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Ameliorative Effect of Aqueous Leaf Extract of *Moringa oleifera* on Reproductive Function Following Cadmium Chloride Induced Oxidative Stress in Male Wistar Rats

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Abstract

Cadmium disrupts the blood-testes barrier, interferes with various antioxidant levels thus enhancing lipid peroxidation and ultimately leading to apoptosis and necrosis of testicular tissue. *Moringa oleifera* is a medicinal plant and a rich source of essential phytochemicals possessing antioxidant properties. The effect of aqueous leaf extract of *M. oleifera* on reproductive function following cadmium chloride induced oxidative stress in male Wistar rats was investigated. Forty adult male Wistar rats were assigned into five groups of eight rats each. Treatment was administered orally daily as follows: Group 1 (control): animal feed and tap water *ad libitum*; Group 2: 5 mg kg^{-1} cadmium chloride for 21 days; Group 3: 500 mg kg^{-1} of *M. oleifera* and 5 mg kg^{-1} of cadmium chloride for 21 days; Group 4: 5 mg kg^{-1} cadmium chloride for 21 days followed by 500 mg kg^{-1} *M. oleifera* for the next 35 days; Group 5: 5 mg kg^{-1} cadmium chloride for 21 days followed by 750 mg kg^{-1} *M. oleifera* for the next 35 days. At the end of treatment, blood was obtained by direct cardiac puncture for fertility hormone assay and testicular tissue specimens were harvested for semen analysis and determination of antioxidant levels. Results obtained indicated that rats treated with the various extracts had significantly increased superoxide dismutase, malondialdehyde and catalase levels, increased serum concentrations of testosterone, follicle stimulating hormone and luteinizing hormone and increased percentage of viable and normal spermatozoa compared to control and only cadmium chloride treated rats ($p < 0.05$). The results obtained suggest that treatments with *M. oleifera* extract could ameliorate possible cellular damage caused by administration of cadmium chloride.

Keywords: alleviated; antioxidant; medicinal; necrosis; peroxidation

Introduction

Moringa oleifera is a plant that belongs to the monogeneric family Moringaceae and is commonly known as horseradish tree, drumstick tree or Ben oil tree (Mishra *et al*., 2011; Ayman *et al*., 2016). The use of medicinal plants in most developing nations is a process that has attracted more concern among health workers and researchers (Otitoju *et al*., 2014) due to the noticeable shift to herbal therapy even among the elites (Zade *et al*., 2013). Many studies have validated the usefulness of *Moringa* as a medicinal herb. For instance, Foidl *et al*. (2001) showed that *Moringa* possesses many valuable properties which make it of great scientific interest. Its rich nutritive value is due to the presence of a variety of essential phytochemicals in its leaves, pods and seeds, and as such every part of the plant can be said to be a store house of nutrients (Gopalakrishnan *et al*., 2016).

Infertility is apparently on the increase: about 15% of couples attempting to conceive are unable to do so within a year of unprotected coital exposure (Zeba *et al*., 2011). Over 20% of infertility in couples is attributed to the male factor (Jarow *et al*., 2002) and oxidative stress has been reported to be a contributory factor (Agarwal *et al*., 2014). Oxidative stress is described as a state associated with increased cellular damage triggered by oxygen and oxygen-derived free radicals known as reactive oxygen species (ROS) (Agarwal *et al*., 2014). In the male reproductive system, this leads to several abnormalities due to the ability of these ROS to cross the blood-testes barrier, thereby leading to testicular cell damage, resulting to decreased spermatogenesis and increased sperm damage (Adebayo *et al*., 2010; Singh *et al*., 2014), impotence or erectile dysfunction (ED), orgasmic difficulties, in addition to reduced libido and retrograde ejaculation, amongst others (Brown *et al*., 2005; Bhasin *et al*., 2007).

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Cadmium is a known reproductive toxicant that accumulates mostly in the testes (Amara *et al.*, 2008). Cadmium has been shown to have adverse effect on gonadal development. For instance, in mouse embryos, reduced genital ridge size, retarded migration of germ cells, aberrant maturation of gamete and sub-fertility have been described after administration of cadmium (Thompson and Bannigan, 2008). Increased accumulation of cadmium in the testes results in oxidative stress, which can be measured using an atomic absorption spectroscopy technique to confirm the presence of hyper chromatic cadmium precipitates in histological sections of the seminiferous tubules of adult male mice treated with cadmium (Amara *et al.*, 2008).

From the aforementioned conditions, it is evident that male sexual function and reproductive activities that leads to fertilization of the female gamete can be grossly impaired due to the toxic effect of cadmium. The aim of the present study is to attempt an evaluation of the effects of *Moringa oleifera* on reproductive functions following cadmium chloride induced oxidative stress using male Wistar rats as models.

Materials and Methods

Plant material and preparation of extract

The *Moringa oleifera* leaves for the study were procured from the herbarium of the University of Port Harcourt, Nigeria and were identified by Dr. C. Ekeke of the Department of Plant Science and Biotechnology of the same institution. The leaves were rinsed in clean tap water to wash away sand and other contaminants. Leaves were then properly air dried and grounded into fine powdered form using a grinding mill. 750 g of the fine powdered *M. oleifera* leaves were soaked in distilled water at room temperature for two days in Soxhlet apparatus. The resulting solution was then filtered into a conical flask with Whatman filter paper number one. The filtrate was hauled together and lypholized using a freeze dryer. The yield aqueous extract was stored at temperature of -12 °C until ready for use.

Acute toxicity study (LD_{50})

The acute toxicity for aqueous leaf extract of *M. oleifera* was determined using the method of Lorke (1989). The LD_{50} value was previously determined in the laboratory to be > 1,000 mg kg^{-1} bw (Ojeka *et al.*, 2016).

Induction of oxidative stress

The method of induction of oxidative stress (El-Demerdash *et al.*, 2004) was followed: 99% pure cadmium chloride (L231151707) was dissolved in saline solution (0.9% NaCl) and administered a dose of 5 mg kg^{-1} bw orally by gavage.

Experimental procedure

Forty male Wistar rats weighing 180-200 g were used. They were divided into five groups (Groups 1-5) consisting of eight rats each. Rats were housed in separate cages in the Animal House of the University of Port Harcourt, Nigeria, under natural day and night cycles at normal room temperature (25-27 °C) and fed *ad libitum* with standard animal feed. They were allowed to acclimatize to their environment for two weeks before experimentation. The rats were subsequently treated as follows:

Group 1: Control group; rats were given only animal feed and tap water *ad libitum*.

Group 2: Positive control group; rats were given 5 mg kg^{-1} bw of cadmium chloride daily for 21 days.

Group 3: Pre-treatment group; rats were given 500 mg kg^{-1} bw of *M. oleifera* followed by 5 mg kg^{-1} bw cadmium chloride 1 hour later, for 21 days.

Group 4: Post-treatment group I; rats were given cadmium chloride 5 mg kg^{-1} bw once daily for 21 days then treated with *M. oleifera* 500 mg kg^{-1} bw for the next 35 days.

Group 5: Post-treatment group II; rats were given cadmium chloride 5 mg kg^{-1} bw once daily for 21 days then treated with *M. oleifera* 750 mg kg^{-1} bw for the next 35 days.

The aqueous leaf extract of *M. oleifera* and cadmium chloride were administered daily using an oral gavage. All rats were treated for the duration as indicated. At the end of the treatment, rats were anaesthetized with chloroform and blood samples were collected by direct cardiac puncture to determine testosterone, follicle stimulating hormone and luteinizing hormone concentrations. The animals were then scarified and the testes immediately harvested for determination of testicular antioxidant level, testicular histology and sperm parameters: sperm count, sperm motility, sperm viability, sperm morphology.

Determination of plasma concentration of reproductive hormones

Serum was obtained by centrifugation of blood at 3,000 rpm for 15 min and stored at -8 °C until ready for analysis. Plasma testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) concentrations were measured by Enzyme-Linked Immunosorbent Assay (ELISA) using specific professional kits, following the procedures described within.

Determination of sperm parameters

Sperm count

As described by Prasad *et al.* (1972), 100 mg of caudal epididymis were minced in 5 ml of normal saline. A drop of the evenly mixed sample was applied to a Neubauer chamber. Counting of both motile and immotile spermatozoa was done per unit area. Values were recorded as millions/mL.

Motility

The caudal epididymis was identified and its content squeezed into 1 ml of normal saline at room temperature (Kaur and Bansal, 2004). One drop of semen suspension was charged into a Makler counting chamber and the number of motile and non-motile spermatocytes was then expressed as a percentage of the total number of the counted spermatocyte (Mahaneem *et al.*, 2011).

Sperm viability

To determine sperm viability, 40 µl of freshly liquefied semen was thoroughly mixed with 10 µl of eosin-nigrosin and 1 drop of this mixture was transferred to a clean slide. At least 200 sperms were counted at a magnification of x 100 under oil immersion. Sperms that were stained pink or red were considered dead and those unstained were considered viable (Raji *et al*., 2003; Kisa *et al*., 2004).

Determination of testicular antioxidants

The left testis was divided into equal parts and stored at -20 °C after freezing in liquid nitrogen. The glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MDA) levels in the testis were determined using commercially available kits and in accordance with the manufacturer instructions.

Testicular histology

The testes of all rats were fixed in 10% formalin, dehydrated stepwise in graded ethanol, cleared in xylene and then embedded in paraffin wax. A section of 5 µ thickness paraffin section was taken from the mid portion of each testicular tissue and stained with haematoxylin and eosin, followed by examination under a light microscope at x 400 magnification. All photomicrographs were interpreted as appropriate by a pathologist with requisite experience.

Statistical analysis

Results were analyzed using one-way analysis of variance (ANOVA) and LSD's post-hoc test with the aid of Statistical Package for Social Science version 20 (IBM SPSS20). The results presented in Tables 1, 2 and 3 are expressed as mean ± SEM with the level of significance set at 95% ($p < 0.05$).

Results

Table 1 shows the effect of administration of aqueous leave extract of *Moringa oleifera* on oxidative stress markers. Malondialdehyde (MDA) level of groups 2 and 5 were significantly higher compared to group 1 ($p < 0.05$). Groups 3 and 4 had significantly lower MDA level in comparison to Group 2 ($p < 0.05$). Superoxide dismutase (SOD) level of Groups 2 and 5 were significantly lower than Group 1 ($p < 0.05$). Groups 3 and 4 had SOD levels significantly higher than Group 2 ($p < 0.05$). Group 3 showed a significant increase in catalase (CAT) level in comparison to group 1; however, group 3 CAT level was significantly lower than that of group 2. Group 5 showed a significant increase in CAT level in comparison to group 2 ($p < 0.05$). Glutathione (GSH) levels were significantly decreased in groups 2 and 4 compared to group 1 ($p < 0.05$).

Table 2 shows the effects of administration of aqueous leave extract of *M. oleifera* on reproductive hormone concentrations. Serum testosterone levels were significantly increased in groups 3 and 4 in comparison to groups 1 and 2 ($p < 0.05$). Serum luteinizing hormone (LH) levels of Group 5 were significantly higher than groups 1 and 2 ($p < 0.05$). Serum follicle stimulating hormone (FSH) levels of group 5 were significantly higher than both groups 1 and 2 ($p < 0.05$).

Table 3 shows the results of the effects of administration of the aqueous leave extract of *M. oleifera* on sperm parameters. There were no significant differences in sperm count and percentage of sperm motility across the various groups. However, the percentage of sperm viability was significantly higher in groups 3 and 4 compared to group 2 ($p < 0.05$). The percentage of normal sperm cells was significantly higher in group 4 compared to group 2 ($p < 0.05$), while the percentage of abnormal sperm cells were significantly higher amongst group 3 compared to group 1 ($p < 0.05$).

Figs. 1 to 5 are photomicrographs of transverse sections of typical testicular tissue from rats of groups 1 to 5 respectively.

Table 1. Effect of aqueous leaf extract of *Moringa oleifera* on oxidative stress markers

	Group 1: Control	Group 2: Positive control	Group 3: Pre-treatment	Group 4: Post-treatment I	Group 5: Post-treatment II
Malondialdehyde (mmol/g)	0.17 ± 0.02	0.44 ± 0.03 [1]	0.27 ± 0.02 [2]	0.21 ± 0.04 [2]	0.48 ± 0.07 [1]
Superoxide dismutase (mmol/g)	0.90 ± 0.03	0.38 ±0.07 [1]	0.80 ± 0.07 [2]	0.85 ±0.03 [2]	0.52 ± 0.12 [1]
Catalase (mmol/g)	2.11 ± 0.17	2.23 ± 0.15	2.21 ± 0.21 [1,2]	3.15 ± 0.42	3.83 ± 0.16 [2]
Glutathione (ng/g)	2.91 ± 0.11	2.57 ± 0.13 [1]	2.71 ± 0.97	2.47 ± 0.08 [1]	2.70 ± 0.50

[1] and [2] indicates significantly different from control and positive control respectively

Table 2. Effect of aqueous leaf extract of *Moringa oleifera* on reproductive hormone concentration

	Group 1: Control	Group 2: Positive control	Group 3: Pre-treatment	Group 4: Post-treatment I	Group 5: Post-treatment II
Testosterone (ng/mL)	1.07 ± 0.27	1.09 ± 0.11	2.40 ± 0.25 [1,2]	2.61 ±0.63 [1,2]	1.77 ±0.56
Luteinizing hormone (mIU/mL)	0.74 ± 0.09	0.62 ± 0.14	1.41 ± 0.16	0.76 ± 0.15	2.67 ± 0.56 [1,2]
Follicle stimulating hormone (mIU/mL)	0.50 ± 0.12	0.52 ± 0.06	0.68 ± 0.04	0.69 ± 0.05	1.23 ± 0.64 [1,2]

[1] and [2] indicates significantly different from control and positive control respectively

Table 3. Effect of aqueous leaf extract of *Moringa oleifera* on sperm parameters

	Group 1: Control	Group 2: Positive control	Group 3: Pre-treatment	Group 4: Post-treatment I	Group 5: Post-treatment II
Spermatocyte count (million/mL)	610.00 ± 102.96	550.00 ± 124.50	730.00 ± 71.76	580.00 ± 147.14	560.00 ± 120.83
Percentage motility (%)	69.00 ± 3.67	62.00 ± 3.74	71.00 ± 4.58	62.00 ± 5.83	63.00 ± 4.90
Percentage viability (%)	76.00 ± 3.67	66.00 ± 2.91	82.00 ± 3.39 [2]	77.00 ± 3.39 [2]	76.00 ± 3.67
Percentage normal spermatocytes (%)	72.00 ± 2.55	66.00 ± 2.92	65.00 ± 2.73	76.00 ±3.67 [2]	68.00 ± 3.39
Percentage abnormal spermatocytes (%)	28.00 ± 2.55	34.00 ± 2.92	38.60 ± 4.52 [1]	24.00 ± 3.67	32.00 ± 3.39

[1] and [2] indicates significantly different from control and positive control respectively

Results show that rats in groups 1, 4 and 5 have normal testicular tissue containing spermatogenic cells and mature spermatozoa. However, slides from groups 2 and 3 showed histologically distorted testicular tissue with seminiferous tubules containing vacuoles and an absence of mature spermatozoa with flagella. Comparatively, rats in group 3 pre-treated with *M. oleifera* before administration of cadmium chloride had much less immature forms of spermatozoa than rats in group 2 treated only with cadmium chloride.

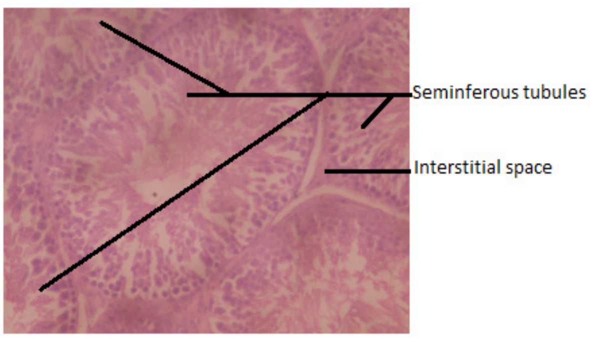

Fig. 1. Photomicrograph of transverse section of typical testicular tissue from group 1 rats (x400 magnification; H&E) Slide show normal testicular tissue containing spermatogenic cells and mature spermatozoa. Interstitial cells of Leydig are essentially normal

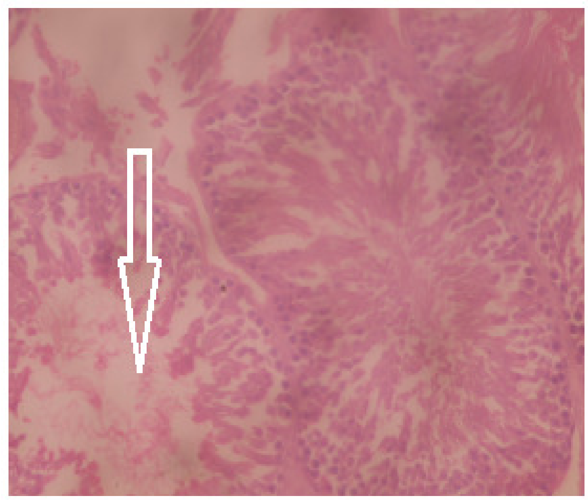

Fig. 2. Photomicrograph of transverse section of typical testicular tissue from group 2 rats (x400 magnification; H&E) Slide shows histologically distorted testicular tissue. Seminiferous tubule contains vacuoles with absent of mature spermatozoa with flagella

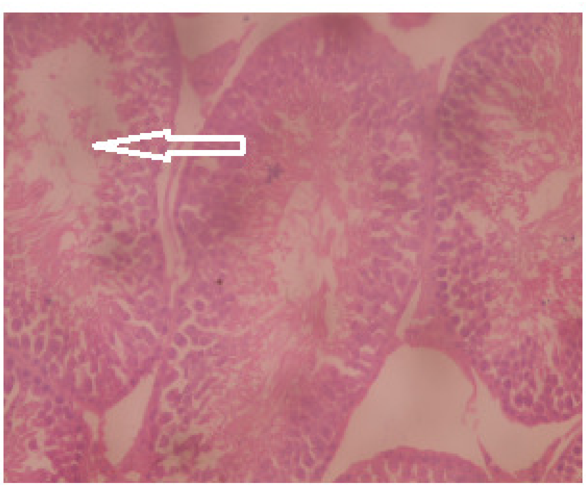

Fig. 3. Photomicrograph of transverse section of typical testicular tissue from group 3 rats (x400 magnification; H&E) Slide shows histologically distorted testicular tissue. Seminiferous tubule contains vacuole with relative absence of mature spermatozoa as compared to Fig. 2

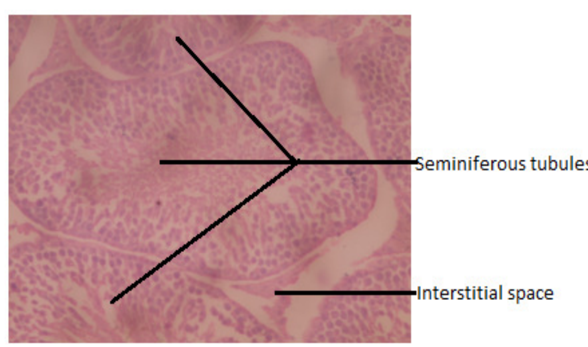

Fig. 4. Photomicrograph of transverse section of typical testicular tissue from group 4 rats (x400 magnification; H&E) Slide show essentially normal testicular tissue containing spermatogenic cells and mature spermatozoa

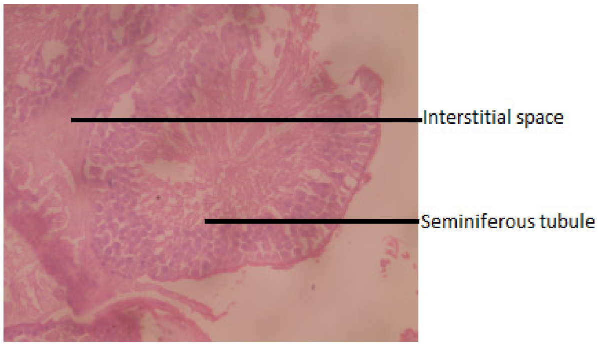

Fig. 5. Photomicrograph of transverse section of typical testicular tissue from group 5 rats (x400 magnification; H&E) Slide shows a normal testicular tissue with essentially normal interstitial cells of Leydig

Discussion

The study evaluated the effect of aqueous leaf extract of *M. oleifera* on some reproductive functions following cadmium chloride induced oxidative stress in male Wistar rats. The results indicate that malondialdehyde levels of groups 2 and 5 rats were significantly higher than group 1 rats, suggesting an alteration in antioxidant levels in rats treated with cadmium chloride. This is similar to the report by El-Sheikh *et al.* (2016) in which malondialdehyde levels was significantly increased in rats treated with paroxetine to induce toxicity. The present results further show a significant decrease in malondialdehyde levels of groups 3 and 4 rats in comparison to group 2 rats, indicating a possible protective capacity of the leaf extract of *M. oleifera.* The significant decrease in malondialdehyde levels seen in the study, amongst groups 3 and 4 compared to group 2 rats, may be attributable to the carotenoids and flavonoids present in *M. oleifera* leaf extract and may be a possible factor contributing to improvement of testicular function earlier described (Afolabi *et al*., 2013). The hereby findings are similar to that of Morakinyo *et al.* (2008) who reported a significant reduction in testicular malondialdehyde level upon administration of aqueous extract of *Zingiber officinale* to experimental rats. Furthermore, *M. oleifera* treated groups 3 and 4 rats had significantly higher values of superoxide dismutase compared to the positive control group 2, suggesting that the extract of *M. oleifera* has the potential to ameliorate the toxic effect of cadmium: this result is comparable with the report of Afolabi *et al.* (2013).

The significantly higher testosterone concentration seen among *M. oleifera* treated groups 3 and 4 rats in comparison to groups 1 and 2 is consistent with the report of Sabha (2016), who described an increased serum testosterone concentration following treatment with *M. oleifera* plant extracts. Further, consistent with the study by Kumar *et al.* (2006), zinc- an important constituent of *M. oleifera*, has been implicated with increasing levels of testosterone in experimental animals following zinc supplementation. Zinc is an important component of proteins involved in testosterone synthesis and secretion (Syarifuddin, 2017). Gonadotropins (follicle stimulating hormone and Luteinizing hormone) are glycoprotein hormones secreted by basophilic cells of the anterior pituitary gland; their secretion is stimulated by gonadotropin releasing hormone (GnRH) secreted by the hypothalamus (Pierce and Parsons, 1981). The secretion of both gonadotropins was markedly increased in group 5 rats, possibly due to the presence of the potent antioxidants: vanillin, tannins, phenolics and especially flavonoids and carotenoids found in *M. oleifera* leaves, which have the ability to alter androgen levels (Gopalakrishnan, 2016). The increased secretion of gonadotropins is consistent with previous reports of Nwamarah *et al.* (2015) and Manhal *et al.* (2017).

The decreased secretion of reproductive hormones following administration of cadmium chloride is an indication of its potential toxic effects either direct on the male gonads or via an indirect effect on the anterior pituitary gland responsible for secretion and release of androgens (Inass *et al*., 2005). However, the increase in the secretion of testosterone, follicle stimulating hormone and luteinizing hormone following treatment with the extract of *M. oleifera* may be inferred to be as a result of the presence of potent antioxidants, especially flavonoids and carotenoids in the extract; these antioxidants could increase the level of secretion of reproductive hormones (Inass *et al*., 2005). The increase in reproductive hormone secretion amongst *M. oleifera* treated rats is consistent with the study of Syarifuddin *et al.* (2017); they reported increased testosterone levels in male rats treated with *M. oleifera* leading to a possible fertility improvement with enhanced spermatogenesis, sperm motility and morphology and an increased libido.

In the present report, no significant changes were noted in other sperm parameters except for an increase in the percentage of sperm viability and the percentage of normal spermatocytes seen amongst group 4 rats. Results obtained from histological analysis essentially confirmed the histological basis for the ameliorative effect of *M. oleifera* leaf extract on reproductive function in male Wistar rats and corroborates with the report of El-Sheikh *et al.* (2016).

Conclusions

Exposure to cadmium causes depletion in levels of natural antioxidants and reactive oxygen species hence oxidative stress. The current study suggests that treatment with aqueous leaf extract of *Moringa oleifera* could ameliorate possible cellular damages caused by exposure to cadmium. We recommend studies to further elucidate the probable ameliorative effects of *M. oleifera* on reproductive function.

Conflict of Interest

The authors declare that there are no conflicts of interest related to this article.

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