Original Research Paper

Serum levels of some Inflammatory Markers in Alloxan induced Diabetic rats Treated with *Terminalia catappa* leaf extract and Exogenous Insulin

ABSTRACT

Background: Inflammation is said to be associated with hyperglycaemia and is implicated in the complications of diabetes.

Aim: This study was designed to investigate the level of some inflammatory markers in blood of diabetic rats administered with aqueous leaf extract of *Terminalia catappa* and exogenous insulin.

Materials and Methods: Thirty five (35) Wistar rats were assigned to 5 groups of 7 animals each. Group 1 served as the control and received 5ml/kg body weight of distilled water and group 2 received orally, 130/kg body weight of aqueous leaf extract of *Terminalia catappa.* Groups 3 (diabetic only), 4 (diabetic + extract) and 5 (diabetic + insulin) were administered 150mg/kg body weight of alloxan solution intraperitoneally to induce diabetes and blood glucose levels ≥200mg/dl after 72 hours were considered diabetic. Then 5ml/kg bodyweight of distilled water, 130mg/kg body weight of *Terminalia catappa* leaf extract was given orally and 0.75U/kg body weight of insulin was administered subcutaneously to respective groups for 14 days.

Result: The results showed significant ($P < 0.05$) increase in serum levels of C-reactive protein, Interleukin-6 and blood fibrinogen in diabetic group compared to control. These inflammatory biomarkers significantly ($P<0.05$) reduced in diabetic group treated with extract and insulin.

Conclusion: Therefore aqueous leaf extract of *Terminalia catappa* can reduce some inflammatory cytokines and ameliorate inflammation in diabetes similar to exogenous insulin.

Key words: C-reactive protein, Interleukin-6, fibrinogen, inflammation, diabetes mellitus, Terminalia catappa, Insulin.

1. INTRODUCTION

It is known that diabetes mellitus is not only a metabolic disorder because several molecules associated with inflammation plays important role in the development of diabetes and it related complications [20]. The role of

inflammation in type 1 and type 2 diabetes mellitus has been clearly established [6, 18]. Studies have shown that subjects who developed diabetes presents with elevated levels of inflammatory markers [19]. Markers of acute phase response which have been reported in type 2 diabetes include sialic acid, alpha-1 acid glycoprotein, c-reactive protein, serum acute phase reactants such as amyloid and mediator cytokine; interleukin-6 [33]. In view of these, targeted anti-inflammatory therapy has been suggested for both prevention and treatment of diabetes [35].

Since there is association of hyperglycaemia, inflammation and vascular complications in diabetes, it is considered that some anti-diabetic drugs may alleviate inflammation by reducing hyperglycaemia [35]. Insulin therapy is reported to possess anti-inflammatory effect because of its ability to reduce the inflammatory cytokines. However, the controversy surrounding this claim suggests investigation of other possible approaches in targeting inflammation alongside glycaemic control. Besides the inconvenience experienced in insulin therapy, economic impact may be the reason for the increase mortality associated with diabetes. Therefore the World Health Organization has advocated the use of natural product due to its availability and affordability [49]. *Terminalia catappa* as a natural plant is said to possess both anti-diabetic and anti-inflammatory properties. A lot of research finding have been reported on its anti-diabetic effect [2] but not much is said about the anti-inflammatory function. The present research is therefore designed to evaluate the status of some inflammatory biomarkers in *Terminalia catappa* leaf extract treated diabetic condition using rat model.

2. MATERIALS AND METHODS

2.1 Preparation of Plant Extract

Fresh leaves of *Terminalia catappa* were collected at the premises of the University of Calabar and the area was free of pesticides and other contaminants. The leaves were authenticated by a botanist at the Department of Botany and Ecological studies, University of Calabar. The leaves were then washed with clean water to remove debris. The water was blotted out and kept overnight at room temperature to dry up. The clean leaves were pulverized and 5000g of the pulverized leaves were soaked in 5 litres of deionised water for 18 hours. The mixture was filtered using muslin cloth and evaporated to dryness using thermostatic water bath at 45^{o}C until a semi solid paste of 204.18g of the extract was obtained after evaporation representing a percentage yield of 4.08%. The extract was stored in refrigerator for later use.

2.2 Preparation of Experimental Animal

Healthy adult male albino Wistar rats weighing between 150-200g were used for the study. The animals were procured from the animal house, Department of Physiology, Faculty of Basic Medical Sciences, University of Calabar. The animals were housed in a well ventilated cage in the animal house and they were allowed to acclimatize for two weeks and maintained in a 24 hours dark and light cycle. The animals were fed with standard pellets (from Guinea Feeds, Plc Nigeria) and have access to water ad libitum.

2.3 Induction of diabetes

Diabetes was induced by intraperitoneal injection of alloxan monohydrate at a dose of 150mg/Kg body weight [22, 25,10]. The animals were assessed for development of diabetes after 72 hours [5] by obtaining blood sample from the tip of the tail. The blood sample was dropped into glucose strip to measure the glucose level using a glucometer (One Touch Ultra (Life Scan Inc, U.S.A). Blood glucose of ≥200mg/dl was considered diabetic (normal range of blood glucose in rat is 80 – 120mg/dl) and were used for the experiments [25, 5].

2.4 Experimental Design

The experimental animals were randomly distributed into five (5) groups of seven (n=7) rats per group as follows:

Group 1: Control group administered with only distilled water orally at a dose of 5ml/kg body weight.

Group 2: Only aqueous extract of *Terminalia Catappa* at a dose of 130mg/kg body weight administered orally.

Group 3: Diabetic group administered with only distilled water orally at a dose of 5ml/Kg body weight.

Group 4: Diabetic group treated with *Terminalia catappa* extract at a dose of 130mg/Kg body weight by oral administration.

Group 5**:** Diabetic group treated with exogenous Insulin at a dose of 0.75U/Kg body weight by subcutaneous administration.

2.5 Rat C-reactive protein assay

Serum C-reactive protein level was analysed by ELISA method. Commercial rat C-reactive protein analysis kit using Sandwich-ELISA (Enzyme Linked Immunosorbent Assay) method. Standards or samples were added to the appropriate microelisastripplate wells and combined to the specific antibody. The absorbance or optical density (OD) is measured spectrophotometrically at a wavelength of 450nm and readings are obtained using Microtiter Plate Reader, the Optical Density value is proportional to the concentration C-reactive protein.

2.6 Rat interleukin-6 assay

Serum from the blood of the experimental animals was used to analyse the Interleukin-6. Interleukin-6 assay was analysed with commercially prepared rat Interleukin-6 analysis kit using Sandwich-ELISA (Enzyme Linked Immunosorbent Assay) method. Standards or samples are added to the appropriate microelisastripplate wells and combined to the specific antibody. The absorbance or optical density (OD) was measured spectrophotometrically at a wavelength of 450nm and readings are obtained using Microtiter Plate Reader, the Optical Density value is proportional to the concentration of Interleukin-6.

2.7 Rat Fibrinogen Assay

Whole blood was collected into plain sample bottle and used for determination of fibrinogen. The fibrinogen level was determined by Enzyme Linked Immunosorbent Assay (ELIZA) method. Standards or samples were added to the appropriate microelisastripplate wells and combined to the fibrinogen antibody. The absorbance

or optical density (OD) was measured by spectrophotometry at wavelength of 450nm and the result read on a microplate immediately. The Optical Density value is proportional to the concentration of fibrinogen. The assay was performed at room temperature (18-25°C).

2.8 Statistical Analysis

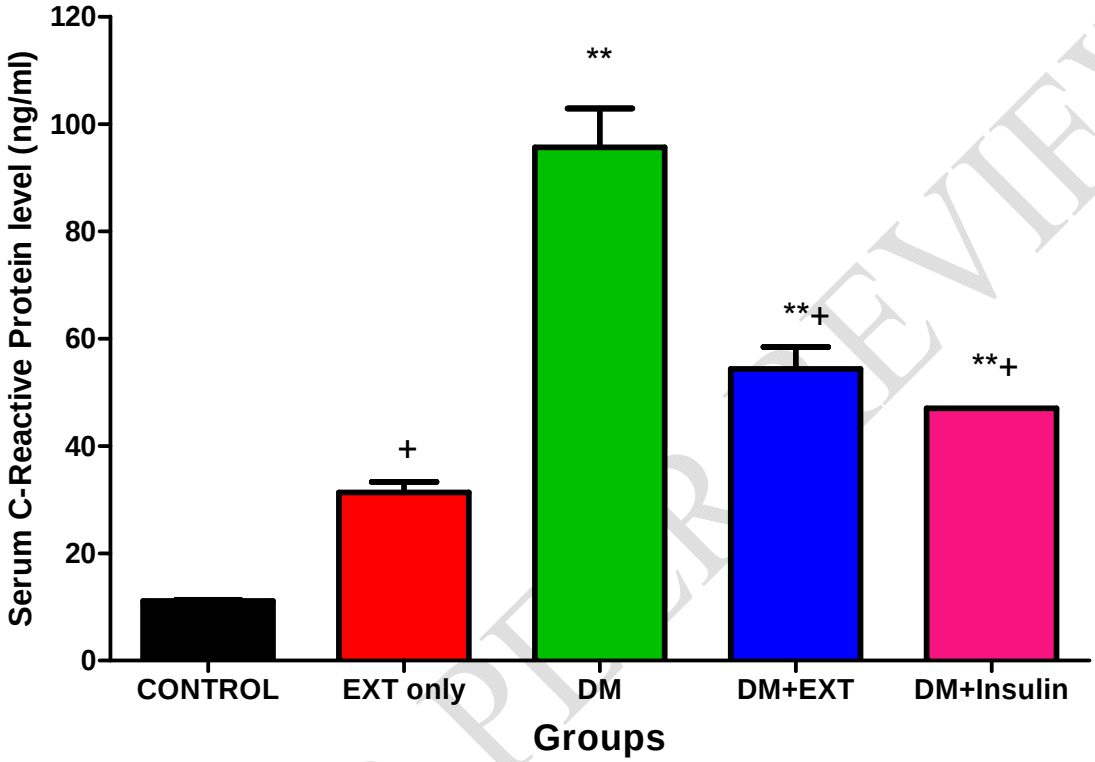
The data obtained from the result was subjected to statistical testing using one way ANOVA followed by Tukey test using Graph Pad Prisms software 6.0. Data were expressed as mean ± standard error of mean (SEM). Results with values of $P< 0.05$ and $P<0.01$ were considered significant when compared to untreated diabetic group and control group respectively.

3. RESULTS

3.1 Serum level of C- reactive protein

The C-reactive protein analysis is shown in (figure 1). The serum level of C-reactive protein in control group was 11.0±0.3ng/ml while the non-diabetic extract fed group significantly increased ($P<0.05$) to a mean value of

31.32±1.9ng/ml when compared to control. In the untreated diabetic group, the serum level of C-reactive protein significantly (P<0.01) increased to 95.67±5.6ng/ml when compared to control. The serum levels of C - reactive protein reduced significantly (P<0.05) to 54.33±3.2ng/ml and 47±0.53 ng/ml in the extract treated diabetic group and insulin treated group respectively when compared to the untreated diabetic group.



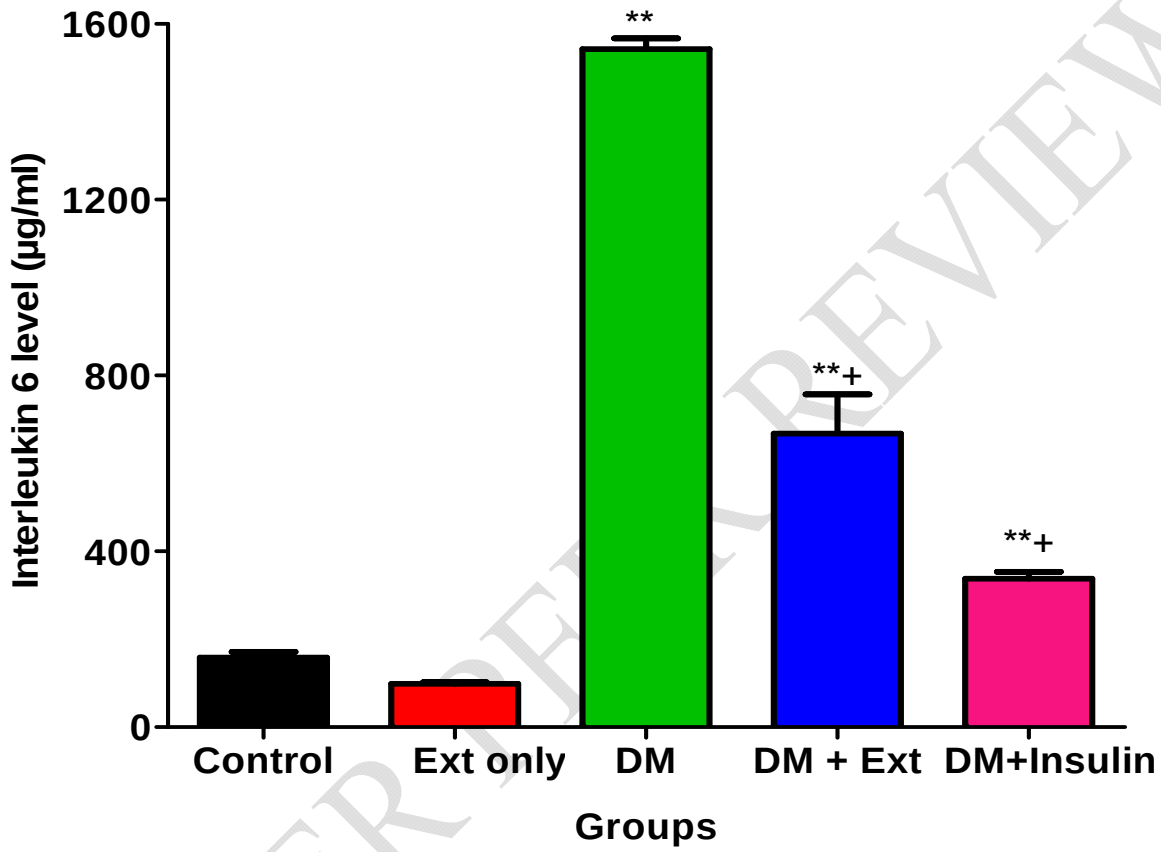
N= 7, Ext= Extract; DM=Diabetic
**** = *P* < 0.01 compared with control group; + = *P* < 0.05 compared with untreated diabetic group**

Fig. 1 Serum C-reactive protein in experimental group compared with control group.

3.2 Serum level of Interleukin - 6

The result of the inflammatory marker interlukin-6 is represented in (figure 2). Serum level of interlukin-6 in the control group was 157.9±13.3 µg/ml while the non diabetic extract treated group level was 98.29±3.4µg/ml and this was significantly lower when compared to control. In the untreated diabetic group, the interlukin-6 was

significantly higher (P<0.01) with mean value of 1543±18.9 µg/ml when compared with control. The interleukin-6 level was observed to significantly reduce (P<0.05) to 667.5±69.2µg/ml in the diabetic group treated with extract when compared with the untreated diabetic group. The mean value in the insulin treated diabetic group also showed a significant reduction (P<0.05) to a mean value of 337.3±12.4 µg/ml which was twice as reduced as that of the extract treated group when compared with the untreated diabetic group.



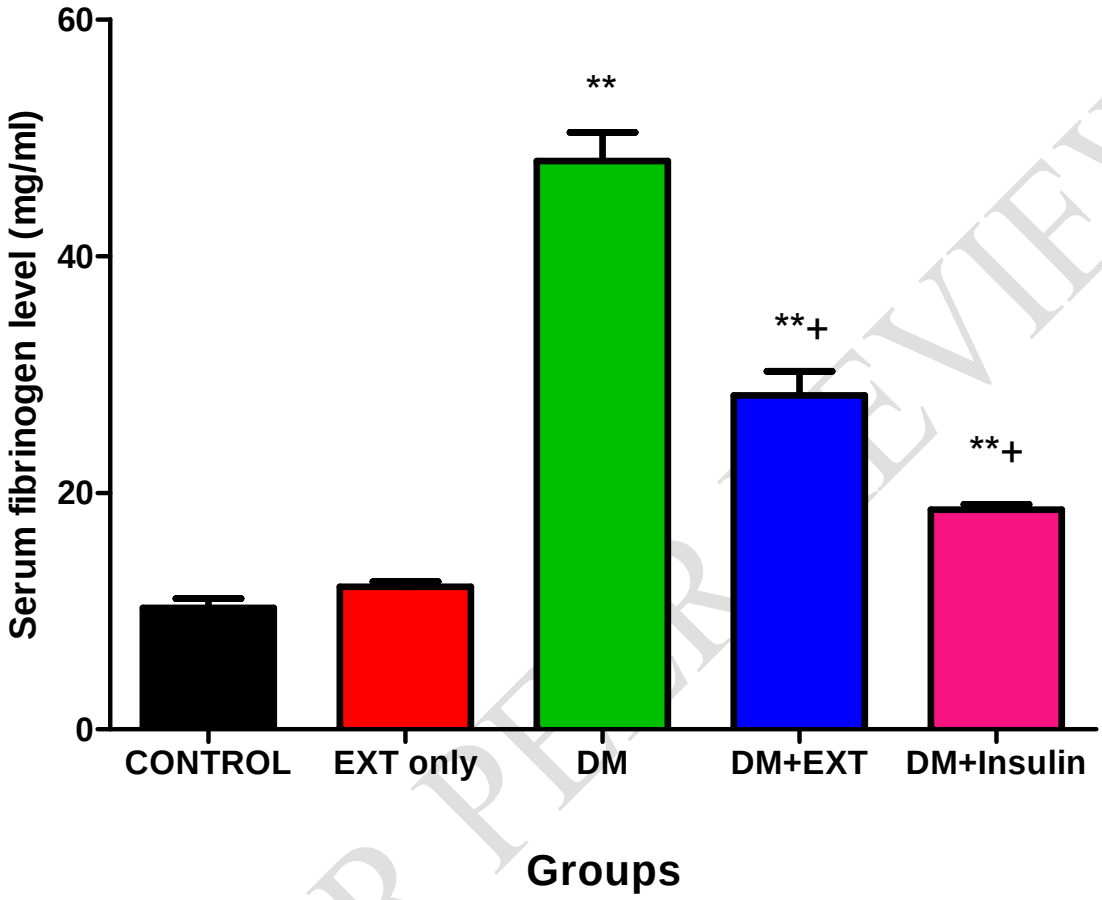
N= 7, Ext= Extract; DM=Diabetic
**** = *P* < 0.01 compared with control group; + = *P* < 0.05 compared with untreated diabetic group**

Fig. 2 Serum level of Interleukin-6 in experimental group compared with control group.

3.3 Serum level of Fibrinogen

The results of serum level of fibrinogen are as shown in (figure 3). The result showed that the serum level of fibrinogen increased from mean value of 10.28±0.77mg/ml in the control to 12.05±0.43mg/ml in extract treated non-diabetic group. The mean value increased significantly (P<0.01) to 48.03±1.9mg/ml in the untreated

diabetic group when compared to control. When the diabetic group were treated with extract there was a significant reduction (*P*<0.05) to a mean value of 28.24±1.6mg/ml when compared to the untreated diabetic group. The mean value was further significantly reduced (P<0.05) to 18.59±0.34mg/ml in the diabetic group with insulin treatment when compared to the untreated diabetic group.



N= 7, Ext= Extract; DM=Diabetic
**** = *P* < 0.01 compared with control group; + = *P* < 0.05 compared with untreated diabetic group**

Fig. 3 Serum fibrinogen level in experimental group compared with control group.

4. DISCUSSION

The levels of inflammatory biomarkers were assessed in diabetic rats treated with aqueous leave extract of *Terminalia catappa* and exogenous insulin. The result showed that the C-reactive protein level was high in the diabetic group compared to control. C-reactive protein is an acute phase protein of inflammation with an

increased expression in systemic inflammation [38]. The result of this study is consistent with other research findings which has established elevated C-reactive protein levels in people with impaired glucose tolerance and frank diabetes [15,50] compared with those without diabetes [17,15,16] although it is also associated with HbA1c in people without diabetes. There are research evidences which support the link between hyperglycemia and inflammation [40] demonstrating simultaneous inflammation, endothelial dysfunction, and insulin resistance at the physiologic level [50, 14]. This implies that inflammation may not only be implicated in the development of diabetes, but also in ongoing levels of hyperglycemia once diabetes is established thereby generating a cycle of cross-talk between inflammation and hyperglycemia and indeed a platform for development of various complications associated with diabetes mellitus.

C-reactive protein is not only related to the development of type 1 diabetes [12, 36, 13], insulin resistance and type 2 diabetes [41,12,36,13,50] but also linked to cardiovascular risk. This inflammatory marker is established as a powerful independent risk factor for atherosclerosis and atherosclerosis-related diseases [29, 46] such as myocardial infarction [38,23,39]. The level is elevated in the blood of patients with essential hypertension [27], abdominal aortic aneurysms [45,44] with enhanced systemic or local arterial strain and it correlates independently with blood pressure [43] and arterial stiffness [24]. The wide spread link between this biomarker of inflammation can explain why diabetes can lead to cardiovascular diseases and other complications.

However, the observed increase was reduced by the aqueous extract of *Terminalia catappa* and insulin in both diabetic extract treated and insulin treated groups respectively. The result is in agreement with current position that natural treatments can help to reduce inflammation in the blood [37]. Some of these natural substances which are useful in lowering C-reactive protein levels and inflammation in the blood are fish oil containing two of the most therapeutic Omega 3 Fatty Acids; the DHA and EPA. These two fatty acids are the most readily absorbed by the body [37]. Ginger can also help reduce inflammation, as it relaxes the muscles surrounding blood vessels and facilitates blood flow throughout the body [11]. Aqueous leave extract of *Terminalia catappa* is therefore a natural product that can lower C-reactive protein significantly as such can ameliorate the hyperglycemia induced inflammation in diabetes mellitus. However, the level of reduction of C-reactive protein in the insulin treated diabetic group was more marked compared to the effect caused by the extract but for the purpose of getting alternative to insulin therapy, *Terminalia catappa* could be a useful anti-inflammatory substitute in diabetes.

To further investigate on the inflammatory biomarkers, interleukin-6 was analysed. The serum level of interleukin 6 was observed to increase outrageously in diabetic group compared to control. Several studies have reported increase in Interleukin-6 in people with features of insulin resistance and overt type 2 diabetes [34]. Interleukin-6 is a well known pro-inflammatory cytokine which is secreted by a variety of tissues such as activated leukocytes, lipocytes, and endothelial cells [36], smooth muscle cells, fibroblasts, lymphocytes and macrophages [32,4]. The downstream effect is mediation of acute phase response by activating C-reactive protein and fibrinogen [36]. In other studies Interleukin-6 has been identified as a factor responsible for the development of insulin resistance, type 2 diabetes and complications of diabetes [26]. As a marker of endothelial dysfunction, Esteve *et al*., 2007 [9] and Nguyen *et al.,* 2012 [31] has reported that Interleukin-6

regulates the expression of adhesion molecules and other cytokines in endothelial cells to cause micro vascular complications in diabetes and mediator of cardiovascular disease development [47]. In this study serum level of interleukin-6 was reduced significantly in the diabetic group treated with the extract. This potential is laudable because of the central role of Interleukin-6 in systemic inflammation. With the extract of *Terminalia catappa* leaves, the deleterious effect of diabetes could be mitigated since the pro-inflammatory cytokine is reduced. Interleukin-6 was also reduced by insulin in this study. This reduction agrees with other research findings which revealed that besides the controlling of blood glucose level, insulin equally targets the cytokines of inflammation. The level of reduction was observed to be more in the insulin treatment than the extract.

Another important biomarker of inflammation is fibrinogen. In this study, the level of fibrinogen in blood was increased in the diabetic group. This result is consistent with the work of Barazzoni *et al.,* 2000 [3] who reported raised level of fibrinogen in type 2 diabetes. Plasma fibrinogen is an important component of the coagulation cascade, as well as a major determinant of blood viscosity and blood flow [21]. Increased fibrinogen content in the blood is considered an indicator for a pro-inflammatory state and a high-risk marker for developing vascular inflammatory diseases, such as hypertension and atherosclerosis and increased risk of cardiovascular disorders such as ischemic heart disease, stroke and other thromboembolism [48, 30]. Similarly, elevated levels of fibrin degradation products, such as D-dimer, are extensively used in clinical practice as indicators for inflammation, markers for increased coagulation activity and risk predictors for thrombotic events [28]. In addition, the peptides released as a part of fibrin formation like fibrinopeptide B, which is cleaved from fibrinogen by thrombin can act as chemo-attractants for leukocytes and thus independently modulate inflammatory responses [42,7].

The elevated blood level fibrinogen observed in the diabetic rats was reduced in the diabetic extract treated group as well as insulin treated group. The insulin induced decrease in the blood fibrinogen level was stronger than that of the extract. A constantly increasing body of evidence supports a prominent role of fibrinogen and degradation products in regulating the inflammatory response in several target tissues [1] and its involvement in micro and macro vascular complications in diabetes [8].

5. CONCLUSION

In conclusion, the results of this research shows that some major biomarkers of inflammation namely; C-reactive protein, Interleukin-6 and fibrinogen were elevated in alloxan induced diabetes mellitus in rats. This suggests the possible link between hyperglycaemia and inflammation in diabetes. The increased blood levels of these biomarkers were remarkably reduced by the aqueous leaf extract of *Terminlia catappa* and exogenous insulin with the effect stronger in insulin than the extract. However, extract of *Terminalia catappa* can serve as a natural anti-inflammatory alternative close to insulin in the management of diabetes.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per International standard or University standard ethical approval has been collected and preserved by the authors.

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