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Citrus Fruit Juice and Anti-Diabetic: Hepato-Protective Effects in Diabetic Wistar Rats

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Abstract: Citrus fruits characterized by flavonoids and limonoids (terpenes) with the juice contains a high quantity of citric acid and sharp flavor. The study aimed to investigate the effect of Citrus Medicus extract on the histo-hepatic architecture and blood glucose parameters in Alloxan induced diabetic wistar rats. A total of Twenty (20) Wistar rats weighing between 75g-120g were used and were classified as control, normoglycemic, hyperglycemic without treatment with Citrus Medicus and hyperglycemic treated with Citrus Medicus. Hyperglycemia was induced with single dose of intraperitoneal injection of Alloxan at 120 mg/kg. Administration was carried for 42 days experimental period using oro-gastric cannula. The weight changes and the blood glucose level were observed. Animals were euthanized 24 hour after the last administration and liver was harvested and process for histological observation using H/E and Van Giessen stains. The result shows that the extract of Citrus Medicus caused significant (p-value < 0.05) decrease in blood glucose and increase in the body weight among the animals treated with Citrus Medicus when compared with hyperglycemia untreated group. Histological observation showed hepatic plates that are arranged, with distinct hepatocytes. Hepatic sinusoid are devoid of blood cloths and are well spaced hence, maintanace of hepatic integrity was observed among the animals treated with Citrus Medicus. However, The de- arrangemnt of the hepatic plates, loss of the hepatocytes, loss of hepatic sinusoid and large deposit of the collagen were demonstrated with presences of blood cloths in untreated hyperglycemia animals. Nuclear shrinkages among the array of hepatocytes was equally observed in the hyperglycemia untreated animals. In conclusion, the extract of Citrus Medicus caused reduction in blood sugar, maintenance of body weight and maintenance of histo-hepatic architecture in Alloxan induced diabetic rats.

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INTRODUCTION

Diabetes and Mellitus are derived from Greek “Diabetes” denotes "a passer through; a siphon" whereas the "Mellitus" denotes "sweet" (Maxwall, 2014). Diabetes Mellitus is a combination of heterogeneous disorders commonly presenting with episodes of hyperglycemia and glucose intolerance, as a result of lack of insulin, defective insulin action, or both (Exposito *et. al*., 2002). Diabetes mellitus is a metabolic disease in which there are high blood sugar levels over a prolonged period or the relative or absolute deficiency of insulin in the body. Diabetes is due to either the pancreas not producing enough insulin or the cells of the body not responding properly to the insulin produced. (WHO, 2014). Symptoms of high blood sugar include frequent urination (polyuria), increased thirst (polydipsia), and increased hunger (polyphagia). Diabetes can cause many complications; acute complications include diabetic ketoacidosis and nonketotic hyperosmolar coma. Serious long-term complications include cardiovascular disease, stroke, chronic kidney failure, foot ulcers, and damage to the eyes (WHO, 2005)

Type 1 Diabetes mellitus results from the pancreas's failure to produce enough insulin. This form was previously referred to as "insulin-dependent diabetes mellitus" (IDDM) or "juvenile diabetes". The cause is unknown. (WHO, 2014.). Type 2 Diabetes Mellitus begins with insulin resistance, a condition in which cells fail to respond to insulin properly. As the disease progresses a lack of insulin also develop. This form was previously referred to as "non-insulin-dependent diabetes mellitus" (NIDDM) or "adult-onset diabetes". The primary cause is excessive body weight and not enough exercise. (WHO, 2014)

Gestational diabetes occurs when pregnant women without a previous history of diabetes develop high blood-sugar level. Prevention and treatment involve a healthy diet, physical exercise, maintaining a normal body weight, and avoiding excessive intake of alcohol, cigarette smoking. Control of blood pressure and maintaining proper foot care are important for people with the disease.

Type 1 Diabetes mellitus must be managed with insulin injections. Type 2 Diabetes mellitus are treated with medications with or without insulin. Insulin and some oral medications can cause low blood sugar. Gestational diabetes usually resolves after the birth of the baby.

In recent time, attention has shifted to the use of botanicals for the treatment of diabetes mellitus. Myriads of researches have been carried out on plants with traditional claim of antihyperglycemic effect like Moringa Oleifra (Adeeyo *et al.,* 2013) and Alium cepa (Yusuf *et. al.,* 2012). Citrus fruits are notable for their fragrance, due to flavonoids and limonoids (terpenes) contained in the rind, and most are juice-laden. The juice contains a high quantity of citric acid giving them their characteristic sharp flavor.

The genus is commercially important as many species are cultivated for their fruit, which is eaten fresh, pressed for juice, or preserved in marmalades and pickles. They are also good sources of vitamin C and flavonoids. The flavonoids include various flavanones and flavones. Citrus species are important in traditional medicine to cure sea-sickness, pulmonary troubles and intestinal ailments. A leaf infusion is used against mouth sores in infants (Sho-ichi et. al., 2012), treating stomach and skin ailments. Therefore the study aims to investigate effects of Citrus medicus fruit juice on the liver of hyperglycemic wistar rats.

MATERIALS AND METHODOLOGY

Animal Model

Twenty (20) Wistar rats weighing between 75g-120g were obtained from animal holding Ladoke Akintola University of Technology, Ogbomoso. They were kept in Laboratory for two (2) weeks of acclimatization and were fed on standard diet (Vital Feeds and Grand Cereals Ltd); Water was given *ad libitum* and maintained under standard conditions. The animal room was well ventilated with a temperature range of 25-27^{o}C under day/night with 12-12 h photoperiodicity. All the experimental procedures were done following the experimental guidelines of Institutional Animal Ethics Committee (IAEC) of Ladoke Akintola University of Technology, Osogbo campus, Osun State.

Preparation of Plant Extra

Citrus medicus fruit was obtained from Corner Odetomi at Ogbomosho South Local Government Area and authenticated at the Department of Pure and Applied Biology of Ladoke Akintola University of Technology, Oyo State. Citrus medicus juice was extracted from the fruit by simple squeezing of the fruit. The extract was squeezed from the fruit daily at the time of administration.

Induction of Hyperglycaemia

Hyperglycaemia was induced in 10 rats overnight-fasted randomly selected rats by a single intraperitoneal administration of Allosan at 120 mg/kg bw (Lal, Korner and Mastsuo 2000). Allosan was dissolved in citrate buffer (0.1m, pH 4.5) just prior to injection. Hyperglycemia was allowed to develop for 72hours (Lenzen, 2008). Animals with Fasting Blood Glucose $\geq$ 250 mg/dl were considered hyperglycemic (Tende *et .al.,* 2011) and were included in this study. Control animals (n= 5) received a single intraperitoneal injection of 0.1M citrate buffer (1ml/kg bw; pH 4.5)

Animal grouping and Extract Administration

Twenty (20) Wistar rats were divided into four (4) groups comprising of five (5) animals each. The animals were induced with Alloxan which caused an increase in their blood glucose level. Group 1 consisted normoglycemic wistar rats administered with 2mls of distilled water. Group 2 consisted normoglycemic wistar rats administered with 2mls of Crude Citrus Medicus Juice. Group 3 comprised of diabetic (hyperglycemia) wistar rats administered with 2mls of distilled water. Group 4 comprised of diabetic (hyperglycemia) Wistar rats administered with 2mls of Crude Citrus Medicus Juice. All the animals were given were given feed and water ad libitum. Administration was done orally using the oral cannula, all animals were treated 9.00am daily for 21 days. The animals were weighed at alternate days using sensitive digital weighing scale. Blood glucose of the Wistar rats were checked weekly with acer glucometer.

Animal Euthanasia

At the end of the 21 days of the experiment, animals were weighed and euthanized by cervical dislocation. The abdominal region was accessed, the liver was harvested, weighed and immediately fixed in 10% formol saline for histological staining.

Tissue Processing for Light Microscopy

Paraffin Wax embedding of the liver was done. At euthanasia, each organ was rinsed in PBS, trimmed free of adipose tissue, cut into smaller pieces (3 mm x 3 mm) and fixed in 10% formosaline, for a maximium of 48hours. Dehydration of the sample was done in graded alcohol, clearing of the sample in two (2) changes of Xylene I and II, Wax Infiltration of the sample and Embedding

(Inclusion) of the sample was done in paraffin wax. 5 μm -thick sections of the liver was cut on a Reichert-Jung 2050 rotary microtome (Cambridge Instruments, Germany). Sections were floated on the water bath at 50ºC, and mounted on pre-washed, sterilized, 25.4 mm x`76.2mm glass slides (Pearls, China). Slides were washed with detergent, rinsed with deionized water, and sterilized with 70% ethanol. Mounted Paraffin sections of the liver were stained in Haematoxylin and Eosin and Verhoeff Van Gieson Staining to demonstrate the type of connective (Bancroft and Stefen, 1982).

Photomicrography

Digital micrographs of the liver sections were obtained to show the morphological changes that occurred in the treated as compared to the control group. The photomicrographs were carried out at the Department of Anatomy, Ladoke Akintola University of Technology Ogbomoso, Oyo State.

Statistical Analysis

All data were expressed as mean ± SEM. The statistical analysis of the result obtained in this was evaluated and tested for significance using t-test at 0.05($P<0.05$). SPSS statistical software was employed.

RESULTS

Figure 1 showed among the group 1 animals an increase in the body weight of the animal at the 1st week and 2nd week and a decline at the 3rd week and an increase at the 4th week. A decrease occurred again at the 5th week then a slight increase at the 6th week. Group 2 showed increase in the body weight of the animal at the 1st and 2nd week a slight decrease at the 3rd week then a rapid increase at the 4th week, a decrease at the 5th week and an increased weight at the 6th week. Group 3 showed weight increase at the 1st and 2nd week and there was a rapid decrease in the body weight of the animals from the 3rd week to the 6th week. Group 4 showed decrease in body weight from the 1st week to the 3rd week and also an increase in the body weight of the animals from the 4th week to the 6th week.

Figure 2 showed among the group 1 an increase in the level of blood glucose of the animal from the 1st week to 2nd week and there was initial decrease at the 3rd week followed by rapid increase at the 4th week, a decrease at the 5th week and a slight increase the level of blood glucose in the 6th week. In group 2 there was an increase in the level of blood glucose of the animal from the 1st week to the 4th week and a rapid decrease in the 5th week, followed by increase in the level of blood glucose in the 6th week. Group 3 showed rapid increase in the level of blood glucose of the animal throughout the weeks of administration. Group 4 showed increase in the level of blood of glucose of the animal at the 1st and 2nd week then rapid decrease occurred from 3rd week till the 6th week.

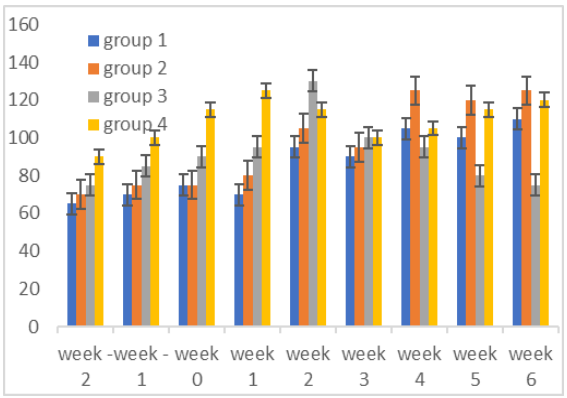

Figure 1: Weight (g) of the Animals

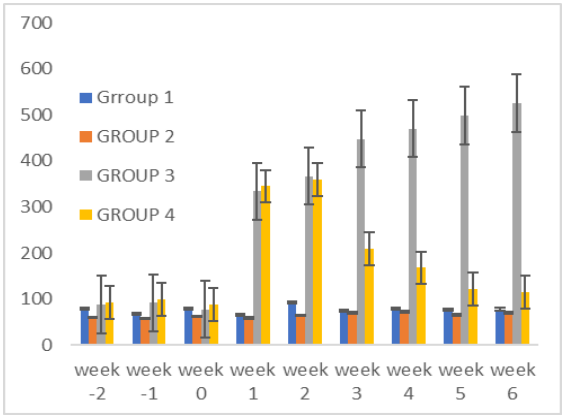

Figure 2: Graphical representation of blood glucose (mg/dl) level of different animal groups

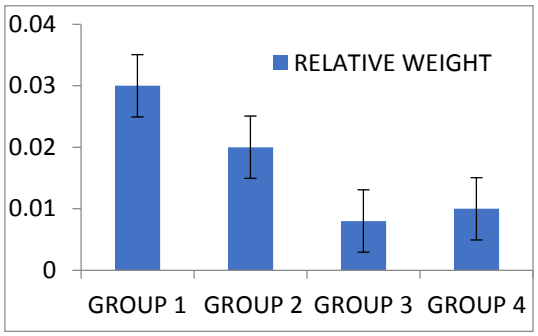

Figure 3: Graphical representation of the group(s) relative weight (g) organ.

Figure 3 showed a relative organ weight among the hyperglycemic animals (group 3) with decrease in the liver organ weight compared with the treated animals' hyperglycemic rats (group 4) and the control animals. Relative weight of the organs were expressed as Average weight of the liver (g)/Average weight of the animal (g)

Plate 1 and 2 showed the hepatic plates that are well arranged occupied with distinct hepatocytes. Hepatic sinusoid are devoid of blood cloths and are well spaced hence, hepatic integrity is maintained.

However, mild loss of hepatocytes and nuclear shrinkages among the array of hepatocytes was observed in plate 2 normaglycemic treated with Citrus Medicus Juice in H/ E stains. The hepatic plates are arranged, with distinct hepatocytes hence, hepatic integrity is observed by the demonstration of the brown coloration of the cytoplasm as revealed by Verhoeff Van Gieson Staining (VGS).

Plate 3 with H/E stains, showed disorganised hepatic plates, with loss of hepatocytes. Hepatic sinusoid are narrowed or reduced, with pesences of blood cloths. Nuclear shrinkages among the array of hepatocytes were equally observed in hyperglycemic hepatic architecture. Plate 4 and 8 are presented with regeneration of hepatocytes and nuclear the array of hepatocytes with hepatic sinusoid devoid of blood cloths and well spaced hence, mainatance of hepatic integrity. Hyperglycemic showed potential histo-hepatic damage as large deposit of the collagen was demonstrated by the RED colouration of the collagen in cytoplasm and cytoplasmic inclusions.

DISCUSSION

Citrus Medicus Juice have been reported to relieve several forms of diseases including; sea-sickness, pulmonary troubles, and intestinal ailments in traditional medicine (Sho-ichi *et. al.*, 2012). Whistler 1996 had giving its uses for treating stomach and skin ailments. The sweet orange bark infusion is used to treat postpartum sickness, serious flu, and internal injuries.

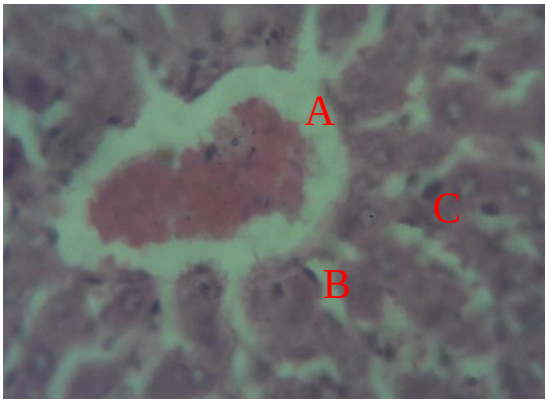

PLATE 1: Photomicrograph of liver in group 1 (water only) H/E, X400 A-central vein B-sinusoid C-hepatocyte. The hepatic plates are arranged, with distinct hepatocytes. Hepatic sinusoid are devoid of blood cloths and are well spaced hence, hepatic integrity is observed

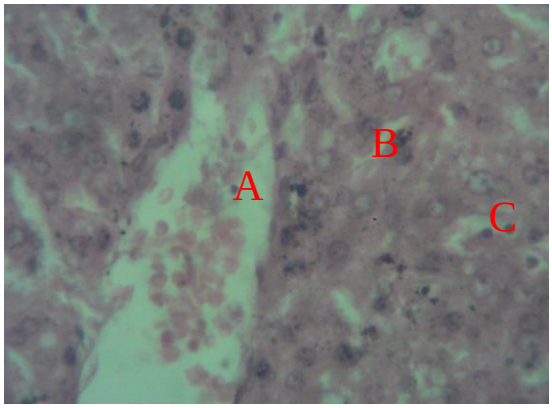

PLATE 2: Photomicrograph of liver in group 2 (Citrus Medicus Juice) H/E, X400 A-central vein B-sinusoid C- hepatocyte. The hepatic plates are arranged, with distinct hepatocytes. Hepatic sinusoid are devoid of blood cloths and are well spaced. However, mild loss of hepatocytes and nuclear shrinkages among the array of hepatocytes was observed

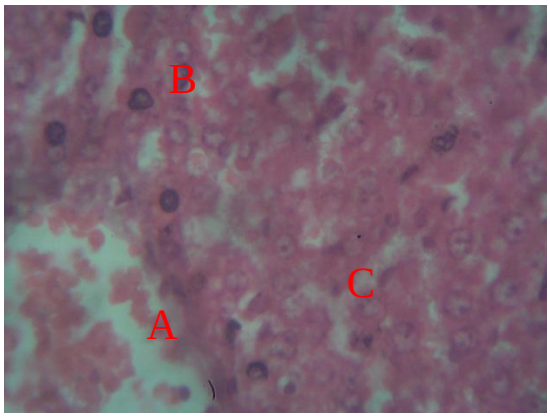

PLATE 3: Photomicrograph of liver in group 3 (Diabetics rats) H/E, X400 A-central vein B-sinusoid C-hepatocyte. The hepatic plates are disorganised, with loss of hepatocytes. Hepatic sinusoid are are narrowed or reduced, with pesences of blood cloths. Nuclear shrinkages among the array of hepatocytes was observed

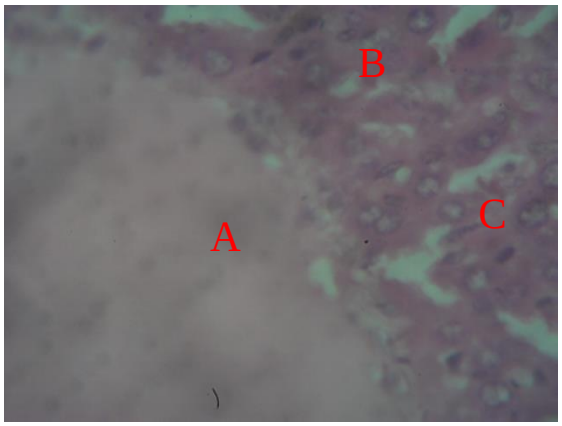

PLATE 4: Photomicrograph of liver in group 4 (Diabetic rats + Citrus Medicus Juice) H/E, X400 A-central vein B-sinusoid C-hepatocyte. The hepatic plates are arranged, with distinct hepatocytes. Hepatic sinusoid are devoid of blood cloths and are well spaced. However, regeneration of hepatocytes and nuclear the array of hepatocytes was observed.

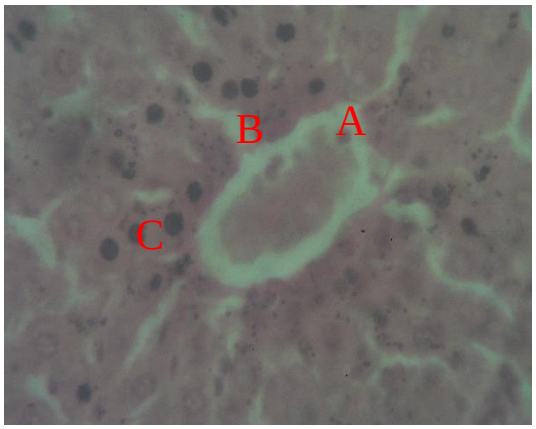

PLATE 5: Photomicrograph of liver in group 1 (water only) VGS, X400 A-central vein B-sinusoid C-hepatocyte. The hepatic plates are arranged, with distinct hepatocytes. Hepatic sinusoid are devoid of blood cloths and are well spaced hence, hepatic integrity is observed by the demonstration of the brown coloration of the cytoplasm

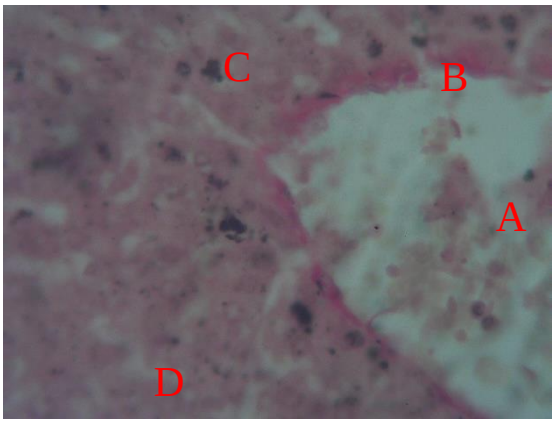

PLATE 6: Photomicrograph of liver in group 1 (Citrus Medicus Juice only) VGS, X400 A-central vein B-sinusoid C-hepatocyte. The hepatic plates are arranged, with distinct hepatocytes. Hepatic sinusoid are devoid of blood cloths and are well spaced hence, hepatic integrity was observed by the brown coloration of the cytoplasm.

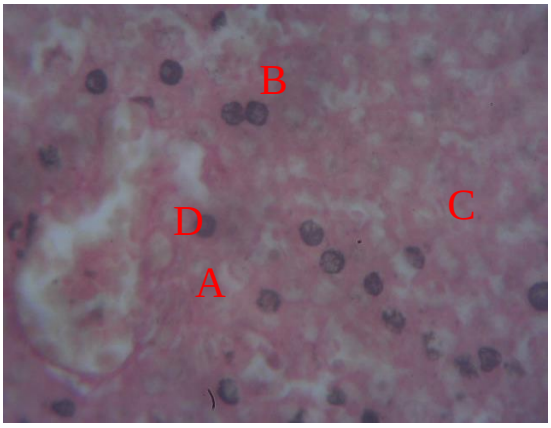

PLATE 7: Photomicrograph of liver in group 1 (Diabetic rats only) VGS, X400 A-central vein B-sinusoid C-hepatocyte. The de- arrangemnt of the hepatic plates, loss of the hepatocytes. Loss of hepatic sinusoid. Large deposit of the collagen was demonstrated by the RED colouration of the collagen. Cytoplasom and cytoplasmic inclusions were demonstrated

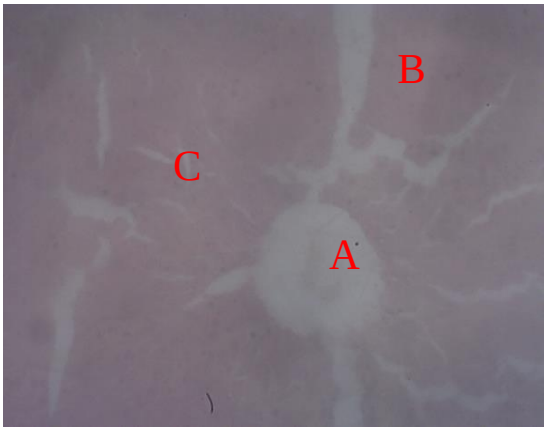

PLATE 8: Photomicrograph of liver in group 1 (Daibetic + Citrus Medicus Juice) VGS, X400 A-central vein B-sinusoid C-hepatocyte. The hepatic plates are arranged, with distinct hepatocytes. Hepatic sinusoid are devoid of blood cloths and are well spaced hence, hepatic integrity is observed

In this study the blood glucose of the control animals (groups 1) and the Citrus Medicus Juice treated animals (group 2) remained normal throughout the period of experiment which showed that water and Citrus Medicus juice have no effect on normoglycemic condition. This is in agreement with the study conducted by Adeeyo *et. el.*, 2013. In hyperglycemia rats (group 3) the level of blood glucose of the animals at the induction of Diabetes Mellitus increased rapidly all through the experimental period. However, hyperglycemia animals treated with Citric Medicus showed decrease in blood glucose level at the sixth week. This reported had demonstrated that Citrus Medicus juice has some anti-hyperglycermic effects in accordance to the work conducted by Nir *et. al.*, 2007 and Diaz-flores, 2006.

Some plants extract like picralima nitida, nauclea latifolia and oxytenanthera abyssinica have been shown to have some anti-hyperglycermic effects according to Krentz and Bailey, 2005. This study also demonstrated increase in body weight of the animals in group 1(normoglycemic wistar rats administered with 2mls of distilled water.) and 2(normoglycemic wistar rats administered with 2mls of Crude Citrus Medicus Juice) all through the experimental period and a decreased in weight was observed in group 3 (diabetic (hyperglycemia) wistar rats administered with 2mls of distilled water) in agreement with the research study by Libman *et. al., 1993*

The histological findings of the liver showed the central vein, hepatocyte and sinusoid appeared normal in group 1 and 2 animals (plates 1, 2, 5 and 6) which revealed that water and Citrus Medicus impacted no adverse effect on the histology of liver. This study observed that untreated hyperglycemia animals showed distorted

hepatocyte both in heamatoxylin and eosin and vangensin stain. There was general distortions of the histo-architecture of the liver (plates 3 and 7). The de- arrangemnt of the hepatic plates, loss of the hepatocytes. Loss of hepatic sinusoid with large deposit of the collagen and cytoplasmic inclusions. This showed that diabetic condition had serious adverse effect on the histology of the liver. It had been shown that hyperglycermic state was associated with deposition of fibrous tissues (Diaz-flores, 2006).

In group 4 animals (plates 6 and 8) the diabetic animals were treated with crude extract of Citrus Medicus juice. The hepatic plates are arranged, with distinct hepatocytes. Hepatic sinusoid are devoid of blood cloths and are well spaced. However, regeneration of hepatocytes and nuclear array of hepatocytes were observed. As well as the arrangment of thepatic plates; with distinct hepatocytes. Hepatic sinusoid are devoid of blood cloths and are well spaced hence, hepatic integrity is observed More importantly, extract of Citrus Medicus juice had some protective effects on the liver of diabetic animals. This could be explained by the antihyperglycermic action possessed by Citrus Medicus juice since the crude extract of the juice was able to bring about antihyperglycermic effect which in turn should be able to prevent degeneration of parenchymal cells that is usually associated with diabetic condition. Adeeyo et al 2013 had showed extract like allo-cepa which had antihyperglycermic effect was able to prevent degenerated cells in diabetic animals. It has also been documented that damage to hepatocytes is associated with Diabetes Mellitus (Jayaprakarsha *et. al.,* 2001). This showed that treatment with Citrus Medicus juice extract potential actions to ameliorate the condition and brought about reduction in hepatocyte damage and maintaining the hepatic integrity of the treated hyperglycemic animals

CONCLUSION

The result obtained from this study showed that Citrus Medicus juice caused a significant increase on the body weight of the animals, decrease in the level of blood glucose of the animals and maintained histo-hepatic architecture of hyperglycemic animals.

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