



Research Article

The Oleo-Gum-Resin of *Commiphora myrrha* Ameliorates Male Reproductive Dysfunctions in Streptozotocin-Induced Hyperglycemic Rats

Mohammadmehdi Hassanzadeh-Taheri^{1,2}, Mehran Hosseini^{1,2}, Davood Dorrani-pour², Mohammad Afshar², Hesam Moodi², Mojtaba Salimi^{2*}

¹Cellular and Molecular Research Center, Birjand University of Medical Sciences, Birjand, Iran.

²Department of Anatomy, Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran.

Article Info

Article History:

Received: 14 April 2019
Revised: 4 June 2019
Accepted: 28 June 2019
ePublished: 20 December 2019

Keywords:

-Apoptosis
-*Commiphora myrrha*
-Diabetes
-Sperm
-Testis

ABSTRACT

Background: The oleo-gum-resin of *Commiphora myrrha* (myrrh) has a long history of therapeutic use in traditional medicine. The aim of this study was to seek for the scientific evidence to determine whether the ethanolic extract of myrrh (EEM) has any beneficial effects on Streptozotocin (STZ) induced testicular impairments, and explore the possible mechanisms underlying such actions.

Methods: Forty-eight severe and complicated diabetic rats (fasting blood glucose above 350 mg/dL), randomly were divided into six equal groups (n=8). Besides, eight healthy rats allocated as a normal control group and only treated with vehicle solution. The diabetic animals orally received the extract (100, 200, 300, and 500 mg/kg), metformin (500 mg/kg) or vehicle solution for 28 days. As a final point, plasma glucose and insulin, circulatory sex hormones, sperm parameters including sperm concentration, motility and viability and also testicular malondialdehyde (MDA) levels were assessed. Furthermore, quantitative histological evaluation of seminiferous tubules area and determination of germinal cells apoptosis were performed.

Results: None of the studied doses of EEM showed anti-diabetic effects. However, the extract mainly at the maximum dose (500 mg/kg) exhibited beneficial effects on reproductive impairments. The EEM treated rats mainly at 500 mg/kg had significantly higher sperm concentration, sperm motility, sperm viability, sex hormones and lower testicular MDA and germ cell apoptosis index than untreated diabetic rats.

Conclusion: These results indicated that EEM may have beneficial effects against reproductive dysfunction induced by diabetes. The mechanisms behind the effects might be associated with the EEM sex hormone booster potential, antioxidant and anti-apoptotic properties.

Introduction

In diabetic patients, sexual dysfunction along with severe and common complications such as nephropathy, retinopathy and neuropathy has frequently been reported.¹ It is well established that diabetes can develop sexual problems in both male and female.² The sexual dysfunction in diabetic patients has been recognized since antiquity. In the tenth century, persian physician Avicenna described "collapse of sexual function" as a complication of diabetes in men.³ Currently, several studies have proven a wide spectrum of structural and functional effects of diabetes on male reproductive system. Diabetes can cause male infertility by reducing sperm concentration, sperm motility, serum gonadotropins and testosterone, and making structural alterations in the testes.⁴

Despite the fact that diabetes-associated reproductive

disorders have widely been studied, the exact responsible mechanisms for male reproductive impairment are not yet completely understood. In the last years, giving antioxidant, testosterone and insulin replacement therapies have been investigated to control the development of diabetes-associated reproductive damages.⁴ Nevertheless, there is still no ideal treatment for diabetic reproductive injury and searching for therapeutic agents with multi-targets efficacy appear vital. The use of medicinal plants and complementary medicine to enhance reproductive function and to stimulate sexual desire is almost as old as the human rise itself. Medicinal plants have a long history of usage and nowadays, global interest in herbal medicine is increasing.^{5,6} Numerous natural products have been employed in the management of diabetes mellitus and its complications.⁷ Among them, some natural products are more popular and widely used

*Corresponding Author: Mojtaba Salimi, E-mail: salimi.mojtaba97@bums.ac.ir

©2019 The Authors. This is an open access article and applies the Creative Commons Attribution (CC BY), which permits unrestricted use, distribution and reproduction in any medium, as long as the original authors and source are cited. No permission is required from the authors or the publishers.

in different traditional medicine systems. The myrrh is a resin made from *Commiphora myrrha* (Nees) Engl., (Burseraceae family) dried sap. It is one of the most researched natural products in the world and appears to be one of the oldest medicines.⁸ Traditional practice and evidence-based research have supported the medicinal properties of myrrh such as anti-inflammatory, wound healing, antiseptic, menstrual stimulant, expectorant, anti-tumour, and anti-parasitic.⁹ There is evidence showing that myrrh exhibited hypoglycemic and hypolipidemic activities in diabetic animals.¹⁰ Moreover, myrrh gum showed potent antioxidant activity in different oxidative stress circumstances.^{11,12} Despite these interests, no scientific literature has been performed regarding its effects on male reproductive function. Therefore, the present study was designed to investigate the ameliorative effects of different oral doses of myrrh on reproductive dysfunctions in diabetic rats.

Methods

Chemicals and reagents

Streptozotocin (STZ), Horseradish peroxidase (HRP)-conjugated peanut agglutinin (PNA) lectin, 3, 3'-diaminobenzidine (DAB), bovine serum albumin (BSA), eosin Y and nigrosin were purchased from Sigma Chemicals Company (St. Louis, MO, USA). Ham's F-10 nutrient mix was procured from Caisson Laboratories (North Logan, UT, USA) and metformin tablet obtained from Merck Sante' S.A.S. (Lyon, France). Alcian blue 8GX dye was procured from Bio Basic (Canada Inc, Canada). The STZ was freshly dissolved in citrate buffer, pH 4.5. Also, the solvent solution containing 0.1% dimethyl sulfoxide (DMSO) (Carlo ERBA, France) in saline was used for dissolving myrrh extracts as well as metformin.

Plant Materials

Dried myrrh gum-resin was purchased from traditional plant market at Birjand (Iran). It was tested (carbon disulfide and nitric acid colour reaction) by comparison with a genuine sample and identified as gum-resin derived from *Commiphora myrrha* (Nees) Engl. A specimen was kept in the herbarium of Agriculture Faculty at the University of Birjand (voucher number: 527).

To prepare the ethanolic extract, the pulverized myrrh was macerated in 80% ethanol 1:10 (w/v) on a magnetic stirrer for 2 days at room temperature. Afterwards, the mixture was passed through the filter paper (Whatman No. 4, England), concentrated under vacuum evaporator, and lyophilized using a freeze-dryer (Dena Vacuum Industry, model FD-5005-BT, Iran).¹³ The extraction yield was about 9.5 %.

Animals

Adult male Wistar rats weighing 180-200g (8 weeks old) were obtained from animal facility of Research Centre of Experimental Medicine at Birjand University of Medical Sciences, Birjand, Iran. The animals were housed in polypropylene cages, temperature-controlled room (22±2

°C) with a 12 h light/dark cycle and free access to commercial animal chow (Behparvar Co, Iran) and tap water during the study. All animal procedures were conducted in accordance with the guide for the care and use of laboratory animals approved by the Ethics Committee of the Brjand University of Medical Sciences (permit code: Ir.bums.REC1396.16). All efforts were made to minimize animal suffering and to reduce the number of used animals.

Induction of diabetes and experimental design

Diabetes was induced by a single intraperitoneal (i.p) injection of STZ (55 mg/kg) into the overnight fasted rats. Age-matched control animals only received an i.p injection of vehicle (citrate buffer). After 72h of the injection, rats with plasma glucose concentration above than 350 mg/dL were considered as severe diabetic.¹⁴ It is worth be noted that, in order to prevent failure in diabetes induction and also animals death before starting the experiment, 18 rats more than the required number (n=48) were subjected to STZ injection.

All diabetic animals left untreated for 4 weeks to develop a complicated form of diabetes.¹⁵ Forty-eight diabetic rats randomly divided into six equal groups (n=8). Group 1 received only vehicle solution (0.1 % DMSO in saline) as diabetic model group (DM), groups 2-5 treated with the ethanolic extracts of myrrh (EEM) at the doses of 100 mg/kg (EEM100), 200 mg/kg (EEM200), 300 mg/kg (EEM300) and 500 mg/kg (EEM500) respectively, and group 6 received metformin at the dose of 500 mg/kg (MET500).¹⁶ The selection of the studied doses of EEM was based on previous studies reported hypoglycemic activity of myrrh in rats.^{10,17} Moreover, eight healthy rats allocated as normal control (NC) group and received just vehicle solution (0.1% DMSO in saline). All the samples were administrated orally to the animals using an intra-gastric tube for a period of four weeks.

Sample collection

At 29th day and after 24 h of the last treatment, the fasted rats were weighed and their blood glucose levels were measured using a glucometer (Accue-Chek, Roche). Subsequently, animals were anaesthetized by an i.p. injection of ketamine: xylazine (65:10 mg/kg)¹⁸ and immediately their blood specimens were collected from the heart. The blood samples were centrifuged for 10 min at 3000 g and the obtained plasma collected. The reproductive organs including testis, epididymides, and prostate were dissected, weighed and fixed in the fixative solution containing 4% paraformaldehyde and 0.1 M glutaraldehyde in 0.1 M phosphate buffer (PBS) (pH 7.4). Besides, a part of the left testis (100 mg) was homogenized in cold PBS (900 µL), and centrifuged (5000 g for 10 min) at 4° C. The collected supernatants were used for lipid peroxidation assay.

Plasma sex hormones, plasma insulin and testicular oxidative stress

Plasma testosterone (T), follicular stimulating hormone (FSH), and luteinizing hormone (LH) levels were

measured using enzyme-linked immunosorbent assay (ELISA) kits (Pishtazteb, Iran) according to the manufacturer's instructions. Plasma insulin levels were determined using an insulin ELISA kit (Mercodia, Sweden). Lipid peroxidation in testicular tissue was evaluated by determination of malondialdehyde (MDA), the main end-products of lipid peroxidation using Thiobarbituric Acid Reactive Substances (TBARS). Briefly, based on reaction with thiobarbituric acid at 90–100 °C, the absorbance of the resulting pink product was measured spectrophotometrically at 535 nm. MDA has been considered equivalent to TBARS.¹⁹

Sperm evaluation

The spermatozoa were collected by mincing cauda epididymis using two NO.11 sterile scalpel blades in 2 mL Ham's F10 medium supplemented with 0.5% BSA, and pre-warmed at 37°C. Following incubation for 30 min, for sperm count, 10 µL of the suspension was diluted in 190 µL cold saline, and then 10 µL of the diluted sample was transferred into each chamber of Neubauer cell counting spaces under 100X magnification. The sperms which lied in the five secondary squares were counted.²⁰

The percentage of motile sperms was evaluated individually as follow: 10 µL of sperm suspension (in medium) were transferred into a pre-warmed microscope slide and covered with a coverslip. The percentage of motile sperms was assessed under 400X magnification in at least 10 microscopic fields and being 200 sperms per rat were evaluated.²¹ Sperm viability evaluated using eosin-nigrosin staining method. Briefly, 10µL of sperm suspension gently mixed with 10µL eosin-nigrosin solution (containing an equal volume of 1.67% eosin Y and 10% nigrosin) and a smear of the suspension was performed and dried at room temperature. The slides were examined by light microscope under 1000X magnification. 200 spermatozoa per rat (10–20 microscopic fields) were evaluated in order to determine viable or non-viable sperms. Stained sperms (pink) were considered as non-viable, while unstained sperms (white) or poorly stained sperm heads evaluated as viable sperms.²²

Testicular histology and lectin histo-chemistry

Tissue samples of the testis were processed for paraffin-embedding and serial sections prepared for staining with hematoxylin and eosin. For each testis, three random slides (9 sections) were analyzed under a light microscope (UPLAN FI, Japan) and for each section; 4–6 unbiased counting frames were sampled. Seminiferous tubule area (µm²), germinal layer area (µm²), and the number of spermatogonia cells were measured using Image J Software (1.44p; National Institute of Health, USA).²³ The ratio of germinal layer area to seminiferous tubule area was calculated for each seminiferous tubule.

To determine in-situ spermatogenic cell apoptosis, the classical lectin histochemistry was performed.²⁴ Briefly, the testicular sections were deparaffinized in xylene and

rehydrated through a descending ethanol series, rinsed in PBS (pH: 7.4) for 5 min and then 30 min incubated in 0.3% H₂O₂ in methanol solution for blocking the endogenous peroxidase activity at a dark chamber. Then, the sections were washed in PBS and incubated in HRP-conjugated peanut agglutinin lectin (PNA) in a moisture chamber at room temperature for 1.5 h. After washing in PBS, the sections were reacted in PBS containing 0.05% DAB and 0.3% H₂O₂ for 5 min at room temperature. Finally, the sections were counterstained with 1% solution of Alcian blue dye in 0.1 M HCl (pH: 2.5), dehydrated, cleared and mounted. For negative control, the next serial section of each specimen was exposed to HRP, DAB, and H₂O₂ without lectin. Germ cells with marked reaction and diffuse stained cytoplasm or nuclear were considered as apoptotic.²⁴ The quantitative assessment of testicular apoptosis was done as follow: the incidence of apoptosis was assessed in 100 seminiferous tubules. The tubule with three or more apoptotic germ cells considered as positive. The apoptosis percentage was calculated by the ratio of the positive tubules to the total number of seminiferous tubules in a cross-section.²⁵

Statistical analyses

Results are expressed as mean ± SD for all parameters. Data distributions were examined for normality and homogeneity of variance by Kolmogorov-Smirnov test. Statistical differences between groups were detected by analysis of variance (ANOVA) followed by Dunnett's test. Statistical significance was inferred at $p < 0.05$. The SPSS software, version 22 was used for all analysis.

Results

Fasting blood glucose, plasma insulin, body and organ weights

The results of the final FBG, plasma insulin, body weight and reproductive organs weight are presented in Table 1. Compared to the NC group, a marked increase in the FBG was observed in diabetic animals ($p < 0.0001$). The elevation only normalized in metformin-treated rats (MET500 mg/kg) efficiently ($p = 0.21$ compared to NC), while the EEM in all doses (EEM 100–500 mg/kg) could not exhibit any hypoglycemic activity. In comparison with NC group, diabetes caused a significant decrease in insulin level ($p < 0.0001$). There was no significant difference between the EEM-treated rats (at all doses) and DM group. Metformin treated diabetic rats exhibited slightly higher plasma insulin than DM group ($p = 0.001$), but the level was still significantly lower than normal level ($p < 0.0001$). In diabetic animals, body weight, weight of testis, epididymis and prostate were significantly lower than NC group ($p < 0.0001$). The EEM treatment at the all doses significantly prevented testis weight loss ($p < 0.0001$). In epididymis weight, both metformin and EEM (100–500mg/kg) significantly improved and normalized it in diabetic animals. There was no significant difference between EEM doses in testes and epididymis weights.

Table 1. Effects of ethanolic extract of myrrh (EEM) on fasting blood glucose (FBG), insulin, body weight, and reproductive organ weights in diabetic rats.

Groups	FBG (mg/dL)	Insulin (ng/uL)	Body weight (g)	Reproductive organs weight (g)		
				Testis	Epididymis	Prostate
Normal control	98.33±8.84	2.55± 0.44	263.33±34.33	1.62±0.23	0.65±0.05	0.46±0.06
Diabetic model	447.33±74.03 *	0.68± 0.12 *	176.66±31.34 *	0.58±0.28 *	0.27±0.10 *	0.21±0.01 *
Metformin (500 mg/kg)	163.16±21.59 #	1.29±0.08 **	168.50±35.54 *	1.15±0.28 **	0.49±0.07 **	0.10±0.08 #
EEM (100 mg/kg)	437.00±50.34 *	0.44± 0.20 *	174.16±17.57 *	1.45±0.20 #	0.42±0.06 **	0.25±0.03 *
EEM (200 mg/kg)	513.66±52.89 *	0.36± 0.18 *	164.33±15.21 *	1.32±0.24 #	0.41±0.06 **	0.32±0.03 **
EEM (300 mg/kg)	457.50±61.86 *	0.59± 0.09 *	163.66±19.27 *	1.34±0.27 #	0.46±0.03 **	0.38±0.10 #
EEM (500 mg/kg)	486.33±42.08*	0.78± 0.16 *	178.33±29.15 *	1.28±0.24 #	0.49±0.008 **	0.39±0.04 #

Values are expressed as mean± standard deviation (n=8). *Represented as $p < 0.05$ in comparison with the normal control group. #Represented as $p < 0.05$ in comparison with the diabetic model group.

Table 2. Effects of ethanolic extract of myrrh (EEM) on plasma levels of testosterone (T), follicular stimulating hormone (FSH), luteinizing hormone (LH), and testicular malondialdehyde (MDA) concentration in diabetic rats.

	T(ng/mL)	FSH (mIU/mL)	LH (mIU/mL)	MDA(nmol/mg protein)
Normal control	1.80 ±0.36	2.02±0.11	3.33 ±0.31	27.12 ±3.07
Diabetic model	0.05 ±0.01*	1.01 ±0.55 *	1.13 ±0.14 *	58.15 ±5.12 *
Metformin (500 mg/kg)	0.47 ±0.07**	1.02 ±0.42 *	3.39 ±0.37 #	33.19 ±2.21 **
EEM (100 mg/kg)	0.33±0.06 *	2.01 ±0.20 #	3.38 ±0.10 #	38.71 ±4.19 **
EEM (200 mg/kg)	0.42±0.04*	2.01 ±0.41 #	4.22 ±0.91 #	37.42 ±5.41 **
EEM (300 mg/kg)	0.47±0.36 **	2.03 ±0.22 #	3.35 ±0.67 #	29.08 ±5.11 #
EEM (500 mg/kg)	0.80±0.25**	2.11 ±0.10 #	3.33 ±0.22 #	25.42 ±4.02 #

Values are expressed as mean± standard deviation (n=8). *Represented as $p < 0.05$ in comparison with the normal control group. #Represented as $p < 0.05$ in comparison with the diabetic model group.

Metformin not only did not affect prostate weight in diabetic animals but also significantly decreased it even more than DM group ($p=0.04$). On the other hand, EEM at the doses of 300 mg/kg ($p=0.001$) and 500 mg/kg ($p=0.001$) could increase and normalize prostate weight in comparison with DM group.

Plasma sex hormones and testicular MDA concentration

Table 2 illustrates the effects of EEM (100-500 mg/kg) on the STZ-induced changes in the plasma concentrations of T, LH, FSH and testicular tissue MDA levels. Compared to the NC group, the plasma levels of T, LH and FSH significantly decreased ($p < 0.0001$) in DM group. The EEM at the doses of 300 mg/kg ($p < 0.0001$) and 500 mg/kg ($p < 0.0001$) as well as metformin ($p < 0.0001$) significantly increased T concentration in diabetic rats; however, in comparison with the NC group the level was still low ($p < 0.001$). The EEM at all doses (100-500 mg/kg) and metformin could significantly increase and normalize LH levels in diabetic animals. There was no significant difference between the EEM doses and metformin. Like LH, the EEM at all doses restored FSH levels in diabetic rats while metformin treated animals had significantly lower FSH level than NC group ($p=0.002$).

Table 3. Effects of ethanolic extract of myrrh (EEM) on epididymal sperm count, motility and viability in diabetic rats.

	Count ($\times 10^7$ /mL)	Motility (%)	Viability (%)
Normal control	20.76 ±1.52	80.80 ±3.27	89.20 ±2.28
Diabetic model	8.63 ±1.31 *	46.40 ±10.13 *	55.40 ±8.59 *
Metformin (500 mg/kg)	17.80 ±2.83 #	74.50 ±3.44 #	78.83 ±5.30 #
EEM (100 mg/kg)	8.16 ±2.22 *	40.76 ±5.58 *	45.20 ±3.96 *
EEM (200 mg/kg)	9.33 ±1.03 *	61.04 ±10.36 #	64.00 ±10.93 **
EEM (300 mg/kg)	17.16 ±2.40 #	62.38 ±1.78 #	65.20 ±6.94 **
EEM (500 mg/kg)	19.16 ±1.72 #	70.80 ±7.66 #	78.60 ±8.08 #

Values are expressed as mean± standard deviation (n=8). *Represented as $p < 0.05$ in comparison with the normal control group. #Represented as $p < 0.05$ in comparison with the diabetic model group.

Diabetic rats exhibited significantly ($p < 0.0001$) higher testicular MDA concentration than the NC group. The EEM at all doses efficiently restored the testicular MDA elevation in diabetic animals in comparison with DM group. Then EEM at doses of 300 and 500 mg/kg normalized MDA elevation with no statistical difference compared to the NC group.

Sperm count and quality

The results of sperm analysis are presented in Table 3. Diabetic rats had significantly lower sperm count ($p < 0.0001$), sperm motility ($p < 0.0001$) and sperm viability ($p < 0.0001$) than non-diabetic rats. Metformin and EEM at the doses of 300-500 mg/kg increased sperm concentration, and improved sperm motility as well as its viability in diabetic rats, however; the EEM only at maximum dose (500 mg/kg) could normalize the alterations.

Histopathological evaluation

In the NC group, light microscopic examination of testis sections revealed normal histological structure with rounded seminiferous tubules in most of which different phases of spermatogenic cells clearly were observed.

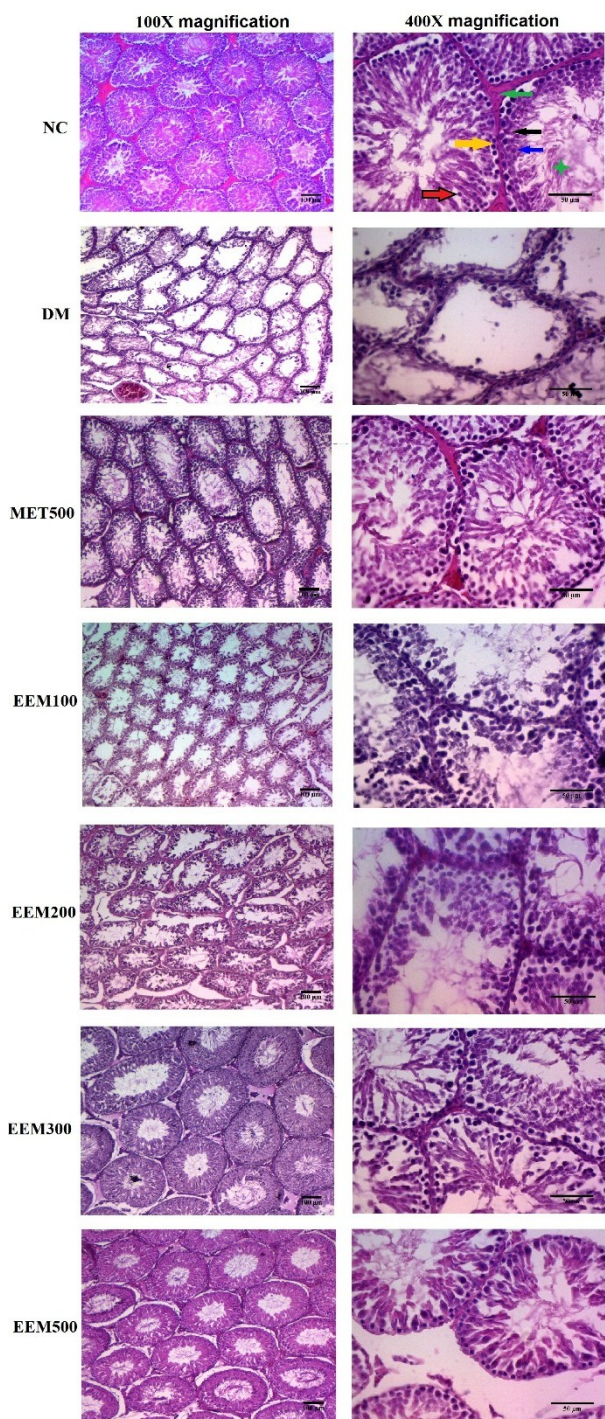


Figure 1. Photomicrographs of the testicular sections of rats belong to the normal control group (NC), diabetic model group (DM), diabetic treated with metformin at the dose of 500mg/kg (MET500) and diabetic groups treated with ethanolic extract of myrrh gum at the doses of 100-500mg/kg (EEM100-500). Hematoxylin and eosin staining with two magnifications 100X (left column, bars=100 μ m) and 400X (right column, bars=50 μ m). In NC group, the epithelium clearly can stratify with spermatogonia (yellow arrow), Sertoli cells (black arrow), primary spermatocytes (blue arrow), spermatids (red arrow), spermatozoa (green star) and active Leydig cells (green arrow).

The epithelium of germinal layer was stratified with the spermatogonia (small rounded cells resting on basement membrane), sertoli cells (column cells with euchromatin

nuclei), primary spermatocytes (large rounded nuclei and greater than spermatogonia) and spermatids (small rounded cells with paler nuclei) radially arranged about 5-7 layers toward the lumen (Figure 1). On the other hand, testicular histopathology of diabetic rats showed extensive shrinkage of seminiferous tubules and massive sloughing of the germinal layer (Figure 1).

The quantitative histopathological assessment clearly showed marked reduction ($p < 0.0001$) in the number of spermatogonia, seminiferous area, germinal layer area and germinal layer to seminiferous area coefficient in diabetic animals with respect to the NC group (Table 4). The number of spermatogonia cells in the EEM treated rats at doses of 200 mg/kg ($p = 0.012$), 300 mg/kg ($p < 0.0001$) and 500 mg/kg ($p < 0.0001$) were significantly higher than DM group. However, EEM at the doses of 300 mg/kg and 500 mg/kg could efficiently restore the spermatogonia number towards the normal level. Furthermore, EEM at the doses of 200 mg/kg ($p = 0.033$), 300 mg/kg ($p < 0.0001$) and 500 mg/kg ($p < 0.0001$) efficiently improved the germinal epithelium area in comparison with DM group. Nevertheless, only at the EEM300 and EEM500 groups the germinal layer area was same as NC group.

Germ cell apoptosis

There were a few PNA-positive germ cells in NC group (Figure 2a), whereas in DM group the number of apoptotic cells was markedly increased (Figure 2b). Compared to NC group, the apoptotic index was significantly higher in DM group ($p < 0.0001$) (Figure 3). The index significantly mitigated in EEM treated rats at the doses of 200-500 mg/kg ($p < 0.0001$). Only in EEM300 and EEM500 groups, the apoptotic index had no significant difference in respect to NC group ($p > 0.05$).

Discussion

In the present study, we provide evidence that EEM might mitigate reproductive dysfunction induced by diabetes in male rats through antioxidant potential and apoptosis inhibition. The results showed that compared to control group, diabetic animals had markedly higher levels of blood glucose, testicular MDA concentration and germinal cells apoptosis, whereas lower plasma insulin, body and reproductive organ weights, plasma T and LH levels, sperm count, sperm motility, sperm viability, and also seminiferous tubules area. These findings clearly show that animal model of diabetes-induced reproductive impairment was well established.¹⁵ Our findings demonstrated that EEM had no hypoglycemic effect. However, diabetic rats treated with EEM, had significantly higher reproductive organ weights, plasma T and LH, and sperm parameters, and lower germinal cells apoptosis and testicular lipid peroxidation than untreated diabetic rats. In the present study diabetic animals were left untreated for 4 weeks to develop a complicated form of the disease. Accumulating evidence suggests that uncomplicated type 1 diabetes is not associated with the hallmarks of male infertility or hypogonadism.¹⁵

Table 4. Effects of ethanolic extract of myrrh (EEM) on the number of spermatogonia per seminiferous tubule, areas of seminiferous tubule (SA), seminiferous epithelium (EA) and EA to SA coefficient in diabetic rats.

	Spermatogonia (number/tubule)	SA (um2)	EA area (um2)	EA/SA×100
Normal control	34.33±2.95	68304.58± 7561.64	56931.69±6042.03	83.63 ±7.22
Diabetic model	21.16±3.55 *	12838.37 ±1303.97 *	6347.50±1451.16 *	49.16 ±8.70 *
Metformin (500 mg/kg)	30.33±4.37 #	29043.92 ±1070.95 #	19619.10±2796.14 #	67.72 ±10.70#
EEM (100 mg/kg)	18.50±4.48 *	10930.86 ±1143.91 *	4457.52±1728.70*	39.87± 10.99 *
EEM (200 mg/kg)	26.66±4.74 #	22245.29 ±3108.69 **	14690.27±3191.15 **	66.06 ±11.62 **
EEM (300 mg/kg)	31.83±2.13 #	48403.91 ±3978.68**	37868.16±4280.03 **	78.18 ±4.76 #
EEM (500 mg/kg)	32.33±1.74 #	52879.90 ±1689.54**	44256.25±2882.310 **	83.67 ±4.27#

Values are expressed as mean ± standard deviation (n=8). *Represented as $p < 0.05$ in comparison with the normal control group. # Represented as $p < 0.05$ in comparison with the diabetic model group.

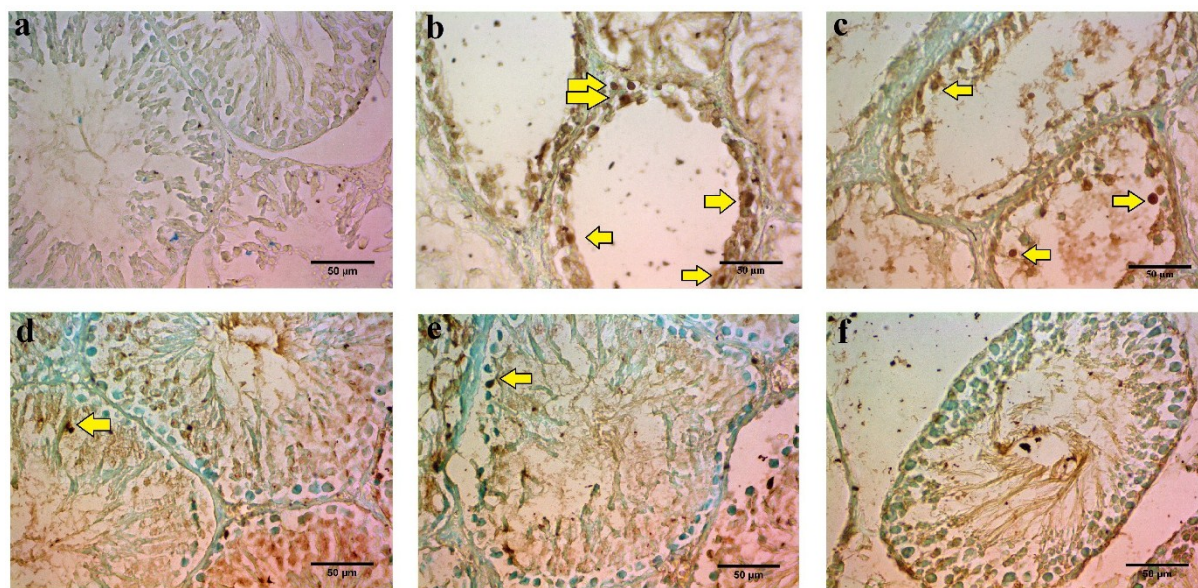


Figure 2. Photomicrographs of the testicular sections of rats belong to the normal control group (a), diabetic model group (b), diabetic groups treated with ethanolic extract of myrrh gum at the doses of 100 mg/kg (c), 200mg/kg (d), 300mg/kg (e) and 500mg/kg (f). PNA lectin histochemistry, 400X magnification, bar=50um. The yellow arrows show apoptotic cell.

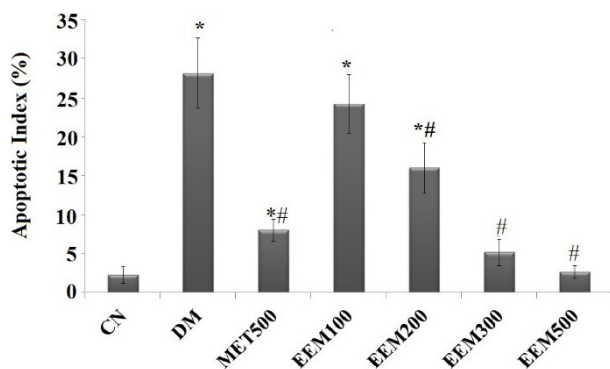


Figure 3. the apoptotic index of seminiferous tubules of the normal control group (CN), diabetic model group (DM), diabetic treated with metformin at the dose of 500 mg/kg (MET500), and diabetic groups treated with ethanolic extract of myrrh gum (EEM) at the doses of 100 mg/kg (EEM100), 200 mg/kg (EEM200), 300 mg/kg (EEM300), and 500 mg/kg (EEM500). Each bar indicates mean± standard deviation. *Represented as $p < 0.05$ in comparison with the normal control group. # Represented as $p < 0.05$ in comparison with the diabetic model group.

Evidence also showing that spermatogenic dysfunction in the early stage of STZ-induced diabetic rats is due to direct STZ cytotoxicity to Sertoli cells, whereas steroidogenic dysfunction is because of insulin

deficiency.²⁶ Therefore, STZ-induced diabetic model, in the early stage, is not a suitable model to study the diabetes-related reproductive dysfunction.

The EEM had no effect on glucose and plasma insulin levels while metformin effectively reduced blood glucose level and also slightly increased plasma insulin concentration in diabetic animals. These findings have not confirmed previous research on hypoglycemic activity of myrrh. Helal *et al.* reported that aqueous extract of *Commiphora myrrh* at the dose of 50 mg/100g body weight significantly reduced blood glucose levels (from 200 mg/dL to 110 mg/dL) after 30 days of administration in rats. Moreover, they reported that the *C. myrrh* extract significantly increased plasma insulin levels from 20 μ U/mL to 40 μ U/mL.²⁷ In the above-mentioned study, rats with FBG around 250 mg/dL were considered as diabetic. Also, the plant part that was used and its route of administration did not mention. The possible reason for these differences may be due to diabetes severity. In the present study, the mean of FBG level of diabetic animals was about 450 mg/dL while in the Helal *et al.* study it was around 250 mg/dL. Generally, in a rat model of diabetes, animals with glycemia between 120 and 300 mg/dL, characterizing moderate or mild diabetes and rats with glycemia above 300 mg/dl consider as severe diabetic.

There is evidence indicating that these phenotypes have different responses to antidiabetic agents.²⁸

As expected, in the present study reproductive organ weights including testis, epididymis, and prostate markedly decreased in diabetic rats. The EEM treatment in a dose dependent manner could effectively ameliorate weight reduction in all organs. It has previously been shown that chronic oral administration of *C. molmol* oleo-gum-resin, a synonym of *C. myrrha*, at dose of 100 mg/kg for 90 days caused markedly increase in weight of testis, cauda epididymis and seminal vesicles in normal mice.²⁹ Like EEM, metformin prevented testicle and epididymis weight loss, but the prostate weight of metformin-treated rats was significantly even lower than diabetic untreated animals. This is in good agreement with a study conducted by Mosli *et al.* in which oral treatment with 500-1000 mg/kg metformin for 14 days significantly inhibited the testosterone-mediated increase in the prostate weight of rats.³⁰

Previous studies indicated that diabetic rats represented a reduction in Leydig cells number and impairment in their function. Therefore, testosterone secretion decreases in diabetic patients or animal models.³¹⁻³³ Results of the present study showed that both of EEM and metformin could improve these parameters. However, it worth to be noted that the T levels failed to revert to normal in both EEM and metformin-treated diabetic animals. The reason for this result are not yet completely understood, but it could be attributed to the chemical composition of EEM and the study period.

Oxidative stress in the testis is a key hallmark for hyperglycemia-induced cell apoptosis. A growing body of evidence suggests that cell death mechanism plays an important role in spermatogenesis dysfunction induced by diabetes.^{34,35} Histological evaluation of testis revealed that in diabetic animals, thickness of germinal epithelium, as well as seminiferous area was declined and germ-cell apoptosis significantly increased. With respect to histological alterations, functional parameters including sperm count, sperm motility and sperm viability significantly decreased in diabetic rats. Furthermore, the testicular lipid peroxidation marker (MDA) significantly increased in diabetic rats. Interestingly, EEM in a dose-dependent manner could mitigate these alterations and in the maximum dose (500mg/kg) could keep histological appearance, germ-cell apoptosis, sperm parameters and MDA concentration to normal. Functional outcome of the histological improvement clearly was demonstrated by significantly increased sperm count, sperm motility and sperm viability in EEM treated diabetic rats. The anti-apoptotic effects of EEM might be attributed with guggulsterone, a steroid obtained from myrrh. There is evidence showing that the use of guggulsterone at the doses of 10-30 μ M significantly reduced the formation of MDA and suppressed caspase-3 activity to reference levels in doxorubicin-induced cytotoxicity and cell death in H9C2 cell line.³⁶

The beneficial effects of EEM found in the present study might be attributed to its phytochemical compounds.

There is evidence showing that the ethanolic extract of *C. myrrha* resin contains 27 organic compounds. Among its organic compounds, limonene, curzerene, germacrene B, isocericenine, myrcenol, beta selinene, and spathulenol are more abundant.³⁷ Limonene is a cyclic monoterpene, and it has two isomers: D and L. which the main active form of limonene is d-limonene. In good agreement with our findings, Bacanlı *et al.* reported that 28 days oral administration of d-limonene (50 mg/kg) to diabetic rats exhibited significantly improvement in the liver, kidney and plasma MDA levels while no considerable effects on FBG and insulin levels.³⁸ The sesquiterpenes such as germacrene and curzerene are responsible for antimicrobial and insecticidal properties of myrrh.³⁷ The EEM lipid peroxidation inhibitory effect may be due to presence of spathulenol in this gum. Recent evidence demonstrated that spathulenol exhibited the highest antioxidant and inhibitory effect against spontaneous lipid peroxidation formation in rat brain homogenate, with IC₅₀ value of 26.17 μ g/mL, which was comparable to a commercial antioxidant butylated hydroxytoluene (34.41 μ g/mLm).³⁹

Among the involved mechanisms behind the development of reproductive dysfunction in diabetes, oxidative stress contributes as the main causative factor for testicular alterations which consequently led to male hypogonadism. Because of germ cells have considerable amount of polyunsaturated fatty acids on their plasma membrane; they are prone to oxidative stress damages.⁴⁰ Previous studies show that most of plant extracts with beneficial effects on reproductive dysfunction induced by diabetes exert their effects through reducing or inhibiting MDA formation as well as increasing catalase activity in testis.⁴⁰ In line with our findings, previous studies demonstrated that myrrh extracts dose-dependently reduced hepatic as well as colonic tissues MDA formation in diabetic and ulcerative colitis rats, respectively.^{10,11}

Conclusion

Taken together, the findings suggested that the mechanisms behind the beneficial effects of EEM might be associated with its sex hormone booster potential, antioxidant and anti-apoptotic properties. The antioxidant and anti-apoptotic activities of EEM at the highest dose (500 mg/kg) were significantly greater than metformin (500 mg/kg).

Acknowledgements

This paper is part of Mr. Mojtaba Salimi M.Sc. thesis which was fully financially supported by Birjand University of Medical Sciences (No.455159). We thank our students; Mr. Ghasem Ivar, Ms. Asma Moghaddam, Ms. Faezeh Rezaei and Ms. Faezeh Vahdati for their support and help.

Conflict of interests

The authors claim that there is no conflict of interest.

References

1. Isidro ML. Sexual dysfunction in men with type 2 diabetes. *Postgrad Med J*. 2012;88(1037):152-9. doi:10.1136/postgradmedj-2011-130069
2. Ding GL, Liu Y, Liu ME, Pan JX, Guo MX, Sheng JZ, et al. The effects of diabetes on male fertility and epigenetic regulation during spermatogenesis. *Asian J Androl*. 2015;17(6):948-53. doi:10.4103/1008-682x.150844
3. Abu Ali RM, Al Hajeri RM, Khader YS, Shegem NS, Ajlouni KM. Sexual dysfunction in Jordanian diabetic women. *Diabetes Care*. 2008;31(8):1580-1. doi:10.2373/dc08-0081
4. Shi GJ, Li ZM, Zheng J, Chen J, Han XX, Wu J, et al. Diabetes associated with male reproductive system damages: Onset of presentation, pathophysiological mechanisms and drug intervention. *Biomed Pharmacother*. 2017;90:562-74. doi:10.1016/j.biopha.2017.03.074
5. Gardiner P, Whelan J, White LF, Filippelli AC, Bharmal N, Kaptchuk TJ. A systematic review of the prevalence of herb usage among racial/ethnic minorities in the United States. *J Immigr Minor Health*. 2013;15(4):817-28. doi:10.1007/s10903-012-9661-z
6. Hassanzadeh-Taheri M, Hosseini M, Salimi M, Moodi H, Dorranipour D. Acute and sub-acute oral toxicity evaluation of *Astragalus hamosus* seedpod ethanolic extract in Wistar rats. *Pharm Sci*. 2018;24(1):23-30. doi:10.15171/PS.2018.05
7. Hassanzadeh-Taheri M, Hassanpour-Fard M, Doostabadi M, Moodi H, Vazifeshenas-Darmiyan K, Hosseini M. Co-administration effects of aqueous extract of turnip leaf and metformin in diabetic rats. *J Tradit Complement Med*. 2018;8(1):178-83. doi:10.1016/j.jtcme.2017.05.010
8. Haffor AS. Effect of *Commiphora molmol* on leukocytes proliferation in relation to histological alterations before and during healing from injury. *Saudi J Biol Sci*. 2010;17(2):139-46. doi:10.1016/j.sjbs.2010.02.007
9. Nomicos EY. Myrrh: Medical marvel or myth of the magi? *Holistic Nursing Practice*. 2007;21(6):308-23. doi:10.1097/01.hnp.0000298616.32846.34
10. Shokoohi R, Kianbakht S, Faramarzi M, Rahmanian M, Nabati F, Mehrzadi S, et al. Effects of an herbal combination on glycemic control and lipid profile in diabetic women: A randomized, double-blind, placebo-controlled clinical trial. *J Evid Based Complement Altern Med*. 2017;22(4):798-804. doi:10.1177/2156587217737683
11. Ashry KM, El-Sayed YS, Khamiss RM, El-Ashmawy IM. Oxidative stress and immunotoxic effects of lead and their amelioration with myrrh (*Commiphora molmol*) emulsion. *Food Chem Toxicol*. 2010;48(1):236-41. doi:10.1016/j.fct.2009.10.006
12. Fatani AJ, Alrojaye FS, Parmar MY, Abuhashish HM, Ahmed MM, Al-Rejaie SS. Myrrh attenuates oxidative and inflammatory processes in acetic acid-induced ulcerative colitis. *Exp Ther Med*. 2016;12(2):730-8. doi:10.3892/etm.2016.3398
13. Hassanzadeh-Taheri M, Hosseini M, Hassanpour-Fard M, Ghiravani Z, Vazifeshenas-Darmiyan K, Yousefi S, et al. Effect of turnip leaf and root extracts on renal function in diabetic rats. *Orient Pharm Exp Med*. 2016;16(4):279-86. doi:10.1007/s13596-016-0249-3
14. Etuk E. Animals models for studying diabetes mellitus. *Agric Biol J N Am*. 2010;1(2):130-4.
15. Maresch CC, Stute DC, Alves MG, Oliveira PF, de Kretser DM, Linn T. Diabetes-induced hyperglycemia impairs male reproductive function: A systematic review. *Hum Reprod Update*. 2018;24(1):86-105. doi:10.1093/humupd/dmx033
16. Rehman K, Saeed K, Munawar SM, Akash MSH. Resveratrol regulates hyperglycemia-induced modulations in experimental diabetic animal model. *Biomed Pharmacother*. 2018;102:140-6. doi:10.1016/j.biopha.2018.03.050
17. Omer SA, Al-Dogmi AM. Toxicologic, hypoglycaemic and hypolipidemic effects of ethanolic and ether extracts of *Commiphora molmol* from Saudi Arabia. *Biomed Res*. 2018;29(11):2300-6. doi:10.4066/biomedicalresearch.43-18-282
18. Hassanzadeh-Taheri M, Hassanzadeh-Taheri M, Jahani F, Hosseini M. Effects of yoghurt butter oils on rat plasma lipids, haematology and liver histology parameters in a 150-day study. *Int J Dairy Technol*. 2018;71(1):140-8. doi:10.1111/1471-0307.12419
19. Serki E, Vazifeshenas Darmiyan K, Ezi S, Bayat J, Shahamat F, Ghiravani Z, et al. Effects of colostrum on sperm parameters, sex hormones and testes histopathological changes in diabetic rats. *Journal of Mazandaran University of Medical Sciences*. 2016;26(141):83-94.
20. Wang Y. Epididymal sperm count. *Curr Protoc Toxicol*. 2003;Chapter 16:Unit16.6. doi:10.1002/0471140856.tx1606s14
21. Atta MS, Almadaly EA, El-Far AH, Saleh RM, Assar DH, Al Jaouni SK, et al. Thymoquinone defeats diabetes-induced testicular damage in rats targeting antioxidant, inflammatory and aromatase expression. *Int J Mol Sci*. 2017;18(5):919. doi:10.3390/ijms18050919
22. Moskovtsev SI, Librach CL. Methods of sperm vitality assessment. In: Carrell D, Aston K. editors. *Spermatogenesis. Methods in Molecular Biology (Methods and Protocols)*. Totowa, NJ: Humana Press; 2013. P. 13-9.
23. Hassanzadeh-Taheri M, Jahani F, Hassanzadeh-Taheri M, Doostabadi M, Doostabadi H, Hosseini M. The impacts of yoghurt butter oil on rat testicular morphology and sexual hormones in a 150-day study. *Comp Clin Pathol*. 2018;27(4):959-65. doi:10.1007/s00580-018-2688-3
24. Seco-Rovira V, Beltrán-Frutos E, Ferrer C, Sánchez-Huertas M, Madrid J, Saez F, et al. Lectin histochemistry as a tool to identify apoptotic cells in

- the seminiferous epithelium of syrian hamster (*Mesocricetus auratus*) subjected to short photoperiod. *Reprod Domest Anim.* 2013;48(6):974-83. doi:10.1111/rda.12196
25. Kanter M, Aktas C, Erboga M. Curcumin attenuates testicular damage, apoptotic germ cell death, and oxidative stress in streptozotocin-induced diabetic rats. *Mol Nutr Food Res.* 2013;57(9):1578-85. doi:10.1002/mnfr.201200170
 26. Xu Y, Lei H, Guan R, Gao Z, Li H, Wang L, et al. Studies on the mechanism of testicular dysfunction in the early stage of a streptozotocin induced diabetic rat model. *Biochem Biophys Res Commun.* 2014;450(1):87-92. doi:10.1016/j.bbrc.2014.05.067
 27. Helal EG, Mahmoud A, El-Badawy EE, Kahwash AA. Effect of *Commiphora myrrha* extract on some physiological parameters and histological changes in diabetic albino rats. *Egypt J Hosp Med.* 2005;20(1):148-62.
 28. Tahara A, Matsuyama-Yokono A, Nakano R, Someya Y, Shibasaki M. Effects of antidiabetic drugs on glucose tolerance in streptozotocin-nicotinamide-induced mildly diabetic and streptozotocin-induced severely diabetic mice. *Horm Metab Res.* 2008;40(12):880-6. doi:10.1055/s-0028-1087167
 29. Rao R, Khan Z, Shah A. Toxicity studies in mice of *Commiphora molmol* oleo-gum-resin. *J Ethnopharmacol.* 2001;76(2):151-4. doi:10.1016/S0378-8741(01)00189-1
 30. Mosli HH, Esmat A, Atawia RT, Shoieb SM, Mosli HA, Abdel-Naim AB. Metformin attenuates testosterone-induced prostatic hyperplasia in rats: A pharmacological perspective. *Sci Rep.* 2015;5(1):15639. doi:10.1038/srep15639
 31. Bahey NG, Soliman GM, El-Deeb TA, El-Drieny EA. Influence of insulin and testosterone on diabetic rat ventral prostate: Histological, morphometric and immunohistochemical study. *J Microsc Ultrastruct.* 2014;2(3):151-60. doi:10.1016/j.jmau.2014.06.002
 32. Bose R, Adiga SK, D'souza F, Salian SR, Uppangala S, Kalthur G, et al. Germ cell abnormalities in streptozotocin induced diabetic mice do not correlate with blood glucose level. *J Assist Reprod Genet.* 2012;29(12):1405-13. doi:10.1007/s10815-012-9873-0
 33. Soudamani S, Malini T, Balasubramanian K. Effects of streptozotocin-diabetes and insulin replacement on the epididymis of prepubertal rats: Histological and histomorphometric studies. *Endocr Res.* 2005;31(2):81-98. doi:10.1080/07435800500229193
 34. Kanter M, Aktas C, Erboga M. Protective effects of quercetin against apoptosis and oxidative stress in streptozotocin-induced diabetic rat testis. *Food Chem Toxicol.* 2012;50(3-4):719-25. doi:10.1016/j.fct.2011.11.051
 35. Rashid K, Sil PC. Curcumin ameliorates testicular damage in diabetic rats by suppressing cellular stress-mediated mitochondria and endoplasmic reticulum-dependent apoptotic death. *Biochim Biophys Acta Mol Basis Dis.* 2015;1852(1):70-82. doi:10.1016/j.bbadis.2014.11.007
 36. Wang W-C, Uen Y-H, Chang M-L, Cheah K-P, Li J-S, Yu W-Y, et al. Protective effect of guggulsterone against cardiomyocyte injury induced by doxorubicin in vitro. *BMC Complement Altern Med.* 2012;12(1):138. doi:10.1186/1472-6882-12-138
 37. Ahamad SR, Al-Ghadeer AR, Ali R, Qamar W, Aljarboa S. Analysis of inorganic and organic constituents of myrrh resin by GC-MS and ICP-MS: An emphasis on medicinal assets. *Saudi Pharm J.* 2017;25(5):788-94. doi:10.1016/j.jsps.2016.10.011
 38. Bacanlı M, Anlar HG, Aydın S, Çal T, Arı N, Bucurgat ÜÜ, et al. D-limonene ameliorates diabetes and its complications in streptozotocin-induced diabetic rats. *Food Chem Toxicol.* 2017;110:434-42. doi:10.1016/j.fct.2017.09.020
 39. do Nascimento KF, Moreira FM, Santos JA, Kassuya CA, Croda JH, Cardoso CA, et al. Antioxidant, anti-inflammatory, antiproliferative and antimycobacterial activities of the essential oil of *Psidium guineense* Sw. and spathulenol. *J Ethnopharmacol.* 2018;210:351-8. doi:10.1016/j.jep.2017.08.030
 40. Shi GJ, Zheng J, Wu J, Qiao HQ, Chang Q, Niu Y, et al. Beneficial effects of *Lycium barbarum* polysaccharide on spermatogenesis by improving antioxidant activity and inhibiting apoptosis in streptozotocin-induced diabetic male mice. *Food Funct.* 2017;8(3):1215-26. doi:10.1039/c6fo01575a