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Original Research Article

The protective effect of alcoholic extract of *Dactylorhiza lancibracteata* roots on the liver tissue in adult rats with polycystic ovarian syndrome (PCOS)

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Abstract

Objective: The role of oxidative stress in PCOS-related non-alcoholic fatty liver disease is well recognized. This study aimed to investigate the protective effect of Salep root (*Dactylorhiza lancibracteata*) alcoholic extract on liver tissue in adult female rats with PCOS.

Materials and Methods: Fifty-six female Wistar rats were divided into seven groups including control, PCO, Salep 320mg/kg, and PCO groups treated with Salep extract (orally) at 40, 80, 160, or 320 mg/kg. PCOS was induced by intramuscular injection of estradiol-valerate (4mg/kg). Water-soluble extract was administered from day 61, once a day for 28 days. Liver function and oxidative stress markers were measured, and stereological analysis was conducted.

Results: PCOS significantly increased aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, triglycerides, glucose, and oxidative stress markers (total oxidant capacity and malondialdehyde, while reducing antioxidant capacity (total antioxidant capacity and glutathione peroxidase). Salep extract dose-dependently ameliorated these changes, with the 320mg/kg showing the greatest effects. Histological and stereological analyses confirmed reduced hepatic damage and inflammation in treated groups.

Conclusion: Salep root extract demonstrated significant hepatoprotective and antioxidative effects in PCOS rats by restoring oxidant–antioxidant balance. These findings support its potential as a natural therapeutic supplement for managing PCOS-associated liver dysfunction, warranting future clinical investigations.

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Introduction

Polycystic ovary syndrome (PCOS), one of the most common causes of female infertility, is associated with various medical symptoms, including decreased or absent ovulation, hyperandrogenism, and insulin resistance (Ashraf et al. 2019; Balen and Rutherford 2007; Fica et al. 2008). OS results from an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense mechanisms. In PCOS, excessive production of ROS leads to lipid peroxidation, DNA damage, and protein modification, which disrupt normal cell function (Mohammadi 2019; Murri et al. 2013; Uçkan et al. 2022). A meta-analysis has shown that OS levels are significantly higher in PCOS patients compared to control women (Murri et al. 2013). High concentrations of homocysteine, asymmetric dimethylarginine (ADMA), and malondialdehyde have been observed in these patients (Bayram et al. 2012; Janati et al. 2022; Murri et al. 2013). Chronic inflammation associated with PCOS exacerbates OS and creates a feedback loop that perpetuates cellular damage and dysfunction in ovarian tissues (Luo et al. 2020; Masjedi et al. 2019). This oxidative damage can impair follicular development and ovulation, thereby contributing to the reproductive symptoms of PCOS (Hyderali and Mala 2015; Mohammadi 2019; Zuo et al. 2016a).

The relationship between PCOS and non-alcoholic fatty liver disease (NAFLD) is complex and multifaceted. Approximately 47% of patients with PCOS are also diagnosed with NAFLD (Paschou et al. 2020; Spremović Rađenović et al. 2022; Vassilatou 2014). OS plays a central role in the pathophysiology of NAFLD. Increased ROS in the liver increases lipid peroxidation and inflammation, leading to liver cell damage and fibrosis (Bovi et al. 2021; Delli Bovi et al. 2021). Factors such as racial and ethnic differences, genetics, cytokines and insulin have been considered in understanding this relationship (Chen et al. 2022; Sharp 2021; Zhao et al. 2023). Both obesity and insulin resistance are common side effects of PCOS and NAFLD. NAFLD contributes to insulin resistance and liver deterioration. Insulin resistance, a common feature of PCOS and NAFLD, further exacerbates this condition by increasing lipolysis in adipose tissue and increasing delivery of free fatty acids to the liver (Arvanitakis et al. 2024; Rudnicka et al. 2022). This leads to hepatic steatosis and progression to more severe liver injury (Holterman et al. 2013; Zhao et al. 2023). In NAFLD, insulin resistance stimulates hepatic stellate cells (HSCs) to produce collagen and fibrinogen, contributing to liver fibrosis (Roehlen et al. 2020). Reactive oxygen species (ROS) produced by insulin resistance and chronic inflammation can exacerbate liver injury and lead to increased levels of liver enzymes including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), as well as increased serum triglycerides and cholesterol (Falzarano et al. 2022; Ma et al. 2011; Vassilatou 2014).

Salep root (*Dactylorhiza lancibracteata* (C. koch) Renz), a member of the Orchidaceae family, contains bioactive compounds such as glucomannan fiber, polyphenols, ferulic acid, quercetin, and steroids. These compounds are known for their antioxidant properties, which can reduce OS and its harmful effects on the liver and ovarian tissue (Atashpour et al. 2017; Kurt and Kahyaoglu 2017).

Glucomannan, a water-soluble fiber found in the root of the Salep, has received particular attention for its role in weight loss, blood sugar control, and cholesterol reduction (Marcolin et al. 2019). Studies have shown that glucomannan can inhibit OS , effectively reduce the levels of ALT and AST, and enhance the antioxidant system in rats fed a high-fat diet by increasing hepatic superoxide dismutase (SOD) and catalase (CAT), while simultaneously decreasing

malondialdehyde levels (Dönmez and Keskin 2008; Dvorska et al. 2007; Liu et al. 2021).

In addition, flavonoids in Salep root exert protective effects against arteriosclerosis and modulate liver enzymes activity, thereby contributing to improved liver function and overall hepatic health (MADANI et al. 2007; Radjabian and FALLAH 2010).

Salep root also contains a diverse set of bioactive compounds that contribute to its therapeutic potential. In addition to polyphenols and flavonoids, its water-soluble fiber content supports metabolic regulation by lowering blood glucose and cholesterol, thereby enhancing antioxidant defense and liver health (Atashpour et al. 2023; Tekinşen and Güner 2010). Additionally, plant flavonoids such as quercetin and kaempferol exhibit strong anti-inflammatory and hormone-regulating effects, which may support ovarian and liver function in PCOS conditions (Pourteymour Fard Tabrizi et al. 2020).

The root also contains polyphenols, including ferulic acid, which is known to reduce oxidative damage and protect liver tissue by modulating liver enzymes such as ALT and AST (Atashpour et al. 2017; Pourahmad et al. 2015). The steroidal compounds found in Salep may further affect endocrine pathways and help restore the hormonal balance disrupted in PCO. Additionally, volatile organic compounds (VOCs) such as caryophyllene, β-cadinene, ocimene, and farnesene—identified in *Dactylorhiza*-related species—may have additional antioxidant and anti-inflammatory effects (Atashpour et al. 2017). These synergistic compounds provide the pharmacological foundation for investigating Salep as a natural intervention in PCOS and associated hepatic disorders.

The aim of this study was to investigate the protective effect of Salep root alcoholic extract on the liver tissue of adult female rats with experimentally-induced PCOS. Considering the common pathophysiology of PCOS and NAFLD and the known antioxidant properties of Salep root, this study seeks to evaluate the potential of Salep root extract as a therapeutic agent in reducing liver damage associated with PCO.

Materials and Methods

Preparation of salep root extract

The tuberous roots of *Dactylorhiza maculata* (IAUJ-DL-1037) were collected from Iran, powdered and mixed with 96% ethanol at a ratio of 5:1 for 24 hr using a rotary mixer. The homogenous solution was then filtered and the ethanol was evaporated to obtain an alcohol-free solid. This solid was dissolved in double distilled water and refrigerated until use (Jahromi et al. 2018; KARGAR et al. 2020).

Animals and housing

Fifty-six female Wistar rats weighing 180 to 200 grams were used in this study. The animals were kept under standard conditions with a light/dark cycle of 12:12 hr at a temperature of 20-25°C and a humidity level of 50-55%. They had free access to food and water. Ethical guidelines for the use of animals in research were followed and the study was approved by the ethics committee of Jahrom University of Medical Sciences (ethics code: IR.JUMS.REC.1394.200).

Experimental design

The animals were randomly divided into seven groups, each group containing eight rats: Control Group, animals without any treatment under normal conditions for 89 days. PCO Groups: animals received 4 mg estradiol valerate (1 dose at first day) and waited for 60 days to ensure stabilization of the PCOS model. Extract Group (SA): Healthy animals received 320 mg of Salep extract for 28 days. Treatment Groups (PCO+Sa (1-4)): After confirmation of PCOS induction, treatment was initiated with Salep extract at different doses for 28 consecutive days (Figure 1). PCO animals received 40, 80, 160, or 320

mg of Salep extract for 28 days. Estradiol Valerate (4 mg/kg) was administered with a single injection into the hamstring muscle. To prove the induction of PCOS, blood samples were collected, and the levels of estrogen, progesterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and free testosterone were evaluated. were evaluated.

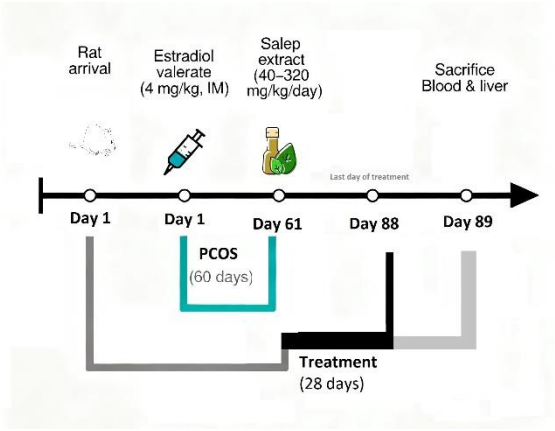


Figure 1. Experimental timeline of PCOS induction and Salep extract treatment in rats. Female Wistar rats (n=56) received a single intramuscular injection of estradiol valerate (4 mg/kg) on day 1 to induce polycystic ovary syndrome (PCOS). After 60 days of PCOS stabilization (days 1–60), animals were treated once daily by oral gavage with Salep root alcoholic extract at doses of 40, 80, 160, or 320 mg/kg/day or vehicle for 28 consecutive days (days 60–88). On day 89, all animals were sacrificed, and blood and liver samples were collected for biochemical, oxidative stress, and stereological analyses. Total study duration: 89 days.

Induction of PCOS

To establish the PCOS model, estradiol valerate was performed under sterile conditions and was based on validated protocols reported in previous studies (Abedi et al. 2024a; Abedi et al. 2024b). The injection causes chronic estrogen exposure, mimicking endocrine imbalance and ovarian dysfunction characteristic of PCOS. The exposure period was 60 days to allow for the full development of cystic ovarian morphology and metabolic changes. Successful induction of PCOS was confirmed by hormonal assays and histopathological examination of ovarian tissues. PCOS model developed systemic metabolic abnormalities including elevated liver enzymes, triglycerides, and markers of oxidative stress that are consistent with the pathology of NAFLD. Therefore, NAFLD features emerged as a secondary outcome of the PCOS model, rather than from a separate induction protocol (Abdollahi et al. 2024).

Sample collection and analysis

At the end of the study (**day 89**), blood samples were collected from the hearts of animals under anesthesia with ketamine (100 mg/kg, intraperitoneal injection) and xylazine (20 mg/kg, intraperitoneal injection). Serum levels of ALT (Man Company-Iran- Lot No.: Pt93336), AST (Man Company-Iran- Lot No.: Ot8106), ALP (Man Company-Iran- Lot No.: AL37S6), Triglyceride (Man Company-Iran- Lot No.: Pt93336) and Glucose (Biorexfars Company-Iran- REF n.: BXC0101A) were measured by a photometric method and using Selectra XL biochemistry auto analyzer- Netherlands. total oxidant capacity (TOC) (Crystal Day Company-China- Lot No.: E1512Ra), TAC (Crystal Day Company-China- Lot No.: E0871Ra), glutathione peroxidase (GPX)(Crystal Day Company-China- Lot No.: E0814Ra) and malondialdehyde (MDA)(Crystal Day Company-China- Lot No.: E0156Ra) were measured by ELISA Kits and using ELISA reader Stat Fax 2100- United States in laboratory of Shahid Motahhari Hospital-Jahrom-Iran. Blood glucose level was measured using a glucometer (Accu Check- Germany) at the first of the experiment.

Histological analysis

After ensuring deep unconsciousness, animals were sacrificed by decapitation using a guillotine. After animals' scarification, by decapitation using a guillotine, their livers were carefully dissected, weighed and fixed in 10% formalin solution. Tissue sections were prepared and stained with hematoxylin-

eosin and Sudan black. The slides were examined using a light microscope (Olympus BX41, United States), and histopathological changes were evaluated.

Stereological study

In the final stage, the weight of the rats was measured before dissection. Then the livers were weighed and the primary volume (V_primary) was determined using Scherle method (Zare et al. 2019). Isotropic uniform random sections were obtained using orientation method. On average, 9-12 slabs were randomly selected from each liver. A circle was punched out of a liver plate using a trocar. All collected slabs and circular pieces were embedded in the same paraffin block. Sections with a thickness of 5 µm and 25 µm were obtained.

After staining 25-micrometer tissue sections with hematoxylin-eosin, they were mounted with a coverslip. The diameter of the circular slice of the liver and the area of the circle were again measured to obtain the amount of liver tissue shrinkage. The degree of contraction was calculated using the following formula:

$$\text{Degree of shrinkage} = 1 - \left(\frac{A_A}{A_B}\right)^{1.5}$$

Where A_A is the area of the circular piece after handling and staining, and A_B is the area of the circular piece before handling and staining.

For estimation of Liver Structure Stereology and Pathology in the Experimental Group the following formula was used. This formula assesses the total volume of hepatocytes, portal triad, sinusoids, central veins, connective tissue (fibrosis), and inflammatory areas:

$$Vv(structure) = \sum_{i=1}^{n} p\,(structure) \Big/ \sum_{i=1}^{n} (reference)$$

Where ∑P (structure) is the number of points hitting the profiles of hepatocytes, sinusoids, central veins, connective tissue, and inflammatory areas, and ∑P (reference) is the number of points hitting the liver.

The total volume of the liver structures was calculated as:

V (structure) = Vv (structure/liver) × V final

The total number of Kupffer cells, hepatocytes and hepatocyte nuclei:

Using Stereolite software and the optical dissection method, the total number of Kupffer cells, hepatocytes and hepatocyte nuclei was evaluated using the following formula:

$$Nv = \frac{\sum_{i=1}^{n} Q}{\sum_{i=1}^{n} P \times h \times \left(\frac{a}{f}\right)} \times \frac{t}{BA}$$

where ∑Q is the total number of Kupffer cells, hepatocytes, and hepatocyte nuclei counted in all dissectors, h is the height of the optical dissector, a/f is the area of the counting frame, ∑P is the total number of counted frames, BA is the microtome block advance to cut the block, and t is the mean final section thickness (Figure 2).

To number and volume of hepatocyte nuclei a counting frame was placed on the liver tissue, and cells within the counting frame were selected by touching the acceptance lines (upper and right borders) and not the rejection lines (lower and left borders). The distance from the center of the cell nucleus to the cell membrane was measured to estimate the cell volume, and the distance from the center of the nucleus to the nuclear membrane was measured to estimate nuclear volume in four vertical directions (L1, L2, L3, and L4). The following formula was used to estimate the volume of hepatocytes and their nuclei:

$$V = \frac{4}{3}\pi \bar{L}_n^3$$

$$\bar{L}_n^3 = \frac{\overline{L_1^3 + L_{2+}^3 L_{3+}^3 L_4^3 + \cdots + n}}{n}$$

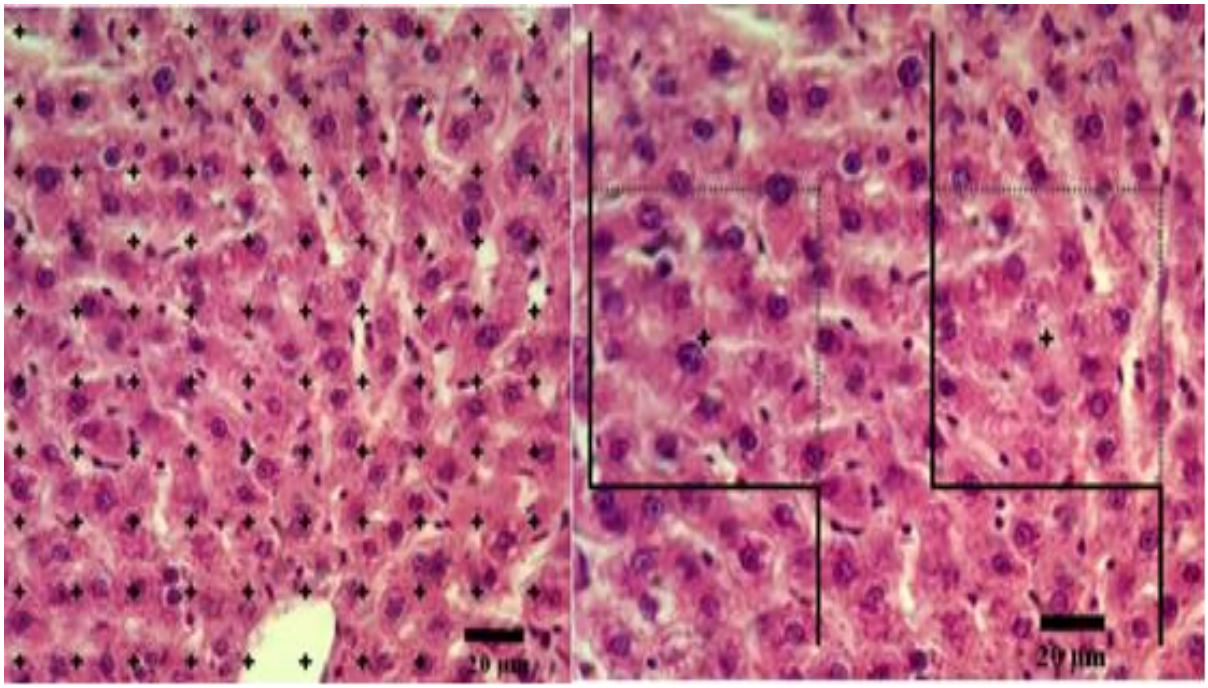


Figure 2. Histological sections of liver tissue stained with hematoxylin and eosin (H&E). The image shows low and high magnification views of hepatocytes, Kupffer cells, and nuclei measured using the optical dissector method. The parameters include ∑Q (total profiles counted), h (dissector height), a/f (frame area), ∑P (number of frames), BA (block advance), and t (section thickness). Scale bars = 20 µm.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8. All data were expressed as mean ± standard error of the mean (SEM). The normality of data distribution was assessed using the Shapiro-Wilk test, and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare between different groups. For comparisons between two specific groups, independent-samples t-test was used. A p-value of less than 0.05 was considered statistically significant.

Results

The results of AST changes showed that in PCO group AST levels were significantly increased compared to the control groups. The consumption of Salep extract caused reductions in AST level compared to PCO group and the dose of 320 mg/kg Salep extract showed the significant effect in reducing AST levels compared to other doses and PCO group. A significant increase in ALP and ALT enzyme levels was observed in PCO group compared to the control group. Treatment with Salep extract significantly reduced these enzyme levels compared to the PCO group at doses of 80, 160, and 320 mg/kg (PCO+Sa2, PCO+Sa3, and PCO+Sa4 groups). High triglyceride (TG) levels in the PCO group were also significantly reduced in PCO+Sa3 and PCO+Sa4 groups compared to the PCO group (Figure 3).

In PCO group, a significant increase in blood glucose levels was observed compared to the control group. Only the PCO+Sa4 group showed a significant decrease in blood glucose concentration compared to the PCO group, with the dose of 320 mg/kg Salep extract having the greatest effect (Figure 4).

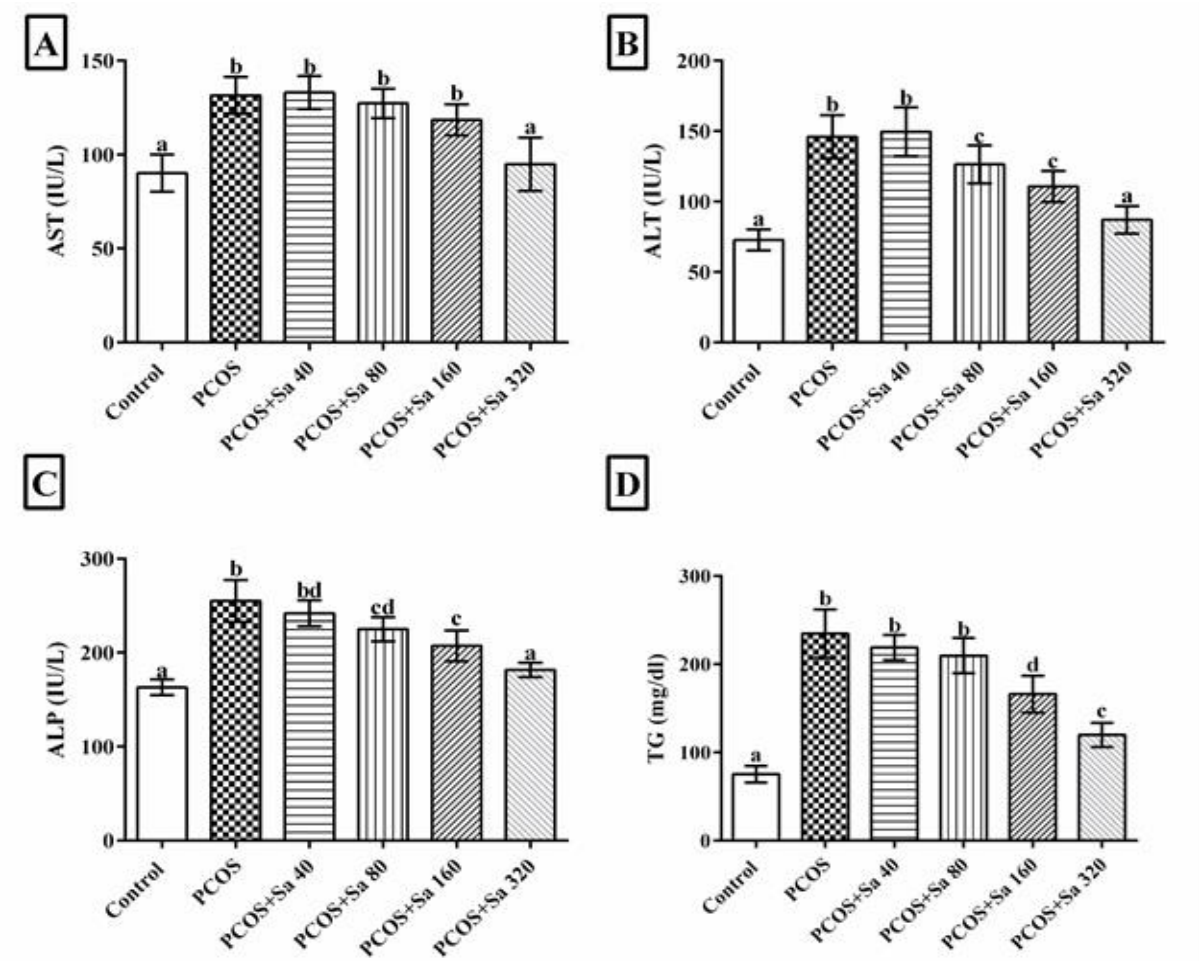


Figure 3. Bar graph showing levels of liver function markers in the control, polycystic ovary syndrome (PCOS), and PCO animals received Salep extract (PCOS+Sa) in various doses (40, 80, 160, and 320 mg/kg)(n=8). Data is expressed as the mean and standard Error. Group means that share at least one common letter are not significantly different from each other ($p>0.05$). $p<0.05$ is considered statistically significant, Independent-Samples T Test. Abbreviations: AST = aspartate transaminase; ALT = alanine aminotransferase; ALP = alkaline phosphatase initial; TG = Triglyceride.

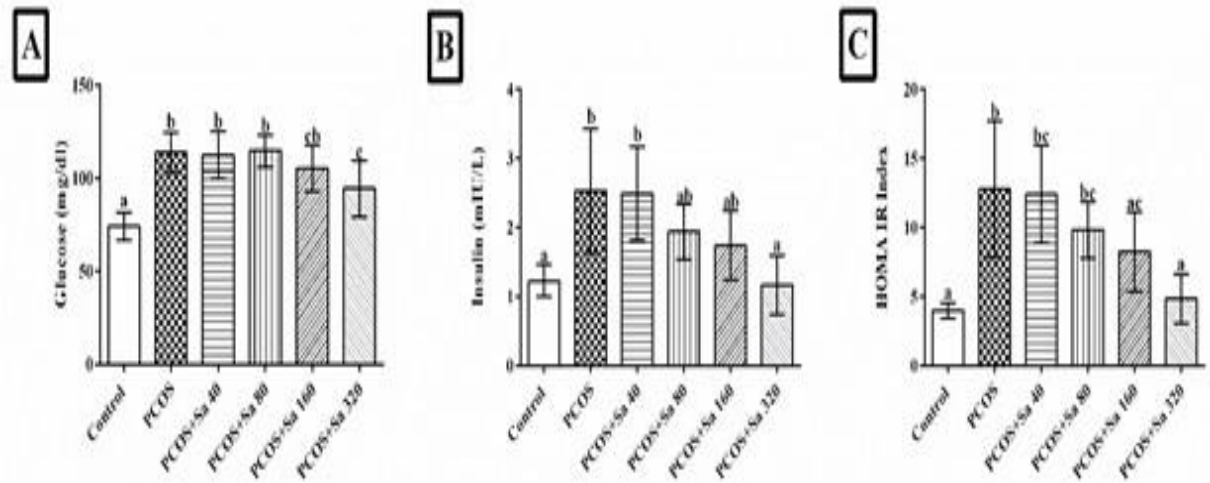


Figure 4. Bar graph showing levels of glucose, insulin, and homeostatic model assessment for insulin resistance (HOMA-IR) in the control, polycystic ovary syndrome (PCOS), and PCO animals received Salep extract (PCOS+Sa) in various doses (40, 80, 160, and 320 mg/kg) (n=8). Data is expressed as the mean and standard Error. Group means that share at least one common letter are not significantly different from each other ($p > 0.05$). $p<0.05$ is considered statistically significant, Independent-Samples T Test. Abbreviations: HOMA IR= Homeostatic model assessment of insulin resistance.

PCO group had higher insulin levels than the control group. Only the PCO+Sa4 group showed a significant decrease in insulin levels compared to the PCO group. The evaluation of the homeostatic model of insulin resistance (HOMA-IR) showed that PCO group showed a significant difference with the control group. Both the PCO+Sa3 and PCO+Sa4 groups showed a significant decrease in HOMA-IR compared to the PCO group (Figure 5).

Compared to the control group, TOC and MDA levels were significantly increased in the PCO group. Treatment with Salep extract at all doses (40, 80, 160, and 320 mg/kg) significantly reduced TOC and MDA levels compared to the PCO group. A significant decrease in serum concentrations of TAC and GPx was observed in the PCO group compared to the control group. Salep extract treatment compensated for the decrease in TAC and GPx levels at doses of 160 and 320 mg/kg (PCO+Sa3 and PCO+Sa4 groups) compared to the PCO group. The higher dose of Salep extract proved to be the most effective in restoring these parameters (Figure 5).

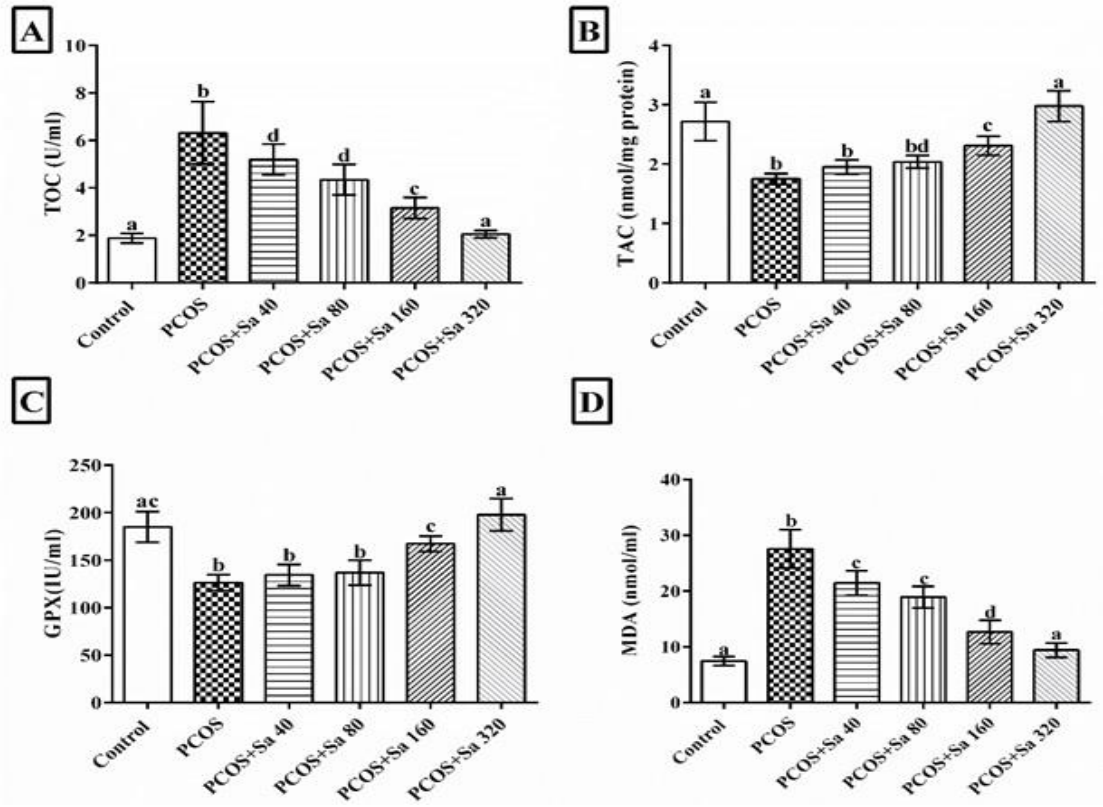


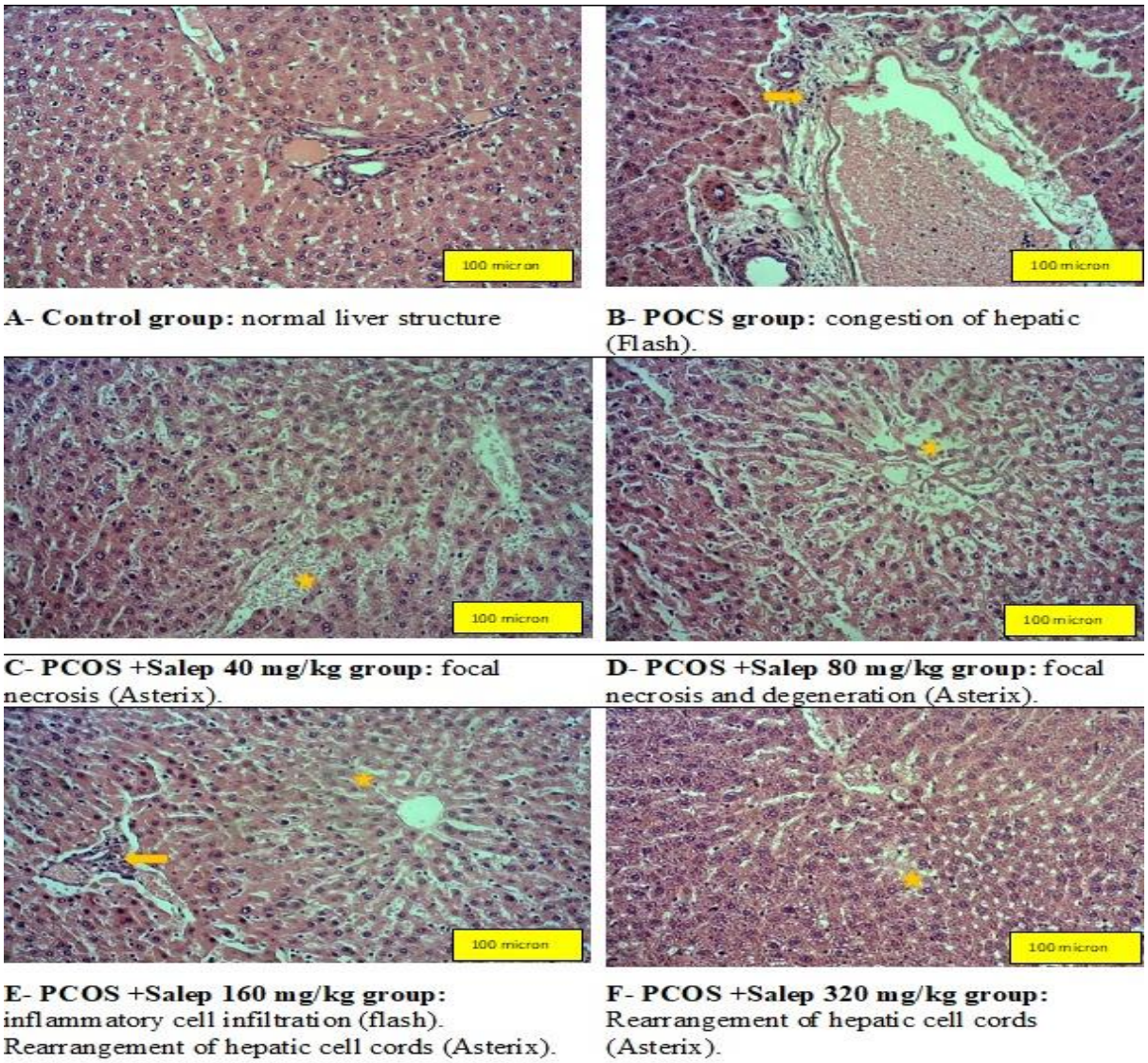
Figure 5. Bar graph showing levels of serum antioxidants, MDA, and total oxidant status (TOS) in the control, polycystic ovary syndrome (PCOS), and PCO animals received Salep extract (PCOS+Sa) in various doses (40, 80, 160, and 320 mg/kg) (n=8). Data is expressed as the mean and standard Error. Group means that share at least one common letter are not significantly different from each other ($p>0.05$). $p<0.05$ is considered statistically significant, Independent-Samples T Test. Abbreviations: GPX = Plasma glutathione peroxidase, TOS = total oxidant status, TAC = Total antioxidant capacity, MDA = Malondialdehyde.

In control animals, normal liver tissue structure and cells were observed (Figure 6-A). Histological evaluations of PCO animals revealed severe liver cell destruction, cellular disorganization, and inflammatory cell infiltration around the portal vein and sinusoidal space with marked congestion. In PCO group, ballooning degeneration was observed in liver cells (Figure 6-B).

In the PCO+Sa1, focal necrosis, disruption of liver cell arrangement, and infiltration of mononuclear inflammatory cells in the sinusoidal space and around the

portal vein were observed (Figure 6-C). In the PCO+Sa2 group, moderate necrosis of liver cells was observed along with liver tissue destruction, cellular disarray of the hepatic cord, and infiltration of mononuclear inflammatory cells (Figure 6-D). 160 mg/kg of Salep extract caused mild liver cell destruction with mild infiltration of inflammatory mononuclear cells (Figure 6-E). In the 320 mg/kg group, liver regeneration and liver cell cord rearrangement were observed (Figure 6-F).

Compared to the control group, the PCO, PCO+Sa1, and PCO+Sa2 groups showed a significant increase in the volume of connective tissue, liver cells, and sinusoids, as well as an increase in the number of Kupffer cells and inflammation. In contrast, PCO+Sa3 and PCO+Sa4 groups showed significantly lower volumes of these tissues compared to the PCO group. The level of inflammation and the number of Kupffer cells were also lower in all groups treated with Salep extract (PCO+Sa1 to PCO+Sa4) compared to the PCO group. Higher doses of Salep extract were more effective, though no significant differences were observed between the doses. The portal vein volume increased significantly only in the PCO group compared to the control group. The central vein volume, hepatocyte nucleus volume, and hepatocyte nucleus count did not show significant differences in any of the studied groups (Figure 6).



A- Control group: normal liver structure

B- POCS group: congestion of hepatic (Flash).

C- PCOS +Salep 40 mg/kg group: focal necrosis (Asterix).

D- PCOS +Salep 80 mg/kg group: focal necrosis and degeneration (Asterix).

E- PCOS +Salep 160 mg/kg group: inflammatory cell infiltration (flash). Rearrangement of hepatic cell cords (Asterix).

F- PCOS +Salep 320 mg/kg group: Rearrangement of hepatic cell cords (Asterix).

Figure 6. Histological analysis in the control, polycystic ovary syndrome (PCOS), and PCO animals received Salep extract (40, 80, 160, and 320 mg/kg) (n=8).

Discussion

In this study, the high levels of AST, ALT and ALP in all PCO groups confirm liver involvement in PCO animals. Our findings align with those of previous studies which have identified elevated liver enzyme levels (AST, ALT, and ALP) in PCO patients, indicating hepatic stress and potential liver damage (Hong et al. 2023; Won et al. 2021). This result is consistent with other studies showing a high prevalence (43%) of NAFLD among PCOS patients, highlighting the systemic impact of PCOS on liver function, which is influenced by several metabolic and specific factors. (Manzano-Nunez et al. 2023a; Spremović Rađenović et al. 2022; Vidal-Cevallos et al. 2023). In a meta-analysis of 36 studies, it was shown that obesity (Body mass index (BMI) and waist circumference), metabolic abnormalities (HOMA-IR, ALT, and triglycerides), and PCOS-specific symptoms (hyperandrogenism and free androgen index) are the most important risk factors for NAFLD in the PCOS population (Manzano-Nunez et al. 2023b).

Our previous results also demonstrated that Salep extract produced dose-dependent changes in serum hormone levels and histological appearance of ovaries of PCO rats. In particular, Salep extract increased the serum levels of progesterone, Gonadotropin-releasing hormone and Follicle-stimulating hormone, while decreasing the levels of estrogen, free testosterone and LH. The hormonal changes observed in these studies, such as increased progesterone and decreased estrogen levels, mirroring findings from our study, suggesting that Salep extract can modulate endocrine function and facilitate ovarian recovery (Abedi et al. 2024a; Kargar et al. 2015). Our previous studies confirm these findings and show increased numbers of primary, secondary and Graafian follicles in PCO animals treated with Salep, indicating reversal of PCO pathology (Kargar Jahromi et al. 2022; Kavoos et al. 2015).

In the experimental groups receiving Salep extract, a significant decrease in the serum concentration of AST, ALT, ALP, TG and blood glucose was observed compared to the PCO group. These changes were dose-dependent and the maximum effect was observed at the highest dose (320 mg/kg). This suggests that Salep extract has a protective effect against PCO-induced liver damage, possibly due to its antioxidant properties.

Oxidative stress (OS) plays an important role in the pathogenesis of PCOS and associated liver damage. Increased oxidative markers have been observed in PCOS patients compared to healthy subjects, contributing to disease progression (Rudnicka et al. 2022; Zuo et al. 2016b). Advanced glycation end products (AGEs) are the result of the Maillard reaction which produces OS that leads to tissue damage and inflammation. AGEs have been suggested as markers of OS in PCOS, causing insulin resistance and inflammation through alterations in steroid biosynthesis, which ultimately impair normal steroidogenesis and folliculogenesis (Mouanness et al. 2022; Sharma et al. 2022; Shen et al. 2020; Twarda-Clapa et al. 2022).

Free radicals play essential roles in reproductive processes such as ovulation, oocyte maturation, and fertilization. However, excessive free radicals, as seen in PCO, lead to oxidative damage (Masjedi et al. 2019; Murri et al. 2013; Papalou et al. 2016). The significant reduction in oxidative markers (TOC and MDA) in Salep-treated groups underscores the potent antioxidant properties of Salep extract. Compounds such as flavonoids, polyphenols (such as quercetin and ferulic acid), and glucomannan play critical roles in neutralizing free radicals and protecting cellular structures from oxidative damage (Atashpour et al. 2017; Kargar Jahromi et al. 2022). For example, Salep extract's ability to reduce ALT and AST levels suggests its hepatoprotective effects, likely due to the presence of ferulic acid and

glucomannan which enhance liver enzyme function and promote liver health by reducing oxidative stress and inflammation (Zhang et al. 2022). Quercetin increases the expression of antioxidant genes and enzymes, further supporting its protective role against oxidative damage (Atashpour et al. 2017; Zhou et al. 2023).

In patients with fatty liver, factors such as cholesterol, TG and liver transaminases are increased and aggravate liver damage (Chrostek et al. 2014; Jia et al. 2023). Our study found similar results with altered TG levels in the PCO group compared to the control group. Administration of Salep extract significantly reduced these levels, demonstrating its potential to reduce lipid peroxidation and liver damage.

As a result, Salep extract reduced liver toxicity and damage in PCO animals and normalized the investigated parameters. PCOS and NAFLD are components of the metabolic syndrome, in which insulin resistance and glucose homeostasis play a key role in these conditions (Rahmatnezhad et al. 2023). Controlling and treating these metabolic disorders is very important. Salep, in combination with supplements such as pumpkin seed extract, may help prevent diabetic complications and support the management of type 2 diabetes (Arzoo et al. 2018).

The findings of this study show the wide consequences of Salep consumption in reducing metabolic and reproductive disorders. This suggests the need for further clinical trials and longitudinal studies in human subjects. Investigating the synergistic effects of Salep extract with other natural supplements, such as pumpkin seed extract, could provide additional protective benefits against metabolic and reproductive disorders. Examining these compounds can pave the way for comprehensive and multimodal therapeutic approaches.

In conclusion, Salep extract can dose-dependently improve liver enzyme levels, lipid profile and hormonal balance in PCO rats. Salep has a direct effect on lipids and liver function and an indirect effect on hormones related to PCO and metabolic syndrome. Antioxidant compounds present in Salep extract play an important role in these protective effects, and these dose-dependent improvements indicate the potential of Salep extract as a therapeutic agent for the management of PCOS and related liver dysfunction.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Ethical Considerations

All experimental procedures involving animals were conducted in accordance with the ethical standards of Jahrom University of Medical Sciences.

Code of Ethics

The study protocol was reviewed and approved by the Institutional Research Ethics Committee of Jahrom University of Medical Sciences (Approval Code: IR.JUMS.REC.1394.200).

Authors' Contributions

H.K.J and H.A provided necessary guidance and supervision throughout the research process. F.K and E.M assisted in various aspects of the project and contributed to the conceptualization and

methodology of the study. P.A and A.A.M helped with data collection. H.H and N.S.J played an important role in data analysis. H.K.J, H.A and B.E took the lead in writing the manuscript and ensured a complete and coherent presentation of the findings. All authors read and approved the article.

References

Abdollahi M, Mirghazanfari SM, Mehri M (2024) Investigating the Possible Ameliorating Impact of Black Seed (Nigella sativa) Hydroethanolic Extract on Liver and Brain Tissue in a Rat Model of Polycystic Ovary. AMHSR 22(22)

Abedi H, Zarrin-Mehr A, Ebrahimi B, Haghshenas H, Parvin N, Jahromi HK (2024a) The effect of aqueous extract of orchid root on the structure of ovary and hypothalamic-pituitary-gonadal hormones in polycystic ovary syndrome rat model: An experimental study. IJRB 22(3):203

Abedi H, Zarrin-Mehr A, Ebrahimi B, Haghshenas H, Parvin N, Jahromi HKJIJoRB (2024b) The effect of aqueous extract of orchid root on the structure of ovary and hypothalamic-pituitary-gonadal hormones in polycystic ovary syndrome rat model: An experimental study. IJRB 22(3):203

Arvanitakis K, Chatzikalil E, Kalopitas G, et al. (2024) Metabolic Dysfunction-Associated Steatotic Liver Disease and Polycystic Ovary Syndrome: A Complex Interplay. J Clin Med 13(14):4243 doi:10.3390/jcm13144243

Arzoo SH, Chattopadhyay K, Banerjee S, Chattopadhyay B (2018) Synergistic improved efficacy of Gymnadenia orchidis root Salep and pumpkin seed on induced diabetic complications. Diabetes Res Clin Pract 146:278-288 doi:10.1016/j.diabres.2018.10.025

Ashraf S, Nabi M, Rashid F, Amin SJEJoMHG (2019) Hyperandrogenism in polycystic ovarian syndrome and role of CYP gene variants: a review. Egypt J Med Hum Genet 20(1):1-10

Atashpour S, Abedi H, Shafiei Jahromi N, et al. (2023) The effect of Salep Aqueous extract high doses on serum level of urea nitrogen, creatinine, uric acid and kidney histopathological changes in adult male wistar rats. Arch Razi Inst 78(5):1451-1461

Atashpour S, Kargar Jahromi H, Kargar Jahromi Z, Zarei S (2017) Antioxidant effects of aqueous extract of Salep on Paraquat-induced rat liver injury. World J Hepatol 9(4):209-216 doi:10.4254/wjh.v9.i4.209

Balen AH, Rutherford AJ (2007) Managing anovulatory infertility and polycystic ovary syndrome. BMJ 335(7621):663-6 doi:10.1136/bmj.39335.462303.80

Bayram F, Kocer D, Ozsan M, Muhtaroglu S (2012) Evaluation of endothelial dysfunction, lipid metabolism in women with polycystic ovary syndrome: relationship of paraoxonase 1 activity, malondialdehyde levels, low-density lipoprotein subfractions, and endothelial dysfunction. J Gynaecol Endocrinol 28(7):497-501 doi:10.3109/09513590.2011.569607

Bovi APD, Marciano F, Mandato C, Siano MA, Savoia M, Vajro P (2021) Oxidative stress in non-alcoholic fatty liver disease. An updated mini review. Front Med 8:595371

Chen Y, Ma L, Ge Z, Pan Y, Xie L (2022) Key Genes Associated With Non-Alcoholic Fatty Liver Disease and Polycystic Ovary Syndrome. Front Mol Biosci 9:888194 doi:10.3389/fmolb.2022.888194

Chrostek L, Supronowicz L, Panasiuk A, Cylwik B, Gruszewska E, Flisiak R (2014) The effect of the severity of liver cirrhosis on the level of lipids and lipoproteins. Clin Exp Med 14(4):417-21 doi:10.1007/s10238-013-0262-5

Delli Bovi AP, Marciano F, Mandato C, Siano MA, Savoia M, Vajro P (2021) Oxidative Stress in Non-alcoholic Fatty Liver Disease. An Updated Mini Review. Front Med (Lausanne) 8:595371 doi:10.3389/fmed.2021.595371

Dönmez N, Keskin EJEJoVS (2008) The effects of aflatoxin and glucomannan on some antioxidants and biochemical parameters in rabbits. Eurasian J Vet Sci 23(1):31-35

Dvorska JE, Pappas AC, Karadas F, et al. (2007) Protective effect of modified glucomannans and organic selenium against antioxidant depletion in the chicken liver due to T-2 toxin-

contaminated feed consumption. Toxicol Appl Pharmacol 145(4):582-587

Falzarano C, Lofton T, Osei-Ntansah A, et al. (2022) Nonalcoholic Fatty Liver Disease in Women and Girls With Polycystic Ovary Syndrome. J Clin Endocrinol Metab 107(1):258-272 doi:10.1210/clinem/dgab658

Fica S, Albu A, Constantin M, Dobri GA (2008) Insulin resistance and fertility in polycystic ovary syndrome. J Med Life 1(4):415-22

Holterman AX, Guzman G, Fantuzzi G, et al. (2013) Nonalcoholic fatty liver disease in severely obese adolescent and adult patients. Obesity (Silver Spring) 21(3):591-7 doi:10.1002/oby.20174

Hong X, Guo Z, Yu Q (2023) Hepatic steatosis in women with polycystic ovary syndrome. BMC Endocr Disord 23(1):207 doi:10.1186/s12902-023-01456-6

Hyderali BN, Mala K (2015) Oxidative stress and cardiovascular complications in polycystic ovarian syndrome. Eur J Obstet Gynecol Reprod Biol 191:15-22 doi:10.1016/j.ejogrb.2015.05.005

Jahromi HK, Pourahmad M, Abedi HA, Jahromi ZKJJot, medicine c (2018) Protective effects of salep against isoniazid liver toxicity in wistar rats. Pars J Med Sci 8(1):239-243

Janati S, Behmanesh MA, Najafzadehvarzi H, Kassani A, Athari N, Poormoosavi SM (2022) Changes of Serum Level of Homocysteine and Oxidative Stress Markers by Metformin and Inositol in Infertile Women with Polycystic Ovary Syndrome: A Double Blind Randomized Clinical Trial Study. Int J Fertil Steril 16(2):102-107 doi:10.22074/IJFS.2021.530040.1125

Jia X, Zhang X, Yan M, et al. (2023) Increased TG to HDL-C ratio is associated with severity of drug-induced liver injury. Sci Rep 13(1):6897

Kargar Jahromi H, Solhjo K, Solhjo KA, Kargar Jahromi Z, Ebrahimian A (2022) The effect of aqueous extract of the roots of Salep plants on the serum concentration of FSH and estrogen hormone in female rats. Pars J Med Sci 13(2):39-44

Kargar JH, Karimi JH, Abedi H, Kargar JZ, Khabbaz KZ EFFECT OF AQUEOUS EXTRACT OF ROOT-TUBERS OF DACTYLORHIZA MACULATE (SALEP) ON HPT-AXIS HORMONES IN ADULT RATS. In, 2015.

KARGAR JH, Solhjoo E, Hasannezhad A, Pourahmadi M, SAHRAEI R, ATASHPOUR S (2020) Comparison Of Aqueous Extract Of Orchid's Root (Dactylorhiza Maculate) With Ibuprofen On Pain Sensory Thresholds Using The Formalin Test In Adult Male Rats. Pars J Med Sci:15-22

Kavoos S, Hossein KJ, Allah SK, Jahromi K (2015) The effect of the aqueous extract of Orchid roots on the serum concentration of progesterone and luteinizing hormone in adult female rats. Pars J Med Sci 13(1):21-6

Kurt A, Kahyaoglu T (2017) Purification of glucomannan from salep: Part 1. Detailed rheological characteristics. Carbohydr Polym 168:138-146 doi:10.1016/j.carbpol.2017.03.060

Liu Y, Dong R, Yang Y, et al. (2021) Protective Effect of Organic Selenium on Oxidative Damage and Inflammatory Reaction of Rabbit Kidney Induced by T-2 Toxin. Biol Trace Elem Res 199(5):1833-1842 doi:10.1007/s12011-020-02279-5

Luo M, Huang JC, Yang ZQ, Wang YS, Guo B, Yue ZP (2020) Hydroxysafflor yellow A exerts beneficial effects by restoring hormone secretion and alleviating oxidative stress in polycystic ovary syndrome mice. Exp Physiol 105(2):282-292 doi:10.1113/EP088147

Ma R, Liu K, Lam P, et al. (2011) Sonographic measurement of mesenteric fat predicts presence of fatty liver among subjects with polycystic ovary syndrome. J Clin Endocrinol Metab 96(3):799-807

MADANI H, TALEB AM, ASGARY S, Mahzouni P, Razban E (2007) Preventive effect of hydroalcoholic extract of Silybum marianum and Fumaria vaillantii in atherosclerosis.

Manzano-Nunez R, Santana-Dominguez M, Rivera-Esteban J, et al. (2023a) Non-Alcoholic Fatty Liver Disease in Patients with Polycystic Ovary Syndrome: A Systematic Review, Meta-Analysis, and Meta-Regression. J Clin Med 12(3):856 doi:10.3390/jcm12030856

Manzano-Nunez R, Santana-Dominguez M, Rivera-Esteban J, et al. (2023b) Non-Alcoholic Fatty Liver Disease in Patients

with Polycystic Ovary Syndrome: A Systematic Review, Meta-Analysis, and Meta-Regression. J Clin Med 12(3) doi:10.3390/jcm12030856

Marcolin E, Forgiarini L, Rodrigues G, et al. (2019) Quercetin Decreases Liver Damage in Mice with Non-Alcoholic Steatohepatitis. BCPT 112:385

Masjedi F, Keshtgar S, Agah F, Karbalaei NJJoR, Infertility (2019) Association between sex steroids and oxidative status with vitamin D levels in follicular fluid of non-obese PCOS and healthy women. JRI 20(3):132

Mohammadi M (2019) Oxidative Stress and Polycystic Ovary Syndrome: A Brief Review. Int J Prev Med 10:86 doi:10.4103/ijpvm.IJPVM_576_17

Mouanness M, Nava H, Dagher C, Merhi Z (2022) Contribution of Advanced Glycation End Products to PCOS Key Elements: A Narrative Review. Nutrients 14(17):3578 doi:10.3390/nu14173578

Murri M, Luque-Ramirez M, Insenser M, Ojeda-Ojeda M, Escobar-Morreale HF (2013) Circulating markers of oxidative stress and polycystic ovary syndrome (PCOS): a systematic review and meta-analysis. Hum Reprod Update 19(3):268-88 doi:10.1093/humupd/dms059

Papalou O, Victor VM, Diamanti-Kandarakis E (2016) Oxidative Stress in Polycystic Ovary Syndrome. Curr Pharm Des 22(18):2709-22 doi:10.2174/1381612822666160216151852

Paschou SA, Polyzos SA, Anagnostis P, et al. (2020) Nonalcoholic fatty liver disease in women with polycystic ovary syndrome. Endocrine 67(1):1-8 doi:10.1007/s12020-019-02085-7

Pourahmad M, Kargar Jahromi H, Kargar Jahromi Z (2015) Protective effect of salep on liver. Hepatitis monthly 15(4):e28137 doi:10.5812/hepatmon.15(4)2015.28137

Pourteymour Fard Tabrizi F, Hajizadeh-Sharafabad F, Vaezi M, Jafari-Vayghan H, Alizadeh M, Maleki V (2020) Quercetin and polycystic ovary syndrome, current evidence and future directions: a systematic review. J Ovarian Res 13(1):11 doi:10.1186/s13048-020-0616-z

Radjabian T, FALLAH HH (2010) Anti-hyperlipidemic and anti-atherosclerotic activities of silymarins from cultivated and wild plants of Silybum marianum L. with different content of flavonolignans.

Rahmatnezhad L, Moghaddam-Banaem L, Behroozi-Lak T, Shiva A, Rasouli J (2023) Association of insulin resistance with polycystic ovary syndrome phenotypes and patients' characteristics: a cross-sectional study in Iran. reprod biol endocrinol 21(1):113

Roehlen N, Crouchet E, Baumert TF (2020) Liver Fibrosis: Mechanistic Concepts and Therapeutic Perspectives. Cells 9(4):875 doi:10.3390/cells9040875

Rudnicka E, Duszewska AM, Kucharski M, Tyczynski P, Smolarczyk R (2022) OXIDATIVE STRESS AND REPRODUCTIVE FUNCTION: Oxidative stress in polycystic ovary syndrome. Reproduction 164(6):F145-F154 doi:10.1530/REP-22-0152

Sharma P, Jain M, Halder A (2022) An Investigation of Steroid Biosynthesis Pathway Genes in Women with Polycystic Ovary Syndrome. J Hum Reprod Sci 15(3):240-249 doi:10.4103/jhrs.jhrs_86_22

Sharp KPH (2021) Non-alcoholic fatty liver disease (NAFLD): Nutrition triggers and weight loss treatment strategies. University of Otago

Shen C-Y, Lu C-H, Wu C-H, et al. (2020) The development of maillard reaction, and advanced glycation end product (AGE)-receptor for AGE (RAGE) signaling inhibitors as novel therapeutic strategies for patients with AGE-related diseases. Molecules 25(23):5591

Spremović Rađenović S, Pupovac M, Andjić M, et al. (2022) Prevalence, risk factors, and pathophysiology of nonalcoholic fatty liver disease (NAFLD) in women with Polycystic Ovary Syndrome (PCOS). Biomedicines 10(1):131

Tekinşen KK, Güner A (2010) Chemical composition and physicochemical properties of tubera salep produced from some Orchidaceae species. Food Chem 121(2):468-471

Twarda-Clapa A, Olczak A, Bialkowska AM, Koziolkiewicz M (2022) Advanced Glycation End-Products (AGEs): Formation, Chemistry, Classification, Receptors, and Diseases Related to AGEs.

Cells 11(8):1312 doi:10.3390/cells11081312

Uçkan K, Demir H, Turan K, Sarıkaya E, Demir CJIJoCP (2022) Role of oxidative stress in obese and nonobese PCOS patients. Int J Clin Pract 2022:4579831

Vassilatou E (2014) Nonalcoholic fatty liver disease and polycystic ovary syndrome. World J Gastroenterol 20(26):8351-63 doi:10.3748/wjg.v20.i26.8351

Vidal-Cevallos P, Mijangos-Trejo A, Uribe M, Tapia NC (2023) The Interlink Between Metabolic-Associated Fatty Liver Disease and Polycystic Ovary Syndrome. Clin Endocrinol Metab 52(3):533-545

Won YB, Seo SK, Yun BH, Cho S, Choi YS, Lee BS (2021) Non-alcoholic fatty liver disease in polycystic ovary syndrome women. Sci Rep 11(1):7085

Zare S, Hossein Dabbaghmanesh M, Noorafshan A, Koohpeyma F, Bakhshayeshkaram M, Montazeri-Najafabady N (2019) Protective effect of vitamin E and vitamin C alone and in combination on testicular damage induced by sodium metabisulphite in rats: A stereological study. Andrologia 51(2):e13193 doi:10.1111/and.13193

Zhang N, Zhou J, Zhao L, Wang O, Zhang L, Zhou F (2022) Dietary ferulic acid ameliorates metabolism syndrome-associated hyperuricemia in rats via regulating uric acid synthesis, glycolipid metabolism, and hepatic injury. Front Nutr 9:946556

Zhao H, Zhang J, Cheng X, Nie X, He B (2023) Insulin resistance in polycystic ovary syndrome across various tissues: An updated review of pathogenesis, evaluation, and treatment. J Ovarian Res 16(1):9

Zhou Y, Qian C, Tang Y, et al. (2023) Advance in the pharmacological effects of quercetin in modulating oxidative stress and inflammation related disorders. Phytother Res 37(11):4999-5016 doi:10.1002/ptr.7966

Zuo T, Zhu M, Xu W (2016a) Roles of Oxidative Stress in Polycystic Ovary Syndrome and Cancers. Oxid Med Cell Longev 2016:8589318 doi:10.1155/2016/8589318

Zuo T, Zhu M, Xu W (2016b) Roles of oxidative stress in polycystic ovary syndrome and cancers. Oxidative medicine and cellular longevity 2016