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| RESEARCH ARTICLE

Expression Levels of Some Genes in Streptozotocin Induced Diabetic Rats Treated with Extracts of *Anona Muricata* and *Vernonia Amgdalina*

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| ABSTRACT
Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion or insulin action. In this study, we investigated the expression levels of some selected genes (Interleukin -1 (IL-1), Tumour necrosis factor (TNF), Glucose transporter 2 (GLUT-2), Phosphofructokinase 1 (PFK-1), and Peroxisome proliferator activated receptor (PPAR) in streptozotocin induced diabetic rats treated with extracts of *V. amgdalina* and *Annona muricata*. Thirty male wistar rats were randomly divided into five groups (1–5): group 1 was the normal control, group 2 served as the diabetic control, group 3 were the diabetic rats treated with glibenclamide (standard drug), while groups 4 and 5 were the diabetic rats treated with 200mg/kg body weight of extracts of *Vernonia amgdalina* and *Annona muricata* respectively. Treatment with extract was for four weeks, after which the animals were sacrificed and the liver tissues collected for gene studies and histological evaluations. Data from the study showed that diabetes resulted in a significant($p \leq 0.05$) increase in the expression of the genes Interleukin -1 (IL-1), Tumour necrosis factor (TNF), with a significant($p \leq 0.05$) decrease in expression of the genes Glucose transporter 2 (GLUT-2), Phosphofructokinase 1 (PFK-1), and Peroxisome proliferator activated receptor (PPAR). Treatment with extracts of *Vernonia amgdalina* and *Annona muricata* demonstrated significant improvements in the expression of Glut-2, PFK-1, PPAR genes and decreased expression of IL-1 and TNF genes. Histological damage caused to the pancreas by streptozotocin was also restored by treatment with the plant extracts.

| KEYWORDS

Diabetes Mellitus, Interleukin -1 (IL-1), Tumour necrosis factor (TNF), Glucose transporter 2 (GLUT-2), Phosphofructokinase 1 (PFK-1), and Peroxisome proliferator activated receptor (PPAR).

| ARTICLE INFORMATION

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1. Introduction

Diabetes mellitus, which is characterized by high blood glucose, causes inflammation, which is shown by an increase in several inflammatory markers, including interleukins, tumor necrosis factor-α(TNF-α), and high sensitivity C reactive protein (hs-CRP) (Nadeem et al., 2013; Node and Inoue, 2012).

Studies reveled that induction of diabetes with STZ showed increased levels of pro-inflammatory markers such as C-reactive protein, tumor necrosis factor, and interleukins. Adipocytes secrete an inflammatory cytokine called Tumor Necrosis Factor-α (TNF-α). By interfering with insulin signaling pathways, its overproduction has been linked to insulin resistance. (Hotamisligil et al., 1993; Hotamisligil et al., 1995).

PPAR-γ agonists down regulate TNF-α expression in adipose tissue and enhances TNF-α induced desensitization to insulin action (Dworzanski et al., 2010; Tyagi et al., 2011). Therefore, these two cytokines influence insulin signaling by upregulating or downregulating PPAR-γ activity, which in turn affects glycemic control. The first committed and rate-determining step of glycolysis is catalyzed by phosphofructokinase-1 (PFK-1).

More than 4000 plants have been studied and identified to have hypoglycemic effects through several mechanisms of antidiabetic activity. *Annona muricata* Linn. (Annonaceae) is commonly known as 'Soursop' or 'Graviola' belong to the Annonaceae family. All parts of this plant has been used medicinally for the treatment/ management of an array of diseases. Several compounds have been isolated from parts of this plant, and their biological activities determined. (Watt and Breyer-Brandwijk, 1962; TDRG, 2002)

The hypoglycemic and anti-hyperglycemic properties of the plant *Vernonia amgdalina* (commonly referred to as bitterleaf in Nigeria) has been reported (Akah and Okafor, 1992; Nimernibo-Uadia, 2003; Akah *et al.*, 2004). However, there is a paucity of information on the mechanism of action of this plant in diabetic models.

A major drawback in the wide spread acceptability of these plants scientifically in the treatment of diabetes is the paucity of information on the mechanisms of action. Hence, this present study was undertaken to determine the expression levels of some genes in diabetic rats treated with extracts of *V. amgdalina* and *Annona muricata* as a step towards elucidating the mechanism of action of these plants. This study, therefore, sought to determine the gene messenger RNA (mRNA) expression level of Interleukin -1 (IL-1), Tumour necrosis factor (TNF), Glucose transporter 2 (GLUT-2), Phosphofructokinase 1 (PFK-1), and Peroxisome proliferator activated receptor (PPAR) in the Streptozotocin induced diabetes.

2. Methodology

2.1 Plant material identification and Authetication

Fresh leaves of *Annona muricata* and *Vernonia amgdalina* were obtained from a garden at Evbuomore quarters in Ugbowo, Benin City. Identification and authentication were done by Prof H.A Akinnibosun of the Department of plant Biology and Biotechnology, University of Benin, Benin City. Herbarium specimens were deposited in the herbarium with numbers assigned UBHa 0205 and UBH_V245 for *Annona muricata* and *Vernonia amgdalina,* respectively.

2.2 Preparation of plant extracts

Fresh leaves of both plants were air-dried for 40 days at 25°C, after which they were blended separately using a mechanical blender. 500g each of the plants were soaked in 2000ML of ethanol for 72hours, filtered, and then freeze- dried. Rats with FBS greater than or equal to 200mg/dl were used for this study.

2.3 Experimental design

Grouping for animals consist of five groups of six rats each.

Group 1(NC) were the normal control rats not induced with diabetes

group 2(DC) were the diabetic control rats induced with diabetes but left untreated

Group 3(PC) were the positive control rats induced with diabetes and treated with 0.5mg/kg b.w of glibenclamide

Group 4(T2) were induced with diabetes and treated with *Annona muricata* (200mg/kg b.w) extract

Group 5(T3) were induced with diabetes and treated with *Vernonia amgdalina* (200mg/kg body weight) extract

Animals in groups 2-5 were induced with diabetes by a single dose intraperitoneal injection with streptozotocin (dissolved in 0.1M Citrate buffer pH 4.5 at a dose of 60mg/kg body weight). Rats with Fasting blood glucose greater than or equal to 200mg/dl were used for this study. Approval for this study was given by the Life Sciences Ethics Committee for Animal Research with approval number LS21046. All experiments were also conducted according to the NIH's guidelines for the care and use of laboratory animals (NIH, 1985).

2.4 Collection of tissue sample

The rats were sacrificed after anaesthetizing with chloroform for 28 days. The liver were excised and kept in the eppendoff tube for RNA isolation.

2.4.1 Total RNA isolation:

Whole tissues were used to extract total RNA using a technique outlined by Omotuyi et al. (2018). In a nutshell, the liver tissues were homogenized in TRI reagent at 4 °C (Zymo Research, USA, Cat: R2050-1-50, Lot: ZRC186885). After centrifugation at 15,000 rpm/15 min, total RNA was separated in chloroform (BDH Analytical Chemicals, Poole, England Cat: 10076-6B) (Abbott Laboratories, Model: 3531, Lake Bluff, Illinois, United States). An equivalent volume of isopropanol was used to precipitate the RNA from the clear supernatant (Burgoyne Urbidges & Co, India, Cat: 67-63-0). The RNA pellet underwent two rinses in 70% ethanol: 30 ml of nuclease-free water (Inqaba Biotec, West Africa, Lot no: 0596C320, code: E476-500ML) and 70 ml of absolute

ethanol (BDH Analytical Chemicals, Poole, England Cat: 10107-7Y). After being allowed to air dry for five minutes, the pellets were dissolved in RNA buffer (1 mM sodium citrate, pH 6.4).

2.4.2 Complimentary DNA (cDNA) conversion:

Before cDNA conversion, total RNA quantity (concentration (µg/ml) = 40 * A260) and quality (≥ 1.8) were measured using a spectrophotometer (Jen-way UV-VIS spectrophotometer model 6305, UK) and the ratio of A260/A280 (A=absorbance). As directed by the manufacturer, DNA contamination was eliminated from RNA using DNAse I treatment (NEB, Cat: M0303S). The M-MuLV Reverse transcriptase Kit (NEB, Cat: M0253S) was used to convert 100 ng of DNA-free RNA into cDNA in a 20 µl final volume (2 µl of N9 random primer mix; 2 µl of 10X M-MuLV buffer; 1 µl of M-MuLV RT (200 U/µl); 2 µl of 10 mM dNTP; 0.2 µl of RNase Inhibitor (40 U/µl); and 10.8 µl of nuclease-free water. O/N, the reaction took place at room temperature. M-MuLV inactivation At 65°C, reverse transcriptase was run for 20 minutes.

2.4.3 PCR amplification and agarose gel electrophoresis:

The following methodology was used to perform PCR amplification. A 25 µl volume reaction mixture, including 2 µl of cDNA (10 ng) and 2 µl of primer (100 pmol), was used for PCR amplification. 8.5 µl of nuclease-free water and 12.5 µl of Ready Mix Taq PCR master mix (One Taq Quick-Load 2x, master mix, NEB, Cat: M0486S). 20 cycles of amplification (denaturation at 95 °C for 30 seconds, annealing (see TM values for each primer pair in table 1.0) for 30 seconds, extension at 72 °C for 60 seconds, and final extension at 72 °C for 10 minutes) were performed after the initial denaturation at 95 °C for 5 minutes. Negative controls, in which the reaction mixture contained no cDNA, were used in every experiment. The amplicons were resolved in Tris (RGT reagent, China, Lot: 20170605) on a 1.5% agarose gel (Cleaver Scientific Limited: Lot: 14170811).-Borate (Lot 20141117, JHD Chemicals, China)buffer with EDTA (pH 8.4).

2.4.4 Amplicon image processing:

As previously reported (Omotuyi et al., 2020), camera-captured in-gel amplicon band images were processed on the Keynote platform and quantified using image-J software. Graph-pad prism was used to plot each graph as mean +/- SEM.

3. Results

3.1 Effects of Annona muricata and Vernonia amgdalina on interleukin -1β on STZ induced rats

Fig 1 and 2 shows the effects of *Annona muricata* and *Vernonia amgdalin*a on interleukin -1β on STZ induced rats. A significant($p \leq 0.05$) increase was recorded in the expression of interleukin -1β in the diabetic control rats when compared with the normal control. However, treatment with extracts of *Annona muricata* and *Vernonia amgdalin*a and standard drug glibenclamide resulted in a significant($p \leq 0.05$) decrease in the expression levels of interleukin -1β compared to the diabetic control groups.

3.2 Effects of Annona muricata and Vernonia amgdalina on TNF-α on STZ induced diabetic rats

The effects of *Annona muricata* and *Vernonia amgdalin*a on TNF-α in STZ induced diabetic rats is shown in fig 3 and 4. The result showed that induction of diabetes to the rats led to a significant ($p \leq 0.05$) increase in the expression of TNF-α genes, while treatment led to a significant $p \leq 0.05$) decrease in its expression.

3.3 Effects of Annona muricata and Vernonia amgdalina on GLUT-2 on STZ induced diabetic rats

Fig 5 and 6 shows the effects of *Annona muricata* and *Vernonia amgdalin*a on expression levels of GLUT−2 genes in STZ induced rats. A significant($p \leq 0.05$) decrease was recorded in the expression of GLUT−2 genes in the diabetic control rats when compared with the normal control. However, treatment with extracts of *Annona muricata*, *Vernonia amgdalin*a, and standard drug glibenclamide resulted in a significant($p \leq 0.05$) increase in the expression levels of GLUT−2 genes compared to the diabetic control groups.

3.4 Effects of Annona muricata and Vernonia amgdalina on the expression of phosphofructokinase genes in STZ induced diabetic rats

Fig 7 and 8 shows the effects of *Annona muricata* and *Vernonia amgdalin*a on expression levels of phosphofructokinase genes in STZ induced rats. A significant($p \leq 0.05$) decrease was recorded in the expression of phosphofructokinase genes in the diabetic control rats when compared with the normal control. However, treatment with extracts of *Annona muricata*, *Vernonia amgdalin*a, and standard drug glibenclamide resulted in a significant($p \leq 0.05$) increase in the expression levels of phosphofructokinase genes compared to the diabetic control groups.

3.5 Effects of Annona muricata and Vernonia amgdalina on the expression of PPAR-γ genes in STZ induced diabetic rats

Fig 9 and 10 shows the effects of *Annona muricata* and *Vernonia amgdalin*a on expression levels of Peroxisome proliferator activated receptor (PPAR-**γ**) genes in STZ induced rats. A significant($p \leq 0.05$) decrease was recorded in the expression of PPAR-γ genes in the diabetic control rats when compared with the normal control. However, treatment with extracts of *Annona muricata*,

*Vernonia amgdalin*a, and standard drug glibenclamide resulted in a significant($p \leq 0.05$) increase in the expression levels of PPAR-γ genes compared to the diabetic control groups.

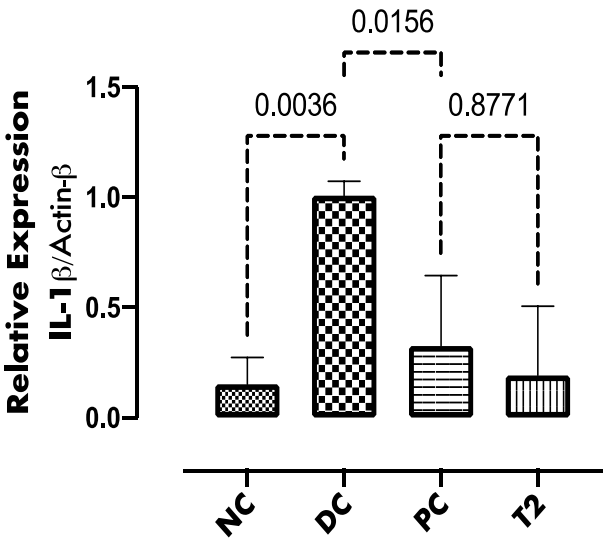

Fig 1: Effects of *Annona muricata* on interleukin -1β on STZ induced rats

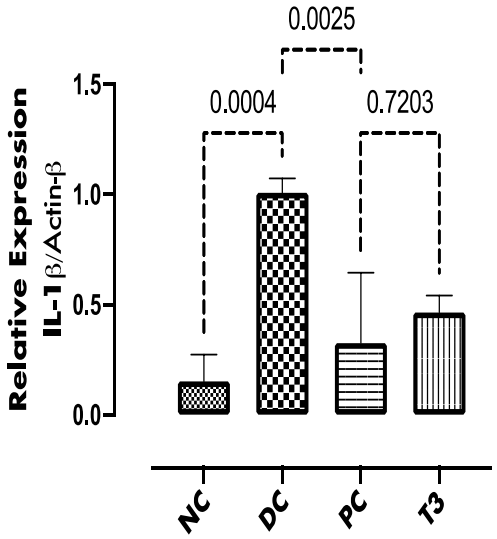

Fig 2: Effects of *Vernonia amgdalin*a on interleukin -1β on STZ induced rats

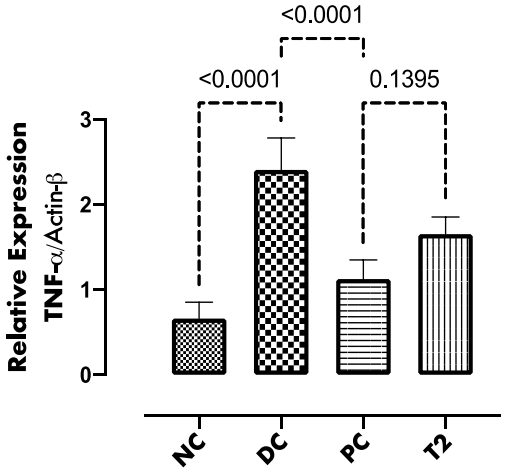

Fig 3: Effects of *Annona muricata* on TNF-α on STZ induced rats

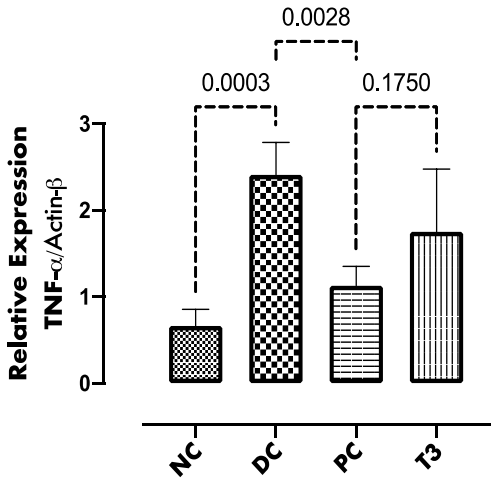

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Fig 4: Effects of *Vernonia amgdalina* on TNF-α on STZ induced rats

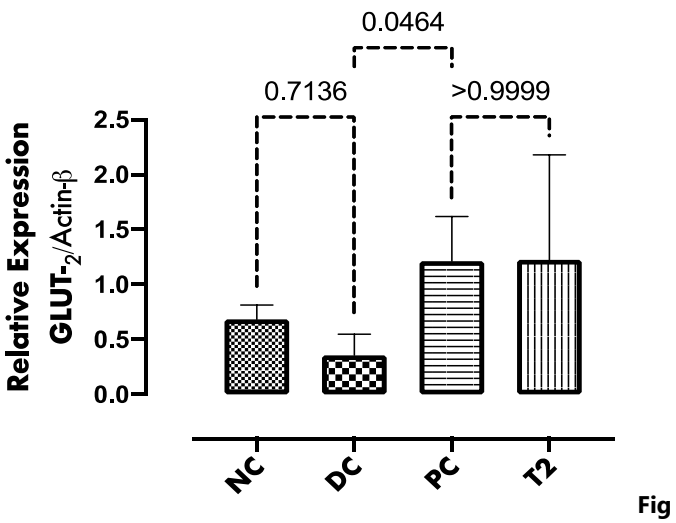

Fig 5: Effects of *Annona muricata* on GLUT-2 on STZ induced rats

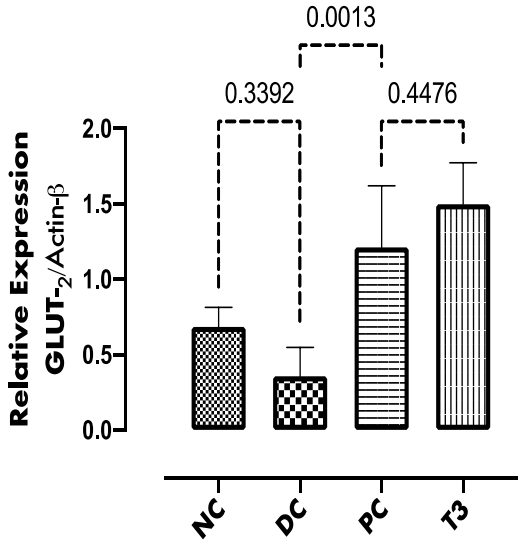

Fig 6: Effects of *Vernonia amgdalina* on GLUT-2 on STZ induced rats

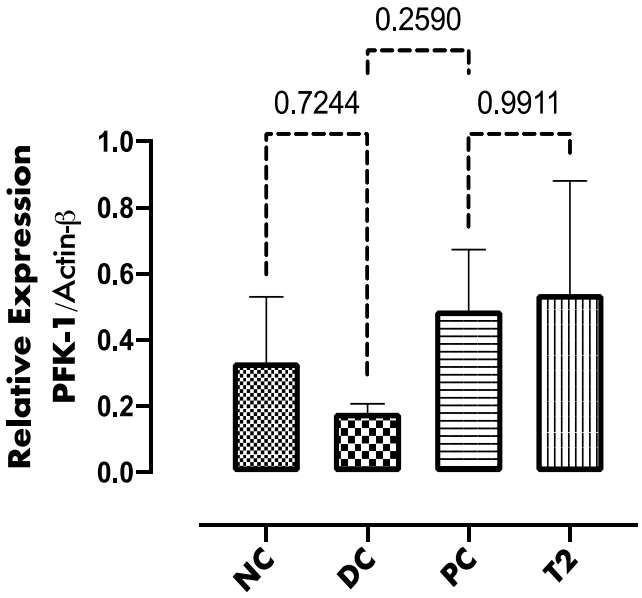

Fig 7: Effects of *Annona muricata* on PFK-1 on STZ induced rats

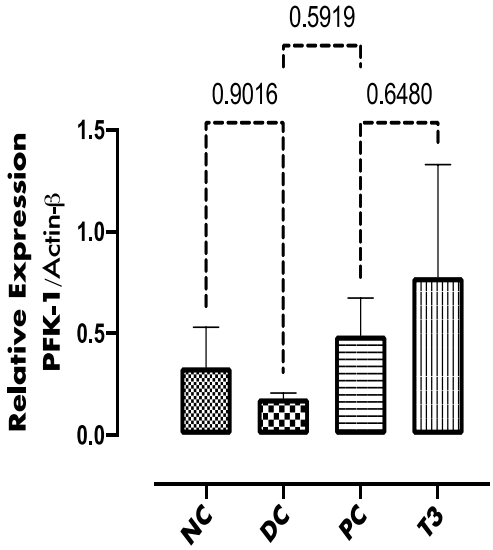

Fig 8: Effects of *Vernonia amgdalina* onPFK-1 on STZ induced rats

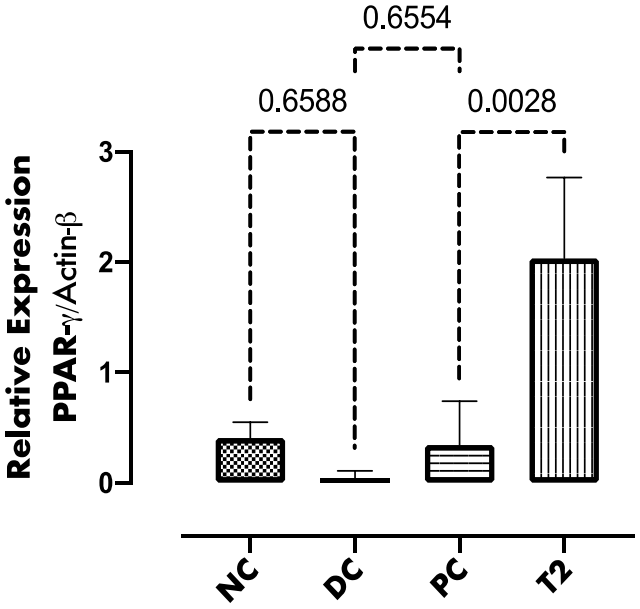

Fig 9: Effects of *Annona muricata* on PPAR-γ on STZ induced rats

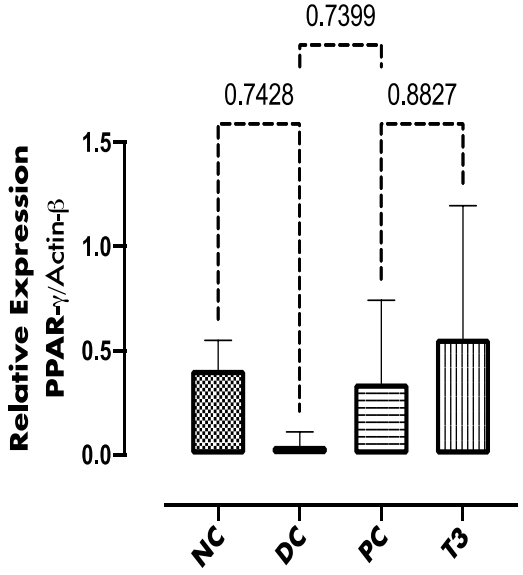

Fig 10: Effects of *Vernonia amgdalina* on PPAR-γ on STZ induced rats

Histology of rat pancreas

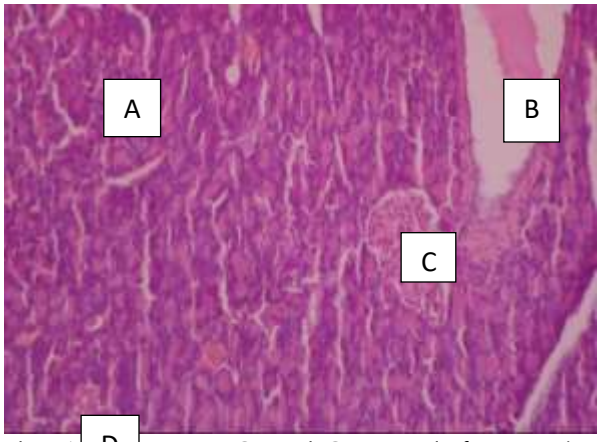

Plate 1: Pancreas. Control. Composed of A: exocrine acini, B, interlobar duct, C, Islet of langerhans, and D, interlobar septum(H&E x 100)

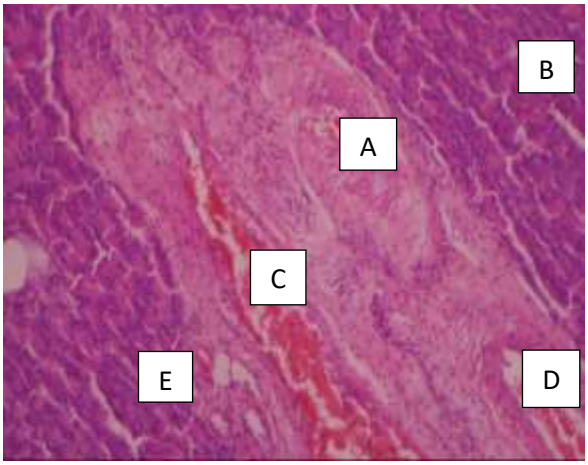

Plate 2: Pancreas of rat given Streptozoztocin (STZ) only showing: A, perivascular infiltrates of inflammatory cells; b, vascular ulceration; C, vascular hypertrophy and D, stenosis as well as E, paucity of islets (H&E x 100)

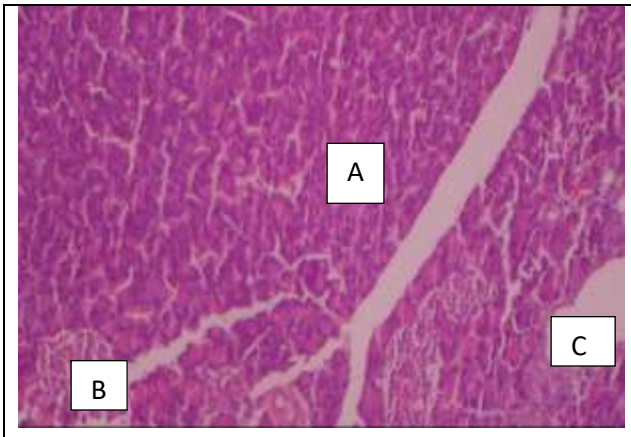

Plate 3: Diabetic rat pancreas given glibenclamide showing A, normal acini, B, regenerating Islets, and C, vascular hypertrophy (H&E x 100)

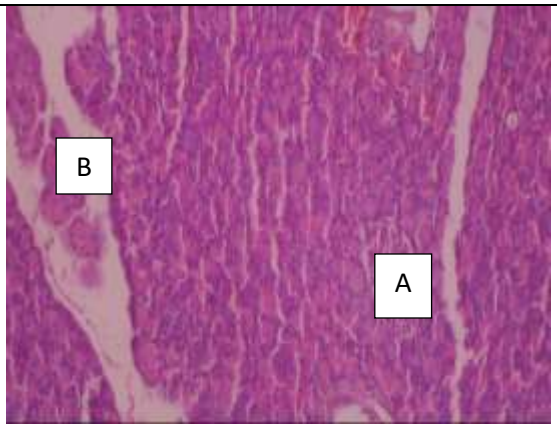

Plate 4: Pancreas of diabetic rat given V. amygdalina showing A: normal acini, B, normal vascular architecture, and C, regenerating islet (H&E x 100)

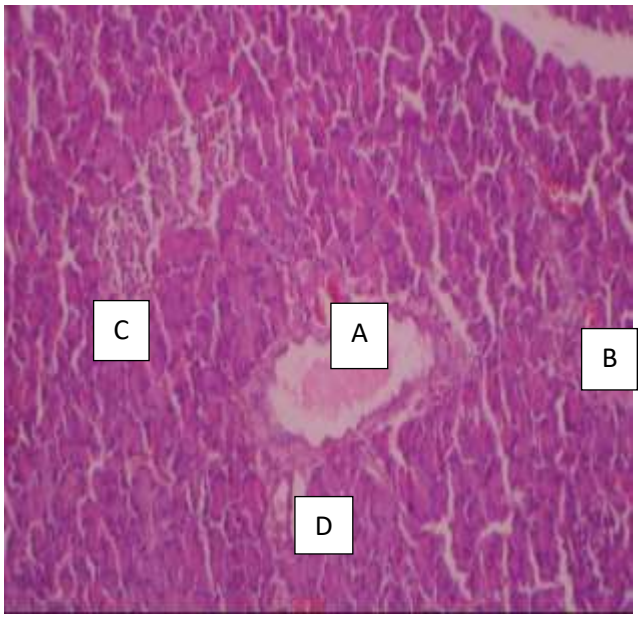

Plate 5: Pancreas of diabetic rat given A. muricata showing A: normal acini, B, regenerating islet, and C, normal blood vessels, and D, interlobar duct (H&E x 100)

4. Discussion

Diabetes was induced in albino Wistar rats using streptozotocin (STZ). The mechanism by which STZ induces diabetes is thought to be through its entry into the β-cells via a glucose transporter (GLUT2), where it causes the alkylation of DNA molecule and its eventual damage (Effing *et al.,* 2013). The Glut-2 gene encodes for the Glucose transporter 2 protein, which plays a crucial role in glucose homeostasis. It is primarily expressed in the liver, pancreatic beta cells, and kidneys (khosla *et al.*, 2000; Chattopadhyay *et al.,* 1987). Reduced Glut-2 expression in pancreatic beta cells was observed in group 2 (diabetes control), which leads to impaired glucose sensing, insulin deficiency, and hyperglycemia, characteristic of Type 1 diabetes(Kern *et al.,* 1990; Slentz *et al.,* 1992). The extracts of the two plants effectively increased the expression of GLUT-2, as observed in group 4 (T2) and 5 (T3) when compared with the diabetes control group. This indicating that *Vernonia amgdalina* and *Annona muricata* has antidiabetics potentials.

These results are consistent with a study on the ameliorative effect of *Annona muricata* extract on hyperglycemia Induced hepatic damage in type 2 diabetic Mice (Yiseul *et al.,* 2021).

Insulin resistance and inflammation are two features of type 2 diabetes mellitus. Interleukin 1 (IL1), a proinflammatory cytokine that regulates critical metabolic processes like insulin production and B-cell apoptosis, is one of the inflammatory markers that has been linked to B -cell destruction (Hend *et al.,* 2023). Lower expression of the IL-1 gene was observed in group 4 and 5 treated with *Vernonia amgdalin*a and *Annona muricata* extracts, respectively, when compared with the diabetic control group (DC). The *Vernonia amgdalina* extract interestingly showed a greater down regulaton effect on the IL-1 gene when compared with the group treated with the standard drug (group 3). These result is in agreement with a report by Goon-Tae and his team (Goon-Tae et al., 2016)

Tumor necrosis factor α(TNF−α) has been linked to the development of insulin resistance, a condition which impairs glucose uptake by cells and leads to higher levels of glucose in the blood (Akash *et al.,* 2018). TNF can influence the secretion and action of other hormones involved in glucose metabolism. For instance, TNF promotes the release of IL-6, which can worsen insulin resistance and impair glucose uptake in the muscles (Jatla *et al.,* 2012). TNF can also increase the breakdown of fats (lipolysis) in adipose tissue. This process releases free fatty acids into the bloodstream, which can further contribute to insulin resistance and glucose intolerance (Jatla *et al.,* 2012). High expression of TNF was observed in the diabetes control group, which is characteristic of type 2 diabetes (Goon-Tae *et al.,* 2016). However, upon treatment with the two extracts, as seen in group 4 and 5, the expression of TNF decreased when compared with the diabetic control.

The gene encoding PFK-1 plays a crucial role in glucose metabolism and glycolysis, a key metabolic pathway in cells. The diabetes control group showed low expression of the PFK-1 gene, which results in reduced activity of phosphofructokinase-1, which alter glucose metabolism and, impact glucose homeostasis, and potentially contribute to the development of diabetes. However, the groups given the two plant showed high expression of the PFK-1 gene, which invariably means increased activity of the enzyme phosphofructokinase-1. The enzyme responsible for catalyzing an essential step in glycolysis, the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate (Chukwudi and Osaretin, 2021). Higher expression levels of the PFK-1 gene leads to enhanced glycolysis, resulting in increased breakdown of glucose and subsequent generation of energy (Berg *et al.,* 2002). This is also similar to a report by Item and his team which investigated how *Vernonia amgdalina* suppress gluconeogenesis and potentiates glucose oxidation via the pentose phosphate pathway in streptozotocin induced diabetic rats (Atangwho *et al.,* 2014).

There are scientific evidence supporting the direct action of PPAR-γ on glucose metabolism as observed in the genes involved in insulin-stimulated glucose disposal (Hai-il and Yong-ho, 2004). High expression of the PPAR gene was observed in the groups treated with *Vernonia amgdalina* and *Annona muricata* extracts when compared to the diabetic control group.

Diabetes caused severe perivascular infiltrates of inflammatory cells, vascular ulceration, vascular hypertrophy and stenosis, and paucity of islets to the pancreas in untreated diabetic rats. Treatment with extracts of *Vernonia amgdalina* and *Annona muricata* restored the normal pancreatic architecture that were damaged by streptozotocin induction (plates 1−5).

5. Conclusion

In conclusion, our study provides evidence of the antidiabetic effects of *Vernonia amgdalina* and *Annona muricata* in streptozotocin-induced diabetic Wistar rats. These natural extracts demonstrated significant improvements in the expression of Glut-2, PFK-1, PPAR genes and decreased expression of IL-1 and TNF genes. Further studies are warranted to elucidate the underlying mechanisms and potential therapeutic applications of *Vernonia amgdalina* and *Annona muricata* in the management of diabetes mellitus.

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Conflicts of Interest: The authors declare no conflict of interest.

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