The Effect of Combined Ethanol leaf extracts of *Annona muricata* and *Artocarpus heterophyllus* on Reproductive Parameters of Type 2 Diabetic Wistar albino rats.

ABSTRACT

The combined effect of ethanol leaf extract of *Annona muricata* and *Artocarpus heterophyllus* on the reproductive parameters of Type II diabetic wistar albino rats were studied. Fifty-six (56) male wistar albino rats were induced with Type II diabetes mellitus using high fat diet and 35 mg/kg body weight streptozotocin (HFD-STZ). The result obtained showed a decreased testosterone level 0.78±.06 ng/ml on the diabetic group while the non-diabetic group had testosterone level of 1.90±.09 ng/ml. Treatment with combined ethanol leaf extract of *A.muricata* and *A. heterophyllus* led to a significant increase in the testosterone level of the treated groups. The motility, viability and the sperm density were also normalised in the treated groups. The result obtained from the present research showed that combined ethanol leaf extract of *A. muricata* and *A. heterophyllus* has ameliorative effect of the reproductive parameters of Type II diabetic male wistar albino rats.

Keywords: Fertility, Diabetes mellitus, *Annona muricata*, *Artocarpus hetrophyllus*, phytochemical

INTRODUCTION

Recently a lot of attention has been focused on African traditional medicine. The administration of herbs in different health conditions is on the increase. Research has revealed that these plants have the potential to cure and prevent various kinds of diseases. Research has also shown that many of these plants can also improve fertility and its related issues due to their numerous phytochemical contents.

Diabetes mellitus (DM) is classified as a metabolic disease and it is characterized by an increase in the blood glucose level (hyperglycemia). It occurs progressively and it can come from different factors (multifactorial)[1]. Usually, when there is an increase in the serum glucose level beyond the normal range, the glucose leaks into the urine and can be detected in the urine of the

patient, a condition known as glycosuria.

As at 2010, DM became one of the World's most dreaded diseases and has affected about 6.4% of the world's population albeit being predicted to affect over 7.7% of the world's population by 2030. DM is not peculiar to any sex or colour, and has been classified as a leading cause of death[2].

Type II DM or non-insulin dependent DM occurs mainly in older individuals. Type 2 diabetes mellitus results from defect in insulin responsiveness in the presence of high glucose level after meal. These results in reduced uptake of glucose to the membranes hence increased blood glucose level[3]. It is mainly found in people who are obese and hypertensive (high blood pressure). It is characterized by different kinds of metabolic disorders which is due to insulin deficiency or impaired insulin action[4]. It has been noted that type II DM has a high prevalence all over the world, with prevalent rate of about 366 million in 2011[5]. Report has shown that this rate may increase by 51%, reaching 552 million by year 2030[5]. The prevalence of type II DM in India alone is about 31 million diabetics cases in 2000, 60 million in 2011 and there is expectations that this rate may increase by 63%, reaching 98 million by 2030[5].

After the creation of man, God ordered man to increase and multiply but due to various diseases and infections infertility has posed as one of the greatest problems facing mankind. Reproduction is a natural experience which must involve both the male and the female, based on God's plan for man it should be simple and easy. Today when we look around us, infertility has become so common amongst couples; some primary and some secondary infertility. Infertility can come from different factors including physical and chemical factors.

Type II diabetes has been linked to a lot of complications, including male infertility resulting from male sexual dysfunction[6]. Research has shown that up to 75% diabetic men have erectile dysfunction[6]. Erectile dysfunction in type II diabetic patients has been linked to inability to control metabolism, vascular alteration induced by diabetes and diabetic neuropathy[6]. Research has also shown reduced testosterone and 17-estradiol levels in testes and plasma in diabetic rats[7]. Researchers have also shown that diabetes can cause testicular dysfunction including reduction in Leydig cells, sperm motility and Sertoli cells[7]. Different Authors have different postulates on the mechanism by which DM cause male infertility; they include reduction of testosterone due to endocrine disorders, neuropathy, and increased oxidative stress[8].These factors have led to defects

especially in male reproductive system which includes abnormalities in sperm production or hormonal imbalance.

Roots and herbs have been used effectively in the treatment of different kinds of diseases and infections, this has drawn the attention of researcher on plants[3]. Many of these plants are rich in bioactive chemicals including alkaloids, flavonoids and essential oils. A lot of plants have proven effective in the treatment of diabetes and its associated complications.

A. muricata and *A.heterophyllus* are one of such plants which have been used over the years locally both as fruits and regimen for different kinds of diseases[3]. *A. muricata* generally called graviola, guanabana[9] or Soursop (shawashop in Eastern Nigeria, West Africa) is a green leafy plant which belong to annonaceae family[10]. *A. muricata* has leaves that are thick and dark green in colour. The fruits are dark greenish, heart shaped and covered with spines. Inside the pulp are black seeds which are not edible and can be more than fifty in one fruit[11].

A. heterophyllus known as Jackfruit in English and Kanthal in Bangladesh is a fruit in the mulberry family known as Moraceae. It grows wildly in the tropics especially in India and Malaysia[12]. Jackfruit tree produce large fruits which weighs up to 35 kg[13, 14]. The back of the fruits are green and spiny while the leaves are dark green in colour, obviate with smooth edges. The inside of the fruits contains white or milky pulp which are sweet and edible and contains up to 500 seeds in one fruit [14,15].

MATERIALS AND METHODS

Materials

All reagents and materials used for this research work were of analytical grade and standards.

Experimental Animals

Fifty six (56) animals (wistar albino rats) were used for this experiment. They were purchased and lodged in the Animal house of Department of Pharmacology, Faculty of Basic Medical Science, Abuja Park of the University of Port Harcourt, Choba, Rivers State. The animals were acclimatized for seven days (7), after which they were nourished with a high fat diet and clean

water. The wistar albino rats weighed 150 to 200 and they were marked for easy identification. The 56 rats were grouped in to seven with 8 rats each.

Collection and Identification of Plants

The leaves of *A. muricata* were obtained from Abuja park of University of Port Harcourt while the leaves of *A. heterophyllus* were obtained from Ozuoba, Obior/Akpo Local Government Area of Rivers State.

Artocarpus heterophyllus and *Anonna muricate* were identified and confirmed botanically by Dr Ekeke Chimezie of the Department of Plant Science and Biotechnology, University of Port Harcourt, Choba, Nigeria and the voucher specimen deposited at the habarium of Department of Plant Science and Biotechnology, University of University of Port Harcourt, Nigeria

Preparation of plant extract

A. muricata and A. heterophyllus leaf extracts**:** Leaves of *A. murica and A. heterophyllus* were washed and shade dried, after which the leaf powder was prepared using home grinder/blender. Two hundred gramms (200g) of the milled *A. muricata and A. heterophyllus* leaves were weighed and soaked in 1000ml of 95% ethanol for 48 hours after which they were sieved using a muslin cloth and subsequently filtered with Whatmann paper size 1. The filtrate was concentrated using Rotary Evaporator at 45^{o} C, the weight of the concentrates were taken and the percentage yield calculated and kept at 4° C until usage.

Preparation of High Fat Diet

A high fat diet (HFD) was prepared with 20% sucrose, 10% margarine (baking fat), 2.5 % egg yolk and 67.5% finisher (animal feed).

Experimental Design for Anti-Diabetic and Anti-Infertility Effect

The rules and regulations guiding animal use were observed. The animals were sorted into seven groups of eight animals and their weights taken before high fat diet was introduced. On day 21 after giving high fat diet, the weights of the animals were also taken. The rats were induced with

diabetes using 35mg/kg body weight of streptozotocin 21 days after giving the animals' high fat diet. Mortality rate was observed and the animals were grouped as follows:

Group 1: Normal control, non-diabetic (NC).

Groups 2: Diabetes control: High fat diet and 35 mg/kg body weight of Streptozotocin (DC).

Group 3: Diabetes + metformin: High fat diet, streptozotocin and known antidiabetic drug (50mg/kg body weight of metformin) (DM)

Group 4: Diabetes + 100 mg/kg of combined ethanol leaf extract of *A. muricata* and *A. heterophyllus* (DSJ1) .

Group 5: Diabetes + 200 mg/kg of combined ethanol leaf extract of *A. muricata* and *A. heterophyllus* (DSJ2)

Group 6: Diabetes + 200 mg/kg of ethanol leaf extract of *A. muricata* (DS)

Group 7: Diabetes + 200 mg/kg of ethanol leaf extract of *A. heterophyllus* (DJ)

Treatment started 72 hours after induction of type II DM. Rats whose blood glucose level were up to 200 mg/dl where selected for the study. The treatment lasted for thirty days before sacrifice.

Sample Collection

Four (4) rats from each of the groups were sacrificed at fifteen (15) days interval during treatment for thirty days. The animals were sedated using cotton wool soaked in diethyl ether in a dessicator. The sedated animals were placed on a dissecting slab, the blood sample were collected from the jugular vein for analysis. Parts of the testes were collected in plain bottles for semen analysis.

Assay of Plasma Glucose Concentration

The plasma glucose was assayed using the multiCarein^{TM} glucose strips.

Serum testosterone assay

Serum collected at termination was used for assaying for total testosterone. Testosterone was measured using a commercial ELISA kit (IBL) which is based on competitive binding of testosterone on immobilised antibody. Horse radish peroxidase was used for colour development and absorbance was measured at 420 nm on a plate reader (Multiskan EX).Values are reported as ng/ml of serum.

Semen analysis

a. Collection of epididymal semen

The cauda epididymis was separated from the testes using a surgical scissor, a small incision was made on the cauda epididymis to extract the spermatozoa from the tubules then it was placed in a slide.

b. Sperm motility, viability and abnormalities

For sperm motility, the semen collected was diluted using about 2 drops of freshly prepared normal saline at the laboratory temperature. This was placed on a slide and was covered with a cover slip. The motility was viewed under x40 magnification

The sperm viability and abnormalities were determined using one step eosin method. A portion of the sperm suspension was added to equal portion of eosin on a microscopic slide. They were mixed together and were covered with coverslip and viewed under times 40 magnifications, normal cell life sperm cells appear whitish while dead sperm cells take up the stain and appear pinkish. Percentage viability was calculated based on the number of life sperm cells.

c. Epididymal sperm count

To count the sperm cell few drops of formal saline (mixture of 10% formaline and 0.9% of normal saline) where added to the spermatozoa. This helps to kill the sperm cells and fix them.

One drop of the diluted sperm suspension was transferred to each counting chamber of the improved Neubauer haemocytometer (Deep 1/10 mm, LABART, Munich, Germany) and the sperms were counted under a light microscope at 40× magnifications.

Statistical Analysis of Data

All data for biochemical analysis were analyzed for statistical differences and in rat treatment groups, by means of one-way ANOVA and post hoc LSD, on SPSS 20. In all, $p<0.05$ was considered significant. Data are presented as mean±S.D (standard deviation)

RESULTS AND DISCUSSION

Table 1: The effect of ethanol leaf extract of *A. muricata*, *A. heterophyllus* and combined ethanol leaf extract of *A. muricata* and *A. heterophyllus* on the glucose levels of type II diabetic rats.

	Glucose (mg/dl)	
Groups	**After 15 days**	**After 30 days**
Normal control	62.33±12.89^{b}	77 ± 5.00^{b}
Diabetes control	232±27.78^{ac}	226.33 ± 12.85^{ac}
Diabetes + Metformin	99.33±1.53^{b}	83.33 ± 12.06^{b}
Diabetes + 100mg combined Extract	65.00±9.85^{b}	96.33±2.30^{b}
Diabetes + 200mg combined Extract	120.33±7.02^{ab}	86.33±7.51^{b}
Diabetes+ 200mg *A. muricata*	136.66±17.95^{ab}	93.33±3.05^{b}
Diabetes + 200mg *A. Heterophyllus*	132.33±29.16^{ab}	98.66±5.03^{b}

Values presented are mean ± Standard deviation (SD) of four determinations. Mean values in each row with different small letter superscripts are Statistical significant (SSF) at $p<0.05$.

Table 2: Effect of ethanol leaf extract of *A. muricate, A. heterophyllus* and combined ethanol leaf extract of *A. muricata* and *A. heterophyllus* on the testosterone levels of type II diabetic rats

Groups	Testosterone (ng/ml)	
	After 15 days	After 30 days
Normal control	1.90±.10	1.90±.09
Diabetes control	0.78±.06[c]	0.75±.04[c]
Diabetes + Metformin	2.75±.17[b]	2.49±.27[b]
Diabetes + 100mg combined Extract	2.96±.64[b]	2.86±.17[b]
Diabetes + 200mg combined Extract	2.43±1.20	2.54±1.04
Diabete+ 200mg *A. muricata*	1.48±.93	2.17±.87
Diabetes + 200mg *A. Heterophyllus*	1.19±.17	2.17±1.03

Values presented are mean ± Standard deviation (SD) of four determinations. Mean values in each row with different small letter superscripts are Statistical significant (SSF) at $p<0.05$.

Table 3: Effect of ELE of *A. muricata, A. heterophyllus* and combined ELE of *A. muricata* and *A. heterophyllus* on the sperm motility of type II diabetic rats

Groups	Sperm motility %	
	After 15 days	After 30 days
Normal control	75.00±5.00[b]	71.67±2.89[b]
Diabetes control	51.67±2.89[a]	48.33±5.77[ac]

Diabetes + Metformin	66.67±5.77	71.67±2.89b
Diabetes + 100mg combined Extract	67.00±7.55	68.33±2.89[b]
Diabetes + 200mg combined Extract	65.00±5.00	68.33±2.89[b]
Diabete+ 200mg *A. muricata*	65.00±8.66	66.66±2.89[b]
Diabetes + 200mg *A. Heterophyllus*	73.33±5.77[b]	75.00±5.00[b]

Values presented are mean ± Standard deviation (SD) of four determinations. Mean values in each row with different small letter superscripts are Statistical significant (SSF) at $p<0.05$.

Table 4: Effect of ethanol leaf extract of *A. muricata, A. heterophyllus* and combined ethanol leaf extract of *A. muricata* and *A. heterophyllus* on the Sperm viability of type II diabetic rats

Groups	**Sperm Viability (%)**	
	After 15 days	**After 30 days**
Normal control	80.00±5.00[b]	80.00±0.00[b]
Diabetes control	51.67±2.89[ac]	56.67±2.89[ac]
Diabetes + Metformin	78.33±2.89[b]	

		78.33±2.89[b]
Diabetes + 100mg combined Extract	76.67±2.89[b]	78.33±2.89[b]
Diabetes + 200mg combined Extract	70.00±8.66[b]	73.33±5.77[b]
Diabete+ 200mg *A. muricata*	73.33±7.64[b]	73.33±5.77[b]
Diabetes + 200mg *A. Heterophyllus*	75.00±5.00[b]	71.67±2.89[b]

Values presented are mean ± Standard deviation (SD) of four determinations. Mean values in each row with different small letter superscripts are Statistical significant (SSF) at $p<0.05$.

Table 5: Effect of ELE of *A. muricata*, *A. heterophyllus* and combined ELE of *A. muricata* and *A. heterophyllus* on the Sperm density (sperm count) of type II diabetic rats

Groups	**Sperm density (X10^6)**	
	After 15 days	**After 30 days**
Normal control	650.00±86.60[b]	703.33±137.96[b]
Diabetes control	55.00±8.66[ac]	50.00±5.00[a]
Diabetes + Metformin	750.00±180.28[b]	716.67±160.73[b]
Diabetes + 100mg combined Extract	550.00±50.00[b]	550.00±86.60[b]
Diabetes + 200mg	383.33±160.73[c]	516.67±76.38[b]

combined Extract		
Diabete+ 200mg *A. muricata*	316.66±152.75[c]	533.33±57.74[b]
Diabetes + 200mg *A. Heterophyllus*	366.67±152.75[c]	533.33±57.74[b]

Values presented are mean ± Standard deviation (SD) of four determinations. Mean values in each row with different small letter superscripts are Statistical significant (SSF) at $p<0.05$.

Table 6: The effect of *A. muricata, A. heterophyllus* and combined ELE of *A. muricata* and *A. heterophyllus* on the abnormal sperm of type II diabetic rats

Groups	Abnormal sperm (%)	
	After 15 days	After 30 days
Normal control	16.67±5.77[b]	16.67±5.77[b]
Diabetes control	41.67±5.77[a]	45.00±8.66[ac]
Diabetes + Metformin	26.67±7.64	25.00±5.00[b]
Diabetes + 100mg combined Extract	25.00±5.00[b]	23.33±2.89[b]
Diabetes + 200mg combined Extract	25.00±5.00[b]	21.67±2.89[b]
Diabete+ 200mg *A. muricata*	28.33±5.77	30.00±5.00[b]
Diabetes + 200mg *A. Heterophyllus*	25.00±5.00[b]	26.67±7.64

Values presented are mean ± Standard deviation (SD) of four determinations. Mean values in each row with different small letter superscripts are Statistical significant (SSF) at $p<0.05$.

Discussion

Type II DM results from defect in insulin response in the presence of high glucose level after meal, leading to reduced uptake of glucose to the membranes hence increased blood glucose level.

Glucose metabolism is important during spermatogenesis, for maintaining basic cell activity, motility and during fertilization [16]. Diabetes is known for its negative effect on male fertility through disruption of spermatogenesis as well as normal erectile function. Findings have shown that male infertility is one of the complications arising from diabetes DM. DM is associated with an increased oxidative stress, which may distort or damages sperm nuclear and mitochondrial DNA [17].

Defect in male reproductive system is part of the complications arising from obesity and type II diabetes[16]. In this study, the effect of type II DM on the male fertility was investigated. Result of this research showed a significant decrease ($p<0.05$) in the testosterone level of the diabetic untreated rats (DC). The reason for reduced testosterone level is not clear. Some scholars suggest that this effect could be as a result of effect of diabetes on aromatase activity which could lead to a reduced formation of testosterone. DM alters the expression of aromatase enzyme expression thus a reduced production of testosterone[18].

The semen of the animals were also analyzed for sperm characteristics (sperm count, motility, morphology and viability). The result showed a low sperm count on the diabetic groups compared with those treated with ethanol leaf extract of *A. muricata* and *A. heterophyllus* and the combined ethanol leaf extracts. Abnormalities in sperm characteristics may be as a result of sperm DNA damage arising from oxidative stress[16]. It could also be due to reduced testosterone level[19]. The result also revealed a low sperm count in the diabetic untreated groups. The antioxidant effects of *A. muricata* have been reported by Adefegha *et al*., 2015**.** The result of this research suggests that the positive effect of *A. muricata* and *A. heterophyllus* on the testosterone levels could be as a result of the antioxidant activity of the plants.

Conclusion

The use of herbs in the treatment of different kinds of disease has been in the fore front of research for some time now. Many of these plants are rich in plant chemicals which have been

used in the treatment of different kinds of diseases. Though mechanism by which these plants chemicals act may not be fully understood but they are effective in the treatment of different kinds of diseases including Type II DM and its associated complications.

Previous studies have shown that the leaves of *A.muricata* and *A. heterophyllus* are rich in phytochemicals especially alkaloids and flavonoids. Phytochemicals are known for their different biological activities including free radical scavenging activity, anticancer, anti-diabetic and antihyperlipidaemic activity among others. Isoflavons, niacin, saponin, lignans, flavonoids, alkaloids, glycosides, tannins and triterpenes have been identified in the leaves of *A. heterophyllus*[20] while *A. muricata* contains alkaloids, tannin, megastigmanes, flavonol, triglycosides, flavonoids, alkaloids, steroids, triterpenoid, phenolic compounds, cyclopeptides, tannins, coumarins, stearic acid, myristique acid, and ellagic acid.[21, 22, 10] This research suggest that combined ethanol leaf extract of *A. muricata* and *A. hetrophyllus* has positive effect on reproductive parameters of Type II diabetic male wistar albino rats.

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