Administration of Combined Methanolic Leaf Extracts of Vernonia amygdalina and Gongronema latifolium Enhanced Glut 2 Expression in the Pancreas and Downregulates Serum Caspase 3 Activity of Streptozotocin-Induced Diabetic Wistar Rats

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Authors’ contributions

This work was carried out in collaboration among all authors. Author MKA designed the study, wrote the protocol and the first draft of the manuscript and together with author NNO performed the statistical analysis. Authors NNO and PEE managed the analyses of the study. Author PEE assisted in obtaining the funding for the research. All authors read and approved the final manuscript.

ABSTRACT

Aim: The study evaluated the effects of the combined extracts of Vernonia amygdalina (VA) and Gongronema latifolium (GL) on pancreatic GLUT 2 expression and caspase 3 activity in streptozotocin (STZ, 45 mg/Kg)-induced diabetic rats.

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**Study Design:** Fifteen Albino rats were used for the study and were placed in 3 groups of 5 rats each: A - normal control, B – Diabetic control and C – experimental group.

**Place and Duration of Study:** The study was carried out in the department of Anatomy, University of Calabar.

**Duration:** 6 months.

**Methodology:** Half of the diabetic rats were treated with VA+GL (400mg/kg, ratio 1:1, DE group) for 28 days, while the other half was untreated and served as diabetic control (DC). Normal control (NC) rats were untreated. After 28 days, the rats were sacrificed and their blood glucose, serum GLUT 2 and caspase 3 activity were measured. Histochemical evaluation of the pancreas was also carried out.

**Results:** Blood glucose concentrations for the 3 groups were 60.31±7.28, 257.00±4.43, and 116.60±10.11 mg/dl for NC, DC and DE respectively. This represented a 4-fold increase in the DC compared with NC and a significant amelioration in the extract-treated DE group compared with DC group. Serum GLUT 2 concentrations were 70 ng/ml in NC, dropped to 8 ng/ml (p<0.05) in the DC and recovered to 20 ng/ml in DE (p<0.05). Serum caspase was 3.2 ng/ml for NC, increased to 8.5 ng/ml in DC (p<0.05) and reduced to 1.8 ng/ml in DE (p<0.05). The histology of the pancreas showed distorted, degenerated and shrunken β-cells mass in DC compared with NC and DE groups. The DE group showed clear signs of regeneration of the islet cells which was corroborated by positive Feulgen’s reaction compared with the DC group.

**Conclusion:** The data suggests that the combined VA+GL extract has the potential to effectively reverse pancreatic damage in diabetes.

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**Keywords:** Vernonia amygdalina; Gongronema latifolium; GLUT 2; Caspase 3; blood glucose.

### 1. INTRODUCTION

Diabetes mellitus is recognized as a group of metabolic disorders with the common element of hyperglycaemia due to either insulin deficiency or impaired effectiveness of insulin action which may be inherited or acquired [1]. The hyperglycaemia is a manifestation of several historical anomalies and depicts a complete deterioration of the endocrine control rather than a pathogenic factor. Individual chemotherapeutic agents act only on part of the pathogenic process and only to a partial extent [2] hence cannot address the problem holistically. The morbidity and mortality from the disease continues to rise despite the availability of huge number of pharmacologic agents for treatment. Thus a multimodal therapeutic approach is required [3].

GLUT 2 is a glucose transporter which facilitates the movement of glucose across cell membranes. It is the principal transporter of glucose in the pancreatic beta cell where it initiates the first step in glucose mediated insulin secretion. It functions as a glucose sensor in pancreatic β-cells of rodents [4] and is found primarily in cellular membranes of liver and the pancreatic beta cells [5]. Caspase 3 on the other hand is a member of the cysteine – aspartic acid protease family, which plays a central role in the execution phase of cell apoptosis [6]. β-cell apoptosis is one of the critical events that contribute to the pathogenesis of type 1 diabetes and apoptosis mediated by caspase 3 has been reported to be the primary mechanism through which β-cells are destroyed. Consequently, caspase 3 mediated β-cell apoptosis is said to be an important step in the development of diabetes [7]. Studies have also shown that although type 1 diabetes is an autoimmune disease which occurs as a result of the selective destruction of insulin producing pancreatic cells by antigen- specific-T cells [8], apoptosis is a fundamental process involved in the destruction of the insulin producing cells [9,8]. β-cell apoptosis is also reported to facilitate cross-presentation of islet cells antigens which is a step critical in the activation of cell specific T cells, a major requirement for the onset of diabetes mellitus [10,11,3].

Vernonia amygdalina Del (African bitter leaf), Azadirachta indica A. Juss (Neem) and Gongronema latifolium (locally known as Utazi in Eastern Nigeria) are some of the plants used traditionally in the management of diabetes in African and Asia [12]. Generally, plants exert their beneficial effect on diabetes via various mechanisms such as: carbohydrate/lipid metabolism modulation in the liver (via induction of key enzymes), influence on β-cells integrity, aldose reductase activity, and antioxidant defence system regulation, and glucose uptake and utilization [13]. Some plants possess phytochemicals that interfere with carbohydrate
digestion and absorption, and may have insulin-like activity or able to inhibit insulinase activity [14]. Thus plants offer exciting opportunities for the development of novel therapeutics.

Vernonia amygdalina (V. amygdalina) Del belongs to the Compositae family and grows extensively in a range of ecological zones in tropical Africa [15]. The antihyperglycemic action of the plant [16], hypoglycaemic effect [12]; hypolipidemic and antihyperlipidemic action of the aqueous leaf extract [17] and its protective effect on kidneys of diabetic rats [17] have been reported. Gongronema latifolium (G. latifolium) on the other hand is a tropical rainforest plant belonging to the Ascepiadaceae family [18]. The hypoglycemic, hypolipidaemic [19] and antioxidant [20] properties of its leaf’s extracts have been articulated. Morebise et al. [21] reported on its anti-inflammatory effects, Hernandez et al. [22] reported on its antioxidant properties whereas and we Akpaso et al. [23] reported on its regenerative properties on pancreatic b-cells. The combined extracts of VA and GL have been reported to improve sperm parameter and testicular damage in STZ induced diabetic rats [24], enhance insulin secretion and reproductive hormone level in diabetic state [25] and the hypoglycaemic potential of the combined extracts have been shown to compare favourably with that of metformin [26]. Despite these reports, the molecular mechanism of the anti-diabetic actions of V. amygdalina and G. latifolium are still not well understood. In particular, the benefits of a combined therapy are not known.

In developed countries such as United States, it is estimated that plant–based drugs constitute as much as 25% of the total drugs available, whereas, in developing countries including China and India, the contribution is as much as 80% [27]. In Africa, remedies made from indigenous plants play a crucial role in the health of millions, with growing number of people relying more and sometimes exclusively on plants for treatment of various illnesses and ailments [28].

2. MATERIALS AND METHODS

Fresh and matured G. latifolia (P.E.S (BOT)/HERB/UC/ 718) and V. amygdalina (P.E.S (BOT)/HERB/UC/188) leaves were bought from local market in Calabar municipality of Cross River State, Nigeria. The leaves were identical with previously deposited specimen in the herbarium unit, Department of Botany, University of Calabar, Calabar, Nigeria. The leaves were washed severally with clean tap water followed with distilled water and thereafter allowed to completely drain. The leaves were then air dried under shade and ambient temperature. The air-dried leaves were homogenized using an electric blender into powder form. The powdered plant materials were respectively soaked in plastic buckets and methanol added, the solvent to solute ratio being 2:1 for 48 hours with intermittent agitation. The solution was filtered using a cloth filter followed by the filtrate being filtered again through Whatman 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 40ºC to total dryness producing 93 g of crude extract each.

2.1 Induction of Diabetes

Diabetes was induced in overnight fasted experimental animals by a single dose of intraperitoneal injection of freshly prepared streptozotocin (STZ) 45 mg/kg body weight reconstituted in 0.1 M sodium citrate buffer (pH4.5-5.0) as solvent. Diabetes was ratified in the STZ treated rats by checking their fasting blood sugar concentration 48hrs after STZ injection using a glucometer (on-call-plus) and rats having fasting blood sugar above 180 mg/dl were regarded to be diabetic and were included in the study.

2.2 Experimental Animals

Fifteen [15] adult albino rats, weighing 80-140 g, were used for this study. The animals were kept in properly ventilated cages and at a room temperature of about 27ºC and 12-hour light/dark cycle. All experiments were conducted in accordance with international guidelines for the care and use of laboratory animals.

2.3 Study Design

The animals were divided into 3 groups of 5 rats each. Group A was the Normal control group which was given tap water. Group B 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. Group C were induced for diabetes and were treated with the combined extracts of VA and GL (400 mg/kg b w twice daily) administered through orogastric tube (Table 1).

After twenty-eight days of treatment the animals were sacrificed using chloroform inhalation. During this process blood was collected by cardiac puncture and organs collected for biochemical and histological assessments.
Table 1. Experimental design

<table>
<thead>
<tr>
<th>Group</th>
<th>Agent administered</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (Normal control)</td>
<td>Feed and water</td>
<td><em>Ad libitum</em></td>
</tr>
<tr>
<td>B (Diabetic Control)</td>
<td>STZ</td>
<td>45 mg/kg single dose</td>
</tr>
<tr>
<td>C (Diabetic + Extract)</td>
<td>VA + GL</td>
<td>400 mg/kg b w (ratio1:1) twice daily for 28 days</td>
</tr>
</tbody>
</table>

2.4 Determination of GLUT 2 and Caspase 3 expression

2.4.1 Sample collection and preparation

Following euthanasia, the pancreas was collected, cleaned of excess blood and weighed. The tissue was then minced to small pieces and homogenized in Phosphate-Buffered Saline (PBS). The resulting suspension was subjected to ultra-sonication and centrifuged for 15 minutes. The supernatant was then collected and used for GLUT 2 assay while the serum was collected for Caspase 3 assay.

2.4.2 Caspase 3 assay

CASPASE-3 ELISA kit applies the competitive enzyme immunoassay technique utilizing a monoclonal anti-CASPASE-3 antibody and a CASPASE-3-HRP conjugate. The assay sample and buffer were incubated together with CASPASE-3-HRP conjugate in pre-coated plate for one hour. After the incubation period, the wells were decanted and washed five times. Thereafter, the wells were incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution was added to stop the reaction, which turned the solution yellow. The intensity of color is measured spectrophotometrically at 450 nm in a microplate reader. The intensity of the color is inversely proportional to the CASPASE-3 concentration since CASPASE-3 from samples and CASPASE-3-HRP conjugate compete for the anti-CASPASE-3 antibody binding site. Since the number of sites is limited, as more sites are occupied by CASPASE-3 from the sample, fewer sites are left to bind CASPASE-3-HRP conjugate. A standard curve is plotted relating the intensity of the color (O.D.) to the concentration of standards. The CASPASE-3 concentration in each sample was then interpolated from the standard curve.

2.4.3 Glut2 assay

This assay is based on the sandwich ELISA principle. Each well of the supplied microtiter plate was pre-coated with a target specific capture antibody. Samples were added to the wells which caused the target antigen to bind to the capture antibody. Unbound sample was washed away. A biotin-conjugated detection antibody was then added which bound to the captured antigen. Unbound biotinylated detection antibody was washed away. An Avidin-Horseradish Peroxidase (HRP) conjugate was then added which bound to the biotin. Unbound Avidin-HRP conjugate was washed away. A TMB substrate was thereafter added which reacted with the HRP enzyme resulting in color development. A sulfuric acid stop solution was added to terminate color development reaction and then the optical density (OD) of the well was measured at a wavelength of 450 nm ± 2 nm. An OD standard curve was generated using known antigen concentrations and the OD of the sample was compared to the standard curve in order to determine its antigen concentration. The investigations were carried out according to the manufacturer's instructions and the kits were obtained from Life Span Bio Sciences, (LSBio) Inc for Rat CASP3/Caspase 3 and Glut2 assays.

3. RESULTS

3.1 Blood Glucose Concentrations

Table 2 shows the blood glucose concentrations for the experimental groups. 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 group was significantly reduced (p<0.05) compared to the DC group at the end of the experiment.

3.2 Serum Caspase 3

Fig. 1 shows the serum concentration of caspase 3 in the pancreatic tissue of the different experimental groups. The level of the enzyme in the diabetic control group was significantly higher (8.5 ng/ml) (p<0.05) compared to the normal control (3.2 ng/ml) and the treatment group (1.8 ng/ml) while that of the treatment group was significantly reduced (p<0.05) compared to the diabetic control.
3.3 Pancreatic GLUT 2

Fig. 2 shows the serum concentration of GLUT 2 in the various experimental groups. In the diabetic control group, there was a significant reduction in the serum level of the enzyme (8 ng/ml) (p<0.05) compared to the normal control (70 ng/ml). In the treatment group, there was a significant increase (20 ng/ml) (p<0.05) in the serum concentration of the enzyme compared to the diabetic control.

3.4 Histological Studies

Staining of the pancreas from the normal control group revealed normal and prominent islet of Langerhans and acinar cells that 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 as the islet cells showed no reaction to Feulgen’s reaction which is indicative of necrosis that had occurred in the cells. However, the acinar cells were strongly positive to Feulgen’s reaction. Macrophages (inflammatory cells) were observed in the islet, the cellularity of the islet cells was reduced and fibrous tissues were seen showing damage to the islet cells (Plate 2).

For the Diabetic+ Extract (400 mg/kg b.w) group that received the combined extracts of VA (200 mg/kg b.w) and GL (200 mg/kg b.w) the islet of Langerhans was prominent and appeared normal with cells that were strongly positive to Feulgen’s reaction but compared to the normal control group, there was reduced cellularity in the islet. Pancreatic duct was observed and the acinar cells appeared normal (Plate 3).

5. DISCUSSION

Diabetes was successfully induced with STZ (45 mg/Kg) as evidenced by the sustained increase in blood glucose concentration in the diabetic control group compared with the normal control group. In addition, GLUT 2 was significantly under-expressed in the diabetic group compared with the normal control, consistent with previous studies [29], and clearly demonstrating successful STZ diabetes. Moreover, most of the islet cells from the diabetic control group were negative for the Feulgen’s reaction, consistent with damage to the DNA, which is a known consequence of STZ toxicity [30].

Table 2. Blood glucose concentrations for the experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Blood glucose (mg/dl)</th>
</tr>
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<tbody>
<tr>
<td>Normal control (normal tap water + feed)</td>
<td>60 ± 7.28</td>
</tr>
<tr>
<td>Diabetic control (Streptozocin, 45 mg/kg b.w single dose)</td>
<td>257 ± 4.43a</td>
</tr>
<tr>
<td>Diabetic+Extract (VA+GL 400 mg/kg b.w, ratio 1:1)</td>
<td>116 ± 10.11*</td>
</tr>
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</table>

Data are represented as Mean ± SEM, n=6

*Significantly different from DC at p<0.05; a= significantly different from NC at p<0.05

Fig. 1. Comparison of Caspase 3 serum concentrations in the different experimental groups

Values are expressed as mean ± SEM, n =5; *significantly different from Normal Control at p<0.05; a = significantly different from Diabetic control at p<0.05
selectively damages the pancreatic beta cells because it shares with glucose the same transporter GLUT 2, which is abundantly expressed in these cells [31,32]. The resulting hyperglycaemia can induce chromatin remodeling and further DNA damage [33], and excessive production of free radicals [34]. STZ also triggers destructive immune and inflammatory reactions within the pancreatic islets by causing the release of glutamic acid decarboxylase autoantigens [35]. In this study inflammatory cells (lymphocytes) were located within the pancreatic islets harvested from the STZ treated group consistent with this possibility. The cytoarchitecture of the pancreatic islets were markedly distorted by STZ treatment, with the tissues shrunken and degenerated.

Treatment with the combined extracts VA+GL substantially reversed the above changes; The Diabetic + Extract (400 mg/kg b.w) group had a much smaller increase in blood glucose, normal islet cytoarchitecture, positive Feulgen’s reaction and increased GLUT 2 expression. VA+GL appeared to offer some protection against STZ induced diabetes and damage to the pancreatic beta cells. Although the molecular mechanism of this protection is not clear, these extracts have been shown to have the potential to stimulate the regeneration of the pancreatic beta cells [23]. The cytoarchitecture of the Islet from the Diabetic + Extract (400 mg/kg b.w) group was less distorted; the cells had histological features similar to those of the normal control group. The beta cells also showed strong positive reaction to Feulgen’s test, indicating possible extract-induced regeneration and recovery from the diabetic insults. This observation is in agreement with previous reports of the potentials of these extracts to cause a regeneration of pancreatic

Plate 1. Photomicrograph of pancreas of normal control animals, stained with H & E (A) and Feulgen’s reaction (B) (X400)

The islet of langerhans and the cells are prominent; Acinar cells are present and there is a pancreatic duct observed in the specimen; AC – Acinar cell, IL – Islet of Langerhans, PD- Pancreatic duct
beta cells in STZ induced diabetic rats [23,36]. That way VA+GL could ensure sustained insulinrelease and lowered blood glucose. The VA+GL treated group also expressed a slightly higher but significant level of GLUT 2 compared with the untreated diabetic group, although the level remained lower compared with normal control. This again may be related to the potential of the extracts to reverse STZ-induced pancreatic beta cell damage.

Caspase - 3 was over expressed in the diabetic control group compared with the normal control group, consistent with previous reports Hashish and Kamal [37] and suggests an increase in apoptotic index. In a study by Kamel et al. [38], it was also found out that Caspase - 3 level was significantly elevated in diabetic rat pancreas while treatment with curcumin resulted in a significant reduction of the enzyme. Curcumin is a plant derivative known to have potent antioxidant properties [39]. Thus, increased reactive oxygen species generation and the simultaneous decrease in antioxidant defense mechanism in diabetic patients appear to contribute to organ damage associated with the disease [34]. V. amygdalina, which is part of the combined extracts used in the current study has a rich antioxidant property capable of ameliorating the damage caused by free radicals to tissues including the pancreas [36]. Thus, the decrease in caspase - 3 activity in the Diabetic +Extract (400 mg/kg b.w) group is consistent with the ability of these extracts to counteract the free radicals and thereby prevent apoptosis.

6. CONCLUSION

This study investigated the effect of combined methanolic leaf extracts of Vernonia amygdalina...
and *Gongronema latifolium* on GLUT 2 and caspase 3 activity in the pancreas and serum respectively, of streptozocin induced diabetic Wistar rats. The results revealed a significant increase (p<0.05) in blood glucose, decrease in GLUT 2 and increase in caspase 3 activity in the diabetic control (STZ) group all of which was reversed after 28 treatment with combined methanolic leaf extracts of *Vernonia amygdalina* and *Gongronema latifolium*.

Methanolic leaf extracts of *Vernonia amygdalina* and *Gongronema latifolium* contain phytochemicals which have antioxidant and other medicinal properties that have the potentials of reversing pancreatic damage and ameliorating diabetic insults in STZ treated animals.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the University of Calabar Faculty Animal Research Ethics Committee (FAREC-FBMS). Approval number: FAREC/PA/010BC31012.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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