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Original Article

Lupeol attenuated the NAFLD and PCOS-induced metabolic, oxidative, hormonal, histopathological, and molecular injuries in mice

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Abstract

Background and purpose: The current study aimed to study the therapeutic effects of lupeol as a nutritional triterpene on non-alcoholic fatty liver disease (NAFLD) and polycystic ovarian syndrome (PCOS) disorders in separate and concurrent models.
Experimental approach: This study was performed in three sets and each set contained 4 groups of female mice (n = 6), including control, NAFLD or PCOS and/or NAFLD/PCOS, lupeol, and metformin (MET). The treatment groups following the induction of disorders were treated with lupeol (40 mg/kg, orally) or MET (500 mg/kg, orally) for 28 days. The insulin resistance index and hormonal assessments were conducted on the collected serum samples. Moreover, oxidative stress biomarkers were measured in the liver and ovaries. Histopathological studies and ultimately any changes in the expression of androgen receptors, toll-like receptor (TLR)-2 and TLR-4 were analyzed.
Findings/Results: Results revealed that lupeol reduced significantly the insulin resistance index in NAFLD and NAFLD/PCOS-positive animals. Lupeol attenuated remarkably the PCOS and PCOS/NAFLD-elevated concentration of testosterone. lupeol recovered the metabolic disorders-induced oxidative stress and restored the disorders-depleted glutathione. The NAFLD/PCOS-induced hepatic damages such as microvesicular or macrovesicular steatosis and atretic follicles number in the ovary were attenuated in the lupeol-treated mice. Serum level of TNF-α was reduced and the expression of androgen receptors, TLR-4 and TLR-2 were downregulated in the lupeol-treated NAFLD/PCOS-positive animals.
Conclusions and implication: The results suggest that lupeol could be a novel nutraceutical for the treatment of metabolic disorders. Lupeol's anti-metabolic disorders effects attribute to its anti-dyslipidemia, antioxidant, and anti-inflammatory properties.

Keywords: Anti-inflammatory; Liver; Insulin resistance; Metabolic disorder; Ovary; Oxidative stress.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the main causes of the hepatic disorder, characterized by the accumulation of lipids, inflammation, and ultimately with fibrosis in non-alcohol consumers. There is a recently-conducted epidemiological study indicating that the prevalence of NAFLD is growing from 18.5% in young adults (12-17 years) to 24% in older young adults (18-24 years) (1).

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Metabolic disorders, obesity, dyslipidemia, and diabetes (type 2) are important risk factors for NAFLD. In addition to genetic susceptibility, high-fat diet, lack of activity and other socio-economic elements are influential factors in the occurrence and severity of NAFLD (2). Moreover, increased levels of free fatty acids due to insulin resistance, reduction of lipolysis, impaired glucose hemostasis, and excessive oxidants production are known molecular mechanisms in NAFLD induction (3).

Polycystic ovarian syndrome (PCOS) at the same time, is the most prevalent endocrine-related disease in women of reproductive age, which was recently based on its close relationship with insulin resistance redefined as another metabolic disorder (4). Following a molecular screening of more than a hundred candidate genes, it has been reported that the genes which are involved in the pathways of steroidogenesis, insulin and glucose metabolism, inflammation, and obesity are associated with PCOS (5,6). Moreover, epigenetic changes such as DNA methylation, histone alterations, and microRNA activities are also playing a crucial role in the development of PCOS. One of the well-known epigenetic factors, which results in PCOS in adulthood is prenatal excessive androgens exposure (7). The insulin resistance-related events result in energy deficiency in the growing follicles and oocytes and ultimately disturbances in folliculogenesis (8).

There is an increasing amount of evidence indicating a tight association between these two metabolic disorders in terms of pathophysiology and also clinical features. Insulin resistance and consequently hyperinsulinemia play a key role in both metabolic disorders of NAFLD and PCOS. As insulin in normal women stimulates the luteinizing hormone-induced androgen and follicle-stimulating hormone-induced estrogen synthesis, therefore, in PCOS cases due to the higher sensitivity of theca cells from polycystic ovaries to insulin, the androgen synthesis is remarkably increased (9). At the same time, insulin resistance results in a marked reduction of fatty acid oxidation and consequently lipid accumulation in different tissues including the liver, which is counted as one of the main etiological factors of NAFLD (10). The prolonged accumulation of lipids in different tissues including the liver results in infiltration of monocytes and inflammatory cytokines secretion (11). There is considerable evidence indicating a central role of androgen excess in the development of PCOS and at the same time there are proven documents demonstrating the beneficial effects of androgen receptor (AR) antagonists in the treatment of PCOS (12). Recent findings revealed that in addition to insulin resistance, excessive androgens also play a key role in the development of NAFLD in women with PCOS (13). Therefore, it is scientifically and practically accepted that there are some overlapping points between these two metabolic disorders, which requires treatment approaches that be able to target those similar abnormalities.

Lupeol (LPL), as a pentacyclic triterpenoid, is found in various fruits and vegetables. A wide range of pharmacological effects including antidiabetic, anticancer, anti-inflammatory, antioxidant, hypolipidemic, and wound healing has been related to the lupeol treatment (14). Among the other properties, its AR inhibitory effect made this compound a potential anticancer agent for prostate cancer therapy (15). Since, there are similar pathologic features of lipid accumulation, oxidative stress, inflammation, and excessive androgens in both metabolic disorders of NAFLD and PCOS, on the other hand medicinally useful effects of lupeol on aforementioned pathologic events have been demonstrated. Therefore, in this study, we hypothesized that lupeol possibly would be able to ameliorate the experimentally-induced NAFLD and PCOS in the mice model.

MATERIALS AND METHODS

Chemicals

Dehydroepiandrosterone (DHEA, D4000) and lupeol (L5632) were purchased from Sigma, USA. The hematoxylin and eosin (H&E) staining kits were supplied by Asia Pajohesh, Iran. Drugs were freshly prepared as solutions in sesame oil on a daily basis. Metformin, fructose, ethylene diamine

tetra acetic acid (EDTA), sulphanilamide, N-(1-naphthyl) ethylene diamine -2HCl, tetramethyl benzidine, 2, 4-dinitrophenylhydrazine (DNPH), thiobarbituric acid, phosphoric acid (85%), guanidine hydrochloride, and ethyl acetate were purchased from Merck (Darmstadt, Germany). N-Butanol was obtained from Carl Roth, GmbH Co. (Karlsruhe, Germany). ELISA kits for the determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Pars Azmoon (Tehran, Iran).

Animal grouping and study design

This study was performed in three steps as follows:

(1) Effects of lupeol on experimentally-induced NAFLD in mice were investigated. Twenty-four female, healthy, and adult mice (4 weeks old and body weight of 25 ± 2 g) were randomly divided into 4 groups of control (C), NAFLD (N), NAFLD plus lupeol (L, 40 mg/kg, orally) and NAFLD plus metformin (M, 500 mg/kg, orally). NAFLD was induced by using a high-fat/high-fructose diet (HFHFr) as reported previously for 8 weeks (16,17). The HFHFr diet contained 11.5% protein, 47.9% carbohydrate (starch: 30.4% and fructose: 17.5%), and 27.8% fat (soybean oil: 2.8%, hydrogenated oil: 5%, and sheep tallow: 20%). The rate of fat-related calories was calculated as 53% of all. The control diet only contained 5% fat (by weight) and 14.5% of entire calories originated from the fat content of the diet. Animals in NAFLD groups, either received the test compounds vehicle (normal saline) or metformin, and/or lupeol for the last 4 weeks of the study period. Both compounds were suspended in normal saline and were given by feeding needle (gavage).

(2) Effects of lupeol on the experimentally-induced PCOS in mice were studied. Like the previous step, this set of experiments also had 4 experimental groups including control (C), PCOS (P), PCOS plus lupeol (L, 40 mg/kg, orally), and PCOS plus metformin (M, 500 mg/kg, orally) (15). Twenty-four female mice (22-day old) were divided randomly into four groups as mentioned before. Test groups (P, M, and L) received DHEA (60 mg/kg/day, intraperitoneally) and the control group received 0.1 mL/mouse/day sesame oil for twenty consecutive days (18). Following PCOS induction, two groups of M and L, were treated with metformin and lupeol for further 28 days and the other two groups only received the test compound's vehicle (saline normal).

(3) The effects of lupeol on the simultaneously and experimentally induced NAFLD and PCOS in mice were investigated. In this set of experiments, twenty-four (22-day old) female mice were divided into 4 groups (n = 6) of control (C), NAFLD +PCOS (N+P), NAFLD + PCOS plus lupeol (N + P + L, 40 mg/kg, orally), and NAPLD + PCOS plus metformin (N+P+M, 500 mg/kg, orally). NAFLD and PCOS were induced as stated earlier. Animals in the test groups (N+P, N+P+M, and N+P+L) received HFHFr diet and DHEA injection for 8 weeks followed by 28 consecutive days of treatment with saline normal, metformin, and/or lupeol.

During the study, all animals were maintained under a controlled temperature (at 23-24 °C), lighting conditions, continuous air renovation, and allowed free access to food and water. All experimental protocols were carefully considered and ultimately approved by the Ethics Committee of Urmia University of Medical Sciences, Urmia, Iran (IR.UMSU. REC.1397.226).

Blood sampling and tissue collection

Both blood sampling and tissue collection from animals were conducted a day after the last treatment. The mice were anesthetized with a cocktail of ketamine and xylazine (ketamine: 90 mg/kg and xylazine: 9 mg/kg, intraperitoneally). After induction of deep general anesthesia, blood samples were collected *via* direct cardiac puncture and were centrifuged at 1500 *g* for 5 min to separate blood serum. The collected serum samples were stored at -20 °C until further analyses. Immediately after the blood collection, the liver and ovaries were dissected out and cleaned with chilled normal saline solution. Thereafter, the right ovary was snap frozen in liquid nitrogen and stored at -80 °C and the left ovary was fixed in 10%

formalin solution and kept at room temperature for histopathology analyses. Moreover, the liver samples were also divided into two parts and either were fixed in 10% formalin for further histopathological analyses or snap-frozen for molecular studies.

Biochemical analyses

The serum levels of ALT, AST, as hepatic functional enzymes, lipid profile (high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TG), and cholesterol, and blood glucose concentration were determined using commercially available Kits (Bayerpaul kit, Tehran, Iran).

At the same time serum level of tumor necrosis factor-alpha (TNF-α) as an inflammatory cytokine was measured by an R&D systems kit (614 McKinley Place NE Minneapolis, MN 55413, USA) according to the manufacturer’s instruction.

Hormonal assays (testosterone and insulin)

The serum levels of free testosterone and insulin hormones were measured using the commercially available ELISA kits (Monobind Inc, Lake Forest, CA 92630, USA) based on the manufacturer's instructions.

Total antioxidant capacity of serum

The ferric reducing/antioxidant power (FRAP) assay was used to analyze the total antioxidant capacity of serum samples according to the previously reported method (19). In this method, in acidic pH (created by acetate buffer, pH 3.6), the produced blue color due to the reduction of Fe^{3+} ions of the Fe (III)-2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) complex and converting them into ferrous (Fe^{2+}) ions is measured at 593 nm by a spectrophotometer.

Malondialdehyde levels in the liver and ovarian tissue

To determine the rate of lipid peroxidation, the amount of malondialdehyde (MDA) in the liver and ovarian tissues were determined by thiobarbituric acid reaction and according to the previously published method (20). The protein content of the samples was also measured according to the Lowry method (21). The MDA content of each sample was expressed as nmol of MDA per mg of protein.

Total thiol molecules assessment

The concentration of total thiol molecules (TTM) was measured in the liver and ovary samples based on the previously published method (22). The supernatant of homogenized samples was reacted with 5,5′ - dithiobis (2-nitrobenzoic acid) (DTNB, 10 mM in pure methanol) and after 15 min incubation at room temperature, the absorbance of the supernatants was assessed at 412 nm. The TTM contents of samples were expressed as nmol per mg of protein.

Nitric oxide measurement

The total nitric oxide (NO) content of the liver and ovary samples was measured according to the Griess reaction (23). In this method, NO is converted into stable nitrite, and then in an acidic environment, nitrite is converted to HNO_2. In reaction with sulphanilamide, HNO_2 produces a diazonium salt, which is in reaction with *N*-(1-naphthyl) ethylenediamine. 2HCl forms an azo dye that can be detected at a wavelength of 540 nm. The NO content of the examined organs was expressed as nmol per mg of protein in samples.

Histopathological examinations (liver and ovary)

Formalin-fixed ovarian and liver tissues after dehydration by standard graded series of ethanol were embedded in paraffin and sectioned into 5-µm sections. Sections were stained using the H&E method and analyzed using a light microscope (Nikon, Tokyo, Japan).

RNA isolation and real-time polymerase chain reaction

Total RNA was isolated from the liver and ovarian tissues using the standard TRIZOL method (24). RNA amounts were quantified spectrophotometrically, and RNA purity was determined by NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) as A260/A280 ratio with expected values between 1.8 and 2. Thereafter, cDNA was synthesized in a 20 µL reaction mixture containing 1 µg RNA,

oligo(dT) primer (1 μL), 5 × reaction buffer (4 μL), RNAse inhibitor (1 μL), 10 mM dNTP mix (2 μL), and M-MuLV reverse transcriptase (1 μL) according to the manufacturer's protocol (Fermentas, GmbH, Germany). The cycling protocol for 20 μL reaction mix was 5 min at 65 °C, followed by 60 min at 42 °C, and 5 min at 70 °C.

Quantitative polymerase chain reaction analysis for toll-like receptor-4 and -2, AR, and GAPDH

Using the Icycler MyIQ system (Bio-Rad, North Carolina, USA), cDNA from all study groups were subjected to a quantitative polymerase chain reaction (Q-PCR) in a total 25 μL reaction mix, which contained 10 μL diluted (1:9, diluted with nuclease-free water) cDNA template, 0.75 μL forward primer, 0.75 μL reverse primer, 12.5 μL IQ SYBR Green super-mix solution, and 1 μL DNA-free water. Q-PCR conditions were run as the following protocol: general denaturation at 95 °C for 3 min, 1 cycle, followed by 40 cycles of denaturation: 95 °C for 20 s; annealing: 61 °C for 30 s; elongation: 72 °C for 30 s. The specific primers for *TLR-4, TLR-2, AR,* and *GAPDH* (Table 1) were designed using NCBI primer BLAST and were manufactured commercially (CinnaGen Co. Tehran, Iran).

Statistical analyses

The results were analyzed using Graph Pad Prism software (Version 7.0; GraphPad Software Inc., San Diego, USA). The data were presented as mean ± SD and compared by one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. *P*-values < 0.05 were considered statistically significant.

RESULTS

Lupeol attenuated the NAFLD/PCOS-elevated hepatic enzymes and lipid biomarkers

lupeol and metformin significantly reduced the NAFLD-elevated hepatic functional enzymes. There were no statistically significant differences ($P > 0.05$) between the treated groups. At the same time, the NAFLD-induced increase in the blood level of lipid biomarkers including TG, TC, and LDL declined with lupeol and metformin administration. Both tested compounds increased significantly the NAFLD-reduced HDL concentration (Table 2).

Induction of PCOS in mice resulted in a significant increase in hepatic enzymes and blood levels of TG and TC. Although the experimentally-induced PCOS elevated slightly and non-significantly the blood level of LDL, the HDL concentration reduced significantly. All changes due to PCOS induction in the measured biochemical factors including hepatic functional enzymes and lipid profile were markedly lower than that of in NAFLD group. Lupeol and MET administration reduced remarkably the PCOS-related elevation of hepatic enzymes (Table 2).

We found that concomitantly-induced NAFLD and PCOS in mice resulted in a huge elevation of both hepatic functional enzyme concentrations and blood lipid factors (TG, TC, and LDL). The mentioned elevation in all items except ALT and LDL concentrations, was higher than those animals, which were NAFLD and/or PCOS positive, separately. Both lupeol and MET lowered significantly all the concurrently NAFLD/PCOS-elevated hepatic enzymes and blood lipid factors (Table 2).

Table 1. Nucleotide sequence and product size for used primers in the quantitative polymerase chain reaction.

Target gene	Gen bank accession number	Primers (5'- 3')
Androgen receptor	NM-013476.4	Forward: TAGGGCTGGGAAGGGTCTAC Reverse: CTATGTTAGCGGCCTCAGGG
Toll-like receptor-2	NM-011905.3	Forward: AGGTGCGGACTGTTTCCTTC Reverse: CTGACCGGTGATGCAATTCG
Toll-like receptor-4	NM-02197.3	Forward: CTGTTCCTCCAGTCGGTCAG Reverse: CGTCGCAGGAGGGAAGTTAG
GAPDH	NM-001289726.1	Forward: TCGTGGATCTGACGTGCCGC Reverse: ACCACCCTGTTGCTGTAGCCGTAT

Table 2. Effects of lupeol on hepatic functional enzymes and serum level of lipid parameters. Data are presented as mean ± SD. *$P < 0.05$ represents significant differences with the control group; and $^{\#}P < 0.05$ versus the respective non-treated (NAFLD, PCOS, or NAFLD + PCOS).

Groups		ALT (U/L)	AST (U/L)	TG (U/L)	TC (mg/dL)	LDL (mg/dL)	HDL (mg/dL)
NAFLD	Control	31.4 ± 4.6	89.1 ± 2.3	110.7 ± 3.1	81.1 ± 3.8	12.9 ± 4.2	83.8 ± 6.5
	NAFLD	49.5 ± 3.6*	156 ± 10.1*	142.9 ± 8.5*	106.3 ± 6.2*	31.7 ±12.2*	64.2 ± 5.3*
	Lupeol	30.7 ± 7.1$^{\#}$	88.1 ± 9.1$^{\#}$	108.7 ± 3.5$^{\#}$	76.8 ± 2.5$^{\#}$	17.4 ± 6.9	80.4 ± 7.9$^{\#}$
	Metformin	25.9 ± 0.9$^{\#}$	83.5 ± 10.1$^{\#}$	109.3 ± 1.8$^{\#}$	79.3 ± 4.1$^{\#}$	17.6 ± 7.8	77.2 ± 4.2$^{\#}$
PCOS	Control	25.5 ± 3.0	87.5 ± 5.4	106.8 ± 3.2	83.4 ± 4.6	13.1 ± 0.9	81.7 ± 7. 5
	PCOS	44.2 ± 4.1*	137.6±12.1*	117.9 ± 5.5*	100.3 ± 7.7*	15.7 ± 1.3	65.7 ± 1.6*
	Lupeol	18.2 ± 2.3$^{\#}$	100.5 ± 5.2$^{\#}$	112.8 ± 3.1	86.0 ± 6.5	13.4 ± 1.1	69.2 ± 3.0
	Metformin	28.4 ± 1.2$^{\#}$	117.8 ± 5.4$^{\#}$	105.6 ± 9.2	85.7 ± 4.1$^{\#}$	12.5 ± 0.8	68.8 ± 4.6
NAFLD + PCOS	Control	27.2 ± 2.4	85.4 ± 1.9	116.3 ± 3.8	81.3 ± 3.8	12.5 ± 1.1	74.7 ± 3.7
	NAFLD + PCOS	38.4 ± 2.2*	162.5 ± 6.1*	220.0 ± [illegible]	130.5 ± 5.2*	31.35 ± [illegible]	68.3 ± 1.7
	Lupeol	25.0 ± 3.4$^{\#}$	90.0 ± 3.5$^{\#}$	152.8 ± 6.4$^{\#}$	116.0 ± 8.2$^{\#}$	16.8 ± 1.4$^{\#}$	80.1 ± 11.3$^{\#}$
	Metformin	23.0 ± 1.8$^{\#}$	106.5 ± 8.5$^{\#}$	147.5 ± 8.5$^{\#}$	106.4 ± 4.1$^{\#}$	22.6 ± 0.6$^{\#}$	85.2 ± 6.0$^{\#}$

NAFLD, Non-alcoholic fatty liver disease; PCOS, polycystic ovarian syndrome; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AR, androgen receptor; TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Table 3. Effects of LPL on serum levels of glucose, Insulin, DHEA, and free testosterone. Data are presented as mean ± SD. *$P < 0.05$ represents significant differences with the control group; and $^{\#}P < 0.05$ versus the respective non-treated (NAFLD, PCOS, or NAFLD + PCOS).

	NAFLD			PCOS			NAFLD + PCOS		
Groups	**FBG**	**FBI**	**IRI**	**Groups**	**DHEAs**	**T**	**Groups**	**IRI**	**T**
Control	108 ± 2.6	8.5 ± 1.0	46.1 ± 1.8	Control	2.1± 0.07	0.48 ± 0.01	Control	42.6 ± 2.5	0.44 ± 0.02
NAFLD	134.3 ± 12.1*	15.1 ± 1.7*	72.5 ± 5.6*	PCOS	2.8 ± 0.36*	0.74 ± 0.06*	NAFLD + PCOS	100.5 ± 13.9*	0.7 ±0.04*
Lupeol	78.7 ± 6.5	10.2 ± 1.9$^{\#}$	43.4 ± 6.1$^{\#}$	Lupeol	1.75 ± 0.05$^{\#}$	0.36 ± 0.05$^{\#}$	Lupeol	50.6 ± 1.7$^{\#}$	0.38 ± 0.01$^{\#}$
Metformin	83.7 ± 11.8$^{\#}$	8.3 ± 1.2$^{\#}$	34.4 ± 6.7$^{\#}$	Metformin	1.6 ± 0.03$^{\#}$	0.39 ± 0.02 $^{\#}$	Metformin	52.0 ± 5.3$^{\#}$	0.50 ± 0.01$^{\#}$

NAFLD, Non-alcoholic fatty liver disease; PCOS, polycystic ovarian syndrome; FBG, fasting blood glucose (nMol/L); FBI, fasting blood insulin (µU/mL); DHEA, dehydroepiandrosterone (ng/mL); T, free testosterone (pg/mL).

Lupeol reduced the NAFLD/PCOS-elevated insulin resistance index and androgen hormones

Induction of NAFLD in mice resulted in higher concentrations of blood glucose and insulin and consequently a higher insulin resistance index (IRI) when compared with the control group. Both lupeol and MET at the given dose levels significantly returned elevated IRI values. On the other hand, in PCOS-positive animals, the serum level of androgens including DHEA and testosterone enhanced remarkably, and the lupeol and MET administration for 28 days attenuated the enhanced concentrations significantly (Table 3). We found that the administration of lupeol and MET markedly decreased the IRI level and also testosterone concentration in the NAFLD/PCOS-positive mice (Table 3).

Lupeol declined the NAFLD/PCOS-elevated oxidative/nitrosative biomarkers

MDA content of target organs (liver and ovary) as a known indicator of lipid peroxidation and the concentration of total thiol molecules as an index of glutathione resources were measured. The aforementioned biomarkers were changed remarkably in both experimental models of NAFLD and PCOS, either in individual or concurrent forms. We found that in either metabolic disease, MDA content was elevated and by contrast, the TTM concentration significantly declined in non-treated groups. At the same time, a 3-4-fold reduction in total antioxidant capacity as a general biomarker of antioxidant status was found in the non-treated groups of the study. Lupeol and MET were able to reduce the MDA content and enhance the

mentioned metabolic diseases-reduced TTM concentrations in both target organs and consequently, they could recover the reduced total antioxidant capacity.

Another analyzed biomarker was the NO concentration in the involved organs of the experimentally-induced NAFLD and PCOS. We found that NAFLD and PCOS in both individual and simultaneous forms resulted in a significant elevation of NO concentration in mentioned organs. The measured concentration of NO in lupeol and MET-received groups remarkably declined (Table 4).

Lupeol reduced the NAFLD/PCOS-increased TNF-α concentration

Serum level of TNF-α as one of the crucial pro-inflammatory factors was measured in all three sets of study. We found that NAFLD and PCOS individually or concurrently elevated the TNF-α concentration significantly. Although lupeol in NAFLD-positive animals and MET in PCOS-positive mice were the potent ones in the reduction of TNF-α concentration, in the animals, which were simultaneously NAFLD- and PCOS-positive, there was no statistically significant difference between the two given compounds. Both tested substances declined the TNF-α concentration in NAFLD and PCOS-positive animals (Fig. 1).

Histopathologic findings

The liver tissue of the control group in the NAFLD set of the study showed a normal histologic appearance. In animals with positive NAFLD, fatty changes in the form of microvesicular steatosis were characterized by small white cavities in the cytoplasm and hypertrophy of hepatocytes. In addition, hepatocyte apoptosis was associated with cell shrinkage, condensed and deeply eosinophilic cytoplasm, and pyknotic nuclear material. There was lobular inflammation associated with the accumulation of inflammatory cells as well as the presence of pigmented macrophages (Kupfer cells). In the group of animals that were treated with lupeol, there were fewer fatty changes and the cytoplasm of hepatocytes was very similar to that in the control group. Inflammatory cell accumulations were observed with a much smaller number of cells. Hepatocyte apoptosis was not recorded. In the MET group, slight fatty changes with microvesicular steatosis were observed along with small masses of inflammatory cells. Hepatocyte apoptosis was not seen (Fig. 2).

Table 4. Effects of lupeol on oxidative/nitrosative biomarkers in the liver and ovary and total antioxidant capacity in serum. Data are presented as mean ± SD. $^{*}P < 0.05$ represents significant differences with the control group; and $^{\#}P < 0.05$ versus the respective non-treated (NAFLD, PCOS, or NAFLD + PCOS).

Groups		MDA (nmol/mg of protein	TTM (nmol/mg of protein)	NO (nmol/mg of protein)	TAC (nmol of Fe_2SO_4)
NAFLD (liver)	Control	12.1 ± 0.4	3.9 ± 0.30	107.0 ± 4.0	38.1 ± 1.8
	NAFLD	23.9 ± 1.1*	0.28 ± 0.02*	151.0 ± 13.1*	12.8 ± 0.8*
	Lupeol	17.0 ± 0.7#	3.6 ± 0.10#	87.0 ± 6.5#	26.9 ± 2.4#
	Metformin	13.7 ± 0.4#	3.3 ± 0.20#	111.0 ± 9.8#	18.9 ± 0.5#
PCOS (ovary)	Control	13.2 ± 1.3	3.1 ± 0.50	109.4 ± 4.1	41.7 ± 0.7
	PCOS	18.5 ± 0.4*	0.45 ± 0.03*	167.7 ± 1.9*	13.5 ± 0.5*
	Lupeol	10.6 ± 1.1#	1.7 ± 0.05#	93.5 ± 2.1#	27.1 ± 2.5#
	Metformin	13.9 ± 0.8#	1.03 ± 0.20#	95.8 ± 9.1#	26.7 ± 0.7#
NAFLD + PCOS (liver)	Control	16.8 ± 0.3	2.6 ± 0.14	126.3 ± 19.1	46.2 ± 1.4
	NAFLD + PCOS	24.7 ± 0.4*	1.4 ± 0.10*	203 ± 7.5*	9.6 ± 1.8*
	Lupeol	19.5 ± 0.8#	1.8 ± 0.05#	165 ± 9.2#	26.7 ± 1.3#
	Metformin	21.3 ± 0.5#	1.75 ± 0.15#	174 ± 12.7#	21.3 ± 0.5#
NAFLD + PCOS (ovary)	Control	13.91 ± 1.30	3.01 ± 0.15	111.2 ± 1.4	46.2 ± 1.4
	NAFLD + PCOS	18.5 ± 0.35*	0.43 ± 0.03*	167.7 ± 4.9*	9.6 ± 1.8*
	Lupeol	10.7 ± 2.05#	1.75 ± 0.05#	93.4 ± 2.01#	26.7 ± 1.3#
	Metformin	13.9 ± 0.80#	1.03 ± 0.27#	95.1 ± 9.1#	21.3 ± 0.5#

NAFLD, Non-alcoholic fatty liver disease; PCOS, polycystic ovarian syndrome; MDA, malondialdehyde; TTM, total thiol molecules; NO, nitric oxide; TAC, total antioxidant capacity.

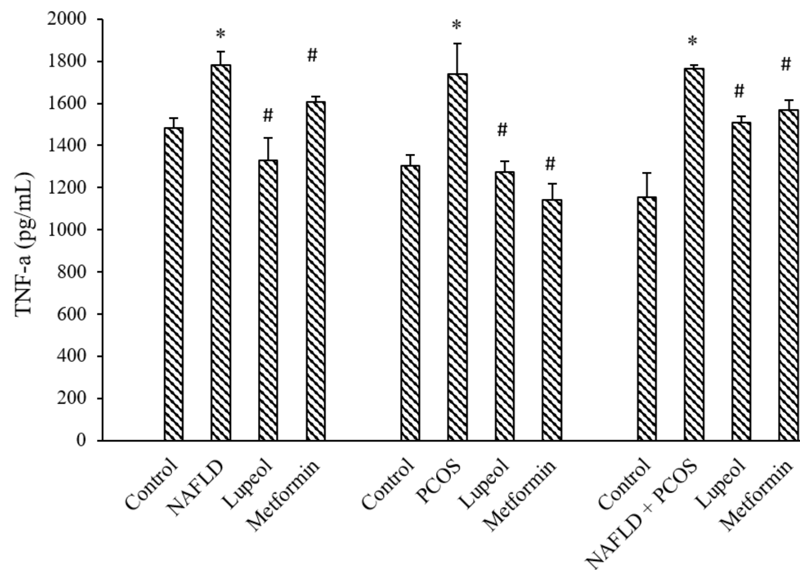

Fig. 1. Effect of lupeol on NAFLD/PCOS-elevated TNF-α concentration. *$P < 0.05$ represents significant differences the control group; and $^{\#}P < 0.05$ versus the respective non-treated (NAFLD, PCOS, or NAFLD + PCOS). NAFLD, Non-alcoholic fatty liver disease; PCOS, polycystic ovarian syndrome; TNF-α, tumor necrosis factor-alpha.

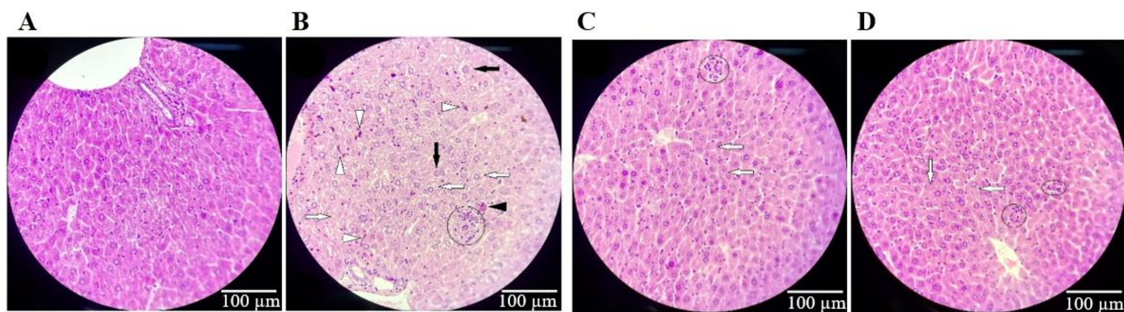

Fig. 2. Liver photomicrographs of (A) control, (B) nonalcoholic fatty liver disease, (C) lupeol, and (D) metformin groups. Microvesicular steatosis (white arrow), hepatocyte hypertrophy (black arrow), hepatocyte apoptosis (black arrowhead), pigmented macrophages (white arrowhead), inflammatory cell infiltration (within circles). Hematoxylin and eosin staining; magnification equals 400×.

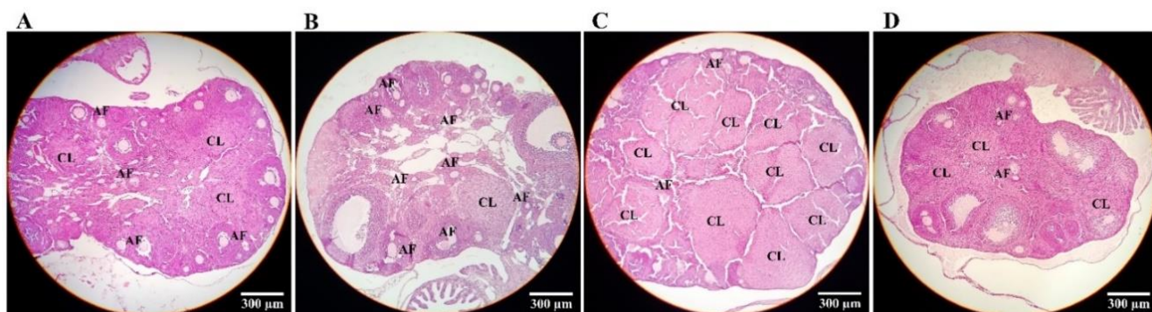

Fig. 3. Photomicrographs from the ovary. (A) The control group; (B) polycystic ovarian syndrome; (C) lupeol; and (D) metformin. AF, Atretic follicle; CL, corpus luteum. Hematoxylin and eosin staining; magnification equals 100×.

Induction of PCOS with DHEA resulted in morphological changes in the ovary such as increasing the number of atretic follicles with abnormal granulosa layer, while administration of lupeol substantially and MET slightly resulted in an increased number of corpora lutea and remarkably reduced the number of atretic follicles (Fig. 3).

In the third set of this study both metabolic disorders (NAFLD and PCOS) were simultaneously induced in mice and following the proposed treatment program, it has been found in the histopathological examinations that the non-treated animals not only showed macrovesicular steatosis in the liver tissue along with infiltrated inflammatory cells (Fig. 4A) but also illustrated an elevated number of atretic follicles (Fig. 4B).

Lupeol regulated the NAFLD and PCOS up-regulated AR and TLRs expression

Induction of NAFLD and PCOS both individually and simultaneously in either target organ resulted in a significant up-regulation of AR expression at the mRNA level. We found the highest rate of AR up-regulation in the ovary of non-treated PCOS-positive animals. Lupeol administration resulted in a substantial down-regulation of AR both in the liver and ovary in both models of study. Metformin, on the other hand, slightly regulated the AR up-regulation only in the ovary (Fig. 5A).

Expression of TLR-2 and -4 as known involved genes in the mediation of inflammatory reactions were analyzed and the results revealed that both genes albeit with minor differences have been upregulated due to experimentally-induced NAFLD and PCOS or both. Lupeol was able to regulate the expression of both mentioned genes in the liver and ovary. Metformin although reduced the NAFLD/PCOS-induced up-regulation of TLR-4 in the liver and ovary, its effect on the expression of TLR-2 was found significant only in the ovary of animals, which simultaneously were NAFLD/PCOS positive (Fig. 5B and C).

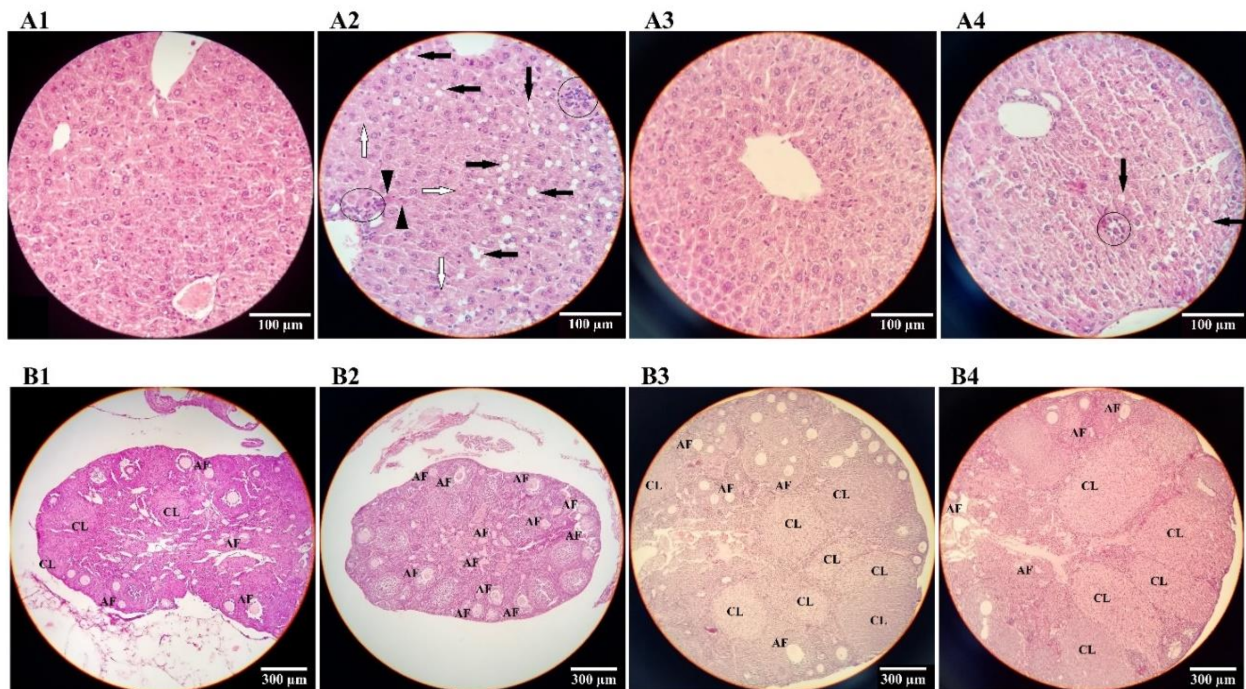

Fig. 4. Photomicrographs from (A) the liver: (A1) control, (A2) NAFLD, (A3) lupeol, and (A4) metformin groups, magnification: 400×; and (B) the ovary tissues: (B1) control, (B2) PCOS, (B3) lupeol, and (B4) metformin, magnification 100×; illustrating macrovesicular steatosis (black arrows) and inflammatory cells infiltration (circles) and the high number of AF in the ovary of the non-treated NAFLD- or PCOS-positive animals. NAFLD, Nonalcoholic fatty liver disease; PCOS, polycystic ovarian syndrome; AF, atretic follicle; CL, corpus luteum. Hematoxylin and eosin staining.

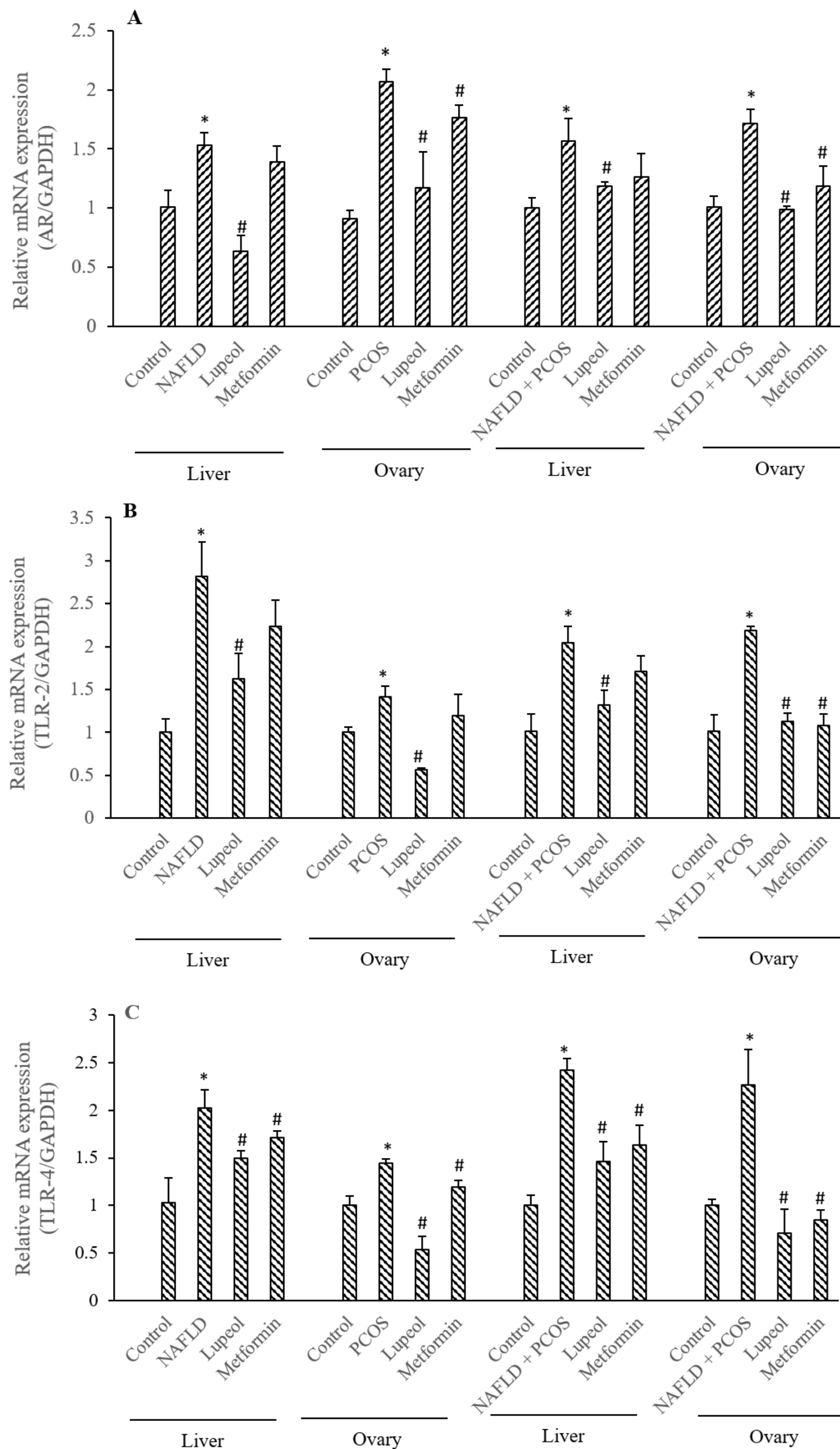

Fig. 5. Effects of lupeol and metformin on the expression of (A) AR, (B) TLR-2, and (C) TLR-4 in the liver and ovary. $^{*}P < 0.05$ represents significant differences with the control group; and $^{\#}P < 0.05$ versus the respective non-treated (NAFLD, PCOS, or NAFLD + PCOS). NAFLD, Non-alcoholic fatty liver disease; PCOS, polycystic ovarian syndrome; AR, androgen receptor; TLR, toll-like receptor.

DISCUSSION

In the current preclinical study, we used two animal models of NAFLD and PCOS to test the possible therapeutic effects of lupeol. Results indicated that lupeol is able to ameliorate the biochemical, oxidative, histopathological, and molecular changes due to both NAFLD and

PCOS in individual and simultaneous models. As the prerequisite of the study, based on changed markers including histopathological findings and other tested endpoints, the accuracy of either experimentally-induced disorders has been confirmed. There are too many approaches to induce the mentioned disorders, while we tried to use the methods, which are very similarly occurring in human life. For example, using high-fat and high-fructose diets to induce NAFLD has been implemented due to the fact that humans also take both high-fat diets and high-fructose ones through high-fructose-containing diets such as corn syrup. Fructose not only stimulates *de novo* lipogenesis, oxidative stress, and insulin resistance but also with increasing the produced endotoxin of intestinal microbiota results in hepatic macrophage activation and ultimately hepatic inflammation (25). It is worth to be noted that the high fructose method alone is not able to produce a NAFLD phenotype and needs to be accompanied by a high-fat diet to supply the other required elements for lipogenesis (26). At the same time, using the DHEA-induced PCOS model, due to representing the human form of PCOS with characteristics such as anovulation, polycystic ovaries, and hyperandrogenism was implemented in the current study (27). Reports are indicating that DHEA-injected female mice showed a significant increase in body weight and fat mass along with impaired glucose tolerance and dyslipidemia, which are the closest feature of clinically PCOS-positive diagnosed women (18).

Our results in the first set of the study which was performed to highlight the possible effects of lupeol on the experimentally-induced NAFLD, revealed that all biochemical changes including the elevated serum level of hepatic functional enzymes along with markedly produced dyslipidemia were attenuated with lupeol and relatively with MET administration. These results indicated that lupeol not only could protect against NAFLD-induced hepatic injuries but also should be able to ameliorate the damages caused by NAFLD as it has been administered for the last 4 weeks of the 12-week study period. As mentioned earlier, HFHFr-induced NAFLD was characterized by stimulated lipogenesis, which reflected a remarkable elevation of lipid profile indices. Lupeol-treated mice, however, showed a remarkable reduction of TG, TC, and LDL suggesting its antidyslipidemic effects, which may partly relate to the capacity of LPL in the reduction of cholesterol absorption (28). It has been previously reported that LPL and its linoleate derivative were effectively able to reduce the high cholesterol-induced renal damage in the rodent model of the study (29). Another key point that was taken into consideration in the current study was to measure the glucose, insulin, and homeostatic model assessment (HOMA) index values to highlight the role of insulin resistance and changes of mentioned indices in the mediation of NAFLD and PCOS and equally effects of LPL administration on their improvement. Our results showed that LPL in experimentally-induced NAFLD effectively alleviated the increased HOMA IR values, indicating its significant effects in the reduction of HFHFr-elevated glucose and insulin levels. To explain how LPL can regulate blood glucose and insulin level, it would be worth to notice to its glycolytic activity and also its capacity in the promoting liver to utilize glucose by increasing insulin secretion in diabetic rats (30).

According to the previous studies that elucidated the molecular events in the NAFLD and PCOS disorders, we found there are other common biomarkers, which change during both disorders including an excessive generation of reactive oxygen/nitrogen species (ROS and RNS), the elevation of inflammatory cytokines, and depletion of cellular glutathione (31,32). Excessive fatty acid oxidation, which is reported in the current study and documented with much previous evidence, results in the abnormal generation of ROS and RNS in particular from mitochondria and reduction of cellular antioxidant capacity (33). An increasing quantity of evidence indicates that an excessive generation of ROS/RNS will in turn increase lipid peroxidation (elevated MDA biomarker), especially in the mitochondrial membrane, which further will cause a remarkable reduction of cellular respiratory activities. Also, it has been reported that the extramitochondrial lipid peroxidation and

consequently cellular respiratory activity reduction will elevate TNF-α and other cytokines production, which has been confirmed in this study (34,35). Our results clearly confirmed all the previous reports as both the generation of ROS/RNS in the experimentally-induced disorders were elevated and the glutathione depletion also was recorded. Our findings indeed showed that LPL with a significant reduction in ROS/RNS generation and recovering the depleted glutathione level resulted in lower TNF-α and hepatic functional enzymes production, confirming its antioxidant and anti-inflammatory effects and also its medicinal use in the treatment of both disorders. The anti-inflammatory effect of LPL on carrageenan-induced acute inflammation in rodents and also on macrophage pro-inflammatory phenotype reduction *in vitro* model have been recently documented (36,37). There are also reports supporting the fact that mitochondria-targeted antioxidant, which is so-called Mito-Q10, with reduction of experimentally-induced PCOS injuries including elevated HOMA-IR and MDA levels and reduced glutathione content, was able to decline the mitochondria-mediated apoptotic cell death in the ovary (38).

Our findings indicated that LPL substantially and MET relatively could recover the histopathological damages in either examined organs of the liver and ovary. The histopathological findings primarily indicate that the presence of microvesicular steatosis in NAFLD-positive animals and macrovesicular steatosis in NAFLD- and PCOS-positive animals could be due to the disorder in lipid metabolism and other metabolic abnormalities including insulin resistance. Moreover, infiltration of proinflammatory cells that are accompanied by elevated hepatic functional enzymes, suggests that in both forms of individual and simultaneous NAFLD + PCOS, there is chronic inflammation. The impaired folliculogenesis on the other hand, which has been demonstrated in non-treated PCOS-positive animals, could be due to excessive androgen production (39). All mentioned histopathological and hormonal changes suggest a chronic insulin resistance that increased insulin secretion promotes the tropic action of luteinizing hormone on theca cells and ultimately elevates androgen production (40). Therefore, increased androgen and insulin secretion results in apoptosis promotion in antral follicles which was demonstrated by a considerable increase of atretic follicles in non-treated PCOS-positive animals (41). Along with other factors including genetic bases, hyperandrogenism in PCOS-positive animals may explain the androgen-related hypertrophy of adipocytes that in turn leads to tissue hypoxia and low-grade chronic inflammation, which ultimately results in follicular atresia (42). It has been recently reported that LPL acts as an anti-inflammatory agent by inhibiting the M1 phenotype of macrophages and promoting the M2 form in rats with diet-induced metabolic syndrome (43).

Since in both individual and simultaneous models of NAFLD and PCOS, we recorded the major characteristics of a metabolic syndrome including dyslipidemia, hyperinsulinemia, and low-grade inflammation; therefore, it merits a mention to examine the expression level of key players at the mRNA level and in both target tissues. An increasing number of reports indicate the activation of TLRs as pattern recognition receptors, stimulating inflammatory reactions, oxidative events, and ultimately insulin resistance. Along with endotoxins (LPS), saturated fatty acids and oxidized LDL are the known stimulators of TLRs (44). Lupeol in all models of study and in both target tissues (liver and ovary) downregulated the NAFLD- and PCOS-up-regulated expression of TLR-2 and TLR-4, suggesting its anti-inflammatory effects at the molecular level and providing considerable evidence to further investigate its capacity as a therapeutic agent for metabolic syndrome cases. Additionally, LPL could regulate the AR expression in the liver and ovary, supporting the idea that hyperandrogenism and insulin resistance are on the same pathway of PCOS induction. Although the inhibitory effect of LPL on androgen receptors in human prostate cancer cells has been reported before, evidence to show the downregulation of AR expression at mRNA level and in the PCOS model is scarce yet (15).

There are a growing number of reports illustrating the closest relationship between two studied disorders and directing the therapeutic strategies toward providing an effective protocol for metabolic syndrome. Recent reports have indicated a high frequency of NAFLD-positive women among those who are suffering from PCOS, which contributes to the excessive production of androgens, confirming a biochemical and clinical relationship between two metabolic disorders (13,45).

CONCLUSION

In this study and 3 sets of experiments, we showed and confirmed the biochemical, hormonal, histopathological and molecular changes in the NAFLD and PCOS individually and in concurrent forms. We found that these two disorders do have some common features including dyslipidemia and elevated HOMA IR, which resemble the symptoms of metabolic syndromes. Moreover, LPL as a dietary triterpene could ameliorate the NAFLD and PCOS-produced damages. Lupeol's ameliorative effects contribute to its anti-lipidemia, antioxidant, anti-inflammatory, and anti-androgenic properties.

Acknowledgments

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

H. Malekinejad designed and performed the experiments, drafted, revised, and discussed the manuscript; Sh. Zeinali-Mogaddam performed some experiments; A. Rezaei-Golmisheh performed experiments and analyzed the data; A. Alenabi and A. Alizadeh helped in the experiment performing and data analyses; F. Malekinejad helped in drafting the first version of the manuscript and some experiments performed; V. Shafie-Irannejad reviewed the manuscript. The finalized article was approved by all authors.

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