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A polyherbal formulation reverses hydrogen peroxide-induced hematological and biochemical aberrations in rats

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DRHM® is a polyherbal formulation [composed of *Cymbopogon citratus* (17%), *Carica papaya* leaves (16%), *Mangifera indica* bark (15%), *Moringa oleifera* leaves (14%), *Citrus limon* (12%), *Psidium guajava (*11%), *Zingiber officinale* root (9%) and *Allium sativum* (6%)] that is indicated for many disease conditions and as a detoxifier. In this study, the effects of DRHM® on hydrogen peroxide (3 mL/kg b.w of 5% H_2O_2, i.p)-induced hematological and biochemical aberrations in rats were evaluated. H_2O_2 significantly ($p<0.05$) reduced hemoglobin level, packed cell volume and red blood cell, white blood cell and platelet counts. It elevated the activities of aspartate and alanine aminotransferases and levels of total bilirubin and malondialdehyde. In addition, H_2O_2 also decreased superoxide dismutase, catalase and glutathione peroxidase activities and reduced glutathione and antioxidant vitamins levels. However, after 14 days of treatment at 1, 2 and 3 mL/kg/d b.w. p.o, DRHM® reversed aberrations in hematological status, enhanced antioxidant status and attenuated lipid peroxidation and hepatic damage induced by H_2O_2, in a dose-dependent manner comparable to silymarin (100 mg/kg/d. b.w.). These findings suggest that the phytoconstituents in DRHM® might be responsible for these ameliorative effects by boosting antioxidant defense system. DRHM® was tolerable up to 10 mL/kg. b.w.

Keywords: Antioxidant, DRHM®, Hematology, Hepatotoxicity, Hydrogen peroxide, Oxidative damage

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The excessive generation of free radicals beyond the level in which natural antioxidant defense of the body can neutralize them results in oxidative stress, a condition that is linked with pathogenesis of cardiovascular diseases, ageing and diabetes[1]. These damages usually occur via., inactivation of key metabolic enzymes and damages to important cellular macromolecules, often resulting in devastating consequences[2]. The liver helps in the detoxification and removal of xenobiotics, some of which are potentially toxic[3]. This exposes the liver to injury leading to the impairment of its functions in the presence of hepatotoxins. The burden of liver diseases is high because damage to this vital metabolic organ has serious implications to the entire health and wellbeing[4]. One major mechanism of xenobiotic-induced liver damage is radical species-mediated, leading to oxidative damage to the hepatocytes. Hepatotoxicity is usually characterized by necrosis of hepatocytes, elevation in lipid peroxidation and decrease in reduced glutathione (GSH) concentration. The serum bilirubin and lipid profile and activities of transaminases and alkaline phosphatase are markedly increased during liver damage[5]. Aberrations in hematological indices such as reduced red blood cell (RBC) and white blood cell (WBC) counts, packed cell volume (PCV) and hemoglobin (Hb) concentration are also recorded in oxidative stress[6]. Interestingly, the dietary supplementation with antioxidants-rich agents has been suggested to be beneficial in abrogating radical species-related cellular assaults[7].

The use of herbal remedies for the management of diseases is increasing globally due to a widespread perception that herbal drugs have little or no side effect, making them a first consideration in some African and Asian populations for treating many diseases[8,9]. Traditional remedies are usually made up of only one part of the plant. However, accumulation of therapeutic experience and the search for improved health outcomes by herbal practitioners over time have resulted in a shift from the use of a single plant or plant parts to the combination of different plants or

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parts of plant for enhanced therapeutic potentials[10]. This involves the use of specific proportions of leaves, stem, seeds and roots of different plants in water, alcohol or other non-toxic solvents. It is believed that the active principles in these plants work synergistically or in combination to produce enhanced therapeutic effect[11].

Nigerian drug stores are enriched with several branded polyherbal formulations. One of such polyherbal formulations that have flooded drug markets in Nigeria and many parts of Africa is DRHM®. It is acclaimed by the manufacturer to be a potent detoxifier and blood booster. It is also claimed to be effective in the management of disease conditions such as malaria, typhoid, viral, bacterial and fungal infections, menstrual problems and low sperm count. Despite the prevalent use of this polyherbal formulation, there is no empirical evidence of the above claims and the safety profile has not been documented, to the best of our knowledge. This study evaluated the effects of DRHM® on H_2O_2-induced hematological and biochemical aberrations in rats. The acute toxicity profile and phytochemical constituents of the polyherbal formulation were also assessed.

Materials and Methods

Materials

All the chemicals used for this study were products of BDH Chemical Ltd. (Poole, England) and Sigma Aldrich (USA); they were all of analytical grades. Drugs used for this study were DRHM® and silymarin. They were purchased from reputable drug stores in Enugu State, Nigeria. Reagents used for this study were products of Randox Laboratories (USA) and QCA (Germany).

Phytochemical analyses of DRHM®

Methods previously described[12] were used for the detection and quantification of the phytochemical constituents of DRHM®.

Determination of acute toxicity profile of DRHM®

This was done using twenty healthy male Swiss albino mice of body weight range 25-30 g. They were sourced from the Department of Zoology and Environmental Biology, University of Nigeria, Nsukka. After seven days of acclimatization to laboratory environment, the mice were fasted overnight and the body weights were measured. Thereafter, the mice were divided into four groups of five mice each. Mice in groups 1-4 were treated with 1, 3, 5 & 10 mL/kg b.w. of DRHM®, respectively. The experimental mice were monitored for 24 h for neurological, behavioural and morphological signs of toxicity. Body weights of the mice were measured 24 h-post DRHM®-treatment to evaluate if there was any significant change in body weight.

Study design for animal experiment

Thirty healthy male Wistar albino rats (180-200 g) used for this study were obtained from the Department of Zoology and Environmental Biology, University of Nigeria, Nsukka. They were maintained under standard husbandry conditions of light (12 h) and darkness (12 h), room temperature of 26±2°C and with free access to commercial rat chow (Vital Grower Feed Nigerian Limited) and portable water *ad libitum*. The rats were ethically handled according to standard institutional, national and international protocols. The study design was approved by the Faculty of Biological Sciences Committee on Research Ethics (UNN/FBS/EC/0218). After acclimatization, the experimental rats were divided into six groups of five rats each: Group 1 served as normal control (NC). Groups 2-6 were intoxicated by single intraperitoneal administration (i.p) of 3 mL/kg b.w of 5% v/v of H_2O_2 on day 0; group 2 that served as H_2O_2 control (HC) was not treated. Groups 3-6 received 1, 2 & 3 mL/kg/d b.w. of DRHM® (based on pilot study result and as well as from manufacturer's recommended dose for human consumers) while group 6 received 100 mg/kg/d b.w. of silymarin orally (p.o) from days 1 to 14. After an overnight fasting, the rats were sacrificed under mild chloroform anaesthesia on day 15. Blood was collected from each rat through the jugular veins in anticoagulated bottle as well as plain tube. Samples collected in anticoagulated tubes were subjected to hematological analyses while samples collected in plain tubes were allowed to clot for 15 min and thereafter centrifuged at 3,000 g for 10 min. Serum from each plain sample tube was subjected to biochemical analyses of liver, lipid peroxidation and antioxidant status.

Determination of hematological and biochemical parameters

The hematological indices were determined using methods described previously[13]. The biochemical parameters evaluated based on standard methods were as follows: Serum activities of aspartate (AST) and alanine (ALT) aminotransferases[14], catalase (CAT)[15], glutathione peroxidase (GPx)[16] and superoxide dismutase (SOD)[17] and serum concentrations of total

bilirubin[18], reduced glutathione[19], vitamins A and C[20] and vitamin E[21] and malondialdehyde (MDA)[22].

Statistical analysis

Statistical analysis of primary laboratory data was performed by one-way analysis of variance (ANOVA) using statistical products and service solutions (SPSS), version 20. The results were presented as mean ± standard deviation (SD) in Tables. Test of significance were set at $p<0.05$.

Results

Phytochemical constituents of DRHM®

Result of the phytochemical analysis of DRHM® is shown in Table 1. The presence of alkaloids (3.50%), steroids (1.00%), terpenoids (1.00%), glycosides (0.50%), anthocyanins (0.46%), anthraquinones (0.43%), saponins (0.40%), flavonoids (0.18%), tannins (0.03%), phenols (0.22%) and carotenoids (0.11%) were detected in DRHM®.

Acute toxicity profile of DRHM®

The polyherbal formulation was tolerable up to 10 mL/kg b.w since there was no significant behavioural and body weight change as well as mortality within the study period (Table 2). This finding indicates that the lethal dose is higher than 10 mL/kg b.w.

Effect of DRHM® on the hematological parameters of H_2O_2-intoxicated rats

Intoxication with H_2O_2 significantly ($p<0.05$) decreased the PCV, RBC, WBC and platelet counts and Hb concentration of the rats in HC when compared with NC. However, treatment of intoxicated rats with graded doses of DRHM® significantly ($p<0.05$) increased, in a dose-dependent manner, the PCV, RBC, WBC and platelet counts and Hb concentration when compared with HC. A similar observation was observed when the levels in rats treated with silymarin were compared with NC and HC. However, the attenuation of H_2O_2-induced alterations in hematological parameters by 3 mL/kg b.w. DRHM® was significantly ($p<0.05$) higher than 100 mg/kg b.w. silymarin (Table 3).

Table 1 — Phytochemical constituents of DRHM®

Phytochemicals	Amount (%)
Saponins	0.40
Tannins	0.03
Alkaloids	3.50
Flavonoids	0.18
Glycosides	0.50
Terpenoids	1.00
Phenols	0.22
Steroids	1.00
Carotenoids	0.11
Anthraquinones	0.43
Anthocyanins	0.46

Table 2 — Acute toxicity profile of DRHM®

Groups	Mortality	Behavioural and body weight changes
1 mL/kg DRHM®	0/5	Not significant
3 mL/kg DRHM®	0/5	Not significant
5 mL/kg DRHM®	0/5	Not significant
10 mL/kg DRHM®	0/5	Not significant

Effects of DRHM® on the liver status of H_2O_2-intoxicated rats

Result presented in Table 4 shows H_2O_2 intoxication significantly ($p<0.05$) increased the serum AST and ALT activities and bilirubin concentration of rats in HC when compared with the NC. Meanwhile, treatment with DRHM® (1, 2 or 3 mL/kg. b.w.) dose-dependently and significantly ($p<0.05$) decreased serum activities of AST and ALT and level of total bilirubin when compared with HC. Similarly, intoxicated rats treated with 100 mg/kg b.w. silymarin had significantly ($p<0.05$) lower serum activities of AST and ALT and level of total bilirubin when compared with HC.

Effect of DRHM® on antioxidant and lipid peroxidation status of H_2O_2-intoxicated rats

The result as presented in Table 5 shows that H_2O_2 intoxication significantly ($p<0.05$) decreased the serum activities of SOD, CAT and GPx of rats in HC relative to NC. Meanwhile, treatment of intoxicated rats with graded doses of DRHM® significantly ($p<0.05$) increased the SOD, CAT and GPx activities when compared with HC. The increase in activities of these enzymes were significantly ($p<0.05$) higher in group 5 than in group 6. Injection of H_2O_2 caused a significant ($p<0.05$) increase in MDA concentration of HC compared with NC while treatment of intoxicated rats with varied doses of DRHM® significantly *(*$p<0.05$) decreased the MDA concentration in groups 3-5. H_2O_2 control (HC) had a significantly ($p<0.05$) lower GSH concentration than NC. Meanwhile, treatment of intoxicated rats with graded doses of DRHM® significantly ($p<0.05$) increased the GSH concentration compared with HC, in a manner comparable to that of intoxicated rats treated with silymarin. H_2O_2-injection significantly ($p<0.05$) decreased the concentrations of vitamins A, C and E in HC when compared with NC. Meanwhile, when intoxicated rats were treated with graded doses of DRHM®, there were significant ($p<0.05$) increases in the concentrations of vitamins A, C and E in groups

Table 3 — Effect of DRHM® on the hematological indices of H_2O_2-intoxicated rats

Groups	PCV (%)	HB (g/L)	RBC count ($x10^9$ cells/L)	WBC count (cells/mm^{-3})	Platelet count ($x10^6$ cells/L)
Normal control (NC)	43.25±0.96^c	14.08±0.83^d	302.50±23.27^c	5250.00±172.25^b	180.00±9.13^d
H_2O_2 control (HC)	30.50±1.41^a	10.58±0.34^a	258.50±17.08^a	4750.00±157.43^a	141.25±8.54^a
H_2O_2 + 1 mL/kg DRHM®	39.25±2.50^b	13.08±0.83^b	282.50±13.23^b	6850.00±182.84^e	161.25±9.31^b
H_2O_2 + 2 mL/kg DRHM®	40.25±0.50^b	13.83±0.43^c	310.00±19.58^c	6550.00±150.23^d	173.75±11.09^c
H_2O_2 + 3 mL/kg DRHM®	43.75±1.00^c	14.58±0.33^e	346.25±11.90^e	5650.00±258.20^c	190.00±4.08^d
H_2O_2 + 100 mL/kg silymarin	40.00±0.58^b	13.33±0.19^c	321.25±18.43^d	5500.00±115.47^c	165.50±3.46^b

Data are mean ± standard deviation (SD) (n = 5). PCV = packed cell volume, Hb = hemoglobin, RBC = red blood cell, WBC = white blood cells. Values with different alphabets as superscripts in a column are significantly different at $p<0.05$.

Table 4 — Effect of DRHM® on the liver status of H_2O_2-intoxicated rats

Groups	AST (IU/L)	ALT (IU/L)	Total Bilirubin (mg/dL)
Normal control (NC)	86.75±2.87^b	54.25±2.75^a	1.38±0.15^b
H_2O_2 control (HC)	108.75±7.89^c	93.25±3.10^d	2.23±0.17^c
H_2O_2 + 1 mL/kg DRHM®	89.50±4.80^b	70.00±4.32^c	1.45±0.13^b
H_2O_2 + 2 mL/kg DRHM®	88.50±3.11^b	61.50±2.75^b	1.08±0.10^a
H_2O_2 + 3 mL/kg DRHM®	87.50±3.56^b	53.00±3.37^a	1.00±0.18^a
H_2O_2 + 100 mL/kg silymarin	81.50±3.42^a	54.00±2.16^a	1.23±0.13^a

Data are mean ± standard deviation (SD) (n = 5). AST = Aspartate aminotransferase; ALT = alanine aminotransferase. Values with different alphabets as superscripts in a column are significantly different at $p<0.05$.

Table 5 — Effect of DRHM® on antioxidant and lipid peroxidation status of H_2O_2-intoxicated rats

Groups	SOD (IU/L)	CAT (IU/L)	GPx (IU/L)	MDA (mmol/L)
Normal control (NC)	10.53±0.26^c	0.93±0.15^b	12.50±0.88^c	4.70±0.70^a
H_2O_2 control (HC)	7.00±0.29^a	0.59±0.28^a	5.53±0.49^a	7.85±0.43^d
H_2O_2 + 1 mL/kg DRHM	9.58±0.81^b	1.11±0.07^b	8.71±1.21^b	5.98±0.10^c
H_2O_2 + 2 mL/kg DRHM	10.85±0.53^c	1.32±0.08^c	12.28±0.99^c	5.33±0.17^b
H_2O_2 + 3 mL/kg DRHM	12.25±0.10^d	1.45±0.08^d	15.05±0.94^d	4.77±0.08^a
H_2O_2 + 100 mL/kg silymarin	10.88±0.48^c	0.98±0.07^b	11.21±1.08^c	5.10±0.56^a

Data are mean ± standard deviation (SD) (n = 5). GPx = Glutathione peroxidase; CAT = Catalase; SOD = Superoxide dismutase; MDA = Malondialdehyde. Values with different alphabets as superscripts in a column are significantly different at $p<0.05$.

3-5 compared with HC. A similar result was obtained when intoxicated rats treated with silymarin (Group 6) were compared with NC and HC (Table 6).

Discussion

This study evaluated the effect of DRHM® on H_2O_2-induced hematological and biochemical aberrations in rats. Although not a free radical, and generally poorly reactive at physiological levels, H_2O_2 is still toxic to most cells. It is capable of inactivating several enzymes, reacts with iron to form much more damaging species such as hydroxyl ion, degrades heme proteins including myoglobin, hemoglobin and cytochrome C and causes oxidative damage to DNA[23]. The presence of important secondary metabolites such as alkaloids, terpenoids, steroids, saponins, anthocyanins, glycosides, anthraquinones, tannins, flavonoids, phenols and carotenoids in DRHM® indicates that it may possess some medicinal values to human health. Alkaloids have been reported to exhibit anticancer and antimalarial effects while flavonoids, tannins, phenolics, saponins and anthocyanins have been reported to possess antimicrobial, antioxidant and anti-inflammatory effects[24]. The presence of these phytochemicals may support the manufacturer's claim that DRHM® is effective in the management of hepatitis and venereal diseases. The result of the phytochemical analysis compares well with the findings of Khawaya *et al.*[25] & Capasso[26] who reported the presence of flavonoids in *Zingiber officinale,* and *Allium sativum,* respectively. Ghosh[27] & Razis *et al.*[28] also reported the presence of

Table 6 — Effect of DRHM on glutathione and antioxidant vitamins concentrations of H_2O_2-intoxicated rats

Groups	Reduced glutathione (mmol/L)	Vitamin A (mg/dL)	Vitamin C (mmol/L)	Vitamin E (mmol/L)
Normal control (NC)	6.73±0.17^c	8.88±0.51^d	3.90±0.08^d	0.51±0.01^d
H_2O_2 control (HC)	2.68±0.78^a	4.03±0.33^a	1.90±0.08^a	0.12±0.01^a
H_2O_2 + 1 mL/kg DRHM	5.65±0.21^b	5.88±0.51^b	2.30±0.52^b	0.47±0.02^c
H_2O_2 + 2 mL/kg DRHM	8.80±0.37^d	7.33±1.12^c	3.18±0.24^c	0.83±0.01^e
H_2O_2 + 3 mL/kg DRHM	9.48±0.46^e	8.50±0.86^d	4.70±0.18^e	1.15±0.03^f
H_2O_2 + 100 mL/kg silymarin	6.05±0.93^c	5.70±0.46^b	2.45±0.21^b	0.40±0.01^b

Data are mean ± standard deviation (SD) (n = 5). GSH = reduced glutathione. Values with different alphabets as superscripts in a column are significantly different at $p<0.05$.

flavonoids, alkaloids and anthraquinones in *A. sativum* and *Moringa oleifera,* which are all components of DRHM®.

The PCV, RBC count and Hb concentration are indices used in determining the status, integrity and functionality of red blood cells. PCV value indicates the transportation capacity of blood; a low PCV value indicates low transportation efficiency of the blood while a low RBC count indicates anaemia[29]. The significant ($p<0.05$) reduction in PCV value, RBC count and Hb concentration in the H_2O_2-intoxicated and untreated rats (HC) suggests an increased erythrocyte destruction and possibly suppression of erythrocyte formation by H_2O_2[30]. The observed significant ($p<0.05$) increase in the above parameters after treatment with the DRHM® suggests that the drug stabilizes the integrity of red cell membranes and prevents attack by free radicals. Similarly, the observed improvement in red cell indices in the H_2O_2-intoxicated and DRHM®-treated rats could also be via the stimulation of erythropoietin production and the consequent erythropoiesis by the polyherbal formulation[31]. This observation agrees with the results of Iranloye[32] who recorded an improvement in hematological indices of rats treated with *A. sativum* extract; one of the components of DRHM®. H_2O_2 also caused a significant ($p<0.05$) decrease in white blood cells of H_2O_2 control rats when compared with the normal control, which suggests a suppressed immune response. The dose-dependent increase in WBC count observed in the H_2O_2-intoxicated and DRHM®-treated as well as silymarin-treated rats indicates an immunostimulatory effect of DRHM®. This study is in line with the findings of Aravinda *et al*.[33] who reported an increase in WBC count by *C. papaya* leaf extract, a component of DRHM®.

From the findings of this study, it was observed that the administration of H_2O_2 caused significant ($p<0.05$) hepatic damage as seen in the elevated serum activities of AST and ALT and concentration of serum total bilirubin. Most toxin-induced liver injuries involve oxidative stress as a mechanism of cellular damage, and may be identified by rapid increase in serum aminotransferases (ALT and AST) activities over days, followed by increases in serum bilirubin and alkaline phosphatase[3]. The increase in serum activities of these liver marker enzymes indicates damage to hepatic cell membrane and leakages of intrahepatic enzymes into circulation. Treatment of intoxicated rats with DRHM® and silymarin normalized the hepatic status by reducing the serum activities of liver marker enzymes towards normal rats. The decrease in liver enzymes' activities indicates stabilization of hepatocyte membrane by DRHM®, preventing the release of intracellular content of liver cells to circulation. This effect could be attributed to the phytochemical content of DRHM®. The result of this study is consistent with the findings of Varsha *et al*.[34] and Shetty *et al*.[35] who reported a decrease in AST and ALT activities in rats treated with *M. oleifera* leaves and *Citrus limon*, respectively which are two components of the polyherbal formulation. Bilirubin is a breakdown product of hemoglobin in liver cells, spleen and bone marrow. As the liver becomes stressed, serum bilirubin level increases, indicating hepatic damage or a damage within the liver's bile duct[13]. In this study, treatment of intoxicated rats with DRHM® significantly ($p<0.05$) reduced the total bilirubin level when compared with intoxicated and untreated group. Kabera *et al*.[24] reported that plant secondary metabolites are effective in protecting against oxidative stress-related diseases. The observed improvement in hepatic function could therefore, be attributed to the phytochemicals present in DRHM®.

Malondialdehyde (MDA) is a common biomarker of lipid peroxidation status[5]; elevation in MDA level indicates the degree of injury in the hepatocytes[36]. It was observed that MDA level increased in serum of HC when compared with NC. Meanwhile, treatment

of intoxicated rats with DRHM® significantly ($p<0.05$) reversed these changes. This suggests that the mechanism of the above hepato-curative effect of DRHM® is probably due to its anti-lipid peroxidation effect. SOD, CAT and GPx are part of the hepatic antioxidant defense system that contribute to the regulation of oxido-reductive homeostasis and mitigation of oxidative attacks on cells[37]. DRHM® significantly ($p<0.05$) increased SOD, CAT and GPx activities in the treated rats. The GSH is an intracellular antioxidant which is also a co-factor to GPx and glutathione reductase. A low GSH concentration exacerbates oxidative assaults, resulting in increased membrane and cell damage. The lower GSH level observed in the intoxicated and untreated rats when compared with normal control might be responsible for the reduced GPx activity. Similarly, a decrease in CAT activity and leads to reduced H_2O_2 decomposition. H_2O_2 reacts with free iron, leading to production of hydroxyl radical that is very toxic to the cells[23]. Treatment of intoxicated rats with DRHM® significantly ($p<0.05$) increased the GSH concentration and this could be responsible for the overall improvement in antioxidant status of the treated rats. This agrees with the earlier observation where plants with hepato-modulatory effects against toxicants have also been shown to improve GSH concentration[38].

Vitamin E is a very important antioxidant in lipid medium; it scavenges free radicals and possibly up-regulates the expression of antioxidant enzymes. Similarly, vitamin A also scavenges free radicals in lipid medium and helps maintain pro-oxidant/ antioxidant balance. Meanwhile, vitamin C neutralizes reactive species in the aqueous medium prior to their initiation of lipid peroxidation; it takes part as a co-factor in many enzymatic reactions and also acts as a plasma localized antioxidant[40]. In addition, vitamin C helps in regeneration of vitamin E to protect the cells in lipid medium. The increase in antioxidant vitamins concentrations observed in the intoxicated and DRHM®-treated rats could be attributed to their presence in some of the plant components of DRHM®. For example, studies have shown that *Moringa* leaf extract is rich in vitamins and carotenoids[41]. In addition, findings from this study suggest that DRHM® is non-lethal up to 10 mL/kg body weight dose. This implies that DRHM® is relatively safe for human consumption at the doses studied.

Conclusion

The results of this study support the existing knowledge that hydrogen peroxide (H_2O_2) induces cellular oxidative stress via enhancing the production and attack of free radicals on cells and weakening of body's antioxidant defense system. The results further added that treatment of H_2O_2-intoxicated rats with DRHM® reverses the indices of hematological and biochemical aberrations likely by boosting antioxidant status and attenuating the cellular oxidative damages caused by H_2O_2 intoxication. The above beneficial bioactivities might be attributed to wide varieties of phytochemicals detected in DRHM®. This makes the herbal drug a potential candidate for the treatment/ management of oxidative-stress related conditions. The polyherbal formulation was tolerable in mice up to 10 mL/kg b.w, showing that the lethal dose is above 10 mL/kg b.w and hence, DRHM® is relatively safe. However, further studies are warranted to evaluate the long-term effects of using this polyherbal formulation especially at the molecular level.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

IUO conceived the idea, JCN, IUO, ECA and CCC did the experiments and statistical analysis, JCN, IUO and CCC wrote the first draft of the manuscript; All authors approved the final manuscript version

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