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# Phytochemical screening and Hypoglycaemic effect of ethanol leaf extract of *Annona squamosa* L. in alloxan induced diabetic rats

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## Abstract
*Annona squamosa* L. commonly known as curstard apple is a medicinal plant found in Nigeria and has diverse ethnomedical uses including management of diabetes. The leaves of *Annona squamosa* L was collected in Damboa road, Maiduguri, Borno State. The leaves were washed and dried under shade at room temperature until constant weight was obtained. The leaves were crushed into coarse powder using wooden pestle and mortar. Defatting was done with petroleum ether, while the extraction was carried out with 95% ethanol. The phytochemical screening and antidiabetic activity of the ethanol extract of *Annona squamosa* leaves were carried out. The phytochemical screening of the *Annona squamosa* revealed the presence of alkaloid, tannin, carbohydrate, saponin, and glycoside. Acute toxicity was carried out and no death was observed at the highest testing dose of 5000 mg/kg. The elevated glucose level in the diabetic rats was significantly reduced by the administration of ethanol leaf extract of *Annona squamosa* within 24 hours.
The result of the study showed that the ethanol leaf extract of *Annona squamosa* L. contains bioactive phytochemical constituents that possess anti-diabetic activity which may be effective in management of diabetes mellitus. This justifies the use of *Annona squamosa* L leaf decoction by the native people for the management of diabetes.

**Keywords:** *Annona squamosa*, phytochemistry, antidiabetes

## Introduction
Diabetes mellitus is one of the most leading causes of death affecting over 100 million people worldwide, and the most common non-communicable endocrine disease characterized by hyperglycaemia and disturbances in carbohydrate, protein and lipid metabolism, due to absolute or relative deficiency in insulin secretion or insulin action (Zimmet *et al.,* 1999). Estimation by International Diabetes Federation showed that in 1985, 30 million people had diabetes, while the figure rose to 150 million in 2000 and 246 million in 2007. It is projected by International Diabetes Federation that by the year 2025, the figure would have risen to 380 million (Rajaei *et al.,* 2015). In the 1960s and 1970s physicians had to stand by helplessly watching their patients overwhelmed by complications of the disease. Prevention of blindness by photocoagulation and renal support treatment for those in renal failure became possible in the 1970s, while development of specialist foot clinics during 1980s succeeded in halving the amputation rate. The sad outcome for pregnancies 20 years after the discovery of insulin when the fetal mortality rate was more than 25% has been transformed so that now more than 95% of these pregnancies succeed (Tiwari and Madhusudana, 2002). The conventional antidiabetic drugs available to manage the disease are still costly and not readily affordable to the majority of the affected population (Mohammed *et al*., 2014) and some of these antidiabetic agents are also associated with some undesirable side effects and contraindications or both ( Kameswara *et al*., 1999; Jouhari *et al*., 2000). Diabetes mellitus can be classified into type 1, type 2 and type 3, but the most common being type 1 and type 2.

The present technology of managing diabetes was undreamt of until the last quarter of the 20$^{th}$century. The introduction of home blood glucose monitoring with new non-invasive technologies now in sight, has made possible the achievement of "tight control", while at the same time advances in understanding and reversing diminished awareness of hypoglycemia are reducing its hazards. The invention of insulin pens and more recently the

development of insulin pumps have contributed in great measure to improving the quality of life of those with the burden of lifelong diabetes (Peter J. Watkins 2003).

## Material and methods

### Plant Collection, Identification and Preparation

The part of the plant used in this research is the leaves of *Annona squamosa* which was collected in Abdulmumin Street, Damboa road, Maiduguri, Borno state, Nigeria. The plant was identified and authenticated by a plant taxonomist, Prof. S.S Sanusi of Biological science department, Faculty of science, University of Maiduguri.

The leaves were air-dried in a well ventilated room until constant weight was obtained. It was then pulverised using a wooden pestle and mortar to a coarse powder ready for extraction.

One kilogram of the powdered leaves was soaked in 1L of petroleum ether for 48 hours, after which it was filtered and the marc (devoid of fatty materials) was used for extraction.

### Phytochemical Screening

The extract was subjected to phytochemical screening to test for the presences of the following secondary metabolites: Alkaloid, Carbohydrate, Flavanoid, Saponin, Tannin, Glycosides, Fatty acids, Resins using standard methods described by Trease and Evans (2002).

### Experimental Animal and Acclimatization

Adult albino rats of both sexes were used for the experiments. The rats were purchased from the animal house section of the Faculty of Pharmacy, University of Maiduguri, Borno State Nigeria. The animals were acclimatized for two weeks, water and food were made available to the experimental animals.

### Lethal Dose ($LD_{50}$ Determination)

The $LD_{50}$ of the ethanol extract of *Annona squamosa* was carried out using modified Lork's method (1983). The route of administration used was oral. This method comprises of two phases as follows:

### Phase I

Three groups of two rats each were administered orally 10 mg/kg, 100 mg/kg, 1000 mg/kg body weight of *Annona squamosa* ethanol leaf extract respectively. They were kept for 24 hrs to determine the sign of acute toxicity and mortality.

### Phase II

Three groups of two rats each were administered 1600 mg/kg, 2900 mg/kg, and 5000 mg/kg body weight of *Annona squamosa* ethanol leaf extract was administration orally respectively. They were kept for 24 hrs to determine the sign of acute toxicity and mortality.

### Alloxan Induced Diabetes

After an overnight fasting, diabetes was induced by intraperitoneal injection of 150mg/kg of alloxan monohydrate (S.D. Fine- Chem. Ltd., Mumbai, India) dissolved in distilled water. After 48 hours of alloxan injection, the glucose levels of the rats were tested. Rats with glucose levels greater than 200 mg/dl were considered diabetic and used for the study as described by Perfumi *et al*., (1996).

### Statistical Analysis

The data generated during the study were expressed as means ± standard error of mean(SEM) and analyzed using student t-test (Graphpad prism 6 Demo) and $p<0.05$ was considered as significant.

## Results

### Phytochemical screening ethanol leaf extract of *Annona squamosa* L.

The phytochemical screening indicates the presence of carbohydrate, cardiac glycosides, tannin, alkaloid, saponin and the absence of anthraquinone and flavonoid (Table 1).

### Acute toxicity study of ethanol leaf of *Annona squamosa* L.

No death was recorded up to the dose of 5000 mg/kg (Table 2), while the $LD_{50}$ was found to be ≥5000mg/kg.

### Alloxan-Induced Experimental Diabetes Mellitus

To determine the optimum time for induction of diabetes mellitus, a time dependent increase of blood glucose was observed following the administration of alloxan monohydrate (150 mg/kg, IP). The average blood glucose was 106.00±2.70. The glucose level was 429.40±20.30 mg/dl after 72 hours post alloxan administration. The blood glucose level increased to 446.60±19.85 mg/dl after 2 hours, then 455.60±17.37 mg/dl, 528.80±36.78 mg/dl, 544.00±37.76 mg/dl after 4, 8, 24 hours post alloxan, respectively. These times were selected to cover the short, intermediate, and long-term actions of anti-diabetic drugs (Fig 1).

### Post Treatment with Insulin

Since the alloxan-induced diabetes is a model of type 1 diabetes mellitus, we used insulin as a standard antidiabetic drug. To compare the efficacy of ethanol extract of *Annona squamosa* at different doses (200, 400, 800, 1600 mg/kg, PO) with insulin (0.64 IU/kg), their time-dependent effects on blood glucose were assessed. The pick effect of insulin was at 4 hours. (Fig 2).

**Effects of 200 mg/kg, PO of the extract**

The effect of ethanol leaf extract of *Annona squamosa* (200 mg/kg, PO) on alloxan (150 mg/kg) induced diabetic rats (n=5). The average basal glucose level was 544.20±28.20 mg/dL. Following the administration of *Annona squamosa* leaf extract (200 mg/kg, PO) there was a decrease in glucose level to 80.80±12.32 mg/dL at two hours after administration of the extract. Similarly there was a significant decrease in glucose levels to 62.40±10.87 mg/dl at 4 hours after administration of extract and subsequent increase to 205.00±71.34 mg/dl after 8 hours of extract administration and ultimately to 405.80±30.53 mg/dl after 24 hours of extracts administration (Figure 3).

**Effects of 400 mg/kg, PO of the extract**

The effect of ethanol leaf extract of *Annona squamosa* (400 mg/kg, PO) on alloxan (150 mg/kg) induced diabetes rats (n=5). The average basal glucose level was 102.20±2.03 mg/dl. On administration of Alloxan (150 mg/kg, IP) the glucose level increased to 490.00±70.52 mg/dl (4.7-fold increase, $p<0.0003$) at 72 hours. Following the administration of *Annona squamosa* leaf extract (400 mg/kg, PO), there was a significant decrease in glucose level to 237.80±47.92 mg/dl at 2 hours after administration of the extract. Similarly there was a significant decrease in glucose levels to 199.60±18.32 mg/dl at 4 hours after administration of extract, an increase to 234.00±77.38 mg/dl after 8 hours of extract administration and finally increase to 369.40±77.31 mg/dl after 24 hours of extract administration (Figure 4).

**Effects of 800 mg/kg, PO of the extract**

The effect of ethanol leaf extract of *Annona squamosa* (800 mg/kg, PO) on alloxan (150 mg/kg) induced diabetic rats (n=5). The average basal glucose level was 101.00±4.79 mg/dl. On administration of Alloxan (150 mg/kg, IP) the glucose level increased to 479.00±66.50 mg/dl (4.6-fold increase, $p<0.0003$) at 72 hours. Following the administration of *Annona squamosa* leaf extract (800 mg/kg, PO), there was a significant decrease in glucose level to 198.60±31.67 mg/dl at 2 hours after administration of the extract. Similarly there was a significant decrease in glucose levels to 144.40±18.32 mg/dl at 4 hours after administration of extract, an increase occured to 179.20±10.13 mg/dl after 8 hours of extract administration and finally increase to 262.00±45.54 mg/dl after 24 hours of extract administration was observed (Figure 5).

**Effects of 1600 mg/kg, PO of the extract**

The average basal glucose level was 539.60±48.46 mg/dl after induction with alloxan (150 mg/kg) for 72 hours. Following the administration of *Annona squamosa* leaf extract (1600 mg/kg, PO), there was also a significant decreased in glucose level to 129.60±27.45 mg/dl at 2 hours after administration of the extract. There was a significant decreased in glucose levels to 63.60±6.12 mg/dl at 4 hours after administration of extract. Similarly a significant increase to 141.60±39.71 mg/dl was observed at 8 hours after administration of extract which significantly increased to 200.00±35.17 mg/dl after 24 hours of extract administration (Fig.6).

**Effects of ethanol leaf extract of *Annona squamosa* in alloxan induced diabetic rats**

**Summary of the results**

Alloxan induced hyperglycaemia after 72hrs of administration in all the study rats. The activity of the ethanol leaf extract of *Annona squamosa* in alloxan induced diabetic rats after 2-8 hours of administration significantly reduced the blood glucose level when compared with the diabetic control ($p<0.05$). It was observed that the activity of insulin after 2 hrs of administration in diabetic rats did not vary significantly with the varying doses of the extract ($p> 0.05$). However, the activity of the extract was not dose dependent at the tested doses of 200, 400, 800 and 1600 mg/kg after 2-8 hours of treatment. The activity of the insulin used as positive control after 4 hours of administration was able to reduce hyperglycaemia significantly than the activity of extract at 400 and 800 mg/kg ($p<0.05$). Interestingly the activity of the extract at the tested doses after 8 hours of administration was found to significantly decrease the hyperglycaemia in the rats when compared with insulin ($p<0.05$). After 24 hours of extract administration the activity was found to be higher ($p<0.05$) and better in the reduction of the blood glucose level when compared with insulin (Fig. 7)

**Table 1 Phytochemical screening of ethanol leaf extract of *Annona squamosa***

| *Phytochemical constituents* | *Results* |
|---|---|
| Carbohydrates | + |
| Cardiac glycosides | + |
| Saponin glycosides | + |
| Anthraquinone glycosides | - |
| Flavonoids | - |
| Tannins | + |
| Alkaloids | + |

+ = present, - = absent

**Table 2: Acute toxicity study of ethanol leaf extract of *Annona squamosa* L**

| *Phase* | *Dose(mg/kg)* | *Observation* |
|---|---|---|
| Phase1 | 10 | - |
| | 100 | - |
| | 1000 | - |
| Phase 2 | 1600 | - |
| | 2900 | - |
| | 5000 | - |
| **$LD_{50}$ = ≥5000 mg/kg** | | |

- = no death

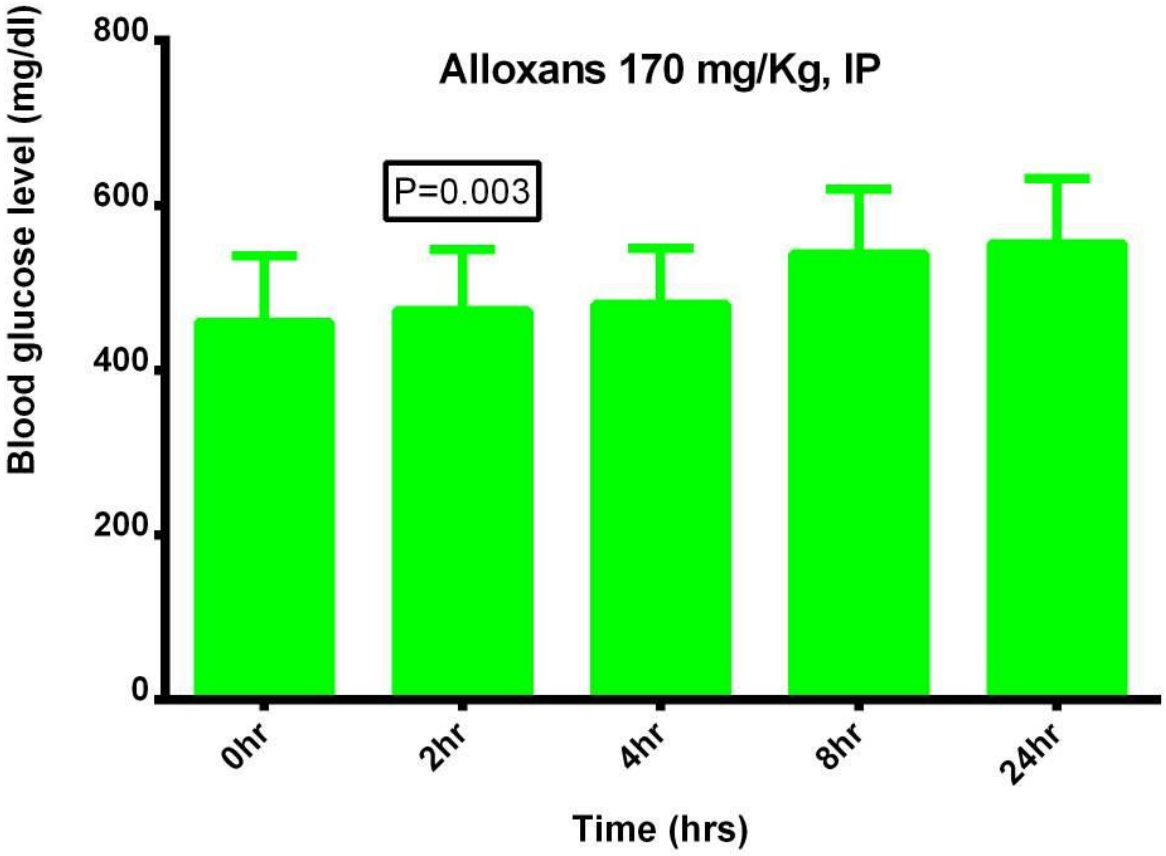


Figure 1: Time-dependent induction of diabetes mellitus with alloxan monohydrate (170 mg/kg, IP) in rats (n=5)

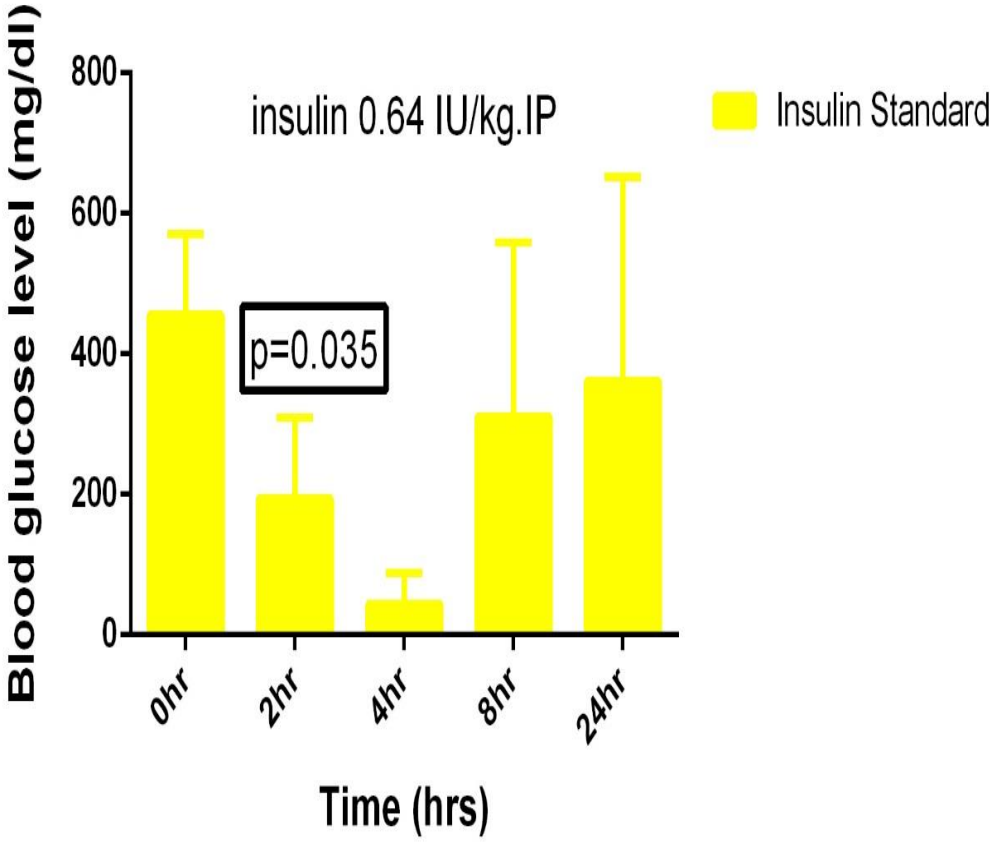


Figure 2: Effect of insulin (0.64 iu/kg) on alloxan induced diabetic rats (n=5)

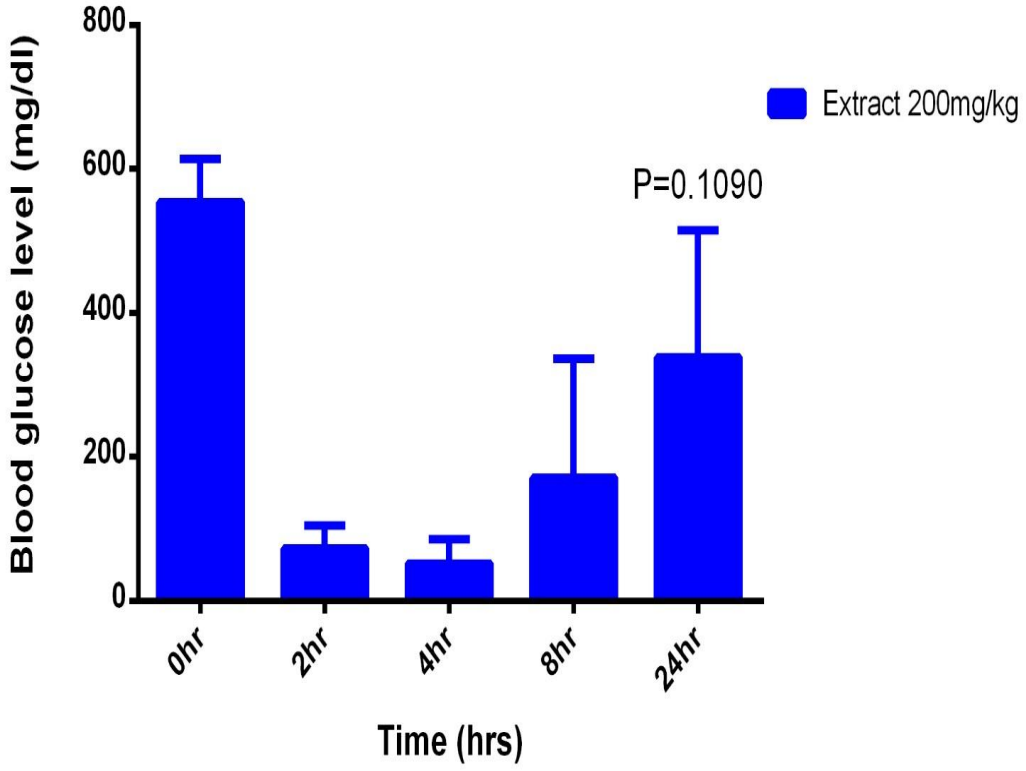


Figure 3: Hypoglycaemic effects of 200mg/kg, PO of the extract

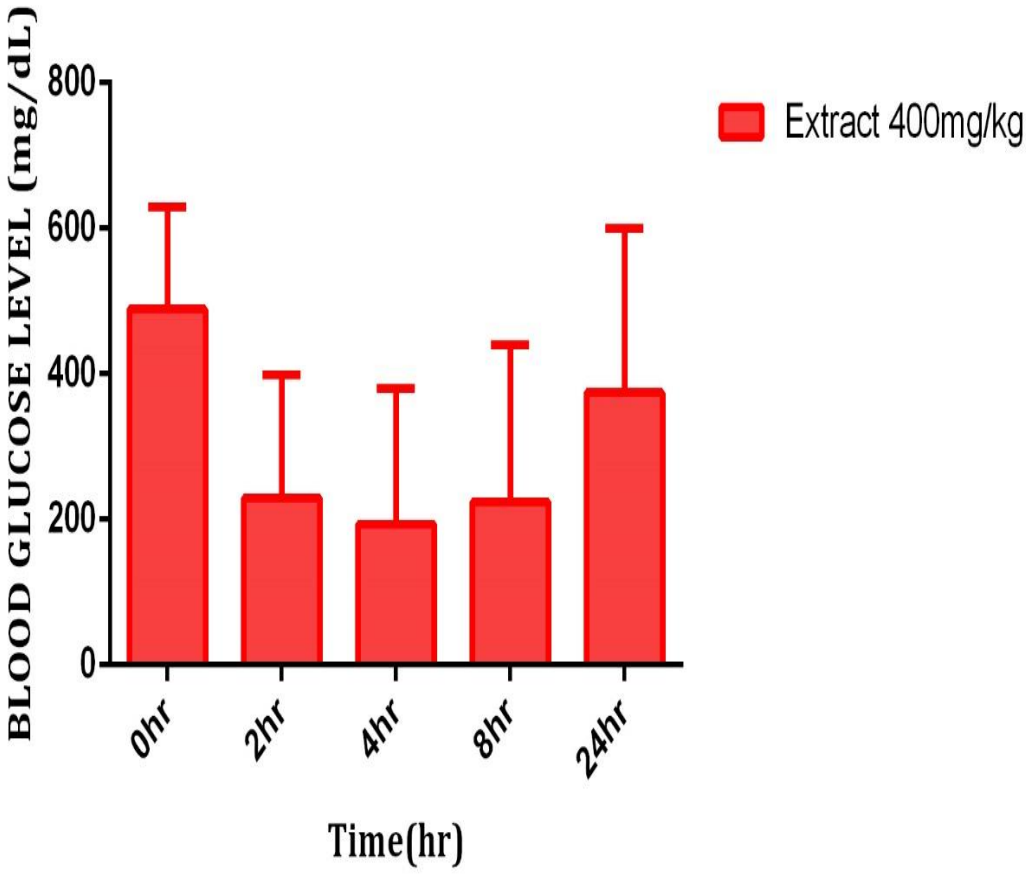


Figure 4: Hypoglycaemic effects of 400 mg/kg, PO of the extract

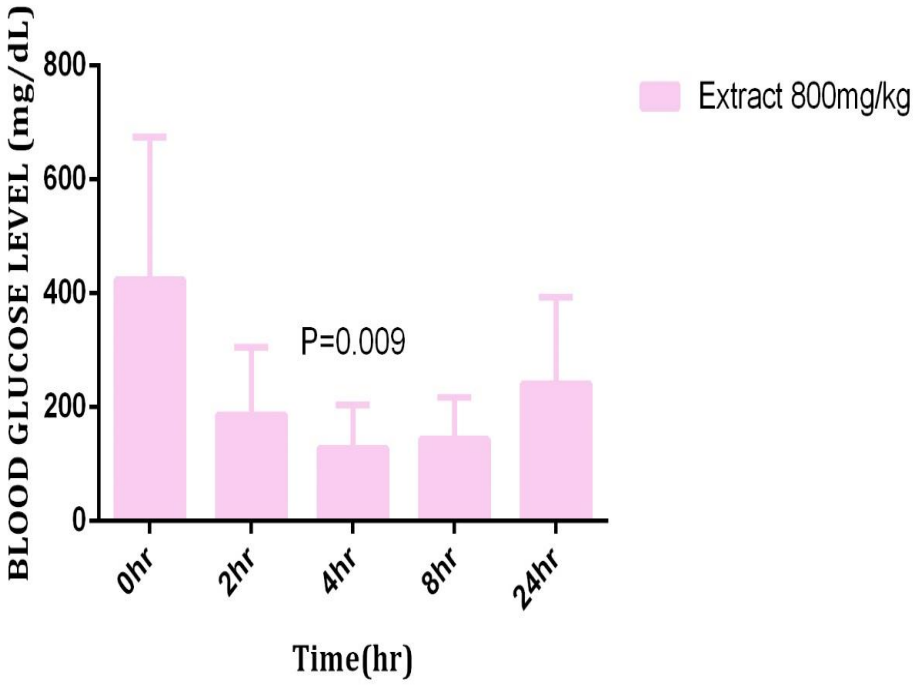


Figure 5: Hypoglycaemic effects of 800 mg/kg, PO of the extract

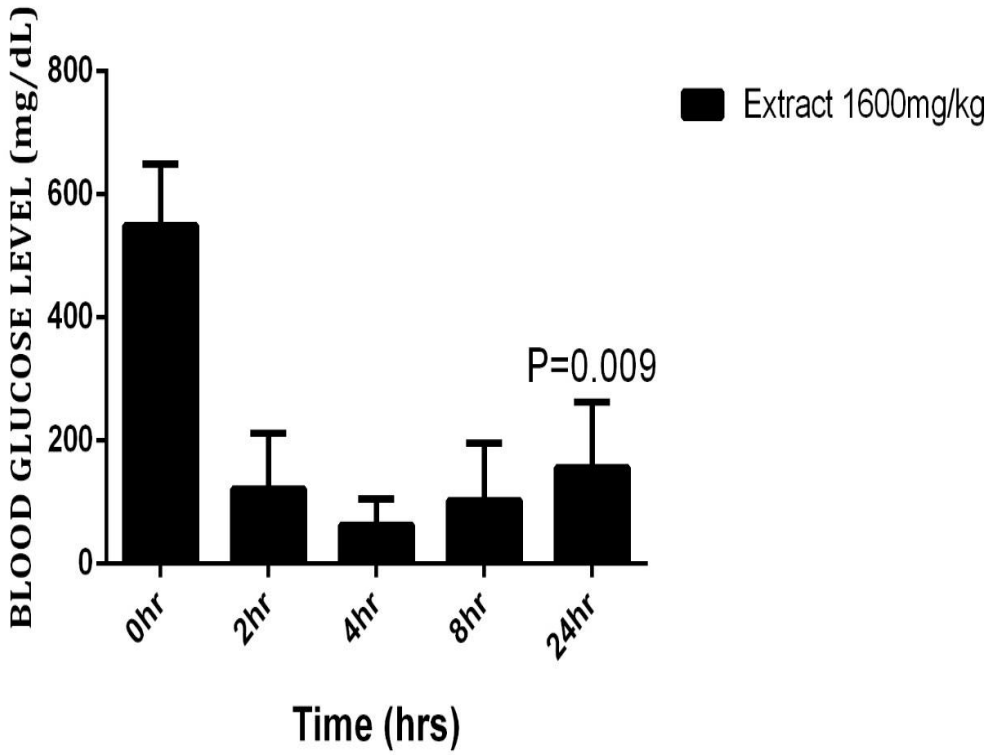


Figure 6: Hypoglycaemic effects of 1600 mg/kg, PO of the extract

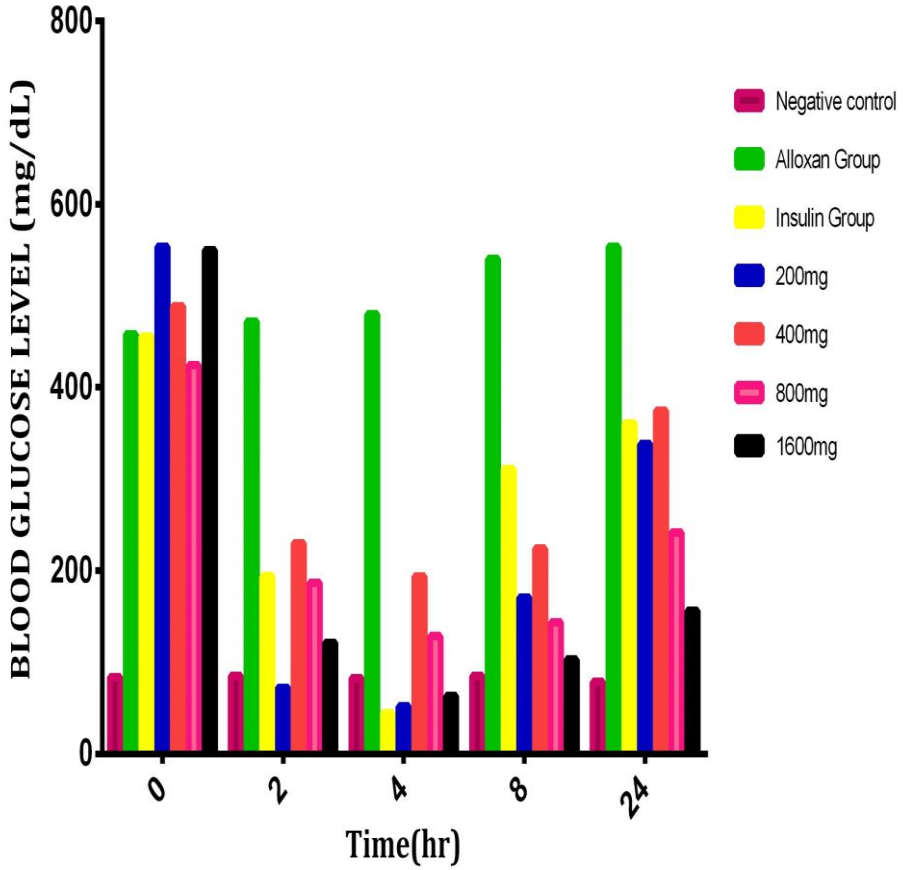


Figure 7: Summary of the experiments

## Discussion

The result obtained from the phytochemical Screening showed that the ethanol leaf extract of *Annona squamosa* L contains carbohydrates, cardiac glycosides, saponins, tannins, alkaloids. The study agrees with that of Kavitha *et al.*, (2013), however they detected the presence flavanoids and anthraquinones and this difference could be attributed to the variation in environmental factors such as the soil type, rainfall and especially the time of collection of the plant. Other factors that could affect the result are the geographical location and climate differences. The presence of the phytochemicals may be responsible for the observed anti-diabetic activity of the plant.

Alloxan monohydrate (a diabetogenic agent widely used to induce Type II diabetes in animals) is selectively toxic to the beta-cells of the pancreas, causing cell necrosis (Sharma, 2007). The cytotoxic action of alloxan monohydrate is mediated by reactive oxygen species, with a simultaneous massive increase in cytosolic calcium concentration, leading to rapid destruction of beta cells which further causes reduction in insulin secretion thereby increasing blood glucose level. This is accompanied by polyuria, reduction in body weight and polyphagia, which could be due to poor glycaemic control and excessive catabolism of protein to provide amino acid for gluconeogenesis during insulin deficiency (Al-khalifa, 2009).

Experimental evidence obtained in this study shows that *Annona squamosa* L. ethanol leaf extract significantly decreases hyperglyceamia induced by alloxan in rats.

It has been observed that the extract has significant hypoglycaemic activity ($(p<0.05)$ as from 200 mg/kg up to 1600 mg/kg when compared to the control.

In the present study, administration of *Annona squamosa* ethanol leaf extract (200 mg/kg-1600 mg/kg) caused significant ($p<0.05$) reduction in the blood glucose level compared to the positive control. Although the hypoglycaemic mechanism of medicinal plants had not been clearly defined, the increase in oxygen free radicals in diabetes could be primarily due to hyperglycaemia resulting from the effects of the diabetogenic agent, alloxan (Ugochukwu, 2003). Plant chemical constituents (carbohydrate, phenol, flavanoid, alkaloid, saponin, glycoside ) have been reported to alleviate diabetic stress by increasing pancreatic secretion of insulin from islet of Langerhans (Une-Hemant, 2014), therefore, they may contribute to its hypoglycaemic activity.

The result of the study showed that the ethanol leaf extract of *Annona squamosa* L. contains bioactive phytochemical constituents that possess anti diabetic activity which may be effective in management of diabetes mellitus. This justifies the use of *Annona squamosa* L leaf decoction by the native people for the management of diabetes.

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## Conflict of Interest

The authors have no conflict of interest over this research

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