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LINUM USITATISSIMUM SEEDS OIL DOWN-REGULATES mRNA EXPRESSION FOR THE STEROIDOGENIC ACUTE REGULATORY PROTEIN AND CYP11A1 GENES, AMELIORATING LETROZOLE-INDUCED POLYCYSTIC OVARIAN SYNDROME IN A RAT MODEL

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The safety and effectiveness of nutricetics suggest that they may offer an alternative to pharmaceutical and surgical therapy for hormone-dependent disorders, such as polycystic ovarian syndrome (PCOS). We investigated the effects of *Linum usitatissimum* seed oil (LSO) on ovarian functionality, its molecular targets, and the oxidative response in hyperandrogenism-induced polycystic ovary. The composition of LSO has been analyzed using ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS). A well-established PCOS rat model orally administered with letrozole daily for 21 days was used to investigate the effect of LSO at doses of 1 and 2 mL/kg body weight for 28 days. The effect on hormonal profile and antioxidant status, histopathology (cell proliferation), and the expression ratio of the steroidogenic acute regulatory protein (StAR) and Cyp11A1 gene were evaluated. LSO exerted beneficial effects on PCOS rat models *via* restoring glutathione (GSH), malondialdehyde (MDA), beta subunit subunit luteinizing hormone (LH), testosterone levels, and histopathological scoring. Furthermore, LSO reversed the elevated StAR and Cyp11A1 genes in the PCOS rat model. This study demonstrated the molecular and cellular mechanisms of the beneficial effect of LSO against the reproductive and metabolic disorders of PCOS.

Key words: *Linum usitatissimum oil, polycystic ovarian syndrome, tandem mass spectrometry, Cyp11A1, antioxidant, action, oxidative stress, malondialdehyde*

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is the most prevalent reproductive endocrinopathy mainly affecting women of childbearing age (1). The PCOS is characterized by reproductive and metabolic abnormalities, including hyperandrogenism, anovulation, menstrual irregularity, insulin resistance and polycystic ovaries (2-5).

The mechanism of arrested follicular growth and thus cyst formation in PCOS is related to the disruption of steroidogenesis, with a high androstenedione to estradiol ratio (6). CYP11A1 catalyzes the rate limiting step of the ovarian steroidogenesis which involves the conversion of cholesterol into pregnenolone (7). CYP11A1 is a potential candidate gene used for studying the pathogenesis of PCOS. It is a membrane bound enzyme located in the inner mitochondrial membrane and is present in all steroid producing tissues. Androgens play crucial role in normal follicular development and function (8). PCOS is associated with hyperandrogenic hormonal state along with upregulation of steroidogenic genes which include StAR, CYP11A, and CYP17 (9).

Some investigations have demonstrated the CYP11A1 gene activity to be a major risk-determining factor for PCOS (10). Another factor that may indicate an exaggeration in androgen biosynthesis in PCOS is the steroidogenic acute regulatory protein (StAR). StAR increases the amount of cholesterol available for conversion into pregnenolone (11). An elevation in the StAR mRNA expression in theca cells from PCOS follicles was reported compared with controls (12-14).

In some cases, conventional pharmaceutical therapies for PCOS may be ineffective and have several side effects, some of which are serious (15). Clomiphene is a commonly used medicine for PCOS, moreover, it is proved to be superior to metformin in achieving live birth in infertile women with PCOS (16). Recently, there has been an increase in women's use of complementary herbal medicine for PCOS (17, 18). Flax or

linseed (*Linum usitatissimum*, family Linaceae), which is a source of oil and fiber, is a commonly cultivated crop in the food and textile industries (19).

The effects of linseed oil omega-3 supplement on the metabolic status of patients with PCOS have been evaluated (20). In addition, the beneficial effects of α-linolenic acid-rich *Linum usitatissimum* seed oil (LSO) on sex steroid hormones, microbiota, and inflammation axis in PCOS have been previously investigated (21). A definite conclusion on the effects and mechanisms of action of LSO in treating PCOS cannot be drawn due to the lack of molecular investigations and scarcity of evidence in the literature. It seems worth investigating and explaining the effects of LSO fatty acids on PCOS. The lipid content of linseed, especially omega-3 fatty acids, has been suggested to have valuable health benefits. Polyunsaturated fatty acids (PUFA) may cause the production of inflammatory mediators, such as cytokines and eicosanoids, and stimulate *in vitro* epithelial cell proliferation (19).

Further research is still required to investigate LSO's various effects and underlying mechanisms at the reproductive as well as metabolic levels. The present study aimed to identify biochemical, histopathological and molecular mechanisms of LSO in the treatment of PCOS in a rat model compared to clomiphene citrate, in addition to metabolomic profiling and identification of potential active metabolites.

MATERIAL AND METHODS

Chemicals

All chemicals in this study were of analytical grade and included high-performance liquid chromatography (HPLC)-grade methanol and formic acid for liquid chromatography-mass spectrometry (LC-MS) (LiChropur™, Sigma-Aldrich, USA), double-deionized water with a conductivity of less than 18.0 MΩ, obtained from a Milli-Q system (Merck KGaA, Darmstadt, Germany), and letrozole (LTZ) (Natco Pharma Limited Hyderabad). Clomiphene citrate (Fertyl-Super tablets; Ar-Ex Laboratories Pvt. Ltd., Goregaon (E), Mumbai) utilized used as a standard ovulation induction drug.

Preparation of linseed oil

Linum usitatissimum seeds were obtained from a medicinal plant store (Haraz) in Cairo, Egypt, and were authenticated at the herbarium of the Botany and Microbiology Department, Faculty of Science, Cairo University, Giza, Egypt. The plant sample (1 kg) was washed with deionized distilled water and dried at 50 ± 1°C. The dried seeds were then ground, and the powder was passed through 16- and 32-mesh sieves to obtain a 0.5-mm particle size. Powdered dried seeds were stored in a refrigerator at 4°C until use. Linseed oil was obtained *via* cold mechanical compression of the seeds, a method that involves the application of pressure (using hydraulic or screw presses) to force out the oil from the plant material. This method is suitable for small- and large (commercial)-capacity operations, as it is more economical compared with other processes. The oil was expressed from 1 kg of seeds using an oilseed expeller followed by filtration and bottling to yield 320 g, which is equivalent to 32 g % on a dry-weight basis (22).

Alkaline hydrolysis

Alkaline hydrolysis was performed using 0.1 N sodium hydroxide at room temperature for 18 h. The oil (1 mL) was dissolved in 0.1 N NaOH in ethanol (70%) and left to stand overnight (18 h) at room temperature. The medium was then neutralized with formic acid, and the reaction mixture was distilled under vacuum at a temperature not exceeding 40°C. The resulting viscous product was then subjected to fractionation to UPLC-MS/MS identification.

UPLC-MS/MS identification

The alkaline-hydrolyzed linseed oil was analyzed *via* UPLC-ESI-MS. The sample was dissolved in MeOH for HPLC at a final concentration of 100 µg/mL and then filtered using a membrane disc filter (0.2 µm) before being subjected to LC-ESI-MS analysis (UPLC-ESI-MS, ACQUITY UPLC System - Waters Corporation, Milford, MA, USA). Column: ACQUITY UPLC-BEH-C18 1.7 µm –2.1 × 50 mm column. Injection volume: 10 µL. The solvent system consisted of (A) water containing 0.1% formic acid and (B) methanol containing 0.1% formic acid. Elution was completed using gradient mobile phase starting from 90% A: 10% B maintained for 2 min. It reached 70% A at 5 min, 30% A at 15 min, and then 10% A at 22 min, which was maintained for 3 min and was subsequently changed to reach 100% B at 26 min, sustained for 3 min, and returned to the initial composition at 32 min with a flow rate of 0.2 mL/min. The analysis were carried out using the negative ionization mode utilizing the XEVO TQD Triple Quadrupole Mass Spectrometer (Waters Corporation, Milford, MA01757 USA). Source temperature 150°C, cone voltage 30 eV, capillary voltage 3 kV, desolvation temperature 440°C, cone gas flow rate 50 L/h, and desolvation gas flow rate 900 L/h. Mass spectra were detected in the ESI between m/z 100 and 1000. The peaks and spectra were processed using the MassLynx 4.1 software and tentatively identified by comparing their retention times (Rt) and mass spectra with the reported data (23).

In vivo experiments

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Cairo University (CU-IACUC; VetCU11112018017). Cyclic, virgin, adult female Wistar albino rats (140 – 160 g) were obtained (Laboratory Animal Colony, Helwan, Egypt). The animals were kept in the animal house of the Faculty of Veterinary Medicine, Cairo University, in standard polypropylene cages and maintained in a controlled environment with the following conditions: 22 ± 3°C temperature, 55 ± 5% humidity, and 12-h light/dark cycle. The rats were allowed to acclimatize for 2 weeks and were provided with a standard diet and water *ad libitum* throughout the study period. Estrous cycle regularity have been checked using vaginal smear technique for all rats.

Experimental protocol

A total of 35 female rats were randomly allocated to 5 groups of 7. The animals in group 1 served as the negative control (normal) and received a daily oral dose (1 mL) of the vehicle (0.5% carboxymethyl cellulose, CMC) for 49 days. For the induction of PCOS, the animals in groups 2 to 5 received LTZ 1 mg/kg dissolved in 0.5% CMC once daily orally for 21 days, guided by a well-established rat model (24) that was recommended as model of choice for investigation of various aspects of PCOS (25). Afterwards, they received the different samples orally for 28 days. Group 2, which served as the positive control (PCOS), received only the vehicle, and group 3 received clomiphene citrate 1 mg/kg (26). Groups 4 and 5 were treated with LSO at doses of 1 and 2 mL/kg, respectively (21, 27). From day 6 of the treatment, daily vaginal smears of all rats were examined to test ovulation. An indiscriminate estrous cycle with a prolonged diestrus phase indicated PCOS (28, 29). On day 50 of the study period, 24 h following last treatment, all the rats were anesthetized with

ketamine 91 mg/kg, i.p. Duplicate blood samples were collected into sodium heparin tubes for plasma separation for hormonal assays. Gel separator tubes were utilized to collect serum samples for blood centrifugation at 3000 g at 4°C for 10 min for biochemical assays. The animals were weighed and then sacrificed, and the ovaries and uteri were excised, cleaned of fat, and weighed. The relative weights of the ovary and uterus were calculated as the ratio of the organ (wet weight, mg) to body weight (g). The ovaries were divided into three sets. Two sets were stored at –80°C to be used for real-time reverse transcription-polymerase chain reaction (RT-PCR) and antioxidant assays. The remaining sets were fixed in 10% neutral buffered formalin for histopathological examination.

Hormonal profile

The serum total testosterone was measured using a commercial ELISA Kit (Chemux Bioscience Inc., San Francisco, USA). The beta subunit chain of luteinizing hormone (LH) level was measured using rat lutropin subunit beta ELISA Kit (EIAab, Wuchan, China) according to the manufacturer's protocols.

Malondialdehyde (MDA) and reduced glutathione (GSH) determinations

Ovarian tissues were separately homogenized in 10-mL cold buffer of 50-mM potassium phosphate, pH 7.5, and 50-mM potassium phosphate, pH 7.5, 1-mM EDTA, for MDA and reduced GSH, respectively. The tissue homogenate was centrifuged at 15000 rpm for 15 min, and the supernatant was utilized to measure the MDA and GSH concentrations according to the standard protocols (30, 31).

Biochemical parameters

The serum glucose level and lipid profile (cholesterol, triglycerides, and HDL-cholesterol) were measured using commercial kits (Spectrum, Egypt), where very-low-density lipoprotein cholesterol (VLDL) concentration = triglycerides/5 and LDL cholesterol concentration = total cholesterol – (HDL + VLDL) .

Histopathological examination

Ovaries from each group were collected and fixed for 48 h in 10% neutral buffered formalin and processed to obtain 5-µm-thick paraffin-embedded sections. The sections were stained with hematoxylin and eosin (H & E) (32), and morphometric analysis of the ovaries was conducted. The number of follicular cysts, the mean diameter of the follicular cysts (µm), and the thickness of the granulosa cell layers (µm) and theca cell layers (µm) were measured using image analysis software (Image J, version 1.46a, NIH, Bethesda, MD, USA).

RT-PCR analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols. Then, 10 µg of RNA was used to synthesize cDNA (Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR). The primer set used for studied genes were designed using BLAST primer and they were shown in *Table 2*.

The primer set used for StAR was sense primer: 5'-CACA CTTTGGGGAGATGCCT-3'; antisense primer: 5'-GAACTTC CAATGGCGTGCAG-3'.

For CYP11A1, we used sense primer: 5'-GCTGG AAGGTGTAGCTCAGG-3'; antisense primer: 5'-TCACTG GTGTGGAACATCTGG-3'.

Real-time PCR was performed using the Power SYBR Green Applied Biosystems 7500 System (Life Technologies, CA, USA) at 94°C for 5 min, followed by 45 cycles at 94°C for 30 s, at 60°C for 30 s, and then held for the final phase at 72°C for 7 min. The GAPDH gene was amplified in the same reaction to serve as the internal control (33). Each assay was repeated twice, and the values were used to calculate the gene/GAPDH ratio, with the value of 1.0 used as the control (calibrator) (34). The normalized expression ratio was calculated by the $2^{-\Delta\Delta Ct}$ method using the MxPro software (35).

Statistical analysis

Different analytical determinations in the biological samples were carried out in triplicate, and the results are expressed as mean ± standard deviation (SD) or standard error, where n = 7 biological replicates. Data for multiple variable comparisons were analyzed *via* one-way analysis of variance (ANOVA) test to analyze the significant differences ($P < 0.05$) between the groups using the SPSS software version 24 package for Windows (SPSS Inc., Chicago, IL, USA). Duncan's *post hoc* test and least significant difference test were used to check the inter-group comparison. (*) indicates a significant difference compared with the control group at $P \leq 0.05$. (#) indicates a significant difference compared with the PCOS group at $P \leq 0.05$. Figures were created using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, California, USA).

RESULTS

LC-ESI-MS for Linum usitatissimum seed oil

In the present study, 16 phytochemical compounds (*Table 1*) have been tentatively characterized in the linseed oil using the data obtained from the LC-ESI-MS^2 and previously reported in the literature. These compounds are summarized along with their retention time, [M-H] – MS and MS^2 data, molecular formula, the proposed assignment, and the relative percentage (SD) of each compound. The detected components in this study were tentatively characterized using their MS and MS^2 data, with the observed spectra compared with those reported. Alkaline hydrolysis has been employed as an effective method for improving the recovery of bioactive compounds from linseed oil (36).

The total ion chromatogram of the alkaline-hydrolyzed linseed oil is presented in *Fig. 1*. The investigated linseed oil is mainly composed of fatty acids. Octadecatrienoic acid (linolenic acid) was the major fatty acid detected, with a total level of about 34% for the detected isomers. This was followed by linoleic and oleic acids, which accounted for 17.45% and 16.01% of the oil composition, respectively. Other detected fatty acids were hydroxyoctadecadienoic acid (hydroxylinoleic acid), hydroxylinolenic acid, hexadecanoic acid (palmitic acid), octadecenedioic acid, and octadecanoic acid (stearic acid), which accounted for 8.7%, 5.8%, 4.7%, 3.1%, and 2.8% of the oil composition, respectively. In addition, phosphatidic acid accounted for 1.4% of the linseed oil composition (*Table 1*).

Relative ovarian and uterine weights and final body weights

In general, the relative weights of both the ovary and uterus were reduced in all groups after treatment compared with the PCOS group. However, these differences were significant only for ovaries ($P < 0.005$). LSO2 showed no significant differences compared with standard clomiphene citrate (*Fig. 2A*). Final body weights in different experimental groups showed in (*Fig. 2B*).

Table 1. Tentatively identified peaks in the LC-MS spectrum of the hydrolysed extract of linseed.

Peak No.	Assignment	Molecular formula	RT (min)	m/z $[M-H]^-$	Product ions MS/MS	Relative percentage (standard deviation)
1	Unknown fatty acid	–	1.02	345.1056	263, 205, 183, 161, 99, 57	0.76 (0.25)
2	Phosphatidic acid	$C_{42}H_{69}O_8P$	5.71	731.4442	Unfragmented	1.40 (0.35)
3	Trihydroxy octadecenoic acid	$C_{18}H_{34}O_5$	9.43	329.2345	211, 183, 171, 139, 127, 99, 57	0.63 (0.16)
4	Octadecenedioic acid	$C_{18}H_{32}O_4$	11.99	311.2201	253, 223, 211	3.07 (0.48)
5	Hydroxylinolenic acid	$C_{18}H_{30}O_3$	14.20	293.2094	275, 249, 231, 209, 181, 171, 157, 135, 83, 67	3.32 (0.33)
6	Hydroxyoctadecadienoic acid (Hydroxylinoleic acid)	$C_{18}H_{32}O_3$	14.71	295.2050	277, 237, 223,137, 121, 43	3.45 (0.12)
7	Hydroxyoctadecadienoic acid (Hydroxylinoleic acid) isomer	$C_{18}H_{32}O_3$	15.10	295.2079	237, 235, 213, 185, 155, 113, 59	5.22 (0.51)
8	Hydroxylinolenic acid isomer	$C_{18}H_{30}O_3$	15.69	293.2094	275, 249, 231, 209, 181, 171, 157, 135, 83, 67	2.43 (0.07)
9	Hydroperoxyl octadecatrienoic acid	$C_{18}H_{30}O_5$	16.30	325.1956	281, 205, 164, 158, 58	0.90 (0.04)
10	Hydroxy octadecenoic acid	$C_{18}H_{34}O_3$	16.59	297.2369	223, 197, 183, 129, 85	0.80 (0.15)
11	Octadecatrienoic acid (α-linolenic acid)	$C_{18}H_{30}O_2$	19.16	277.2116	209, 185	21.55 (3.99)
12	Octadecatrienoic acid isomer (γ-linolenic acid)	$C_{18}H_{30}O_2$	19.30	277.2116	209, 185	12.40 (1.38)
13	Otadecadienoic acid (Linoleic acid)	$C_{18}H_{32}O_2$	20.95	279.2268	211, 126	17.45 (1.04)
14	Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$	22.57	255.2293	211, 191, 158, 152, 121, 59	4.67 (0.46)
15	Octadecenoic acid (Oleic acid)	$C_{18}H_{34}O_2$	22.86	281.2420	219, 207, 201, 89, 182, 124, 76	16.01 (1.12)
16	Octadecanoic acid (Stearic acid)	$C_{18}H_{36}O_2$	25.15	283.2573	255, 246, 223, 148	2.83 (0.34)

Table 2. The genes accession number and primer sets.

Gene	Forward primer	Reverse primer	Accession number
StAR	5'-CACACTTTGGGGAGATGCCT-3'	5'-GAACTTCCAATGGCGTGCAG-3'	XM_032918521.1
CYP11A1	5'-GCTGGAAGGTGTAGCTCAGG -3'	5'-TCACTGGTGTGGAACATCTGG-3'	NM_017286.3

Hormonal profiles

In the PCOS-induced group, the LH and testosterone levels were significantly higher compared with those in the control group. Conversely, the rats treated with LSO at doses of 1 and 2 mL/kg for 28 days exhibited significantly reduced LH and testosterone levels compared with the PCOS-induced group. The two LSO groups exerted superior hormonal effects which was comparable to clomiphene group or even better in LSO2 group regarding testosterone levels, as presented in *Fig. 3A* and *3B*.

Oxidative stress biomarkers in the ovary

In LTZ-induced PCOS, the MDA levels significantly increased, whereas the GSH activity was significantly decreased compared with the control group. LSO at doses of 1 and 2 mL/kg improved ovarian MDA levels and GSH activities toward their normal levels (*Fig. 4A* and *4B*). For MDA levels, LSO2 group was even superior to clomiphene group.

Glucose and lipid profile

The glucose and lipid profile (total cholesterol, triglyceride, LDL cholesterol, VLDL cholesterol) of the PCOS untreated group exhibited significantly higher values than the control group. With regard to the LSO 1 mL/kg-treated group, the glucose and lipid profile demonstrated significantly lower values compared with the PCOS untreated group, whereas in the LSO 2 mL/kg-treated group, normal levels were restored. However, clomiphene group showed insignificant effects on glucose and lipid profile compared to PCOS. (*Fig. 5A-5F*).

Histopathology of ovaries

The normal control group demonstrated normal ovarian histology. Multiple follicles in different developmental stages were observed with normal granulosa, theca, and interstitial stromal cell layers. Moreover, various corpora lutea were present (*Fig. 6A* and *6B*). The PCOS group had numerous ovarian cysts and small follicles at the early developmental stage with the

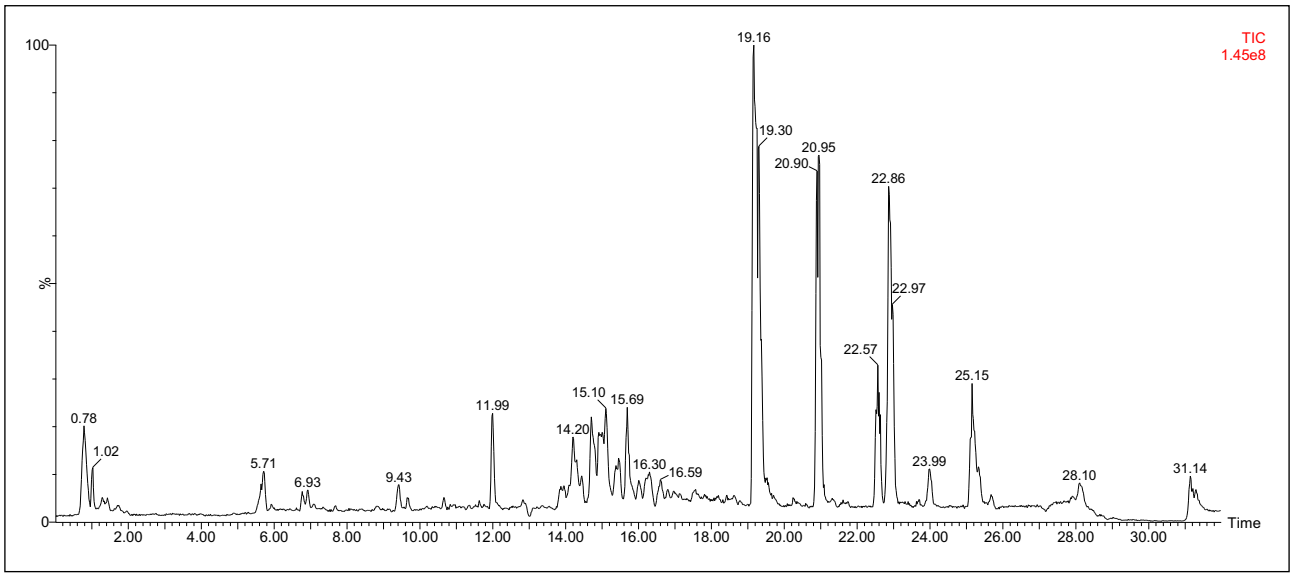

Fig. 1. Total ion chromatogram of hydrolyzed linseed oil.

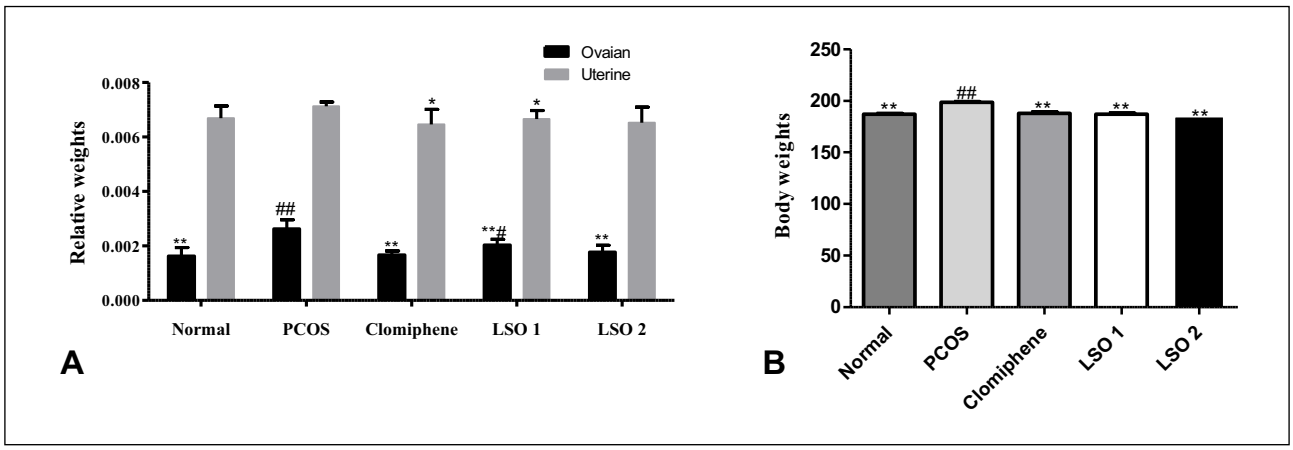

Fig. 2. Effect of LSO at doses of (1 and 2 ml/kg, respectively) on (A): relative ovarian and uterine weights, (B): body weights in letrozole induced PCOS rat model. (Mean ± SD, n = 7), (*) and (**) indicated the significant difference compared to the PCOS group at $P < 0.05$ and < 0.005, respectively, whereas (#) and (##) indicated the significant differences compared to the normal group at $P < 0.05$ and < 0.005, respectively.

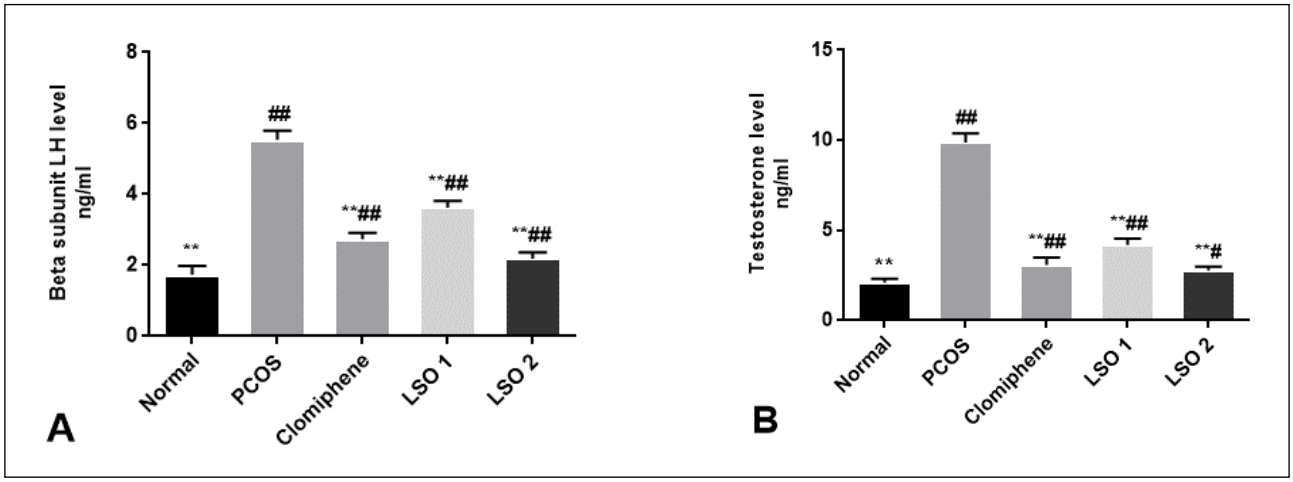

Fig. 3. Effect of LSO at doses of 1 and 2 ml/kg on (A): beta subunit LH level, (B): testosterone level in letrozole induced PCOS rat model. (Mean ± SD, n = 7). (*) and (**) indicated the significant difference compared to the PCOS group at $P < 0.05$ and < 0.005, respectively, whereas (#) and (##) indicated the significant differences compared to the normal group at $P < 0.05$ and < 0.005, respectively.

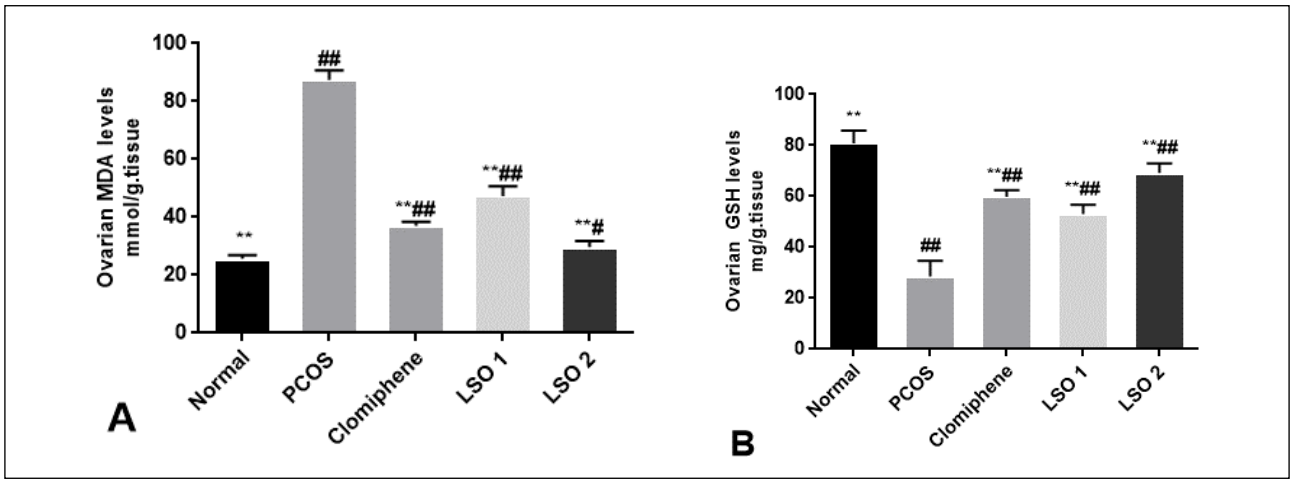

Fig. 4. Effect of LSO at doses of 1 and 2 ml/kg on ovarian (A): MDA level, (B): GSH activity in letrozole induced PCOS rat model. (Mean ± SD, n = 7); (*) and (**) indicated the significant difference compared to the PCOS group at $P < 0.05$ and < 0.005, respectively, whereas (#) and (##) indicated the significant differences compared to the normal group at $P < 0.05$ and < 0.005, respectively. LSO 1, LSO 2: linseed oil at dose of 1 and 2 ml/kg b.w, respectively.

absence of a corpus luteum in all cases. The granulosa cell layers in the cystic follicles were very thin and flattened (*Fig. 6C* and *6D*), and most of the cells had undergone necrosis and apoptosis. The granulosa cell layers in some ovarian cysts were absent; only the theca interna and theca externa cells were present. In other ovarian cysts, the granulosa cell layers were hyperplastic and folded. The theca cell layers exhibited marked proliferation of both the theca interna and theca externa cells. The groups treated with clomiphene (*Fig. 6E* and *6F*), LSO 1 mL/kg (*Fig. 6G* and *6H*), and LSO 2 ml/kg (*Fig. 6I* and *6J*) demonstrated a marked reduction in the number of ovarian cysts with restoration of the granulosa cell layer thickness and marked reduction of both the theca cell layers compared with the PCOS group. The histomorphometric study was performed on ovaries during follicular phase on the tertiary follicles in both treated and normal groups and on the follicular cysts in PCOS group. The number and diameter of the cystic follicles significantly decreased ($P < 0.05$) and the thickness of the granulosa layer significantly increased. However, the thickness of the theca layer significantly decreased compared with those of the PCOS group ($P < 0.05$) (*Fig. 7A-7D*).

Quantitative real-time PCR for StAR and CYP11A1 genes

The StAR mRNA demonstrated a significant upregulation in the PCOS treated group (8.5-fold) compared with the negative control. LSO co-treatment at both doses modulated the expression level of the StAR gene, as they demonstrated a significant decrease in the gene expression compared with the PCOS group. The LSO 2 mg/kg exhibited an insignificant upregulation of the StAR gene compared with the negative control. The mRNA of the CYP11A1 gene was significantly upregulated in the PCOS treated group (6.3-fold) compared with that of the negative control. The LSO co-treatment with both doses modulated the expression level of the CYP11A1 gene, as they demonstrated a significant decrease in the gene expression similar to that of clomiphene compared with the PCOS group. Both doses showed an insignificant increase in the expression level compared with the negative control (*Fig. 8*).

DISCUSSION

Conventional therapy as clomiphene is an effective therapeutic remedy for PCOS that help increasing fertility and probability of pregnancy, however it may be associated with several side effects including breast cancer and uterine cancer specially in long term use (37). Recently, herbal remedies have been used to mitigate the adverse effects of conventional remedies and demonstrated to be effective with no risk of side effects (15, 37, 38). The present study clearly showed that treatment with LSO has alleviated PCOS associated disorders in rats, with superior activity as compared to clomiphene especially regarding metabolic disorders in addition to reproductive disorders.

PCOS is an endocrine disorder associated with cysts, hyperandrogenism, insulin resistance, and glucose intolerance. It is considered to be a risk factor for type 2 diabetes mellitus, hypertension, cardiovascular disorders, and cancer (39-41). Thirty three percentage of women with PCOS affected with metabolic syndrome, particularly in those over 25 years old or with central obesity (42, 43). Eventhough, (44) suggesting that hormonal factors might be considered as predictable risk factors for obesity developed in PCOS. The LTZ rat model is an effective method used to induce and study various aspects of PCOS with histological and biochemical features (41, 44, 45). Normally, the development and maturation of ovarian follicles require LH and FSH to work in harmony. LH stimulates theca cells to produce androgen, whereas FSH is responsible for promoting the aromatization of the androgen to estradiol by granulosa cells (39). In LTZ-induced PCOS, the aromatization of androgen into estrogens in the granulosa cells is inhibited, which leads to androgen accumulation and decreased estrogen production. Estrogen reduction lessens the negative feedback on LH production in the pituitary, which results in increased LH levels (46). In our study, the LTZ-induced group demonstrated high LH and testosterone levels, whereas the LSO-treated groups exhibited significantly decreased levels. This is consistent with another studies, one recorded a significantly higher LH/FSH ratio and testosterone level in the PCOS group compared with the LSO group (21), while others recorded significant rise in progesterone and a decrease in testosterone and estradiol compared to PCOS by combination of spearmint and flaxseed extract (47) and by hydroalcoholic extract of flaxseed (48). That could be contributed to the effect of linseed Ω-3 PUFAs which reported to had significant role in ameliorating hormonal lipid profile in PCOS rats (49).

In PCOS, oxidative stress induced in the reproductive tissues may be responsible for hyperglycemia and insulin resistance.

These cause excessive lipid peroxidation, increased levels of free radicals, and antioxidant exhaustion (50, 51). Our results indicated a significant increase in the MDA levels and a reduction in the GSH activity, which are in agreement with the studies on liver MDA (52) and serum and skin MDA and GSH (53) in rats. The provoked improvement in the ovarian antioxidant status which appear in decrease MDA level and increase GSH activity in LSO-treated groups may be attributed

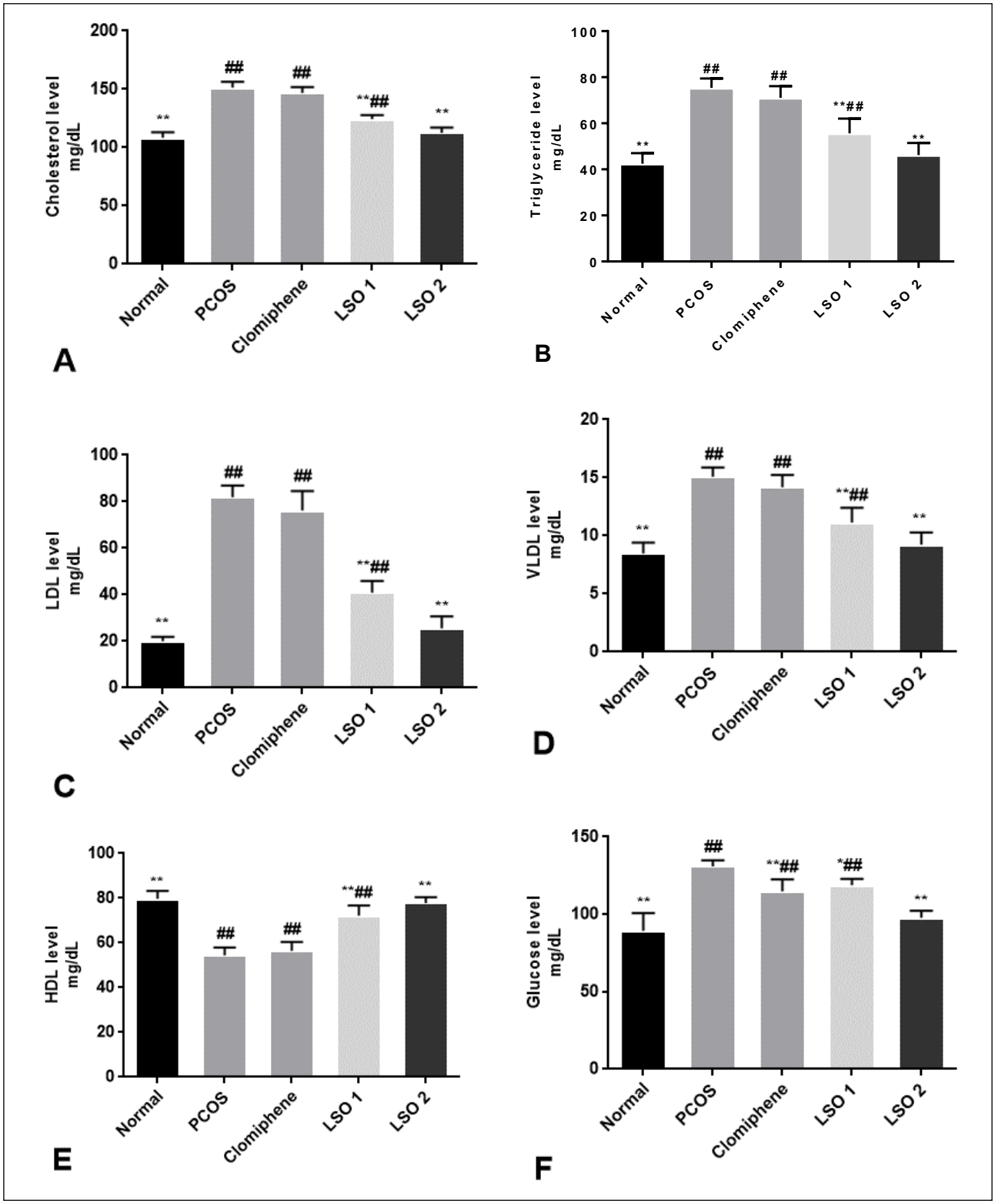

Fig. 5. Effect of LSO at doses of 1 and 2 ml/kg on (A): cholesterol level, (B): triglycerides level, (C): LDL level, (D): VLDL, (E): HDL level, (F): glucose level in letrozole induced PCOS rat model. (Mean ± SD, n = 7), (*) and (**) indicated the significant difference compared to the PCOS group at $P < 0.05$ and < 0.005, respectively, whereas (#) and (##) indicated the significant differences compared to normal group at $P < 0.05$ and < 0.005, respectively.

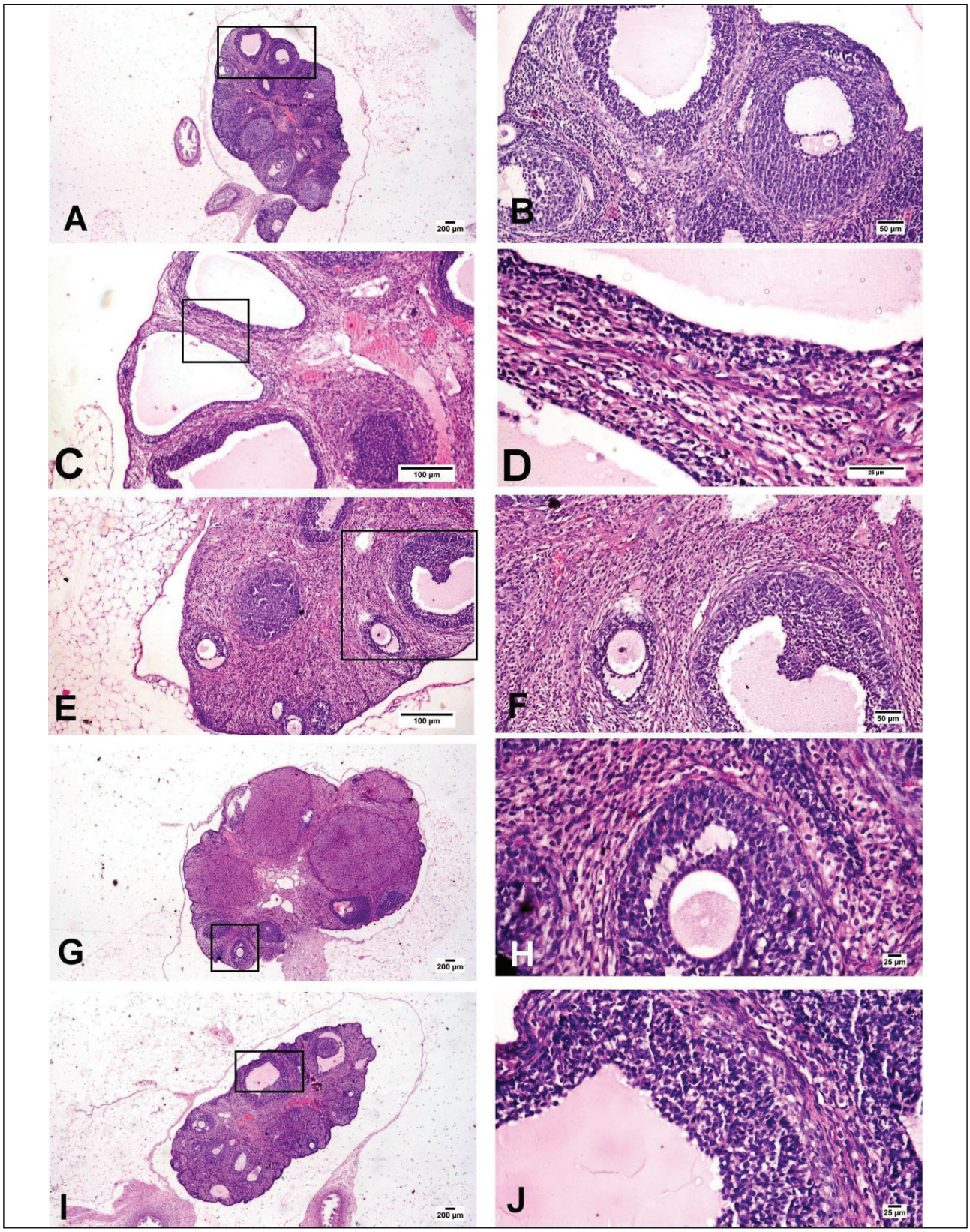

Fig. 6. Photomicrographs of ovaries of rats in different experimental groups. The right column contains higher magnification images from the region in the left column outlined by the black rectangle. (A): ovary of normal control group showing normal histology (×40). (B): normal secondary and tertiary follicles (×200). (C): ovary of PCOS group showing multiple ovarian cysts (×100). (D): ovarian cyst with thin flatten granulosa cell layer (×200). (E): and (F): ×100 and ×200, respectively). Clomiphene treated group showing reduction in the number of ovarian cysts, restoration of granulosa cell thickness and presence of corpora lutea. (G): and (H): ×40 and ×400, respectively) LSO 1 ml/kg treated group showing marked reduction in the number of ovarian cysts and restoration of granulosa cell thickness. Presence of corpora lutea. (I) and (J), ×40 and ×400 respectively) LSO 2 ml/kg treated group showing reduction in the number of ovarian cysts and restoration of granulosa cell thickness. Presence of corpora lutea. Hematoxylin and eosin (H & E).

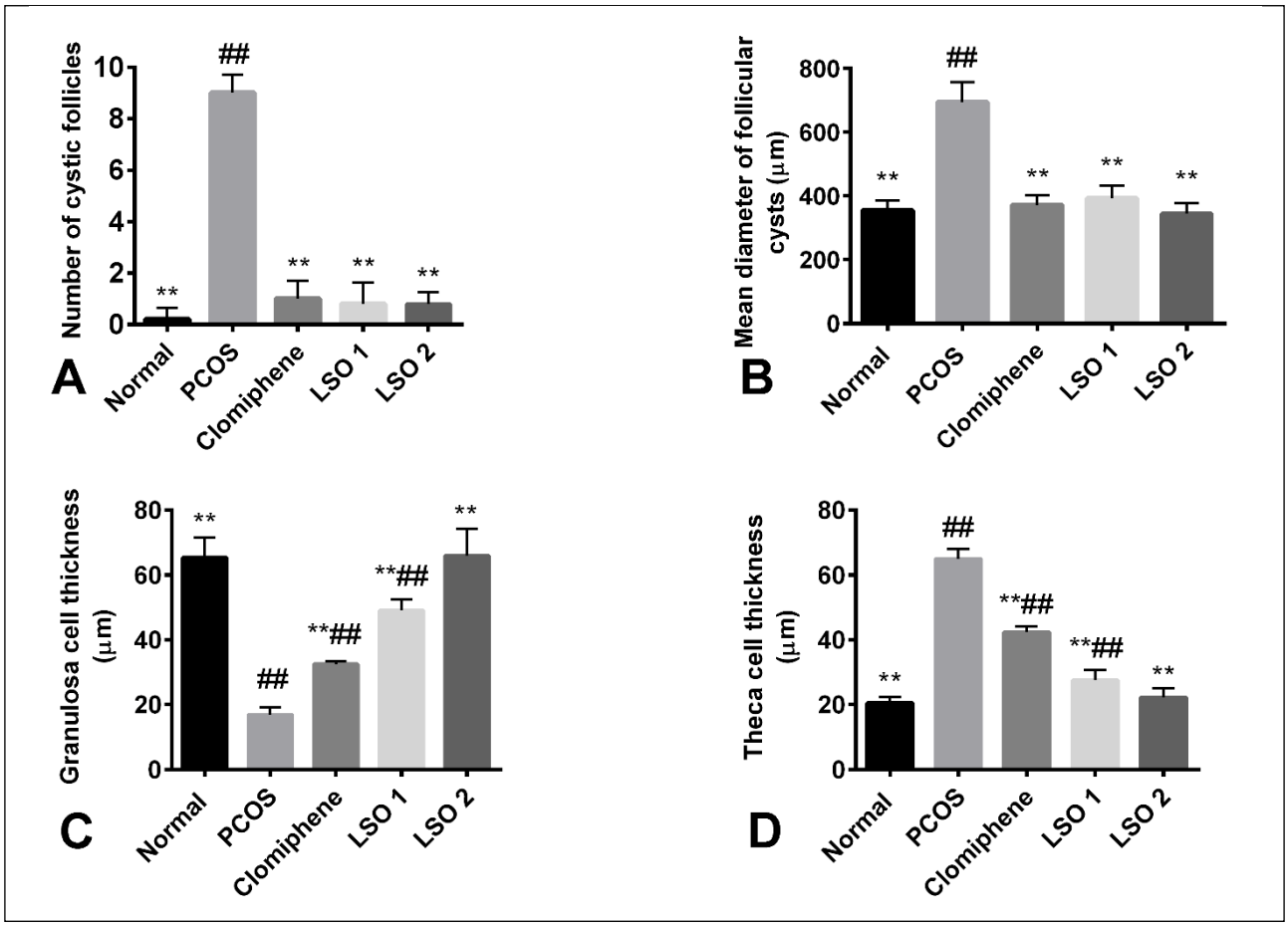

Fig. 7. Morphometrical analysis of ovaries of rats in the different experimental groups. (A): number of ovarian cysts per ovary. (B): mean diameter of follicular cysts (µm). (C): granulosa cell thickness (µm). (D): theca cells thickness (µm). Mean ± SD, n = 7, (*) and (**) indicated the significant difference compared to the PCOS group at $P < 0.05$ and < 0.005, respectively, whereas (#) and (##) indicated the significant differences compared to the negative control group at $P < 0.05$ and < 0.005, respectively. LSO 1, LSO 2: linseed oil at dose of 1 and 2 ml/kg b.w., respectively.

to its components, such as polyunsaturated fatty acids like omega-3 fatty acids and rich phenolic compounds (40).

The metabolic disorders in PCOS may be caused by hormonal disturbances, especially high testosterone concentrations, which leads to pancreatic β cell dysfunction, insulin resistance, and thus hyperglycemia and dyslipidemia (54). Our results showed a significant elevation in glucose and all lipid profile parameters, including cholesterol, triglyceride, LDL, VLDL, and HDL reduction in LTZ-induced PCOS rats compared with normal controls. Hyperglycemic and dyslipidemia effects were improved in the LSO-treated groups in a dose-dependent pattern, in agreement with previously reported effects for omega 3 fatty acids of LSO (49) and flaxseed supplementation (55). This may be due to LSO decreasing effect on testosterone levels (56) or linseed fatty acids and fiber content that can improve insulin sensitivity. In addition, omega-3 fatty acids may increase the level of adiponectin, which has antiatherosclerotic, antidiabetic, and anti-inflammatory properties (20, 55). On the contrary, clomiphene group exhibited no beneficial effects on PCOS associated metabolic disorders, as it showed insignificant differences in glucose and lipid profile compared to PCOS. That was consistent with what previously reported for clomiphene (57, 58).

Women with PCOS have multiple ovarian cysts with hypertrophy of the theca cell layers, which lead to excess androgen secretion (59). The histopathological analysis in our PCOS rat model showed multiple ovarian cysts, a thin granulosa cell layer, decreased follicular development, and thickened hyperplastic theca cell layers with the absence of corpora lutea compared with the normal group. In the current study, LSO treatment restored the components and morphologies of ovarian follicles to normal, which indicated that LSO regulates several factors related to the development of ovarian follicle and corpus luteum and reduces the cystic follicles after the induction of PCOS. Consistent with our results, the presence of multiple mature Graafian follicles with corpora lutea in the rats' ovaries has previously been reported after treatment with LS extract (60). These results indicated a possible treatment for PCOS. Thus, we hypothesized that LSO could be useful for PCOS management.

PCOS ovaries showed up-regulation of the CYP11A m-RNA expression relative to the normal theca cells (61). Thus the *CYP11A* is considered a potential promising biomarker for PCOS (62). The upregulation of CYP11A1 causes the proliferation of theca cells derived from polycystic ovaries (61). The CYP11A locus is involved in the progress of PCOS due to the correlation between PCOS development and the pentanucleotide repetitions in the sequencing of 50UTR (TTTTA) and the susceptibility to PCOS (63). In the current study, CYP11A upregulation was observed. Increased PCOS is

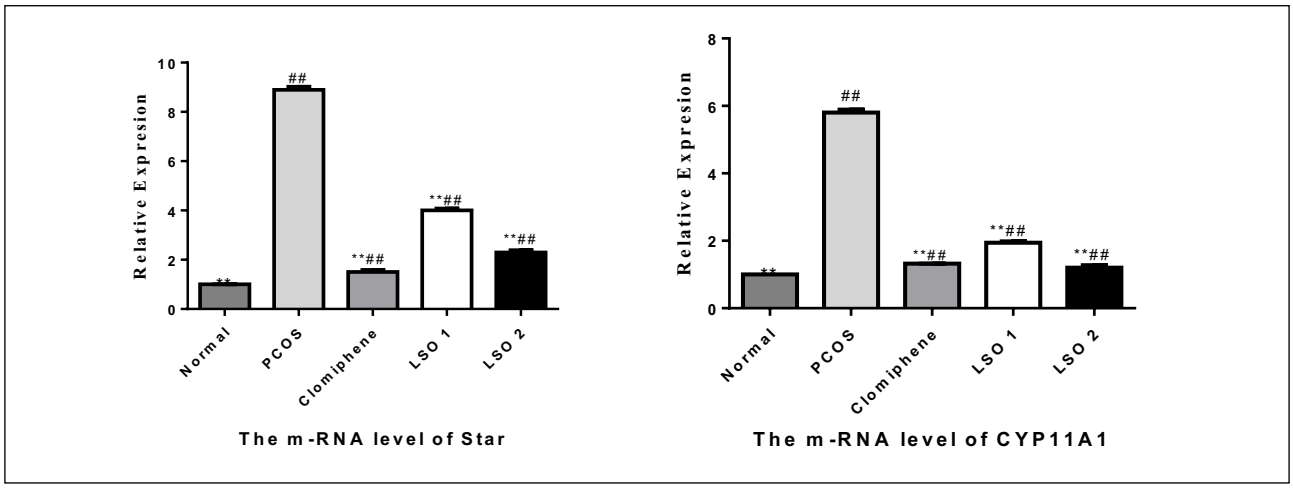

Fig. 8. The m-RNA level of (A): StAR gene, (B): CYP11A1 gene, in the different experimental groups. Data represented as Mean ± SE, n = 7. (*) and (**) indicated the significant difference compared to the PCOS group at $P < 0.05$ and < 0.005, respectively, whereas (#) and (##) indicated the significant differences compared to the negative control group at $P < 0.05$ and < 0.005, respectively. LSO 1, LSO 2: linseed oil at dose of 1 and 2 ml/kg b.w., respectively. LSO 1, LSO 2: LSO at doses of 1and 2 ml/kg b.w., respectively.

associated with the upregulation and polymorphisms of CYP11A (64).

The StAR gene encodes the StAR protein, which binds to cholesterol and facilitates its cellular uptake for androgen synthesis. It regulates testosterone biosynthesis and is therefore responsible for the increase and decrease in plasma testosterone levels (65). Fluctuating androgen biogenesis is considered to be an important feature in patients with PCOS (66). We observed StAR mRNA upregulation in the PCOS group.

The objective of PCOS treatment is to restore ovulation (67). However, the role of LSO in regulating the expression of the key genes implicated in steroidogenesis remains undetermined and needs to be assessed (68). Immunohistochemical studies have reported an increase in the expression of the StAR gene in rats' polycystic ovaries (69, 70). On a molecular basis, we found that LSO restores the expression of the CYP11A1 and StAR genes involved in steroidogenesis (71). Moreover, LSO improves the ovarian size and thickness of the theca cells and considerably downregulates the StAR, CYP11A1, and Hsd3b1 mRNA levels. LSO functions either by direct action on the ovary or by affecting the hypothalamic-pituitary axis (72). Upregulation of the StAR gene by estrogen has also been reported (73), suggesting that reduced estrogen production may explain the downregulation of the StAR gene in LTZ-treated rats (74).

The present study revealed for the first time the potential molecular mechanisms of LSO in the alleviation of the effects of PCOS-associated reproductive disorders. LSO improved the ovarian size and thickness of the theca cells by downregulating the StAR and Cyp11A1 genes involved in steroidogenesis. LSO also offsets ovarian hyperandrogenism and oxidative stress, which may be involved in the pathogenesis of PCOS, and alleviated PCOS-associated metabolic disorders. While LSO reproductive effects were comparable to clomiphene and superior only for LSO2 regarding testosterone levels, granulosa and theca cell thickness, its metabolic effects were absolutely superior to clomiphene. These effects could be attributed to the high content of linolenic, linoleic, and oleic acids. However, further studies is required to identify other possible modulating mechanism of LSO for ameliorating PCOS.

Abbreviations: CMC, carboxymethyl cellulose; CYP11A1, cytochrome P450 family 11 subfamily A member 1; LS, linseed; LSO, linseed oil; GnRH, gonadotropin-releasing hormone; GSH, glutathione; HDL, high-density lipoprotein; HDMS, high-definition mass spectrometer; LDL, low-density lipoprotein; LTZ, letrozole; LH, luteinizing hormone; MDA, malondialdehyde; PCOS, polycystic ovary syndrome; StAR, steroidogenic acute regulatory protein; VLDL, very low-density lipoprotein.

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