Scientia Africana, Vol. 22 (No. 3), December, 2023. Pp 219-230
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EXPLORING THE UNDERLYING MECHANISM OF ETHYL ACETATE EXTRACT OF *ANNONA MURICATA* LEAVES VIA THE NRF2 AND NF-KB PATHWAY IN TYPE 1 DIABETIC WISTAR RATS

[a]Oriakhi, K[*]., Ogbomo, R.O., Anyanwu, C.I., and Agu, K.C
[a]Department of Medical Biochemistry, School of Basic Medical Sciences, College of Medical Sciences, University of Benin, Benin City, Edo State, Nigeria
***Corresponding author:** Kelly.oriakhi@uniben.edu, +2347032979016

Received: 16-10-2023
Accepted: 05-12-2023

https://dx.doi.org/10.4314/sa.v22i3.20

Journal Homepage: http://www.scientia-african.uniportjournal.info
Publisher: Faculty of Science, University of Port Harcourt.

ABSTRACT

Type 1 diabetes mellitus is an autoimmune disorder characterized by the destruction of pancreatic beta cells, leading to impaired insulin production and regulation. Recent research has shown that medicinal plants with antioxidant and anti-inflammatory properties hold promise as potential therapeutic agents for managing complications of the disease. The aim of this study is to investigate the anti-diabetic, antioxidant, and anti-inflammatory effect of ethyl acetate extract of *Annona muricata* leaves in streptozotocin (STZ)-induced diabetes in Wistar rats. Thirty five (35) male Wistar rats weighing 150-180g were used for this experiment. Group 1 (Normal control), Group 2 (STZ 40 mg/kg only Diabetic control), Group 3 (STZ+ metformin 500mg/kg bw), Group 4 (STZ + ethyl acetate extract 250 mg/kg bw), Group 5 (STZ + ethyl acetate extract (500 mg/kg bw). Fasting blood samples were collected by cardiac puncture for glucose estimation and liver tissues excised for both biochemical and molecular studies. The study demonstrated the extract's ability to modulate the NF-κB pathway, leading to the suppression of inflammatory responses in diabetic rats. These findings suggest that the ethyl acetate extract of *A. muricata* leaves may ameliorate type 1 diabetes by promoting antioxidant defense and reducing inflammatory processes through the NRF2 and NF-κB pathways. This research sheds light on the potential of natural compounds as adjunct therapies for type 1 diabetes and provides a foundation for further investigations into the development of novel treatments targeting oxidative stress and inflammation in autoimmune diabetes.

Keywords: *Annona muricata; antioxidants; anti-inflammatory; diabetes; streptozotocin*

INTRODUCTION

Diabetes mellitus is a prevailing lifestyle disease that contributes significantly to the global burden of illnesses (WHO, 2016). It is characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or a combination of both (Akram & Hisham, 2015). The metabolic abnormalities in carbohydrates, lipids, and proteins arise from the pivotal role of insulin as an anabolic hormone. Dysregulation of insulin levels and resistance in target tissues, particularly skeletal muscles, adipose tissue, and to a lesser extent, the liver, involving insulin receptors, signal transduction pathways, and effector enzymes or genes, contribute to these metabolic irregularities.

The clinical presentation and severity of symptoms are influenced by the type and duration of diabetes. While some patients, especially those with type 2 diabetes, may remain asymptomatic during the early stages of the disease, others, especially children with absolute insulin deficiency, may experience symptoms such as polyuria, polydipsia, polyphagia, weight loss, and blurred vision. If left uncontrolled, diabetes can lead to serious complications such as stupor, coma, and, if untreated, may result in death due to ketoacidosis or, rarely, non-ketotic hyperosmolar syndrome (Craig et al., 2009; Galtier, 2010).

Oxidative stress plays a crucial role in the initiation and advancement of diabetes and its associated complications (Forman & Zhang, 2021). The disruption in the equilibrium between the generation of reactive oxygen species (ROS) and the body's defense mechanisms against them results in harm and malfunction of cells. These reactive oxygen species (ROS) comprise both free radicals such as superoxide ($O_2^{\bullet-}$), and non-radical species such as hydrogen peroxide (H_2O_2) (Gülçin, 2012; Weseler & Bast, 2010). These molecules, continuously produced in the cell, are involved in physiological events such as cell differentiation, primary immune defense, and signaling (Gülçin, 2012; Poli et al., 2004; Shah & Sauer, 2006). In the context of diabetes, persistent high blood sugar levels (chronic hyperglycemia) and dysfunction of the mitochondria collectively contribute to an elevated production of ROS, further intensifying oxidative stress (Badawi et al., 2010). This heightened oxidative load detrimentally affects several aspects of diabetes, which impaired the functioning of beta cells and increased resistance to insulin, which disrupts the regulation of glucose levels (Tangvarasittichai, 2015). Additionally, the harm induced by oxidative stress extends to blood vessels, impairing their function and fostering the development of diabetic vascular complications, including retinopathy, nephropathy, and cardiovascular diseases. Furthermore, various organs and tissues throughout the body, such as the kidneys, nerves, and eyes, become susceptible to the damaging effects of oxidative stress, culminating in diabetic nephropathy, neuropathy, and retinopathy (Evan et al., 2002).

Accumulating evidence from both laboratory studies *(in vivo* and *in vitro*) suggests that elevated glucose levels instigate chronic inflammation and oxidative stress. This, in turn, leads to the upregulation of various pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, and IL-18 (Escobar-Morreale *et al*., 2017). Moreover, in a state of elevated glucose concentration, activated endothelial cells (ECs) release pro-inflammatory cytokines as a result of local autocrine and paracrine signaling between neighboring cells, setting off a self-perpetuating cycle, as demonstrated by Wang et al. (2017). Additionally, the surface expression of adhesion molecules on ECs, including monocyte chemoattractant protein 1 (MCP-1) and intercellular adhesion molecule-1 (ICAM1), recruit's monocytes, which, in conjunction with inflammatory cytokines, sustain a persistent state of chronic inflammation and contribute to endothelial dysfunction (Buravkova *et al*., 2018). This dysfunction may be the culprit for the complications associated with type 1 diabetes.

Furthermore, in response to chronic hyperglycemia, a series of events including glucose oxidation, nonenzymatic glycation of proteins, and subsequent oxidative degradation of these glycated proteins result in an excessive production of free radicals (Khan *et al*., 2018). To counteract the pathological damage and the development of insulin resistance stemming from hyperglycemia-induced oxidative stress associated with diabetes mellitus, antioxidant defense mechanisms play a critical role, as emphasized by Luna and Estevez (2018). Addressing oxidative stress and the inflammatory state in diabetes mellitus, various strategies have been developed to alleviate this burden. One such approach is

Scientia Africana, Vol. 22 (No. 3), December, 2023. Pp 219-230
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antioxidant therapy, and it's worth noting that *Annona muricata* leaves exhibit robust antioxidant properties, as reported by Agu et al. (2018). In this article, the multifaceted nature of diabetes mellitus, the pharmacological activities of *Annona muricata*, and its potential therapeutic role in diabetes management were explored.

MATERIALS AND METHODS

Plant collection

The leaves of *Annona muricata* were collected from trees in household gardens in Benin City and around the University of Benin vicinity, Edo state, Nigeria. The plant was identified by Dr. Bamidele of the Department of Plant Biology and Biotechnology, University of Benin, and authenticated by Professor Idu of the same Department. A voucher specimen number UBHa 0205 was deposited at the Herbarium of the Department of Plant Biology and Biotechnology, University of Benin. The leaves were air dried, pulverized and stored at room temperature in an airtight polythene bag prior to use.

Preparation of extract

The pulverized plant material (1 kg) was extracted with 3l of ethyl acetate for 48 h on an orbit shaker. The solvent mixture was filtered using a Buckner funnel and Whatman No. 1 filter paper. The filtrate was concentrated to dryness with a freeze dryer to give a yield of (18.21 g) and later reconstituted in distilled water due to its solubility in water to give the required concentrations needed in this study.

Chemicals

Chemicals, reagents, and solvents used during this research were of analytical status. Ethyl acetate, sodium chloride, sodium hydroxide, hydrochloric acid, and chloroform, were purchased from Merck (Darmstadt, Germany). Phosphate buffer saline, streptozotocin, citric acid, were products of Sigma Chemical Co. (USA).

Animals

The rats used were adult male Wistar rats weighing between 100g - 150g. The rats were bred in Department of Anatomy, University of Benin, Benin City, housed in the Department of Biochemistry animal house, and were acclimatized for one week before the study. They were fed standard rat chow and water *ad libitum*. Written approval no: CMR/REC/2014/57 for the study was obtained from the Research Ethics Committee Guideline Principles on Handling of Animals of the College of Medicine, University of Benin.

Induction of rats with streptozotocin (STZ)

Streptozotocin (40mg/kg) was injected intraperitoneally using an insulin syringe into the rat after an overnight fast. To establish hyperglycemia (≥200mg/dl) fasting blood glucose was checked after three days by collecting blood samples from the tail tip of the Wistar rat.

Experimental design

The experiment spanned a period of fourteen (14) days, with a total of 35 (thirty-five) rats divided into five (5) groups of seven (7) rats each, with treatment including; Group 1 serving as the normal (background/vehicle control) receiving 2 ml of water only; Group 2 was pathologically induced with STZ (40 mg/kg) only serving as the negative control; Group 3 received STZ + Metformin (Met) for 14 days, with Met, as the positive control; Group 4 received STZ + 250 mg/kg of ethyl acetate extract of *A. muricata* leaf for 14 days, as the lower dose treatment; Group 5 received STZ + 500 mg/kg of ethyl acetate extract of *A. muricata* leaf for 14 days, as the higher dose treatment.

Biochemical assays of blood (plasma) parameters

At the end of the 14th day of experimental treatments, the rats were weighed and then euthanized. Blood samples were collected with sterile syringes into heparinized sample bottles, for biochemical analysis. Assays were

done using ready-to-use standard assay kits from Randox® (Randox Laboratory, UK), including glucose oxidase activity, which was done to monitor serum glucose concentration.

Statistical analysis

Data were entered into the Microsoft Excel spreadsheet (v.10) prior to descriptive analysis. The data are presented as mean ± SEM and were analyzed using Duncan's multiple range analyses of variance, ANOVA were performed using Statistical Package for Social Sciences, SPSS®, Version 21.0, IBM Corp., Armonk, NY, USA. Values of $p < 0.05$ were considered significant.

RESULTS

Effect of ethyl acetate extract of *A. muricata* leaf on Fasting Blood glucose in Streptozotocin-Induced Diabetic Rats

The study investigated the effects of ethyl acetate extract obtained from *Annona muricata* leaves on fasting blood glucose levels in diabetic rats induced with streptozotocin (STZ), as outlined in Table 1. Initially, the blood glucose levels in the normal control, diabetic control, and diabetic treated groups were found to be comparable, demonstrating similar baseline glucose levels across the groups. Three days following the administration of streptozotocin, both the diabetic control and diabetic treated groups exhibited a substantial and statistically significant ($p < 0.05$) rise in blood glucose levels when compared to the normal control group. This increase in blood glucose levels is a well-documented response to STZ-induced diabetes, and no noteworthy differentiation was observed between the two diabetic groups at this early stage. The most remarkable observation in this investigation was the considerable normalization of blood glucose levels in the diabetic treated group, occurring fourteen days after the administration of the ethyl acetate extract derived from *A. muricata* leaves. Notably, the blood glucose levels in the diabetic-treated group at this juncture were not only significantly reduced in comparison to the diabetic-control group but had also returned to levels akin to those in the normal control group. This normalization strongly suggests the potential of the ethyl acetate extract of *Anona muricata* to effectively ameliorate hyperglycemia in diabetic rats. Crucially, it is imperative to acknowledge that throughout the entire duration of the fourteen-day study, the blood glucose levels in the diabetic control group remained significantly elevated ($p < 0.05$) in comparison to both the normal control and diabetic treated groups, underscoring the persistent hyperglycemic condition experienced by the diabetic control rats.

Table 1: Effect of ethyl acetate extract of *A. muricata* leaves on the fasting blood glucose level in streptozotocin-induced diabetic rats

Group/ Treatment	Glucose (mg/dl) Day 0	Glucose (mg/dl) Day 3 (After induction)	Glucose (mg/dl) Day 7	Glucose (mg/dl) Day 14
Group 1	81.41±2.20^{a}	82.20±2.00^{a}	84.10±1.34^{a}	77.67±0.52^{a}
Group 2	80.20±2.24^{a}	*320.00±5.43^{b}	*322.20±2.20^{c}	*336.10±3.20^{c}
Group 3	80.24±2.34^{a}	**317.25±4.50^{b}	**101.32±0.33^{c}	**100.40±1.20^{c}
Group 4	79.80±2.20^{a}	**330.20±8.02^{b}	**219.70±0.41^{c}	**80.20±1.05^{d}
Group 5	81.60±2.14^{a}	**271.21±2.37^{b}	**94.20±1.25^{c}	**76.36±0.23^{c}

Data were expressed as mean ± SEM, n=7 Values with different alphabets along the same row are statistically significant ($p < 0.05$). *Mean is significant ($p < 0.05$) when compared with the control. ** mean is significant ($p < 0.05$) when compared with the diabetic control group. Group 1, Normal control, Group 2, Diabetic control (STZ only), Group 3, Positive control (Metformin), Group 4, ethyl acetate extract (250 mg/kg bw), Group 5, ethyl acetate extract (500 mg/kg).

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Effect of ethyl acetate extract of *A. muricata* leaves on non-enzymatic antioxidant (GSH, MDA) levels and enzymatic antioxidant (SOD, CAT) activities of the liver in streptozotocin-induced diabetic rats

The evaluation of the ethyl acetate extract obtained from *Annona muricata* leaves is depicted in Fig 1A -1D. The data showed that rats within the diabetic control group exhibited a substantial reduction ($p < 0.05$) in SOD, CAT activities and GSH levels when juxtaposed with the normal control group, signifying an impairment in the antioxidant defense mechanisms within the diabetic milieu. Intriguingly, rats treated with the ethyl acetate extract from *Annona muricata* leaves exhibited a significant, dose-dependent increase ($p < 0.05$) in SOD, CAT activities and GSH level when compared to the diabetic control group (Group 2). Similarly, rats subjected to metformin treatment caused a significant increase ($p < 0.05$) in SOD, CAT activities and GSH levels when compared to the diabetic control group.

Notably, the rats subjected to STZ induction displayed a noteworthy and statistically significant elevation ($p < 0.05$) in MDA levels compared to the normal control group, signifying a substantial upsurge in oxidative stress within this diabetic cohort. Intriguingly, rats administered with the ethyl acetate extract from *Annona muricata* leaves showcased a remarkable and dose-dependent reduction ($p < 0.05$) in MDA levels concerning the diabetic control group (Group 2). This reduction substantiates the potential of the extract as an effective agent in mitigating oxidative stress, suggestive of its antioxidative capacity.

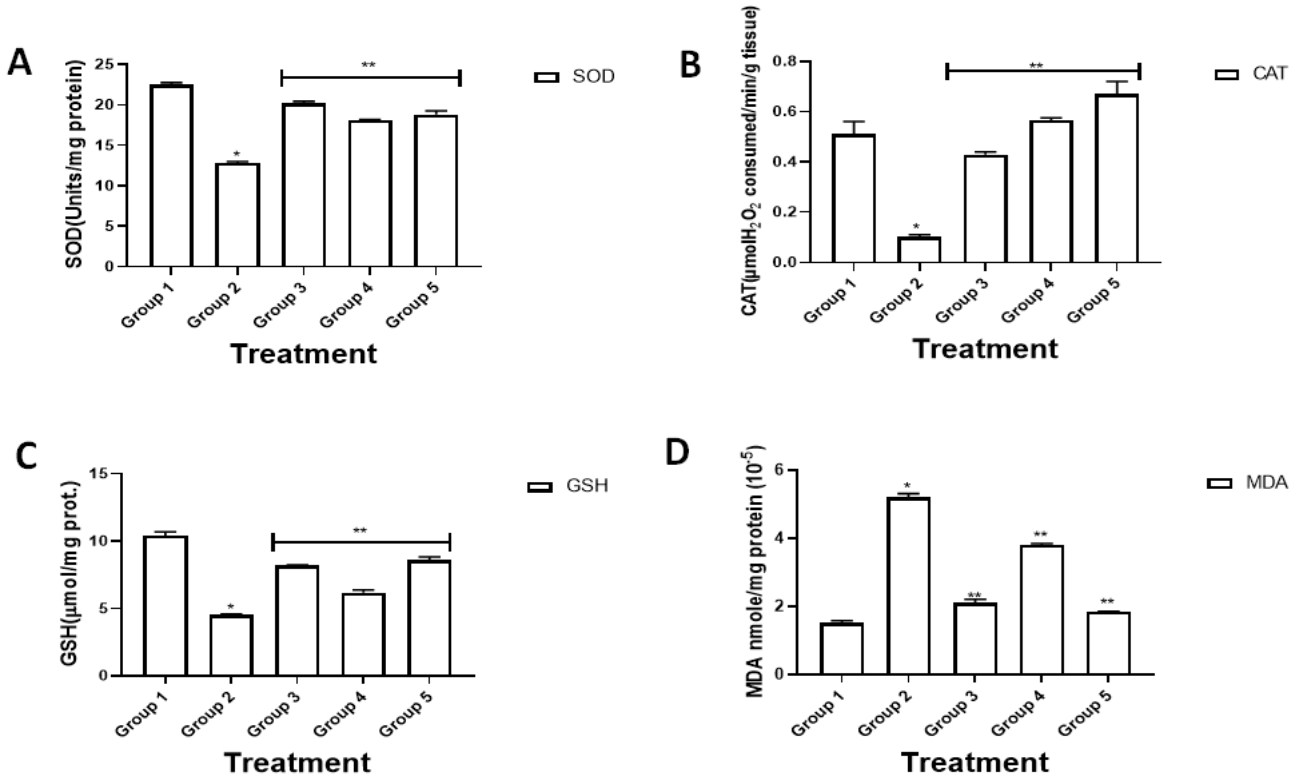


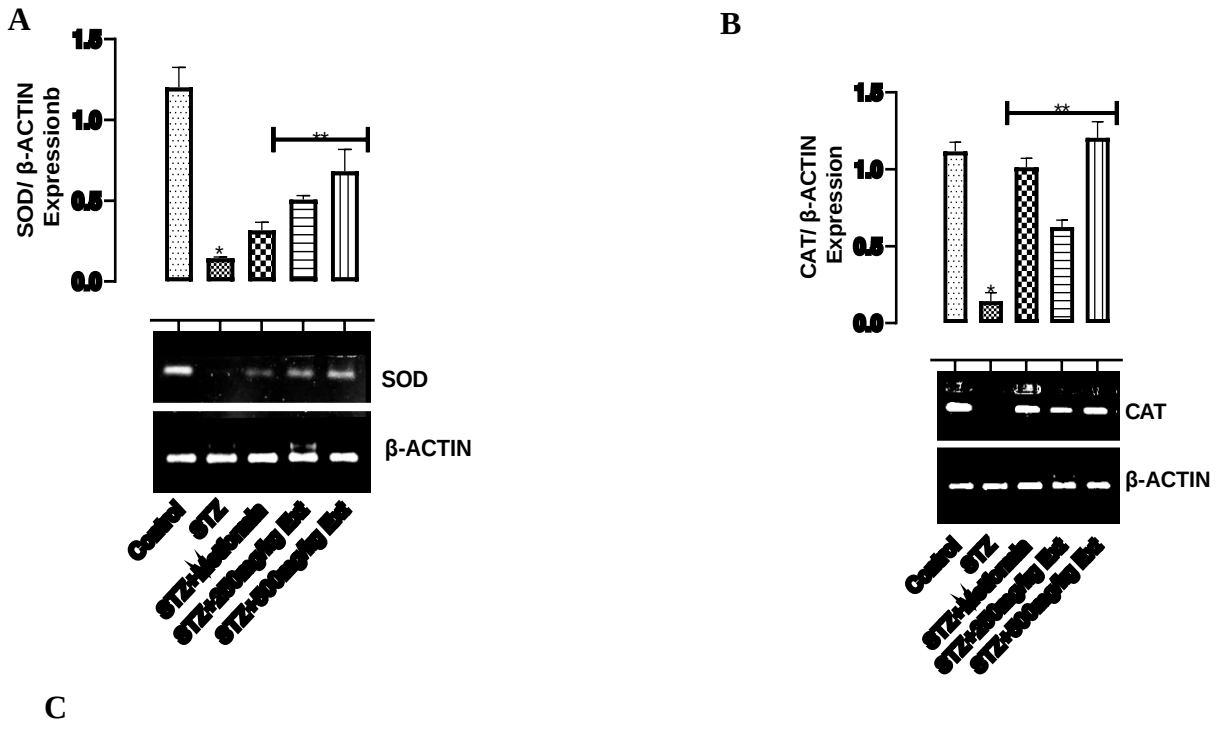
Fig 1: Effect of ethyl acetate extract of *A. muricata* leaves on liver **A**) superoxide dismutase (SOD) and **B**) catalase (CAT) activities; **C**) reduced glutathione (GSH) and **D**) malondialdehyde (MDA) levels in streptozotocin-induced diabetic rats' data were expressed as mean ± SEM, n=7 *Mean is significant ($p <$ 0.05) when compared with the control. ** mean is significant ($p <$ 0.05) when compared with the diabetic

control group. Group 1, Normal control, Group 2, Diabetic control (STZ only), Group 3, Positive control (Metformin), Group 4, ethyl acetate extract (250 mg/kg bw), Group 5, ethyl acetate extract (500 mg/kg).

Effect of ethyl acetate extract of *A. muricata* leaves on liver antioxidant enzymes and nuclear factor erythroid 2-related factor 2 (Nrf2) in STZ-induced diabetic Wistar rats.

The effect of ethyl acetate extract of *A. muricata* leaves on liver antioxidant enzymes and nuclear factor erythroid 2-related factor 2 (Nrf2) in STZ-Induced diabetic Wistar rats is shown in Fig 2A-C. It was observed that rats induced with STZ significantly decrease the expressions of SOD, CAT and Nrf2 genes when compared to normal control (group 1). However, rats treated with 250 and 500 mg/kg body weight extracts of ethyl acetate of *A. muricata* leaves significantly increased the expressions of SOD, CAT and Nrf2 genes when compared to diabetic control (group 2).

Similarly, rats administered metformin showed a relatively high expression of SOD, CAT and Nrf2 genes when compared to diabetic control (group 2). It is worth noting that the lower concentration (250 mg/kg bw) of ethyl acetate extract of *A. muricata* leaves significantly stimulates Nrf2 gene.



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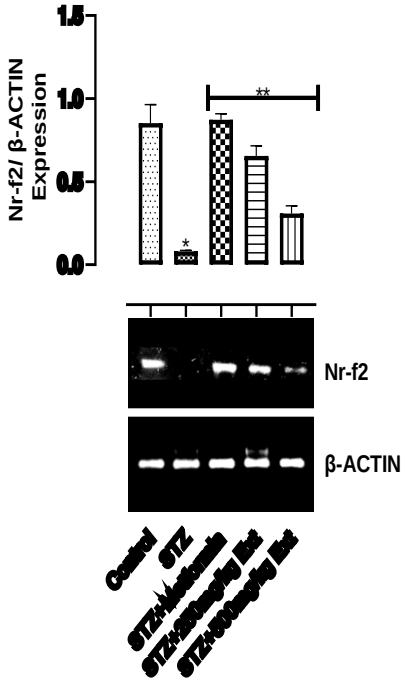

Fig 2: Effect of ethyl acetate extract of *A. muricata* leaf on liver (A) Superoxide Dismutase (SOD); (B) Catalase (CAT); (C) Nuclear factor erythroid 2–related factor 2 (Nrf2) in streptozotocin-induced diabetic rats. Data were expressed as mean ± SEM, n=7 *Mean is significant ($p < 0.05$) when compared with the control. ** mean is significant ($p < 0.05$) when compared with the diabetic control group. Group 1, Normal control, Group 2, Diabetic control (STZ only), Group 3, Positive control (Metformin), Group 4, ethyl acetate extract (250 mg/kg bw), Group 5, ethyl acetate extract (500 mg/kg)

Effect of ethyl acetate extract of *A. muricata* leaves on the liver Tumor Necrosis Factor (TNF-α) and Nuclear factor kabba B (NF-κB) in streptozotocin-induced diabetic rats

The effect of ethyl acetate extract of *A. muricata* leaves on TNF-α, IL-1β and NF-κB in STZ-induced diabetic Wistar rats is shown in Fig 3A-C. It was observed that rats induced with STZ significantly increased the expressions of TNF-α, IL-1β and NF-κB genes when compared to normal control (group 1). However, rats treated with 250 and 500 mg/kg body weight extract of ethyl acetate of *A. muricata* leaves significantly increased the expressions of TNF-α, and IL-1β genes when compared to diabetic control (group 2). The expression of NF-κB gene was only stimulated with the lower dose (250 mg/kg bw) extract of ethyl acetate of *A. muricata* leaves.

Similarly, rats administered metformin showed a relatively high expression of TNF-α, IL-1β and NF-κB genes when compared to diabetic control (group 2).

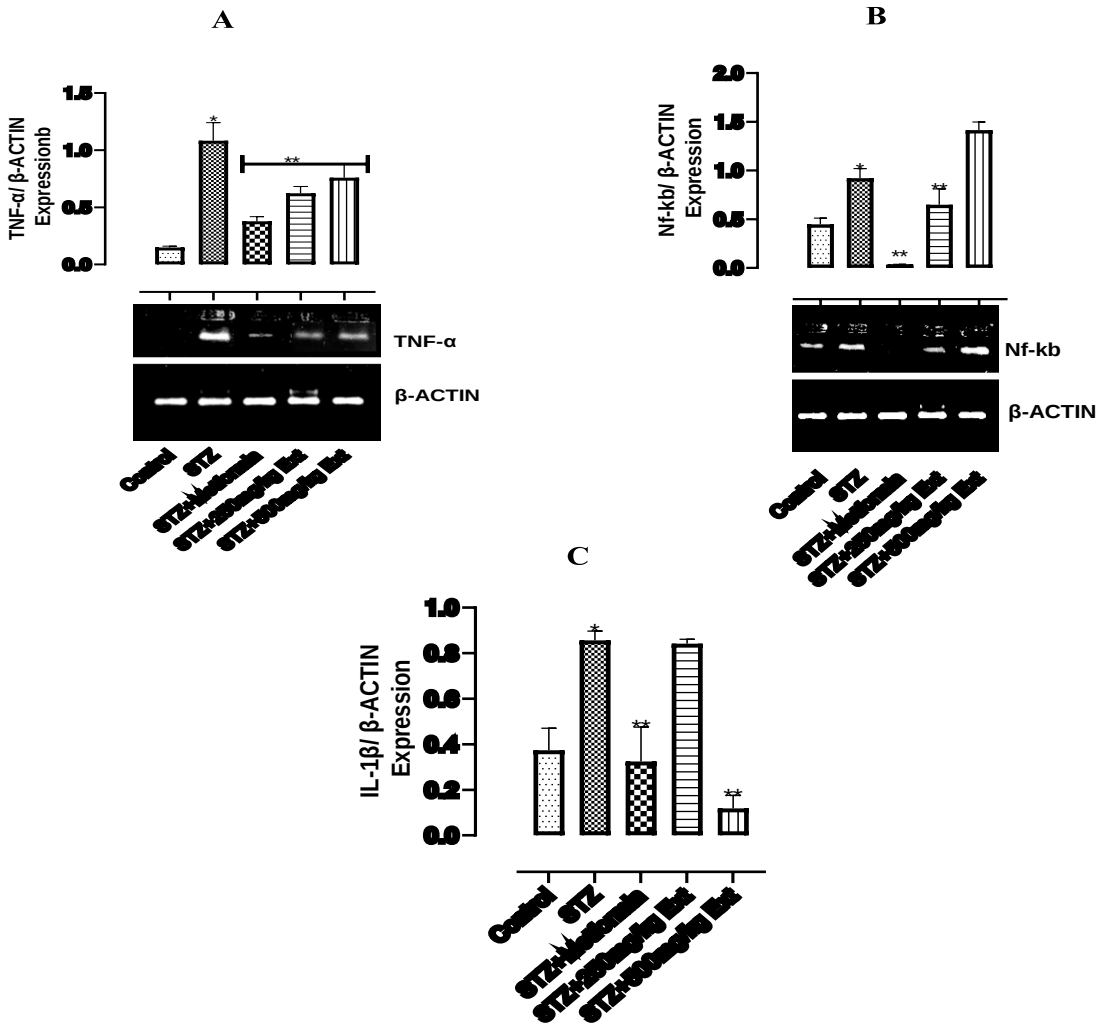

Fig 3: Effect of ethyl acetate extract of *A. muricata* leaf on liver (**A**) Tumor Necrosis Factor (TNF-α); (**B**) Interleukin 1β(IL-1β); (C) Nuclear factor kabba B (NF-κB) in streptozotocin-induced diabetic rats. Data were expressed as mean ± SEM, n=7. *Mean is significant ($p < 0.05$) when compared with the control. ** mean is significant ($p < 0.05$) when compared with the diabetic control group. Group 1, Normal control, Group 2, Diabetic control (STZ only), Group 3, Positive control (Metformin), Group 4, ethyl acetate extract (250 mg/kg bw), Group 5, ethyl acetate extract (500 mg/kg bw)

DISCUSSION

Medicinal plants have been employed for centuries in the treatment and management of various diseases, thanks to their rich repertoire of chemical compounds (Adewole & Ojewole, 2009). Among these plants, *Annona muricata* L., also known as soursop, holds a prominent place in herbal medicine in tropical regions of South and North America, as well as in West Africa, particularly in Western Nigeria (Adewole & Ojewole, 2009). This versatile plant has demonstrated efficacy in the treatment of various conditions, including cardiovascular diseases, cancer, and diabetes mellitus. Diabetes mellitus is known to cause an imbalance in lipid profiles, leading to lipid peroxidation and an increased risk of atherosclerosis (Guigliano *et al*., 1995). Elevated levels of free radicals, lipid peroxidation, and reduced antioxidant status signify heightened oxidative stress in diabetic patients (Baynes, 1991; Hiramatsu & Arimori, 1988). Oxidative stress, defined as an imbalance between pro- and antioxidant species, can result in molecular and cellular damage (Conti *et al*., 2016). To counter this danger, the body

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employs antioxidants, both enzymatic and non-enzymatic. Catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) are the three main antioxidant enzymes that are part of the body's defense mechanism against ROS (Birben *et al*., 2012). This study is aimed to investigate the potential anti-diabetic and antioxidant effects of the ethyl acetate extract of *Annona muricata*. Since streptozotocin is known to work by destroying β-cells (Szkudelski & Szkudeska, 2002).

The study evaluated the effect of the ethyl acetate extract of *Annona muricata* leaves on fasting blood glucose levels in streptozotocin (STZ)-induced diabetic rats. The results showed a significant increase in blood glucose levels in the diabetic control and diabetic-treated groups compared to the normal control three days after STZ administration. However, the diabetic-treated rats exhibited a normalization of blood glucose levels fourteen days after treatment with the ethyl acetate extract. The diabetic control group maintained significantly elevated glucose levels compared to the normal control and diabetic-treated groups during the study period. These findings suggest that *Annona muricata* leaf extract contains phytochemicals effective in ameliorating hyperglycemia associated with diabetes mellitus. Furthermore, the treated groups showed improved glucose homeostasis compared to the untreated diabetic rats. This correlated with the findings of Florence et al. (2014) that extracts from medicinal plants may protect the beta cells of the pancreas from destruction by promoting peripheral glucose absorption. The ethyl acetate extract also exhibited anti-inflammatory activities by significantly decreasing ($p<0.05$) TNF-α and IL-1, in a dose dependent manner when compared to the diabetic control. However, ethyl acetate extract at a dose of 250 mg/kg significantly reduced NF-kB gene expression. This study agreed with Al-Dwairi et al. (2018), that soursop leaves also contain quercetin compounds (flavonoid group) which can increase the binding of Glucagon like peptide 1(GLP-1) with GLP-1R in fat tissue so that it will cause an increase in cyclic adenosine monophosphate (cAMP) production in fat tissue. An increase in cAMP will reduce the expression of the NF-kB gene which in turn reduce the pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α. The study also assessed the levels of reactive oxygen species (ROS) and malondialdehyde (MDA), a marker of lipid peroxidation and a free radical that can cause cellular and DNA damage (Wo´zniak et al., 2007). The rats induced with STZ exhibited significantly increased MDA levels compared to the normal control. However, treatment with the ethyl acetate extract of *Annona muricata* leaves led to a dose-dependent decrease in MDA levels, akin to the effects observed with metformin, a standard anti-diabetic drug. These results indicate that the leaf extract possesses antioxidant properties, mitigating oxidative stress and lipid peroxidation in diabetic rats. In a previous study, it was observed that aqueous and organic extracts from the leaves of *A. muricata* possess strong DPPH and FRAP scavenging capabilities. These findings underscore the effectiveness of *A. muricata* extracts in neutralizing free radicals generated during diabetes mellitus. Furthermore, the presence of phytochemicals (phenols, flavonoids and saponins) in *A. muricata* leaf extracts are well-known for their antioxidative properties (Adewole & Ojewole, 2009; Agu & Okolie, 2017).

The study further explored the enzymatic antioxidant activities, including glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) contents in hepatic tissues of STZ-treated diabetic rats. The diabetic control group showed reduced GSH, CAT, and SOD levels. However, treatment with the ethyl acetate extract of *Annona muricata* leaf resulted in a significant increase in GSH, CAT, and SOD activities in a dose-dependent manner, similar to the effects of metformin treatment. The upregulation of CAT and SOD gene expressions in the liver further supported the normalization of antioxidant

activities by *Annona muricata* treatment. A similar result was observed in another study, that A. muricata leave extract significantly increased reactive oxygen species gene expression in rats induced with monosodium glutamate. Still, treatment with the extract of *A. muricata* leaves dramatically reduced the ROS gene compared to the negative control(Shukry et al., 2020).

In summary, the study demonstrates that the ethyl acetate extract of *Annona muricata* possesses both anti-diabetic and antioxidant activities. The extract effectively mitigated oxidative damage induced by STZ treatment in the liver, thereby contributing to improved glucose homeostasis in diabetic rats. The findings suggest that *Annona muricata* holds promise as a beneficial therapeutic agent in managing diabetes mellitus and modulating associated antioxidant activities.

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