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EUGENOL AMELIORATES LETROZOLE INDUCED POLYCYSTIC OVARY SYNDROME IN RATS

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ABSTRACT

Background: It is now evident that there is a strong association between oxidative stress and chronic low grade inflammation in polycystic ovary syndrome (PCOS) pathogenesis. Therefore, simultaneous targeting of these pathways by eugenol which has dual anti-inflammatory and anti-oxidant potential might be a therapeutic alternative approach to the current treatment. This study was designed to evaluate the dual targeting effects of eugenol against oxidative stress and inflammatory aspects of PCOS in rats. **Methods:** Female albino rats were administered letrozole (1 mg kg^{-1} daily) orally for 21 days for the induction of PCOS, followed by administration of eugenol (100 mg kg^{-1} and 250 mg kg^{-1}, oral) for 15 days. Body and ovary weight were measured. Estrous cycle assessment. C-reactive protein (CRP), prostaglandin E2 (PGE2), tumor necrosis factor α (TNF-α), serum insulin, estradiol, progesterone and testosterone were evaluated. Furthermore, antioxidant activity was tested using Malondialdehyde (MDA), catalase and superoxide dismutase (SOD). **Results:** administration of eugenol significantly decrease the elevated weight of body and ovary and normal sexual cycle. Eugenol treatment significantly decreased levels of inflammatory response markers CRP, TNF-α and PGE2 in PCOS rats. In addition, eugenol significantly reduced oxidative stress. Insulin and sex hormones were detected more or less near normal control levels after eugenol administration in PCOS rats. **Conclusion:** The study provides evidence for the potential ameliorative effects of eugenol against the biochemical and inflammatory aspects of PCOS.

KEYWORDS: eugenol, clove oil, polycystic ovary, letrozole, herbal medicine.

Abbreviations
CAT: catalase
CRP: C-reactive protein
MDA: Malondialdehyde
PCOS: polycystic ovary syndrome
PGE2: prostaglandin E2
SOD: superoxide dismutase
TNF-α: tumor necrosis factor –α

1. INTRODUCTION

Polycystic ovary syndrome is the most frequent endocrinological disorder that affects women of childbearing age, leading to metabolic alterations, such as hyperandrogenism, obesity, menstrual irregularities, insulin resistance and polycystic ovaries. Several genetic and environmental factors have been correlated with manifestations of this syndrome (Reis et al., 2017). While PCOS is the core pathology, it has contributory complications such as hyperinsulinemia, abdominal obesity, hypertension, and metabolic syndrome which are associated with increased risk of type 2 diabetes and cardio-vascular disease in later life (Moran et al., 2010). Although the etiology of PCOS remains unclear (Reis et al., 2017), there is ample evidence suggesting that uncontrolled steroidogenesis might be the primary abnormality in this disorder. Furthermore, increased oxidative stress and inflammatory cytokines in the blood and histological samples of women with PCOS directed investigators to suspect that inflammatory responses, oxidative and nitrosative stress might play pivotal roles in the pathogenesis of PCOS (Escobar-Morreale et al., 2011;Murri et al., 2013; Showell et al., 2013).

In PCOS patients, the elevated oxidative stress causes disturbance in antioxidants balance leading to harmful effects of reactive oxygen species including endometriosis, infertility, abortion, birth defects, preeclampsia, injury to ovarian epithelium's DNA, excessive apoptosis and alteration in cell signaling process (Agarwal et al., 2005). PCOS is associated with increased systemic inflammation. This is evidenced by elevated CRP and TNF-α (Kelly et al., 2001).

In this regard, ovarian cystogenesis and follicular atresia leading to ovarian dysfunction in PCO rats might correlate with higher concentration of the serum TNF-α

and the interplay between oxidative stress and pro-inflammatory cytokines, particularly TNF-α (Rezvanfar et al., 2012a,b, 2014, 2015). Therefore, their specific involvement in the pathogenesis of PCOS provides invaluable opportunity to block the disease progression using pharmacological agents with dual antioxidant and anti-TNF- α activities (Rezvanfar et al., 2012a,b).

Many studies have indicated the implication of secondary metabolites from medicinal plants on the regulation of reproductive functions (Telefo et al., 2004; Jha et al., 2010). These metabolites act on main organs of the reproductive system to inhibit or induce ovarian folliculogenesis. Their biological activities are often evaluated on reproductive organs of female rats which have long been used as a model system for studying, *in vivo*, the inducing effect of pharmacological compounds (Tohei et al., 2000) and medicinal plants (Butterstein et al., 1985) on ovarian folliculogenesis. Unfortunately, there is minimal evidence that complementary and alternative therapy is safe and efficacious. Therefore, new treatment strategies including complementary and alternative therapy need to be evaluated to alleviate PCOS, regulate hormones, and improve quality of life in PCOS patients (Minhee et al., 2014).

Eugenol, an active principle of cloves, is also widely distributed in various other plants (eg. basil, cinnamon, etc). Eugenol has attracted considerable attention because of its potential for many pharmaceutical applications including anti-inflammatory, anti-tumorigenic and anti-oxidant properties (Prasad et al., 2016; Dubey et al., 2017). The radical-scavenging and anti-inflammatory activities of eugenol have been shown to modulate chronic diseases in vitro and in vivo (Fujisawa and Murakami, 2016). Dimers of eugenol and its related compounds showed large antioxidant activities and high electrophilicity values and also exerted efficient anti-inflammatory activities (Fujisawa and Murakami, 2016). Eugenol is known to interact with different proteins as well as DNA molecules and to influence their functional properties (Bi et al., 2012). Recently, the inhibition effect of eugenol on key enzymes related to diabetes and hypertension was reported in vitro and in vivo model system (Mnafgui et al., 2015).

This study aimed to investigate the possible effects of eugenol in rat PCO after hyperandro-genization with letrozole using biochemical and inflammatory parameters.

3. MATERIALS AND METHODS

Animals

Female albino rats weighing180-200 g and exhibiting normal estrous cycle were taken from Animal house, Faculty of Medicine, Assiut University. Animals were housed in animal place with room temperature being maintained at 25±2°C. Animals were fed on a commercial pellet diet and kept under normal light/dark cycle. Animals were given food and water *ad libitum*.

Induction of PCOS

PCOS was induced by oral administration of aromatase inhibitor, letrozole (1 mg kg^{-1}) dissolved in 0.9% NaCl once daily for 21 days (Kafali et al., 2004 and Rezvanfar et al., 2012). During the experiment, the estrous cycle phases were monitored by the analyses of relative proportion of leukocytes, epithelial and cornified cells in vaginal smear. A vaginal smear was taken daily to determine the phase of the estrous cycle throughout the entire treatment up to the day of the autopsy. The eugenol doses (100 mg kg^{-1} and 250 mg kg^{-1}, oral) were administered from day 22 to the day 36 of the experiment (15 days).

Animals randomly divided into the following groups consisting of six rats in each group.
Group I: Normal control group (0.9% NaCl once daily, orally) (1.0 ml/rat).
Group II: PCOS group + sesame oil (1.0 ml/rat)
Group III: PCOS + eugenol group (100 mg kg^{-1}/day, orally) (Abraham, 2001).
Group IV:PCOS + eugenol group (250 mg kg^{-1}/day, orally). (Abraham, 2001).

Rats were weighed on 37th day, 24 h later the termination of the experiment, blood samples were collected from the heart and serum was separated by centrifugation and stored at – 80˚C until biochemical and hormonal analysis. The animals were sacrificed by cervical dislocation. The abdomen was then rapidly dissected and ovaries were removed, cleansed gently with normal saline and weighed. Ovaries were homogenized in 10-volume ice cold potassium phosphate buffer (50 mM, pH 7.4), sonicated, and centrifuged. The supernatants were then transformed into several Eppendorf tubes for biochemical assays and all were kept at -80 ˚c until analyses (Rezvanfar et al., 2012a,b).

Assay of oxidative stress

SOD was determined by a colorimetric method as described by Nishikimi et al., (1972) using a diagnostic kit supplied by Biodiagnostic Company (Cairo-Egypt).

Catalase was determined by a colorimetric method as described by Aebi (1984) using diagnostic kit supplied by Biodiagnostic Company (Cairo-Egypt).

Malondialdehyde, the oxidative stress product of lipid peroxidation, reacts with thiobarbituric acid under acidic conditions at 95°C to form a pink-colored complex with an absorbance at 532 nm (Ohkawa et al., 1979).

Evaluation of inflammatory and metabolic parameters

-The level of CRP was determined using ELISA kit catalog No. 557825 for the quantitative measurement of rat CRP in serum.
- Ovarian PGE2 was quantified using a rabbit antiserum and results were expressed as pg mg^{-1} protein. To report

the concentrations of PGE per protein, Bradford method was used to measure protein content using concentrated Comassie blue as reagent and BSA as the standard.

-Tumor necrosis factor-α was measured, using a sandwich enzyme immunoassay kit protocol supplied by the manufacturer of the antibodies (Multisciences Biologic Company, Hangzhou, China) and resultant optical density determined, using a microplate reader (Thermo Multiskan MK3) at 450 nm.

-Serum insulin concentration as a metabolic biomarker determined by ELISA Kit as described previously (Pakzad et al., 2013). According to the manufacturer's instructions, after 8 h fasting for all the studied rats, the serum insulin level was measured and reported as ng ml^{-1}.

-Sex steroid evaluation: The levels of steroid hormones were measured in all groups by competitive ELISA kits. Assay sensitivity was 0.008 ng ml^{-1} for estradiol, 0.04 ng ml^{-1} for progesterone and 0.07 ng ml^{-1} for testosterone. For each hormone, intra- and inter-assay CV% were ≤10%.

Chemicals

1. **Eugenol** (Sigma Aldrich Company, England). Eugenol in pure oily solution, bottle contain100 ml and freshly diluted with sesame oil.
2. **Letrozole** Sigma Aldrich Company, England.
3. **Sesame oil** (Nile Co. for pharmaceuticals, Cairo, Egypt). It will be used for dilution of eugenol, 1.0 ml/ rat/day.

Statistical analysis

Statistics was performed using the statistical graph pad prism 5. One way analysis of variables (ANOVA) was used. Significant differences between the groups were determined using a Newman-keuls test. Data were expressed as means ± standard deviation of the mean (SD) and the level of significance between groups were considered significant (*) at $p<0.05$

RESULTS

The body and ovary weights (g)

The PCO rats gained significantly more body and ovarian weights in comparison with the control group whereas treatment of PCO rats with eugenol (100 and 250 mg kg^{-1}) caused significant reduction in body and ovarian weights near results as the control group (Table 1).

Table (1): Effect of eugenol (100 and 250 mg kg^{-1}) on the body and ovary weights.

parameters	Normal control	PCOS	PCOS + eugenol (100 mg kg^{-1})	PCOS + eugenol (250 mg kg^{-1})
Body weight(g)	221.13±3.16	256.37±3.56$^{\#}$	232.50±2.44*	230.58±3.16*
Ovarian weight(g)	0.042±0.004	0.059±0.003$^{\#}$	0.044±0.001*	0.043±0.002*

Values are expressed as mean ± S.D., n = 6 animals in each group $^{\#}$ Significant result at $p<0.05$ from normal control * Significant result at $p<0.05$ from PCOS control group.

Sexual cycle

All rats in control group exhibited regular estrous cycles with an expected time of 4–5 d. However, in the PCO control group were completely acyclic and exhibited constant estrous state as compared to normal control group. Data indicated that when the PCO rats were concurrently treated with eugenol (100 and 250 mg kg^{-1}), they exhibited a normal sexual cycle to a large extent as seen in the normal control group.

Effect of eugenol on oxidative stress biomarkers:

Activities of antioxidant enzymes were significantly reduced in PCOS group as compared to normal control group. This reduction in enzymes activity was significant in the values of catalase and SOD. Alteration in the ovarian enzyme activities with the treatment of eugenol (100 and 250 mg kg^{-1}) showed statistically significant increase ($P < 0.05$) as compared to PCOS control group. Compared with control, higher levels of serum MDA of PCOS rats were found. Eugenol treated PCOS rats showed significant decrease in serum MDA. (Table 2)

Table (2): Effect of eugenol (100 and 250 mg kg^{-1}) on antioxidant profile.

Groups	MDA (nmol ml^{-1})	CAT U/mg protein	SOD U/mg protein
Group-I	33.42±3.03	30.87±4.02	7.68±0.49
Group-II	47.86±1.98$^{\#}$	10.93±1.01$^{\#}$	2.92±0.16$^{\#}$
Group-III	37.71±2.50*	26.14±3.11*	6.48±0.15*
Group-IV	36.11±1.33*	29.95±1.66*	7.32±0.41*

Values are expressed as mean ± S.D., n = 6 animals in each group $^{\#}$ Significant result at $p<0.05$ from normal control * Significant result at $p<0.05$ from PCOS control group.

Effect of eugenol on inflammatory biomarkers

As shown in table (3), serum CRP and TNF-α was significantly higher in letrozol induced PCO rats as compared with normal control. However, treatment with eugenol significantly reduced CRP concentration in PCOS rats. The concentrations of ovarian PGE2 significantly increased in PCOS rats. Treatment with eugenol significantly decreased the concentration of PGE2 compared with PCOS rats.

Table (3): Effect of eugenol (100 and 250 mg kg^{-1}) on inflammatory biomarkers.

Groups	CRP ($mg\ L^{-1}$)	PGE2 Pg/mg protein	TNF-α ($pg\ ml^{-1}$)
Group-I	0.213±0.018	98.01±12.07	14.76±1.21
Group-II	2.89±0.23#	316.55±40.91#	51.24±3.99#
Group-III	1.65±0.13*	125.21±5.17*	26.95±1.34*
Group-IV	1.59±0.12*	99.23±6.04*	22.69±1.27*

Values are expressed as mean ± S.D., n = 6 animals in each group # Significant result at p<0.05 from normal control * Significant result at p<0.05 from PCOS control group.

Effect of eugenol on serum insulin

Serum insulin concentration was detected significantly higher in hyperandrogenized PCO rats when compared with normal control. In contrast, treatment of PCO rats with eugenol decrease the elevation of insulin significantly and retained it close to that of normal control as shown in figure (1).

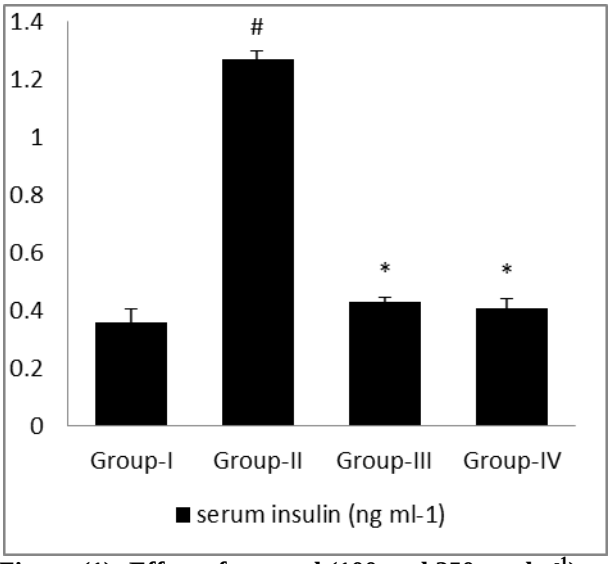

Figure (1): Effect of eugenol (100 and 250 mg kg^{-1}) on serum insulin.

Values are expressed as mean ± S.D., n = 6 animals in each group # Significant result at p<0.05 from normal control * Significant result at p<0.05 from PCOS control group.

Effect of eugenol on sex steroid concentration

The serum concentrations of testosterone, progesterone and estradiol are shown in figures (2-4). In comparison with controls, rats of PCO group exhibited hyperandrogenization due to significant increase in serum testosterone concentration. In contrast, letrozole-induced PCO rats showed significant decrease in serum progesterone and estradiol when compared with the control group. However, comparable to the control group, normal serum concentrations of testosterone, progesterone and estradiol were detected in PCO rats after treated with eugenol.

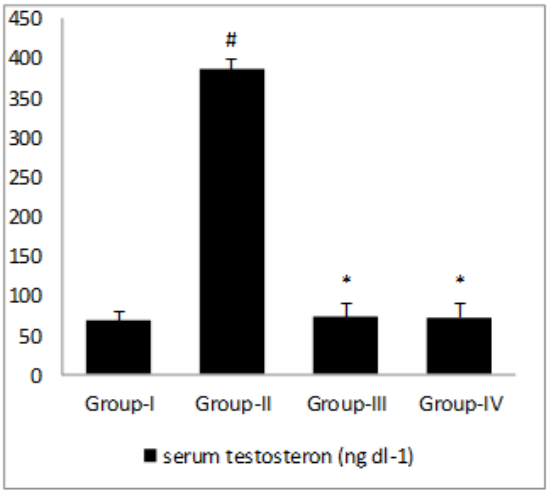

Figure (2): Effect of eugenol (100 and 250 mg kg^{-1}) on serum testosterone.

Values are expressed as mean ± S.D., n = 6 animals in each group # Significant result at p<0.05 from normal control * Significant result at p<0.05 from PCOS control group.

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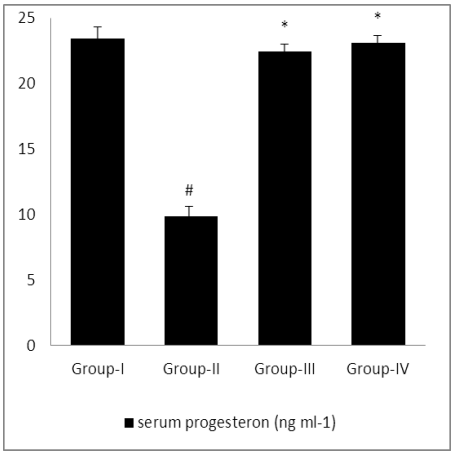

Figure (3):Effect of eugenol (100 and 250 mg kg^{-1}) on serum progesterone

Values are expressed as mean ± S.D., n = 6 animals in each group # Significant result at p<0.05 from normal control * Significant result at p<0.05 from PCOS control group.

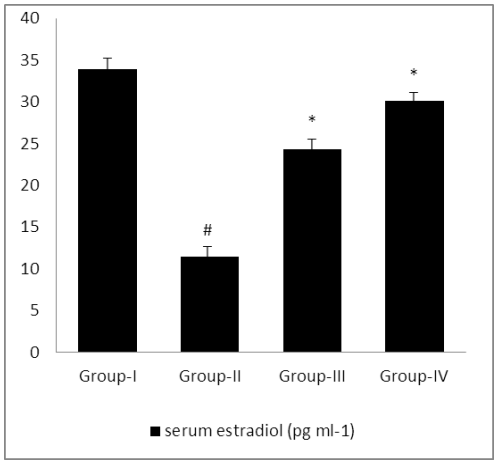

Figure (4):Effect of eugenol (100 and 250 mg kg^{-1}) on serum estradiol

Values are expressed as mean ± S.D., n = 6 animals in each group # Significant result at p<0.05 from normal control * Significant result at p<0.05 from PCOS control group.

DISCUSSION

This study demonstrated the effects of eugenol in hyperandrogenism induced PCO rats by endocrine profiles, steroidogenesis and alteration of selected markers involved in oxidative stress and inflammation. The present data showed that eugenol significantly decrease selected inflammatory biomarkers and oxidative stress biomarker, in addition to preserved antioxidant potential in letrozole-induced PCO rats. Interestingly, these effects were consistent with the maintenance of physiological ovarian steroidogenesis and normal sexual cycling activity when compared with the PCO group.

Letrozole, a non-steroidal aromatase inhibitor, was used to induce PCOS in the rats. Subsequently the increased weights of rats and irregular estrous cycle in the positive control substantiated the induction of PCOS and also signified that the rat model is anestrous, as it imitated anovulatory characteristic (Manneras et al., 2007). The biochemical and histological characteristics of letrozole-induced PCO in rats have been extensively discussed in previous studies (Rezvanfar et al., 2012a,b, 2014, 2015). It was found that cystogenesis in the letrozole-induced hyperandrogenemia was associated with several alterations in biochemical factors. Those measured alterations included: an increase in MDA and peroxynitrite production, over-activity of inflammatory cytokines like TNF-α and PGE and reduced activity of enzymatic antioxidants like SOD and CAT (Rezvanfar et al., 2012a,b).

Letrozole reduces conversion of androgens to estrogens in the ovary and results in a condition termed hyperandrogenemia, in which serum estradiol concentration significantly decreases and consequently non-aromatizable androgens such as testosterone increase. Hyperandrogenism as a key regulator in the pathogenesis of a majority of PCOS cases developed cystogenesis by impairing maturation of developing follicles in the ovaries (Goodarzi et al., 2011). Experimental models and clinical studies data suggest that hyperandrogenism is progenitor of chronic low-grade inflammation that in turn directly stimulates excess ovarian androgen production (Gonzàlez, 2012). It can arrest follicular development via apoptosis in granulosa cells resulting in poor oocyte quality and ultimately progressive follicular atresia (Sasson et al., 2002; Jonard & Dewailly, 2004) through higher serum TNF-α concentrations (Escobar-Morreale et al., 2011; Murri et al., 2013) and oxidative stress (Belgorosky et al., 2010).

The present results indicate that eugenol treatment can inhibit increased body and ovarian weight in letrozole induced PCOS. Eugenol seems to be one of the effective components in cinnamon extract (Nagababu et al., 2010). Administration of eugenol exhibited normal estrous cycle like that obtained with cinnamon supplementation which improves menstrual cyclicity in women with PCOS (Kort and Lobo, 2014).

In recent decades, more studies have shown that oxidative stress and low grade inflammation are the two main pathways in the pathogenesis of PCOS (Rezvanfar et al., 2016). Elevated serum MDA levels in PCO rats suggest enhanced lipid peroxidation leading to tissue damage and inability of antioxidant defense mechanisms to prevent free radical attack. This peroxidative damage to membranes may lead to the leakage of enzymes and metabolites into the blood circulation (Adefegha et al., 2014). Eugenol completely inhibits both iron and Fenton reagent-mediated lipid peroxidation (Nagababu et al., 2010). The present results show that administration of eugenol increases CAT and SOD of letrozole induced PCO in rats. It was observed that eugenol specifically exhibited higher inhibitory effect on hydrogen peroxide than other reactive oxygen species, and also blocked DNA oxidation and lipid peroxidation induced by hydroxyl radical (Hyang and Moon-Moo 2013).

Eugenol has an interesting structure in which an aromatic moiety is linked to a hydrocarbon chain, a hydroxyl group and a methoxy group. A molecule with such a unique structure is predicted to participate in multiple non-covalent interactions with globular proteins, provided that it finds an appropriate binding domain within the protein molecule. Both methoxy and hydroxyl groups are known to be vital for eugenol's antioxidant property. It has been proposed the hypothesis that eugenol reduces two or more 1,1-diphenyl-2-picryl hydroxyl radicals, despite the availability of only one hydrogen from a hydroxyl group. The formation of

dimers of eugenol (dehydrodieugenol) with two phenolic hydroxyl groups originated from eugenyl intermediate radicals has also been proposed as mechanism between eugenol and 1,1-diphenyl-2-picryl hydroxyl radicals. (Gulçin, 2011). According to the results of Gulçin (2011) study, eugenol has the most powerful antioxidant and radical scavenging activity. Eugenol appears to possess multiple antioxidant activities (dimerization, recycling, and chelating effect) in one molecule, thus having the potential to alleviate and prevent chronic diseases (Fujisawa and Murakami, 2016).

The present study demonstrated that eugenol treatment decrease serum CRP and TNF- α compared to the PCOS control. Ovarian PGE2 levels in PCOS rats was evaluated as a measure of inflammatory process. C-reactive protein is an inflammatory marker, which is a member of the group of acute phase proteins and the level of CRP increases in response to inflammation (Rhodes et al., 2011). Cyclooxygenase-2 is a major contributor to increase the level of PGE2. Prostaglandins is known to be associated with the oxidative stress as overexpression of COX-2 induces generation of free radicals and lipid peroxides. Nuclear factor kappa-B is a signaling molecule acting upstream of cyclooxy-genase-2 expression and also regulates the production of pro-inflammatory cytokines TNF-α and PGE2. Eugenol inhibits cyclooxy-genase-2 and nuclear factor kappa-B (Murakami et al., 2003; Okada et al., 2005; Fujisawa and Murakami, 2016).

Results of the present study show significant decrease in serum insulin level in PCOS rats after eugenol administration. Excess insulin can cause disruption of folliculogenesis through stimulating androgen production by theca cells and elevating serum testosterone levels (Dumesic & Richards, 2013). Eugenol exerts insulin sensitizing effects via phosphorylation of phospho-glycogen synthase kinase-3 and AKT (Ser473) (Sartorius et al., 2014). Eugenol has been associated with improved glucose homeostasis in a wide array of studies; this demonstrates the ability of eugenol to influence key endocrine parameters (Sartorius et al., 2014;Srinivasan et al., 2014).

It was observed decreased serum testosterone and elevated estradiol level in eugenol treated PCOS rats. These findings are in harmony with results obtained by Farzaneh et al.,(2012).

In previous studies, letrozole-treated rats exhibited lower serum Progesterone (Baravalle et al., 2006), indicating possible luteal phase defects like those found in some PCOS women (Joseph-Horne et al., 2002; Meenakumari et al., 2004). In the present study, eugenol corrected serum Progesterone level might be through increasing the number of corpus luteum and a direct stimulatory effect on corpus luteum function. Therefore, maintenance of normal estrus cyclicity, following eugenol treatment of PCO rats could be attributed to preserving of steroid status and enabling ovarian function to be normalized.

There is a potential relationship between oxidative/ nitrosative stress and inflammatory response in letrozole-induced hyperandrogenic PCO rats (Rezvanfar et al., 2012a). Therefore, the beneficial effects of eugenol in PCO are probably returned to its strong antioxidant potential and down regulation of inflammatory responses.

Another hypothesis for the beneficial effect of eugenol to protect the follicles from being cyst is eugenol might decrease the levels of matrix metalloproteinase-9 via inactivation of ERK (Hyang and Moon-Moo, 2013) and matrix metalloproteinase-2 (Manikandan et al., 2010) which are enzymes involved in the pathogenesis of PCO (Lewandowski et al., 2006; Rezvanfar et al., 2014).

CONCLUSION

The current results suggest that application of eugenol with dual antioxidant and anti-inflammatory effects might be effective in the management of PCOS. So that it can be used as an adjunct therapy sideways to currently used drugs in PCOS management.

REFERENCES

1. Abraham, S. K. (2001):″Anti genotoxicity of Tran-canethole and Eugenol in mice″. food and chemical toxicology; 39: 493-498.
2. Adefegha SA, Oboh G, Adefegha OM, Boligon AA, Athayde ML.(2014): Antihyperglycemic, hypolipidemic, hepatoprotective and antioxidative effects of dietary clove (Szyzgium aromaticum) bud powder in a high-fat diet/streptozotocin-induced diabetes rat model. Volume 94, Issue 13 October Pages 2726–273
3. Aebi H, (1984):Catalase. In: L. Packer (Ed), methods in enzymology. Academic pres, Orlando, 105: 121-126.
4. Agarwal A, Gupta S, Sharma RK.(2005): Role of oxidative stress in female reproduction. Reprod Biol Endocrinol. 2005;3(1):1.
5. Baravalle C, Salvetti NR, Mira GA, Pezzone N, Ortega HH (2006): Microscopic characterization of follicular structures in letrozole-induced polycystic ovarian syndrome in the rat. Arch Med Res; 37: 830–839.
6. Belgorosky D, Sander VA, Yorio MP, Faletti AG, Motta AB. (2010). Hyperandrogenism alters intraovarian parameters during early folliculogenesis in mice. Reprod Biomed Online 20:797–807.
7. Bi S., Yan L., Wang Y., Pang B. & Wang T. (2012):Spectroscopic study on the inter- action of eugenol with salmon sperm DNA *in vitro*. Journal of Luminescence 132, 6.
8. Bradford M Ms (1976).: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 72, 248-54.

9. Butterstein GM, Schladler MH, Lysogorski E, Robin L, Sipperly S. (1985):A Naturally occurring plant compound, 6-Methoxybenzoxazolinone, stimulates reproductive responses in rats. Biol Reprod. ;32:1018–1023.
10. Dubey K, Anand BG, Shekhawat DS, Kar K (2017):Eugenol prevents amyloid formation of proteins and inhibits amyloid-induced hemolysis. Sci Rep. 2017 Feb 1;7:40744. doi: 10.1038/srep40744.
11. Dumesic DA, Richards JS. (2013). Ontogeny of the ovary in polycystic ovary syndrome. Fertil Steril 100:23–38.
12. Escobar-Morreale HF, Luque-Ramı´rez M, Gonza´lez F. (2011).Circulating inflammatory markers in polycystic ovary syndrome:A systematic review and metaanalysis. Fertil Steril 95:1048–58.
13. Farzaneh Dehghani, Aseyeh Heshmatpour, Mohammad Reza Panjehshahin, Tahereh Talaei-Khozani (2012): Toxic effects of water/alcoholic extract of *Syzygium aromaticum* on sperm quality, sex hormones and reproductive tissues in male mouse. IUFS J Biol 2012, 71(2):95-102
14. Fujisawa S, Murakami Y (2016): Eugenol and Its Role in Chronic Diseases. Adv Exp Med Biol. 2016;929:45-66.
15. Gonzàlez F. (2012). Inflammation in polycystic ovary syndrome: Underpinning of insulin resistance and ovarian dysfunction. Steroids 77:300–5.
16. Goodarzi, M.O., Dumesic, D.A., Chazenbalk, G., Azziz R. (2011*)* Polycystic ovary syndrome: etiology, pathogenesis and diagnosis. Nature Reviews. Endocrinology, 7, 219–231.
17. Gulçin, I. (2011): Antioxidant activity of eugenol: a structure-activity relationship study. Journal of medicinal food 14, 975–985.
18. Hahn S, Janssen OE, Tan S, Pleger K, Mann K, Schedlowski M, et al. (2005) Clinical and psychological correlates of quality-of-life in polycystic ovary syndrome. Eur J Endocrinol 153(6): 853–860
19. Hyang Nam, Moon-Moo Kim, (2013): Eugenol with antioxidant activity inhibits MMP-9 related to metastasis in human fibrosarcoma cells**.** Food and Chemical Toxicology, Volume 55, May 2013, Pages 106–112.
20. Jha U, Asad M, Asdaq BMS, Das KA, Satya Prasad SV.(2010): Fertility inducing effect of aerial parts of *Coccinia cordifolia* L. in female rats. J Ethnopharmacol. ;127:561–564.
21. Jonard S, Dewailly D. (2004). The follicular excess in polycystic ovaries, due to intra-ovarian hyperandrogenism, may be the main culprit for the follicular arrest. Hum Reprod Update 10:107–17.
22. Joseph-Horne R, Mason H, Batty S, White D, Hillier S, Urquhart M,(2002): Luteal phase progesterone excretion in ovulatory women with polycystic ovaries. Hum Reprod; 17(6): 1459–1463.
23. Kafali H, Iriadam M, Ozardalı I, Demir N.(2004): Letrozole-induced polycystic ovaries in the rat: a new model for cystic ovarian disease. Arch Med Res. 2004;35(2):103-108.
24. Kelly CC, Lyall H, Petrie JR, Gould GW, Connell JM, Sattar N. (2001):Low grade chronic inflammation in women with polycystic ovarian syndrome. J Clin Endocrinol Metab. 2001;86(6):2453-5.
25. Kort DH, Lobo RA (2014): Preliminary evidence that cinnamon improves menstrual cyclicity in women with polycystic ovary syndrome: a randomized controlled trial. Am J Obstet Gynecol. Nov;211(5):487.e1-6.
26. Lewandowski KC, Komorowski J, O'Callaghan CJ, , Tan BK, Chen J, Prelevic GM, et al. (2006). Increased circulating levels of matrix metalloproteinase-2 and -9 in women with the polycystic ovary syndrome. J Clin Endocrinol Metab 91:1173-7.
27. Manikandan P, Murugan RS, Priyadarsini RV, Vinothini G, Nagini S. (2010): Eugenol induces apoptosis and inhibits invasion and angiogenesis in a rat model of gastric carcinogenesis induced by MNNG. Life Sciences Volume 86, Issues 25–26, 19 June 2010, Pages 936–941
28. Manneras L, Cajander S, Holmang A, Seleskovic Z, Lystig T, Lönn M, et al.,(2007): A new rat model exhibiting both ovarian and metabolic characteristics of polycystic ovary syndrome. Endocrinology. 2007;148(8):3781–91.
29. Meenakumari KJ, Agarwal S, Krishna A, Pandey LK. (2004). Effects of metformin treatment on luteal phase progesterone concentration in polycystic ovary syndrome. Braz J Med Biol Res 37:1637–44.
30. Minhee Jang, Min Jung Lee, Jin Moo Lee, Chun-Sik Bae, Sung-Hoon Kim, Jong Hoon Ryu et al.,(2014): Oriental Medicine Kyung-Ok-Ko Prevents and Alleviates Dehydro-epiandrosterone-Induced Polycystic Ovarian Syndrome in Rats. PLoS One. 9(2): e87623.
31. Mnafgui K, Hajji R, Derbali F, Gammoudi A, Khabbabi G, Ellefi H, et al., (2015). . Anti-inflammatory, Antithrombotic and Cardiac Remodeling Preventive Effects of Eugenol in Isoproterenol-Induced Myocardial Infarction in Wistar Rat. Cardiovascular toxicology, doi: (2015).10.1007/s12012-015-9343-x
32. Moran LJ, Misso ML, Wild RA, Norman RJ. (2010). Impaired glucose tolerance, type 2 diabetes and metabolic syndrome in polycystic ovary syndrome: A systematic review and meta-analysis. Hum Reprod Update 16:347–63.
33. Murakami Y, Shoji M, Hanazawa S, Tanaka S, Fujisawa S (2003): Preventive effect of biseugenol, a eugenol ortho dimer, on lipopolysaccharide-stimulated nuclear factor kappa B activation and inflammatory cytokine expression in macrophages. Biochem Pharmacol 66:1061–1066.
34. Murri M, Luque-Ramı´rez M, Insenser M, Ojeda-Ojeda M, Escobar-Morreale HF. (2013): Circulating markers of oxidative stress and polycystic ovary

syndrome (PCOS): A systematic review and meta-analysis. Hum Reprod Update 19:268–88.
35. Nagababu E, Rifkind JM, Boindala S, Nakka L.(2010): Assessment of antioxidant activity of eugenol in vitro and in vivo. Methods Mol Biol. 2010;610:165-80. doi: 10.1007/978-1-60327-029-8_10.
36. Nishikimi, M., Rao, N.A., and Yagi, K. (1972): The occurrence of superoxide anion in the reaction of reduced phenazine methosulphate and molecular oxygen. Biochemical Biophysical Research Communications, Vol. 46, No. 2, pp. 849-854.
37. Ohkawa H, Ohishi N, Yagi K. (1979): Assay of lipid peroxide in animal tissue by thiobarbituric acid reaction. Ann Biochem. 95:351–358.
38. Okada N, Hirata A, Murakami Y, Shoji M, Sakagami H, Fujisawa S (2005): Induction of cytotoxicity and apoptosis and inhibition of cyclooxygenase-2 gene expression by eugenol-related compounds. Anticancer Res 25:3263–3269
39. Pakzad M, Fouladdel S, Nili-Ahmadabadi A, Pourkhalili N, Baeeri M, Azizi E et al. (2013): Sublethal exposures of diazinon alters glucose homostasis in Wistar rats; biochemical and molecular evidences of oxidative stress in adipose tissues. Pest Biochem Physiol 105:57–61.
40. Prasad SN, Bharath MM, Muralidhara (2016): Neurorestorative effects of eugenol, a spice bioactive: Evidence in cell model and its efficacy as an intervention molecule to abrogate brain oxidative dysfunctions in the streptozotocin diabetic rat. Neurochem Int. May;95:24-36. doi:10.1016/j.neuint.2015.10.012. Epub Oct 28.
41. Reis GV, Gontijo NA, Rodrigues KF, Alves MT, Ferreira CN, Gomes KB (2017): Vitamin D receptor polymorphisms and the polycystic ovary syndrome: A systematic review. J Obstet Gynaecol Res. 2017 Jan 27. doi: 10.1111/jog.13250.
42. Rezvanfar MA, Ahmadi A, Shojaei-Saadi HA, Baeeri M, Abdollahi M.(2012): Molecular mechanisms of a novel selenium-based complementary medicine which confers protection against hyperandrogenism-induced polycystic ovary. Theriogenology. 2012;78(3):620-631.
43. Rezvanfar MA, Rezvanfar MA, Ahmadi A, Shojaei-Saadi HA, Baeeri M, Abdollahi M. (2012b). Molecular mechanisms of a novel selenium-based complementary medicine which confers protection against hyperandrogenism-induced polycystic ovary. Theriogenology 78:620–31.
44. Rezvanfar MA, Saadat S, Shojaei Saadi HA, Mansoori P, Saeedi S, Gooshe M. et al. (2015). Cellular and molecular mechanisms of pentoxifylline's beneficial effects in experimental polycystic ovary. Theriogenology 83:968–77.
45. Rezvanfar MA, Sarah S., Parisa M, Sepideh Saadat, Maziar Goosheh, Habib A. et al., (2016):Dual targeting of TNF-a and free radical toxic stress as a promising strategy to manage experimental polycystic ovary. Pharm Biol, 54(1): 80–90
46. Rezvanfar MA, Shojaei Saadi HA, Gooshe M, Abdolghaffari AH, Baeeri M, Abdollahi M. (2014). Ovarian aging like phenotype in the hyperandrogenism-induced murine model of polycystic ovary. Oxid Med Cell Longev 2014:948951
47. Rezvanfar MA1, Rezvanfar MA, Ahmadi A, Saadi HA, Baeeri M, Abdollahi M. (2012a). Mechanistic links between oxidative/nitrosative stress and tumor necrosis factor alpha in letrozole-induced murine polycystic ovary: Biochemical and pathological evidences for beneficial effect of pioglitazone. Hum Exp toxicol 31:887-97.
48. Rhodes B, Fürnrohr BG, Vyse TJ. (2011): C-reactive protein in rheumatology: biology and genetics. Nat Rev Rheumatol. 7: 282–289.
49. Sartorius T, Peter A, Schulz N, Drescher A, Bergheim I, Machann J, et al., (2014): Cinnamon extract improves insulin sensitivity in the brain and lowers liver fat in mouse models of obesity. PLoS One. Mar 18;9(3):e92358. doi: 10.1371/journal.pone.0092358
50. Sartorius T, Peter A, Schulz N, Drescher A, Bergheim I, Machann J, et al., (2014): Cinnamon Extract Improves Insulin Sensitivity in the Brain and Lowers Liver Fat in Mouse Models of Obesity. PLoS One.9(3): e92358.
51. Sasson R, Winder N, Kees S, Amsterdam A. (2002): Induction of apoptosis in granulosa cells by TNF alpha and its attenuation by glucocorticoids involve modulation of Bcl-2. Biochem Biophys Res Commun 294:51–9.
52. Showell M, Brown GJ, Clarke J, Hart RJ. (2013): Antioxidants for female subfertility. Cochrane Database Syst Rev 5:8,
53. Srinivasan S, Sathish G, Jayanthi M, Muthukumaran J, Muruganathan U, Ramachandran V.(2014): Ameliorating effect of eugenol on hyperglycemia by attenuating the key enzymes of glucose metabolism in streptozotocin-induced diabetic rats. Mol Cell Biochem. Jan;385(1-2):159-68.
54. Telefo PB, Moundipa PF, Tchouanguep FM. (2004): Inductive effects of the leaf mixture extract of *Aloe buettneri*, *Justicia insularis*, *Dicliptera verticillata* and *Hibicus marcranthus* on in vitro production of estradiol. J Ethnopharmacol. 2004;90:225–230.
55. Tohei A, Sakamoto S, Kogo H.(2000) Dexamethasone or Triamcinolone increases follicular development in immature female rats. Japan J Pharmacol. 2000;84:281–286.