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Quercetin modulates granulosa cell mRNA androgen receptor gene expression in dehydroepiandrosterone-induced polycystic ovary in Wistar rats via metabolic and hormonal pathways

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Abstract:
Background: It is estimated that about 5–10% of women suffer from polycystic ovarian syndrome (PCOS) which is a major cause of female reproductive dysfunction. This study examined the role of quercetin on dehydroepiandrosterone (DHEA)-induced PCO in Wistar rats.
Methods: Twenty-eight pre-pubertal female Wistar rats that are 21 days old weighing 16–21 g were sorted into four groups (n = 7). Group I served as control and was given distilled water only, Group II were injected with 6 mg/100 g BW of DHEA in 0.2 mL of corn oil subcutaneously, Group III received 100 mg/kg BW of quercetin orally and Group IV received 6 mg/100 g BW of DHEA in 0.2 mL of corn oil subcutaneously and 100 mg/kg BW of quercetin orally. Rats were sacrificed after 15 days by cervical dislocation method. Blood samples and ovaries were collected for hormonal, biochemical, and histopathological analysis and expressions of mRNA androgen receptor gene were determined using RT–qPCR. All data were analysed using one-way ANOVA.
Results: A significant decrease ($p < 0.05$) in the antioxidant and metabolic enzyme activity in the DHEA treated group was observed when compared with control. DHEA co-administration with quercetin showed a significant decrease in malondialdehyde and cytokines when compared with DHEA treated group. Also a significant increase in progesterone, metabolic and antioxidant enzyme activity was observed. The histopathology demonstrates a reduction in cystic and atretic cells, improved expression of BCl2, E-Cadherin and a decrease in Bax.
Conclusions: Quercetin alleviated DHEA-induced PCO. These effects could be attributed to its antioxidant property.

Keywords: dehydroepiandrosterone, mRNA androgen receptor, PCOS, quercetin, Wistar rats
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Introduction

Infertility may be linked to an underlying medical ailment that may affect the fallopian tubes, inhibit ovulation process or causes hormonal dysfunctions. In women of reproductive age (18–44), polycystic ovarian syndrome

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(PCOS) is a prevalent endocrine disorder implicated in anovulatory infertility and menstrual cycle abnormalities with a projected value ranging of 5–10% [1], [2], [3]. Women affected by PCOS are characterized by increased androgen levels, chronic inflammatory conditions and oxidative stress (OS) [4]. Though the pathophysiological mechanism of the polycystic ovary (PCO) has not been fully elucidated [5], numerous investigations have shown that OS has significantly implicated in PCO than normal. Reactive oxygen species (ROS) is also observed to be significantly correlated with hyperandrogenism and chronic inflammation cascades [6]. OS is as a result of an imbalance between the production of free radicals and the ability to counteract the destructive effects by the endogenous antioxidants [7].

Dehydroepiandrosterone (DHEA) also known as androstenolone is a very common human steroidal hormone secreted by the adrenal glands, brain, and gonads [8], [9], [10]. DHEA serves as a metabolic precursor in the production of the primary sex hormones [11]. DHEA is synthesized from cholesterol by the external layer of adrenal glands, known as the adrenal cortex, though it is also produced from the testes and ovaries in small quantities. It flows in the blood by attaching to sulphur as DHEA sulphate, which stops the hormone from being broken down. In women, DHEA is an essential source of estrogens providing about 75–100% of oestrogens before and after menopause. Women with the PCOS and genetic adrenal hyperplasia have shown increased amounts of DHEA or DHEA sulphate. Furthermore, the levels may rise in individuals with adrenal gland carcinoma [12].

Quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone) is nearly a ubiquitous in plants polyphenolic flavonoid, with a potent antioxidant property, because of its capacity to scavenge free radicals and chelate with transition metal [13]. In addition, De Groot [14] reported the anti-inflammatory suppressive property of quercetin. Transcription factors may be initiated by the presence of free radicals leading to the production of pro-inflammatory cytokines, often prominent in patients suffering from chronic inflammatory diseases. Quercetin is one of the most widely used bioflavonoids in the management of inflammatory disorders and metabolic [13]. Tumor necrosis factor alpha (TNF α), a pro-inflammatory marker known to perform a major role in the pathophysiology of inflammatory diseases is controlled by OS [15]. Quercetin has also been shown to inhibit TNF-α synthesis, gene and protein expression [14]. A decrease in the synthesis of TNF-α with quercetin indicates that bioflavonoids possess great ability to regulate the immune system and possess anti-inflammatory properties. Quercetin-induced decrease TNF-α production can lead to the activation of anti-inflammatory cytokines through suppression of NF κβ, therefore quercetin could be used as an anti-inflammatory agent. Pharmacological interventions and the use of antioxidants are considered treatments for PCOS to normalize hyperandrogenism and anovulation. There are no studies to our knowledge that have assessed the effects of quercetin on mRNA androgen receptor (AR) gene expression in rats with PCOS. To test this hypothesis, we examined the ovaries from rats with PCOS to evaluate the effects of quercetin on the expression of relative concentrations of mRNA AR gene in the DHEA induced PCO in Wistar rats.

Materials and methods

Twenty-eight pre-pubertal female Wistar rats that are 21 days old weighing 18 ± 1.58 g were obtained from the Department of Human Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Ibadan and housed in cages in a well-ventilated animal house of Bingham University, Karu, Nasarawa State, Nigeria. They were given rat pellets and water ad libitum. The ethical approval on the animal act right was obtained from the Institutional Animal Care Committee of the same Institution. The experimental procedures were carried out in accordance with Institutional Animal Ethics Committee (IAEC) guidelines.

Animal treatments and tissue collection

Twenty-eight immature female Wistar rats were sorted in no-specific order into four experimental groups (n = 7): The rats in Group I served as control and were given distilled water daily, Group II were injected with 0.2 mL of corn oil containing 6 mg/100 g of DHEA subcutaneously daily to induce PCOS condition [16], Group III were given quercetin (100 mg/kg orally daily) and Group IV were injected with 0.2 mL corn oil containing 6 mg/100 g of DHEA subcutaneously and 100 mg/kg quercetin orally, respectively. All treatments were done for 15 days. Blood samples were collected 24 h after the last administration on the day of sacrifice through the retro-orbital venous sinus[17] and serum was obtained for the determination of female sex hormones (Progesterone and estrogen). Rats were sacrificed by cervical dislocation method, followed by the collection of ovaries and cleaning-off of adherent connective fat tissues for further biochemical study. One ovary from each rat was rapidly submerged in RNA later and stored at 4 °C till the mRNA androgen receptor gene expression was de-

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termined using real-time reverse transcriptase polymerase chain reaction (qPCR). The other ovary was fixed in Bouin's fluid and processed for histopathological analysis.

Experimental protocols

Lipid peroxidation assay

According to the method described by Kartha and Krishnamurthy [18], malondialdehyde (MDA) levels were evaluated. MDA was assayed as a marker of lipid peroxidation and ROS by implication. Samples were transferred to a microcentrifuge tube containing thiobarbituric acid (TBA) and incubated. After incubation, the mixture was centrifuged for 10 min at 2000 rpmfollowed by absorbance measurement at 532 nm. TBA reactive substances were expressed in nanomoles of MDA/gram of wet tissue. Lipid peroxidation was evaluated by estimating the TBA reactive substances (TBARS) generated during the oxidative process [19]. MDA bis-(dimethyl acetal) was used as the external standard.

Evaluation of tissue superoxide dismutase activity

Superoxide dismutase (SOD) was analysed using the technique of Beauchamp and Fridovich [20] modified by Misra and Fridovich [21], and the activity was expressed as unit/mg of protein. The ability of SOD to terminate the autoxidation of epinephrine at pH 10.2 makes this process a simple assay for SOD estimation. The superoxide ($O2^{\cdot}-$) radicals produced by the xanthine oxidase reaction causes oxidation of epinephrine to adrenochrome and the yield produced per introduced $O2^{\cdot}-$ increases with pH and concentrations of epinephrine [22]. These outcomes led to the fact that autoxidation of epinephrine proceeds via a two-pathway process, of which only one is free radical dependent involving the $O2^{\cdot}-$ and is henceforth subject to inhibition by SOD.

Evaluation of tissue catalase activity

Sinha [23], reported the protocol of catalase activity used in this study, which is based on the condensation of dichromate to chromic acetate and perchromic acid (unstable intermediate) in the presence of acetic acid and H_2O_2. The amount of chromic acetate generated is determined by colorimetric method at 570–610 nm; a region where dichromate has no absorbency and will not interfere with the evaluation of chromic acetate. The H_2O_2 is split for period of time and the reaction inhibited at a particular time with the addition of a dichromate/acetic acid/H_2O_2 is evaluated by measuring chromic acetate by colorimeter.

Determination of tissue glutathione-S-transferase activity

According to the method of Habig et al. [24], glutathione-S-transferase (GST) activity was evaluated based on the principle that glutathione-S-transferase indicates a relatively high-level activity in the presence of the substrate; 1-chloro-2, 4-dinitrobenzene (CDNB). Conjugation of CDNB with reduced glutathione, causes the absorption to maximum and shifts to a longer wavelength measured at 340 nm which is a direct measurement of the enzymatic response.

Reduced glutathione assay (GSH)

Reduced glutathione was determined by the protocol described by Jollow et al. [25], in which 1, 2-dithio-bis nitrobenzoic acid (DTNB) is employed as the substrate. The yellow color developed was read instantly at 412 nm and expressed as μmol GSH/g tissue.

Total protein (Biuret reagent)

The tissue protein content was determined by the method described by Lowry et al. [26]. This is based on the principle that copper ion reacts with the peptide bonds of proteins in an alkaline medium to form a pink to

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purple complex. Potassium sodium tartrate and potassium iodide inhibit the precipitation of the hydroxide of copper and auto reduction of copper from solution, respectively.

$$\text{Protein} + \text{Cu}^2 \overset{\text{Alkaline pH}}{\longrightarrow} = \text{Cu} - \text{Protein complex}$$

Intensity of the color is directly proportional to the protein level and is measured at 546 nm using spectrophotometry (F93 Drawell fluorospectrophotometer).

Cytokines

Test principle tissue vascular endothelial growth factor (VEGF)

An antibody specific to vascular endothelial growth factor (VEGF) (Elabsciennce Cat. No E-EL-215896T) precoated with micro enzyme linked immunosorbent assay (ELISA) plate was used according to Sandwich-ELISA principle. Standards, samples, and substrate solution were transferred into the micro ELISA plate wells. A biotinylateddetection antibody, specific for VEGF and Avidin Horseradish Peroxidase (HRP) conjugates were introduced and incubated. A stop solution was added to the enzyme-substrate mixture to give a yellow colored complex and the optical density (OD) was measured at 450 nm. The OD value was compared to the concentration of VEGF from the standard curve to determine the OD of the samples.

Test principle tissue tumor necrosis factor alpha (TNF-α)

ELISA kit Bioassay technology laboratory TNF alpha (Cat. No E0003Ra) was used in this study, in which the TNF-α was added to the TNF-α monoclonal antibody pre-coated well and incubated. Then, biotin-conjugated anti-Rat TNF-α antibody and streptavidin-HRP was introduced to bind to the rat TNF-α, and washed to remove the unbound biotin-conjugate and unbound Streptavidin-HRP. Addition of substrate solution causes the development of color proportion to the amount of Rat TNF-α. An acid stop solution was added to the mixture to terminate the reaction and absorbance measured at 450 nm.

Estimation of serum progesterone and estrogen levels

The concentrations of progesterone and estrogen hormone in the serum samples were determined through a tube-based enzyme immunoassay (EIA) technique. The protocol used in hormone testing followed the technique described by the kit manufacturers (Immunometrics Limited UK) and met the WHO research program standards for reproductive studies.

Determination of ovarian proton pump (ATPase) activity

Na^+/K^+-ATPase, Calcium ATPase, and Hydrogen ATPase activities were analysed based on an adjustment of the method published by Evans [27]. Spectrophotometer was used to measure the levels of inorganic phosphate in the ovarian tissue homogenate as per the technique described by Bonting [28], [29].

Histological techniques

Histological examination was carried out on the tissues fixed in Bouin's fluid. Fixed tissue blocks were sectioned for routine hematoxylin and eosin (H&E) staining. The fixed organs were up to 0.5 cm thickness and sectioned transversely on a slab and dehydrated in a vial containing 70% alcohol. The tissues were then passed through 90% alcohol and xylene for different durations before they were transferred for infiltration into two stages of molten paraffin wax for 1 h each in an oven at 65 °C. Subsequently, the tissues were embedded and serial sectioned using a rotary microtome at six microns (6 μ), transferred onto the albumenized slides and dried on a hot plate for 2 min. The resulting slides were dewaxed with xylene and passed through 70% and 50% alcohol, respectively, then through water for 5 min and stained with hematoxylin and eosin.

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Immunohistochemistry (Bax, BCl2, and E-Cadherin)

Immunohistochemical studies were carried out by cutting samples into 7 μm sections, fixing in 10% formalin, dehydrating and embedding in paraffin. To determine the protein expression level of Bax, BCl_2, and E-Cadherin, preparations from the groups were used; a negative control was performed (a slide without primary antibody) with a standard three-step immunohistochemical procedure. The catalog number for Rabbit Bax (A0207), BCl_2 (A0208) and E-Cadherin (A3044) antibodies were used as a primary antibody. Then biotinylated secondary antibody was added, and followed by the HRP conjugated with streptavidin. Since streptavidin has great affinity to biotin, after adding a chromogen (DAB) a reddish color appears which is measured by spectrophotometer.

mRNA Androgen receptor gene expression study using qPCR

The total RNA Mini Kit was used for this study; and the samples were homogenized efficiently using a micropestle in a microcentrifuge tube. Cells are lysed and RNase inactivated with an optional on-column DNase treatment with the aid of chaotropic salt and detergents. RNA in the chaotropic salt is bound by the glass fiber matrix of the spin column.

Agarose gel electrophoresis

Extracted RNA was done on 1% agarose gel electrophoresis. Ethidium bromide was used to stain the gel and visualized under blue-light transilluminator. Approximately, 1% agarose gel and buffer were swirled gently to ensure complete dissolution. The colloidal solution obtained was heated at medium heat to a clear solution in an oven for 3 min and allowed to cool to about 50 °C. About 0.5 μg/mL of ethidium bromide was added to the gel, mixed thoroughly, transferred into the gel tray set with combs and allowed to solidify for about 20 min. The gel was submerged in a running buffer (1× TAE) subsequently, the amplicons were loaded in the wells created by the comb. The genomic RNA extracted from each of the samples was mixed with a 6× loading dye in the ratio of 5 μL of the sample/amplicon to 1 μL of loading buffer. This was combined individually and loaded into the wells.

cDNA synthesis

One microgram of each of the extracted RNA samples was converted to cDNA using the Bioline SensiFAST cDNA synthesis kit (in line with manufacturer's protocol). The reaction contains 1 μL of Reverse Transcriptase and 4 μL of 5× TransAmp Buffer. Nuclease-free water was added to make reaction volume up to 20 μL. The thermocycling conditions were as follows: Annealing at 25 °C for 10 min, reverse transcription at 42 °C for 15 min, an additional 48 °C for 15 min, because of the highly structured RNA and lastly, inactivation of the enzyme at 85 °C for 5 min.

Real-time polymerase chain reaction

The synthesized cDNA was amplified using the Bioer LineGene 9600 Real-Time PCR machine. This was done in 10 μL reactions consisting of 2 μL of the template, 2 μL of the Solis Biodyne 5× qPCR mix, 0.25 μL each of the forward and reverse primers and 5.5 μL of nuclease-free water. This process was carried out for all the samples and a 'no template control' reaction was made in which water was substituted for a template. The qPCR conditions were as follows: initial activation at 95 °C for 12 min, denaturation at 95 °C for 15 s, annealing at 65 °C for 20 s and elongation at 72 °C for 20 s. Same cycling conditions were used for the housekeeping gene βActin, but the annealing temperature was changed to 64 °C. The electrophoresis for total RNA was run at 75 volts for 1 h, after which it was viewed and photographed under blue light transilluminator. Solis biodyne 100 bp DNA ladder was run alongside the extracted RNA samples.

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Data analysis

The results were expressed as Mean ± standard error of mean (SEM), and subjected to statistical analysis using the ANOVA Graph-Pad Prism software version 6, and Tukey post-hoc analysis for data analysis. p-Value < 0.05 was considered significant and $p < 0.01$ indicates highly significant difference while $p > 0.05$ indicate non-significant differences.

Results

Effects of oral administration of quercetin on ovarian MDA level in DHEA-induced PCO in Wistar rats

The results obtained revealed that the Group IV co-administered with quercetin and DHEA caused a significant decrease ($p < 0.05$) in MDA level when compared with the Group II that was administered only DHEA. Group II showed a significant increase ($p < 0.05$) in MDA level when compared with the control, Group I (Figure 1).

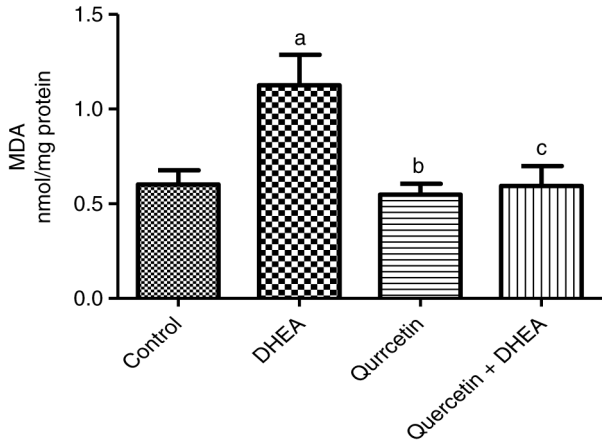


Figure 1: The effects of oral administration of 100 mg/kg quercetin on MDA level in DHEA-induced PCO in Wistar rats. Values are represented in mean ± SEM n = 7, [a]$p < 0.05$ when compared with control value [b]$p < 0.05$ when compared with Group II (DHEA) [c]$p < 0.01$ when compared with Group II (DHEA)

Effects of oral administration of quercetin on ovarian antioxidant levels in DHEA-induced PCO in Wistar rats

The results showed that Group II (DHEA) caused a significant decrease ($p < 0.05$) in the level of antioxidant when compared with control, Group I. Group IV (quercetin and DHEA) revealed an increase in the level of antioxidant as compared with Group II (Table 1).

Table 1: The effects of oral administration of 100 mg/kg quercetin on antioxidant enzymes in DHEA-induced PCO in Wistar rats.

Groups	SOD U/mL/mgprotein	CAT µmole/min/mgprotein	GST µmole/min/mgprotein	GSH mg/mL/mgprotein
I	1.50 ± 0.07	103.3 ± 3.19	0.04 ± 0.01	7.12 ± 0.70
II	1.52 ± 0.04	92.9 ± 0.39[a]	0.06 ± 0.00[a]	6.09 ± 0.48
III	1.62 ± 0.05	106.7 ± 3.11	0.08 ± 0.01	7.22 ± 0.64
IV	1.88 ± 0.10[b]	122.2 ± 6.74[b]	0.09 ± 0.01[b]	7.77 ± 0.74

Values are represented in mean ± SEM, n = 7, [a]$p < 0.05$ when compared with control (Group I) value. [b]$p < 0.05$ when compared with Group II (DHEA). SOD, superoxide dismutase; CAT, catalase; GST, glutathione-S-transferase; GSH, reduced glutathione.

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Effects of oral administration of quercetin on serum estrogen and progesterone levels in DHEA-induced PCO in Wistar rats

The results obtained showed that Group II (DHEA) caused a significant decrease ($p < 0.05$) in the level of progesterone and significant increase ($p < 0.05$) in estrogen levels as compared with control (Group I). Group IV (quercetin and DHEA) caused a significant increase ($p < 0.05$) in the level of progesterone when compared with Group II (Figure 2).

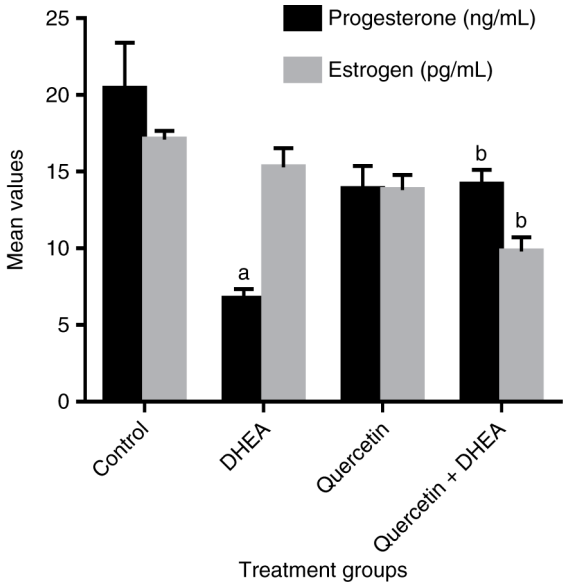


Figure 2: The effects of oral administration of 100 mg/kg quercetin on progesterone and estrogen level in DHEA-induced PCO in Wistar rats.
Values are represented in mean ± SEM, n = 7, [a]$p < 0.05$ when compared with control value and [b]$p < 0.05$ when compared with Group II (DHEA)

Effects of oral administration of quercetin on ovarian proton pump ATPase enzymes activity (Na^+/K^+, Ca^{2+} and H^+ ATPase) levels in DHEA-induced PCO in Wistar rats

The results obtained showed that Group IV (quercetin and DHEA) caused a significant increase ($p < 0.05$) in proton pump enzymes activity when compared with Group II (DHEA).Also, Group II (DHEA) showed a reduction in the activity of these enzymes as compared with the control, Group I (Table 2).

Table 2: The effects of oral administration of 100 mg/kg quercetin on ATPase enzymes activity in DHEA-induced PCO in Wistar rats.

Groups	Na^+/K^+ ATPase	Ca^{2+} ATPase	H^+ ATPase ($\pi\mu$mol/mgprotein/hr/10^{-3})
I	0.49 ± 0.04	4.72 ± 0.19	0.44 ± 0.00
II	0.41 ± 0.01	4.02 ± 0.06[a]	0.42 ± 0.01[a]
III	0.46 ± 0.02	4.61 ± 0.15	0.45 ± 0.15
IV	0.53 ± 0.03[b]	5.84 ± 0.25[b]	0.51 ± 0.03[b]

Values are represented in mean ± SEM, n = 7, [a]$p < 0.05$ when compared with control (Group I) value [b]$p < 0.05$ when compared with Group II (DHEA).

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Effects of oral administration of quercetin on ovarian inflammatory cytokines (TNF-α and VEGF) levels in DHEA-induced PCO in Wistar rats

The results showed that Group II (DHEA) caused a significant increase ($p < 0.05$) in TNF-α and VEGF levels when compared with control group. However, Group IV (quercetin and DHEA) caused a significant decrease ($p < 0.05$) in inflammatory cytokines level when compared with Group II (DHEA) (Figure 3).

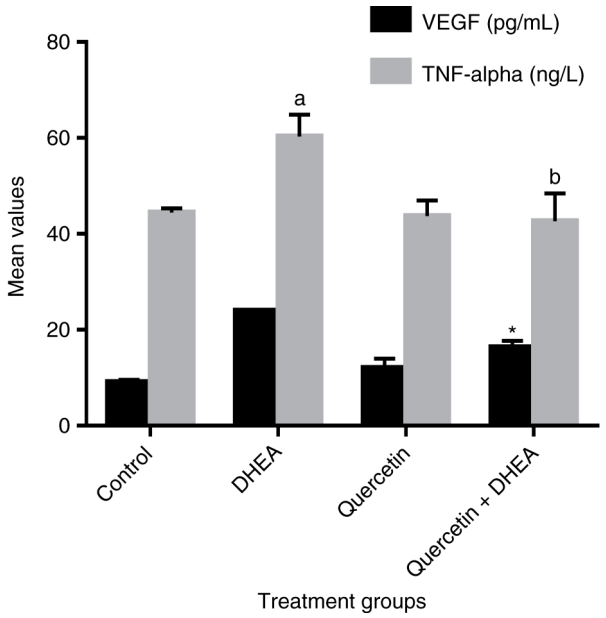


Figure 3: The effects of oral administration of 100 mg/kg quercetin on VEGF and TNF-α level in DHEA-induced PCO in Wistar rats.
Values are represented in mean ± SEM, n = 7, [a]$p < 0.05$ when compared with control value and [b]$p < 0.05$ when compared with Group II (DHEA)

Effects of oral administration of quercetin on the granulosa cell mRNA androgen receptor gene expression levels in DHEA-induced PCO in Wistar rats

The results obtained showed that DHEA treated group (Group II) showed a significant expression ($p < 0.05$) in mRNA androgen receptor gene in the ovarian granulosa cells when compared to the control group (Group I). However, Group IV (quercetin and DHEA) caused a significant decrease ($p < 0.05$) in mRNA androgen receptor gene expression as compared to Group II (DHEA) (Figure 4 and Figure 5, Table 3).

Table 3: List of primers and probe sequences for the gene expression.

AR	
RNAR F	ATGCTGGGCCTGTAGCCCCCT
RNAR R	CAGGCAGGTCTTCTGGGGTGGG
B-Actin	
RNACTB F	CCTCCGTCGCCGGTCCACACC
RNACTB R	TCTTGCTCTGGGCCTCGTCGC

F, forward primer; R, reverse primer.

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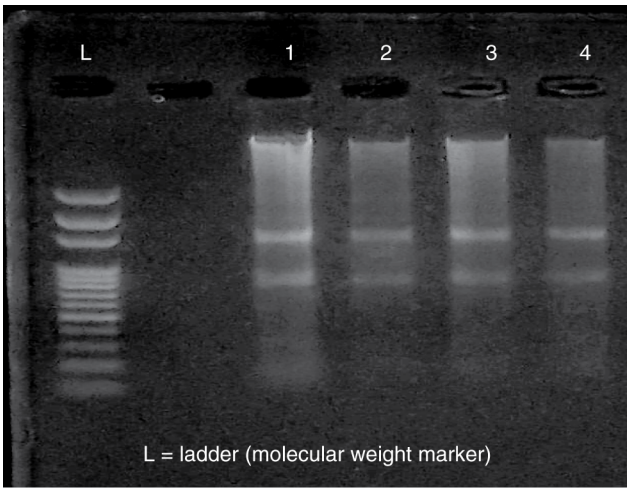


Figure 4: Gel image for total RNA.

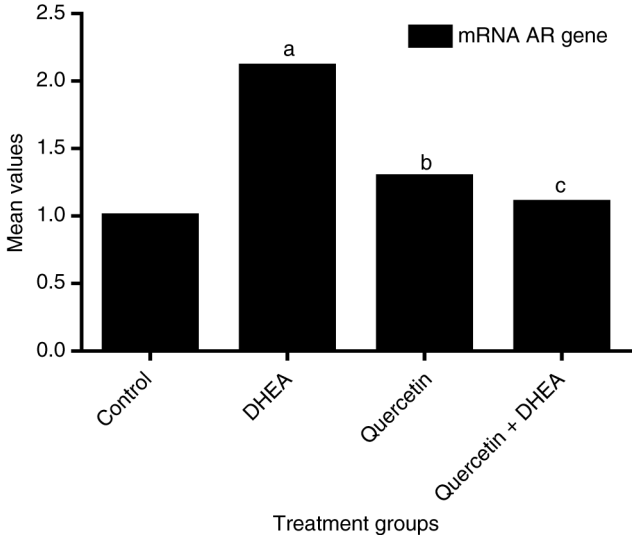


Figure 5: mRNA androgen receptor gene fold change values (2ˆ-ddCt) for quercetin and DHEA treatment. Values are represented in mean ± SEM, n = 7, [a]p < 0.05 when compared with controlvalue, [b]p < 0.05 when compared with Group II (DHEA) and [c]p < 0.01 when compared with Group II (DHEA).

Discussion

PCO remains the major endocrine disorders in women causing an ovulatory infertility [30]. It has been linked with endometrial, cervical, and ovarian cancer [16]; [31]. OS induction in PCO is linked to cancer pathogenesis [32]. ROS could cause genetic disorders by destroying nuclear integrity, causing chromosomal damage with impairment in the repair mechanism. Consequently, alterations in protooncogenesis and cancer suppressor genes then hijack cell multiplying out of control [33]. Also, OS could cause changes by DNA methylation, silencing tumor suppressor genes [34]; [35]. The most significant mechanisms and series of events by which free radicals impede with cellular physiology are by lipid peroxidation leading cellular damage. To protect this cellular damage, organisms have developed antioxidant defense mechanisms [32]. This is not sufficient in the face of excessive production of ROS, therefore the need arises to supplement with endogenous antioxidant.

Each cell is under constant surveillance to maintain the integrity of its genome. Genomic lesions in a cell must be repaired before the onset of DNA replication and cell division. Apoptosis and progression are two intimately linked phenomena. Uncontrollable cell proliferation perturbs the cellular homeostasis and this can lead to malignancies as well as organ dysfunction with developmental abnormalities. The biological pathways controlling cell fate is sequentially organized at the molecular level. An oncogene-derived protein, Bcl_2 confers negative control in the pathway of cellular suicide machinery. A Bcl_2–homologous protein, Bax promotes cell death by competing with Bcl_2. While Bax-Bax homodimers act as apoptosis inducers, Bcl_2–Bax heterodimer formation evokes a survival signal for the cells as seen in Figure 4–Figure 11.

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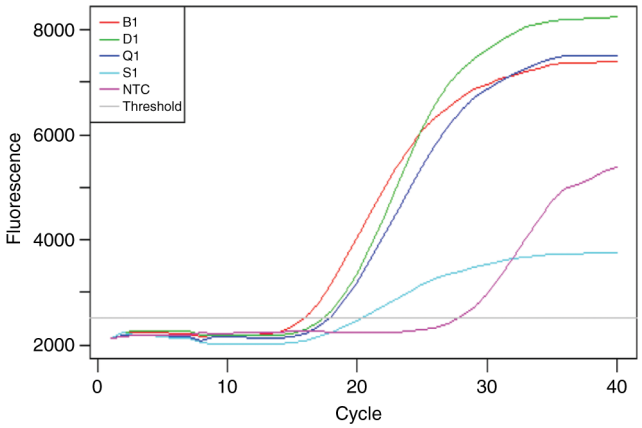


Figure 6: Amplification curve for quercetin treatment (Androgen receptor).

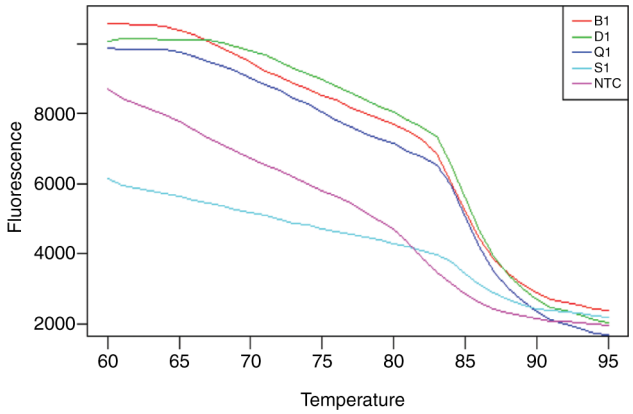
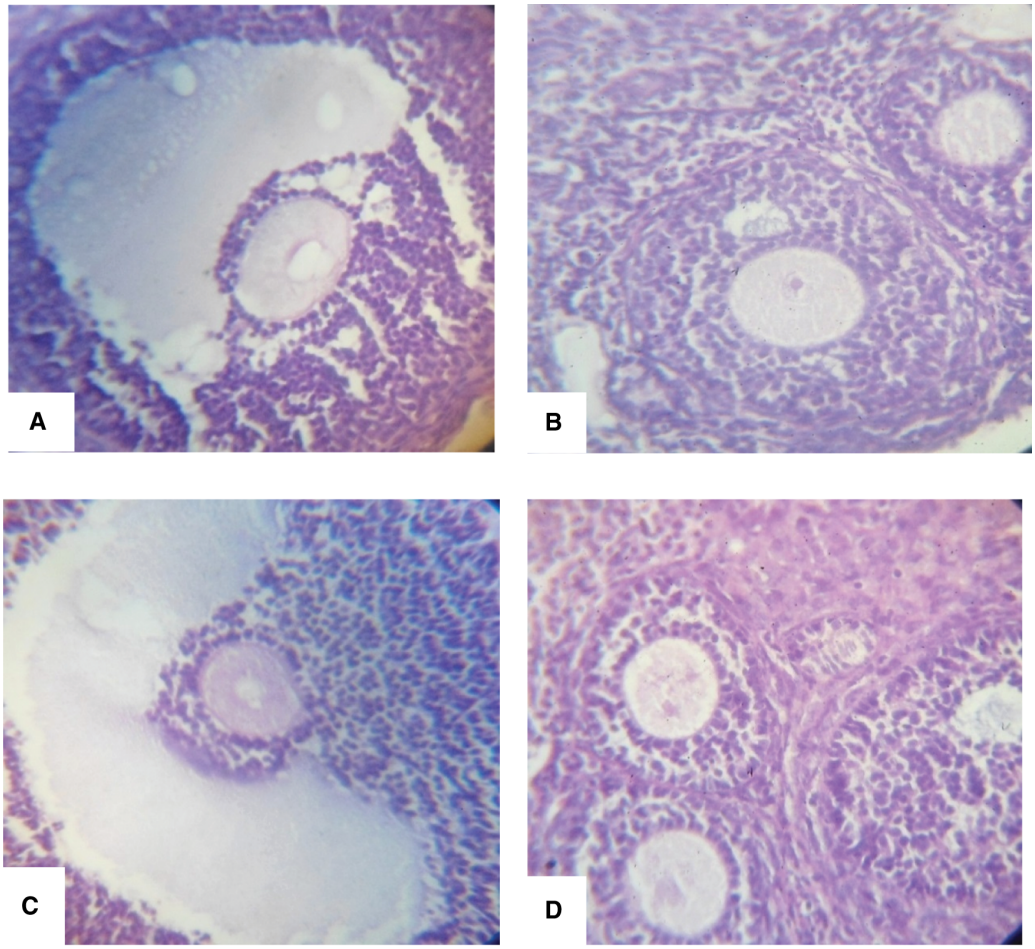


Figure 7: Melt curve for quercetin treatment (Androgen receptor).



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Figure 8: A–D represents histological sections of ovaries from DHEA exposed rats and control following 15 days treatment using haematoxylin and eosin stain. ×40 magnifications.
(A) Photomicrograph section of ovary from control group (Group I) showing normal follicle of different stages with well-organized surface epithelium but without cystic follicle. The stromal cells of the ovarian follicles and corpus luteum are also well developed. (B) Photomicrograph section of ovary from DHEA treated rats (Group II) showing an elevated number of cystic follicles with a thicker theca cell layer and a marked higher level of collagen especially in region around the follicle. (C) Photomicrograph section of ovary from quercetin exposed rats showing normal histoarchitecture of the ovary. The interstitial tissues are also normal. (D) Photomicrograph section of ovary from quercetin and DHEA exposed rats (Group III) showing a decrease in the level of cystic follicles with a thinner theca cell layer and a reduced level of collagen especially in region around the follicle.

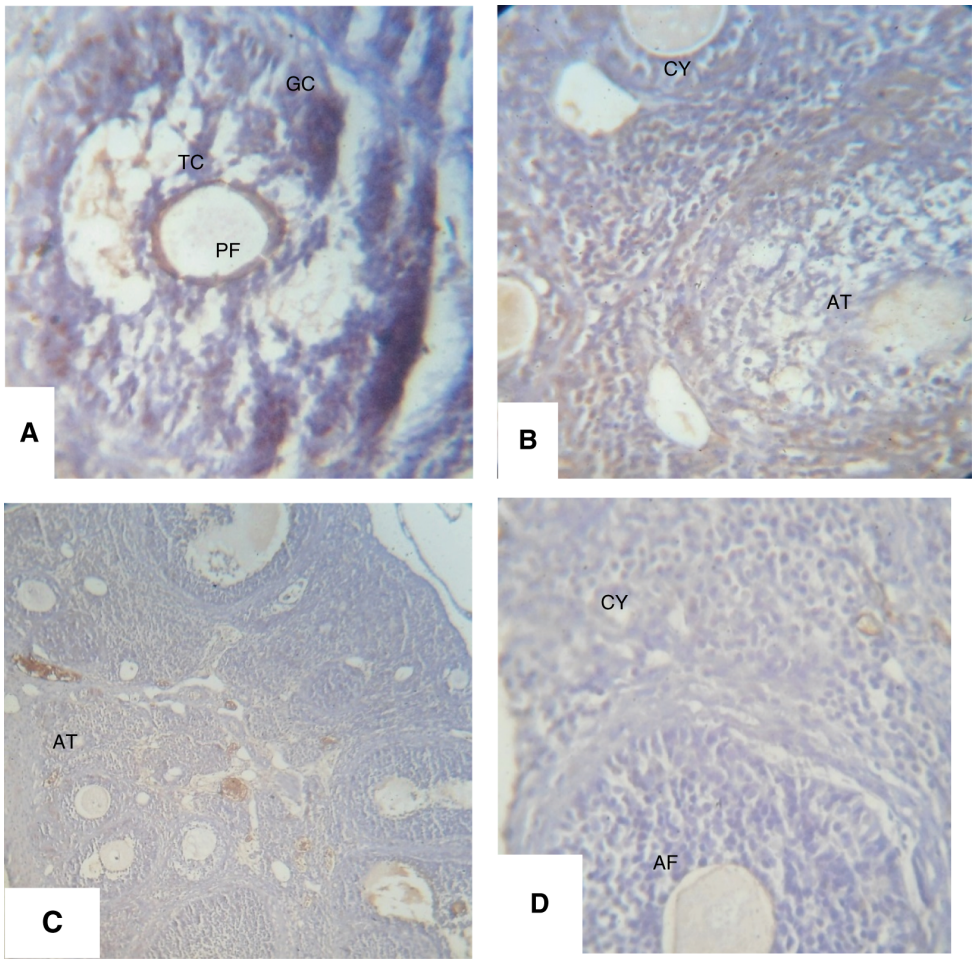


Figure 9: A–D represents immunohistochemistry of *Bax* apoptotic protein staining of ovaries from Group II (DHEA) rats and control following 15 days treatment. X40 magnifications.
(A) Photomicrograph section of ovary from control group (Group I) showing normal preantral follicles (PF), granulosa cells (GC), and theca cells (TC). Bax expression is mainly localized in granulosa cells of antral follicles. (B) Photomicrograph section of ovary from Group II DHEA exposed rats showing an increasing number of cystic follicles (CY), presences of atretic follicle (AT) and Bax expression is greater in preantral and antral follicles. (C) Photomicrography section of ovary from quercetin exposed rats, Group III showing normal preantral, antral follicle and stroma (ST). The granulosa cells and oocytes (Oo) are also normal. (D) Photomicrography section of ovary from quercetin and DHEA exposed rats (Group IV) showing a decrease in amount of cystic follicles (CY). Bax expression is decreased in preantral and antral follicles (AF).

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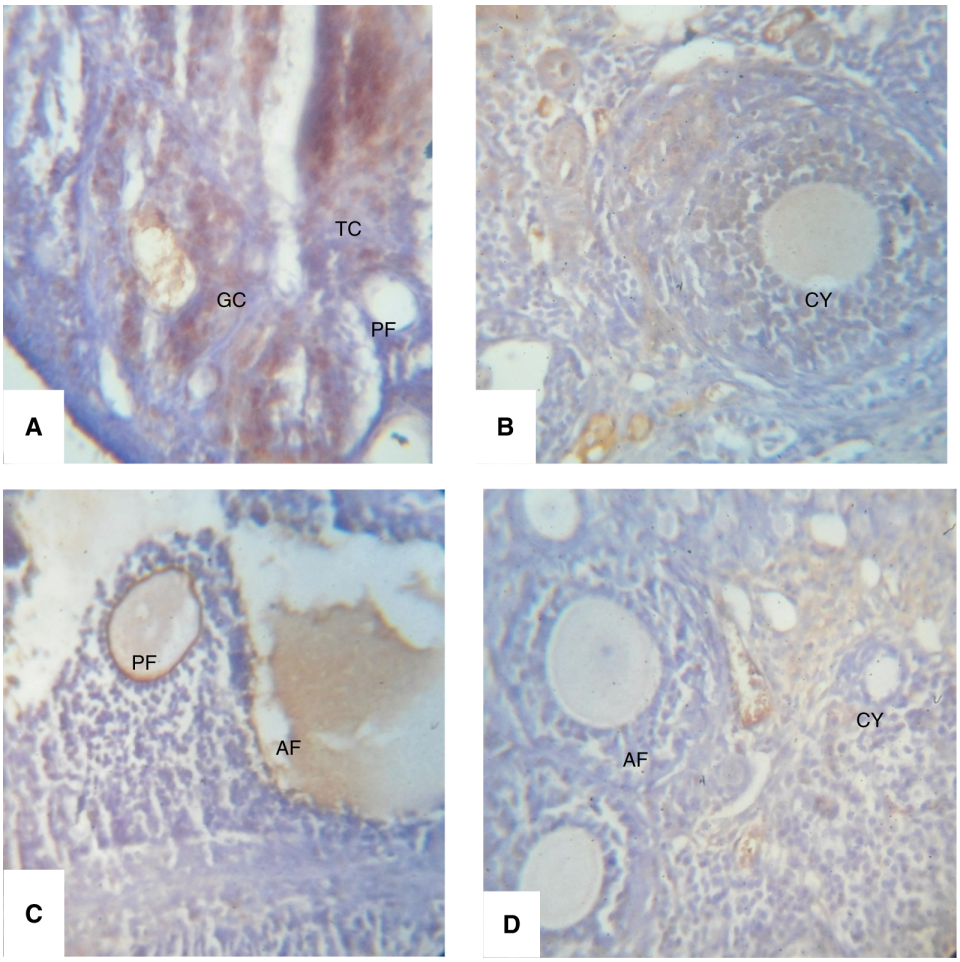


Figure 10: A–D represents immunohistochemistry of Bcl-2 anti-apoptotic protein staining of ovaries from Group II DHEA treated rats and control following 15 days treatment. X40 magnifications.
(A) Photomicrograph section of ovary from control group (Group I) showing normal preantral follicles (PF), granulosa cells (GC) and theca cells (TC) with Bcl-2 expressions are mainly localized in granulosa cells of antral follicles. (B) Photomicrograph section of ovary from Group II, DHEA treated rats showing an increasing amount of cystic follicles. Bcl-2 protein was either absent in preantral follicles or weakly expressed in antral follicles. (C) Photomicrography section of ovary from Group IIIquercetin treated rats showing normal preantral and antral follicle with Bcl-2 expression mainly localized in granulosa cells of antral follicles. (D) Photomicrography section of ovary from Group IV, quercetin and DHEA treated rats showing a decrease in amount of cystic follicles. Bcl-2 expression was decreased in preantral and antral follicles (AF).

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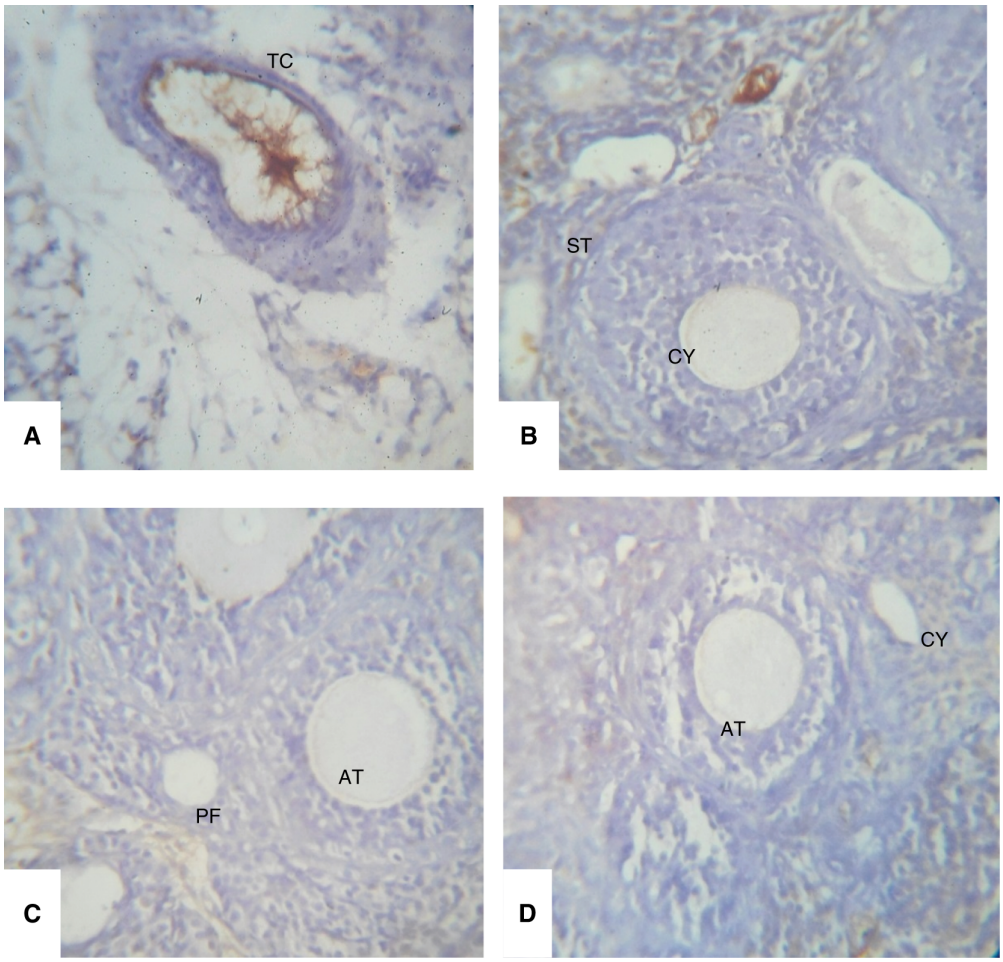


Figure 11: A–D represents immunohistochemistry of *E- Cadherin* (adhesion molecule) staining of ovaries from DHEA (Group II) treated rats and control following 15 days treatment. X40 magnifications.
(A) Photomicrograph section of ovary from control group showing normal expression of E-cadherin which was largely confined to areas of the interstitium, theca, and surface epithelium. (B) Photomicrograph section of ovary from DHEA exposed Group II rats showing an increasing amount of cystic follicles (CY). Expression of E-Cadherin which was largely confined to areas of the stroma indicates the reduction in the growth of the follicle. (C) Photomicrography section of ovary from quercetin exposed Group III rats showing normal preantral and antral follicle. The granulosa cells are also normal with well-expressed E-Cadherin indicating the growth and development of the follicles. (D) Photomicrography section of ovary from quercetin and DHEA treated Group IV rats showing a decrease in quantity of cystic follicles. Expression of E-Cadherin was decreased in preatral and antral follicle, but well expressed in the granulosa cells.

OS reflects dysfunction between synthesis and inhibition of reactive oxygen/nitrogen species [36]. ROS accumulated in-vivo would induce cell [37], protein [38] and lipid damage [39]. In this study, Group II rats treated only with DHEA, revealed a significant increase ($p < 0.05$) in MDA level compared to the control, Group I. This may be due to increased production of ROS and lipid peroxidation [40]. Group IV co-administered with quercetin and DHEA showed a significant decrease ($p < 0.05$) in MDA level compared to Group II (DHEA). This is in line with work done by Sakanashi et al. [41] who revealed that quercetin possesses antioxidant properties. As shown in Table 1, Group II demonstrated a significant decrease ($p < 0.05$) in the antioxidant enzymes activity. Group IV revealed a significant increase ($p < 0.05$) in antioxidant enzyme activities. Pharmacological intervention and the use of antioxidants are considered treatments for PCOS to normalize hyperandrogenism and anovulation. There are no studies to our knowledge that have evaluated the effects of quercetin on mRNA androgen receptor gene expression in rats with PCOS.

Quercetin is one of the most potent polyphenols known to protect the body against oxidative damage during physiological oxygen breakdown induced by exogenous damage [14]. Chronic inflammation is considered as an important aspect of the cystic ovary, suggested being involved in pathology and development of PCO [42]. Inflammatory markers such as TNF, interleukin-6 (IL-6) rise in patients with PCOS [42]. A tight link exists between oxidative damage and inflammatory processes making it difficult to separate between inflammation and OS [43]. OS could cause the releasing inflammatory markers and inflammatory reaction as shown in (Figure 3), through the stimulation of signaling pathways of nuclear factor-Kappa B, activated protein 1, and hypoxia-inducible factor 1 [44]. Results obtained showed that Group II (DHEA) caused a significant increase ($p < 0.05$)

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in the inflammatory cytokine markers VEGF and TNF- α levels as compared with control group. This is in agreement with the increase in ovarian VEGF levels in PCOS patients, supporting that DHEA model used in this study to evaluate changes in angiogenesis in PCOS [45], [46]. Quercetin co-administered with DHEA that is Group IV, revealed a significant decrease ($p < 0.05$) in VEGF and TNF- α levels compared with Group II. Oxidative damage and inflammation appear to cause an increase in androgen levels in PCOS by; enhancing the activation of steroidogenesis in the ovarian, stimulating androgen production and antioxidant [47].

TNF- α is linked with inflammation, shown to possess the capacity to support the proliferation of mesenchymal cells of follicular tissue. DNA damage and methylation caused by OS play an important role in the early phase of malignant tumor pathogenesis and conversion through the activation of protooncogene and silencing antioncogene. Therefore, oxidative damage in PCOS could be linked to genetic instability and increase the risk of cancers formation. OS has been shown to be accompanied by inflammation, obesity, and hyperandrogenemia, which are the common characteristics and potential risk for PCO and cancer. TNF-α is involved in the regulation of ovarian function, exerting an influence on proliferation, differentiation, follicle maturation, steroidogenesis and apoptosis. TNF-α is expressed by the oocyte, thecal cells, granulosa cells, and the corpus luteum in the ovary. Alterations in TNF-α levels are correlated to poor oocyte quality. Increased concentrations of TNF-α in women with PCOS are also significantly inverse correlated to ovarian concentrations of estrogen (E2), which are indicative of poor oocyte quality.

Oxygen free radicals or ROS (ROS) are involved in many physiological functions where they act as mediators in a variety of signal transduction pathways. An excess of these substances can cause cellular damage which can cause apoptosis. In women with PCOS, elevated levels of ROS in the ovary and reduced antioxidant capacity are closely associated with reduced oocyte quality and ovulatory dysfunctions. Also decreased in mitochondrial O_2 consumption and glutathione (GSH) levels, along with increased ROS production, explains the observed apoptotic induction seen in PCOS group. The ovarian cells of women with PCOS are increased in this inflammatory state, which occurs more often in response to increased TNF α. Physiological hyperandrogenism generates increased levels of ROS from the ovarian cells, which then activate the release of TNF-α and increase inflammatory transcription factor NF-kappa B. As a result, the concentrations of TNF-α, a known mediator of inflammation and apoptosis, are further increased. The resulting OS creates an inflammatory environment that promotes damage and contributes to hyperandrogenism.

Quercetin is a natural antioxidant; with anti-cancer activities that have been proved by many in-vivo studies, thereby making it a potential anti-tumor agent. Other studies have shown that quercetin plays a significant role in the inhibition of endometrial, prostate, ovary, and lung tumor cancer cells. Quercetin is a potent antioxidant; because it is a good free radical scavenger and transition metal chelating agent, hence a strong lipid peroxide inhibitor and an anti inflammatory agent. This explains the ability of quercetin to stimulate transcription factors that produce pro-inflammatory cytokines, which are often found in patients suffering from inflammatory conditions. TNF-α is a pro-inflammatory marker linked with the pathophysiology of inflammatory diseases and response associated with OS damage [48]. Our observations on inflammatory cytokines also provide additional new evidence highlighting the critical relationship between inflammatory pathways and androgen receptor gene expression in the granulosa cells of the ovary of Wistar rats treated with DHEA as shown in the results obtained in Figure 3 and Figure 5).

Quercetin caused significant inhibition of TNF-α synthesis and androgen receptor gene expression of the granulosa cell in the ovary of Wistar rats treated with DHEA. This is in line with Taylor et al. [49]. Reduction in the TNF-α synthesis in combination with quercetin signifies that flavonoids possess the ability to regulate the immune reaction with potential anti-inflammatory response. Quercetin caused the decreased production of TNF-α resulting in the activation of anti-inflammatory markers through the in stimulation of NF-KB. Persistent exposure to estrogen in the absence of adequate progesterone, which could be induced by an anovulatory response, is known as a contributory feature to hyperplasia and cancer pathophysiology in PCO [50]. Estrogens binds with the nuclear receptor, thereby stimulating the production of different growth factors such as insulin-like growth factor, epidermal growth factor, and vascular endothelial growth factor and activate extracellular signal-regulated kinases signaling process to stimulate endometrial and ovarian proliferation and cancer [51], [52]. Furthermore, metabolites of estrogen can induce ovarian and endometrial cancer through binding with DNA thereby causing more DNA damage which is linked with OS damage. In OS damage, estrogen intermediate metabolites, including 2- hydroxyl estrone (2- OHEI), 4- hydroxyl estrone (4- OHEI) and 16 α- hydroxyl estrone (16 α OHEI) could not be methylated and removed. This is then oxidized to semiquinones and quinones compounds [51]. These abnormal metabolites of estrogen with very high electron affinity bind to the nucleophilic group of DNA through covalent bond resulting in DNA mutation and cause cancer formation. DHEA treated group (Group II) showed a significant decrease ($p < 0.05$) in progesterone level and a significant increase ($p < 0.05$) in estrogen levels when compared to the control group (Group I) while the group co-administered with quercetin and DHEA (Group IV) produced a significant elevation ($p < 0.05$) in progesterone level compared to Group II. Likewise, a significant reduction ($p < 0.05$) in estrogen level was observed in Group IV as

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compared with the Group II. This could be due to the anti-estrogenic effect of quercetin on the ovary. Androgens possessing a central position in PCOS, are closely related to the ovarian morphology, and are sufficient to cause PCOS-like states in both female animal models and women. The most evident neuroendocrine feature, regulating abnormal ovarian follicle development in PCOS is due to increased luteinizing hormone (LH) pulsatility regarding both frequency and amplitude, with relatively low follicle stimulating hormone (FSH) secretion. Increased LH pulse frequency increases theca cell production of androgens, while the lower FSH levels impair follicle maturation and consequently ovulation. The cause of LH hypersecretion in PCOS is probably due to enhanced pituitary sensitivity to gonadotropin releasing hormone (GnRH) or to changes in GnRH secretion patterns rather than increased GnRH secretion. It appears to be a result of an acquired impaired sensitivity of the hypothalamic pulse generator to the negative feedback of estrogen and progesterone in PCOS, possibly by chronic estrogen exposure. Levels of FSH in PCOS appear to be low or within the lower follicular range, and response to GnRH is relatively similar to ovulatory controls. Altered sex steroid production, metabolic dysfunction, and obesity may all contribute to the changes in LH secretion pattern. Hyperandrogenemia itself may cause hypothalamic desensitization to progesterone/estrogen negative feedback that further increase gonadotropin secretion and hence ovarian androgen production, causing a self-driven viscous circle.

Also the biosynthesis of androgens is mediated by microsomal P450c17 which catalyses 17–20 lyase activity. Alterations in P450c17 at transcriptional and post-transcriptional level have been implicated in the aetiology of PCOS. These women show, indeed, relative inhibition of 17–20 lyase activity with respect to 17-hyroxylase, leading to an increased 17OHP/A ratio. Administration of GnRH or hCG in women with PCOS causes excessive production of 17OHP. Low aromatase activity has also been demonstrated in women with PCOS. Aromatase is a granulosa cell enzyme that converts androgens into estrogens. It may be partly responsible for hyperandrogenism in this syndrome. Quercetin as a chief member of flavonoid protects cellular injury directly by scavenging free radicals and prevents from atherosclerosis by inhibiting low density lipid (LDL) from oxidation. Moreover, it has decreased cholesterol and increased high density lipids (HDL-C) levels. Quercetin contains large amounts of certain phytoestrogens that has anti-androgenic property, which causes decrease in androgens levels in patients or animals with PCOS by inhibiting NF-kB pathway, and improved ovarian tissue symptoms including the number of developing follicles, granulosa layer thickness, and the number of corpus luteum through antioxidant and anti-inflammatory properties. Also quercetin may cause increase in aromatase activity, and decrease in testosterone through aromatizing testosterone and converting it into estradiol as seen in the results.

Calmodulin is known to transport calcium ion across membranes and also causing the initiation of numerous physiological cellular process. Quercetin appears to regulate calmodulin/calcium activity via this pathway, and functions at a cell membrane with a membrane stabilizing action [53]. Results from several studies have implicated decreased proton pump (ATPases) activity in female reproductive dysfunction. OS is a contributory feature to impaired ATPase activity [54] which has been found to be susceptible in the presence of free radicals and lipid peroxides [55] causing membrane lipid peroxidation and inflammation. Further, lipid peroxidation has been found to alter magnesium ATPase, calcium ATPase and Na^+/K^+-ATPase functions by alternating specific active sites [56]. ROS deplete glutathione and other protective antioxidants which may greatly influence and contribute to the increased levels of reactive species, hence, accounting on the diminished activity of Na^+/K^+-ATPase [57]. Calcium/calmodulin-dependent membrane enzyme such as ATPases (Na^+/K^+, Ca^{2+}, and H^+ ATPase) are modulated by quercetin whereby phospholipases regulates membrane permeability. Proton pumps maintain trans-membrane gradients for ions and generate a driving force for the secondary transport of metabolic substrates such as amino acids and glucose.

Na^+/K^+, Ca^{2+}, and H^+ ATPases are responsible for proper cellular function and for preserving the ionic gradient across the cell membrane, membrane potential, and osmotic equilibrium, thus permitting the transportation of Na^+, K^+, Ca^{2+}, Mg^{2+} ions through the membrane [58]. The group co-administered with quercetin and DHEA revealed a significant rise ($p < 0.05$) in proton pump enzyme activity as compared with the DHEA group. Our findings demonstrate, for the first time, a functional link between quercetin and membrane-bound ATPases. The modulation of the activity of the three membrane-bound ATPases by the quercetin seen in this study suggests vital roles for the antioxidant in the maintenance of ovarian functions via antioxidant and hormonal regulatory pathways. To date, no single medication exist for PCOS, it tailors according to the symptoms [27]. In PCOS positive rats, alterations in the oxidant-antioxidant profile have been observed. However, the role of OS in the pathogenesis of PCOS can never be neglected, because of its involvement in metabolic disorders like infertility, hormonal imbalance and metabolic dysfunction. It causes hyperplasia in ovarian mesenchyme that further contributes to pre-eclampsia, endometriosis, abortion, PCOS, lessened fertility, and dysgenesis. Currently, plant extracts are being widely used to treat female reproductive dysfunction. Flavonoids are plant based compounds which are of great interest due to their expansive pharmacological activities. Quercetin is one among the most commonly consumed dietary flavonoids with an average of 25–50 mg/day. It belongs to a set of plant based non-steroidal compounds known as phyto-estrogen. Quercetin is found to have antioxi-

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dant potential, organ protection, anticancer activity, anti-diabetic, anti-inflammatory effects, and restoration of bone loss in post-menopausal women. It protects LDL from oxidation, lipid peroxidation and avoids redox disproportion in cells.

Conclusions

Quercetin alleviated DHEA-induced PCO in Wistar rats. These effects could be ascribed to the antioxidant, anti-inflammatory, and hormonal modulatory mechanisms.

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Ethical approval: The ethical approval on the animal act right was obtained from the Institutional Animal Care Committee of the same Institution. The experimental procedures were carried out in accordance with Institutional Animal Ethics Committee (IAEC) guidelines.

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