Annona muricata leaves extract mitigates the testicular oxidative stress induced by doxorubicin in male rats

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ABSTRACT

Background: Doxorubicin (DOX) is a strong antineoplastic drug that is used for the treatment of several types of cancer and has toxic effects. Annona muricata fruits and leaves possess phytotherapeutic bioactive constituents have potency in the treatment of several diseases. Aim: This study aims to evaluate the therapeutic role of Annona muricata extract (AME) against the testicular damage induced by doxorubicin in male rats. Material &methods: Thirty-two male adult albino rats were used in this study. The rats were divided into four groups (n = 8): the control group, the AME-supplemented group (100 mg/kg b.wt. daily for consecutive 28 days), the DOX-treated group (2 mg/kg twice a week for 4 weeks), and the DOX-treated group that was supplemented with AME. Results: Treatment of male rats with DOX showed a remarkable increase in the levels of serum pro-inflammatory markers, decreased sexual hormones, and decreased testicular antioxidant activities. The treatment of DOX-injected rats with ASE revealed a pronounced return to normalcy of the oxidative stress markers, as evidenced by a remarkable decrease in the testicular MDA and an improvement of SOD, CAT, and GSH activities. On the other hand, the rats administered DOX and AME had considerably decreased serum levels of TNF-α and IL-6 and improved serum FSH, LH, and testosterone levels. AME successfully restored the testicular histopathological signs induced by DOX through amelioration of the architecture of disrupted seminiferous tubules with AME. Conclusion: Annona muricata extract has a potential alleviation role against DOX-induced oxidative stress and inflammation in the testicular tissues.

Keywords: Annona muricata, testes, oxidative stress, testosterone, COX-2

INTRODUCTION

Doxorubicin (DOX) is an anti-cancer medication that is frequently used to treat a variety of cancers, including testicular, breast, lung, ovarian, and uterine cancers, in addition to leukemia (1,2). Treatment with DOX has been shown to raise the risk of significant dose-dependent toxicity in various non-target tissues, including the brain, kidneys, heart, and testicles, even though it can be used effectively against malignant tumors (3). DOX can dramatically suppress spermatogenesis, which eventually results in infertility (4). Oxidative stress, lipid peroxidation, and cellular death are the main mechanisms that have been suggested to be responsible for DOX-induced testicular damage (5). However, the findings of recent research point to oxidative stress as one of the primary causes (6). Studies conducted on testicular tissue in rats given DOX treatment revealed elevated MDA levels, lowered superoxide dismutase, glutathione
peroxidase (GPx), and glutathione (GSH) levels, and enhanced cell death due to DNA fragmentation (5). Although DOX helps fight cancer cells, its harmful effects on healthy cells, like testicular tissue, are concerning (7). Thus, to help mitigate DOX-induced tissue damage, non-pharmacological methods, including antioxidant supplementation, are crucial (8).

In the context of reproduction, oxidative stress is a primary factor in male infertility (9). ROS causes lipid peroxidation and DNA fragmentation, disrupting both the survival of lipids and DNA in isolated spermatozoons (10). So, antioxidants may be important in the treatment of male infertility (11).

Some researchers have recently directed their attention toward the use of medicinal plants as an alternative to traditional drugs in the treatment or alleviation of various diseases. Because of the abundance of phytochemicals found in plants, there is strong proof that plants may successfully control male fertility worldwide (12,13). Medicinal plants, on the other hand, are recognized for their superior therapeutic approach to resolving fertility problems resulting from sedentary lifestyles and environmental factors (14). This is due to their extensive phytochemical content. Annona muricata L. (Graviola) is a tropical tree that belongs to the Annonaceae family. A. muricata is recognized for its medicinal usefulness due to its phytotherapeutic and bioactive components. A. muricata leaf extract against doxorubicin-induced testicular damage in male rats.

MATERIALS & METHODS

1. Chemicals

1.1. Doxorubicin: Doxorubicin as DOX hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA).

1.2. Annona muricata (Graviola) dry extract® (Code number: 912943735) was purchased from Origini Naturali Company (Quarrata, Pistoia, Italy).

1.3. The rest of the chemicals required for the estimation of biochemical parameters were obtained from Biodiagnostic, Cairo, Egypt.

2. Experimental animals

In this study, thirty-two male Wistar albino rats weighing between 170-180 g were utilized. The animals were housed in wire-bottom cages in a room with standard lighting, a 12-hour light-dark cycle, 25 ± 1°C, and 50% relative humidity. They also had access to tap water and an abundant, well-balanced feed. The rats were randomly divided into four groups (n = 8) following a week of acclimatization as follows:  

Control group: They were kept untreated and given a saline solution (the solvent of DOX).

AME group: The rats were supplemented orally with AME (100 mg/kg b.wt. for 28 consecutive days) using gavage (18).

DOX-treated group: The rats were treated with DOX dissolved in normal saline (2 mg/kg/twice/week; I.P. for 4 weeks). (19).

DOX&AME-treated group: The rats were treated with DOX for four weeks, followed by treatment with AME at the same doses in groups 2 and 3. Graviola extract dissolved in normal saline (0.9%) was used.

Sample collection and tissue preparation

At the end of the experimental period (4 weeks for groups 1-3 and 8 weeks for group 4), the rats were anesthetized, and the blood was collected in glass tubes. The serum was separated by centrifugation at 3000 rpm for 10 min and stored at −80°C for further analysis.
biochemical analysis. The animals were dissected, and the testes were removed immediately, washed in normal saline, and cut into two halves. One half was fixed in 10% neutral buffered formalin for histological and immuno-histochemical investigation, and the other half was kept frozen for estimation of testicular oxidative stress and antioxidants.

All procedures were performed following the guidelines of the Standing Committee for Scientific Research - Jazan University.

3. Investigated parameters

3.1. Measurement of serum FSH, LH, and testosterone

Using commercial enzyme immunoassay kits, as directed by the manufacturer (DRG Diagnostics GmbH, Marburg, Germany), assays were carried out to assess the concentrations of testosterone, FSH, and LH. The FSH and LH concentrations were expressed as mlU/mL, and the testosterone was expressed in ng/mL.

3.2. Measurement of serum TNF-α and IL-6

The level of serum TNF-α was determined by enzyme-linked immunosorbent assay (ELISA) using ready-made chemical reagents provided by Eastbiopharm, China (20). The serum IL-6 levels were assessed by ELISA. The specific steps were as described in the ELISA kit (21).

3.3. Determination of SOD, CAT, GSH, and MDA activities in the testicular tissues

Superoxide dismutase (SOD) in the testicular tissues was assessed according to the method of Asada et al, which depends on the suppression of photochemical reduction of nitro blue tetrazolium (NBT) at pH 8.0. (22). The quantity of superoxide dismutase needed to provide 50% inhibition of the photochemical reduction of NBT is recognized as a single unit of enzyme. Using a UV-Vis spectrophotometer against a blank, the absorbance was calculated at 580 nm. Testicular catalase (CAT) activity in testis homogenate was calculated using the Aebi method. (23). CAT-specific activity has been reported in terms of mmole of H2O2 consumed/min/mg protein. Catalase activity is measured by a variation in absorbance at 240 nm during a given time. The SOD and CAT activity was expressed as U/mg protein Testicular reduced glutathione (GSH) was measured with a coloring agent, 5,5-dithio-bis (2-nitrobenzoic acid) (Ellman’s reagent) (24). At 412 nm, a spectrophotometer was used to determine the absorption. The amount of GSH was determined using a standard curve. The GSH activity was expressed as ng/g protein. Malondialdehyde (MDA) was assessed according to Ohkawa et al. using commercial kits (Biodiagnostic, Cairo, Egypt). At 532 nm, the colorimetric absorbance was calculated. The MDA activity was given as ng/g protein (25).

3.4. Histological and immunohistochemical; investigation of testis

One-half of each testis was processed for the preparation of histological sections. In brief, the testicular samples were fixed in 10% formalin for 24 hours. Following two passes of distilled water washing, the samples were dehydrated using increasing concentrations of ethyl alcohols (60, 70, 80%, and absolute). Subsequently, the samples were cleaned in xylene and immersed in paraffin for 24 hours at 56° in a hot air oven. Using a microtome, paraffin blocks were sectioned at 4-5 μm thickness. The obtained testicular sections were collected on glass slides, deparaffinized, and stained by hematoxylin and eosin stain (26).

Testicular cyclooxygenase-2 (Cox-2) was demonstrated by immunohistochemistry on additional embedded paraffin sections. In brief, the testicular sections were deparaffinized, hydrated in descending grades of ethyl alcohol, followed by immersion in citrate buffer (10 mM, pH 6.0) for 3.5 min, then treated with H2O2 in the dark for about 30 min. After immersing the slides in PBS (10 mM, pH 7.2), they were treated for a whole
night at 4°C in a moist chamber with polyclonal anti-rabbit (COX-2) antibody (Cell Signalling #4842, dilution 1:50). For 20 minutes at 37°C, the sections were immersed in Poly HRP conjugate (Zymed, USA, Cat No. 87-8963). Following that, the slides were submerged for five minutes at 37°C in diaminobenzidine (Sigma, USA, D-5637). Finally, the testicular sections were counterstained with hematoxylin, then dehydrated in an ascending series of ethanol, and coated with mounting medium.

The histological and immunohistochemically prepared sections were examined and photographed using a light microscope (Leica Microsystems GmbH, Wetzlar, Germany) with a Leica camera.

Statistical analysis:
The statistical analysis one-way ANOVA is followed by post hoc test means in the same row with a different superscript(*). The data are expressed as mean± standard error \(n=8 \text{ per group}\). are significantly different when \(p<0.05\) * significant at value <0.05, **significant at \(p=0.01\) and ***significant \(p=0.001\) in comparison with control.

RESULTS
1. Changes in the activities of serum FSH, LH, and testosterone
The obtained results revealed that the levels of serum FSH, LH, and testosterone (P<0.01) was recorded among DOX-treated rats if compared with the control. AME treatment successfully increased the levels of declining hormones induced by DOX but was still significantly lower than the control. (Figure 1)

2. Changes in the activities of serum pro-inflammatory cytokines (IL-6 and TNF-α)
In the current study, the serum samples from rats treated with DOX exhibited a remarkable rise in TNF-α and IL-6 levels when compared to the control group. Nevertheless, when AME was given to rats treated with DOX, there was a highly significant (P<0.01) decline in the serum levels of IL-6 and TNF-α, even though the levels were still significantly greater than those of the control group (Figure 2).

3. Changes in the levels of CAT, SOD, GSH, and MDA in testicular tissues
SOD, CAT, GSH, and MDA levels in the testicular tissue of rats treated with AME did not significantly differ from the control group. Comparing the DOX-treated rats to the control group, the levels of MDA were significantly higher (P <0.001), but the levels of SOD, CAT, and GSH were significantly lower (P <0.001). Rats treated with DOX and given AME for 28 consecutive days exhibited a complete recovery of their SOD and GSH levels to the control level. On the other hand, the levels of CAT and MDA improved when compared to the DOX group, although they were still not statistically significant (P <0.05) compared to the control group (Figure 3&4).
Fig. 1: The mean serum levels of FSH, LH, and testosterone among the different studied groups. 
(a: significant with the control, b: significant with the DOX-treated group)

Fig. 2: The mean serum levels of IL-6 and TNF-α among the different studied groups. 
(a: significant with the control, b: significant with the DOX-treated group)
Fig. 3: The mean levels of testicular SOD, GSH, and MDA among the different studied groups. 
(a: significant with the control, b: significant with the DOX-treated group)

Fig. 4: The mean levels of testicular, CAT among the different studied groups. 
(a: significant with the control, b: significant with the DOX-treated group)

4. **Histological observations**

The testicular sections of the control and AME-supplemented rats showed normal histological architecture, whereas the seminiferous tubules contained numerous spermatogenic cells and appeared well-organized (Figure 5A-B1). On the other hand, the testicular sections from DOX-treated rats showed complete atrophy of seminiferous tubules, little intertubular hemorrhage, and a remarkable lack of mature spermatozoa in the tubular lumens. Additionally, prominent apoptosis and vacuolation in the early spermatogenic stages appeared near the basal lamina of seminiferous tubules, in addition to the appearance of some congested testicular capillaries (Figure 5C-C1). In rats treated with DOX followed by treatment with AME, most of the testicular histopathological features caused by DOX markedly disappeared (Figure 5 D-D1).
Fig.5: Photomicrograph of histological sections through the testes of the control group (image A-A1), AME group (image B-B1), DOX-treated group (image C-C1), and DOX&AME group (image D-D1). In the control and AME groups, the testicular sections show the normal histological architecture of seminiferous tubules. In DOX-treated rats, the seminiferous tubules appear atrophied with lacking sperm arrows) In the DOX &AME group of rats, the testicular sections showed remarkable amelioration that tended to be more or less a control. (Stain: Hx&E )

**Abbreviations and symbols:** Basal lamina (BL), Intertubular space (ITS), Sertoli cell (SC), spermatogenic stages (SPS), Spermatogonia(Sg), primary spermatocytes(PS), secondary spermatocytes (SS), Spermatozooa (SZ), Vacuoles (V), Leydege cells (LCs), apoptotic cells (red square) absence of sperms (*), hemorrhage (*), lost spermatogenic cells (*)
5. Immunohistochemical observation of Cyclooxygenase-2 (COX-2)

The testicular sections from control and AME-treated rats displayed negative immune expression for the COX-2 antibody in the developing stages of sperm; however, this expression appeared very weak in the interstitial (Leyedged) cells (Figure 6A and B). Comparatively with the control, the DOX-treated rats showed an intensely positive reaction to the COX-2 antibody all over the seminiferous tubules (Figure 6A C). On treatment with AME in DOX-treated rats, the testicular sections illustrated weak immune reactivity for COX-2, especially in the interstitial cells (Figure 6D).

**Fig.6:** Photomicrograph of testicular sections of control (Panel A), AME (Panel B), DOX (Panel C), and DOX and AME (Panel D) rats stained with COX-2 antibody. In control and AME groups, the testicular sections appear negatively or very weakly stained with COX-2 antibody however, in DOX-treated rats, the section appears strongly stained. In DOX and AME-treated rats, the testicular section shows weak to moderate expression for COX-2 antibody. Arrowheads point to the localization of COX-2 antibody reactivity (Stain: COX-2 antibody, Scale bar=100µm).
DISCUSSION

Doxorubicin is a potent antitumor drug that is frequently used to treat a variety of cancers (27). Nonetheless, it has major adverse impacts on organs that are not meant to be targeted, like the testis (28). Accordingly, the current work evaluates the impact of Annona muricata leaf extract on biochemical and histological aspects following DOX-induced testicular dysfunctions in male rats.

TNF-α and IL-6 are proinflammatory proteins that are one of the families of cytokines that help organisms react to infectious and chemical agents and increase (29,30). Our results showed that the AME extract normalized the levels of TNF-α and IL-6 that were elevated by doxorubicin. The primary cause of this is the anti-inflammatory compounds found in AME, including tannins, alkaloids, saponins, and flavonoids, which prevent the formation of prostaglandins and, as a result, suppress the production of TNF-α and IL-6 (31,32).

The hypothalamic gonadotropin-releasing hormone regulates the pituitary gland's secretion of gonadotropins, such as FSH and LH (33). Gonadotropins, FSH and LH, are mediated in the testes by two distinct transmembrane receptors, FSHR and LHR, respectively. Sertoli cells (SCs) in the seminiferous tubules of the testes express FSHR preferentially, while Leydig cells (LCs) express LHR in the interstitial space. Thus, by controlling SC factors, FSH directly affects spermatogenesis, while LH indirectly does so through the androgen receptor. The LCs produce testosterone in a pulsatile manner in response to LH signaling, whereas the SCs create a nonsteroidal hormone in a nonpulsatile manner in response to FSH (34). During adolescence and maturity, the primary function of the gonadotropins FSH and LH is to maintain proper spermatogenesis and sperm production. However, in the present study, DOX administration reduced the production of gonadotropins (LH and FSH) and testosterone, possibly due to the disturbed hypothalamus-pituitary-gonadal axis (35). Another study reported that testosterone level reduction induced by DOX may be generated by an increase in ROS levels or by ROS directly impairing Leydig cells (36).

AME treatment significantly raised the hormone levels disrupted by DOX, according to our research. The efficacy of A. muricata to counteract ROS activities produced by DOX is linked to its flavonoid and saponin concentrations. The findings obtained are consistent with earlier studies that observed a notable rise in testosterone levels upon Annona muricata treatment following testicular dysfunction(37-39). Moreover, the physiology underlying the notable rise in testosterone levels following AME treatment is associated with the concentrations of flavonoids and saponins. However, The data concerned with significant improvement in the activities of FSH and LH under treatment with AME in this study go parallel with findings (40) who found remarkable improvement in the levels of gonadotropins after treatment with AME in arsenic trioxide-induced testicular dysfunction in rats. Previous reports attributed the potential ameliorative role of A.muricata against disrupted male gonadotropins to the presence of flavonoids and saponins which act as a free radical scavenging element (41,42).

When there is an imbalance between the oxidant and antioxidant systems, the antioxidant system is depleted, which leads to the generation of reactive oxygen species (ROS) and damage to different organs and tissues (43). Because testicular tissue has a greater content of unsaturated fatty acids than other tissues and a very rapid rate of cell division and mitochondrial oxygen consumption, ROS generation is crucial in the initial stages of male infertility (44). According to our research, DOX triggers lipid peroxidation, as seen by the significantly elevated testicular MDA level. Additionally, DOX significantly reduced the levels of the testicular antioxidants SOD, CAT, and GSH, which are crucial for protecting cells from
oxidative damage. On the other hand, AME decreased MDA levels and increased SOD, CAT, and GSH levels. Oxidative stress is one of the major mechanisms of DOX-induced testicular damage; the DNA damage induced by DOX leads to increased production of ROS, resulting in lipid peroxidation, depletion of antioxidants, and cellular apoptosis (45). Our results are in agreement with previous studies that investigated DOX-induced testicular toxicity (46,47).

The results of this study, which emphasize the ameliorative effect of AME on testicular antioxidant activities, are consistent with those of Alsenosy et al. who found that *Annona muricata* significantly reduced testicular oxidative stress caused by streptozotocin in diabetic rats (39). According to earlier studies, *Annona muricata* leaf extract includes several non-enzymatic (Vitamin C and E) and enzymatic (catalase and SOD) antioxidants (Kyathanahalli et al., 2014). As a result, AME may be able to inhibit oxidative stress. Tocopherol, or vitamin E, is also an essential lipophilic antioxidant that may avoid Sertoli cell death, as demonstrated by Johnson and Sinclair (48). The research showed that vitamin E could suppress lipid peroxide (one cause of oxidative stress) in mitochondria. Researchers identified and isolated about two hundred antioxidant compounds from *Annona murmurica: ta*, alkaloid phenols, ols, and acetogenins (49), bulatacin, asimisin, and squamosin (50), phenols, flavonoid vitamins, and carotenoid (51). This explains the potential antioxidant effects of AME.

In the current investigation, DOX exhibited atrophied seminiferous tubules with spermatogenic cell stages that had deteriorated and obvious spermatozoa loss in their lumen. Furthermore, there was a noticeable decrease in Leyedig cells and a congested capillary outside the tubules. This indicates that DOX may be involved in the induction of infertility. Comparable histological findings in testicular damage caused by DOX have been reported in recent years (36,52). Since DOX depletes GSH, it causes potent oxidative stress on the testis that damages testicular cells, as was previously mentioned.

Nevertheless, the testicular architecture improved after treatment with *Annona muricata* extract, as evidenced by the treated groups' enhanced spermatogenesis and powerful healing with active spermatid and spermatogonia. Flavonoids, which have an antioxidant impact, are the physiology behind these effects of the extract. Similar to the results of this investigation, Uno et al. found that *Annona muricata* had a curative effect after testicular dysfunction caused by caffeine (53).

Cyclooxygenases (COX) are essential enzymes that help produce prostaglandin (PG) from polyunsaturated fatty acids and arachidonic acid. The distribution of COX-2 in adult and fetal human reproductive tissues has been documented in previous research (54). Although it is well known that inflammatory tissues have significantly higher levels of COX-2, other stimuli and hormones, including cytokines and steroid hormones, can also cause this protein to be produced (55). There are conflicting reports about the presence of COX-2 in human testes. For example, it has been demonstrated that in testicular biopsies of men with various forms of defective spermatogenesis, COX-2 is highly expressed, whereas in the testes of men with normal spermatogenesis, it lacks (56). In the current investigation, the DOX-induced rats exhibited upregulation of COX-2 in the spermatogenic series; however, this expression was mitigated by AME. This demonstrates the anti-inflammatory role of *A. muricata* induced by DOX in testicular tissues. This result coincided with a notable rise in the serum levels of rats given DO and decreasing levels in animals given AME.

**Conclusion**

Based on our findings, treatment of male rats with doxorubicin at a dose of 2 mg/kg b.wt. twice
a week for four weeks was implicated in the induction of oxidative stress and inflammation in the testicular tissues. The treatment of DOX-injected rats with ASE revealed a pronounced return to normalcy of the oxidative stress and inflammatory markers, as evidenced by a remarkable decrease in the testicular MDA and an improvement of SOD, CAT, and GSH activities as well as TNF-α and IL-6 in serum. Additionally, AME modulated the altered levels of gonadotropins and testosterone in the serum. These findings suggest the strong antioxidant, anti-inflammatory, and cytoprotective impact of Annona muricata leaf extract against DOX-induced testicular dysfunctions. More work is needed to assess the mechanism of action of AME.

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