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Research Article

# Effect of Ethyl Acetate Fraction of Lycopene on Immunohistochemistry of Cystatin C in Kidney of Diabetic Wistar Rats

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## Abstract

This research investigated the effect of ethyl acetate fraction of lycopene (EAFL) on immunohistochemistry of Cystatin C in kidney of streptozotocin-induced diabetic wistar rats.

Diabetes Mellitus (DM) is defined as a metabolic disease characterized by chronic hyperglycemia due to absolute or relative insulin deficiency which complications affect virtually all organ systems. The prevalence of the disease has become increasingly rampant worldwide despite avertable, DM and its complications are leading causes of death globally. Thirty six adult male Wistar rats weighing between 120-160 g were used for this study. The rats were divided into 6 groups (A, B, C, D, E and F.) of 6 rats each. Group A was the normal control that received 20 ml/kg/day of citrate buffer intraperitoneally (i.p) for 30 days . Groups B, C, D and E were induced with 60 mg/kg of streptozotocin in citrate buffer intraperitoneally once daily for three days and observed for one week. Group B animals were left untreated, while Groups C, D, E, and F were treated with ethyl acetate fraction of lycopene (EAFL) at dosages of 20, 40, 60 and 20 mg/kg/day via oral route respectively for 30 days. At the end of the experimental period the rats were sacrificed through cervical dislocation. It was concluded that EAFL preserves the macro-anatomy of the kidney and reversed the chronic hyperglycemic state characterizing DM. The distortion in renal histological morphology was reversed and restoration of the histoarchitectural alteration in the treated diabetic animals were observed. Moreover, the renal cystatin C antigen antibody reactions were only accentuated in the untreated diabetic group while no immune reactivity were observed in high dose EAFL.

**Keywords:** Ethyl Acetate; Lycopene; Immunohistochemistry; Kidney; Diabetes; Wistar Rats

## Introduction

Diabetes mellitus is a common metabolic health problem all over the world. Globally, the prevalence of diabetes in adults has risen rapidly from 4.7% in 1980 to 8.5% in 2014 [17,18]. Hyperglycemia is one of the features of diabetes which increases diabetes risk and associated complications including heart attack, stroke, kidney disease, limb amputations, poor vision, and nerve damage [9]. Diabetes and its complications are leading causes of death worldwide [16]. It has been estimated that in Africans about 80% of cases of diabetes are undiagnosed. This may be because diabetes is often asymptomatic or produces only mild symptoms, which may be ignored or attributed to other causes [6].

Diabetic kidney disease or Diabetic nephropathy (DN) is the leading cause of end stage renal disease (ESRD) worldwide, in the United States it accounts for 44% of all new cases [10]. In the Kidneys, elevated blood pressure and high blood glucose levels can damage the small blood vessels, consequently the glomeruli become leaky which results in kidney dysfunction and this is referred to as diabetic nephropathy or diabetic kidney disease [14]. Tomato is one of the most chief vegetables globally, it is rich in vitamins, macro and micronutrients, essential amino acids, sugars, dietary fibres, vitamin B and C [5,6]. A prospective study of tomato products by Giovannucci showed that tomatoes fresh, contained the highest lycopene content (mg/100g wet basis) of all plant sources of lycopene [7].

Lycopene is one of the major carotenoids found in the human serum, with plasma levels ranging from 0.22 to 1.06 nmol/ml [4]. It is exogenous but widely distributed in the human body, its unique chemical properties have shown lycopene to possess superior antioxidant abilities in comparison to other carotenoids, it is a potent

antioxidant [13]. Its singlet oxygen-quenching property and its ability to trap peroxyl radicals is twice as high as that of β- carotene and 10 times higher than that of α-tocopherol and butylated hydroxyl toluene. Studies also showed that it inhibits reactive oxygen species generation in a dose-dependent manner [15].

Appreciable percentage of Diabetics progressed to DN within one year; however, they did not experience microalbuminuria or macroalbuminuria [20]. Glomerular filtration rate (GFR) has been found to be superior to Albuminuria excretion rate (AER) or Urinary Albumin Creatinine ratio (ACR) in the early diagnosis of DN. However, recently serum cystatin C has been considered a new biomarker for the diagnosis of kidney damage, several studies have shown that serum cystatin C is a better marker of GFR than serum creatinine [2].

## Materials and Methods

### Animal care and management

Thirty six adult male Wistar rats weighing between 120-160 g were used for this study. The animals were procured from the College of Health Sciences, Obafemi Awolowo University, Ile-Ife animal house, kept in plastic cages. They were maintained under natural conditions of light, temperature and humidity, they were acclimatized for two weeks before commencing the experiment, they were fed on high fat rat pellets and given free access to feed and clean water. Animals were fed for two weeks after which diabetes was induced. 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.

### Induction of experimental diabetes

The animals were injected with streptozotocin 60mg/kg intraperitoneally, which was dissolved in 0.1M sodium citrate buffer (PH 6.3). All the animals were fasted for 16 hrs with free access to water, at the end of the fasting, initial glucose levels were determined and recorded and another glucose level measurement and recording was done at 72 hours after the end of the induction and fasting period to confirm diabetes. Afterwards the ethyl acetate fraction of lycopene was administered to the test groups C, D, and E at a graded doses of 20, 40 and 60 mg/kg/ day for four weeks respectively. Then group F was given EAFL at 20 mg/kg/day.

### Biochemical studies

The blood levels of glucose were measured with fine test ®digital glucometer (Advantage) and test strips using glucose oxidase method. The blood samples were obtained from the dorsal vein of the tail of the rats to determine the glucose level on the 3rd day, this was again determined on day 28.

### Plant materials

The fruits of *Lycopersicum esculentus* (salad tomatoes) were procured at Odo Ogbe market in Ile-Ife. This was authenticated in Department of Botany O.A.U Ife with voucher Number IFE 17767. The fruits were washed, rinsed cleaned and kept at room temperature and crushed with electrically powered blender to get the pulp and juice, the former were then further processed accordingly via solvent extraction method to obtain lycopene fraction.

### Extraction of ethyl acetate fraction of tomatoes (*Lycopersicum esculentus*)

- **Materials:** Electrically powered warring Blender, cheese cloth, transparent container, knife, foil paper.
- **Apparatus:** Rotary Evaporator, measuring cylinder, beakers and weighing balance.
- **Solvent:** Ethyl-acetate.
- **Sample:** Wet Tomato

### Method of extraction

The tomato was weighed using electric weighing balance and the weight determined, then homogenized using a warring blender. The homogenized tomato was then extracted with ethyl acetate. After 48 hours, the mixture was filtered, and the filtrate was collected into a bottle. The filtrate was re-filtered until homogenous extract is obtained using cheese cloth. The homogenous extract was then concentrated to dryness with the use of rotary evaporator and later transferred into a petri dish and the weight taken.

The percentage yield was determined. The extract appeared as a honey-like gel.

$$\text{Percentage yield was thus} = \frac{\text{Weight obtained}}{\text{Weight of sample}} \times 100\%$$

### Animal treatment

The rats were divided into 6 groups (A, B, C, D, E and F.) of 6 rats each. Group A was the normal control that received 2 ml/kg/day of citrate buffer intraperitoneally (i.p) for 3 days. Groups B, C, D and E were induced with 60 mg/kg of streptozotocin in citrate buffer intraperitoneally once daily for three days and observed for one week. Group B animals were left untreated, while Groups C, D, E, and F were treated with ethyl acetate fraction of lycopene (EAFL) at dosages of 20, 40, 60 and 20 mg/kg/day via oral route respectively for 30 days. At the end of the experimental period, the rats were sacrificed through cervical dislocation.

## Experimental design

| SN | Groups | Agents | Dose | Route | Duration |
|---|---|---|---|---|---|
| 1 | A | Citrate buffer | 60 mg/kg | Oral | 30 days |
| 2 | B | Streptozotocin | 60 mg/kg | Intraperitoneal | 3 days |
| 3 | C | Streptozotocin + EAFL | 60 mg/kg + 20 mg/kg/day | Intraperitoneal, Oral | 3 days 30 days |
| 4 | D | Streptozotocin + EAFL | 60 mg/kg + 40 mg/kg | Intraperitoneal, Oral | 3days 30days |
| 5 | E | Streptozotocin + EAFL | 60 mg/kg + 60 mg/kg | Intraperitoneal, Oral | 3 days 30 days |
| 6 | F | EAFL. | 20 mg/kg/day | Oral | 30 days |

**Table a**

## Determination of relative organ weight

The animals were weighed twice weekly and the mean weight calculated then the relative organ weight was calculated by measuring the organ weight at sacrifice then this was divided by the mean body weight.

$$\text{Relative organ weight (\%)} = \frac{\text{Weight of the organ(g)} \times 100\%}{\text{Mean weight of the animals(g)}}$$

Mossa., *et al.* 2015.

## Histological and immunohistochemical studies

The Kidneys were fixed in 10% neutral buffered formalin. Tissues were processed with paraffin embedding and sections of 5µm were cut and stained; Haematoxylin and Eosin stain was used to demonstrate general tissue histoarchitecture.

## Immunohistochemistry

Tissue sections were de-parafinized at 60 °C for 30 min or in xylenes and hydrated with the following series of washes, each of the wash was performed in a separate glass jar containing 200-250ml: xylene (5min), xylene (5min), xylene (5min), 100% ethanol (1 min), 100% ethanol (1 min), 100% ethanol (1 min) 95% ethanol (vol/vol 1min), 95% ethanol (vol/vol 1min), 70% ethanol (vol/vol 1min) at room temperature in a ventilation hood. Slides were washed in the distilled water for 5 min, at room temperature and quenched in endogenous peroxidase with 200-250 ml 3% $H_2O_2$ (vol/vol) in a glass jar for 10 min and washed in 200-250 ml Phosphate Buffered Saline (PBS) in a glass jar twice for 5 min. The tissue samples were encircled using a hydrophobicPen, non-specific background staining, was blocked with 300µl of normal horse serum (vol/vol, 50µl normal horse serum in 5 ml PBS for 30 min in a humidity chamber. The serum was drained from the slides and primary antibody was applied in a volume of 300µl. Polyclonal antibody 1:200 made in Dako Antibody Diluent. Control sections were incubated in 300 µl antibody diluents only, slides were incubated in humidity chamber for 1 hr, washed in 200-250 ml Phosphate Buffered Saline in a jar twice for 5 min and secondary antibody was applied in a volume of 300 µl, biotinylated goat- anti-rabbit- mouse IgG (1:500) for 1 hr (5 ml PBS, 75 µl horse serum, 25 µl biotinylated goat-anti rabbit igG) in a humidity chamber. Then the slides were washed twice in phosphate buffered saline for 5 min. Approximately 300 µl Vector Elite ABC (avidin biotin complex) reagent were applied for 1 hour, washed in 200-250 ml phosphate buffered saline in a glass jar twice for 5 min, developed signal in approximately 300µl DAB plus for 1-2 min and was rinsed in 200-250 ml distilled water in a glass jar for 1 min.

Then counterstained in 200-250 ml 100% Harris Hematoxylin (wt/vol) in distilled water for 3 min, rinsed in 200-250 ml tap water in a glass jar for 1 min, blued in 200-250 ml ammonia water in a glass jar for 5 min and rinsed in 200-250ml water in a jar for 1 min. Slides were further dehydrated for 1 min in 200-250 ml with the following series of washes in a glass jar: 70% ethanol, 95% ethanol, 100% ethanol, 100% ethanol, 100% ethanol, xylenes, xylenes in a ventilation hood and mounted with 2-3 drops of mounting media (1:1 per mount/xylene) in a ventilation hood.

## Cystatin C Quantitative Analysis.

Staining intensity was done with Image J software and the immunohistochemical stained Cystatin C photomicrographs were imported into image J software. Colour deconvolution and adjustment to RGB was done with the threshold adjusted to the area of interest. The images were analysed and quantified by the software and the values generated an excel sheets which were analyzed statistically.

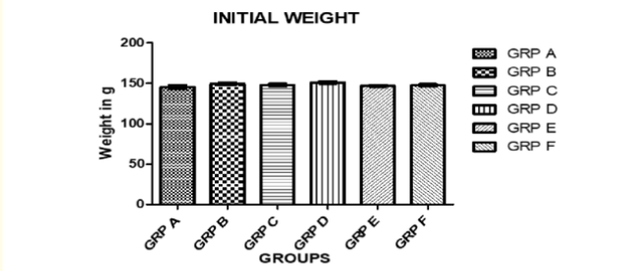
## Statistical analysis

One way ANOVA was used for analysis of the data generated, then Student Newman-Keuls (SNK) test was used for multiple comparisons. Graph pad prism 5 (Version 5.03 Graph Pad inc.) was the statistical package used for the analysis of data. Significant difference was set at $p< 0.05$.

# Results

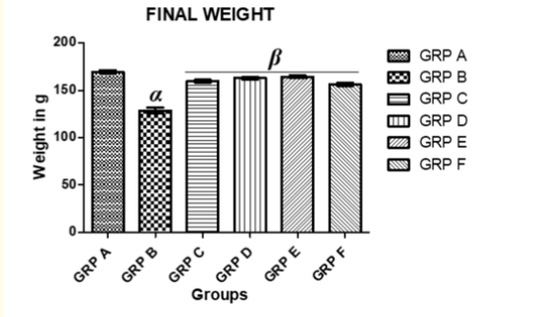
## Body weight

There was decrease in the mean body weight of the rats towards the 1st week of the experiment in test groups B, C, D, E and F, whereas the treated groups improved and this accompanied a steady increment in the weight gain once the intervention started, till the end of the experiment (Figure 1 and Figure 2).




The graph showing the mean initial body weight of the rats across the groups 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 1**

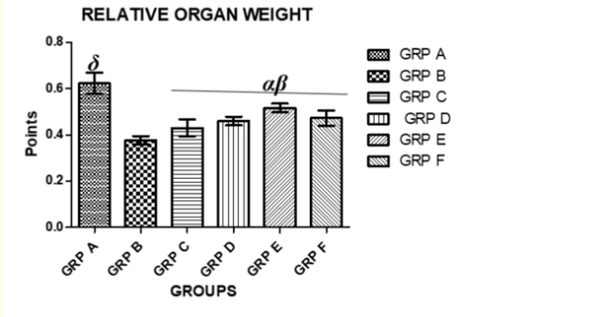



The graph showing the mean final weight of the rats across the groups after treatment.
Results presented as Mean ± SEM, n=6 (p<0.05)
*α Significantly different from normal control at p < 0.05*
*β Significantly different from toxic control at p < 0.05*
*δ Significantly different from C,D,E and F at p<0.05*

**Figure 2**

## Relative organ weight

The relative organ weight of the animals in the test groups was noted to be reduced generally when compared to the normal control group, the animals in the untreated group was observed to have even more reduction in relative organ weight when compared to the intervening groups (Figure 3).

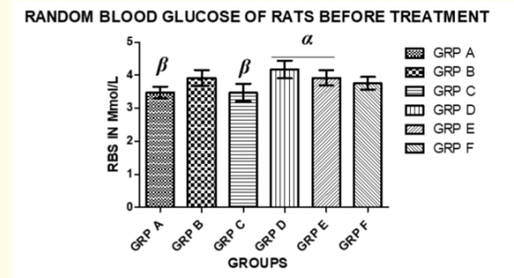



The graph showing the mean relative organ weight of the rats across the group after treatment.
Results presented as Mean ± SEM, n=6 (p<0.05)
*α Significantly different from normal control at p < 0.05*
*β Significantly different from toxic control at p < 0.05*
*δ Significantly different from C,D,E and F at p<0.05*

**Figure 3**

## Level of blood glucose

At approximately 72 hours after administration of STZ, the mean glucose levels were markedly elevated and this increase was persistent in the untreated group (B) while the blood glucose levels in the intervening groups were significantly ($p < 0.05$) reduced when compared with the untreated group (Figure 5). (Figure 4-6)

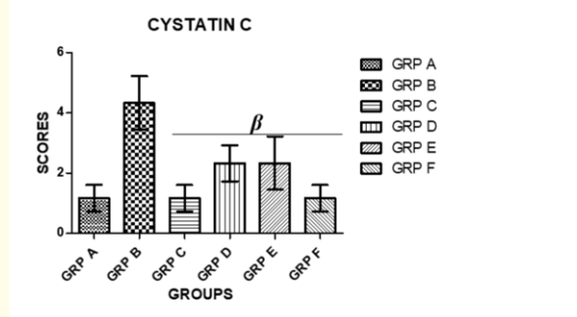



The graph showing the mean Random blood glucose levels of the rats across the group after treatment. Results presented as Mean ± SEM, n=5 (p<0.05)
*α Significantly different from normal control at p < 0.05*
*β Significantly different from toxic control at p < 0.05*
*δ Significantly different from C,D,E and F at p<0.05*

**Figure 4**


The graph showing the mean Random blood glucose levels of the rats across the group after treatment. Results presented as Mean ± SEM, n=5 (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**




The graph showing the mean Renal Cystatin count of the rats across the group after treatment.
Results presented as Mean ± SEM, n=5 (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**

## Immunohistochemistry

The Cystatin C immunohistochemistry of the rats' kidney was done, the tissue sections of diabetic groups showed areas of antigen antibody reactions when compared to the normal control group. The untreated group showed more intense immunoreactive areas when compared to the treated groups. The Cystatin C quantification showed more immunoreactive β-cells in the untreated groups than the treated groups when compared with the normal control. The mean of the cystatin C color intensity (Mean ± SEM) is as shown in fig 6. There was significant effect ($p < 0.05$) of EAFL on the cystatin C immunoreactivity in the kidney of the treated diabetic groups when compared with the untreated diabetic group.

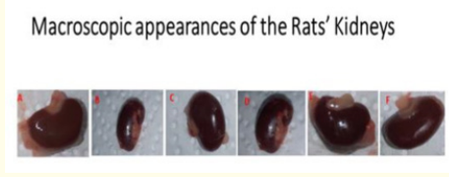



**Plate 1**

The macroscopic appearances of the representative kidneys of the rats from various groups; A-F. MAG: X1.

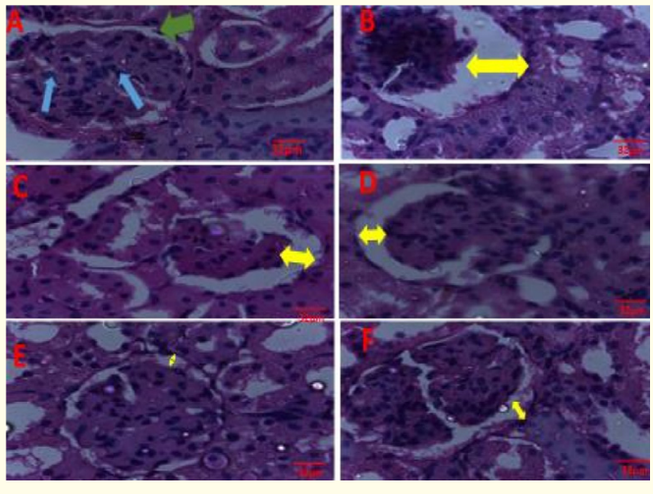



**Plate 2**

Representative photomicrographs of kidney tissue from central cortex on light microscope showing histoarchitectural differences across the groups: A is the normal control group that was not induced nor treated, B is the positive control treated with STZ only. Groups C, D and E received STZ+20, 40, 60mg/kg of ethyl acetatae fraction of lycopene respectively, while F is STZ+ 5mg/kg of Glibenclamide. The yellow arrows show the gaps in the urinary space across the group, green and blue arrows shows the podocyte and mesangial cells respectively…. H and E Stain X 1000.

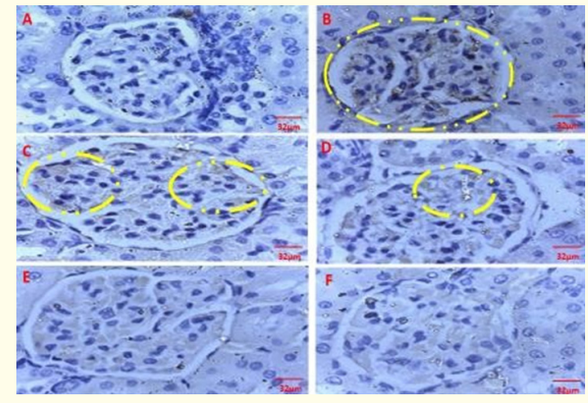



**Plate 3**

Sample photomicrographs of kidney tissue showing immunostained sections in different groups, reactive areas are darkly stained. A is the normal control group; B is the positive control treated with STZ only. Groups C, D and E received STZ+20, 40 and 60mg/kg of EAFL respectively, while F is STZ + 5mg/kg of Glibenclamide… Cystatin C immunostain X 1000.

## Discussion

Dietary therapy has been severally recommended in the management of diabetes mellitus whereas it received little cognizance. Diabetes ensues when the body does not properly process food to be used as energy, because there are combinations of various disturbances in the metabolic system of the body due to chronic hyperglycemia associated with abnormal metabolism of carbohydrates, fats and protein [1]. The incidence of diabetes rose from 108 million in 1980 to 422 million in 2014. The mortality associated with diabetes mellitus between 2000 and 2016 was noticed to increase by 5% [8]. Its incidence is increasing rapidly and it is estimated that by 2030 this number will almost double [19].

This research investigated the effect of ethyl acetate fraction of lycopene on immunohistochemistry of cystatin C in kidney of streptozotocin-induced diabetic wistar rats.

The study showed that chronic hyperglycemia led to relative reduction in weight gain as well as marginal decrease in organ size and weight especially in the untreated group (B) when compared with normal control group (A). There were however, increase in both the body weight as well as the relative organ weight in the test groups C, D, E and F with remarkable weight gain observed in all groups except the toxic group (B). This is in keeping with the work done by Chinwe., *et al.* 2015 who documented that diabetes significantly reduces body weight of experimental animal in untreated group when compared with the normal control and the test groups,

with intervention agent which restores the body weight loss. It is also in keeping with the research by Bruckner., *et al.* 1986 and Koporec., *et al.* 1995 and Japan Bioassay Research Center 1998; Nagano., *et al.* 1998 which was later reviewed by Bruckner and Koporec in 2006 who stated in their works that weight loss probably may be due to reduction in food intake via the satiety and appetite center in the hypothalamus [11].

Findings from this study also revealed that there were significant reduction ($P < 0.05$) in blood glucose levels towards normal in EAFL treated Wistar rats after 30 days of intervention, the random blood glucose level was tending towards normal reference value (Figure 4 and Figure 5) and this is directly proportional to the dose of the lycopene administered, this is in keeping with the work done by Kuhad., *et al.* 2008 who reported that lycopene has significant, dose-dependent antidiabetic action in streptozotocin-induced diabetic Wistar rats

[12].

The microanatomy of the kidneys was examined in all groups and the histoarchitecture revealed distortion in renal corpuscular morphology in untreated diabetic group (group B) which shows distortion in the renal corpuscles, characterized by presence of thick glomerular membrane, mesangial cells (blue arrows in plate 2) were noticed to expand when compared to the normal control (group A). The urinary spaces in toxic group B were also noticed to be wide in the toxic group when compared to normal control with gradual reduction in the bowman's space which improves directly proportionally to the dose of lycopene. Administration of lycopene in graded doses after diabetes induction was noticed to gradually reverse the initial distortion by chronic hyperglycemic state when compared to untreated diabetic group, administration of lycopene in graded doses gradually reversed the alteration seen in renal corpuscles. The groups C, D and E showed reversal of the histological distortion with near normal histoarchitectural organization when compared with the normal controls (A and F), this may be due to free radical scavenging property which course was probably through decongestion of renal vascular channels and regeneration of the renal corpuscles in the treated groups treated with AEFL, this is in keeping with the work done by Kuhad., *et al.* 2008 who reported that lycopene has significant, dose-dependent antidiabetic action in streptozotocin-induced diabetic Wistar rats.

## Conclusion

From the findings above we then conclude that EAFL preserves the macro-anatomy of the kidney and reversed the chronic hyperglycemic state characterizing DM. The distortion in renal histological morphology was reversed in the treated diabetic animals and restored the histoarchitectural alteration. Moreover, the renal cystatin C antigen antibody reactions were only accentuated in the untreated diabetic group while no immune reactivity was observed in high dose EAFL. The EAFL potentials exhibited here may be of immense benefit in treating diabetes and its attendant complications if and when researched further.

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