WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 7, 39-51. Research Article ISSN 2277– 7105

HORMONAL PROFILE AMELIORATION BY THE MIXTURE OF VITEX-AGNUS CASTUS AND METFORMIN ON POLYCYSTIC OVARY REMEDY

Shereen Said Elshaer[1], Hend Mohamed Anwar*[2] and Mamdouh Salah El-Din Zohni[3]

[1]Research Assistant, at National Organization for Drug Control and Research, Biochemistry Department.

[2]Assistant Professor at Faculty of Pharmacy (Girls), Al-Azhar University, Biochemistry Department.

[3]Assistant Professor at National Organization for Drug Control and Research, Biochemistry Department.

Article Received on
22 April 2015,

Revised on 16 May 2015,
Accepted on 07 June 2015

***Correspondence for Author**
Dr. Hend Mohamed Anwar
Assistant Professor at Faculty of Pharmacy (Girls), Al-Azhar University, Biochemistry Department.

ABSTRACT

Aim: This study was aimed to ameliorate the effect of Metformin (Met) in Polycystic Ovary (PCO) treatment. **Materials and Method**: Forty rats were classified into 4 groups (10 Rat each): Control group, Dehydroepiandrosterone (DHEA) group, DHEA+ Met group and DHEA + Met + Vitex Agnus-Castus (VAC) group. After 30 days of injection; Luteinizing Hormone (LH), Follicular Stimulating Hormone (FSH), Prolactin (PRL) and some biochemical parameters were assayed in serum. **Results**: The results showed an elevated FSH and PRL but depressed LH levels in DHEA group. But significant changes were demonstrated in DHEA + Met group. Obviously, very highly significant ameliorations were obtained in DHEA + Met + VAC group. **Conclusion:** This study confirms that the treatment by the mix of Met and VAC ameliorating the hormonal levels and providing a new therapeutic modality for PCO.

KEYWORDS: Polycystic ovarian syndrome, Metformin, DHEA, and Vitex Agnus-Castus.

1. INTRODUCTION

Polycystic ovary syndrome (PCOS) which affecting15–20% of women is mainly one of the familiar endocrine disorder.[1] It is characterized by diverse causes including anovulation,

oligomenorrhea, follicular cysts, hyperandrogenism, hyperestrogenism and changeable levels of gonadotropins in the blood. Regardless of abundant severe investigations that were carried out on PCOS, to date, a distinct single etiology of this disease remind unclear.[2] Hyperinsulinemia affects the progress of cystic ovaries in the rat by causing an increase in the sizes of the cystic follicles, as well as the ovary.[3] It has been found that adrenal androgen excess presents in about 25-60% of women with PCOS, in which dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfconjugate (DHEAS), and androstenedione are the mainly general androgens that are elevated.[4] DHEA was used to induce PCOS in female rats by injection subcutaneously (Sc) (6mg/100g).[3]

Metformin (Met) is valuable in the decreasing of both insulin resistance and circulating androgens in addition to restoring ovulation.[5] Also, it is an insulin sensitizer acting in the liver and the peripheral tissues that ameliorate the metabolic and reproductive defects in PCOS. It is usually used for the remedy of patients influenced by type 2 diabetes mellitus (DM). Since many women with PCOS are insulin resistant. Metformin was established in clinical practice to cure these patients also. Herbal extract of *Vitex Agnus Castus* (Chaste berry) (a deciduous shrub that is native to Mediterranean, Europe, and central Asia) was used with metformin to improve its action. This combination of treatment was introduced due to the prospective of VAC fruit extract in the management of several female conditions, including menstrual disorders, premenstrual syndrome (PMS), and menopause.[6] Currently, the hydro-alcoholic of vitex fruit extract is used all over the world for the treatment of premenstrual syndrome and luteal insufficiency-complaints. The actions of vitex owing to the brain neurotransmitter systems such as dopaminergic and estrogenic like effects resulting in decreasing of prolactin secretion from the anterior pituitary.[7] It considered one of the most important herbs for PCOS treatment as it helps in stimulating and normalizing the function of the pituitary gland, which regulates luteinising hormone (LH) secretion. Also, VAC has been shown to improve fertility especially during cycles with no ovulation (anovulation) by elevating the significantly decreased levels of estrogens and androgens.[8] In addition, it has found that some active constituents which act on the pituitary gland elucidating its actions on hormonal levels.[9] Several studies have shown that VAC fruit extracts can also bind to opiate receptors and this could clarify why administration of VAC lessens PMS symptoms.[10] VAC fruit extracts have glycosides, diterpenes, flavonoids and essential oils.[11] *In vitro* studies revealed that the diterpenes have dopaminergic action, the glycosides have an indirect effect on hormones and The flavonoids have an effect on estrogen receptors.[12] Consistent with a

German investigation, an alcohol extract of the entire berry is more active than separates of single components.[13]

In this study the effect of *Vitex Agnus Castus* extract and metformin co-administration was tested to evaluate their ameliorative effects and to establish the therapeutic use of herbal-metformin combination for PCOS–metabolic syndrome subjects.

2. MATERIALS AND METHODS

2.1 Chemicals

All drugs and chemicals were obtained from Sigma chemical company (St Louis, USA). *Vitex agnus castus* herb was obtained from El lebidy Farm, Cairo, Egypt.

2.2 Preparation of the hydro-alcoholic extract of vitex fruit

About 200gm of Vitex agnus castus herbal material was air-dried at shade and grinded into fine powder. Later, it was boiled with 1.5 liters of 70% ethanol in a magnetic stirrer for eight hours. After that, it was lyophilized and the crude extract was obtained. It was stored in the refrigerator for biological studies.[14]

2.3 Animals

In the current study, 40 Female Sprague-Dawley strain rats (weighing 130-150gm) were obtained from the animal house of National Organization for Drug Control and Research (NODCAR) Cairo, Egypt. Vaginal smears were taken for at least 2 estrous cycles to eliminate non-cyclic animals. Animals were housed for at least one week in laboratory room prior to testing under standard housing conditions (room temperature 24-27ºC) with altering 12 hr light and dark cycles and free access to food (standard pellets diet) and water was allowed *ad libitum* unless otherwise specified. The rats were grouped into 4 groups (10 rats each), Corn oil control group (Group I), DHEA group (GroupII) (received DHEA soluble in corn oil; 6mg/100g/day, sc.), Met + DHEA group (Group III) (received metformin soluble in water 52.5mg/kg/day, po. and DHEA 6mg/100g/day, sc.) and Met+ DHEA + VAC group (Group IV) (received same doses of Met and DHEA but VAC 13mg/kg/day, po.).

At the end of experiments (30 days), animals were anesthetized using ether, then blood samples gathered in non-heparinized tubes. Tubes were centrifuged at 3000 r.p.m. for 15 minutes at 4°C and the generated sera stored at -20°C till analysis. Different biochemical parameters were assayed using commercial kits. Glucose level was estimated using Glucose

KIT Cromatest, triglycerides level was estimated using TGS KIT Vitro scint and cholesterol using cholesterol Biolabo Kit. Hormonal levels of FSH, LH and PRL were estimated using Accu Bind ELISA Microwells Kit.

The ovaries were secluded. All tissues were washed in ice-cold isotonic saline and blotted dry between two filter papers. The segment of the ovary was homogenized in ice-cold 1.15% KCl to make 10% (w/v) homogenate with Glass-Col motor driven homogenizer (USA) and the homogenate was used for determination of the antioxidant capacity of the used VAC-Met combination *in vivo* in terms of malondialdehyde (MDA)[15] and superoxide dismutase (SOD).[16] The other section of the ovary was homogenized in ice-cold 5% sulfosalicylic acid to make 10% (w/v) homogenate for the estimation of glutathione (GSH).[17]

2.4 Histological study

Autopsy samples were taken from the ovary of different groups of rats and fixed in 10% formalin for twenty-four hours. The obtained tissue sections were collected on glass slides, and stained by hematoxylin and eosin stains for the histopathological examination through the electric light microscope.[18]

2.5 Statistical Analysis

The results were expressed as the mean ± standard error of the mean (SEM) for ten animals in each group. Differences between groups were assessed by one-way analysis of variance (ANOVA). Subsequent multiple comparisons between the different groups were analyzed by Dunnett 2 sided multiple comparison tests. Data were statistically analyzed using the statistical package for social science (SPSS 6.0 software). Values at $P < 0.05$ were considered significant.[19]

3. RESULTS

3.1 Descriptive Statistics

Table 1 showed the descriptive statistics of the metabolic changes in the studied groups. Significant changes of glucose, cholesterol, triglycerides levels (p value< 0.05) were obtained in DHEA group when compared to oil control group (Group. I). In the other hand, Met + DHEA and DHEA+ Met+ VAC groups demonstrated a significant decrease of glucose, cholesterol and triglycerides levels (p value< 0.05) when compared to DHEA group.

Table (1): descriptive statistics of metabolic changes of the studied groups.

Data / Parameter	Mean±SE			
	Oil	**DHEA**	**Met+DHEA**	**Met+VAC+DHEA**
Glucose mg/di	63.95±2.8	76.33±2.7*	59.3±1.7*	65.13±2.7
Cholesterol mg/dl	75.9±2.5	93.16±3.1*	80.6±2.5*	62.5±3.3*
Triglycerides mg/dl	39.4±2.9	98.6±5.8***	51.3±3.5**	42.6±3.***

* Significance difference at *P* value <0.05.

** Significance difference at *P* value <0.01.

*** Significance difference at *P* value <0.001

DHEA = Dehydroepiandrosterone

Met = Metformin

VAC = 70% hydro-alcoholic extract of *Vitex agnus castus*

Results showed in Table 2 and indicated that DHEA group had a significant increase in FSH, PRL levels but a decrease in LH levels when compared to the oil control group (Oil group). While Met + DHEA and DHEA+ Met +VAC groups (treated group) were demonstrated a very highly significant decrease of FSH and PRL levels when, compared to levels of DHEA group.

Table (2): FSH, LH and PRL levels in the studied groups.

Data / Parameter	**Mean±SE**			
	Oil	**DHEA**	**Met+DHEA**	**Met+VAC+DHEA**
FSH mlU/ml	**40.5±5.1**	**75.23±11.7*** **	**45.4±2.5*** **	**46.5±0.8**** **
LH mlU/ml	**6.1±0.5**	**2.35±0.4*** **	**2.8±0.1**	**2.95±0.5**
PRL ng/ml	**6.33±1.7**	**15.6±3.3*** **	**3.4±0.75*** **	**3.87±0.3*** **

*** Significance difference at *P* value <0.001.

FSH= Follicle stimulating hormone.

LH= Luteinizing hormone.

PRL= Prolactin.

3.2 Receiver Operated Characteristics analysis (ROC)

Receiver operated characteristics analysis (ROC curves) was employed in order to define the most active drug used for PCOS cure. ROC analysis of serum levels of FSH, LH and PLC was performed between DHEA group and Met + DHEA group, and between DHEA group and Met + VAC + DHEA group. As smaller area as more significant amelioration regarding

FSH and PRL. In contrast, as larger AUC (area under the curve) of LH as a more significant improvement. These results were illustrated in figure 1(A-F).

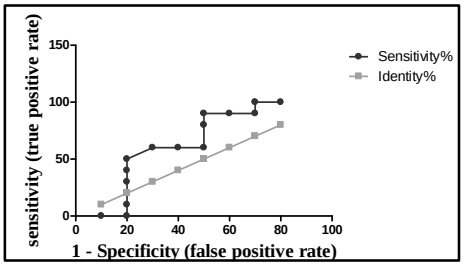

Figure (1A): ROC of FSH in Met+DHEA group (AUC: 0.65, cutoff: 47.95).

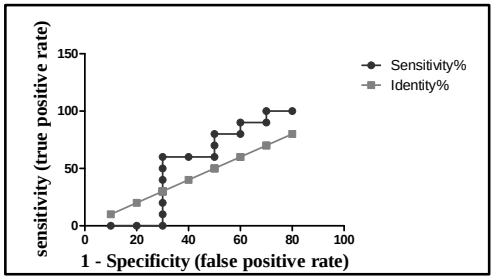

Figure (1B): ROC of FSH in Met+VAC+DHEA group (AUC: 0.59, cutoff: 50.5).

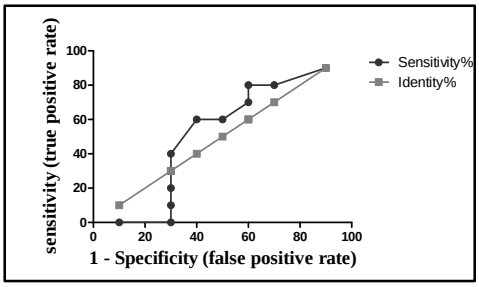

Figure (1C): ROC of LH in Met+DHEA group (AUC: 0.52, cutoff: 2.7).

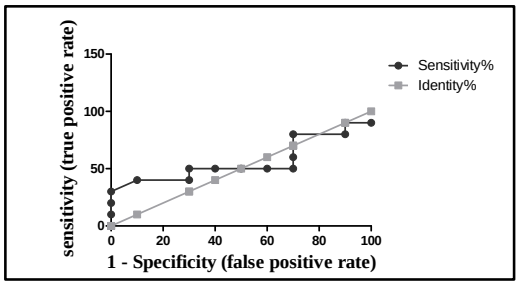

Figure (1D: ROC of FSH in Met+VAC+DHEA group (AUC: 0.56, cutoff: 1.8).

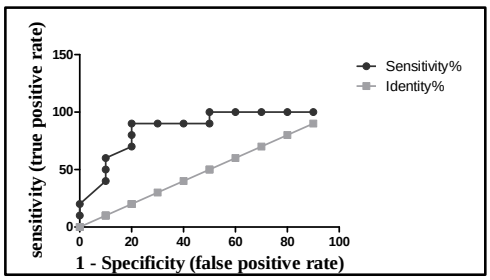

Figure (1E): ROC of PRL in Met+DHEA group (AUC: 0.86, cutoff: 7.2).

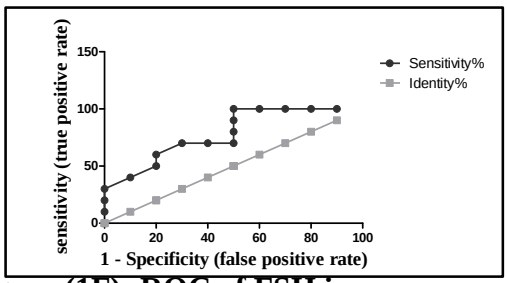

Figure (1F): ROC of FSH in Met+VAC+DHEA group (AUC: 0.78, cutoff: 7.9).

Figure 1: (A-F) ROC analysis of the studied group AUC=area under the curve.

3.3 Antioxidant Studies

Table 3 revealed that DHEA group had highly significant changes ($p< 0.01$) in oxidative stress parameters as presented by a decrease in the levels of GSH and SOD but increase in MDA ovary tissues content, when Compared to control group. Obviously, a very high significant increase in the levels of GSH and SOD was detected in both treated groups (Met +DHEA and Met + VAC +DHEA groups). Also, a significant decrease in MDA ovary tissues ($p< 0.001$), when compared to DHEA group.

Table (3) Oxidation parameters in the studied group.

Data / Parameter	Mean ± SE			
	Oil	**DHEA**	**Met + DHEA**	**VAC + Met + DHEA**
GSH µmol/g	58.1±1.8	22.7±1.2***	35.7±1.3*	53±5.9***
MDA µmol/g	10.2±1.4	23.5±2.4***	12.1±1.7***	8.8±1.7***
SOD U/g protein	49.1±4.6	12.7±1***	43.9±1.3**	29.03±2.6***

* Significance difference at P value <0.05.

** Significance difference at P value <0.01.

*** Significance difference at P value <0.001

GSH=glutathione.

MDA=malondialdehyde.

SOD=superoxide dismutase

3.4 Histological Examination of Ovaries: figure (2A-D)

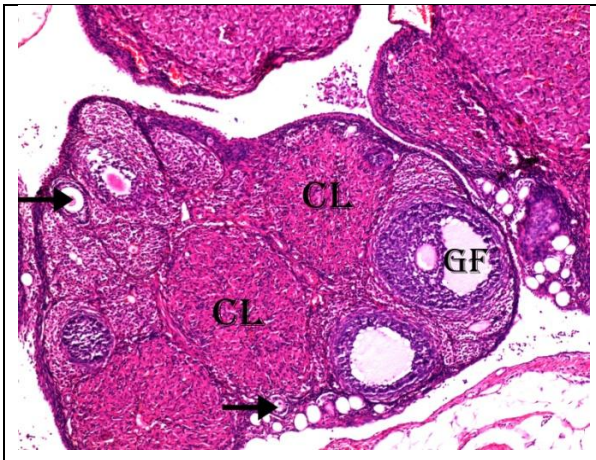

Figure (2A): Photomicrograph of ovary tissues of normal rats, demonstrating intact and normal graffian follicle (GF), corpora lutea (CL), and primary follicles (Arrow). H&E, X: 40.

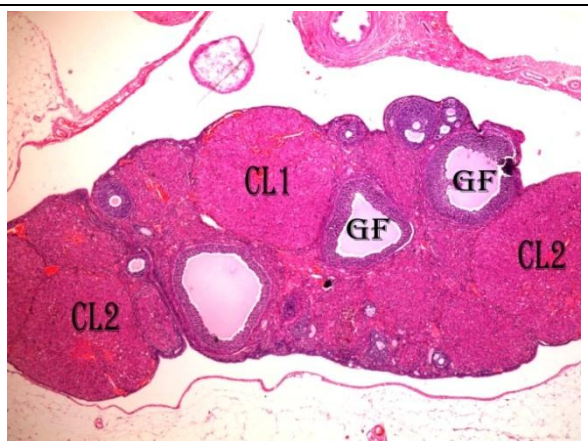

Figure (2B): Photomicrograph of ovary tissues of DHEA treated group, showing many cystic corpora lutea (CL1), graffian follicle (GF) with no ova, corpora lutea (CL2) with regression. H&E, X: 40.

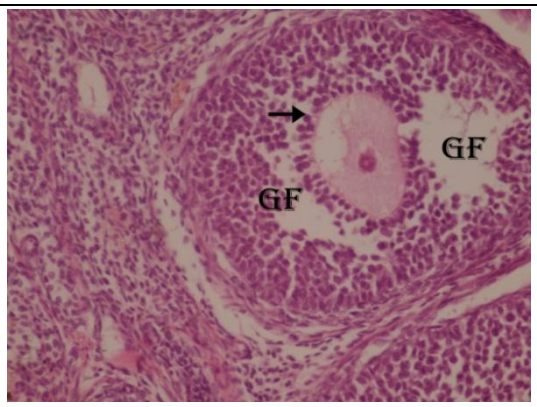

Figure (2C): Photomicrograph of ovary tissues of DHEA+Met treated group, showing normal graffian follicle (GF), intact granulose cells (Arrow). H&E, X: 400.

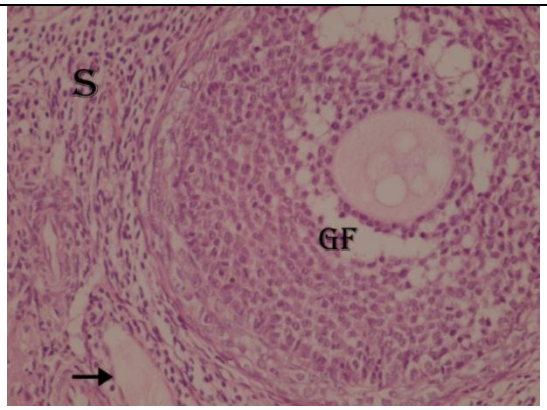

Figure (2D): Photomicrograph of ovary tissues of MET + VAC + DHEA treated group, demonstrating intact graffian follicles (GF), stroma(S), lymphatics vessels (Arrow), H&E, X: 400.

4. DISCUSSION

In this study, DHEA (one of the androgens) was used to induce PCOS causing metabolic disturbances as an increase in plasma glucose, cholesterol and triglycerides levels. This pointed out by Iuhas[20] who reported that both insulin resistance and hyperandrogenemia contribute to this abnormal lipid profile, where androgen decreases lipoprotein lipase activity in abdominal fat cells, and insulin resistance impairs the ability of insulin to exert its antilipolytic effects**.** Also, disturbance in hormonal profile (DHEA group) and this could be ascribed to adrenocorticotrophic hormone (ACTH) which reduces LH secretion.[21] This is unusual result in PCOS, but it occurs in women who suffering from PCOS over 20 years, and also for women which have a decrease in ovary size.[22]

The sex hormones dehydroepiandrostenedione (DHEA) is produced in the adrenal glands, and in abundant peripheral locations. Although adrenal androgen secretion increases in response to adrenocorticotropic hormone (ACTH), androgens do not influence ACTH secretion, and ACTH acts principally accommodating role in the adrenal androgen physiology. ACTH stimulates ovaries tissue to secrete FSH.[23] As well as DHEA could induce the over production of active oxygen in ovarian tissue.[24] This lead to a very high significance decrease in the level of GSH and SOD but remarkable significance increase in MDA. Our results were in harmony with Kandasamy[25] who have documented an increased oxidative stress in patients with PCOS. Elevations in MDA (a byproduct of lipid peroxidation), have been previously reported in PCOS patients.[26] Significantly, all abnormalities were ameliorated in Met + DHEA treated group, where Met decrease both

hepatic glucose assembly and its intestinal absorption, and cause an increase in peripheral uptake and its utilization and thus resulting in improved insulin sensitivity.[27] This assumption is in harmony with Heibashy.[16]

Metformin suppresses androstenedione production by a direct effect on ovarian theca cells and decreases FSH.[28] Recently, it has been demonstrated that metformin treatment increased AMPK activity in rat granulosa cells, resulting in consequent reduction of steroid synthesis However, it is still unclear whether this effect is AMPK-dependent or not.[29]

Oxidation parameters analysis revealed that metformin can antagonize the action of DHEA and prevent damage to ovarian tissues[30] and produced an enhanced chronic activation of AMPK which is necessary to increase the ovarian GSH content.[31] It is also obvious that metformin remedy, significantly reduces the high content of malondialdehyde (MDA) and total reactive oxygen species generation and returns the reduction of both enzymatic and non-enzymatic antioxidants.[32]

VAC has an estrogenic similar influence[11] which is in harmony with van Die[33] who reported that increase the level of luteinizing hormone (LH) while gently suppressing the secretion of (FSH) follicle stimulating hormone due to its dopaminergic nature which attributed to the highest quantity of its diterpenes.[12]

Also, VAC can act as opiate system, decreasing the gonadotropic releasing hormone (GnRH) which acts on the pituitary gland lead to decrease the release of LH and FSH.[34]

The aforementioned results of Met + VAC + DHEA treated group, which showed a very high significance amelioration in oxidative parameters were in harmony to Maltaş, who stated that VAC has potential natural antioxidant activity/.[35]

The Met + VAC +DHEA combination remedy have higher significant amelioration than Met + DHEA, which supported by ROC analysis and revealed lower AUC regarding FSH and PRL but higher AUC regarding LH than those of Met + DHEA group. Worthy mentioning, VAC has a synergistic effect with metformin in PCOS treatment.

5. CONCLUSION

The present study concluded that Metformin and vitex mixture showed beneficial and synergistic effect in the treatment of PCOS as they together give improvements in most of the parameters estimated, in addition to increasing the antioxidant potency and amending histological changes of ovaries tissues produced by metformin.

Further investigations to find out the mechanism of the herbal extracts effect is needed to complete our understanding of the reproductive endocrinological effects for herbal medicine for these common conditions.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

6. REFERENCES

1. Mamata J, Sasikumar Menon SS. Anti-androgenic effect of Symplocos racemosa Roxb. against letrozole induced polycystic ovary using rat model. J Coast Life Med., 2013; 1(4): 309-314.
2. Razi M, Akhtari K, Najafpour AR, et al. Effect of bilateral uterine artery ligation on follicular atresia in ovaries of mature female rabbits; histomorphometric and histochemical study. Iran J Reprod Med., 2010; 8(3): 101-110.
3. Talhouk R, Tarraf C, Kobrossy L, Shaito A, Bazzi S, Bazzoun D, El-Sabban, M Modulation of Cx43 and gap junctional intercellular communication by androstenedione in rat polycystic ovary and granulosa cells in vitro. J Reprod Infertil., 2012; 13(2): 21-32.
4. Narurkar MD, Patil S, Vaidya RA. Explore Testesterone Lowering Potential of Glycyrrhiza Glabra Root Extract in Hyperandrogenic Female Rats Induced with Polycystic Ovary. Int Res J Invent Pharm Sci., 2014; 2(5): 212-222.
5. Jang M, Lee MJ, Lee JM, Bae CS, Kim SH, Ryu JH, Cho IH. Oriental medicine Kyung-Ok-Ko prevents and alleviates dehydroepiandrosterone-induced polycystic ovarian syndrome in rats. PLoS One., 2014; 9(2): e87623.

6. Bachrach ZY. Contribution of Selected Medicinal Plants for Cancer Prevention and Therapy. Sci J Fac Med., 2012; 29(3): 117-123.
7. Webster DE, He Y, Chen SN, Pauli GF, Farnsworth NR, Wang ZJ. Opioidergic mechanisms underlying the actions of Vitex agnus-castus L. Biochem Pharmacol., 2011; 81(1): 170-177.
8. Paulsen CE, Carroll KS. Cysteine-mediated redox signaling: Chemistry, biology, and tools for discovery. Chem Rev., 2013; 113: 4633-4679.
9. Mirabi P, Hanieh S, Esmaeilzadeh S, Mojab F. Effect of Medicinal Herbs on Primary Dysmenorrhoea- a Systematic Review. Iran J Pharm Res., 2014; 13(2013): 757-767.
10. Yaghmaei P, Oryan S, Fatehi Gharehlar L, Salari A-A, Solati J. Possible Modulation of the Anexiogenic Effects of Vitex Agnus-castus by the Serotonergic System. Iran J Basic Med Sci., 2012; 15(2): 768-776.
11. Aksoy AN, Gozukara I, Kucur SK. Evaluation of the efficacy of Fructus agni casti in women with severe primary dysmenorrhea: A prospective comparative Doppler study. J Obstet Gynaecol Res., 2014; 40(3): 779-784.
12. Chen SN, Friesen JB, Webster D, Nikolic D, van Breemen RB, Wang ZJ, Fong H, Farnsworth NR, Pauli GF. Phytoconstituents from Vitex agnus-castus fruits. Fitoterapia., 2011; 82(4): 528-533.
13. Csupor D, Boros K, Hohmann J. Low Potency Homeopathic Remedies and Allopathic Herbal Medicines: Is There an Overlap? PLoS One., 2013; 8(9): 4-8.
14. Hasan F, Qaragholi MF, Abdull-wahed KLNA. EFFECT OF ALCOHOLIC EXTRACT OF VITEX AGNUS SEEDS ON SOME FUNCTION OF FEMALE REPRODUCTIVE SYSTEM. Int J Sci Nature., 2014; 5(2): 254-257.
15. Karabulut AB, Cakmak M, Kiran RT SI. Oxidative Stress Status, Metabolic Profile and Cardiovascular Risk Factors in Patients with Polycystic Ovary Syndrome. Med Sci., 2012; 1(1): 27-34.
16. Heibashy MIA, Mazen GMA, Shahin MI. Metabolic Changes and Hormonal Disturbances in Polycystic Ovarian Syndrome Rats and the Amelioration Effects of Metformin and/or Cinnamon Extraction. Journa Am Sci., 2013; 9(12): 54-62.
17. Rahal A, Mehra D, Rajesh S, Singh V, Ahmad H. Prophylactic Efficacy of Podophyllum hexandrum in alleviation of immobilization stress-induced oxidative damage in rat. J Nat Prod. 2009; 2: 110-115.

18. Linares R, Hernández D, Morán C, Chavira R, Cárdenas M, Domínguez R, Morales-Ledesma L. Unilateral or bilateral vagotomy induces ovulation in both ovaries of rats with polycystic ovarian syndrome. Reprod Biol Endocrinol., 2013; 11(1): 68-76.
19. Wu C, Lin F, Qiu S, Jiang Z. The Characterization of Obese Polycystic Ovary Syndrome Rat Model Suitable for Exercise Intervention. PloS one., 2014; 9(6): e99155.
20. Iuhas C, Costin N, Mihu D. Lipid Parameters in Patients with Polycystic Ovary Syndrome. Appl Med Informatics., 2012; 31(4): 27-32.
21. Hassani F, Karami M, Nadoushan MRJ, Yazdi PE. Nitric Oxide-Induced Polycystic Ovaries in The Wistar Rat. Int J Fertil Steril., 2012; 6(2): 111-116.
22. Dewailly D, Lujan ME, Carmina E, Cedars MI, Laven J, Norman RJ, Escobar-Morreale HF. Definition and significance of polycystic ovarian morphology: A task force report from the androgen excess and polycystic ovary syndrome society. Hum Reprod Update., 2014; 20(0): 334-352.
23. Teede H, Deeks a, Moran L. Polycystic ovary syndrome: a complex condition with psychological, reproductive and metabolic manifestations that impacts on health across the lifespan. BMC Med. 2010;8:41-50.
24. Zhao F, Li K, Zhao L, Jian L, Qi S, Jing Z, Hebin W, Shuhua Z. Effect of Nrf2 on rat ovarian tissues against atrazine-induced anti-oxidative response. Int J Clin Exp Patho., 2014; 7(6): 2780-2789.
25. Kandasamy S, Sivagamasundari RI, Bupathy A, Sethubathy S, Gobal V, Nadu- T. Evaluation of Insulin Resistance and Oxidative Stress in Obese Patients with Polycystic Ovary Syndrome. Int J Appl Biol Pharm Technol., 2010; 1(2): 391-398.
26. Ouladsahebmadarek E, Khaki A. Ultra-structural Study by Transmission Electron Microscopy: Effect of Omega-3 on Ovary Cell Organelles after Experimental Induced Poly Cystic Ovary Syndrome. Int J Women's Heal Reprod Sci., 2014; 2(3): 186-194.
27. Inzucchi SE, Bergensta RM, Buse JB, Diamant M, Ferrannini E, Nauck M, Peters AL, Tsapas A, Wender R, Matthews DR. Management of hyperglycemia in type 2 diabetes: a patient-centered approach: position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) - with comments. Diabetes Care., 2012; 35: 1364-1379.
28. Falbo A, Morena R, Tiziana R, Antonietta D, Achille T, Fulvio Z, Francesco O, Stefano P. Serum and follicular anti-Mullerian hormone levels in women with polycystic ovary syndrome (PCOS) under metformin. J Ovarian Res., 2010; 3(21): 16-21.

29. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F. Cellular and molecular mechanisms of metformin: an overview. Clin Sci (Lond)., 2012; 122(6): 253-270.
30. Rojas LBA, Gomes MB. Metformin: an old but still the best treatment for type 2 diabetes. Diabetol Metab Syndr., 2013; 5(1): 6-20.
31. Takayama H, Hirofumi M, Hisakazu I, Keita C, Yoshiro S, Koji M, Atsushi T et al. Metformin suppresses expression of the Selenoprotein P gene via an AMP-activated kinase (AMPK)/FoxO3a pathway in H4IIEC3 hepatocytes. J Biol Chem., 2014; 289(1): 335-345.
32. Luchetti CG, Paz D, Motta AB. Metformin Prevents the Increase of Nitric Oxide and Lipid Peroxidation Induced By Dehydroepiandrosterone in Early Pregnant Mice. Reprod Sci J., 2013; 5: 14-22.
33. Van Die MD, Burger HG, Teede HJ, Bone KM. Vitex agnus-castus extracts for female reproductive disorders: a systematic review of clinical trials. Planta Med., 2013; 79: 562-575.
34. Mahmoud RMISMS and HM. Effect of Vitex agnus Custus (VAC) on premenstrual syndromes among nursing students. J Am Sci., 2012; 8(4): 144-153.
35. Shah NA, Khan MR, Naz K, Khan MA. Antioxidant potential, DNA protection, and HPLC-DAD analysis of neglected medicinal jurinea dolomiaea roots. Biomed Res Int., 2014; 2014: 1-10.