Modulation of steroidogenesis by *Actaea racemosa* and vitamin C combination, in Letrozole induced polycystic ovarian syndrome rat model: Promising activity without the risk of hepatic adverse effect

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Abstract

Background Complementary remedies such as the Chinese herb ‘Sheng Ma’ (Black cohosh; *Actaea racemose* ‘AR’) are being sought to surmount the shortcomings of conventional hormonal and surgical therapies developed in the treatment of polycystic ovary syndrome (PCOS). However, the AR hepatotoxicity urges a cautionary warning to be labelled on its products as recommended by the United States Pharmacopeia where 4 out of 7 hepatotoxic cases in Sweden were possibly associated with black cohosh products.

Methods We investigated the efficacy, safety and molecular targets of black cohosh ethanolic extract and/or vitamin C on ovarian functionality, oxidative response in the hyperandrogenism-induced PCOS alongside with holistic profiling of its arsenal of secondary metabolites using UPLC-Qtof-MS. A well-established rat model using oral letrozole, daily, for 21 days was employed then the rats received the AR extract with and without vitamin C for 28 days. The hormonal evaluation, antioxidant status, histopathological examination, immunohistochemical analysis, cell proliferation, apoptosis, and the expression ratio of the aromatase (Cyp19α1) gene were evaluated.

Results Beneficial effects were exerted by AR in PCOS rats via the improved antioxidant status, hormonal profile, lipid profile, glucose level, liver functions, curtailed percentage of apoptotic cells and the induced Ki-67 expression in the granulosa, theca cell layers and interstitial stromal cells. Notably, AR combination with vitamin C was not only more effective to reverse the dysregulated levels of testosterone, luteinising hormone, and mRNA level of Cyp19α1 gene in the PCOS rat, but also safer, while the combination regulated both ovarian and hepatic MDA and GSH levels with a histologic improvement noticed in livers and ovaries. In addition, the untargeted metabolomic profiling enabled the identification of 61 metabolites allocated in five major chemical classes.

Conclusion This study demonstrated the benefit of the combination between AR and vitamin C in mitigating the reproductive and metabolic disorders of PCOS with the elimination of AR hepatotoxic risk.

1. Background

Polycystic ovary syndrome (PCOS) is a complex metabolic-endocrine disorder (1) that affects 4–18 % of women at the age of reproduction (2). The genetic predisposition to PCOS is uncertain, and there is no genetic screening test endorsed (3). PCOS characterized by anovulation, menstrual irregularity, amenorrhea, hirsutism and infertility (2). Furthermore, various metabolic and clinical complications have been reported such as insulin resistance and diabetes, obesity, extensive coronary artery disease, hypertension, endometrial hyperplasia, ovarian and breast cancers (4, 5). In PCOS, the secretion and metabolism of estrogens and androgens are disturbed (6). The excess androgen level is a leading cause of PCOS (7) where, the elevated Gonadotropin-releasing hormone (GnRH) during PCOS stimulates the ovarian thecal cells, in turn, more androgens are produced.

Various side effects are associated with chemical and hormonal PCOS therapies such as hyperplasia, uterine bleeding and uncertain risks (8, 9) along with considerable cost (10), nonetheless, these therapies may not be effective in some cases. Therefore, several studies are focused on the investigation of complementary herbal medicine as a possible treatment of PCOS (8, 11). For instance, promising effects of hops, dong qui, ginseng , liquorice, black cohosh and kelp against PCOS were reported (12).

Black cohosh (*Actaea racemosa* (AR) or formerly; *Cimicifuga racemosa*) is a common herbal treatment in Europe for treating a variety of women's health disorder and a common Chinese herb in the history of traditional Chinese medicine (13). Preclinical and clinical investigations provided evidence on the efficacy of AR for treatment of oligo/amenorrhea, hyperandrogenism, mammary cancer and PCOS in woman, with a limited quantity of preclinical data, and variable quality of clinical evidence (14). Though the positive effect of AR on peri-menopausal women cannot be denied, its efficacy on menopausal symptoms' treatment is undefined and further investigations are necessary (15). Recent studies revealed conflicting evidence concerning AR safety especially on the liver and its efficacy (16-18). The Medical Products Agency in Sweden found that 4 of the 7 hepatotoxic cases were possibly associated with black cohosh products, but 3 of those were confused by the use of other liver injury related drugs (19). The consumption of black cohosh was correlated with acute liver injury (20), where the AR hepatotoxicity warning should be labelled on its products package as recommended by the United States Pharmacopeia (21).

Considerable protective action of vitamin C/ascorbic acid against hepatotoxicity has been reported (22, 23). Moreover, antioxidants such as vitamin C has been associated with fertility for many years. Ascorbic acid showed a chemo-protective effect against degenerative changes in ovarian tissue (24), however, its precise physiological role in reproduction has been undetermined.

The present study was established to pinpoint the mechanism and molecular targets of AR for the treatment of PCOS alongside with any anticipated combinatorial benefits with vitamin C, and the investigation of the concerning hepatic safety. In addition to UPLC-HDMS profiling of AR with ion mobility spectroscopy (IMS) for more confidence level of identification to support further chemometric and/or bioactive molecular network study identifying potential active metabolites.

2. Material And Methods

2.1. Preparation of AR extract

Black cohosh powdered roots and rhizomes (0.5 kg) were obtained from medicinal plant store (Haraz) in Cairo, Egypt and were exhaustively extracted by 70% ethanol (2 × 1.5 L). Solvents were evaporated under vacuum, in a rotary evaporator (Büchi, Switzerland), at 45–55 °C (25). The dried ethanol extract of *Actaea racemosa* was weighed, and the percentage yields calculated. For pharmacological assays, the dried extracts were dissolved in 0.5% Carboxy Methyl Cellulose (CMC), which was found not to interfere with any assay.

2.2. UPLC-MS fingerprinting of the AR extract

The AR extract was dissolved in acetonitrile and centrifuged at 13,000 g for 15 min at 4 °C. The supernatant was filtered through a 0.2 µm PTFE filter. The metabolomic analysis was performed in Acquity UPLC (Waters, USA) coupled with SYNAPT G2-S (Waters, USA) mass spectrophotometer. The sample injection volume was 5 µL, and the flow rate was set at 400 µL/ min. Chromatographic separation was achieved using ACQUITY UPLC HSS T3 Column (1.8 µm, 2.1 X 150 mm, Waters Corporation, Milford, USA). The column temperature was kept at 45 °C and gradient elution was implemented utilizing water with 0.1 % formic acid (A), and acetonitrile with 0.1% formic acid (B). Initially, 1% of the mobile phase B was used for 2 mins, and linearly inclined as the following gradient: 35-60% B (2-4 min), 60-80% B (4-8 min), and 99% B (8-8.5min) and finally declined to 10% B till reach 11.5 min.

G2-S high definition mass spectrometer (HDMS) (Waters Corp, Manchester, England) equipped with Z-spray source controlled by MassLynx v4.1 was used for mass spectrometry analysis in both positive and negative ESI ionization modes using HDMS mode of operation. The scanning mode parameters were: source temperature; 120 °C, desolvation temperature; 500 °C, cone gas flow; 50 L/h, desolvation gas flow; 1000 L/h, collision energy ramp; 20-50 eV, capillary voltage; 2.5kV, and acquisition mass range; 50-1200 m/z (26). Data acquired in a profile mode and corrected with separate lock mass spray switching between the injected samples and external reference permitting the MassLynx to continuously ensure mass analysis accuracy. Leucine enkephaline (1 ng/µL) was used as an external reference in 1:1 acetonitrile-water containing 0.1% formic acid at flow rate 5 ul/min via a lock-Spray interface, generating a reference ion for positive ion mode $[M-H]^+$ and negative ion mode $[M-H]^-$ of 556.2771 and 554.261 m/z, respectively (27).

Three technical replicates were implemented in a randomized batch sequence. To enable proper column equilibration and conditioning, the mobile phase run for 1.5 h, followed by six quality control (QC) samples before each batch analysis. In line with the published guidelines and to overcome the UPLC-MS analytical drifts, QC samples were injected at regular intervals during the experimental sequence (28). Features were considered reproducible if their coefficient of variation (CV) among the samples were < 25%, and the fold change (FC) > 2, ANOVA p-value and Q value < 0.01 against blank samples.

Progenesis QI software (Waters Corp., USA) was used for data processing and putative identification of metabolites of interest by comparison with metabolomic profiling CCS library, LipidBlast and Progenesis Metascape imported databases including HMDB, MONA, and GNPS.

2.3. In vivo experiments

Virgin, cyclic, adult female Wistar Albino rats (160–200 g) were employed for the study. Animals were acquired from Laboratory Animal Colony, Helwan, Egypt and housed in the animal house of faculty of veterinary medicine, Cairo University, and allowed to acclimatize for two weeks. During the study, all animals were caged in standard polypropylene cages and maintained in a controlled environment of (22

± 3) °C temperature, (55 ± 5) % humidity and a 12 h light/ dark cycle. Rats were provided with standard diet and water ad libitum.

2.4. Experimental protocol

Forty-eight female rats were randomly allocated into six groups of 8 each. Animals of group one served as negative control and received a daily oral dose of (1ml) the vehicle (0.5% CMC) for 49 days. The induction of PCOS was done guided by an established rat model described by (29). Animals of groups 2 to 6 received letrozole (LTZ) (Natco Pharma Limited Hyderabad) at a dose of 1 mg/kg dissolved in 0.5% CMC once daily for 21 days for induction of PCOS. Then, received orally the different samples (vehicle, standard and extracts) for 28 days, Group 2 (positive control) received only the vehicle; Group 3 received Clomiphene Citrate in 0.5% of CMC with a dosage of 1 mg/kg (Fertyl-Super tablets was procured from Ar-Ex Laboratories Private Limited, Goregaon (E), Mumbai) as a standard ovulation induction drug (30). Groups (4-6) were treated for 28 days with AR extract; 7.14 mg/kg (31), vitamin C; 500 mg/kg (32) and AR extract with vitamin C, respectively. From the Day 6 of the treatment, daily vaginal smears of all rats were observed to test ovulation where, indiscriminate estrous cycle with prolonged diestrus phase indicated PCOS (33, 34). On the 50^{th} day of the study, all the rats were anesthetized with ketamine 91 mg/kg, i.p. Duplicate blood samples were collected into sodium heparin tubes for separation of plasma and into gel separator tubes for collection of serum samples. The serum was separated by blood centrifugation at 3000 g at 4º C for 10 min and used for different biochemical assays. The animals were then sacrificed, ovaries and uteruses excised, cleaned of fat and weighed and divided into triplicates; two sets; stored at –80º C to be used for real-time reverse transcriptase-PCR and antioxidant assays. Other sets of the ovaries in addition to liver tissue were fixed in 10% neutral buffered formalin for histopathological examination. After weighing the body and ovaries of each animal at the day of scarification, the relative weight of ovary was calculated as the ratio of the ovary (wet weight, mg) to body weight (g).

2.5. Hormonal profile

The serum total testosterone was measured using a commercial ELISA kit (chemux bioscience Inc, USA) and beta subunit chain of luteinising hormone (LH) level were measured using rat lutropin subunit beta ELISA kit (EIAab, China) following the instructions of the manufacturer.

2.6. Malondialdehyde (MDA) and antioxidants biomarkers determinations

Ovarian and liver tissue were separately homogenized in 10 ml cold buffer (50 mM potassium phosphate, pH 7.5 for MDA and 50 mM potassium phosphate, pH 7.5. 1 mM EDTA for reduced glutathione (GSH)) per gram tissue using glass homogenizer then tissue homogenate was centrifuged at 15000 rpm for 15

min. The supernatant was used to measure reduced glutathione (GSH) and MDA concentrations according to the standard protocols (35, 36).

2.7. Biochemical parameters

Serum glucose level, lipid profile (Cholesterol, triglycerides, and HDL cholesterol) and liver enzymes (ALT and AST) were measured using commercial kits (spectrum, Egypt), where;

VLDL cholesterol concentration = Triglycerides/5

LDL cholesterol concentration = Total cholesterol – (HDL + VLDL)

2.8. Histopathological examination

Ovaries and liver from the different groups were collected and fixed in 10% neutral-buffered formalin and processed to obtain 3-4 µm paraffin-embedded sections. The sections were stained with hematoxylin and eosin (H&E) and the morphometric analysis of the ovaries was performed (37, 38). The number of follicular cysts, average follicular cyst (mean), diameter, the thickness of the follicular wall, the thickness of granulosa cell layer and thickness of theca cell layer were measured.

2.9. Immunohistochemical analysis

The tissue sections deparaffinized, rehydrated and pre-treated with 10 mM citrate buffer for antigenic retrieval. Sections were incubated overnight at 4° C in a humidified chamber with the following primary antibody: rabbit monoclonal anti-Ki 67 antibody (Dako, Denmark) in 1:25 dilution. The tissue sections were incubated with a biotinylated goat anti-rabbit and mouse antibody (Thermo Scientific, USA), Streptavidin peroxidase (Thermo scientific, USA) and 3,3’-diaminobenzidine tetrahydrochloride (DAB, Sigma). The slides were counterstained with Mayer’s hematoxylin then dehydrated and mounted. The primary antibody was replaced by PBS for negative controls. The stained sections were analysed by Leica Qwin 500 Image Analyzer (Leica, Cambridge, England). In each field, the immunolabeled area (dark brown) was recorded. The percentage of positively stained area in the granulosa cell layer, theca cell layers and interstitial stromal cell layer was calculated (39).

2.10. Quantitative real-time PCR for Aromatase (Cyp19a1) gene

Total RNA was extracted from the female rat ovarian tissue samples using RNeasy mini kit (Qiagen, Hilden, Germany). The quantity and purity of total RNA were measured by the NanoDrop Spectrophotometer. The cDNA synthesis was carried out using reverse transcriptase (Invitrogen,

California, USA) and oligo-dT following the manufacturer protocol. The cDNA samples were then submitted to qPCR using the following primer pairs for Cyp19α1 gene forward CTGCTGATCATGGGCCTCCT and reverse (5'-CTCCACAGGCTCGGGTTGTT -3'). The cDNA was amplified by 40 cycles of denaturation at 95° C for 45 s, annealing at 59° C for 45 s and extension at 72° C for 45 s. The 95° C step was extended to 4 min along the first cycle. The amplicon size was confirmed by 2% agarose gel electrophoresis stained with SYBR Safe DNA gel stain (Invitrogen). The β-actin gene was furtherly amplified in the same reaction to serve as the reference gene (40). Each measurement was repeated 3 times, and the values were used to calculate the gene/β-actin ratio, with a value of 1.0 used as the control (calibrator) and the normalized expression ratio was calculated (41).

2.11. Statistical analysis

The different analytical measurements in the biological samples were carried out in tripclicate and results are expressed as the mean ± SD. Data for multiple variable comparisons were analysed by one-way analysis of variance (ANOVA) test to analyse the significant differences ($P < 0.05$) between groups using SPSS version 24 package for Windows.

3. Results

3.1. Identification of metabolites via UPLC-QTof-MS

Roots and rhizomes extract of black cohosh were analysed in both positive and negative ESI modes using UPLC-High Definition MS (HDMS) system which combines the quantitative time of flight (QTof), HDMS^{TM}, and StepWave^{TM} ion optics. It utilizes the ion mobility mass spectrometry (IM-MS) based measurements and separations with the high-performance tandem mass spectrometry. Notably, IM-MS and collision cross-section CCS measurement recognized as a valuable tool for a variety of studies (42). Metabolites were tentatively identified based on their accurate mass, isotope distribution, fragmentation patterns, comparison with common mass libraries and the dictionary of natural products (CRC) and reported literature, as well.

The untargeted metabolomic analysis enabled the putative identification of 61 compounds in both ionization modes and their structures were allocated as thirteen flavonoids and its glycosides, twelve fatty acids/lipids, ten tannins, seven triterpenes, six steroids, two miscellaneous terpenoids, two carbohydrates and one phenolic acid derivative (Table S1, Figure S1-S43).

3.2. Relative ovarian and uterine weights

In general, the relative weights of both organs were reduced in all groups after treatment compared from the PCOS group. Nevertheless, these differences were significant for ovaries and insignificant for uterus.

3.3. Hormonal profiles

Induction of PCOS significantly increased LH and testosterone concentrations compared to control untreated group. However, rats treated with AR extract and its combination with vitamin C for 28 days showed significantly lowered LH and testosterone levels. The combination group provoked superior hormonal effect, in contrast to vitamin C which displayed the least effect (Figure 2A and B).

3.4. Oxidative stress biomarkers in ovary and liver tissues

Induction of PCOS significantly increased MDA levels, while GSH activity was significantly decreased compared to the normal group. *Actaea racemosa* hydroethanolic extract with, and without vitamin C restored normal ovarian MDA and GSH activities (Figure 3A, B). While, the hepatic MDA and GSH activities were demonstrated in (Figure 4A, B). The exerted hepatic oxidative stress by AR was prevented in combination with vitamin C.

3.5. Biochemical parameters

The changes in lipid profile (total cholesterol, triglyceride, LDL cholesterol, VLDL cholesterol) and glucose concentrations were reported in Table 1. Furthermore, ALT and AST activities were significantly inclined in AR and PCOS groups when compared with the control group. They were significantly lowered in vitamin C group and to a lesser extent in the combination group, respectively.

3.6. Histopathology of ovaries

The normal control group showed normal ovarian histology in the form of multiple follicles in the different stage of development with normal granulosa, theca and interstitial stromal cell layers in addition to various corpora lutea (Figure 5A, B). PCOS group revealed numerous ovarian cysts in addition to small follicles in the early developmental stage and without any evidence of corpora lutea (Figure 5C). The follicular walls in the cystic follicle contained a very thin layer of flattening granulosa cells (Figure 5D) that most of them exhibited necrosis and apoptosis. The theca cell layers showed marked proliferation of theca interna and theca externa cells. Some ovarian cysts did not contain any granulosa cells with only theca interna and theca externa cells. Other ovarian cysts, the granulosa cell layers were hyperplastic and folded. The interstitial stromal cell layer was very thick with proliferated and hyperplastic cells forming cord-like structure. The same ovarian architecture was observed in vitamin C treated group (Figure 6C, D). The groups treated with clomiphene (Figure 5E, F), AR extract (Figure 6A, B) and AR combined with vitamin C (Figure 6E, F) revealed significant reduction in the number and the diameter of ovarian cysts

with a significant restoration of the granulosa cell layer thickness and marked reduction of both theca cells and interstitial stromal cell layers comparing to PCOS treated groups (Figure 7A, B, C, D).

3.7. Immunohistochemical analysis of Ki-76 expression on ovaries

Figures (8 and 9) summarised the results of immunohistochemical analysis of Ki-67 expression in the granulosa cell, theca cell and interstitial stromal cells of the ovaries in the different treated group. The ovary of the normal control group showed strong immunopositivity to Ki-67 expression in the granulosa cell layer of the follicles (Figure 8A). Both PCOS and Vit C treated groups, revealed weak immunoreactivity in the granulosa cell layer and strong immunopositivity in the theca cell and interstitial stromal cell layers (Figure 8B, E). The groups treated with clomiphene, AR and AR combined with vitamin C showed strong immunopositivity in the granulosa cell layer of follicles with weak immunoreactivity in the theca cell and interstitial stromal cell layers (Figure 8C, D, F).

3.8. Histopathological examination of liver

The livers of control and vitamin C treated groups revealed normal hepatic architecture (Figure 10A, E). The livers of PCOS and clomiphene treated groups showed moderate and mild vacuolar degeneration with single hepatocyte necrosis, respectively ((Figure 10B, C). The liver of AR treated groups revealed moderate vacuolar degeneration, apoptosis, moderate hepatocellular necrosis (Figure 10D) and sinusoidal dilation with leukocytosis. However, the group treated with AR combined with vitamin C exhibited marked improvement of the previously described hepatic lesions induced by AR extract treatment alone. The liver showed mild vacuolar degeneration and sporadic cell necrosis (Figure 10F).

3.9. Quantitative real-time PCR for Aromatase Cyp19α1

To evaluate the mRNA expression level of an enzyme related to the steroid biosynthesis pathway such as aromatase (Cyp19α1), we performed real-time PCR. Figure (11) shows the mRNA expression level measured by quantitative PCR method in ovarian tissue from different groups for Cyp19α1 gene. Quantitative real-time PCR analysis showed that the Cyp19α1 mRNA expression level was significantly downregulated in the PCOS group compared to control. Interestingly, the treatment of AR extract and/or vitamin C significantly induced the expression level for Cyp19α1. The combination treatment with AR extract and vitamin C is more effective in upregulating mRNA expression of Cyp19α1 than each treatment alone.

4. Discussion

Regardless the growing literature on the AR effectiveness for menopausal symptoms, definite assumptions on its exact mechanism of action cannot be derived owing to the methodological limitations of the available studies, in particular the lack of blinding and long-term follow-up alongside with the product and dosage variation (43). In the present study, the plethora of active secondary metabolites such as triterpenes, steroids, alkaloids, flavonoids, and tannins of the black cohosh root ethanolic extract have been tentatively identified using UPLC-HDMS (Table S1). An extra dimension of separation was achieved via IMS based on the size, shape and charge of the analytes, where characteristic CCS values were reported and could have been used in combination with other molecular identifiers to enhance the confidence level of metabolite recognition.

The efficacy of LTZ rat model in the induction of PCOS is well established and represents human PCOS in various aspects (44-46). Oxidative stress may be engaged in the pathogenesis of PCOS (47). Induced oxidative stress in ovaries by LTZ (48) was similar to that observed in natural PCOS (6). Our results indicated a significant increase in MDA level and reduction in GSH activity. In PCOS, augmented formation of MDA may be due to the incremented oxidation of biomolecules resulting in excessive lipid peroxidation, and thus deterioration of tissue by free-radical mediated mechanism (49). The provoked enhancement in ovarian MDA and GSH activities by AR may be associated with its global mechanisms for PCOS treatment. Furthermore, the effect of AR and vit C combination on the ovarian MDA and GSH were statistically significant compared from the single treatments. On the other hand, the effect of the combination on the hepatic MDA and GSH was only significant against AR, not vit C indicating the hepatoprotective effect of the vit C in the combination and the hepatotoxic effect of AR alone.

In LTZ model of PCOS, the conversion of androgen substrates into estrogens in the granulosa cells was blocked leading to the accumulation of androgen (44), which in turn can cause early luteinization of the ovarian granular cell layer, ceasing follicular development with eventual anovulation or poor ovulation (50). In our study, LTZ treated group exhibited numerous ovarian cysts without any evidence of corpora lutea and the follicular walls contained a very thin layer of flattening granulosa cells with marked proliferation of theca interna and theca externa cells (Figure 6), similar results were previously reported by (29, 38).

Previous studies listed that ovarian cysts were formed by apoptosis of both ovarian oocytes and granulosa cells where Ki-67 can be used as an indicator for cellular proliferation (51). In our work, we observed higher expression of Ki-67 in the theca cell layer and in the interstitial cells which responsible for excess androgen production in the PCOS group (29). The groups treated with AR with and without vitamin C showed significant intense immunolabeling of Ki-67 in the granulosa cell layer with weak immunolabeling of Ki-67 in the theca cell layers and interstitial cells which indicated their role in the reduction of the high androgen level induced in PCOS. This finding outlined that AR may be a useful therapeutic approach in case of PCOS by protecting the granulosa cell layer from apoptosis and necrosis and it could help in their maintenance and proliferation with androgen level reduction.

On the other hand, the androgen aromatisation to estradiol in dominant follicles is conducted in the granulosa cells by the aromatase enzyme. Aromatase (Cyp19α1) is a key steroidogenic enzyme that separately catalyses the conversion of testosterone to estradiol and estrone. Aromatase is encoded by the Cyp19α1 gene (52). Intriguingly, the Cyp19α1 encoding gene can be included as a major determinant of risk for PCOS (53). Our findings unveiled a significant downregulation of CYP19α1 mRNA expression level in PCOS ovary tissues. These results were consistent with previously published data (45, 53, 54). Remarkably, the AR and vit C combo showed superior and significant gene upregulation versus its components. CYP19α1 expression could be regularly suppressed in PCOS ovaries owing to the promoter hypermethylation such as the promoter hypermethylation of Cyp19α1 which may play a key role in the PCOS pathogenesis (55). In addition, the testosterone was reported as a crucial factor responsible for aromatase downregulation in PCOS with the downregulation in both aromatase mRNA and protein levels in cultured luteinized granulosa cells (54). The present study proposed the reversal of PCOS downregulation of the steroidogenic CYP19α1 gene expression as a new mechanism for AR's treatment effects on PCOS with superior effect when combined with vit C.

The reported elevation in testosterone and LH hormones in LTZ induced PCOS rat models (8, 56, 57) were significantly restored by AR (Figure 2A and B). Similar data were reported on the treated cells isolated from ovariectomized rats (14, 58) and on the ovariectomized rats (59). That probably mediated by interfering with the LTZ inhibitory effect on androgen aromatization, preventing excessive androgens accumulation in the ovaries as mentioned before. Moreover, AR has an estrogen-like effect, where it works directly on the hypothalamus to decrease GnRH and the subsequent reduction of LH hormone (60, 61).

The hormonal disorders of PCOS are combined with metabolic disorders. High testosterone concentrations in PCOS lead to pancreatic β cell dysfunction, insulin resistance, and thus hyperglycemia and dyslipidemia (62, 63), moreover, the androgens have an antilipolytic effect (64). Consistently, our results showed hyperglycemia and significant elevation in all lipid profile parameters; cholesterol, triglyceride, LDL, VLDL and reduction in HDL in LTZ induced PCOS rats compared with normal control (Table 1), (65-67). Hyperglycemic and dyslipidemic effects observed in black cohosh rats were related to PCOS, while AR does not affect lipid profile and glucose in women (68, 69). Vitamin C exerted obvious enhancement in glucose and lipid metabolism, and increased the HDL level, which were previously detected in both diabetic patients (70) and rats (71).

Hepatic safety of AR stills a debatable issue. We observed a significant surge in ALT and AST activities, and hepatic MDA levels with a reduction in hepatic GSH activity in AR group when compared to normal control, vitamin C and the combination group (Table 1, Figure 4A, B). Similar findings for AR induced hepatic oxidative damage was reported (20, 72, 73), while this was contradicted in other studies (18, 74). The increased hepatic lipid peroxidation with AR treatment was previously observed by (75). Although, no reduction was observed in the hepatic GSH with AR 0.6 mg/kg b. wt in ovariectomized female rats, a marked reduction in the hepatic GSH of male rats treated with AR (300 mg/kg) was previously stated (76). This discrepancy could be related to lower dosing and hyperandrogenism (75). The mechanism

explaining AR hepatotoxicity is still unclear, nevertheless, AR contains hepatotoxic constituents such as salicylates and alkaloids, as well as, hepatoprotective triterpene glycosides (43).

One interesting finding from our study is that although vitamin C exerted the minor ovarian effect on PCOS, it had a protective effect when combined with AR which diminished the adverse effect of AR on liver tissue. Although, there was mild deterioration in liver demonstrated by elevated ALT and AST levels in LTZ treated rats but without inducing oxidative stress, which is consistent with the literature (77). The protective effect of vitamin C may be due to its scavenging effect to oxidant molecules that consequentially enhanced the antioxidant capacities. In agreement with the later results, other vitamin C studies in PCOS patients displayed lower concentration of ROS than in controls (47).

5. Conclusions

The present study demonstrated the potentiality of *Actaea racemosa* and vitamin C combination to mitigate the reproductive and metabolic disorders of PCOS with curtailed AR hepatotoxic risk. The AR arsenal of secondary metabolites inhibited the androgen aromatization in the letrozole-induced PCOS rats, offset the ovarian oxidative stress, which may be involved in the pathogenesis of PCOS, with enhanced hormonal profile, lipid profile, glucose level and liver functions. Furthermore, AR or its vitamin C combo declined the apoptotic cell percentage, augmented Ki-67 expression alongside with a significant downregulation of CYP19-α1 mRNA expression level. Our results of steroidogenesis modulation without the risk of hepatic adverse effect by AR and vitamin C combination warrant further studies of this combination in polycystic ovarian syndrome.

Abbreviations

ALT; alanine aminotransferase, AR; *Actaea racemose,* AST; aspartate amino transferase, CMC; Carboxy Methyl Cellulose, CV; coefficient of variation, GNPS; Global Natural Products Social molecular networking, GnRH; Gonadotropin-releasing hormone, GSH; glutathione, HDL; high density lipoprotein, HDMS; high definition mass spectrometer, HMDB; Human metabolites database, IMS; Ion mobility spectroscopy, LDL; low density lipoprotein, LTZ; letrozole, LH; luteinising hormone, MDA; Malondialdehyde, MONA; Massbank of North America, PCOS; Polycystic ovary syndrome; QC; quality control, Vit. C, Vitamin C, vLDL; very low-density lipoprotein.

Declarations

Competing interests

The authors declare no known conflict of interests.

Ethics approval and consent to participate

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Cairo University (CU-IACUC; VetCU11112018017).

Consent for publication

Not applicable

Availability of data and materials

The datasets supporting the conclusion of this article are included within the article and its additional files.

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Supplementary information

Electronic_supplementary_information.docx. Table S1 Metabolomic profiling of *Actaea racemosa* using UPLC-MS^n, List of the identified metabolites in negative mode; structures, MS^n ions and/or fragmentation tree (figure S1-S12) and the identified metabolites in positive mode; structures, MSn ions and/or fragmentation tree (Figure S13-S43).

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Tables

Table (1): Effect of AR extract and/or vitamin C on lipid profile, glucose level, liver function tests in letrozole induced PCOS rat model. Values are presented as Mean ± SD, where means carrying different superscript letters are significantly different at $P < 0.05$ (n=5).

Parameters	Normal	PCOS	Clomiphene	AR	Vit. C	AR and Vit. C
Cholesterol (mg/dL)	104.8± 4.5^{a}	148.78± 1.6^{c}	141.5 ± 3.2^{c}	143.3± 5.94^{c}	115.9± 2.64^{b}	121.8± 4.73^{b}
Triglyceride (mg/dL)	44.03± 4.1^{a}	72.24± 3.81^{d}	74.51± 4.1^{d}	70.99 ± 3.69^{d}	53.52 ± 0.86^{b}	67.23± 3.11^{c}
HDL (mg/dL)	76.57± 3.1^{a}	58.42± 1.1^{c}	57.66 ± 1.8^{c}	55.11 ± 5.08^{c}	66.23 ± 1.38^{b}	63.44 ± 3.02^{b}
LDL (mg/dL)	19.46± 2.2^{a}	75.92± 0.4^{c}	73.54± 1.12^{c}	72.14± 1.49^{c}	29.17 ± 1.03^{b}	31.69± 2.13^{b}
VLDL (mg/dL)	8.80 ± 0.8^{a}	14.44± 0.8^{c}	15.30± 0.92^{c}	15.79± 0.74^{c}	10.5 ± 0.17^{b}	11.65 ± 0.62^{b}
Glucose (mg/dL)	82.06± 4.3^{a}	128.4 ± 0.8^{c}	123.03± 1.39^{c}	130.13 ± 2.5^{c}	104.4 ± 2.9^{b}	107.34± 1.2^{b}
ALT(U/L)	19.27± 1.1^{a}	36.32± 3.76^{c}	31.44 ± 2.1^{b}	94.85 ± 6.20^{d}	22.35 ± 1.77^{a}	24.22 ± 1.89^{ab}
AST (U/L)	34.16 ± 1.3^{a}	51.43 ±4.85^{c}	44.46 ± 2.47^{b}	93.96 ± 7.14^{d}	36.41 ± 3.17^{a}	40.82 ±2.2^{ab}

Figures

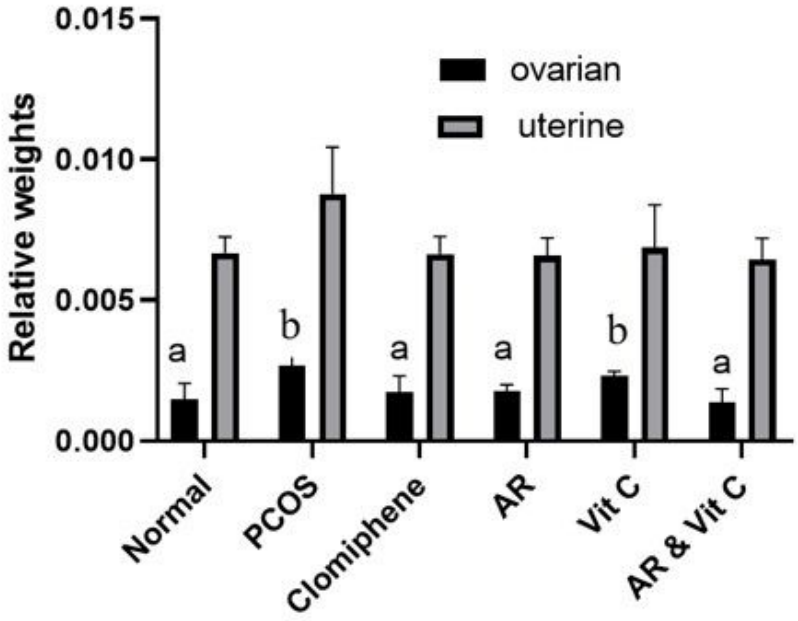

Figure 1

Effect of AR extract and/or vitamin C on relative ovarian and uterine weights in letrozole induced PCOS rat model. Values are presented as Mean ± SD, where means carrying different letters are significantly different at P < 0.05 (n=5).

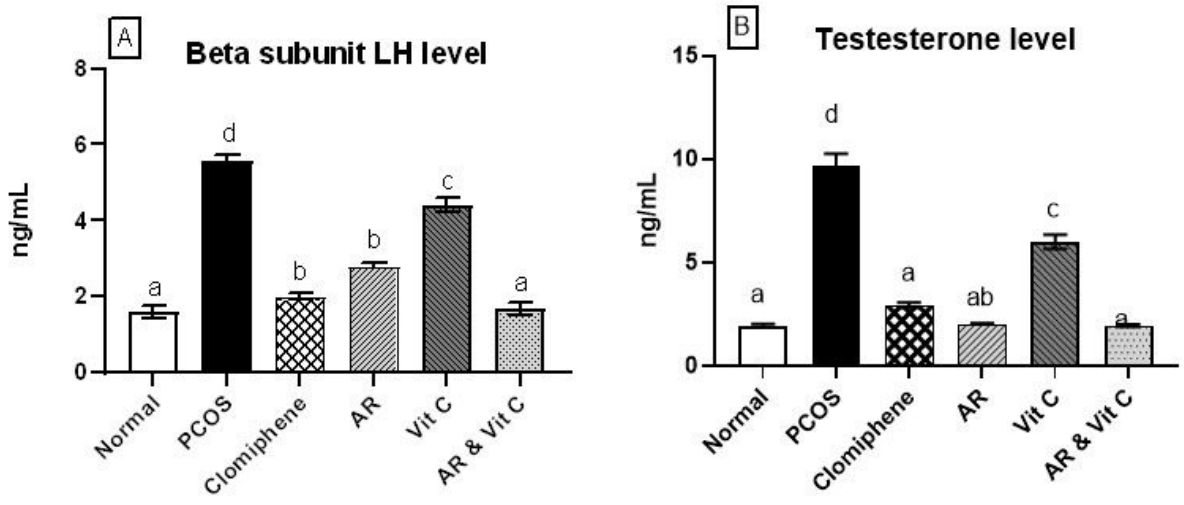

Figure 2

Effect of AR extract and/or vitamin C on A) Beta subunit LH level B) testosterone level in letrozole induced PCOS rat model. Values are presented as Mean ± SD, where means carrying different letters are significantly different at P < 0.05.

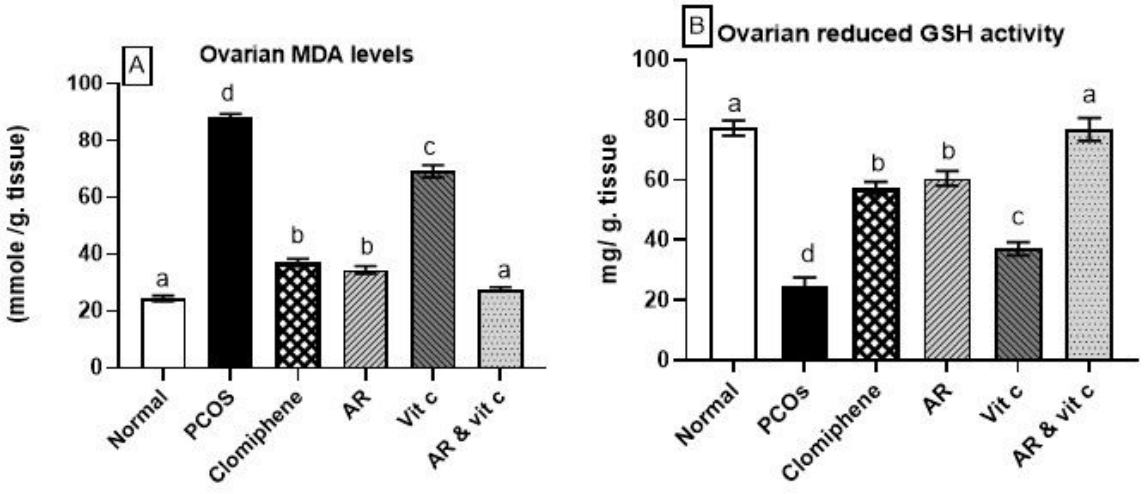

Figure 3

Effect of AR extract and/or vitamin C on ovarian A) MDA level B) GSH activity in letrozole induced PCOS rat model. Values are presented as Mean ± SD, where means carrying different letters are significantly different at $P < 0.05$ (n=5).

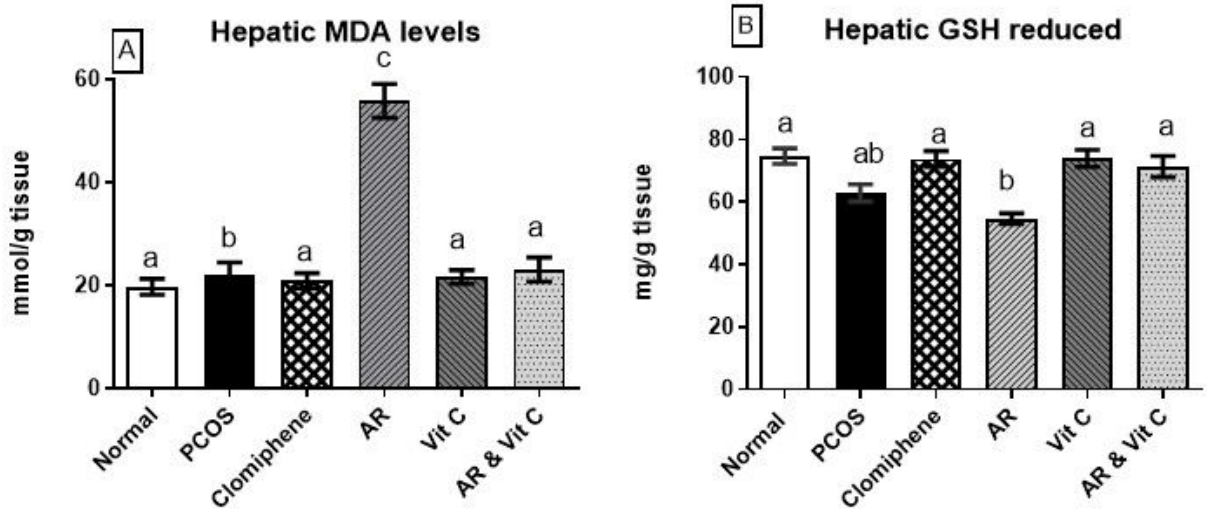

Figure 4

Effect of AR extract and/or vitamin C on hepatic A) MDA level B) GSH activity in letrozole induced PCOS rat model. Values are presented as Mean ± SD, where means carrying different letters are significantly different at $P < 0.05$ (n=5).

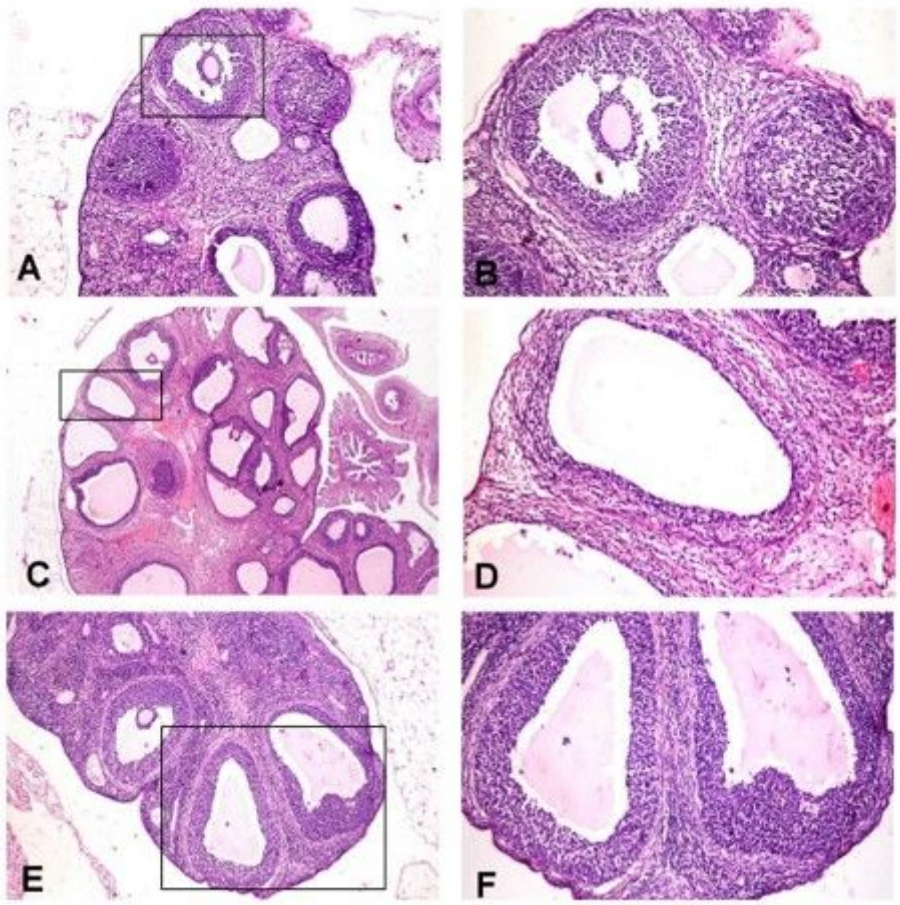

Figure 5

Photomicrographs of ovaries in different experimental groups. Rectangle refers to an area with higher magnification in the next picture. (A) Ovary of normal control group showing the normal histological picture. (B) Normal secondary follicle. (C) Ovary of PCOS showing multiple ovarian cysts. (D) Ovarian cyst with a thin layer of flattens granulosa cells. (E-F) Clomiphene treated group showing a reduction in the number of ovarian cysts and restoration of granulosa cell thickness. Hematoxylin and eosin (H&E).

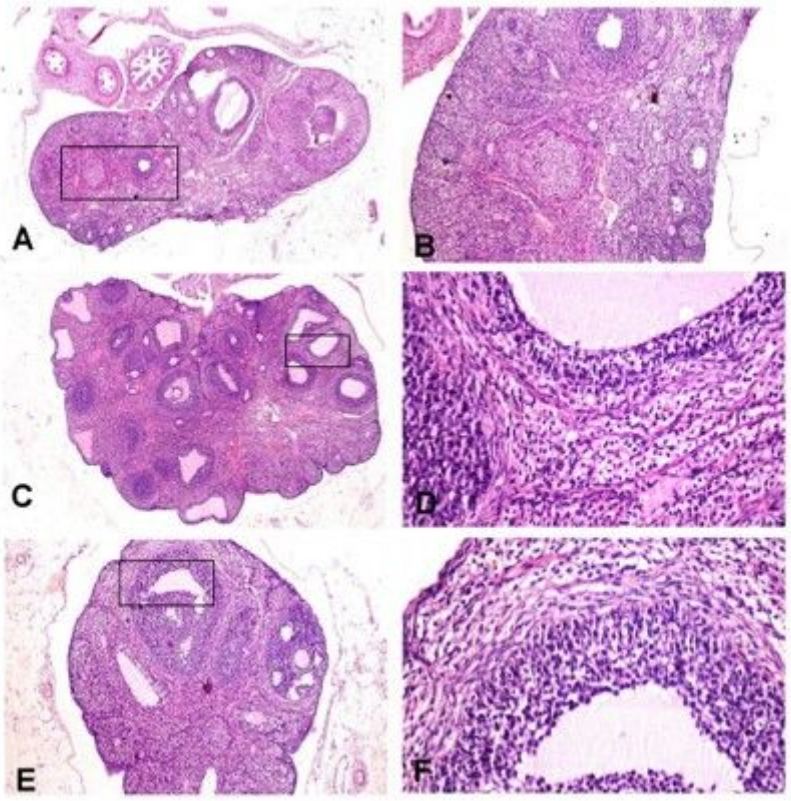

Figure 6

Photomicrographs of ovaries in different experimental groups. (A) Ovary of AR group showing a marked reduction in the number of ovarian cysts with the presence of growing follicles. (B) Presence of corpora lutea. (C) Ovary of vitamin C treated group showing polycystic ovary. (D) Ovarian cyst with degenerated and necrosed granulosa cell layer. (E) AR combined with Vit. C treated group showing a marked reduction in the number of cysts with the presence of secondary follicles and corpora lutea. (F) Follicle showing proliferated granulosa cell layer. (H&E).

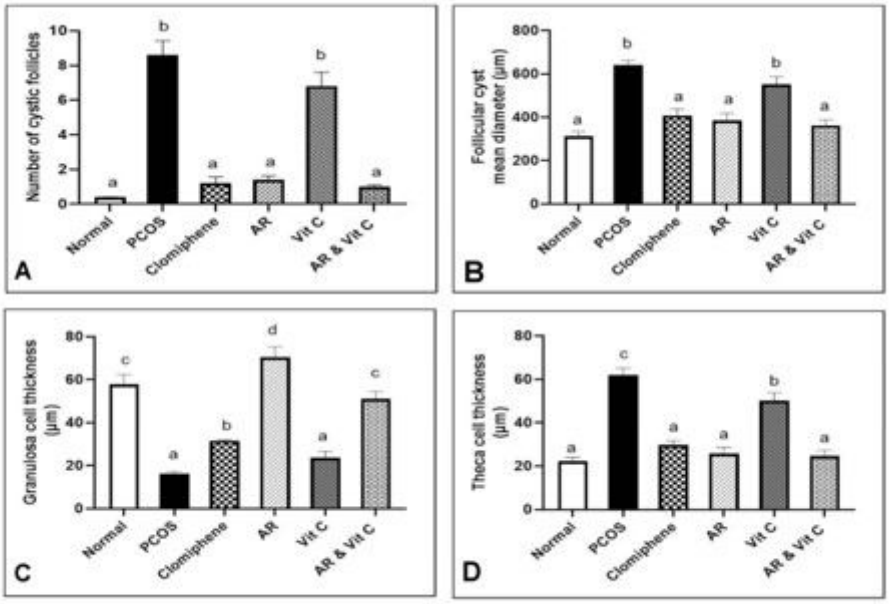

Figure 7

Morphometrical analysis of ovaries in the different groups. (A) Number of ovarian cysts per ovary. (B) Mean diameter of follicular cysts. (C) Granulosa cell thickness. (D) Theca cells thickness. Values with different superscripts are significantly different ($P < 0.05$).

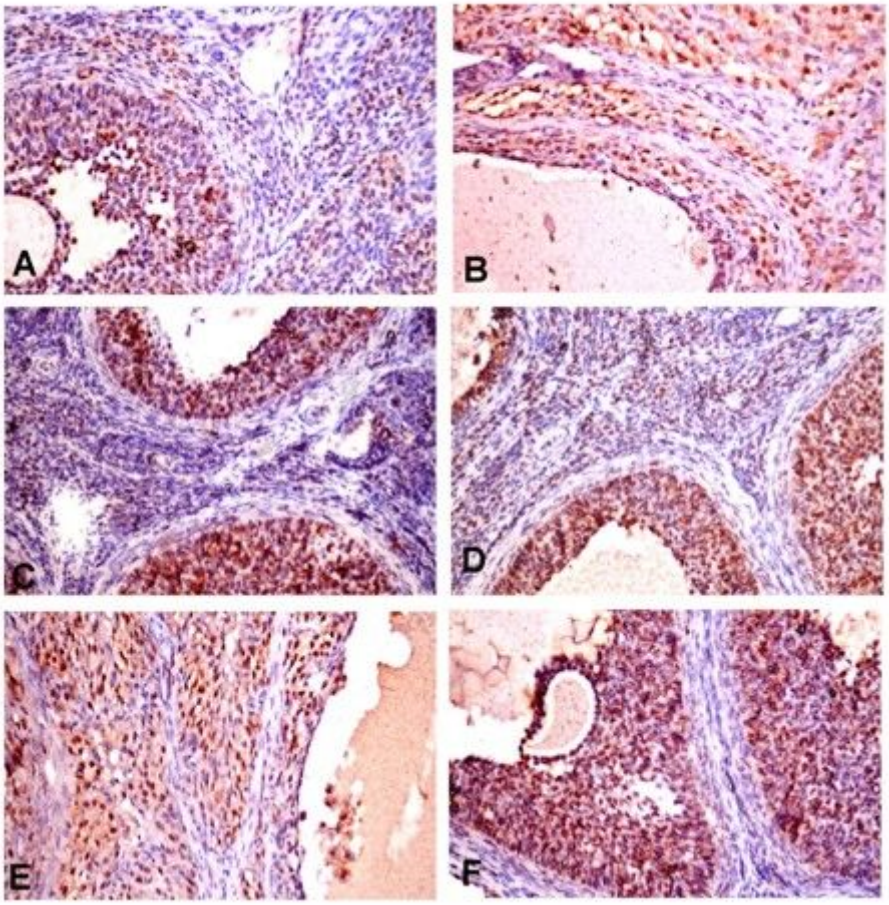

Figure 8

Immunohistochemical evaluation of Ki-67 in the ovary (X 200). (A) Normal control group showing strong immunopositive staining in the granulosa cell layer with weak immunoreactivity in the theca cells and stromal cells. (B) PCOS group showing strong immunopositive reaction in theca cells and interstitial stromal cells. (C) Clomiphene treated group showing strong immunopositive staining in the granulosa cell layer. (D) AR treated group showing strong immunolabeling in granulosa cell layer and slight immunopositive staining in theca cells and stromal cells. (E) Vitamin C group showing intense immunolabeling in the theca cells and interstitial stromal cells. (F) AR combined with vitamin C group showing very strong immunopositive staining in granulosa cell layer with a faint reaction in the theca cells and interstitial stromal cells.

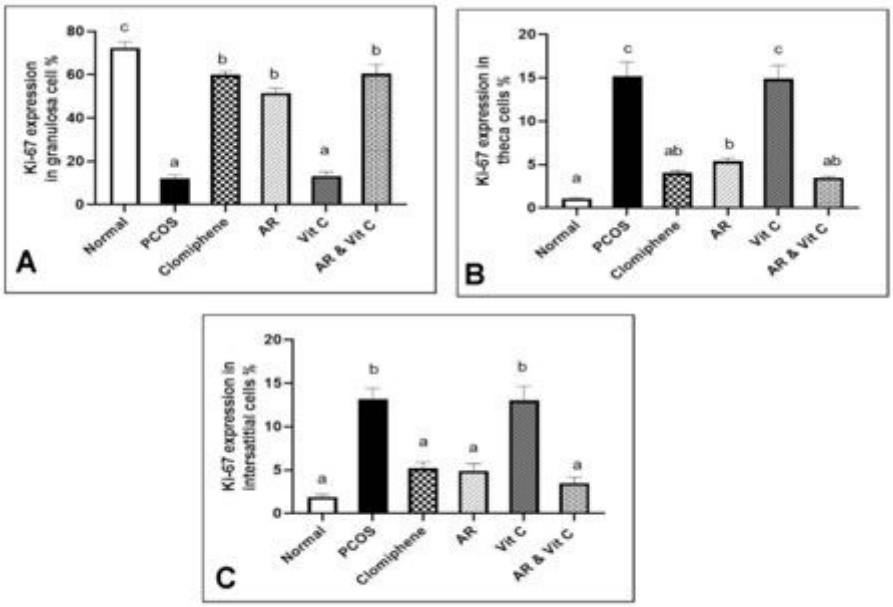

Figure 9

Image analysis of Ki-67 in the ovaries of the different groups. (A) Ki-67 expression in the granulosa cell layer. (B) Ki-67 expression in the theca cell layers. (C) Ki-67 expression in the interstitial stromal cells. Values with different superscripts are significantly different ($P < 0.05$).

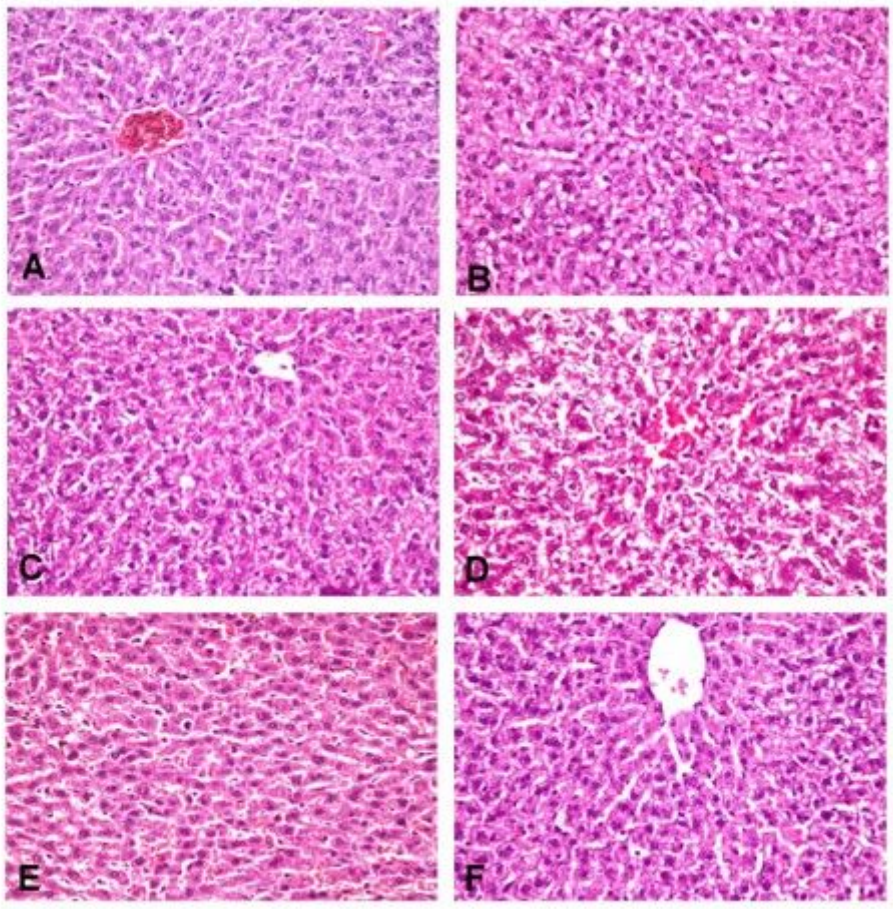

Figure 10

Photomicrographs of the liver in the different groups (H&E X 400). (A) Normal control group showing normal hepatic architecture (B) PCOS group showing moderate vacuolation of hepatocytes. (C) Clomiphene treated group showing mild vacuolation of hepatocytes. (D) AR treated group showing hepatocellular necrosis with sinusoidal dilation and disorganization of hepatic cords. (E) Vit. C group showing normal histological findings. (F) AR combined with Vit. C group showing mild vacuolation of hepatocytes with sporadic cell necrosis.

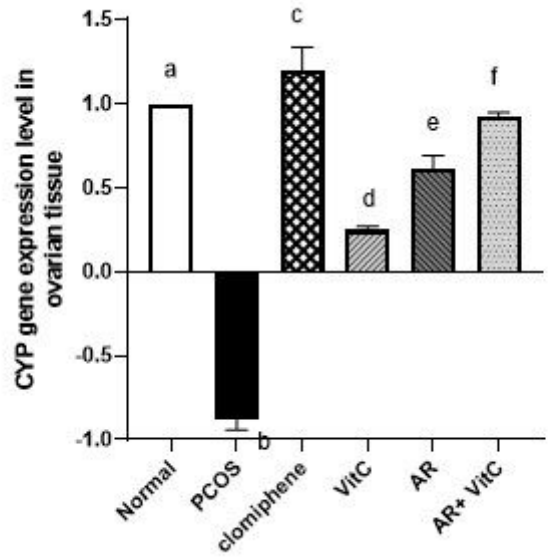

Figure 11

Effect of AR extract and/or vitamin C on the expression level of Cyp19α1 gene in PCOS rat model. Values are presented as mean ± SD. Means carrying different letters are significantly different at $P < 0.05$.

Supplementary Files

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