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A COMPARATIVE STUDY BETWEEN THE EFFECT OF LICORICE ROOTS, MARJORAM AND DATE PALM POLLEN AQUEOUS EXTRACTS AND METFORMIN ON POLY CYSTIC OVARY SYNDROME IN RATS

Doaa Khalaf Mahmoud[1], Zakia Mostafa Abdelkader[3] and Mai Elsayed[2]*

[1]Demonstrator in Biochemistry and Nutrition Department, Faculty of Women for Arts, Science and Education, Ain Shams University.
[2]Lecturer of Biochemistry and Nutrition, Faculty of Women for Arts, Science and Education, Ain Shams University.
[3]Prof. of Food Science and Technology, Biochemistry and Nutrition Department, Faculty of Women for Arts, Science and Education, Ain Shams University, Cairo, Egypt.

***Corresponding Author: Prof. Mai Elsayed**
Lecturer of Biochemistry and Nutrition, Faculty of Women for Arts, Science and Education, Ain Shams University.

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ABSTRACT
The present study was designed to comparethe effect of licorice roots, marjoram, date palm pollen (DPP) aqueous extracts and metformin on polycystic ovary syndrome (PCOS) in reproductive-aged female rats. PCOS was induced by oral administration of letrozole given daily in a dose 1mg/kg b. wt. for 21days. After PCOS induction, the experimental groups were classified as follow; **G1**: Healthy control, **G2**: PCOS control rats, **G3**: PCOS+Licorice roots, **G4**: PCOS+Marjoram, **G5**: PCOS+DPP.**G6**: PCOS+Mixture of herbs by a ratio (1:1:1). Herbal extracts were administrated orally in a dose of (300mg/kg b.wt./day) for 30 days. **G7**:PCOS+Metformin (200mg/kg b.wt/day) for 30 days. Estrous cycle phases were measured every day to follow up. The present results revealed that PCOS causeddisturbances in estrous cycle, serum sex steroid profile (luteinizing hormone, follicle stimulating hormone, estrogen, progesterone, and testosterone), lipid profile, glucose homeostasis, antioxidant status, lipid peroxidation and caspase-3 protein (apoptosis marker). On the other hand, oral administration of licorice roots, marjoram, DPP and mixture of herbs as well as metformin significantly restoring parameters up to the normal level as compared to PCOS control group. Meanwhile, herbal administration showed better ameliorative effects as compared to metformin. These results were confirmed by ovarian microscopic examination which showedpartial disappearance and reduction of ovarian cysts. In conclusion, the current results suggested that licorice roots, marjoram, DPP and their mixture have hypolipidemic, antioxidant, anti-apoptotic and hormonal regulatory effects on PCOS. Generally, the most ameliorative effect was observed in mixture administered group. This may be due to the synergistic action between the mixtures of phytochemicals present in herbs.

KEYWORDS: PCOS, Licorice, Marjoram, date palm pollen, Metformin, oxidative stress, insulin resistance.

1- INTRODUCTION
Polycystic ovary syndrome (PCOS) is the most common endocrine disorder among women of fertile age, which causes an ovulation in animal and women. The prevalence of PCOS varies between 2.5 and 7.5% of population. Its hyperandrogenic manifestations include menstrual irregularity, acne, hirsutism and oligo-ovulation/anovulation. Metabolic abnormalities such as dyslipidemia, insulin resistance that persist in PCOS are responsible for making the patient more prone to diabetes, obesity, cancer, and infertility as well as coronary heart diseases.[1]

Letrozole is an aromatase inhibitor which decreases the conversion of androgens to estrogens, leading to an accumulation of androgens in the ovary.[2] Previous reports have shown that letrozole can induce PCOS in rats that developed many characteristics of human PCOS, including abnormal follicles, hyperglycemia, oxidative stress, and altered sex hormones levels as luteinizing hormone (LH), follicle stimulating hormone (FSH), estrogen (E2), progesterone (PROG), and testosterone.[3,4,5]

The therapeutic treatment of PCOS involved the use of several drugs such as metformin and clomiphene citrate, but they are commonly associated with serious side effects such as vomiting, nausea, congestive heart failure and osteoporosis. A new therapeutic approach with fewer side effects, easy availability, and broad spectrum is required. Previous studies reported the efficiency of some plants such as licorice roots[6], marjoram[7] and date

palm pollen[8] in the restoration of ovarian function in rats with PCOS.

Licorice roots *(Glycyrrhizaeglabra)* are used as medicine and flavoring agent to disguise the unpleasant flavor of other medications.[9] Besides its main active component, glycyrrhizic acid, licorice also consists of high amount of flavonoids, saponins, triterpenes, isoflavonoids and chalcones. Glycyrrhizic acid is used as herbal treatment of various diseases due to its anti-inflammatory, neuro-protective, anti-carcinogenic and antiviral features. Isoliquiritigenin (ISL), chalcone and liquiritigenins (LTG's) are the most common flavonoids in licorice that has anti-inflammatory, antioxidant, antitumor activities, liver protective effect as well as estrogenic effect.[10]

Marjoram *(Origanum majorana* L.*)* edible parts are the dried leaves and flower tops, which are found throughout the world. Marjoram contains phenolic terpenoids, flavonoids, tannins, phenolic glycosides and sitosterol. The antiviral, bactericidal, antiseptic and antifungal effects of marjoram are attributed to ursolic acid and essential oil and in particular to thymol and carvacrol.[11] The antioxidant and anti-tumors activities of marjoram have recently been determined. Marjoram is used for treatment of many diseases especially those related tomenopause complications. Regarding the Iranian and Jordanian traditional medicine, marjoram was used to treat complications of menstruation and PCOS.[12,13]

Date Palm pollen (DPP) (*Phoenix dactylifera L.* Palmae) is a useful traditional medicinal plant. Palm pollen ingredients contain various vitamins (e.g. vitamin A, E and C) as well as elements (e.g. zinc, copper, selenium, cobalt, iron, nickel and manganese); it also contains essential and non-essential amino acids, fatty acids, flavonoids, sterols estradiol, beta sitosterol, estrone, estradiol, estriol α-amirin, tri-terpenoidal, saponins, and a crude gonadotrophic substance. In traditional medicine, Palm pollen has been recommended to treat infertility in women.[14,15]

The present study was conducted to investigate the effect of oral administration of aqueous extracts of licorice, marjoram, and date palm pollen against the effect of metformin on letrozole-induced PCOS in female rats.

2- MATERIALS AND METHODS

2.1. Materials

2.1.1-Chemicals

Letrozole and carboxymethyle cellulose (CMC) were purchased from sigma Aldrich Company for chemicals, USA. Metformin was purchased from Chemical Industries Development (CID) Company.

2.1.2. Plant materials

Licorice roots, marjoram leaves and date palm pollen grains were purchased from Ministry of agriculture, Cairo, Egypt.

2.1.3. Animals

Eighty five healthy adult virgin female white albino rats weighing 160±10 used for the experimental study were obtained from National Research Center, Cairo, Egypt.

2.2. Methods

2.2.1. Preparation of aqueous herbal extracts

Licorice roots, marjoram leaves and date palm pollen grains were cleaned by tab water and desiccated by oven at 25C, then powdered by electric grinder and the fine powder was sieved. The licorice roots powder was extracted by mixing powdered plant with distilled water and boiled at 100°c in a flask with continuous mixing for 10 minutes, and finally it was left for 15-20 minutes to cool. Then the mixture was filtered through a filter paper and then kept at 4°c until used.[16] Marjoram leaves powder was extracted by steeping them in boiled water for 20 min, followed by straining for 5-10 minutes then filtered and kept in refrigerator until used.[17] The powder of date palm pollen grains was freshly suspended in distilled water and kept in refrigerator until used.[18]

2.2.2. Quantitative determination of total polyphenols, total flavonoids and total antioxidant capacity in licorice roots, marjoram and DPP

Total phenolic content, total flavonoids and total antioxidant capacity were determined according to Kähkönen et al[19], Barros et al[20] and Mattila et al[21], respectively.

2.2.3. Estrous cycle follows up by using vaginal smear

Rats were maintained on standard commercial pellets diet and tap water *ad libitum*, and kept individually in stainless cages for one week in constant condition. After acclimation period the two normal estrous cycles of eighty five female rats have been determined to ensure their regular menstruation cycle, this was achieved by examination the animal vaginal smear for two weeks before induction of the disease. The swab was taken by inserting plastic micro pipette contains 0.2 ml normal saline into the vagina and the sample placed on microscope slide, the smear on the slide was dried in room temperature and stained by methylene blue, then put the slide under microscope(x40 objective lens) to classify it into one of four phases of estrous.[22]

2.2.4. Induction of PCOS

After two weeks of normal estrous cycle follow up, seventy rats were induced PCOS by administration of letrozole (1mg/kg body weight) dissolved in 1% CMC and given orally for 21 days according to Kakadia et al.[23] The remaining fifteen rats were administered CMC orally. On the other hand, the length of phases of estrous cycle was determined for all rats along the periods of letrozole administration. At the end of 21days, random samples were collected to determine testosterone, LH, FSH, progesterone, estrogen (E2) and insulin hormones to ensure the prevalence of PCOS, also some rats were sacrificed to perform the microscopic examination of ovaries.

2.3- Experimental design

After prevalence of PCOS, seventy rats were divided into seven groups each group consisted of 10 rats. The rat groups were divided as follow: **G (1)** healthy control rats**, G (2)** PCOS control rats**, G (3)** rats induced PCOS and administered licorice roots extract.[22] **G (4)** rats induced PCOS and administered marjoram leaves extract[7], **G (5)** rats induced PCOS and administered date palm pollen extract[24], **G (6)** rats induced PCOS and administered mixture of licorice, marjoram and date palm pollen extracts by a ratio (1:1:1), herbal extracts were administrated in a dose of (300mg/kg b.wt./day), finally, **G (7)** rats induced PCOS and administered metformin (200mg/kg b.wt./day).[25] The oral administration of herbal extracts as well as metformin lasted for 30 days.

2.4. Sample collection

After 12hours fasting with water *ad libitum* rats were scarified and blood samples were collected from hepatic portal vein in two centrifuge tubes; the first tube was heparinized to collect whole blood used in determination of reduced glutathione (GSH) level. The second tube was used (containing fluoride) for separation of serum by allowing blood samples left for 15 minutes at temperature of 34ºC, and then centrifuged at 4000 rpm by EBA8 centrifuge for 20 min, and then part of fresh serum samples were used immediately for the determination of blood glucose level and other part was kept in plastic vials at - 20°C until used for biochemical analysis.

The ovaries were separated, rinsed, washed by saline solution and blotted on filter paper to remove water residue and weighed. Part of the ovary specimens were taken and immediately fixed in formalin 10% for microscopic examination, while the rest of specimens were stored frozen at -20°C until used for the tissue biochemical analysis.

2.5. Biological measurements

The body weight of animals was recorded weekly to monitor the body weight changes. The relative weight of ovaries was calculated according to Al-Waeli and Al-Khalisy.[26]

2.6. Estrous cycle detection and period's phases length determination

The duration of the estrous cycle and the number of days that spent at the stage of the cycle (phase length) was estimated as the following[22]:

The estrous cycle phases length within hours = Total hours of each phase cycle / total number of animals

The estrous cycle phases length in days = The phase estrus cycle length in hours/ 24 hrs.

2.7. Biochemical parameters

2.7.1. Determination of hormonal status

Serum LH,FSH, E2, PROG and testosterone assay were performed following quantitative sandwich enzyme-linked immune sorbent assay (Elisa) technique according to the method described by Knobil[27], Simoni et al.[28], Tsang et al[29], Pedersen et al[30] and Chen et al[31], respectively using Bioassay technology laboratory, England.

2.7.2. Determination of glucose homeostasis status

The determination of serum glucose was performed according to enzymatic colorimetric method.[32] The serum insulin level was determined using Ray Bio rat insulin ELISA Kit.[33] Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was calculated in serum using the values of fasting serum glucose concentration and fasting serum insulin levels.[34]

2.7.3. Assessment of antioxidant/oxidant markers

Erythrocyte and ovarian GSH concentration, serum catalase (CAT) activity, serum and ovarian malondialdehyde (MDA) levels were measured calorimetrically according to Beutler et al.[35], Aebi[36] and Ohkawa et al.[37]**,** respectively.

2.7.4. Determination of apoptosis marker (ovarian caspase 3-protein)

The determination of ovarian caspase 3-protein was performed by Rat Caspase 3 (CASP3) ELISA Kit according to kaushal et al.[38]

2.7.5. Determination of lipid profile levels

Serum total cholesterol (TC) and triacylglycerols (TAGs), were determined following the colorimetric methods of Allain et al.[39], and Fassati and Prencip[40], respectively. Serum high-density lipoprotein cholesterol (HDL-C) was determined[41]; in addition, very low density lipoprotein cholesterol (VLDL-C) and low density lipoprotein cholesterol (LDL-C) concentrations were calculated according to Friedewald et al.[42]

2.8. Microscopic examination of ovarian samples

Ovarian samples were collected and preserved in 10% formalin solution for microscopic examination. Part of ovary specimens from each group were embedded in paraffin wax and the microscopic sections of 5µm were taken and stained with hemosylin and eosin H&E stain for microscopic examination.[43]

2.9. Statistical analysis

Values were statistically analyzed by one way ANOVA, using SPSS statistics version 17.0 according to Levesque.[44]

3- RESULTS

3.1. Determination of phytochemicals and antioxidant analysis of licorice roots, marjoram and date palm pollen aqueous extracts

Table (1) showed the values of total polyphenols measured in mg as gallic acid equivalent; GAE %, and total flavonoids in mg as catechin equivalent; CE % as well as total antioxidant capacity indicated by the 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activity.

Data showed that total phenols and total antioxidant contents were higher in marjoram than licorice roots and date palm pollen; on the other hand total flavonoids were higher in the licorice roots as compared to marjoram and DPP.

Table (1): Bioactive constituents and antioxidant capacity of the licorice roots, marjoram and date palm pollen extracts (Mean ± SE).

Bioactive components	Licorice roots	Marjoram leaves	Date palm pollen
Total phenols (mg as GAE)	110.5±0.17	151.20±0.21	40.10±0.19
Total flavonoids (mg as CE)	212.00±0.12	190.00±0.31	67.00±0.11
Total antioxidant capacity (mg%)	85.88±0.10	90.48±0.13	85.51±0.14

3.2. Effect of oral administration of licorice roots, marjoram and date palm pollen aqueous extracts as well as metformin on food intake, body weight change and relative ovaries weight

The data presented in table (2) indicated that PCOS control group showed significant elevation ($P \leq 0.05$) in food intake, body weight and relative weight of ovaries as compared to healthy control group.

Food intake, body weight and relative weight of ovaries were significantly decreased ($P \leq 0.05$) in all treated groups as compared with PCOS control group. Oral administration of herbal mix showed significant reduction in food intake as compared to other treated groups. Moreover, there were no significant change in body weight and relative ovaries weight between herbal extracts administered groups. On the other hand, herbal treated groups showed marked reduction ($P \leq 0.05$) in food intake, body weight, and relative ovaries weight as compared with metformin administered group.

Table (2): Effect of oral administration of herbal extracts as well as metformin on food intake, body wt. change and relative ovaries weight in experimental groups.

NO	Parameters / Groups	Food intake (g)	body weight change (g)	Relative ovaries weight (%)
G1	**Healthy(-ve control)**	754.20 ±3.98^{a}	63.40±1.15^{a}	0.030±0.0007^{a}
G2	**PCOS (+ve control)**	995.25±3.80^{b}	87.40±1.84^{b}	0.061±0.0008^{b}
G3	**PCOS+ Licorice**	804.55±4.31^{c}	71.02±1.58^{c}	0.048±0.006^{c}
G4	**PCOS+ Marjoram**	819.08±3.80^{d}	71.3±1.47^{c}	0.046±0.001^{c}
G5	**PCOS+ DPP**	801.46±3.41^{c}	70.70±1.51^{c}	0.045±0.005^{c}
G6	**PCOS+ Mixture of herbs**	787.96±1.79^{e}	71.50±1.66^{c}	0.045±0.001^{c}
G7	**PCOS+ Metformin**	857.96±2.71^{f}	76.30±1.31^{d}	0.050±0.001^{d}
	LSD	8.25	3.58	.002

*Values are represented as mean ± SE, (n=10), there was no significance difference between means have thesame letter in the same column, ($P \leq 0.05$).

3.3. Effect of oral administration of licorice roots, marjoram and date palm pollen aqueous extracts as well as metformin on Estrous cycle period's phases length in experimental groups

During letrozole administration for PCOS induction**,** estrous cycle period's phases length was measured in days. The data presented in table (3a) indicated that letrozole administration caused a statistical significant elevation ($P \leq 0.05$) in the days of estrous cycles as compared to healthy control group(during the period of induction).

Table (3a): Estrous cycle between healthy control group and letrozole treated group in days.

NO	Groups/Parameters	cycle 1	cycle 2	cycle 3	cycle 4
G1	**Healthy (-vecontrol)**	4.49±0.01^{a}	4.50±0.02^{a}	4.51±0.024^{a}	4.54±0.02^{a}
G2	**PCOS (+ve control)**	4.640.007^{b}	4.92±0.01^{b}	5.35±0.01^{b}	6.28±0.006^{b}

*Values are represented as mean ± SE, (n=10), there was no significance difference between means have the same letter in the same column, ($P \leq 0.05$).

From the data obtained in Table 3b, it was obvious that, the oral administration of aqueous extracts of licorice roots, marjoram, date palm pollen and mixture of their mix, as well as, metformin to rats induced PCOS caused

a significant reduction (P<0.05) in days of estrous cycle especially in cycle four and cycle five as compared to PCOS control group. In addition herbal administrations caused significant reduction (P<0.05) in days of estrous cycle especially in cycle four and cycle five as compared to metformin administered group.

Table (3b): Effect of oral administration of herbal extracts as well as metformin on estrous cycle period's phases length in days in experimented groups.

No	Parameters / Groups	cycle 1	cycle 2	cycle 3	cycle 4	cycle 5
G1	Healthy (-vecontro)	4.49±0.016^{a}	4.50±0.028^{a}	4.51±0.024^{a}	4.54±0.023^{a}	4.55±0.018^{a}
G2	PCOS (+ve control)	6.25±0.013^{b}	6.28±0.012^{b}	6.30±0.009^{b}	6.31±0.009^{b}	6.33±0.007^{b}
G3	PCOS + Licorice	6.02±0.017^{c}	5.73±0.016^{c}	5.48±0.008^{c}	4.79±0.016^{c}	4.61±0.015^{c}
G4	PCOS + Marjoram	6.11±0.013^{d}	5.77±0.024^{c}	5.51±0.011^{c}	4.89±0.01^{d}	4.65±0.014^{c}
G5	PCOS + DPP	6.10±0.014^{d}	5.73±0.013^{c}	5.48±0.018^{c}	4.77±0.02^{cde}	4.59±0.022^{ac}
G6	PCOS + Mixture	5.95±0.012^{e}	5.52±0.011^{d}	5.21±0.012^{d}	4.74±0.017^{e}	4.58±0.007^{ac}
G7	PCOS + Metformin	6.16±0.012^{f}	5.39±0.047^{e}	5.24±0.018^{d}	5.001±0.013^{f}	4.79±0.022^{d}
	LSD	0.367	0.037	0.059	0.037	0.037

*Values are represented as mean ± SE, (n=10), there was no significance difference between means have the same letter in the same column, (P≤ 0.05).

3.4. Effect of oral administration of licorice roots, marjoram and date palm pollen aqueous extracts as well as metformin on serum hormonal status in experimental groups

According to the data summarized in table (4) induction of PCOS caused significant elevation (P<0.05) in serum levels of LH, testosterone and LH/FSH ratio, while caused significant depletion (P<0.05) in serum FSH, E2 and PROG levels as compared to healthy control group.

Oral administration of aqueous extracts of licorice roots, marjoram, date palm pollen and mixture as well as metformin to rats with PCOS caused a significant decrease (P<0.05)in serum levels of LH, testosterone and LH/FSH ratio, while caused significant increase (P<0.05) in serum FSH, E2 and PROG levels as compared to PCOS control group. The present results reported that there were significant depletion (P<0.05)in serum levels of LH, testosterone and LH/FSH ratio, as well as significant elevation (P<0.05) in serum FSH, E2 and PROG levels in groups administered aqueous extracts of licorice roots, marjoram, date palm pollen and mixture as compared to rats administrated metformin.

Generally, the best ameliorative effect on serum hormonal status was showed in mixture administered group as compared to licorice roots, marjoram, and date palm pollen administered separately. In addition, administration of herbal extracts significantly ameliorated the hormones levels when compared to metformin administered group.

Table (4): Effect of oral administration of herbal extracts as well as metformin on serum LH, FSH, E2, PROG, Total testosterone levels and LH/FSH ratio in experimental groups.

NO	Parameters / Groups	LH (mlU/ml)	FSH (mlu/ml)	LH/FSH ratio	Estrogen (E2) (pg/ml)	Progesterone (ng/ml)	Total testosterone (ng/ml)
G1	Healthy (-ve control)	0.34±0.007^{a}	0.64±0.013^{a}	0.53±0.01^{a}	75.17±0.92^{a}	22.31±0.28^{a}	1.07±0.02^{a}
G2	PCOS (+ve control)	0.95±0.012^{b}	0.29±0.007^{b}	3.30±0.07^{b}	52.09±0.34^{b}	8.28±0.18^{b}	3.02±0.12^{b}
G3	PCOS + Licorice	0.55±0.009^{c}	0.51±0.011^{c}	1.07±0.02^{c}	67.11±0.37^{c}	18.54±0.24^{c}	1.60±0.02^{c}
G4	PCOS + Marjoram	0.58±0.005^{d}	0.52±0.006^{c}	1.11±0.02^{c}	64.44±0.55^{d}	17.8±0.12^{d}	1.72±0.02^{d}
G5	PCOS + DPP	0.55±0.01^{c}	0.51±0.013^{c}	1.03±0.01^{c}	65.71±0.37^{e}	18.55±0.21^{c}	1.60±0.02$^{c, d}$
G6	PCOS + Mixture	0.58±0.018^{d}	0.55±0.016^{d}	1.06±0.07^{c}	67.59±0.38^{c}	19.49±0.18^{e}	1.53±0.01^{c}
G7	PCOS + Metformin	0.72±0.01^{e}	0.42±0.006^{e}	1.70±0.02^{d}	59.82±0.45^{f}	13.78±0.15^{f}	2.01±0.03^{e}
	LSD	0.02	0.02	0.10	1.22	1.53	0.11

*Values are represented as mean ± SE, (n=10), there was no significance difference between means have the same letter in the same column, (P≤ 0.05).

3.5. Effect of oral administration of licorice roots, marjoram and date palm pollen aqueous extracts as well as metformin on serum glucose homeostasis in experimental groups

According to the data presented in table (5), there were significant increase (P<0.05)in serum levels of glucose, insulin and HOMA-IR in PCOS control group as compared to healthy control group.

The current results revealed that oral administration of aqueous extracts of licorice roots, marjoram, date palm pollen and their mixture as well as metformin caused significant reduction (P<0.05) in serum levels of glucose,

insulin and HOMA-IR as compared to PCOS control group. The ameliorative hypoglycemic and reducing insulin resistance effects of licorice roots, marjoram, date palm pollen and their mixture as well as metformin were approximately equal.

Table (5): Effect of oral administration of herbal extracts as well as metformin on serum glucose, serum insulin and HOMA-IR levels in experimental groups.

NO	Parameters / Groups	Glucose (mg/dl)	Insulin (µIU/ml)	HOMA-IR
G1	Healthy (-ve control)	75.43±0.94^{a}	7.03±0.14^{a}	1.31±0.035^{a}
G2	PCOS(+ve control)	153.06±0.96^{b}	11.71±0.14^{b}	4.42±0.059^{b}
G3	PCOS+ Licorice	87.76±1.03^{c}	9.21±0.11^{c}	1.99±0.032^{c}
G4	PCOS+ Marjoram	91.60±1.94^{d}	9.54±0.10^{d}	2.15±0.05^{d}
G5	PCOS+ DPP	88.27±1.79^{c}	9.35±0.12$^{c, d}$	2.03±0.047^{c}
G6	PCOS+ Mixture	88.99±2.04^{c,d}	9.48±0.12$^{c, d}$	2.08±0.048^{c,d}
G7	PCOS+ Metformin	92.96±1.02^{d}	9.82±0.10^{e}	2.25±0.035^{d,e}
	LSD	3.46	0.29	0.11

*Values are represented as mean ± SE, (n=10), there was no significance difference between means have the same letter in the same column, (P≤ 0.05).

3.6. Effect of oral administration of licorice roots, marjoram and date palm pollen aqueous extracts as well as metformin on antioxidant and lipid peroxidation status in experimental groups

According to the data summarized in tables (6a and 6b),PCOS induction caused significant reduction (P<0.05) in serum CAT activity as well as erythrocyte and ovarian GSH levels as compared to healthy control group. Moreover, PCOS caused significant elevation (P<0.05) in serum and ovarian MDA levels as compared to healthy control group. The present study showed that there were significant elevation (P<0.05) in serum CAT activity as well as erythrocyte and ovarian GSH levels, while there were significant depletion (P<0.05) in serum and ovarian MDA levels in all treated groups as compared to PCOS control group.

Moreover, there were marked elevation in blood and ovarian antioxidant markers including GSH level and CAT activity in all groups administered herbal extracts in respect to metformin administered group. While, there was significant reduction in MDA level (serum and ovarian) in all groups administered herbal extracts as compared to metformin administered group. Generally, the best antioxidant effect was observed in mixture administered group.

Table (6a): Erythrocyte GSH level, serum catalase activity and serum MDA level in experimental groups.

NO	Parameters / Groups	GSH (mg/dl)	Catalase (U/g)	MDA (mmol/L)
G1	healthy (-ve control)	17.7±0.14^{a}	680.32±1.80^{a}	62.33±3.018^{a}
G2	PCOS(+ve control)	9.58±0.16^{b}	521.23±0.97^{b}	97.051±1.007^{b}
G3	PCOS+Licorice	16.29±0.23^{c}	696.41±1.96^{c}	72.94±1.08^{c}
G4	PCOS+Marjoram	17.03±0.15^{d}	705.52±2.77^{d}	70.78±0.57^{c}
G5	PCOS+Date palm pollen	16.41±0.31^{c}	706.05±2.054^{d}	70.02±0.69^{c}
G6	PCOS+Mixture	18.04±0.23^{a}	711.95±3.27^{e}	69.34±0.77^{c}
G7	PCOS+Metformin	13.62±0.16^{e}	601.8±2.13^{f}	77.36±0.62^{d}
	LSD	0.49	3.23	5.301

*Values are represented as mean ± SE, (n=10), there was no significance difference between means have the same letter in the same column, (P≤ 0.05).

*** Table (6b): Ovarian GSH and MDA levels in experimental groups.**

NO	Parameters / Groups	GSH (mg/g tissue)	MDA (nmol/g tissue)
G1	**healthy (-ve control)**	43.24±0.42[a]	116.22±0.73[a]
G2	**PCOS(+ve control)**	12.77±0.13[b]	201.26±0.68[b]
G3	**PCOS+Licorice**	32.37±0.57[c]	147.50±1.25[c]
G4	**PCOS+Marjoram**	36.17±0.76[d]	141.31±0.77[d]
G5	**PCOS+Date palm pollen**	35.11±0.63[d]	145.70±0.28[c]
G6	**PCOS+Mixture**	35.66±0.78[d]	145.82±0.83[c]
G7	**PCOS+Metformin**	25.60±0.58[e]	159.56±0.89[e]
	LSD	1.39	1.94

* Values are represented as mean ± SE, (n=10), there was no significance difference between means have the same letter in the same column, (P≤ 0.05).

3.7. Effect of oral administration of licorice roots, marjoram and date palm pollen aqueous extracts as well as metformin on ovarian caspase 3-protein in experimental groups

The data presented in table (7) revealed that PCOS caused significant increase (P<0.05) in ovarian caspase3-protein level as compared with healthy control group. Meanwhile, oral administration of licorice roots, marjoram, date palm pollen aqueous extracts and their mixture, as well as metformin caused significant decrease(P<0.05) in ovarian caspase3-protein levels as compared to PCOS-control rats.

The current results showed significant reduction (P<0.05) in ovarian caspase3-protein level in groups received herbal extracts as compared to metformin administered group. On the other hand, there was no significant change in ovarian caspase3-protein levels between groups received licorice, marjoram, and date palm pollen.

Furthermore, oral administration of herbal mixture aqueous extract caused significant decrease (P<0.05) in ovarian caspase3-protein level as compared with groups received marjoram or date palm pollen aqueous extracts. On the other hand, there was no significant change in ovarian caspase3-protein levels between groups received licorice and herbal mixture extract.

Table (7): Ovarian caspase 3- protein levels in experimental groups.

No	Parameters / Groups	Caspase 3- protein (pmol/L)
G1	**Healthy (-ve control**	2.53±0.07[a]
G2	**PCOS (+ve control)**	8.84±0.25[b]
G3	**PCOS + Licorice**	4.50±0.12[cd]
G4	**PCOS + Marjoram**	4.66±0.16[c]
G5	**PCOS + Date palm pollen**	4.64±0.13[c]
G6	**PCOS + Mixture**	4.17±0.13[d]
G7	**PCOS + Metformin**	6.04±0.12[e]
	LSD	**0.36**

*Values are represented as mean ± SE, (n=10), there was no significance difference between means have the same letter in the same column, (P≤ 0.05).

3.8. Effect of oral administration of licorice roots, marjoram and date palm pollen aqueous extracts as well as metformin on serum lipid profile in experimental groups

Results showed that there were significant elevation (P<0.05) in serum TC, TAGs, LDL-C and VLDL-C levels, as well as significant reduction (P<0.05) in serum HDL-C level in PCOS-control group as compared to healthy control group as shown in table (8). The present results revealed that oral administration of licorice roots, marjoram, date palm pollen extracts, and their mixture as well as metformin showed hypolipidemic effects that were manifested by significant decrease(P<0.05) in serum TC, TAGs, LDL-C and VLDL-C levels and significant elevation (P<0.05) in serum HDL-C levels as compared with PCOS- control group.

Oral administration of different herbal aqueous extracts caused significant reduction (P<0.05) in serum TC, TAGs, LDL-C and VLDL-C levels and significant elevation (P<0.05) in serum HDL-C level as compared to a metformin administered group.

Generally, the data showed that there was no significant change in serum lipid profile between all herbal extracts administered groups.

Table (8): Serum lipid profile (TC, TAGs, HDL-C, LDL-C, and VLDL-C) levels in experimental groups.

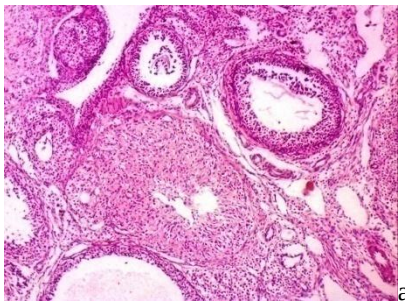
NO	Parameters / Groups	TC (mg/dl)	TAGs (mg/dl)	HDL-C(mg/dl)	LDL-C (mg/dl)	VLDL-C (mg/dl)
G1	**Healthy(-ve control)**	145.8±1.85^{a}	81.005±0.79^{a}	70.74±0.54^{a}	58.85±1.74^{a}	16.20±0.16^{a}
G2	**PCOS (+ve control)**	196.8±1.98^{b}	179.34±0.76^{b}	23.48±0.58^{b}	137.45±2.08^{b}	35.86±0.15^{b}
G3	**PCOS+ Licorice**	161.9±2.80^{c}	103.48±0.76^{c}	54.52±0.63^{c}	86.67±3.22^{c}	20.69±0.15^{c}
G4	**PCOS+ Marjoram**	158.1±1.35^{cd}	100.57±0.58^{d}	55.64±1.12^{c}	82.34±1.68^{c,d}	20.11±0.11^{d}
G5	**PCOS+ Date palm pollen**	162.7±2.06^{c}	104.18±0.82^{ce}	54.30±0.69^{c}	87.56±1.98^{c}	20.83±0.16^{c}
G6	**PCOS+ Mixture**	157.2±2.09^{d}	102.08±1.35^{c}	55.99±1.21^{c}	80.78±2.86^{d}	20.42±0.27^{cd}
G7	**PCOS+ Metformin**	176.4±2.11^{e}	107.82±0.98^{f}	51.42±0.80^{d}	103.41±2.59^{f}	21.56±0.19^{e}
	LSD	4.90	2.11	1.98	5.61	0.42

*Values are represented as mean ± SE, (n=10), there was no significance difference between means have the same letter in the same column, ($P \leq 0.05$).

3.9. Effect of oral administration of licorice roots, marjoram and date palm pollen aqueous extracts as well as metformin on ovarian microscopic examination in experimental groups

Microscopic examination of ovarian tissue of rats from healthy control group showed normal histological structure with numerous follicles of different types (Graafian follicles and Corpus luteum) as shown in fig. (1). Moreover, ovarian tissues of rats with PCOS showed marked ovarian cyst, congestion and necrosis of interstitial blood vessel and interstitial inflammatory cells infiltration as well as vacuolation of lutein cells of multiple corpora lutea as shown in fig. 2 (A and B). Rats with PCOS and administered herbal extracts of licorice roots, marjoram, date palm pollen and mixture shown in figures (3, 4, 5, 6 respectively), showed strands of fine fibroblasts proliferation between the follicles and showed numerous follicles of different types and multiple corpus luteum. On the other hand, metformin administered group showed vacuolation of lutein cells, necrosis of follicle (large arrow) and interstitial inflammatory cells infiltration as shown in fig. (7).

Oral administration of palm pollen extract could improve the induced PCOS symptoms, where the average number of primary, preantral, antral, and Graafian follicles, and corpus luteum were reduced in all PCOS treated groups compared to those in the control group. However, the number of follicles and corpus luteum were increased in the licorice roots, marjoram and palm pollen group compared to those in the PCOS control group.



a

Fig. (1)

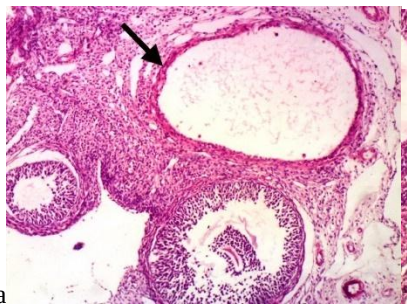


Fig.2 (A)

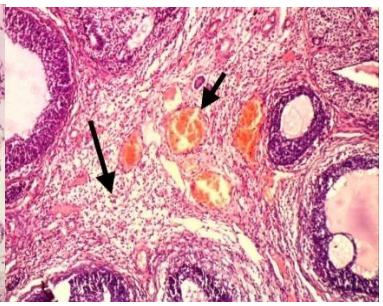


Fig. 2 (B)

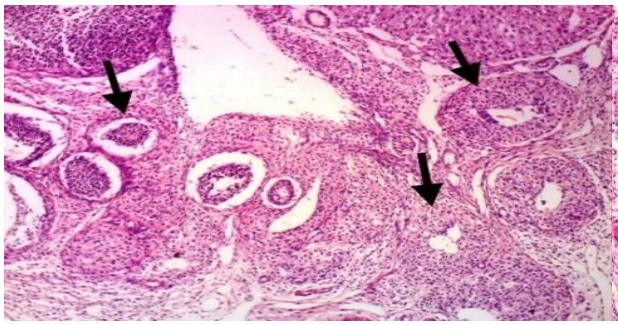


Fig. (3)

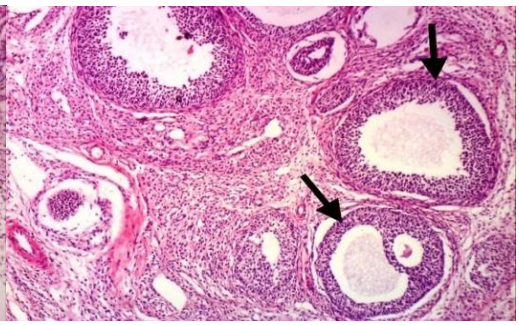


Fig. (4)

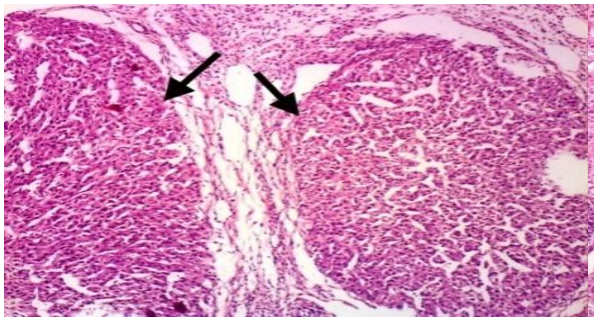


Fig.(5)

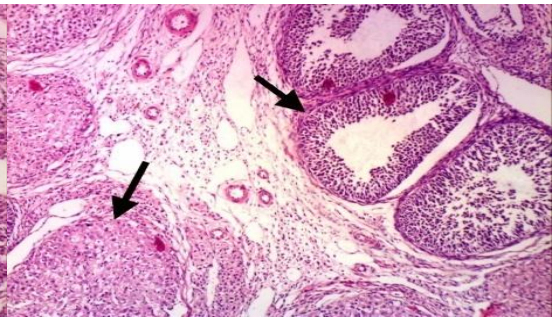


Fig. (6)

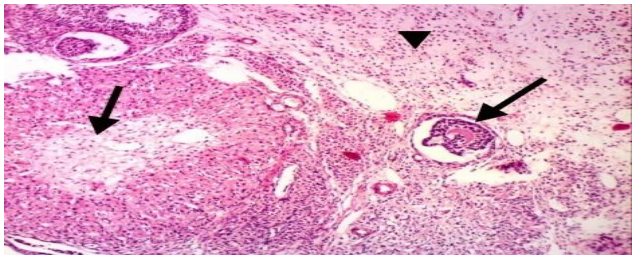


Fig. (7)

1- DISCUSSION

The current study clearly aimed to examine the effect of aqueous extracts of licorice roots, marjoram, DPP and their mixture to alleviate the reproductive and metabolic consequences caused by PCOS and compared with the metformin drug.

Letrozole (an aromatase inhibitor) acts by inhibition of aromatase activity, leading to low conversion of androgens to estrogens, resulting in an excessive accumulation of androgens in the ovary. The hormonal changes negatively affect or stop the maturation of follicles, leading to anovulation. Letrozole-induced PCOS model is a good method because animals developed many characteristics of human PCOS, including hyperandrogenism and abnormal follicles, hyperglycemia, oxidative stress and altered sex hormones (testosterone, estrogens, LH and FSH) levels.[45] As expected, it was observed in the current study that letrozole easily induced PCOS in rats after 21 days of continuous administration.

The estrus cycle is negatively affected in rats with PCOS, mainly due to the alteration of steroid hormones, which regulate ovarian function. The oral administration of letrazole to induce poly cyctic ovary in rats caused irregularity in all phases of estrous cycle, such as prolonged the hours of proestrous, metaestrus and diestrus in addition decreased and disappeared the hours of estrus as well as increased the days of estrous cycle. These altitudes of androgen levels stamped on the hypothalamus result in a non-cyclic discharge of gonadotrophins and abnormality in all estrous phases.[46] Phytochemical studies have demonstrated the presence of sterols, estrogen-like compounds and steroidal saponin glycoside in licorice, marjoram and DPP grains which help to restore menstrual cycle, regulate hormonal balance and alleviate PCOS symptoms.[45,46]

Abdominal obesity worsens the clinical features of menstrual irregularity, increases infertility and increases serum androgens and luteinizing hormone. Increase in body weight is associated with increased androgen levels in women with PCOS. A complex interrelationship thus exists between abdominal obesity, insulin resistance, androgen level and LH level in the etiology and pathogenesis of PCOS.[47] In our study, the increase in body weight and presence of estrous cycle irregularities after oral administration of letrozole; suggest that development of PCOS in rats due to increased androgen and LH hormone. In the current study, a significant decrease in body weight was observed in treated groups. The reduction of body weight through licorice, marjoram, DPP, and their mixture consumption is probably due to a combination of enhanced lipolysis and inhibition of lipogenesis, which also contributes to the reduction of hyper-insulinemia.

The hyperlipidemia observed in untreated PCOS rats was also correlated with an upward trend in body weight and a significant increase in ovarian weight which could be due to the anabolic properties of letrozole, associated with fat accumulation and multiple cysts formation in the ovary, respectively.[48] The significant reduction in ovarian weight in PCOS rats treated with herbal extracts could indicate normal follicle formation and uterotonic effects of licorice roots, marjoram leaves and DPP.

Measurement of sex hormones levels; testosterone, LH, and estradiol; is recommended for diagnosing PCOS. Indeed, elevated serum testosterone and LH concentrations and low estradiol, progesterone, and FSH levels are the most consistent hormonal features to diagnose PCOS in woman. In the current study, PCOS showed high LH and testosterone levels, low estradiol and FSH concentrations, compared with healthy control.

These results matched those of some researchers[49,50] and further confirm the PCOS condition.

The androgen-lowering effects of licorice roots, marjoram and DPP have been demonstrated in an animal study examining hormone concentration in female rats, which reported significantly reduced free and total testosterone levels.[6] The influence of the glycyrrhizic acid which establish in herbal product participated in ruling the hormones and activation the ovulation, licorice roots extract in vitro produced endorsement the formation of estradiol and activation of aromatase by glycyrrhizic acid, therefore the estrous cycles after administered rats with licorice roots extract it can be returned to the regular sequences, these results agreed with Takeuchi et al.[51]

Licorice blocks the activity of 3-β- hydroxyl steroid dehydrogenase, 17β- hydroxysteroid dehydrogenase and 17-20 lyase enzymes taking role in the metabolism and synthesis of androgen and oestrogen. It is claimed that licorice extract reduces serum testosterone hormone by stressing 17β-hidroxysteroid dehydrogenase enzyme which catalyzes the transformation of androgenic steroids into testosterone hormone.[52]

Isoflavones are a subgroup of phytoestrogens, natural plant substances with structure similar to 17-β-estradiol and capable of binding to estrogen receptors (ERs). Isoflavones possess higher affinity to ERβ than to ERα and may have a potency to activate both genomic and non-genomic estrogen signaling pathways. In addition, isoflavones interact with the metabolism of steroid hormones. Recently, isoflavones have come into focus of interest due to several reports about the positive effect on human health, in particular prevention of hormone-dependent cancers, cardiovascular diseases, osteoporosis, adverse menopausal manifestations and age related cognitive decline. Isoflavones may bring new insights in to the mechanisms of physiological regulations and increase the possibilities of medical interventions.[53]

Palm pollen extract increases the number of secondary and antral follicles and also increases the levels of ovarian hormones in rats. Stimulatory effects of Palm pollen on ovary have related by researches to the presence of compounds such as glycosidal flavonoids, saponins, alkaloids and steroidal compounds.[54] In addition to the positive effects of establishment of hormonal balance between gonadotropin and ovarian hormones in improving PCOS symptoms, administration of various kinds of antioxidants such as vitamin E and selenium is also considered as another common treatment approach to manage PCOS. Owing to its contents, e.g. estrogen-like compounds, sterols, estrone-like compounds, and steroidal saponin glycoside, DPP is believed to increase female fertility.[55]

Hyperinsulinemia is a key element in the pathogenesis of PCOS. Insulin sensitizing drugs including metformin has been used as a treatment for this syndrome. It prevents hepatic glucose release and improves peripheral tissue sensitivity to insulin, reducing the androgen synthesis by ovarian theca cells. Metformin also suppresses ovarian steroidogenesis.[56]

Licorice root administration caused significant reduction in serum glucose levels that may be due to by inhibition of α-glucosidase and α-amylase enzyme activities leads to a reduction in disaccharide hydrolysis which has beneficial effects on glycemic index control.[57] Glycyrrhizic acid (GA) produced a significant decrease in fasting blood glucose and mean serum insulin concentration in obese rats after 28 days of GA treatment. This could be due to the activity of GA on inhibiting 11β- hydroxyl steroid dehydrogenase type 1 (11β-HSD 1), which in turn decreases the hexose-6-phosphate dehydrogenase (H6PDH) activity and glucocorticoid production resulted in decrease the conversion of oxaloacetate into phosphoenol pyruvate and carbon dioxide in the gluconeogenesis pathway, as well as glucose-6-phosphatase that hydrolyzes glucose-6-phosphate into a phosphate group and free glucose.[58]

Marjoram partially affects peroxisome proliferator-activated receptors (PPAR-γ) expression and its action is dependent on lipolysis through its action on lipoprotein-lipase (LPL) expression but not on lipogenesis. Insulin integrates hepatic carbohydrate metabolism by increasing the biosynthesis of enzymes of glycolysis, glycogenesis, and pentose oxidative pathway; and by inhibiting gluconeogenesis.[59] However, in present study because of insulin resistance and hyperinsulinemia, cells are not able to consume glucose under the effect of insulin but cells increased its internal gluconeogenesis by the effect of marjoram.

In addition, marjoram cause significant reduction of testosterone, which is considered mainly to be an adrenal product, suggests an androgen-lowering effect of marjoram tea, particularly for the adrenal androgens. The phenolic content of marjoram aqueous extract mainly includes caffeic acid derivatives, such as rosmarinic acid, as well as glycosides of luteolin and hydroquinone. Rosmarinic acid and luteolin are among the phenolic compounds reported to exhibit an insulin-sensitising effect via the activation of PPARs.[60]

The normo-glycemic effect of DPP may be due to its minerals, phenolics and phytoestrogens constituents. The minerals that are present in DPP have a vital role in management of hyperglycemia such as magnesium that plays a key role in regulation of insulin action and insulin-mediated-glucose uptake. In addition, zinc induces the insulin formation and release, while chromium potentiates the insulin action, and selenium, which has been shown to stimulate glucose uptake, regulates glycolysis and pentose phosphate pathways. Phenolics present in DPP are considered to be a potent inhibitor of alpha glycosidase and alpha amylase, leading

to reduction of carbohydrates' digestion and absorption that may counteract the hyperglycemia present in case of PCOS.[61]

In PCOS, high level of androgens induces insulin resistance through the activation of the lipolytic cascade and by modifying muscle histology. Hyperandrogenism also leads to dyslipidemia. Free testosterone levels have been implicated in lowering HDL-C levels. Androgens through the interaction with androgen receptor (AR), decrease the catabolic removal of LDL-C.[62]

Licochalcone A. (LA) a constituent of licorice roots suppressed hepatic triacylglycerols accumulation. Licochalcone A restrained lipogenesis via suppression of sterol regulatory element binding protein1 (SREP1C) and target enzymes (glycerol3phosphate acyltransferase, stearoyl COA desaturase 1 and fatty acid synthase) transcription. Licochalcone A up-regulated gene expression of proteins such as PPAR-α and fatty acid transport (FAT/CD36) which are responsible for lipolysis and fatty acid transport. Chalcones of licorice roots decrease the levels of plasma total cholesterol and TAGs. Chalcones showed strong inhibition against pancreatic lipase. Saponins which are present in the root of licorice and DPP are known to lower TAGs by inhibiting pancreatic lipase activity.[63]

Phytosterols present in herbs are well known for their ability to inhibit absorption of cholesterol and lowering of serum cholesterol by two main processes, preferential uptake in the gut for plant sterols versus cholesterol, and improving elimination of cholesterol, fat solubility of phytosterolesters followed by their effective intestinal hydrolysis, preferentially of unsaturated fatty acid esters, allows sufficient micelle solubilization of un-esterified plant sterols for prevention of cholesterol absorption, and subsequent lowering of their serum concentrations.[64]

Saponins form strong insoluble complexes with cholesterol and bile making them unavailable for absorption, this mixture is then removed from the body through the normal elimination process, increased bile acid excretion may cause compensatory increase in bile acid synthesis from cholesterol in the liver. As the body needs more cholesterol for bile acid production that used for digestion, the liver removes cholesterol from the blood stream through increase the hepatic LDL-receptor levels, increase hepatic uptake of LDL-cholesterol and aid its catabolism to bile acids, thus lowers serum cholesterol.[65]

Saponins are known to inhibit pancreatic lipase, leading to greater fat excretion due to reduced intestinal absorption of dietary fats, by this mechanism saponins can lower TAGs level, furthermore, the decline in VLDL cholesterol levels in treated groups could be directly correlated to a decline in TAGs levels of these groups, as it is well established that VLDL particles are the main transporters of TAGs in plasma.[66]

Marjoram hypolipidemic mechanism might be explained by the presence of flavonoids, which exerts lipid-lowering effects by reducing the activity of 3-hydroxy-3-methylglutaryl-CoA enzyme and increasing the liver receptors.[67]

Many studies reported oxidative stress as one of the pathological factor for PCOS. Increased oxidant levels may alter the stereo diagnosis in ovaries contributing to increased androgen production and polycystic ovaries. In the present study, it was observed that the PCOS animals exhibited elevated oxidative stress markers MDA and reduced endogenous antioxidants in ovary. Catalase activity and GSH level were significantly diminished in the PCOS group and concomitant treatment with herbal treatment restored their activities. Lipid peroxidation is generally used as one of the marker for oxidative tissue damage, as it induces free radical damage to the components of cell membrane which leads to cell necrosis and inflammation.[68]

Palm pollen contains a variety of natural antioxidants including different kinds of vitamins and minerals such as zinc and selenium. The results of a study indicated that levels of reactive oxygen species (ROS) in ovarian tissue are increased in PCOS and the balance between oxidant and antioxidant system is disturbed in this condition. Natural growth of Theca interstitial layer is necessary for normal ovarian function and oxidative substances and free radicals impair regular growth and apoptosis in this layer. It has been known as there is a direct relationship between reduced oxidative stress and increased maturation of oocytes in women with PCOS and infertile women. So, antioxidants can improve PCOS symptoms by reduction of oxidative stress.[69]

Moreover, licorice root has also been shown to have a significant free radical quenching effect. Glabridin is reported to be a potent antioxidant towards LDL oxidation. There are several studies suggesting that licorice has anti-inflammatory, apoptotic, angiogenic, and estrogen-like effects.[70]

The protective role of marjoram, which could be due to the anti-oxidative effect of flavonoids present in the plant which act as strong superoxide radical and singlet oxygen quenchers. The biological mechanisms of flavonoids have been attributed to their antioxidant properties through several possible mechanisms, such as their ability to scavenge free radicals, break radical chain reactions, directly reducing peroxides, and stimulating the anti-oxidative defense enzyme activities.[71]

Phenolic compounds react with lipid radicals to form non-reactive radicals, interrupting the propagation of chain reactions. These compounds are able to donate an electron or a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation and stabilizes the resulting phenoxyl radical. Phenolic compounds exert their antioxidant abilities by

scavenging peroxyl and alkoxyl radicals and by chelation of transition metal ions present in trace quantities.[72]

Furthermore, letrozole injection negatively affected the ovarian architecture by decreasing the number of corpus luteum and increasing cystic and atretic follicles. On the other hand, Palm pollen extracts almost restore normalcy of the induced PCO structure.[73] The microscopic examination of ovarian tissues showed significant improvement in ovarian architecture (reduction of cystic follicles and elevated number of corpus luteum) after herbal and metformin administration, thus corroborating our findings, licorice roots, marjoram, DPP and their mixture are potential agents for the treatment of reproductive and metabolic consequences related to PCOS.

The absence of corpora lutea reflected the failure of the ovulation due to disturbances of feedback mechanism between ovary and pituitary gland leading to hormonal imbalance (LH/FSH).[74] As observed in the current study, a decline in estrogen level in untreated PCOS rats is correlated with multiple cysts formation and low number of corpus luteum in the ovary.

Metformin inhibits the production of hepatic glucose, decreases lipid synthesis, increases fatty acid oxidation and inhibits gluconeogenesis resulting in a decrease in circulating insulin and glucose. Metformin enhances insulin sensitivity at the cellular level and also appears to have direct effects within the ovary. Therefore, it would seem logical to anticipate that insulin lowering and insulin-sensitizing treatments, such as metformin, should improve symptoms and reproductive outcomes for women with PCOS.[75] In the present study herbal extracts were used as comparative agents, and to recommend the similar effect of metformin with no side effects. Metformin side effects include vomiting, nausea, gastrointestinal disturbances congestive heart failure and osteoporosis.[25]

The therapeutic effects of herbal extracts were characterized by the restoration of estrus cyclist, the reduction of blood glucose level and oxidative stress as well as the improvement of lipid profile and sex hormones.

2- CONCLUSION

It can be concluded that, that letrozole-induced PCOS in rats was associated with reproductive and metabolic disorders. Licorice roots, marjoram leaves and date palm pollen restore estrus cyclist, reduce blood glucose level and oxidative stress, improve lipid profile and sex hormones, and prevent ovarian damage in PCOS rats after 30 days of treatment. These plants might be considered as an alternative therapeutic remedy to treat reproductive and metabolic disorders in patients with PCOS and lead to augment positive consequence when all herbs mixed together. These herbs are available widely in most of the area and these can be used easily and conveniently by the community persons. They are effective as anti-PCOS compounds with no side effects.

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