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Effect of *Byrsocarpus Coccineus (Connaraceae)* Aqueous Leaf Extract on Pancreatic Islets Volume in Type 2 Diabetic Male Wistar Rats.

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Summary: Diabetes mellitus is a devastating illness associated with alterations in the pancreatic islet of Langerhans and the islet volume constitutes a significant variable in diabetic investigations. This study investigates the volume of pancreatic islets in type 2 diabetic rats following treatment with aqueous extract *Byrsocarpus coccineus* leaves. Twenty-five male Wistar rats weighing between 100-120g were divided into five groups. Group 1 served as the normal control given distilled water while type 2 diabetes mellitus was induced by a high fat diet feeding for five weeks and a single dose of streptozotocin (35mg/kg, *i.p.*) in groups 2,3,4 and 5. After confirmation of diabetes, animals in groups 2,3,4 and 5 were administered normal saline, 50 mg/kg metformin, 800 mg/kg *Byrsocarpus coccineus* leaves leaf extract (BCLE) and 400 mg/kg BCLE respectively for 21 days. The pancreas was harvested, fixed in neutral buffered formalin and processed for stereological and histological analysis. Isotropic uniform random samples were obtained with the orientator grid. Serial sections were cut with a rotatory microtome and stained with Haematoxylin and Eosin. Pancreatic islet volumes were measured with the aid of a Cavalieri estimator grid. The result showed significant ($p<0.05$) increase in the blood glucose level of the diabetic control group, when compared to the normal control. But blood glucose levels from groups 4 and 5 were significantly ($p<0.05$) decreased when compared to the diabetic control. Pancreatic islet volume estimations showed a significant decrease in the diabetic control group when compared to the normal control ($p<0.05$), while pancreatic islet volumes in groups 3, 4 and 5 were significantly increased when compared to the diabetic control group ($p<0.05$). In a likewise manner the histology of the pancreas of the diabetic control shows damaged pancreatic islet cells and surrounding tissue that was reduced in all the treated group. In conclusion the aqueous extract of *Byrsocarpus coccineus* has shown an anti- hyperglycaemic effect in the experimental rats and increased the volumes of the pancreatic islet cells as well as ameliorated the pathological changes in the pancreas.

Keywords*: Byrsocarpus coccineus*, Pancreatic islet volume, Stereology, Type 2 diabetes mellitus

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INTRODUCTION

Diabetes mellitus is the most common endocrine disorder in man, is estimated to affect 4% of the world's population; a doubling of this figure is expected in the near future, especially in the African and Asian continents (Engelgau *et al*., 2003). Most Nigerians living with diabetes have suboptimal glycemic control and have chronic complications (Chinenye and Young 2011). In Nigeria, the national prevalence of diabetes mellitus was estimated to be 6.8% in adults older than 40 years (Abubakaria and Bhopalb, 2008). Earlier in a survey of type 2 diabetes mellitus in Giwa and Markarfi local government areas, both sub-urban communities in Kaduna state in the northern part of Nigeria showed a prevalence rate of 1.6% (Bakari *et al*., 1999) and Dahiru *et.al.* (2008), reported prevalence rate of 2.0% in Dakace village, a semi-urban community in Zaria local government area in Kaduna state. A study of the prevalence of diabetes mellitus in Nigeria showed that type 2 diabetes mellitus is the most common type of diabetes accounting for about 90% of cases (Familoni *et al*., 2008).

The endocrine islets are scattered among the exocrine acini in the pancreas of human and mammals, hormone-secreting cells containing insulin, glucagon, somatostatin, and pancreatic polypeptide are present in the islets of Langerhans. The insulin containing cells are most numerous (60-80% of the islets) and are generally located in the central part of the islets (Knop *et al.*, 2010). Normal islet cell mass and function was initially, associated with type 1 diabetes, which is

characterised by the loss of β-cell mass due to loss of functional β-cell mass is now also associated with type 2 diabetes (T2D) (Bonner-Weir and O'Brien, 2018). Studies have observed that type 2 diabetes in human and rodent pancreas specimens show a loss of desmosomes and adherens junctions between islet and acinar cells, due to fibrosis and remodeling of the islet-acinar interface, that may result in an impaired function (Hayden *et al.,* 2008)

Byrsocarpus coccineus is a shrub plant that belongs to a family connaracea and commonly found across west and tropical Africa. Scientific study of *Byrsocarpus coccineus* revealed that the aqueous leaf extract possesses anti-inflammatory (Akindele *et al*., 2007), anxiolytic activity (Akindele *et al*., 2010) and anti-oxidant effect (Andrew *et al*., 2017). A number of study have reported the anti-hyperglyceamic effect of *Byrsocarpus coccineus (Dada et al., 2013*) in this regard the changes in pancreatic islets volume of diabetic animals following treatment with aqueous extract *Byrsocarpus coccineus* (leaves) may be an important consideration since, the β-cell composition in human islets has been reported as percentage on the basis of cell number or cell volume (Chen *et al.,* 2017) Additionally, studies by Dada *et al*. (2013) reported, an increased insulin level with the high dose of extract *Byrsocarpus coccineus*. Also enhanced insulin secretion concurs with β-cell function. Therefore, the analysis of the volume of pancreatic islets in diabetic rats treated with *Byrsocarpus coccineus* will further substantiate the anti-diabetic effect of the plant.

MATERIALS AND METHODS

Drugs, chemicals, reagents and other materials

Metformin tablets (Pharmatex Nigeria Ltd), dextrose solution, Streptozotocin (STZ) (Sigma-Aldrich, St Louis, USA). Glucose test kit (Accu-Check performa; Roche diagnostics Germany), Animal-derived fat, (Zaria Abattoir after Veterinary screening). Ketamine hydrochloride injection 50mg/ml; Batch no: N-5629, Manufactured by Kwality Pharmaceuticals PVT. LTD. India. Neutral buffered formalin, Orientator 44 grid, Cavalieri Estimator grid and Rotatory Microtome.

Plant material and extraction

The leaves of *Brysocarpus coccineus* were collected from the Galadimawa / Birnin-Gwari road in Giwa Local Government Area of Kaduna state, Nigeria. The leaves were identified and authenticated in the department of Botany, Ahmadu Bello University, Zaria. A voucher specimen with number 590 was assigned. Plant material was shade-dried, processed and aqueous extraction was done using maceration method as described by Brian *et al*. (1975) in the Department of Pharmacognosy, Ahamdu Bello University, Zaria, Kaduna state, Nigeria. About 150g of *Byrsocarpus coccineus* leaves yielded 15.43g of extract.

Animals and Animal Handling

Twenty-five apparently healthy adult male Wistar rats weighing between 100g- 120g were used. They were housed in plastic cages under good laboratory conditions. The rats were allowed to acclimatize for two (2) weeks before commencement of the research. Animals were fed with pelletized form of Growers' mash (protein 13%, fat 8%, fiber 15%, calcium 0.9%, phosphorus 0.35% and metabolisable energy – 2550Kcal/Kg) purchased from vital feeds, Zaria-Kaduna state, and water *ad libitum*. Rats were handled humanely according to the laws guiding the use of laboratory animals for scientific research of Ahmadu Bello University Zaria.

Induction of Experimental Type 2 Diabetes Mellitus

Obesity was first induced by feeding the animals in groups 2-5 with high fat diet (100g animal fats mixed with 100g of livestock growers' mash for five (5) weeks. The animal fat was obtained from the renal pad of slaughtered cows. At the end of 5_{th} week the animals were fasted for 12 hours, but allowed water *ad-libitum* and a freshly prepared streptozotocin at a dose of 35mg/kg body weight in a citrate buffer with a P_H of 4.5 was intra-peritoneally injected (Iliya *et al.,* 2016). Diabetes was allowed to develop and stabilized in the STZ-treated rats over a period of 72 hours. Hyperglycaemia in the rats was confirmed by conducting a glucose tolerance test on the fasted rats over a 2 hours period with the aid of a glucometer (Accu-Check Active Roche®). At the end of the tolerance test the animals were given dextrose solution (2g glucose in 100ml distilled water) to prevent hypoglycemia. Rats that displayed a sustained rise of ≥200mg/dl in blood glucose level were confirmed to be diabetic (Iliya *et al*., 2017).

Experimental design

After successful diabetic induction, the animals were grouped and treated as follows:

Group 1 (normal control): Administered 1ml/kg distilled water

Group 2 (Diabetic control): Administered 1ml/kg distilled water

Group 3 (Diabetic-Metformin): Administered 50mg/kg metformin

Group 4 (Diabetic High Dose BCLE): Administered 800 mg/kg *Byrsocarpus coccineus* leaf extract

Group 5 (Diabetic Low Dose BCLE): Administered 400 mg/kg *Byrsocarpus coccineus* leaf extract

The LD_{50} of Akpan *et al*. (2012) was adopted in this study. All treatments were done by oral gavage for 21 days.

At the end of the duration of treatment the rats were anaesthesized with an injection of Ketamine Hydrochloride at a dose of 50mg/kg body weight intra-peritoneally. A vertical incision was made along the rat's abdominal wall. The thoracic diaphragm was

incised to gain entry into the thoracic cavity and viscera. A cardiac puncture was done and 1ml of blood was collected into plain tubes and centrifuged at 10 rad/sec for 7 minutes to obtain the serum, which was stored at -20$_o$C and later used to measure the serum glucose level in the rats.

Sample Collection and Sectioning

Thereafter normal saline was used to flush the rat body systems for a period of 30 minutes with after which normal saline was replaced with the neutral buffered formalin solution for another 30 minutes. At the end of the perfusion-fixation, the pancreas was carefully dissected out and subjected to preparation techniques for stereological estimation of pancreatic islet volumes and histological analysis.

Isotropic Uniform Random Sampling (IURS)

IURS was performed using the Orientator Grid. At first the pancreas was placed at (a) center of the circle with equal division and a random number (2) was calculated and selected from the random number table and the sample was cut. Secondly (b) each part of the cut sample was again placed on a second circle with unequal divisions and another random number (6) was selected and the sample were cut here (Ali *et al.*, 2012). The method is shown in figure 1 below.

After IURS, samples were processed using normal routine histological techniques (Bancroft, 2002). Tissues were embedded in molten paraffin wax in cassettes. Sections were cut using a Rotatory Microtome (Leica) at 10µ. A random number 5 was calculated and selected from the random number table and 10 sections were systematically picked from the ribbon of cut sections. Floated out in a warm bath, mounted on charged slides, air-dried and stained with Haematoxylin and Eosin. Photomicrographs were taken with a microscope digital camera at 510 mega-pixel (DCM ScopePhoto® China) and a light microscope (Leitz Wetzlar, Germany) at ×250 magnification.

Islet of Langerhans volume estimations

A test point counting grid (cavalieri estimator) was superimposed on the pancreatic tissue sections and single test points hitting the pancreatic islets were counted and summed.

The volume changes of the pancreatic islets were calculated as previously described by Gundersen *et al.* (2002) *using* the following imputations:

V (mm3) = $\bar{T}$x *A P*x ∑pi (where $\bar{T}$= distance from the 1$_{st}$ section to the 10$_{th}$ section; *A P* = area per point; ∑pi=sum of test points)

Noise due to errors in the sampling was calculated thus: noise= 0.0724 x B/√Ax √n x ∑pi

Variations due to the systematic random sampling of the serial sections was calculated: VARsurs=3(A-noise)-4(B+C) +C

Total variance (TVAR) = noise + VARsurs

Coefficient of error due to the entire sampling process (CE) was calculated: CE=√TVAR/∑pi.

Statistical Analysis

Results were expressed as Mean ± SEM. One-way ANOVA was used to test for statistical significant difference at $p<0.05$. Tukey post-hoc test was used to determine where the difference lies. All statistics was done using SPSS (version 20).

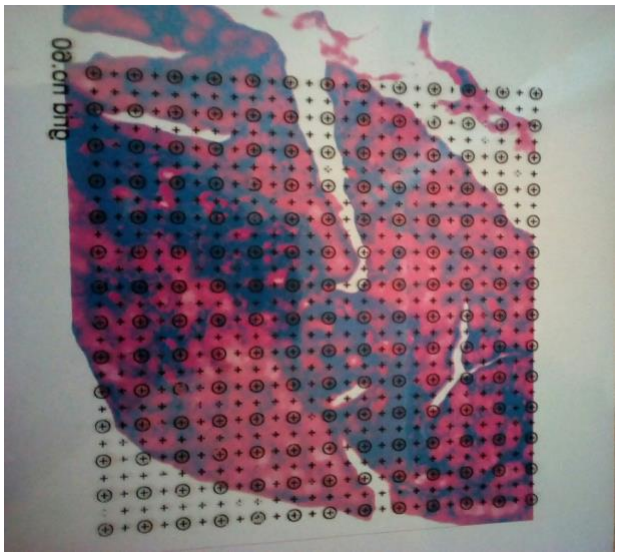

Plate 1:
The Cavalieri Estimator Grid used for the study.

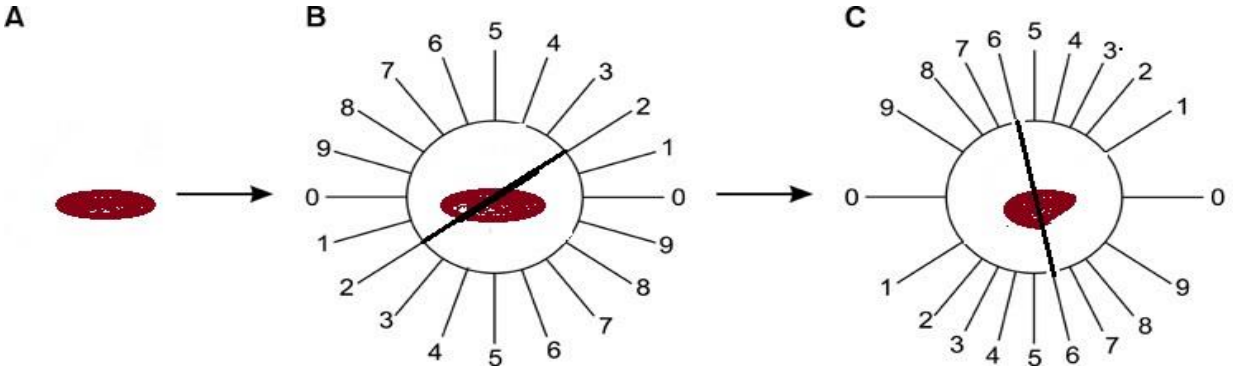

Figure 1: Isotropic Uniform Random Sampling (Ali et al., 2012)

RESULTS

Serum Blood Glucose Level:

Figure 2 shows the effect of *Byrsocarpus coccineus* leaves leaf extract (BCLE) on the blood glucose levels of the normal and diabetic rats. The result indicated that there was a significant increase ($p<0.05$) in the blood glucose level of the diabetic rats when compared to the normal control. Treatment interventions with standard drug as well as different dosages of BCLE significant decreased ($p<0.05$) the blood glucose level when compared to the diabetic control.

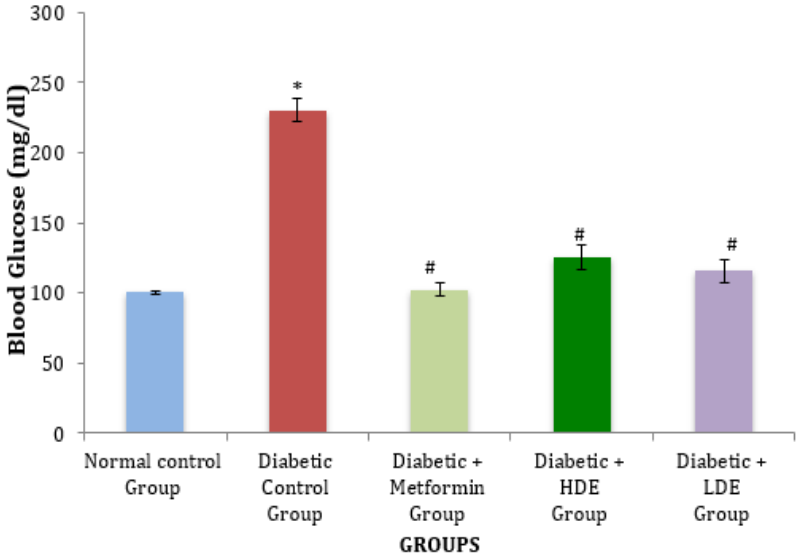

Figure 2.
Effect of *Byrsocarpus coccineus* Leave Extract on Serum Fasting Blood Glucose in High-fat Diet and STZ-induced Diabetic Rats.

Table 1:
Effect of *Byrsocarpus coccineus* Leave Extract on Volume Estimation of Pancreatic Islets in High-fat diet and STZ-induced Diabetic Rats

Groups	Volume mm3	Noise	VAR_{SURS}	Total Variance	Coefficient of Error	Mean ± SEM	P
1	22620	1317.52	–245243.56	–243926.04	0.65	75.4 ± 4.217	0.01
2	5220	149.55	–14745.65	–14596.11	0.69	12.2 ± 0.964*	
3	15990	812.11	–87533.03	–86720.93	0.69	53.3 ± 2.591	
4	11580	487.867	–68708.60	–68220.73	0.67	42.89 ± 2.176	
5	15540	688.97	–104966.91	–104277.94	0.62	51.8 ± 4.657	

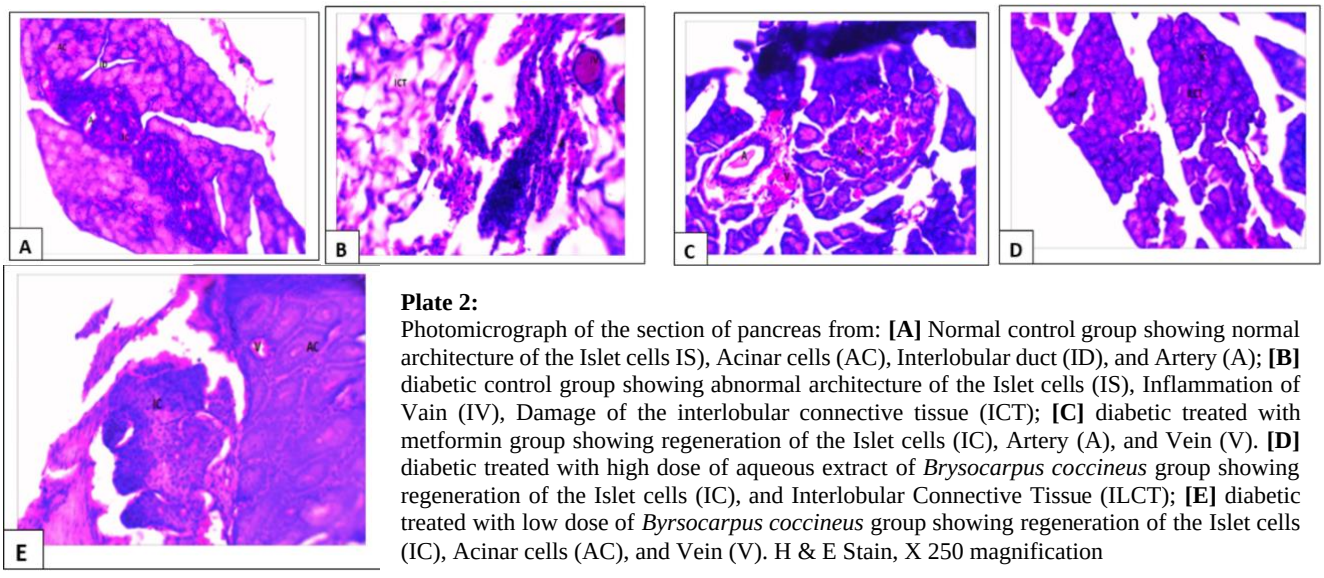

Plate 2:
Photomicrograph of the section of pancreas from: **[A]** Normal control group showing normal architecture of the Islet cells IS), Acinar cells (AC), Interlobular duct (ID), and Artery (A); **[B]** diabetic control group showing abnormal architecture of the Islet cells (IS), Inflammation of Vain (IV), Damage of the interlobular connective tissue (ICT); **[C]** diabetic treated with metformin group showing regeneration of the Islet cells (IC), Artery (A), and Vein (V). **[D]** diabetic treated with high dose of aqueous extract of *Brysocarpus coccineus* group showing regeneration of the Islet cells (IC), and Interlobular Connective Tissue (ILCT); **[E]** diabetic treated with low dose of *Byrsocarpus coccineus* group showing regeneration of the Islet cells (IC), Acinar cells (AC), and Vein (V). H & E Stain, X 250 magnification

Volume Estimation of pancreatic islets:

As shown in table 1, a significant decrease ($p<0.05$) in the estimated volume of pancreatic islets in diabetic control group when compared to normal control group. There was a significant increase ($p<0.05$) in the volume of pancreatic islets in the diabetic metformin group (group 3); high dose of BCLE at 800mg/m (group 4) and low dose of BCLE at 400mg/ml (group 5) when compared to diabetic control.

Histological Evaluation of Pancreatic Tissue

Plate 2 shows the result the histology of the pancreas. In plate 2a, the section of pancreas from normal control group shows normal architecture of the Islet cells (IS), Acinar cells (AC), Interlobular duct (ID), and Artery (A). Plate 2b, the diabetic control group revealed damaged pancreatic islet cells, the exocrine pancreatic cells (acinar cells and ductal cells), the interlobular duct, interlobular connective tissue, and blood vessels. But there was a gradual increase in the regeneration of the pancreatic islet cells, interlobular duct and connective tissue in the diabetic metformin group (plate 2c); high dose of BCLE at 800mg/m (Plate 2d) and low dose of BCLE at 400mg/ml (plate 2e)

DISCUSSION

Loss of pancreatic islet cell mass is associated with type 2 diabetes (T2D) (Bonner-Weir and O'Brien, 2018). The present study aimed at evaluating the volume of pancreatic islets and pancreatic pathological changes in type 2 diabetic rats following treatment with aqueous *Byrsocarpus coccineus* leaves extract (BCLE). Our findings showed that the aqueous extract BCLE attenuated the hyperglycemic conditions of the diabetic rats as was reported by Dada *et al.* (2013). The anti-hyperglycaemic potential of the extract observed in our study was better in the group treated with the lower dose (400 mg/kg body weight), which was not the case in respect to study by Dada *et al.* (2013). Nevertheless *Byrsocarpus coccineus* has been confirmed in this study to possess anti-hyperglycaemic activity. In line with the outcome of the glycemic control, the pancreatic islets volume estimation results showed that the aqueous extract of the leaves of *Byrsocarpus coccineus* elicited an increase in the volume of the pancreatic islets in the treated rats. The increase in the islet volumes was also slightly better at the lower dose of the extract. An increase in the total number of islet cells may reflect an increase in the total volume of the islets and may provide a clue to the improvement in the glycaemic control observed in the groups treated with the extract. Skau *et al.* (2001) had initially reported that an increase in islet volumes could be due to growth of existing islet cells or production of new ones from intra-islet stem/progenitor cells and β-cells are primary sources for these types of new cells. It can be deduce that the aqueous extract of *Byrsocarpus coccineus* leaves possibly contain biomolecules that has the potential to stimulate remnant of β-cells of the damaged pancreatic islets or influence a regeneration of the β-cells in these islets. With respect to the studies reported by Dada *et al*. (2013) that the high dose of extract *Byrsocarpus coccineus* increased insulin level, it can be might inferred that the reduced blood glucose levels observed in the extract treated group is a direct consequence of insulin action Also, Streptozotocin injections at low dose coupled with a high-fat food regimen like the one used in this study can expose an animal to type 2 diabetes mellitus with a selective destruction of the islet of Langerhans β-cells thus leading to a decrease in the blood insulin level and thus the poor glycaemic control observed in the diabetic control rats (Reed *et al*., 2000; Imam and Ismail, 2012).

In addition, the hyperglycemic condition and the decrease in the volumes of pancreatic islets of the rats in the diabetic control group was evident by the result of the histology that revealed an abnormal architecture of the Islet cells (IS), with Inflammation, and Damage of the interlobular connective tissue and Blood vessel.

It can be concluded that the aqueous extract of *Byrsocarpus coccineus* possess potent anti-diabetic efficacy in type 2 diabetic rats by restoring pancreatic damaged β-cells which carry a direct linear relationship with the total islet volumes thus the increases in the pancreatic islet volumes and attenuating the hyperglycaemic condition.

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