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# Protective Effects of Curcumin, Coumarin and Honey against Diclofenac Sodium-induced Testicular Dysfunction in Adult Mice

Ibtisam M.H. El Mileegy[1], Nasser S. Abou Khalil[1], Asmaa S.M. Abdelnazir[2], Mahmoud Abd-Elkareem[3], Elham A. Abd-Allah[4], Eman A. Negm[2]*

[1]Department of Medical Physiology, Faculty of Medicine, Assiut University, Assiut 71526, Egypt.

[2]Department of Physiology, Faculty of Veterinary Medicine, Assiut University, Assiut 71526, Egypt.

[3]Department of Cell and Tissues, Faculty of Veterinary Medicine, Assiut University, Assiut 71526, Egypt.

[4]Department of Zoology, Faculty of Science, New Valley University, 72511 New Valley, Egypt.

***Correspondence**
Eman A. Negm
Department of Physiology, Faculty of Veterinary Medicine, Assiut University, Assiut 71526, Egypt.
E-mail address: Emannegm@vet.aun.edu.eg

## Abstract

Although diclofenac (DS) is used in treating a wide variety of painful and inflammatory situations in humans and animals, its chronic administration is accompanied by side effects. Curcumin, coumarin, and honey are suggested to be promising strategies owing to their antioxidant, anti-apoptotic and cytoprotective properties. Thus, this study aimed to highlight the potential protective effects of these natural products on the testis of DS-challenged mice and its mechanistic tools. Thirty adult male mice were divided into five groups: control, DS, DS + curcumin, DS + coumarin and DS + honey. DS-exposed mice were characterized by a decrease in plasma FSH. 17beta-estradiol and FSH increased in the plasma of all the protected groups. Plasma LH increased in DS+curcumin and DS+coumarin groups compared to the DS group. DS increased testicular lipid peroxides and plasma total antioxidant capacity (TAC) and decreased testicular reduced glutathione (GSH) and superoxide dismutase (SOD). The plasma testosterone levels were within the standard limit in all experimental groups. Curcumin, coumarin, and honey decreased LPO and increased GSH and SOD, whereas coumarin and honey only decreased TAC. The natural products normalized the integrity of the basement membrane of the seminiferous tubules. Immuno-staining of glutathione reductase (GR) and SOD2 was increased in all groups relative to the control. Curcumin-, coumarin- and honey- supplemented groups, showed few numbers of apoptotic spermatogenic cells similar to the control group. The studied natural products provided efficient protective strategies against DS-induced testicular deterioration by their antioxidant, cytoprotective, and anti-apoptotic effects.

KEYWORDS
Coumarin, Curcumin, Diclofenac, Honey

## INTRODUCTION

Diclofenac sodium (DS) is one of the most prescribed drugs for alleviating pain and a wide array of inflammatory disorders (Ledakowicz *et al*., 2019). The in vivo metabolism of DS results in production of reactive oxygen species (ROS) (Gómez-Lechón *et al*., 2003). The overgeneration of ROS can threaten the cellular microenvironment by causing oxidative stress, damage to nucleic acids, enzyme inhibition and finally leading to death by cellular apoptosis (Hickey *et al*., 2001; Inoue *et al*., 2004). A broad spectrum of studies revealed that exposure to DS leads to nephrotoxicity (Ahmed *et al*., 2017), hepatotoxicity (Alabi *et al*., 2017; Adeyemi and Olayaki, 2018; Olayaki *et al*., 2018) and reproductive toxicity (Vyas *et al*., 2019). The reproductive toxicity which recently attracted attention is not yet fully researched although testis is considered as one of the primary target organs for DS attack due to its high oxygen consumption rate, abundance of polyunsaturated fatty acids, and poor reserve of antioxidant enzymes (Lewis and Aitken, 2005; Agarwal *et al*., 2008). Adedara *et al*. (2021) and Waly *et al*. (2022) found that DS caused elevation in lipid peroxidation along with a reduction in total antioxidant capacity and enzymatic antioxidants in the testicular tissues of DS-exposed rats. Besides that, DS has high endocrine disruption potential causing a disturbance in the hypothalamic-pituitary-gonadal axis and reduction in sperm functional characteristics (Adeyemi *et al*., 2019; Vyas *et al*., 2019). The role of DS in targeting the main points of apoptotic pathways could be implicated in the pro-apoptotic effect of DS on the testis. This outcome could be due to increased transcript level of pro-apoptotic caspase 3 (Orabi *et al*., 2020), Bcl-2 and caspases 3 and 9, and decreased transcript level of anti-apoptotic Bax (Huang *et al*., 2016).

Consequently, it is plausible that antioxidant and cytoprotective agents, suppressing ROS overproduction and inhibiting the apoptotic pathways, could be appreciated as an adjuvant supplement during DS chemotherapeutic protocol. The side effects of synthetic antioxidant compounds (Park and Kim, 2017) and the low acceptability for consumers (Kulawik *et al*., 2013; Anraku *et al*., 2018) give a driving force to focus on antioxidants derived from natural sources. In this regard, curcumin, coumarin and honey are considered to be a highly hopeful candidate owing to its antioxidant, anti-apoptotic and cytoprotective nature (Payá *et al*., 1992; da Silva *et al*., 2016; Cheraghi *et al*., 2017; Mohebbati *et al*., 2017; Huang *et al*., 2018) giving a strong rationality to hinder the multiple pathophysiological mechanisms of DS. Therefore, this


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study aims to highlight the possible protective effects of these natural products on DS-induced testicular damage in adult mice and their substantial underlying pathways.

## MATERIALS AND METHODS

*Drugs and chemicals*

Diclofenac sodium ampoules (Voltaren ® 75 mg/3 mL, Novartis Pharma S.A.E. Cairo, Egypt) was obtained from the local registered medical store. Curcumin from Curcuma longa (Turmeric) powder (Diferulylmethane, (E,E)-1,7-bis(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, Diferuloylmethane) CAS Number: 458-37-7 was obtained from Sigma Chemical Company (St. Louis, MO, USA). Coumarin ≥99% (HPLC), CAS Number: 91-64-5 C9H6O2 was purchased from Sigma Chemical Company (St. Louis, MO, USA). Honey (Hadramy Mountain Sidr Honey) was obtained from Imtenan Health Shop, Assiut, Egypt. Carboxymethyl cellulose (purity is 98%, CAS: 9005-64-5) was purchased from Alpha global search company.

*Experimental animals*

Thirty male mice at 5-6 weeks of age and weighing 35.0±5.0 grams were obtained from the Animal House, Faculty of Medicine, Assiut University, Assiut, Egypt, and bred under natural photoperiods, a temperature of 20-25°C and relative humidity of 55.0±5.0%. They were fed with commercial pelleted feed and water ad libitum.

*Experimental design*

After an adaptation period of one week, mice were randomly allocated into five groups six animals each. One group received no treatment and was kept as a negative control. The second group (DS) was injected intraperitoneally with DS at a dose of 10 mg/kg BW (Waly *et al*., 2022) daily during the second and third weeks of the experiment. The third group (DS + curcumin) was supplemented with curcumin at a dose of 100 mg/kg BW (Zha *et al*., 2018) dissolved in 0.5% carboxymethyl cellulose. The fourth group (DS + coumarin) was supplemented with coumarin at a dose of 30 mg/kg BW (Bilgin *et al*., 2011) dissolved in 0.5% carboxymethyl cellulose. The fifth group (DS + honey) was supplemented with honey at a dose of 5 g/kg BW (Afroz *et al*., 2014) dissolved in distilled water. All the protective agents were administrated daily using an oral gavage throughout the experiment, concomitant with the injection of DS during the second and third weeks. The experimental procedure was reviewed by the Institutional Review Board of the Faculty of Medicine, Assiut University, Egypt (17101891). It fulfilled all requirements as governed by the Declaration of Helsinki.

*Collection and preparation of samples*

At the end of the experiment, mice were subjected to overnight fasting. Blood samples were collected from the jugular vein into EDTA-containing tubes. The plasma was obtained after centrifugation at 3000 rpm for 10 minutes and stored at -20 °C to measure pituitary gonadotrophic hormones, sex hormones and the lipid profile. Mice were killed by cervical dislocation, testes were quickly harvested, and one testis was fixed in 10% neutral buffered formalin for histopathological and immunohistochemical investigation. For evaluation of redox outcomes, the other testis was stored at -20°C. Samples from the testis were homogenized in phosphate buffer (pH 7.4) to give 10% w/v homogenate. The homogenates were centrifuged at 10000 rpm for 15 minutes, and the supernatants were preserved frozen at -20 °C for the consequent oxidant/antioxidant analysis.

*Biochemical measurements*

Plasma testosterone level was estimated by ELISA technique using a microplate enzyme immunoassay kit (Catalog number: BC-1115) according to the manufacturer's protocol (BioCheck, Inc., Foster City, USA) with a minimum detectable concentration of 0.05 ng/ml.

Plasma luteinizing hormone (LH) level was measured by ultra-sensitive LH ELISA kit (Catalog number: LH550F) obtained from Calbiotech Inc. (Spring Valley, USA). The procedure has a sensitivity 0.0094 mIU/ml, intra-assay coefficient of variation 7.74% and inter-assay coefficient of variation 7.82%. Plasma follicle-stimulating hormone (FSH) level was measured by FSH ELISA kit (Catalog number: CSB-E06867h, CUSABIO TECHNOLOGY LLC, Houston, USA) using quantitative sandwich enzyme immunoassay technique with minimum detectable limit less than 1mIU/ml, and intra- and inter-assay precision less than 15%. Plasma 17beta-Estradiol (E2) level was measured by ELISA kit (Catalog number: RE52041, IBL International GmbH, Hamburg, Germany). The analytical sensitivity of the kit is 10.6 pg/ml, with intra- and inter-assay coefficient of variation 8.97% and 10.87%, respectively. Total protein level in the supernatant of tissue homogenate was measured following Lowry *et al*. (1951). Lipid peroxides (LPO) were measured according to a previously published protocol (Ohkawa *et al*., 1979). Nitric oxide (NO) was measured using the method of Ding *et al*. (1988). Total antioxidant capacity (TAC) was measured using a colorimetric kit (Catalog number: TA 2513, Biodiagnostic, Giza, Egypt). Reduced glutathione (GSH) content was estimated according to the method of Beutler *et al*. (1963). Superoxide dismutase (SOD) activity was determined based on its ability to inhibit the autoxidation of epinephrine at alkaline medium (Misra and Fridovich, 1972). Catalase (CAT) activity was measured according to the method of Lück (1963). All the measured oxidant/antioxidant parameters were corrected with the total protein levels in the testicular homogenate. According to the manufacturer's instructions, plasma total cholesterol (TC) (Catalog number: 230002), triglyceride (TG) (Catalog number: 314002) were estimated by commercially available colorimetric kits (Egyptian Company for Biotechnology, Cairo, Egypt). Testosterone, E2, LH, and FSH were measured using an ELIZA reader (ELx800UV, Bio Tek Instruments, Inc, USA), while the other biochemical parameters were measured using a spectrophotometer (S1200, Unico, USA).

*Histological examination*

The formalin-fixed testes samples were dehydrated in ascending grades of ethanol, cleared in methyl benzoate, and then embedded in paraffin wax. Paraffin sections at 5 µm in thickness were cut and stained with the following histological stains:

A. Haematoxylin and Eosin (HX&E) for general histological examination (Bancroft and Gamble, 2008).

B. Crossmon's trichrome technique to stain collagen fibers (Abd-Elkareem *et al*., 2020).

*Immunohistochemistry of glutathione reductase and superoxide dismutase 2*

For immunohistochemical detection of glutathione reductase

(GR) and superoxide dismutase 2 (SOD2) in the testis, we used polyclonal anti-superoxide dismutase 2 and anti-glutathione reductase antibodies respectively (Chongqing Biospes Co., Ltd, China) and Power-Stain™ 1.0 Poly horseradish peroxidase (HRP) 3,3′-Diaminobenzidine (DAB) Kit (Genemed Biotechnologies, Inc, 458 Carlton Ct. South San Francisco, CA 94080, USA) (Sayed *et al*., 2019; Abd-Elkareem *et al*., 2021).

*TUNEL assay*

Investigation of apoptosis was done using In Situ Cell Death Detection Kit, Fluorescein (Sigma-Aldrich), according to a recent study (Waly *et al*., 2022).

*Statistical analysis*

Data were represented as mean ± standard error of the mean (SEM). The results were analyzed by one-way analysis of variance (ANOVA) followed by Duncan post-test using SPSS program version 16 (SPSS Inc., Chicago, USA). Differences of $p < 0.05$ were considered to be statistically significant.

## RESULTS

*Biochemical findings*

Effects of curcumin, coumarin, and honey on the plasma level of pituitary gonadotrophic and gonadal hormones in DS-challenged mice

Table 1 illustrates the changes in plasma levels of pituitary-gonadal hormones following supplementation with curcumin, coumarin, and honey in mice challenged with DS. There is a non-significant decrease in the plasma level of LH in the DS group relative to the control one. The supplementation with curcumin and coumarin significantly increased the plasma LH level in DS-exposed mice. DS-challenged mice were characterized by a significant reduction in the plasma FSH level compared to the control group. Supplementation with curcumin, coumarin and honey significantly increased the plasma FSH level compared to the DS group. There were non-significant differences between the protective agents-supplemented groups regarding plasma LH and FSH levels. A significant rise in the plasma testosterone level was found in the DS-exposed mice compared to the control group. Administration of each coumarin and honey failed to cause any significant difference in the plasma testosterone level versus the DS group. In contrast, the DS+curcumin group had significantly lower plasma testosterone levels than the DS group. It was found that the plasma testosterone level was significantly higher in both DS+coumarin and DS+honey groups than that of the DS+curcumin group without a significant difference between DS+coumarin and DS+honey groups. The plasma E2 level of the DS group did not significantly change compared with that of the control group. Curcumin, coumarin, and honey supplementation significantly increased the plasma level of E2 compared to the DS group. There were no significant differences in plasma E2 levels between all the protective agents received groups when compared with each other.

Effects of curcumin, coumarin and honey on the testicular redox balance in DS-challenged mice

Table 2 shows the changes in the oxidant/antioxidant parameters in the DS-challenged mice following supplementation with curcumin, coumarin, and honey. A significant rise in testicular LPO level was found in DS group relative to the control one. Oral supplementation of curcumin, coumarin and honey caused a significant decrease in the testicular LPO level in the mice suffering from DS burden. The testicular LPO level of the DS+honey group was significantly lower than that of DS+coumarin group. There was an insignificant difference between the testicular LPO level of the DS+coumarin group and that of the DS+curcumin group and the testicular LPO level of DS+curcumin and DS+honey groups. There were insignificant differences among all the experimental

Table 1. Effects of curcumin, coumarin and honey on the levels of pituitary gonadotrophic and gonadal hormones in mice with diclofenac sodium-induced testicular impairment.

| Parameter \ Group | Control | DS | DS+Curcumin | DS+Coumarin | DS+Honey | *P* value |
|---|---|---|---|---|---|---|
| Plasma LH level (mU/ml) | 0.68±0.06$^{ab}$ | 0.58±0.04$^{b}$ | 0.78±0.03$^{a}$ | 0.84±0.04$^{a}$ | 0.70±0.07$^{ab}$ | 0.021 |
| Plasma FSH level (mU/ml) | 1.52±0.05$^{b}$ | 1.30±0.07$^{c}$ | 1.64±0.07$^{ab}$ | 1.78±0.06$^{a}$ | 1.58±0.06$^{ab}$ | 0.001 |
| Plasma testosterone level (ng/ml) | 2.15±0.07$^{b}$ | 7.92±0.61$^{a}$ | 0.78±0.13$^{b}$ | 7.267±0.66$^{a}$ | 8.73±1.54$^{a}$ | 0 |
| Plasma E2 level (pg/ml) | 21.37±0.88$^{ab}$ | 20.02±0.78$^{b}$ | 22.78±0.48$^{a}$ | 23.04±0.36$^{a}$ | 23.19±1.02$^{a}$ | 0.032 |

DS: diclofenac sodium; LH: luteinizing hormone; FSH: follicle-stimulating hormone; E2: 17beta-estradiol
Data are expressed as the mean ±SEM of 6 mice per group.
$^{a\text{-}c}$Different letters in the same row indicate significant difference at $p < 0.05$ (one-way ANOVA followed by Duncan post-test).

Table 2. Effects of curcumin, coumarin and honey on oxidant/antioxidant parameters in mice with diclofenac sodium-induced testicular impairment.

| Parameter \ Group | Control | DS | DS+Curcumin | DS+Coumarin | DS+Honey | *P* value |
|---|---|---|---|---|---|---|
| Testicular LPO level (nmol/mg protein) | 1.66±0.03$^{b}$ | 2.21±0.12$^{a}$ | 1.38±0.13$^{bc}$ | 1.58±0.11$^{b}$ | 1.16±0.11$^{c}$ | 0 |
| Testicular NO level (nmol/mg protein) | 9.13±0.84 | 6.68±0.72 | 6.05±0.79 | 6.99±0.66 | 9.59±2.61 | 0.283 |
| Plasma TAC (nmol/ml) | 0.04±0.01$^{bc}$ | 0.07±0.01$^{a}$ | 0.06±0.01$^{ab}$ | 0.04±0.01$^{c}$ | 0.03±0.00$^{c}$ | 0.004 |
| Testicular SOD activity (nmol/mg protein) | 7.86±0.15$^{c}$ | 6.24±0.25$^{d}$ | 8.25±0.56$^{c}$ | 10.17±0.55$^{b}$ | 12.51±0.29$^{a}$ | 0 |
| Testicular GSH level (nmol/mg protein) | 36.65±2.84$^{ab}$ | 24.61±1.607$^{c}$ | 43.29±2.27$^{a}$ | 31.89±2.48$^{b}$ | 40.25±2.61$^{a}$ | 0 |
| Testicular CAT activity (nmol/mg protein) | 1.49±0.42 | 1.57±0.24 | 1.74±0.36 | 1.98±0.14 | 1.85±0.29 | 0.657 |

DS: diclofenac sodium; LPO: lipid peroxides; NO: nitric oxide; TAC: total antioxidant capacity; SOD: superoxide dismutase; GSH: reduced glutathione; CAT; catalase
Data are expressed as the mean ±SEM of 6 mice per group.
$^{a\text{-}d}$Different letters in the same row indicate significant difference at $p < 0.05$ (one-way ANOVA followed by Duncan post-test).

groups regarding the testicular NO level. A significant rise in the plasma TAC was found in the DS-exposed mice compared to the control group. Curcumin administration did not cause any significant change in the plasma TAC versus the DS group, while DS+coumarin and DS+honey groups had significantly less plasma TAC than the DS group. By comparing between the protective agents received groups, it was found that the plasma TAC was significantly greater in the DS+curcumin group than in each of DS+coumarin and DS+honey groups. Still, there was an insignificant difference between DS+coumarin and DS+honey groups. DS-challenged mice showed a significant reduction in testicular SOD activity compared to the control group. Curcumin, coumarin, and honey supplementation led to a significant increase in the activity of testicular SOD compared to the DS group. In the DS+honey group, the testicular SOD activity was significantly higher than that of the DS+coumarin group or DS+curcumin group. The testicular SOD activity of the DS+coumarin group was significantly higher than that of DS+curcumin.

The testicular GSH level of the DS group was significantly lower than that observed in the control group. Administration of curcumin, coumarin, and honey significantly increased the testicular GSH level compared to the DS group. Testicular GSH levels of both DS+curcumin and DS+honey groups were significantly higher than that of the DS+coumarin group. An insignificant difference was found between the DS+curcumin group and DS+honey group. Concerning the testicular CAT activity, comparison between all the experimental groups revealed an absence of significant differences.

Effects curcumin, coumarin and honey on plasma total cholesterol and triglycerides in DS-challenged mice

Table 3 shows the changes in plasma TC and TG in the DS-treated mice following supplementation with curcumin, coumarin, and honey. No significant differences were observed in plasma TC or TG levels when comparing the different experimental groups.

*Histopathological findings*

Effects of curcumin, coumarin and honey on the histological features of the testis of DS-exposed mice

The histological examination of the testes in the control group revealed the typical testis structure, consisting of seminiferous tubules (ST) separated by several interstitial cells of Leydig. ST were lined by stratified germinal epithelium and Sertoli cells. This germinal epithelium was made of spermatogenic cells in multiple phases of development (Fig. 1A & 2A). DS-challenged group showed degeneration in ST, spermatogenic cells, and Sertoli cells along with a slight proliferation of Leydig cells (Fig. 1B & 2B). Curcumin- and honey-treated groups showed almost typical ST, spermatogenic cells, and interstitial cells of Leydig and Sertoli cells (Fig. 1C, 1E & 2C & 2E). Concerning coumarin-treated group, it showed degenerated seminiferous tubules, degenerated spermatogenic cells, and Sertoli cells (Fig. 1D & 2D). All protective agents-supplemented groups exhibited slight proliferation of

Table 3. Effects of curcumin, coumarin and honey on plasma total cholesterol and triglycerides in mice with diclofenac sodium-induced testicular impairment.

| Parameter / Group | Control | DS | DS+Curcumin | DS+Coumarin | DS+Honey | *P* value |
|---|---|---|---|---|---|---|
| Serum TC level (mmol/l) | 122.83±14.39 | 98.27±21.98 | 129.88±31.36 | 82.03±14.04 | 35.17±9.14 | 0.097 |
| Serum TG level (mg/dl) | 125.31±41.11 | 56.57±25.84 | 84.09±22.34 | 48.45±9.03 | 66.20±22.31 | 0.316 |

DS: diclofenac sodium; TC: total cholesterol; TG: triglycerides
Data are expressed as the mean ±SEM of 6 mice per group. (One-way ANOVA followed by Duncan post-test)

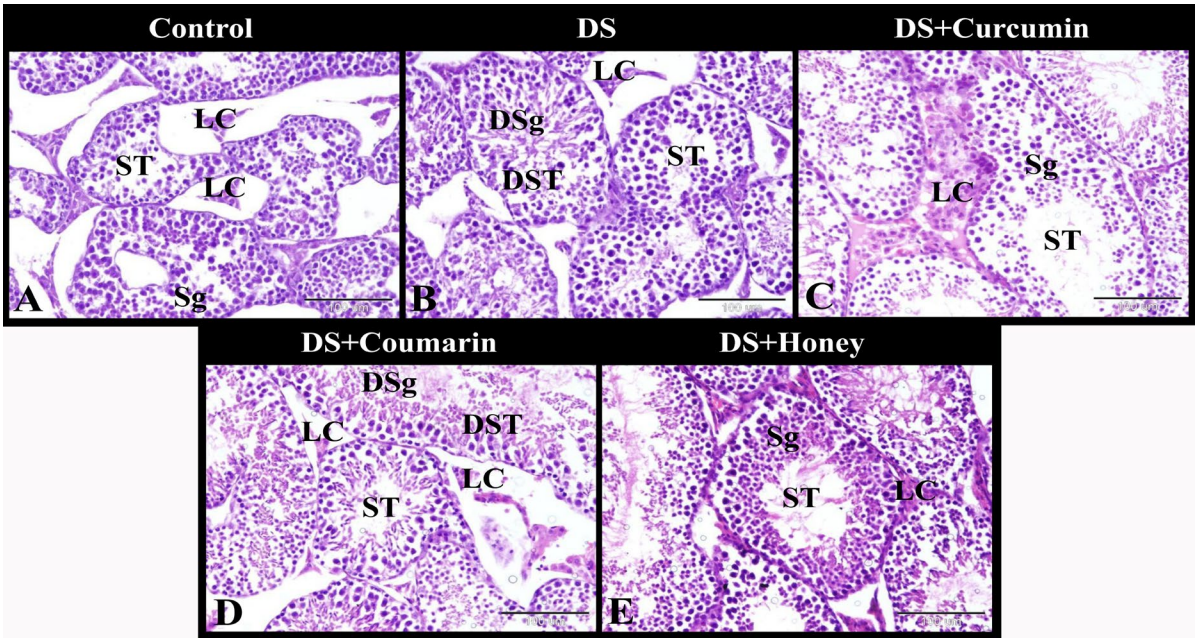


Fig. 1. Photomicrograph of paraffin sections showed the ameliorative effect of curcumin, coumarin and honey on DS induced testicular damages in mice. A: Control group showed the normal histology of the testis; normal seminiferous tubules (ST), Leydig cells (LC), and spermatogenic cells (Sg). B: DS treated group showed degenerated seminiferous tubules (DST), degenerated spermatogenic cells (DSg), slight proliferation of Leydig cells (LC). C: DS+ Curcumin treated group showed nearly healthy seminiferous tubules (ST), spermatogenic cells (Sg) and slight proliferation of Leydig cells (LC). D: DS+Coumarin treated group showed nearly healthy seminiferous tubules (ST), spermatogenic cells (Sg), some degenerated seminiferous tubules (DST), degenerated spermatogenic cells (DSg), and slight proliferation of Leydig cells (LC). E: DS+Honey treated group showed nearly healthy seminiferous tubules (ST), spermatogenic cells (Sg) and slight proliferation of Leydig cells (LC). Original magnification; A-E X200, scale bar = 100 µm, Hematoxylin and Eosin stain.

Leydig cells. Using Crossmon's trichrome technique, we found that the control group showed standard content of the peritubular collagen fibers around ST (Fig. 3A). Whereas the DS-treated group showed few peritubular collagen fibers around ST (Fig. 3B). Curcumin-, coumarin- and honey-treated groups showed nearly regular and continued peritubular collagen fibers around ST (Fig. 3C-E). The PAS staining procedure was utilized to assess the structural integrity of the ST basement membranes. The control group showed regular, continued strong PAS-positive basement membrane of ST (Fig. 4A). While the DS-treated group showed weak PAS-positive basement membrane of ST (Fig. 4B). In contrast, the curcumin-treated group showed approximately regular and continued PAS-positive basement membrane of ST (Fig. 4C). Whereas the coumarin-treated group showed nearly regular and continued moderately PAS-positive basement membrane of ST (Fig. 4D). Honey-treated group showed nearly regular, continous, and strong PAS positive basement membrane of ST (Fig. 4E).

Immunohistochemistry of glutathione reductase and superoxide dismutase 2

Immunostaining of GR and SOD2 revealed that the control group showed negative GR and SOD2 immuno-staining in the germ and Leydig cells (Fig. 5A & 6A, respectively). In contrast, the DS-treated group displayed mild GR and SOD2 immuno-staining in the spermatogenic and Leydig cells (Fig. 5B & 6B, respective-

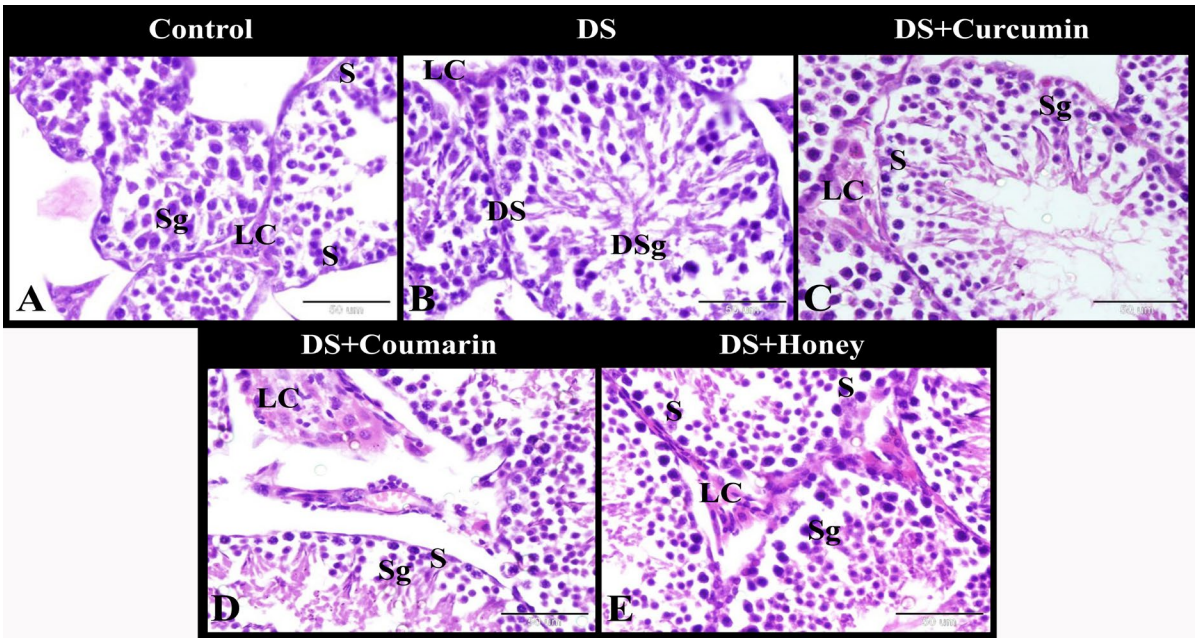


Fig. 2. Photomicrograph of paraffin sections showed the ameliorative effect of curcumin, coumarin and honey on DS induced testicular damages in mice. A: Control group showed the normal Leydig cells (LC), Sertoli cells (S) and spermatogenic cells (Sg). B: DS treated group showed degenerated spermatogenic cells (DSg), degenerated sertoli cells (DS) and slight proliferation of Leydig cells (LC). C: DS+ Curcumin treated group showed nearly healthy spermatogenic cells (Sg), Sertoli cells (S) and slight proliferation of Leydig cells (LC). D: DS+Coumarin treated group showed nearly healthy spermatogenic cells (Sg), Sertoli cells (S) and slight proliferation of Leydig cells (LC). E: DS+Honey treated group showed nearly healthy spermatogenic cells (Sg), Sertoli cells (S) and slight proliferation of Leydig cells (LC). Original magnification; A-E X400, scale bar = 50 µm, Hematoxylin and Eosin stain.

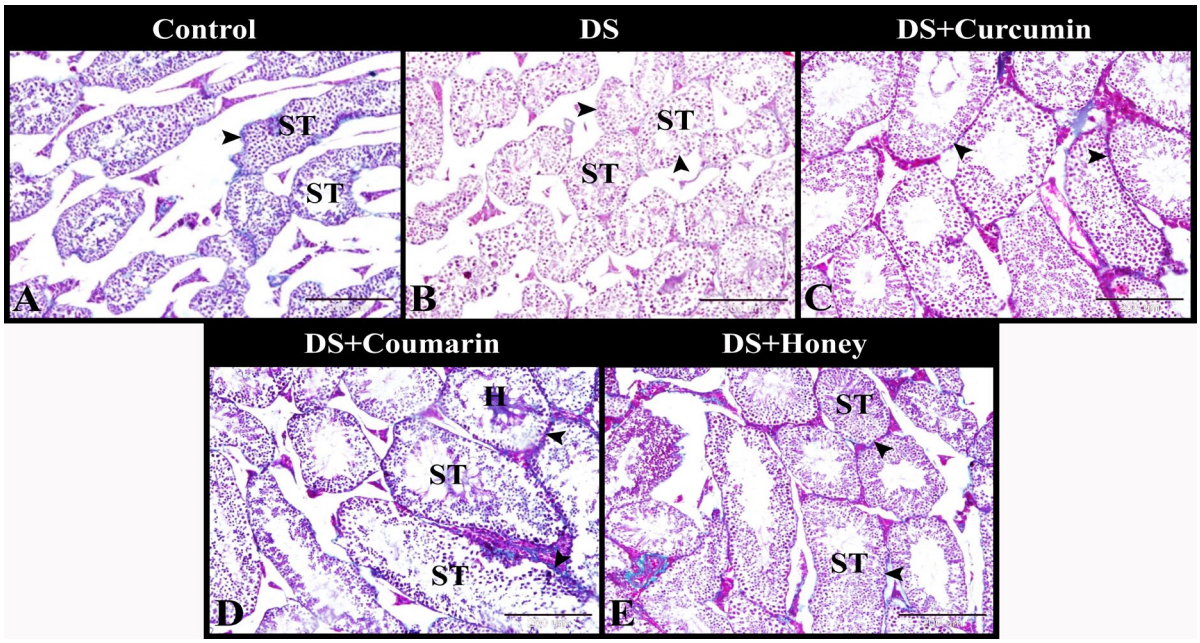


Fig. 3. Photomicrograph of paraffin sections showed the ameliorative effect of curcumin, coumarin and honey on DS induced testicular damages in mice. A: Control group showed normal peritubular collagen fibers (arrowhead) around the seminiferous tubules (ST). B: DS treated group showed few peritubular collagen fibers (arrowhead) around the seminiferous tubules (ST). C: DS+ Curcumin treated group showed nearly regular and continued peritubular collagen fibers (arrowhead) around the seminiferous tubules (ST). D: DS+Coumarin treated group showed nearly regular and continued peritubular collagen fibers (arrowhead) around the seminiferous tubules (ST). Note the hyalinized center of the seminiferous tubule. E: DS+Honey treated group showed nearly regular and continued peritubular collagen fibers (arrowhead) around the seminiferous tubules (ST). Original magnification; A-E X100, scale bar = 200 µm, Crossmon's trichrome technique.

ly). Also, the curcumin-, coumarin- and honey-treated groups showed mild GR and SOD2 immuno-staining in the germ and Leydig cells (Fig. 5C-E & 6C-E, respectively).

Effects of curcumin, coumarin, and honey on the DNA fragmentation in the testis of DS challenged in mice

The control group showed few numbers of apoptotic spermatogenic cells (Fig. 7A), however the DS group showed greater number of apoptotic spermatogenic cells compared to the control group (Fig. 7B). Whereas the curcumin-, coumarin- and honey-treated groups showed few numbers of apoptotic spermatogenic cells similar to the control group (Fig. 7C-E).

## DISCUSSION

The present investigation showed that exposure of adult male mice to DS resulted in a significant decrease in the plasma FSH level without a significant change in the plasma LH level. A close inspection of the experimental animal research denotes contradictory data about the effects of DS burden on the levels of gonadotrophins in males. One of this research revealed a marked reduction in both LH and FSH levels (Adedara *et al.*, 2021), a second one proved that DS did not exert any impacts on the levels of gonadotrophins (Adeyemi *et al.*, 2019), while the others re-

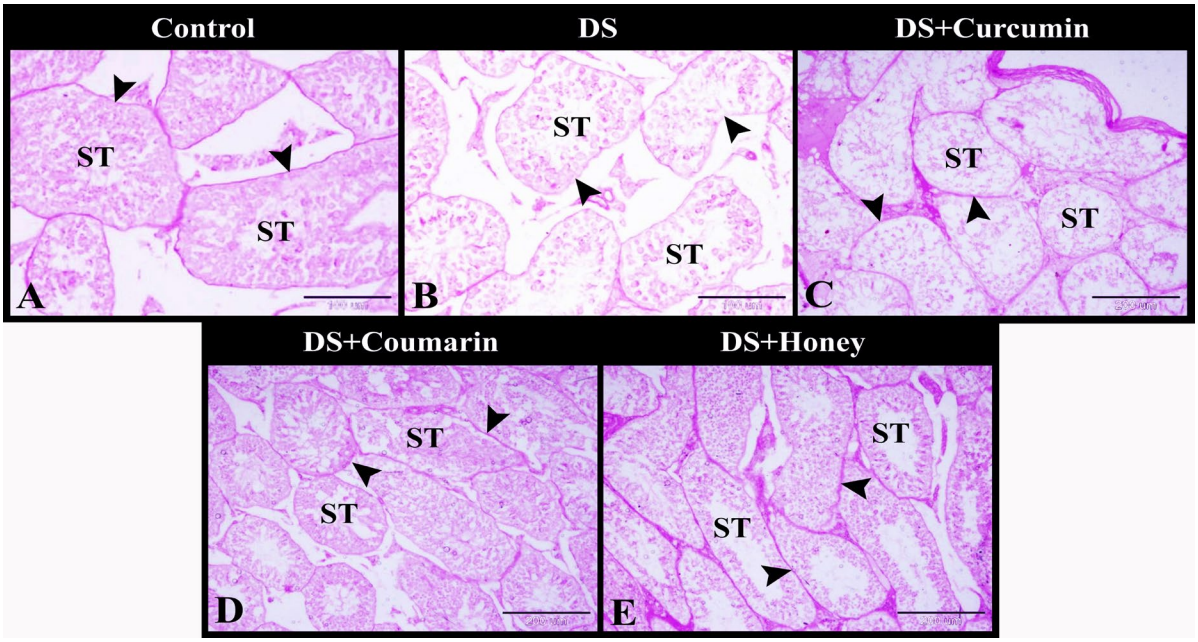


Fig. 4. Photomicrograph of paraffin sections showed the ameliorative effect of curcumin, coumarin and honey on DS induced testicular damages in mice. A: Control group showed the normal regular continued strong PAS positive basement membrane (arrowheads) of the seminiferous tubules (ST). B: DS treated group showed weak PAS positive basement membrane (arrowheads) of the seminiferous tubules (ST). C: DS+ Curcumin treated group showed nearly regular and continued PAS positive basement membrane (arrowheads) of the seminiferous tubules (ST). D: DS+Coumarin treated group showed nearly regular and continued moderately PAS positive basement membrane (arrowheads) of the seminiferous tubules (ST). E: DS+Honey treated group showed nearly regular and continued strong PAS positive basement membrane (arrowheads) of the seminiferous tubules (ST). Original magnification; A-E X100, scale bar = 200 µm, periodic acid-Schiff (PAS) stain.

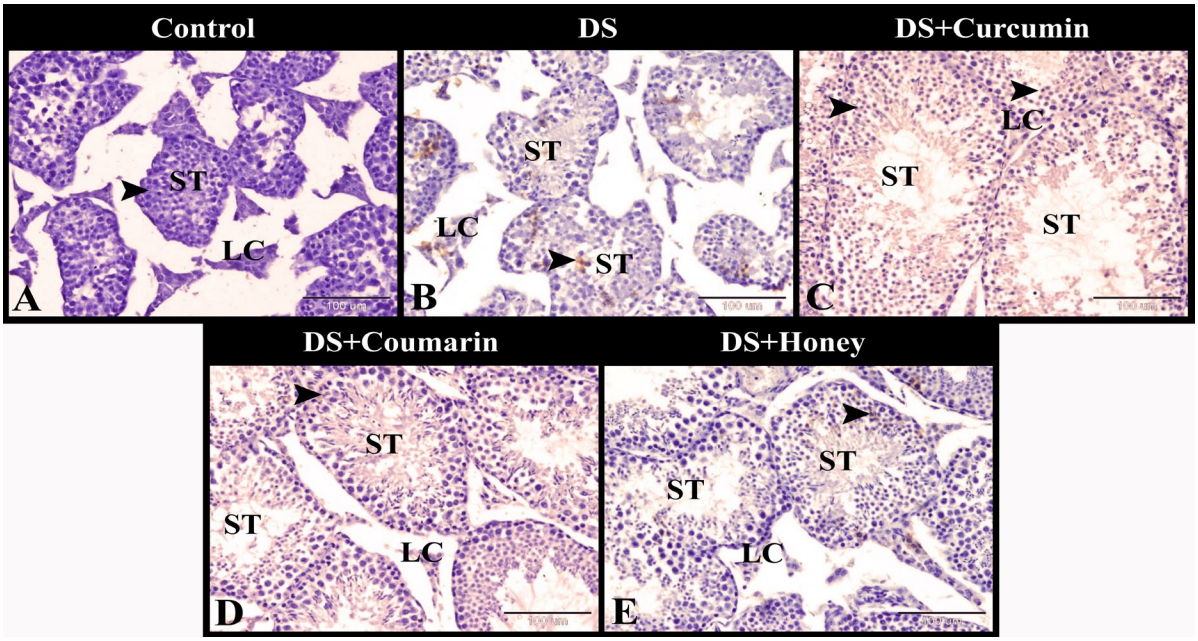


Fig. 5. Photomicrograph of GR immunostaining showed the ameliorative effect of curcumin, coumarin and honey on DS induced testicular damages in mice. A: Control group showed negative GR immunostaining in the spermatogenic cells (arrowheads) of the seminiferous tubules (ST) and in the Leydig cells (LC). B: DS treated group showed mild GR immunostaining in the spermatogenic cells (arrowheads) of the seminiferous tubules (ST) and in the Leydig cells (LC). C: DS+ Curcumin treated group showed mild GR immunostaining in the spermatogenic cells (arrowheads) of the seminiferous tubules (ST) and in the Leydig cells (LC). D: DS+Coumarin treated group showed mild GR immunostaining in the spermatogenic cells (arrowheads) of the seminiferous tubules (ST) and in the Leydig cells (LC). E: DS+Honey treated group showed mild GR immunostaining in the spermatogenic cells (arrowheads) of the seminiferous tubules (ST) and in the Leydig cells (LC). Original magnification; A-E X200, scale bar = 100 µm, GR immunostaining.

ported that DS significantly decreased LH level without affecting FSH (Owumi *et al*., 2020; El-Megharbel *et al*., 2021). These conflict outcomes may be due to differences in the animal models or the experimental protocol. In this study, the decrease in plasma FSH could be attributed to a reduction in gonadotropin-releasing hormone (GnRH) secretion (Kaprara and Huhtaniemi, 2018). The drop in FSH output in the DS group could be a contributing factor in inducing testicular redox disturbance in light of the ability of FSH to antagonize oxidative injury (Shen *et al*., 2017). As shown by the histopathological part of this study, the reduction in FSH results in disturbances in spermatogenesis owing to its prominent role in initiating and maintaining spermatogenesis and nourishing the developing germ cells by stimulating Sertoli cell (Jones and Lopez, 2013; Griswold, 2018). The apoptosis in the testicular tissue can be explained by the fact that FSH suppression induces spermatogonia and spermatocyte apoptosis (Ruwanpura *et al*., 2008).

Surprisingly, the plasma testosterone level increased in DS administrated mice in this study when compared to the control, a finding that contradicts several other studies (Adeyemi *et al*., 2019; Owumi *et al*., 2020; El-Megharbel *et al*., 2021) but it is matched with a recent experiment (Waly *et al*., 2022). It should be considered that the testosterone levels in all the experimental groups including DS were within the standard limit (Waly *et al*., 2022). The inability of DS to trigger an alteration in the steroidogenic potential of the testis denotes that Leydig cells are quite resistant to chemotherapies and cytotoxic-associated injury (Nayak *et al*., 2020). For instance, Leydig cells stay resistant to DNA fragmentation even when exposed to high doses of cadmium (Cupertino *et al*., 2017) and although the germ cells are intensely vulnerable to irradiation, Leydig cells are more relatively resistant (Brignardello *et al*., 2016).

As approved histologically, Leydig cell hyperplasia provides solid evidence about the boosting effect of DS on the biosynthesis of testicular hormones relative to the period of exposure and the dose of chemotherapy in the current experimental design. It was hypothesized that the testicular oxidative load could be responsible for the induction of Leydig cell proliferation (Al-Bader and Kilarkaje, 2015). The redox disruption in the present animal model potentially causes up-expression of phospholipase D, an enzyme implicated in cell proliferation, which triggers Leydig cell hyperplasia and stimulates the cellular cholesterol transportation system encouraging the androgen biosynthesis in the Leydig cells (Lee *et al*., 2011). Waly *et al*. (2022) hypothesized that DS causes modulation in testosterone secretion in two phases and that the early phase involves a decline in testosterone levels. This outcome highlights the significance of exploring the potential endocrine disorders caused by DS along the reproductive axis across several time points to monitor the fluctuations in the sexual hormonal profile, regulation of the LH receptors on Leydig cells, and transcript levels of sexual pituitary-gonadal enzymes.

Administration of all investigated natural substances in this study to DS-challenged mice led to an apparent increase in the plasma FSH levels. Also, curcumin and coumarin caused a marked increase in plasma LH levels. These findings are in accordance with earlier reports (Gholami *et al*., 2018; Akomolafe and Aluko, 2020; Alotaibi *et al*., 2020; Belhan *et al*., 2020) and could be attributed to up-regulation in the transcript levels of LH and FSH receptors (Banihani, 2019; Alotaibi *et al*., 2020; Akomolafe and Aluko, 2020; Allam *et al*., 2022). The increase in FSH level caused by the protective agents may have a role in reducing apoptosis as shown by TUNEL assay owing to the anti-apoptotic nature of FSH in Sertoli and germ cells secondary to activation of protein kinase B/AKT (an anti-apoptotic pathway that promotes proliferation and increases cell survival) and reduction of ROS generation (Gonzalez-Robayna *et al*., 2000; Tesarik *et al*., 2002; Tsai-Turton and Luderer, 2006).

It was observed in this study that curcumin administration resulted in lower plasma testosterone levels in comparison with the DS group. This result is consistent with Ide *et al*. (2018) and contradictory to many other studies that proved curcumin's effectiveness in increasing testosterone levels (Cheraghi *et al*., 2017; Jiang *et al*., 2019; El-Sherbiny *et al*., 2022). LH does not mediate the decrease in plasma testosterone level as its secretory potential is not reduced in the DS+curcumin group. However, it could be mediated by an increase in FSH as it increases androgen binding proteins (Dorrington and Armstrong, 1979), decreasing free plasma testosterone.

All the protective agents increased the plasma E2 level in this study, which agrees with that observed in other studies (Zhang *et al*., 2018; Usman *et al*., 2021). Upon close examination of the results of pituitary gonadotrophic and gonadal hormones in the natural products received groups, it can be noted that E2 levels rise is in concomitant with an increase in the FSH level, corre-

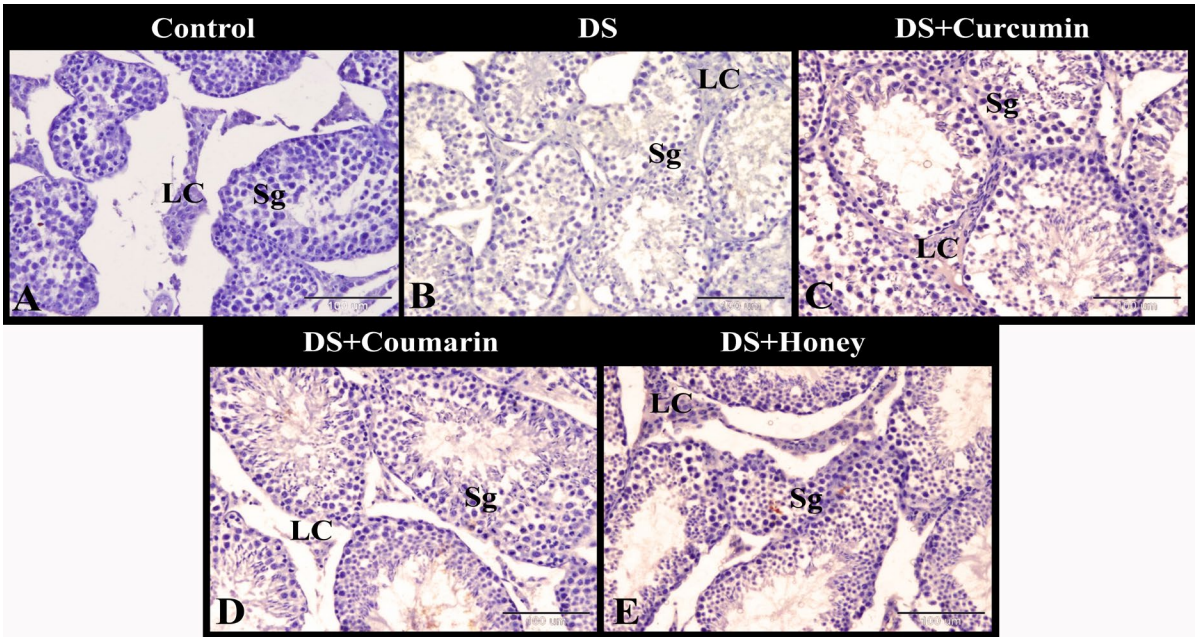


Fig. 6. Photomicrograph of SOD2 immunostaining showed the ameliorative effect of curcumin, coumarin and honey on DS induced testicular damages in mice. A: Control group showed negative SOD2 immunostaining in the spermatogenic cells (arrowheads) of the seminiferous tubules (ST) and in the Leydig cells (LC). B: DS treated group showed mild SOD2 immunostaining in the spermatogenic cells (arrowheads) of the seminiferous tubules (ST) and in the Leydig cells (LC). C: DS+ Curcumin treated group showed mild SOD2 immunostaining in the spermatogenic cells (arrowheads) of the seminiferous tubules (ST) and in the Leydig cells (LC). D: DS+Coumarin treated group showed mild SOD2 immunostaining in the spermatogenic cells (arrowheads) of the seminiferous tubules (ST) and in the Leydig cells (LC). E: DS+Honey treated group showed mild SOD2 immunostaining in the spermatogenic cells (arrowheads) of the seminiferous tubules (ST) and in the Leydig cells (LC). Original magnification; A-E X200, scale bar = 100 μm, GR immunostaining.

sponding to the ability of FSH to stimulate testosterone/E2 conversion by the Sertoli cells (Carreau and Hess, 2010). This also implies that the decrease in FSH in the DS group leads to a delay in this hormonal conversion causing increase in testosterone and a relative reduction in E2. Conversely the increase in FSH in DS+curcumin group results in the opposite outcome. Surprisingly, the other protective groups did not follow the same hormonal pattern, most probably due to the sensitivity of Sertoli cell aromatase towards other hormones and growth factors (Schteingart *et al*., 1995) which could be modulated by coumarin and honey. It was known that E2 decreases pulse amplitude and responsiveness to GnRH thus, inhibits LH secretion (Hayes *et al*., 2000). Although E2 increased in the protected groups in this study, LH in the same groups didn't decrease. Thus, it was concluded that E2 may not have a negative feedback impact on the hypothalamic-pituitary axis and may not inhibit the production of both FSH and LH, as suggested by Fattahi *et al*. (2013).

DS induced a disturbance in the testicular redox status in this study, similar to that observed earlier (Mousa *et al*., 2020; Adedara *et al*., 2021). The elevated LPO reflects the oxidative deterioration of polyunsaturated lipids caused by ROS (Cipak Gasparovic *et al*., 2017; Niknahad *et al*., 2017). DS stimulates ROS output by targeting the mitochondrial respiratory chain (van Leeuwen *et al*., 2012), induction of nicotinamide adenine dinucleotide phosphate-oxidases (Li *et al*., 2008), and upregulation of lipoxygenase pathway (Kusuhara *et al*., 1999) and cytochrome P450 (van Leeuwen *et al*., 2012). Excessive lipid oxidation could alter the physicochemical properties of the cellular membrane, resulting in a covalent alteration in the proteins and nucleic acids (Gaschler and Stockwell, 2017). This response is regarded as a contributing factor in the histological abnormalities and DNA fragmentation in the testicular tissue of the DS group.

Reduction in the testicular GSH level following DS burden is matched with another finding (Mousa *et al*., 2020). The intracellular antioxidant GSH is central to male fertility (Nayernia *et al*., 2004). GSH depletion in this experiment is consistent with that seen in the testes of rats (Mousa *et al*., 2020), and with the exhaustion of the glutathione redox system in DS-induced nephro-, hepato- and gastrointestinal dysfunction rat models (Prince, 2018; Simon and Evan Prince, 2018; Simon *et al*., 2019). Under ROS overloading, GSH is transformed to its oxidized form (Zitka *et al*., 2012), resulting in a consumption of GSH reservoir. Given that cysteine is an essential building block in de novo GSH synthesis (Meister *et al*., 1986), the ability of DS to suppress hepatic γ-glutamyl transpeptidase (Grillo *et al*., 2008), an enzyme that converts glutathione into cysteine, is regarded as a leading cause of GSH reduction. Furthermore, DS transacylates GSH to produce diclofenac-S-acyl-glutathione, which is excreted in bile (Grillo *et al*., 2003). Form another perspective, depletion of GSH under DS challenge in this study could be incriminated in the observed apoptosis in the testicular tissues because the efflux of intracellular glutathione secondary to diminishment in the extracellular GSH is associated with an increase in caspase-3 activity (Circu *et al*., 2009). Once caspase-3 is stimulated, it initiates an augmented apoptotic pathway by activating other caspases, resulting in quick cell death (Elmore, 2007).

Monitoring the biodegradation routes of DS denotes that excessive inhibition of glutathione conjugation causes impairment in the mitochondrial transmembrane, resulting in antioxidant suppression (Huang *et al*., 2017). In the current study, testicular SOD activity was reduced in the DS group, similar to that observed in rats (Mousa *et al*., 2020). It is most likely due to decreased transcript level of SOD (Jung *et al*., 2012). Considering that SOD dismutase the damaging superoxide anion, its depletion causes impairment in the fertilizing potential of sperms (Tsunoda *et al*., 2012).

In spite of the decline in the levels of both testicular SOD and GSH in the DS-treated group, it is surprising that there was a marked increase in the plasma TAC. This result may be contradictory to the findings of other studies indicating exhaustion of TAC in different models (Iftikhar *et al*., 2015; Adeyemi *et al*., 2019). This conflict should be explained by the difference in the measurement site, e.g., testicular tissue versus plasma, and the type of outcome measure, e.g., individual antioxidant versus the overall integrative antioxidant ability of the plasma. The testicular microenvironment is highly vulnerable to the attack of reactive oxidants owing to the high rate of cell division, low stock of antioxidants, cell competition for oxygen consumption secondary to the weakened testicular microvasculature and the abundance of unsaturated fatty acids (Asadi *et al*., 2017). Subsequently, the testis is more susceptible to depletion of its antioxidant reservoir than the body fluids such as plasma. In addition, TAC not only represents the summation of all constituents of redox stabilizers but also indicates the accumulative synergistic action of whole antioxidants found in the sample (Ghiselli *et al*., 2000). In the same line with earlier studies (Ibegbulem *et al*., 2016; Abd-Elka-

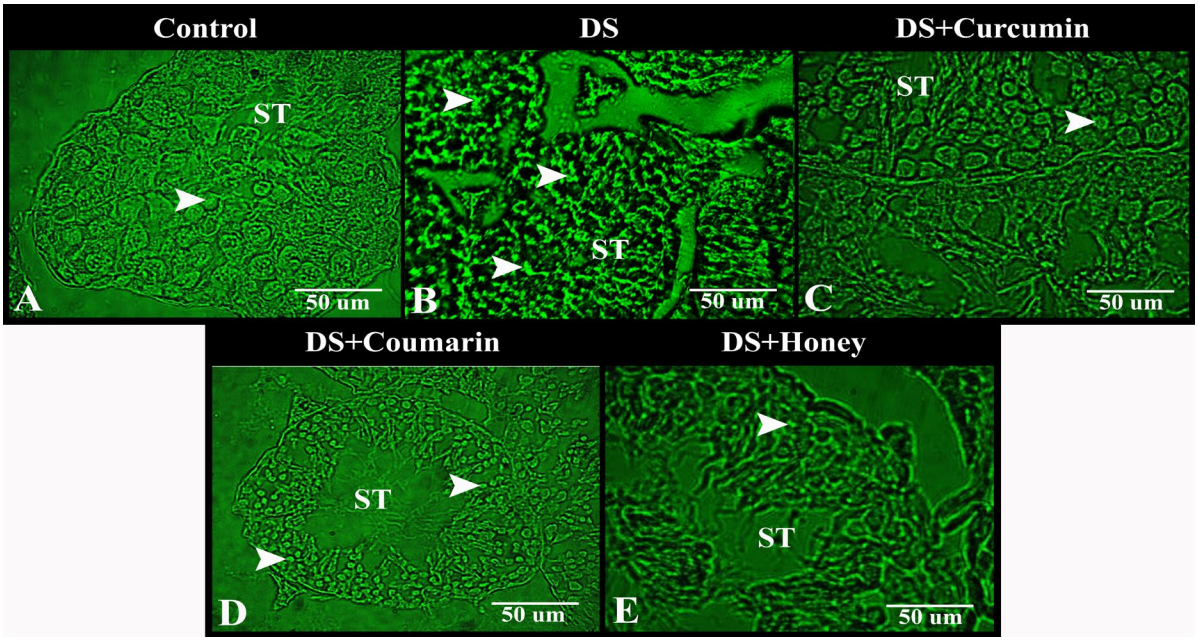


Fig. 7. Fluorescent Photomicrograph of TUNEL assay in paraffin sections showed the ameliorative effect of curcumin, coumarin and honey on DS induced testicular damages in mice. A: Control group showed few numbers of apoptotic spermatogenic cells (arrowhead) in the seminiferous tubules (ST). B: DS treated group showed high number of apoptotic spermatogenic cells (arrowhead) in the seminiferous tubules (ST). C: DS+ Curcumin treated group showed few numbers of apoptotic spermatogenic cells (arrowhead) in the seminiferous tubules (ST). D: DS+Coumarin treated group showed few numbers of apoptotic spermatogenic cells (arrowhead) in the seminiferous tubules (ST). E: DS+Honey treated group showed few numbers of apoptotic spermatogenic cells (arrowhead) in the seminiferous tubules (ST), scale bar = 50 µm.

reem *et al*., 2021), TAC was raised as a compensatory adaptive response following exposure to oxidative stress inducers. The shift in the redox equilibrium towards the pro-oxidant side enhances the antioxidant defense mechanism by motivating redox-sensitive transcription factors and its downstream signaling avenues (Done and Traustadóttir, 2016).

Curcumin, coumarin, and honey supplementation with DS to mice in this study was beneficial in re-establishing the testicular redox stability, as shown by the decreased LPO level and increased SOD activity and GSH level. Normalization of the testicular redox balance following curcumin administration is in harmony with the findings of numerous studies (Ilbey *et al*., 2009; Sudjarwo *et al*., 2017; Yang, Y.J. *et al*., 2019). The oxidant/antioxidant rebalance associated with coumarin supplementation is similar to that found in other oxidative stress-related animal models (Allam *et al*., 2022; Mahmoud, 2016; Türk *et al*., 2021). The honey's positive antioxidant results agree with that of others (El Rabey *et al*., 2019; Ara *et al*., 2021a).

The preventive effects of curcumin, coumarin, and honey on lipid peroxidation are in line with the other researchers (Fabunmi *et al*., 2021; Huyut *et al*., 2021; Allam *et al*., 2022). In addition to the free radical scavenging properties of the studied natural antioxidants (Symeonidis *et al*., 2009; Barzegar and Moosavi-Movahedi, 2011; Garg *et al*., 2020), curcumin also inhibits xanthine oxidase (one of the most important biological free radical producers) (Shen and Ji, 2009), and up-regulates heme oxygenase-1 (a suppressor of oxidant-induced chain reactions) (Yang *et al*., 2017).

The ability of curcumin, coumarin, and honey to support the antioxidant capability of the testicular microenvironment might be due to the stimulation of nuclear factor erythroid 2-related factor 2, which, in turn, positively regulates the transcript level of γ-glutamyl cysteine synthetase (Abd El-Twab *et al*., 2016; Alvarez-Suarez *et al*., 2016; Mahmoud *et al*., 2017; Yang S.H., *et al*., 2019).

All the investigated natural products in this study increased testicular SOD activity which might be due to up-regulation of SOD expression (Ahmad *et al*., 2013; Rungratanawanich *et al*., 2018; Xu *et al*., 2019) and inhibition or scavenging of superoxide radical generation (Payá *et al*., 1993; Mishra *et al*., 2004; Hegazi *et al*., 2009).

In the current study, all the experimental groups had insignificant changes in both plasma TC and plasma TG levels. The non-significant effect of DS on both TC and TG is similar to Vyas *et al*. (2019), while it contradicts what Maity *et al*. (2012) found. This contradiction may be due to the use of different concentrations of diclofenac in each study. Curcumin does not affect TC or TG levels, as proved by many studies (Alwi *et al*., 2008; Baum *et al*., 2007; Shin *et al*., 2011). Alternatively, different studies demonstrated that curcumin decreased blood lipids (Belhan *et al*., 2020; Pourmahmoudi *et al*., 2021; Qin *et al*., 2017). The conflict could be due to different duration of treatment in each investigation. The non-significant change caused by coumarin on both plasma TC and TG in this study contradicts what was previously known about the hypolipidaemic action of coumarins (Allam *et al*., 2022; Dharmarajan and Arumugam, 2012; Kim *et al*., 2014; Yao *et al*., 2018). Depending on its source, honey may increase or decrease TC and TG levels, as shown by Mohammadimanesh *et al*. (2019).

The changes in testicular histological characteristics after the DS challenge are consistent with prior research (Adeyemi *et al*., 2019; Vyas *et al*., 2019; Mousa *et al*., 2020). The degenerative changes in the germinal cells may be linked to abnormalities in the Sertoli cell, which provides a suitable supportive background for germ cell attachment and growth (Monsees *et al*., 2000; Vyas *et al*., 2019; Altindağ and Rağbetli, 2021). The ability of DS to elicit redox disturbance (Adeyemi *et al*., 2019; Mousa *et al*., 2020) could be implicated in breaking down the tight junctions between Sertoli cells and increasing the leakage of the blood-testis barrier (Chen *et al*., 2018) as confirmed by loss of integrity of ST basement membrane.

Collagens are scaffolding proteins that provide structural support to Sertoli cells in the seminiferous epithelium and maintain cytoarchitecture potency (Li *et al*., 2020). It was found that the DS group has irregular and interrupted peritubular collagen fibers and weak PAS-positive basement membrane of ST. This observation is compatible with what was seen by Waly *et al*. (2022) and may be due to the ability of ROS to activate collagenase (Wlaschek *et al*., 1995; Tyrrell, 2012).

Curcumin, coumarin, and honey supplementation restored the testicular histo-architecture. The ability of the studied natural therapeutic approaches to enhance the testicular histological patterns is matched with their effects on other testicular dysfunction models (Fetouh and Azab, 2014; Lin *et al*., 2015; Mahmoud, 2016; Karimi *et al*., 2019; Ara *et al*., 2021b). Their beneficial histological impacts are attributed to their ability to improve antioxidant defenses, reduce ROS generation, and suppress apoptosis (Gholami *et al*., 2018; Chen *et al*., 2019; Akomolafe and Aluko, 2020; Alotaibi *et al*., 2020). The notable increase in E2 levels in all protective groups in this study in comparison with the DS group denotes improved functional capability of Sertoli cells which are responsible for the development, proliferation and maturation of the germ cells during spermatogenesis (Gerber *et al*., 2016), explaining the return of spermatogenic cells to the healthy features. The increased SOD level in all the protected groups is beneficial in restoring the testicular collagen because SOD has a significant role in preventing collagen oxidative fragmentation during redox imbalance (Petersen *et al*., 2004).

Targeting crucial essential points of the programmed cell death pathway is implicated in the pro-apoptotic impact of DS on the testis by stimulating Akt, Bid, cytochrome c, and caspase pathway (Inoue *et al*., 2004; Orabi *et al*., 2020), together with decreased transcript abundance of anti-apoptotic Bax (Huang *et al*., 2016). ROS over generation under DS burden plays a fundamental role in triggering apoptosis. The oxidative challenge causes increased outflow of cytochrome c and apoptogenic mediators from the mitochondria and eventually stimulates apoptosis (Patil *et al*., 2010).

The anti-apoptotic influences of the protective agents used in this study is matched with the results of several other studies (Gholami *et al*., 2018; Chen *et al*., 2019; Akomolafe and Aluko, 2020; Alotaibi *et al*., 2020; Türk *et al*., 2021). Both curcumin and coumarin exhibit anti-apoptotic activity by increasing the transcript levels of anti-apoptotic factors and reducing those of pro-apoptotic ones (Chen *et al*., 2019; Akomolafe and Aluko, 2020; Alotaibi *et al*., 2020; Türk *et al*., 2021). Honey active ingredients such as flavonoids and quercetin inhibit mitochondrial-mediated intrinsic apoptotic pathway, increase the protein level of Bcl-2, repair DNA damage, and up-regulate proliferating cell nuclear antigen in the testicular tissues (Kanter *et al*., 2012; Ye *et al*., 2020).

## CONCLUSION

The adverse testicular consequences of DS are ameliorated by supplementing adult male mice with curcumin, coumarin, and honey. These natural products rebalanced the redox potential, blocked the programmed cell death, and provided cytoprotection. These findings are important in opening windows toward utilizing these natural products as candidate strategies against DS-related abnormalities and touching a new ground for exploring their efficiency in combating the other adverse impacts of DS.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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