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ANTIDIABETIC AND HEPATOPROTECTIVE EFFECT OF *HURA CREPITANS* SEED EXTRACT IN ALLOXAN-INDUCED DIABETIC ALBINO RATS

***[1]IGWENYI, I. O., [2]AGU, E. A., [1]AWOKE, J. N., [1]EDWIN, NZUBECHUKWU, [3]FAMUREWA, A. C., [3]OBASI, N. A. AND [4]OBASI, D. C.**

[1]Department of Biochemistry, Ebonyi State University; [2]Chemical Pathology Unit, Federal Teaching Hospital, Abakaliki; [3]Department of Medical Biochemistry, Federal University, Ndufu Alike Ikwo; [3]Department of Chemical Science, Evangel University, Akaeze, Ebonyi State, Nigeria

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ABSTRACT

The *Hura crepitans* (Sandbox tree) seed extract (HSE) is used traditionally in the treatment of certain diseases and conditions like diabetes mellitus and its complications in Nigeria; however, there are no systematic investigations that have confirmed its antidiabetic potential. The study investigated the antidiabetic and hepatoprotective effects of ethanol extract of *Hura crepitans* seed in alloxan-induced diabetic rats so as to ascertain its therapeutic value, safety and possible toxicity. Diabetes was induced in overnight fasted albino rats by a single intraperitoneal injection of alloxan (100 mg/kg) and observed for 72 hrs for confirmation of diabetes. Diabetic rats were orally treated with ethanol extract of *Hura crepitans* (200 and 400 mg/kg body weight) for 15 days. Metformin (150 mg/kg body weight), a standard antidiabetic drug, was used as a positive control drug. Fasting blood glucose level was measured along the experimental period. Liver function parameters: aspartate transaminase (AST), alanine transaminase (ALT), total bilirubin, total protein and albumin levels of control and diabetic rats were evaluated at the end of the treatment. Treatment of experimental diabetes with the administration of HSE to diabetic rats for 15 days significantly reduced ($p<0.05$) fasting blood glucose levels compared to the diabetic control rats. The hypoglycemic effect of HSE was comparable to that of metformin. The diabetic condition induced liver damage evident by marked increase ($p<0.05$) in serum AST and ALT activities and total bilirubin level, as well as pronounced ($p<0.05$) decrease in serum total protein and albumin. The investigation revealed that *Hura crepitans* seed extract administration attenuated the liver damage induced by diabetic condition in rats. The

present findings suggest that HSE has antidiabetic and hepatoprotective effects in experimental diabetic rats that may offer health benefits in the management of diabetes and its complications.

Keywords: *Hura crepitans*, antidiabetic potential, metformin, hypoglycemia, ethanol extract, liver enzymes

INTRODUCTION

Diabetes is becoming increasingly more common throughout the world. The incidence has continued to grow and takes an enormous human monetary toil each year. It is a global health disorder affecting millions of people worldwide with an increasing incidence and prevalence (Onyechi *et al*., 2013). According to International Diabetes Foundation (2012), the number of diabetes cases in the world stands at 365 million people representing around 8.5 percent of the global population. Diabetes if left uncontrolled or untreated can lead to further metabolic and anatomical disturbances, among which are lipidemia, hypercholesterolemia, loss of weight, ketoacidosis, arteriosclerosis, gangrene, pathological changes in the eye, neuropathy, renal disease and coma (Sharma and Kumar, 2010).

Many therapeutic agents such as insulin, sulfonylureas etc, and standard drugs such as metformin and glitazones have been used to achieve glycemic control. Due to their side effects, failure of the drugs and cost of management, many people have resorted to the traditional medicinal plants due to their low cost, ease of accessibility and less side effects (Sharma and Kumar, 2010). Many medicinal plants have been reported of having hypoglycemic property such as *Rubus ellipticus, Ocinium santum, Vinca rosea, Allium sativum,* Neem etc (Sharma and Kumar, 2010).

The World Health Organization which is an organ of the United Nation approved that antidiabetic drugs used in the treatment and the management of diabetes that are of natural source should be used both in orthodox and traditional medicine (WHO, 1999). Other remedies are being verified to reduce glucose level and manage diabetic consequences. The plant *Hura crepitans* (sandbox tree) belongs to the family Euphoriaceae. Fowomola and Akindahunsa (2005) have reported that the consumption of the seed can result to throat burning, vomiting and others. The seeds can also be used to feed animals when prepared very well due to its high protein content. Adedire and Ajayi (2003) have also revealed its food potential and physiochemical properties of the seed of *Hura crepitans*. The seed oil has good qualities for industrial application (Olatidoye *et al*., 2010). However, the information on the medicinal value or therapeutic applications such as the antidiabetic potentials of *Hura crepitans* seed is very scarce hence, this investigation. Consequently, the aim of this investigation is to evaluate the

antidiabetic potentials of ethanol extract of *Hura crepitans* seed on alloxan induced diabetic albino rats.

Alloxan is a toxic glucose analogue, which selectively destroys insulin-producing pancreatic β-cells and causes insulin-dependent diabetes mellitus (T_1DM), when administered to animal species (Etuk, 2010). Alloxan is a urea derivative which causes selective necrosis of the pancreatic islet of β-cell thereby inducing diabetes in animals. Alloxan initiate diabetes through its capacity to destroy the pancreatic beta cell (Sharma and Kumar, 2010). *In vitro* studies have shown that alloxan can selectively destroy pancreatic beta cells. This is achieved with the presence of reactive oxygen species, with a continuous massive increase in cytosolic calcium concentration, leading to a fast destruction of beta cells (Oberley, 1988).

Metformin is an insulin sensitizing agent with potent antihyperglycemic properties. These properties of metformin are mainly attributed to suppression of hepatic glucose production, especially hepatic gluconeogenesis, and increased peripherial tissue insulin sensitivity (Sharma and Kumar, 2010).

Studies have shown that plant extracts have the ability to cause antihyperglycaemic effect by supporting regeneration of beta cells or protecting these cells from destruction, by restricting glucose load as well as by promoting unrestricted endogeneous insulin action. Plant extracts can also cause antihyperglycaemic effect by acting on beta cell to release insulin or activate the insulin receptors to absorb the blood sugar and stimulate the peripherial glucose consumption (Sharma and Kumar, 2010).

MATERIALS AND METHODS

Sample Collection and Preparation: *Hura crepitans* seed pods were collected from Fatilami Abubakar Park, Abakaliki Ebonyi State, Nigeria where it is planted as shade tree. The shells were broken and the cotyledons carefully removed from the pods. There were sun dried and ground to smaller particle size. The ground cotyledons were weighed and soaked in absolute ethanol for 24 hours and filtered. The filtrate was collected and the ethanol evaporated using the hot air oven at 60 degree Celsius to get the crude extract.

Chemicals: All chemicals used were of analytical grade. Alloxan monohydrate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Biochemical kits for AST and ALT assays were purchased from Randox laboratory (Crumlin, Co. Antrim, UK). Accu-chek active glucometer and strips (Roche Diagnostic, Mannheim, Germany).

Experimental animals: Thirty male healthy albino rats of Wistar strain (150 – 180 g) were obtained from a private animal holding facility in Nsukka, Enugu State, Nigeria. The animals were housed in the Animal House of the Department of Biochemistry, Ebonyi State University, Nigeria. They were maintained

under standard environmental conditions of temperature, humidity and light (12-hr light: 12-hr dark cycle) and fed on standard rat pellets (Vital feeds, Jos, Nigeria) and water *ad libitum*. The animals were acclimatized to the animal house for 1 week prior to the induction of experimental diabetes. The rats received humane care and handled in humane manner according to the approved animal experimental procedures in the NIH Guidelines for the Care and Use of Laboratory Animals (NRC, 1985).

Induction of experimental diabetes: Experimental diabetes was induced in overnight fasted rats by an intraperitoneal injection of 100 mg alloxan/kg body weight of rat. Alloxan monohydrate was dissolved in sterile distilled water (Mohammad *et al.*, 2012). Fasting blood glucose was measured in rats 72 hours after the injection. Rats with fasting blood glucose level >200 mg/dl were considered diabetic and included in the study. Animals used for normal control were injected with equivalent volumes of same sterile water.

Experimental design: The experimental rats were randomly divided into 5 groups of 6 rats each with Group 1 as normal control rats and treated with distilled water while groups 2 to 5 were induced with diabetes; group 2 were diabetic control rats that were treated with distilled water; group 3 was made up of diabetic rats treated with metformin (150 mg/kg body weight); group 4 animals were diabetic rats treated with HSE (200 mg/kg b.w.); and group 5 was composed of diabetic rats treated with HSE (400 mg/kg b.w.). The HSE and the drug metformin were given in aqueous solution daily by oral gavage for 15 consecutive days. The fasting blood glucose was measured on days of the treatment to evaluate the antidiabetic activity of the extract. The rats were fasted for 12 hrs and the blood from tail vein was collected for fasting blood glucose estimation using Accu-chek active glucometer and strips (Roche Diagnostic, Mannheim, Germany).

Estimation of liver function parameters: At the end of the experimental period, the animals were fasted overnight and then sacrificed following anesthesia. Blood was collected in plain sample tubes for the estimation of liver function parameters. The serum samples was obtained after centrifugation at 3500 g for 10 minutes. Serum marker of liver function such as such as aspartate transaminase and alanine transaminase, as well as serum total proteins and albumin were estimated spectrophotometrically, using enzymatic colorimetric assay kits (Randox, Crumlin, Co. Antrim, UK) following standard methods. The methods of Reithman and Frankel (1957) were employed in the analysis of liver enzymes (AST and ALT). Jendrassik and Grof method was used for the estimation of the bilirubin level while the Biuret method was used for the total protein estimation. Bromocresol Green solution method was used for the estimation of albumin (Burtis *et al*., 2006).

Statistical analysis: All values were presented as mean ± standard deviation (SD) for six (6) rats in each of the five groups. The significant difference in the means of all parameters was determined using one-way analysis of variance (ANOVA; 95% confidence interval) followed by Tukey's post hoc test and $P < 0.05$ was considered statistically significant.

RESULTS

Percentage Yield Of The Extract: The percentage yield of the extract = 3.64%.

Table 1 indicates a reduction in glucose level of the group treated with metformin and the extract of the seed. However, there was a dose – dependent effect as 400mg/kg body weight of extract was observed to be more effective in reducing the glucose level.

Table 2 shows a significant reduction ($P<0.05$) of AST in the group treated with metformin and the seed extract. There was a significant increase in the ALT ($P>0.05$).

Table 1: Effect Of Ethanol Extract Of Hura Crepitans Seed On Blood Glucose Level (mg\dl) Of Alloxan – Induced Diabetic Albino Rats

Grpoup	Fasting blood sugar	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15
Normal control	90.7 ±6.00	92.3 ±2.00	93.8 ±9.00	94.0 ±5.00	91.8 ±8.00	80.2 ±3.00	89.7 ±4.00	86.1 ±9.00	85.9 ±8.00
Non-treated diabetic rats	98.1 ±9.00	215.3 ±3.00	219.5 ±5.00	228.8 ±4.00	232.9 ±5.00	243.4 ±7.00	260.9 ±80	278.4 ±7.00	310.4 ±5.00
Diabetic + treated with metformin	101.8 ±5.0	252 ±4.00	178.6 ±5.00	160.4 ±3.00	153.6 ±40.0	124.8 ±2.00	106.3 ±4.00	100.2 ±3.00	94.6 ±6.00
Diabetic+200mg\kg body weight extract	91.8.2 ±8.00	299,7 ±6.00	290.6 ±5.00	256.4 ±4.00	233.3 ±3.00	220.8 ±4.00	215.7 ±6.00	128.5 ±8.00	124.5 ±5.00
Diabetic+400mg\kg body weight extract	95.4 ±8.00	281.4 ±3.00	224.5 ±2.00	200'3 ±9.00	195.7 ±2.00	189.8 ±7.00	175.6 ±6.00	121.4 ±5.00	107 ±4.61

NB: Values represent mean ± standard deviation (n=6)

Table 2: Effect Of Ethanol Extract Of Hura Crepitans Seed On Activities Of Liver Enzymes Of Alloxan–Induced Diabetic Albino Rats

Grpoup	AST (iU/L)	ALT (U/L)
Normal control	46±1.60	15±1.50
Non-treated diabetic rats	77±1.70	26±1.00
Diabetic + treated with metformin	68±1.20	34±1.20
Diabetic+200mg\kg body weight extract	48±1.70	37±1.00
Diabetic+400mg\kg body weight extract	31±1.60	19±1.40

NB: Values represent mean ± standard deviation (n=6)

Table 3: Effect Of Ethanol Extract Of Hura Crepitans Seed On The Liver Function Of Alloxan – Induced Diabetic Albino Rats

Group	T. Bil (mg\dl)	T. Protein (g\L)	Albumin (g\L)
Normal control	0.60±0.10	6.7±0.20	3.8±0.10
Non-treated diabetic rats	0.65±0.20	6.8±0.50	3.4±0.20
Diabetic + treated with metformin	0.45±0.30	6.3±0.10	3.5±0.20
Diabetic+200mg\kg body weight extract	0.45±0.22	7.2±0.80	3.5±0.80
Diabetic+400mg\kg body weight extract	0.35±0.20	6.7±0.40	3.7±0.20

NB: Values represent mean ± standard deviation (n=6)

DISCUSSION

Most common standard drugs used in the management of diabetes have presented with a lot of side effects. The need to develop drugs devoid of side effect has lead to increased demand for natural products with antihyperglycemic activity with little or no side effects. Current pharmacological strategies against hyperglycemia in diabetes are limited and involve insulin and classes of oral antidiabetic agents that stimulate pancreatic insulin secretion and action or modulate blood glucose level (Srinivasan and Ramarao, 2007). However, these agents have been associated with inadequate efficacy and number of serious adverse effects. Herbal remedies have become an important component of human health care due to their low cost, affordability and availability, with fewer side effects than prescription drugs (Gao *et al*., 2007). A growing body of evidence indicates that natural components in medicinal plants may possess effective antihyperglycemic effect and prevent diabetic complications (Patel *et al.,* 2012; Ashraf and Zare, 2015). Although anecdotal reports claim antidiabetic property of *Hura crepitans*, there are no systematic investigations in existing literatures to support this claim. Thus, hypoglycemic and hepatoprotective effects of ethanol extract of *Hura crepitans* seed in alloxan-induced diabetic rats was examined and the effect compared with a biguanide drug, metformin, in a period of 2 weeks.

In the current study, the continuous treatment with *Hura crepitans* seed extract (HSE) produced a significant ($p<0.05$) dose-dependent decrease in blood glucose levels in treated diabetic rats compared to untreated diabetic rat control (Table 1). It was further observed that the hypoglycemic effect of HSE was comparable to that of standard drug, metformin (Table 1). Research findings have established the contribution of chronic hyperglycemia to the development of glycation products, oxidative damage and diabetic complications such as retinopathy, neuropathy, nephropathy and other macrovascular complications (*Patel et al*., 2012; Choi *et al*., 2016). Effective control of blood glucose levels remains the cornerstone for preventing diabetic complications and for improving the quality of life of patients (Choi *et al*., 2016). The doses (200 mg/kg and 400 mg/kg) of HSE administered in the present study demonstrate capacity for glycemic control in diabetes rats. The mechanism underlying the beneficial hypoglycemic effect is currently unclear. However, previous studies suggest that plant extracts containing bioactive antioxidants that may improve hyperglycemia via modulation of enzymes associated with glucose metabolism or improve pancreatic insulin secretion and sensitivity (Choi *et ai*., 2016; Krishnasamy *et al*., 2016). According to the findings of Adindu *et al*., (2015), leaves, stem bark and roots of *H. crepitans* are rich in phytochemicals such as flavoniods, carotenoids,

alkaloids, and lignans. Although the current study considered the seed extract of *H. crepitans*, the antioxidant phytochemicals in HSE may contribute to the hypoglycemic effect observed in the present study. However, the dearth of previous studies regarding *H. crepitans* prevents comparison of obtained data. Nevertheless, findings from this study support previous work by Okoli *et al*., (2010) and Shabeer *et al*., (2009) which showed hypoglycemic effects of *Phyllanthus niruri* and *Phyllanthus simplex* in the same family of Euphorbiaceae as *Hura crepitans*. Our results suggest that HSE may modulate hyperglycemia in diabetes.

It was therefore observed that induction of diabetes in rats caused alterations in liver functions as evident by significant increase in serum activities of AST, ALT and total bilirubin, as well as a remarkable decrease in serum levels of total protein and albumin. Elevated serum levels of AST and ALT are indicative of cellular leakage and loss of functional integrity of the hepatic cell membranes implying hepatocellular damage (Kabbaoui *et al*., 2016). Diabetes melitus has been associated with oxidative stress produced by chronic hyperglycemia, which promotes the pathogenesis of functional liver abnormalities in diabetes (Adeyemi *et al*., 2014; Elbe *et al*., 2015). It is well-known that oxidative stress resulting from overproduction of reactive oxygen radicals affects the structural integrity of cellular components, particularly the membranes (Elbe *et al*., 2015). Therefore by implication, oxidative stress possibly induced by diabetes in the rats may contribute to the observed alterations in serum hepatic enzymes, total bilirubin, protein and albumin as observed in this study. Similar findings have been obtained in previous investigations of diabetes rat models (Adeyemi *et al*., 2014; Kabbaoui *et al*., 2016). Alterations in serum liver function parameters were dose-dependently attenuated after 2 weeks treatment with HSE to diabetic rats. It is noteworthy to indicate that the hepatoprotective effect of HSE was higher in comparison to metformin in diabetic rats (Table 2). The hepatoprotective effect of HSE might be attributable to the antioxidant beneficial health potential of free radical scavengers that may be present in HSE.

CONCLUSION

It is hereby reported for the first time that the ethanol extract of *Hura crepitans* seed has potential antidiabetic effect in alloxan-induced diabetic rats comparable in efficacy to a standard antidiabetic drug, metformin. In addition, the ethanol extract also demonstrated hepatoprotective potential against diabetic complication by attenuating liver damage in the present study. However, further investigations are needed to unravel the bioactive components of *Hura crepitans* seed extract involved in the antidiabetic and hepatoprotective activities as

well as the elucidation of their mechanism of action.

REFERENCES

[1] Adedire, M. A. and Ajayi, O. A. (2003) Potential of sandbox Hura crepitans seed oil for protection of cowpea seeds from Bruchidae infestation. Journal of Plant Diseases and Protection, **110**(6): 1 - 9.

[2] Adeyemi, D. O., Ukwenya, V. O., Obuotor, E. M. and Adewole, S. O. (2014). Anti-hepatotoxic activities of *Hibiscus sabdariffa* L. in animal model of streptozotocin diabetes-induced liver damage. *BMC Complement Altern Med*., **14**:277

[3] Adindu, E. A,, Elekwa, I. and Ogwo, J. I. (2016). Phytochemical comparative screening of aqueous extracts of the leaves, stem barks, and roots of *Hura crepitans* (L) using GC – FID. *IOSR Journal of Biotechnology and Biochemistry,* **2**(1): 2455-2463.

[4] Ashraf, H. and Zare, S. (2015). Preventive effects of aqueous extract of berberis integerrima bge. root on liver injury induced by diabetes mellitus (type 1) in rats. *Iranian J. Pharm. Res*., **14** (1): 335-343.

[5] Burtis, C. A., Ashwood, E. R. and Bruns, D. E. (2006) Tietz Textbook of clinical chemistry 4th edn Saunder company Philadelphia 801-803.

[6] Choi, K. H., Lee, H. A., Park, M. H. and Han, J. M. (2016). *Morus alba* fruit extract containing anthocyanins improves glycemic control and insulin sensitivity via activation of AMP-activated protein kinase in diabetic C57BL/Ksj-db/db mice. *J. Med. Food,* **19**(8): 1–9.

[7] Elbe, H., Vardi, N., Esrefoglu, M., Ates, B., Yologlu, S. and Taskapan, C. (2015). Amelioration of streptozotocin-induced diabetic nephropathy by melatonin, quercetin, and resveratrol in rats. *Human Exp Toxicol*, **34**(1): 100–113.

[8] Etuk, E.U. (2010). Animal Models for Studying diabetes mellitus. *Agriculture and Biology Journal of North America,* **1**(2): 130-134.

[9] Fowomola, M. A. and Akindahunsi, A.A. (2005). Protein quality indices of sandbox (*Hura crepitans*) seeds. *Journal of Food, Agriculture and Environment,* **3**(3&4):16-19.

[10] Fowomola, M. A and Akindahunsi, A. A. (2010). Effect of sand box (*Hura crepitens*) seed meals on Rat's liver's enzyme levels. *Continental Journal of Biomedical science,* **4**(3): 50 - 54.

[11] Gao, D., Li, Q., Li, Y., Liu, Z., Liu, Z., Fan, Y., Han, Z., Li, J. and Li, K. (2007). Antidiabetic potential of oleanolic acid from *Ligustrum*

lucidum Ait. Can. J. Physiol. Pharmacol., **85**: 1076–1083.

[12] Kabbaoui, M. E., Chda, A., Mejrhit, N., Azdad, O., Farah, A., Aarab, L., Bencheikh, R. and Tazi, A. (2016). Antidiabetic effect of *Thymus satureioides* aqueous extract in streptozotocin-induced diabetic rats. *International Journal of Pharmacy and Pharmaceutical Sciences*, **8**(9): 140-145.

[13] Krishnasamy, G., Muthusamy, K., Chellappan, D. R. and Subbiah, N. (2016). Antidiabetic, antihyperlipidaemic, and antioxidant activity of Syzygium densiflorum fruits in streptozotocin and nicotinamide-induced diabetic rats. *Pharm. Biol*., **54**(9): 1716–1726.

[14] Muhammad N. O., Akolade, J. O., Usman, L. A. and Oloyede, O. B. (2012). Haematological parameters of alloxan-induced diabetic rats treated with leaf essential oil of *Hoslundia opposita (vahl). EXCLI J*., **11**: 670-676.

[15] National Research Council, (1985). Guide for the Care and the Use of Laboratory Animals, 20, National Institute of Health, Bethesda pp. 85–123.

[16] Oberley, L. W. (1988). Free Radicals and diabetes. *Free Rad. Bol. Med,* **5**(8): 113-134.

[17] Okoli, C. O., Ibiam, A. F., Ezike, A. C., Akah, P. A. and Okoye, T. C. (2010). Evaluation of antidiabetic potentials of *Phyllanthus niruri* in alloxan diabetic rats. *African Journal of Biotechnology,* **9**: 248-259.

[18] Onyechi, A. U., Ibeanu, V. N., Eme, P. E. and Ossai, O. C. (2013). Nutrient and Phytochemical composition of formulated diabetic snacks made from two Nigerian foods: *Afzelia africana* and *Detarium microcarpium* seed flour. *Pakistan Journal of Nutrition,* **12**(2): 108-113.

[19] Olatidoye, O. P., Adeleke, A. E., Adegbite, S. A. and Sobowale, S.S. (2010).Chemical composition and Nutritional Evaluation of sandbox (*Hura crepitans*) seed flour for domestic consumption and industrial utilization in Nigeria. *Journal of Medical and Applied Biosciences,* **5**(2): 125 – 129.

[20] Patel, D. K., Kumar, R., Laloo, D. and Hemalatha, S. (2012). Diabetes mellitus: An overview on its pharmacological aspects and reported medicinal plants having antidiabetic activity. *Asian Pac. J. Trop. Biomed.,* **2**(5): 411-420.

[21] Reithmen, S. and Frankel, S. (1957) "Procedures of Transaminase Analysis". *American Journal of Chemical Pathology,* **28** (1): 56

[22] Sharma, U. S. and Kumar, A. (2010). Anti diabetic effect of *Rubus ellipticus* fruit extract on alloxan-inducecd diabetic rats. Journal of Diabetology, **17**(4): 376–379.

[23] Shabeer, J., Srivastava, R. S. and Singh, S. K. (2009). Antidiabetic and antioxidant effects of various fractions of Phyllathus simplex in alloxan diabetic rats. Journal of Ethnopharmacology, 2009; 124: 34-38.

[24] Srinivasan, K. and Ramarao, P. (2007). Animal models in type 2 diabetes research: An overview. *Indian J Med Res*., **125**: 451-472.

[25] World Health Organisation. (1999). "Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications". *Annals of Internal Medicine*. **28** (3): 423-429.