



Combined Therapy of *Moringa oleifera* and *Ocimum gratissimum* Reversed Testicular Damage in Diabetic Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author PEE project conception and design, coordination and interpretation of data. Author EEE experimentation and acquisition of data; preparation of draft manuscript. Author BIAM analysis and interpretation of data and micrographs, graphics, preparation of final manuscript and coordination. Author GOI extraction and fractionation methodologies, statistical analysis, interpretation of data. Author EHI experimental design, protocols and interpretation of data. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Introduction: Testis is an important male reproductive and endocrine organ whose structure and function are altered in diabetes complicated disorders.

Aim: This study evaluated the protective effect of *Moringa oleifera* (MO) and *Ocimum gratissimum* (OG) on diabetic rat testes.

Methodology: Thirty six rats, weighing between 120-180g, were divided into six groups of 6 rats each. Groups 1 and 2 representing Normal (NC) and Diabetic Control (DC) received 0.5ml of dimethylsulphoxide. Group 3 received 5IU/kg b.w insulin; groups 4, 5 and 6 received 500mg/kg b.w of MO, 500mg/kg b.w of OG and 250mg/kg b.w of each extract respectively. Fasting blood glucose (FBG), serum testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH) and histology of the testes were analysed after 28 days treatment.

Results: MO, OG and the combination extract normalized the levels of FBG. Only the

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Moringa extract normalized the levels of testosterone, LH and FSH compared with DC. The OG extract had no effect on the level of the three sex hormones but provided a potentiating effect on the FSH level in the MO + OG group. The results were confirmed by histological studies which showed damage on the testes for the DC and OG and reversal of damage to the testes in MO and MO + OG groups.

Conclusion: The combined extracts more than *Moringa* extract alone, had ameliorative effects on testicular architecture and spermatogenesis in diabetes and provide a cheap alternative to treating diabetes associated testicular damage and sexual dysfunction.

Keywords: Diabetes; testicular damage; sex hormones; phytotherapy; combined therapy; *Moringa oleifera*; *Ocimum gratissimum*.

ABBREVIATIONS

STZ: Streptozotocin; MO: *Moringa oleifera*; OG: *Ocimum gratissimum*; NC: normal control; DC: diabetic control; FBG: Fasting blood glucose; LH: Luteinizing hormone; FSH: Follicle stimulating hormone.

1. INTRODUCTION

Diabetes mellitus is characterised by absolute or relative deficiencies in insulin secretion and/or insulin action associated with chronic hyperglycemia and disturbances of carbohydrate, lipid and protein metabolism [1-3]. Persistent hyperglycemia causes increased production of free radicals especially reactive oxygen species (ROS) from glucose auto-oxidation and protein glycosylation [4-7] resulting in part in increased susceptibility to lipid peroxidation that play a role in the progression of the symptoms of diabetes [8].

Diabetes is also associated with gender-specific changes in sex steroid hormones [9-13]. Men with type 1 and 2 diabetes have lower than normal testosterone levels [12] associated with inappropriately low luteinizing hormone and follicle-stimulating hormone concentrations [14-15]. On the other hand women with diabetes tend to have too little estrogen and/ or too much testosterone (a low E:T ratio) [13]. These imbalances in sex steroid hormone levels associated with diabetes may negatively impact upon sexual function [13,16]. For instance women with type 1 diabetes often show impaired ovarian function, delayed age at menarche, sexual dysfunction, menstrual irregularities, and high risk of adverse pregnancy outcomes, including abortions, stillbirths, and congenital anomalies while men with type 1 diabetes commonly present with erectile dysfunction, which has been shown to correlate with the decline in renal function [13]. Reductions in levels of testosterone and follicle stimulating hormone (FSH) have been responsible for suppressed potency, hormonal imbalance and sexual dysfunctions in males [17-18]. Men and women with low levels of sex hormone-binding globulin (SHBG), a protein that regulates sex hormones are at higher risk of diabetes and genetics may play a role in determining both the levels and the risk [13,19-20]. Thus low circulating level of SHBG is a strong predictor of type 2 diabetes.

Currently available therapies for diabetes include insulin and various oral antidiabetic agents such as sulfonylureas, biguanides and glinides. Although this oral hypoglycemic agents/insulin is effective in controlling hyperglycemia, they have prominent side effects and fail to significantly alter the course of diabetic complications [21-22]. Thus there is a need to search for improved and safe natural anti-diabetic agents. Phytochemicals from traditional medicinal plants have been identified as presenting an exciting opportunity for the

development of new types of therapeutics especially on account of their cost effectiveness, relative accessibility and availability and low incidence of drug toxicity [23-24]. To this end the World Health Organization has recommended the development of herbal medicine [25] and several African and Asian nations are now encouraging traditional medicines as an integral component of their public health care programs [24] as indigenous medicines are relatively inexpensive, locally available and are readily accepted by the local population. Nowadays, bioassay-guided fractionation of medicinal plants is a routine feature in the attempt to isolate bioactive components from natural sources and a wide array of plant derived active principles representing numerous chemical compounds have demonstrated activity consistent with their possible use in the treatment of non insulin dependent diabetes mellitus, NIDDM [24,26]. Following leads from traditional medicinal practitioners we have demonstrated in our laboratory the anti diabetic activities, singly and in combination of *Vernonia amygdalina*, *Azadiracta indica*, *Peristrophe bicalyculata* [27-29], *Nauclea latifolia* and *Gongronema latifolium* [7].

Although various plant extracts have been shown to be useful in the cure of diabetes mellitus, few of them were tested for their effects on body tissues of diabetic patient. Given the leads on the hypo-cholesterolemic and hypoglycemic activities of *Moringa oleifera* and *Ocimum gratissimum* and thus their antidiabetic potential [30], this work aims to examine if the two plants, singly or in combination, can reverse the low levels of the sex hormones testosterone, Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) and testicular damage associated with diabetes.

Moringa oleifera Lam (also known as the horseradish tree and drumstick tree) is the most widely cultivated species of a monogeneric family, the *Moringaceae*. Native to the sub-Himalayan tracts it is now widely cultivated and has become naturalized in many locations in the tropics [31]. The relative ease with which it propagates through both sexual and asexual means and its low demand for soil nutrients and water after being planted makes its production and management easy [32]. Moringa preparations have been cited in the scientific literature as having nutritional, antimicrobial, hypotensive, antispasmodic, antiulcer, anti-inflammatory, hypo-cholesterolemic, and hypoglycemic activities, as well as having considerable efficacy in water purification by flocculation, sedimentation, antibiosis and even reduction of *Schistosoma cercariae* titer [31 and references therein]. The plant has also been used extensively for treating inflammation, cardiovascular disease, liver disease and hematological, hepatic and renal function [33]. Moringa leaf extracts have also been shown to have ameliorating effect on chromium-induced testicular toxicity in rats [34] and to enhance sexual activity in mice [35].

Ocimum gratissimum, African basil/sweet basil, is a plant belonging to the *Lamiaceae* family. The *Ocimum* species are widely found in tropical and subtropical regions and commonly used as food spice and traditional herb. The herb has been recommended for the treatment of various diseases [36]. Its hypoglycemic efficacy has been reported [37-39] as has its therapeutic potentials on hepatic disorders [40].

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

Fresh matured *Moringa oleifera* (MO) and *Ocimum gratissimum* (OG) leaves were harvested from the Endocrine laboratory farm, University of Calabar, Nigeria and were authenticated at

the Department of Botany, University of Calabar, Nigeria. Voucher specimens were deposited in the Department of Botany herbarium, University of Calabar.

The leaves were rinsed severally with clean tap water to remove dust particles and debris and thereafter allowed to dry completely. About 2kg portion of each leave was then taken for preparation of the plant extracts.

2.2 Preparation of Plant Extract

Two kilograms (2kg) each of *M. oleifera* and *O. gratissimum* was separately homogenized with a manual blender in 4.0 liters of 80% (v/v) ethanol. The blends were allowed for 48 hours in a refrigerator at 4°C to allow for thorough extraction of the plants' active components. These were then filtered with a cheese cloth and later with Whatman No. 1 filter paper to obtain a homogenous filtrate. The filtrates were then concentrated *in vacuo* at low temperatures (37-40°C) to about one tenth the original volume using a rotary evaporator. The concentrates were allowed open in a water bath at 40°C to allow for complete dryness yielding 80.00g (4.0%) and 75.50g (3.8%) of greenish brown substance for OC and MO respectively.

2.3 Experimental Animals

Thirty six adult male albino Wistar rats with a body weight of 120-180g obtained from the animal house of the Department of Biochemistry, University of Calabar, Nigeria were used for this study. The animals were placed in standard cages maintained in 12-hour light: dark cycle under standard conditions (temperature 25±5°C, relative humidity 50±5%). All animals received standard laboratory animal's chow and water *ad libitum* during the whole period of experiment.

2.4 Experimental Induction of Diabetes

Diabetes was induced in thirty overnight fasted rats by a single intraperitoneal injection of streptozotocin, STZ (Sigma, ST. Louis, MO, USA), 40 mg per kg of body weight, dissolved in 0.1 M sodium citrate buffer (pH = 4.5). The animals were confirmed diabetic if the glucose level of blood of fasted animals, collected from tail vein after 48 hours of injection of STZ, was above 120 mg/dl as determined with an automated glucose analyzer device (One Touch Glucometer, Lifescan, California, USA).

2.5 Experimental Design

Thirty six rats were divided into six different experimental groups of 6 animals each. Two groups, normal control group (NC) and diabetic control group (DC) received placebo, 0.5ml DMSO; the other four diabetic groups, the treated groups, received respectively Insulin (administered at a dose of 5Ukg⁻¹b.w.s.c once per day to simulate human regimens), *M. oleifera* (MO), *O. gratissimum* (OG) and a combination of *M. oleifera* + *O. gratissimum* (MO+OG). The plant extracts, reconstituted in DMSO were administered orally, via gastric intubations, at a dose of 500mg per kg of body weight for single extract treatment and 250 mg each per kg of body weight in combined extracts treatment. The dosage of the extract was determined from preliminary studies in our laboratory. The duration of treatment was 28 days after induction of diabetes. At the end of the experimental period, the animals were fasted for 12h, then anaesthetized under chloroform vapour and dissected. Whole blood was

obtained by cardiac puncture into sterile plain tubes and allowed to clot for about 2h and thereafter centrifuged (3,000g for 10min) to remove cells. Serum recovered was used for the Biochemical assays. The testes were also surgically removed for histological study.

2.6 Biochemical Assay of Sex Hormones

Testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) concentrations were determined by ELISA based assays using commercial kits (Monobind inc. USA) according to the manufacturer's protocol.

2.7 Tissue Preparation for Histological Study

Dissected testes were immediately fixed in 10% formaldehyde in buffered solution containing 54 mM NaH_2PO_4 and 28 mM Na_2HPO_4 (pH 7.4) kept at 4°C. After fixing for 48 hours, a transverse section was made at the middle part of each testis and kept immersed in the fixative for the completion of tissue fixation. The formaldehyde-fixed samples were then embedded in paraffin and sectioned into slices 6-7 μm thick. The tissue slices were mounted onto albumin-precoated glass slides, de-paraffined with xylol and stained with the hematoxylin and eosin (H&E). The stained sections were studied under a light microscopy.

2.8 Morphometric Analysis

Morphometric analyses of tissue sections of testes prepared as above were performed from images obtained at $\times 400$ magnifications and digitalized using an Olympus DP70 digital camera (Olympus Europe, Hamburg, Germany).

2.9 Statistical Analysis

Results were analyzed using SPSS. All data were reported as mean \pm SEM. For the evaluation of significant differences, the comparison of means between any two experimental groups was done by computer program for student-t test (paired t-test). Differences were considered to be statistically significant if $P = 0.5$.

3. RESULTS

3.1 Effect of Treatment on Blood Sugar Level

After treatment with STZ, there was a significant increase in blood glucose level in the diabetic rats relative to normal control ($P = 0.5$). The glucose levels during the treatment period were significantly lower in the extract treated groups ($P = 0.5$) than in the diabetic control (Fig. 1). These values were even lower than that of insulin suggesting a hypoglycemic effect by the two plants singly and in combination.

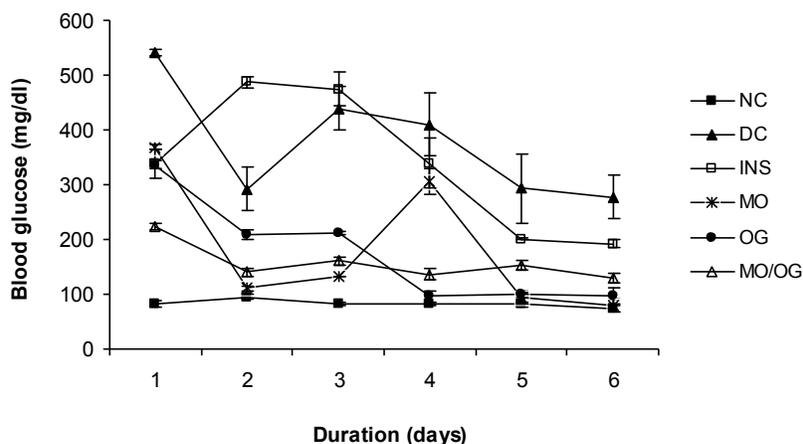


Fig. 1. Comparison of daily blood glucose concentration of the different experimental groups. Values are mean of six values. Normal control(NC); Diabetic control(DC); Insulin(Ins); *Moringa*(MO); *Ocimum*(OG).

3.2 Effect of Treatment on Sex Hormone Levels and Testicular Architecture

There was a significant decrease in the levels of testosterone, FSH and LH in the diabetic control relative to normal control ($P = 0.5$) (Figs. 2 and 3). Treatment with *Moringa* significantly reversed the levels of the three sex hormones towards normal values. These values were comparable or even better than the reversals by insulin. Also of note is that insulin could not reverse the depressed levels of LH in the diabetic rats. OG singly had no effect on the levels of any of the three hormones. Although the MO+OG combination reversed the levels of the three sex hormones, these levels, except for FSH, can be reasonably ascribed to the MO effect alone as the values were lower than that for MO alone. There was, however, a potentiating effect by the combination of the two plants extract on the level of FSH. This result is supported by photomicrographs of tissue sections of the testis in normal, diabetic and diabetic-treated groups (Plates 1-6). Micrographs of tissue sections in testis from normal control rats (Plate 1) showed closely packed seminiferous tubules lined by a distinct germinal epithelium with developing spermatogonia at various stages of development. The Diabetic rats had functional testes, with their seminiferous tubules lined by spermatogonia but with sparse primary and secondary spermatocytes with degenerate spermatozoa in the lumen of the tubules (Plate 2). Photomicrographs of testis from diabetic groups treated with Insulin, MO and MO + OG (Plates 3,4 and 6) showed partial recovery in spermiogenesis with more remarkable recovery observed for the MO/OG group even though there was no synergistic effect on testosterone and LH for the combined groups except for FSH. The OG treated group exhibited a sparse lumen containing atrophic, degenerate spermatocytes and mature spermatozoa similar to DC (Plate 5).

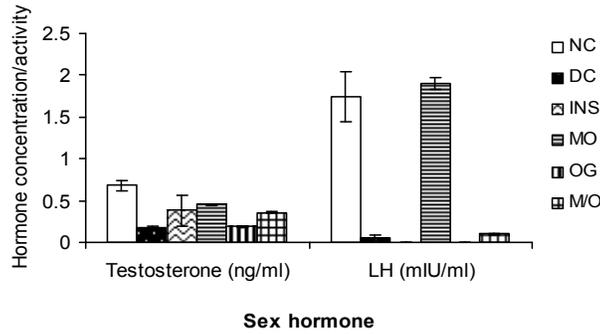


Fig. 2. Effect of treatment on Testosterone and Luteinizing hormone levels in diabetic rats. Values are the mean±SEM of six experimental rats in each group. Normal control (NC); Diabetic control (DC); Insulin(Ins); *Moringa* (MO); *Ocimum* (OG).

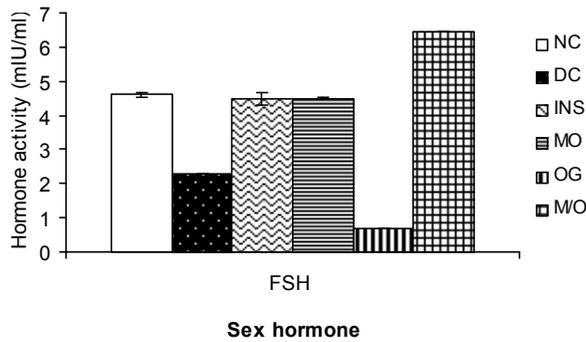


Fig. 3. Effect of treatment on Follicle stimulating hormone levels in diabetic rats. Values are the mean±SEM of six experimental rats in each group. Normal control (NC); Diabetic control (DC); Insulin(Ins); *Moringa* (MO); *Ocimum* (OG).

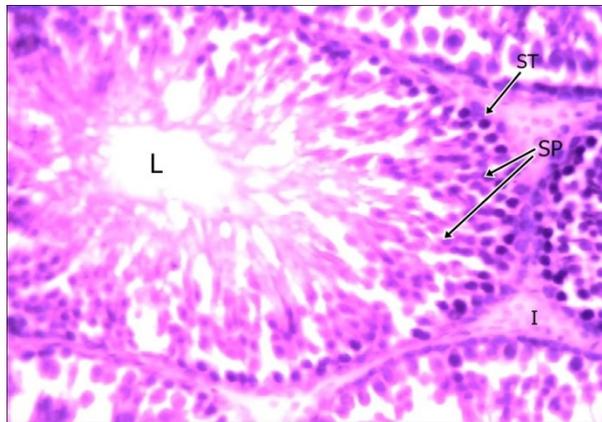
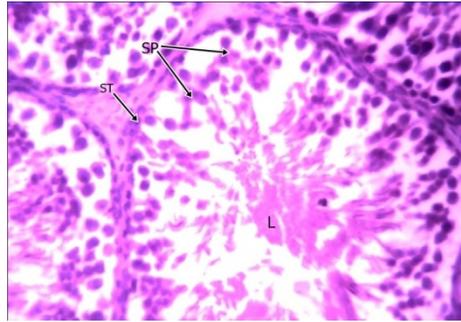
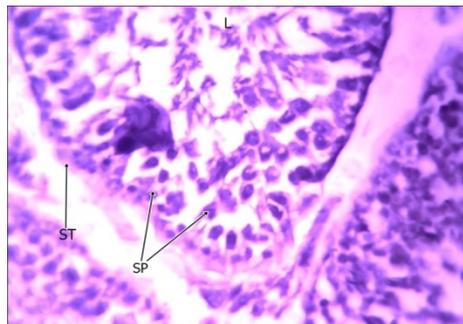


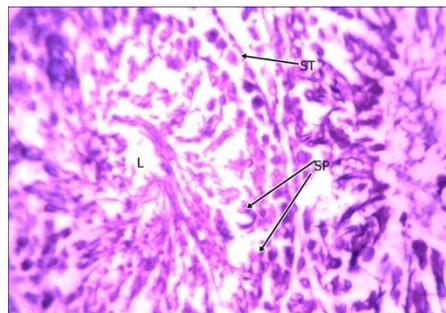
Plate 1. Photomicrograph of normal rat testis that received placebo showing closely packed seminiferous tubules (ST) lined by a distinct germinal epithelium with densely packed spermatogonia and developing spermatocytes and spermatozoa. Haematoxylin and Eosin (mag. X 400)
I = Interstitium; L = Lumen



**Plate 2. Photomicrograph of diabetic rat testis that received placebo showing closely packed seminiferous tubules (ST) lined by spermatogonia and sparse primary and secondary spermatocytes (SP) with degenerate spermatozoa in the lumen. Haematoxylin and Eosin (mag. X 400)
I = Interstitium; L = Lumen**



**Plate 3. Photomicrograph of diabetic rat testis treated with insulin with seminiferous tubules (ST) consisting of spermatogonia and spermatocytes (Sp) at various stages of maturation. Some spermatozoa in the lumen appear to be atrophic and degenerate with pyknotic nuclei. The basement membrane is partly intact. Haematoxylin and Eosin (mag. X 400).
I = Interstitium; L = Lumen**



**Plate 4. Photomicrograph of diabetic rat testis treated with *M. oleifera* showing seminiferous tubules (ST) with spermatogonia and spermatocytes/spermatozoa at various stages of maturation. As with insulin, a few atrophic and degenerated spermatozoa cells with pyknotic nuclei, ostensibly from the diabetic induction, are evident; basement membrane (BM) is partly intact. Haematoxylin and Eosin (mag. X 400)
Lumen = Lumen**

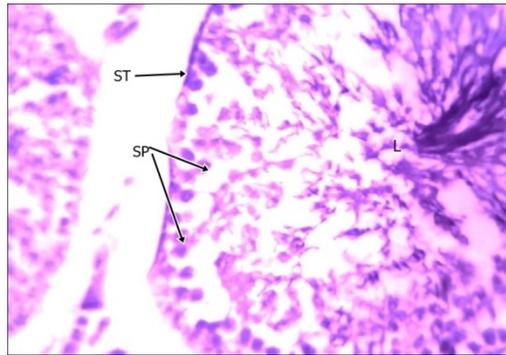


Plate 5. Photomicrograph of diabetic rat testis treated with *O. gratissimum* showing seminiferous tubules (ST) with sparse and degenerate spermatogonia, spermatocytes and spermatozoa; basement membrane (BM) is partly intact. Haematoxylin and Eosin (mag. X 400)

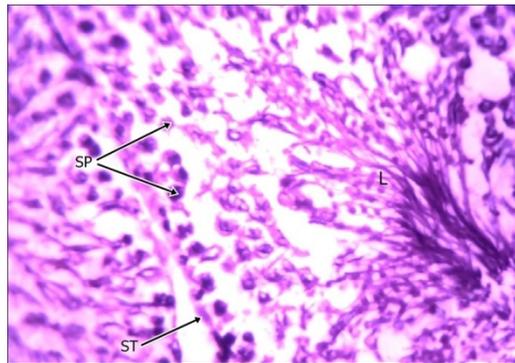


Plate 6. Photomicrograph of diabetic rat testis treated with *M. oleifera* and *O. gratissimum* showing a preserved architecture, seminiferous tubules (ST), intact basement membrane (BM). There is obvious spermatogenesis with developing spermatogonia (Sp), spermatocytes and spermatozoa at various stages of maturation. Haematoxylin and Eosin (mag. X 400)

4. DISCUSSION

The vast majority of studies on the male STZ-induced diabetic rat show decreased levels of testosterone associated with diabetes [13,14 and references there in] similar to the results obtained in the present study. Decreases in the serum levels of FSH and LH associated with diabetes, as reported in this study, have also been reported in earlier studies [14,41-42].

STZ is commonly used for the induction of type I diabetes in experimental rats. STZ causes diabetes by rapid depletion of β cell in pancreatic islets of Langerhans, leading to a reduction in insulin secretion and consequently to hyperglycaemia [7,43]. It has also been shown that the morphologic alterations observed in the testes of STZ-diabetic rats are not caused by a direct effect of the drug, but rather by diabetes [44] implying that this model of diabetes is a useful tool to study the insulin-related modulation of testicular function [14].

The STZ induced reduction in insulin secretion leading to hyperglycemia was manifested in the present study by a significant increase in Fasting Blood Glucose (FBG) of DC compared with NC. Glycemic control is important for the management of diabetes [1]. The hypoglycemic activity of MO and OC, which was even better than that of insulin, makes them good candidates for management of diabetes singly and in combination.

Overall testicular function is controlled by two independent, synchronized functions - the biosynthesis of androgens by Leydig cells, and the production of spermatozoa in the epithelium of seminiferous tubules [14]. Androgens (testosterone and androsterone) control male libido and spermatogenesis [14,45]. It has been suggested [14] that diabetes-related hypoinsulinemia may have a major effect on tubular function by altering serum FSH levels; the absence of the stimulatory effect of insulin on Leydig cells and to an insulin-dependent decrease in FSH in turn decreases LH levels. LH controls normal Leydig cell function [46-48]. Reduced control of LH on Leydig cells in STZ-induced diabetes causes both a decrease in total Leydig cell number and impairment in Leydig cell function, the combination of these two factors inducing a decrease in androgen biosynthesis and a decrease in serum testosterone levels leading to a reduction in sperm output and fertility [14]. Moreover, FSH is known to also influence sertoli cell function (cells lining the semen-producing tubules of the testis that provides support and nourishment for developing sperm); a reduction in FSH level affects sertoli cell function ultimately leading to malnourished and degenerate sperm cells.

There is a wide discrepancy concerning the effects of insulin treatment on LH and FSH levels in diabetic rats, from a total recovery of LH and FSH to a lack of recovery of LH and FSH [14 and references therein] buttressing the fact that the regulation of testicular function is the result of multiple mechanisms that include the combined effects of insulin/glucose, LH, and FSH. Our results show that while insulin treatment resulted in a recovery in testosterone and FSH levels there was no recovery at all in the LH level explaining some of the atrophic and degenerated spermatogonia cells seen in photomicrographs of testis of diabetic animals treated with insulin.

The preserved architecture of seminiferous tubules (ST), intact basement membrane (BM) and developing spermatogonia, comparable to NC, observed for the MO+OG treated group could be explained by the particularly high levels of FSH in this group (higher than in NC) leading to an enhanced response of the epithelium of seminiferous tubules to FSH stimulation. The follicle-stimulating hormone regulates spermatogenesis and influences sertoli cell function. The increased level of spermatogenesis and increased support and nourishment by sertoli cells occasioning increased FSH levels in the MO+OG group probably explains the sustained production, healthy state and preserved integrity of spermatocytes and spermatozoa in this group, comparable to normal but better than the MO group where a few atrophic and degenerate spermatozoa were evident.

Chromium induces testicular toxicity through oxidative stress and the protective efficacy of *Moringa* may be due to the antioxidant properties of its phytochemical constituents which have been shown to influence oxidative stress [34]. It is likely that the antioxidants present in the leaves of the plants, *Moringa oleifera* and *Ocimum gratissimum*, acting in concert with the antioxidant system present in the epididymis preserved and enhanced the process of spermatogenesis [35] ostensibly by its ability to reverse the levels of FSH and LH and ultimately testosterone.

5. CONCLUSION

Diabetes is accountable for the reduction in sex hormone levels and deleterious effect on the testes. However, *Moringa oleifera* when administered singly and in combination with *Ocimum gratissimum* leaf extract reversed the low level of testosterone, FSH and LH in diabetic animals towards normal and had ameliorative effects on diabetes-induced testicular damage as evidenced from histological observation. A combination of the two plant extracts thus provides a cheap alternative to treating diabetes associated testicular damage and sexual dysfunction. Further studies will aim at isolating the active principle(s) responsible for the reversal of the diabetes induced decrease in sex hormone and testicular damage.

CONSENT

Not applicable to this work.

ETHICAL APPROVAL

All authors hereby declare that the research has been determined exempt from review by the University animal research and ethics review committee and that the principles of laboratory animal care were followed.

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COMPETING INTERESTS

The authors affirm that there is no conflict of interest in the publication of this article.

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