

Original Research Article

Hepatotoxicity assessment of D'General Bitters: Histological and biochemical evaluation in Wistar rats

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Abstract

Purpose: To investigate the hepatotoxic potential of D'General Bitters in Wistar rats, by analysing its histopathological and biochemical safety profile.

Methods: Twenty male Wistar rats were divided into a control group and three study groups of five rats each. The rats in the study groups received 0.2, 0.4 and 0.8 mL (low, moderate and high doses) of D'General Bitters, respectively, for six weeks. At the termination of the experiment, the liver of the rats was harvested, histologically processed and stained with haematoxylin and eosin. The rat serum was analysed for alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) activities. In addition, body weight assessments were conducted.

Results: Histological examination revealed well-preserved hepatic architecture with no signs of necrosis, steatosis, or fibrosis. Serum AST and ALT levels remained within normal ranges, indicating no hepatocellular injury. However, ALP level of the moderate dose group was significantly lower when compared with the control group ($p = 0.048$). Body weight increased significantly in the moderate-dose group ($p = 0.011$), while relative liver weight showed no significant ($p > 0.05$) change across groups.

Conclusion: D'General Bitters does not induce significant hepatotoxic effects under the administered conditions. However, the decrease in alkaline phosphatase (ALP) activity indicates a notable biochemical alteration, warranting further investigation into its long-term safety and hepatobiliary effects.

Keywords: Dose response, Liver enzymes, Natural products, Polyherbals, Toxicology

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INTRODUCTION

Herbal medications have been widely used for centuries as alternative therapeutic agents for various ailments [1]. The World Health Organization (WHO) has noted that approximately 80 % of the global population relies on herbal formulations for primary

healthcare [2]. In Nigeria, the use of herbal formulations (especially *bitters*) has increased because of the claim that it enhances sexual performance, improve digestion and overall well-being, and detoxify the liver [3]. However, despite these claims and rising popularity, the safety of many herbal products marketed in Nigeria remains largely unverified, raising concerns

about potential hepatotoxicity and implications of their chronic use or abuse [4].

One of such popular herbal formulations in Nigeria is *D'General Bitters*, marketed widely as a general health tonic and constitutes a mixture of honey, citrus, alcohol, water, and other natural herbal extracts [4]. Like many polyherbal beverages, it contains multiple bioactive compounds such as proteins, alkaloids, flavonoids, reducing sugars, glycosides, and amino acids [5]. These bioactive compounds could exert both beneficial and toxicological effects (either in combination or independently) on hepatic function [6]. While certain phytochemicals present in this herbal drink, such as flavonoids, have demonstrated hepatoprotective activity, some others, such as alkaloids, have been implicated in liver toxicity, fibrosis, and hepatocellular necrosis [7,8].

Previous studies have reported that *D'General Bitters* did not cause significant histological and gross morphological damage to the kidney [9] and testes [10]; but induced dose-dependent structural and biochemical changes to the pancreas [11], induced biochemical changes to the kidney [4], and significantly disrupted testicular hormonal balance and sperm morphology [10] of adult male Wistar rats.

The liver is an organ that plays an important role in the metabolism and detoxification of xenobiotics, including drugs, herbal products, and toxins [8]. Hepatotoxicity arising from exposure to toxic compounds can lead to functional alterations of the liver, notable in forensic and postmortem investigations [6]. In forensic pathology, unexplained liver failure often necessitates toxicological screening to identify potential hepatotoxic agents, including herbal formulations, as contributing factors to morbidity or mortality [12]. Toxicological screening on the liver includes histopathological and biochemical assessment, which may be supplemented by oxidative stress assessment to fully characterize the hepatotoxic effect.

Histopathological assessments provide insight into the structural changes within hepatic tissues, helping to distinguish between different aetiologies of liver injury. Biochemical assessment of the liver provides insights into the hepatocellular damage and cholestatic injury [5]. Key biochemical markers of liver function include alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and total direct bilirubin. Abnormal elevations of these enzymes in toxicological investigations often

point toward toxic liver insults, including drug-induced liver injury (DILI) and/or herbal-induced liver injury (HILI) [12].

Herbal-induced liver injury (HILI) has become a growing public health and forensic concern due to the increasing consumption of herbal products with undocumented toxicological profiles [13]. In many postmortem cases, herbal intoxication is a major cause of liver dysfunction, primarily due to the lack of awareness and standardized screening protocols in forensic investigations. Previous studies have demonstrated that prolonged administration of certain herbal bitters can lead to hepatocellular damage, characterized by significant increases in liver enzymes and histopathological abnormalities such as sinusoidal congestion, hepatocyte vacuolation, and inflammatory infiltration [12].

The increasing cases of acute and chronic liver failure associated with abuse of these popular herbal beverages highlight the need for systematic toxicological investigations on them. This study, therefore, investigated the toxicological implications of *D'General Bitters* in male Wistar rats.

EXPERIMENTAL

Animals

A total of twenty (20) healthy male Wistar rats sourced from a local farm in Nsukka, Enugu State, Nigeria, with initial weight range of 100 – 150 g. A veterinarian was employed to confirm that the rats were healthy upon arrival at the research facility (Animal Farm of the College of Health Sciences, Nnamdi Azikiwe University, Nnewi campus, Anambra State, Nigeria). The rats were allowed to acclimatize for two weeks with access to feed and water *ad libitum*. The health status of rats was closely monitored throughout the experiment.

Protocol

The study protocol was approved by the Ethics Committee of the Faculty of Basic Medical Sciences, College of Health Sciences, Nnamdi Azikiwe University, with approval reference number: NAU/CHS/NC/FMBS/571, dated 26th July 2023. The guidelines were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (8th edition, 2011) [14] and reported in accordance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).

The herbal drink was purchased from the authorized distributor at Nkwo Nnewi market, Nnewi, Nigeria. The animal feed (Top Feeds Grower's Mash Super-Deluxe, manufactured by Eastern Premier Feed Mills Ltd., a division of Premier Feeds Mills Co. Ltd., Plateau State, Nigeria) was purchased from a local trader in Nnewi, Nigeria. The analytical reagents used included Dragendorff's reagent, Benedict's reagent, and other high-purity chemicals, which were supplied by Syntro Bioresearch Inc., USA. All materials were of analytical grade, ensuring their suitability for the study procedures.

The rats were divided into four groups of five per group and housed in spacious cages with a controlled 12-hour light/dark cycle. The sample size was selected to provide sufficient data for statistical reliability while adhering to the 3Rs principles of animal ethics [15]. Group A served as the control, while groups B, C, and D served as the study groups, respectively. Group A received only water and standard feed; Group B received 0.2 mL of *D'General Bitters*, Group C received 0.4 mL of *D'General Bitters*, and Group D received 0.8 mL of *D'General Bitters* daily. The administration of *D'General Bitters* to the rats lasted for 6 weeks. Prior to the administration of the herbal drink, the rats were weighed using a 6000 g capacity scale (model WT6000GT, WANT Balance Instrument Company Ltd., China). During both the acclimatization and study periods, all the rats had *ad libitum* access to standard pelleted feed and clean water. The cages and environment were cleaned and disinfected daily.

Acute toxicity test

This study adopted the published toxicity test result for *D'General Bitters* in male Wistar rats [4]. The published result for LD₅₀ was 2154.17 mg/kg. Hence, the study groups B, C, and D received 0.2, 0.4 and 0.8 mL of *D'General Bitters*, respectively, for low, moderate and high doses.

Animal euthanasia

The rats were euthanized via overdose of inhalant anaesthesia (CO₂ gas). The gas flow rate was set to displace 10 – 30 % of the chamber volume per minute to minimize distress. The animals were closely monitored until unconsciousness was achieved, followed by confirmation of euthanasia through the complete cessation of respiratory, cardiac activity and reflexes, including corneal reflexes. The carcasses of the Wistar rats were disposed of in accordance with institutional biosafety protocols.

The euthanasia was conducted in full compliance with the Institution's Animal Welfare Regulations and Ethical Guidelines, in accordance with established protocols for study studies involving Wistar rats [14].

Blood collection and organ harvesting

Rats were positioned on a flat board, with their limbs gently secured using optical pins. Blood samples were collected via ocular puncture and allowed to clot in non-additive plastic tubes for 30 minutes. The abdomen was then carefully dissected using a dissection kit, and the liver was excised, rinsed in a plain tube containing phosphate buffer, and fixed in 10% formalin for histological examination.

Upon complete blood clotting, the blood samples were centrifuged at 2500 rpm for 10 minutes using an 800D Electric Centrifuge (4000 rpm, 6x20 mL rotor capacity). The resulting clear serum was carefully aspirated and stored at -2°C until further biochemical assay. The procedure for blood collection followed an established protocol for animal study studies [16]. This procedure for histological examination followed an established protocol for organ harvesting of rat models [14].

Biochemical assay

The biochemical assay analysed the aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) levels of the Wistar rats. The liver function tests were performed following standard protocols used in rat study models [4].

Histology analysis

Liver samples were processed through fixation, dehydration, clearing, embedding, sectioning, and staining with haematoxylin and eosin (H&E). Photomicrographs were captured using an Amscope 14MP digital camera mounted on a NOVEX compound microscope (produced by Euromex Microscope BV, Arnhem, the Netherlands), equipped with Hi-PLAN objectives. Image labelling was performed with Photoscape v3.7. All histological procedures adhered to established protocols for rat study studies [9].

Statistical analysis

The statistical analysis for this study was performed using IBM SPSS Statistics software (version 25). The descriptive statistics are presented as the mean ± standard error of the mean (SEM). One-way ANOVA was used to

analyse the differences in the mean between the control and study groups. The assumption of homogeneity of variance was verified with Levene's test. For ANOVA results reaching statistical significance, post hoc analysis was applied to identify specific group differences. A *p*-value of less than 0.05 was considered statistically significant for all tests.

RESULTS

Results of histological analysis

Histological analysis demonstrated preserved hepatic architecture in all groups. In the control group (Figure 1 AI and AII), the liver exhibited distributed portal triads around the central vein (Figure 1 AII), with moderate portal inflammation and interface involvement (piecemeal necrosis),

but without signs of portal fibrosis or steatosis. Liver sections from Groups B (Figure 1 BI and BII), C (Figure 1 CI and CII), and D (Figure 1 DI and DII), which received low, moderate, and high doses of *D'General Bitters*, respectively, showed intact hepatic architecture with no signs of portal inflammation, fibrosis, necrosis, or steatosis.

Biochemical findings

Serum AST and ALT levels showed no statistically significant differences between the control and study groups (Table 1), indicating no hepatocellular injury. However, ALP levels showed a statistically significant decrease in Group C compared to the control ($p = 0.048$), while no statistically significant differences were observed in Groups B or D.

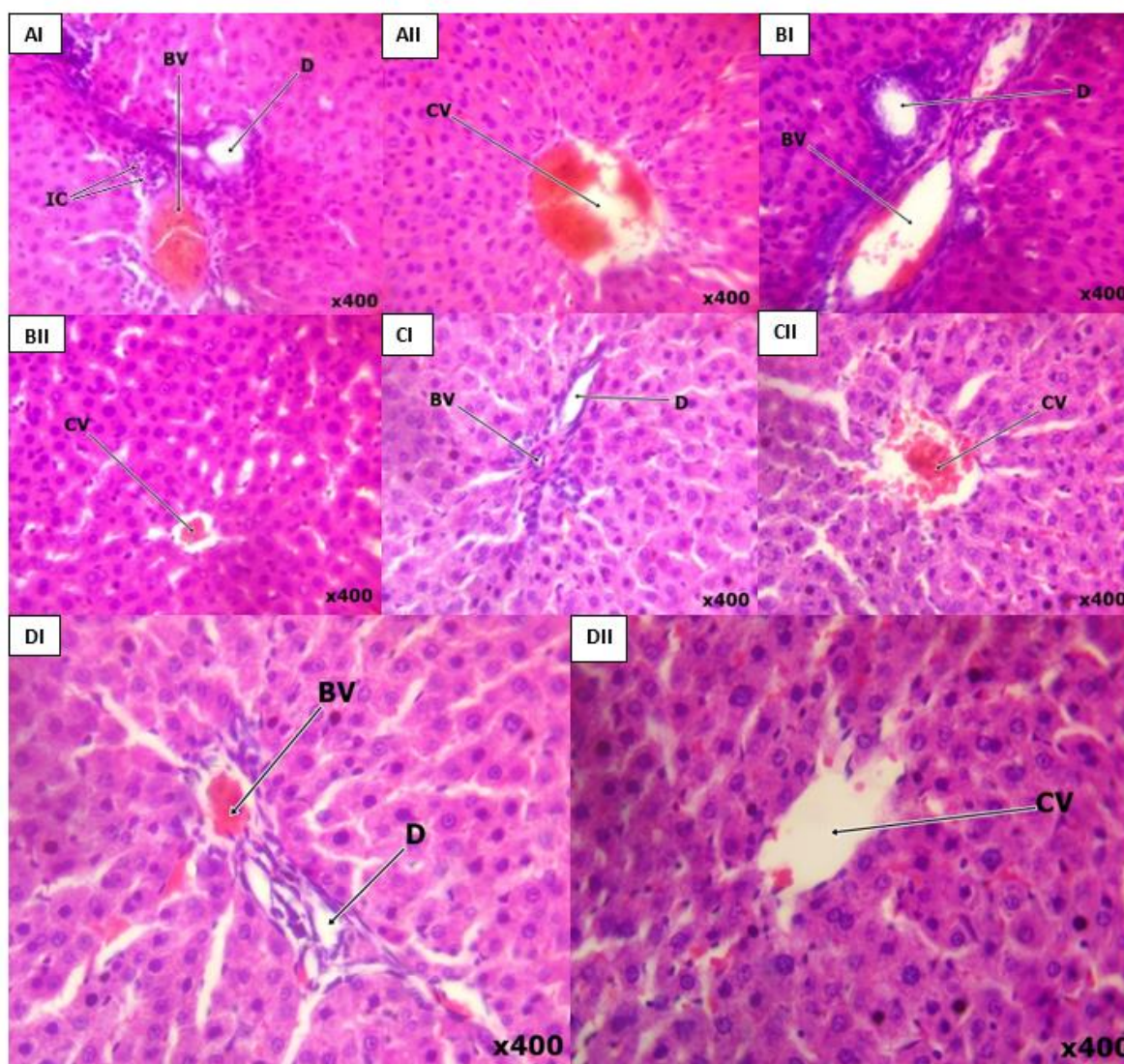


Figure 1: (AI – DII) Representative photomicrographs of the liver of rats in Groups A – D; I = porta triad image of the liver, II = central vein image of the liver, D = Hepatic duct, IC = Inflammatory cells, CV = Central vein, BV = Blood vessel; 400x magnification; H&E stain

Table 1: Results of AST, ALT and ALP levels of the rats

Group		Mean ± SEM	P-value	F value
AST (IU/L)	Control (A)	29.00±0.00		4.000
	B	28.00±0.00	0.705 [#]	
	C	26.00±0.00	0.205 [#]	
	D	29.00±0.00	1.000 [#]	
ALT (U/L)	Control (A)	21.00±0.00		0.500
	B	20.00±0.00	0.705 [#]	
	C	19.00±0.00	0.293 [#]	
	D	20.00±0.00	0.705 [#]	
ALP (IU/L)	Control (A)	99.00±0.00		68.000
	B	98.01±0.00	0.500 [#]	
	C	89.98±0.00	0.048 [*]	
	D	102.11±0.00	0.128 [#]	

Values were significant at $p \leq 0.05$. *: significant, #: not significant

Table 2: Result of the effect of *D' General Bitters* on body weight and relative liver weight

Group	Body weight (g)	P-value	Relative liver weight (g)	P-value
A	214.67±10.11		3.23±0.07	
B	249.00±9.23	0.079 [#]	3.39±0.21	0.153 [#]
C	270.67±17.61	0.011 [*]	3.49±0.02	0.589 [#]
D	243.00±9.23	0.136 [#]	3.13±0.05	0.596 [#]
F-ratio	3.653		1.879	

Values were considered significant at $p < 0.05$. *: significant, #: not significant, SD: standard deviation, SEM: standard error of the mean

Morphometric findings

Analysis of body weight changes showed an increase in groups B, C, and D compared to group A (Table 2). The changes observed in groups B and D were not statistically significant, but the change in group C was statistically significant ($p = 0.011$). On the other hand, result of the relative liver weight revealed an increase in groups B and C, but a decrease in group D when compared to group A. However, these changes were not statistically significant (Table 2).

DISCUSSION

This study provides crucial insights into the hepatotoxicity and safety profile of *D'General Bitters*, which is a widely consumed polyherbal beverage in Nigeria. The toxicological assessments, which included histopathological and biochemical analyses, provide a comprehensive evaluation of its effects on the liver of adult male Wistar rats.

The histology results of the liver tissues showed a well-preserved histo-architecture, with no significant structural pathology such as hepatocellular necrosis, steatosis, or fibrosis. This suggests that *D'General Bitters* does not induce overt structural liver damage, contrasting with reports that implicated certain polyherbal beverages with hepatic toxicity [6,7]. In addition,

the histology results from this study contrast with the histology results of the same herbal drink on the pancreas of adult male Wistar rats [11] but correlate with the reports on the same herbal drink on the kidney and testes of adult male Wistar rats [9,10]. This discrepancy suggests a selective tropism of *D'General Bitters* across different tissues or organs, where each tissue has a different metabolic activation or detoxification pathway. It may also suggest that the liver, being a strong metabolic organ, possesses detoxification enzymes that neutralize pathogenic phytochemicals in *D'General Bitters*. The liver's robust detoxification enzymes may successfully neutralize phytochemicals that are pathogenic to other organs like the pancreas.

The result of the biochemical analyses further supported these findings. Serum AST and ALT levels remained within normal ranges, indicating an absence of significant hepatocellular injury, which aligns with the histology results. However, the isolated, statistically significant decrease in ALP activity in the moderate-dose group (Group C) is a notable finding. While ALP is a classic marker for cholestasis and bile duct injury when elevated [17], a decrease is less commonly reported in toxicology. This reduction could suggest an inhibitory effect on the synthesis or release of this enzyme at the moderate dose, rather than a cholestatic injury. The non-linear, dose-specific nature of this effect (absent in low and high doses) warrants further investigation

into the complex pharmacokinetic interactions of this herbal drink and the liver.

Body weight analysis corroborated these results, showing a significant increase in the moderate-dose group (Group C) compared to controls, while changes in the low and high-dose groups were not significant. This suggests a possible biphasic, hormetic effect on metabolism, increasing cellular resistance to future stress, and appetite stimulation of the groups that received a moderate dose of the herbal drink. Importantly, this weight gain was not associated with adverse histopathological or hepatocellular enzyme (AST, ALT) changes, indicating it is likely not a pathological outcome. Furthermore, the absence of a statistically significant change in relative liver weight across all groups supports the absence of pathological hepatomegaly or oedema, reinforcing the formulation's metabolic safety under the tested conditions.

Limitations of the study

While this study provides a clear toxicological profile in male Wistar rats, its scope introduces certain constraints. The use of only male Wistar rats precludes an assessment of sex-dependent differences in metabolism, which are known to influence hepatotoxic outcomes. Furthermore, the six-week exposure period and the selected panel of biochemical markers, though appropriate for this preliminary assessment, suggest avenues for further research. Further studies should investigate the effects of this herbal drink on long-term (chronic) exposure and include oxidative stress assessment.

CONCLUSION

Under the conditions of this study, *D'General Bitters* does not induce significant histopathological damage or hepatocellular injury (as indicated by preserved liver architecture and normal AST/ALT levels) in male Wistar rats. However, the significant decrease in ALP activity in the moderate-dose group points to a dose-specific, non-linear, hormetic response on hepatobiliary markers. The morphometric results reinforce the conclusion that, though there was a change in ALP level of the moderate dose group, it is a hormetic response. However, there is need for further studies on long-term exposure of this herbal drink to the liver.

DECLARATIONS

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Ethical approval

Is stated in the method.

Use of Artificial intelligence/Large language models

The authors confirm that AI-based tools were used solely for language editing and manuscript polishing purposes (e.g., grammar, phrasing, and clarity). No AI tools were used to generate content, interpret data, or influence study design, results, or conclusions. All intellectual contributions, study analyses, and interpretations reported in the manuscript are the original work of the authors.

Availability of data and materials

The datasets generated during and/or analysed during the current study are available within the text.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the final manuscript. DNO conceptualized the study, designed the study, wrote the study protocol, supervised the experiment, carried out the histology analysis and wrote the first draft of the manuscript; and assisted DCE to manage the literature searches. DCE managed the literature searches, carried out the biochemical assay, and reviewed the manuscript. CDN carried out the experiment, managed the animals and assisted ACU to curate the data. ACU curated the data, carried out the data analysis and assisted CDN to manage the animals.

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