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Evaluation of the effect of ethanol extract of *Phaseolus vulgaris* seeds on haematological parameters of alloxan-induced Wistar diabetic rats

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Abstract

The study was designed to evaluate the effect of ethanol extract of *Phaseolus vulgaris* seeds on haematological parameters in alloxan -induced diabetic rats. Packed cell volume (PCV), total cholesterol, haemoglobin levels were evaluated. The phytochemical tests were carried out on the extract using standard phytochemical procedures. The condition of hyperglycaemia was attained by a single dose of alloxan (200 mg/kg$_{bw}$). The rats were divided into six groups containing five rats each. The groups were administered 150 mgkg, 250 mgkg and 350 mgkg doses of ethanolic extract of *Phaseolus vulgaris seed* per kg body weight respectively. Administration in all instances was by gavage using intubation cannular. These treatments were repeated for five consecutive days. To a positive control group of five rats was administered 5 mg of glibenclamide (a standard anti-diabetic drug) per kg body weight for five consecutive days. Another group of five rats used as negative control did not receive any treatment. The non-diabetic group received neither alloxan nor the extract. The 24-hour acute toxicity test of the orally administered ethanolic extract of *Phaseolus vulgaris* seeds showed that the extract is non-toxic because no death was recorded. All the doses were significantly ($P<0.05$) effective in reducing blood sugar level of alloxan induced hyperglycaemic rats when compared to the control diabetic rats that were treated with glibenclamide (a standard antidiabetic drug). The phytoconstituents identified in the extracts are saponin, condensed tannins, flavonoids, alkaloids, cardiac glycosides and phenolic group. The PCV levels showed a significant increase ($P<0.05$) when compared to the negative control which received no treatment. Similarly, for total cholesterol levels, there was no significant increase compared to the control groups. The haemoglobin levels increased slightly with the administration of the extract when compared to the control group. These results suggest that the seed extract of *Phaseolus vulgaris* has no negative effect on the haematological parameters evaluated. The seeds could be of benefit in the treatment and management of diabetes mellitus.

Keywords: Haematological; Diabetes mellitus; Alloxan; Glibenclamide

1. Introduction

The use of medicinal plants to prevent and treat diabetes mellitus successfully over the years has attracted the attention of scientists globally. In the rural communities, many people depend solely on medicinal plants for the treatment of diabetes due to its easy accessibility, affordability and availability even when the efficacy of the herbal remedy has not been established. Many traditional plant treatments for diabetes mellitus are used throughout the world and some of these plants have been assayed while a good number of them are yet to receive scientific scrutiny (Agunu, *et al*., 2005). *Phaseolus vulgaris* is one of the most versatile medicinal plants having a wide spectrum of biological activity. In the rural area, the traditional healers make use of the seeds of *phaseolus vulgaris* to treat diabetes. Scientific reports also support the hypoglycemic activity of *Phaseolus vulgaris indica* seeds(Chattopadhyay,1998).

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Diabetes mellitus occurs when insulin is not secreted in sufficient amounts or does not efficiently stimulate its target cells. As a consequence, blood glucose levels becomes so elevated that the glucose “spills over” into the urine, providing a convenient, diagnostic test for the disease . Yet, despite these high blood glucose levels, cells “starve” since insulin-stimulated glucose entry into cells is impaired.

Diabetes mellitus is a metabolic disease resulting from insulin deficiency; characterized by a failure in glucose transport from the blood into cells at normal glucose concentrations. Diabetes mellitus can be as a result of insufficient response of the body to circulating insulin (Lawrence, *et al*., 2008).

The normal fasting blood sugar levels according to WHO range between 70 and 110 or 60 and 100 mg/dl of blood, but this value may increase to 120 or 140 mg/dl after a carbohydrate meal (post prandial blood sugar level). These normal values are maintained by the action of insulin (a peptide hormone released by the β-cells of the pancreatic islet of langerham).

Kidney bean (*Phaseolus vulgaris*) which is high in dietary fiber, is cooked before to make it edible for human consumtion. Dried beans from this species, harvested when the pods have fully matured and dried, may be sold dry or already partially cooked and canned, are high in protein, vitamins B and C, and various minerals (including iron, magnesium, phosphorus, and potassium). Dried beans are soaked and cooked for several hours, and cooked into numerous soups, stews, and meat dishes, including French *cassoulet*, Mexican *frijoles*, North American *chili* (or *chili con carne*), and the Brazilian black bean *feijoada*. Dried beans, including this species, are an important source of protein in many parts of Africa, southeast Asia, and South America(Lawrence, *et al*., 2008).

1.1. Packed cell volume (haematocrit)

The haematocrit (Ht or HCT, hematocrit), also known as packed cell volume (PCV) or erythrocyte volume fraction (EVF), is the volume percentage (%) of red blood cells in blood. It is normally about 45% for men and 40% for women. It is considered an integral part of a person's complete blood count results, along with hemoglobin concentration, white blood cell count, and platelet count. The packed cell volume (PCV) can be determined by centrifuging heparinized blood in a capillary tube (also known as a microhematocrit tube) at 10,000 RPM for five minutes. This separates the blood into layers. The volume of packed red blood cells divided by the total volume of the blood sample gives the PCV. Because a tube is used, this can be calculated by measuring the lengths of the layers (Briggs and Brooker, 1953).

With modern lab equipment, the hematocrit is calculated by an automated analyzer and not directly measured. It is determined by multiplying the red cell count by the mean cell volume. The hematocrit is slightly more accurate as the PCV includes small amounts of blood plasma trapped between the red cells. An estimated hematocrit as a percentage may be derived by tripling the hemoglobin concentration in g/dL and dropping the units. In cases of dengue fever, a high hematocrit is a danger sign of an increased risk of dengue shock syndrome.

Polycythemia vera (PV), a myeloproliferative disorder in which the bone marrow produces excessive numbers of red cells, is associated with elevated hematocrit. Chronic obstructive pulmonary disease (COPD) and other pulmonary conditions associated with hypoxia may elicit an increased production of red blood cells. This increase is mediated by the increased levels of erythropoietin by the kidneys in response to hypoxia (Briggs and Brooker, 1953). Professional athletes' hematocrit levels are measured as part of tests for blood doping or erythropoietin (EPO) use; the level of hematocrit in a blood sample is compared with the long-term level for that athlete (to allow for individual variations in hematocrit level), and against an absolute permitted maximum (which is based on maximum expected levels within the population, and the hematocrit level that causes increased risk of blood clots resulting in strokes or heart attacks). (Biswas, *et al*., 2002)

1.2. Total cholesterol levels (TC)

According to the lipid hypothesis, abnormal cholesterol levels (hypercholesterolemia) — actually higher concentrations of LDL particles and lower concentrations of functional HDL particles — are strongly associated with cardiovascular disease because these promote atheroma development in arteries (atherosclerosis). This disease process leads to myocardial infarction (heart attack), stroke, and peripheral vascular disease. Since higher blood LDL, especially higher LDL particle concentrations and smaller LDL particle size, contribute to this process more than the cholesterol content of the HDL particles, LDL particles are often termed "bad cholesterol" because they have been linked to atheroma formation. On the other hand, high concentrations of functional HDL, which can remove cholesterol from cells and atheroma, offer protection and are sometimes referred to as "good cholesterol". These balances are mostly genetically determined, but can be changed by body build, medications, food choices, and other factors. Resistin, a protein secreted by fat tissue, has been shown to increase the production of LDL in human liver cells and also degrades LDL receptors in

the liver. As a result, the liver is less able to clear cholesterol from the bloodstream. Resistin accelerates the accumulation of LDL in arteries, increasing the risk of heart disease. Resistin also adversely impacts the effects of statins, the main cholesterol-reducing drug used in the treatment and prevention of cardiovascular disease.

Conditions with elevated concentrations of oxidized LDL particles, especially "small dense LDL" (sdLDL) particles, are associated with atheroma formation in the walls of arteries, a condition known as atherosclerosis, which is the principal cause of coronary heart disease and other forms of cardiovascular disease (Grover, *et al*., 1998). In contrast, HDL particles (especially large HDL) have been identified as a mechanism by which cholesterol and inflammatory mediators can be removed from atheroma. Increased concentrations of HDL correlate with lower rates of atheroma progressions and even regression. A 2007 study pooling data on almost 900,000 subjects in 61 cohorts demonstrated that blood total cholesterol levels have an exponential effect on cardiovascular and total mortality, with the association more pronounced in younger subjects. Still, because cardiovascular disease is relatively rare in the younger population, the impact of high cholesterol on health is still larger in older people.

1.3. Hemoglobin (Hb)

Haemoglobin (Hb or Hgb) is the protein in your red blood cells that carries oxygen. A low hemoglobin count is a below-average concentration of the oxygen-carrying hemoglobin proteins in your blood. A low hemoglobin count is generally defined as less than 13.5 grams of hemoglobin per deciliter (135 grams per liter) of blood for men and less than 12 grams per deciliter (120 grams per liter) for women (Abbas, *et al*., 1992) In children, the definition varies with age and sex. The threshold differs slightly between medical practices. A low hemoglobin count is a commonly seen blood test result. In many cases, a low hemoglobin count is only slightly lower than normal, isn't considered significant and causes no symptoms. A low hemoglobin count can also be caused by an abnormality or disease. In these situations, a low hemoglobin count is referred to as anemia (Grover, *et al*., 1998).

2. Methodology

2.1. Preparation of the extract

The seeds were dried at room temperature for two weeks. The dried seeds were ground into powder using corona manual grinding machine. 1910 g of the ground seeds powder of *phaseolus vulgaris* were soaked in four litres of 80 % ethanol for 24 hrs. It was sieved and filtered using whatman no 1(125mm) filter paper. The filtrate was concentrated to dryness using rotary evaporator, the weight after concentration of the filterate was 71 g.

2.2. Phytochemical screening

Phytochemical tests were carried out on the ethanolic extract using standard phytochemical tests as described by Harbone (1973), Sofowora (1993), Trease and Evans (1996). The following phytochemicals were assayed for:

- Anthracine Glycosides
- Saponins
- Tannins
- Flavonoids
- Cyanogenic Glycosides
- Alkaloids
- Cardiac Glycosides
- Phenolic Group

2.3. Tests for anthracine glycosides

Principle: Anthracine glycosides under acid condition react with ferric chloride and carbon tetrachloride when heated in the presence of ammonia to give a pink colour.

Procedure: To a test tube containing 5ml of dilute H_2SO_4, 5ml of 5% ferric chloride was added. Then 1.0ml of the extract was added into the same test tube. The solution was properly mixed by shaking. It was boiled over water bath for five minutes and separated. The lower layer was mixed with equal volume of carbon tetrachloride. Then 5.0ml of dilute ammonia was added to the lower organic layer and shaked gently.

2.3.1. Test for tannins

2.3.2. Bromine water test

- **Principle:** Condensed tannins react with bromine water at room temperature to give greenish to red colour.
- **Procedure**: Exactly 1.0ml of bromine water and 1.0ml of extract were pipette into a test tube and observed for colour change.

2.3.3. Acid test

- **Principle:** Phlobotannin under acidic conditions react with dilute hCl to give a red colour or precipitate.
- **Procedure:** Exactly 3.0ml of extract was added to 2.0ml of HCl in a test tube. The mixture was observed for colour change or formation of precipitates.

2.3.4. Lead acetate test

- **Principle:** Addition of lead acetate solution to tannins precipitates the acidic substance as their salts and removes them from the aqueous solution. Phlobotannins react with lead acetate at room temperature togive a dark blue to black precipitate.
- **Procedure:** Exactly 3 drops of 5% lead acetate solution were added into a test tube containing 2.0ml of extract. It was then observed for presence of precipitate.

2.3.5. Test for flavonoids

Ferric chloride test for phenolic nucleus

- **Principle:** Phenolic nucleus reacts with ferric chloride at room temperature to give greenish brown or black colour or precipitate.
- **Procedure:** To a test tube containing 1.0ml of extract, 1.0ml of 10% ferric chloride was pipette. It was mixed properly and observed carefully for colour change to greenish brown or black.

Sodium hydroxide test

- **Principle:** At room temperature and under alkaline pH, flavonoids react with NaOH to form observable precipitates.
- **Procedure:** Exactly 1.0ml of dilute NaOH solution was pipette into a test tube containing 1.0ml of extract. It was mixed properly and observed for colour change or presence of precipitates.

Lead acetate test

- **Principle:** At room temperature, flavonoids react with lead acetate to give a yellow colour or precipitate.
- **Procedure:** To a test tube containing 1.0ml of extract, 1.0ml of 10% lead acetate solution was pipette. The solution was mixed properly and observed for colour change or formation of precipitates.

2.3.6. Test for alkaloids

Mayer's reagent test

- **Principle:** Basic alkaloids react with complexes such as Wagner's reagent (A solution of iodine, Potassium iodide and distilled water) and Meyer's reagent (A solution of mercuric Chloride, Potassium iodide and distilled water) to form insoluble salts i.e precipitates.
- Alkaloids react with Mayer's reagent under acid condition to give a cream colour or precipitate.
- **Procedure:** Exactly 5.0ml of 2% HCl was pipette into a test tube containing 1.0ml of extract. The solution was heated in a water bath for 10 minutes and then filtered. Then 1.0ml of the filtrate was pipette into a test tube. Exactly 1.0ml of Mayer's reagent was pipette into the same test tube. The solution was mixed properly and carefully observed for cream colour change or precipitates.

2.3.7. Test for cardiac glycosides

Huppert salkowski test

- **Principle:** On dehydration using H_2SO_4, Cholesterol yields a red compound called cholesterylene.

- **Procedure:** Exactly 1.0ml of extract was pipette into a test tube containing 2.0ml of chloroform. The solution was mixed properly. Then, 1.0ml of concentrated H_2SO_4 was introduced carefully. The solution separated into two years and was carefully observed for colour change.

2.3.8. Test for saponins

Emulsion test

- **Principle:** When saponin solution is mixed with oil and heated, it emulsifies after rigorous shaking. Saponins emulsify olive oil .
- **Procedure:** Exactly 5.0ml of distilled water was pipette into a conical flask containing 1.0ml of extract and boiled. The soluble fraction of the mixture was decanted into a test tube while hot. Then 2 drops of olive oil were added into this test tube. The mixture was shaked and observed for presence of emulsion.

Frothing test

- **Principle:** Saponins when heated and shaken in water maintains a stable froth.
- **Procedure:** To a conical flask containing 1.0ml of extract, 5.0ml of distilled water was added and then boiled. The soluble fraction of the mixture was decanted into a test tube while hot; to 1.0ml of this, 3.0ml of distilled water was added. The solution was shaked vigorously and observed for froth.

Test for cyanogenic glycosides

- **Principle:** Cyanogenic glycosides react with sodium picrate, changing its colour from yellow to brick red.
- **Procedure:** Using a thread, a piece of picrate paper was suspended in a conical flask containing 5.0ml of extract. The flask was stoppered, heated in a water bath and observed for colour change.

2.4. Experimental animals

Exactly forty (40) wistar albino rats and 13 mice were purchased from the animal house at the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu, Anambra State. They were maintained and housed in cages in the Department of Applied Biochemistry Laboratory, Nnamdi Azikiwe university, Awka. They were allowed to acclimatize with the environment for one week before use. The animals were kept on guinea growers mash pellets that was obtained from Eke market, Awka. The animals were weighed and their glucose levels were measured accordingly using One Touch basic Glucometer and test strips (Code-12).Blood was collected using orbito-recto method (that is from the tail). Diabetes mellitus was induced intraperitoneally with alloxan (200 mg/kg).

2.5. Determination of median lethal dose (LD50) of Phaseolus vulgaris

The Median Lethal Dose (LD_{50}) was determined using Wistar Albino Mice. Test animals were randomly divided into three (3) groups of three mice each and administered graded doses of 10 mg,100 mg,1000 mg of ethanolic extract of *Phaseolus vulgaris* per kg body weight respectively. When there was no mortality recorded, another three groups of one mice each were administered with an increased dose of 1600 mg, 2900 mg and 5000 mg. The *Phaseolus vulgaris* extract was administered by gavage using an intubation canular and were monitored for 24 hours for changes in behaviour and mortality, however no mortality was recorded.

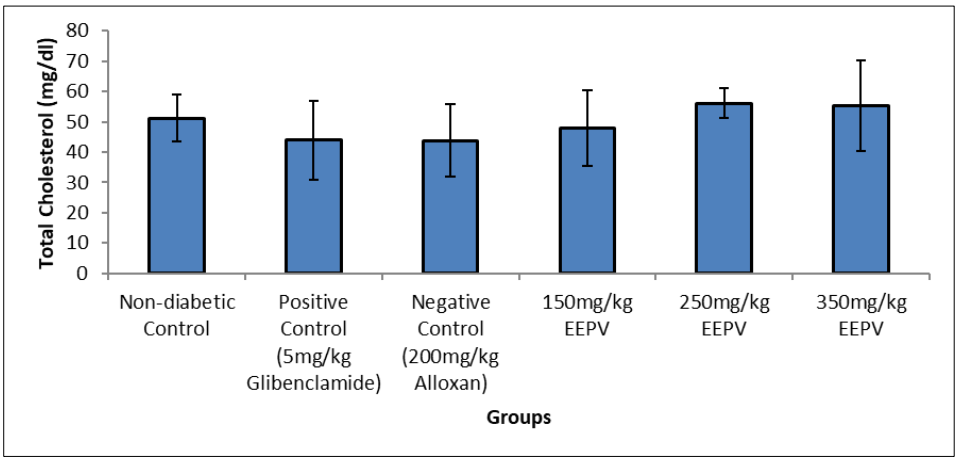
3. Results

Table 1 Phytochemical test for the ethanolic extract *Phaseoulus vugaris* seeds

Phytochemical constituents	Test	Ethanolic extract
Anthracine Glycosides		-
Saponin	Frothing test	+++
	Emulsion test	+++
Tannins	Bromine Water test	-
	Acid test	+
	Lead Acetate test	++

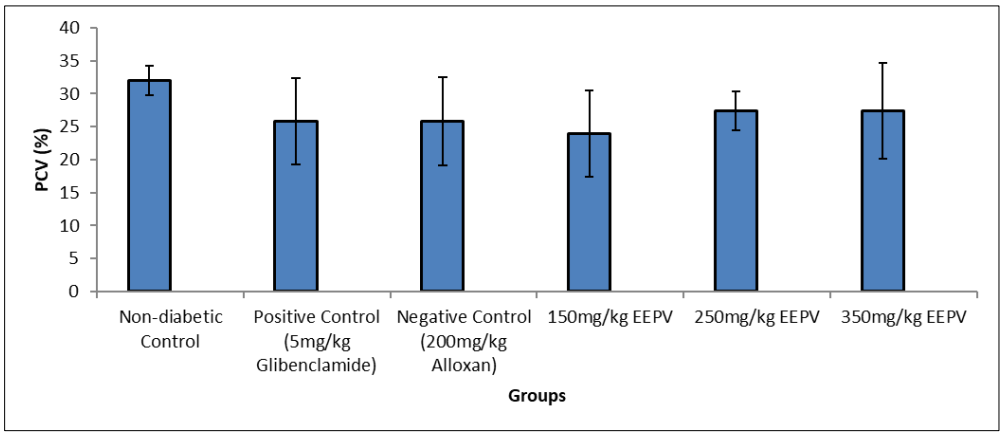
Flavonoids	Ferric Chloride test for Phenolic nucleus	++
	Lead Acetate test	+++
	Sodium Hydroxide test	+++
Cyanogenic Glycosides		
Alkaloids	Wagner's reagent test	++
	Meyer's reagent test	++
Cardiac Glycosides	Huppert Salkowski test	
Phenolic Group (Tyrosine Millions method)		-

+ Present ++ Moderately ==========+++ present; +++ Abundantly present - Absent



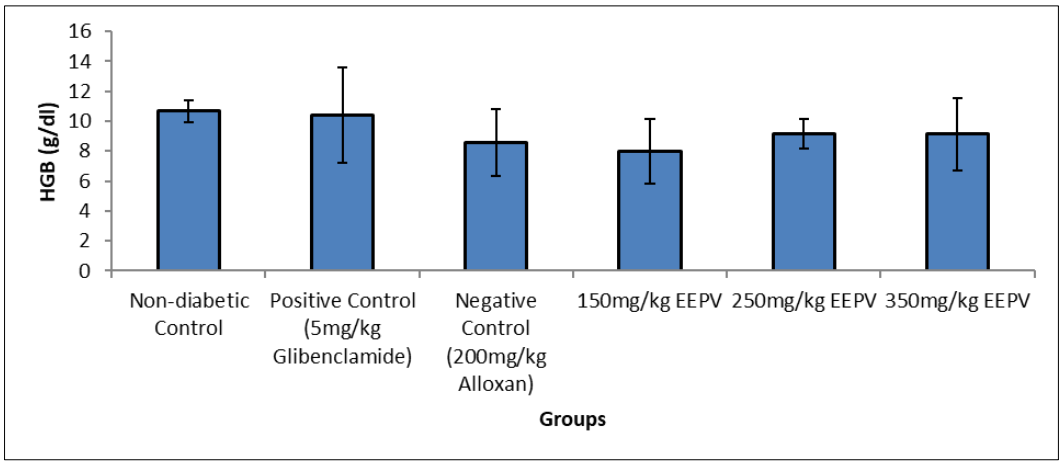
(Data represented as Mean ± SEM; * = significantly different compared to the Negative Control (200mg/kg Alloxan) group)

Figure 1 Total cholesterol levels of the test groups



Data represented as Mean ± SEM; * = significantly different compared to the Negative Control (200mg/kg Alloxan) group)

Figure 2 Packed cell volume levels of the test groups



(Data represented as Mean ± SEM; * = significantly different compared to the Negative Control (200mg/kg Alloxan)

Figure 3 Haemoglobin levels of the test groups

4. Discussion

Diabetes mellitus (DM) is the commonest endocrine disorder that affects more than 100 million people worldwide (6% of the population) and in the next 10years it may affect about five times more people than it does now (Abdullahi *et al*., 2001). WHO Expert Committee on diabetes encourages further investigation into traditional methods of treatment and also emphasizes the need to ensure safety and quality control of ingredients used. The presence of abnormal high blood glucose level is the criterion on which the diagnosis of diabetes is based. The therapeutic goal of treatment within each type of diabetes is to normalize insulin activity and blood glucose levels in an attempt to reduce the development of the vascular and neuropathic complications. The acute toxicity study of the orally administered ethanolic extract of *Phaseolus vulgaris seed* showed that the extracts are not toxic, the extent of hypoglycemic effect varied considerably among different groups of rats given different doses of ethanol extract of *Phaseolus vulgaris* seed.

Phytochemical screening of ethanolic seed extract of *Phaseolus vulgaris* revealed the presence of saponins, condensed tannins, flavonoids, alkaloids, and phenolic group. Saponins are capable of neutralizing some enzymes in the intestine that can become harmful, building the immune system and promoting wound healing (Okigbo, *et al*., 2006). Tannins play important role as stable and potent antioxidants (Akinpelu and Onakoya, 2006) and thus show that antioxidants are important in prevention of diabetes mellitus. Tannins hasten the healing of wounds and inflamed mucous membrane (Okwu and Okwu, 2004). Flavonoids are potent water-soluble antioxidants and free radical scavengers which prevent oxidative cell damage and have strong anticancer activity (Salah, *et al*., 1995; Del-Rio, *et al*., 1997; Okwu, 2004). Flavonoids also lower the risk of heart diseases. Alkaloids have been documented to possess analgesic, antispasmodic and bacteriocidal effects (Salah, *et al*., 1995). Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Jorgensen, *et al*., 1999). The presence of these phytochemicals supports the medicinal uses of the seed extract of *phaseolus vulgaris* as an antidiabetic agent. Throughout the treatment days, ethanolic extract of *P. vulgaris* at 350mg/kg showed reductions in glucose levels but was not statistically significant ($p>0.05$) when compared to the negative control group.

From figure 1, 2 and 3, the treated groups showed no statistical significant reduction ($p<0.05$) in the mean level of total cholesterol (TC), packed cell volume (PCV) and haemoglobin when compared to their control groups, in other words, the ethanolic extract of phaseolus vulgaris does not have a negative effect on blood cholesterol level, packed cell volume (PCV) and maintains a good balance of haemoglobin levels.

5. Conclusion

The results of this study indicate that the ethanol extract of *Phaseolus vulgaris* seeds may be useful for the treatment and management of diabetes. Also the study has shown that the seed extract has no negative effect on the haematological parameters evaluated in alloxan-induced wistar diabetic rats.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

The Department of Biochemistry Ethical Review Committee, Ebonyi State University, Abakaliki, Nigeria, approved the study design and assigned approval Number.

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