ORIGINAL RESEARCH

Cauliflower (*Brassicae oleracea*) extract restores hormonal imbalance, resumes follicular maturation, and down regulates oxidative stress in oestradiol valerate-induced polycystic ovary syndrome in Sprague-Dawley rats

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ABSTRACT

Background: Polycystic ovary syndrome is a common cause of anovulatory infertility.
Objectives: This study investigated the ameliorative effect of ethanolic extract of cauliflower in oestradiol valerate-induced polycystic ovary syndrome in Sprague-Dawley rats.
Method: Twenty five female rats weighing between 120-160 g were used. They were grouped into five (Groups 1-5; N=5). Polycystic ovary syndrome was induced using oestradiol valerate (16 mg). Groups1and 2 served as negative and positive control respectively, groups 3-5 received cauliflower (200, 500 and 1025 mg/kg body weight) for four weeks. Animals were sacrificed; blood and ovarian tissues were collected for biochemical analysis and histology.
Results: The relative weight of the ovary was increased significantly in all the groups compared to the control. This significantly reduced when high dose cauliflower group was compared to EV-group. MDA and SOD levels increased significantly in EV-only groups compared to control. Treatment with cauliflower successfully reduced MDA and SOD levels in comparison to EV-only groups. On the other hand, GSH and catalase did not show any significant differences when the treatment groups were compared to control. FSH and progesterone reduced significantly while LH, testosterone, and oestrogen levels increased significantly in EV-only groups compared to control. Treatment with cauliflower was able to ameliorate these effects. Histological sections showed cystic follicles in EV-only treated group compared to control. Treatment with cauliflower extract in increasing doses was able to rescue growing follicles from cystic formation.
Conclusion: Cauliflower extract restores hormonal imbalance, recues follicles from cystic formation, and combats oxidative stress in oestradiol valerate-induced polycystic ovary syndrome in Sprague-Dawley rats.

Keywords: Oestradiol Valerate; Polycystic Ovarian Syndrome; Cauliflower; Ovary; Hormones

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is one of the most frequent endocrine diseases that leads to infertility in women of reproductive age[1]. It is a disorder with a high heterogeneity, and its clinical features mainly include hyperandrogenism, anovulation, presence of

multiple ovarian cysts, irregularities in the menstrual cycle, and variable levels of gonadotropins[2,3]. Imbalance between pro-oxidant molecules and antioxidant defensive system may result in PCOS; hence, antioxidants can play an important role in reducing infertility associated with this disease[4]. Features of PCOS may manifest at any age, ranging from childhood, teenage years, early adulthood, and middle life. Depending on the phenotypic and physiological characteristics that are to be investigated, a number of experimental models have been proposed to induce PCOS in neonatal, prepubertal, or adult rats. These models include steroidal and nonsteroidal drug administration (dehydroepiandrosterone, letrozole, dihydrotestosterone, and oestradiol valerate (EV)-administration)[5,6].

Oestradiol valerate (EV) is a long-acting oestrogen and on administration to experimental rodents, causes hypothalamic-pituitary dysregulation of gonadotropin releasing hormone, resulting in improper release and storage of luteinizing hormone[7]. This ultimately results in a rapid appearance of the polycystic ovarian condition due to its disturbances in metabolic and physiologic processes[8]. To study the relationship between the PCOS and how it affects infertility, the most commonly used PCOS model is generated by a single injection of EV in prepubertal rats, which results in a polycystic ovary morphology, irregular oestrous cycles[9,10], alterations in basal and pulsatile luteinizing hormone concentrations and follicle stimulating hormone concentrations and an increased androgen response to human chorionic gonadotropin stimulation[11]. The ovaries of rats injected with EV was said to show increased neural sympathetic activity[11.12]. This increase was said to be due to changes in the homeostasis of ovarian catecholamines that begin before the development of cysts and persist after their formation[11].

Considerable attention has been placed on edible plants in recent years, particularly those that are abundant in phytochemicals, or secondary metabolites. As a result, there is growing interest in the antioxidant properties of such dietary phytochemicals[13]. The cruciferous veggies like broccoli, cabbage and cauliflower are superb sources of phytochemicals which also includes glucosinolates and their by-products, phenolics, antioxidant, vitamins and dietary minerals[14,15]. Cauliflower (*Brassica oleracea*) have been said to have multiple pharmacological activities including phytoestrogenic, anti-inflammatory, antimicrobial, antidiabetic and hypo-lipidemic effects in addition to its anti-cancer properties[16-18]. It is also reported to contain major antioxidants such as vitamin C, vitamin E, carotenoids and polyphenols, especially flavonoids, which all provide protection against free radicals which can damage both sperm and egg cells, enzyme regulation, apoptosis control, and the cell cycle[19,20]. Broccoli with its rich antioxidant properties have been reported to modulate gonadotropin levels and hyper-androgenemia, improve ovarian morphology, and down-regulate oxidative stress in PCOS-induced rat model[21]. There is a dearth of literature on the effect of cauliflower on PCOS. Hence, this study was carried out to investigate the ameliorative effect of cauliflower extract on EV-induced PCOS in Sprague-Dawley rat.

MATERIALS AND METHODS

Animals

A total of 25 young adult virgin Sprague-Dawley rats weighing between 120-160 g were used for this experiment. They were procured from the Animal House of the College of Medicine, University of Lagos, and were kept in wire meshed cages in the department of Anatomy, University of Lagos. They were divided into 5 groups (Group 1-5) of five animals per group. The animals were fed with standard rat chow procured from Animal care, Mushin, Lagos State, Nigeria, and were allowed free access to tap water. They were maintained under 12 hours light/12 hours darkness and were allowed two weeks to acclimatize to the prevailing laboratory conditions. Body weight changes of the

animals were taken weekly throughout the study. All protocols regarding the care and use of experimental animals and tissue collection were observed and approved by the Health Research Ethics Committee of the College of Medicine University of Lagos, Nigeria.

Drug and induction of polycystic ovarian syndrome

Oestradiol valerate (EV) tablets (2 mg), manufactured by Zydus Healthcare through Bayer Zydus Pharma Pvt Ltd. Kolshet Road, Thane, India, with Batch no: ZHP3659 were purchased from a registered Pharmacy in Lagos, Nigeria. The tablets were crushed and dissolved in distilled water. PCOS was induced by a previously described method[16]; a single intramuscular injection of EV was given at a dose of 16 mg/kg.

Preparation of cauliflower extract

Fresh cauliflower was purchased from a local market in Lagos, Nigeria. The plants were authenticated at the department of Botany, University of Lagos, Nigeria, and were kept in the Herbarium with voucher number (LUH: 8507). The curds were separated from the stem leaves; twenty bunches of the curd were shredded into small pieces and were freeze dried at -40°C, and thereafter grounded into powder. Cauliflower powder weighing 500 g was placed in a round bottom flask attached with a condenser and extracted with 70% ethanol at 70ºC. The extraction was repeated with 2 L of 70% ethanol and the solvent was collected and filtered. The solvent was condensed in a rotary evaporator at 55ºC, and then placed in an oven till dryness was achieved. The extract was dissolved in distilled water to obtain the administered doses for this study (200, 500 and 1025 mg/kg body weight). The choice of dosage selection was based on previous works[17,18].

Experimental design

Twenty five Sprague-Dawley rats (n=5 per group) were randomly divided into five main groups and cauliflower extract was given orally using oro-gastric cannula between 8-10 a.m. daily for a period of 4 weeks.

Group 1 received distilled water as control
Group 2 received 16 mg/kg of EV alone
Group 3 received 16 mg/kg of EV and 200 mg/kg of cauliflower (CF) (low dose)
Group 4 received 16 mg/kg of EV and 500 mg/kg of CF (medium dose)
Group 5 received 16 mg/kg of EV and 1025 mg/kg of CF (high dose)

Animal sacrifice and sample collection

At the end of the experiments, animals were sacrificed through cervical dislocation, and the abdomen was exposed. Blood was collected from the apex of the heart via cardiac puncture for serum and hormonal analysis; thereafter ovaries were excised, trimmed of fat, and weighed. The ovaries were used for both histological and biochemical analysis.

Preparation of ovary tissue samples for histological assessment

Routine histological processing using Haematoxylin and Eosin (H & E) staining method was carried out. The ovaries were fixed in 10% paraformaldehyde and dehydrated by passing through ascending grades of alcohol. They were cleared in xylene and were dipped quickly into molten paraffin wax before finally embedded in molten paraffin wax in order to form hard blocks. The hard block containing the tissue was then sectioned using the rotary microtome at 5 μm thickness. The sections were then floated in water bath at 400ºC and transferred to a glass slide and stained with H & E stains. All stained ovary sections were examined under a LEICA research microscope (Leica DM 3000, Switzerland) connected to a digital camera (Leica ICC50). Digital photomicrographs of stained sections of the ovarian structures were taken at various magnifications (x40 and x100).

Preparation of ovary tissue samples for biochemical analysis

The ovarian tissues were homogenized with 0.1 M phosphate buffer (pH 7.2), laboratory sand (acid washed sand) was added to each ovary in the mortar and blended together to

obtain a homogenate. The homogenate was centrifuged at 2500 rpm speed for 15 minutes and the supernatant was decanted and stored at -20^{o}C and used for further analysis.

Analysis of oxidant status of oxidative stress markers

Superoxide dismutase (SOD) activity was analysed using the method described by Mccord and Fridovich[19]. Catalase activity was determined by the method described by Sinha[20]. Reduced glutathione (GSH) was estimated following the procedure described by Beutler and colleagues[21].

MDA, the marker most frequently used for lipid peroxidation, was determined by the fluorometric method with 2-thiobarbituric acid (TBA) as described by Conti and colleagues[22].

Analysis of serum hormones

Hormones was analysed from the serum as given below. Briefly, serum was thawed on ice; diethyl ether 5 v/v was added and thoroughly mixed. This mixture was centrifuged under 1000 g for 3 minutes to separate phases. Upper layer (ether) was then stocked on a clean tube, repeated twice for optimal extraction. A precision curve was run for a coefficient value of $r^2 > 0.96$. Oestradiol and oestrogen assays (Roche Diagnostics GmbH) were performed on the same day. Sensitivity was 5 pg/mL and 0.25 ng/mL respectively. CV was 2.73 % and <1 % respectively. Follicle stimulating hormone (FSH) and Luteinizing hormone (LH) serum levels were not extracted before the assay and were measured by enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's instructions (Elabscience Biotechnology Co. Ltd, Wuhan, China). Sensitivity was 0.938 mIU/mL and CV <10%.

Statistical analysis

One way ANOVA and student t-test were used to analyse the data using Graph Pad Prism version 6.0. The results were presented as mean ± SEM with significant level determined at $P<0.05$.

RESULTS

Effect of cauliflower extract on ovarian weights

Ovarian weight increased significantly in the EV-only group when compared to control. Co- administration of EV and cauliflower also produced a significant increase in ovarian weights. However, ovarian weight reduced significantly when the high dose group was compared to the EV-only, low, and medium dose cauliflower groups (Table 1).

Cauliflower extract and oxidative stress markers

The MDA level in EV-only group showed a significant increase ($p<0.05$) when compared to control. However, there was a significant decrease in MDA level in a dose dependent manner when cauliflower extract was co-administered with EV compared to EV-only group. There was a significant increase ($p<0.05$) in the level of SOD among the EV-only treated group when compared to the control group. However, there was a significant decrease in SOD level in a dose dependent manner when cauliflower extract was co-administered with EV compared to EV-only group. There was no significant difference in GSH and catalase levels across the groups compared to the control (Table 2).

Cauliflower extract and serum hormones

The FSH and progesterone levels in EV-only group showed a significant decrease ($p<0.05$) when compared to control. However, there was a significant increase ($p<0.05$) in FSH and progesterone levels in a dose dependent manner when cauliflower extract was co-administered with EV compared to EV-only group (Table 3). Furthermore, there was a significant increase ($p<0.05$) in LH, testosterone and oestrogen levels in EV-only group when compared to the control. However, the combined administration of EV and cauliflower extract in a dose dependent manner showed a significant decrease ($p<0.05$) in LH, testosterone and oestrogen levels when compared to EV group (Table 3).

Table 1: Effect of cauliflower extract on ovarian weights in EV- induced PCOS rats.

Groups	Ovarian weight (g)
Control	0.11±0.004
EV-only	0.14±0.005*
EV + low dose	0.14±0.005*
EV + medium dose	0.14±0.002*
EV + high dose	0.12±0.002*$^{\#\alpha\beta}$

Values are expressed as Mean ± Standard Error of Mean (S.E.M), n=5.*P<0.05= significant when compared to control, $^{\#}$P<0.05= significant when compared to EV-only treated group. $^{\alpha}P$<0.05= significant when compared to EV + low dose treated group. $^{\beta}P$<0.05= significant when compared to EV+ medium dose treated group. EV= Oestradiol valerate

Table 2: Effect of ethanol fraction of cauliflower extract on oxidative stress markers in the ovary of EV- induced PCOS rats.

Groups	MDA (µ/mg)	GSH (µ/mg)	SOD (µ/mg)	CAT (µ/mg)
Control	0.27 ±0.02	13.64 ± 3.70	1.55 ± 0.02	18.08 ± 2.66
EV-only	0.62 ± 0.09*	11.96 ± 2.02	4.61 ± 0.11*	21.53 ± 2.93
EV + low dose	0.36 ± 0.06	12.27 ± 1.28	2.79 ± 0.47$^{\#}$	20.56 ± 2.73
EV + medium dose	0.34 ± 0.04	12.94 ± 2.51	2.33 ± 0.32$^{\#}$	17.59 ±2.89
EV + high dose	0.30 ± 0.01$^{\#}$	13.63 ± 1.01	1.89 ± 0.02$^{\#}$	18.08 ± 0.69

Values are expressed as mean ± Standard Error of Mean (S.E.M), n=5. *p<0.05=when compared to control, $^{\#}$p<0.05=when compared to EV-only group, EV= Oestradiol valerate

Table 3: Effect of ethanol fraction of cauliflower extract on hormones in EV-induced PCOS rats.

Groups	FSH (mIU/mL)	LH (IU/L)	Progesterone (ng/mL)	Testosterone (ng/dL)	Oestrogen (pg/mL)
Control	2.75 ± 0.75	1.70 ± 0.10	15.90 ± 0.60	2.53 ± 0.26	1.83 ± 0.12
EV-only	0.80 ± 0.80	2.15 ± 0.05*	11.48 ± 0.43*	6.20 ± 0.06*	3.67 ± 0.12*
EV + low dose	0.70 ± 0.7	2.05 ± 0.05	12.60 ± 0.60	6.17 ± 0.09*	3.53 ± 0.12*
EV + medium dose	1.75 ± 0.05	1.75 ± 0.75	12.66 ± 1.18	6.00 ± 0.12*	3.43 ± 0.09*
EV + high dose	1.85 ± 0.05	1.60 ±0.10#	13.61 ± 1.48	5.20 ± 0.12#*	3.20 ± 0.06*

Values are expressed as mean ± Standard Error of Mean (S.E.M), n=5. *p<0.05= when compared to control, $^{\#}$p<0.05= when compared to EV-only group, EV= Oestradiol valerate

Histological analysis of ovaries from EV and cauliflower treated rats compared to control

Ovarian histology of the control rats showed normal healthy follicles at different stages of follicular development (Fig. 1). Histological sections from EV-only group consisted of numerous large cysts with thin granulosa layer, very few corpora lutea and hyperplasia of theca interna cells (Fig. 2). Photomicrographs from the low dose cauliflower treated group demonstrated atretic follicles, fewer cystic follicles, and more corpora lutea when compared to EV-only treated group. Cystic follicles were absent in the medium and high dose treatment groups which also demonstrated presence of normal healthy growing follicles, atretic follicles, and many more corpora lutea (Figs. 3-5).

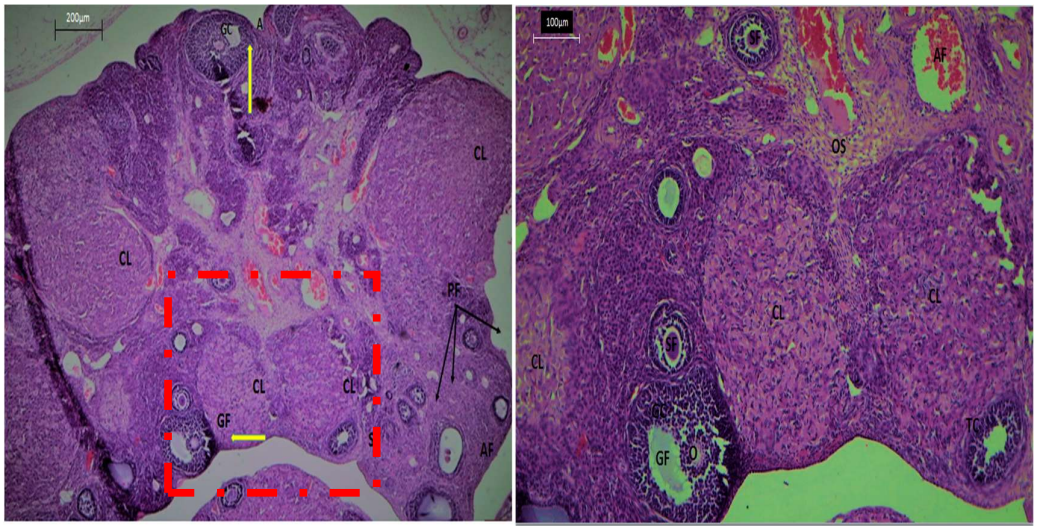

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Figure 1: H & E Photomicrograph of cross-section of normal control rat ovary, CL- Corpus luteum, GF- Graafian follicle, SF- Secondary follicle, PF- Primary follicle, Yellow Arrow- Oocyte, A- Antrum, OS- Ovarian stroma

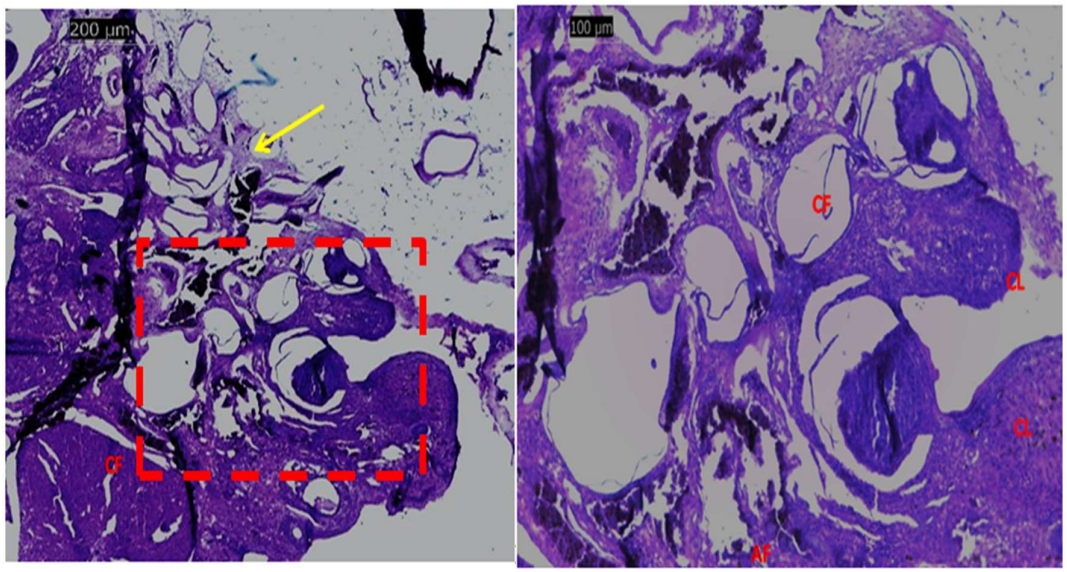

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Figure 2: H & E Photomicrograph of cross-section of ovary of rat treated with oestradiol valerate-only showing CF-Cystic follicle, CL-Corpus luteum, AF-Atretic follicle, Yellow arrow-degenerating follicle.

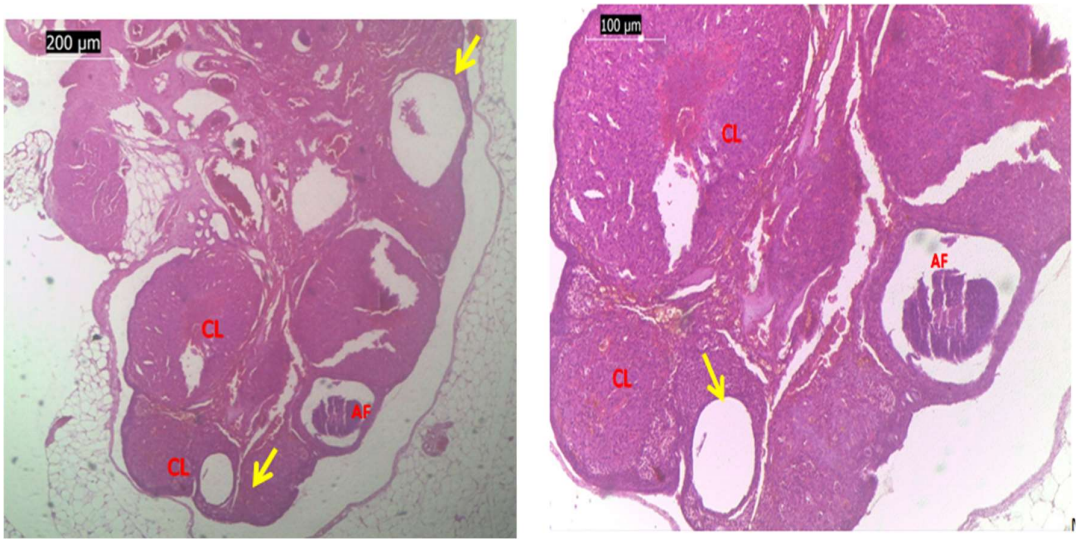

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Figure 3: H & E Photomicrograph of cross-section of ovary of rat treated with oestradiol valerate + low dose (200 mg/kg) cauliflower showing Yellow Arrow- Cystic follicle, CL-Corpus luteum, AF- Atretic Follicle.

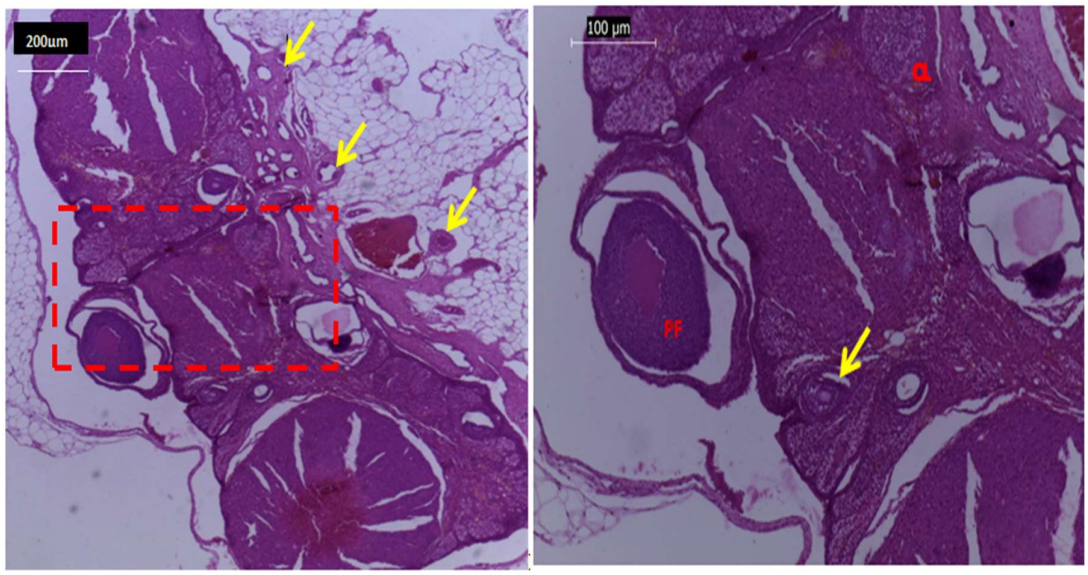

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Figure 4: H &E Photomicrograph of cross-section of ovary of rat treated with oestradiol valerate + medium dose (500 mg/kg) cauliflower showing CL- Corpus Luteum, AF- Atretic Follicle, Yellow Arrow-Primary Follicle

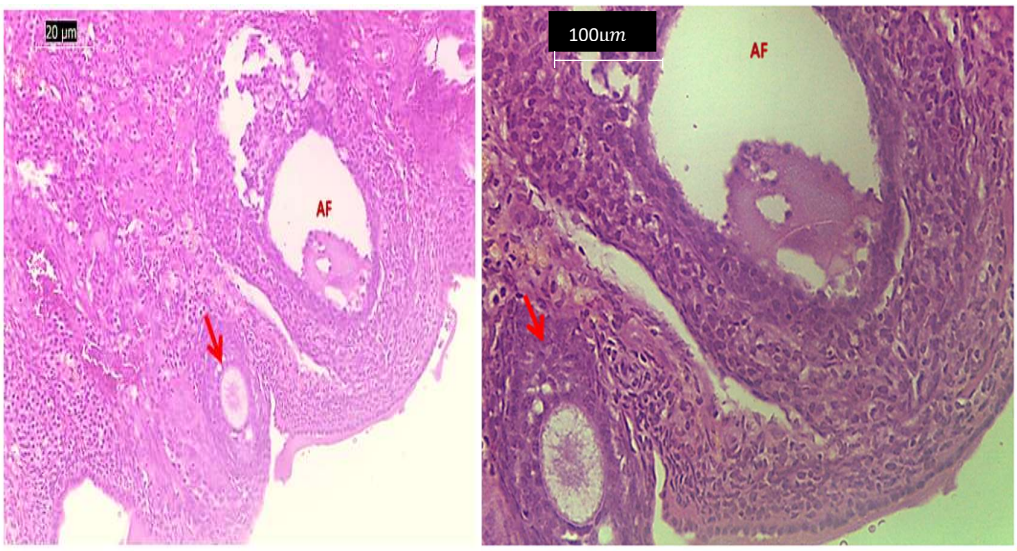

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Figure 5: H &E Photomicrograph of cross-section of ovary of rat treated with oestradiol valerate + high dose (1025 mg/kg) cauliflower showing Red Arrow- secondary follicle, AF- atretic graafian follicle.

DISCUSSION

In this present study, ovarian weights increased significantly in all the treated groups when compared with control. Studies have shown that hyperandrogenism may contribute to the increase in ovarian weights by increasing the thickness in ovarian cortex and ovarian volume leading to polycystic ovaries[23,24]. However, the high dose cauliflower group showed a significant decrease in relative ovarian weight when compared to all other treatment groups suggesting the anti-androgenic properties of cauliflower extract at high doses.

It is established that serum levels of MDA reflect the extent of lipid peroxidation and tissue damage[25,26]. High MDA levels is an indicator of chronic oxidative state. MDA levels are reported to increase in PCOS condition because of its association with insulin resistance, hyperandrogenism, and dyslipidemia[27,28]. In this present study, the MDA level in EV-only group showed a significant increase ($p<0.05$) when compared to control. However, there was a significant decrease in MDA level in a dose dependent manner when cauliflower extract was co-administered with EV compared to EV-only group. Our findings are similar to the results reported in previous literature[25,29,30] thereby corroborating the scavenging properties of cauliflower extract in mopping up free radicals caused by the PCOS condition.

GSH is an important antioxidant present in millimolar concentrations in living cells and assumes the role of intracellular radical scavenger. There was no significant difference in GSH level across the groups when compared to the control which was contrary with the studies reported by other researchers[25,31]. These authors suggested that lower GSH levels may be as a result of the association between insulin resistance and an impaired mitochondrial oxidative metabolism. *Brassicae olecerae* vegetables contains phytochemicals which are known to possess antioxidant activities and these antioxidants such as water-soluble vitamin C and phenolic compounds as well as lipid-soluble vitamin E and carotenoids contribute to both the first and second defence lines against oxidative stress[32,33]. GSH level in the cauliflower treated groups did not show any significant difference when compared with control.

There was a significant increase ($p<0.05$) in the level of SOD among the EV-only treated group when compared to the control group. However,

there was a significant decrease in SOD level in a dose dependent manner when cauliflower extract was co-administered with EV compared to EV-only group. Our finding was contrary with previously reported studies in which significantly increased SOD activity in patients with PCOS were recorded[25,30]. Kuşçu and Var[30] in their study suggested that the elevations observed in SOD activities might be due to a compensatory response by the body's defence mechanisms to higher circulating levels of oxidants.

In this present study, no remarkable change in catalase activities was observed. Catalase increased in EV-only group when compared to control but decreased in the cauliflower treated groups reaching values comparable to control at the high dose. Phenolics contained in cauliflower have been reported to possess many useful properties among which is their antioxidant properties[34,35].

Circulating levels of androgens are typically elevated in women with PCOS[36]. Testosterone is the main circulating active androgen, and the total serum testosterone concentration is the first-line recommendation for assessing androgen excess in women[37,38]. There was a significant increase in the level of testosterone in all the treatment groups compared to control. The significant decline in plasma concentration of testosterone in the high dose cauliflower treated group compared to the EV-only group might be indicative of the anti-androgenic properties of cauliflower extract. The result of this study is in agreement with the study of Nofal and colleagues[15] in which broccoli significantly reduced testosterone levels in EV-induced PCOS rats.

LH levels were significantly increased in EV-only group as seen in PCOS models which is thought to occur as a result of increased frequency of hypothalamic gonadotropin-releasing hormone (GnRH) pulses. Furthermore, studies have clearly demonstrated that the theca cell is the primary source for androgen production in PCOS [39]. In addition to increased LH secretion, the theca cell also exhibits increased responsiveness to LH stimulation[41]. The LH secretion pattern in PCOS probably reflects the unrestrained activity of the hypothalamic GnRH pulse generator which appears to be insensitive to negative feedback by progesterone[42].In addition, studies also suggests that primary defect of theca cell function also comprises the mechanism of androgen excess in PCOS. Treatment with ethanolic extract of cauliflower reduced LH levels significantly at the high dose when compared to the EV-only group indicating that cauliflower may have a positive influence on the GnRH pulse frequency and most likely on theca cell function. The result of this study is in agreement with the study of Nofal and colleagues[15] in which broccoli significantly reduced LH levels in EV-induced PCOS rats.

Significantly reduced FSH levels were seen in EV-only group in this study mimicking the PCOS phenotype which indicates follicular growth arrest. Thus follicles are trapped before the point of dominant follicle selection hence resulting in the appearance of cystic follicle thereby causing anovulation. Treatment with the ethanolic extract of cauliflower remarkably increased the FSH levels indicating the possibility of normal resumption of follicular development which might subsequently lead to ovulation. The result of this study is in agreement with the study of Nofal and colleagues[15] in which broccoli significantly increased FSH levels in EV-induced PCOS rats.

Previous studies utilizing the EV model has demonstrated high oestrogen levels[43-47]. In this present study, oestrogen increased significantly in all the groups when compared to control. High levels of oestrogen reported in EV models are a consequence of anovulation[48]. Abnormal increase in oestradiol level is correlated with multiple cysts formation, abnormal oestrous cyclicity and anovulation[49]. Oestrogen secretion in PCOS women is characterized by chronic secretion without the cyclic pattern that accompanies an ovulatory cycle. Unfortunately, treatment with cauliflower only caused a slight decrease in oestrogen levels when compared to the EV-only group. Nofal *et al.,*[15], on the other hand reported significant reduction in oestrogen levels in EV-induced PCOS rats treated with broccoli.

The decrease in the level of progesterone in the PCOS-positive group seen in this study could be

attributed to the very few corpus luteum observed in the histological sections from this group. Corpus luteum is responsible for progesterone secretion. Treatment with ethanolic extract of cauliflower increased the number of corpus luteum and progesterone levels. The result of this study is in agreement with the study by Nofal and colleagues[15] in which broccoli significantly increased progesterone levels.

The ovarian histology of the control group in this study showed normal healthy follicles at different stages of follicular development while the EV-only group showed very thin granulosa layer, numerous large cysts filled with follicular fluid, few corpora lutea and hyperplasia of internal theca cells. In PCOS, ovarian hyperandrogenism, insulin resistance, and altered intrafollicular paracrine signalling disturb the activation, growth, and selection of follicles. This leads to accumulation of small antral follicles giving it the polycystic morphology[50]. These findings are as a result of the presence of biologically active levels of FSH, increased LH, and lack of interplay between granulosa and theca cells as seen in PCOS models[51-53]. Treated groups with ethanolic extract of cauliflower was able to ameliorate the features seen in the EV-only group. With increasing doses, cystic follicles disappeared, giving way to the presence of normal healthy follicles at different stages of development and increased corpora lutea indicating restoration of normal ovulation.

EV rat model of PCOS does not share all features with human PCOS, however, the results obtained from this study revealed that ethanolic extract of cauliflower attenuated PCOS features in this animal model. Furthermore, cauliflower improved the ovarian morphology, and hormonal imbalance caused by the PCOS disorder and down regulated oxidative stress as seen in healthy models.

CONCLUSION

In conclusion, the present study shows that oral administration of the ethanolic extract of cauliflower has great potentials in the treatment and management of PCOS in women of childbearing age which can be attributed to the antioxidant and anti-inflammatory potential of the *Brassicae* family.

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