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Original Article

Ameliorative effect of Ruzu herbal bitters on high-fat diet induced non-alcoholic fatty liver disease in Wistar rats

[Efecto mejorador de los amargos de hierbas de Ruzu en la enfermedad del hígado graso no alcohólico inducida por una dieta alta en grasas en ratas Wistar]

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Abstract

Context: One of the world's most widespread and frequent liver diseases is the non-alcoholic fatty liver disease (NAFLD).

Aims: To evaluate the preventives activities of Ruzu herbal bitters (RHB), which is an anti-obesity therapeutic concoction used widely in Nigeria on high–fat diet (HFD) induced NAFLD in albino Wistar rats.

Methods: A total number of twenty-five rats were isolated and divided equally into five groups. Group 1, the normal control group was placed on normal rat diet and normal saline (1 mL/kg body weight daily) for twelve weeks. The remaining four groups 2-5 were placed on HFD for twelve weeks; adding to the following treatment schedules by oral gavage: group 2 received pioglitazone 4 mg/kg daily, group 3 received RHB 0.6 mL/kg daily, group 4 received normal saline 1 mL/kg daily and group 5 received fenofibrate 10 mg/kg daily (s.c). The animals were sacrificed and biochemical markers of liver function, lipid profile, glycemic index, and histopathological assessment of the liver of the rats were determined.

Results: Rat treated with RHB and other treated groups significantly ($p<0.05$) reduced the liver index, fasting blood glucose, and activities and concentrations of liver function enzymes and molecules when compared to untreated NAFLD group. Scoring of hepatic steatosis also showed the ameliorative role of the treatment on NAFLD.

Conclusions: This study reveals that RHB and other treatment options assessed could prevent HFD–induced NAFLD and could be explored as another therapeutic approach to fenofibrate and pioglitazone in NAFLD management.

Keywords: high–fat diet; liver; non-alcoholic fatty liver disease; pioglitazone; Ruzu herbal bitters.

Resumen

Contexto: Una de las enfermedades hepáticas más extendidas y frecuentes del mundo es la enfermedad del hígado graso no alcohólico (NAFLD).

Objetivos: Evaluar las actividades preventivas de los amargos de hierbas Ruzu (RHB), que es un brebaje terapéutico contra la obesidad utilizado ampliamente en Nigeria en la NAFLD inducida por una dieta alta en grasas (HFD) en ratas Wistar albinas.

Métodos: Se aisló un total de veinticinco ratas y se dividió por igual en cinco grupos. Grupo 1, el grupo de control normal recibió una dieta normal para ratas y solución salina normal (1 mL/kg de peso corporal al día) durante doce semanas. Los cuatro grupos restantes 2-5 se colocaron en HFD durante doce semanas; añadiendo a los siguientes programas de tratamiento por sonda oral: el grupo 2 recibió 4 mg/kg de pioglitazona al día, el grupo 3 recibió 0,6 mL/kg de RHB al día, el grupo 4 recibió 1 mL/kg de solución salina normal al día y el grupo 5 recibió 10 mg/kg de fenofibrato al día (s.c.). Los animales fueron sacrificados y se determinaron marcadores bioquímicos de función hepática, perfil lipídico, índice glucémico y evaluación histopatológica del hígado de las ratas.

Resultados: Las ratas tratadas con RHB y otros grupos tratados redujeron significativamente ($p<0,05$) el índice hepático, la glucosa en sangre en ayunas y las actividades y concentraciones de las enzimas y moléculas de la función hepática en comparación con el grupo NAFLD no tratado. La puntuación de la esteatosis hepática también mostró el papel mejorador del tratamiento en NAFLD.

Conclusiones: Este estudio revela que la RHB y otras opciones de tratamiento evaluadas podrían prevenir la NAFLD inducida por HFD y podrían explorarse como otro enfoque terapéutico para el fenofibrato y la pioglitazona en el manejo de la NAFLD.

Palabras Clave: amargos de hierbas de Ruzu; dieta rica en grasas; enfermedad del hígado graso no alcohólico; hígado; pioglitazona.

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INTRODUCTION

The chronic liver disease, non-alcoholic fatty liver disease (NAFLD) is one of the major causes of liver diseases in childhood, adolescence, and adults (Zaitone et al., 2011). NAFLD is a prevalent type of fatty liver malady, developed not as a result of excessive alcohol intake and is associated with insulin resistance and obesity (Pastori et al., 2015). NAFLD is characterized by hepatic steatosis (HS) ≥5%, when there is non-appearance of some other competing liver diseases cause, for example use of medications, hemochromatosis, chronic viral hepatitis, Wilson's disease, autoimmune hepatitis, and substantial alcohol intake (Mishra and Younossi, 2012). The genesis of NAFLD shows as accumulation of massive droplets in the liver cells, which eventually stimulates the induction of type 2 diabetes, obesity, hyperlipidaemia, resistance of insulin and another metabolic syndrome (Birkenfeld and Shulman, 2014). In NAFLD, triacylglycerides accumulates in the liver as HS because of an imbalance between storage and removal of lipids (Taylor, 2008). This eventually progresses into steatohepatitis, then to fibrosis and cirrhosis (Dowman et al., 2010). Patients with NAFLD with simple steatosis rarely transform to other forms of liver diseases, however about 20% of individuals with non-alcoholic steatohepatitis (NASH) advances to cirrhosis and fibrosis over a period of 15 years (Angulo, 2010). The pathogenesis underlying steatosis and its progression to NASH is proposed as a two-hit hypothesis (Day and James, 1998). According to this hypothesis, there is firstly the induction of liver fat accumulation and then insulin resistance, which eventually prompts the progression of steatosis to non-alcoholic steatohepatitis.

Globally, the prevalence of NAFLD has been directly proportional to the prevalence of obesity, diabetes, and metabolic syndrome (MS) (Alisi et al., 2012). It has been estimated that the burden of NAFLD, by the year 2021 will increase to about 60% (Akala and El-Saharty, 2006). It must be noted that the major cause of morbidity and mortality in NAFLD is cardiovascular disorders. It is considered as the most common liver disease in Western societies and affects up to 35% of the population in several countries (Clark, 2006). Notably, 1-5% of patients with simple steatosis can eventually develop actual cirrhosis; and 10-15% of patients with NASH can progress to cirrhosis and eventually to hepatocellular carcinoma (Ascha et al., 2010).

Globally, there are at least 400 million obese adults in low-, middle- and high-income countries (Ogden et al., 2006). Previous studies have estimated that 75% of those with obesity have NAFLD (Clark, 2006). The prevalence of NAFLD in the United States is reported to be between 10% and 30%, with similar rates reported from Europe (23%), South America (31%), Middle East (32%) and Asia (27%). The diagnosis, treatment, and management of NAFLD are a major problem in Africa. However, in Nigeria, NAFLD prevalence is between 9.5–16.7% in individuals with diabetes and 1.2–4.5% in individuals without diabetes (Onyekwere et al., 2011). At present, there is lack of consensus on the management of NAFLD, and consequently no drug is currently indicated for the treatment of NAFLD. However, since NAFLD is a multifactorial disease, approaches that combine reducing visceral adiposity, insulin resistance and hyperinsulinemia among others have been indicated as possible way out. Lifestyle intervention (diet, exercise) represents the mainstay of treatment (Rajwal and McClean, 2017).

Ruzu herbal bitters (RHB) is a poly-herbal mixture prepared in Nigeria and widely used as an anti-obesity concoction. It has unverified claims for the management of obesity, diabetes, hypertension, arthritis and infertility, liver toning capacity, among others. It is an aqueous preparation of *Curculigo pilosa* root (40%), *Uvaria chamae* stem (20%) and *Citrullus colocynthis* bark (40%). The phytochemical analysis of RHB showed that the product is rich in saponins (0.77 mg/mL), alkaloids (0.76 mg/mL), flavonoids (0.73 mM), and cardiac glycosides (0.32 mM), while phenols (0.09 mg/mL), steroids (0.09 mg/mL) and tannins (0.01 mg/mL) are least amongst the phytochemicals analyzed (Obasi et al., 2020). *C. pilosa* has been used tradi-

tionally to treat impotence, arthritis, gastrointestinal and heart diseases (Nie et al., 2013). A thorough examination of the phytochemical screening of *C. pilosa* shows that it possesses antioxidant, neuroprotective and hepatoprotective activity (Nie et al., 2013). *U. chamae* has been reported for treating severe abdominal pains, diarrhea, sickle cell anemia, cough, urinary tract, and cerebral infections as well as possesses hepatoprotective activity (Oluremi et al., 2010). *C. colocynthis* plant is widely recognized for its wide range of medicinal applications and uses, as well as its pharmaceuticals and nutraceutical potentials (Aldhahi and Hamdy, 2003). The saponin extract of *C. colocynthis* fruits at different doses lowers the fasting blood glucose levels in alloxan diabetic rabbits significantly when administered orally (Abdel-Hassan et al., 2000). The antilipidemic, antioxidant and hepatoprotective activity of RHB has been attributed to the presence of *U. Chamae* and *C. Colocynthis* present in it (Ogunlana et al., 2018). Despite these studies, there is a paucity of information on the effect of RHB in the treatment/prevention of NAFLD. This study is therefore aimed at evaluating the protective activities of RHB in an experimental NAFLD animal model induced by high fat diet.

MATERIAL AND METHODS

Chemicals and reagents

Ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), thiobarbituric acid (TBA), pyrogallol, trichloro acetic acid (TCA), sodium hydroxide, hydrochloric acid were purchased from Sigma-Aldrich. Ruzu herbal bitters (RHB) was obtained from A2W Global Ltd., Lagos, with National Agency for Food and Drug Administration and Control (NAFDAC) Registration Number A7-1102L. Pioglitazone hydrochloride (>99% purity) was obtained from Tokyo Chemical Industry, Development Co. Ltd., Shanghai. Fenofibrate and cholesterol were also obtained from Fisher Scientific. All other chemicals and reagents used in the study were of analytical grade.

Experimental animals

Twenty-five Male albino rats of Wistar strain weighing between 130-170 g were housed in a neat and well-ventilated propylene cages and maintained under standardized laboratory conditions (12 h light/dark cycle, 24°C) and provided free access to normal and high-fat diet and drinking water *ad libitum*. Animals were procured from Lagos State University Teaching Hospital Idi araba, Lagos State, Nigeria. They were allowed to acclimatize for two weeks before commencement of experiment. All experiments and protocols described in the present study were approved by the Institutional Animal Ethics Committee, an arm of the Covenant Health Research Ethics Committee of the University with approval number (CU/HREC/ATS06/19. The experimental procedures and animals care were performed in accordance with the "Guide for the care and use of laboratory animals" and "Committee for the purpose of control and supervision on experimental animals" (CPCSEA) in order to minimize pain and discomfort.

Experimental diet

The diets used in this study were of two types, the normal rat chow and high-fat diet (HFD), which were compounded and manufactured from Graceline Feed Ltd., Ota, Ogun State. The formulations were as described in Ogunlana et al. (2020).

Experimental design

Animal model

The experiment was carried out using twenty-five albino male Wistar rats that were housed at constant temperature with exposure to 12 h of dark/light condition and free access to food and water *ad libitum*. Animals were allowed to acclimatize for two weeks before the commencement of the experiment. The animals were weighed before the commencement of treatment and weekly throughout the duration of study. Thereafter, the animals were randomly grouped into five of five animals in each group. Table 1 shows experimental design and treatments of animals.

Table 1. Experimental design and treatment of animals.

Group	Name	Treatment of animals
1	Normal control (Normal)	Fed standard rat diet and given normal saline (1 mL/kg body weight (BW)/day) by gastric intubation for twelve weeks
2	Pioglitazone (PIO)	Fed high fat diet and given pioglitazone (4 mg/kg BW/day) by gastric intubation for twelve weeks
3	Ruzu herbal bitters (Ruzu)	Fed high fat diet and given Ruzu (0.6 mL/kg BW/day); an equivalent of the prescribed adult human dosage) by gastric intubation for twelve weeks
4	Non-alcoholic fatty liver disease (NAFLD)	Fed high fat diet and given normal saline (1 mL/kg BW/day) by gastric intubation for twelve weeks
5	Fenofibrate (FENO)	Fed high fat diet and given fenofibrate (10 mg/kg BW/day) by sub-cutaneous injection for twelve weeks

The physical appearance and daily activities of the rats, such as eating patterns and signs of abnormalities, were observed and recorded. Body weight of the animals were measured weekly throughout the duration of the experiment. At the end of the twelve weeks, fasting blood glucose evaluation was carried out using standard diagnostic kit (Accu-Check Diagnostics, England), thereafter, the animals were sacrificed using mild anesthesia (sodium pentobarbital 50 mg/kg (2.5 mL/kg, i.p.) and blood collected from the heart using heparinized syringes.

Sample preparation

Plasma was obtained from whole blood collected in heparinized tube by centrifugation (Thermo Scientific Legend Micro 21 centrifuge, Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A) at 3000 rpm for 10 min. Liver was excised, rinsing with normal saline and homogenized in ice-cold homogenization buffer (0.25 M sucrose, 10 mM Tris-HCL, 1 mM EDTA and 10 mM Hepes-NaOH at pH 7.4) using a Teflon pestle homogenizer (Thomas Pestle tissue homogenizer, Thomas Scientific, Swedesboro, New Jersey, U.S.A). The homogenate was centrifuged at 12 000 g for 10 min in 4°C temperature. The supernatant was collected and frozen at 20°C for enzymatic assays. A portion of liver was stored in 10% neutral buffered formalin for histology.

Determination of liver function parameters

Liver function biomarkers including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), triglycerides, total bilirubin, direct bilirubin, indirect bilirubin, total protein, albumin, and cholesterol were carried out according to manufacturer's instructions (Randox Laboratories, UK). Readings were taken by spectroscopic methods of analysis using UV/Visible spectrophotometer (Thermo Fisher Scientific, Genesys, USA).

Histological examination

Neutral buffered formalin (10%) fixed liver tissues were embedded in paraffin wax, sectioned, stained using hematoxylin and eosin (H&E), viewed under the microscope, and analyzed by a pathologist according to the method as described by Ogunlana et al. (2013). Briefly, fixed liver tissues were dehydrated and cleared in methanol and xylene, respectively. Wax infiltration and embedding were carried out in rotary microtome. Thereafter, tissues sections (5 µm) were made, mounted on glass slides, and stained with stained with H&E. The slides were analyzed under the light microscope (Olympus BX63 with a DP72 camera, Olympus Corporation, Tokyo, Japan) at 400× and sections were observed for vascular congestion and inflammation and scored.

Statistical analysis

Data were analyzed using statistical package for social science (SPSS version 23). The analyzed data was documented as mean ± SEM of five replicates in each group. Analysis of Variance (ANOVA) will be carried out to test for the level of homogeneity at $p<0.05$ among the groups. Duncan's Multiple Range Test (MRT) will be used to separate the heterogeneous group.

RESULTS

Liver index

Fig. 1D shows the liver index of rats in all the groups. There was significant ($p<0.05$) increase in the relative liver weight of all the groups in comparison with normal control.

Fasting blood glucose

Fig. 1A shows the fasting blood glucose of the experimental on the last day of the experiment. NAFLD animals had significantly ($p<0.05$) increased fasting blood glucose levels in comparison to normal control, pioglitazone (PIO), Ruzu herbal bitters (Ruzu), and fenofibrate (FENO). However, the significant decrease in the blood glucose levels of rat treated with Ruzu was not comparable to animals treated with pioglitazone and fenofibrate.

Biochemical markers of liver function biomarkers in the blood

The concentrations of albumin (ALB) (Fig 1K), total cholesterol (TC) (Fig. 1E) and triglycerides (TRIG) (Fig. 1F), total bilirubin (Fig. 1H), direct and indirect bilirubin (Fig. 1I-J) were significantly reduced in treatment groups in comparison to negative control, NAFLD group. In addition, the activities of aspartate aminotransferase (AST) (Fig. 1B), alanine aminotransferase (ALT) (Fig. 1C) and alkaline phosphatase (ALP) (Fig. 1G) were significantly ($p<0.05$) reduced in normal control and other treatment groups when compared to the NAFLD group (Fig. 1).

Histological architecture and scoring of hepatic steatosis

Fig. 2 illustrates the histological architecture and grading of the liver steatosis of the liver of the rat. Score of hepatic steatosis was used to assess the level of liver damage or its improvement. Level of hepatic steatosis was scored from 0, 1 to 5, reflecting no steatosis (0), mild steatosis (1), mild to moderate (2), moderate (3), moderate to severe (4) and severe steatosis and cellular infiltration (5). Normal control group and rats treated with fenofibrate had no visible steatosis and scored 0, while rats treated with pioglitazone and Ruzu had mild to moderate and moderate level of hepatic steatosis, respectively. However, the NAFLD rats has severely diffused hepatic steatosis characteristics of the disease state.

DISCUSSION

The general unverified use of non-alcoholic Ruzu herbal bitters in Nigeria, West Africa and elsewhere for a number of health-related issues, which involves detoxification of the liver among other reasons, has motivated pharmacological studies of the decoction. HFD induction of NAFLD has been used to study the ameliorative roles of medicinal plants and plant phytochemicals in laboratory animals. High fat diet (HFD) feeding in rats for twelve weeks in the present study induced severe steatosis, portal inflammation, elevated glycaemic index, blood lipids and liver function enzymes, which are characteristics of NAFLD. The typical histopathological non-alcoholic steatohepatitis lesions observed in this study is consistent with the study of Zaitone et al. (2011).

A significant liver index, as observed in the NAFLD group demonstrates a substantial weight gain as reported previously (Elshazly, 2015; Ogunlana et al., 2018; 2020). Weight gain in experimental animal may result from lipid bioaccumulation in the hepatocytes and blood circulation. The marked increase in fasting blood sugar (FBS) observed in the NAFLD group may predispose the

test animals to prediabetes state. The two major risk factors of NAFLD are dyslipidemia and insulin resistance (Fernandez–Miranda et al., 2008).

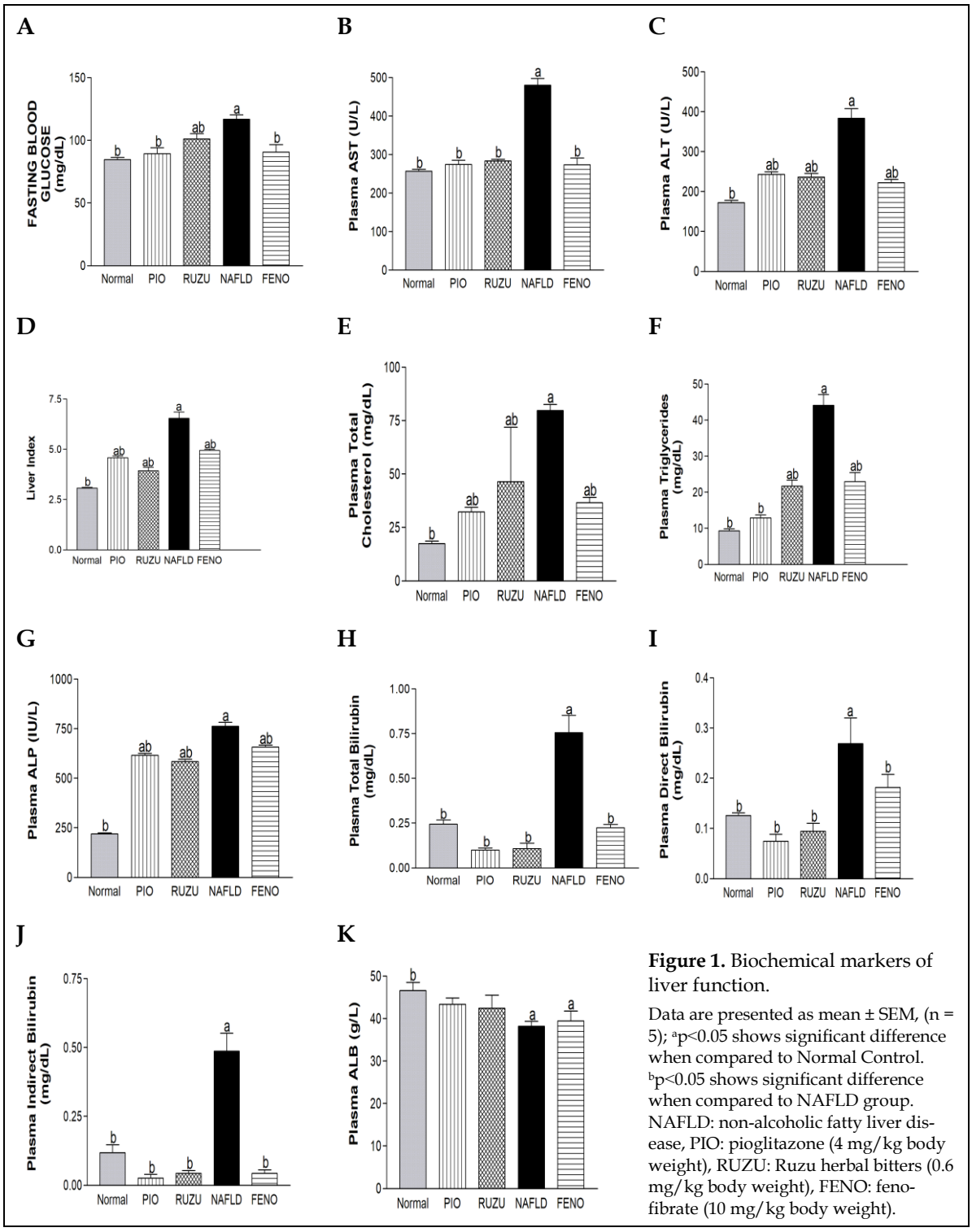

Figure 1. Biochemical markers of liver function.

Data are presented as mean ± SEM, (n = 5); [a]p<0.05 shows significant difference when compared to Normal Control. [b]p<0.05 shows significant difference when compared to NAFLD group. NAFLD: non-alcoholic fatty liver disease, PIO: pioglitazone (4 mg/kg body weight), RUZU: Ruzu herbal bitters (0.6 mg/kg body weight), FENO: fenofibrate (10 mg/kg body weight).

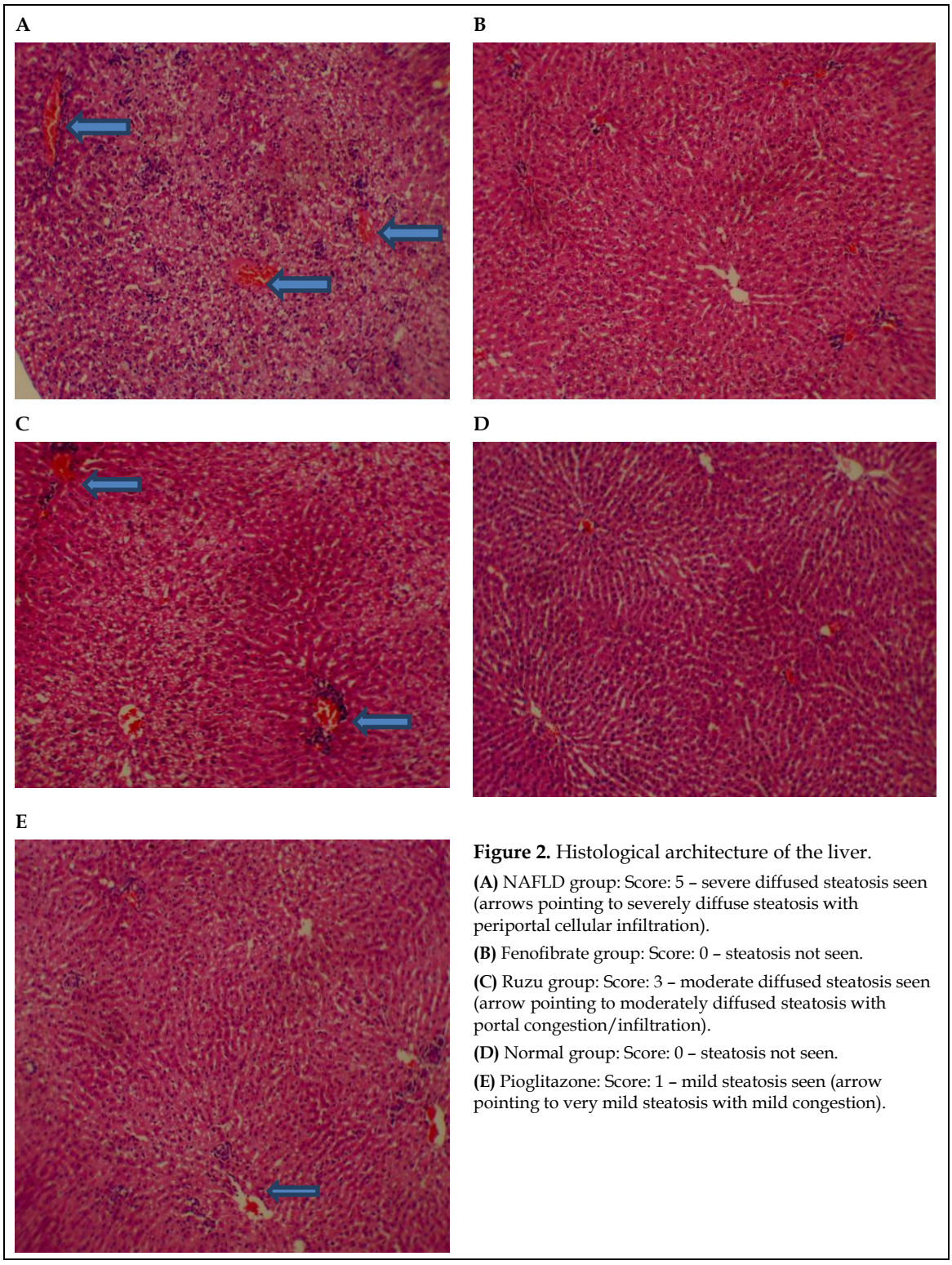

Figure 2. Histological architecture of the liver.

(A) NAFLD group: Score: 5 – severe diffused steatosis seen (arrows pointing to severely diffuse steatosis with periportal cellular infiltration).

(B) Fenofibrate group: Score: 0 – steatosis not seen.

(C) Ruzu group: Score: 3 – moderate diffused steatosis seen (arrow pointing to moderately diffused steatosis with portal congestion/infiltration).

(D) Normal group: Score: 0 – steatosis not seen.

(E) Pioglitazone: Score: 1 – mild steatosis seen (arrow pointing to very mild steatosis with mild congestion).

The increase in FBS is ameliorated by treatment with pioglitazone, Ruzu and fenofibrate. The insulin enhancing activity of Ruzu, pioglitazone and fenofibrate in reducing FBS and consequently causing a reduction in plasma insulin has been reported previously. Ruzu and others may play a

protective role of the endocrine system by improving insulin sensitivity of the tissues to glucose uptake and thereby, improving lipid and glucose metabolism (Fernandez–Miranda et al., 2008; Kale et al., 2018; Ogunlana et al. 2018).

The increase in the levels of plasma conjugated and unconjugated bilirubin as a result of diet induced NAFLD was significantly reduced in all the treatment groups. Unconjugated hyperbilirubinemia can result from increased production, impaired conjugation, or impaired hepatic uptake of bilirubin, a bile pigment produced from the degradation of hemoglobin. Hepatocellular disease such as NAFLD can cause a mixture of plasma unconjugated and conjugated hyperbilirubinemia due to both impaired bilirubin conjugation and canalicular excretion. Defects in conjugated bilirubin excretion may cause isolated conjugated hyperbilirubinemia without cholestasis (Harb and Thomas, 2007). The hepatocellular disease characterized by conjugated and unconjugated hyperbilirunemia was reversed by hepatoprotective activity of Ruzu, pioglitazone and fenofibrate. This report was in agreement with previous report (Ogunlana et al., 2018).

In addition, the significant elevation in the total plasma cholesterol and triglycerides concentrations observed in NAFLD group were reversed in treatment groups. This observation was in consonance with the findings of other researchers (Gomathy et al., 1989; Šeböková et al., 2002; Xu et al., 2006; Yalniz et al., 2007; Guo et al., 2012; Ogunlana et al., 2018). *C. colocynthis,* a major constituent in Ruzu, induced a dose dependent suppression of the intracellular triglyceride accumulation during adipogenesis. The downregulation and inhibition of main transcription factors of adipogenesis, which include CCAAT/enhancer binding protein α (C/EBPα), peroxisome proliferator activated receptor γ (PPARγ), and sterol regulatory element-binding protein 1c (SREBP-1c) were reported (Jemai et al., 2020). Equally, the expression of PPARα, a transcription factor for lipid catabolism and its associated genes, which include CPT1A, CPT2 and CYP4b10 were decreased in NAFLD (Wahlang et al., 2014). Hypolipidemia activity may result from the decrease in the absorption of intestinal cholesterol by plant phytochemicals (Chan and Tang, 1995). Hence, Ruzu may play a significant antilipidemic role in the treatment of obesity owing to its powerful effects on fat, by the activation of lipid oxidation genes, inactivation of adipogenesis genes and/or by reducing the intestinal absorption of cholesterol. Hence, promoting hepatic lipid catabolism and protective roles against steatosis.

The reversal in the marked elevation observed in the activities of AST and ALT in NAFLD groups were demonstrated in all the treatment groups. This is in agreement with previous reported works (Pan et al., 2006; Yalniz et al., 2007; Fernandez–Miranda et al., 2008; Hamed et al., 2017; Ogunlana et al., 2018). The liver function parameters are biomarkers for the assessment of the functionality and integrity of the hepatocytes. The hepatoprotective activities of Ruzu may be due to the presence of plant phytochemicals in its constituents. The hepatoprotective activity of *C. colocynthis* has been documented in animals (Arjaibi et al., 2017; Sari et al., 2019). This activity has been linked to the presence of cucurbitacins, a major phytochemical and others in *C. colocynthis.* Antioxidant, free radical scavenging, and anti-inflammatory activities of the phytoconstituents in *C. colocynthis* and Ruzu have been reported (Tannin-Spitz et al., 2007; Arjaibi et al., 2017; Ogunlana et al., 2020). In the current study, the histopathological findings of the hepatocytes further revealed the protective and hypolipidemic effects of Ruzu and other treatment options.

CONCLUSIONS

The results of this study demonstrate that Ruzu herbal bitters possesses hepatoprotective, hypoglycemic and hypolipidemic activities, hence, was able to comparatively with standard drugs ameliorates HFD induced NAFLD in Wistar rats. Thus, Ruzu herbal bitters may be considered as an alternative therapy to pioglitazone and fenofibrate in the clinical management of NAFLD.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest with respect to the research, authorship, or publication of this article.

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Contribution	Ogunlana OO	Adetuyi BO	Adekunbi TS	Adegboye BE	Iheagwam FN	Ogunlana OE
Concepts or ideas	x					x
Design	x					x
Definition of intellectual content	x					
Literature search		x	x			
Experimental studies			x	x	x	
Data acquisition			x	x	x	
Data analysis	x	x	x		x	
Statistical analysis	x	x	x		x	
Manuscript preparation	x	x	x			
Manuscript editing	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x

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