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Comparative Effect of Aspirin, Meloxicam and *Terminalia catappa* Leaf Extract on Serum Levels of Some Inflammatory Markers in Alloxan Induced Diabetic Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Author EEB designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors AEA and DUO managed the analyses of the study. Author AEA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: The association between diabetes mellitus and inflammation is established but the use of non-steroidal anti-inflammatory drugs is not without some health risk.
Aim: The study was aimed at comparing the levels of some inflammatory biomarkers in diabetic rats treated with aqueous leaf extract of *Terminalia catappa*, non steroidal anti-inflammatory drugs (NSAIDs) and exogenous insulin.
Materials and Methods: Thirty six (36) Wistar rats were assigned to 6 groups of 6 animals each. Group 1 and 2 served as normal and diabetic controls and received orally 5ml/kg body weight of distilled water. Group 3 was diabetic treated orally with 130mg/kg body weight of aqueous leaf extract of *Terminalia catappa*. And groups 4, 5 and 6 were administered orally with aspirin (30mg/kg), meloxicam (2mg/kg) and 0.75U/kg body weight of insulin subcutaneously. Diabetes was induced with intraperitoneal injection of 150mg/kg body weight of alloxan solution and diabetes confirmed after 72 hours with blood glucose levels ≥200mg/dl. The experiment lasted for 14 days

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and blood was collected by cardiac puncture for serum analysis of C-reactive protein, Interleukin-6 and Fibrinogen by ELISA method.
Results: 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 biomarker were significantly (P<0.05) reduced by the extract, aspirin, meloxicam and insulin.
Conclusion: The reduced levels of C-reactive protein, Interleukin-6 and fibrinogen by aqueous leaf extract of *Terminalia catappa* was significant compared to aspirin and meloxicam. This may present the extract as a potent anti-inflammatory agent and could complement the function of insulin in diabetes treatment.

Keywords: C-reactive protein; Interleukin-6; fibrinogen; inflammation; diabetes mellitus; Terminalia catappa; Insulin; aspirin; meloxicam; NSAIDs.

1. INTRODUCTION

Several studies have reported the existing association between hyperglycaemia, inflammation and diabetes complications [1]. Investigations on various markers of inflammation in different population groups have confirmed this association [2,3]. Biomarkers of inflammation such as interleukin-6, C-reactive protein, tissue necrosis factor, fibrinogen and even total white blood cell count [4] are increased in cases of injury, infection [5] or obesity [6]. It is now well appreciated that low grade chronic inflammation is central to the pathology of the pancreatic islet in type 1 diabetes and also plays an important role in the pathogenesis of type 2 diabetes, including obesity-related insulin resistance, impaired insulin secretion, and diabetes-related vascular complications [7]. The development of overt diabetes of any category results in hyperglycemia. Other findings have shown reverse causality in which hyperglycemia is itself pro-inflammatory through the generation of oxidative stress [8]. Thus there is a vicious cycle of inflammation – hyperglycemia and hyperglycemia – inflammation association. Following the first line of causality and effect, since inflammation leads to poor glycaemic control, then treatment of inflammation with non-steroidal anti-inflammatory drugs (NSAIDs) may help improve glycaemic control. On the other hand, hypoglycemic and anti-diabetic drugs can reduce inflammation by reducing the hyperglycemia. But accumulated evidence on association between inflammation and complications of diabetes [9,10] has attracted the consideration of targeting inflammation to ameliorate diabetes, prevent its progression and diminish vascular complications [11]. However, the effects of immunomodulatory treatments are not limited to tissues involved in disease pathophysiology and thus might have unwarranted side effects. It is reported that some anti-diabetic agents may alleviate systemic and tissue-specific inflammation [12,13,14].

The anti-inflammatory effects of anti-diabetic agents might be mediated via their metabolic effects on hyperglycemia and hyperlipidemia or by directly modulating the immune system [1]. For example, glitazones that reduce insulin resistance, metformin which improves insulin sensitivity partly through activation of Adenosine Monophosphate-activated Kinase (AMPK), a key regulator of cellular energy homeostasis to exert both anti-inflammatory and antioxidant effects [15] and exogenous insulin which increases glucose uptake and disposal by stimulation of insulin dependent glucose transporter are all found to reduce inflammation [16]. Moreover, vasoactive drugs such as statins and Adenosine Converting Enzyme (ACE) inhibitors/angiotensin receptor antagonists often prescribed to people with diabetes also counteract inflammation and reduce the risk of diabetes complications in type 2 diabetes [4,6]. The complications of diabetes are divided into macrovascular (myocardial infarction and stroke) [13] and microvascular (nephropathy and retinopathy) complications. Several researches has supported that reduction in hyperglycemia may ameliorate the microvascular complications but such certainty is not attained in macrovascular complication [17]. This is because many cytokines have been released thus activating some inflammatory pathways which may not only be inhibited by glycaemic control. Therefore the use of drugs targeting some more specific biomarker and inflammatory signaling pathways may provide the needed reduction in morbidity and mortality resulting from diabetic complications. The use of pharmaceutical agent in treatment of disease is not without some side effect. With such side effects in addition to economic burden and unavailability of the anti-diabetic drugs, World

Health Organization encouraged the option of exploring the efficacy of medicinal plants in the treatment of diseases [18]. Among many ethnopharmacological plants, *Terminalia catappa* (*Combretaceae*) is useful in treatment of different ailments [19]. The plant is also called Almond tree or Tropical Almond and found commonly in the Tropics [20]. Extracts from the leaves and bark of this plant have been reported as antioxidant [21], anti-diabetic [22] and anti-inflammatory [23]. The current adjunct therapeutic target for diabetes is on inflammation and scientific exploration on anti-inflammatory potentials of this plant becomes necessary to investigate the possible link to its anti-diabetic property and compare it to the actions of non-steroidal anti-inflammatory drugs. This research aims at comparing the effect of *Terminalia catappa* leaf extract and non steroidal anti-inflammatory drugs (NSAIDs) on some inflammatory biomarkers in diabetic rats.

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 photographs of the leaf as taken directly are shown in plates 1 and 2 in the result section of this article. The leaves were authenticated by a botanist (Mrs E. G. Udoma) at the Department of Botany and Ecological studies, University of Calabar, Nigeria and it is documented in the herbarium with voucher number UUPH 22(a). 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 weighting 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, Nigeria. 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 [24,25,26]. The animals were assessed for development of diabetes after 72 hours [27] 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 [25,27].

2.4 Experimental Design

The experimental animals were randomly distributed into Six (6) groups of six (n=6) 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: Diabetic group administered with only distilled water orally at a dose of 5ml/Kg body weight.
Group 3: Diabetic group treated with aqueous leaf extract of *Terminalia catappa* at a dose of 130mg/Kg body weight by oral administration.
Group 4: Diabetic group treated orally with 30mg/kg body weight of aspirin.
Group 5: Diabetic group treated orally with 2mg/kg body weight of meloxicam.
Group 6: Diabetic group treated with exogenous Insulin at a dose of 0.75U/Kg body weight by subcutaneous administration.

2.5 Acute Toxicity Test (LD_{50})

The toxicity test of the *Terminalia catappa* aqueous leaf extract was carried out by Lorke's method [28] on mice weighing 15 and 25g. The animals were randomly divided into seven (7) groups of six mice per group. The extract was respectively administered to groups 1 to 7 at a dose of 500mg//Kg, 800mg/Kg, 1000mg/Kg, 1200mg/Kg, 1400mg/Kg, 1500mg/Kg, 1600mg/Kg body weight intraperitoneally. The animals were observed for physical signs of

toxicity such as gasping, writhing, palpitation and death after 24 hours. The median leathal dose (LD_{50}) was calculated as square root of maximum (most tolerable) dose producing 0% mortality (x) and minimum (least tolerable) dose producing 100% mortality (y) using the formula: LD50 = √xy.

2.6 Ethical Approval

The experimental protocol received full approval from Faculty Animal Research Ethics Committee (FAREC-FBMS), Faculty of Basic Medical Sciences University of Calabar, Nigeria with approval number 021PY30417.

2.7 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 [29]. Standards or samples were added to the appropriate microelisa strip plate 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 and the Optical Density value is proportional to the concentration of C-reactive protein.

2.8 Rat Interleukin-6 Assay

Serum from the blood of the experimental animals was used to analyse the Interleukin-6. Interleukin-6 assay was carried out with commercially prepared rat Interleukin-6 analysis kit using Sandwich-ELISA (Enzyme Linked Immunosorbent Assay) method [30]. Standards or samples are added to the appropriate microelisa strip plate 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 and the Optical Density value is proportional to the concentration of Interleukin-6.

2.9 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 (ELISA) method [31]. Standards or samples were added to the appropriate microelisa strip plate 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).

2.10 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 was expressed as mean ± standard error of mean (SEM). Results with values of $P < 0.05$ were considered significant.

3. RESULTS

3.1 Photograph of the *Terminalia catappa* Leaf

Plate 1 shows the leaves of *Terminalia cattapa* clustered at the end of the twigs and plate 2 shows a smooth coriaceous single green *Terminalia catappa* leaf.

Plate 1

Plate 2

Source: Direct photograph taken by Ben and colleagues, 7/2/2019

3.2 Toxicity Test (Ld_{50})

The result of the acute toxicity test showed that 0% mortality was obtained at 1200mg/Kg body weight and 100% mortality was at 1400mg/body weight. Thus the LD50 was √1200 x 1400 = 1296.15mg/Kg (approximately 1300mg/Kg). The experimental dose was obtained as 10% of the lethal dose being 130mg/Kg body weight.

3.3 C- Reactive Protein

The C-reactive protein analysis is shown in (Fig. 1). The serum level of C-reactive protein in control group was 30.33±2.51mg/dl while in the diabetic none treated group, the serum level significantly (*P*<0.05) rise to 110.33±6.24mg/dl. This was reduced significantly to 49.0±1.63mg/dl, 68.25±3.89mg/dl and 84.4±2.48 g/dl in the diabetic extract, aspirin and meloxicam treated groups respectively. Insulin treated group also show significant (*P*<0.05) reduction to a mean value of 71.67±1.95mg/dl.

3.4 Interleukin – 6

The result of the inflammatory marker, interlukin-6 is represented in (Fig. 2). Serum level of Interlukin-6 in the control group was 48.0±2.44ng/ml and 143.33±11.69ng/ml diabetic group. Diabetic extract treated group has a serum level of 78.5±1.84ng/ml and this was significantly (*P*<0.05) lower than the diabetic group. The diabetic aspirin and meloxicam treated groups have respectively the mean values of 88.6±2.47ng/ml and 86.6±2.24ng/ml which was significant when compared to diabetic group. The mean value in the insulin treated diabetic group also showed significant (*P*<0.05) reduction to a mean value of 90.67±4.29ng/ml.

3.5 Fibrinogen

The results of serum level of fibrinogen are as shown in (Fig. 3). The result showed that the serum level of fibrinogen increased from mean value of 106.33±1.33mg/dl in the control to 297.67±47.64mg/dl in diabetic group. The mean value reduced significantly (*P*<0.05) to 199.5±4.29mg/dl diabetic extract treated group. Also there was a significant reduction to a mean value of 255.2±14.46mg/dl, 267.2+16.02mg/dl and 222.67+4.81mg/dl in diabetic groups which received aspirin, meloxicam and insulin treatments respectively.

4. DISCUSSION

The levels of inflammatory biomarkers in diabetic rats treated with aqueous leave extract of *Terminalia catappa*, Non-Steroidal Anti-inflammatory Drugs (NSAIDs) and exogenous insulin were analysed. The result showed that C-reactive protein level was high in the diabetic group compared to control. The result of this study is consistent with our previous result [32] and with other research findings which has established elevated C-reactive protein levels in people with impaired glucose tolerance and frank diabetes [33,34]. However, the observed increase was reduced by the aqueous leaf extract of *Terminalia catappa*, aspirin, meloxicam

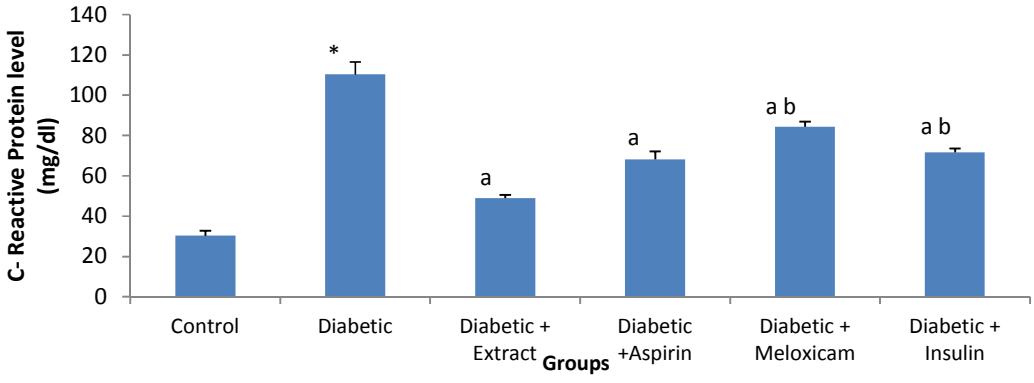

Fig. 1. Serum C - reactive protein in experimental group compared with control, diabetic control and diabetic + extract groups

*Values are mean ± SEM. * =Significant change compared with control group (P< 0.05)*
a= Significant change compared with diabetic control group (P< 0.05)
b= Significant change compared with diabetic + extract group (P< 0.05)

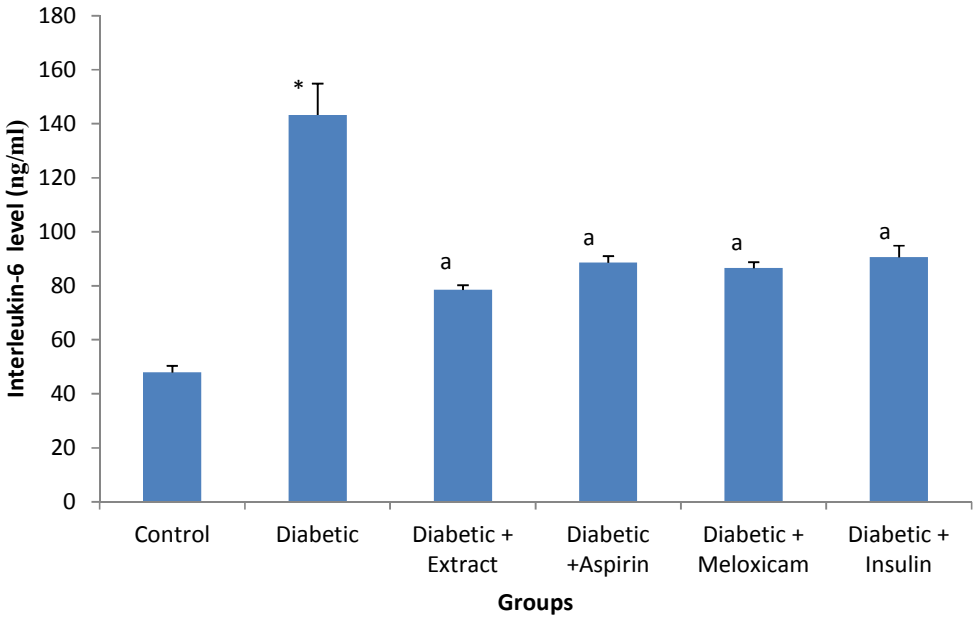

Fig. 2. Serum level of Interleukin-6 in experimental group compared with control and diabetic control groups

*Values are mean ± SEM. * =Significant change compared with control group (P< 0.05)*
a=Significant change compared with diabetic control group (P< 0.05)

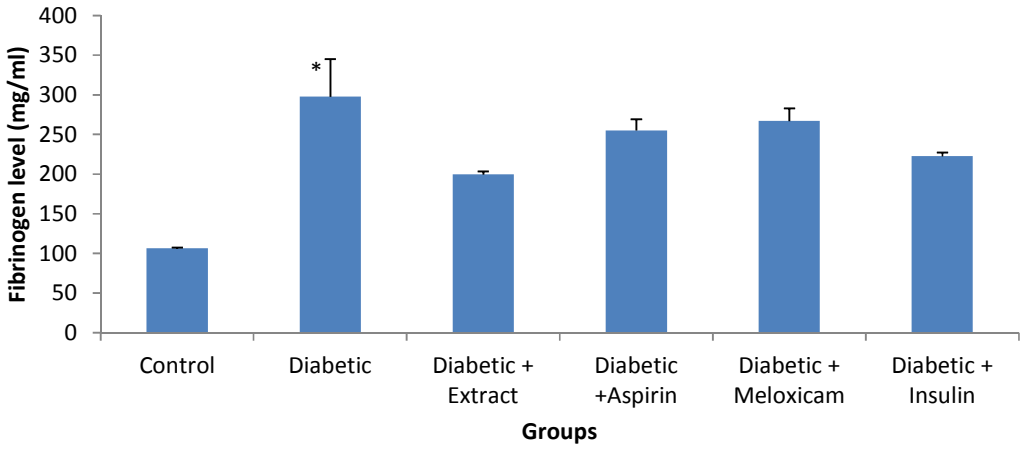

Fig. 3. Serum level of fibrinogen in experimental group compared with control and diabetic control groups

*Values are mean ± SEM. * =Significant change compared with control group (P< 0.05)*
a=Significant change compared with diabetic control group (P< 0.05)

and insulin in their respective diabetic treated groups. These results reveal that the reduction in C-reactive protein and Interleukin-6 by the extract was higher than that of aspirin and meloxicam. Moreover, fibrinogen level follows a similar pattern of being increased significantly in the diabetic group and was lowered significantly by the extract, aspirin, meloxicam and insulin, with insulin having higher effect compared to the other agents. Both aspirin and meloxicam are

non-steroidal anti-inflammatory drugs used in the treatment of inflammation besides other functions as analgesia and anti-pyretic. These anti-inflammatory drugs inactivate cyclooxygenase (COX) enzymes with meloxicam being more COX-2 specific blocker than COX-1 [35]. Blocking COX enzymes leads to suppressed production of prostaglandins and thromboxanes [36] as the mechanism of reducing inflammation.

The level of reduction of these biomarkers varies between the two anti-inflammatory drugs. It was observed that C-reactive protein was more reduced by aspirin than meloxicam while the level of reduction of Interleukin-6 and fibrinogen were similar between the two drugs. The irreversible effect of aspirin on COX-1 enzyme [37] makes the drug different from other anti-inflammatory drugs in addition to regular pathways such as the formation of nitric oxide free radicals in the body as independent mechanism in reducing inflammation [38], uncoupling of oxidative phosphorylation in mitochondria [39] and signal modulation through NF-kB [40]. The present study cannot explain if the specificity in inactivation of the COX isoforms; COX-1 and COX-2 may play a role in the effectiveness of aspirin in the reduction of the acute phase inflammatory marker, C-reactive protein compared to meloxicam despite the similarity in the reduction of pro-inflammatory cytokine; Interleukin-6.

It was observed that insulin reduced C-reactive protein and Interleukin-6 and fibrinogen with comparatively higher effect on fibrinogen than the other agents. Following the established association between hyperglycaemia and inflammation, the anti-inflammatory effect of insulin will first involve lowering of blood glucose and this might show existing positive link between hyperglycaemia, fibrinogen level and risk of cardiovascular disease in diabetes. It is reported that insulin suppresses important inflammatory mediators namely: Intercellular adhesion molecule-1(I-CAM-1), Monocyte Chemoattractant Protein-1 (MCP-1) expression, necrosis factor NF-kB binding [41,42] in human aortic endothelial cells *in vitro* via nitric oxide signalling pathway [43,44,45]. It is suggested that Toll like receptors (TLRs) which plays an important role in tissue inflammation and damage such as cardiac ischemia and atherosclerosis [46] is also suppressed by insulin. Although the reduction of C-reactive protein and Interleukin-6 by insulin in this study is not as much as the non steroidal anti-inflammatory drugs, the significant effect of insulin as shown in this study on fibrinogen may suggest the usefulness of insulin in thrombosis more than the non steroidal anti-inflammatory drugs.

In view of these findings, NSAIDs such as aspirin or meloxicam adjunct to insulin therapy in diabetes may be advocated. But reports on various side effects of these drugs are documented. Gastrointestinal toxicity and increased risk of cardiovascular events such as heart attack and stroke are associated with many non steroidal anti-inflammatory drugs [47]. Meloxicam is specifically contraindicated in persons with hypertension and diabetes despite its modification for gastrointestinal tolerability thereby ruling out its use in diabetes condition. Moreover, recent clinical findings have revealed the development of NSAIDs induced enteropathy [48,49]. It is reported that the permeability of the small intestinal mucosa in a single dose of NSAID is within 12 hours and this is by uncoupling of the mitochondrial phosphorylation which breaks the integrity of the mucosa junction normally protected by COX1 and COX 2 [50,51]. However, this enteropathy is asymptomatic and physicians continue to treat with this drug because the side effect is vague [52].

The reduction of these biomarkers of inflammation by the aqueous leaf extract suggests that the extract is in agreement with current position that natural treatments can help to reduce inflammation in the blood [53]. But the higher level of reduction observed may suggest that the extract activates the pathways used by these agents and other pathways not known to express its anti-inflammatory actions. However this assertion requires a more empirical investigation to ascertain the signalling pathway(s) involved.

5. CONCLUSION

The serum level of C-reactive protein, Interleukin-6 and fibrinogen were significantly reduced by the aqueous leaf extract of *Terminalia catappa* compared to aspirin and meloxicam. And the effect of aspirin was more marked on C-reactive protein than meloxicam while the two affected Interleukin-6 and fibrinogen similarly. Therefore aqueous leaf extract of *Terminalia catappa* may be a potent anti-inflammatory agent than non steroidal anti-inflammatory drugs and could complement the function of insulin in diabetes treatment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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