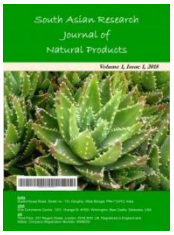

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# Effect of Processing Unripe Plantain (*Musa paradisiaca*) Extracts on Some Biochemical Parameters in Alloxan-Induced Wistar Rats

**C. D. Luka[1*], J. C. Oyeocha[1], M. K. Kwarpo[2] and G. Istifanus[1]**

*[1]Department of Biochemistry, University of Jos, Nigeria.*
*[2]Department of Biochemistry, Plateau State University, Bokkos, Nigeria.*

***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors CDL and GI designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors JCO and MKK managed the analyses of the study. Author GI managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

Unripe plantain is used for management of diabetes mellitus in Nigeria, the possible effect of its methods of processing on some biochemical parameters has not been investigated. The objective of this study is to determine the effect of methods of processing unripe plantain on blood glucose, Total Cholesterol, Triglyceride, HDL, LDL, serum Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alanine Phosphatase (ALP), total protein, albumin, creatinine, uric acid and urea levels in alloxan-induced diabetic rat models. Twenty male albino Wistar rats were used and were divided into 5 groups of 4 rats each. Group 1 (normal control) received standard rat feeds; group 2 (diabetic control) received standard rat feeds; groups 3,4 and 5 received boiled, roasted and dried unripe plantain pellets respectively. In the result, a significant ($P<0.05$) reduced blood glucose levels were seen for those experimental animals given dried, boiled and roasted extracts respectively. Triacylglycerol levels were significantly decreased ($p<0.05$) for those diabetic rats

**Corresponding author: E-mail: carrll42@yahoo.com;*

administered dried, boiled and roasted extracts respectively. The Total cholesterol levels were significantly decreased ($p<0.05$) for those administered dried, roasted and boiled extracts respectively. The LDL-cholesterol levels were also significantly decreased ($p<0.05$) for those treated with dried, roasted and boiled extracts respectively. The HDL-cholesterol were significantly increased for the diabetic administered dried extract, roasted extract and a significant decrease for those administered boiled extract. There were significant increase ($p<0.05$) in the levels of serum ALT, AST and ALP in the diabetic control group compared to the normal control are observed. On administration of unripe plantain, the values of these enzymes significantly decreased ($p<0.05$). The boiled extract showed a greater decrease in the level of ALT and ALP whereas the dried extract showed a greater decrease in the level of AST. There were also increase in the level of urea and uric acid in the diabetic control compared to the normal control group is observed. However the dried extract showed a greater decrease in the levels of both urea and uric acid. The creatinine level is seen to increase in the diabetic control group when compared to the normal control group and showed greater increase on administration of unripe plantain, for the group fed with roasted extract having the highest level of creatinine.

*Keywords: Diabetes mellitus; unripe plantain; alloxan.*

## 1. INTRODUCTION

One of the most challenging diseases of the 21st century is Diabetes. Diabetes mellitus is a metabolic disease characterized by hyperglycemia, resulting from partial or total destruction of pancreatic beta cells [1]. It affects the essential biochemical pathways of the body (carbohydrate, protein and lipid metabolism) and its prevalence is rising globally, which includes the rural Nigeria populations [2,3]. Due to the inability of the modern therapy to control all the pathophysiological aspects of the disorder, as well as the enormous cost it poses on the economy of the developing nations of the World, alternative strategies are urgently needed [4].

The global concern for the diversification of the uses of plant foods to improve normal and therapeutic nutrition for diabetes control has shifted scientists' interest to enhancing the potential sources of beneficial constituents in plant foods. Plant foods have generated increasing research interest because of their anti-diabetic potentials. The diets/medicinal plants that are commonly used in the management of diabetes in Nigeria include: acha (*Digitaria exiles*), breadfruit (*Treculia africana*) and beans (*Phaseolus vulgaris*) [5]. However, diabetic patients have often complained of the monotony of staying on a particular diet (personal communication) and this has therefore increased the research into other plants.

Plantain (*Musa paradisiaca*) belongs to the *musica*l family and it is cultivated in many tropical and sub-tropical countries of the world. Plantain is a source of starchy staple for millions of people in Nigeria. It contains low quantities of minerals and sugars; this can be seen in an unripe plantain. Scientifically, unripe plantain has being documented as a hypoglycemic plant [6]. In folklore medicine, unripe plantain is used in the management of diabetes, renal and liver dysfunction [7]. Although, unripe plantain is used to manage diabetes mellitus in Nigeria, the possible effect of its methods of processing on some biochemical parameters, including renal and hepatic dysfunction has not been investigated. This study was aimed at examining the effect of methods of processing unripe plantain on some biochemical parameters in alloxan-induced diabetic rats.

## 2. MATERIALS AND METHODS

### 2.1 Preparation of Plant Materials

Matured freshly cut unripe green plantain (*Musa paradisiacal)* where purchased from a local market in Jos Plateau state, Nigeria. The bunch of *Musa paradisiacal* was rinsed with water to remove latex and dirt. These were divided into three portions and prepared differently. To the first portion, it was boiled in boiling water for 30 minutes, allowed to cool before being peeled and cut into pieces, air-dried at room temperature for 6-7 days. The dried pieces were pulverized using a milling machine to obtain a fine powder. The second portion was roasted using a charcoal oven until it is browned. It was peeled, cut into piece pulverized using a milling machine to obtained a fine powder. The last portion was air-dried at room temperature for 6-7 days. The dried plant food was milled into powder.

## 2.2 Preparation of Extracts

Each of the processed plantain powder was used for the extraction process. 100 g of each powdered plant material was weighed using a weighing balance and transferred into different beakers. Each was dissolved with 1L of distilled water and allowed to stay for twenty-four hour for maximum extraction of the active ingredient(s). The dissolved plant was filtered and the filtrate was kept in an oven at 60°C. This was done to ensure that it undergo evaporation until it becomes dried. The dried extracts were separately transferred into airtight containers and stored in the refrigerator.

## 2.3 Experimental Designs

The experimental animals used were twenty (20) healthy Wistar rats (weighing 185-200 kg) obtained from the animal house of University of Jos, Nigeria. They were allowed to acclimatize for 2 weeks, after which they were maintained under a constant 12 hours light and dark cycle and at room temperature.

## 2.4 Induction of Experimental Animals

The animals were induced with freshly prepared saline solution of Alloxan and injected into the animals intraperitoneally. After 48 hours of induction of Alloxan, the animals were tested to confirm if they were diabetic.

## 2.5 Grouping and Administration of Extract

The plant (plantain extract) from the differently processed method were administered orally to the animals with 150 ml/kg body weight measurement. This was given to the experimental animals (albino rats) that were divided into 5 groups of 4 each as follows:

Group 1: normal rats administered standard feed pellets (Normal control)
Group 2: diabetic control rats administered standard feed pellets (Diabetic control)
Group 3: diabetic rats administered boiled plantain extract
Group 4: diabetic rats administered roasted plantain extract
Group 5: diabetic rats administered dried plantain extract.

The extract was administered for the period of 8 days following an interval of 48 hours fasting period. The administration was stopped at the eighth day, the rats were anesthetized with chloroform and their blood samples collected.

Ethical issues were observed in line with the regulations of animal usage and approval was obtained as required in the University of Jos ethical committee guide.

## 2.6 Methods

### 2.6.1 Determination of glucose

Glucose reacts with O'toludine in a glacial acetic acid with heat of field N-glucosyl amine which is blue green in color. The absorbance is measured at 025 nm [8].

### 2.6.2 Determination of cholesterol level

The Total Cholesterol was determined by Liebermann Burchard's method [9].

### 2.6.3 Determination of creatinine

Creatinine reacts with picric acid to produce a coloured compound creatinine adenine picrate which was photometrically measured. The intensity of the colour is a function of the creatinine in the blood. The total serum proteins, albumin, uric acid and urea were determined using Biosystems Diagnostic kits as described by [10,11].

### 2.6.4 Determination of serum urea

The ammonia reacts with phenol in the presence of hypochlorite to form indophenols which give a blue compound in alkaline solution [12].

## 2.7 Haematological Analysis

The haematological analysis carried out were Packed Cell Volume, haemoglobin, platelet count, white blood cell count, red blood cell count, using standard procedures [13].

## 2.8 Statistical Analysis

Data were subjected to analysis using the Statistical Package for Social Sciences (SPSS), version 15.0. Results were presented as the means standard deviations of triplicate experiments. One way analysis of variance (ANOVA) was used for comparison of the methods. Differences between methods were

considered to be significant at P <0.05 using the Duncan Multiple Range Test.

## 3. RESULTS

The administration of alloxan at a dosage of 65 mg/kg body weight to the rats produced a stable diabetic condition within few days in most of the experimental rats. Administration of unripe plantain resulted in 510%, 55% and 113% decrease in blood glucose compared to the diabetic control in the groups administered boiled, roasted and dried extracts respectively (Table 1).

**Table 1. Result of effect of plantain extracts on blood glucose level (mmol/l) in diabetic rat models**

| Treatment groups | Boiled extract | Roasted extract | Dried extract |
|---|---|---|---|
| A Normal Control | 5.56±0.01 | 5.56±0.01 | 5.56±0.01 |
| B Diabetic Control | 19.84±0.02[a] | 19.84±0.02[a] | 19.84±0.02[a] |
| C Diabetic Treated | 3.25±0.02[ab] | 12.82±0.02[ab] | 9.32±0.02[ab] |

*Values are expressed as mean ± SD, n = 4 for each group.*
*[a]values are significantly different from normal control group (P<0.05)*
*[b]values are significantly different from diabetic control group (P<0.05)*

**Table 2. Result of effect of plantain extracts on lipid profile concentrations in diabetic rat models**

| Treatment groups | TG | TC | LDL | HDL |
|---|---|---|---|---|
| A Normal control | 1.37±0.02 | 3.68±0.01 | 2.26±0.36 | 1.54±0.02 |
| B Diabetic control | 2.53±0.18[a] | 5.84±0.01[a] | 3.24±0.01[a] | 0.47±0.01[a] |
| C Diabetic + boiled extract | 1.73±0.01[ab] | 4.43±0.01[ab] | 2.61±0.01[ab] | 0.08±0.01[ab] |
| D Diabetic + roasted extract | 1.89±0.01[ab] | 4.82±0.01[ab] | 2.93±0.02[ab] | 0.74±0.02[ab] |
| E Diabetic + dried extract | 1.53±0.01[ab] | 4.02±0.01[ab] | 2.24±0,02[ab] | 0.94±0.01[ab] |

*Values are expressed as mean ± SD, n = 4 for each group.*
*[a]values are significantly different from normal control group (P<0.05)*
*[b]values are significantly different from diabetic control group (P<0.05)*

**Table 3. Result of effect of plantain extracts on enzymes of rat models**

| Treatment groups | ALT(mmol/L) | AST (mmol/L) | ALP (mmol/L) |
|---|---|---|---|
| A Normal Control | 11.31±0.01 | 16.84±0.02 | 236.62±0.02 |
| B Diabetic Control | 48.18±2.46[a] | 79.87±0.01[a] | 642.24±0.01[a] |
| C Diabetic + boiled extract | 31.46±0.01[ab] | 60.16 ±0.09[ab] | 86.00±0.70[ab] |
| D Diabetic + roasted extract | 39.85±0.03[ab] | 64.41 ±0.01[ab] | 431.00±0.70[ab] |
| E Diabetic + dried extract | 36.36±0.02[ab] | 49.42 ±0.01[ab] | 411.00± 0.01[ab] |

*Values are expressed as mean ± SD, n= 4 for each group.*
*[a]values are significantly different from normal control group (P<0.05)*
*[b]values are significantly different from diabetic control group (P<0.05)*

**Table 4. Result of effect of plantain extracts on creatinine, urea and uric levels in the experimental rat models**

| Treatment Groups | Creatinine (mmol/L) | Urea (mmol/L) | Uric acid (mmol/L) |
|---|---|---|---|
| A Normal Control | 91.54±0.01 | 7.02 ± 0.01 | 226.08±0.01 |
| B Diabetic Control | 173.97±1.01[a] | 22.82 ± 0.01[a] | 564.24±0.02[a] |
| C Diabetic + Boiled Extract | 182.54 ±0.02[ab] | 10.42±0.01[ab] | 396.04± 0.01[ab] |
| D Diabetic + Roasted Extract | 194.64 ±0.02[ab] | 12.82±0.02[ab] | 401.64± 0.01 [ab] |
| E Diabetic + Dried Extract | 177.54±0.01[ab] | 9.32±0.02[ab] | 371.84± 0.01[ab] |

*Values are expressed as mean ± SD, n= 4 for each group.*
*[a]values are significantly different from normal control group (P<0.05)*
*[b]values are significantly different from diabetic control group (P<0.05)*

**Table 5. Result of effect of plantain extracts on the total protein and albumin concentrations in rat models**

| Treatment groups | Total protein g/L | Albumin g/L |
|---|---|---|
| A Normal Control | 75.60±0.01 | 35.62±0.02 |
| B Diabetic Control | 68.42±0.02[a] | 28.47±0.02[a] |
| C Diabetic + Boiled Extract | 72.08±0.01[ab] | 32.34±0.04 [ab] |
| D Diabetic + Roasted Extract | 70.38±0.01[ab] | 30.30±0.01[ab] |
| E Diabetic + Dried Extract | 74.04±0.01[ab] | 33.18±0.01[ab] |

*Values are expressed as mean ± SD, n= 4 for each group.*
*[a]values are significantly different from normal control group (P<0.05)*
*[b]values are substantially different from diabetic control group (P<0.05)*

**Table 6. Result of effect of plantain extracts on some hematological parameters in diabetic rat models**

| Treatment groups | PCV | HB | WBC | PLT | RBC |
|---|---|---|---|---|---|
| Normal Control | 32.00±0.81 | 7.62±0.01 | 5260±1.63 | 242000±0.81 | 5360±201.33 |
| Diabetic Control | 30.00±0.81[a] | 3.24±0.01[a] | 9500.00±0.8[a] | 318000.00±163[a] | 9525.00±49.33[ab] |
| Diabetic + Boiled Extract | 35.00±0.81[ab] | 8.32±0.01[ab] | 7400.00±0.81[ab] | 26400±0.81[ab] | 4898.00±0.81[ab] |
| Diabetic + Roasted Extract | 33.00±2.94[ab] | 4.51±0.01[ab] | 7450.00±0.00[ab] | 26600±3.26[ab] | 7500±1.41[ab] |
| Diabetic + Dried Extract | 35.00±2.89[ab] | 5.31±0.01[ab] | 6600±0.00[ab] | 255000±0.81[ab] | 6600.00±0.81[ab] |

*Values are expressed as mean ± SD, n= 4 for each group.*
*[a]values are significantly different from normal control group (P<0.05)*
*[b]values are substantially different from diabetic control group (P<0.05)*

The serum Alanine aminotransferase, Aspartate aminotransferase and Alanine phosphatase enzyme levels of the diabetic control were significantly increased ($p<0.05$) compared to the non-diabetic control group. Administration of unripe plantain extracts decreased considerably ($p<0.05$) the levels of these enzymes, especially in the group fed boiled extract (31.46 mmol/L) followed by the group fed dried extract (36.36 mmol/L), and then the group fed roasted extract (39.85 mmol/L) for ALT. For AST, the level of decrease is more in the group supplied dried extract (49.42 mmol/L**)** as against the boiled (60.16 mmol/L) and roasted (63.41 mmol/L) plantain extracts respectively. There is a similar decrease in ALP in the trend 86 mmol/L for boiled, 411 mmol/L for dried and 431 mmol/L for roasted plantain extract respectively (Table 3).

There is a significant increase in creatinine level in the diabetic control group compared to the non-diabetic control group. On administration of unripe plantain extracts, there were significant increases ($p<0.05$) especially in the group administered roasted extract (194.64 mmol/L). Also, there is substantial increase in the level of urea in the diabetic control group compared to the non-diabetic control group. However, on the administration of unripe plantain, a significant decrease ($p<0.05$) was observed as follows: 9.32 mmol/L, 10.42 mmol/L and 12.82 mmol/L for groups fed dried, boiled and roasted extracts respectively. A significant increase is observed in the level of uric acid in the diabetic control group (564.24 mmol/L) compared to the non-diabetic group (226.08 mmol/L). After treatment with unripe plantain extracts, uric acid level decreased significantly to 396.04 mmol/L, 401.64 mmol/L and 371.84 mmol/L for the groups treated with boiled, roasted and dried extracts respectively.

A significant decrease was observed in the level of total protein in the diabetic control group

(68.42 g/L) compared with the non-diabetic control group (75.60 g/L). After administration of unripe plantain extracts, there was noticeable significant increase ($p<0.05$) especially in the group administered dried extract (74.04 g/L) followed by boiled extract (72.08 g/L) and then the group conducted roasted extract (70.38 g/L). Similarly, there is a significant decrease of Albumin level in the diabetic control (28.47 g/L) compared with the non-diabetic control (35.62 g/L). Administration of unripe plantain significantly increase ($p<0.05$) this value to 32.34 g/L, 30.30 g/L and 33.18 g/L for groups treated with boiled, roasted and dried plantain extracts respectively.

## 4. DISCUSSION

Different factors can affect or influence blood glucose level. These include the physical form of the food, degree and type of processing example, cooking method and time, amount of heat and moisture used [14], and also the type of starch (amylose versus amylopectin). Findings from this study indicate that the boiled plantain extract has the highest hypoglycemic effect while the roasted extract has the least; thus confirming the ability of unripe plantain to ameliorate hyperglycemia. This further explains that the result of moist heating improves the strength of the food substance to enhance the effect of hyperglycemia than drying without direct heating (drying) and dry heating (roasting). Boiling of the plantain allowed the starch granules to swell, gelatinise and increase the availability of amylase digestion and thereby growing starch availability [15].

Serum total cholesterol, LDL cholesterol and Triglyceride levels of the diabetic control rats are significantly ($p<0.05$) higher than that of the non-diabetic control rats with the decreased level of HDL-Cholesterol (Table 2). This is an indication that diabetes mellitus is associated with elevated levels of Total cholesterol, LDL-Cholesterol and Triglyceride with reduced level of HDL-Cholesterol. The result is shown in the table also indicates that the diabetic animals when fed with unripe plantain have significant ($p < 0.05$) decrease in the levels of cholesterol compared with the controls. This is an indication that plantain, however, reduces cholesterol [16]. Increase in cholesterol is a risk factor associated with arteriosclerosis and cardiovascular diseases [17]. The experimental group fed with dried plantain extract had the most pronounced effect in lowering serum cholesterol level while the roasted plantain extract had the least. Therefore, dried plantain extract has the highest ability to enhance the impact of arteriosclerosis and cardiovascular disease than the boiled and the roasted extracts. From the same table, triglyceride and Low-density lipid (LDL) concentrations are significantly decreased ($p<0.05$) in all the diabetic treated groups, with the group treated with dry extract being the most decreased. High levels of LDL-Cholesterol and triglyceride have been associated with heart disease [18]. In the medical term, high cholesterol and triglyceride levels are referred to as lipid disorder, which increases the risk of atherosclerosis and also heart disease, stroke and high blood pressure [17]. The consumption of unripe plantain has been shown to reduce triglyceride level [19].

Measurement of enzymatic activities of aminotransferases (AST and ALT) and phosphatases is of clinical and toxicological importance as changes in their actions are indicative of tissue damage by toxicants or in disease conditions [10,20,21]. Aminotransferases such as ALT and AST and ALP are common liver enzymes whose activities are a sensitive indicator of liver cell injury and are helpful in recognising hepatocellular diseases such as diabetes. In Harris et al. [22] studies, it was shown that individuals with type 2 diabetes mellitus (T2DM) have a higher incidence of liver function abnormalities than individuals who do not suffer from diabetes mellitus. This study indicates an increase in the level of the diagnostic enzymes (AST, ALT and ALP) in the serum of alloxan diabetic rat models which is attributable to the toxicity of alloxan to the tissue that expresses GLUT2 transporters such as hepatocytes and renal tubular cells [23]. The effect of alloxan on the levels of these diagnostic enzymes (AST, ALT and ALP) in the serum of alloxan diabetic rat models has remained unravelled. While some authors reported increased activities of AST, ALT [24] and ALP [25] in the liver of alloxan diabetic rat models, some others reported no alteration in the levels of these enzymes in the serum of the diabetic rats. The increase observed in the level of these enzymes in the serum of the diabetic control rat models could be as a result of leakage of these enzymes from the liver cytosol into the bloodstream [26] which indicates the hepatotoxicity of alloxan. However, Treatment of the diabetic animals with unripe plantain was able to decrease significantly ($p<0.05$) the levels of these enzymes in the serum of these rat

models indicating the ability of unripe plantain to repair liver damage. The boiled plantain extract has the highest capacity to decrease the levels of AST and ALP, while the dry extract has the highest ability to reduce the activity of ALT.

It is the function of the kidney to remove urea from the blood. In kidney impairment, the urea level builds up in the blood because the kidneys are unable to clear the area from the bloodstream [27]. In this study, the serum urea levels of the diabetic control group increased significantly ($p<0.05$) compared with the non-diabetic control. This could be as a result of kidney impairment in diabetic control rats. However, treatment of the diabetic animals with unripe plantain resulted to the significant decrease ($p<0.05$) of urea in the serum of the diabetic rats (Table 4). This shows that plantain may possess protective effects on the kidney. The dried unripe plantain has the most useful ability to enhance the impact of urea on the organs of the diabetic animals while the roasted plantain extract has the least effect.

Creatinine, a metabolite of creatine is generated from muscle and excreted by the kidney. However, in kidney impairment, creatinine is poorly cleared and therefore builds up in the blood [27]. The outcome of this study significantly highlighted that consumption of unripe plantain (be it boiled, roasted or dried) as seen in Table 4, can induce an elevation of creatinine level, which by implication, suggests that unripe plantain has a higher propensity to cause renal failure due to increase in creatinine level. The diabetic rats treated with roasted plantain extract have the highest level of creatinine, while those treated with the dry extract has the least.

The uric acid level of the diabetic control group is higher when compared to the non-diabetic group. This could be as a result of renal failure, resulting to reduced clearance of uric acid by the kidney, since it is the function of the organ to clear out uric acid from the blood. High levels of uric acid can lead to a kidney stone or cause solid crystals to form within joints. This creates a painful condition called gout. If gout remains untreated, these uric acid crystals can build up in the joint or nearby tissues, forming hardy lumpy deposit called tophi. On administration of unripe plantain, the levels of uric acid significantly decrease ($p<0.05$). This could be as a result of the renal protective effect of unripe plantain. Precisely, the dried plantain extract has the highest uric acid-reducing ability while the roasted extract has the least.

The decrease in the serum protein of the diabetic control rats is an indication of proteinuria which is an important clinical marker of diabetics nephropathy, and this decrease can be attributed to increasing protein catabolism while the increase in the serum protein level of the diabetic rats fed unripe plantain extracts is an indication of the protective action of unripe plantain against nephrotoxicity [28] and also unripe plantain is an excellent source of protein [29]. The study shows that the dried plantain extract has the highest ability to enhance the effect of nephropathy in the diabetic rats.

The low serum albumin level of the diabetic control rats could be attributed to their low serum protein levels suggesting the impaired renal function for the rats of this group, or it may also recommend an impaired liver function for this group. The elevation of the serum albumin levels of the diabetic rats fed unripe plantain suggests that plantain can be used in the management of diabetic renal dysfunction. However, the dried plantain extract suggests better management of renal dysfunction compared to the boiled and roasted extracts.

Packed cell volume (PCV), white blood cells (WBC), Platelet, Red Blood Cell (RBC) and HB are of diagnostic importance. A decrease in PCV generally means red blood cell loss from cell destruction, blood loss or failure of bone marrow production. Consumption of plantain could protect the red blood cells due to its content of blood-forming nutrients such as iron. This explains why the PCV of the diabetic control and non-diabetic control (Table 6) is significantly different from the diabetic treated. Distinctly, the boiled and dried extracts have higher PCV values.

## 5. CONCLUSION

The knowledge of an active processing method for dietary staples to control and enhance the effect of complications of diabetes is essential in the treatment of diabetes. The findings in this study are useful to health care providers and nutritionist in diabetes education. This is because diet management is crucial to control spikes in blood glucose levels. The research indicates that unripe plantain can be used in the management of complications arising from diabetes mellitus. The boiled plantain extract had the highest

hypoglycemic effect of all the processed test extracts and can be used to decrease the impact of hyperglycemia. It can be used to improve the aberrations of the enzymes activities of diabetes mellitus, whereas, the dried plantain extract has the highest TG and LDL-cholesterol lowering effect and can effectively be used to manage the heart-damaging effect of diabetes mellitus. The use of dried plantain in the dietary management of diabetes mellitus could be a breakthrough in search of plants that could prevent the development of diabetic nephropathy.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

Ethical approval was gotten from the ethical Committee University of Jos Department of Pharmacology.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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