A Comparative Study on the Effect of Combined Methanolic Leaf Extracts of *Vernonia amygdalina* and *Gongronema latifolium* and Metformin on the Pancreatic beta cells of Streptozocin Induced Diabetic Wistar Rats

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ABSTRACT--- T***he effects of methanolic leaf extracts of Vernonia amygdalina (VA) (Bitter leaf) and Gongronema latifolium (GL) (Utazi) and metformin on the histology and the DNA of the pancreas of streptozocin (STZ) induced diabetic rats were investigated in this study. 24 male albino rats which were divided into four groups of six rats each were used for this work: A (Normal control) and B (Diabetic control) received tap water, C received 5mg/kg of Metformin daily, D received combined extract of VA and GL, 400mg/kg twice daily. Groups B – D animals were induced for diabetes by intraperitoneal injection of 45mg/kg of Streptozocin, Following sacrifice, the pancreas and blood were collected for histopathological and biochemical studies. The results revealed normal exocrine and endocrine pancreas in group A. In group B, the pancreatic islets cells were shrunken with signs of degeneration and inflammation. In the group that received metformin, the islet appeared shrunken. In group D, the pancreas showed prominent and normal acinar and islet cells, suggestive of a possible regeneration. Feulgen's reaction showed damage to the DNA of group B which was almost completely reversed in group D. The blood glucose in group B was significantly higher (P<0.05) compared to group A. In all the treatment groups, the blood glucose was significantly reduced (p<0.05) compared to group B. Administration of methanolic leaf extracts of Vernonia amygdalina and Gongronema latifolium especially in combination restores pancreatic beta cells damaged by STZ compared to metformin.***

Keywords--- Diabetes mellitus, metformin, *Vernonia amygdalina*, *Gongronema latifolium*, pancreatic beta cells

1. INTRODUCTION

Diabetes Mellitus is a metabolic disorder having the characteristic feature of hyperglycaemia, which is usually due to an absolute or relative lack of insulin, impaired effectiveness of insulin action or tissue insensitivity to insulin (1).The hyperglycaemia associated with diabetes mellitus results in the spillage of glucose into the urine, that is why diabetes mellitus is usually referred to as the " sweet -urine" disorder (2). Insulin, a hormone produced by the pancreatic beta cells functions to maintain a strict control of the blood glucose. The hormone makes it possible for the tissues and cells of the body to make use of glucose for energy but in the absence of insulin or where its action is impaired due to tissue insensitivity, cells and tissues are unable to uptake glucose causing an accumulation of glucose in the blood, which is responsible for the symptoms of diabetes.

The symptoms include: - weight loss, excessive thirst, excessive urination, increased hunger and general fatigue (2; 3).

Two main types of Diabetes mellitus exist- Type 1 and Type 2. Type 1 diabetes, also known as Insulin Dependent Diabetes Mellitus (IDDM) accounts for about 10% of all cases of diabetes. Previously known as juvenile onset diabetes, it usually occurs in those below 40years of age (2; 4). In type 1 diabetes, there is usually a deficiency of insulin secretion due to disorders affecting and destroying the beta cells of the pancreas and it is reported to have a genetic predisposition and an autoimmune basis; sufferers require a daily dose of insulin to survive (28). Type 2 diabetes affects about 90% of people with diabetes mellitus and people that are 40 years and above (29). It occurs as a result of tissue insensitivity to insulin leading to impaired insulin action and in some cases, insulin levels may be high (28).
Diabetes mellitus is a chronic disease which can lead to many other health problems that are debilitating and sometimes life-threatening. Despite the type of diabetes, poorly managed diabetes is associated with debilitating complications such as microangiopathy, nephropathy, retinopathy, cardiovascular diseases (30), infertility and problems with conception. Some of the factors implicated in the development of diabetic complications are non-enzymatic glycosylation,

intracellular glucose reduction via aldose reductase, lipoprotein modification and free radical damage, due to sustained hyperglycaemia.

Although, copious knowledge exists due to researches done on diabetes and chemotherapeutic agents, the morbidity and mortality associated with diabetes is still at high levels. People suffering from diabetes are more vulnerable to infections, especially respiratory tract infections; blindness, kidney failure and lower limb amputation is caused by diabetes mellitus in industralised nations (31). According to (32), the disease ranks as number 7 among the conditions that cause death in America.

Various reports exist on the prevalence of diabetes mellitus. As at 2013, the global estimate was said to be 8.3% with about 382 million suffering from the condition. This estimate will most likely increase to about 592 million by 2035 (33). The region with the highest prevalence of 11% is North America and the Carribean with 37 million sufferers, the Middle East and North Africa has a prevalence of 9.2% with thirty five million people while the prevalence in the Western Pacific region is 8.6%, close to the global prevalence (34).

It has been reported that the use of plant derived extracts may actually result in the treatment of diabetes mellitus, these plants are said to contain various herbal and non-herbal properties such as phytonutrients and phytochemicals which can act on several targets through diverse modes and mechanisms (5). Whereas, some of these plants are very rich in antioxidants, thus providing antioxidant defense (6), some delay gastric emptying rate while some enhance insulin secretion

There has been an expansion of conventional medicine in the last 3 decades; however, a lot of Africans, particularly Nigerians are still patronizing traditional medicine. In addition, evidence has shown that some herbal remedies are highly efficacious, potent with minimal side effects and highly affordable, thus the need to explore and carryout researches in this area (7).

Vernonia amygdalina Del belongs to the compositae family and is widely grown in the tropical forest of sub-saharan Africa (8). The plant is reported to have hypoglycaemic effect (9; 10), hypolipidaemic effect (11) and protective effect on the kidneys of diabetic rate (11). *Gongronema latifolium* belongs to the ascepiadeceae family of plants. According to (12, 27), the leaf extracts of the plant has hypoglycaemic, hypolipidaemic and antioxidant properties. (13) reported its anti-inflammatory activities and the regenerative effect of the combined leaf extracts of VA and GL, on pancreatic beta cells of wistar rats has been reported by (10).

STZ is one of the agents that are used to induce experimental diabetes. STZ enters the beta cells through the glucose transporter, GLUT 2 and causes alkylation of DNA which induces DNA damage, resulting in damage to the pancreatic beta cells thus presenting a prototype of Type 1 diabetes (14, 36).

2. MATERIALS AND METHODS

2.1 Preparation of plant materials

Fresh and matured leaves of *Gongronema latifolia and vernonia amygdalina* were purchased from Marian market (IkaIka Oqua market) in Calabar municipality of Cross River State, Nigeria. The leaves were rinsed severally with clean water and thereafter allowed to completely drain. The leaves were then air dried under shade in the under ambient temperature. The dried leaves were homogenized into powder form using an electric blender. The powdered plant materials were respectively soaked in plastic buckets and ethanol added; the solvent to solute ratio being 2:1 for 48 hours with intermittent agitation. The mixture was filtered first using a chess cloth followed by the filtrate being filtered again through Whattman No1 filter paper of pore size 0.45micrometer. The filtrate was placed in beakers and allowed to concentrate in a water bath by evaporation at 40°C to complete dryness yielding 93g of crude extract each.

2.2 Experimental animals

Twenty-four (24) male albino rats, weighing 80-140g, were used for the work and were kept in the animal house of the Department of Biochemistry, Faculty of Basic Medical Sciences, University of Calabar. The animals were obtained from the animal house of the Department of Pharmacology, University of Calabar. They were allowed to acclimatize for 2 weeks prior to experimentation, kept in properly ventilated cages, which were cleaned every two days, and at a room temperature of about 27^{o}C and 12 hour light/dark cycle.

The animals were divided into four (4) groups of 6 rats each. The first group was the Normal control group which was given tap water, designated as NC. The second group was the Diabetic control group. The animals in this group were induced for diabetes and were given normal feed and water. They received no treatment and were designated DC. Group A animals were induced for diabetes and were treated with Metformin (5mg/kg daily). Group B were induced for diabetes and were treated with the combined extracts of VA and GL (400mg/kg twice daily). Group C were induced for diabetes and were treated with VA extract (200mg/kg twice daily) while Group D were induced for diabetes and were treated with GL extract (200mg/kg twice daily) only. Metformin and plant extracts were administered through orogastric tube.

2.3 Induction of Diabetes

Diabetes was induced in overnight fasted experimental animals by a single dose of intraperitoneal injection of freshly prepared streptozocin (STZ) 45mg/kg body weight reconstituted in 0.1M sodium citrate buffer (pH4.5-5.0) as solvent.

Diabetes was confirmed in the STZ treated rats by measuring their fasting blood glucose concentration 48hrs after STZ injection using on-call-plus glucometer and rats having fasting blood glucose of more than 180mg/dl were considered diabetic and included in the study (Table 1).

Table 1: Experimental Design

Group	Agent administered	Quantity
Normal control	Normal tap water	Ad. Libitum
Diabetic control	STZ	45mg/kg
C	Metformin	5mg/kg
D	VA + GL	400mg/kg (200mg/kg each)

2.4 Statistical analysis

Oneway analysis of variance (ANOVA) and Post HOC LSD tests were used for the statistical analysis. Results are expressed as Mean ± Standard Error of Mean (SEM)

3. RESULTS

3.1 Blood glucose

Table 2 below shows the mean basal and final blood glucose of experimental animals. There was a significant increase ($p<0.05$) in the blood glucose of the DC group compared to the NC group. The blood glucose level of the normal control group and the treatment groups was significantly reduced ($p<0.05$) compared to the DC group at the end of the experiment. Compared to the basal values, the percentage decrease in the blood glucose was greater in the group treated with the combined extracts of VA and GL than in the Metformin (VA+GL – 112% and 109% for metformin).

Table 2: Mean Basal and Final blood glucose of the different experimental groups

Groups	Initial blood glucose (mg/dl)	Final blood glucose (mg/dl)
Normal control	68.16 ± 2.48	60.33 ± 7.28
Diabetic control	156.16 ± 4.86	257.00 ± 4.43[a]
Metformin	206.40 ± 9.16	97.60 ± 12.06[*]
VA+ GL	228.33 ± 3.57	116.60 ± 10.11[*]

Values are expressed as Mean ± SEM, n=6
*significantly different from DC at $p<0.05$
a= significantly different from NC at $p<0.05$

3.2 Haematoxylin & Eosin and Feulgen's reaction

Results of the normal control group revealed normal and prominent islet of Langerhans and acinar cells. The nuclei in the islet cells and the acinar cells were prominent and were strongly positive to Feulgen's reaction. A pancreatic duct was also observed in the specimen (Plate 1). In the diabetic control group, the Islet of Langerhans appeared shrunken and necrotic. Macrophages (inflammatory cells) were observed in the islet, the cellularity of the islet cells was reduced and fibrous tissues were seen indicating damage to the islet cells. The islet cells showed no reaction while the acinar cells were strongly positive to Feulgen's reaction (Plate 2).

For the group that received metformin, the islet of langerhans was present but appeared shrunken. There were fibrous tissues in the islet and with Feulgen's reaction, there were strongly positive cells interspersed with weakly positive cells in the pancreatic islet. The acinar cells were prominent and appeared normal (Plate 3). For the group that received the combined extracts of VA and GL, the islet of Langerhans was prominent and appeared normal. The pancreatic islet was prominent with cells that were strongly positive to Feulgen's reaction but compared to the normal control group, there was slightly reduced cellularity in the islet. Pancreatic duct was observed and the acinar cells appeared normal (Plate 4).

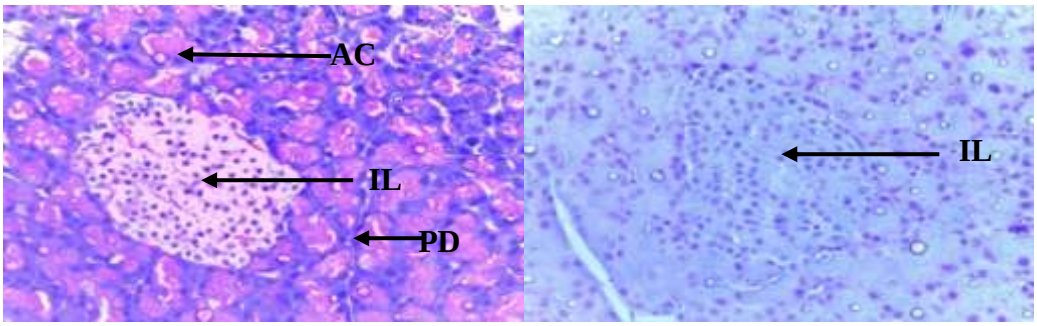

Plate 1: Photomicrograph of Normal control animals stained with H&E (X400) and Feulgen's reaction (X400). Showing well circumscribed islet with prominent cells, and deeply stained nuclei indicating strong positivity to Feulgen's reaction. Acinar cells and pancreatic duct are also present. **IL** – Islet, **AC** – Acinar cell, PD – Pancreatic duct

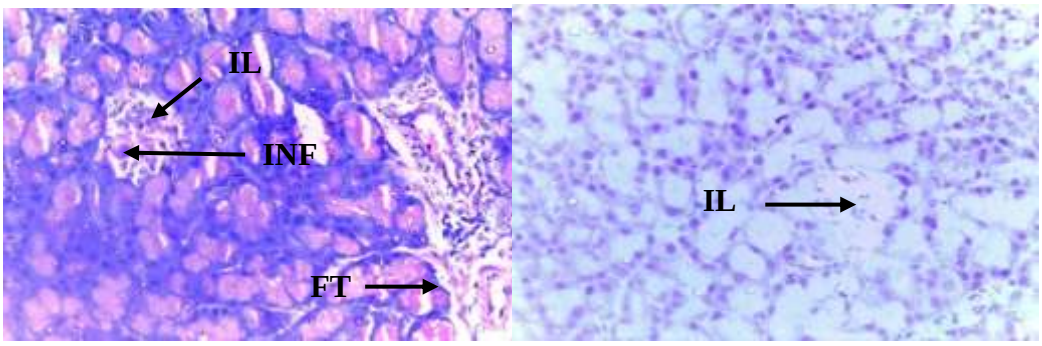

Plate 2: Photomicrograph of the pancreas of diabetic control animals stained with H&E and Feulgen's reaction (X400). Showing shrunken and pyknotic islet with cells that are negative to Feulgen's reaction. There is also reduced cellularity of the islet, fibrous tissues and inflammatory cells are present. The Acinar cells are normal. **IL** – Islet, **INF** – Inflammatory cell, **FT** – Fibrous tissue

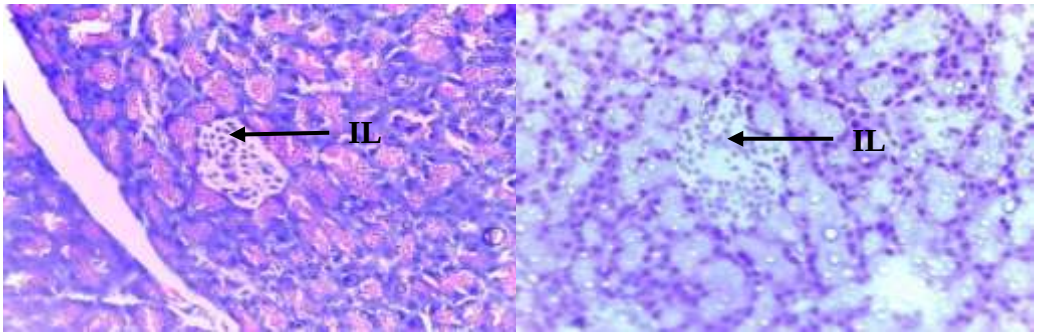

Plate 3: Photomicrograph of group C animals given 5mg/kg of metformin stained with H&E and Feulgen's reaction (X400). The islet appears shrunken and degenerated and the cells are weakly positive to Feulgen's reaction

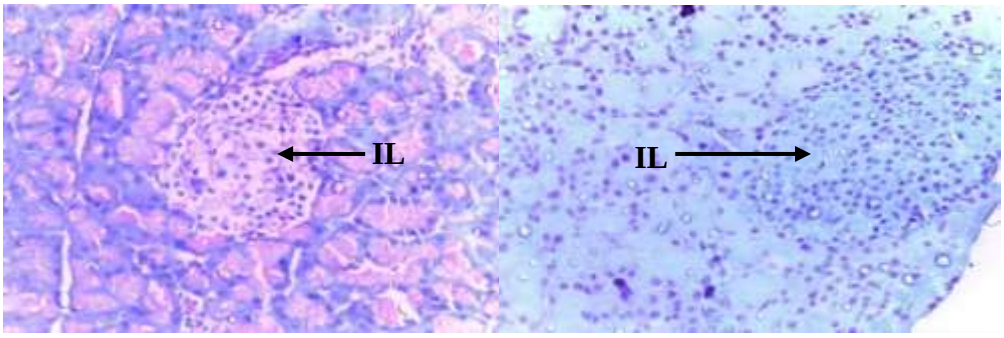

Plate 4: Photomicrograph of Group D animals, given 400mg/kg of combined extracts of VA and GL stained with H&E and Feulgen's reaction (X400). Showing a well circumscribed islet, similar to that of the normal control, with prominent islet cells that are strongly positive to Feulgen's reaction.

4. DISCUSSION

In the diabetic control group, there was a significant increase in the blood glucose compared to the normal control and all the treatment groups (metformin, combined extracts of VA&GL). This suggests that the insulin producing cells of the pancreas were destroyed in the DC group. This is in line with a study by (15) who reported that Streptozocin which was used for diabetes induction in this study damages pancreatic beta cells resulting in hypoinsulinaemia and hyperglycaemia. Streptozocin, according to (14) selectively destroys the beta cells of the pancreas thus presenting a prototype of Type 1 Diabetes.

The blood glucose levels in the group that received the plant extracts and the metformin was significantly reduced compared to the DC group at the end of the experiment. Medicinal plants are said to be used in the management of diabetes mellitus due to their blood sugar lowering effects, consequent upon stimulation of insulin release and their antioxidant properties. The reduction observed in the groups that were given the plant extracts may have been due to the stimulation of insulin release by the extracts or a possible regeneration of the pancreatic beta cells. An earlier study carried by (10) showed that the extracts of VA and GL, especially when used as combined extracts have the potential of causing a regeneration of pancreatic beta cells destroyed by Streptozocin. Flavonoid-rich, saponin-rich and glycoside-rich fractions of *Vernonia amygdalina* have also been found to lower blood glucose and ameliorate pancreatic damage (16, 28) due to their antioxidant free radical scavenging activity which could counteract the generation of free radicals, one of the factors responsible for STZ induced diabetes. The antioxidant properties of medicinal plants help to scavenge reactive oxygen species or stop its generation. This may be one of the mechanisms through which phytochemicals reverse pancreatic damage leading to an improvement in blood glucose levels (17, 27). Metformin on the other hand causes a reduction in blood sugar due to its effect on tissue sensitivity to insluin, its mechanism of action is increasing tissue sensitivity to insulin through its effect on insulin receptor expression and tyrosine kinase activity, and enhancing glucose uptake by the tissues (18). Metformin also reduces blood glucose by decreasing hepatic glucose production primarily by inhibiting gluconeogenesis (19).

On H&E, the pancreas of the normal control animals revealed normal histological features in both the endocrine and exocrine pancreas. In the diabetic control group, there was a marked distortion of the cytoarchitecture of the pancreatic Islet. The Islet appeared shrunken and degenerated with signs of necrosis. Lymphocytes were identified in the Islet showing signs of inflammation. For the Feulgen's test for DNA, it was observed that most of the cells in the DC group were negative for the reaction (the cells did not pick up the stain) in the islet cells of the pancreas suggesting damage to the DNA in the nuclei of the cells with consequent cellular damage. The acinar cells of the pancreas were however strongly positive to the reaction. According to (14), Streptozocin (STZ) which was used to induce diabetes in this work is known to exert its toxic effect on cells primarily by damaging the cell's DNA. STZ selectively damages the beta cells of the pancreas due to its similarity to glucose molecules which causes it to be transported into the cells by the glucose transporter, GLUT 2 and pancreatic beta cells have a relatively high level of GLUT 2 (20;15). STZ also causes the release of glutamic acid decarboxylase autoantigens which induces immune and inflammatory reactions in tissues. In this situation, the destruction of beta cells with the resultant hyperglycaemia is associated with inflammatory cells in the pancreatic islets (21, 22), as was observed in this study. Besides STZ, the DNA damage observed in this study may have also been due to hyperglycaemia. In a study by (23), it was discovered that hyperglycaemia induces chromatin remodeling in mouse hepatocytes. It also affects the action and expression of sirtuins which further promotes chromatin remodeling and consequently, DNA damage. Another mechanism of DNA damage secondary to hyperglycaemia is excessive production of free radicals. Increased ROS generation and the simultaneous decrease in antioxidant defense mechanism in diabetic patients may be responsible for the organ damage associated with diabetes (23). In the groups that received the combined extracts of VA and GL, there was marked improvement in the cytoarchitecture of the pancreatic Islet and the cells. The histological features of the cells were almost similar to that of the normal control. With Feulgen's reaction, the cells showed strong positivity to the reaction. This suggests a possible regeneration of the pancreatic beta

cells and a reversal of the diabetic insults on the cells by the extracts. This observation agrees with previous studies which reported on the potentials of extracts of *Vernonia amygdalina*, *Gongronema latifolium* and *Azadirachta indica* to cause a regeneration of pancreatic beta cells of STZ induced diabetic rats. (25, 10). This effect of the plant extracts was more pronounced when the extracts were used in combination. In the groups that were treated with metformin, the cells were weakly positive to the reaction. The reaction of the cells to Feulgen's test may have been due to the potential of metformin in reducing hyperglycaemia. Metformin which is the most commonly used Biguanide acts by causing a reduction in glucose output from the liver while at the same time causing an increased uptake of glucose by the skeletal muscles and other cells (26) thus reducing blood glucose levels. The staining intensity of cells treated with Feulgen's reaction is dependent on the amount of viable DNA in the cells. These results therefore show the potentials of plant extracts to reverse DNA damage caused by streptozocin in pancreatic beta cells compared to metformin.

5. SUMMARY AND CONCLUSION

This study elucidated the effect of methanolic extracts of the leaves of VA and GL in combination and metformin administration on the histology of the pancreas of STZ induced diabetic male Wistar rats. 24 male Wistar rats were used for the study. The histology of the pancreas was evaluated using Haematoxylin and Eosin stain and Feulgen's reaction for DNA. Results revealed hyperglycaemia and DNA damage in pancreatic islet cells in the diabetic control group. These effects were reversed on administration of the plant extracts and the blood glucose level was reduced to almost normal levels on treatment with metformin although there was no appreciable difference in cytoarchitecture of the pancreas in the metformin treated group compared to the diabetic control group. In the groups that received the combined extracts, the reversal of diabetic insults on the histology of the pancreas was close to that of the normal control. This effect is suggested to be due to the presence of secondary metabolites (phytochemicals) in the plants.

In conclusion therefore, combined methanolic leaf extracts of VA and GL has the potential of reversing DNA damage caused by streptozocin in pancreatic islet cells, compared to metformin administration.

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