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Antioxidant effect of *Ferula hermonis* Boiss on acrylamide induced testicular toxicity in male rats

Nisreen Abdullah Rajeh* & Areej Mohammad Al-Shehri

Department of Anatomy, Faculty of Medicine, King Abdulaziz University, P O Box: 80215, Jeddah 21598, Kingdom of Saudi Arabia

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Acrylamide (ACR) is potentially carcinogenic to humans. It is also a food toxicant, geno-neuro and reproductive toxicant to wide variety of laboratory animals. ACR stimulates release of free radicals, which cause oxidative stress inducing many toxic effects in body, particularly testis. Antioxidants are known to decrease the toxic effect of ACR and *Ferula hermonis* Boiss root extract is a known natural antioxidant. This study investigates the antioxidant effect of *Ferula* sp. root extract on acrylamide induced testicular toxicity in male rats. Sixteen adult male virgin Wister rats were used and divided into four groups (control, ACR, ACR+*Ferula* sp., *Ferula* sp.). Histopathological studies for the right testis and caudal sperm count were carried out. Significant reduction in sperm count and testosterone serum level was found in ACR treated group. However, histopathological study did not show significant difference between ACR and ACR+*Ferula* sp. groups. ACR produced histopathological changes in testes and liver of rats. *Ferula* sp. root extract can be used as a weak antioxidant against ACR induced testicular toxicity. We recommend restriction of ACR in food and use of *Ferula* sp. plants with caution especially in patients with impotence.

Keywords: Impotence, Natural antioxidant, Oxidative stress, Reproductive toxicity, Serum testosterone, Sperm count

Acrylamide (ACR) is a vinyl organic compound, highly soluble in water, easily absorbed and distributed inside the human body. It is an essential industrial compound used for various manufacturing purposes such as in the formation of several polymers e.g., polyacrylamide. Polyacrylamide is mostly used in water treatment, paper and plastic industry, mineral production and is significantly used in electrophoresis in laboratory analysis[1]. The International Agency for Research on Cancer (IARC) has classified ACR as "a potential carcinogen to human". The toxic effect of acrylamide on humans is well studied[2-4]. It is also being considered as neuroreproductive toxicant in a wide variety of laboratory animals[3,4]. Many studies have demonstrated the toxic effect of acrylamide in the reproductive performance of many organisms[5], and also highlighted the potential dangers that ACR poses to health and the diseases in humans[6,7]. ACR is also a food toxicant, highly formed in carbohydrate rich food such as fried potato, coffee, cookies and breakfast cereals when exposed to high temperature during cooking in which Maillard reaction occurs between asparagine amino acids and glucose, producing acrylamide[8,9].

ACR is metabolized by two main pathways; glutathione conjugation or glycidamide epoxidation[3]. The reactive toxic metabolite of ACR, known as glycidamide is more toxic toward proteins and DNA than acrylamide[8]. Cytochrome P450 E1 (CYP2E1) is the main enzyme involved in glycidamide epoxidation[3,10,11].

Earlier, in a study conducted by Shirashi[12], ACR was considered a specific germ cell mutagen. Unscheduled DNA synthesis (UDS) took place in an *in vivo* assay in rat spermatocytes[13], and was also found in an *in vivo* study conducted on mouse germ cells[14]. A study revealed that chromosomal aberrations were not found in spermatogonia[15]. In contrast, aberrations were found in spermatocytes[16]. Numerous dominant lethal mutation assays have been conducted in both mice and rats. All studies have reported the induction of dominant lethal mutations by ACR[17].

Despite the tremendous advancement in allopathic medicine, a vast percentage of the world population counts on folklore medicine for curing the ailments related to liver, reproductive and other disorders.

*Correspondence:
Phone: 012 6401000, Ext: 23039
E-mail: nrajeh@kau.edu.sa; drnisreen73@gmail.com

Nonetheless, not much of these medicinal plants plus formulations used are scientifically evaluated for their activity[18]. Although the use of plants for the treatment of many diseases is ancient but it is still handy among the tribal and local people in Saudi Arabia and other parts of the Middle East. The background for the usage of these plants is that, these contain various dietary nutrients like phenolic compounds and vitamins which possess strong antioxidant capacities. Therefore, these compounds are used as antioxidants to prevent the pathogenesis of many disorders which is the aftereffect of oxidative DNA damage[19]. The plant polyphenolics are used as natural antioxidants in folk medicine as a remedy for liver disorders, an analgesic and pain reliever for headache, colic and colds, brain related diseases; improve memory, reproductive disorders, skin diseases and other ailments[20,21].

Ferula hermonis Bioss commonly known as *'Shilsh-el-zallouh', 'Hashishat-al-kattira'* or 'The Lebanese viagra', is a plant that belongs to Apiaceae family and grows abundantly in the middle East[22]. It was first discovered in biblical Mount Hermon in Southern Lebanon[23]. For many years, the root extract of this plant has been used in the treatment of sexual impotence in males. Nowadays, the root extract of this plant is commercially available and is being used by many populations in Middle East to improve sexual performance[24]. Several studies have been conducted on the root of this plant to identify its active ingredients and mechanism of action[25,25]. Plenty of naturally occurring vitamins and minerals are present in the root of *F. hermonis*[22]. However, four aromatic esters were identified to be the main active ingredients of this root extract, where ferutinin was the primary and active one[26]. One study reported that a single oral dose of acetonic extract of *F. hermonis* can increase serum testosterone concentration in adult male rat up to three-folds[27].

Acrylamide is a compound that can stimulate release of free radicals, which may cause oxidative stress inducing many toxic effects in testis and body. Further, *F. hermonis* root is a known antioxidant. The urgency for finding natural antioxidant for acrylamide reproductive toxicity is mandatory. In this work, *F. hermonis* root extract is used as a natural antioxidant against acrylamide reproductive toxicity. Therefore, the aim of this study was to investigate the antioxidant effect of *F. hermonis* on the induced testicular toxicity of acrylamide.

Materials and Methods

Materials

Plus one acrylamide (PAGE) grade with purity >99.95 was purchased from Pharmacia Biotech (Uppsala, Sweden). Unless otherwise mentioned, all other chemicals and materials of molecular biology grade (BHD laboratory supplies, Analar®, England) were used in carrying out the study.

Preparation of *Ferula hermonis* root extract

Ferula hermonis roots were purchased from a local market in Jeddah. The roots were soaked in boiled hot water for half an hour and the extract was then cooled before use, it was freshly prepared daily.

Animals and treatments

A total of 16 adult male virgin Wister rats (250±20 g), 60 days old on arrival, were brought from King Fahad Medical Research Center (KFMRC) in Jeddah, Kingdom of Saudi Arabia (KSA), and were housed 4 per cage under controlled temperature, humidity and light. The dose of acrylamide, prepared daily by using distilled water, used was 60 mg/kg body wt./day for five consecutive days. Rats were dosed with ACR by oral gavage using metallic needle curved-ball ended (Size PS-18). Control group was orally gavaged with 1 mL of distilled water.

Experimental protocol

All animal care procedure and treatments were carried out at KFMRC, Jeddah, KSA with the approval of the Unit of Biomedical Ethics, King Abdulaziz University (KAU), Medical College, Jeddah, KSA in accordance with the guidelines of the KAU. These guidelines are in compliance with the national and international laws and policies (National Institutes of Health Guiding Principles on the Care and Use of Laboratory Animals, USA).

The animals were randomly divided into four groups (n=4 each) as follows: Group I wherein rats received one mL of normal drinking water by oral gavage;

Groups II, III and IV received ACR (60 mg/kg body wt./day), ACR (60 mg/kg body wt./day) + *Ferula hermonis* root extract, and *Ferula hermonis* root extract alone, respectively by oral gavage for five consecutive days. Group I served as the control. After five days of treatment followed by three days of observation, blood was collected and centrifuged at 3200 g for 10 min. After three days of observation, animals were killed by cervical dislocation, and right testis of all rats was isolated for further experimental evaluation.

Histopathological processing

Testes and liver of all rats were fixed by 10% neutral buffered formalin for 24 hours. Following fixation of tissues by previous methodology, tissues were then processed using standard methods for histology. Tissues were briefly embedded in paraffin blocks, sectioned at approximately 3-5 µm thickness and then stained with hematoxylin & eosin (H & E). Stained sections were mounted with Di-N-Butyle Phthalate in Xylene (DPX) and were examined for histological changes using light microscopy (Olympus BX51TF) at the indicated magnifications and representative images were captured with Olympus DP 72 camera.

Testosterone ELISA

Blood of 16 adult male virgin Wister rats was collected in pane tubes of red top caps to obtain their serum for promoting testosterone hormonal analysis by means of an automated analysis administration used the ADVIA Centaur and ADVIA Centaur XP Systems provided by Siemens Healthcare Diagnostics for both quantitative and competitive immunoassay of chemiluminescent technology. According to the system findings, the correlation coefficient (r) was equal to 0.99 which indicated the inverse relation between testosterone levels and the amount of light unit (RLUs) detected by the system.

Caudal sperm count

Two µL from each caudal tissue suspension (diluted 1:20) was taken, and sperm number was manually counted using a Makler Counting Chamber (Sefi-Medical Instruments, Haifa, Israel) in a strip of ten squares. In case of oligospermia, 3-4 strips were counted and their mean was used. The resultant number was multiplied by the dilution factor (10) and represented their concentration in millions/mL of suspension. Counting was undertaken using a Leica, DM 1000 light microscopy at 20X magnifications.

Statistical analysis

All statistical analysis was performed using SPSS (Statistical Package for the Social Sciences) version 16.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ±1 SEM. Differences among the groups were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni test as a post hoc for multiple comparisons. A P-value of less than 0.05 was considered as criterion for a statistically significant difference.

Results

Effect of *Ferula hermonis* root extract on ACR induced reduction in sperm count

This study showed that there was a significant reduction in sperm count (P <0.05) between the control and all other groups. However, there was no statistically significant difference between the ACR treated group and the group treated with ACR + *F. hermonis* (Fig. 1A).

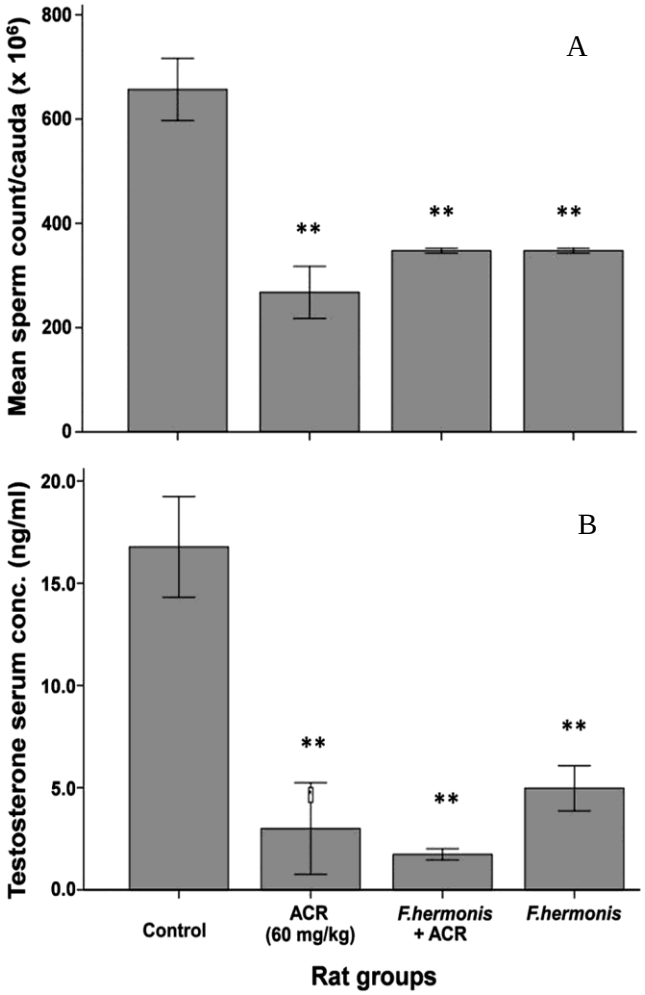

Fig. 1 — Effect of *Ferula hermonis* on ACR induced (A) sperm count reduction; and (B) serum testosterone concentration. [(A) Significant reduction in sperm count (P <0.05) between the control and all other groups was observed. But, the difference in the sperm count between the ACR treated group and the group treated with ACR + *Ferula hermonis* was not significant. (B) Serum testosterone concentration reduction was observed in all the groups when compared to the control group (P <0.005). However, no statistically significant difference was detected between the ACR and ACR + *Ferula hermonis* groups. The symbol '**' represents statistical significance from normal control: P <0.001]

Effect of *Ferula hermonis* root extract on ACR induced serum testosterone reduction

As shown in Fig. 1B, there was a significant reduction of testosterone hormone in all groups when compared to the control (P <0.005). However, no statistically significant difference was detected between the ACR and ACR + *F. hermonis* groups.

Effect of *Ferula hermonis* root extract on ACR induced testicular histopathology

As shown in Fig. 2 A & B, section of testes from control rats showed normal testicular histology with apparent normal leydig cells in the interstitial space. Interestingly, testes of ACR treated rats showed seminiferous tubule atrophy (Fig. 2C) with many multinucleated giant cells of different size in the lumen of seminiferous tubule (Fig. 2 D & E). Testes of rats treated only with root extract showed atrophy in seminiferous tubules (Fig. 2F). After *F. hermonis* root extract treatment to ACR treated rats, testes showed mild improvement in general histology with reduction in multinucleated cell number and improved atrophy while areas of atrophy between cells is still seen (Fig. 2G).

Effect of *Ferula hermonis* root extract on ACR induced hepatic histopathology

We next attempted to study the protective role of *F. hermonis* in liver against the ACR toxicity. The results showed degenerative changes with sinusoidal dilatation (Fig. 3 A-C) in the hepatic cells post ACR treatment. Interestingly, the degenerative liver showed dramatic improvement after root extract treatment (Fig. 3D).

Discussion

The present work was performed to evaluate the testicular toxicity of ACR in adult male rats and to investigate the antioxidant action of *Ferula hermonis* on ACR reproductive and genotoxicity. A high dose of acrylamide (60 mg/kg/day) for five days was chosen to induce the reproductive and genotoxic effects of acrylamide in male rats. The reason for using this high dose, which is much higher than the estimated food intake of ACR by general population, was its effectiveness in eliciting a strong response over a short period (5 days).

In this work a significant reduction in total sperm count per cauda was detected between control group

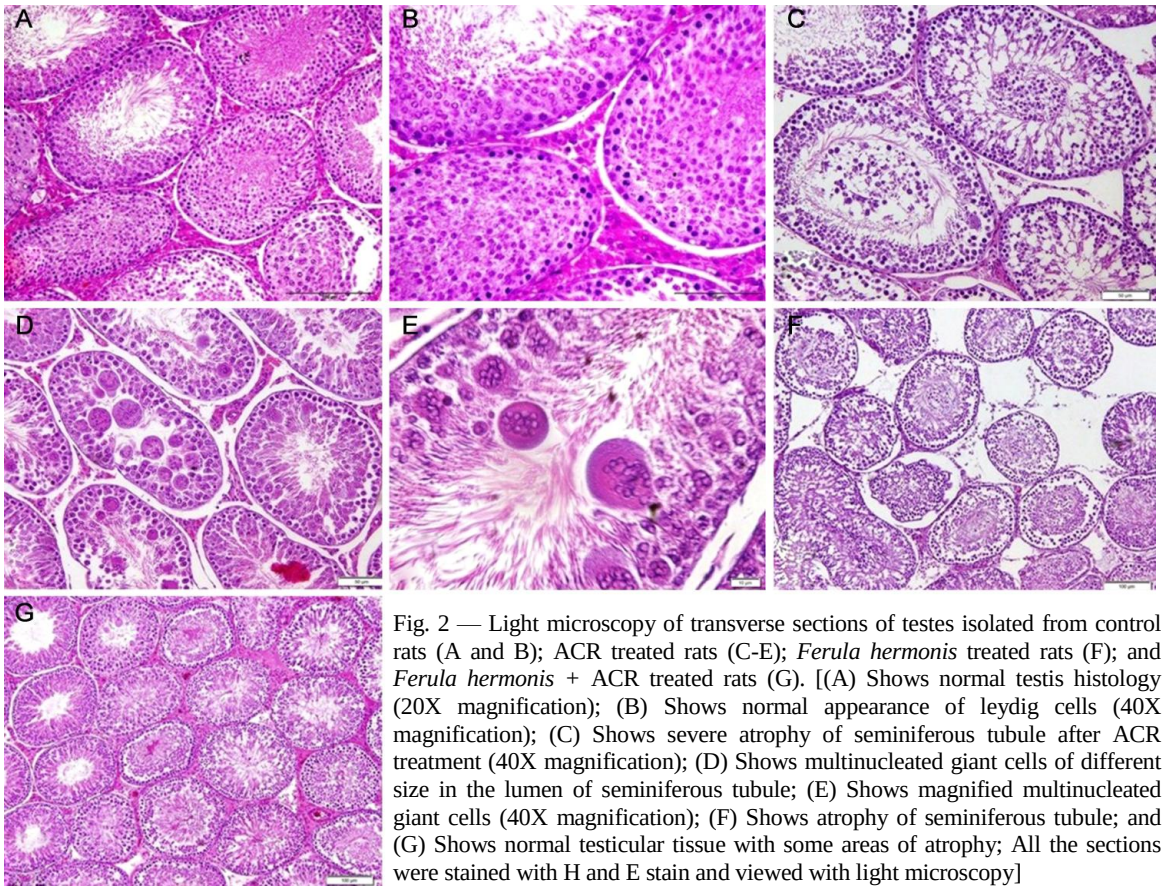

Fig. 2 — Light microscopy of transverse sections of testes isolated from control rats (A and B); ACR treated rats (C-E); *Ferula hermonis* treated rats (F); and *Ferula hermonis* + ACR treated rats (G). [(A) Shows normal testis histology (20X magnification); (B) Shows normal appearance of leydig cells (40X magnification); (C) Shows severe atrophy of seminiferous tubule after ACR treatment (40X magnification); (D) Shows multinucleated giant cells of different size in the lumen of seminiferous tubule; (E) Shows magnified multinucleated giant cells (40X magnification); (F) Shows atrophy of seminiferous tubule; and (G) Shows normal testicular tissue with some areas of atrophy; All the sections were stained with H and E stain and viewed with light microscopy]

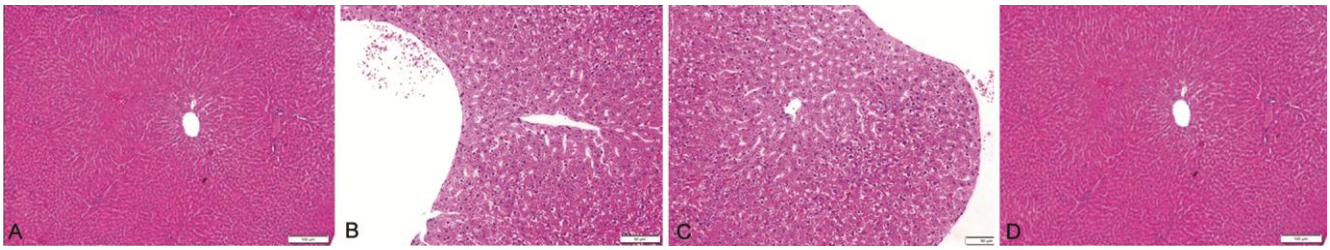

Fig. 3 — Light microscopy of transverse sections of liver isolated from ACR treated rats (A-C); and *Ferula hermonis* + ACR treated rats (D). [(A) Shows normal liver tissue and normal sinusoids; (B) Shows liver with sinusoidal dilatation; (C) Shows hepatic degeneration; and (D) Shows normal liver tissue. All the sections were stained with H and E stain and viewed with light microscopy]

and all other groups. However, there was no statistical difference between the group treated with ACR and the group treated with ACR+*F. hermonis*. Acrylamide has been reported previously to induce reproductive toxicity in male rats and our results support these studies[28,29]. This reproductive toxicity was in the form of reduction in serum testosterone concentration and reduction in sperm count per cauda. On the structural level of the testis, ACR was shown to induce many abnormal histological changes in the seminiferous tubules and epididymis, mainly in the form of germ cell degeneration, tubular atrophy, and sloughed germ cells in the lumen of the tubule, basal and intercellular vacuolations and leydig cells atrophy. Multinucleated giant cells were also seen with nuclear vacuolations of round spermatids. All of these abnormal histological changes were previously reported and are consistent with the current study plan for generation of ACR mediated toxicity[30,31]. The findings of this study are in accordance with the one where it was observed that acrylamide induces degeneration of seminiferous tubules that ultimately leads to decrease in total sperm count and increase in dead sperm count[32]. Incomplete spermatogenesis, where seminiferous tubules lack spermatids, spermatozoa, vacuolar degeneration of spermatogonia and Sertoli cells was also observed in one of the studies[28]. Again, the histological changes like multiple giant cell formations, degeneration of germinal epithelial cells, coagulative necrosis and depletion of germinal epithelium with hyalinization of luminal contents were observed[33].

The current finding tends to support the results reported in previous studies, which demonstrated that male rats receiving ACR at doses of 5, 15, 30, 45 or 60 mg/kg/day for 5 consecutive days by oral gavage showed a significant reduction in sperm reserves in a dose dependent manner, even at the lowest dose of ACR (5 mg/kg/day)[28,31]. The results of the current study were also consistent with the observations made by Sakamoto & Hashimoto[29] in the study conducted on male mice, which showed a significant reduction in sperm count and an increase in abnormal sperm morphology following the administration of the highest dose of ACR (1.2 mM) in the drinking water. In contrast to the latter, two studies reported that ACR had no significant effect neither on epididymal sperm motility nor on sperm concentration in rat testis after oral administration of ACR at doses of 5, 15, 30, 45 or 60 mg/kg/day for 5 days[34].

Moreover, current study indicated a significant reduction in serum testosterone concentration in all groups in comparison to control group. The reduction in serum testosterone following ACR exposure is consistent with the reports of a study, which demonstrated a significant reduction in testosterone concentration by using radioimmunoassay in sera of ACR treated rats at doses of 30, 45, and 60 mg/kg/day for 5 days followed by 3 days of observation[31]. Another study also observed that ACR treatment to rats declines the serum total testosterone and progesterone levels significantly but raises serum estradiol significantly[33]. Moreover, testosterone concentration in the culture medium of leydig cells after incubation for 24 h, decreased significantly in all ACR treated rat groups, thus indicating that testosterone reduction was due to influence of acrylamide, presumably caused by the observed dose dependent leydig cell death. As a result of increased leydig cell death, testosterone level in the testis is likely to be decreased, resulting in a reduction in spermatogenesis.

In the current sub-acute study, rats treated with *Ferula hermonis* alone for five consecutive days showed significant reduction in sperm count per cauda and in serum testosterone level when compared to control. But unfortunately, this study did not report any significant change in these parameters in ACR treated rats after *F. hermonis* treatment. However,

liver showed dramatic improvement in general structure after *F. hermonis* treatment with mild improvement in testis histology. Similarly, *F. hermonis* when administered alone to rats or concomitantly with bee honey normalized testicular weight reduction caused by the exposure of rats to γ- radiation. So, it reveals that *F. hermonis* has a significant protective potential against γ-radiation induced oxidative testicular damage[35]. The results of the current study were consistent with the observations of a study conducted on male mice for 6 weeks, where after chronic oral administration of *F. hermonis* it showed a significant reduction in the level of serum testosterone and partially impaired fertility[36]. Also, histopathological degenerative changes and a significant reduction in estrogen receptor were observed in testes, epididymis, and seminal vesicle of treated mice. And the authors reported that, although *F. hermonis* roots are recommended to improve erectile and fertility problems, it should be used for short periods and with extreme caution, and further clinical studies to assess safety and efficacy are needed[36].

In contrast to our findings and to the other study, one study conducted on rats by administration of single oral dose of the active single component of *F. hermonis*, ferutinin, in sexually potent sluggish/impotent rats in a dose of 2.5 mg/kg body wt./day, reported a significant increase in serum testosterone concentration[36]. However, after the subchronic administration of this active compound of *Ferula hermonis* for 10 days, a significant reduction in serum testosterone level was reported[37]. The authors hypothesized that the acute administration of ferutinin has a stimulating influence on hypothalamus-pituitary-gonadal axis, with subsequent increase in testosterone release. However, repeated daily use has a negative consequence on male reproductive functions with a reduction in serum testosterone concentration due to inhibition of hypothalamus-pituitary-gonadal axis. In the current study, *Ferula hermonis* roots treatment did not show complete protection against ACR testicular toxicity, although it showed good protection for the liver as seen in liver histology.

Conclusion

Our findings show that the treatment of ACR to rats for 5 days in a sub-acute study results in histological changes in rats' testes with statistically significant difference regarding serum testosterone and sperm count between ACR treated group and control. Also, ACR produced significant pathological changes in the testes that were not completely reversed by concomitant gavage of rats with *Ferula hermonis* root extract, which itself lead to significant negative changes on serum testosterone, sperm count and testis histology. This study provides evidence that *Ferula hermonis* herbal treatment after acrylamide testicular toxicity was mildly effective as a potential natural pharmacological alternation to manage testes pathologies produced by ACR exposure. Extra studies are needed in human. Further investigations are required to elucidate the molecular basis of the antioxidative role of *F. hermonis* against ACR induced testicular toxicity. We recommend restriction of ACR exposure either occupationally or in food containing product, and using of *F. hermonis* plants with caution in patients with impotence. Further, the protective effect of *F. hermonis* on liver also needs to be investigated.

Conflict of interest

The authors declare no conflict of interest.

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