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PROTECTION OF TESTIS THROUGH ANTIOXIDANT ACTION OF OF ARTEMISIA ANNUA (KAYSOM) IN ALLOXAN-INDUCED DIABETIC ALBINO RATS

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ABSTRACT

Background: Diabetes mellitus is a common metabolic disease and widely distributed all over the world. Most diabetic patients were associated with reproductive impairments.Previous studies reported negative complications of diabetes on the male reproductive system with associated gonadal dysfunction. Medicinal plants are effective enhancer of oxidative stress associated with diabetes mellitus. The current study was aimed at assessing the importance of supplementation of Artemisia annua (Kaysom) extract in reducing the metabolic abnormalities accompanied with alloxan-induced diabetes in male albino rats. **Material and Methods**: This study was conducted on thirty male albino rats with an average 100-110 g body weight. The rats were divided into three equal groups including control, diabetic and diabetic treated with Kaysom extract. One dose of alloxan (120 mg/kg body weight) was used to induce diabetes in rats. Diabetic rats were administered "28.5 mg/kg body wt. twice/day" Kaysom extract orally for 30 days. **Results:** It has been detected that the morphological testicular changes noted in diabetic groups had been considerably improved after treatment with Kaysom extract compared with the control group**. Conclusion**: These results explained that Kaysom extract improves diabetes induced oxidative damage in testis as well as provides protection to testis.

KEYWORDS: Testis, Artemisia annua (Kaysom), Antioxidant, alloxan, Albino rat.

INTRODUCTION

Diabetes mellitus is a common metabolic disorder worldwide. It is linked to disturbances in carbohydrate, fat, and protein metabolism.[1] Experimentally, in male rats the induction of diabetes had been found to be associated with change in the function of the reproductive system. Induction of diabetes in rats was used as an in vivo model to study the effects of diabetes on the various organs.[2,3] It has been proved that induction of diabetes affects the testicular functions due to the lack of insulin and therefore the weakness of insulin regulatory action on Sertoli and Leydig cells.[4] There are several previous studies showed changes in the reproductive system structure in diabetic cases.[5,6,7]

Diabetes mellitus is a multivariate disease with imperfection in reactive oxygen species (ROS) scavenging enzymes.[8] The main reason of a number of long term complications of diabetes is chronic hyperglycemia. Protein glycation, the most important source of free radicals, is leaded by hyperglycemia. ROS produced by protein glycation and glucose oxidation mediates the pathogenic effects of high glucose. ROS can directly charged molecular and cellular damage by activating many cellular stress-sensitive pathways, which direct to late complication of diabetes.[9] Many plant secondary metabolites have shown antioxidant potential and ameliorative effect on oxidative stress induced damage in diabetes.[10]

Traditional medicine practices, are considered responsible for an impartial role in primary health care despite modern medicine accessibility.[11]

Artemisia Annua is one of the most important medicinal plant species with high content of essential oils and flavonoids.[12] The plant has the Arabic name Qaysom and which grows in the limestone wadis of the north eastern desert and Red Sea regions.[13]

Moreover, many effects such as anti-inflammatory, anti-oxidative effects, antihyperlipidemia and antihypertensive effects of Kaysom treatment have been reported.[14,15,16]

The present study, was aimed to study the antidiabetic effects of the aqueous extract of Artemisia annua

(Kaysom) on the testis histology in the alloxan-induced diabetic male albino rats.

MATERIAL AND METHODS

Plant Material

Preparation of aqueous extract of Artemisia annua: Plants were collected from El-Arbaeen valley, Saint Catherine, Wadi Gebal, South Sinai, Egypt. The leaves and stems were dried and separately ground into coarse powder. The aqueous extract of Artemisia annua has been prepared by boiling 2g of Artemisia annua with 200ml water for 5 min, kept cool to room temperature then filtered.The extract was daily prepared and stored in in glass containers refrigerator. The Diabetic rats were treated orally with Artemisia annua twice daily at 8 am and 8 pm for 30days (28.5mg/kg twice /day).

Animals

In this study, 30 healthy, adult male Wistar albino rats (150–200g,3months old) were used. They obtained from Animal house, Collage of pharmacy, Prince Sattam bin Abdulaziz University, KSA. They were preserved under controlled standard animal house conditions (temperature: 26–28 °C; photoperiod:12 h natural light and 12 h darkness; humidity: 80–90%) with easy access to food and water ad libitum at Animal Care Facility at, Prince Sattam bin Abdulaziz University. The pellet diet consisted of 23 % protein, 5% lipids, 4% crude fiber, 8% ash, 1% calcium, 0.6% phosphorus, 3.4% glucose, and 55% nitrogen-free extract(carbohydrates). The rats were kept under observation for about 2 weeks before the start of the experiment for adaptation. Diabetes mellitus was induced in animals by single dose of alloxan (120 mg/kg B. W. dissolved in saline), injected intraperitoneally to induce diabetes mellitus.[17]

Ethical considerations

All procedures performed in studies involving animal models were in accordance with the ethical standards of the institutional ethics committee of Prince Sattam bin Abdulaziz Univesity and animal house committee (IRB, PSAU-2017 ANT 1/34PI).

Experimental Design

Three experimental groups, ten rats for each, were used as follows:

1. Group I (Control group): Non-diabetic control rats.
2. Group II (Diabetic group): Rats were injected intraperitoneally with a single dose of alloxan (120 mg/kg dissolved in saline solution).
3. Group III (Diabetic group + Kaysom): Diabetic rats treated orally with Artemisia annua extract (28.5 mg/kg twice /day) for 30 days.

Histological studies: The rats from the control, diabetic and treated groups were anesthetized by intraperitoneally administration of 90 mg/kg ketamine and were sacrificed by cervical dislocation after one month. Small pieces of the testis were taken for the histological studies. The specimens were prepared *via* fixation in 10% neutral buffered formalin solution and Carnoy's fluid. For histological study, paraffin sections were stained with Harris's haematoxylin and eosin (H&E).[18] For detection of collagen fibers, paraffin sections were stained by using Mallory's trichrome stain.[18] All the stained sections were examined by using light microscope, photographed and all the detected variations between the three groups on the level of the microscopic findings had been scientifically discussed.

Immunohistochemical study: Sections of testes were deparaffinized with xylene, followed by antigen retrieval by heating in citrate buffer (10 mM, 20 min). This was followed by endogenous peroxidase blocking in 3% H2O2 for 10 min and incubation with anti-caspase–3 (1:100; Abcam, Ab4051). After washing the slides with phosphate buffered saline, the sections were incubated with the related secondary antibodies at room temperature for 1 h, followed by detection with 3-amino-9-ethylcarbazole, a chromogen. The slides were mounted in paramount aqueous mounting medium.

Morphometric analysis: The image analyzer (ImageJ 1.46r) was used to obtain the following morphometric data:

The mean diameter of the seminiferous tubules for the different groups using H&E - stained sections at 400x magnification.

The mean thickness of the seminiferous tubular diameters for the different groups using Mallory's trichrome-stained sections at 400x magnification.

The area percentage of the collagen fibers in the seminiferous tubules for the different groups using Mallory's trichrome-stained sections at 400x magnification.

The mean apoptotic changes of the seminiferous tubular germinal cells nuclei for the different groups of the study using Feulgen-stained sections at 400x magnification.

The mean number of the caspase-3+ve expressed cells of the seminiferous tubular germinal cells for the different groups of the study using caspase-3 immnuostained sections at 400x magnification.

Statistical analysis: All statistical analyses were performed *via* Paleontological Statistics Version 3.0 (PAST 3.0) statistical software.[19] The obtained data were expressed as mean ± standard deviation (SD) and analyzed using analysis of variance (ANOVA)-Bonferroni with $p<0.05$ considered statistically significant.

RESULTS

Histological results: Sections of the control adult albino rat testis showed several seminiferous tubules with interstitial tissue in between (Fig.1). The seminiferous tubules were lined with spermatogenic cells in different

spermatogenic stages and mature sperms in the lumen. Seminiferous tubules (S. Ts) had two cell types: Spermatogenic and Sertoli cells (Fig.1). Spermatogenic cells were arranged from the basal to the adluminal compartments in the following order; spermatogonia, spermatocytes (primary and secondary), spermatids and spermatozoa.While spermatogonia were basal in position and were small had small rounded nuclei, primary spermatocytes were next to it and were large and had large rounded central nuclei. Next to it, secondary spermatocytes and spermatids were detected (Fig. 1). Spermatids have smaller nuclei with pale chromatin. The late elongated spermatids were identified via their elongated deeply stained nuclei. Sertoli cells with ovoid nuclei were detected in between spermatogenic cells resting on the basement membrane of S.Ts (Fig. 1). Within the interstitial connective tissue (I. T), the blood vessels were surrounded by polygonal or rounded Leydig cells that had granular cytoplasm, single or doubled nuclei and appeared singly or in groups (Fig.1). The histological investigations of testicular tissue in the untreated diabetic rats demonstrated irregularity of the S. Ts shapes with significant decrease their diameters in comparison to the control group (Figs. 2 & Table 1). Also, the germinal epithelium showed obvious disorganization of the germinal epithelium with abnormal cellular attachment. Moreover, the spermatogonia cells were the major cell type that was seen (Fig. 2, & Table 1). Also, multinucleated cells with two or three nucleus were detected in S. Ts (Fig. 2). Moreover, the interstitial connective tissue had an amorphous material with marked destruction of the connective tissues with subsequent widening of the I.T spaces (Fig. 3, & Table 1). The treated group with Kaysom showed a considerable significant recovery of the S.Ts diameters in comparison to the diabetic group (Fig 2,& Table 1). It showed also a considerable recovery of the I.T to normal levels with significant increase in the amount of the collagen fibers in comparison to the diabetic group (Figs. 3, & Table 1).

Immunohistochemical Results

The immunohistochemical investigations of the testicular tissue for the control group showed mild expression of Caspase-3 immunostaining with few caspase-3 +ve cells in the S. Ts (Fig. 4,& Table 1). In diabetic group, a marked significant increase in the number of caspase-3+ve cells was observed in the S. Ts in comparison to the control ones (Fig. 4 & Table 1). The treated group with Kaysom showed a marked significant increase in the number of caspase-3+ve cells was observed in the S. Ts in comparison to the control ones (Fig. 4,& Table 1).

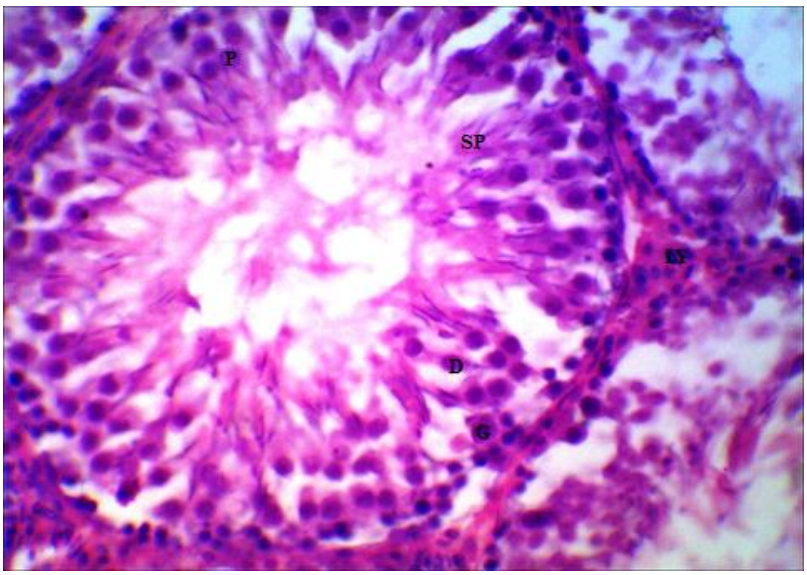

Fig. 1: Light photomicrograph of a section in a rat testicular tissue from the control group shows the normal association of the germ cells and normal architecture of the interstitial tissue. G= Spermatogonium, P=Primary spermatocytes, D=Spermatid, SP=Sperm, Ly=Lydig cell, H&E (400×).

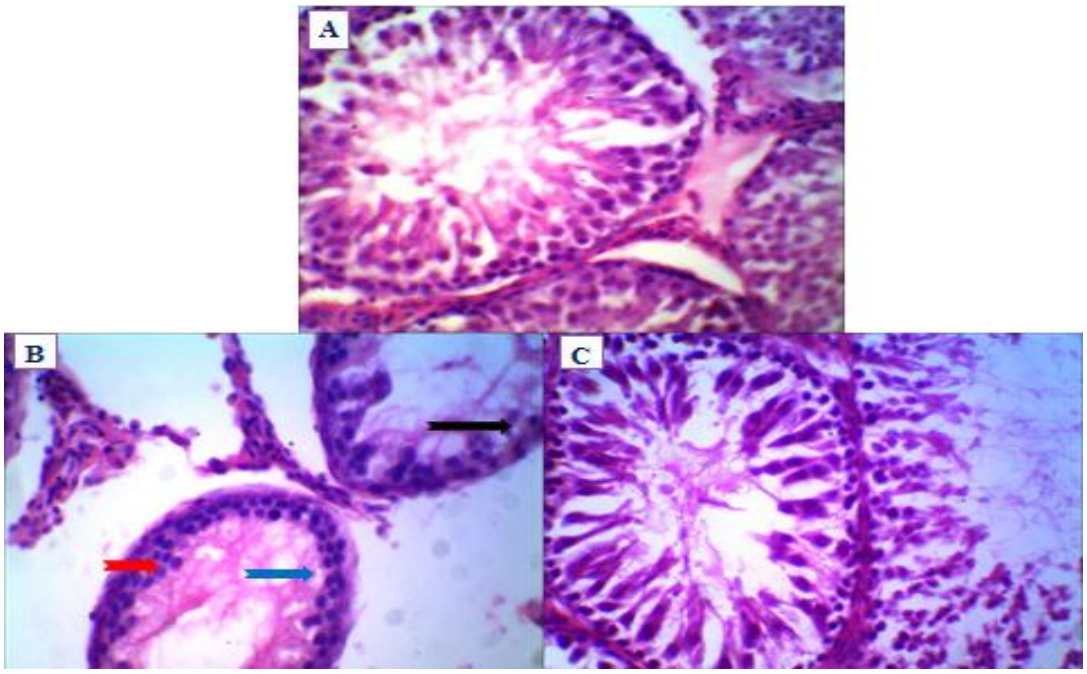

Fig. 2: A) Light photomicrograph of a section in a rat testicular tissue from the control group. The seminiferous tubules (S.Ts) have ordinary shape. S.Ts epithelium is structurally intact and shows normal association of germ cells. B) Light photomicrograph of a section in a rat testicular tissue form untreated diabetic rat. The S. Ts have irregular shape and the germinal epithelium is disorganized. Depletion of germ cells, pyknotic germ cells (black arrow) and karyolysis (blue arrow) are seen. The giant cell formation with two or three nucleus (red arrow) is seen in the lumen of irregular shaped seminiferous tubule (ST). C) Light photomicrograph of a section in a rat testicular tissue treated with Kaysom extract. The S.Ts have partial recovery to normal structure.H&E (400×).

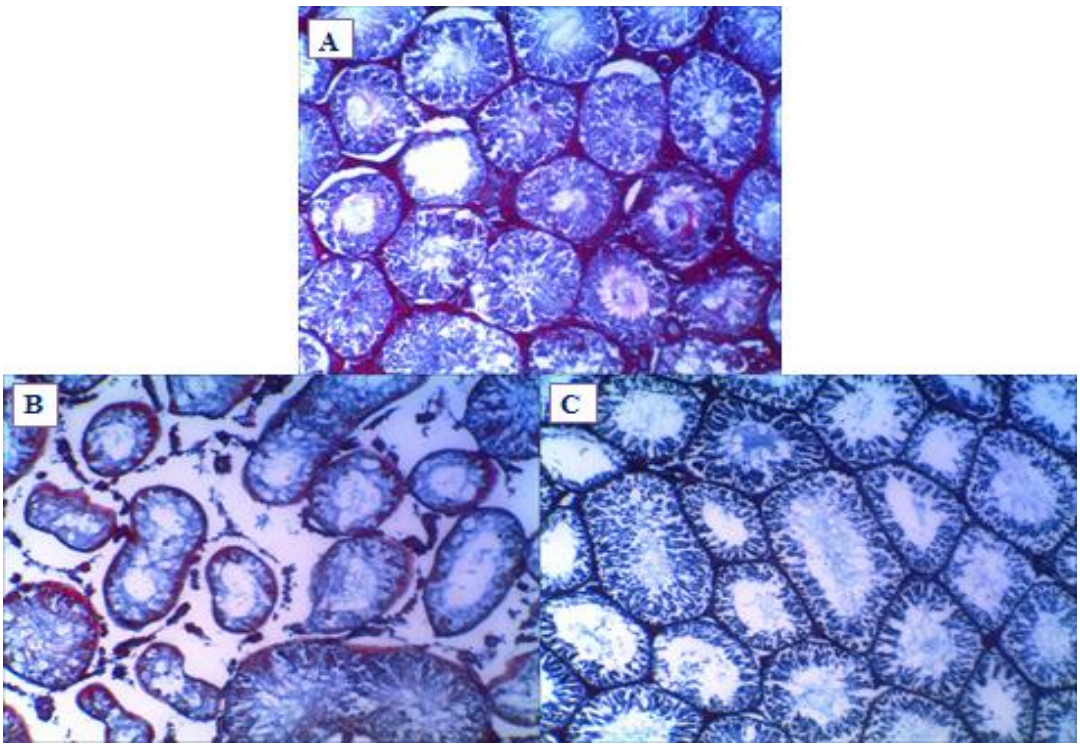

Fig. 3. A) Light photomicrograph of a section in a rat testicular tissue from the control group shows normal distribution of collagen fibers in the interstitial tissue (I.T) around the seminiferous tubules. B) Light photomicrograph of a section in a rat testicular tissue form untreated diabetic rat shows marked reduction of collagen fibers in the I.T around the seminiferous tubules, and an increase diameter of the interstitial spaces. C) Light photomicrograph of a section in a rat testicular tissue treated with Kaysom shows mild distribution of collagen fibers in the I.T around the seminiferous tubules in comparison to the control group. Mallory's trichrome (200×).

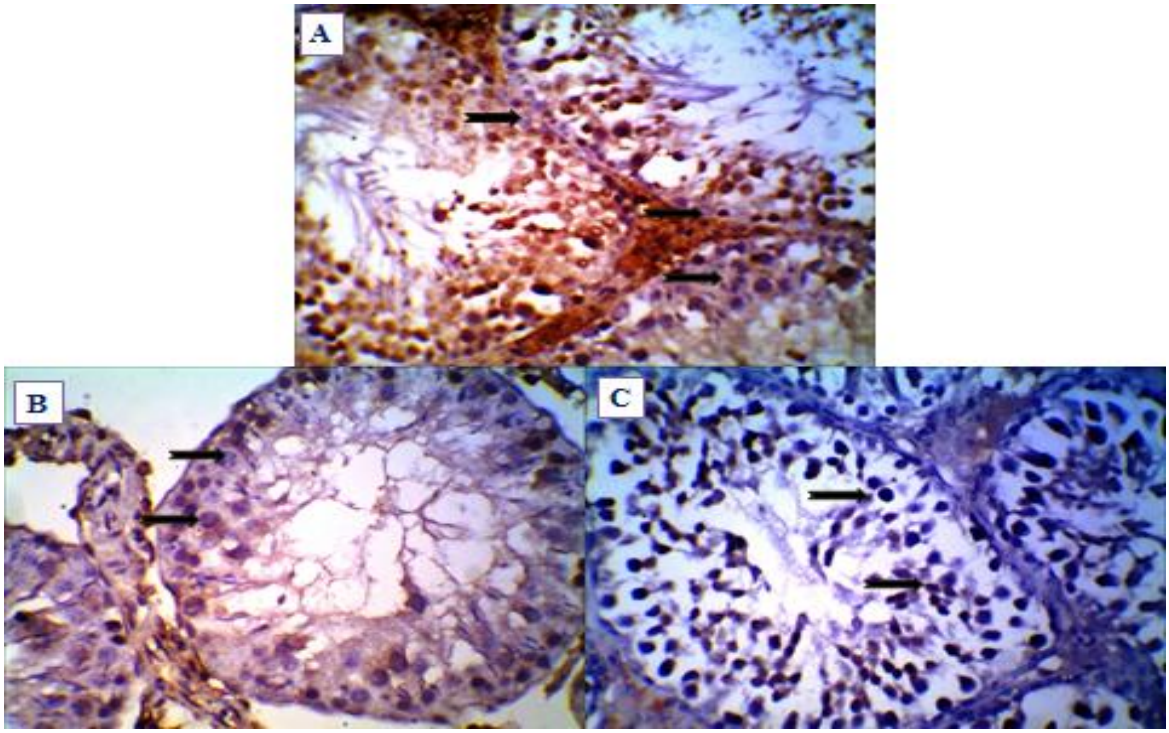

Fig. 4: A) Light micrograph of testicular tissue of a rat from the control group. The seminiferous tubules (S.Ts) germinal cells show mild expression of Caspase-3 immunostaining (black arrows). B) Light micrograph of testicular tissue of a rat from the untreated diabetic rat. The S.Ts germinal cells show marked expression of Caspase-3 immunostaining (black arrows). C) Light photomicrograph of a section in a rat testicular tissue treated with Kaysom shows marked expression of Caspase-3 immunostaining (black arrows) in the S.Ts germinal cells. Caspase-3 immunostaining (400×).

Table 1: Diameters of the seminiferous tubules, thickness of the seminepherous tubular interstitial diameters, percentage of the collagen fibres of the seminiferous tubular interstitial diameters, apoptotic changes of the seminepherous tubular germinal cells nuclei and number of the caspase-3+ve cells of the seminiferous tubular germinal cells for the different groups of the study expressed as mean ± SD.

Paramaters / Study Groups	Mean diameters of the seminiferous tubules	Mean thickness of the seminiferous tubular interstitial diameters	Mean area percentage of the collagen fibres of the seminiferous tubular interstitial diameters	Mean apoptotic changes of the seminiferous tubular germinal cells nuclei	Mean number of the caspase-3+ve cells of the seminiferous tubular germinal cells
Group I (Control)	187.11± 13.92	95.89± 7.57	142.03± 23.25	31.073± 1.48	13.00± 1.94
Group II (Diabetic)	125.61± 31.17 **a	179.70± 22.50 **b	63.97± 16.65 **a	13.08± 4.03 **a	35.81± 2.77 **b
Group II (Kaysom)	157.13± 19.98 **c	68.64± 11.05 **d	56.8911± 10.52 **a	15.87± 3.49 **a	32.92± 2.55 **b

*Significantly different from the control group ($P < 0.05$).
**Significantly different from the control group ($P< 0.001$).
[a]Significant decrease in the parameters levels of the treated group in comparison to the control group.
[b]Significant increase in the parameters levels in the treated group in comparison to the control group.
[c]Significant increase in the parameters levels of the treated group in comparison to the diabetic group.
[d]Significant decrease in the parameters levels in the treated group in comparison to the diabetic group.

DISCUSSION

Diabetes is a major health issue influencing major population worldwide. Several research findings in diabetic men and animal models signified that DM may increase the oxidative stress in testis, alter the endocrine metabolism and spermatogenesis process, which in turn affect male reproductive functions causing male infertility.[20,21]

The results of this study revealed that the alloxan had a destructive effect on seminiferous tubule cells in the testis of rats. on the other hand, in alloxan diabetic rats, these tubules were dilated and the spermatogenic cells irregularly arranged, spermatogenesis was arrested with highly reduc tion in the number of spermatids. Kaysom extract is abundant with many flavonoids such as afroside, cirsimartin, chrysoplenol and cirsiliol. There is evidence that hyperglycemia results in the generation of

reactive oxygen species, leading to oxidative stress in various tissues, including reproductive system. An important link between oxidative stress, inflammatory response and insulin activity is now well established. The ability of antioxidants to protect against the deleterious effects of hyperglycemia and also to improve glucose metabolism and intake must be considered as leads of choice in diabetes treatment.

Diabetic associated-tissue injury and its subsequent complications are most probably induced by free radicals.[22] Prior studies on diabetic subjects showed decreased testosterone levels and vacuolization in the germinal epithelium (spermatogonia and spermatocytes) with subsequent decrease in the testicular weight, sperm number and motility.[23,24] Other studies pointed to increasing S. Ts thickness and germ cell depletionin both the diabetic human and rats.[25,26] In addition, the present study showed similar results as irregularity of the S.Ts shapes with significant decrease in S.Ts diameters. Furthermore, similar to several previous studies results.[27] On the other hand the current study showed degeneration and necrosis of the S. Ts, giant cell formation and interstitial changes in the diabetic rats.

Many herbal extracts or derivatives with high antioxidant activity are useful for diabetes treatment and other metabolic syndrome.[28] Thus, antioxidant therapy is one of the major strategies for diabetes treatment.[28] Our results showed that Kaysom has significant protective effects against the apoptotic changes. This protective effect may be due to their antioxidant properties that has been shown in previous studies.[28] Abnormal high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms may damage the cellular organelles and enzymes, increases lipid peroxidation and leads to insulin resistance.[29]

Antioxidant defense by antioxidant enzymes is a protective mechanism against oxidative stress that scavenge harmful ROS in male reproductive organs and playing an important role in maintaining spermatogenesis and reproductive functions.[30]

In conclusion, considerable improvements in the testicular tissue morphological changes that were observed in diabetic groups had been detected after Kaysom treatment in comparison to the control group.

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