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# Aqueous peel extract of Citrus aurantifolia attenuated tramadol-mediated testicular oxidative stress, apoptosis, inflammations and histo-architectural degeneration in a rat model

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**SUMMARY**

Tramadol is one of the most commonly abused drugs. It has been reported to impair testicular function in models. Citrus aurantifolia is a citrus fruit used extensively in beverages and herbal preparations. This study investigated the modulating role of aqueous zest extract of Citrus aurantifolia (AZECA) against tramadol-mediated testicular toxicity in rat. Twenty-five male rats were assigned to 5 groups and orally treated with distilled water (negative control) for 28 days, 30mg/kg of tramadol (positive control) for 14 days, 30 mg/kg of tramadol for first two weeks and 50 (treatment 1) and 100 mg/kg (treatment 1) of AZECA for the last 2 weeks. Biochemical markers were evaluated and testes were stained routinely for morphometry. Results shows a significant ($p<0.05$) decrease in testosterone, FSH and LH, SOD, CAT and a significant increase MDA, IL-1 and TNF-∝ evidenced by a significant ($p<0.05$) reduction in volumetric proportion of interstitium and seminiferous tubules-tunica propria, macrophages, blood vessels and lymphatic space, macrophages, connective tissue, diameter, cross-sectional area and number of profiles of seminiferous tubules per unit area in group treated with tramadol-alone. These parameters were significantly ($p<0.05$) negated in groups that had AZECA supplementation. This study has demonstrated that treatment with AZECA exerted a potent testiculo-protective activity against tramadol-induced testicular injury in rats.

**Key words:** Testes – Oxidative stress – Infertility – Citrus aurantifolia – Tramadol

## INTRODUCTION

Infertility occur in 8-12% of couples. However, its incidence varies from different regions (Panti et al., 2014). Reproductive failure has very far-reaching social implications in Nigeria, where the main reason for marriage is child-bearing (Ikechebelu et al., 2003). Some medications have been known to affect male reproduction through central hormonal effects, direct gonadotoxic effects, effects on sperm function or on sexual function (Brezina et al., 2012; Samplaski et al., 2015).

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TMD is an opioid pain medication used to treat both acute and chronic pain of moderate to moderately severe intensity, along with a variety of diseases including osteoarthritis, neuropathic pain, lower back pain and migraine. There are also indications that it could affect premature ejaculation (Kurkar et al., 2015). Prolonged use of this opioid has been signaled as a causative factor for testicular degeneration, impaired sperm quality and fluctuation of androgen hormones secretion (Abdellatief et al., 2014; Azari et al., 2014; Sawy and Malak, 2015). As with many other countries, in Nigeria TMD is one of the many drugs being abused, especially by youths, and this has become a significant problem to the national public health. Statistics show that males constitute 97% of TMD users, with 67% being below 37 years of age in terms of age composition (Ibrahim et al., 2017).

In many African countries, 80% of the population use traditional medicine based on plants to improve its health status (Njamen et al., 2013). Citrus peel is no exemption. Citrus fruit is one of the most important fruit crops grown in the world (Tao et al., 2007). We have reported the testiculo-protective and curative roles of Citrus paradisi peel in experimental models (Akunna et al., 2017). The same could be said about lime. Citrus aurantifolia peel has been reported to contain essential oils (Nallely et al., 2012), potent antioxidants and free radical scavenging potentials (Tundis et al., 2012), beneficial in treatment of cardiovascular, hepatic, osteoporosis and urolithiasis diseases, and it acts as a fertility promoter (Narang and Jiraungkoorskul, 2016). Citrus aurantifolia in its natural state is widely used in West Africa, particularly in Nigeria, where it is employed in herbal medicine to treat several illnesses. It forms an essential ingredient in the preparation of most herbal concoctions (Enejoh et al., 2015).

In this study, we evaluated the therapeutic role of aqueous zest extract of Citrus aurantifolia against TMD-mediated testicular oxidative stress, apoptosis and inflammations in rat models.

## MATERIALS AND METHODS

### *Plant material and aqueous extraction*

Five hundred (500) lime fruits purchased from Ibadan were peeled with a grater. The white portion of the peel under the zest (mesocarp) was generally avoided by limiting the peeling depth. The peel extraction technique was done in accordance to the report of Akunna et al. (2017).

### *Chemicals and animals treatments*

The TMD (containing 100 mg TMD hydrochloride) used in this study was purchased from Alben Health Care Industries Ltd, USA). The biochemical assays for hormone and testicular antioxidant enzymes were carried out according to the procedures stated in the kits supplied by Randox Laboratory Ltd., UK. Thiobarbirutic acid (TBA) was obtained from Sigma Aldrich, USA. Commercial kits for cytokins and nitric oxide were from R&D systems, USA. The kits for testosterone and follicle stimulating hormone were supplied by NIADDK–NIH (USA). All other reagents used were obtained commercially and of analytical grade.

### *Animals*

Twenty-five adult male Sprague Dawley rats (120 -190 g body weight) were used for the experiment. The animals were housed in well-ventilated cages (six rats per cage; 12 h dark:12 h light cycle). All animals were given rat chow and tap water ad libitum. The experiment and use of animals were approved by the University Committee on Research and Ethics, Bowen University, Osun State. The study is consistent with the standard of the use of laboratory animals (American Physiological Society, 2002).

### *Experimental protocol*

The animals were randomly divided into 4 groups (n=5):

Group 1 (negative control): rats treated with 5ml/kg of normal saline for 28 days. Group 2 (Positive control): rats treated with 30mg/kg of TMD for 14 days. Groups 3 and 4: rats treated with 30 mg/kg of TMD for the first two weeks and 50 and 100 mg/kg of AZECA for the last 2 weeks, respectively. TMD and AZECA were administered orally in solution.

After the experimental duration, animals (fasted) were anesthetized 300mg/kg and 30mg/kg of ketamin and xylazine respectively 48 hours after last doses. Blood was collected through cardiac puncture into a plain bottle. The blood was centrifuged (3000 g for 10 min), and serum was separated for analysis of testicular function. The testis was isolated, washed in saline water, dried and weighed. The testes were homogenized in 0.1 M Phosphate buffered solution (1:5 w/v, pH 6.4) and centrifuged (4000 g for 20 minutes). The supernatant obtained was used for analysis of lipid peroxidation, antioxidant enzyme activity, reduced gluthathione and pro-inflammatory markers, and apoptotic protein. The testes were fixed in 10% buffered formalin for histopathological studies.

### *Biochemical analysis*

Determination of testicular inflammatory mediator level: Tissue homogenate interlukin-1(IL- □), interlukin-6 (IL-6) and tumor necrosis factor-∝ (TNF-∝) were measured in testicular homogenate using ELISA kits according to the manual.

### *Hormonal Assays*

Testosterone TT, Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). Testicular testosterone in homogenate were determined by the enzyme immunoassay technique based on the

principle of competitive binding between TT and TT-horseradish peroxidase conjugate for a constant amount of rabbit anti-TT, as previously described (Tietz, 1995). LH and FSH were done according to the procedure adapted by Amballi et al. (2007). World Health Organization (WHO) matched reagent program protocol (manual) for EIA kits (protocol/ version of December 1998 for LH, FSH).

### *Oxidative stress markers*

Lipid peroxidation (MDA) in the tissue was estimated colorimetrically by thiobarbituric acid reactive substances (TBARS) method of Buege and Aust (1978). Concentration was calculated using the molar absorptivity of malondialdehyde, which is 1.56 x105 M-1 cm-1 and expressed as nmol/mg protein. Catalase (CAT) activity was measured according to the method of Aebi (1983). Enzyme activity was expressed as units/mg protein. Superoxide Dismutase (SOD) activity was measured according to the method of Winterbourn et al. (1975). Enzyme activity was expressed as units/ mg protein.

### *Histological preparation*

The organs were processed for histological analysis. Prior to embedding, it was ensured that the mounted sections were orientated perpendicular ("vertical sections") to the long axes of the testes. Serial sections of 4 µm thick were obtained from a solid block of tissue, fixed on clean slides to which Mayer's egg albumin had been coated to cement the sections to the slides properly and were stained. For histological and histochemical study, sections were stained with H&E (GHS132 and HT1102128, Sigma-Aldrich) for detection of glycogen, after which they were passed through a mixture of equal concentration of xylene and alcohol. Following clearance in xylene, the sections were oven-dried between 35°C and 40°C (Sheehan and Hrapchak, 1987).

### *Morphometry and Stereology*

Morphometrical and stereological analyzes were performed using the software Image-Pro Plus 4. We captured images with a light microscope ((Eclipse 80i, Nikon, Japan). For each testis, seven "vertical sections" from the polar and the equatorial regions were sampled (Qin and Lung, 2002). Seven "vertical sections" per testis were selected by a systematic sampling method that ensured fair distribution between the polar and equatorial regions of each testis. Volumetric proportion of the interstitium and seminiferous tubules-tunica propria, epithelium and lumen, leydig cells, connective tissue cells and fibers, macrophages, blood vessels and lymphatic space, tubulesomatic index (TSI), diameter of seminiferous tubules, total seminiferous tubule length as described by Karine et al. (2011). Cross-sectional area (AC) of the seminiferous tubules (ST) of the testes were determined from the formula AC= πD2/4, (where π is equivalent to 3.142 and D the mean diameter of the seminiferous tubules), number of profiles (NA) of ST in a unit area of testis; number of profiles of ST per unit area was determined by using the unbiased counting frame proposed by Gundersen (1977), numerical Density (NV) of ST was determined with the modified Floderus equation: NV =NA/ (D +T) (Gilliland et al., 2001) where, NA is the number of profiles per unit area, D is the diameter and T the average thickness of the section.

### *Statistical analysis*

All data were expressed as mean ± SD of number of experiments (n = 4). The level of homogeneity among the groups was tested using Analysis of Variance (ANOVA), as done by Snedecor and Cochran (1980) with Scheffe's post hoc test. The level of significance was considered at $p < 0.05$. Analysis of data was done using both electronic calculator and Statistical Package for Social Sciences (SPSS)/ PC computer program (version 20.0 SPSS, Cary, NC, USA).

## RESULTS

### *Hormone analysis*

As shown in Table 1, there was a significant decrease in testicular testosterone level, follicle stimulating hormone and luteinizing hormone in the group treated with TMD-alone when compared to the negative control group. There was a significant increase at ($P<0.05$ and $p<0.01$) in the levels of these hormones in group 3 and group 4 rats when compared to the group that were treated with TMD alone. Also, the levels of these hormones was significantly different between groups 3 and 4.

### *Oxidative stress marker*

As shown in Table 2, there was a significant ($p<0.05$) decrease in the level of SOD, CAT and a significant increase in MDA level in rats treated with TMD when compared to the negative control group. There was a significant increase in the level

**Table 1.** Effect of AZECA and TMD on the hormonal profiles of models

| Groups | TT(x10 ng/g protein) | FSH(ng/ml) | LH(ng/ml) |
|---|---|---|---|
| Group 1 | 3.18±0.09 | 0.33±0.003 | 0.82±0.01 |
| Group 2 | 1.04±0.02[a] | 0.14±0.02a | 0.22±0.05[ac] |
| Group 3 | 2.03±0.01[b] | 0.30±0.01[b] | 0.76±0.03[b] |
| Group 4 | 3.00±0.04[bc] | 0.36±0.01[bc] | 0.84±0.02[bc] |

[a,b,c] represent significant difference at p≤0.05 when compared to group 1 (5 ml/kg NS), group 2 (30 mg Tram) and group 3 (30 mgTram+50mgAZECA) respectively (n= 5). TT= Testosterone, FSH= Follicle stimulating hormone, LH =Lutenizing hormone

**Table 2.** Effect of AZECA and TMD on oxidative stress markers

| Groups | SOD (U/mg protein) | MDA (nmol/mg) | CAT (U/mg protein) |
|---|---|---|---|
| Group 1 | 18.72±0.34 | 0.91±0.02 | 16.84±0.29 |
| Group 2 | 13.74±0.89[a] | 1.80±0.05[a] | 11.39±0.86[a] |
| Group 3 | 16.79±0.29[b] | 0.95±0.06[b] | 15.71±0.94[b] |
| Group 4 | 16.71±0.32[b] | 0.97±0.03[b] | 15.02±0.35[b] |

[a,b,c] represent significant difference at p≤0.05 when compared to group 1 (5 ml/kg NS), group 2 (30 mg Tram) and group 3 (30 mgTram + 50 mgAZECA) respectively. (n= 5) SOD: superoxide dismutase, MDA: malondialdehyde, CAT: catalase.

**Table 3.** Effect of AZECA and TMD on testicular level of interlukin-1β(IL-β), interlukin-6 (IL-6) and tumor necrosis factor-α (TNF α-) in rat

| Groups | IL-1 β (ng mg protein) | TNF-α (ng mg protein) | IL-6 (ng mg protein) |
|---|---|---|---|
| Group 1 | 18±0.4 | 86±1.3 | 102±1.3 |
| Group 2 | 48±0.6[a] | 118±3.2[a] | 142±3.2[a] |
| Group 3 | 28±0.2[b] | 81±1.1[b] | 113±1.2[b] |
| Group 4 | 17±0.1[bc] | 62±2.4[bc] | 84±2.4[bc] |

[a,b,c] represent significant difference at p≤0.05 when compared to group 1 (5 ml/kg NS), group 2 (30 mg Tram) and group 3 (30 mgTram + 50 mgAZECA) respectively. (n= 5) interlukin-1β(IL-β), interlukin-6 (IL-6) and tumor necrosis factor-α(TNF-α).

of SOD, CAT and a significant decrease in level of MDA in rats treated with AZECA (group 3 and 4) when compared to positive control group. However, there was no significant difference between group 3 and 4.

### *Inflammation markers*

There was a significant (p<0.05) increase in the level of IL-1□, TNF-∝ in the group of rats treated alone with 30mg/kg body weight of TMD when compared to the negative control. There was a reduction in the level of these markers after AZECA supplementation. However, there was no significant difference in the level of the markers between groups 3 and 4 (Table 3).

### *Histo-morphometric and stereological analysis*

There was a significant (p<0.05) reduction in volumetric proportion of the interstitium and seminiferous tubules-tunica propria, macrophages, blood vessels and lymphatic space, macrophages, connective tissue, TSI, diameter, cross-sectional area and number of profiles of seminiferous tubules per unit area in group treated with TMD when compared to the negative control group (Table 4). These parameters were significantly (p<0.05) increased in the groups that were treated with AZECA. However, there was no significant difference bet-

**Table 4.** Effect of AZECA and TMD on testicular Histo-morphometry and stereology

| Volumetric proportion in testis (%) | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|
| ST | 73.11 ± 0.12 | 56.01 ± 1.1[a] | 67.21 ± 1.01[b] | 62.41 ± 8.04[b] |
| Interstitium | 14.15 ± 0.2 | 7.2 ± 0.1[a] | 9.2 ± 0.21[b] | 11.4 ± 3.1[b] |
| Lymphatic space | 8.08 ± 0.35 | 6.2 ± 0.15[a] | 8.1 ± 0.15[b] | 10.3 ± 1.2[b] |
| Blood vessels | 3.08 ± 0.35 | 1.7 ± 0.11[a] | 2.0 ± 0.15[b] | 2.12 ± 0.35[b] |
| Leydig cells | 2.7 ± 0.41 | 1.8± 0.21[a] | 2.7± 0.11[b] | 3.21± 0.15[b] |
| Macrophages | 0.61 ± 0.11 | 0.43 ± 0.11[a] | 0.61 ± 0.11[b] | 0.81 ± 0.21[b] |
| Connective tissue | 0.8 ± 0.21 | 0.7 ± 0.01[a] | 0.91 ± 0.03[b] | 1.01 ± 0.06[b] |
| **Volume(mL)** | | | | |
| ST | 1.72 ± 0.5 | 1.21 ± 0.1[a] | 1.82 ± 0.1[b] | 1.81 ± 0.21[b] |
| Interstitium | 0.2 ± 0.05 | 0.1 ± 0.02 | 0.09 ± 0.02 | 0.12 ± 0.01 |
| Lymphatic space | 0.23 ± 0.02 | 0.21 ± 0.01 | 0.24 ± 0.12 | 0.20 ± 0.12 |
| Blood vessels | 0.08 ± 0.01 | 0.06 ± 0.01[a] | 0.09 ± 0.001[b] | 0.1 ± 0.001[b] |
| Leydig cells | 0.32 ± 0.01 | 0.26± 0.03[a] | 0.35± 0.02[b] | 0.31± 0.02[b] |
| Macrophages | 0.003 ± 0.001 | 0.002 ± 0.001 | 0.003 ± 0.04 | 0.09 ± 0.04 |
| Connective tissue | 0.007 ± 0.005 | 0.007 ± 0.001[a] | 0.009 ± 0.002[b] | 0.06 ± 0.02[b] |
| TSI | 0.72 ± 0.21 | 0.51 ± 0.01 | 0.62 ± 0.01 | 0.73 ± 0.01 |
| Tubular Diameter (µm) | 275.42 ± 3.5 | 205.34 ± 4.1[a] | 232.17 ± 3.2[b] | 254.17 ± 1.2[b] |
| Germinal epithelia height(µm) | 27.1 ± 1.2 | 15.3 ± 3.1[a] | 25.2 ± 0.2[b] | 26.5 ± 1.3[b] |
| Cross-sectional area of ST Ac (x 103µm$^3$ ) | 20.2 ± 1.4 | 12.2 ± 2.2[a] | 18.1 ± 2.2[b] | 19.1 ± 4.3[b] |
| Number of profiles of ST/unit area NA (x10-8 µm-2) | 29.1 ± 0.8 | 20.3 ± 1.01[a] | 25.5 ± 1.2[b] | 28.6 ± 3.1[b] |

[a,b,c] represent significant difference at p≤0.05 when compared to group 1 (5 ml/kg NS), group 2 (30 mg Tram) and group 3 (30 mgTram + 50 mgAZECA) respectively. (n= 5).

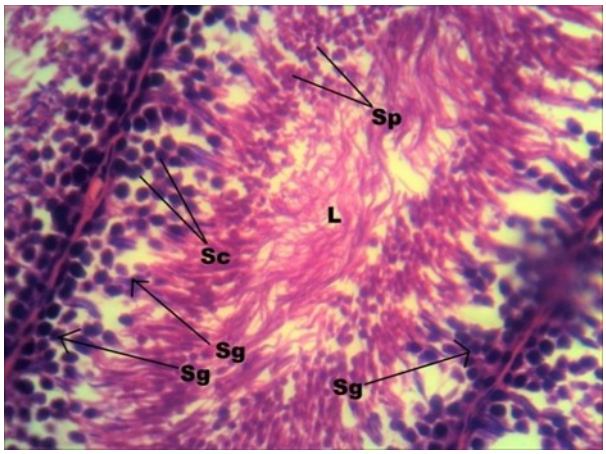


Figure 1A

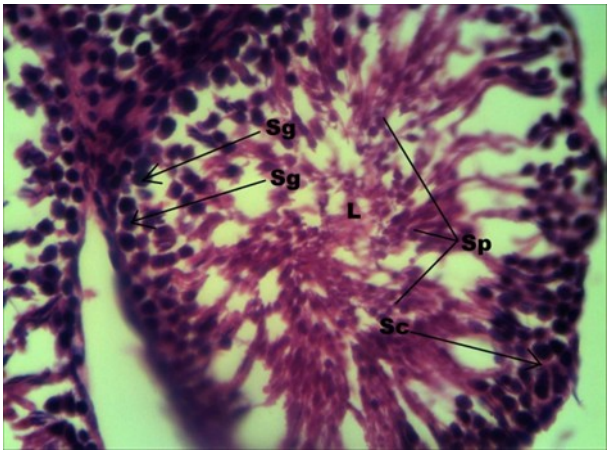


Figure 1B

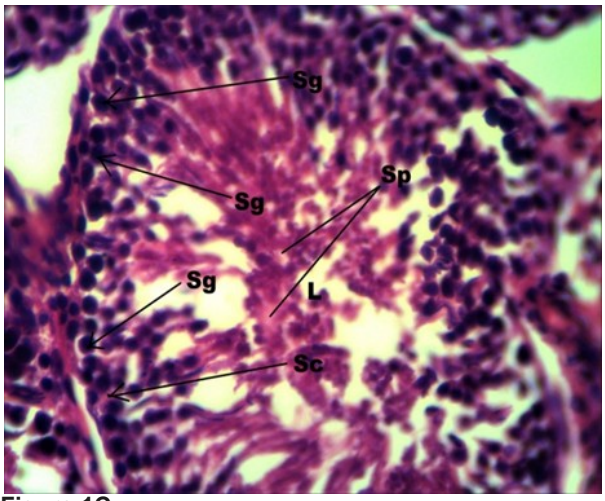


Figure 1C

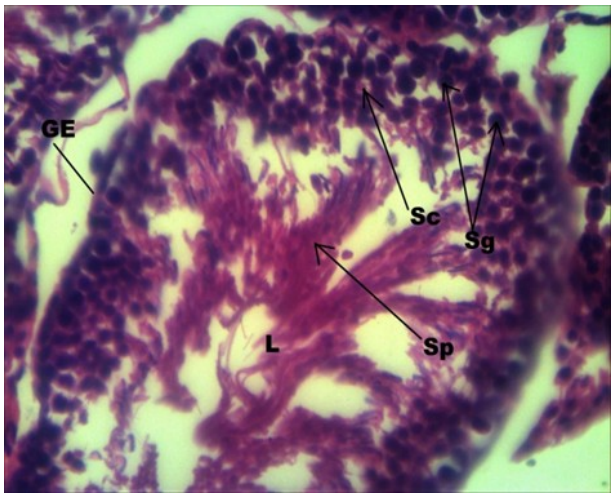


Figure 1D

**Fig 1A-1D.-** Histological features of control (1A) and experimental models (1A-1D). Mag: x40, Stain: H&E). I= interstitial cells, Sc= Sertoli cells, Sg= spermatogonial cells, L= lumen, GE= germinal epithelium, Sp= spermatocyte. **1A**: Negative control (5ml/kg of normal saline); **1B**: Positive control (30 mg/kg of TMD); **1C**: 30 mg/kg of TMD+ 50 mg/kg of AZECA; **1D**: 30 mg/kg of TMD+ 100 mg/kg of AZECA.

ween the treatment groups 3 and 4.

***Histological and immunohistochemical results***

Figure 1a shows the photomicrograph of the testes of rats in group A (5ml/kg body weight NS) with evidence of normal testicular architecture with numerous spermatozoa radiating towards the lumen and an elevated germinal epithelium. There is also an evidence of intact interstitial cells and Sertoli cells. Figure 1b shows the testes of rats in group B (30 mg/kg body weight TMD) with evidence of distorted testicular architecture with almost empty lumen and a decreased germinal epithelium. The interstitial cells and Sertoli cells are also degenerated when compared to the animal in group A. Figures 1c and 1d shows the histological features of testes of rats in group C and D rats (30 mg/kg bwt. TMD + 50 mg/kg bwt. and 100 mg/kg of AZECA, respectively). There testicular architecture is mildly distorted with spermatozoa in lumen and a decreased germinal epithelium. Few interstitial cells, Sertoli cells and spermatonial cells were intact when compared to the animal in group B.

For histochemical studies, there was a weak PAS reaction in seminiferous tubular profile of rats in group B (Fig. 2B) when compared to control group (Fig. 2A) that had strong reactions in well circumscribed basal lamina and spermatogenic epithelium. However, group C (Fig. 2C) and D (Fig. 2D) rats showed moderate PAS reaction in the basal lamina and interstitium, weak reaction in sperms with no reaction in the spermatogenic cells when compared to the negative control group A (Fig. 2).

## DISCUSSION

TMD is a centrally acting opioid analgesic mainly used as a first-line therapy for the treatment of mild to severe muscular and skeletal pains, osteoarthritis in cases of contraindication for NSAIDs and for pain resistance for the other analgesics (El-Baky and Hafez, 2017). Nonetheless, it has been repea-

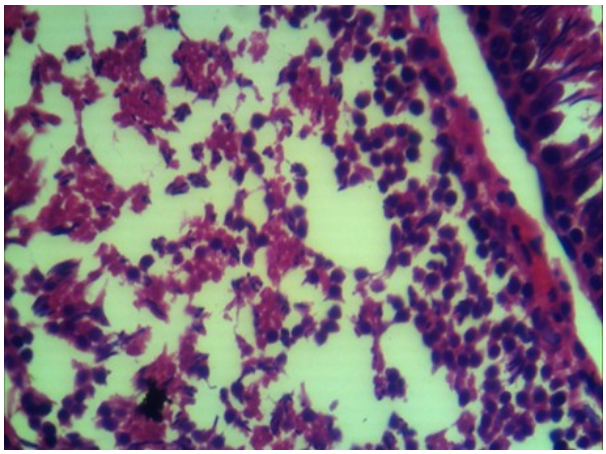

Figure 2A

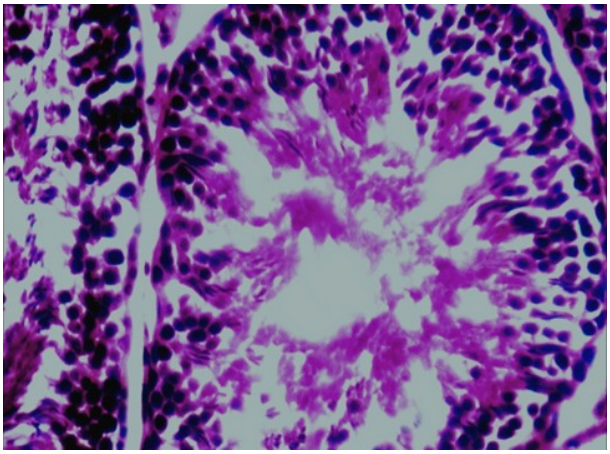

Figure 2B

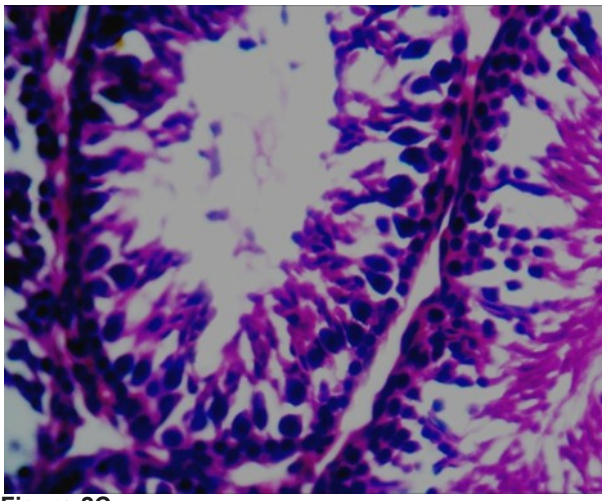

Figure 2C

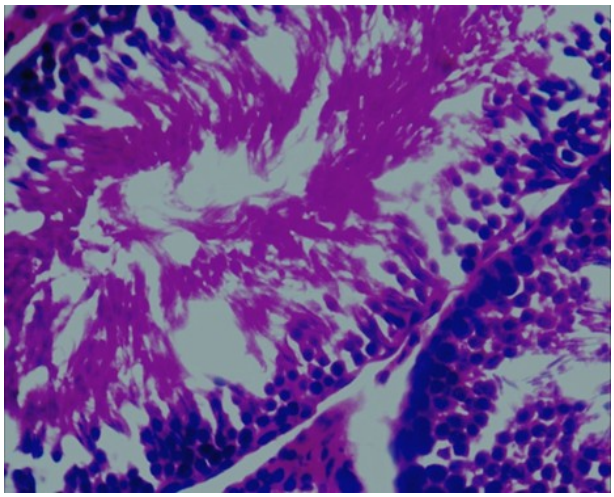

Figure 2D

**Fig 2A-2D.-** Histological features of control with higher PAS content (2A) when compared to group B rats (2B). The animals in group C and D (2C-2D) showed a higher PAS reaction when compared to the group B rats. Mag: x40, Stain: PAS). **2A**: Negative control (5 ml/kg of normal saline); **2B**: Positive control (30 mg/kg of TMD); **2C**: 30 mg/kg of TMD+ 50 mg/kg of AZECA; **2D**: 30 mg/kg of TMD+ 100 mg/kg of AZECA.

tedly linked to testicular toxicity. In this study we evaluated the therapeutic role of aqueous peel extract on TMD-induced testicular toxicity.

Body composition requires that energy intake match energy expenditure and that nutrient balance be achieved. Results showed that rats treated with TMD and AZECA had a non- significant decrease in the body weight when compared to negative control group of rats (El- Baky and Hafez, 2017). This could have come as a result of reduced food intake due to their inhibitory effect on appetite centers of the hypothalamus (Oka et al., 2015), or through alteration of obesity-related genes, anti-inflammation activity, anti-oxidative stress capacity hence regulating appetite, metabolism, or absorption of calories (Lu et al., 2012).

Although pathophysiology of testicular toxicity includes several mechanisms, reports have linked inflammatory signaling in TMD-induced toxicity (Mohameda and Mahmoud, 2019). In this study, TMD-treatment consequently raised testicular IL-1B, IL-6. Famurewa et al. (2018) implicated ROS as a prime trigger of inflammatory responses via activation of transcription factors. Interestingly, there was a significant reduction in the level of IL-1B, IL-6 in AZECA-treated group. This suggests a possible anti-inflammatory role in models which could be due to suppression of mRNA expression of these pro-inflammatory mediators as shown in our result. It is important to note at this point that our results on body weight were not in accordance with the report of Ahmed and Kurkar (2014), stating an increase in body weight with the administration of TMD for 8 weeks. Inflammation cytokines, such as IL-6 and TNF-α has been said to directly contribute to the development of obesity (Lu et al., 2012). These could explain the varying result from this study.

TMD-induced testicular degeneration has been linked to abnormalities of hypothalamic-pituitary function and decreased testosterone production. Long term exposure to opioids decreased gonadal hormones in humans, as well as in experimental animals (Adam et al., 1993; Abdellatief et al.,

2014). Rats treated with TMD had a significant dose-dependent decrease in TT, FSH and LH. This is in line with the reports of Abdellatief et al. (2014), Ahmed and Kurkar (2014), Youssef and Zidan (2016) and El-Baky and Hafez (2017). TMD could act as a mitogen in the Leydig cells, suppress protein expression of the LH receptor and the 17-betahydroxy steroid dehydrogenase enzyme thereby, decreasing androgen secretion by Leydig cells, thereby interfering with spermatogenesis and reducing male fertility. It could also be through the HPGA-axis, which plays a crucial role in the development and regulation of immune and reproductive system.

Gonadotropin-releasing hormone secreted from the hypothalamus stimulates the release of FSH and LH from the anterior pituitary gland. Interference in the hypothalamus secretion of GnRH by TMD could have led to subsequent decrease in these hormones (Abdellatief et al., 2014; Ahmed and Kurkar (2014). However, there was a significant increase in the level of the aforementioned hormones when rats were treated with low and high dose of AZECA when compared to rats treated alone with TMD. Reduction in germ cell death via suppression of apoptosis and secretion of TT and FSH has been opined since these hormones are known to inhibit autophagy in sustentacular cells. AZECA could have exerted these effects through antioxidant and anti-inflammatory responses. Our study showed that administration of TMD-alone resulted in development of oxidative stress demonstrated by a progressive decrease in testicular enzymatic antioxidant activities—SOD, CAT, a consequent progressive increase in MDA level of TMD-only treated rats (Ahmed and Kurkar, 2014; El-Baky and Hafez, 2017; Ibrahim and Salah -Eldin, 2019). SOD is the first line of defense against oxidative stress with dismutation of superoxide radicals to H2O2 and molecular oxygen, CAT converts hydrogen peroxide to oxygen and water. A decrease in these enzymes would lead to the high production of ROS, which reacts with various intracellular DNA, lipids and proteins. Lipid peroxidation may eventually result in the dysfunction and structural damage of the cell due to peroxidation of polyunsaturated fatty acids in their plasma membranes by TMD (Ibrahim and Salah-Eldin, 2019).

MD-treatment could result in increased ROS and arrest of cell cycle for DNA repair or induction of cellular apoptosis. This is so as ROS has been reported to oxidize trans-membrane protein and unsaturated fatty acids by binding on them, thereby distorting the permeability of cell membrane (Akunna et al., 2017).

AZECA has antioxidant properties which can protect peroxidative damage induced by various types of oxidants (Jeon et al., 2001; Boshtam et al., 2011). It has been reported to contain high concentration of apigenin, rutin, quercetin, kaempferol, monoterpenes and sesquiterpenes and nobiletin. n-Hexane fractions of both peel and leaves showed a good acetylcholinesterase inhibitory activity with IC50 values in the range 91.4-107.4 µg mL−1 (Loizzo et al., 2012).

Infertility has been linked to reduction in number of Sertoli, Leydig and spermatogenic cells (Akunna et al., 2017). Morphometric parameters enable effective quantification of histological and histochemical features of a tissue (El-Ghawet, 2015). Evidenced in our study were significant alterations in testicular architecture in TMD-group. Following our reports in this study, administration of AZECA showed marked improvement of the morphometric parameters. Oxidants like TMD could cause their cellular effect through binding and interacting with estrogen receptors and androgen and thyroid hormone receptors at low and high doses respectively. They could also stimulate myoid cells to produce more collagen fibers around basal lamina causing them to become thickened, irregular and corrugated in shape.

There is a direct relationship between the size of the testicular interstitium and the number of Leydig cells, which is directly proportional to the testicular androgen level (Akunna et al., 2014). TMD is attributed to Leydig cell degeneration, leading to reduced testosterone secretion, hence affecting testicular volume, spermatogenic arrest, sloughed germinal epithelium, Sertoli cell death and thickened and irregular basement membrane (Youssef and Zidan, 2016). We opined that the reduction observed in both interstitial volume and volumetric proportion was due to a reduction in the lymphatic space volume. Surrounding the blood vessels and Leydig cell is the lymphatic space, which is linked to testosterone and nutrient distribution transport from source via blood vessels to the seminiferous tubules. The reduction in testosterone level could happen as a result of TMD mediated toxicity on Leydig cell, and unavailability to needing spermatogenic cells due to lymphatic space shrinkage (Chieregatto, 2005; Karine et al., 2011).

## CONCLUSION

AZECA has a modulating effect on TMD-induced testicular toxicity, as shown by the oxidative, hormonal and histomorphometric evidence.

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