**Zataria multiflora** ameliorates cisplatin-induced testicular damage via suppression of oxidative stress and apoptosis in a mice model

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**ABSTRACT**

**Objective(s):** Cisplatin (CP), as an anti-neoplastic drug, causes testicular damage. *Zataria multiflora* Boiss (ZM), a medicinal plant, has antioxidant and anti-inflammatory properties. The aim of this study was to investigate the effects of ZM against CP-induced testicular toxicity.

**Materials and Methods:** In this experimental study, thirty-two adult male mice were randomly divided into four groups. The control group received normal saline with oral gavage during 7 days; ZM group received ZM (200 mg/kg) during 7 days by gavage; CP group received CP (10 mg/kg) intraperitoneally (IP) in the 5th day of study; ZM + CP group received ZM during 7 days and CP was injected in 5th day. Sperm parameters, biochemical (MDA, GSH, and PC) levels, serum testosterone levels, and histopathological and immunohistochemical assays of testis were examined one day after the last drug treatment.

**Results:** CP treatment caused significant damage via changed sperm parameters (sperm motility, count, viability rate, and abnormalities), increased oxidative stress (increased MDA and PC levels, and decreased GSH level), histological changes (degeneration, necrosis, arrest of spermatogenesis, congestion, and decrease in thickness of the germinal epithelium, diameter of seminiferous tubules, and Johnsen’s Score), decreased serum testosterone level, and increased caspase-3 immureactivity. ZM preserved spermatogenesis and mitigated the toxic effects of CP on the testis tissue. In addition, treatment with ZM significantly reduced caspase-3 immureactivity.

**Conclusion:** The findings of this study suggest that ZM as a potential antioxidant compound and due to free radicals scavenging activities has a protective effect against CP-induced testicular toxicity.

**Introduction**

Cisplatin (cis-diaminedichloroplatinum-II, (CP)), an anti-neoplastic agent, is commonly used in the treatment of various solid cancers (1). One of the side effects of CP in cancer treatment is testicular toxicity (2). The testis is an organ that has a high proliferative rate and adverse effects of chemotherapy on the testis are very intense (3). Reduction of sperm parameters (count, viability, and motility), inhibition of spermatogenesis, testicular atrophy, and infertility were proven in human and animal studies (4, 5). Pathogenesis of testicular damage following CP exposure is usually attributed to oxidative injury. Increase in reactive oxygen species (ROS) production causes an imbalance of antioxidant-oxidant status. Oxidative stress with membrane lipid peroxidation caused the breakage of cell integrity and reproductive toxicity (6). Previous research demonstrated that administration of antioxidants protects normal tissue against oxidative stress-induced damage (7, 8).

*Zataria multiflora* Boiss (ZM) (Shirazi avishan extract), a member of the Lamiaceae family, is one of the largest families of herbal plants growing in the world. This plant has multiple thin, hard, fork-shaped leaves (9, 10). ZM is a medicinal herb that is used for treatment of various diseases. Pharmacological properties such as anti-angiogenesis (11), anti-inflammatory (12), radioprotective (13), chemoprotective (14), anti-ulcerogenic (15, 16), anti-bacterial, and antioxidant (17) properties have been reported in numerous studies. The main biologically active components of ZM are phenolic compounds, mainly thymol and carvacrol (18, 19). Previous researches showed that thymol by having antioxidant and anti-inflammatory properties suppressed lipid peroxidation and subsequently protected normal organs against toxicity induced by oxidative stress (20) and chemotherapy (21).

Hence, ZM has the potential to protect against CP-induced testicular damage, and to the best of our knowledge, no such study has been done previously. The aim of this study was to investigate the effects of ZM on testicular damage-induced CP. Therefore, the present study investigated the protective effect of ZM against CP-induced testicular injury in mice by biochemical,
histological, and immunohistological assessment.

Materials and Methods

Chemicals
Cisplatin (1 mg/ml; Oncotec Pharma Produktion GmbH-ALLEMAGNE, code: 5622539) was purchased from a pharmacy.

Plant material and extraction
In the flowering season, dried aerial parts of ZM were collected in the city of Firozabad in the Fars province of Iran. The homogeneous powder was macerated in 70% ethanol for 72 hr (1:10 w/v), after which the hydroalcoholic extract of dried ZM was processed by removing the solvent using a rotary evaporator (Heidolph, Germany). The ZM extract was standardized based on thymol as the major active ingredient according to our previous reports (22, 23).

HPLC analysis
Analyses were developed by HPLC system (Knauer Assoc., Germany), equipped with ODS-C18 column (5 μm, 250 × 4.6 mm i.d.). A mobile phase was prepared consisting of acetonitrile:H2O:acetic acid (65:34:1), at a flow rate of 1 ml/min. Detection was done at 274 nm, and the chromatographic data analyses were done using the ChromGate software (Germany). The filtered ZM extract was injected into the HPLC system and chromatogram was recorded. In this mobile phase, the peaks of thymol and carvacrol (as the two main phenolic compounds in ZM) were successfully separated in the ZM extract and pure peak of thymol was reached for quantification. Thymol standards were quantified by preparing in a methanol solution (HPLC grade, Merck, Germany), and serial dilutions were carried out by double-distilled water. Various standard concentrations of thymol were used in the HPLC system to draw standard calibration curves. The amount of thymol in ZM extract is expressed as micrograms per gram of dry extract weight (mg/g).

Experimental animals
Thirty-two adult male BALB/c mice (25–30 g) were used in the study. The mice were obtained from Animal Research Center of Mazandaran University of Medical Sciences, Sari, Iran, and were kept in suitable conditions (23±2 °C, 55±5% humidity, 12-hr dark/light cycle). A mobile phase was prepared consisting of acetonitrile:H2O:acetic acid (65:34:1), at a flow rate of 1 ml/min. Detection was done at 274 nm, and the chromatographic data analyses were done using the ChromGate software (Germany). The filtered ZM extract was injected into the HPLC system and chromatogram was recorded. In this mobile phase, the peaks of thymol and carvacrol (as the two main phenolic compounds in ZM) were successfully separated in the ZM extract and pure peak of thymol was reached for quantification. Thymol standards were quantified by preparing in a methanol solution (HPLC grade, Merck, Germany), and serial dilutions were carried out by double-distilled water. Various standard concentrations of thymol were used in the HPLC system to draw standard calibration curves. The amount of thymol in ZM extract is expressed as micrograms per gram of dry extract weight (mg/g).

Study design
In experimental study, the mice were randomly divided into 4 groups (B/group):
Group I: Mice were received physiological saline (same volume as other groups) for 7 consecutive days.
Group II: Mice were received 200 mg/kg ZM daily for 7 consecutive days with oral gavage.
Group III: Mice were injected IP with a single dose of CP at the dose of 10 mg/kg on the fifth day of the study.
Group IV: Mice were received 200 mg/kg ZM daily for 7 consecutive days and on the fifth day of the study were injected with a single dose of CP.

Evaluation of sperm parameters
The cauda epididymis sample was minced by anatomical scissors into 1 ml of Dulbecco’s Modified Eagle’s medium (DMEM) pre-warmed with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a petri dish. Afterward, the pieces of epididymides were incubated for 15 min to allow the migration of all spermatozoa into DMEM. Then epididymal tissues were removed, the cell suspension obtained was used to assess sperm parameters. For evaluation of the sperm motility, one drop of cell suspension was dropped onto a slide, covered by a coverslip and the percentage of sperm motility according to the move type was assessed routinely by a light microscope at 200 magnification. The motility estimations were performed in three different fields for each sample, and average numbers were considered as the final motility score (8).

To determine the percentage of sperm morphology, 10 μl of the sperm suspension and 10 μl of Eosin were mixed. After 1 min incubation, smear was prepared with a 12 μl drop on a glass slide. After drying, sperm morphology was checked. Abnormal shapes of the head and tail of sperm were analyzed in the prepared slides and mean data were recorded.

To assess sperm viability 20 μl of sperm suspensions with 20 μl of 1% Eosin-Y were mixed and after 3 to 4 min stained and unstained cells were counted using hemocytometer with ×40 magnification on an inverted microscope. Each sample was measured at least three times.

Mitochondrial preparation of testicular tissues
After homogenization and centrifugation of fresh testicular tissues, mitochondria were separated (25). Briefly, the testes were removed and minced in a cold mannitol (225 mM) solution and then homogenized...
and centrifuged (1500 × g, 10 min) at 4 °C. The supernatant was removed for further centrifugation (11000 × g, 10 min) and then it was discarded. The sedimented mitochondrial pellet was gently washed and then suspended in the isolation medium (0.225 M D-mannitol, 75 mM sucrose, and 0.2 mM EDTA, pH 7.4) and then centrifuged again (11,000 × g for 10 min). Final mitochondrial samples were suspended in Tris buffer. For assessment of ROS generation in mitochondria, it was used of respiration buffer (0.32 mM sucrose, 10 mM Tris, 20 mM Mops, 50 μM EGTA, 0.5 mM MgCl\textsubscript{2}, 0.1 mM KH\textsubscript{2}PO\textsubscript{4}, and 5 mM sodium succinate). Mitochondria were freshly prepared for each experiment and were used within 4 hr of isolation. The mitochondrial samples (0.5 mg mitochondrial protein/ml) were used in this study.

**Protein concentration**

The concentration of protein in mitochondrial was measured by the Coomassie blue protein binding method (26). BSA was used as the standard for evaluation of the protein content.

**Biochemical analysis**

**Evaluation of malondialdehyde (MDA) formation in mitochondria**

The concentrations of testicular lipid peroxidation were measured by measuring MDA using the thiobarbituric acid with a spectrophotometric method. First, 0.2 ml of sample and 0.25 ml phosphoric acid (0.05 M) were mixed and then 0.3 ml of 0.2% thiobarbituric acid (TBA) was added. Samples were preserved in a boiling water bath for 30 min. The sample tubes were placed in the ice-bath and then 0.4 ml of n-butanol was added to each sample. Then they were centrifuged (3500 rpm) for 10 min and MDA was measured based on reactions with thiobarbituric acid (an MDA-TBA complex). Created MDA in each sample was calculated on reactions with thiobarbituric acid (an MDA-TBA complex). Created MDA in each sample was calculated.

**Evaluation of protein carbonyl (PC) content in mitochondria**

Protein carbonyls were measured by Fathi et al. procedure (25). The protein carbonyl was measured using 2,4-dinitrophenylhydrazine (DNPH) reagent. After determination of tissue protein, 500 μl of trichloroacetic acid (20% w/v) was added to the sample and stored at 4 °C for 15 min. Then precipitated protein was centrifuged at 6500 × g for 10 min and the supernatant was discarded. Soluble protein (0.5 ml) was reacted with DNPH 10 mM (0.5 ml) in HCl 2 M for 1 hr at room temperature. The precipitate was washed with 1 ml of a mixture of ethanol and ethyl acetate 1: 1 (v/v) and then centrifuged at 6500 × g for 10 min and the supernatant was removed. The final protein deposition was solubilized in 200 μl guanine hydrochloride solution and centrifuged at 16000 × g for 5 min to remove any trace of insoluble material. The protein carbonyl was assessed spectrophotometrically by reading the absorption at a wavelength of 365 nm with an absorption coefficient of 22,000 M-1 cm-1 expressed as a nmol of DNPH per milligram of protein.

**Measurement of glutathione (GSH) content**

The content of glutathione in the samples was determined by a spectrophotometer (UV-1601 PC, Shimadzu, Japan) with 5,5’-dithiobis-2-nitrobenzoic acid (DTNB) as an indicator at 412 nm and expressed as μM (25).

**Testosterone analysis**

Levels of testosterone in the serum were determined by using radioimmunoassay according to manufacturer’s instructions (Mouse Testosterone ELISA Kit, Bioassay, cat. no. E0260MO). Testosterone levels in the samples were calculated from testosterone standard curves using a linear regression method and expressed as nmol/L. All samples were carried in duplicate.

**Histopathological examinations**

For microscopic evaluation and to determine the effect of CP exposure on the testis, the tissue samples were fixed in 10% buffered formalin, processed, and embedded in paraffin. Then sections with 5 μm thickness were stained with Hematoxylin and Eosin (H & E) and were investigated under a light microscope (Olympus, Japan). Histopathological findings were investigated with Johnsen scoring system, where five sections per animal and 10 seminiferous tubules per section were assessed using a score of 1–10 under ×40 magnification (27) (Table 1).

**Histomorphometric assay**

For quantitative assessment, the mean diameter of seminiferous tubule (ST) and thickness of the epithelium of the seminiferous tubules (from the basement membrane to lumen) in 10 tubules per testicular section and 10 sections per group were measured at ×10 magnifications by using the calibrated OLYSIA software (Soft Imaging System, version 3.2, Japan). ST that were round or close to round were randomly selected for measurement. All samples were measured by a histologist in a blind fashion (28).

**Immunohistochemical assay**

The immunohistochemical technique was performed according to the instructions of the kit manufacturer (Abcam Company, USA). After deparaffinization and rehydration, endogenous peroxidase activity was blocked by 0.3% H\textsubscript{2}O\textsubscript{2} in methanol for 15 min. Then, tissue sections were incubated at 4 °C overnight with primary antibodies (anti-caspase-3 rabbit polyclonal antibody, 1:100 in PBS, v/v, Abcam, lat: GR224831-2). Then they were incubated with secondary antibodies conjugated with horseradish peroxidase (Mouse and Rabbit IgG conjugated with horseradish peroxidase, 1:100 in PBS) and 3,3′-diaminobenzidine for development of immunoreactive signals. Sections were then counterstained with Haematoxylin for 10–15 seconds before dehydration and mounting.

**Table 1. Johnsen scoring system histopathological examination of testicular tissue**

| Score | Description | Description |
|-------|-------------|-------------|
| 1     | No cells    | No cells    |
| 2     | Sertoli cells without germ cells | Sertoli cells without germ cells |
| 3     | Only spermatogonia | Only spermatogonia |
| 4     | Only a few spermatocytes | Only a few spermatocytes |
| 5     | Many spermatocytes | Many spermatocytes |
| 6     | Only a few early spermatids | Only a few early spermatids |
| 7     | Many early spermatids without differentiation | Many early spermatids without differentiation |
| 8     | Few late spermatids | Few late spermatids |
| 9     | Many late spermatids | Many late spermatids |
| 10    | Full spermatogenesis | Full spermatogenesis |
Rabbit specific HRP/DAB, Abcam, Lot: GR2623314-4) for 10 min; sections were incubated with diaminobenzidine tetrahydrochloride for 5 min (29). Then, the samples were dehydrated and mounted. The primary antibody was omitted for negative controls. For the quantitative analysis, immunohistochemical photomicrographs were assessed by densitometry using MacBiophotonics ImageJ 1.41a software. The positive staining severity was assessed as the ratio of the stained area to the entire field of assessment.

Statistical analysis
Statistical analysis of the obtained data was performed using SPSS version 19 (Chicago, USA). All of the data with normal distribution are presented as the mean±standard deviation (M±SD). One-Way ANOVA and Tukey’s test were used. Statistically significant differences were accepted as P<0.05.

Results
HPLC characterization of the ZM extract
Carvacrol and thymol as main phenolic compounds from Z. multiflora were identified using the HPLC method. In ZM extract, thymol and carvacrol showed 8.2 and 7.7 min retention times, respectively. The total thymol content of the extract was 9.24±0.11 mg per gram dried extract (Figure 1).

Oxidative stress biomarkers
MDA, GSH, and PC levels as indicators of oxidative stress in testes are presented in Figure 2.
In the CP group, MDA significantly (P<0.05) increased compared with the control and ZM groups. Whereas ZM treatment in CP-treated mice significantly decreased the elevated MDA levels in comparison with the CP group (P<0.05).
In the CP group, the GSH significantly (P<0.05) was decreased compared with the control and ZM groups, which shows an impaired antioxidant system; while disruption of the endogenous antioxidant system was significantly minimized (P<0.05) in animals that were treated with ZM.
Protein carbonyl contents, as an index for oxidative modification of proteins, were increased significantly in testes of CP-treated mice (P<0.05) as compared with control and ZM groups. ZM treatment in CP-treated mice mitigated elevated protein carbonyl content in the testis tissue (P<0.05) compared with the CP group. Overall, CP-induced oxidative stress in testes and ZM decreased oxidative stress.

Sperm parameters
The mean of testicular weight and sperm parameters in all groups are presented in Table 2. The results showed that CP caused a significant (P<0.05) decrease in testicular weight and ZM treatment could not prevent this reduction when compared to the CP group. As well, CP caused significantly decreased sperm count, motility, and sperm viability, and increased sperm abnormality when compared to control and ZM groups (P<0.05). ZM treatment in CP-treated mice had protective effects on sperm count, motility, viability rate, and sperm morphology when compared to the CP group (P<0.05).

Testosterone level
Serum testosterone levels are presented in Table 3.
Serum testosterone levels were significantly decreased in the CP group compared with the control and ZM groups (P<0.05). ZM treatment in CP-treated mice increased testosterone levels, but the amount was not significant compared to the control group.

Histopathological evaluations

The photomicrographs of testes are shown in all groups (Figure 3). Normal spermatogenic cells, Sertoli and Leydig cells, and precise spermatogenesis with abundant spermatids can be seen in the control group. (Figure 3I). ZM treated testes were similar to those of the control group (Figure 3II). CP-induced impairment in the testes. Testicular atrophy and disruption of spermatogenesis with disorganization in the germinal epithelium layer of seminiferous tubules (ST), desquamation, necrosis and degeneration, diffuse interstitial edema, exfoliation and formation of giant cells by spermatocyte and spermatids, congestion, and destruction in Leydig cell were seen in seminiferous tubules in the CP group (Figures 3III, IV, and VI). Whereas in testes pre-treated with ZM preserved spermatogenesis, decreased necrosis and degeneration of seminiferous tubules, edema, and congestion were observed compared to the CP group (Figure 3V).

Johnsen's mean scores of all groups are shown in Figure 4. CP decreased testicular injury score. Scores of the ZM+CP group were higher compared to the CP group (P<0.05).

Histomorphometrical findings

Morphometric findings are presented in Table 4. In CP-treated mice, a significant difference was found in the thickness of the epithelium and atrophy of seminiferous tubules (30.32±7.43 and 142.7±18.07, respectively) (P<0.05) as compared to control (60.38±10.18 and 191.7±23.01, respectively) (P<0.05). ZM pretreatment increased the mean epithelial thickness and tubular diameter in CP-treated mice (44.62±9.77 and 170.6±21.61, respectively). These changes in thickness of the epithelium and diameter of seminiferous tubules were statistically significant compared to the CP-treated mice (P<0.05).

Immunohistological findings

Positive cells in immunohistochemical staining for caspase-3 are stained brown. Caspase-3 immunoreactivity was not observed in seminiferous tubules in the control and ZM groups. In the CP group, severe to moderate caspase-3 immunoreactivity was detected in spermatogonia cells and in the primary spermatocyte and spermatid cells it was medium to weak (Figure 5A). Treatment with ZM improved these changes in ST (Figure 5B).

The histograms of the semi-quantitative analysis of caspase-3 staining in all groups are shown in Figure 6. The most intense immunoreactivity of caspase-3 was confirmed by semi-quantitative analysis in CP-treated mice (36.17±17.34) compared with the other groups (P<0.05). ZM treatment decreased the severity of immunoreactivity.
MDA as the final product of lipid peroxidation is the best index of oxidative stress (30). In the present study, CP treatment caused a significant increase in MDA levels compared to the control group in tests. Also, CP treatment led to significant reduction in GSH and increased PC levels in testes when compared to the control group. The increase in lipid peroxidation and PC contribute to elevation of free radicals. Glutathione (GSH), as a strong antioxidant, has a protective role in injury against oxidative stress. GSH moderates cellular damage that is related to increased ROS and modulates apoptosis (8).

ZM, by having phenolic compounds and with antioxidant property, inhibits the oxidation process and prevents free radicals formation in the body (31). Thymol and carvacrol are the main biologically active components of ZM (18), which in previous studies revealed protective effects against cisplatin-induced nephrotoxicity (32) and doxorubicin-induced cardiotoxicity (33). ZM with antioxidant (34), anti-inflammatory (35), and anti-apoptotic (36, 37) activities protects the body against chemotherapy. Also, in a previous study ZM exhibited synergistic effect in radiotherapy on cancer cells (23). In most studies with antioxidants are used as pre-treatment, post-treatment, or combined treatment. We administrated ZM 5 days before and 3 days after receiving CP. CP significantly decreased lipid peroxidation as an indicator of oxidative stress compared to the CP group. In previous studies ZM effectively scavenged ROS and inhibited the production of oxidative damage induced by radiotherapy and CP (13, 38). This study is consistent with another study that showed ZM with free radicals scavenging can inhibit lipid peroxidation (14). Histopathological findings revealed CP caused disruption of seminiferous tubules, necrosis, vacuolation, desquamation of tubular epithelium, edema of interstitial tissue, and reduced spermatogenesis activity. Following exfoliation and necrosis of spermatogenesis cells, giant cells were formed by spermatocytes and spermatids in the central lumen of the seminiferous tubules. This is consistent with previous studies, which showed same histological changes in testicular tissue following CP treatment (2, 4) and ZM was able to significantly attenuate CP-induced testicular damage in mice. Previous studies revealed significant protection by ZM against cyclophosphamide-induced genotoxicity and acetic acid-induced colitis (12, 14). In a previous study, ZM markedly reduced DNA damage induced by cyclophosphamide in mice bone marrow cells at the optimum dose of 200 mg/kg (14). Also, Aksu et al. in their study showed carvacrol as one of the active components of ZM, mitigates apoptosis and degeneration of testicular tissue induced by CP (37).

Furthermore, the mean JS in the CP group has a
lower value compared to the control group. Testicular degeneration in the CP-treated group could be attributed to oxidative stress and increased ROS. This finding is consistent with the results of others (37). Value of JS in the CP + ZM group was statistically higher than in the CP group. This finding can be related to the antioxidant properties of ZM and active components, which by decreasing oxidative stress had a protective effect on the testicular tissue structure.

Histomorphometric findings on testes showed CP treatment led to loss of germ cells and decreased spermatogenesis cell lines, reducing the epithelial thickness and diameter of seminiferous tubule (ST). These results were in accordance with the previous studies (39, 40). The massive cellular loss in seminiferous epithelium leads to decrease in the morphometric parameters. This reduction has been seen in this study also. In the testes there exists a positive relationship between the tubular diameter and the spermatogenic activity (41). The cell density of ST in the CP group significantly decreased, whereas the cell density of the interstitial tissue significantly increased. However, in the animals treated with ZM, the cell density of ST significantly increased and the cell density of interstitial tissue decreased.

Germinal epithelial damage by ST induced chemotherapeutic agents leads to oligospermia or azoospermia (42). Our results showed that CP significantly decreased sperm count, sperm motility, and sperm viability rate, and increased sperm abnormality compared to the control group. These changes confirm spermatotoxic effects of CP and our results were consistent with other studies (43-45). ROS hurts macromolecules of the cell membrane such as polyunsaturated fatty acids and subsequently damages the cell body. The effects of ROS on sperm membrane, lead to death, abnormality, and reduced sperm motility (46). So, these improvements in sperm parameters after ZM administration may be attributed to the free radical scavenging effect of ZM. ZM, as an antioxidant, at a dose of 200 mg/kg mitigated spermatotoxicity parameters.

Oxidative stress by increasing the ROS level induces the apoptotic activities (47). Also, CP through the production of ROS activates the p38-mediated p38α MAPK pathway induces apoptosis (48). Immunohistochemical findings on the testes showed CP treatment led to testicular degeneration and increased ROS and immunoreactivity level of caspase-3. Our findings were similar to others studies (8, 49, 50). Treatment with ZM attenuated immunoreactivity level of caspase-3 in the testes.

Conclusion

Briefly, biochemical, histopathological, and immunohistochemical findings showed that CP caused testicular degeneration and changes in sperm parameters and induced apoptosis in testes. Our results highlight the protective effect of ZM against CP-induced testicular toxicity and spermatotoxicity by decreasing ROS and apoptosis. It can be concluded that ZM extract may be useful in the inhibition of oxidative stress and apoptosis in cancer patients receiving CP.

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Conflicts of Interest

There are no conflicts of interest in this study and publication.

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