THE FLAVONOID- RICH FRACTION OF *VERNONIA AMYGDALINA* LEAF EXTRACT REVERSED DIABETES-INDUCED HYPERGLYCEMIA AND PANCREATIC BETa CELL DAMAGE IN ALBINO WISTAR RATS.

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**ABSTRACT**

Medicinal plant bioactive constituents are used as complements in the management of diabetes mellitus. This study investigated the effect of treatment with flavonoid-rich fraction of *Vernonia amygdalina* (VA) on blood glucose and pancreatic islet histopathology of STZ-induced diabetic rats. Forty-eight Wistar rats were randomly assigned to eight treatment groups. Forty-two fasted rats were rendered hyperglycemic by a single intraperitoneal injection of STZ (40mg/kg.bw). Methanol crude extract, 30% methanol flavonoid-rich fraction, 50% methanol saponin-rich, 100% methanol glycoside-rich and butanol fractions of VA were administered by gastric intubation to five groups of the hyperglycemic rats at 200mg/kg.bw for crude extract and 75mg/kg.bw for fractions respectively while insulin was given intramuscularly at 5 IU/kg.bw. Diabetic control and normal control groups were administered DMSO. Hyperglycemic rats treated with the crude extracts and fractions were hypoglycemic by the end of the 28 days treatment period. The 30% methanol flavonoid-rich and 100% methanol glycoside-rich fractions showed greater anti-hyperglycemic activity of all the fractions. Pancreatic islet histology of diabetic control rats showed damaged islets shrunken in cell mass compared with the non-diabetic control rats which had numerous islet cell mass and well stained nuclei. Treatment with the flavonoid-rich fraction presented total recovery of the islet cell mass, better than the treatment with insulin and methanol crude extract which showed only partial recovery. This observation indicates that the flavonoid-rich fraction of VA leaf extract reverses hyperglycemia and regenerates pancreatic islet cell mass
destroyed in diabetes mellitus and consequently may be responsible in full or in part for the antihyperglycemic effect of *Vernonia amygdalina*.

**KEYWORDS:** Hyperglycemia, Beta cells, *Vernonia amygdalina*, flavonoid-rich fraction, diabetes mellitus.

**INTRODUCTION**

The human population worldwide seems to be in the midst of an epidemic of diabetes. As of 2014, the number of diabetic cases was estimated to be 387 million with type 2 making up about 90% of cases. Between 2012 and 2014, diabetes is estimated to have resulted in 1.5 to 4.9 million deaths per year.[1] This number may probably double by the year 2030. Diabetes mellitus is a common metabolic disorder resulting from defects in insulin secretion or decreased sensitivity of tissues to insulin. It is a metabolic disorder with severe socio-economic importance associated with disturbances of carbohydrate, lipid and protein metabolism.[2] Defects in insulin secretion leads to hyperglycemia. Further decrease in insulin secretion can lead to chronic hyperglycemia thereby causing increased release of free radicals especially reactive oxygen and nitrogen oxygen species.[3] This increased production of ROS in diabetes and decreased destruction by antioxidants is responsible for the destruction of beta cells of the islets of Langerhans.[4]

The conventional approach of using insulin and oral antihyperglycemic agents like metformin and glibenclamide in the management of diabetes have been effective but poses a lot of risk and complications on patients. This has led to the search for better and non-risky alternatives in the management of diabetes and has drawn the attention of nations to the use of bioactive components of medicinal plants. These bioactive compounds, referred to as phytochemicals, include the flavonoids, saponin, tannins, glycosides, alkaloids, anthocyanin amongst others. *Vernonia amygdalina* DEL (VA) commonly called bitter leaf is widely used for its therapeutic and nutritional purposes.[5] It is a perennial shrub belonging to the *Asteraceae* family. The leaves are green with a characteristic odour and bitter taste. They are well distributed in tropical Africa and Asia, commonly found along drainage lines and natural forest or commercial plantation.[6] *Vernonia amygdalina* (VA) is indigenous to south-east Nigeria where it is commonly used for the preparation of soup. It is also used in traditional medicine as an anti-malaria, purgative, anti-parasitic and anti-helminthic, and also in the treatment of wound and control of blood glucose levels.[6] It has also been shown in our laboratory and elsewhere to reverse hyperglycemia, hyperlipidemia, hepatotoxicity and
nephrotoxicity associated with diabetes.[7] A probable regenerative action of the hitherto destroyed beta cells has been suggested as a possible mechanism for the antihyperglycemic action of Vernonia amygdalina.[7-9] This work seeks to establish which of the active fraction of Vernonia amygdalina is responsible for the anti-hyperglycemic activity and validate the suggestion that regeneration of the beta cells may be responsible for the antiglycemic action of the plant.

METHODS

Collection of Plant Materials: Fresh mature leaves of Vernonia amygdalina were harvested from the Endocrine Laboratory farm, University of Calabar, Calabar, Nigeria. The leaves were authenticated at the Department of Botany, University of Calabar and Voucher specimen deposited in the department’s herbarium.

Preparation of Plant Extract: The leaves of Vernonia amygdalina were rinsed severally with clean tap water to remove dust particles and debris then allowed to drain and dry completely. About 5kg of the leaves was homogenized, using a manual blender, and then soaked in 80% (v/v) methanol for 48hrs at a temperature of 4°C. The extract was filtered using a cheese material and thereafter with Whatman No 1 filter paper. The filtrate was evaporated in a rotary evaporator and allowed to dry completely in a water bath at 40°C.

Fractionation of Crude Extract: The methanol extract was fractionated on a Silica gel (mesh 50-120) column with the following solvents in order of increasing polarity: butanol, 30%, 50% and 100% methanol. Each fraction was run on thin layer chromatography (TLC) to obtain a profile of its constituents.

Determination of constituent phytochemicals of fractions: Chemical analysis were carried out on the bitter leaf fractions to identify its phytochemical constituents; tannins, flavonoids, alkaloids, saponins, glycosides using standard procedures as described by Trease and Evans.[10]

Experimental animals: Forty eight male albino Wistar rats weighing between 140-200g were employed for the study. These animals were divided into eight groups of 6 rats each. Before the experiment, the animals were allowed one week acclimatization and housed in cages under room temperature (25±2°C), relative humidity (50±5%) and a 12 hour light/dark
cycle. The animals were allowed free access to rat chow and tap water ad libitum throughout the experimental period.

**Acute Toxicity Test (LD$_{50}$):** Oral acute toxicity of the phytochemical fractions of *Vernonia amygdalina* was determined in mice as described by Lorke.$^{[11]}$

**Induction of Diabetes:** Streptozotocin (STZ) was used to induce diabetes in 42 overnight fasted rats by injecting the animals intraperitoneally with 40mg/kg body weight of the drug dissolved in 0.1M sodium citrate buffer (pH 4.5). Animals were confirmed diabetic if blood glucose level of the fasted animals was above 120mg/dl, as determined using a glucose strip read on an Accu-Check Active glucometer (Accu-Check, Roche Diagnostics, Germany) 72hours after day of induction.

**Experimental design**
Forty eight adult male rats were divided into 8 groups of 6 rats per group. The treatments were as follows.

**Table 1:** Distribution and treatment of experimental animals in the respective experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No of Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal control (0.5ml DMSO)</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Diabetic control (0.5ml DMSO)</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Vernonia amygdalina extract (200mg/kg b.w)</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Insulin (5i.u/kg, b.w)</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>30% methanol VA fraction (75mg/kg, b.w)</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>50% methanol VA fraction (75mg/kg, b.w)</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>100% methanol VA fraction (75mg/kg, b.w)</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>Butanol VA fraction (75mg/kg, b.w)</td>
<td>6</td>
</tr>
</tbody>
</table>

Treatment was administered twice daily (12 hourly) for 28days except the insulin group

**Estimation of blood glucose:** Blood glucose was estimated in overnight fasted rats using Accu-Check Active glucometer (Accu-Check, Roche Diagnostics, Germany). Blood was obtained from the dorsal vein of the tail before induction of diabetes and every 4days after induction of diabetes mellitus up to 28 days.
Body Weight: Body weights of the rats were taken prior to induction of diabetes at day 0 of treatment and every three days for 4 weeks.

Histological Studies: The pancreas was surgically removed and fixed in formaldehyde and embedded in molten paraffin. The fixed pancreatic tissue was sectioned (5-micron thickness) and sections stained with basic dyes, Hematoxylin and Eosin (H&E). Pancreatic sections were specifically stained for beta cells by the aldehyde fuchsin procedure. Slides of stained sections were viewed under a microscope at a magnification of 400x and images digitalized using an Olympus Camera.

Statistical analysis: Blood glucose levels were expressed in mg/dl as mean ±SEM. The data were statistically analyzed using ANOVA with multiple comparisons versus control group by Dunnett’s method. Differences were considered to be statistically significant if $p<0.05$.

RESULTS

Phytochemical Analysis: The result of the phytochemical screening is shown in table 2. There was a preponderance of flavonoids in the 30% methanol fractions while saponins were found mainly in the 50% methanol fraction and to a lesser extent in the butanol fraction. Tannins were also found in 50% methanol fraction and glycosides separated in the 100% methanol fraction with moderate separation in the 50% and butanol fractions while the alkaloids separated in both the 30% and 50% methanol fractions. The separation of the various phytochemicals using the four solvent systems was borne out by the TLC profiles.

Table 2: Results of Phytochemical Analysis of Fractions of Vernonia amygdalina

<table>
<thead>
<tr>
<th>Constituent</th>
<th>n-butanol fraction</th>
<th>30% methanol fraction</th>
<th>50% methanol fraction</th>
<th>100% methanol fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponin</td>
<td>Frothing test</td>
<td>+++</td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>Emulsifying test</td>
<td>+++</td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>Hemolysis test</td>
<td>++</td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>Wagner’s reagent</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Meyer’s reagent</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Draggendorff’s reagent</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Flavonoids</td>
<td></td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Tannins</td>
<td>+ferric chloride</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>+lead acetate</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>+ dilute H2SO4</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Glycosides</td>
<td></td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>
(-) => Not present (+) => Present in small concentration.

(++) => Present in moderately high concentration.

(+++) => Present in high concentration

(++++) => Present in very high concentration.

**Effect of Treatment on blood glucose concentration:** Three days after treatment with STZ, all animals rendered diabetic had significant increase in blood glucose concentration when compared to the normal control (p<0.05). However, significantly lower levels of blood glucose were observed for the crude extract and fraction treated groups (p<0.05) than in the diabetic control during the treatment period. The 30% methanol flavonoid-rich and 100% methanol glycoside-rich fractions showed greater anti-hyperglycemic activity of all the fractions. By the end of the 28th day, hypoglycaemia was attained in the diabetic rats treated with crude extract, 30% methanol (flavonoid rich) and 100% methanol (glycoside rich) fractions. These values were even lower than that of insulin treated groups.

![Figure 1: Comparison of initial and final fasting blood glucose concentrations in the different experimental groups. Values are expressed as mean ± SEM, n = 6.](image)

**Effect of Treatment on Histology of the Pancreatic Tissues:** The micrographs of the pancreas for the various treatment groups is shown in Figures 2-9. The untreated diabetic rats showed damaged and shrunken islets of the pancreas with sparsely populated cell mass compared to normal control group which showed islets of Langerhans with intact capsule, cell densely populated and centrally concentrated. Intervention with the 30% methanol fraction (flavonoid rich) showed regeneration of islet cell mass while partial reversal was
observed in the groups that received 50% (saponin rich) methanol fraction, 100% methanol fraction (glycoside rich) and butanol fraction (saponin rich).

**Fig 2**: photomicrograph of normal control X 400

Section of the pancreas showing both endocrine and exocrine components. The exocrine part consist of prominent acini lined by cuboidal epithelium and the lumen filled with mucinous secretion. The endocrine part consist of islet cell of Langerhans with intact capsule and containing cords of oval to round cells separated by capillaries. The cells are densely populated and concentrated centrally.

AC - Acini; IL- Islets of Langerhans; BV- Blood Vessel; N- Nucleus

**Fig 3**: photomicrograph of diabetic control X400

Section of the pancreas shows shrunken and sparsely populated islet cell of Langerhans with intact capsule. The cells are arranged in cords and nests separated by capillaries. Their
nuclei are deeply basophilic with a thin rim of cytoplasm. The cells are concentrated peripherally.

AC - Acini; IL- Islets of Langerhans; N- Nucleus

Fig 4: photomicrograph of insulin group X400

Section of the pancreas showing prominent islet cell of Langerhans with intact capsule containing cords and nest of sparsely populated oval to round cells. The cells at the periphery have deeply stained nuclei surrounded by thin rim of cytoplasm while the centrally clustered cells have abundant eosinophilic cytoplasm and deeply stained nuclei. The acini are prominent with intact epithelial lining and congested blood vessels are seen.

AC - Acini; IL- Islets of Langerhans; BV- Blood Vessel; N- Nucleus

Fig 5: photomicrograph of crude VA extract X400
Section of the pancreas showing densely populated islet cell of Langerhans with intact capsule containing cords and nest of oval to round cells separated by capillaries. The cells are evenly spread with deeply stained nuclei and abundant cytoplasm. The acini are prominent with intact epithelial lining.

AC - Acini; IL - Islets of Langerhans; BV - Blood Vessel; N - Nucleus

Fig 6: photomicrograph of 30% methanol fraction X 400

Section of the pancreas shows prominent islet cell of Langerhans with intact capsule containing cords and nest of densely populated oval to round cells. The cells at the periphery have deeply stained nuclei surrounded by thin rim of cytoplasm while the centrally clustered cells have abundant eosinophilic cytoplasm and deeply stained nuclei. These are separated by capillaries and evenly distributed. The acini are prominent with intact epithelial lining and congested blood vessels are seen.

AC - Acini; IL - Islets of Langerhans; BV - Blood Vessel; N - Nucleus

Fig 7: photomicrograph of 50% methanol fraction
Section of the pancreas shows prominent islet cell of Langerhans with intact capsule containing cords and nest of densely populated oval to round cells separated by congested capillaries. The cells are evenly distributed. The acini are prominent with intact epithelial lining and congested blood vessels are seen.

AC - Acini; IL - Islets of Langerhans; BV - Blood Vessel; N - Nucleus

Fig 8: photomicrograph of 100% methanol fraction X400

Section of the pancreas shows islet cell of Langerhans with intact capsule containing cords and nest of oval to round cells with deeply stained nuclei separated by capillaries. The cells are evenly spread with deeply stained nuclei and abundant cytoplasm. The acini are prominent with intact epithelial lining.

AC - Acini; IL - Islets of Langerhans; BV - Blood Vessel; N - Nucleus

Fig 9: photomicrograph of butanol fraction X400
Section of the pancreas shows islet cell of Langerhans with intact capsule containing cords and nest of sparsely populated oval to round cells with deeply stained nuclei separated by capillaries. The acini are prominent with intact epithelial lining.

AC - Acini; IL- Islets of Langerhans; BV- Blood Vessel; N- Nucleus

DISCUSSION

This work seeks to establish the active fraction(s) of the crude *Vernonia amygdalina* extract responsible for its anti-hyperglycemic activity and if indeed the structural integrity of the pancreas was restored by this anti-hyperglycemic fraction(s) employing histological studies. The Rf profile of phytochemicals in the various fraction shows that fractionation was in deed effected by the solvent systems employed in the fractionation. The preponderance of flavonoids in the 30% methanol fraction, glycosides in the 100% methanol fraction and saponins in the butanol and 50% methanol fractions is consistent with the study of Akah *et al.*[12] and Cushine and Lamb.[13]

Diabetes resulted in significant hyperglycemia relative to normal control. This is in agreement with other published works.[8,14-16] The hyperglycemia was as a result of the non-production of insulin occasioning the destruction of the beta cells of the pancreas in the STZ-induced type 1 diabetes. The result of this study showed that all the phytochemical fractions exhibited significant (P<.05) reduction of hyperglycemia. Comparison of the initial and final concentrations of fasting blood glucose clearly showed the 30% methanol flavonoid rich and 100% methanol glycoside-rich fraction to have greater anti-hyperglycemic activity of all the fractions. Hypoglycemic phytochemicals have been reported to include flavonoids, alkaloids, saponin, tannins, and cardiac glycosides.[17,18] The bitter tasting saponins in higher plants have also been associated with hypoglycemic activity.[19] This ostensibly may have been due to the antioxidant activity of these phytochemical fractions.

A probable regenerative action of the hitherto destroyed beta cells has been suggested as a possible mechanism for anti-hyperglycemic action of *Vernonia amygdalina.*[8,9,17] The histological examination of the pancreas of the non-treated diabetic rats in this work showed damaged islet of Langerhans shrunken in cell mass and sparsely populated with intact capsule and well stained nuclei. Report from various studies had indicated that absence of insulin, occasioning the destruction of the insulin-producing beta cells, and chronic exposure of tissues to high glucose concentration is responsible for the development of diabetes and its complications.[20,21]
Incessant hyperglycemia results in a rise in the generation of free radicals especially reactive oxygen species (ROS) and nitrogen oxygen species (NOS) from glucose auto-oxidation and protein glycosylation which interferes with the steady state between ROS production and cellular defense activity (oxidative stress) resulting in increased sensitivity to lipid peroxidation which plays a role in the gradual advancement of the symptoms of diabetes. This unsteady state can lead to cell malfunction and destruction resulting in tissue damage. The elevated level of ROS in diabetes might be due to a rise in the generation and or reduced destruction by antioxidant. The level of these antioxidants has the power to sway the vulnerability of different tissues to oxidative stress and this is linked to the progression of complications in diabetes. This is also responsible for the danger posed on the beta cells of the islets of Langerhans.

The reversal of the pancreatic damage was complete in the groups treated with 30% (flavonoid rich) methanol fraction while partial reversal was observed in the groups that received 50% (saponin rich) methanol fraction, 100% methanol fraction (glycoside rich) and butanol fraction (saponin rich). The ability of the flavonoids, glycosides and saponin to ameliorate partly or completely this pancreatic lesion lies in their antioxidant properties. It has been reported that the antioxidant and free radical scavenging activity of flavonoids found in *Vernonia amygdalina* could counteract the generation of free radicals responsible for STZ induced diabetes. The antioxidant property helps to scavenge the reactive oxygen species or stops its generation process. Thus the mechanism by which these phytochemicals reversed the pancreatic damage may have been through the arrest or scavenging of free radicals responsible for the pancreatic lesion thereby enhancing regeneration of pancreatic islet cells.

Polyphenols have been shown to affect glycemia through mechanisms including the inhibition of glucose absorption in the gut or uptake by peripheral tissues through the inhibitory action of alpha glucosidase. Ong et al. has suggested polyphenols as been responsible for the antihyperglycemic effect of *Vernonia amygdalina* most probably through increasing GLUT 4 translocation and inhibiting hepatic G6Pase.

Flavonoids comprise the most studied group of polyphenols. This group which has a common basic structure consisting of two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle has been shown to protect tissue injury by reactive oxygen species. The antidiabetic potential of flavonoids have been shown to be mainly
through their modulatory effects on glucose transporter by enhancing GLUT-2 expression in pancreatic β cells[D].

Taken together this work has shown that the 30% fraction with its relative abundance in flavonoids can completely reverse the STZ induced destruction of the pancreas and the subsequent hyperglycemia occasioning the induction of diabetes. This fraction can thus be an effective supplement in the formulation of a dose regimen for the management of diabetes.

REFERENCES


