

Original Research Article

Histopathological effects of D'General Bitters on the pancreas of male adult Wistar rats

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Abstract

Purpose: To investigate the histopathological effect of D'General Bitters (a herbal drink) on the pancreas of adult male Wistar rats.

Methods: Twenty male Wistar rats (90 – 160 g) were randomly divided into four groups (n = 5). Group A (control) received food and water only, while Groups B, C and D were administered 0.2, 0.4 and 0.8 mL of D'General Bitters daily, respectively, for six weeks. Blood samples and organs were collected for analyses of serum lipase, amylase and insulin levels.

Results: Pancreatic histology in control group showed normal architecture. Group B showed mild β -cell reduction, while Groups C and D exhibited moderate β -cell reduction. Acinar cells and α -cells of the Islets of Langerhans remained normal across all groups. Serum lipase and insulin levels differed significantly between control and experimental groups ($p > 0.05$). Serum amylase levels were significantly ($p > 0.05$) different in Groups C and D compared to control, but were non-significant in Group B.

Conclusion: D'General bitters induce dose-related biochemical and histological changes in the pancreas of Wistar rats, suggesting potential pancreatic toxicity with prolonged or high-dose use. Public health education and regulatory scrutiny are recommended to ensure safe use of such herbal formulations.

Keywords: Acinar cells, Beta cells, Diabetes, Diarrhoea, Pancreatitis

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INTRODUCTION

Most herbal bitters are produced from bitter herbs or ingredients [1]. Such ingredients include herbs, roots, tree barks, fruits, seeds and flowers, usually mixed with aromatics. Hence, most herbal bitters contain about 50 % alcohol. In recent times, bitter drinks (bitters) have been used to add flavour to sour and sweet drinks [2]. These Bitters are produced with the aim that they are medicinal and could heal anything from

indigestion to malaria [3]. Bitters occupy an unusual record in history, especially in Africa, the Caribbean and Asia, where it is used as patent and conventional medicine and some folklore believes that the bitter, awful taste increases their potency for healing [4].

The use of herbal medicinal products has increased tremendously over the last three decades, with about 80 % of people relying on them for primary healthcare all over the world [4].

In addition, therapies involving herbal drugs have also shown promising potential for efficacy. However, most of these herbals have not undergone clinical trials and most have not been certified by health or drug regulatory bodies [5]. The proliferation of herbal bitters and their increasing consumption and abuse have been adjudged as one of the leading causes of diseases, and therefore a major health concern in Nigeria [6,7]. A good number of these herbal drinks are poorly produced, yet the producers claim that the bitter ingredients are secret recipes that have high therapeutic effects [8]; hence, taking advantage of the gullibility of the ignorant populace, unaware of their health risks.

The proliferation of herbal drinks in Nigeria and the high incidence of pancreatic diseases raise concerns about the need to evaluate the pathological effects of popular herbal drinks in Nigeria. D'General Bitters is a popular herbal drink consumed by many Nigerians. It contains phytochemicals such as flavonoids, alkaloids, glycosides, proteins, reducing sugars and amino acids [9]. Studies have shown that herbal drinks could be very harmful to some vital organs of the body, especially the pancreas [10]. A recent study on the biochemical effects of D'General Bitters on the kidney of male Wistar rats showed elevated creatinine and urea levels in certain groups which suggests that D'General Bitters may impact kidney function, while the reduction in uric acid levels in some groups may suggest a potential therapeutic effect on purine metabolism [9]. This study, however, is aimed at investigating the histopathological effects of D'General Bitters herbal drink on the pancreas of adult male Wistar rats.

EXPERIMENTAL

Animals

This study used 20 male adult Wistar rats, each weighing between 90 g and 160 g. The animals were obtained from a farm in Nsukka, Enugu State, Nigeria. Prior to the study, they underwent a two-week acclimatization period, during which they had unrestricted access to food and water. A veterinarian assessed the health of the rats before their transfer to the research facility under humane conditions. The rats were housed in well-ventilated stainless-steel cages with sufficient space, maintained at a controlled temperature and exposed to a 12-hour light/dark cycle. Their health was monitored regularly, and they were provided with a balanced diet and clean water. Sawdust was placed on the cage floors to promote hygiene and reduce the risk of

infection, and the cages were cleaned daily to maintain a sanitary environment.

Protocol

The rats were divided into four groups of five rats each. Group A served as control group while Groups B, C and D served as the experimental groups. Rats in group A were fed with water and feed only, Group B received 0.2 mL of D'General Bitters daily, Group C received 0.4 mL of D'General Bitters solution daily and Group D received 0.8 mL of D'General Bitters daily. The rats were weighed before the commencement of the experiment using a 6000 g capacity weighing scale (model number WT6000GT; Want Balance, China). The rats were fed *ad libitum* on standard pelleted mash and clean tap water during the entire course of the acclimatization and experiment periods. The cages and environment were kept clean and disinfected daily. All the procedures were carried out in strict compliance with the guidelines of the Ethics Committee of the Faculty of Basic Medical Sciences of Nnamdi Azikiwe University, Nnewi Campus, Nigeria (approval no. NAU/CHS/NC/FMBS/573).

Acute toxicity test

This study employed the OECD Guideline 425 (Up-and-Down Procedure), which involves sequential dosing based on previous outcomes to estimate the LD₅₀ with greater accuracy while minimizing animal use [11]. The study aimed to evaluate the acute oral toxicity of D'General Bitters in rats, conducted in two phases. In Phase I, nine rats were divided into three groups of three animals each and observed for 24 hours for signs of morbidity and mortality. No adverse effects were observed, and all rats remained healthy, enabling progression to Phase II. In the Phase II studies, three additional rats were administered single doses and monitored for morbidity and mortality over 24 hours.

Animal euthanasia

Animals were euthanized through an overdose of inhalant anaesthesia using CO₂ gas. To ensure comfort and minimize distress, the gas flow rate was regulated to displace 10 – 30 % of the chamber's volume per minute. The animals were observed for signs of unconsciousness, followed by verification of euthanasia after the complete cessation of respiratory and cardiac activity. Confirmation of euthanasia was performed by checking for the absence of a heartbeat, respiration, and reflexes such as corneal reflexes.

Blood collection and organ harvesting

Blood samples were collected from each rat eye using ocular puncture and placed into sterile plastic tubes. The samples were then allowed to sit for 30 minutes to ensure full clotting. Once clotted, they were centrifuged at 2500 rpm for 10 minutes using an 800D Electric Centrifuge Machine. The resulting clear serum was carefully separated and stored in a refrigerator until it was needed for assay. The rats were placed on a plane board, and their limbs were humanely held firm to the boards with optical pins. The abdomen of each rat was carefully dissected with the aid of a dissecting kit, while the pancreas was harvested, washed in a plain tube containing normal saline and then placed in a 10 % formal saline for histological analysis.

Histology analysis

The pancreas samples were fixed, dehydrated, cleared, embedded, sectioned and stained using standard tissue processing procedures [7]. Photomicrographs were taken with Amscope 14MP digital microscope camera fitted on Novex compound microscope with Hi-Plan objectives. The labelling was done with Photoscape v3.7 software.

Determination of serum lipase activity

Serum lipase activity was determined using a colorimetric enzymatic method as described by Burtis *et al* [12]. Three test tubes were arranged for blank (B), standard (ST) and sample (S) and 1000 μ L of lipase reagent 1 (R1) was added to all the test tubes. Thereafter, 20 μ L each of standard and sample was added to the respective test tube, while 20 μ L of distilled water was added to blank. The solution was mixed and incubated for 5 min at 37 °C. This was then followed by the addition of 250 μ L of lipase reagent 2 (R2). The solution was mixed and incubated for 2 min at 37 °C. The initial absorbance (A1) was determined 120 s after the addition of R2, while the final absorbance (A2) was read after another 120 s at 580 nm. Concentration of lipase (CL) was calculated using Eq 1.

$$CL = \{(A2-A1)_B / (A2-A1)_{ST}\} C_{ST} \dots\dots (1)$$

Determination of serum amylase

Serum amylase activity was determined using the classical starch-iodine colorimetric method as described by Reitman and Frankel [13]. For the determination of serum amylase (CA), three test tubes for control (C), test (T) and blank (B) were

arranged. One thousand microliters (1000 μ L) of starch substrate were added to all the test tubes. Tube C and T were incubated at 37 °C for 3 min. Twenty microliters each of control serum and test serum were added to the respective test tubes. The solution was mixed very well and incubated at 37 °C for 7.5 min. Working iodine reagent (1000 μ L) was added to all the test tubes, mixed thoroughly and final volume was made up to 10 mL with distilled water in each tube. The absorbance was read at 660 nm using a spectrophotometer and amylase concentration was calculated according to Eq 2.

$$CA = \{(Abs_B - Abs_{T/C}) / Abs_B\} \times 1480 \text{ U/i} \dots\dots (2)$$

Determination of rat insulin (Ins) levels

Rat insulin levels were determined using a sandwich enzyme-linked immunosorbent assay (ELISA), following the protocol described in the Abcam Rat Insulin ELISA Kit [14]. Briefly, 100 μ L of rat serum sample was added to each well of a 96-well microplate pre-coated with a monoclonal antibody specific for rat insulin. The plate was covered and incubated at 37 °C for 90 minutes. After incubation, the wells were aspirated and washed three times with 300 μ L of wash buffer to remove unbound substances. Next, 100 μ L of biotinylated detection antibody was added to each well, followed by incubation at 37 °C for 60 minutes. The wells were washed again three times, after which 100 μ L of avidin-horseradish peroxidase (HRP) conjugate was added and incubated at 37 °C for 30 minutes. After five additional washes, 90 μ L of TMB substrate solution was added to each well and incubated in the dark at 37 °C for 15 minutes. The reaction was terminated by adding 50 μ L of stop solution, resulting in a yellow colour. The optical density was measured immediately at 450 nm using a microplate spectrophotometer. A standard curve was generated by plotting the absorbance values against the known insulin concentrations, and sample concentrations were interpolated from this curve.

Determination of relative organ weight

The relative organ weight (ROW) was calculated by determining the final body weight (BW) and respective organ weights (OW) of the rats immediately after harvest as shown in Eq 3.

$$ROW = (OW/BW)100 \dots\dots (3)$$

Statistical analysis

Data obtained from this study were analysed using the Statistical Package for Social Sciences

(SPSS), International Business Machines Corporation (IBM) series 25. The confidence level for the hypothesis test was set at 95 %. Data were descriptively and inferentially analysed and presented as mean $\pm$ standard deviation (SD). The difference between control group and the experimental groups was analysed using a two-way ANOVA, followed by LSD *post hoc* comparison and the values were significant at $p < 0.05$.

RESULTS

Histopathological effects of D'General Bitters on the pancreas

The histology result of the pancreas of the rats in control group (Figure 1a) shows the lobular arrangement of highly cellular glandular tissue composed of acinar cells, ductular structures and Islets of Langerhans. The Islets of Langerhans had peripheral arrangement of α -cells and abundant β -cells in the central area. The acinar cells contained abundant granular eosinophilic cytoplasm in the apical aspect with basally located nuclei. The interlobular ducts were

surrounded by a variably thick rim of dense fibrous tissue (Figure 1a). Also, the histology result of the pancreas of rats in groups B - D showed Islets of Langerhans with peripheral arrangement of α -cells and mildly reduced β -cells in the central area (Figure 1b) and moderately reduced β -cells in the central area in Groups C - D (Figure 1c, d). The acinar cell contained abundant granular eosinophilic cytoplasm in the apical aspect, with basally located nuclei and interlobular ducts were surrounded by a variably thick rim of dense fibrous tissue.

Effects of D'General Bitters on serum amylase, lipase and insulin

The result of the effect of D'General Bitters on serum amylase levels of the rats showed that there was an increase in serum amylase levels of the low and high dose rat groups, whereas there was a decrease in serum amylase levels of the rats administered moderate dose of the herbal drink when compared to control group. These changes were only statistically significant for groups C ($p = 0.022$) and D ($p = 0.004$; Table 1).

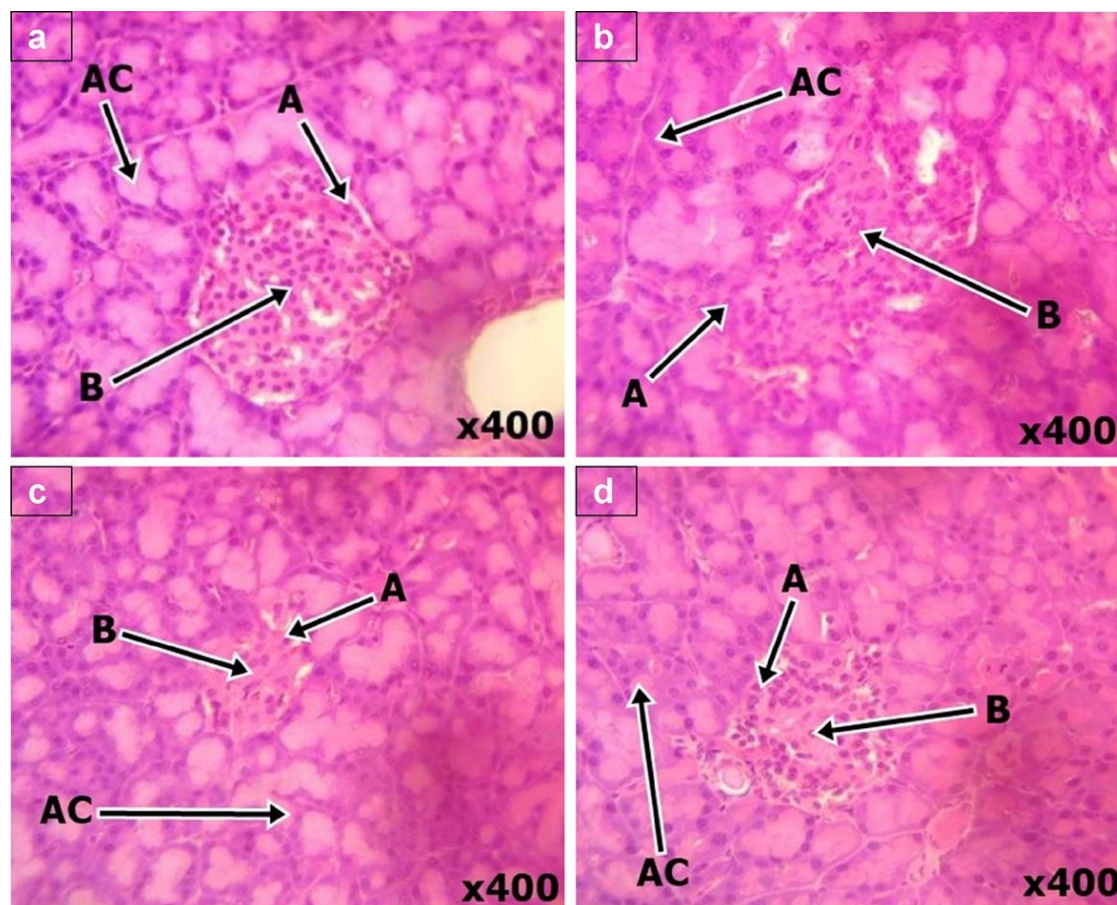


Figure 1: (a – d) Representative photomicrograph of the pancreas of rats in Groups A – E, respectively. AC=Acinar cells, A= α -cell, B= β -cell

Table 1: Effect of D'General Bitters on serum amylase, lipase, and insulin of the rats

Group	Amylase (U/L)	P-value	Lipase (U/L)	P value	Insulin (Miu/L)	P-value
A	220.10±1.42		25.23±2.59		5.45±0.11	
B	225.76±3.26 ^a	0.211	62.25±4.55 [#]	0.001	4.03±0.06 [#]	0.011
C	208.33±9.48 [#]	0.022	57.15±1.98 [#]	0.012	3.62±0.12 [#]	0.001
D	237.07±1.32 [#]	0.004	47.98±1.81 [#]	0.001	3.77±0.13 [#]	0.001
F ratio	16.249		93.063		18.275	

[#]p = significant, ^ap = not significant

Table 2: Effect of D'General Bitters on the final body weight and relative organ weight

Group	Body weight (g)	P-value	Relative organ weight (g)	P-value
A	213.00±17.69		0.0036±0.0001	
B	241.25±18.41 ^a	0.151	0.0035±0.0001 ^a	0.818
C	247.50±36.42 ^a	0.086	0.0032±0.0006 ^a	0.370
D	239.50±15.15 ^a	0.175	0.0039±0.0001 ^a	0.696
F-ratio	1.316		0.646	

[#]p = significant, ^ap = not significant

Effects of D'General Bitters on rat morphology

The result of the final body weight of the rats showed that there was a non-significant difference between control group and experimental groups. The result of the relative organ weight also showed a statistically non-significant difference between control and experimental groups (Table 2). Also, the results show that there was a significant increase ($p < 0.05$) in the serum lipase levels for all the test groups. In addition, at high doses of the herbal drink, serum lipase levels were lower than those of the moderate and low dosage groups. These changes across the test groups were statistically significant when compared to control group (Table 1). Furthermore, a dose-dependent, statistically significant decrease in the serum insulin concentration was seen in all groups compared to control ($p = 0.011$ in Group B), ($p = 0.001$ in Group C) and ($p = 0.001$ in Group D; Table 1).

DISCUSSION

Pancreatic diseases, especially diabetes, have been reported as the leading cause of death in recent times [15]. Studies have identified several factors, amongst which alcoholic herbal drinks have frequently been noted [16]. Attention should be drawn to other possible causes of diabetes and other pancreatic diseases in Africa. This study has drawn attention to the possible pathological effects of this popular polyherbal drink (D'General Bitters) on the pancreas.

Investigations on rat serum showed significant difference between control group and the test groups in the amylase and lipase levels, which are some of the enzymes produced by the acinar cells of the pancreas that play a significant role in

the production and release of insulin and amylin, enzymes that regulate blood glucose levels. This means that any pathology that affects the β -cells of the pancreas could lead to diabetes and/or its associated complications. Similarly, a study has also reported that herbal drinks are one of the primary causes of diabetes in Africa [17]. The rat serum lipase test showed a significant decrease in the test groups when compared to control group, and these differences were statistically significant ($p < 0.05$). This means that even though the pathology on the acinar cells has not manifested structurally, the herbal drink has induced some physiological changes to the pancreas. Reduced lipase levels lead to impaired fat absorption, which could lead to diarrhoea and fatty stools [18]. It has also been reported that disruption of the acinar cells could alter enzyme activities during food digestion and further lead to diarrhoea or may be associated with cystic fibrosis [19].

The result on amylase levels showed a significant difference between control group and the groups administered with medium and high doses of D'General Bitters herbal drink. However, the mean test result of the rats in the low and high dose groups showed an increase in amylase level, whereas the group that received a moderate dose had a decreased amylase level. This result suggests a non-linear (biphasic) dose-response to the herbal drink. This pattern is common with complex herbal mixtures, which may act differently at various doses due to multiple interacting phytochemicals. Elevated amylase levels, as seen in the low and high dose groups, suggest pancreatitis due to pancreatic irritation or enzyme leakage [20]. Conversely, decreased amylase levels in the moderate dose group may point to exocrine pancreatic insufficiency, where enzyme production is impaired [21]. In addition, a previous study

associated herbal and/or energy drinks with pancreatitis, noting that they contain phytochemical constituents like those found in D'General Bitters [22]. These findings suggest that D'General Bitters may disrupt normal pancreatic function in a dose-dependent manner.

The morphological assessment of the rats' pancreas revealed no major differences between control and treatment groups. This suggests that the herbal formulation has not yet induced overt structural alterations in the tissue architecture of the pancreas. Rather, the observed effects appear to be predominantly physiological, indicating the potential onset of early or subclinical pathological changes. This finding supports the hypothesis that the pathology may be at an incipient stage, where functional disruptions precede visible morphological damage.

Limitations of the study

In this study, rats were administered the herbal mixture for a duration of 12 weeks. Additional studies should be conducted to examine the long-term effects of D'General Bitters and similar herbal formulations on other vital organs, using both animal models and human subjects. Such study should also explore the mechanisms underlying the observed pancreatic alterations.

CONCLUSION

Administration of D'General Bitters shows a dose-dependent reduction in pancreatic β -cells within the Islets of Langerhans, with moderate reductions in rats administered moderate and high doses. These changes suggest potential impairment of insulin production. D'General Bitters, when consumed in increasing doses, may induce pancreatic dysfunction by compromising both the endocrine (insulin secretion) and exocrine (digestive enzyme production) roles of the pancreas.

DECLARATIONS

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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. Author ODN conceptualized the study, designed the study, supervised the experiment, wrote the experimental protocol and wrote the first draft of the manuscript. Author AUS carried out the histology analysis, curated the data and carried out the data analysis. Author NAP managed the literature searches, carried out the experiment and managed the animals.

Ethical approval

The ethical approval was obtained from the Ethical Committee of the Faculty of Basic Medical Sciences, College of Health Sciences, Nnamdi Azikiwe University, Nnewi campus. The certification number is NAU/CHS/NC/FMBS/573, on the 4th of August 2023.

Availability of data and materials

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

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