The benefit of *Silybum marianum* in ethanol-induced reprotoxicity of male Wistar rat

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This study investigates the toxic effects of ethanol (Eth) on the reproductive system of male rats and the possible protective role of *Silybum marianum* seeds-infused solution (SMI) over six consecutive weeks of administration. Animals were divided into the following groups: control, SMI positive control (200 mg/kg/day), Eth1 (1 g/kg/day), Eth2 (2 g/kg/day), Eth1+SMI, and Eth2+SMI. Plasma testosterone concentration, epididymal spermatozoa biology, and testicular and epididymal MDA, GSH and GPx levels were evaluated. The results indicated a significant decrease in testis and epididymis weight, testosterone level, sperm concentration, sperm vitality and sperm motility (total motility, progressive motility, curvilinear velocity, straight-line velocity, velocity average path, beat cross frequency, and lateral head displacement) in both Eth1 and Eth2 compared to the control groups and the combined-treatment groups (Eth1+SMI and Eth2+SMI). Furthermore, results showed a significant elevation in MDA concentration with a significant decrease of testicular and epididymal GSH concentration and GPx activity in theEth1 and Eth2 groups compared to the combined-treatment groups. The administration of SMI succeeded in improving the parameters cited above in the combined-treatment groups compared to the Eth1 and Eth2 groups, and bring them to the levels seen in the control groups. To conclude, SMI has clearly protected reproductive indices against ethanol-induced reprotoxicity in male rats

Keywords: Ethanol. Reprotoxicity. Protective role. Spermatozoa. Rat. *Silybum marianum*.

INTRODUCTION

Male infertility is a major clinical problem in andrology. Substantiations on worldwide reduction in human sperm quality have been increasing in the course of the last twenty years (Sengupta *et al.,* 2018). A recent comprehensive systematic review and meta-regression analysis found an overall 50-60% diminution in sperm count among men from North America, Europe, Australia and New Zealand (Levine *et al.,* 2017).

Several lifestyle risk factors, such as smoking, alcohol consumption, obesity, and psychological stress, can influence male fertility (Durairajanayagam, 2018). Ethanol consumption is one of the lifestyle habits that have become very common in the world even though there are many studies have demonstrated the deleterious effects of alcohol on reproductive systems in both humans and animals. The World Health Organization (WHO, 2018) has reported that alcohol consumption has increased since 2000 in almost all regions of the world, with consumers drinking an average of 32.8 grams of pure alcohol per day.

Clinical and experimental studies have demonstrated the harmful effects of ethanol intake on male fertility parameters such us testicular atrophy and the reduction in seminiferous testicular diameter (Dosumu, Osinubi, Duru, 2014), declines in semen production, sperm quality, sperm motility, and serum testosterone levels (Oremosu, Akang, 2015), and dysfunction in spermatogenesis (Shayakhmetova *et al.*, 2014). It is reported that mechanisms by which ethanol induced reproductive system disorders are mainly related to oxidative stress (OS) (Akbari *et al.,* 2017). Wherein, OS plays an

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important role in inducing male infertility (Agarwal, Majzoub, 2016).

Recently, the use of antioxidants has been an interesting subject over the last years to adjust the imbalance between pro-oxidants and antioxidants (Agarwal, Majzoub, 2016). Many cultures have long used plants and their bioactive substances in traditional medicine to treat many diseases (Tutar *et al.,* 2018).

Due to its special location and climate, Algeria has a rich and varied vegetation; many of these species have been used in medicine since ancient times to treat various ailments and diseases. Milk thistle (*Silybum marianum*), known in Algeria as *chouk ahmar*, is one of the 123 classified botanical families in Algeria. It has been known for its traditional therapeutic value as a treatment for liver diseases (Bhattacharya, 2011). It has been reported that the extract of milk thistle seed contains a high concentration of an active compound named silymarin (Greenlee *et al.,* 2007), which had shown antioxidant effect by scavenging reactive oxygen species (ROS) and inhibiting the peroxidation of lipids (Yaman *et al.,* 2018). In addition to its ROS-neutralizing properties, silymarin also increases the efficacy of physiological antioxidants (Surai, 2015). Moreover, silymarin can inhibit virus infection, modulate cellular metabolism, and reduce inflammation (Lovelace *et al.,* 2015). In addition, silymarin and silybin extracted from *Silybum marianum* have been found to improve blood testosterone levels (Oufi, Al-Shawi, Hussain, 2012) and sperm function (Attiaa *et al.,* 2017; Fatehi *et al.,* 2018).

In the present study, we sought to investigate ethanol-induced reprotoxicity in the male Wistar rat and to demonstrate the potential mitigating effects of *Silybum marianum* seeds-infused solution against alcohol toxicity.

MATERIAL AND METHODS

Plant and ethanol preparation

S*ilybum marianum* (Milk thistle) dry seeds were harvested in July from Ain-Berda, (Annaba province) and were stored in a glass box until use. Ten grams of the crushed seeds were poured over 100 mL of boiling distilled water and left to infuse for 30 minutes (Raskovic *et al.,* 2002). After filtration, the infused solution of seeds (SMI) to animals at a dose of 200 mg/kg/day by gavage. The infusion was prepared fresh every day during the experimental period. Absolute ethanol 99.8% (Honeywell Laboratory, Germany) was diluted in distilled water to reach the desired concentration of 40%, and then two doses were prepared (Eth1 and Eth2).

Experimental protocol

In this experiment, 36 male adult Wistar rats aged 90-120 days and weighing 200-220g obtained from Pasteur Institute (Algiers) were reared in the animal house of University of Badji Mokhtar-Annaba. Rats were fed a standard diet (GAC-ORAC, Bejaia, Algeria) and received drinking water *ad libitum*. Rats were equally divided into six groups; the first group represented the control, while the second group received SMI (200 mg/kg/day). The third and the fourth groups were respectively treated with 1 g/kg/day (Eth1) and 2 g/kg/day (Eth2) of ethanol. The rats of the fifth (Eth1+SMI) and the sixth (Eth2+SMI) groups were firstly treated respectively with 1g/kg/day and 2g/kg/day of ethanol, after one hour, animals were given SMI (200 mg/kg/day). During the treatment, bodyweight were recorded weekly. After six consecutive weeks of the experiment, the rats were decapitated under ether anesthesia and then organs, blood and epididymal fluid were obtained. Animals' treatments were authorized by the Ethical Committee of Animal Sciences at the University of Badji Mokhtar-Annaba before the experimental work was initiated. Experiments were carried out in accordance with international guidelines for the care and use of laboratory animals.

Organs and blood collection

After dissection, testis and epididymis were removed, cleaned of adipose tissue, rinsed in 0.9% NaCl, weighed to determine their mean absolute weight, and then stored in a freezer at -80°C until their later use for determining oxidative stress parameters. Blood was collected in heparinized tubes, centrifuged at 3000 rpm for 15 minutes, and then the plasma was collected and stored at -80 °C for the testosterone assay.

Sperm collection

In this study, we used epididymal fluid was used to study the biology of spermatozoa. An opening was performed with a scalpel at the level of the epididymis cauda to obtain sperm (Martinez-Pasteur *et al.,* 2006). Approximately 1μl of sperm was diluted in a physiological solution of NaCl 0.9% to study the concentration and the motility, while another 1μl was diluted in a hypo-osmotic solution to evaluate spermatozoa vitality.

Measurement of sperm concentration and motility

Concentration and sperm motility were immediately measured by the automated Computer-Assisted Sperm Analysis Method (CASA) using Sperm Class Analysis version 6.2.0.0 (SCA®, Microptic, Barcelona, Spain). 5μl of sperm diluent at 37 °C was poured into a pre-warmed GoldCyto slide and then placed under a Nikon Eclipse microscope (Nikon E200-LED), using objective x4 negative-phase contrast combined with a phase condenser contrast. The motility/concentration module of sperm class analysis determined the following parameters: concentration, total motility, progressive motility, and kinematics parameters such as; curvilinear velocity (VCL): the real point-to-point trajectory of a spermatozoon from its starting point to the point of arrival divided by the time elapsed. Velocity straight-line path (VSL): The distance between the first and last tracked point of the spermatozoa trajectory divided by the time elapsed. Beat Cross Frequency (BCF): derivation of the true frequency of flagella beat and frequency of rotation of the head. Lateral head displacement (ALH): The maximum value of the distance of any point on the track from the corresponding average path, multiplied by two. Measuring the kinematics parameters give us a more great detail about the sperm motility function.

Measurement of sperm vitality

The hypo-osmotic swelling test was used to evaluate spermatozoa vitality. This test is based on the semi-permeability of the intact sperm tail membrane in the presence of a hypo-osmotic extracellular medium. Live spermatozoa produce an influx of water into their tails

to rebalance the osmotic pressure on either side of the membrane, causing subsequent tail swelling and curling. Dead spermatozoa lose this osmoregulation ability and, therefore, do not swell in a hypo-osmotic environment. The percentages of live and dead spermatozoa were calculated manually in 100 counted spermatozoa.

Measurement of testosterone

Plasma testosterone concentration was measured by the enzyme-linked immunosorbent assay (ELISA) method as mentioned in the instructions manual of commercial kit (manufactured by DGR Instruments Gmbh, Germany).

Measurement of oxidative stress parameters

In this study, the oxidative stress parameters were evaluated in the testis and the epididymis. Lipids peroxidase was measured as Malondialdehyde (MDA) level, according to the method of Ohkawa *et al.,*(1979), while Reduced Glutathione levels (GSH) and Glutathione Peroxidase activity (GPx) were respectively evaluated according to the methods of Weckbercker and Cory (1988), and Flohe and Gunzler (1984).

Statistical analysis

The obtained results were expressed as means ± standard deviation. The differences between the groups were tested for statistical significance by one-way analysis of variance (ANOVA), followed by the Tukey test for multiple comparisons (Minitab 18). Statistical significance was set at $p < 0.05$.

RESULTS

Body weight, Organs' weights and testosterone concentration

The variations of initial body weight, final body weight and body weight gain indicated no significant difference was observed between all groups (table I). Table II shows a significant decrease in testes and

epididymis relative weight of rats of Eth1 and Eth2 groups compared to the other treatments groups, while it demonstrates no significant changes in Eth1+SMI and Eth2+SMI groups compared to the control. Likewise, testosterone concentration has decreased significantly in ethanol-treated groups compared with the other groups. Meanwhile the combined-treatment groups have significantly increased plasma testosterone compared to Eth1 and Eth2 groups, with no noticeable changes when compared to the control.

TABLE I - Values of body weight (g) (initial body weight, final body weight and body weight gain) of Wistar rat expressed as mean ± SD after six consecutive weeks of treatment

	Control	SMI	Eth1	Eth2	Eth1+SMI	Eth2+SMI
IBW (g)	219.83±6.6[a]	220.67±8.3[a]	220.33±11.6[a]	220.33±9.4[a]	219.83±8.7[a]	219.67±12.8[a]
FBW (g)	300.67±6.9[a]	302.33±15.9[a]	311.33±13.4[a]	312.67±15.9[a]	298.00±17.7[a]	300.0±29.6[a]
BWG (g)	81.60±7.02[a]	82.40±12.14 [a]	90.60±10.01 [a]	90.00±17.45 [a]	74.40±20.38 [a]	79.2±23.9 [a]

Means do not share the same letter are significantly different (p< 0.05),According to one-way ANOVA followed by Tukey test. IBW: Initial Body Weight; FBW: Final Body Weight; BWG: Body Weight Gain; SMI: *Silybum marianum* seeds-infused solution; Eth1:01 g/kg/day; Eth2: 02 g/kg/day

TABLE II - Values of the reproductive indices (testis and epididymis weight, and plasma testosterone concentration) of Wistar rat expressed as mean ± SD after six consecutive weeks of treatment

	Control	SMI	Eth1	Eth2	Eth1+SMI	Eth2+SMI
RTW (g)	0.55±0.014[a]	0.59±0.028[a]	0.48±0.014[b]	0.46±0.005[b]	0.56±0.019[a]	0.55±0.036[a]
REW (g)	0.23±0.009[a]	0.24±0.010[a]	0.19±0.007[b]	0.17±0.014[b]	0.23±0.015[a]	0.23±0.0257[a]
T (ng/ml)	6.10±0.58[ab]	6.84±0.41[a]	4.15±0.83[c]	3.13±0.45[c]	5.70±0.67[b]	5.55±0.59[b]

Means do not share the same letter are significantly different (p< 0.05),According to one-way ANOVA followed by Tukey test. RTW: Relative Testis Weight; REW: Relative Epididymis Weight; T: testosterone; SMI: *Silybum marianum* seeds-infused solution; Eth1:01 g/kg/day; Eth2: 02 g/kg/day

Spermatozoa biology parameters

Results of the spermatozoa biology are summarized in Table III. Results indicate that ethanol significantly reduced spermatozoa concentration, vitality and sperm motility (total motility, progressive motility, VCL, VSL, VAP, BFC, and ALH) compared to the control group and the other treatment groups. On the other hand, all spermatozoa biology parameters were significantly higher in Eth1+SMI and Eth2+SMI groups compared to Eth1 and Eth2 groups, with no differences observed when compared with the control.

TABLE III - Values of spermatozoa biology parameters of Wistar rat expressed as mean± SD after six consecutive weeks of treatment

	Control	SMI	Eth1	Eth2	Eth1+SMI	Eth2+SMI
CON (m/ml)	771.7±49.0[ab]	838.8±34.8[a]	625.0±57.9[c]	481.5±66.1[d]	733.9±24.10[b]	717.8±55.7[b]
TMOT (%)	74.85±4.24[b]	81.91±4.07[a]	42.61±1.80[c]	33.96±4.66[d]	71.15±4.27[b]	70.14±4.01[b]
PMOT (%)	10.61±0.68[ab]	11.71±0.55[a]	4.24±0.85[c]	2.98±0.89[c]	9.61±0.92[b]	9.66±0.79[b]
VCL (μm/s)	81.15±4.51[a]	81.19±2.21[a]	72.92±3.74[b]	71.07±4.31[b]	79.89±1.99[a]	79.81±4.45[a]
VSL (μm/s)	18.36±1.84[ab]	20.40±1.49[a]	13.68±1.75[c]	11.98±1.53[c]	16.59±2.09[b]	17.09±3.82[b]
VAP (μm/s)	39.84±0.95[ab]	41.42±1.49[a]	33.83±0.90[c]	32.73±1.03[c]	38.64±2.39[ab]	37.52±2.11[b]
BCF (Hz)	4.19±0.11[a]	4.45±0.18[a]	3.54±0.33[b]	3.36±0.18[b]	4.09±0.27[a]	4.09±0.40[a]
ALH (μm)	4.62±0.11[a]	4.59±0.12[a]	4.23±0.15[b]	4.06±0.11[b]	4.52±0.173[a]	4.50±0.14[a]
Vitality (%)	62.02±1.83[b]	68.50±2.41[a]	34.31±3.28[c]	27.29±3.11[d]	59.77±3.49[b]	58.22±6.20[b]

Means do not share the same letter are significantly different ($p < 0.05$), According to one-way ANOVA followed by Tukey test. SMI: *Silybum marianum* seeds-infused solution; Eth1:01 g/kg/day; Eth2: 02 g/kg/day; CON: Sperm concentration; TMOT: Total motility; PMOT: Progressive motility; VCL: Curvilinear Velocity; VSL: Velocity straightline path; VAP: Velocity Average Path; BCF: Beat Cross Frequency; ALH: Lateral head displacement.

Stress oxidative parameters

As illustrated in Figure 1, results indicate a significant elevation in the testicular and epididymal level of MDA, with a significant decrease in GSH level and GPx activity of animals given ethanol (1g/kg/day and 2g/kg/day) compared to the other groups. However, the combined-treatment show a significant reduction in MDA concentration with a significant increase in GSH level and GPx activity of Eth1+SMI and Eth2+SMI treated rats compared to Eth1 and Eth2 groups, with no significant changes compared to the control.
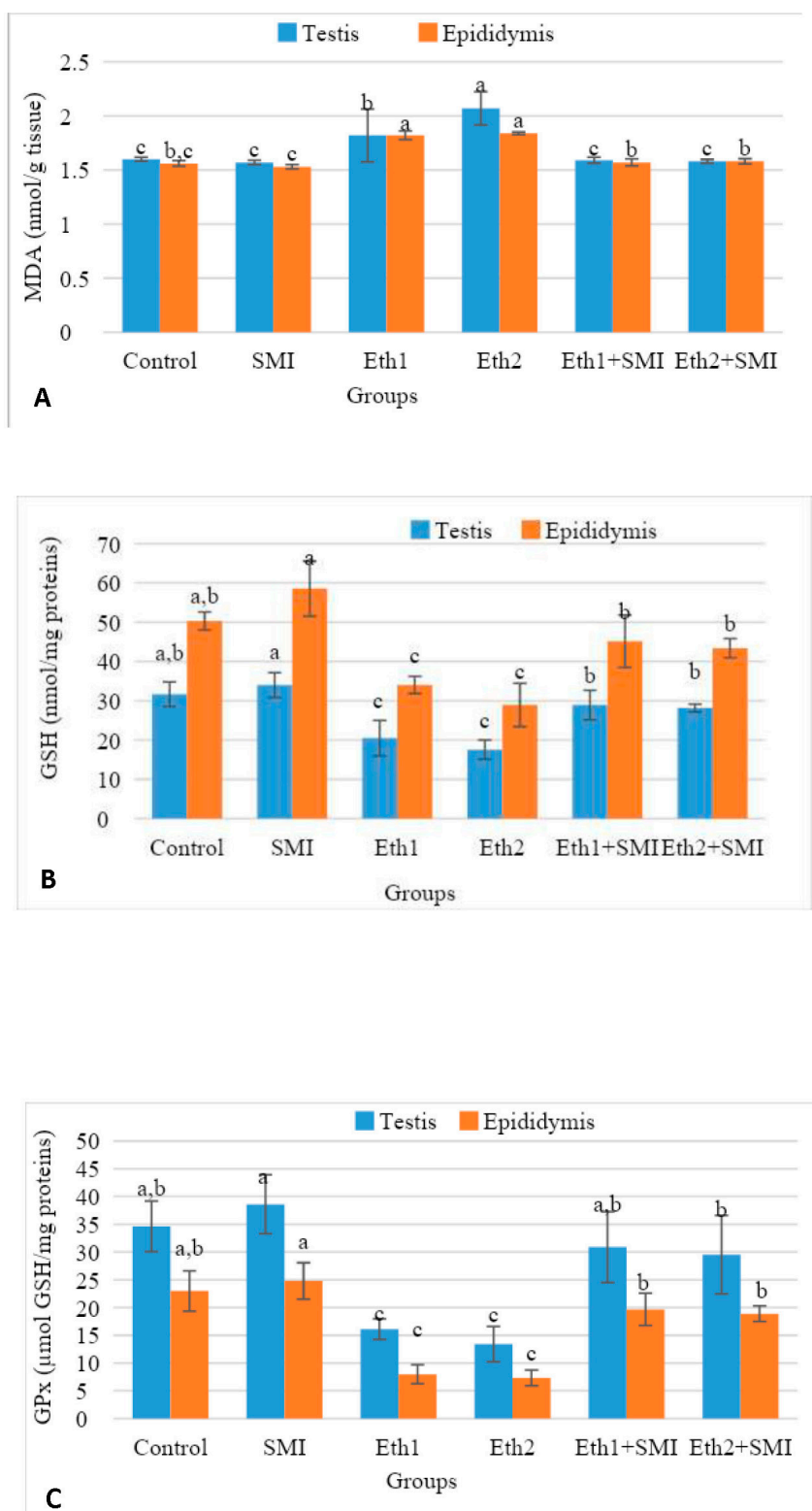
FIGURE 1 - Variation of oxidative stress parameters (mean ± SD) in testis and epididymis of Wistar rat of control group and treated groups after six consecutive weeks of treatment. A: Variation of tissue MDA concentration in testis and epididymis; B: variation of glutathione reductase levels in testis and epididymis; C: variation of glutathione peroxidase level in testis and epididymis. Means do not share the same letter are significantly different ($p < 0.05$), according to one-way ANOVA followed by Tukey test. SMI: *Silybum marianum* seeds-infused solution; Eth1:01 g/kg/day; Eth2: 02 g/kg/day.

DISCUSSION

In this study, attention was focused on the effects of ethanol on reproductive parameters of Wistar rats, and demonstrate the feasibility of using *Silybum marianum* seeds-infused solution as a preventive agent. Our results indicated that both doses of ethanol provoked a remarkable decrease in the testicular and epididymal relative weights. These findings are consistent with those of previous investigations (Saihia, Khelili, Boulakoud, 2015; Akbari *et al.,* 2017). There are several possible explanations for the obtained results; one of them is the direct effect of ethanol on reproductive organs. A recent study found that the administration of ethanol induced pathological lesions in the epididymis structure of male rats by reducing cell height and enlarging the interstitial space (Al-Bairuty, Al-shmgani, Taha, 2016). Such effects may be the reason of the decreasing epididymis weight observed in this study. Besides, considering that the epididymis stores mature sperm, the observed diminution in its weight may be due to a reduction of sperm concentration. In the testes, ethanol has been found to induce changes in the diameters of the seminiferous tubules, apoptosis of germ cells, and testicular atrophy (Maneesh *et al.,* 2005), which lowers testicular weight. Moreover, the decrease in the reproductive organs' weights could stem from the decline in testosterone level. Our results indicated a noticeable decline in this hormone after six weeks of exposure to ethanol. These findings align with those of a previous study (Saihia, Khelili, Boulakoud, 2015), which suggested that ethanol may enhance the conversion of androgens to estrogens by the aromatase (Muthusami, Chinnaswamy, 2005). Furthermore, it has been shown that alcohol consumption causes an increase in cortisol hormone levels, which inhibits the production and release of testicular testosterone, ending with total suppression of testosterone levels (Venkat *et al.,* 2009).

In contrast, in the combined-treatment groups, the testicular and epididymal weights increased significantly compared to those of ethanol-treated groups. These findings can be explained by the strong antioxidant properties of *Silybum marianum* components silymarin and silybin, which are the primary active ingredients.

Silybin was found to enhance spermatid diameter and the number of primary spermatocytes in mice (Oufi, Al-Shawi, Hussain, 2012). Furthermore, it was stated that silymarin significantly raised the percentage of seminiferous tubules and the spermatogenesis indices (Moshtaghion *et al.,* 2013).

Similarly, plasma testosterone concentration, along with organ weight, increased to the normal levels when *Silybum marianum* seeds-infused solution was administered after ethanol. Previous research has reported that the administration of silybin (Oufi, Al-Shawi, Hussain, 2012) and silymarin (Abedi *et al.,* 2016) to rats caused a significant rise in blood testosterone levels. Even thought that there is little evidence about how SMI boosts testosterone levels. *Silybum marianum* seeds may increase serum testosterone levels is via silymarin, which functions as an aromatase inhibitor, blocking the conversion of androgens to estrogens and thereby bringing testosterone to its normal level (Khalil, 2002). Meanwhile, silybin may stimulate Leydig cell function by enhancing the production of testosterone (Oufi, Al-Shawi, Hussain, 2012).

In the current study, ethanol caused a clear toxic alteration in sperm concentration, vitality, and in the motility parameters (total motility, progressive motility, curvilinear velocity (VCL), straight-line velocity (VSL), velocity average path (VAP), beat cross frequency (BFC), and lateral head displacement (ALH). Ethanol was demonstrated to make deleterious effects on reproductive ability in both rats and rabbits by reducing semen production and sperm motility (Alirezaei, Jelodar, Ghayemi, 2012; Saihia, Khelili, Boulakoud, 2015). The reduction observed in sperm concentration and vitality could be reasonably assumed to oxidative stress induced by ethanol. Ethanol stimulates reactive oxygen species production and lipid peroxidation (Saleh, Agarwal, 2002).These free radicals may attack testicular germ cells, causing necrosis and spermatogenesis alterations, decreasing spermatozoa numbers (Abedi *et al.,* 2016). Similarly, oxidative stress was found to induce apoptosis, membrane lipid peroxidation and DNA fragmentation, which impairs spermatozoa function (Lampiao*et al.,* 2013). In these circumstances, elevated apoptosis and impaired membrane integrity induced by lipid

peroxidation appear to be the mechanisms behind the diminution of sperm concentration and vitality caused by ethanol.

The reduction in sperm motility parameters induced by ethanol in the current study seems to be a signal of a failures in the acquisition and maintenance of sperm motility caused by ethanol (El-Ashmawy, Saleh, Salama, 2007). These findings could be explained by the effect of ethanol on spermatozoa tail morphology, since it is a very important organ for spermatozoa motility. Accordingly, sperm flagella morphology was reported to have an important correlation with increases or decreasesinsperm motility (Sadighi Gilani, Sadighi Gilani, 1998). Moreover, it was shown that ethanol increases the portion of spermatozoa with abnormal flagella (Muthusami, Chinnaswamy, 2005). Sperm motility, velocity parameters (VCL, VSL and VAP), ALH and BCF all reflect the mitochondrial function as an energy producer. The likely explication of the deleterious effects of ethanol on sperm motility parameters that we observed in this study may be linked to mitochondrial dysfunction, which reduces the energy required for flagella movement.

In contrast, SMI has showed remarkable protective properties of sperm parameters against ethanol toxicity. The protective role of silymarin and silybin on sperm function has been reported in some studies (Attiaa *et al.,* 2017; Fatehi *et al.,* 2018). Such protection can be attributed to the antioxidant proprieties of silymarin, which scavenges ROS and inhibits the peroxidation of lipids (Yaman *et al.,* 2018). By reducing oxidative stress, silymarin can protect cells against apoptosis (Adhikari *et al.,* 2013), thus increasing the number of live spermatozoa. Moreover, silymarin and silybin were proved to protect the integrity of mitochondria (Surai 2015) that produce the energy required for the movement of flagella, which enhances sperm motility and vitality.

The present study indicated a remarkable increase in the levels of MDA, accompanied with a reduction in GSH and GPx in testis and epididymis after six weeks of exposure to ethanol. Previous studies have found elevated lipids peroxidation and a decrease in the activities of antioxidant enzymes after ethanol consumption in male rats (Akomolafe *et al.,* 2015;

Siervo *et al.,* 2015). The generation of free radicals and the depletion of antioxidants (Gheorghiu *et al.,* 2004) may cause the increase in testicular and epididymal MDA production during alcohol consumption. GSH is an endogenous antioxidant that plays a key in detoxification of ethanol. The lower levels of GSH could be explained by its consumption by catalase and glutathione peroxidase during the degradation of hydrogen peroxide and lipid peroxide (Arivazhagan, Thilagavathy, Pannerselvam, 2000). It was reported that ethanol seems to be involved in inhibiting GSH transport into the mitochondria (Dosumu, Osinubi, Duru, 2014). Thus, the decline in GPx activity observed in this study could result from reduced GSH levels, since GSH is one of the antioxidant enzymes that work on lowering oxidative damage by eliminating toxic H_2O_2 (Panda, Ashar, Srinath, 2012).

In this study, SMI has reversed the effects of ethanol by reducing MDA concentration and raising GSH levels and GPx activity in rat reproductive organs. These results are in good agreement with previous research that has demonstrated the advantage of using *Silybum marianum* extract or one of its active compounds (silymarin or silybin) to protect reproductive and other organs against oxidative stress induced by various chemicals (Yaman *et al.,* 2018; Khoei *et al.,* 2019). It has been shown that *Silybum marianum* participates in antioxidant defense by preventing free radical formation through the inhibition of specific enzymes responsible for free radical generation (Surai, 2015). It can also promote protein synthesis such as glutathione, which increases GPx activity (Fatehi *et al.,* 2018). Likewise, silymarin was found to preserve GSH concentration by increasing the levels of methionine, S-adenosylmethionine, and cysteine metabolites that are needed for GSH synthesis (Kim *et al.,* 2016).

CONCLUSIONS

This experiment can be added to a body of research that has discussed the use of natural compounds against the effects of ethanol toxicity on male fertility. Alcohol intake provoked a declines in the weight of reproductive organs, lower testosterone concentration, alteration in most sperm functions, elevations in MDA, and decline

in anti-oxidant capacity (GSH and GPx). On the other hand, the administration of *Silybum marianum* seed-infused solution with ethanol successfully maintained most of these parameters at normal levels. Future investigations are needed to clarify the mechanisms by which SMI mitigates ethanol toxicity in the male reproductive system.

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