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Antioxidant and Ameliorative Effects of *Basella alba* L. On Letrozole-Induced Polycystic Ovarian Syndrome in Rats

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ABSTRACT

Basella alba L. (BA) is utilized in Nigeria to manage Polycystic Ovarian Syndrome (PCOS), according to an ethnobotanical study. However, this claim has not been supported by any scientific data. This study investigated the effect of the methanol leaf extract of the plant in alleviating polycystic ovary conditions in rats. The methanol extract of BA was subjected to a brine shrimp lethality assay. Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and the DPPH assay were used to measure antioxidant activity. To induce PCOS in thirty female Wistar rats (180-200 g), letrozole (1 mg/kg) was used. For 15 days, BA leaf methanol extract (100 mg/kg body weight) and Clomiphene citrate (1 mg/kg body weight), a standard medication, were given. Microscopically, the ovaries underwent histopathological investigation. ELISA was used to assess luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol levels. One-way ANOVA was used to analyze the data, and $P < 0.05$ was set as the threshold for Dunnett's Multiple Comparison Test. LC_{50} value for the extract was 17.29±0.15 µg/mL. The DPPH radical-scavenging activity of the ethyl acetate fraction was the highest. When PCOS animals were given BA, the ovarian stroma was normal and the granular cells were leutinized. The level of estradiol increased but the level of LH was not significantly different between the BA-treated group (0.23±0.03 mIU/mL) and the untreated PCOS group (0.23±0.01 mIU/mL). *Basella alba* leaf displayed ameliorative effects on polycystic ovary conditions in rats. This support the use of this plant in the management of PCOS.

***Keywords*:** Antioxidant Assay, Brine shrimp lethality assay, polycystic ovarian syndrome, Histopathology

Introduction

An endocrine and reproductive disorder called polycystic ovarian syndrome (PCOS), which affects 5–18% of women, is multifactorial.[1] PCOS affects one out of every six infertile women in Nigeria.[2] According to the Rotterdam criteria, the diagnosis is made if two of these three symptoms are present: hyperandrogenism, anovulation, and polycystic ovaries.[1] The hyperandrogenism in PCOS is due to excessive stimulation of the theca cells by gonadotropin-releasing hormone.[3] It is characterized by insulin resistance, inflammation, oxidative stress, obesity, type II diabetes, hyperlipidemia, cardiovascular dysfunctions, depression, anxiety, impaired follicular development, absent or irregular menstruation, and fertility problems.[4,5] Imbalance in the level of reproductive hormones (estrogens, follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone and progesterone) is observed in women with PCOS.[6] Insulin and P13k-Akt signalling pathways have also been discovered to be involved in the pathogenesis of PCOS with hyperinsulinemia indirectly increasing androgen levels.[7-9]

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High androgen and LH levels disrupts normal ovarian functions leading to the development of multiple cysts.[10]

Genetics, sedentary lifestyle, birth control pills, stress, consumption of processed foods are the common causes of PCOS.[11] Management and treatment include medications (metformin, clomiphene citrate, GnRH agonists, antiandrogens) and dietary and lifestyle modifications targeting metabolic and hormonal dysfunction.[12,13] However, the symptoms and hormonal dysfunctions are not totally addressed by these treatments. Long-term use of these medications is expensive and can result in side effects such as abdominal cramps, dizziness, weight gain, thromboembolism.[14] Hence the search for cost-effective herbal treatments with little or no side effects.

Traditionally, medicinal plants have been utilized to manage and treat various diseases and they have been discovered to greatly improve PCOS condition by decreasing the androgen level, enhancing insulin sensitivity and improving ovulation.[13,15] *Basella alba* of the family Basellaceae is a leafy vegetable. It is also known as Malabar spinach, climber spinach and 'amunu-tutu'. It has antioxidant, anti-inflammatory, antiulcer, antibacterial, wound healing, CNS depressant, antiproliferative, and antimicrobial activities due to the presence of various phytochemicals.[16,17] According to an ethnobotanical survey by Ogunlakin and Sonibare,[18] *Basella alba* leaf juice is used traditionally for managing and treating irregular menstrual cycle and female infertility but there is no scientific evidence to justify its application. Hence this study was designed to investigate the effects of *Basella alba* in alleviating the polycystic ovary conditions in wistar rats.

Materials and Methods

Chemicals and reagents

All solvents used for the study, which includes *n*-hexane, chloroform, dichloromethane, ethyl acetate and methanol, were purchased from BDH Ltd, England. The reagents, such as Folin–Ciocaltaeu reagent, Sodium bicarbonate, $FeCl_3$, CH_3COOK, $AlCl_3$, Vitamin C (ascorbic acid), Gallic acid, Quercetin, PCA, Rutin, and l, l-diphenyl-2-picryl-hydrazyl (DPPH) were bought from Merck, Germany. Brine Shrimp Egg was bought as Artemia salina cysts, from a pet shop in United State of America.

Plants collection and extraction

In August 2017, leaves of *Basella alba* were collected in Oluponna, Osun state, Nigeria, and were identified in the Bowen University Herbarium with voucher number of BUH:039. They were air-dried and using an electric grinding machine, they were pulverized into a coarse powder. The powdered sample (1 kg) was macerated in 10 L of methanol at room temperature for 72 hours while being sometimes stirred and shaken. Next, the mixture was filtered through a new cotton plug and Whatman (Number l) filter paper. The crude extract was concentrated using a rotary evaporator at 40 °C *in vacuo*. The percentage yield was calculated.

Solvent-solvent partitioning

After being dissolved in a 3:1 mixture of methanol and water, the crude extract of *Basella alba* (leaf) was put into several separation funnels. A 50 mL aliquot of this extract was partitioned with n-hexane, dichloromethane (DCM), and ethyl acetate. The fractions from each aliquot of the n-hexane, DCM, and ethyl acetate fractions were combined and evaporated *in vacuo* to produce fractions that were kept in airtight containers for subsequent preliminary testing. The weights of the fractions were established, and each fraction's percentage yield was computed using those weights.

Brine shrimp lethality assay

For the samples to be evaluated for toxicity, dried methanol extract (30 mg) and the reference medication (cyclophosphamide) were reconstituted in MeOH (3 mL) to create a stock solution of l0 mg/mL (10,000 µg/mL). Serial dilution of a part of the stock solution produced concentrations of 1000, 500, 100, 10, and 1 µg/mL. Ten nauplii were chosen after 48 hours of hatching and transferred into each vial with different sample concentrations using a disposable Pasteur pipette. This arrangement was left on for a full day. Sea water was used as a control. Nauplii were regarded as dead when they stopped moving and settled at the bottom of the test tubes.[19] Finney's Probit analysis was used to determine the fatal doses of plant extract and the standard, LD_{50}, resulting in 50% shrimp larva death at 95 percent confidence intervals.

Measurement of total phenolic content (TPC)

The total phenolic content of the methanol extract and fractions was evaluated using the Folin-Ciocalteu spectrophotometric technique.[15,20] To 1 mL aliquots of each test sample (100 g/mL), five milliliters of the Folin-phenol Ciocalteu's reagent were applied. Then, each vial's combination received 4 mL of a Na_2CO_3 solution in distilled water (7.5 g/100 mL), which was then incubated for 30 minutes at 27 °C in the dark. 1 mL of methanol was used to make a blank. Three times each sample was analyzed. Using a UV-VIS spectrophotometer, the mixture's absorbance at 765 nm was measured after 30 minutes of incubation. (Spectrumlab 752S). Using a linear dosage response regression curve created from the absorbance of gallic acid, the total phenolic content was determined. Milligramme (mg) of Gallic acid equivalent/g of the dry weight of extract was used to express the TPC results.

Measurement of total flavonoid content (TFC)

This study used aluminum chloride colorimetry using the Woisky and Salatino [21] modified technique. The calibration curve was drawn using quercetin as the standard, which was diluted in ethanol (100-6.25 g/mL). Additionally, 30 mg of each test substance was reconstituted in 30 mL of methanol to create test samples. Separately, one milliliter of each diluted quercetin solution or test sample (1.0 mL) was combined with 2.0 milliliters of water, 2.0 milliliters of methanol, 0.10 milliliters of aluminum chloride, and 0.10 milliliters of potassium acetate. The same amount of 1% aluminum chloride was used in the blank, but pure water was used instead. Three times each sample was analyzed. A UV-VIS spectrophotometer was used to measure the reaction's absorbance at 455 nm following 30 minutes of incubation at 27 °C. (Spectrumlab 752S). Each test samples' total flavonoid content was calculated using the equation derived from the quercetin calibration curve and reported as mg of quercetin equivalent per g of extract.

Diphenyl picryl hydrazyl (DPPH) radical scavenging assay

Using a slightly modified version of Bursal and Gülçin's method [22], the free radical scavenging capacity of the methanol extract and fractions was assessed. For the DPPH test, 3 mL of 0.004% of 1, 1-diphenyl-2-picryl-hydrazyl-hydrate (DPPH), and 2 mL of *B. alba* crude extract, solvent fractions, as well as standards (ascorbic acid and rutin) were added separately. Methanol in the amount of two milliliters was introduced as the test sample to the control. The reaction mixtures were heated in the dark for 30 minutes at 27 °C after being vigorously shaken. Each vial's absorbance was measured at 517 nm with a Spectrumlab 752S UV-VIS spectrophotometer, and the results were represented as percentages of free radical scavenging activity (%FRSA).

$$\%\text{FRSA} = \{1 - \left(\frac{\text{ABS}}{\text{ABD}}\right)\} x\ 100$$

Where ABS is the solution's absorbance with the sample, and ABD is the solution's absorbance with DPPH. Using the graphed relationship between scavenging activity and the concentration of the test substances, the percentage inhibition concentration (IC_{50}) value was determined (using linear regression analysis).

In vivo PCOS study

Doses for plant extract

For treating irregular menstruation and related gynecological diseases, human therapeutic dosages of *B. alba* leaf powder were mentioned in an ethnobotanical study as ranging from 1 to 9 g.[18] Using a conversion factor based on body surface area (conversion factor = 0.018), the dosage for rats was estimated. This was done by multiplying it by a number that took into account the animal's body surface area and dividing it by the weight of an adult male human, which is 60 kg.[23,24] So, in this investigation, a dosage level of 100 mg/kg body weight was employed.

Selection and grouping of animals for assay

Twenty female rats (weighing between 150 and 200 g) without gestation but with a normal estrous cycle were divided into four groups of five at random. To induce PCOS, 15 of these rats received oral administration with letrozole at a dose of 1 mg/kg every day for 21 straight days using 0.5% carboxymethyl cellulose (CMC) as a vehicle. The following samples were administered to the groups: For 15 days, group one was given 100 mg/kg of *B. alba* extract, whereas group two was given 1 mg/kg of clomiphene citrate (Colid, Pfizer Pharmaceuticals, USA). Two milliliters of 5% w/v CMC in distilled water were given to groups three (untreated disease control) and four.[24]

Determination of oestrous cycle pattern

The oestrous cycle's stages were identified using vaginal cytology.[15, 26, 27] To obtain vaginal lavage, a Pasteur pipette was gently introduced into the rat's vagina and filled with 0.1 mL of normal saline (0.9% NaCl). To see how the cells were distributed, the retracted vaginal fluid was put on a glass slide and immediately inspected under a microscope with an x 10 objective. This was carried out every day between 7 and 9 a.m. during the trial.

Blood and organ collection

The animals were euthanized for laparotomy after 15 days of therapy (24 hours after the final dose) with 2% sodium pentobarbital (30 mg/kg). To reduce diurnal variation, animals were put to sleep between 0900 and 1100 h, and the tissue architecture of their ovaries was

examined.[15] The amount of blood drawn, 8 mL, was used to calculate the concentrations of estradiol, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) in each group.

Hormonal analysis
Luteinizing and follicle-stimulating hormones were assessed in serum samples using Dialab's ELISA, while estradiol levels in serum samples were determined using the ELISA (Fortress Diagnostics Limited, Unit 2C Antrim Technology Park, Antrim, BR4l lQS, United Kingdom).

Histopathological evaluation of ovary
A routine technique was used to examine the ovaries.[28] It was stained using the haematoxylin and eosin method. The tissues were evaluated, cut into pieces no more than 4 mm thick, and then put in cassettes with labels already on them. These ovarian tissue fragments were immersed in 10% formal saline for 24 hours. An automatic tissue processor (Leica TP l020) was used to process the tissue automatically. The tissues were dehydrated using dehydrating agents including l0% formal saline and alcohol (70%, 80%, 90%, and 95%). The tissues were poured into a metal mold, submerged in molten paraffin wax, and hardened on a cold plate. The manufactured tissue block was taken out of the mold and shaped with a 6 m rotating microtome to disclose the tissue surface. At 4 m intervals, surfaces were placed on the ice and trimmed (ribbon section). The sections floated on a hotplate (Raymond Lamb) set to 60° C for one hour and were picked up with clean, labeled slides. They were then examined under a light microscope using x l00 and x 400 objectives.

Statistical analysis
The mean and standard error of the mean were used to present the values. GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego, California, USA, was used to analyze the data using one-way analysis of variance (ANOVA) and compare the group means using Dunnett's Multiple Comparison tests and Bonferroni tests. P values of 0.05 or higher were regarded as significant.

Results and Discussion

Plant extraction and toxicity study
The crude extract, and hexane, DCM, and ethyl acetate fractions yields were 13.91, 11.80, 8.20, and 15.20%, respectively as shown in Table 1.

The brine shrimp lethality assay (BSLA) is a widely used method for determining the toxicity of medicinal herbs.[29] The LC_{50} values were 29.41±0.12 µg/mL and 224.74±0.35 µg/mL for *B. alba* leaf methanol extract and cyclophosphamide (standard), respectively (Table 2). Meyer's toxicity index[30] showed that the methanol extract of *Mormodica charantia* had an LC_{50} > l000 µg/mL (non-toxic). The toxicity levels of this plant extract were further classified using Clarkson's criterion for preliminary assessment of toxicity.[31] It has been revealed that most bioactive principles that cause toxicity are occasionally biosynthesized in medicinal herbs alongside other medicinally important principles.[32] The recent attention in toxicity assessment of medicinal herbs and phytomedicines provides reassurance that phytotoxicity should not be regarded as a negative feature of herbal medicine, but rather as a useful tool in drug research and development.
Numerous human diseases, including cancer, inflammatory arthritis, diabetes, and atherosclerosis, are exacerbated by oxidative damage. Synthetic antioxidants' use has been restricted because of their potential health risks, as well as their low solubility and considerable antioxidant activity.[15,33,34] This necessitates the discovery of organic antioxidants with improved solubility and safety as a replacement. Numerous plant species have recently been studied in the search for new antioxidants.[33, 35] Accessible and less toxic phyto-antioxidants that are significant therapeutic constituents of food have been effective in reducing the vulnerability of reactive oxygen species (ROS).[36] The extract of *B. alba* crude extract, hexane, DCM, and ethyl acetate fractions had IC_{50} values of l56.7l ± ll.03, l72.40 ± 2.22, 7.l7 ± l.50, and 4.23 ± 0.4l µg/mL, respectively (Table 3). Because of the existence of -OH functional group, phenols are a major constituent of medicinal plants that serve a variety of therapeutic purposes, including free radical scavenging. Several studies have revealed a comparative relationship between phenolics as well as anti-oxidant ability.[35,37] The hydroxyl functional groups in the skeleton of the phenolic compound structure could be responsible for the high scavenging property of the extract and solvent fractions studied in this study. The DCM fraction contained the most total phenolics (5l23.34 ± 4.7l µg GAE/g), while methanol extract contained the most total flavonoids (304.07 ± l.09 mg QE/g) as shown in Table 3. The findings support previous research findings that established a parallel relationship between phenolics and antioxidant activity.[38]

Table 1: The percentage yield of the plants crude and solvents fractions

Plants	% yield (w/w) MeOH	% yield (w/w) *n*-hexane fraction	% yield (w/w) DCM fraction	% yield (w/w) ethyl acetate fraction
Basella alba	l3.9l	ll.80	8.20	l5.20

The percentage yields of the solvent fractions were calculated and expressed as the percentages of the ratio of solvent fractions to the crude extract

Table 2: The LC_{50} (µg/mL) of *B. alba* leaf methanol extract

Extracts	LC_{50} (µg/mL)
Basella alba	29.4l ± 0.l2***
Cyclophosphamide	224.74 ± 0.35

Data are shown as means with a standard error of the mean (n = 3). One-way ANOVA, then Dunnett's Multiple Comparison Test at a P value of 0.05. The extract was compared to the reference standard (cyclophosphamide), and the level of significant difference was indicated with a ***.

In vivo study
Letrozole-induced PCOS animal model was used in this study because it imitates clinical characteristics such as anovulation, hyperandrogenism, follicular cysts and metabolic dysfunctions observed in women with PCOS.[39, 40] In this study, PCOS induction was confirmed by irregular estrous cycle through vaginal smear (Figure 1). The average duration of a normal estrous cycle in a rat is four days. In the disease control group, a reduction in estrus and increase in diestrus duration was observed compared with the normal control group showing an irregular estrus cycle. This is consistent with reported scientific findings.[10,15,41,42] Letrozole-induced estrus irregularity could be caused by hyperandrogenism, imbalance in steroidal hormones responsible for follicular development and ovulation.[42] The estrus cycle was restored following treatment with *Basella alba.* This is consistent with the traditional claim of *Basella alba* being used for treating menstrual irregularities.[19]
Letrozole was administered to the illness control group in this study, and the difference between that group's level of estradiol and the normal control group's was considerable (Table 4). Letrozole is a non-steroidal aromatase inhibitor that reduces the ovary's androgen conversion to estrogens, which lowers estradiol production and raises testosterone synthesis.[43] It has been determined that an increase in testosterone levels is what causes PCOS. The lack of the corpus luteum, which produces estradiol, may also be to blame for the drop in estradiol levels.[42] But compared to the disease control group, there was a discernible rise in the estradiol content in the *Basella alba*-treated group. In the illness control group compared to the normal control group, there was a significant increase in LH concentration and a drop in FSH level.

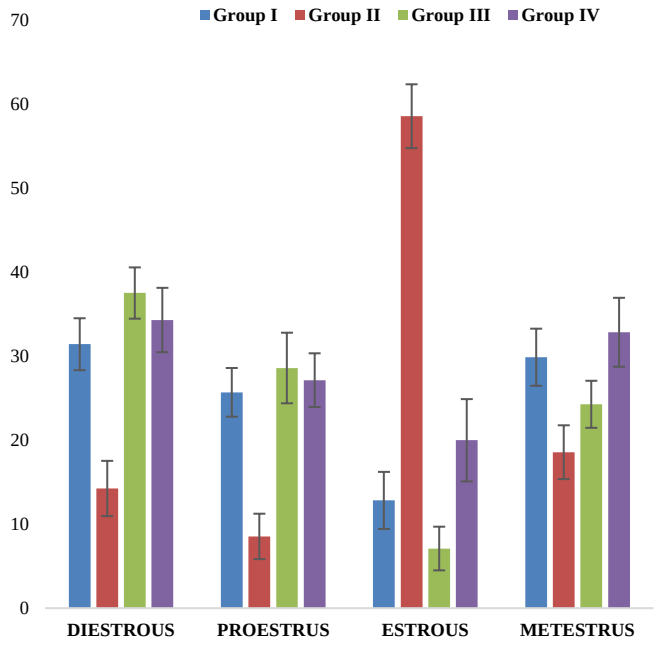

Figure 1: Estrous cycle phase index in albino rats throughout the first five days of therapy.
Estrous phase index (%) = Days with a clear phase smear x 100/total treatment time (l5 days). The data indicate the mean with SEM for the animals, with n = 5. Group I received l00 mg/kg of *Basella alba*, Group II received l mg/kg of clomiphene citrate administered orally, Group III is a disease control group, while Group IV received a normal control group.

This difference between LH and FSH could be the result of increased pulsatile GnRH production and overstimulated anterior pituitary gland caused by excess androgens.[44,45,46] When compared to the disease control group, the *Basella alba*-treated group had significantly higher FSH levels but no appreciable variation in LH concentration. Comparable investigations by Ndeingang *et al*.[10] and Pachiappan *et al*.[41] found that the illness control group had higher levels of LH and lower levels of FSH and estradiol than the normal control group.
In PCOS, there is an increase in antral follicle count, ovarian stroma thickness, ovarian thickness, and theca cell hyperplasia.[47] This study found that the Basella alba-treated group had no fibrosis, whereas the disease control group's granular cells had hyperplasia and the ovarian stroma was normal. (Figure 2). This demonstrates that in the PCOS rats, *Basella alba* restored ovarian morphology.

Conclusion

This study presented the first scientific data on the effect of *Basella alba* leaf on PCOS. Treatment with *B. alba* methanol extract caused upsurge in the level of estradiol. The results of the *in vivo* study revealed that the leaf extract have curative effect on irregular estrual cycle and hormonal imbalance associated with PCOS. The crude extract of *Basella alba* leaf had beneficial effects on polycystic ovary conditions in female rats. This support the use of this plant in the management of PCOS.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 3: *Basella alba* leaf extract and fractions ability to scavenge free radicals (DPPH IC_{50}), as well as their TPC and TFC values

Extracts	Solvents	DPPH (IC_{50}) (µg/mL)	TPC (µg GAE/g)	TFC (mg QE/g)
Basella alba	Crude	l56.7l ± ll.03***/***	277.l7 ± 3.89***	304.07 ± l.09^{H}
	Hexane	l72.40 ± 2.22***/***	358l.67 ± 20.04^{H}	224.07 ± 0.0l***
	DCM	7.l7 ± l.50^{NS}/^{NS}	5l23.34 ± 4.7l ^{NS}	223.8l ± 0.05^{NS}
	Ethyl acetate	4.23 ± 0.4l^{NS}/^{NS}	50l6.67 ± 4.7l***	205.47 ± 0.l4^{H}
Ascorbic acid		2.76 ± 0.0l		
Rutin		20.6 ± 9.26		

Data are provided as mean with SEM (n = 3). Dunnett's Multiple Comparison Test at P<0.05, then one-way ANOVA. Comparisons were made between the highest TPC and TFC extracts from each solvent, designated by the letter "H," and other extracts from that solvent. At P<0.05, the degrees of significance are indicated by the letters * (significant), ** (moderately significant), and *** (very significant). NS denotes that there are no appreciable differences between "H" or the solvent extract and fractions. Each sample's IC50 DPPH was compared to standards (ascorbic acid and rutin). The order of relevance from rutin and ascorbic acid is shown by the asterisks separated by slashes (/). NS stood for minimal deviation from the standards.

Table 4: Effect of the test sample on estradiol, FSH, and LH.

Parameters	Group I	Group II	Group III	Group IV
LH (mIU/mL)	0.23 ± 0.03*	0.23 ± 0.0l*	0.23 ± 0.0l*	0.22 ± 0.0l
FSH (mIU/mL)	l.l0 ± 0.23*	0.75 ± 0.05*	0.73 ± 0.03*	0.93 ± 0.l9
Estradiol (pg/mL)	7.l8 ± 0.49*	7.63 ± 0.89*	5.70 ± 0.77*	9.84 ± l.44

Data are provided as the mean with SEM (n = 5). ANOVA evaluation was followed by Bonferroni analysis. * Mark p < 0.001 vs Group IV. (Normal control). Group I received l00 mg/kg of Basella alba, Group II received l mg/kg of clomiphene citrate administered orally, Group III received a disease control group, and Group IV received a normal control group.

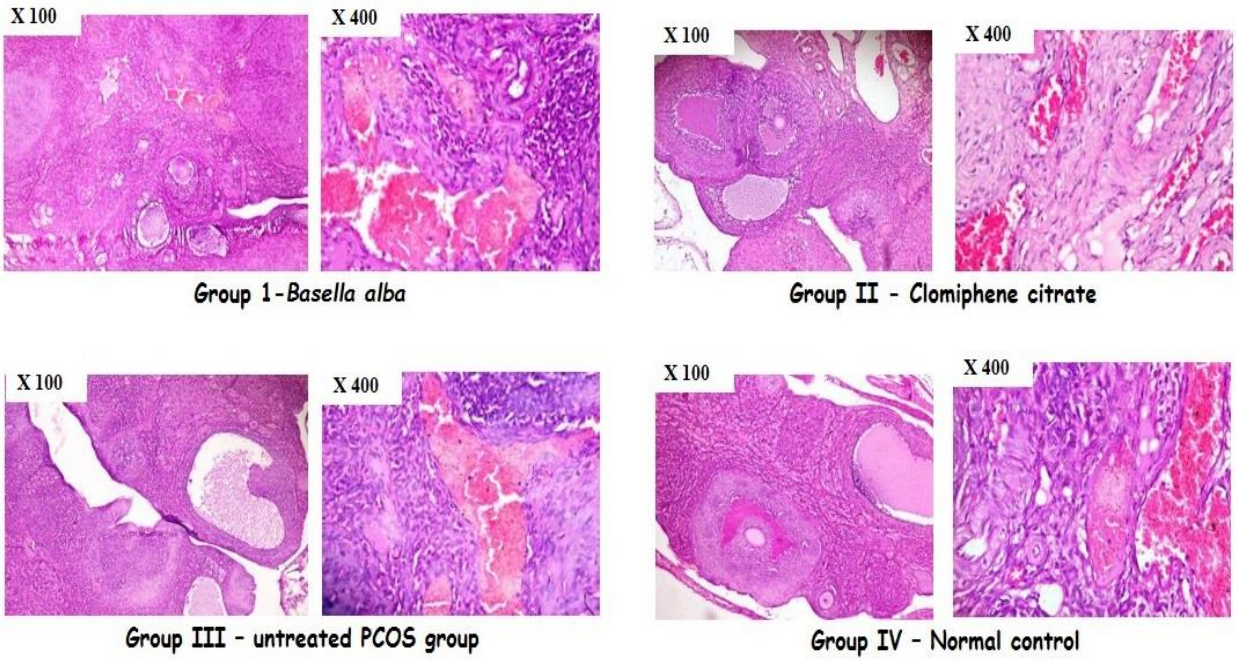

Figure 2: Animal experimentation subjects' ovary photomicrographs.
Group I contains Basella alba at a dose of 1000 mg/kg body weight; Group II has clomiphene citrate at a dose of 1 mg/kg bw; Group III contains a disease-free group; and Group IV contains a healthy group.

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