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Evaluation of *Caesalpinia bonducella* Extract (HAECB) on Ovarian Cysts in Polycystic Ovarian Syndrome-Induced Wistar Female Rats

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ABSTRACT

This study was conducted to evaluate the effect of *Caesalpinia bonducella* extract (HAECB) on ovarian cysts in Polycystic Ovarian Syndrome-Induced Wistar Female Rats. Female Wistar rats were divided into 5 groups consisting of 6 rats each. PCOS was induced in all the animals except normal group on the administration of letrozole (0.75mg/kg p.o) for 21days daily. In order to ensure PCOS, blood sample was collected through cardiac puncture and ovaries are excised for histopathalogical on 22nd day. After 21 days, group III and group IV were orally administered 200 mg/kg and 400mg/kg of HAECB (Hydroalcoholic extract of *Caesalpinia bonducella* extract) respectively, Group V was treated with clomiphene citrate (CC) (1mg/kg.p.o) for 21 days. During induction and treatment period estrus cycles have been evaluated. At the end of the experiment period, blood samples were obtained on 43rd (21 days for induction of PCOS after 21 days for treatment) day to evaluate serum levels of FSH, LH, testosterone and estradiol. The ovaries were also removed and studied with light microscopy after cutting of the tissues. In letrozole induced PCOS decreased FSH and testosterone, elevated level of LH and estradiol, large size of cysts and increased number of follicular cysts were seen. The results of HAECB treatment revealed the normal cycle in estrus cycle. FSH, LH, testosterone and estradiol levels in the group III and group IV showed a significant decrease compared to the PCOS group. Group III and group IV (HAECB treated groups) reverse the altered FSH LH, testosterone and estradiol level near the control level and reduction in the size of cysts and less number of atretic follicles were seen when HAECB treated groups compared to the PCOS group. HAECB can improve tissue symptoms and adjusted the levels of sex hormones in polycystic ovary syndrome. Overall, from the obtained results, it is determined that the *Caesalpinia bonducella* extract (HAECB) exhibited a good antiandrogenic effect by reducing the letrozole levels in PCOS-induced conditions.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is characterized by endocrine, metabolic, and genetic disorders, chronic absence of ovulation of polycystic ovary, and clinical and biochemical presentations of hyperandrogenism [1]. The symptoms of PCOS include clinical ones (menstrual disorders, hirsutism, acne, baldness and infertility), changes in endocrine hormones (increased levels of androgen, estrogen, and prolactin and decreased level of progesterone), and metabolic disorders (insulin resistance, diabetes, dyslipidemia, and type 2 diabetes). However, in some cases, estradiol level does not change. It is often associated with psychological impairments, including depression and other mood disorders and metabolic derangements, chiefly insulin resistance and compensatory hyperinsulinemia, which is recognized as a major factor responsible for altered androgen production and metabolism. Most women with PCOS are also overweight or obese, further enhancing androgen secretion while impairing metabolism and reproductive functions and possibly favoring the development of the PCOS phenotype. PCOS is one of the most common gynaecological disorders in reproductive-age women with the incidence likelihood of 4–12%. The prevalence rate of this disease was reported 5.6–8% in Europe. According to the latest studies, the prevalence of PCOS in Iran is 19.5% based on Rotterdam criteria and 6.8% based on the NIH criteria. The cause of infertility is lack of ovulation in approximately 75% of the cases. Currently, clomiphene citrate, metformin, and tamoxifen are the most widely used drugs to treat PCOS.

The plant *Caesalpinia bonducella* (synonym: Caesalpinia Crista Linn.) has been used in different system of traditional medication for the treatment of diseases and ailments of human beings [2]. It is reported to contain various Alkaloids, Glycosides, Terpenoids and Saponins. It has been reported as anti-asthmatic, antidiabetic, anti-inflammatory, anti-oxidant, anti-bacterial, anti-filarial, anti-tumor, anxiolytic, immunomodulatory, hypoglycemic activity. *Caesalpinia bonducella* is an Indian herb reported in Ayurveda, the ancient Hindi medicine system of India. *Caesalpinia bonducella* belonging to Family: Caesalpiniaceae found throughout India and tropical countries of the World.

METHODS

Collection and authentication and extraction

The seeds of *Caesalpinia bonducella* were collected from local source, Tamil Nadu in November. The plant material was identified and authenticated by Dr. P. Jayaraman, Retd. Professor. Presidency College, Chennai-600005, Tamilnadu. **Reference No. PARC/2018/3879.** The seeds of *Caesalpinia bonducella* was collected and ground into coarse powder. 450g powder of *Caesalpinia bonducella* seeds was extracted with 4500 ml of hydroalcohol (ethanol:water (3:1 ratio)) by using a Soxhlet apparatus for 24 hours.[3]

The yield of the extract is 2.47% (w/w). The Hydroalcoholic extract of *Caesalpinia bonducella* (HAECB) is preserved in a sterile airtight glass container at 4^0C and used for further studies.

Drugs and Chemicals

All reagents and chemicals were purchased from SD fine chemicals; Standard drug Clomiphene citrate (CC) and letrozole were purchased from Sun pharma.

Experimental animals and grouping

Adult female wistar albino rats of weighing 150-200 gms were used for this study. The animals were procured from the animal house of C.L. Baid Metha College of Pharmacy, Thoraipakkam, Chennai-97. They were housed six per cage under standard laboratory conditions at a room temperature at 22 ± 20° C with 12 hr light / dark cycle and provided with standard pellet chow and water ad libitum. Animals were quarantined for 5 days before the start of the experiment. The study was conducted on 30 healthy adult female rats after the approval of the Institutional Animal Ethical Committee (**IAEC Reference no:9/321/PO/Re/S/01/CPCSEA/dated 12/10/18).**

Initially, 24 animals were induced PCOS with letrozole for 21 days. After confirmation of PCOS induction, animals were randomly divided into 4 different groups consisting of six animals for drug treatment as shown in Table 1, and the separate untreated group was maintained as normal control.

Table 1: Treatment Plan

Groups	Groups and treatment
Group I	Normal control
Group II	PCOS induced by letrozole 0.75mg/kg p.o for 21 days
Group III	PCOS induced by letrozole 0.75mg/kg p.o for 21 days, after 21 days treated with HAECB (200mg/kg p.o) for 21 days
Group IV	PCOS induced by letrozole 0.75mg/kg p.o for 21 days, after 21 days treated with HAECB (400mg/kg p.o) for 21 days
Group V	PCOS induced by letrozole 0.75mg/kg p.o for 21 days, after 21 days treated with Clomiphene citrate 1 mg/kg p.o for 21 days

Induction of PCOS

After quarantine, the rats were administered with letrozole for 21 days for PCOS induction. During this period vaginal smears were collected daily for estrus cycles determination. On the 22nd day blood samples was collected through cardiac puncture under mild anesthesia from all the group of animals to analyse the serum levels of FSH, LH, testosterone and estradiol and then rats were killed by euthanasia using chloroform. Uterus and ovaries were excised and weighed. Serum hormonal levels and histopathological changes in ovaries were examined to ensure the PCOS findings.

Evaluation of ovarian cysts

The HAECB suspended in aqueous solution to achieve 100 mg/ml stock solution. Estrus cycle was observed daily for all the groups. On 43rd day at the end of the treatment period, blood samples were collected to estimate various hormonal parameters (estimation of FSH, LH, testosterone and estradiol levels) and animals from each group were sacrificed, and vital organs (uterus and ovary) were excised and subjected for histopathological studies as shown in Fig 6.

Hormonal assays

Blood was collected and serum was separated. Serum levels of FSH, LH, testosterone and estradiol were estimated with commercial double-antibody RIA kits.

Histopathology

The ovaries of rats from all groups were removed dissected out and washed out with ice-cold saline and preserved in 10% formalin solution. Paraffin blocks were prepared and tissue was stained with hematoxylin and eosin, and subjected to histopathological study. The area of the largest follicle and the thickness of its follicular wall that is the thecal cell layer and granulose cell layer were measured. The ovarian follicles and corpora lutea at different stages of development and the theca and granulosa cell layers were analysed in detail.

Statistical analysis

The results were analyzed in terms of Mean ± Standard error of Mean (SEM). For statistical analysis, multiple comparisons of data were made using one and two way analysis of variance (ANOVA) followed by Dunnet‟s test was used for post hoc analysis. Software program GraphPad PrismVersion 8.0 was used for all data analysis.

RESULTS

Preliminary Phytochemical Analysis of Hydroalcoholic Extract of *Caesalpinia Bonducella* (HAECB)

The result of preliminary phytochemical analysis of hydroalcoholic extract of *Caesalpinia bonducella* (HAECB) showed presence of various phytochemical constituents such as proteins, amino acids, alkaloids, tannins and flavonoids and absence of carbohydrate, glycosides, phenols, saponins, fixed oils, steroids, terpenes, gums and mucilage.

To study the effect of estrus cycle on letrozole induced PCOS

Estrus cycle was examined by vaginal smear method. In the PCOS condition, the incidence of estrus phase and its duration is very low due to low amount of estradiol, the main cause for the development of PCOS.

Normally estrus cycle of rats is in sequential order such as estrus, metaestrous, diestrous, and proestrous phase, respectively as shown in Fig 5. However, letrazole treated rats showed irregularity in its phases because of the physiological disturbance due to polycystic ovary condition. Most of the letrozole-treated rats showed persistent days of diestrous phase during the 21 days of treatment when compared to normal rats.

The estrous cycle was restored to regular in all the HAECB treated PCOS animals. Diestrous phase was reduced significantly, and estrus phase was extended in terms of days in the treated group when compared to control. Thus, *Caesalpinia bonducella* has potential effect by reverting the reproductive cycle towards nomal in PCOS rats.

Hormonal parameters

In the present investigation, the PCOS induction after letrazole administration was confirmed by decreased level of FSH, testosterone and elevated level of LH, estradiol.

Effect of HAECB on Follicular stimulating Hormone in PCOS induced Female rats

When compared to Group I, Group II($p < 0.0001$), Group III($p < 0.0001$), Group IV($p < 0.0001$) significantly decreased FSH in blood, non significant decrease in FSH in blood in Group V.

When compared to Group II, non significant decrease in FSH in blood in Group III and Group IV, GroupV($p < 0.0001$) significantly increased FSH in blood.

When compared to Group V, Group III($p < 0.0001$), Group IV($p < 0.0001$) significantly decreased FSH in blood. (Table 2, Figure 1)

Effect of HAECB on Luteinizing Hormone in PCOS induced rats

When compared to Group I, Group II($p < 0.0001$) significantly increased LH in blood, Group III($p < 0.0001$), Group IV($p < 0.0001$), Group V($p < 0.0001$) significantly decreased LH in blood.

When compared to Group II, Group III($p < 0.0001$), Group IV($p < 0.0001$), Group V($p < 0.0001$) significantly decreased LH in blood.

When compared to Group V, Group III($p < 0.0001$), Group IV($p < 0.0001$) significantly decreased LH in blood. (Table 2, Figure 2)

Effect of HAECB on Testosterone in PCOS induced rats

When compared to Group I, Group II($p < 0.0001$), Group III($p < 0.0001$), Group IV($p < 0.0001$), Group V($p < 0.0001$) significantly decreased testosterone in blood.

When compared to Group II, Group III($p < 0.0001$), Group IV($p < 0.0001$) significantly decreased testosterone in blood, non significant decrease in testosterone in blood in Group V.

When compared to Group V, Group III($p < 0.0001$), Group IV($p < 0.0001$) significantly decreased testosterone in blood. (Table 2, Figure 3)

Effect of HAECB on Estradiol in PCOS induced rats

When compared to Group I, Group II($p < 0.0001$) significantly increased estradiol in blood, Group III($p < 0.0001$) significantly decreased estradiol in blood, Group IV($p < 0.0001$), Group V($p < 0.0001$) significantly increased estradiol in blood.

When compared to Group II, Group III($p < 0.0001$), Group IV($p < 0.0001$) significantly decreased estradiol in blood, Group V($p < 0.0001$) significantly increased estradiol in blood.

When compared to Group V, Group III($p < 0.0001$), Group IV($p < 0.0001$) significantly decreased estradiol in blood. (Table 2, Figure 4)

Table 2: Influence of HAECB on hormonal parameters in letrozole induced PCOS female rats

Parameters	Group I Normal control	Group II PCOS Induced (letrozole 0.75mg/kg only)p.o	Group III HAECB- I (200mg/kg) p.o	Group IV HAECB- II (400mg/kg) p.o	Group V Standard (1mg/kg Clomiphene Citrate) p.o
FSH (ng/ml)	23.3±1.688	14.81±1.822a****	13.91±0.938 a****b^{ns} c****	13.91±0.893a ****b^{ns} c****	21.6±0.991a^{ns} b****
LH(ng/ml)	27.1±0.923	29.18±0.541a****	22.5±0.260 a****b****c****	20.43±0.307a ****b****c********	24.58±0.292a******** b****
Testosterone(total)(ng/ml)	2.24±0.272	1.18±0.352a********	0.63±0.021 a****b****c****	0.22±0.018a******b****c******	1.05±0.033a******b^{ns}
Estradiol (ng/ml)	56.13±0.581	68.36±0.320a****	53.88±0.813 a****b****c****	63.11±0.738a ****b****c********	87.4±0.368a******b****

All the values are expressed as mean ± SEM n=6. ****p<0.0001

Group I Vs Group II,Group III, GroupIV, Group V is considered as a.

Group II Vs Group III, Group IV, Group V is considered as b.

Group V Vs Group III, Group IV is considered as c (one- way ANOVA followed by Dunnet's

Multiple Comparison) Graph Pad prism 8.1 version

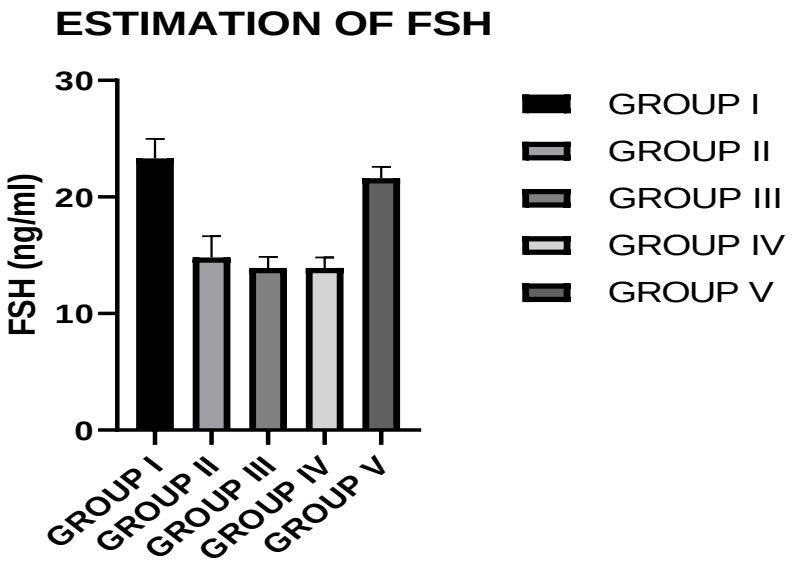
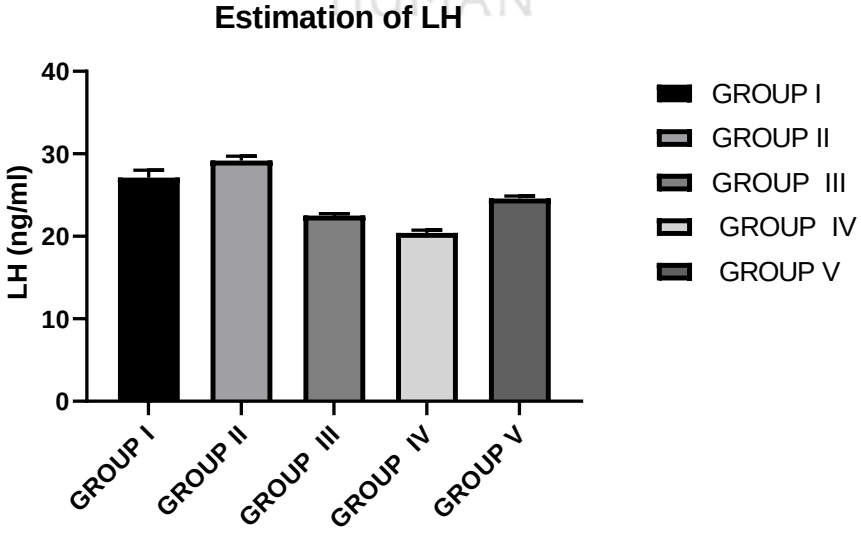


figure 1



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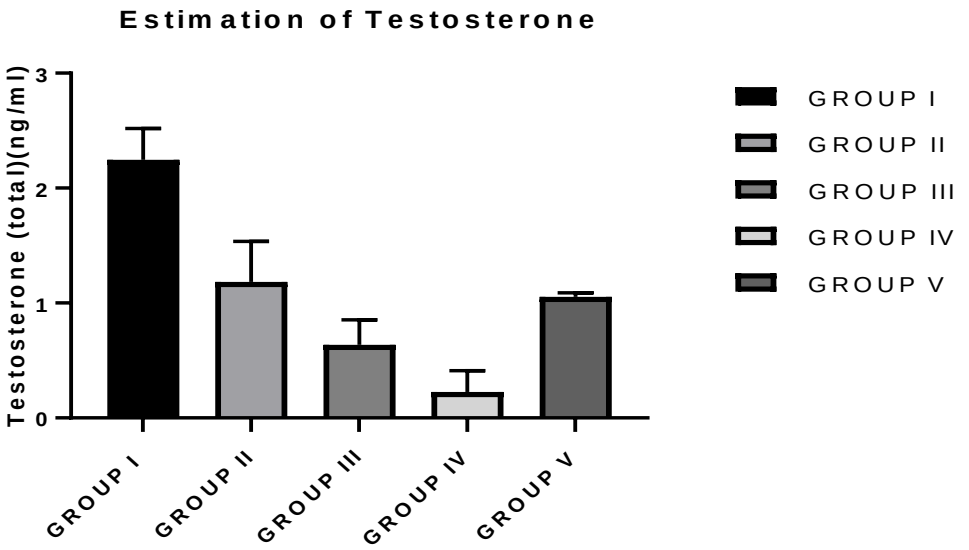

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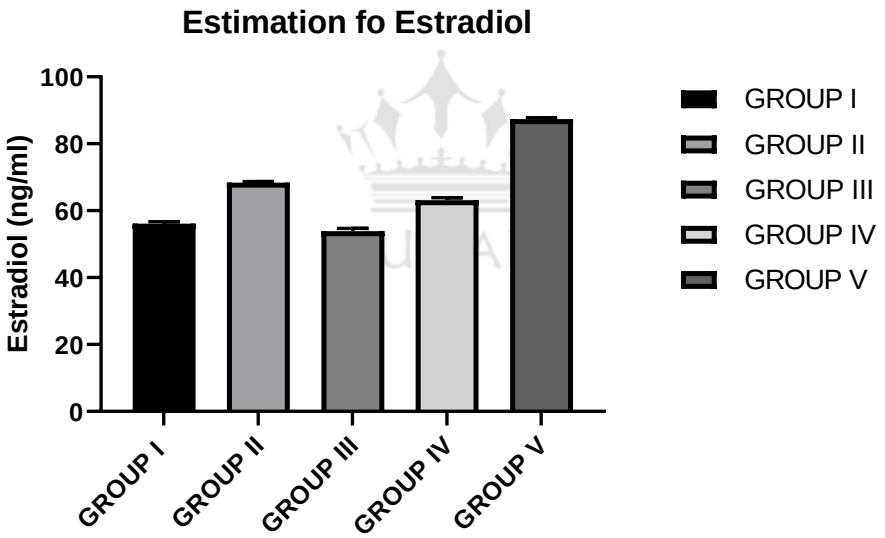

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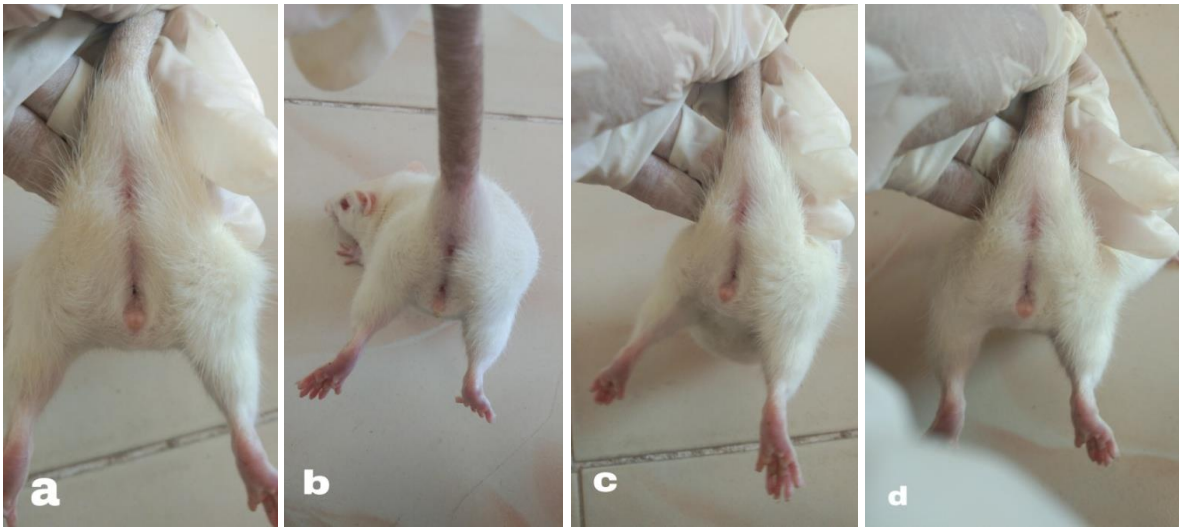
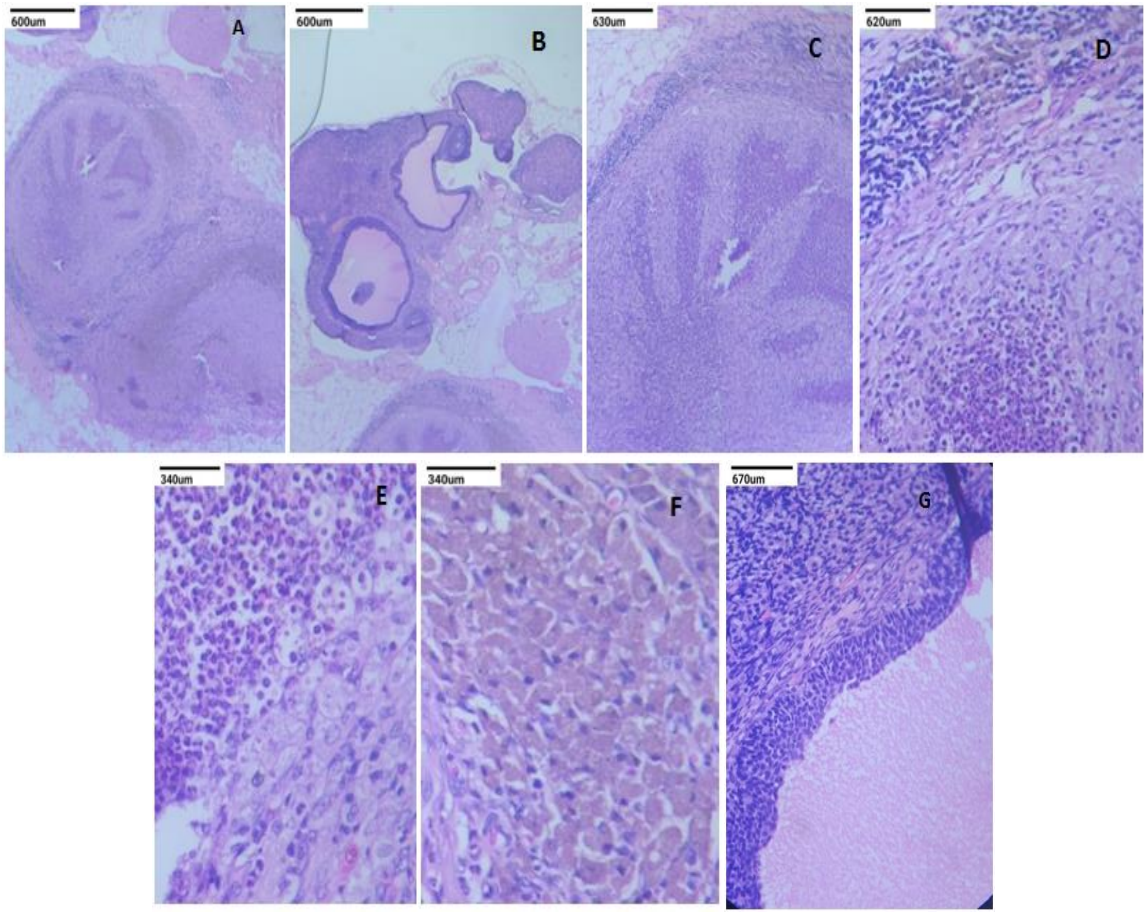


Fig 5: Photographs of vaginal smear for confirmation of reproductive phases. a. Proestrus b. Estrus c. Metestrus d. Diestrus

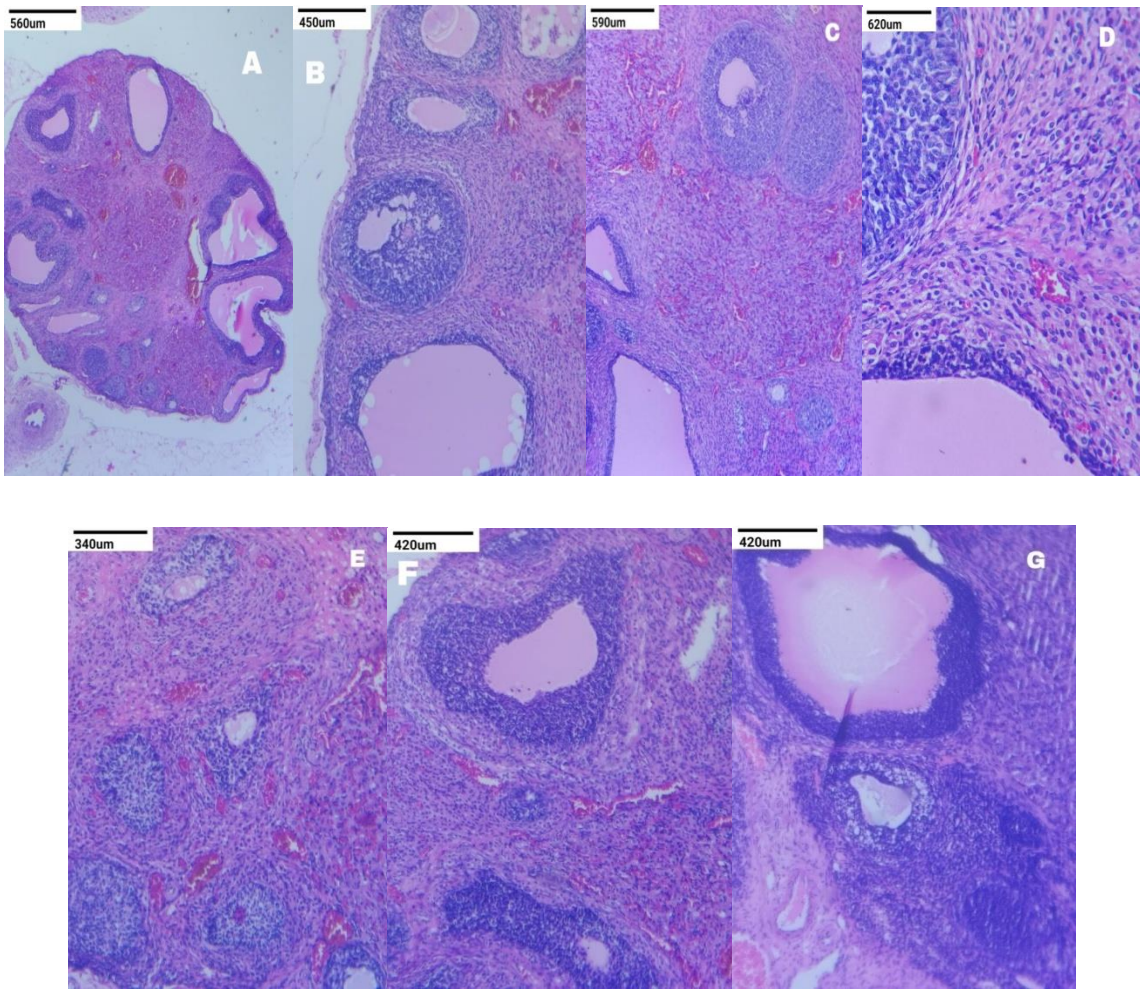
Group I: Ovary



A. Ovary showing space in the stroma. **B.** Two large follicles and one primordial follicles **C**. Higher magnification of area in A. **D.** Higher magnification of area in C. **E.** A higher

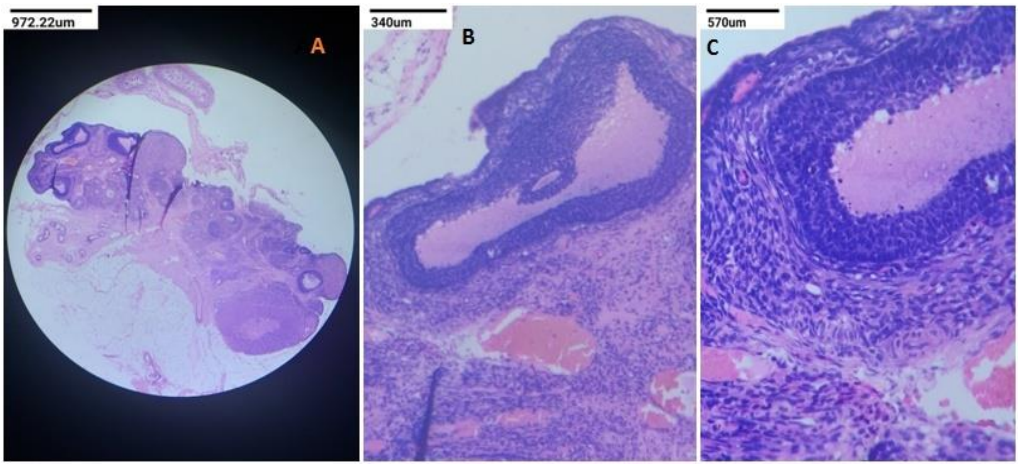
magnification view of the cells forming the corpus luteum, the large amounts of cytoplasmic vacuolization is typical of steroid hormone producing cells. **F.** Higher magnification of D.**G.** Follicle wall with granulosal cell layer

Group II: Ovary



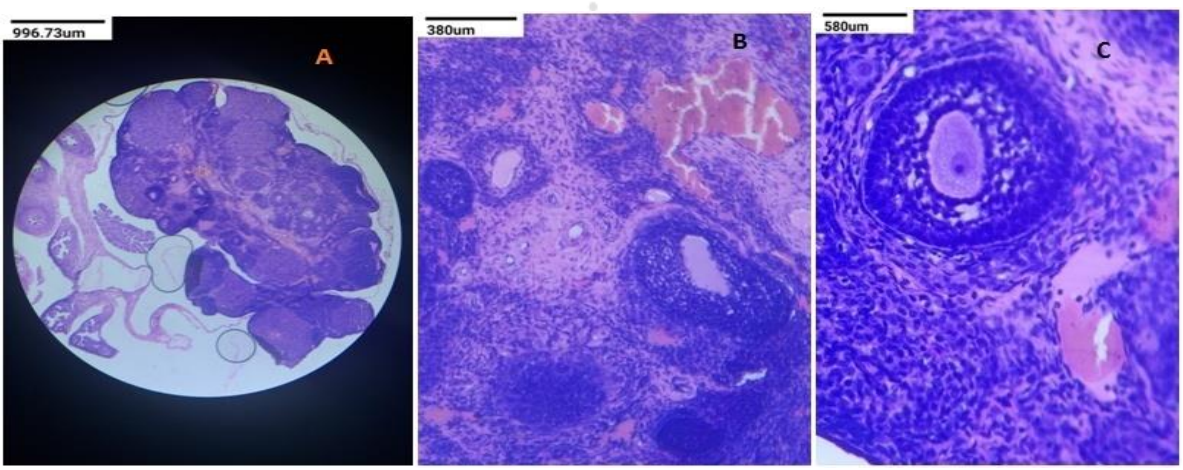
A. Ovary showing cysts. **B.** Hyperluteinized theca cells and decreased granulosa cell layer. **C**. The cyst wall has a vasularized layer of leutenized cells and group of granulose cells. **D.** Higher magnification of B showing stroma **E.** Non vascularized luteinized membrane granulosa and vascularised luteinized granulose. **F.** Cyst with decreased granulose layer and thick hyperluteinized theca layer. **G.** Highly vascularized cyst.

Group III: Ovary



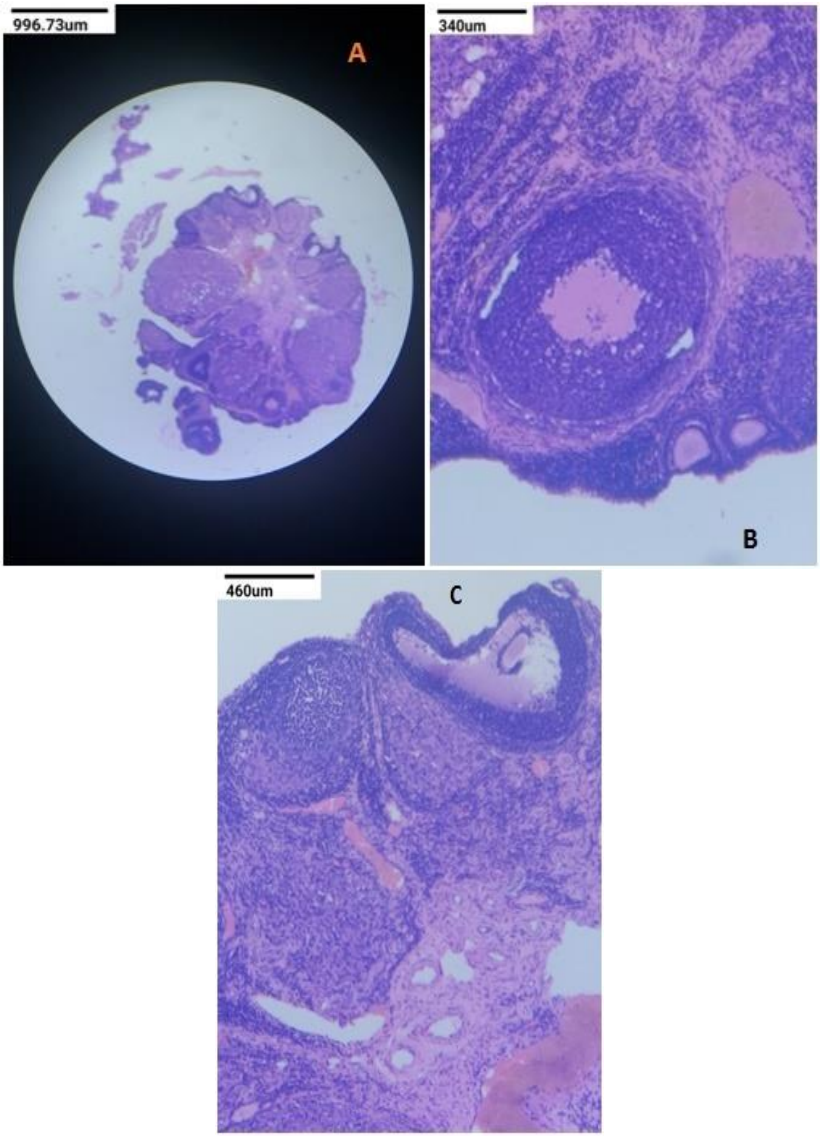
A. Ovary showing mature follicles **B.** Follicular cyst with the oocyte.**C.** Higher magnification view of B

Group IV: Ovary



A. Ovary showing less number of cysts. **B.** Follicular cysts are decreased in size when compared to Group III. **C.** Graafian follicules (mature follicule) having the oocyte surrounded by granulosa cells with follicular antrum and is surrounded by theca interna, theca externa.

Group V: Ovary



A. Ovary showing follicles. **B.** Cortical area of ovary showing primordial follicles. **C.** Higher magnification of A

Fig 6: Histopathological studies of ovaries

DISCUSSION

Polycystic ovary syndrome (PCOS) is a complex condition characterized by elevated androgen levels, menstrual irregularities, and/or small cysts on one or both ovaries.[4] The disorder can be morphological (polycystic ovaries) or predominantly biochemical (hyperandrogenemia) and it affects 5–10% of women of reproductive age. Research suggests

that 5% to 10% of females 18 to 44 years of age are affected by PCOS, making it the most common endocrine abnormality among women of reproductive age in the U.S.[5]

Clinical signs of PCOS include elevated luteinizing hormone (LH) and gonadotropin–releasing hormone (GnRH) levels, whereas follicular-stimulating hormone (FSH) levels are muted or unchanged. As a result of the increase in GnRH, stimulation of the ovarian thecal cells, in turn, produces more androgens.[6] Follicular arrest can be corrected by elevating endogenous FSH levels or by providing exogenous FSH. Elevated circulating androgen levels are observed in 80–90% of women with oligomenorrhea.

Hyperandrogenism, a clinical hallmark of PCOS, can cause inhibition of follicular development, microcysts in the ovaries, an ovulation, and menstrual changes.[7] During the follicle development reduces progesterone and estrogen levels due to regression of the corpus luteum.[8] Consequently, release from negative feedback suppression allows a small but steady increase in FSH and LH levels, which stimulates the growth phase of ovarian follicles.[9] Hormonal contraceptives, selective estrogen receptor modulator (SERM), insulin sensitizers, gonadotropins, and ovarian surgery have been shown to be useful for improving PCOS symptoms in women.[10]

The plant *Caesalpinia bonducella* has been used in different system of traditional medication for the treatment of diseases and ailments of human beings. All components of the plant have medicinal properties so it is a valuable medicinal plant which is utilized in traditional system of medicine. The phytochemical screening of seeds of *Caesalpinia bonducella* revealed the presence of bioactive compounds such as Triterpenoids, Flavonoids, glycosides, saponins, tannins, alkaloids and amino acids and it has been reported to have some pharmacological activities.

Letrozole being a non-steroidal aromatase inhibitor blocks the conversion of testosterone to estradiol. This results in the reduction in estradiol production and elevated level of testosterone. Letrozole, an aromatase inhibitor has increased the circulatory androgen levels. Clomiphene citrate, a selective estrogen receptor modulator (SERM) has agonist and antagonist activity. Treatment of clomiphene citrate has been shown to improve ovulation induction in female rats.

In the present study, letrozole induced aromatase inhibition in female rats in order to induce conditions similar to those of PCOS in women and then examined the hormonal

concentrations and histolopathological changes in the ovaries for follicular phase in Letrozole-induced PCOS female rats.

Decreased or normal FSH and increased LH are due to GnRH pulsatile secretion, that is at hypothalamic level and high estrogen environment at pituitary level.[11] Clinically intense androgenization due to excess androgen production is observed in PCOS. FSH which is responsible for the development of eggs into mature follicles by acting on immature follicular cells of the ovary [12] in normal individual. In this study FSH level are significantly decreased FSH level in HAECB treated female rats than letrozole induced female rats.

Elevated LH increase androgen production. Luteinizing hormone which is responsible for the secretion of progesterone and estrogen[13] has elevated level in PCOS. The decrease in the sex hormone binding protein in the liver, increase in insulin response in the ovary and the effect of high LH, induce the increase in androgen secretion in the ovary. After that, follicle growth and maturation are suppressed. In this study, LH level are significantly decreased in HAECB treated female rats than letrozole induced female rats. It showing the PCOS induced rats reverted to have normal condition of estrus cycle.

Clinically, intense androgenization due to excess androgen production is observed in PCOS. Insulin resistance leads to hyperinsulinemia, reduces SHBG and raises free circulating testosterone. Elevated levels of free testosterone account for the vast majority of abnormal findings in the laboratory examination. The elevated levels of free or total testosterone were not converted to estradiol in PCOS. HAECB treatment show significantly decreased total testosterone level than letrazole induced female rats.

Estrogen is normal or slightly elevated in PCOS. Estrogen secretion in PCOS women is characterized by chronic secretion without the cyclic pattern that accompanies an ovulatory cycle. Serum estradiol (E_2) levels may vary in PCOS but are usually in the mid-follicular phase range. Estradiol levels are constantly in the early to mid follicular range without the normal mid-cycle increases.[14] In contrast, serum levels of estrone (E_1) are usually greater than those of E_2 which is the reverse of the E_1:E_2 ratio seen in normal women. Estrone levels are increased [15] because of extraglandular aromatization of increased circulating androstenedione levels[16] due to hyperandrogenemia. Hyperandrogenemia induces the increase in testosterone, androstenedione, dehydroepiandrosterone (DHEA), DHEA-S, 17-hydroxyprogesterone and estrone (E1) (excess androgen converted to E1 by peripheral fat).

The decreased SHBG levels typical of PCOs result in increased non-SHBG bound or bioavailable estradiol as well as T levels.

In this study letrozole is treated for induction of PCOS, thus the conversion of androgen (4-androstenedione) were not converted to testosterone and then to estradiol (E2). In another metabolic pathway of androgen (4-androstenedione) were not converted to estrone (E1) and then to estriol, estriol (E3) which is converted to estradiol. Thus present suggested to have increase in the estradiol level in letrozole induced PCOS female rats when compared to normal. HAECB treatment show significantly decreased estradiol level than letrazole induced female rats.

Although HAECB reverse the altered LH, FSH and testosterone level near the control level. The estrus cycle reverted to regular and normal. Vaginal smear of HAECB treated rats showed regularity in reproductive cycle.

The HAECB shows modulation of pathogenicity induced by letrozole induced PCOS. (Fig 6)

Histopathological observations showed number of atretic follicles, cystic formation in the negative control. HAECB showed reduction in the size of cysts and less number of atretic follicles. The different dose of HAECB showed normal granulose layer well-defined thecal layers and matured follicles. There is also disappearance of cysts in a dose dependent manner resulting in formation of a number of corpora lutea suggesting the restoration of ovulatory condition along with follicles at different developmental stages, showed that HAECB restored regular estrus cycle.

CONCLUSION

The estrus cycle is restored to regular in the animals treated with HAECB. Thus, it can be concluded that this extract has a potential effect on PCOS bringing the reproductive cycle and other complications to normal. The findings of the study confirmed the HAECB causes an increase in estradiol levels and reverse the altered LH, FSH and testosterone level near the control level. HAECB showed remarkable antiandrogenic effect by degenerating the immatured follicular cysts, and decreased the cysts size. Thus, helped in regulating normal ovulation. Therefore, this extract shows further study is required to establish the mechanism of action of HAECB.

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