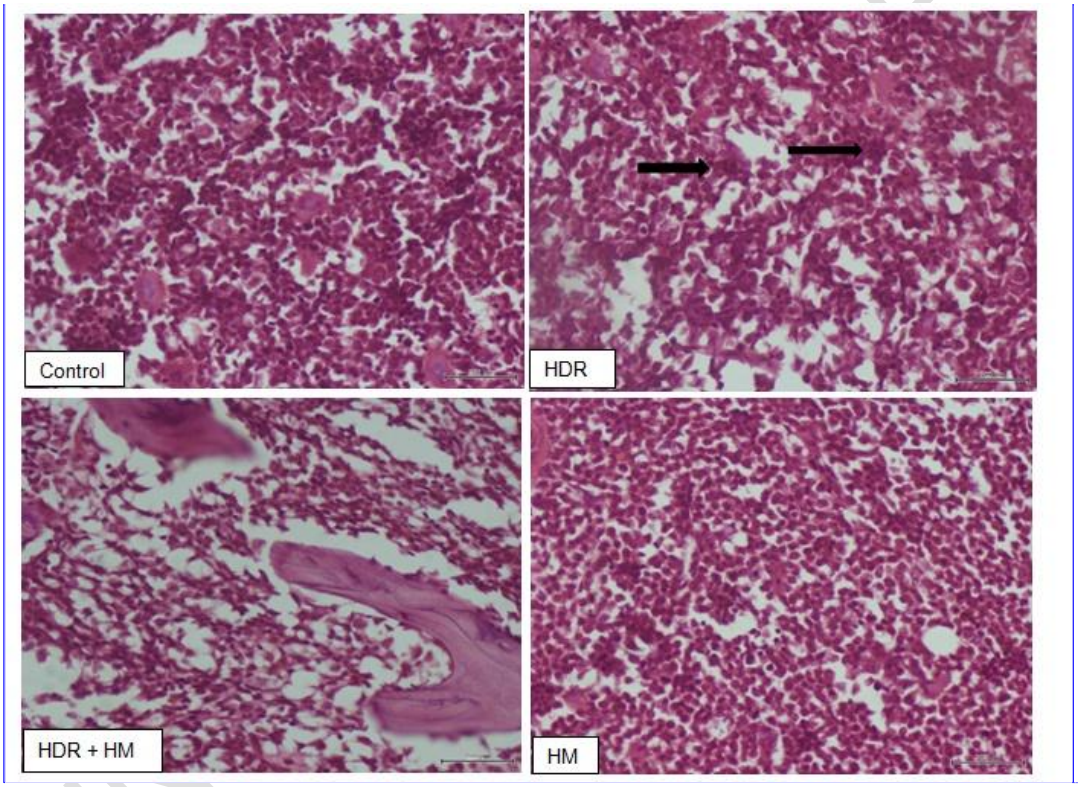

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Figure 4: Photomicrograph showing the effect of methanolic extract of *Harunganamadagascariensis* on bone marrow architecture of Benzene-initiated pre-leukemic rats with arrows showing dysplastic cells.

4. DISCUSSION

Leukemia is a type of cancer that is traceable to environmental risk factors such as the exposure to benzene [31, 32, 33]. The resultant toxic effect due to human contact with benzene fumes ranges from early reversible hematotoxicity with characteristic features of anemia, leucopenia and thrombocytopenia to irreversible bone marrow damage leading to leukemia that resulted from prolonged exposure to high benzene doses [34,33]. Some studies have established connection between benzene-induced haematinic derangement and oxidative stress [35]. However, *Harungana madagascariensis* is a medicinal plant with a proven anticancer, antioxidant and anti-sickling activities with some pharmacological studies that supported its usage in treatment of anaemia as it restored hemoglobin level and pack cell volume [24, 19, 17, 20, 21]. This research investigated the anti-leukemic effects of methanol extract of *Harungana madagascariensis* against the toxicological influence of benzene on the hematology of rats through the determination of hematological parameters, blood cell morphology and genomic instability.

This study shows that administration of benzene induced hematological imbalance gearing towards leukemogenesis evidenced by anaemic indices indicated by reduction in hemoglobin (Hb), red blood cells, packed cell volume and increase in white blood cell with concormitant observable cellular deforrmities such as poikilocytosis and anisocytosis in the blood film of benzene exposed animals when compared to the control animals. The erythrocyte deformability in conjuction with reduction in RBC count, Hb level and percentage PVC are notable indication of anemia in animals [36].

Benzene metabolite specifically hydroquinone was reported to impairs granulocyte maturation and induce neutrophilia, a condition that led to increased neutrophils in the peripheral compartment by mechanism that be due to intense mobilization of segmented cells from the bone marrow, [37, 38]. Therefore, benzene bioactivation may be responsible for increase in white blood cell counts was noted in benzene-exposed rats in this study and report from other work [39]. The occurrence of blasts in the peripheral blood film of leukemic rats in the present study is an indication of undifferentiated blood-forming cells in the blood mobilized from the marrow that had been associated with leukemia [40].

The occurrence of hypersegmented neutrophils is mostly linked to megaloblastic anemia that is classically pathognomonic of vitamin B12 and folic acid deficiency [41]. It is also observed in alcohol abuse, iron deficiency aenemia, myelodysplastic syndromes under chemotherapy and heatstroke [42,43]. This morphologic changes is usually a consequent of impaired DNA synthesis stemmed from inadequate substrate or altered replication from toxin or chemotherapy effect. Macrocyte and polychromatophilia are manifestation of new blood formation as polychromasia is a disorder linked to high immature red blood cells in the blood consequent to premature release from bone marrow. Polychromasia in adult human have been suggested to result from disturbance of haematopoiesis in the form of aplastic anaemia, myelophthistic and megaloblastic and was also notable in microangiopathic hemolytic anemia [44,45]. Hypersegmentation, macrocyte and polychromasia were observed in animals intoxicated with alcoholic benzene mixture. These observations were similar to presentations observed in human diagnosed with heatstroke [43]. However, there was an alleviative effect on animal administered with *Harungana madagascariensis*.

The blood morphology result also showed the presence of nucleated red blood cells in peripheral blood of rats exposed to benzene mixture. Nucleated red blood cells (NRBCs) was reported be normal occurrence in the peripheral blood of neonates which however disappear during pregnancy [46,47]. In certain pathological conditions, there tend to be insufficiency in erythrocyte antioxidant system [48]. Nucleated red blood cells (NRBCs) are present in the peripheral blood of a high number of hematological diseases and are related to ineffective erythropoiesis or stress erythropoiesis most especially to severe hypoxic stress and injury to the microenvironment of bone marrow exemply in myelofibrosis and leukemia [49]. In certain pathological conditions, there tend to be insufficiency in erythrocyte antioxidant system [48]. The appearance of NRBCs has been shown in the blood of patient with severe diseases and a reliable parameter that cumulated into life-threatening hypoxic and inflammatory injuries in some patients [50, 51]. Furthermore, the realease of NRBC into blood circulation of trauma patients has been associated with failure in bone marrow [52]. However, the administration of extract of *Harungana madagascariensis* offered a mitigative influence on anaimal intoxicated with benzene mixture.

The mechanism of benzene-induced hematological toxicity involved irreversible oxidative injury to macromolecules such as lipids, proteins and DNA that may ultimately initiate carcinogenesis [53,54]. The studies had revealed that resultant increased in myeloid cell growth by Benzene metabolite leads to increased formation of reactive oxygen species in vitro and that benzene was implicated in increased levels of oxidized DNA and raised DNA binding capacity potential for activator protein-1 (AP-1) which is known transcription factor target of oxidative stress in benzene exposed mice [55,56,57,58]. The result of our work showed a significant reduction in the level of total thiol with corresponding significant increase in advanced oxidation protein products and frequency of formation of micronucleated polychromatic erythrocytes in rats exposed to benzene mixture. The determination of plasma total thiol and AOPPs levels have been used to assess antioxidant status where AOPPs level correlates negatively to total thiol contents [59,60]. The involvement of oxidative stress by overwhelming benzene or its active metabolite leading to reduction in antioxidant sulphydry protein content due to their usage and subsequent oxidative damage on plasma protein evidenced by increased advanced oxidation protein

products is established in this study. This agrees with our previous and finding of other researchers [61,35]. However, the administration of extract of *H. madagascariensis* restored the level of plasma total thiol content level to near control level and mediated the reduction in advanced oxidized protein products in benzene-exposed rats.
Also, our result indicated the presence of bone marrow dysplasia and hypercellularity with simultaneous induction of clastogenicity inferred from significant increase in the formation of micronucleated polychromatic erythrocyte in the bone marrow of rats exposed to benzene when compared to the control animals. Morphological influence of benzene leading to pre-leukemic appearance of dysplasia and hypercellularity in myeloid tissue had been reported in both human and murein model [37,35]. Benzene metabolites were shown to promote myeloblast proliferation but inhibited myelocyte maturation whose mutation without subsequent DNA repair could lead to development of leukemia [62, 63]. Benzene metabolic intermediates such as benzoquinones was reported to induce genotoxicity and cytotoxicity through oxidative DNA damage and DNA strand breaks in the bone marrow cells among other diverse mechanisms [64,65,66,67]. The result however further indicated that administration of H. madagascariensis extract significantly ameliorated the benzene-induced clastogenicity and improve the architecture of bone marrow cell in treated rats which may probably due to antioxidant potential of the plant extract.

5. CONCLUSION

The methanolic extract of *Harungana madagascariensis* offered protection against benzene-induced haematinic and myeloid disturbance.

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