Histological and Histomorphometric Effects of Ethyl Acetate Fraction of Lycopene on the Liver and Pancreas of Experimentally-Induced Diabetic Wistar Rats

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ABSTRACT

Objectives: The aim of the study was to assess the effects of ethyl acetate fraction of lycopene (EAFL) on the histoarchitecture and histomorphometry of liver and pancreas of experimentally-induced diabetic Wistar rats.

Materials and Methods: Sixty adult male Wistar rats weighing between 180 and 200g were used for this study. The animals were kept in the animal holding of the department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife. They were fed on high fat rat diet for 4 weeks and given water ad libitum. The animals were given humane care according to the guidelines of Health Research Ethics Committee, Institute of Public Health, Obafemi Awolowo University. They were divided into 6 groups (A, B, C, D E and F) of 10 rats each. Group A was the normal control that received 2ml/kg/day of distilled water orally for 28 days. Group B was induced with 25 mg/kg/day of streptozocin administered intraperitoneally for 5 days. Group C, D and E were the test groups that received EAFL 20, 40, 60mg/kg/ day per oral, respectively for 28 days after induction of diabetes. Group F was the test group treated with Insulin 5 IU/kg/day subcutaneously for 28 days after induction of diabetes. At the end of the experimental period the rats were given 2 weeks of recovery period, then sacrificed under ether anaesthesia. The blood samples were collected by cardiac puncture into plain bottle for serum analysis. The organs of target were harvested, pancreas and liver were fixed in Bouin’s fluid and 10% neutral buffered formalin respectively. Tissues of the organs were processed for paraffin embedding and sections of 5μm were cut and stained.

Results: The immunohistochemical, histological and biochemical evaluations of liver and pancreas revealed, actual distortion to the target organs as shown by immunoreactivity in the pancreas tissue of the toxic group whereas the test groups that were treated with EAFL did not markedly exhibit immunoreactivity, the group with the highest dose of EAFL expressed a near normal immunoreaction, likewise, the toxic group showed a marked histoarchitectural liver and pancreas tissue distortion due to the chronic hyperglycemic state when compared with the diabetic groups and among which the group treated with the highest dose of EAFL responded best, as evidenced by the reversal of the liver and pancreas tissue distortions.

Conclusion: The findings of this study showed that, EAFL may proffer better treatment option for diabetic liver complications if and when further researched to compare various synthetic antidiabetic medications with this intervening agent, which may eventually be a generally acceptable alternative therapy to abate the menace of diabetes and its associated complications.
Keywords
Diabetes, Liver, Pancreas, Wistar rats, Ethyl Acetate fraction of Lycopene.

Introduction
Liver fibrosis and steatosis have been found to be associated with Type 2 diabetes mellitus while nonalcoholic fatty liver disease (NAFLD) has been increasingly diagnosed globally which has been recognized as a source of public health concern. Liver disease comprises a wide range of histological features that vary from simple steatosis to severe forms of fibrosis, steatohepatitis and even cirrhosis [1-3]. The pathogenesis of this disease progression is poorly understood, but several anomalies have been strongly linked to its onset, such as central obesity, insulin resistance, chronic inflammation, increased uptake of fatty acids by the liver and lipotoxicity [4,5] NAFLD has been considered to be a hepatic manifestation of metabolic syndrome (MetS) and, as such, is strongly related to type 2 diabetes mellitus (T2DM). Some studies have correlated the severity of insulin resistance with the development of severe forms of NAFLD. Moreover, NAFLD has been described as a reliable predictor of development of T2DM [6-9].

Diabetes mellitus (DM) is a chronic disease that is characterized by a relative or absolute lack of insulin, resulting in hyperglycemia. Diabetes is also a disorder of the metabolism of carbohydrates, protein, lipids and fats, characterized by chronic hyperglycemia [10]. Its manifestation of prolonged high blood glucose leads to sign and symptoms of diabetes-specific non-alcoholic fatty liver disease, kidney, eye and peripheral nerve disorders consequently leading to hepatopathy, nephropathy, retinopathy and peripheral neuropathy [11-13].

Research indicated DM to be associated with a number of liver abnormalities, such as abnormal glycogen deposition, non alcoholic fatty liver disease (NAFLD), fibrosis, cirrhosis, hepatocellular carcinomas (HCCs), abnormal elevated hepatic enzymes, acute liver disease and hepatitis [14,15]. Statistics by WHO as far back as 2013, revealed global prevalence of 347 million people living with diabetes, China has the highest incidence of diabetes having 98.4 million people affected, 8 out of 10 deaths occur in the low and middle income countries [16]. In Africa about 19.8 million people are affected, Nigeria is ranked highest with 3.9 million in the number of people living with diabetes (PLWD) as at 2013. By the year 2035, the projection estimated as percentage of PLWD will increase to 58% [17].

Type 2 diabetes is the commonest form of diabetes responsible for 90-95% of all diabetic cases approximately 50% of the sufferers are above 65yrs old [18]. There are numerous problems associated with current management of diabetes, besides being difficult and tedious, it is expensive and unaffordable by majority of African and Asian populations, other difficulty in management include; noncompliance with medication and polypharmacy. Current treatment for DM include the use of insulin synthetic drugs like; sulfonylurea, metformin, alpha glucosidase inhibitors and thiazolidinediones with life style adjustments. These drugs are valuable but are limited in pharmacokinetic actions, secondary failure rates, and associated side effects [19-21]. From the foregoing, WHO projected about 80% of world’s population rely mainly on herbal medicines for their primary health care [22,23]. Over the years, gastrointestinal disorders have been rampant in people living with diabetes (PLWD), medicinal plants screening for novel bioactive compound is therefore an alternative in the management of DM, due to the fact that herbal medicines are biodegradable, safe, cheap with fewer side effects, the effects of ethyl acetate fraction of lycopene on the histology and histomorphometry of liver and pancreas of experimentally induced diabetic Wistar rats have not been fully documented hence this study. This study contributed to the knowledge of beneficial action or otherwise of ethyl-acetate fraction of lycopene (EAFL) in managing hepatic complication of experimentally induced diabetic Wistar rats.

Materials and Methods
Sixty (60) adult male Wistar rats weighing between 180-200g were used for this study. The animals were kept in the animal holding of the department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife. They were fed on high fat rat diet for 4 weeks and given water ad libitum. Ethical clearance for the study was obtained from health research ethics committee (HREC), institute of public health (IPH) Obafemi Awolowo University (OAU) Ile-Ife. The animals were given humane care according to the guidelines of HREC, IPH, OAU.

They were divided into 6 groups (A, B, C, D E and F) of 10 rats each. Animals were kept and maintained under standard laboratory condition of temperature, humidity and light, they were fasted for 16 hours but with access to water. The end of the fasting hours was taken as 0 hour, the glucose levels of the animals were determined and recorded and the animals were monitored and stabilized for two weeks, thereafter ethyl acetate fraction of lycopene was administered. Group A was the normal control that received 2ml/kg/day of distilled water orally for 28 days. Group B was induced with 25mg/kg of streptozocin dissolved in 0.1M sodium citrate buffer (pH 6.3) intraperitoneally for 5 days. Group C, D and E were the test groups that received EAFL 20mg/kg/day, 40mg/kg/day, 60mg/kg/day peroral, respectively for 28 days after induction of diabetes. Group F was the test group that was treated with Insulin 5 IU/kg/day subcutaneously for 28 days after induction of diabetes. At the end of the experimental period the rats were given 2 weeks of recovery period, then sacrificed under ether anaesthesia. The blood samples were collected by cardiac puncture into plain bottle for serum analysis. The organs of interest were harvested and fixed in 10% neutral buffered formalin. Tissues of the organs (pancreas and liver) were processed for paraffin embedding and sections of 5μm were cut and stained with Hematoxylin & Eosin for general histoarchitecture of the organs. Periodic Acid Schiff stain with diastase control was done to reveal the capillary basement membranes of the central vein and the portal triad. Aldehyde fuchsin stain was done to evaluate and quantify beta cells. Masson Trichrome Stain was done for evaluation of collagen fibres.

Data so generated were analyzed by using descriptive and inferential statistics. Graph pad prism 5 (version 5.03 raph Pad Inc.) statistical package was used for data analysis.
Results

Plate 1: Sample photomicrographs of 5μm sections of pancreas tissue showing stained sections. Beta cells are stained dark brown shown in yellow arrows. A is the normal control group that was not induced nor treated. B is the positive control with STZ only. Groups C, D and E received STZ+20, 40, 60mg/kg of EAFL respectively, while F is STZ + 5 IU Insulin... Aldehyde fuchsin stain.

Plate 2: Sample photomicrographs of 5μm sections of pancreas tissue showing stained sections. Beta cells are stained purple shown with yellow arrows. A is the normal control group that was not induced nor treated, B is the positive control with STZ only. Groups C, D and E received STZ+20, 40, 60mg/kg of EAFL respectively, while F is STZ+ 5 IU Insulin with Hematoxylin and Eosin
Plate 3: Sample photomicrographs of 3um sections of pancreas tissue showing stained sections. Collagen fibres are shown in yellow circumscribed outline stained bluish green. A is the normal control group that was not induced nor treated, B is the positive control with STZ only. Groups C,D and E received STZ+20, 40, 60mg/kg of EAFL respectively, while F is STZ+ 5IU Insulin Masson Trichrome stain.

Plate 4: Sample photomicrographs of sections of pancreas tissue showing immunostained sections in 4 weeks groups, reactive areas are darkly brown stained represented with yellow arrows. A is the normal control group that was not induced nor treated, B is the positive control with STZ only. Groups C,D and E received STZ+20, 40, 60mg/kg of lycopene respectively, while F is STZ+ 5 IU Insulin…. Interleukin 2 immunostain.
Plate 5: Sample photomicrographs of 5um sections of Liver tissue showing stained sections. Hepatic cells are stained purple shown in yellow arrows, the necrotic hepatocytes shown in green arrows, edematous hepatocytes are shown in blue arrows. A is the normal control group that was not induced nor treated, B is the positive control with STZ only. Groups C, D and E received STZ+20, 40, 60mg/kg of EAFL respectively, while F is STZ+ 5 IU Insulin with Hematoxylin and Eosin.

Plate 6: Sample photomicrographs of 5um sections of pancreas tissue showing stained sections. Collagen fibres are stained bluish green shown in yellow circumscribed regions in groups B, C, D and F. A is the normal control group that was not induced nor treated, B is the positive control with STZ only. Groups C, D and E received STZ+20, 40, 60mg/kg of EAFL respectively, while F is STZ+ 5 IU Insulin with Hematoxylin and Eosin.

Plate 7: Sample photomicrographs of 5um sections of pancreas tissue showing stained sections. Glycogen deposition are stained purple shown in yellow arrows. A is the normal control group that was not induced nor treated, B is the positive control with STZ only. Groups C, D and E received STZ+20, 40, 60mg/kg of EAFL respectively, while F is STZ+ 5 IU Insulin with Periodic acid-Schiff stain.
**Figure 1:** Bar Charts Showing Mean Animal weight before the experiment.

The graph showing the mean weight of the rats across the group after treatment.
Results presented as Mean ± SEM, n=6 (p<0.05)

α Significantly different from normal control at p< 0.05
β Significantly different from toxic control at p< 0.05
δ Significantly different from C,D,E and F at p< 0.05

**Figure 2:** Bar Charts Showing Mean Animal weight after the experiment.

The graph showing the mean weight of the rats across the group after treatment.
Results presented as Mean ± SEM, n=6 (p<0.05)

α Significantly different from normal control at p< 0.05
β Significantly different from toxic control at p< 0.05
δ Significantly different from C,D,E and F at p< 0.05

**Figure 3:** Bar Charts Showing Glutathione Peroxidase levels.

The graph showing the mean Glutathione peroxidase levels of the rats across the group after treatment.
Results presented as Mean ± SEM, n=6 (p<0.05)

α Significantly different from normal control at p< 0.05
β Significantly different from toxic control at p< 0.05
δ Significantly different from C,D,E and F at p< 0.05
Figure 4: Bar Charts Showing Mean Insulin levels.
The graph showing the mean Insulin levels of the rats across the group after treatment.
Results presented as Mean ± SEM, n=6 (p<0.05)
α Significantly different from normal control at p< 0.05
β Significantly different from toxic control at p< 0.05
δ Significantly different from C,D,E and F at p< 0.05

Figure 5: Bar Charts Showing Mean Random Blood Sugar levels.
The graph showing the mean RBS levels of the rats across the group after treatment.
Results presented as Mean ± SEM, n=6 (p<0.05)
α Significantly different from normal control at p< 0.05
β Significantly different from toxic control at p< 0.05
δ Significantly different from C,D,E and F at p< 0.05

Figure 6: Bar Charts Showing Mean cell count in the Pancreas.
The graph showing the mean pancreas cell count levels of the rats across the group after treatment.
Results presented as Mean ± SEM, n=6 (p<0.05)
α Significantly different from normal control at p< 0.05
β Significantly different from toxic control at p< 0.05
δ Significantly different from C,D,E and F at p< 0.05
Discussion

Nutritional therapies or treatments have been recommended for the management of diabetes mellitus but the beneficial effects of their phytochemicals on the affected organs are being under reported. Therefore this study assessed the histological and histomorphometric effects of ethyl acetate fraction of lycopene on the liver and pancreas of experimentally-induced diabetic wistar rats.

In this study, chronic hyperglycemia led to weight loss in group B when compared to group A. There were however, increase in absolute body weight in the test groups C, D, E and F that is, there was significant weight gain observed in all groups except group B, according to figures 1 and 2, there was initial increase intake of feed in the untreated diabetic group (B) as excessive eating is a feature of diabetes however, as hyperglycemia worsened as a result of no intervention there was reduced appetite, which may be due to reduction in food intake through the satiety and appetite regulatory center in the hypothalamus. This is in keeping with the work done by Chinwe et al., 2015 who reported that diabetes significantly reduces body weight of experimental animal in diabetic condition, as compared with the normal control and the test groups with intervention agent, which restores the body weight. This study has found comparable body weight gain of the experimental animals with normal control and test groups to show statistically significant increase except in the untreated diabetic group (B). Figure 3 is the levels of glutathione peroxidase which showed group B to be statistically reduced significantly when compared with the test groups C, D, E, F and group A. Generally, it is noted that the treated group reflected similar level of antioxidant comparable to the normal control and this explained the reversal of the histopathological features seen in the liver and pancreas tissues of the untreated diabetic group (B) as evidenced in the immunohistochemical and histological photomicrographs in plates 4 and 5 above.

Moreover in figure 5, at week 4 after induction of diabetes and commencement of intervention, there was further rise in the random blood glucose level with a mean value of 29.3 Mmol/L in group B, whereas the RBS level in group A was within normal limit of 4.2 Mmol/L, while after commencement of treatment the glucose levels in the test groups (C, D, E and F) were noticed to reduce significantly, the least value being noticed in group E and F which were still in diabetic range. That is, significantly lower glucose level was observed in group E with 60mg/kg than in group D with 40mg/kg and it is more significantly reduced in group D with 40mg/kg than group C with 20mg/kg, while the glucose level in the normal control remains within normal blood glucose range when compared with the untreated diabetic group which random glucose levels were increasing unabated. From this study, administration of EAFL in graded doses after induction of diabetes with four weeks of treatment gradually reversed the chronic hyperglycemia, by the antioxidant properties possessed by EAFL as shown in figure 3. The reversal was also in line with sensitization of the remnant insulin in the pancreas with improvement in the level of insulin as shown in figure 4, this increase glucose uptake by the pancreas by opening K+ sensitive ATP channels. This study as indicated in figure 4 showed insulin levels in the test groups C, D, E and F to increase significantly (<0.05) when compared to the untreated diabetic group B which had the lowest level of insulin in all the groups. According to figure 4 during intervention, the group treated with Insulin (group F) has the highest level of insulin level compared to test groups C, D and E that were treated with graded doses of EAFL at 20, 40, and 60 mg/kg, this explained why group F has the lowest random blood glucose level next to the normal control group (A).

From image J histomorphometry beta cell count, there were cell loss that led to reduced cell count in the toxic group (B) whereas there were no significant change in other test groups C, D, E and F. Moreover, the staining intensity has been evidently reduced in the toxic group (B) which made it significantly different (<0.05) from the normal control and the tests groups C, D, E and F. The quantification of the cell count done revealed that there was significant difference (<0.05) in the treated diabetic groups C, D, E and F when compared with the untreated diabetic group B, the group E administered with the highest EAFL dose showed the highest number of cells recorded. This implies that there was pancreatic and or liver cell regeneration during treatment with highest dose of EAFL.

The microanatomy of pancreas tissue was also done under aldehyde fuchsin stain across the groups, the histological outline revealed changes in the beta cells within the islet of Langerhans in the diabetic untreated groups, the treated groups C, D, E and F showed fewer beta cells with cell loss while, there was total beta cell loss due to beta cell destruction by STZ toxicity, with many necrotic cells seen in untreated diabetic group while in normal control group (A) the beta cells were intact and of normal size, shape and population when compared with the diabetic groups. Group A showed substantial number of cells and the pancreas cell count across the treated group increased, with increase dosage of the intervening agent, this showed that administration of EAFL in graded doses after diabetes induction was noticed to gradually reverse the initial cell loss by chronic hyperglycemic state when compared with untreated diabetic group (B). The group E and F showed increase in number of cell in the islet of Langerhans which may be due to cell regeneration within the pancreas after treatment with the intervening agent (EAFL). The groups E and F showed histological outline with near normal histoarchitectural organization in term of cell density when compared with normal control group (A). This may be due to cytoprotective and antioxidant effect of EAFL.

The microanatomy of the pancreas tissue was done under hematoxylin and eosin staining technique, the histarchitecture revealed changes in tissue of the untreated diabetic group (B) with distortion in the pancreatic layout marked with loss of islet cells when compared with the normal control group (A). The distortion in the pancreatic histology of untreated diabetic group (B) was more when compared with the treated group in their histological features,
characterized by presence of necrotic islet cells and effacement of beta cells of islet of Langerhans, the untreated diabetic group (B) further showed disorganized histomorphology of pancreas with little or no islet cells but fibrous stroma however, the treated diabetic groups C, D, E and F showed evidence of recovery and histological improvements in the islet cells structure with appearance of more number of cells in the pancreas parenchyma. Administration of EAFL in graded doses after diabetes induction was noticed to significantly reverse the initial distortion occasioned by chronic hyperglycemic state on the islet cells, when compared with the untreated diabetic group (B) also, administration of EAFL in graded doses after diabetic stabilization significantly reversed the alteration seen in pancreatic histoarchitecture of the test groups C, D, E and F when compared with the untreated diabetic group (B). The groups E and F showed reversal of the histological distortion with near normal histoarchitectural organization. This may be due to free radical scavenging property or antioxidant effect of EAFL this is as shown in the biochemical reports of this study in figures 3 which is in keeping with the work done by Kuhad et al.,2008 who reported that EAFL has significant, dose-dependent antidiabetic action in streptozotocin-induced diabetic Wistar rats. Streptozotocin did not only selectively alter the pancreatic insulin secreting B cells but also distort histoarchitecture of liver of the untreated/toxic group (B).

The histology of the pancreas under Masson Trichrome staining technique was examined in all groups of the experimented animals as shown in plate 3 above, the histoarchitecture of the pancreas in the untreated group showed a general reduction in collagen fibres in the untreated diabetic group (B) when compared with the normal control group (A), with intervention by administration of treatment agents there were evidences of improved collagen fibres density in the stroma of the pancreatic tissue in treated diabetic groups C, D, E and F when compared with histological sections of the untreated diabetic group (B) and control group (A).

Administration of the intervening agent for four weeks showed appearance and increase in collagen density in the treated diabetic groups C, D, E and F. The increase in collagen density was not so marked with the insulin group when compared with histological sections of group E and normal control group A, more collagen fibres in these groups may be due to the continuous and prolonged inhibition of reactive oxygen species formation by blocking the protein kinase C pathway through antioxidant effects of EAFL on liver tissues as evidenced by biochemical increase in the antioxidant assay of GPx in EAFL treated diabetic rats. This is in agreement with the work done by Jain et al., 2020. Who reported in his work that lycopene has ability to reverse diabetes- induced tissue injury through increased levels of serum antioxidants.

The microanatomy of the pancreas in experimental animals were examined under interleukin 2, immunohistochamical stain across groups, the histological layout of untreated diabetic group B, showed areas of reactivity in groups B, through immunohistochemical stain reactivity; immunohistological outline of the toxic group is more intense when compared with other treatment groups while, no reactivity was observed in groups C, D, E and F. interleukin 2 was adopted as a proinflammatory marker and with the immune reaction observed in plate 4, the reactivity may be due to the inflammatory pathogenesis of diabetes, which is similar to the report of Muhammad et al., 2012, that inflammatory mechanisms through insulin resistance, decrease insulin secretion from B cell dysfunction which provokes the pathogenesis of type 2 diabetes. It is evident that the immunostaining of antigen for the pancreas of diabetic rats in the untreated diabetic group (B) showed reaction to the antibodies of the interleukin 2 immunomarkers to the antigens present in the pancreas tissue after four weeks of treatment, this corroborates the work done by Araujo et al., 2020 who posited that there is in-situ production of cytokines and chemokines in diabetes complications.

A significant increase in the relative percentage of liver was observed in this study. The microanatomy of liver in the control group (A) did not show any histological changes during the period of the experiments, the liver lobules were observed to be normal with polygonal hepatocytes having regular nucleus and cytoplasm (plate 5). In the diabetic groups B, C, D, E and F. The EAFL treated groups C, D, E and F animals were observed four weeks after diabetes induction, the liver showed several alterations including mild degree of fatty changes, cloudy swelling, mild infiltration of lymphocytes with hemorrhage, while the untreated diabetic animals (B) showed more progressive changes as evidenced by severe congestion, necrotic foci, hydropic changes, aggregation of lymphocytes between the hepatocytes (plate 5). Results showed several morphological and histological alterations in liver tissues, indicated by increase in the liver percentage of liver weight, glycogen reduction, associated with lipid deposition, inflammatory cells infiltration and Kupffer cells hyperplasia. Furthermore, this study illustrated the worsening of liver histology over a short time in STZ diabetic rats.

The microanatomy of liver demonstrated by the masson trichrome stain revealed a marked collagen fibre deposition in the untreated diabetic group (B) as shown in the yellow circumscribed area in plate 6, there were areas of collagen deposition in group C, D and F but not as much as that of untreated diabetic group (B), while there was near normal outline in group E. The collagen deposition is a marker of structural abnormality of the liver tissue which indicates the triggering events of chronic liver disease (fibrosis of the liver), also there was minimal collagen deposition in the treated groups C, D, and F which showed that the histoarchitectural layout in the liver tissues of treated groups C, D and F were gradually reversing towards normal whereas, there was no significant or almost normal collagen content in the treated group E, which was treated with the highest dose of EAFL.

The microscopic structure of the liver tissue under periodic acid Schiff stain revealed a significant thickening of the basement membrane as a major diabetic microvascular angiopathy, in the wall of the central vein in the test groups (B, C, D, E and F) when compared to the non-diabetic/normal group (A). The thickness is more marked in the untreated diabetic group (B), than the treated
diabetic groups (C, D, E and F). This corroborates the finding of Sayon et al., 2010 who reported in his work that chronic hyperglycemia is the principal causal factor mediating changes in basement membranes of small blood vessels in diabetes and as such, are foundamental histoarchitectural alterations observed.

**Conclusion**

This study concluded that diabetes caused pancreas and liver pathologies which resulted in histological and histomorphometric alterations in the hepatocytes and pancreatic beta cell histoarchitectural layout. The ethyl acetate fraction of lycopene as intervening agent, was able to ameliorate the deleterious diabetic effects by reversing the biochemical and histological alteration initially caused and eventually restoring the histoarchitectural and biochemical alterations of the diabetic animals under study even better than the standard synthentic drug, hence EAFL may play a significant role in the management of diabetes and its attendant complications. This as well substantiates tradomedical use of tomatoes as nutritional therapies in the management of diabetes mellitus.

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Universitas Airlangga, Subraya, Indonesia.

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