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Ocimum Gratissimum Linn Causes Dose Dependent Hepatotoxicity in Streptozotocin-Induced Diabetic Wistar Rats

Adejoke Y Onaolapo[1], Olakunle J Onaolapo[2]

[1]Department of Human Anatomy, Ladoke Akintola University of Technology, Ogbomoso, Oyo state, Nigeria; [2]Department of Pharmacology and Therapeutics, Ladoke Akintola University of Technology, Ogbomoso, Oyo state, Nigeria

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Key words: Ocimum gratissimum; Streptozotocin; Diabetes Mellitus; Hepatotoxicity.

Correspondence: Adejoke Y. Onaolapo. Department of Human Anatomy, Ladoke Akintola University of Technology, Ogbomoso, Oyo state, Nigeria. Phone: 2348062240434 E-mail: olakunleonaolapo@yahoo.co.uk, adegbayibiy@yahoo.com

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Abstract

Aim: Hepatoprotective effects of Ethanolic extract *Ocimum gratissimum* leaves (*O. gratissimum*) in streptozotocin-induced diabetic rats was studied.

Material and Methods: Thirty-six adult rats were assigned into six groups (A, B, C, D, E and F) of six rats each. Group A was the control, while diabetes was induced in groups B, C, D, E and F. Fasting blood glucose was determined and rats with blood glucose concentration >18.0 mmol/L were considered diabetic. Group A (non diabetic control) and F (diabetic control) received normal saline orally, group B received a Metformin at 25 mg/kg while groups C, D and E had *O. gratissimum* at 400, 600 and 800 mg/kg. Treatment was daily for a period of 28 days for all groups. Fasting blood glucose was monitored weekly, serum levels of Alanine transaminase (ALT) and Aspartate transaminase (AST) were measured on day twenty eight. Animals were sacrificed, liver sections processed for histological study. Statistical analysis was carried out using a one way ANOVA followed by a post-hoc test.

Results: Results showed reduction in blood sugar and increase in biochemical and histologic markers of hepatic injury.

Conclusion: Ethanolic extract of *O. gratissimum* causes dose dependent hepatic injury despite its glucose lowering ability.

Introduction

Diabetes mellitus is a chronic metabolic disorder with numerous complications. It is characterized by chronic high blood glucose levels leading to increased morbidity and mortality. Diabetes if not controlled results in structural and functional changes in various target tissues and organs [1]. The worldwide prevalence of DM has risen dramatically over the past two decades, from an estimated 30 million cases in 1985 to 177 million in 2000 [1]. Based on current trends, it is projected that ~435 million individuals will have diabetes by the year 2030 [2].

In modern medicine, the beneficial effects of standard medications on glycemic levels are well documented, however, the preventive activity of medications against the progressive nature of diabetes and its complications was modest but not always effective [3]. Insulin therapy affords glycemic control in type 1 diabetes, yet its shortcomings such as, short shelf life, the requirement of constant refrigeration, fatal

hypoglycemia in event of excess dosage, reluctance of patients to take insulin injection and above all resistance due to prolonged administration limits its usage [4].

Similarly, treatment of type 2 diabetes patients with sulfonylureas and biguanides is almost always associated with side effects [5]. Hence, the search for drugs with low cost, more potential and with fewer side effects is being pursued in several laboratories around the world.

Hepatobiliary disorders, such as inflammation, necrosis or fibrosis of non-alcoholic fatty liver disease, cirrhosis, hepatocellular carcinoma, hepatitis C, acute liver failure, and cholelithiasis are complications of diabetes [6, 7].Therefore combating diabetes mellitus goes far beyond mere glycaemic control.

Streptozotocin is frequently used to induce experimental type 1 diabetes [8]. The cytotoxic action of STZ is mediated by free radicals and STZ has toxic and carcinogenic effects on the pancreas, liver and kidneys [9].

Ocimum gratissimum is an herbaceous plant belonging to the Labiatae family [10]. The plant is indigenous to tropical areas especially India and it is also found in West Africa, it is widespread throughout tropical countries including Brazil, where it is popularly known as "alfavacão, alfavaca and alfavaca-cravo" [11]. In Nigeria, it is found in the savannah and coastal areas. It is known by various names in different parts of the country, in the southern part of Nigeria, the plant is called "effinrin-nla" by the Yoruba speaking tribe. It is called "Ahuji" by the Igbos in the eastern part, while in the Northern part of Nigeria; the Hausas call it "Daidoya" [10]. *O. gratissimum* has been used extensively in the traditional system of medicine in many countries. In the North east of Brazil, it is used for medicinal, condiment and culinary purpose. The flowers and the leaves of this plant are rich in essential oils so it is used in preparation of teas and infusion. In the coastal areas of Nigeria, the plant is used in the treatment of epilepsy, high fever and diarrhea [12]. In the Savannah areas decoctions of the leaves are used to treat mental illness while *O. gratissimum* is used by the Ibos of Southeastern Nigeria in the management of the baby's cord, to keep the wound surfaces sterile, it is also used in the treatment of fungal infections, fever, cold and catarrh [13].

Extensive research has been done on the various potential scientific uses of O. gratissimum, a plant that also has extensive therapeutic use in traditional medicines in South America and Africa, which include its use in treating bacterial infections, diarrhoea [12], respiratory-tract infections, pneumonia, fever and coughs [14], anti-diarrheal effects in experimental animals [15, 16], high antiviral indices against HIV-1 and HIV-2 [17].The essential oil of this species also presented interesting activities such as insecticidal [18], antibacterial [19-21], antifungal [22], and as a relaxant on isolated ileum from guinea pig [23]. Hypoglycemic and antidiabetic activity in rats has also been studied; Egesie and colleagues concluded that the plant was an effective antidiabetic agent [24]. There have been reports on the hypoglycemic potential of extracts of *O. gratissimum* as well as its effects on liver morphology, however there is very little information on the effects of this herb on the liver in the diabetic state; our intention in this study was to evaluate this.

Materials and Methods

Plant Material

Fresh leaves of *Ocimum gratissimum* Linn were collected from Ogbomoso, Oyo state, Nigeria in August 2010. The plant was identified by Dr Ogunkunle of the Department of Biology, Ladoke Akintola University of Technology, Ogbomoso and a voucher specimen was deposited in the herbarium of the department (LAU2396).

Chemicals And Drugs

Normal saline, 5% ethanol, Streptozotocin (STZ (Sigma St. Louis, USA)), 0.1M citrate buffer pH 4.5, Metformin (Bristol-Myers Squibb.). All chemicals and drugs used were of analytical grade.

Preliminary Phytochemical Screening of Plant Fraction

Preliminary screening of the extract was performed for the presence of secondary metabolites, using the following reagents and chemicals: alkaloids with Mayer's and Dragendorff's reagents [25, 26] ; flavonoids with the use of Mg and HCl [27]; tannins with 1% gelatin and 10% NaCl solutions and saponins with ability to produce suds.

Preparation of Extract of Ocimum gratissimum

Ocimum gratissimum leaves were first separated from the stalk, rinsed with water to remove dirt; air dried at room temperature and ground to fine powder using an electric blender (Christy and Norris - 47362.England) at

the Department of Pharmacognosy of the Obafemi Awolowo University, Ile – Ife. Extraction was performed by adding 800mg of ground powdered in 5 liters of ethanol in a sterile flask, mixture was swirled to ensure effective mixing and a stopper used to avoid loss of volatile liquid at ambient temperature (28 ± 2°C). The mixture was extracted by agitation on a rotary shaker. After 48 hrs, the mixture was decanted. The filtrate was then poured into stainless trays and the extract was allowed to evaporate to dryness at room temperature (28 ± 2°C) for 2-3 days by using a vacuum evaporator (RE 100B Bibby Sterilin, United Kingdom). The dry residue was stored until ready to use. The percentage yield of extract was 10.33%w/w.

Animals

Thirty-six healthy adult male Wistar rats purchased from the Empire Animal Farms in Osogbo, Osun State, Nigeria were used with weight in the range of 180 to 200 g. The animals were randomly allocated into six groups. The animals were housed in metallic cages measuring 64" x 36" x 32" (6 rats in each cage). All animals had free access to food and water *ad libitum*. They were maintained under standard laboratory conditions i.e. a well aerated room with alternating light and dark cycles of 12 hours each and at room temperature of 25°C. The experimental protocol was approved by the Institutional Animal Ethics Committee of the Ladoke Akintola University of Technology, Ogbomoso. All rules applying to animal safety and care were observed.

Acute Toxicity Test

The 50% lethal dose determination for each of the fractions was conducted separately using modified method of Lorke [28]. Details as previously published [29].

Induction of Diabetes Mellitus

Diabetes mellitus was experimentally induced in 36 rats by a single intraperitoneal injection of [30] of Streptozotocin (STZ) (Sigma St. Louis, USA) dissolved in 0.1M sodium citrate buffer pH 4.5 [31]. An equivalent volume of citrate buffer instead of Streptozotocin was administered to a group of six animals which served as control. Seventy-two hours post induction of diabetes, blood glucose was determined from the tail vein after an overnight fast. Rats with blood glucose levels of > 18 mmol/L were considered diabetic and used.

Experimental Method

The animals were assigned into six groups A, B, C, D, E and F of six rats each. Group A, the non diabetic control received normal saline orally. Animals in group F served as the diabetic control and were also administered normal saline orally. Animals in group B received Metformin daily at a dose of 25 mg/kg body weight [32]. Groups C, D and E received daily oral doses of ethanolic extracts of *Ocimum gratissimum* leaves at 400, 600 and 800 mg/kg. All treatments were administered for a period of 28 days. Samples for blood glucose estimation were determined weekly using the glucose oxidase method [33]. Alanine and Aspartate transaminase (ALT, AST) levels were measured on the 28th day.

Furthermore, sections of the right lobe of the liver were obtained, processed, sectioned and stained with Hematoxylin & Eosin (H&E), Periodic Acid-Schiff (PAS) and Periodic Acid Schiff-diastase for histological and histochemical studies respectively. An Olympus BX50 digital light microscope was used to examine the slides and acquire photomicrographs.

All the data for all biochemical parameters were analyzed by analysis of variance (ANOVA), and post-hoc tests (Student Newman Keuls) were used to determine the source of a significant effect. Results are expressed as Mean ± S.E.M., $p<0.05$ is taken as accepted level of significant difference from control.

Collection and Analysis of Blood Samples for Liver Biochemistry

Blood was collected from each diabetic and non diabetic rat on the 29th day by intracardiac puncture; rats were fasted for 8 hours before samples were collected. Samples were collected into lithium heparinised bottles, blood was allowed to clot and serum separated by centrifugation at 3500 rpm for 10 minutes using a hematocrit centrifuge (JICA, Japan). The serum was assayed either immediately or stored at –20 ^{o}C. Alanine and Aspartate transaminase (ALT, AST) levels were determined by the spectophotomeric method described by Berg Meyer and Bernt, 1974 [34].

Results

Preliminary phytochemical screening

Freshly prepared extracts subjected to preliminary phytochemical screening test revealed the presence of carbohydrates, reducing sugars, lipids,

flavonoids, alkaloids, steroids, tannins, cardiac glycosides and resin.

Physical Examination of The Animals

Animals were observed for changes in their physical characteristics all through the experimental period. Animals in group A (non diabetic control) showed no deterioration in their grooming, eating or drinking behaviours, they also showed no change in locomotion, sleep patterns or social behaviours' whereas animals in groups B, C, D, E and F with experimentally induced diabetes showed a deterioration in grooming, eating and water drinking behavior within 24-48 hours after administration of streptozotocin, this was followed by an increase in appetite, increased water consumption and an increase in urine production as evidenced by soiling of the animal coat. These changes were noticed to have abated within 48 hours of commencement of Metformin in group B animals and Ocimum *gratissimum* extract at all doses. Animals in group F (diabetic control) showed continued deterioration in physical appearance all through the experimental period.

Gross examination of liver showed yellowish-brown discoloration in livers taken from animals in groups C, D and E while livers from animals in groups A and B showed normal liver coloration.

Effects of Ocimum Gratissimum on Blood Glucose

The mean blood glucose levels monitored weekly for a period of 28 days in all experimental groups are presented in Fig 1. There was a statistically significant ($F(5, 35) = 42.23$, $p<0.05$) reduction in blood glucose in the *O. gratissimum* experimental groups compared to the diabetic control. A steep descent is seen at higher doses. When comparing initial blood glucose levels (day 0) to the blood glucose level observed on day 28, 17.2%, 17.06%, 15.87% reduction in blood glucose levels is seen in groups C, D and E respectively. Metformin administration also resulted in a significant drop in the blood glucose level its administration resulted in a 28% reduction in blood glucose between days 0 and 28.

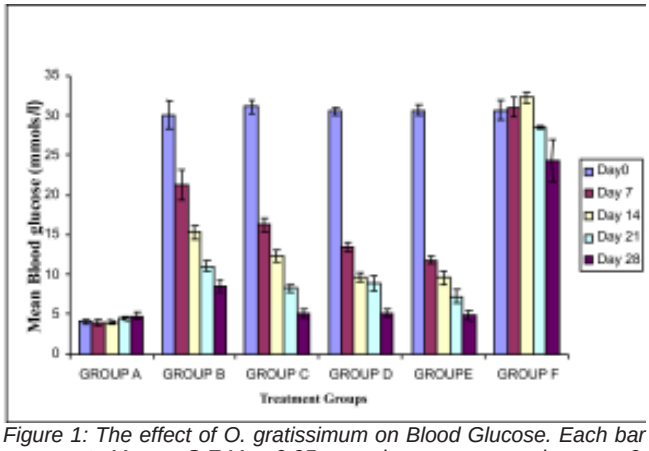

Figure 1: The effect of O. gratissimum on Blood Glucose. Each bar represents Mean ± S.E.M p<0.05 group by group comparison, n = 6. Groups A- non diabetic control, B- Metformin , C- 400 mg, D- 600 mg, E-800 mg, F Diabetic control.

Effects of Ocimum Gratissimum *on Liver Transaminases (Ast, Alt)*

Figure 2 shows the mean Aspartate Transaminase (AST) and Alanine Transaminase (ALT) values in all experimental groups. There was a statistically significant rise in serum AST and ALT in the diabetic control group compared to the non diabetic control animals. The diabetic state resulted in a fivefold rise in the liver transaminases. The ethanolic extract of *O.gratissimum* was noticed to have resulted in a significant ($F(5, 35) = 48.14$, $p<0.05$), ($F(5, 35) = 10.03$, $p<0.05$) (AST and ALT respectively) four to fivefold rise in liver transaminase values in groups D and E compared to the non diabetic control. Metformin caused a mild reduction in liver transaminases compared to the non diabetic control although this was not statistically significant.

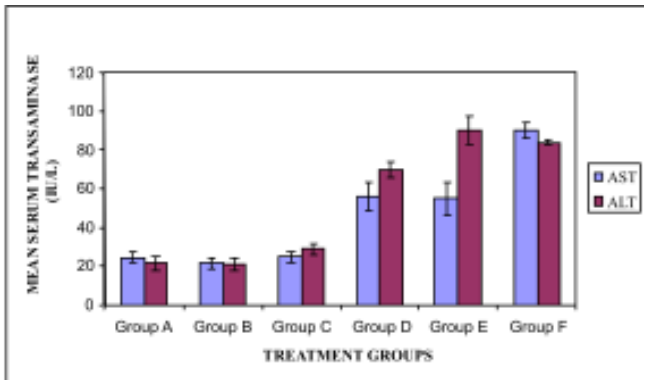

Figure 2: The effects of O.gratissimum on Liver Transaminase Levels. Each bar represents, Mean ± S.E.M, p<0.05 group by group comparison, n = 6. Groups A- non diabetic control, B- Metformin , C- 400 mg, D- 600 mg, E-800 mg, F Diabetic control.

The Effect of Ocimum Gratissimum *on Relative Weight of the Liver*

Table 1 show the mean relative weights of the liver in all experimental groups. There was a statistically significant dose dependent increase in liver weight in rats in groups C, D and E that received ethanolic extract

of *O. gratissimum* compared to the non-diabetic control animals (24.7 ± 0.30, 28.69 ± 0.13, and 30.08 ± 0.11 vs. 23.04 ± 1.20). Animals in group B (Metformin + diabetes) showed an increase in liver weight which was however not statistically significant (24.7 ± 1.12 vs. 23.04 ± 1.20).

Table 1: Showing mean ± S.E.M. of weights of animals and weights of liver at time of sacrifice. Groups A- non diabetic control, B- Metformin, C- 400 mg, D- 600 mg, E-800 mg, F Diabetic control.

Groups	Mean weight at sacrifice	Mean Liver weight	Relative liver weight gm/kg bodyweight
A	210 ± 0.62	4.84 ± 0.52	23.04 ± 1.20
B	134 ± 2.22	3.32 ± 0.48	24.78 ± 1.12
C	122 ± 1.23	3.50 ± 0.33	28.69 ± 0.32
D	118.3 ± 0.62	3.55 ± 0.28	30.08 ± 0.13
E	115.8 ± 0.72	3.65 ± 0.34	31.73 ± 0.11
F	131.8 ± 2.70	3.49 ± 0.28	26.50 ± 0.11

Result of Liver Histopathology

Examination showed grossly normal livers in groups A and B. Livers taken from animals in groups C, D and E were enlarged, they also had yellowish brown discoloration on the external surface otherwise structure was grossly normal. Liver from group F animals were enlarged.

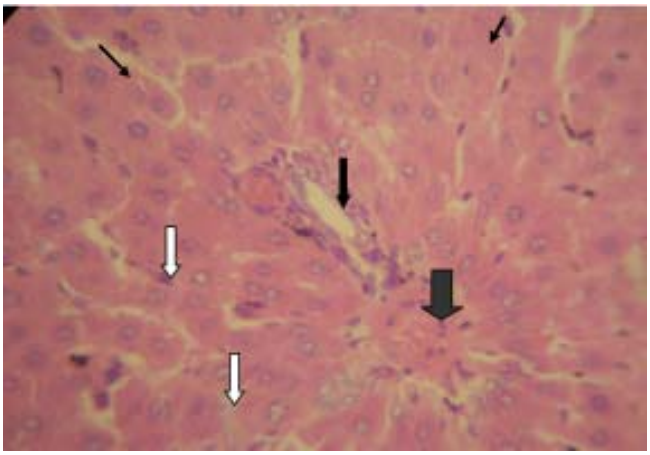

Figure 3: Group A (non diabetic) showing normal liver architecture: hepatocyte with prominent nuclei (white arrow), sinusoids (very thin arrows), portal vein (thin black arrow) and hepatic artery (thick black arrow).X400 (H& E).

Slides made from sections of liver from group A (Fig. 3) animals revealed normal liver architecture with radially arranged cords of hepatocytes around a terminal hepatic venule. There were intervening sinusoidal spaces between each cord and plate of hepatocytes; normal central veins were also seen. These are in keeping with normal hepatic histology. Liver slides from groups B

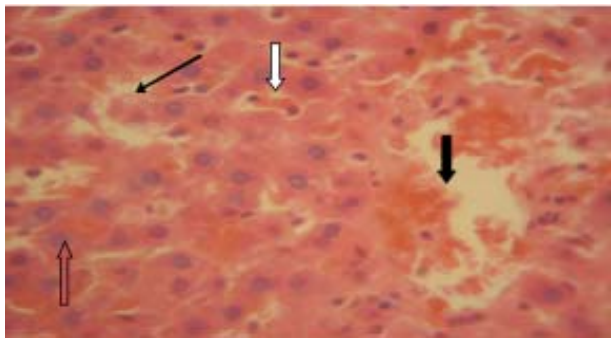

Figure 4: Group B (Metformin)m showing mild loss of normal liver architecture: hepatocytes with pale nuclei (brown arrow) normal hepatocytes with deeply staining nuclei (white arrow), ruptured central vein (black arrow) and dilated sinusoids (thin arrows). X400 (H& E).

(Fig. 4) animals showed mild loss of normal liver architecture with numerous radially arranged cords of hepatocytes with normal nuclei, a few hepatocytes with

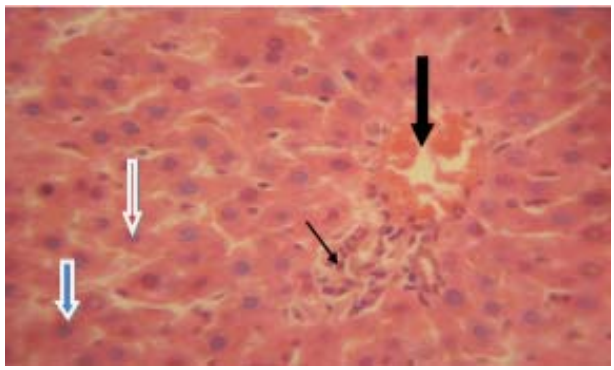

Figure 5: Group C (400 mg/kg) showing mild loss of normal liver architecture: numerous swollen hepatocytes with pale nuclei (brown arrow), some hepatocytes with pyknotic nuclei (blue arrow), normal central vein (black arrow) and mononuclear cell infiltration (thin arrow). X400 (H& E).

either pale or deeply staining nuclei. There was also mild central vein congestion this features are in keeping with probable recovery from STZ insult.

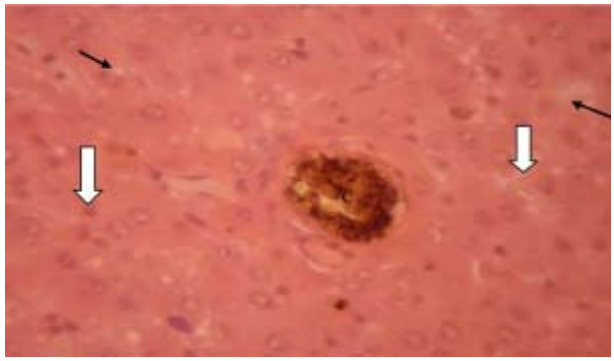

Figure 6: Group D (600 mg/kg) showing severe loss of normal liver architecture: numerous swollen hepatocytes with pale nuclei (white arrows), dilated central vein filled with blood and cellular debris (C) and vacuolization all over (thin arrow). X400 (H& E).

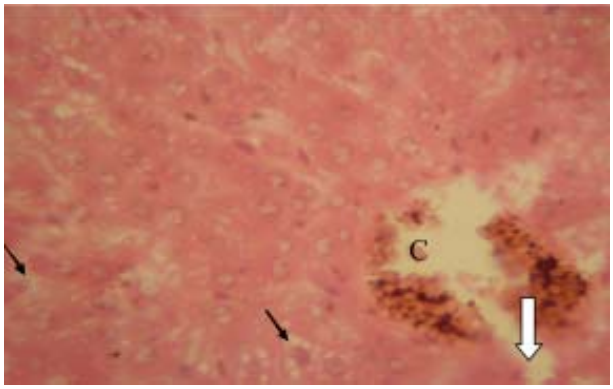

Figure 7: Group E (800 mg/kg) showing severe loss of normal liver architecture: numerous shrunken hepatocytes with pale nuclei, dilated central vein filled with cellular debris (C), central vein rupture (white arrow) fatty infiltration of hepatocytes (thin arrow). X400 (H& E).

Examination of the liver slides of animals in group F (Fig. 8) showed loss of normal liver architecture: numerous shrunken hepatocytes with pale nuclei and fat

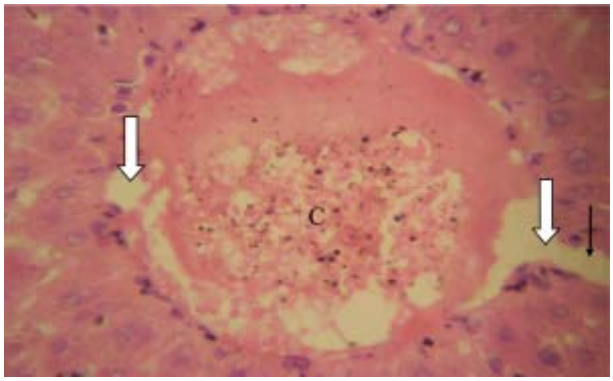

Figure 8: Group F (diabetic control), showing loss of normal liver architecture: numerous shrunken hepatocytes with pale nuclei, central vein rupture (white arrows). Dilated central vein filled with blood and cellular debris (C) dilated sinusoids (thin arrow).X 400 (H& E).

infiltrates, centrilobular hepatocyte degeneration. Congested central vein filled with blood and cellular debris, dilated sinusoids. These features are in keeping

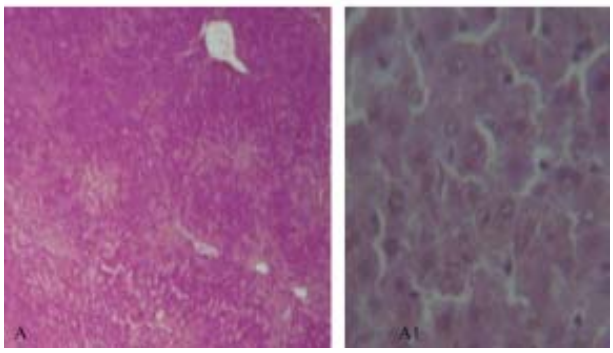

Figure 9: Groups A, A1 showing PAS and Diastase control slides with evidence of deeply staining PAS positive granules (glycogen) which are dissolved by the diastase in the control slide A1.X400.

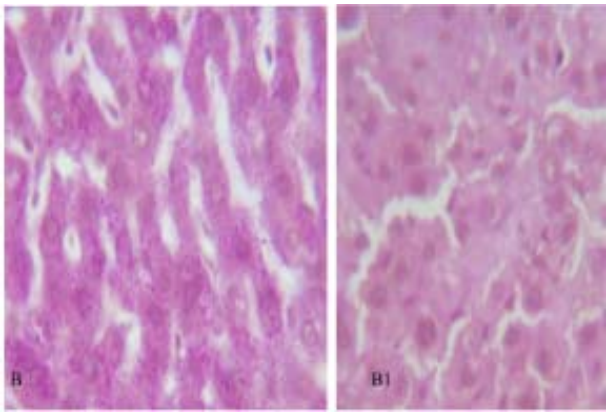

Figure 10: Groups B B1 showing PAS and Diastase control slides with a few deeply staining PAS positive granules (glycogen) which are dissolved by the diastase in the control slide B1.X400.

with those documented for STZ-induced liver injury. Slides from animals in groups C (Fig. 5),showed features in keeping with normal histology although there is an

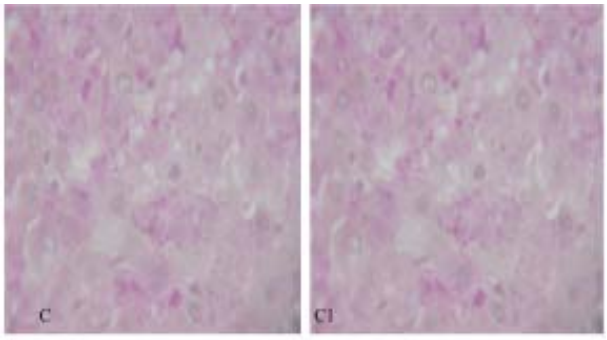

Figure 11: Groups C, C1 showing PAS and Diastase control slides with absence of deeply staining PAS positive granules (glycogen) as evident by no difference in colour compared to the diastase control slide C1.X400.

aggregation of inflammatory cells seen in a few of the slides examined. Group D (Fig. 6) and E (Fig. 7), revealed varying degrees of liver damage as evidenced

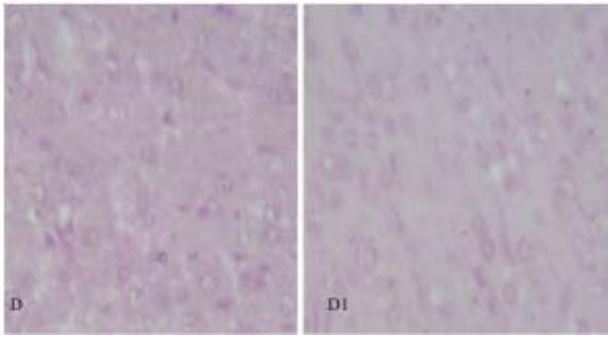

Figure 12: Groups D, D1 showing PAS and Diastase control slides with absence of deeply staining PAS positive granules (glycogen) as evident by no difference in colour compared to the diastase control slide D1X 400.

by loss of normal liver architecture, numerous shrunken hepatocytes with pale nuclei and fat infiltrates. There are also some hepatocytes with pyknotic nuclei. Congested central veins filled with blood and cellular debris, numerous fat cells replacing dying hepatocytes are also evident.

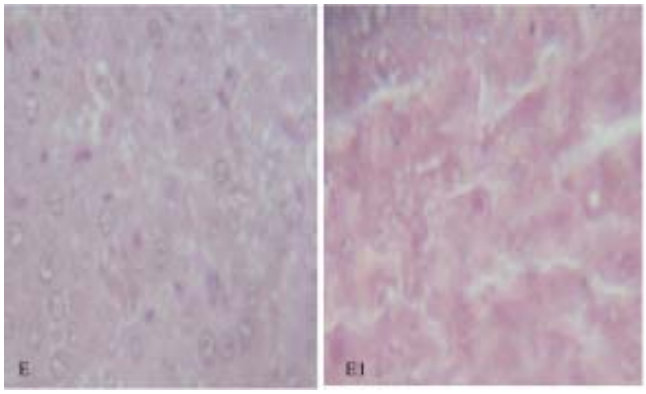

Figure 13: Groups E, E1 showing PAS and Diastase control slides with absence of deeply staining PAS positive granules (glycogen) as evident by no difference in colour compared to the diastase control slide E1. X 400.

Periodic acid Schiff and Periodic acid Schiff - diastase control slides for group A (Fig. 9) animals revealed normal liver architecture with hepatocytes that appear mostly mononuclear with extensive cytoplasm densely stained with coarse granular PAS-positive deposits. Similar deposits were not seen in the diastase-controlled section these features are in keeping with demonstrable glycogen deposits. The slides for groups B (Fig. 6, Fig. 10) animals revealed normal liver architecture with moderate PAS staining. These are in keeping with presence of some glycogen deposits corroborated by loss of staining in the diastase control slides. PAS slide for group F (Fig. 11) animals showed pale cytoplasm which did not stain positively for glycogen. Groups C (Fig. 12), D (Fig. 13) and E (Fig. 14) slides showed progressively worsening pallor of the cytoplasm and reduction in degree of staining for PAS positive glycogen granules.

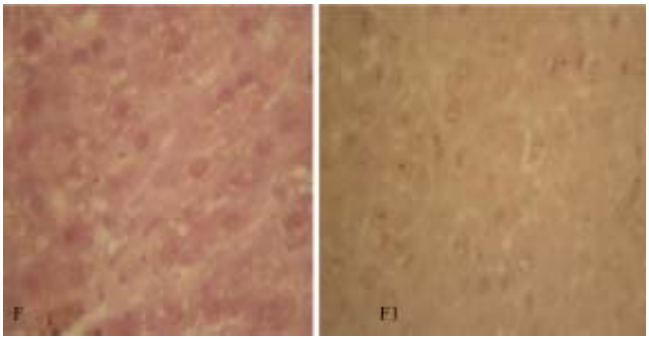

Figure 14: Groups F, F1 showing PAS and Diastase control slides with absence of deeply staining PAS positive granules (glycogen) as evident by almost no difference in colour compared to the diastase control slide F1. X400.

Discussion

Diabetes mellitus is a serious risk factor for the development of multiple organ damage as a result of multiple and complex mechanisms. The intention of this study was to evaluate the ability of *Ocimum gratissimum* to protect against changes that occur in the liver as a result of diabetes mellitus, however, we observed that *Ocimum gratissimum* does not protect against STZ induced morphological and biochemical changes in the liver.

Ocimum gratissimum treatment significantly decreased serum glucose levels in the STZ-induced rats at all doses and this was comparable to the response seen with Metformin theraphy but not better at controling blood glucose levels at the doses tested. This study confirmed that ethanolic extract of *O. gratissimum* like the aqueous and methanolic extracts previously studied [12, 24, 30] has hypoglycaemic effects.

AST and ALT are important and critical enzymes in the biological processes. These enzymes are involved in the breakdown of amino acids into a-keto acid, which is routed for complete metabolism through the Krebs cycle and electron transport chain. The increased gluconeogenesis and ketogenesis observed in diabetes may be due to high levels of activity of these transaminases [35].

Consequently, they are considered specific indicators for liver damage [36]. The increase in AST and ALT levels in serum of STZ-diabetic animals reflects impaired liver function. The decrease in those enzyme levels in STZ-diabetic rats given Metformin shows that Metformin prevented liver damage.

A yellowish brown color of the liver was observed in animals who were administered *O.gratissimum* at 600 and 800 mg/kg this could be due to fatty liver formation. Fatty liver and hyperlipidemia in streptozotocin-induced shrews were reported by Ohno et al. 2000 [37] and Muhammad Zafar in 2009 [31].

Histopathological findings revealed vacuolation, pycnotic nuclei, rupturing in the central vein, dilation in some sinusoids and mononuclear cell infiltration in the

diabetic control compared to the non-diabetic control group. In the group given Metformin, it was observed that vacuolization, pycnotic nuclei, hyperaemia and mononuclear cell infiltration were decreased although sinusoidal dilation was present in some of the Metformin slides. It was also noticed that the rupturing of the epithelium of some central veins was still continuing. Animals that received *O. gratissimum* showed worsening liver histology with increasing doses of extract. The liver showed mild to severe loss of architecture, vacuolations, and rupture of the central vein, mononuclear cell infiltration and massive liver cell death. The liver also showed reduction in PAS positive (glycogen) granules which are indicative of continuing gluconeogenesis and ketogenesis. Metformin treatment however showed an improvement in hepatic glucogenesis as evident by the staining of PAS positive granules

The documented effects of diabetes on hepatic microanatomy include hypertrophy and autophagic vacuoles in hepatocytes. The hepatocyte nuclei are usually enlarged and sometimes showed irregular contours and intranuclear inclusions [6]. Cytoplasmic changes observed in the STZ-induced diabetes included markedly depressed glycogen granules and poorly developed rough endoplasmic reticulum [38]. Glycogen synthase phosphatase activity in the liver is lowered in insulin-deficient animals and could be the basis for the defective deposition of glycogen from glucose in the livers of diabetic animals [39, 40].

The study concluded that *O. gratissimum* reduces blood glucose at all doses but worsens Streptozotocin- induced hepatic injury at high doses.

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