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Ameliorative effect of *Allium sativum* extract on busulfan-induced oxidative stress in mice sperm

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Abstract

Busulfan is known to cause several adverse effects including reproductive toxicity in humans. Garlic *(Allium sativum)*, a widely distributed medicinal plant, is highly regarded for its medicinal activities including antioxidant property. This study was conducted to assess whether garlic extract could serve as protective agents against testicular toxicity during busulfan treatment in a mice model. Seventy-two adult male mice were randomly divided into nine groups. In groups 1,2 and 3, distilled water, busulfan, and dimethyl sulfoxide and in the treatment groups hydro-alcoholic extract of garlic was administered orally at different doses per day (groups 4, 5 and 6; 200, 400, 800 mg kg^{-1} respectively). Groups 7, 8 and 9 were treated with the extract (200, 400 and 800 mg kg^{-1}, respectively) plus busulfan. Following euthanasia, blood samples and epididymal sperm were collected. The busulfan-treated group showed significant decreases in sperm quality parameters, and serum levels of testosterone, LH and FSH was observed in the busulfan-treated mice. In addition, the TAC levels and antioxidant enzymes activities were reduced and malondialdehyde (MDA) levels were increased in the busulfan-treated mice. Notably, garlic extract co-administration caused a considerable recovery in sperm quality parameters, TAC levels, antioxidant enzymes activities, hormonal changes and MDA level. Based on our results, garlic has antioxidant effects against busulfan-induced testicular damages in mice.

اثر بهبود دهنده عصاره *آلیوم ساتیووم* بر تنش اکسیداتیو ناشی از بوسولفان در اسپرم موش

چکیده

بوسولفان به واسطه آثار جانبی متعدد نظیر سمیت تولیدمثلی در انسان شناخته می شود. سیر به عنوان یک گیاه دارویی با پراکندگی گسترده به واسطه فعالیت های دارویی از جمله خاصیت آنتی اکسیدانت مطرح می باشد. این مطالعه به منظور ارزیابی کارایی عصاره سیر به عنوان عامل محافظتی در برابر سمیت بیضه ای در طول درمان با بوسولفان در موش انجام شد. هفتاد و دو موش نر بالغ به صورت تصادفی به ۹ گروه تقسیم شد. در گروههای ۱، ۲ و ۳ به ترتیب آب مقطر، بوسولفان و دی متیل سولفوکساید و در گروه های ۴، ۵ و ۶، عصاره هیدروالکلی سیر روزانه و به صورت خوراکی در دوزهای مختلف (به ترتیب ۲۰۰ ، ۴۰۰ و ۸۰۰ میلی گرم بر کیلوگرم) تجویز شد. گروههای ۷، ۸ و ۹، به ترتیب با ۲۰۰ ، ۴۰۰ و ۸۰۰ میلی گرم بر کیلوگرم عصاره به همراه بوسولفان درمان شدند. متعاقب آسان کشی، نمونه های خون و اسپرم اپیدیدیمی جمع آوری گردید. گروه تحت درمان با بوسولفان کاهش های معنی داری در پارامترهای کیفیت اسپرم و سطوح سرمی تستوسترون، LH و FSH نشان داد. بعلاوه، در موش های تحت درمان با بوسولفان سطوح TAC و فعالیت های آنزیم های آنتی اکسیدانت کاهش و سطوح مالون دی آلدهید افزایش یافت. تجویز همزمان عصاره سیر موجب بهبود پارامترهای کیفیت اسپرم، سطوح TAC، فعالیت های آنزیم های آنتی اکسیدانت، تغییرات هورمونی و سطح MDA شد. براساس نتایج ما، سیر واجد آثار آنتی اکسیدانت علیه صدمات بیضه ای ناشی از بوسولفان در موش ها می باشد.

واژه های کلیدی: *آلیوم ساتیووم*، بوسولفان، موش، تولیدمثل، اسپرماتوزوآ

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Introduction

Busulfan is an effective chemotherapy drug widely used for cancer treatment.[1] Busulfan as a cytostatic agent absorbs from the gastro-intestinal tract and quickly disappears from blood with a half-life of 2 to 3 hr. It is also potentially carcinogenic and teratogenic and has many side effects on gonadal function and different body organs such as skin, bladder, liver and nervous system.[1,2] Spermatogenesis in mammals is a complex process depending on spermatogonial stem cells. Unlike other drugs, busulfan is a potent agent that preferentially kills spermatogonial stem cells.[3,4] From the mechanisms by which busulfan can damage the cells of different organs, production of reactive oxygen species (ROS) are important and they have major impact in development of oxidative stress.[5,6] It seems busulfan inhibits the spermatogenesis process, especially by oxidative damage. Other mechanism suggested that busulfan increases the level of CK-18, a surface marker on Sertoli cell. The elevation of this marker causes spermatogenesis disorder and infertility.[7]

To protect molecules against toxic free radicals and other ROS, cells have developed antioxidant defenses including the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which destroy toxic peroxides and small molecules including glutathione (GSH). External sources of antioxidant nutrients that are essential for antioxidant protection include antioxidant vitamins C and E, vitamin A and the mineral selenium, a component of selenium-dependent GPx.[8,9]

Medicinal plants have been used for treatment and prevention of certain diseases throughout the world; however, the effects of some plants have been studied scientifically.[10] Phytochemicals from plant-rich diets including garlic, provide important additional protection against oxidant damage.[11,12] Allium species such as onion and garlic are consumed as seasonings, foods, spices and local drugs. *Allium sativum* is extended throughout the world.[10] Compounds of garlic are vitamins A, C, B6, B1 and B2, sulfur, ajoine and alicine.[11] Reducing cardiovascular and cancer risk factors, stimulation of immune activities, antioxidant activity and antiviral and antimicrobial effects are biological responses of garlic.[13,14] The antioxidant effects of garlic extract on reproductive performance have been studied previously.[15,16]

To the best of our knowledge, there is no scientific study concerning the effect of *A. sativum* hydro-alcoholic extract on busulfan-induced male reproductive damages. This study was designed to investigate the effect of *A. sativum* (garlic) hydro-alcoholic extract on changes of serum concentrations of testosterone, LH and FSH hormones, semen parameters, antioxidant enzyme activities and total antioxidant capacity (TAC) and malondialdehyde (MDA) levels in adult male mice treated with busulfan.

Materials and Methods

Extract preparation. In this experimental study, *A. sativum* samples were obtained from the Agricultural and Natural Research Center of west Azerbaijan province. Identification of genus and species samples was done by plant taxonomy experts from Faculty of Science, Urmia University, Urmia, Iran. Hydro-alcoholic extract of *A. sativum* was obtained using the method of Erdemoglu *et al*.[17]

Drugs preparation. Busulfan (1,4-Butanediol dimethanesulfonate; Sigma, St. Louis, USA) was first dissolved in dimethyl sulfoxide (DMSO; Sigma), then an equal volume of sterile water was added to reach a final concentration of 5.00 mg mL^{-1}.

Animals. Adult male mice with the age range of 8 to 10 weeks and average weight of 26.00 ± 2.00 g were provided from Animal House of Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. Animals were kept under a controlled environmental condition at room temperature (22.00 ± 2.00 ˚C) with humidity of 5.00 ± 10.00% and a 12/12 hr photoperiod. This project was carried out in accordance with international guidelines for the care and use of laboratory animals and approval of the ethics committee of Faculty of Veterinary Medicine, Urmia University (3/T. DT/1813- 2016).

Experimental design. After one week of acclimatization period, the mice were divided randomly into nine groups of eight mice each. Animals within different treatment groups were maintained on their respective diets for 35 days as follows: group 1 (control), received distilled water (0.1 mL per day), group 2 (busulfan), received busulfan (20 mg kg^{-1}; IP),[18] group 3 (sham), received a single dose of DMSO (10 mg mL^{-1}; IP),[19] group 4, 5 and 6 was treated orally with 200, 400, and 800 mg kg^{-1} hydro-alcoholic extract of *A. sativum* per day, respectively. In groups 7, 8 and 9, hydro-alcoholic of *A. sativum* (200, 400, and 800 mg kg^{-1} per day, respectively) [20] plus busulfan (20 mg kg^{-1}, IP) was administered orally.

Semen collection. Epididymal sperms were collected by slicing the cauda region of the epididymis in 5 mL of human tubal fluid and incubated for 30 min at 37 ˚C in an atmosphere of 5.00% CO_2 to allow sperm to swim out of the epididymal tubules. After collection, the sperm count, motility, viability, morphology and DNA integrity and semen TAC were evaluated using conventional methods.

Sperm count assessment. In order to count sperms, a 1:20 dilution was prepared in a 1 mL microtube through pouring 190 µL of distilled water and addition of 10 µL sperm mixture. Then, 10 µL of the mixture was dropped on a Neubauer slide and the sperms were counted.[21]

Sperm motility evaluation. In order to evaluate sperm motility, 10 µL sperm suspension was placed on a pre-heated slide and covered with a slip and then the motility was observed under a light microscope (Nikon, Tokyo, Japan) with 400× magnification.[22]

Sperm viability. Sperm viability was evaluated as follows: 20 µL of 0.05% Eosin Y-nigrosin was added into an equal volume of sperm suspension. After 2 min incubation at room temperature, slides were observed by a light microscope with magnification of 400×. Dead sperms were stained pink but the live ones took no color. Viable sperms (n = 400) were counted in each sample and the viability percentage was computed.[23]

Sperm morphology. To evaluate sperm morphology in the present study, aniline blue staining method was implemented and abnormal morphologies percentage was determined. Especially, the cytoplasmic residual of sperms was considered as an abnormal morphology.[24]

Sperm DNA damage determination. Fragmentation of sperm DNA was applied as a biomarker for male infertility. Acridine orange (AO) staining was used, after challenging at low pH, to distinguish between denatured, native and double-stranded DNA regions in sperm chromatin[25] and high level of fluorescent can be observed in denatured DNA. Thick smears were placed in Carnoy's fixative (methanol/acetic acid 1:3) for 2 h for fixation.[26] The slides were removed from the fixative and left on the outside to be dried for 5 min at laboratory temperature. Then, slides were placed in a stock solution of 1 mg of AO in 1000 mL distilled water and stored in a dark place at 4 ˚C.[27] After 5 min staining, sperms were examined using fluorescent microscope (model BX51; Olympus, Tokyo, Japan). Green-colored sperms were observable as normal sperms and yellow-red ones were considered as sperms with abnormal or damaged DNA.[28]

Hormonal assay. Serum concentration of testosterone was measured by enzyme-linked immunosorbent assay (ELISA) as described in the instructions provided by manufacturer's kit (Demeditec Diagnostics GmbH, Kiel, Germany).

Serum levels of LH and FSH were determined by ELISA using specific commercial kits (Amersham, Buckinghamshire, UK) according to a previous study.[29]

TAC assay. The TAC of the semen was measured by ferric reduction antioxidant power (FRAP) assay.[30] Cellular supernatants (100 µL) was added to 1 mL of fresh FRAP reagent [(2,4,6-Tri(2-pyridyl)-s-triazine; Sigma)] and incubated in 37 ˚C for 10 min at dark condition. Reading of the blue-colored reagent was then taken at 595 nm every 20 sec for 10 min. Aqueous solution of Fe^{II} ($FeSO_4.7H_2O$; Merck; Darmstadt, Germany) and appropriate concentration of freshly prepared ascorbic acid were used as blank and standard solutions, respectively.

Assessment of antioxidant activity. For biochemical tests, after thawing of the testicular tissue, one g of the samples was homogenized in ice-cold 20 mM Tris-HCl Buffer (pH = 7.40; Sigma) and then centrifuged for 10 min in 4 ˚C at 4000 *g*. For examination of antioxidant enzymes activities, CAT level was determined as described by Sinha,[31] GPx level was determined according to Paglia and Valentine [32] and SOD level was determined according to Nishikimi *et al*.[33]

Measurement of GSH. To measure reduced GSH, testicular tissue was homogenized in phosphate-EDTA buffer (pH= 8.00; Merck) and 25% HPO_3 and centrifuged for 10 min at the temperature of 4 ˚C at 15,000 rpm. Reduced GSH level was estimated according to Rajesh and Latha.[34]

Measurement of MDA. A volume of 300 µL of 10% trichloro-acetic acid (Sigma) was added to 150 µL of the sample, centrifuged at 1000 rpm for 10 min at 4 ˚C and then incubated in 300 µL of 67.00% thiobarbituric acid (TBA; Sigma) at 100 ˚C for 25 min. Five min after cooling the solution, pink color was appeared because of MDA-TBA reaction and evaluated using a spectrometer (Novaspec II; Biochrom Ltd., Cambridge, UK) at a wave length of 535 nm.[35]

Statistical analysis. The results are expressed as the mean ± standard error of mean. Differences between the groups were assessed by analysis of variance using SPSS (Version 17.0, SPSS Inc., Chicago, USA). Statistical significance between groups was determined by Tukey's multiple comparison post hoc test and the *p*-values less than 0.05 were considered to be statistically significant.

Results

Average sperm count. The results revealed that the number of sperms was decreased significantly ($p < 0.01$) in busulfan group in comparison with the control group. Group 6 revealed a significant enhancement compared to control group ($p < 0.01$). There was no significant difference in sperm count between group 5 and group 6 as well as group 8 and group 9 (Table 1).

Table 1. Effects of *Allium sativum* extract on sperm parameters in study groups.

Groups	Count (10^6)	Motility (%)	Viability (%)	Morphology (%)	DNA integrity (%)
Control	21.77 ± 0.14^a	75.19 ± 0.23^a	89.23 ± 0.33^a	88.72 ± 0.41^a	1.23 ± 0.14^a
Busulfan	14.32 ± 0.19^b	58.38 ± 0.44^b	67.08 ± 0.45^b	63.41 ± 0.05^b	19.17 ± 0.34^b
Sham	21.75 ± 0.01^a	74.69 ± 0.09^c	89.21 ± 0.22^c	86.34 ± 0.18^c	1.25 ± 0.51^a
***A. sativum* (200 mg kg^{-1})**	22.18 ± 0.07^c	76.34 ± 0.01^d	91.00 ± 0.01^d	88.20 ± 0.08^a	1.09 ± 0.01^c
***A. sativum* (400 mg kg^{-1})**	22.85 ± 0.09^d	78.09 ± 0.19^e	92.60 ± 0.25^e	88.91 ± 0.17^d	0.89 ± 0.27^d
***A. sativum* (800 mg kg^{-1})**	22.90 ± 0.12^d	78.22 ± 0.26^f	93.20 ± 0.16^e	89.42 ± 0.20^e	0.87 ± 0.17^d
***A. sativum* (200 mg kg^{-1}) + Busulfan**	19.20 ± 0.23^e	71.07 ± 0.09^g	72.05 ± 0.12^f	68.28 ± 0.07^f	7.62 ± 0.24^e
***A. sativum* (400 mg kg^{-1}) + Busulfan**	20.03 ± 0.31^f	71.96 ± 0.07^h	72.08 ± 0.07^f	72.08 ± 0.36^g	6.33 ± 0.21^f
***A. sativum* (800 mg kg^{-1}) + Busulfan**	20.08 ± 0.01^f	72.80 ± 0.31^a	75.36 ± 0.24^g	75.36 ± 0.31^h	4.17 ± 0.07^g

Different superscript letters indicate significant differences between groups.

Sperm motility. Comparison of sperm motility in busulfan group with control group showed significant decrease ($p < 0.01$). However, significant variations ($p < 0.01$) were observed among *A. sativum* supplemented groups and control group (Table 1).

Sperm viability. The comparison of sperm viability in busulfan group with control group showed that it was significantly reduced ($p < 0.01$), (Fig. 1). However, with administration of *A. sativum* in group 7, group 8 and group 9, this reduction was improved, but there was significant difference ($p < 0.01$) between control and busulfan groups. Also, no significant variations ($p < 0.01$) were observed between group 7 and group 8 (Table 1).

Sperm morphology. The mean percent of sperm with normal morphology in busulfan group was significantly lower ($p < 0.01$) than control group, but with administration of *A. sativum* in busulfan groups, this reduction was improved. Also, in treatment groups (groups 4-6), in spite of high percentages of normal sperm, it was significantly different ($p < 0.01$) from the control (Table 1).

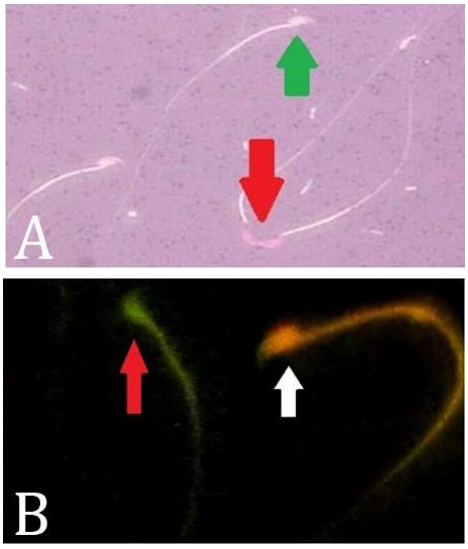

Fig. 1. A) Dead sperm (red arrow) with red color head and viable sperm (green arrow) with colorless head are observable (Eosin/nigrosin staining, 400×); **B)** Green-colored normal sperm (red arrow) and yellow-colored damaged sperm (white arrow) can be seen (Acridine orange staining, 1000×).

Sperm DNA damage. The percentage of sperm with damaged DNA in busulfan group was significantly increased ($p < 0.01$) compared to the control group (Fig. 1). However, in groups 4-9, it was reduced in comparison with busulfan group, yet it was significantly higher than control group ($p < 0.01$). The percentage of sperm with damaged DNA in sham group was almost the same as the control group (Table 1).

Level of TAC. Comparisons of the TAC levels are shown in Table 2. The TAC levels in groups 4-9 were significantly higher ($p < 0.01$) than busulfan group.

Hormonal levels. Treatment with busulfan caused a significant decrease ($p < 0.01$) in serum levels of testosterone, LH and FSH as compared to the control group. The administration of hydro-alcoholic extract of *A. sativum* along with busulfan significantly restored serum levels of testosterone, LH and FSH towards the control values (Table 2).

Antioxidant enzyme activities. In this study, intraperitoneal (IP) administration of busulfan significantly ($p < 0.01$) decreased tissue GSH contents compared to the control group (Table 2). Also, oral treatment with hydro-alcoholic extract of *A. sativum* significantly ($p < 0.01$) increased tissue GSH contents in testes compared to busulfan group (Table 2). In this study, IP administration of busulfan in mice significantly ($p < 0.01$) decreased the activity of antioxidant enzymes (GPx, CAT and SOD) in testes compared to the control group. Oral administration of hydro-alcoholic extract of *A. sativum* significantly ($p < 0.01$) increased the activity of GPx, CAT and SOD in testes compared to the busulfan group (Table 3).

Lipid peroxidation. The level of MDA, as a major product of lipid peroxidation was significantly increased ($p < 0.01$) in busulfan group compared to the control group. There was a significant restoration in treatment groups (groups 7-9). Also, in some treatment groups (groups 4-6), there was a significant decrease ($p < 0.01$) in MDA levels compared to control groups (Table 2).

Discussion

This study was planned to investigate the protective role of *A. sativum* hydro-alcoholic extract against busulfan-induced testicular toxicity in male mice. The obtained results showed that IP administration of busulfan in male mice induced reproductive toxicity, increased MDA levels and decreased serum levels of testosterone.

Table 2. Effects of *Allium sativum* extract on TAC, GSH, MDA and testosterone, LH and FSH levels in study groups.

Parameters	Control	Busulfan	Sham	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
TAC (µmol L^{-1})	1.20± 0.25^{a}	0.54 ± 0.34^{b}	1.17± 0.12^{c}	1.40± 0.21^{d}	1.82± 0.37^{e}	2.10± 0.05^{f}	0.69± 0.17^{g}	0.83± 0.20^{h}	0.99 ± 0.28^{i}
Testosterone (µmol L^{-1})	6.11± 0.11^{a}	3.24 ± 0.18^{b}	6.00± 0.09^{a}	6.12± 0.33^{a}	6.18± 0.07^{a}	6.21± 0.19^{a}	4.17± 0.26^{c}	4.77± 0.05^{d}	5.37 ± 0.28^{e}
LH (mIU mL^{-1})	2.43± 0.46^{a}	0.93 ± 0.69^{b}	2.42± 0.85^{a}	2.45± 0.61^{a}	2.79± 0.97^{c}	3.16± 0.51^{d}	1.14± 0.43^{e}	1.39± 0.70^{f}	1.76 ± 0.89^{g}
FSH (mIU mL^{-1})	3.75± 0.84^{a}	1.48 ± 0.70^{b}	3.75± 0.33^{a}	3.83± 0.61^{c}	3.89± 0.97^{d}	4.19± 0.50^{e}	1.64 ± 0.41^{f}	1.95± 0.68^{g}	2.20 ± 0.29^{h}
MDA (µmol mg^{-1})	3.26 ± 0.01^{a}	8.13 ± 0.20^{g}	3.40 ± 0.17^{c}	3.21 ± 0.16^{d}	3.18 ± 0.39^{e}	3.12 ± 0.04^{f}	5.94 ± 0.23^{g}	5.19 ± 0.06^{h}	4.47 ± 0.24^{j}
GSH (U mg^{-1} Proteins)	64.10 ± 1.28^{a}	45.12 ± 0.79^{b}	64.08 ± 0.95^{a}	66.23 ± 1.34^{a}	69.38 ± 1.19^{c}	72.11 ± 0.62^{d}	49.27 ± 0.47^{e}	53.61 ± 1.05^{f}	53.94 ± 1.23^{f}

TAC: Total antioxidant capacity; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; MDA: Malondialdehyde; GSH: Glutathione. Different superscript letters indicate significant differences between groups.

Table 3. Effect of *Allium sativum* extract on activities of testicular SOD, GPx and CAT in study groups.

Parameters	Control	Busulfan	Sham	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
SOD (U mg^{-1})	1467 ± 1.28^{a}	1025± 1.03^{b}	1437 ± 1.37^{c}	1481 ± 0.74^{d}	1503± 1.13^{e}	1549± 1.05^{f}	1103 ± 0.78^{g}	1165 ± 0.43^{h}	1219± 1.29^{i}
GPx(U mg $^{-1}$)	5.11 ± 0.47^{a}	3.19 ± 0.91^{b}	5.12 ± 1.04^{a}	5.21 ± 0.27^{b}	5.47 ± 0.72^{c}	5.93 ± 1.14^{d}	3.87 ± 0.48^{e}	4.18 ± 0.19^{f}	4.49 ± 1.07^{g}
CAT(U mg^{-1})	1.27 ± 0.68^{a}	0.71 ± 0.18^{b}	1.25 ± 0.73^{a}	1.31 ± 0.30^{a}	1.40 ± 0.45^{c}	1.49 ± 0.60^{d}	0.77 ± 0.31^{e}	0.79 ± 0.22^{e}	0.89 ± 0.39^{f}

SOD: Superoxide dismutase, GPx: Glutathione peroxidase, CAT: Catalase.
Different superscript letters indicate significant differences between groups.

In this study, observed lowered sperm quantity and quality in busulfan group compared to control and sham groups were in agreement with the results of others.[36-38] The inhibition of spermatogenesis in busulfan group in this study can be the result of decreased testosterone level. In addition, free radical products in the testicular tissue may exert detrimental effects on spermatogenesis.[39]

In this study, lowered serum levels of testosterone, LH and FSH in busulfan-treated mice were in agreement with previous reports indicating a dropped level of aforementioned factors following IP administration of busulfan in male mice.[36,40] However, Dehghani *et al.* have reported no significant changes in serum testosterone level of male mice following IP administration of busulfan.[38] In another study, Gerl *et al*. have reported that busulfan can affect exocrine and endocrine compartments of testis and a persistent impairment of Leydig cell function can occur following administration of high doses of busulfan.[41] In addition, findings of this study showed that IP injection of busulfan can lead to antioxidant enzymes activities reduction and testicular lipid peroxidation increase. The mechanism of the detrimental effect of IP injection of busulfan can be attributed to its specific direct toxic effects on the testis and it is not the result of its general toxicity and/or non-specific indirect effects through lowering serum testosterone level.[42] The oxidative stress in testes increased MDA level and concomitantly decreased the activities of protective antioxidant enzymes (SOD, CAT and GPx) in testicular tissue. The busulfan-induced oxidative stress observed in this study was similar to previous reports in male reproductive system.[37] Oxidative stress in testicular tissue can lead to serum testosterone level disturbance and testicular damages as well.[36,38]

In our study, oral administration of *A. sativum* hydro-alcoholic extract in busulfan treated animals significantly increased serum levels of FSH, LH and testosterone and improved sperm quantity and quality. Also, the findings of this study demonstrated that administration of *A. sativum* hydro-alcoholic extract caused a significant improvement in reproductive parameters of male mice compared to busulfan treated group. In addition, there was a significant increase in activities of antioxidant enzymes (SOD, GPx and CAT) and a decrease in testicular lipid peroxidation following administration of *A. sativum* hydro-alcoholic extract in this study. Several studies have reported that *A. sativum* contains a large amount of beneficial nutritional components and detectable active antioxidants including alliin, allicin, gamma-glutamyl cysteine, dially sulphide and diallyl disulphide.[43,44]

The increased level of serum testosterone, LH and FSH due to administration of *A. sativum* in this study could be responsible for sperm quantity and quality improvement as it has been established that androgen hormone is essential for spermatogenesis.[44] In addition, the improvement of sperm quantity and quality via administration of *A. sativum* hydro-alcoholic extract may be also attributed to its antioxidant activity.[15,46,47]

The improvement of semen characteristics in this study following administration of *A. sativum* hydro-alcoholic extract was in agreement with previous findings reporting that an improvement in male reproductive function following *A. sativum* administration can be attributed to its powerful antioxidant properties.[15,46,48,49] In another study, Oi *et al*. have showed that administration of garlic powder in rats increases the testicular testosterone concentration.[50] Also, Mirfard *et al*. demonstrated that administration of hydro-alcoholic extract of garlic can increase serum concentrations of LH, FSH and testosterone hormones following cyclophosphamide administration.[49]

In conclusion, it has been clearly determined that antineoplastic or cytotoxic chemotherapy drugs like busulfan, causes a decrease in FSH, LH and testosterone level, antioxidant status and sperm motility, normal morphology, count and viability and an increase in levels of MDA in male mice. Also, our findings showed that oral administration of *A. sativum* hydro-alcoholic extract after IP injection of busulfan can reduce the reproductive toxicity of busulfan and help the improvement of reproductive parameters of normal male mice. Our results also revealed that the above mentioned changes will be dose dependent with *A. sativum* hydro-alcoholic extract.

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Conflicts of interest

The authors declare that they have no particular conflicts of interest.

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