

Immunohistochemical Effects of Ethyl Acetate Fraction of *Lycopersicum Esculentus* on the Kidney in Experimentally Induced Diabetic Wistar Rats

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Received: 22 Jul 2022; Accepted: 28 Aug 2022; Published: 03 Sep 2022

Citation: Arayombo BE, Adewole OS. Immunohistochemical Effects of Ethyl Acetate Fraction of *Lycopersicum Esculentus* on the Kidney in Experimentally Induced Diabetic Wistar Rats. Trends Int Med. 2022; 2(2): 1-5.

ABSTRACT

Diabetic nephropathy (DN) as a leading cause of end-stage renal failure which has become source of concern recently. The aim of this study was to investigate the effect of EAFL on the histomorphology and evaluate its effect on inflammatory markers in Streptozotocin- Induced diabetic Wistar rats. This study was conducted using thirty male Wistar rats. The animals were divided into five groups of 6 rats each (n=6). Group A was the control animals (administered with 20 ml/kg/day of citrate buffer orally (p.o) stat for 4 weeks. Group B was induced with 65 mg/kg of streptozotocin in citrate buffer intraperitoneally stat and observe for four weeks without treatment. Group C,D and E were the test groups that received 65 mg/kg of streptozotocin in citrate buffer intraperitoneally stat and 20, 40 and 60 mg/kg/day of EAFL respectively for four weeks after induction of diabetes. Descriptive and inferential statistics were used to analyze the data.

The results showed reduction in serum insulin levels in diabetic groups which was reversed to normal when the intervening agent (EAFL) was administered. The histology of the kidneys showed distortion in renal corpuscles which was reversed when EAFL was administered. The immunohistochemistry of kidney tissues in the normal group (A) showed areas of Ag-Ab immunological reactivity, however, there was no reactivity in the diabetic groups.

The study concluded that EAFL has anti glycemc and anti inflammatory effect on the kidneys of Streptozotocin-induced diabetic Wistar rats.

Keywords

Immunohistochemistry, Ethyl Acetate Fraction of Lycopene (EAFL), Diabetes Mellitus, Wistar rats, Kidney.

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder of the endocrine system. It is a group of metabolic diseases characterized by hyperglycemia which results from defects in insulin secretion or action or both [1]. The term diabetes describes a group of metabolic disorders characterized and identified by the presence of hyperglycaemia in the absence of treatment [2]. The heterogeneous aetio-pathology includes defects in insulin secretion, insulin action, or both, and disturbances of carbohydrate, fat and protein metabolism. The long-term specific effects of diabetes include retinopathy, nephropathy and neuropathy, among

other complications. People with diabetes are also at increased risk of other diseases including heart, peripheral arterial and cerebrovascular disease, obesity, cataracts, erectile dysfunction, and non-alcohol fatty liver disease. They are also at increased risk of some infectious diseases, such as tuberculosis [3].

Diabetes may present with characteristic symptoms such as polyphagia, polydipsia and polyuria, blurring of vision, and weight loss. Genital yeast infections frequently occur. The most severe clinical manifestations are ketoacidosis or a non-ketotic hyperosmolar state that may lead to dehydration, coma and in the absence of effective treatment, death. However, in T2DM symptoms are often not severe, or may be absent, owing to the slow pace at which the hyperglycaemia is worsening. As a result, in the absence of biochemical testing, hyperglycaemia sufficient

to cause pathological and functional changes may be present for a long time before a diagnosis is made, resulting in the presence of complications at diagnosis [2]. It is estimated that a significant percentage of cases of diabetes (30–80%, depending on the country) are undiagnosed [4].

Diabetes comprises many disorders characterized by hyperglycaemia. According to the current classification there are two major types: type 1 diabetes (T1DM) and type 2 diabetes (T2DM). The distinction between the two types has historically been based on age at onset, degree of loss of β cell function, degree of insulin resistance, presence of diabetes-associated autoantibodies, and requirement for insulin treatment for survival [5].

DM has poised to affect the developing countries of the world much more than their developed counterparts and has thus assumed pandemic proportions globally. As early as the beginning of 20th century, DM was termed by Cook as being an infrequent disorder in Africans. However, there is now compelling data to indicate an increasing incidence and prevalence of DM in the continent [6]. The estimated prevalence of diabetes in Africa is 1% in rural areas, and ranges from 5% to 7% in urban sub-Saharan Africa [6].

Diabetes is found in every population in the world and in all regions, including rural parts of low- and middle-income countries. The number of people with diabetes is steadily rising, with WHO estimating there were 422 million adults with diabetes worldwide in 2014. The age-adjusted prevalence in adults rose from 4.7% in 1980 to 8.5% in 2014, with the greatest rise in low- and middle-income countries compared to high-income countries [6]. In addition, the International Diabetes Federation (IDF) estimates that 1.1 million children and adolescents aged 14–19 years have T1DM (8). Without interventions to halt the increase in diabetes, there will be at least 629 million people living with diabetes by 2045 [7]. High blood glucose causes almost 4 million deaths each year [6], and the IDF estimates that the annual global health care spending on diabetes among adults was US\$ 850 billion in 2017 [7].

The effects of diabetes extend beyond the individual to affect their families and whole society. It has broad socio-economic consequences and threatens national productivity and economies, especially in low- and middle-income countries where diabetes is often accompanied by other diseases.

Tomato (*Lycopersicon esculentum*) is one of the most important vegetables worldwide, it is rich in minerals, vitamins, essential amino acids, sugars, dietary fibers, vitamin B and C, iron and phosphorus [8].

Lycopene is the pigment principally responsible for the distinctive red color of ripe tomato and tomato products [9]. It is found in various tissues throughout the body such as the liver, kidney, adrenal glands, testes, ovaries, pancreas and the prostate gland [10].

Far from being inert, by virtue of its unique chemical properties, studies have shown lycopene to possess superior antioxidant abilities in comparison to other carotenoids, it is a potent antioxidant [11].

Materials and Methods

Animal Care and Management

Thirty adult male Wistar rats weighing between 150-200 g were used for the study. The animals were maintained under standard laboratory conditions of light, humidity and temperature. The animals were acclimatized for two weeks thereafter was the commencement of the research work. They were fed with standard rat pellet and given water *ad libitum*. All the experiment and animals were handled in accordance with the “Guide for the Care and Use of Laboratory Animals” prepared by Health Research Ethics Committee(HREC), institute of public health (IPH) Obafemi Awolowo University(OAU) Ile-Ife.

Experimental design

The rats were divided into 5 groups (A, B, C, D and E) of 6 rats each. Group A is the normal control that received 20ml/kg/day of citrate buffer orally (p.o) stat for 4 weeks. Group B was induced with 65mg/kg of streptozotocin in citrate buffer intraperitoneally stat and observe for 4 weeks without treatment. Group C is the test group that received 65mg/kg of streptozotocin in citrate buffer intraperitoneally stat and EAFL 20mg/kg/day p.o for 4 weeks. Group D is the test group that received Lycopene 40mg/kg/day p.o for 4 weeks after induction of diabetes. Group E is the test group that received Lycopene 65mg/kg/day p.o for 4 weeks after induction of diabetes.

Induction of Experimental Diabetes

Streptozotocin (65mg/kg body weight) was dissolved in 0.1 mol/L Sodium citrate buffer (pH 6.3) was intraperitoneally injected to animals in groups B,C,D, and E while group A was administered with equivalent volume of 0.1 mol/L sodium citrate buffer. All animals were fasted for 16hrs before the start of the experiment while the animals were still allowed free access to water. After the 16hrs fasting period which was taken as 0 hr –initial glucose levels were determined and recorded, after 72 hrs, the ethyl-acetate fraction of lycopene was then administered orally through gavages of varying concentrations in different groups 20,40,60 mg/kg/rat/day for groups C, D and E respectively.

Animal Sacrifice

The experimental animals were treated humanely and sacrificed through cervical dislocation after the last administration of intervening agent with two weeks of recovery period. Blood samples were collected retro-orbitally. The rats were opened up and the kidneys were excised and fixed appropriately for light microscopy.

Blood glucose level determination

The blood levels of glucose were determined using fine test @digital glucometer (Advantage) and test strips via biochemical method of glucose oxidase. The blood samples were obtained by

jabbing the dorsal vein of the rat's tail to determine the glucose level on the 3rd day, this was again measured on day 28. The other portion of blood sample was collected into plain specimen bottles and allowed to clot, and separated by centrifugation using CENLBR-3L-1 bench top low speed centrifuge (Universal 32, Made in Germany) at 2000g for 10 min. The supernatant obtained was used for the determination of serum insulin assay. Serum insulin was biochemically assayed using the method of double antibody enzyme immunoassay as described in the kits from Bio-source Belgium.

Histological Techniques

The Kidneys were fixed in 10% neutral buffered formalin. Tissues were passed through ascending grades of alcohol, infiltrated and processed with paraffin embedding and sections of 3µm were cut and stained; Haematoxylin and Eosin stain demonstrated general histoarchitecture. Immunohistochemical stain demonstrated the presence of antigen-antibody complex and localization of inflammatory markers with IL- 6.

Immunohistological Techniques

Sections were deparaffinized at 60°C for 30 min or in xylenes and hydrated with the following series of xylene and graded ethanol washes, each wash was performed in a separate glass jar containing 200-250ml, at room temperature in a ventilation hood. Slides were washed in the distilled water at room temperature quenched in endogenous peroxidase with 3% H₂O₂ in a glass jar and washed in Phosphate Buffered Saline (PBS) in a glass jar twice. The tissue samples were encircled using a PAP-Pen, nonspecific background staining, was blocked with normal horse serum in a humidity chamber. The serum was drained from the slides and primary antibody was applied, 1:200 made in Dako Antibody Diluent. Control sections were incubated in antibody diluents only, slides were incubated in humidity chamber for 1 hr, washed in 200-250ml Phosphate Buffered Saline in a jar twice for 5 min and secondary antibody was applied, biotinylated goat-antirabbit- mouse IgG (1:500) for 1 hr. in a humidity chamber. The activity of the antibody-peroxidase complex was visualized by incubation with DAB (3,3'-diaminobenzidine – tetrachloride) kit. Sections were counterstained with hematoxylin and mounted with DPX.

Photomicrography

The photomicrographs of the slides prepared for histology and immunohistochemistry were viewed and taken using LEICA DM 750 microscope coupled to a digital camera (LEICA ICC50) and a desktop computer.

Statistical Analysis

Data were presented as mean ± SEM and analyzed using descriptive and inferential statistics. One-way ANOVA was used to analyze data, followed by Student Newman-Keuls (SNK) test for multiple comparison. Graph pad Prism 5 was the statistical package used for data analysis. P-value ≤0.05 was considered statistically significant.

Results

Table 1: Body weight of the Animals (Initial and Final).

SN	Groups	Initial body weight	Final body weight
1	A	155.6 ± 2.391	179.4 ± 1.301 β
2	B	169.4 ± 1.290	148.8 ± 3.322 $\alpha\delta$
3	C	157.9 ± 1.573	169.9 ± 1.259
4	D	161.1 ± 1.430	173.0 ± 1.010 α
5	E	156.9 ± 1.009	174.4 ± 1.613

Table 2: Random blood glucose level.

SN	Groups	R.B.S before EXP	R.B.S after STZ Induction	R.B.S After Intervention
1	A	3.475 ± 0.1793	4.042 ± 2.687 β	4.408 ± 0.415 β
2	B	3.917 ± 0.2393	25.38 ± 1.911 $\alpha\delta$	27.96 ± 1.047 $\alpha\delta$
3	C	3.475 ± 0.2722	20.52 ± 2.415	12.97 ± 1.210
4	D	4.175 ± 0.2643	23.91 ± 2.310 α	11.14 ± 0.854 α
5	E	3.925 ± 0.2310	26.85 ± 1.267	9.817 ± 0.861

Table 3: Serum Insulin Levels.

SN	Groups	Insulin level
1	A	6.928 ± 0.2782 β
2	B	2.817 ± 0.4150 $\alpha\delta$
3	C	7.340 ± 0.3670
4	D	7.375 ± 0.2821 α
5	E	7.863 ± 0.4129

Results presented as mean ± SEM (n= 5)

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 and E at p<0.05

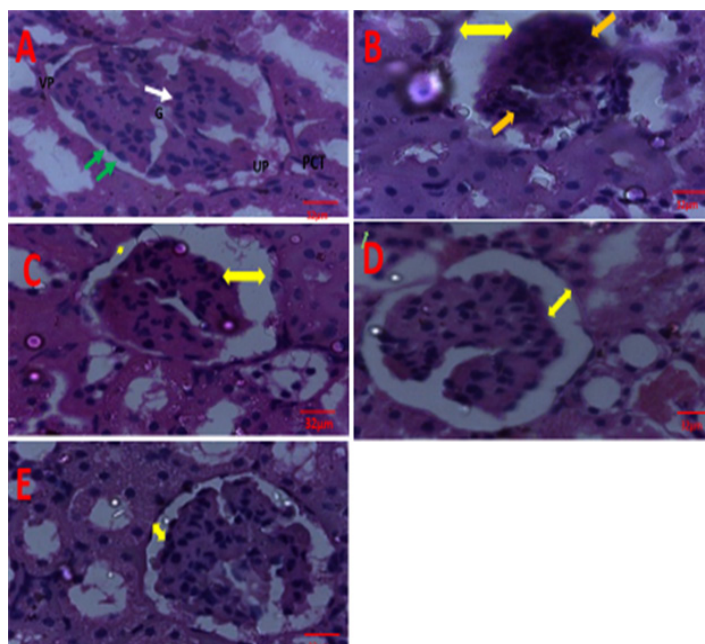


Plate 1: Photomicrograph of Hematoxylin and Eosin-stained kidney tissue. Sample photomicrographs from central cortex of 3µm kidney on light microscope showing histo-architectural differences across the groups: A is the normal control group that was not induced nor treated, B is the positive control induced with STZ but not treated. Groups C, D and E received STZ+20, 40 and 60mg/kg of lycopene respectively. The yellow arrows show the gaps in the urinary space across the group, green and white arrows point to the podocyte and mesangial cells respectively.... H and E Stain X 1000.

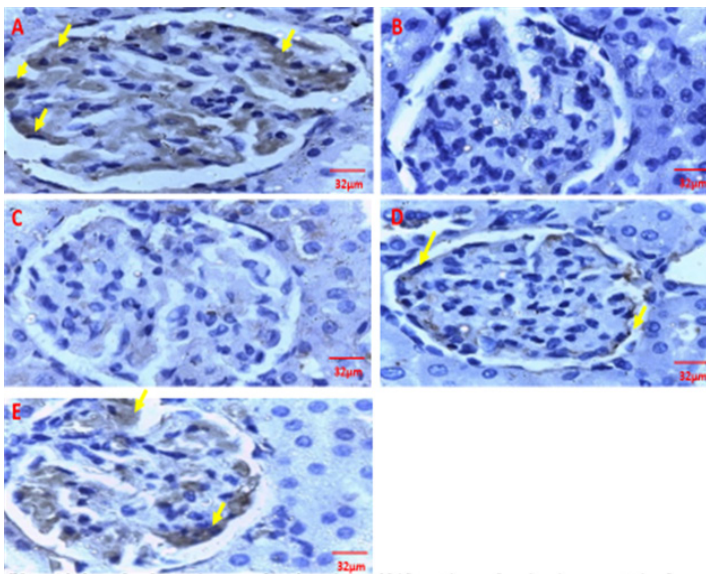


Plate 2: Photomicrograph of IL-6 immuno-stained kidney tissue.

Sample photomicrographs of sections of kidney tissue showing immunostained sections in various groups moderately reactive areas are darkly 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 and 60mg/kg of lycopene respectively... Interleukin 6 immunostain X 1000.

Discussion

The present study showed that there was significant weight loss ($p < 0.05$) in the untreated diabetic Group B when the initial weight of the animals was compared with the final weight and when this was compared with the normal control and the treated groups, though there were initial weight loss in the treated diabetic groups C, D, and E, there was however marginal weight gain when the intervening agent (EAFL) was administered when compared with the normal control where there was increase in the weight of the rats over the period of the experiment in the non-diabetic normal control (Group A) this is as shown in table 1, which is in keeping with the work done by Chinwe *et al.*, [12], who documented that diabetes significantly ($p < 0.05$) reduces body weight of experimental animal in toxic group (negative control) as the study duration increases compared with the normal control and the test groups with intervening agent which restores the body weight.

Results from this study also revealed according to table 2, that there was significant statistical reduction ($p < 0.05$) in blood glucose levels of the treated diabetic groups C, D and E when compared with the untreated diabetic group B Wistar rats, after four weeks of intervention, the random blood glucose level in the treated groups were tending towards normal when compared with the normal control of the normal reference value and this varies directly with the dose of the lycopene administered, this corroborates the research done by Kuhad *et al.*, [13] who stated that lycopene has significant, dose-dependent antidiabetic action in streptozotocin-induced diabetic Wistar rats.

Findings from this study also showed that the serum insulin level was reduced in the 4th week of induction of diabetes in the toxic group (B) when compared to the normal group (A) and the test groups C, D, and E as shown in the above table (3). There was further reduction in the serum insulin level as the chronic hyperglycemia continues in the toxic group compared to the test groups C, D, and E which values improved with time and intervention with Ethyl Acetate Fraction of Lycopene. This is in agreement with the work done by Sun *et al.*, [14] who reported that renal function and biochemical perturbation as a result of glomerular hyper filtration and renal injury are one of the factors implicated in the pathophysiology of diabetic nephropathy.

The microanatomy of the kidneys was examined in all groups and the histoarchitecture revealed changes in positive control (group B) which showed distortion in the renal corpuscles, characterized by presence of thick glomerular membrane, mesangial cells were noticed to expand when compared to the normal control (group A). The urinary spaces in toxic group B was also noticed to be wider in the toxic group when compared to normal control which improved directly proportionally to the time and dose of lycopene. Administration of EAFL in graded doses after diabetes induction was noticed to gradually reverse the initial distortion by chronic hyperglycemic state when compared to untreated diabetic group, also, administration of EAFL in graded doses after two weeks of diabetic stabilization with four weeks of treatment gradually reversed the alteration seen in renal corpuscles, group E showed reversal of the histological distortion with near normal histoarchitectural organization. This may be due to antiglycemic and free radical scavenging property of EAFL this is in keeping with the work done by Kuhad *et al.*, [13] who reported that lycopene has significant, dose-dependent antidiabetic action in streptozotocin-induced diabetic Wistar rats.

The immunohistochemistry of kidney in diabetic animals were examined across groups and the immunostaining of normal group showed areas of interleukin 6 reactivity in group A, while there was no reactivity observed in untreated diabetic group (B) of animals. Interleukin reactivity showed that there may be natural anti-inflammatory defense mechanism in the tissues of the normal control groups, interleukin 6 was adopted as an anti-inflammatory marker and with the immune reaction observed in normal control group A, in plate 2, this supports the report of Patrice *et al.*, 2017 who stated that anti-inflammatory mediators inhibit development of type 2 diabetes.

Conclusion

This study concluded that ethyl acetate fraction of Lycopene has antiglycemic, anti-inflammatory effects on the kidneys of streptozotocin-induced diabetic Wistar rats.

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