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Original Article

# Betel Leaf Extract Amends Dehydroepiandrosterone Induced PCOS Related Hormonal Abnormality and Histopathological Alterations in Rat Model

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## ABSTRACT

**Introduction:** PCOS is a hormonal disorder with missed or irregular periods at the reproductive ages of women, which was mainly due to increased androgen levels. **Objective:** To evaluate the antiandrogen activity of EEBL (ethanolic extract of betel leaf) in DHEA induced PCOS (polycystic ovary syndrome) and improving ovulation rate, consequently its effects on hormonal and biochemical profile of the blood serum and Histopathology of the ovary. **Methods:** Divide the 30 immature (4-week-old) female Sprague Dawley rats into 5 groups. Four groups except the control group were injected each morning with dehydroepiandrosterone (DHEA) (6 mg/100 g body weight/0.2 ml sesame oil) for 20days. The control group was injected with 0.2ml sesame oil for 20days. Pretreatment completed after 21$^{st}$ day then animals are subjected to post-treatment with EEBL (LD-100, HD-200mg/kg, p.o) and CC (100 µg/kg, p.o) from 21 to 41 days. After the treatment animals are subjected to biochemical, hormonal and histopathological examinations. **Results:** In negative control group SOD, Catalase were decreased. Total protein, SGOT, SGPT, TG, LDL and cholesterol levels were increased than the control group. Hormones LH and Testosterone levels increased. FSH, estradiol, and progesterone levels were decreased when compared with the control group. Histopathology has revealed that the presence of cysts in the negative control group and recovery of cysts seen in treatment groups. **Conclusion:** Treatment with EEBL is effectively attenuated to the DHEA induced PCOS and it is significant in comparison results with clomiphene citrate attributing its therapeutic potential towards the treatment of PCOS.

**Key words:** DHEA, PCOS, Betel Leaf, Clomiphene citrate, Anti-androgenic activity, Rats.

## INTRODUCTION

Ovaries, where a woman's eggs are produced, ovaries have tiny fluid-filled sacs called follicles/cysts. As the egg grows, the follicle builds up fluid. When the egg matures the follicle breaks open the egg is released, and the egg travels through the fallopian tube to the uterus (womb) for fertilization is an ovulation process.[1] PCOS (Polycystic ovarian syndrome) is a problem in which a woman's hormones are out of balance. It can cause problems with the menstrual periods and make it difficult to get pregnant.[2-5] PCOS causes changes in the look (acne, hair growth, and obesity). If it is not treated, over time it can lead to diabetes and heart disease.[6]

Polycystic ovarian syndrome (PCOS) or Stein-Leventhal syndrome is the most common female endocrinological abnormality, affecting 4-8% of women in their reproductive years. Approximately 1 in 15 women experience PCOS.[7,8] A key sign of PCOS is irregular or missed periods. Imbalanced hormone levels of testosterone, Progesterone, and FSH are seen.

Dehydroepiandrosterone (DHEA), a metabolic intermediate in the biosynthesis of androgens. Increased levels of androgens seen in PCOS. Treatment of PCOS by clomiphene citrate, metformin, tamoxifen and troglitazone, non-steroidal estrogen agonist, oral contraceptives, antiandrogens, gonadotropin-releasing hormone agonist, insulin stabilizers, spironolactone, and laparoscopy. Current therapy of PCOS has mild to severe side effects including hot flushes, arthritis, joint or muscle pain and psychological side effects such as irritability, mood swings, depression and bloating makes to think towards safe and alternative drugs.[9]

Medicines from natural sources may be an attractive and alternative therapy with an exact known mechanism to develop a potent, safe and cost-effective therapeutic agent for this disease. *Betel leaf* a plant widely used in Ayurveda and it's having tannins and flavonoids have been shown to possess anti-inflammatory, anti-oxidant activity useful in Headache, Obstructed Urination, Weakness of Nerves, Sore Throat, Respiratory Disorders, Constipation, in the problem of breast milk secretion, Contraceptive, and Anti-diabetic.[10-12] Hence the current study has been undertaken to reveal the potential benefits of ethanolic extract of *Betel leaf* for the management of PCOS in DHEA induced PCOS in rats.

## MATERIALS AND METHODS

### Drugs and chemicals

Ethanolic Extract of Betel Leaf; Dehydroepiandrosterone (DHEA), Total Cholesterol, LDL and Triglyc-

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erides kits were purchased from ERBA Diagnostics. All others reagents of analytical grade were purchased from SD fine chemicals Ltd. India.

## Experimental animals

Sprague –Dawley rats, weighing 100g, at the age of 4 weeks old female rats were used in the study. They were maintained under standard laboratory pellet chow diet; Provimi Limited (India), provided water ad libitum and were kept under standard conditions at 23-25°c, 35 to 60% humidity, and 12hr light /dark cycle. The rats were acclimatized to the laboratory conditions a week prior to the experiment. The experimental protocol was duly approved by the institutional animal ethics committee (IAEC) and care of the animals was carried out as per the guidelines of Committee for the purpose of control and supervision of experiments on animals (CPCSEA) (Protocol. No: I/IAEC/LCP/012/2014/SD-30).

## Experimental design

Immature rats (approximately 21-22 days old) are treated with daily s.c, with DHEA injections (6mg/100g body weight in 0.2ml of sesame oil) for 20 days. This dose of DHEA is sufficient to induce a hyperandrogenism state similar to that of PCOS in women. The control group was injected with 0.2ml sesame oil for 20days. Pretreatment was completed after 21$^{st}$ day then animals are subjected to post-treatment with EEBL (LD-100, HD-200mg/kg, p.o) and CC (100 µg/kg, p.o) from 21 to 41days. After the treatment animals are subjected to biochemical, hormonal and histopathological examination.[13-17]

# BIOCHEMICAL PARAMETERS

## Preparation of ovarian homogenate

10% of ovarian homogenate was prepared in 0.1 M Tris HCl buffer (pH-7.8) and centrifuged at 10,000 rpm for 30 min at 4 µC. The supernatant was used as a source for estimating the antioxidants, liver enzymes, hormones, and total protein.

### *Estimation of superoxide dismutase*

Superoxide dismutase (SOD) activity was determined by the Pyrogallol oxidation method. One unit SOD activity is defined as the amount of enzyme that inhibits the rate of auto-oxidation of Pyrogallol by 50%. The reaction is initiated by adding Pyrogallol and the change in optical density was recorded at 420 nm.[18]

### *Estimation of catalase*

The rate of decomposition of $H_2O_2$ to water and molecular oxygen is proportional to the activity of catalase. The sample containing catalase is incubated in the presence of a known concentration of $H_2O_2$. After incubation for exactly one minute, the reaction is stopped with ammonium molybdate. The amount of $H_2O_2$ remaining in the reaction is then determined by the oxidative coupling reaction between molybdate and $H_2O_2$.[19]

### *Estimation of protein estimation*

The standards and reagents were added in the order represented in the table above. For unknown samples: Total protein (TP): To 20 µl of TP, 100 µl of 2% Na2Co3 in 0.1N NaOH and 880 µl water was added to make the volume to 1 ml from this 50 µl was used as a sample. Total soluble protein (TSP): To 20 µl TSP, 980 µl of water was added making the volume to 1 ml. From this 50µl was used as a sample. Total soluble protein (TSP): To 20 µl TSP, 980 µl of water was added making the volume to 1 ml. From this 100 µl was used as a sample for protein estimation. For protein estimation, the unknown samples and reagents were used in the same order as in standards (given in the table above). TP, TSP, and standard samples were all taken in duplicates.[20]

### *Estimation of assay of TBARS content*

The method of Utley *et al.*, with some modification was used to estimate the rate of Lipid peroxidation (LPO). Homogenate (0.25 ml) was pipetted into 15×100 mm test tubes and incubated at 37°C in a metabolic shaker for 1 hr. An equal volume of homogenate was pipetted into a centrifuge tube, placed at 0°C and marked at 0 h incubation. After 1 hr of incubation, 0.5 ml of 5% (w/v) chilled trichloroacetic acid (TCA), followed by 0.5 ml of 0.67% TBA (w/v) was added to each test tube and centrifuge tube, and centrifuged at 1000 × g for 15 min. Thereafter, the supernatant was transferred to other test tubes and was placed in a boiling water bath for 10 min. The absorbance of pink color produced was measured at 535 nm in a spectrophotometer (Shimadzu-1601, Japan). The TBARS content was calculated by using a molar extinction coefficient of $1.56 \times 10^5$ $M^{-1}$ $cm^{-1}$ and expressed as nmol of TBARS formed $min^{-1}$ $mg^{-1}$ of protein.[21]

## Ovarian histopathology

Isolated ovaries are fixed in 10% Neutral Buffered Formalin. They were subjected to tissue processing by dehydration through an ascending ethanol series, clearing in xylene and embedding completely in paraffin wax into blocks. The blocks were then serially sectioned at 5 mm thickness using microtome and were mounted on polylysine coated slides, deparaffinised using xylene, rehydrated and stained with hematoxylin and eosin, dehydrated, cleared and mounted on DPX under glass coverslips. The slides were then observed under a light microscope connected to a camera to capture images**.**

## Statistical analysis

All data are presented as Mean ± S.E.M. The significance of difference among the groups was assessed using one-way analysis of variance (ANOVA) followed by Tukey's test using Graph pad PRISM software and *P<0.05* was considered significant.

# RESULTS

## Effect of EEBL on serum SOD

The effect of EEBL on SOD was shown in the Table 1. The SOD levels were significantly increased (*P<0.001*) in group II compared with group I. Group II and group IV shown a significantly (*P<0.01; P<0.001*) decreased in SOD levels when compared with group II. Moreover, group IV showed statistical more significant decreased (*P<0.001*) in the level of SOD when compared with group III.

## Effect of EEBL on serum CATALASE

The effect of EEBL on catalase level shown in the Table 1. the catalase levels were significantly increased (*P<0.001*) in group II compared with group I. Group III and group IV shown a significantly (*P<0.05; P<0.001*) decreas1e in catalase levels when compared with group II. Moreover, group IV showed statistical more significant decreased (*P<0.001*) in the level of catalase when compared with group III.

## Effect of EEBL on serum total protein

The effect of EEBL on TOTAL PROTEIN was shown in the Table 1. the total protein levels were significantly increased (*P<0.001*) in group II compared with group I. Group III and group IV shown a significantly (*P<0.01*) decreased in total protein levels when compared with group II.

## Effect of EEBL on serum SGOT & SGPT

The effect of EEBL on SGOT & SGPT was shown in the Table 1. The SGOT& SGPT levels were significantly (*P<0.001*) increased in group II compared with group I. Group III and group IV shown a significantly (*P<0.05; P<0.01*) decrease in SGOT levels when compared with group II.

## Effect of EEBL on serum triglycerides

The effect of EEBL on triglycerides was shown in Table 2. The TG levels were significantly increased (*P<0.001*) in group II compared with group I, there were significantly (*P<0.001; P<0.001*) decreased TG levels in group III &group IV when compared with group II.

## Effect of EEBL on serum cholesterol

The effect of EEBL on cholesterol was shown in Table 2. in this study the cholesterol levels were significantly increased (*P<0.001*) in group II compared with group I. Group III and group IV shown a significantly (*P<0.01; P<0.001*) decreased in cholesterol levels when compared with group II. Moreover, group IV showed statistical more significant decreased (*P<0.001*) in the level of cholesterol when compared with group III.

## Effect of EEBL on serum HDL

The effect of EEBL on HDL was shown in the Table 2. The LDL levels were significantly increased (*P<0.001*) in group II compared with group I. Group III and group IV shown a significantly (*P<0.01; P<0.001*) decreased in LDL levels when compared with group II. Moreover, group IV showed statistical more significant decreased (*P<0.001*) in the level of LDL when compared with group III.

## Effect of EEBL on hormones

The effect of EEBL on Hormones was shown in the Table 3. Testosterone & LH levels were significantly increased (*P<0.001*) in group II compared with group I. Progesterone, Estradiol & FSH levels were decreased (*P<0.001*) in group II compared with group I. Group III and group IV shown a significantly (*P<0.05, P<0.01; P<0.001*) altered the hormones levels when compared with group II.

**Table 1: Antioxidants and liver enzymes.**

| Groups | SOD (U/min/mg protein) | CATALASE (u/mg protein) | TOTAL PROTEIN (mg/ml) | SGOT (IU/L) | SGPT (IU/L) |
|---|---|---|---|---|---|
| Group I (SO) | 2.33 ± 0.16 | 46.48 ± 3.28 | 4.90 ± 0.30 | 28.00 ± 1.22 | 32.00 ± 0.83 |
| Group II (DHEA 6mg/100gr) | 0.49 ± 0.21### | 18.15 ± 2.36### | 14.20 ± 1.06### | 61.00 ± 3.50### | 66.00 ± 0.83### |
| Group III (DHEA+EEBL100mg/kg) | 1.17 ± 0.29$$ | 29.77 ± 2.88$ | 9.22 ± 1.59$$ | 35.60 ± 3.09$ | 31.80 ± 0.66$$$ |
| Group IV (DHEA+EEBL200mg/kg) | 2.53 ± 0.277&&& | 38.09 ± 1.99&&& | 2.60 ± 0.40&& | 41.80 ± 7.37&& | 27.80 ± 0.37&& |
| GROUP V (DHEA+ Standard (CC) 100ug/kg) | 2.52 ± 0.22*** | 40.02 ± 1094*** | 4.60 ± 0.24*** | 30.60 ± 0.24*** | 26.80 ± 0.58*** |

Values are expressed as mean ± SEM of n=6 animals. Symbol represents the statistical significance done by ANOVA, followed by Tukey's tests. #P<0.05, ##P<0.01, ###P<0.001 indicates comparison of negative control group with control group. $P<0.05, $$P<0.01, $$$P<0.001 indicates comparison of low dose group with negative control. ***P<0.001 indicates comparison of high dose with a negative control.

**Table 2: Lipid parameters.**

| Groups | TRIGLYCERIDES (mmol/L) | TOTAL CHOLESTROL (mg/dl) | LDL (mg/dl) |
|---|---|---|---|
| Group I (SO) | 0.94 ± 0.18 | 71.63 ± 1.92 | 52.02 ± 1.14 |
| Group II (DHEA 6mg/100gr) | 2.40 ± 0.15### | 90.56 ± 1.08### | 78.73 ± 0.73### |
| Group III (DHEA+EEBL100mg/kg) | 1.31 ± 0.16$$$ | 77.92 ± 2.84$ | 69.32 ± 1.61$$ |
| Group IV (DHEA+EEBL200mg/kg) | 0.96 ± 0.10&&& | 68.42 ± 3.9&&& | 55.84 ± 2.24&&& |
| GROUP V (DHEA+ Standard (CC) 100ug/kg) | 0.88 ± 0.38*** | 73.53 ± 3.20*** | 56.80 ± 3.20*** |

Values are expressed as mean ± SEM of n=6 animals. Symbols represents the statistical significance done by ANOVA, followed by Tukey's tests. #P<0.05, ##P<0.01, ###P<0.001 indicates comparison of negative control group with control group. $P<0.05, $$P<0.01, $$$P<0.001 indicates comparison of low dose group with negative control. ***P<0.001 indicates comparison of high dose with a negative control.

**Table 3: Reproductive hormones.**

| Groups | TESTOSTERONE (ng/dl) | PROGESTERONE (ng/ml) | ESTRADIOL (pg/ml) | FSH (IU/L) | LH (IU/L) |
|---|---|---|---|---|---|
| Group I (so) | 0.8117 ± 3.073 | 9.17 ± 0.668 | 156.6 ± 13.18 | 11.48 ± 0.945 | 2.175 ± 0.356 |
| Group II (DHEA 6mg/100gr) | 6.933 ± 0.953### | 0.555 ± 0.246 ## | 23.75 ± 7.112### | 2.418 ± 0.909$$$ | 19.79 ± 2.43### |
| Group III ( DHEA+EEBL 100mg/kg) | 2.490 ± 0.539$$$ | 3.383 ± 0.671 $ | 51.52 ± 10.48$ | 6.55 ± 0.701 | 2.342 ± 0.381$$$ |
| Group IV (DHEA+EEBL 200mg/kg) | 0.9.3 ± 0.166&&& | 8.68 ± 1.55&& | 132.4 ± 12.93&&& | 10.85 ± 0.99&&& | 1.817 ± 0.443&&& |
| Group v (DHEA +CC 100ug/kg) | 0.733 ± 0.1331*** | 8.967 ± 2.321** | 142.5 ± 13.34*** | 11.36 ± 0.99*** | 1.835 ± 0.209*** |

Values are expressed as mean ± SEM of n=6 animals. Symbols represents the statistical significance done by ANOVA, followed by Tukey's tests. #P<0.05, ##P<0.01, ###P<0.001 indicates comparison of negative control group with control group. $P<0.05, $$P<0.01, $$$P<0.001 indicates comparison of low dose group with negative control. ***P<0.001 indicates comparison of high dose with a negative control.

### Effect of EEBL on histopathology of ovaries

Figure 1 indicates that secondary oocytes inside the secondary follicles appeared normal. Figure 2 indicates that the ovary showing multiple cysts. Figures 3 & 4 Treatment with EEBL successfully restored the ovarian follicles in comparison with the PCOS group.

## DISCUSSION

PCOS is an increased incidence worldwide is a matter of great concern today. Although there are few medications that are being used in the management of PCOS, there are not completely effective in treating the condition and also women using them are prone to a higher risk of their side effects.[18] Abnormal follicular maturation or acceleration of follicular atresia is reported with elevated intraovarian androgen levels. Therefore, intraovarian androgen excess resulting from hyperandrogenemia may result in abnormal follicular development and polycystic ovary condition.

The working of this model was confirmed by regular examination of vaginal smears and the presence of persistent vaginal cornification. Due to the administration of DHEA, there was a marked increase in testosterone levels when compared to control animals indicating the hyperandrogenism status in PCOS condition. Betel leaf extract was able to normalize serum Testosterone levels similar to that of Clomiphene citrate by its antiandrogenic effect.[13]

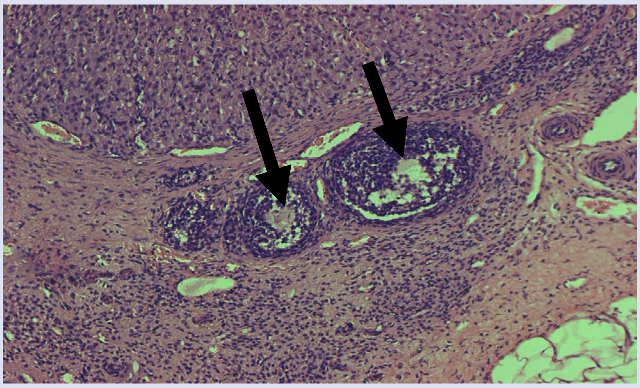


**Figure 1:** Section of the ovary from control animal showing large follicles (H&EX10).

Secondary oocytes inside the secondary follicles appeared normal- Black arrow.

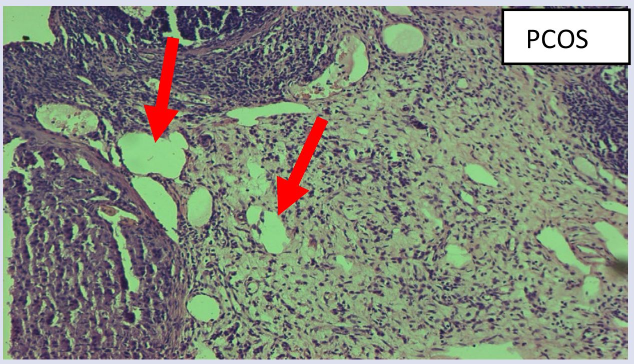



**Figure 2:** Section of the ovary from PCOS induced animal showing multiple cysts (H&EX10).

Multiple cystic conditions are noticed in the medullary region of ovary - Red arrow.

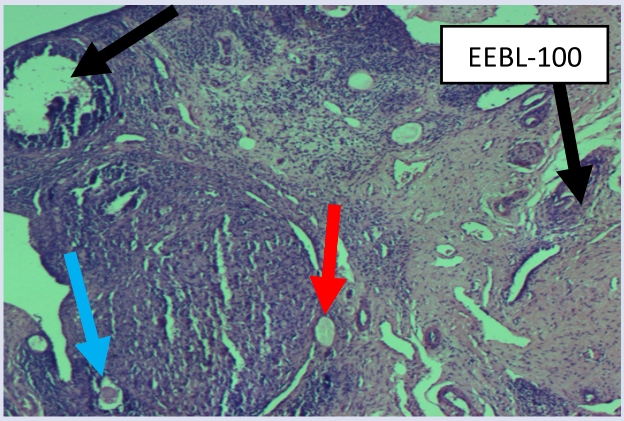



**Figure 3:** Section of the ovary from EEBL treated (100 mg/kg) (H&E X10).

- The cortex region appeared normal. Normal secondary follicles with secondary oocytes noticed in cortex region – Black arrow
- Primary follicles with primary oocytes appeared normal – Blue arrow
- Few cysts appeared in LD EEBL – Red arrow.

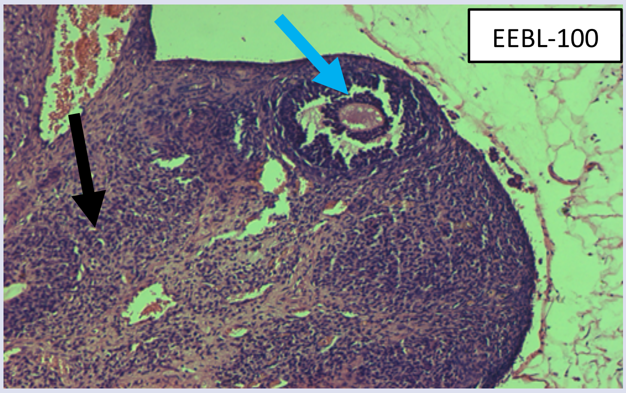



**Figure 4:** Section of the ovary from EEBL treated (200 mg/kg) animal showing follicles (H&EX10).

- Corpus luteum appeared normal – Black arrow
- Secondary follicles with secondary oocytes appeared normal – Blue arrow

Anovulation is due to decreased progesterone levels.[9] Serum levels of Progesterone and Estradiol were decreased in PCOS induced group, treatment with Betel leaf extract successfully restored Progesterone and Estradiol level to normal.[19,20]

Dyslipidemia was seen in PCOS, imbalances in the lipid profile decreased HDL, increased TG, TC and LDL are observed in biochemical examination.[22,23] In negative control group showed a notable increase in TC, TG's and LDL levels. Betel leaf extract showed its antihyperlipidemic action by considerably decreasing serum TC, TG's, LDL levels.

Oxidative stress is one of the pathological factors for PCOS.[24] Oxidative stress lead to decreasing antioxidant levels seen PCOS. Increased oxidant levels may increase androgen production.[25] In the present study, it was observed that the PCOS animals exhibited elevated oxidative stress markers like SOD, Catalase. SOD and Catalase activity was significantly diminished in the PCOS group and concomitant treatment with betel leaf extract restored their activities.

Women's with PCOS have increased the risk for liver diseases, increased SGPT and SGOT are seen in negative control group, treatment with

Betel leaf extract effectively attenuated the imbalances of liver enzymes associated with PCOS by its Hepatoprotective nature.[26]

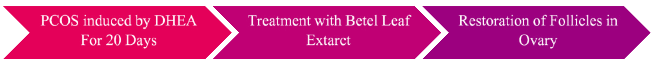
Histopathology of ovary in negative control group reveals that the presence of multiple cysts and they are in immature state seen in the medullary region of ovary. Treatment with Betel leaf extract has restored multiple into normal and immature follicles into primary and secondary oocyte state. HD of Betel leaf extract has shown that corpus luteum into normal and appearance of secondary follicles with secondary oocyte.[9]

## CONCLUSION

Betel leaf extract has shown its beneficial effect similar to Clomiphene citrate in treating PCOS related anovulation and multiple cysts. In our research Betel leaf successfully restored the antioxidants, hormonal and lipid profile. These ameliorative effects may be ascribed to its potential towards the management of PCOS and improving fertility rate.

## HIGHLIGHTS

1. Dehydroepiandrosterone given for 20days has induced PCOS (Polycystic Ovary Syndrome) in rats.
2. Treatment with Betel Leaf extract effectively ameliorated the PCOS induced hormonal defects.
3. Hormones like Progesterone, Estradiol, and FSH are restored to normal and testosterone was deceased**.**
4. In Ovary Histopathology clearly states that the presence of multiple cysts in the PCOS group, treatment with betel leaf extract proven its therapeutic efficacy by the generation of follicles.

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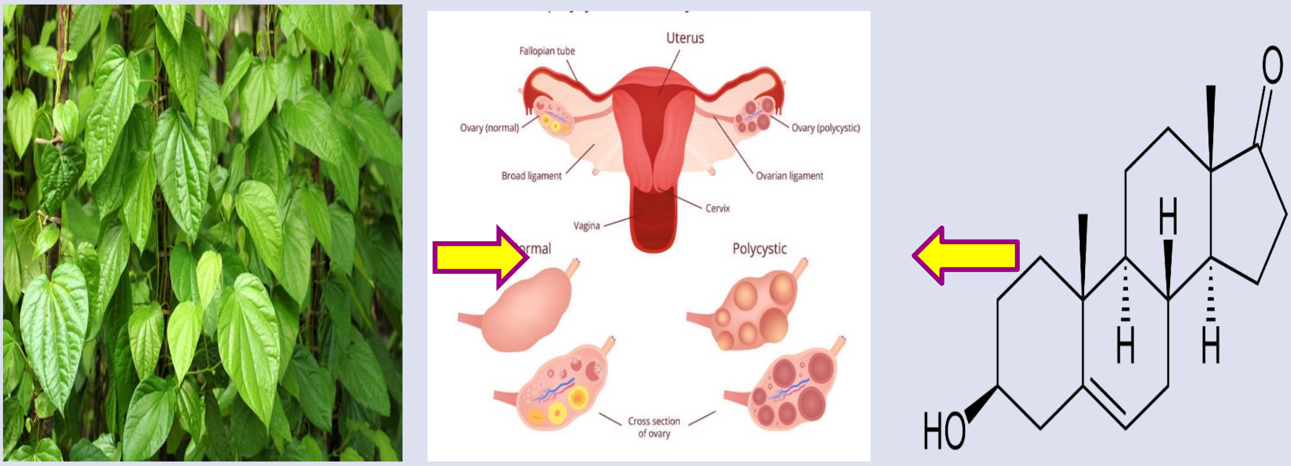
## GRAPHICAL ABSTRACT

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