Histological study on the effect of aqueous extract of citron leaf on pancreas of hyperglycemic wistar rats

Received 26 September, 2019  Revised 15 November, 2019  Accepted 22 November, 2019  Published 10 December, 2019

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The present study was carried out to assess some effects of Citrus medica leaf extract treatment on the following parameters in hyperglycemic Wistar rats: diabetic hyperglycaemia and microanatomy of pancreas. The male adult Wistar rats (40) weighing between 160-200g were indiscriminately classified into four consisting of 10 rat namely: (A) normal control, (B) hyperglycemic only, (C) hyperglycemic treated with citron and (D) citron only. Aqueous leaf extracts of citron were administered orally at 400mg/kg body weight daily for 6 weeks. After 6 weeks extract of administration, animals were sacrificed by cervical dislocation. Organ weight was also taken while the pancreas was processed for light microscopy. Data were analysed using one way ANOVA. p<0.05 was considered significant. After 6 weeks the glucose level of hyperglycemic+Citron and Citron only groups significantly lowered, increase in body weight and relative pancreatic weight were observed relative to the hyperglycemic group. The histological examination of the pancreas of hyperglycaemic rats showed reduction islet of Langerhans with fibrotic tissue while hyperglycemic+Citron rats was similar to control rats. Aqueous leaf extract of citron has the ability to ameliorate the damage initiated by hyperglycemia on the pancreas of adult male Wistar rats.

**Key words:** Pancreas, streptozotocin, Citrus medica, hyperglycemia, Wistar rat.

INTRODUCTION

In adult humans, the pancreas weighs about 80g and lies retroperitoneal in the transpyloric plane (Banks et al., 2010). The second and third portions of the duodenum curve around the head of the pancreas (Verspohl, 2009). The spleen is adjacent to the pancreatic tail. The regions of the pancreas are the head, neck, body and tail (Banks et al. 2010). The distal end of the common bile duct passes through the head of the pancreas and joins the pancreatic duct entering the duodenum (Barnby, 2015). The arterial blood supply to the pancreas are from the splenic and pancreaticoduodenal arteries branches of gastroduodenal artery which have the corresponding veins that drain into the portal system of veins (Verspohl, 2009). In view of its dual blood supply, ischemia to the pancreas from vascular obstruction is uncommon (Banks et al., 2010). The pancreas is innervated by both the parasympathetic and sympathetic nerve fibers (Rayan et al., 2014). The efferent parasympathetic fibers are contained within the branches of the vagus nerve that originates in the dorsal vagal complex (tenth cranial nerve nucleus) in the medulla of the brain. The terminal branches of the vagus nerve synapse with intrapancreatic ganglia while the sympathetic
innervation originates in the lateral grey matter of the thoracic and lumbar segment of the spinal cord (Frank et al., 2003). The bodies of the postganglionic sympathetic neurons are located in the hepatic and celiac plexuses of nerve (Ryan et al., 2014). The postganglionic nerve fibers innervate the blood vessels of the pancreas (Ryan et al., 2014).

The endocrine pancreas refers to those cells within the pancreas that synthesize and secrete hormones. It takes the form of many small clusters of cells called the islets of Langerhans or, more simply, islets (Franks et al., 2003). Humans have roughly one million islets (Burns and Edil, 2012). In standard histological sections of the pancreas, islets are seen as relatively pale-staining groups of cells embedded in a sea of darker-staining exocrine tissue (Burns and Edil, 2012).

The pancreatic islets house three major cell types, each of which produces a different endocrine product:

- **Alpha cells** (α cells) which secrete the hormone glucagon.
- **Beta cells** (β cells) which produce insulin and are the most abundant of the islet cells.
- **Delta cells** (δ cells) which secrete the hormone somatostatin, which is also produced by a number of other endocrine cells in the body.

The different cell types within an islet are not randomly distributed - beta cells occupy the central portion of the islet and are surrounded by a “rind” of alpha and delta cells (Ryan et al., 2014). Aside from the insulin, glucagon and somatostatin, a number of other “minor” hormones have been identified as products of pancreatic islet cells (Frank et al., 2003). Islets are richly vascularized, allowing their secreted hormones ready access to the blood circulation. Although the islets comprise only about 1-2% of the mass of the pancreas, they receive about 10 to 15% of the pancreatic blood flow (Frank et al., 2003). Additionally, they are innervated by parasympathetic and sympathetic neurons, and nervous signals clearly modulate the secretion of insulin and glucagon (Burns and Edil, 2012).

Hyperglycemia is the technical term for high blood glucose or blood sugar. High blood glucose happens when the body has too little insulin or when the body cannot use insulin properly (Pais et al., 2007), other conditions that can cause hyperglycemia are pancreatitis, cushing’s syndrome, unusual hormone-screating tumors, pancreatic cancer, certain medications and severe illnesses (Sommerfield et al., 2004). Temporary hyperglycemia is often benign and asymptomatic in nature. Blood glucose levels can rise well above the normal values for significant periods without producing any permanent effects or symptoms (Sommerfield et al., 2004). However, chronic hyperglycemia at levels more than slightly above the normal values can produce a very wide variety of serious complications over a period of years, chronic hyperglycemia that persists even in fasting states is most commonly caused by Diabetes mellitus (Sommerfield et al., 2004).

Diabetes mellitus is a disorder of glucose metabolism whereby the body is not properly making use of glucose in the blood stream, therefore compromising a necessary function for cell nutrition and function (Faramarz et al., 2012). Type 1 diabetes also called insulin-dependent diabetes (Baynes et al., 2009). It used to be called juvenile-onset diabetes, because it often begins in childhood and accounts for about 10% of all diabetic cases, affecting approximately 20 million people Worldwide (Baynes et al., 2009). Type 2 diabetes is also known as adult-onset diabetes, accounting for about 95% of the cases of Diabetes mellitus in adults (Kia Soo et al., 2012). Gestational diabetes is known to be Diabetes triggered by pregnancy which account for about 2% to 10% of Diabetes mellitus pregnancies (Kia Soo et al., 2012).

Diabetes mellitus had been connected with disabling and life threatening complications such as retinopathy, neuropathy, nephropathy, dermopathy, cardiomyopathy and hepatopathy (Faramarz et al., 2012).

After the classic work of Banting and Best, (1922) on insulin, indefinite number of findings had accumulated over the years. Currently, botanicals are being screened for their efficacy and safety in the management of Diabetes mellitus and its complications (Shibib et al., 1993; Choudhury et al., 2017). In this regard, there is laboratory-based evidence that the fruit juice of Momordica charantia reverses hyperglycemia in rats by decreasing gluconeogenesis and increasing insulin secretion (Shibib et al., 1993; Choudhury et al., 2017). Additional potential herbal sources of new chemical entities for the management of Diabetes mellitus include Coccina indica (Kimble et al., 1996), Gymnema sylvestre (Baskaran et al., 1990) and Panax quinquefolius (ginseng) (Sievenpiper et al., 2004). Others include Annona muricata (Adewole and Caxton-Martins, 2006), Hypoxis hemerocallidi (Ojewole, 2006), Berberis lyceum (Gulraifet al., 2007) Aloe vera (Noor et al., 2008), Trichosanthes cucumerina (Adeeyo et al., 2011) and Allium cepa (Yusuf et al., 2012).

Chronic hyperglycemic oxidative stress is implicated in the pathogenesis of these complications (Jeanette et al., 2005). Some reports have shown that antioxidants may protect people from the disabling effects of Diabetes mellitus by mopping up free oxygen and superoxide radicals (Jeanette et al., 2005). It has also been shown by Opara (2004) that depletion of antioxidant appears to be a major risk factor for developing complications, and that antioxidant supplements lowered the risk. Besides, impaired insulin levels or action in Diabetes mellitus predisposes to dyslipidemia and increased risk of atherosclerosis (Rosetti and Goldberg, 2002). In view of the fact that emphasis is now on herbal therapy, searchlight is on herbs that are edible of which Citron is qualified. The Citron is a large fragrant citrus fruit with a thick rind, botanically classified as Citrus medica (Kalpesh et al., 2012). It is one of the four original citrus fruits from which most other citrus types developed through natural hybrid speciation or artificial hybridization (Taha et al., 2012). A source of confusion is that citron or similar words in...
French, Lithuanian, Hungarian, Finnish, Latvian, the West Slavic languages, and all Germanic languages but English are false friends, as they refer to the lemon (Sharangouda and Patil, 2010). Indeed, into the 16th Century, the English name Citron included the lemon and perhaps the lime as well. In most Arabic languages it is called Turunj, or similar, but in Syria and many other Islamic countries it is called Kabbad; in Japanese it is called Bushukan (Klein, 2014). In Nigeria Citrus medica is called several names in different languages such as ‘Osan jaganhin’ (Ayeloja and Bello, 2006), ‘Oronbo nla’ (Adeogun et al., 2014) in Yoruba, also in Igbo it is called ‘Oloma oyinbo’ and ‘Babban lemu’ in Hausa (Ayeloja and Bello, 2006).

From ancient through medieval times, the citron was used mainly for medicinal purposes: to combat seasickness, pulmonary troubles, intestinal ailments, scurvy and other disorders. Citron juice with wine was considered an effective antidote to poison, as Theophrastus reported. In the Ayurvedic system of medicine, the juice is still used for treating conditions like nausea, vomiting, and excessive thirst (Bairagi et al., 2011). Citron has been used in the treatment and management of many diseases for many years, both in traditional and complementary medicine in Asia, Europe and some parts of Africa (Kalpesh et al., 2012). It has also been reported to be effective in the management of chronic metabolic disorders such as Diabetes mellitus (Archana et al., 2011). A number of biological effects, such as anticancer, antimycobacterial, antiviral, apoptosis-inducing and immunomodulatory activities have been reported for Citrus medica (Kabra et al., 2012). The major constituents of Citrus medica are the lecithins, polypeptides known as viscotoxins and a number of phenolic compounds (e.g., digallic acid, O-coumaric acid) found in their free state or as glycosides (Bhmya and Begum, 2009). Phytochemical properties of fruit decoction, peels (rind of fruit) and leaves of Citrus medica Linn had been found to have alkaloids, flavonoids, phenols, carbohydrates and mucilage; the peels had alkaloids, flavonoids, steroids, phenols and carbohydrates; the leaves contained alkaloids, flavonoids, vitamins C, steroids and glycosides (Taha et al., 2012). The flavonoids reported from the fruits are hesperidin: 3,5,6-trihydroxy -4,7 -di methoxy flavone; 3,5,6-trihydroxy-3',4,7- trimethoxy flavones (Bhmya and Begum, 2009). Citronleaf has abundance of antioxidants (Taha et al., 2012). The study is therefore aimed at investigating some ameliorative effects of aqueous extract of citron leaf on the pancreatic histo-architecture of hyperglycemic wistar rats.

**MATERIALS AND METHODS**

The authentication of Citron leaves was done at Forestry Research Institute of Nigeria (FRIN), Jericho hill, Ibadan and voucher number (No. FHL 110913) was assigned. Citron aqueous leaf extracts were prepared as reported by Taha et al., (2012). Fresh leaves of citron were air-dried (under shade). The leaves were ground to coarse powder using an electric blender. The powdered sample of 400 g was soaked in 4000 mls of distilled water for 24 hours in a measuring cylinder. The mixture was homogenised in Explosion Proof Blender for two minutes, then filtered using a whatman filter (Grade 1 circles, diameter 15 mm, Z274844). The filtrate in the round bottom flask was put on a heating mantle (Barnstead/ eletrothermal) at 100°C for 7 hours. The concentrate formed was taken to an oven at 50°C for 1 hour; the final residue of about 80 g was a dark green mass which was stored at room temperature of 25°C until reconstituted for uses.

**Animals and animal management**

A total number of Forty (40) adult presumably healthy male Wistar rats (Rattus norvegicus) were used for this study, weighing about 160-200g of 8 to 10 weeks old. Animals were kept in four cages (10 rats per cage) and housed in the animal holdings of the Department of Anatomy, Faculty of Basic Medical Sciences, Animal House, Obalisi Onabanjo University Ikenne. They were maintained on standard animal feeds (Weight-gate pelleted feeds) and allowed access to clean water and feeds freely (ad libitum).

**Induction of hyperglycemia in rats**

The animals were acclimatised for four weeks and blood glucose level was monitored before induction of hyperglycaemia. Hyperglycaemia was induced in twenty overnight-fasted indiscriminately designated rats by a single intraperitoneal administration of Streptozotocin at 70 mg/kg bw (Lal et al., 2000). Streptozotocin (STZ) was dissolved in citrate buffer (1ml, pH 4.5) just prior to injection. Hyperglycaemia was allowed to develop for 72 hours (Lenzen, 2008). Rats with Fasting Blood Glucose ≥ 250 mg/dl were considered hyperglycemic (Tende, 2011). Control and citron only rats received a single intraperitoneal injection of 1ml citrate buffer (1ml/kg bw; pH 4.5).

**Experimental design**

The Forty (40) animals were divided into four groups of ten (10) animals each. The group A is the control group were normoglycemic animals that received neither STZ nor citron leaf extract, Group B was hyperglycemic rats that received distilled water solely, Group C was hyperglycemic rats that received citron extract only and Group D was normoglycemic group that received solely citron extracts.

**Citron (citrus medica) mode of administration**

The dose of the aqueous extracts of citron employed in these studies was adopted from the report of Taha et al.(2012). Physiological saline was used in dissolving citron daily and was administered orally with use of oro-gastric cannula to Group C rats (n=10) at 400 mg/kg bw (at 9.00 – 10.00 a.m. every day) for a maximum period of 6 weeks,
Group D rats (n=10) were administered 400 mg/kg bw of citron extracts.

**Measurement of blood glucose**

The blood glucose was evaluated in nightlong fasted rats at 9:00 – 10:00 hours using Glucose oxidase method of one touch ultra 2 glucometer (Accu-Chek Compact Plus). Blood was obtained from the median caudal vein of the tail by snipping the tip of the tail. The blood glucose level was monitored from twenty-eight (28) days (acclimatization period) before the induction of hyperglycaemia and for 42 days of treatment (Adewole and Caxton-Martins, 2006).

**Measurement of the body weight (g)**

Body weights (g) of the rats were recorded for four weeks (acclimatization period) before induction of hyperglycaemia and on a weekly basis throughout the experimental treatment for a period of 6 weeks. Body Weight was taken with a weighing scale (Venus VT 30 SL); (Adeeyo et al., 2011).

**Animal sacrifice**

After 42 days of treatment the animals were sacrificed by cervical dislocation. Laparotomy was performed, the pancreas was harvested, rinsed in phosphate buffered solution and fixed in 10% formol saline for histological procedures namely; Haematoxylin and Eosin stain and Gomori stain (Bancroft and Steven, 1992).

**The relative pancreatic weight (%)**

The relative pancreatic weight of the rat was evaluated as the ratio of respective weight of the pancreas and the terminal body weight of the same rat, the unit was recorded as percentage (%) using sensitive weighing balance (SonyF3G brand); (Adeeyo et al., 2011).

**Photomicrography**

Photomicrography of histological sections of the Pancreas were taken with an Olympus Microscope (New York, United State of America) coupled with camera at Department of Human Anatomy, Kampala International University, Gongo La mboto, Dar es Salaam, Tanzania.

**Statistical analysis**

Data were analysed using Excel 2016 (Microsoft Corporation, U.S.A). Data were expressed as mean ± standard error of the mean (mean±SEM). Mean values were compared using one way ANOVA. P values less than 0.05 (P<0.05) were taken to be statistically significant. All graphs were drawn with Excel 2016 (Microsoft Corporation U.S.A).

**RESULTS**

**average body weights of the rats (g).**

Figure 1 showed the weekly changes in body weight of rats in various groups. The body weight of rats in numerous groups throughout the 4 weeks of acclimatization were normal with no significant difference when compared to Group A (control) (P>0.05). At the second week, there was a rise of 8.3% in the average body weight of the rats in Group B of hyperglycemic only (174.16± 3.21g), however once the second of treatment, their average body weight started decreasing and by the sixth week (166.07± 3.28g) there was a decrease of 3.7% compared to the initial average body weight at week 0 (170.64±3.14g) with significant difference when compared to Group A P<0.05. In Group C of hyperglycemic+Citrus medica, there was a rise of 7.9% at second week of treatment (173.06± 3.79g), however at sixth week (178.95±3.37g) the rise was 12.1% when compared with their weight at week 0 (169.92±3.88 g) with no significant difference when compared to Group A(P>0.05).

**relative pancreatic weight (%)**

The relative weight of the pancreases in various groups were shown in Figure 2. There was a significant decrease in pancreatic weight in Group B (0.538±0.032) compared to Group A (0.916±0.072) (P<0.05). In Group C (0.810±0.045) the relative pancreatic weight was not significantly different from Group A(P>0.05) and there was no significant different between Group D (0.981±0.064) and Group A when compared.

**blood glucose levels on weekly basis (mg/dl)**

Figure 3, the levels of glucose of different groups of rats on weekly basis. The blood glucose level of rats in numerous groups throughout 4 weeks of acclimatization were normoglycemc in nature with no significant difference when compared with rats in Group A (P>0.05). The Group B rats were hyperglycaemic at week 0 (253.16±5.77 mg/dl) and remained therefore till the end of the sixth week of treatment (367.53±7.11 mg/dl). The worth was considerably significantly increase from Group A (74.65± 3.38 mg/dl) (P<0.05). Whereas the Group C (265.15±6.05 mg/dl) rats had high blood glucose level at week zero up to week three that was considerably significantly increased compared with Group A rats at P<0.05. From the fourth week the blood glucose levels of Group C (98.17± 3.98 mg/dl) were similar to Group A (74.65± 3.38 mg/dl) that weren’t significantly different at P>0.05.

**HISTOLOGICAL FINDINGS**

Using Haematoxylin and eosin (H&E) and Gomori stains to study the histology of pancreas in the various grouped rats
**Figure 1**: Histogram chart of Average Body weight on weekly basis. Data were expressed as Mean±SEM. P<0.05*

**Figure 2**: Bar chart of Relative pancreatic weight in Percentage (%). Data were expressed as Mean±SEM. P<0.05*

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**LEGEND:**
- NC: Normal Control
- HG: Hyperglycemic
- HGC: Hyperglycemic+Citrus medica
- CM: Citrus medica only
Figure 3: Histogram chart of Blood Glucose on weekly basis. Data were expressed as Mean±SEM (P<0.05*).

Figure 4: Photomicrograph of the Pancreas showing the pancreatic islet at day 42. (Arrow black: Islet of langerhans; a: Acini, *: blood vessel). A normal control, B hyperglycemic, C hyperglycemic+Citrus medica and D Citrus medica only. H&E stain. Mag. X400

The control group pancreatic islets appeared normal with normal cytoarchitectures showing lots of basophilic cells occupying the central portions of the islets and surrounded by other acidophilic cells. The acini were also well distributed. (Figure 4A and 5A). In the hyperglycemic group (B) of rats, the pancreatic islets appeared smaller with few
cellularity. There was also some degree of fibrosis in this group (Figure 4B and 5B); The hyperglycemic + Citrus medica group pancreatic islet appeared a bit small with cellularity and the acini were well distributed. (Figure 4C and 5C) and Citrus medica only group (D) of rats appeared normal with numerous cells present in the pancreatic islet and the acini were well distributed (Figure 4D and 5D).

**DISCUSSION**

The present study evaluated the possible ameliorative effects of citron on the hyperglycemia effects of STZ on the pancreas, treatment of STZ induced hyperglycaemia rats with aqueous leaf extract of Citrus medica, at a dose of 400mg/kg/d, produced normoglycaemia in 83.2% of rats by the end of the third week of treatment and all the animals had become normoglycaemic by the end of the forth week. Normoglycemia was maintained in these rats from fourth week to the end of experiment. Antihyperglycemic activities of Citrus medica leaf extract had been reported earlier by Taha et al. (2012), which was an agreement with the results of this study.

Antihyperglycemic activities are usually achieved through accentuation of release of insulin from B cells of islets of Langerhans of pancreas, prevention of uptake of glucose from gastrointestinal tract as seen in alpha-gluconidase or pancreatic amylase enzyme inhibitors, prevention of gluconeogenesis and glucogenolysis (Adewole et al., 2007). Citrus medica antihyperglycemic activities had been reported to be due to the presence of alpha gluconidase (Ahmad et al., 2008) and pancreatic amylase enzyme inhibitors (Nizam et al., 2014). These enzymes inhibit the digestion of glucose into an absorbable product, hence the inability of blood glucose to increase after glucose intake. The presence of these inhibitors was reported in plants like Morus alba, which was able to exhibit antihyperglycaemic activity (Sudha et al., 2011). Also the antihyperglycemic activity of Citrus medica leaf extract was due to the presence of antioxidants like flavonoids, phenol in it (Taha et al., 2012). Duong et al. (2003) reported that flavonoids and phenols were powerful hydrosoluble antioxidants in biological fluids. The antioxidants were able to prevent further destruction of beta cells in the pancreatic islets. The declining of the body weight of the rats in hyperglycemic group after second week might due to the low level of insulin which had been reported by Raheleh et al, 2016 that low level of insulin

**Figure 5:** Photomicrograph of the Pancreas showing the pancreatic islet at day 42. (Arrow black: Islet of langerhans; a: Acini, *: blood vessel). A normal control, B hyperglycemic, C hyperglycemic+Citrus medica and D Citrus medica only. Gomori stain. Mag. X400.
leads to inability of the body to breakdown glycogen for energy and the body begins to breakdown its own stored fat for energy, this causes rapid weight loss. In hypertyglycemic Citrus medica group there was an increase in the body weight from the second week of the treatment this might due to the presence of antioxidant in the Citrus medica which had been reported by Rahelehet al., 2016 that is capable of reducing blood glucose level which was an agreement with this study.

The histological findings showed that hypertyglycemic group (Figure 4B and 5B) islet reduced with paucity of cells and presence of fibrotic tissue due to the STZ given to these group which was taken up by β cells, the STZ caused the death of these cells by DNA fragmentation (Suvattanee et al., 2014). In the hypertyglycemic Citrus medica group (Figure 4C and 5C) there is reduction in the islet with numerous and viable cells present. In this group, citron treated, early reversal of hyperglycaemia might have brought about increase in G-6-PDH levels with associated increased production of Ribose-5-Phosphate, resultant DNA production and β cell proliferation. However, the finding was in agreement with the observations of Sudha et al. (2011) that β cells still possessed regenerative potentials even in hyperglycemic state. The real mechanism of improvement in islet morphology and cell mass in citron treated group is not clear, but the morphological improvement seen in this group was absent in hyperglycemic group.

**CONCLUSION**

Aqueous leaf extract of Citrus medica possess antihyperglycemic effect and this underscore the potential of this herbal therapy in the management of type 1 diabetic hyperglycaemia.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of the paper.

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