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Full-length Research Article

Kolaviron Ameliorates Phenylhydrazine-Induced Hepatotoxicity and Genotoxicity but Induces Splenic Follicular Damage in Wistar Rats

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Summary: Phenylhydrazine (PHZ) induces oxidative stress via reactive oxygen species generation and lipid peroxidation, leading to organ and DNA damage. Kolaviron (KV), a biflavonoid complex extracted from Garcinia kola seeds has demonstrated antioxidant and cytoprotective properties. The study aimed to evaluate the effects of kolaviron on phenylhydrazine-induced hepatotoxicity, splenic pathology, and genotoxicity in Wistar rats. Twenty-eight male rats were divided into four groups: Control (distilled water), PHZ (40 mg/kg intraperitoneally for 3 days), PHZ+KV (PHZ at 40 mg/kg+KV at 100 mg/kg orally for 14 days), and KV alone (100 mg/kg orally for 14 days). Hepatotoxicity, splenic pathology, and genotoxicity were assessed through biochemical analyses, oxidative stress markers, antioxidant parameters, histopathology, and micronucleus testing in bone marrow cells. Results showed that PHZ significantly elevated liver enzymes ($p<0.05$), increased malondialdehyde levels, decreased antioxidant enzymes, and caused periportal fibrosis. Treatment with KV normalised these liver parameters and mitigated histopathological damage. In the spleen, PHZ induced lymphoid follicular hyperplasia, whereas in the PHZ+KV group, we observed lymphoid follicular necrosis; however, splenic oxidative stress and antioxidant levels remained similar across all groups. For genotoxicity, PHZ significantly increased the number of micronucleated polychromatic erythrocytes and reduced the polychromatic erythrocytes/normochromatic erythrocytes ratio ($p<0.05$), whereas KV treatment restored these to near-control levels. Kolaviron showed significant hepatoprotective and genoprotective effects against PHZ-induced toxicity, primarily by modulating oxidative stress parameters. However, KV induced splenic follicular necrosis, indicating potential organ-specific toxicity. Further research should investigate the optimal dosing regimens and mechanisms underlying both KV's protective and adverse effects.

Keywords: *Kolaviron, phenylhydrazine, hepatotoxicity, genotoxicity, oxidative stress, splenic pathology.*

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INTRODUCTION

Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the body's antioxidant defences (Cichoż-Lach & Michalak, 2014). Drug-induced liver injury (DILI) is a major clinical challenge everywhere in the world. One of the main ways drugs damage the liver is by inducing oxidative stress, in which harmful molecules called ROS damage hepatocytes (Björnsson, 2016). Phenylhydrazine (PHZ) is commonly used in research because it can induce oxidative stress by generating ROS and promoting lipid peroxidation (Pandey *et al*., 2014). Moreover, exposure to PHZ has been associated with genotoxic effects, including DNA strand breaks and chromosomal abnormalities, making it a critical model for investigating substances that may exert hepatoprotective and genoprotective effects (Rungemorris *et al*., 1994; Pandey *et al*., 2014).

The liver is primarily involved in metabolising and detoxifying foreign substances; hence, making it especially susceptible to chemical injury (Gu and Manautou, 2012). Typical indicators of liver toxicity include elevated serum aminotransferases, histopathological alterations, and imbalances in the cellular redox state (Zimmerman, 1999). Moreover, some harmful chemicals that damage the liver can also harm the spleen, which filters blood and supports the immune system. This is especially true for chemicals that break down red blood cells, like PHZ. (Elmore 2006; Odi *et al*., 2022). Despite progress in understanding DILI mechanisms, effective treatments remain limited. This highlights the urgent need to identify new liver-protective substances that act through different mechanisms (Andrade *et al*., 2019).

Natural substances such as polyphenols, flavonoids, and carotenoids, having antioxidant properties, have attracted significant attention as prospective hepatoprotective agents

(Madrigal-Santillán *et al*., 2014). Kolaviron (KV), a biflavonoid complex obtained from the seeds of Garcinia kola, has shown considerable antioxidant, anti-inflammatory, and cytoprotective effects in various experimental studies. Specifically, in vitro studies have demonstrated KV's free radical scavenging capacity and membrane stabilizing properties (Farombi *et al*., 2013), while in vivo studies in rodent models have shown its protective effects against oxidative tissue damage (Adaramoye *et al*., 2005; Olaleye & Farombi, 2006). A number of studies have shown that KV can provide protection to the liver against different kinds of chemical toxins, some of which include dimethyl nitrosamine (Farombi *et al*., 2009), 2-acetylaminofluorene (Farombi *et al*., 2000), and acetaminophen (Akintonwa and Essien 1990). A number of studies have shown that KV can provide protection to the liver against different kinds of chemical toxins, some of which include dimethyl nitrosamine (Farombi *et al*., 2009), 2-acetylaminofluorene (Farombi *et al*., 2000), and acetaminophen (Akintonwa and Essien 1990). KV helps in the protection of the liver mainly by scavenging harmful free radicals, increasing the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) to maintain cellular redox balance, and reducing inflammatory processes by inhibiting pro-inflammatory cytokines and mediators (Erukainure *et al*., 2021).

While ample evidence supports KV's hepatoprotective capabilities, there remains a dearth of data on its effectiveness against PHZ-induced hepatotoxicity and genotoxicity. Additionally, the influence of KV on PHZ-exposure-related splenic pathology remains largely unexamined. Most research has focused primarily on hepatic parameters, with little evaluation of systemic effects, particularly of splenic structure and function, which play a supportive role in the body's response to xenobiotic-induced stress (Liezmann *et al*., 2012).

We aimed to assess the effects of KV on PHZ-induced hepatotoxicity, splenic pathology, and genotoxicity in Wistar rats. We hypothesised that KV would mitigate PHZ-induced hepatotoxicity, splenic pathology, and genotoxicity in Wistar rats through its antioxidant and cell-protecting abilities. By studying both the liver and spleen, the research gives a deeper look into how PHZ affects different organs and how KV might help protect it. Also, by checking for DNA damage, the study shows whether KV can help prevent genetic harm, which is important if it's going to be used to help stop diseases like cancer.

MATERIALS AND METHOD

Experimental animals and ethical considerations: Twenty-eight healthy adult male Wistar rats weighing between 130 and 150 grams were sourced from the Animal House of the Faculty of Veterinary Medicine at the University of Ibadan, Nigeria. The animals were housed in cages with wood shavings as bedding, which were changed twice weekly, under strictly controlled environmental conditions, maintaining a temperature of 22 ± 2°C, relative humidity of 55 ± 5%, and a 12-hour light/dark cycle. Throughout the study, the rats had unrestricted access to a standard rodent diet supplied by Ladokun Feeds Limited, Nigeria, and filtered water. Prior to the commencement of experimental procedures, a 14-day acclimatization period was observed to allow the animals to adjust to the laboratory conditions. The study received ethical approval from the University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC) with approval number: NHREC/UIACUREC/05/12/2022A.

Study design and treatment protocol: The rats were randomly allocated using the lottery method (drawing of lots) into four experimental groups, each consisting of seven animals. The sample size of seven rats per group was considered adequate as it exceeds the number used in similar hepatoprotective studies by Farombi *et al.,* 2009 (n=6) and Farombi *et al.,* 2000 (n=5), and is sufficient to detect significant differences in biochemical and histological parameters while adhering to the ethical principle of using the minimum number of animals necessary. Randomization ensured uniform distribution of weight and health status across groups. To minimize potential biases, all animals were handled by the same personnel, treatments were administered at the same time each day, and the investigator performing biochemical analyses was blinded to group allocation. Group A served as the control and received 0.2 mL of distilled water daily via oral gavage for a period of 14 days. Group B was administered PHZ at a dose of 40 mg/kg body weight intraperitoneally for three consecutive days to induce oxidative stress and hepatotoxicity. Group C received the same PHZ regimen followed by KV at a dose of 100 mg/kg body weight orally for 14 days to evaluate its protective effects. Group D was treated exclusively with KV at 100 mg/kg orally for 14 days to assess its standalone impact.

Sample collection and tissue preparation: On the 15th day of the experiment, blood samples were promptly collected from the rats via the retroorbital venous plexus using non-heparinized capillary tubes and transferred into sterile tubes. Following blood collection, the animals were humanely euthanized by cervical dislocation in accordance with institutional ethical guidelines. The blood was allowed to clot at room temperature (20-25°C) for 30-60 minutes before centrifugation at 4,000 ×g for 10 minutes at 4°C to separate the serum, which was then aliquoted and stored at −80°C for subsequent biochemical analyses. A post-mortem examination was performed immediately after euthanasia, during which the liver and spleen were carefully excised, cleaned of extraneous tissue, and weighed to determine organ-to-body weight ratios. Each organ was divided into two portions: one portion was homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4) using a Potter-Elvehjem homogenizer, and the resulting homogenate was centrifuged at 10,000 ×g for 30 minutes at 4°C to obtain the post-mitochondrial fraction (PMF) for oxidative stress assays. The remaining portion was fixed in 10% neutral-buffered formalin for histopathological examination.

Liver function tests: Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities were quantified using commercial kits (Quimica Clinica Applicada, Spain) on a Mindray BS-200E autoanalyser. For ALT and AST determination, serum samples (10 µL) were incubated with

reagent mixtures containing L-alanine or L-aspartate and α-ketoglutarate at 37°C. The resulting pyruvate or oxaloacetate reacted with 2,4-dinitrophenylhydrazine to form coloured hydrazones, which were measured at 505 nm (Reitman-Frankel method). ALP activity was assessed using p-nitrophenyl phosphate as substrate in diethanolamine buffer (pH 9.8); liberated p-nitrophenol was measured at 405 nm (Babson's method). Enzyme activities were expressed as international units per litre (U/L) using manufacturer-provided calibrators.

Oxidative stress and antioxidant defence: Oxidative stress parameters and antioxidant defences were systematically evaluated in post-mitochondrial fractions (PMF). Lipid peroxidation was quantified by measuring malondialdehyde (MDA) levels using the thiobarbituric acid reactivity assay according to Varshney and Kale (1970), and the absorbance of the resulting pink chromogen was measured spectrophotometrically at 532 nm. Hydrogen peroxide (H_2O_2) generation was determined spectrophotometrically following Wolff's (1994) xylenol orange method. Antioxidant enzyme activities were assessed through three key assays: superoxide dismutase (SOD) activity was measured via the epinephrine autoxidation inhibition method (Misra & Fridovich 1976); glutathione peroxidase (GPX) activity was determined by monitoring NADPH oxidation (Beutler *et al.,* 1963); and glutathione-S-transferase (GST) activity was evaluated using the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (Habig *et al.,* 1974). Cellular redox status was further characterized by quantifying reduced glutathione (GSH) levels through the 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) reaction as described by Ellman (1959). All assays were performed in triplicate with appropriate controls, and absorbance readings were obtained using a Shimadzu UV-1800 spectrophotometer.

Genotoxicity assessment: The potential genotoxic effects of PHZ and the protective role of KV were evaluated using the *in vivo* micronucleus test as previously described by Ola-Davies *et al.,* (2019). Twenty-four hours after the final treatment, the rats were euthanized, and bone marrow cells were extracted from both femurs by flushing with foetal bovine serum. The cell suspension was centrifuged at 1,500 ×g for 10 minutes at 4°C, and the pellet was resuspended in a minimal volume of serum. Smears were prepared on pre-chilled microscope slides, air-dried, fixed in absolute methanol, and stained sequentially with May-Grünwald and Giemsa stains to differentiate between polychromatic erythrocytes (PCEs, immature erythrocytes with residual RNA) and normochromatic erythrocytes (NCEs, mature erythrocytes without RNA). For each animal, 2,000 polychromatic erythrocytes (PCEs) were scored under a light microscope to determine the frequency of micronucleated PCEs (MnPCEs) and the PCE/NCE ratio, which served as indicators of chromosomal damage and bone marrow cytotoxicity, respectively.

Histopathological examination: Liver and spleen tissues fixed in 10% neutral-buffered formalin were processed using standard paraffin-embedding techniques. Sections of 5 µm thickness were cut with a rotary microtome, mounted on glass slides, and stained with haematoxylin and eosin (H&E). A blinded pathologist evaluated the stained sections under a light microscope for structural alterations, including hepatic damage (e.g., fibrosis, inflammatory infiltration, vascular congestion) and splenic changes (e.g., lymphoid hyperplasia, follicular necrosis). Representative photomicrographs were captured at 400× magnification to document morphological findings.

Data analysis: All data were analysed using the Statistical Package for Social Sciences (SPSS®, version 26). Data was normally distributed based on the Shapiro-Wilk test. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was employed to compare differences among groups. Results were expressed as mean ± standard deviation (SD), and a p-value less than 0.05 was considered statistically significant.

RESULTS

Hepatic and splenic antioxidant defence parameters: In the liver, Group D (kolaviron alone) exhibited significantly lower GPX activity (58.00 ± 3.55 U/mg protein) compared to Groups A (control: 658.09 ± 1879.99 U/mg protein), B (phenylhydrazine: 577.27 ± 3.78 U/mg protein), and C (phenylhydrazine + kolaviron: 511.56 ± 8.80 U/mg protein) ($p < 0.05$). However, no significant differences were observed in hepatic GSH levels among groups ($p > 0.05$). In the spleen, GPX and GSH levels remained comparable across all groups, with no statistically significant variations ($p > 0.05$) (Table 1).

No significant differences were observed in hepatic or splenic GST activity among groups ($p > 0.05$). Similarly, SOD activity in the liver and spleen showed no significant variations, except for a non-significant trend of higher splenic SOD in Groups B (144.56 ± 16.99 U/mg protein), C (141.92 ± 47.58 U/mg protein), and D (145.53 ± 26.85 U/mg protein) compared to Group A (101.58 ± 58.64 U/mg protein) ($p > 0.05$) (Table 2).

Table 1:
Hepatic and splenic antioxidant defence parameters (GPX and GSH)

Group	Liver (Mean ± SD)		Spleen (Mean ± SD)	
	GPX (U/mg protein)	GSH (µmol/g)	GPX (U/mg protein)	GSH (µmol/g)
A	658.09 ± 1879.99[a]	28.66 ± 5.90[a]	60.40 ± 5.34[a]	33.13 ± 5.91[a]
B	577.27 ± 3.78[a]	27.13 ± 3.98[a]	62.52 ± 7.31[a]	25.62 ± 2.50[a]
C	511.56 ± 8.80[a]	34.23 ± 9.80[a]	57.44 ± 6.22[a]	32.72 ± 8.14[a]
D	58.00 ± 3.55[b]	35.63 ± 18.66[a]	61.33 ± 7.28[a]	32.91 ± 8.88[a]

Values with different superscript letters ([a,b]) within columns are significantly different ($p < 0.05$), A= Control (distilled water), B= Phenylhydrazine (40 mg/kg, 3 days), C= Phenylhydrazine + Kolaviron (40 mg/kg + 100 mg/kg, 14 days), D= Kolaviron alone (100 mg/kg, 14 days), GPX= Glutathione Peroxidase, GSH= Reduced Glutathione

Table 2:
Hepatic and splenic antioxidant defence parameters (GST and SOD)

Group	Liver (Mean ± SD)		Spleen (Mean ± SD)	
	GST (µmol/min/mg)	SOD (U/mg protein)	GST (µmol/min/mg)	SOD (U/mg protein)
A	0.10 ± 0.06[a]	60.61 ± 41.47[a]	0.02 ± 0.05[a]	101.58 ± 58.64[a]
B	0.12 ± 0.03[a]	58.77 ± 21.90[a]	0.01 ± 0.04[a]	144.56 ± 16.99[a]
C	0.08 ± 0.05[a]	66.32 ± 23.13[a]	0.03 ± 0.05[a]	141.92 ± 47.58[a]
D	0.11 ± 0.04[a]	36.40 ± 8.62[a]	0.03 ± 0.03[a]	145.53 ± 26.85[a]

Values with different superscript letters ([a,b]) within columns are significantly different ($p < 0.05$), A= Control (distilled water), B= Phenylhydrazine (40 mg/kg, 3 days), C= Phenylhydrazine + Kolaviron (40 mg/kg + 100 mg/kg, 14 days), D= Kolaviron alone (100 mg/kg, 14 days), GST (Glutathione S-Transferase), SOD (Superoxide Dismutase)

Table 3:
Hepatic and splenic oxidative stress markers

Group	Liver (Mean ± SD)		Spleen (Mean ± SD)	
	H_2O_2 (µmol/g)	MDA (nmol/g)	H_2O_2 (µmol/g)	MDA (nmol/g)
A	120.69 ± 50.88[a]	24.68 ± 71.04[a]	80.37 ± 16.38[a]	2.02 ± 0.47[a]
B	144.88 ± 44.38[a]	1.58 ± 0.26[b]	102.22 ± 28.31[a]	2.13 ± 0.86[a]
C	139.06 ± 21.19[a]	1.35 ± 0.52[b]	123.91 ± 35.56[a]	1.31 ± 0.37[a]
D	105.16 ± 11.13[a]	1.60 ± 1.04[b]	92.59 ± 31.42[a]	1.54 ± 0.42[a]

Values with different superscript letters ([a,b]) within columns are significantly different ($p < 0.05$), A= Control (distilled water), B= Phenylhydrazine (40 mg/kg, 3 days), C= Phenylhydrazine + Kolaviron (40 mg/kg + 100 mg/kg, 14 days), D= Kolaviron alone (100 mg/kg, 14 days), H_2O_2= Hydrogen Peroxide), MDA= Malondialdehyde)

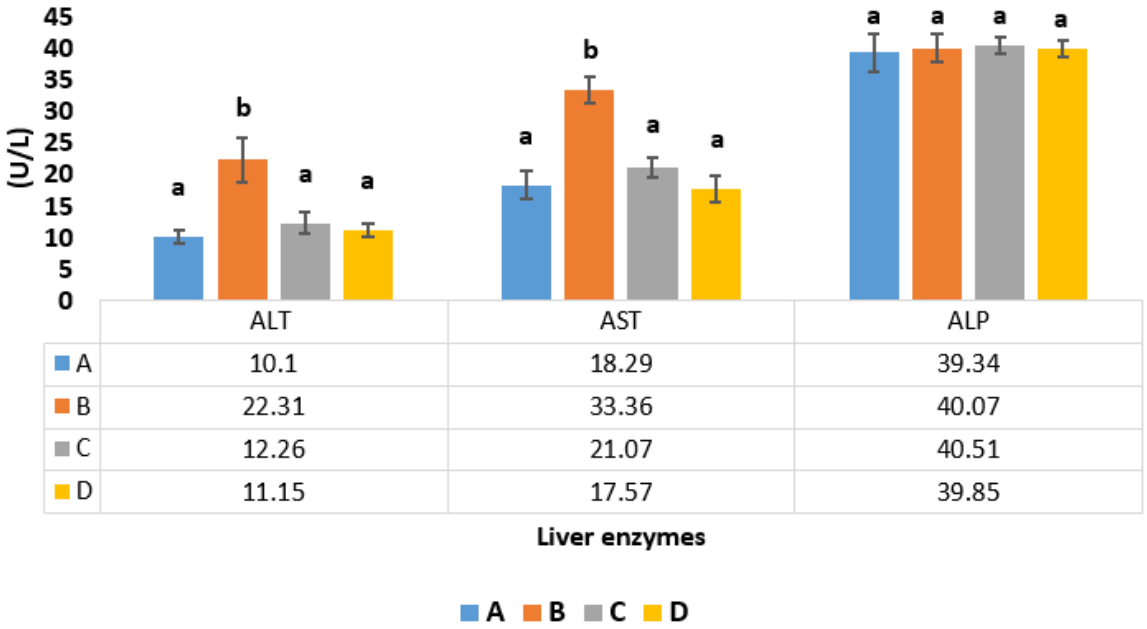

Figure 1:
Liver enzyme profiles (Mean ± SD)
Bars with different letters (a, b) are significantly different ($p < 0.05$). A= Control (distilled water), B= Phenylhydrazine (40 mg/kg, 3 days), C= Phenylhydrazine + Kolaviron (40 mg/kg + 100 mg/kg, 14 days), D= Kolaviron alone (100 mg/kg, 14 days), ALT= Alanine Aminotransferase, AST= Aspartate Aminotransferase, ALP= Alkaline Phosphatase

Hepatic and splenic oxidative stress markers: In the liver, MDA levels were significantly lower in Groups B (1.58 ± 0.26 nmol/g), C (1.35 ± 0.52 nmol/g), and D (1.60 ± 1.04 nmol/g) compared to Group A (24.68 ± 71.04 nmol/g) ($p < 0.05$), suggesting reduced lipid peroxidation in treated groups. However, hepatic H_2O_2 levels did not differ significantly among groups ($p > 0.05$). In the spleen, both H_2O_2 and MDA levels remained comparable across all groups ($p > 0.05$) (Table 3).

Liver enzyme profiles: Group B (phenylhydrazine) showed significantly elevated ALT (22.31 ± 3.49 U/L) and AST (33.36 ± 2.18 U/L) compared to Group A (control: ALT = 10.10 ± 1.01 U/L; AST = 18.29 ± 2.22 U/L) ($p < 0.05$). However, Groups C (phenylhydrazine + kolaviron) and D (kolaviron alone) exhibited ALT and AST levels comparable to controls ($p > 0.05$). ALP activity did not differ significantly among groups ($p > 0.05$) (Figure 1).

Genoprotective effects of kolaviron: The micronucleus test revealed that Group B (phenylhydrazine) had a significantly higher frequency of micronucleated polychromatic erythrocytes (MnPCEs: 3.20 ± 0.30 per 1000 PCEs) compared to Group A (control: 1.33 ± 0.10 per 1000 PCEs) ($p < 0.05$). Groups C (phenylhydrazine + kolaviron) and D (kolaviron alone) showed MnPCE frequencies similar

to controls ($p > 0.05$). The PCE/NCE ratio, an indicator of bone marrow cytotoxicity, was significantly reduced in Group B (0.62 ± 0.05) compared to other groups ($p < 0.05$), suggesting phenylhydrazine-induced genotoxicity. Kolaviron treatment restored this ratio to near-normal levels, demonstrating its genoprotective potential (Table 4).

Liver and spleen histopathology: Histopathological examination of liver tissue from control rats (Group A) showed normal hepatic architecture with intact central and portal veins, indicating no evidence of liver damage (Plate 1a). In PHZ-treated rats (Group B), moderate periportal fibrosis and portal vein congestion was observed, suggesting liver injury due to PHZ-induced toxicity (Plate 1b). Rats treated with PHZ followed by kolaviron (Group C) exhibited mild periportal fibrosis and inflammatory infiltration, along with central vein congestion, indicating partial attenuation of PHZ-induced liver damage by kolaviron (Plate 1c). In the kolaviron-alone group (Group D), liver architecture remained normal, confirming that kolaviron at the tested dose (100 mg/kg) does not induce hepatotoxicity (Plate 1d).

Histopathological examination of the spleen in control rats (Group A) showed normal lymphoid follicle structure with distinct white and red pulp, indicating no pathological changes (Plate 2a). PHZ-treated rats (Group B) exhibited prominent lymphoid follicular hyperplasia, suggesting an immune response or stress-induced splenic activation (Plate 2b). In rats treated with PHZ followed by kolaviron (Group C), lymphoid follicular necrosis was observed, indicating possible PHZ-induced immune cell damage, with kolaviron failing to fully correct this effect (Plate 2c). The kolaviron-alone group (Group D) showed concurrent lymphoid follicular hyperplasia and necrosis, suggesting that kolaviron may have immunomodulatory effects but could also induce mild splenic toxicity at the tested dose (Plate 2d).

Table 4:
Genoprotective effects of kolaviron against phenylhydrazine-induced micronuclei formation in rat bone marrow erythrocytes

Group	MnPCEs/1000 PCE (Mean ± SD)	PCE/NCE Ratio
A	1.33 ± 0.10^{a}	0.98 ± 0.08
B	3.20 ± 0.30^{b}	0.62 ± 0.05
C	1.31 ± 0.14^{a}	0.94 ± 0.07
D	1.35 ± 0.19^{a}	0.97 ± 0.09

Values with different superscript letters (^{a,b}) within columns are significantly different ($p < 0.05$), A= Control (distilled water), B= Phenylhydrazine (40 mg/kg, 3 days), C= Phenylhydrazine + Kolaviron (40 mg/kg + 100 mg/kg, 14 days), D= Kolaviron alone (100 mg/kg, 14 days), MnPCEs = Micronucleated polychromatic erythrocytes, PCE = Polychromatic erythrocytes, NCE = Normochromatic erythrocytes.

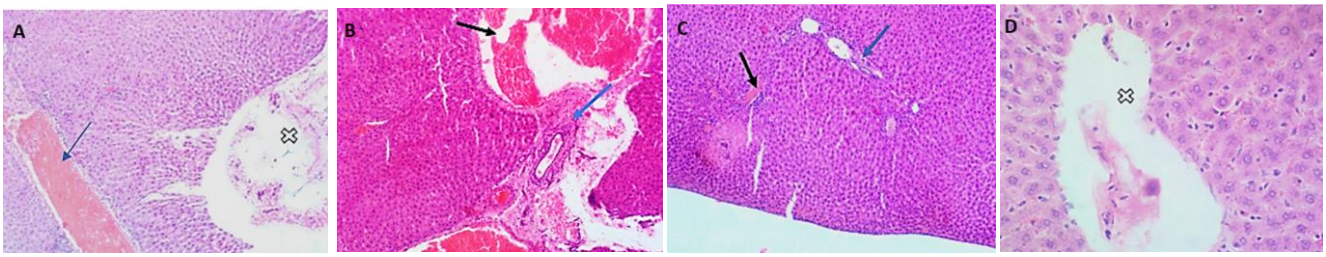

Plate 1: (a)-(d) Histopathology of the Liver in PHZ and Kolaviron Treatment Study. (a) Liver section of a control rat (Group A) showing normal hepatic architecture with no significant lesions, central vein (asterisk), and portal vein (arrow) (H&E, 100×); (b) Liver section of a rat treated with PHZ (40 mg/kg, i.p., Group B) showing moderate periportal fibrosis (blue arrow) with congested portal vein (black arrow) (H&E, 100×); (c) Liver section of a rat treated with PHZ (40 mg/kg, i.p.) followed by kolaviron (100 mg/kg, orally, Group C) showing mild periportal fibrosis and inflammatory cellular infiltration (blue arrow) with congested central vein (black arrow) (H&E, 100×); (d) Liver section of a rat treated with kolaviron alone (100 mg/kg, orally, Group D) showing normal hepatic architecture with no significant lesions and central vein (asterisk) (H&E, 400×).

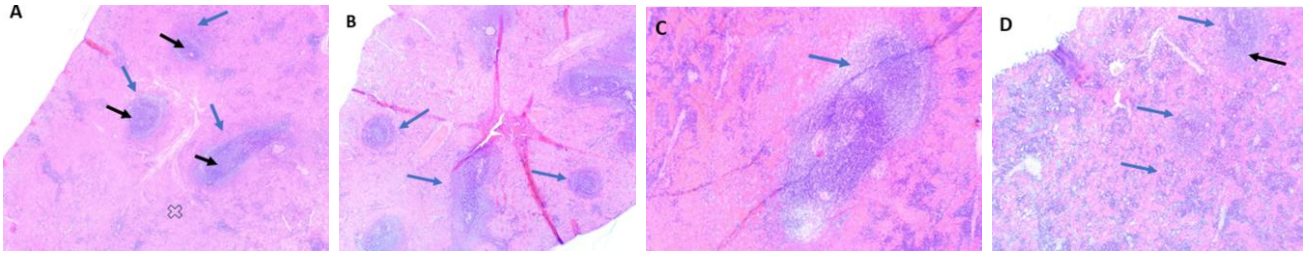

Plate 2: (a)-(d) Histopathology of the Spleen in PHZ and Kolaviron Treatment Study. (a) Spleen section of a control rat (Group A) showing normal architecture of lymphoid follicles (blue arrows) with white pulp (black arrows) and red pulp (asterisk) (H&E, 100×); (b) Spleen section of a rat treated with PHZ (40 mg/kg, i.p., Group B) showing prominent lymphoid follicular hyperplasia (blue arrows) (H&E, 100×); (c) Spleen section of a rat treated with PHZ (40 mg/kg, i.p.) followed by kolaviron (100 mg/kg, orally, Group C) showing lymphoid follicular necrosis (blue arrow) (H&E, 100×); (d) Spleen section of a rat treated with kolaviron alone (100 mg/kg, orally, Group D) showing concurrent lymphoid follicular hyperplasia (blue arrows) with necrosis of the follicles (black arrow) (H&E, 100×).

DISCUSSION

This study investigated the hepatoprotective, genoprotective, and potential splenic effects of kolaviron against phenylhydrazine (PHZ)-induced toxicity in Wistar rats. Our key findings demonstrated that kolaviron effectively mitigated PHZ-induced liver damage and genotoxicity through antioxidant mechanisms. However, an unexpected finding was the organ-specific toxicity observed in the spleen, where kolaviron induced follicular necrosis both when administered alone and in combination with PHZ. These contrasting effects—hepatoprotection versus

splenic toxicity—highlight the complex pharmacological profile of kolaviron and underscore the importance of comprehensive toxicological evaluation of natural products before clinical application.

The significantly elevated serum ALT and AST levels observed in Group B (phenylhydrazine-treated rats) provide clear biochemical evidence of substantial hepatocellular injury. Aminotransferases, particularly ALT, are primarily located in hepatocyte cytoplasm and are released into circulation when hepatocellular membrane integrity is compromised (Andrade *et al*., 2019). The elevation of these enzymes reflects the severity of liver cell damage induced by PHZ-mediated oxidative stress. Phenylhydrazine, a potent oxidizing agent, generates reactive oxygen species (ROS) through its metabolism, leading to lipid peroxidation, protein oxidation, and ultimately hepatocellular necrosis (Misra and Fridovich 1976; Pandey *et al*., 2014).

Remarkably, kolaviron treatment (Group C) normalized these liver enzyme levels to near-control values, indicating significant preservation of hepatocellular membrane integrity. This biochemical improvement was further supported by the reduction in hepatic malondialdehyde (MDA) levels observed in the kolaviron-treated groups. MDA, a terminal product of lipid peroxidation, serves as a reliable biomarker of oxidative stress-induced cellular damage (Varshney and Kale 1990). The lower MDA levels in Groups C and D compared to the control group suggest that kolaviron effectively attenuates oxidative stress, potentially through direct free radical scavenging and enhancement of endogenous antioxidant systems. Interestingly, the absence of significant differences in most antioxidant enzyme activities such as GSH, GST, and SOD across groups suggests that kolaviron's protective mechanism may rely more heavily on direct antioxidant activity rather than modulation of enzymatic antioxidant defenses. However, the significantly lower hepatic GPX activity in Group D (kolaviron alone) compared to other groups requires further investigation, as this finding is difficult to interpret without additional mechanistic studies.
The biochemical evidence of hepatoprotection was definitively confirmed by histopathological examination of liver tissue. Group B (PHZ-treated) exhibited moderate periportal fibrosis and portal vein congestion, classical histological features of chemical-induced hepatotoxicity (Zimmerman 1999; Gu & Manautou, 2012). Periportal fibrosis represents the liver's response to chronic oxidative injury, characterized by excessive deposition of extracellular matrix proteins, particularly collagen, in the periportal region. Portal vein congestion reflects hemodynamic alterations and endothelial damage induced by oxidative stress. In contrast, Group C (PHZ + kolaviron) showed only mild periportal fibrosis and inflammatory infiltration with central vein congestion, indicating substantial attenuation of PHZ-induced liver damage. Critically, Group D (kolaviron alone) maintained normal hepatic architecture comparable to controls, confirming that kolaviron at 100 mg/kg does not induce hepatotoxicity. This histological evidence validates the biochemical findings and establishes kolaviron as a genuine hepatoprotective agent against oxidative liver injury.

These findings align with extensive previous research demonstrating kolaviron's hepatoprotective properties across various hepatotoxic models. Farombi (2000) demonstrated kolaviron's ability to protect against carbon tetrachloride-induced hepatotoxicity by preserving microsomal enzyme activities, including aniline hydroxylase, aminopyrine N-demethylase, ethoxyresorufin O-demethylase, and p-nitroanisole O-demethylase, and by reducing lipid peroxidation, which corroborates our observation of reduced hepatic damage and MDA levels. Similarly, Alabi and Akomolafe (2020) reported that kolaviron diminished diclofenac-induced liver toxicity through suppression of inflammatory events and upregulation of antioxidant defenses, while Ola and Adewole (2021) observed that kolaviron ameliorated sodium valproate-induced hepatotoxicity by attenuating oxidative stress markers and restoring antioxidant enzyme activities. Ayepola *et al*. (2013) additionally reported that kolaviron lowered serum levels of hepatic enzymes in streptozotocin-induced diabetic rats, consistent with our findings on ALT and AST reduction. The consistency of these findings across diverse hepatotoxic models suggests that kolaviron's hepatoprotective mechanism is broadly applicable to oxidative stress-mediated liver injury, regardless of the specific toxic agent.

The micronucleus assay revealed significant genoprotective effects of kolaviron against PHZ-induced DNA damage. Group B exhibited a markedly elevated frequency of micronucleated polychromatic erythrocytes (MnPCEs) and a reduced PCE/NCE ratio, both hallmarks of genotoxicity and bone marrow cytotoxicity (Rungemorris *et al*., 1994). Micronuclei arise from chromosomal fragments or whole chromosomes that fail to incorporate into daughter nuclei during cell division, serving as sensitive biomarkers of chromosomal damage and genomic instability. The restoration of MnPCE frequencies and PCE/NCE ratios to near-control levels in Groups C and D demonstrates kolaviron's potent genoprotective capacity.

The genoprotective mechanism of kolaviron can be attributed to its robust antioxidant properties. Oxidative stress is a primary driver of genotoxicity through multiple mechanisms: direct oxidation of DNA bases, particularly the formation of 8-oxo-7,8-dihydroguanine (8-oxoguanine), a mutagenic DNA lesion; induction of single- and double-strand DNA breaks through ROS-mediated attack on the sugar-phosphate backbone; and promotion of chromosomal aberrations through oxidative disruption of mitotic spindle function and centromere integrity (Pandey *et al*., 2014). By effectively scavenging ROS and reducing oxidative stress, kolaviron likely mitigates these oxidative insults to genetic material, thereby preventing the DNA and chromosomal damage that manifests as micronucleus formation.
These findings align with previous studies demonstrating kolaviron's genoprotective effects. Ola and Adewole (2021) reported that kolaviron inhibited sodium valproate-induced DNA fragmentation and micronuclei formation in bone marrow cells, while Nwankwo *et al*. (2000) demonstrated that kolaviron inhibited aflatoxin B1-induced genotoxicity in human hepatoma cells. The consistency of these findings across different genotoxic models reinforces kolaviron's potential as a chemopreventive agent against oxidative and chemical-induced DNA damage.

In stark contrast to the hepatoprotective and genoprotective effects, kolaviron exhibited adverse effects on splenic tissue. Histopathological examination revealed that while Group B (PHZ alone) showed only lymphoid

follicular hyperplasia—a compensatory immune response to oxidative stress—Group C (PHZ + kolaviron) exhibited lymphoid follicular necrosis. Most critically, Group D (kolaviron alone) displayed concurrent lymphoid follicular hyperplasia and necrosis, indicating that kolaviron itself induces splenic damage independent of PHZ exposure. This organ-specific toxicity is particularly concerning because necrosis, unlike hyperplasia, represents irreversible cellular death and tissue damage.

This finding is not entirely unprecedented. Akingboye *et al*. (2015) reported dose-dependent splenic toxicity of ethanolic *Garcinia kola* extract in Wistar rats, where high doses (0.6 ml and 0.9 ml) caused distortion and mild degeneration of spleen cells with increased relative spleen weight and histological lesions, while low doses (0.3 ml) showed no adverse effects. This dose-dependent pattern suggests that the splenic follicular necrosis observed at 100 mg/kg kolaviron may represent a threshold effect, in which the dose exceeds the spleen's tolerance despite remaining within the hepatoprotective range. The increased relative spleen weight observed by Akingboye *et al*. (2015), alongside cellular distortion, suggests potential inflammatory or compensatory responses preceding cellular damage, which may explain the concurrent hyperplasia and necrosis observed in our Group D.

The mechanisms underlying kolaviron-induced splenic toxicity remain poorly understood and warrant comprehensive investigation. One possible explanation relates to tissue-specific metabolism and accumulation. The spleen's unique immunological functions and high metabolic activity in lymphoid follicles may render it particularly vulnerable to certain phytochemicals. Kolaviron or its metabolites may accumulate preferentially in splenic tissue due to differences in blood flow, cellular uptake mechanisms, or metabolic capacity compared to the liver. The spleen receives approximately 5% of cardiac output and serves as a major site of immune cell activation and proliferation, creating a metabolically demanding environment that may be sensitive to xenobiotic stress.

Another potential mechanism involves direct cytotoxicity to immune cells. The biflavonoid structure of kolaviron may interact adversely with specific immune cell populations within lymphoid follicles, particularly rapidly dividing B and T lymphocytes. Flavonoids can interfere with topoisomerase enzymes, cell cycle regulation, and mitochondrial function in certain cell types. If lymphocytes are particularly sensitive to these effects, this could explain the selective follicular necrosis observed. Additionally, paradoxically, antioxidants can exhibit pro-oxidant activity under certain conditions, particularly in environments with high oxygen tension and metabolic activity. The spleen's red pulp is highly oxygenated due to its role in erythrocyte processing, and this oxidative environment may convert kolaviron's antioxidant properties into pro-oxidant effects, leading to oxidative damage specifically in splenic tissue.

Kolaviron may also possess immunomodulatory properties that, at certain doses, disrupt normal immune homeostasis within the spleen. This could trigger inappropriate apoptotic signaling or inflammatory cascades leading to follicular damage. Akingboye *et al*. (2015) suggested that high doses of *Garcinia kola* extract may cause alterations in the cytoarchitecture of the spleen, though the exact mechanism was not elucidated. Further studies examining kolaviron's tissue distribution, metabolism, dose-response relationships, and effects on immune cell viability are essential to elucidate these mechanisms and establish a safe therapeutic window.

The splenic toxicity of kolaviron observed in our study contrasts sharply with studies on other natural compounds tested against PHZ-induced toxicity. Olukanni *et al*. (2023) reported that Moringa oleifera ameliorated phenylhydrazine-induced splenic toxicity by improving antioxidant status and reducing inflammatory markers. Similarly, Innih *et al*. (2020) demonstrated the protective effect of Spondias mombin against phenylhydrazine-induced spleenotoxicity, while Zangeneh *et al*. (2019) and Obayuwana and Obayuwana (2022) showed ameliorative effects of Ocimum basilicum and Brassica nigra, respectively.

This fundamental difference can be attributed to several factors. First, kolaviron's unique biflavonoid structure, containing GB-1, GB-2, and kolaflavanone, may confer distinct pharmacokinetic and pharmacodynamic properties compared to the phytochemical profiles of other plant extracts. While most hepatoprotective plants contain diverse mixtures of flavonoids, polyphenols, and other antioxidants that may distribute more evenly across tissues, kolaviron's specific biflavonoid configuration may result in tissue-selective effects. Second, the differential organ responses suggest that hepatoprotection and splenoprotection may require different mechanisms. While kolaviron's antioxidant capacity is sufficient for liver protection, it may lack specific factors necessary for splenic protection or may even contain components that are particularly toxic to lymphoid tissue. Third, variations in treatment duration (our study employed 14 days of treatment) and the severity of PHZ-induced damage may influence the manifestation of splenic effects compared with other studies.

Importantly, the absence of significant changes in splenic oxidative stress markers such as H_2O_2 and MDA, and antioxidant parameters including GPX, GSH, GST, and SOD across all groups suggests that the splenic toxicity does not result from overwhelming oxidative stress, but rather from alternative mechanisms unrelated to the oxidative parameters we measured. This further distinguishes the splenic response from the liver response, where oxidative stress markers clearly correlated with tissue damage and kolaviron's protective effects were manifested through antioxidant mechanisms.

The dual nature of kolaviron's effects—hepatoprotective and genoprotective on one hand, but splenotoxic on the other—raises important safety considerations for potential therapeutic applications. The spleen plays critical roles in immune surveillance, antibody production, removal of senescent erythrocytes, and hematopoiesis (Elmore 2006; Liezmann *et al*., 2012). Splenic follicular necrosis could potentially compromise these functions, leading to immunosuppression, increased susceptibility to infections, or altered hematological parameters. While no significant hematological abnormalities were noted in the current study, longer-term exposure or higher doses might reveal more severe consequences.

The hepatoprotective effects observed suggest that kolaviron could potentially benefit patients with oxidative stress-induced liver injury, particularly those undergoing treatments with hepatotoxic drugs. However, the splenic

toxicity observed at 100 mg/kg necessitates extreme caution. Before any clinical application can be considered, several critical questions must be addressed. First, what is the minimum effective dose for hepatoprotection? Second, is there a dose threshold below which splenic toxicity does not occur? Third, what is the therapeutic window, if any, where hepatoprotection can be achieved without splenic damage? Fourth, are the splenic effects reversible upon discontinuation of kolaviron? These questions can only be answered through comprehensive dose-ranging studies and long-term toxicological evaluations.

This study has several limitations that should be acknowledged. First, the fixed-dose approach (100 mg/kg kolaviron) prevents assessment of dose-response relationships. Given the evidence from Akingboye *et al*. (2015) suggesting dose-dependent splenic toxicity, a comprehensive dose-ranging study is urgently needed to identify whether lower doses, such as 25 to 75 mg/kg, can maintain hepatoprotective efficacy while eliminating splenic toxicity. This would help establish a therapeutic window for safe application.

Second, while we examined several oxidative stress and antioxidant markers, we did not assess all possible mechanisms through which kolaviron might exert its effects. Future studies should investigate inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-10, to elucidate the inflammatory mechanisms underlying both hepatoprotection and splenic toxicity. Apoptotic markers including caspase-3, Bax, Bcl-2, and TUNEL staining should be examined to elucidate whether splenic necrosis involves programmed cell death pathways. Immunomodulatory parameters such as lymphocyte subpopulations, antibody production, and cytokine profiles need to be assessed to evaluate functional immune consequences. Tissue distribution and pharmacokinetic studies are necessary to determine if kolaviron accumulates differentially in liver versus spleen. Additionally, metabolomic profiling could identify specific metabolites responsible for organ-specific effects.

Third, the 14-day treatment duration, while sufficient to demonstrate effects, may not reveal long-term consequences. Chronic toxicity studies with extended treatment periods and recovery phases are needed to assess the reversibility of splenic damage and potential cumulative effects.

In conclusion, this study demonstrates that kolaviron effectively protects against PHZ-induced hepatotoxicity and genotoxicity through antioxidant mechanisms, as evidenced by normalised liver enzymes, reduced lipid peroxidation, improved histological architecture, and decreased micronucleus formation. These findings support kolaviron's potential as a hepatoprotective and genoprotective agent. However, the concurrent observation of splenic follicular necrosis, occurring both with kolaviron alone and with PHZ, indicates significant organ-specific toxicity that cannot be overlooked. This dichotomy underscores the complexity of natural product pharmacology and highlights the critical importance of comprehensive toxicological evaluation across multiple organ systems.

The results provide further evidence for the role of oxidative stress in phenylhydrazine-induced toxicity and confirm kolaviron's antioxidant properties in vivo. However, the splenic toxicity observed at 100 mg/kg raises substantial safety concerns that necessitate thorough dose-optimization studies, mechanistic investigations, and comprehensive toxicological evaluation before any clinical applications can be considered. Future investigations should implement a dose-optimization strategy with multiple kolaviron concentrations, such as 25 to 200 mg/kg, to determine whether lower doses can maintain hepatoprotection while eliminating splenic toxicity, and to establish the therapeutic window for safe clinical application. Additionally, research should focus on elucidating the detailed molecular mechanisms underlying both kolaviron's protective effects in the liver and its toxic effects in the spleen, particularly examining inflammatory pathways, apoptotic signalling, immune cell function, and tissue-specific metabolism. Only through such comprehensive investigation can the true therapeutic potential and safety profile of kolaviron be established.

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