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RESEARCH ARTICLE

Protective role of aqueous extract of *Moringa oleifera* leaf against oxidative stress and histological alterations in testes of rats under insulin deficient condition

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ABSTRACT

The current study investigates the effect of Aqueous Extract of *Moringa oleifera* (AEMO) leaf on the oxidative stress, antioxidant status and histopathological changes in testes of rats under insulin deficient condition. Male Wistar rats were used in this study. Diabetes (insulin deficient) was induced in rats by a single intraperitoneal injection of Streptozotocin. AEMO was administered at a dose of 200 mg/kg body weight for a period of 60 days. At the end of the experimental period, the rats were sacrificed by cervical dislocation. Relative organ weight was calculated. Oxidative stress was assessed by the extent of lipid peroxidation (LPO) and the levels of reduced Glutathione (GSH) in testes. The status of antioxidant enzymes was estimated by determing the activities of Glutathione Reductase (GR), Glutathione- S-Transferase (GST), Sorbitol dehydrogenase (SDH), Catalase (CAT) and Superoxide dismutase (SOD). Histological changes in testes were studied. Statistical significance of the results was evaluated by Duncan's Multiple Range Test. In this study, diabetic rats showed significant decrease in absolute and relative weights of testes, increased LPO and decreased levels of GSH. The activities of antioxidant enzymes- GR, GST, SOD, CAT were reduced. In contrast, the activity of SDH, a polyolpathway enzyme, increased. Histological picture of testes of D group showed pathological changes. However, administration of AEMO showed beneficial effect which was reflected by reduced oxidative stress, enhanced activities of antioxidant enzymes in testes. AEMO also protected the testes against the histological changes induced by oxidative stress under insulin deficient condition. The study revealed that AEMO ameliorated oxidative stress, exhibited antioxidant potential, and played a role in the protection of testicular structural integrity under insulin deficient condition.

KEYWORDS: *Moringa oleifera*, Testes, antioxidants, oxidative stress, Histopathology.

INTRODUCTION

Diabetes mellitus is a disorder of carbohydrate, fat and protein metabolism characterized by chronic hyperglycemia resulting from defects in insulin secretion accompanied by various degrees of insulin resistance. The number of diabetics was 171 million in 2000 which might increase to 360 million in the year 2030 (WHO, 2000). The rapid increase in type-1 diabetes strongly suggests that the action of the environment on susceptibility genes contributes to the evolving epidemiology of type-1 diabetes (Gillespie, 2006).

During diabetes, persistent hyperglycemia causes increased production of free radicals especially reactive oxygen species, ROS, (George and Mary, 2004). Normalizing ROS generation showed to prevent the long-term complications of diabetes (Nishikawa, 2000). Although there is a controversy about the antioxidant status in diabetes, several studies report decreased plasma or tissue concentrations of superoxide dismutase, CAT, GSH and ascorbic acid in diabetic animals (Hink et al., 2001). Thus over production of ROS with decreased antioxidant potential intensifies the oxidative stress in diabetes.

Insulin dependent diabetes mellitus or type 1 DM is associated with several forms of long term complications. These include microvascular complications (retinopathy, nephropathy and neuropathy) and macrovascular complications (coronary artery disease, peripheral vascular disease and cerebrovascular disease).

Many studies have shown that the secondary metabolites produced by plants possess antioxidant property, which plays a major role against functional and cellular damage caused by ROS (Pari et al., 2007). Several phytochemicals were reported to act against the deleterious effects of oxidative stress (Tiwari, 2001; Gabrieli, 2005; Ramesh et al., 2012).

In the present study the efficacy of aqueous extract of *Moringa oleifera* (AEMO) leaf in combating the oxidative stress in testes of insulin deficient rats was investigated. *Moringa oleifera* Lam (syn Pterigosperma Geartn) belongs to the monogeneric family Moringaceae and it is one of the best known, most widely distributed and naturalized species (Nadkarni). It is popularly known as drumstick or horseradish. *Moringa oleifera* was well known to the ancient world, but only recently it has been 'rediscovered' as the "Miracle tree" with a tremendous variety of potential uses. Leaves, immature pods, flowers and fruits of this plant are edible and are highly nutritive. *Moringa* leaves have been reported to be a rich source of β-carotene, protein, Vitamin C, calcium, potassium and essential amino acids which make them an ideal source of dietary supplement (Makkar, 1996). Studies of AEMO for trace elements by Particle Induced X-ray Emission (PIXE) technique revealed the presence of many physiologically and biochemically important trace elements (Gowrishankar et al., 2010).

Moringa leaves possess many medicinal uses. Earlier work in our lab has shown that aqueous extract of *Moringa* leaves corrected hyperglycemia, hyperlipidemia in both type 1 and type 2 diabetic rat models (Sai Mangala, 2012). The leaves possess strong antioxidant and radical scavenging activities and enhance the process of spermatogenesis in mice (Lilibeth, 2010), inhibit the growth of pathogenic microorganisms (Caceres, 1991), reported to be useful in treating hyperthyroidism (Tahiliani, 2000).

As a result of scientific evidence, *M. oleifera* has gained significance as a natural source of phytochemicals.

MATERIALS AND METHODS

Chemicals

Thiobarbituric acid, pyrogallol, Streptozotocin were obtained from Sigma Chemical Co., St Louis, MO, USA. All other chemicals and solvents were procured from Sisco Research Laboratories (p) Ltd., Mumbai. India.

Plant material

Aqueous Extract of *Moringa oleifera* leaf (AEMO) powder (Product Code P/DSM/MOOL-01, Batch Number P8060947) was purchased from Chemiloids (manufactures and exporters of herbal extracts, Vijayawada, Andhra Pradesh, India).

Animals

Male albino Wistar rats of age 4-5 weeks with a body weight of 150-160 g were procured from Sri Venkteswara Enterprises, Banglore and acclimatized for 7 days to animal house maintained at a temperature of 22 ±2ºC.The study was approved by Animal Ethics Committee of Sri Krishadevaraya University, Anantapur (*Reg. no. 470/01/a/CPCSEA*). The animal room was regulated by a 12/12 h light/ dark schedule. Two animals were housed per cage. All rats were fed on a standard pellet diet and water.

Induction of diabetes

Diabetes was induced in rats by a single intraperitoneal injection of freshly prepared Streptozotocin (STZ) with a dosage of 55 mg/ kg body weight, in 0.05 M citrate buffer pH 4.5 in a volume of 0.1 ml. STZ was first weighed individually in Eppendorf tubes for each animal according to the bodyweight and then solubilized in buffer just prior to injection. Seventy-two hours after STZ administration, plasma glucose level of each rat was determined for confirmation of diabetes. They were allowed for a window period of 5 days before commencement of treatment. Rats with fasting plasma glucose greater than 300 mg/dl were considered diabetic and included in the present study.

Experimental Design

About 12 rats were made diabetic by STZ injection and 12 rats served as controls and all rats were maintained on standard pellet diet. Each set of animals (Control and type-1 DM) was further subdivided into two groups thus comprising a total of four groups: control group (C-group), control rats administered with AEMO (C+MO-group), STZ diabetic group (D-group) and STZ diabetic rats administered with AEMO (D+MO group). Rats in the Groups-C+MO and D+MO were administered with the aqueous extract of *M. oleifera* leaf at a dose of 200 mg/kg body weight through gastric intubation for a period of 60 days. At the end of experimental period the rats were sacrificed by cervical dislocation. Immediately after scarification the rats were dissected and the testes removed and weighed and saved for further study.

Relative testicular weight.

The relative weight of testes was calculated from body weight and absolute testes weight.

Oxidative stress markers and Antioxidant Enzymes

A 10% tissue homogenate was prepared in 0.15 M KCl using pestle and mortar at 4^0C. The extent of lipid peroxidation (LPO) was determined by assaying malondialdehyde (MDA) formation according to method of Utley et al.(1967). Total reduced glutathione (GSH) content was measured following the method of Ellman's (1959). The protein con

tent of the homogenate was estimated by the method of Lowry et al (1951).

A portion of the homogenate was centrifuged at 4°C in Eppendorf centrifuge at 12,000 rpm for 45 minutes. The clear supernatant was used for the assay of Glutathione reductase (GR; E.C 1.6.4.2); Pinto and Bartley (1969), Glutathione-S-transferase (GST; E.C 2.5.1.18) Habig et al.(1974), Catalase (CAT; E.C 1.11.1.6); Beers and Sizer (1952), Superoxide dismutase (SOD; E.C 1.15.1.1); Soon and Tan (2002), and Sorbitol dehydrogenase (SDH; E.C 1.1.1.14); Asada and Galambos (1963) .

Histological study

Immediately after separation, the testes were weighed and fixed in 10% formalin and later were embedded in paraffin. Sections of 3 microns thickness were cut from tissue blocks by microtome. Using a heated tissue separator (water bath), the sections were uniformly separated on a glass slide and drained. Harris' Hematoxylin and Eosin stain was used for staining the tissue sections following the procedure from the Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology (1960).

Statistical Analysis

The results were expressed as means ±S.E.M. Data were analysed for significant differences using Duncan's Multiple Range (DMR) test ($p<0.05$) Duncan(1955).

RESULTS

Table 1 shows the body weight, absolute and relative testes weights of the experimental groups. D group showed significantly decreased relative testes weight by 30.6% compared to control. The relative organ weights of C+MO did not deviate from that of control. However, administration of AEMO restored the altered relative testes weight observed in D group to near normal values.

Table 1: Effect of AEMO on the relative organ weight of testes in experimental rats.

Group→	C	C+MO	D	D+MO
Body weight(g)	278 ± 2.29^{a}	277 ± 2.29^{a}	135.0 ± 2.0^{b}	221.0 ± 1.68^{c}
Testes weight(g) (Absolute)	3.46 ± 0.07^{a}	3.45 ± 0.06^{a}	1.17 ± 0.03^{c}	2.66 ± 0.08^{d}
Relative weight	1.24 ± 0.02^{a}	1.25 ± 0.02^{a}	0.87 ± 0.01^{d}	1.20 ± 0.02^{ac}

Values are mean ± S.E.M., (n= 6 animals). Values with different superscripts with in the row are significantly different at $p<0.05$(Duncan's Multiple Range Test).

The extent of LPO and the levels of GSH, GR and GST are shown in table 2. The activities of SDH, SOD and CAT are shown in table 3. D group showed 68.1% increase in LPO and 59.9% decrease in GSH levels compared to C group. The activities of antioxidant enzymes GR and GST were decreased by 49.6 and 37.9 % respectively and SOD and CAT were decreased by 53.5 and 60.0% respectively in D group compared to C. In contrast, the activity of SDH, the polyol pathway enzyme, was increased by 75.8% in D group compared to C. Administration of AEMO for 60 days resulted in a decreased LPO with a recovery of 72% in D+MO group and the GSH levels were restored to normal with 100% recovery. There was 75% and 97.5% recovery in GR and GST activities and 82.3 and 71.8 and 70.5% recovery in SOD, CAT and SDH activities respectively (Fig-1). The control group which received the AEMO also showed a significantly increased GSH, increased activities of GR, SOD and CAT and decreased LPO.

Table 2: Effect of AEMO administration on LPO, GSH, GR and GST in testes of experimental rats.

Group	C	C+MO	D	D+MO
LPO(A)	10.3 ± 0.10^{a}	8.8 ± 0.08^{b}	17.2 ± 0.14^{c}	12.2 ± 0.10^{d}
GSH(B)	3.6 ± 0.02^{a}	4.3 ± 0.01^{b}	1.4 ± 0.01^{c}	3.6 ± 0.02^{a}
GR (C)	6.0 ± 0.02^{a}	6.4 ± 0.01^{b}	3.0 ± 0.04^{c}	5.3 ± 0.01^{d}
GST(D)	215.8 ± 0.54^{a}	214.3 ± 0.64^{a}	133.8 ± 0.85^{b}	213.9 ± 0.34^{a}

A:n mol MDA formed/min/mg protein; B:µg/mg protein; C: µmol NADPH oxidised/min/mg protein; D: µmol CDNB-GSH conjugate formed/min/mg protein.Values are mean±S.E.M., (n= 6 animals). Values with different superscripts with in the row are significantly different at $p<0.05$(Duncan's Multiple Range Test).

The photomicrograph of section of testes of experimental rats is shown in Fig. 2(a—d). The control rat testis shows normal histological picture. The seminiferous tubules (ST) are uniformly arranged with well-defined interstitial tissue (IT). The spermatogenesis pattern appears to be normal with fully matured sperms in the centre of ST. C+MO group also shows normal picture of testis. The photomicrograph of sections of testes of diabetic rats shows pathological changes. The size of the ST in D group is smaller when compared to C group. There is a moderate degeneration of ST with increased lumen size. Many ST are empty with very few/no spermatozoa. The ST appears isolated with the massive edema of interstitial tissue. The diabetic group administered with AEMO shows improved relative testicular weight and the photomicrograph of sections of D+MO group shows normal picture with intact ST and improved interstitial tissue compared to D group. The ST show good number of spermatozoa in the lumen and different stages of spermatogenesis are seen.

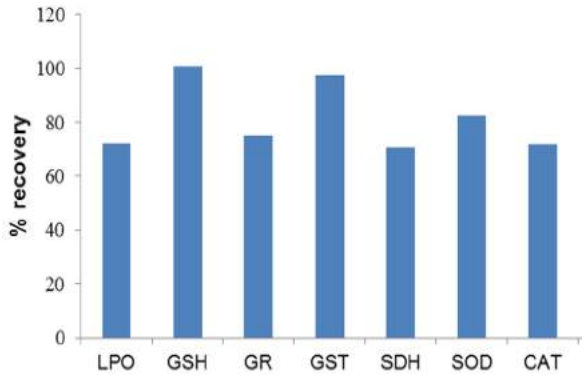

Figure 1: Percent recovery in LPO, GSH levels and antioxidant enzyme activities in testes of experimental rats.

DISCUSSION

Exposure to environmental toxins (Jacobson and Miller, 1998), X-irradiation (Manda et al, 2007) and high concentrations of certain metals (Wellejus et al, 2000) have been shown to increase the testicular oxidative stress. Studies have shown increased concentration of ROS and oxidative stress in testes of STZ diabetic rats (Thyagaraju et al., 2008). The diabetes related testicular dysfunction has been attributed to lack of insulin. The regulatory action of insulin on Leydig cells (Khan et al; 1992) and sertoli cells (Mita et al; 1985) has been reported. Mechanisms that contribute to increased oxidative stress in diabetes may include not only the persistent hyperglycemia, increased non-enzymatic glycosylation (glycation) and autooxidative glycosylation but also metabolic stress and the status of antioxidant defense systems. The oxidative stress in testes leads to an increase in germ cell apoptosis (Samanta and Chainy, 1997) which may decrease spermatogenesis. Oxidative stress has been reported to be associated with severe changes in structure and function of testes, two weeks after the onset of diabetes (Maiorino and Ursini, 2002).

Table 3: Effect of AEMO administration on the activities of SDH, SOD and CAT in testes of experimental rats

Group→	**C**	**C+MO**	**D**	**D+MO**
SDH(A)	4.9 ± 0.02^{a}	5.0 ± 0.04^{b}	8.6 ± 0.04^{c}	$5.9\pm0.02^{\mathbf{d}}$
SOD(B)	22.2 ± 0.25^{a}	23.9 ± 0.20^{b}	10.3 ± 0.11^{c}	20.1 ± 0.13^{d}
CAT(C)	1.7 ± 0.02^{a}	1.8 ± 0.02^{b}	0.69 ± 0.003^{c}	$1.43\pm0.01^{\mathbf{d}}$

A: µmol NADH oxidised/min/mg protein, B: units/mg protein, C: µmol of H_2O_2 consumed/min/mg protein. Values are mean ± S.E.M., (n= 6 animals). Values with different superscripts with in the row are significantly different at $p<0.05$(Duncan's Multiple Range Test).

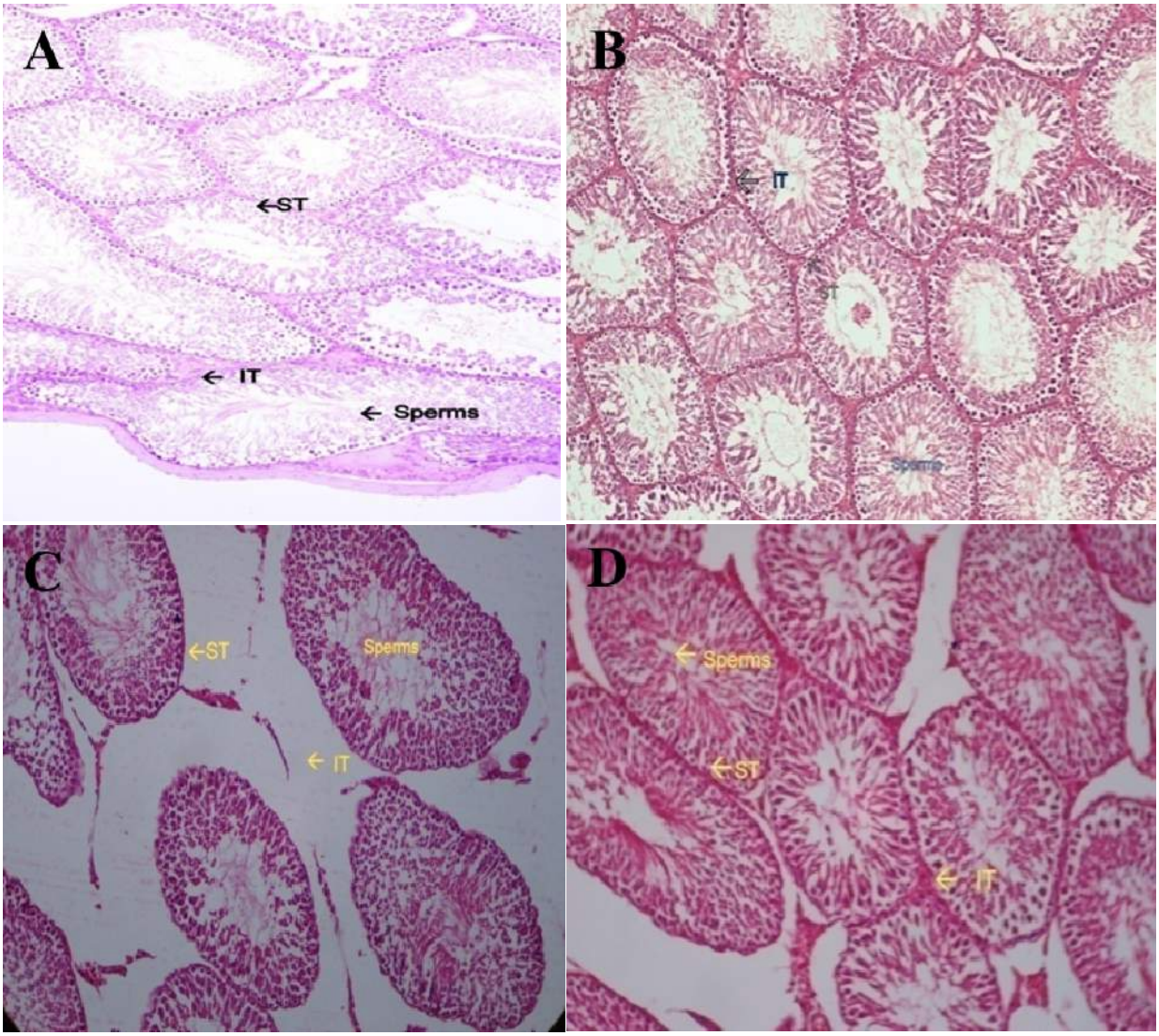

Figure 2: A. C-group (Mag x200). **B.** C+MO group (Mag x200). **C.** D group (Mag x200). **D.** D+MO group (Mag x200). ST: Seminiferous tubule; IT: Interstitial Tissue.

In the present study an increased LPO was observed in testes of D group but administration of AEMO prevented the increased tissue LPO in D+MO rats. The lipid lowering and insulin sensitizing effects of AEMO along with its anti-hyperglycemic affect (Sai Mangala et al., 2012) could have inhibited LPO generation in D+MO group. Age related testicular oxidative stress is documented (Syntin et al., 2001). In this study the C+MO group showed decreased LPO compared to C group which clearly indicates that AEMO prevented the age related oxidative stress too. In vitro and *ex-vivo* studies have revealed that the water extract of *M. oleifera* leaf significantly inhibited TBARS formation in $CuSo_4$ induced rabbit and human LDL oxidation (Pilaipark, 2008).The lyophilized hydroalcoholic extract of *M. oleifera* leaf prevented the increase in lipid peroxidation in rats with Isoproterenol induced myocardial damage (Nandave, 2009).

In the current study enhanced activity of SDH, a polyol pathway enzyme, has led to the depletion of GSH content with subsequent decreased activities of glutathione dependent en-

zymes GR and GST in D group. Polyol pathway leads to depletion of $NADPH_2$ which is required by GR to regenerate GSH. Depleted plasma GSH and tissue GSH is well documented in type 1 diabetic animal models (Venkateswaran and Pari, 2003: Uma Bhandari and Ansari 2008). Decreased activity of GST in D group could also be explained by the low content of GSH since GSH is a substrate and cofactor of GST (Domingues, 1998). However, AEMO treatment activated the compensatory mechanism against the oxidative stress which was reflected by enhanced activities of GR and GST in D+MO group.

SOD and CAT are widely distributed in all animal cells. SOD a Cu/Zn containing enzyme is a major defense for aerobic cells in combating the toxic effect of superoxide radicals (Mc Cord, 1969). Catalase, a haemoprotein, reduces H_2O_2 produced by dismutation reaction and prevents generation of hydroxyl radical. D group showed decreased activities of SOD and CAT when compared to C group. Restoration of SOD and CAT activities in D+MO reveal the scavenging activity and the beneficial action of AEMO against pathological alterations caused by the presence of superoxide radical and H_2O_2. Earlier studies also indicated decreased activities of antioxidants both GSH dependant and GSH independent in STZ diabetic rats (Li, 2007). The antioxidant and radical scavenging properties of *M. oleifera* leaf are well documented (Pari et al., 2007; Atawodi, 2010).

In the present study diabetic rats showed abnormal histological alterations of testes which are consistent with those of previous studies (Adewole et al., 2007). Clinical and experimental studies have shown that diabetes mellitus has adverse effects on male sexual and reproductive functions in humans and animals (Penson, 2004; Ricci et al., 2009). Impairment of spermatogenesis, reduced sperm count, sperm motility, seminal fluid volume and low testosterone levels were found in diabetic subjects (Sexton, 1997). Testosterone which is produced in testes is required for normal sperm development. Many conditions like cryptorchidism (Chaki et al.,2005), ageing (Zirkin and Chaen, 2000) and IR injury (Turner et al., 2005) have shown increased ROS production and oxidative stress resulting in acute reduction of testicular testosterone production and increased germ cell apoptosis.

Apoptosis, a programmed cell death, is a form of cell death that serves to eliminate dying cells in proliferating or differentiating cell population. Apoptosis control is critical for normal spermatogenesis in the adult testes (Sinha, 1999). The oxidative stress is recognized as a strong mediator of apoptosis. Diabetic condition was reported to enhance apoptosis of germ cells (Guneli et al., 2008) and also increased LPO was reported to impair membrane functions by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors(Chen et al., 2002). Thus in the current study the increased oxidative stress with compromised antioxidant status has led to the tissue damage in D group. However, the administration of AEMO improved the histological picture with concurrent enhanced spermatogenesis in D+MO group. Lilibeth (2010) reported that the administration of hexane extract of *M. oleifera* enhanced male reproduction in mice. Some herbs like *Tribulus terresteris* also showed beneficial effects and improved testicular function in rats (Esfandiari, 2010). Numerous epidemiological studies suggest that herbs/diets rich in phytochemicals and antiox-

idants execute a protective role in health and disease (Vinson et al., 2001).

Thus the results of the present study clearly show that AEMO exhibited protective role with its enhanced radical scavenging activity and antioxidant potential and exerted beneficial action against pathological alterations in testes caused by oxidative stress under insulin deficient condition. Further studies can be done on the benefits of *Moringa* leaf as an antioxidant in the treatment of male infertility.

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