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RESEARCH ARTICLE

In Vivo Study on the Therapeutic Potential of *Nothapodytes nimmoniana* (J. Graham) Mabb. in DHEA-induced Polycystic Ovary Syndrome in Female Albino Mice

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Abstract

Background: Polycystic Ovary Syndrome (PCOS) is the most common endocrine disorder among women of reproductive age, characterised by elevated androgen levels, chronic inflammation, and oxidative stress. Conventional therapies for PCOS, such as hormonal drugs and insulin sensitisers, often pose side effects and may not fully address the complex nature of the disorder. In recent years, there has been a growing interest in herbal medicine as a safer and more holistic alternative. **Aim:** The present study aims to evaluate the therapeutic potential of *Nothapodytes nimmoniana* methanolic leaf extract in the management of PCOS induced by Dehydroepiandrosterone (DHEA) in female albino mice, with a focus on its impact on reproductive, hormonal, and histopathological parameters. **Methods:** The investigation focused on the effects of its methanolic leaf extract of *N. nimmoniana* (MLENN), a medicinal plant of significant ethnobotanical value, on hormonal balance, antioxidant status, and the histoarchitecture of ovarian and uterine tissues in a DHEA-induced PCOS mice model. Female albino mice received MLENN at 200 and 400 mg/kg body weight for 20 days, with clomiphene citrate as the standard drug. Hormonal levels (LH, FSH, estrogen, progesterone, testosterone), antioxidant enzymes (SOD, CAT, GPX) and tissue morphology were assessed using histological and Scanning Electron Microscopy (SEM) techniques. **Results:** This study highlights the potential of *N. nimmoniana* as a promising natural resource for addressing PCOS through a multi-pronged approach involving significant reduction in serum LH, estrogen, and testosterone levels while increasing FSH and progesterone ($p<0.05$). PCOS-induced mice showed restored oestrous cyclicity and enhanced antioxidant activity (SOD, CAT, GPX). Histological and SEM analyses revealed reduced cyst formation, thinner theca layers, increased corpora lutea, thicker granulosa layers, and normalisation of uterine histoarchitecture. The 400 mg/kg MLENN dose showed the most pronounced therapeutic effects in all the experimental parameters.

Major Findings: This is the first report demonstrating the efficacy of MLENN in ameliorating PCOS symptoms in mice. The methanolic extract of *N. nimmoniana*, particularly at 400 mg/kg, significantly improves hormonal profile, oxidative stress markers, and reproductive tissue structure, indicating its potential as a natural therapeutic agent for PCOS.

Keywords: Antioxidant, Hormone Profile, Lipid Profile, *Nothapodytes nimmoniana*, PCOS, SEM

Abbreviations: PCOS - Polycystic Ovary Syndrome; LH - Leutenising Hormone; FSH - Follicle Stimulating Hormone; SOD - Super Oxide Dismutase; CAT - Catalase; GPX - Glutathione Peroxidase; LDL - Low Density Lipoprotein; SEM - Scanning Electron Microscopy; DHEA - Dehydroepiandrosterone; IAEC - Institutional Animal Ethical Committee; NIH - National Institutes of Health; WHO - World Health Organisation; ELISA - Enzyme-Linked Immunosorbent Assay; OECD - Organisation for Economic Co-operation and Development; AST - Aspartate Transaminase; ALT - Alanine transaminase; HDL - High-Density Lipoprotein; TC - Total Cholesterol; MLENN - Methanolic Leaf Extract of *N. nimmoniana*.

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1. Introduction

In recent times, women across all age groups have increasingly experienced various gynaecological disorders, often attributed to factors such as chronic stress, inadequate healthcare access, and nutritional deficiencies. Polycystic Ovary Syndrome (PCOS) is a gynaecological condition linked with hormonal imbalance and ovarian dysfunction, usually beginning during adolescence. PCOS was first reported by Stein and Laventhal in 1935, and globally, its prevalence is estimated to range between 8.7% and 21%[1-3]. PCOS may be characterised by (i) clinical or biochemical signs of elevated androgens, (ii) ongoing absence of ovulation, and (iii) the elimination of other related conditions. According to the Rotterdam criteria, a diagnosis of PCOS requires the presence of at least two out of three key indicators: elevated androgen levels, irregular or absent ovulation, and the presence of polycystic ovaries[1,4]. Although the exact aetiology of this condition is unknown, environmental and genetic factors play a major role. Poor dietary habits and a lack of physical activity are major contributors to PCOS progression[5]. World Health Organisation (WHO) estimates that PCOS occurs in 8-13 % of women at reproductive age; over 50 % of all cases remain undiagnosed[6]. Experts claim that in India, 10% of women are affected by PCOS[7]. In Assam, studies have shown that PCOS affects adolescents and 62.71% of women aged 24-29 in urban regions and 51.02% in rural areas[8]. The condition is also linked with increased oxidative stress, which plays a significant role in metabolic disturbances, insulin resistance, and hormonal irregularities. The metabolic enzymes, lipids, and endocrine hormones can be a prominent biomarker to evaluate a case of PCOS biochemically[9]. Though PCOS is incurable, it can be treated through a multifaceted approach that can help to manage the condition, which comprises pharmacological and non-pharmacological interventions. In the field of pharmaceuticals, letrozole is widely recognised as an effective agent for inducing ovulation in women with PCOS-related infertility. Metformin, a first-line medication for Type 2 diabetes, has shown its efficiency in controlling body weight in animal studies induced for PCOS. Clomiphene citrate, a traditional ovulation induction chemical, is used in gavage in prolonged studies due to its suitability of bearing a non-steroidal nature and its capacity to bind with estrogen receptors, then restore FSH levels and finally induce ovulation[10]. However, such chemicals have also been shown to have adverse effects on influencing other metabolic components.

In contrast, plant-derived compounds structurally and functionally similar to endogenous estrogens have emerged as a promising alternative due to their multi-targeted and gentler mode of action[11]. Medicinal plants have played a significant role in traditional healthcare systems in India since ancient times. Their therapeutic potential is largely attributed to the diverse range of phytochemicals they contain. Numerous bioactive compounds have been isolated and studied for their pharmacological properties. Indigenous communities across India possess extensive ethnobotanical knowledge and have long utilised medicinal herbs to treat various ailments, including gynaecological disorders. According to the World Health Organisation, approximately 80% of the global population relies on traditional medicine for primary healthcare[12]. Notably, the Northeastern Region (NER) of India is recognised as a biodiversity hotspot, rich in medicinal flora, where traditional healing practices remain widely prevalent[13]. *N. nimmoniana*, locally known as Gandheli in Assam, is a medicinally important deciduous tree belonging to the family Icacinaceae[14]. The species holds considerable ethnobotanical significance and is well-documented for its pharmacological activities, including anti-inflammatory, anti-helminthic, anti-tumour, antimicrobial, antioxidant, antimalarial, and cytotoxic effects. These properties are largely attributed to its rich reservoir of bioactive compounds such as camptothecin, alkaloids, flavonoids, and terpenoids[15,16]. Reaching a height of 4 to 10 meters, this endangered species has been traditionally used by indigenous populations, who consume its leaves and fruits for their therapeutic benefits. Notably, camptothecin — a potent anti-cancer alkaloid—has been successfully isolated from the plant in various studies[17,18]. Though native to the Sahyadri range, *N. nimmoniana* is also distributed across other Indian states, including Maharashtra, Kerala, Goa, Jammu and Kashmir, Tamil Nadu, and Assam[14,15]. In Assam, early records by Kanjilal (1914) documented its presence in Tinsukia and Lakhimpur districts, and it was later rediscovered by Sarma in 2013 after nearly a century of presumed extinction[14,19].

In light of this, an attempt has been made to evaluate and understand the efficacy of *N. nimmoniana* in PCOS

induced by DHEA in a subject animal model. Once granted as extinct, the rediscovery of this important medicinal plant had already shaken the floor of the pharmacological and ethno-medicinal domain, our experimental results can be supportive a token of its various mysterious chemical activities.

2. Materials and Methods

2.1 Collection and Authentication of the Plant

The fresh *N. nimmoniana* leaves were collected from Rojabari under Digboi Division of Assam (Latitude: 27.429095°, Longitude: 95.613598°) on November 12, 2022. The plant species was identified and authenticated voucher specimen with accession no. (101534,101535) was prepared as per the guidelines of the Botanical Survey of India (BSI), Shillong. MLENN will be written as MLENN in all the upcoming sections.

2.2 Preparation of Crude Plant Extract

The collected *N. nimmoniana* leaves were washed properly and shade-dried for 15 days at room temperature. Dried leaves were ground into powder form and stored in plastic zip-lock bags for further use. 30 g of powdered leaf material was extracted using the cold maceration process in 300 mL methanol at a 1:10 ratio and stirred for 48 h[20]. The agitated mixture was then filtered with the help of Whatman filter paper. The solvent from the filtrate was then removed with a rotary evaporator until it reached a semi-solid consistency, and stored at 4°C for further analysis.

2.3 Experimental Animals

Healthy and active adult female Swiss albino mice were obtained from the Animal House Facility at Gauhati University, maintaining 25°C as constant room temperature with 50% humidity, light and dark exposure in a 12:12 ratio of hours in cyclic alternates, provided food and water available ad libitum. The Institutional Animal Ethical Committee (IAEC) at Gauhati University granted animal ethical certificate for the present experiment, with a registration number IAEC/2024/Ethical-Per/PhD/2024-6 and the animals were maintained following the guidelines in *"The Guide for the Care and Use of Laboratory Animals"*; a publication of the National Institutes of Health (NIH) with number 80-23;1978. On the 25th Postnatal Day,

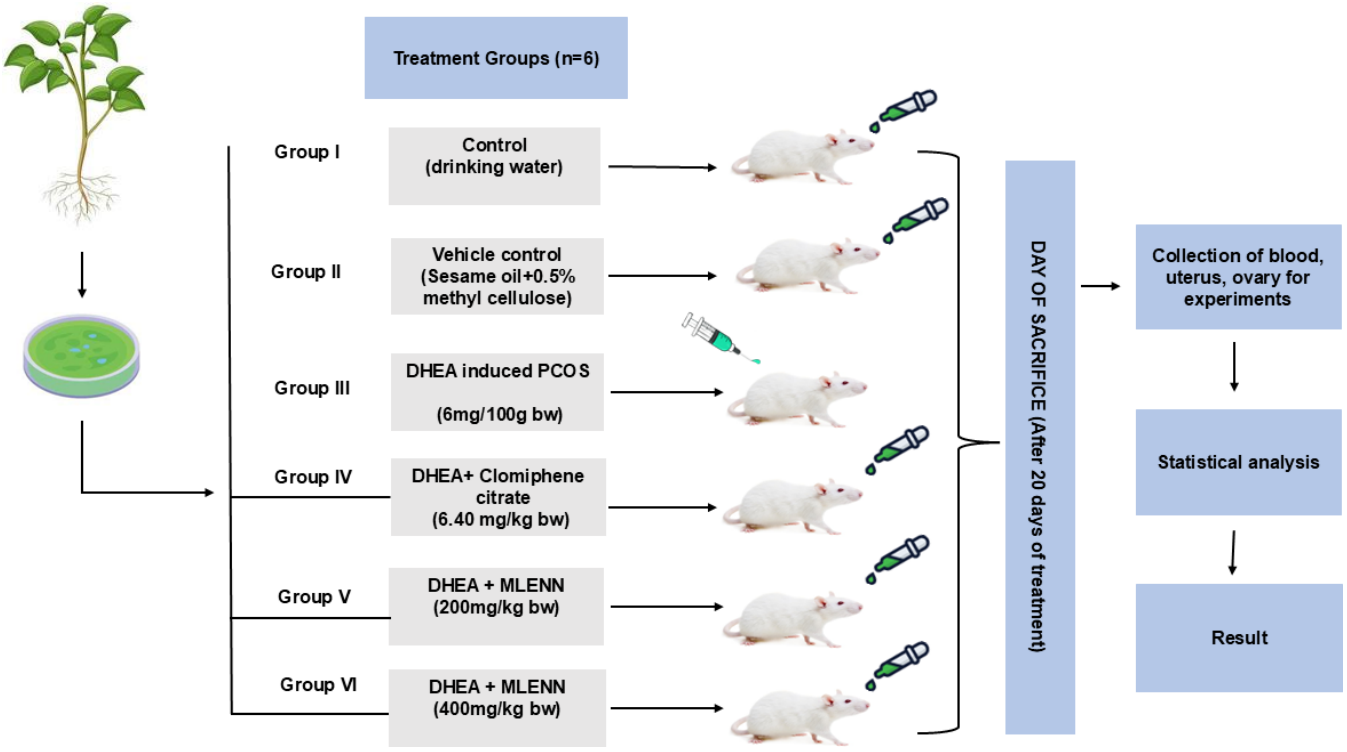


Figure 1. Schematic flow diagram of the experimental design.

these animals with discriminable weights were divided randomly into six groups and treated for 20 more days (from day 1 to day 20). The whole PCOS mouse model was constructed in a modified form of the available methodology[21-23] and is presented in Figure 1. On completion of 20 days of treatment strategies, metabolic as well as reproductive features were estimated.

2.4 Chemicals and Reagents

All the chemicals and reagents used in the present study were of high purity and were purchased from different reputable chemical companies. Methyl cellulose (Molecular formula: R H or CH_3; CAS No. 9004-67-5) was purchased from Sigma-Aldrich, and sesame oil (CAS No. 8008-74-0, Product code S3547) was obtained from Sigma-Aldrich. Giema's stain solution (Brand name: Qualikems, Product code G0Q8610) was procured from Jaldhara and Company, Guwahati, India. The Enzyme-Linked Immunosorbent Assay (ELISA) kits for serum FSH, LH, Progesterone, Estrogen, and Testosterone were procured from Zenith India, Guwahati, India. Again, AST, ALT, Total cholesterol, Total uterine Protein, HDL-C, LDL-C kits were procured from Aspen Laboratories Private Limited, Delhi, India. Chemicals for *in vivo* and *in vitro* antioxidant assay, and analytical grade chemicals used in histological preparation were brought from Merck Limited, Mumbai, India.

2.5 Acute Oral Toxicity

Acute oral toxicity was performed following the standard protocol of the Organisation for Economic Co-operation and Development (OECD), 425, Ministry of Social Justice and Empowerment, Government of India, 2008[24]. MLENN was administered orally to mice (n = 6) at a dose of 2000 mg/kg using a gavage needle, and the percentage of mortality, if any, was recorded over 24 h. Prior to oral administration of MLENN, the mice were exposed to a 24-h fasting period. One-hour post-treatment with the leaf extract, the animals were monitored for any observable changes in behaviour. The control group was administered distilled water as a vehicle. The animals were kept under observation initially for the first 24 h, and later, physiological and behavioural changes were observed for 72 h. No mortality and no additional clinical signs of toxicity were detected up to the dose of 2000 mg/kg body weight. Hence, 2000 mg/kg bw was considered as the LDs cut-off value, and therefore, its $1/10^{th}$ and $1/5^{th}$ values, viz. 200 mg/kg bw and 400 mg/kg bw, were selected for dose response and *in vivo* studies[25].

2.6 Experimental Design

A total of 36 adult female albino mice were divided into six groups (n = 6) in each group. Group I: normal untreated mice (control), Group II: vehicle control group (0.1 mL sesame oil and 100 µL 0.5% methyl cellulose), Group III: DHEA induced PCOS group (6 mg/100g bw), Group IV: DHEA+C.c.(6.40 mg/kg bw clomiphene citrate)[10], Group V: DHEA+MLENN (200 mg/kg bw) and Group VI: DHEA+MLENN (400 mg/kg bw).

2.7 Induction of PCOS

DHEA (6 mg/100g) dissolved in 0.1 ml of sesame oil was injected subcutaneously into mice, and 100 µL of 0.5% methyl cellulose was given once daily using a gavage needle for 20 days to develop PCOS[10,26,27].

2.8 Oestrous Cycle Determination

The estrus cycle of all experimental animals was monitored by characterisation of cell type in the vaginal smear as per OECD guidelines[28], starting from the 13^{th} day after the initial injection, and continued for 20 consecutive days. 0.1 mL of saline is pipetted into a sterilised dropper, and with the assistance of a fine probe, the tip of the dropper is inserted into the vaginal orifice to a depth of 1-2 mm and the saline is washed into the vagina and allowed to back out twice. Once it has been left to air dry for a few minutes, it is analysed using a microscope.

2.9 Collection of Tissues

Experimental animals were sacrificed under light anaesthesia using diethyl ether, followed by cervical dislocation. Blood samples were also collected through cardiac puncture and left to stand for coagulation. Samples were then centrifuged at 10 rpm for 15 min, and serum samples were collected and stored at -20°C for analysis[29].

2.10 Biochemical Assays

2.10.1 Estimation of Aspartate Transaminase (AST) and Alanine Transaminase (ALT)

The International Federation of Clinical Chemistry (IFCC), as described by Bergmeyer *et al.*, provided the

methodology for estimating the AST level and ALT level, respectively[30,31].

2.10.2 Estimation of Cholesterol, Lipoprotein, and Total Protein Profile

The TC (total cholesterol), utilising the cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) methodology, was estimated by commercially available kits. HDL (High-Density Lipoprotein) cholesterol and LDL (Low-Density Lipoprotein) cholesterol were measured as per the standard protocol[32]. The Biuret method was used to estimate the Total Protein (TP).

2.10.3 Estimation of Serum FSH, LH, Estrogen, Progesterone, Testosterone

The serum concentrations of Luteinising Hormone (LH), Follicle-Stimulating Hormone (FSH), progesterone, estrogen, and testosterone of treatment groups were analysed using an ELISA kit, following the manufacturer's instructions[33].

2.11 Antioxidant Activity Assessment

The serum antioxidant enzyme Super Oxide Dismutase (SOD)[34,35], Catalase (CAT)[36,37] and Glutathione Peroxidase (GPX)[38] activities were determined by following a standard method using the modified method.

2.12 Histological Studies

Following blood collection, the ovaries and uterus of mice were removed for histological analysis. Following the removal of the adhering connective adipose tissue, the tissues of the ovaries were fixed for at least 48 h by embedding them in a 10% formaldehyde solution[39,40]. The tissues were subjected to serial alcoholic dehydration, cleared in xylene, and infiltrated before embedding in paraffin wax. 5 µm-thick sections of trimmed paraffin blocks were sliced through the microtome machine. The routine hematoxylin and eosin staining technique was used in staining after stretching in warm water (40°C). The sections were then mounted with DPX and observed under a microscope (Leica DM 3000).

2.13 Scanning Electron Microscopy (SEM) Analysis

For the SEM study, all samples were pre-fixed in 2% gluteraldehyde in "0.1 M Na-cacodylate buffer" (pH 7.2) at 4°C for 4 h, rinsed in "*Na-Na-cacodylate buffer*" for 15-30 min. They were then dehydrated in a graded series of acetone, and tetramethylsilane was used to dry them using the procedure described by Dey *et al*[41]. The samples were mounted on brass stubs (10 mm x 10 mm) using double-coated adhesive, coated with a gold facilitative coating using a JFC-1100 (Jeol) Ion-Sputter Coater, with a lower vacuum (10-3 Torr) in the sputtering chamber and examined under SEM(JSM-6360, JEOL), with an accelerating voltage of 15-20 kV.

2.14 Ovarian Follicular Count

For this study, ovarian follicles of treatment groups were counted and classified into primary, preantral, antral, cystic, and corpus luteum based on the morphological classification by Pedersen and Peters[42], Myers *et al.*[43] and the criteria of Johnson and Everit[44,45]. The average percentage of atretic follicles and healthy follicles was counted. Ovarian differential follicle counts were made from every 10th section. The follicles were classified into: (A) Primary follicles possessed an oocyte surrounded by a single layer of cuboidal granulosa cells (B) Preantral follicles with no visible antrum (C) Both small and large antral follicles possessed a clearly defined antrum and a segmented cavity with two or more compartments (D) Cystic follicles were the large size follicles with translucent, thin-walled, unilocular and smooth-surfaced (E) Corpus luteum.

2.15 Statistical Analysis

The results were expressed as mean ± SEM (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test, along with the Chi-square test. The combination index was evaluated using Microsoft Excel. Probability calculation was done with the help of https://statisty.app/. Graphs were developed by using GraphPad Prism version 10.2 for Windows (GraphPad Software, San Diego, California, USA) and Matplotlib using Python. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1 Effect of MLENN on the Body Weight of DHEA-Induced PCOS Female Swiss Albino Mice after 20 Days of Treatment

After 20 days of subcutaneous administration of DHEA, there was a significant ($p<0.05$) increase in

body weight of the PCOS group when compared to the control group. However, compared to the DHEA-induced group, the body weight of PCOS mice treated with Clomiphene citrate (C.C.), DHEA+ MLENN (200 mg/kg), and DHEA+ MLENN (400 mg/kg) for 20 days was dramatically reduced ($p<0.05$) (Figure 2).

3.2 Effect of MLENN on the Oestrous Cycle of PCOS-induced Female Mice

The estrus cycle of the control and vehicle control mice was regular, lasting four to five days, and each stage of the estrus cycle was normal in length (Figure 3). DHEA-induced PCOS mice exhibited irregular estrus cyclicity, with a persistent diestrus state leading to longer estrus cycles. However, the regularisation of the estrus cycle following MLENN (400 mg/kg b.w.) administration led to the restoration of a normal cycle length (Figure 2). Compared to the PCOS-induced group, treatment with MLENN (200 mg/kg b.w.) for 20 days resulted in a substantial reduction in the duration of estrus cyclicity. Compared to the untreated PCOS group, mice administered clomiphene citrate exhibited a progressive normalisation of oestrous cyclicity, as evidenced by a significant increase in the average number of cycles over a 20-day period, indicating restoration of regular reproductive function (Figure 4).

3.3 Effect of MLENN on the Serum Liver Enzymes in All the Experimental Groups

3.3.1 Effect of MLENN on AST, ALT

Post-induction of PCOS, there was an increase in levels of AST and ALT ($p<0.05$). The treatment of the PCOS group with clomiphene citrate and MLENN (200 mg/kg and 400 mg/kg) decreased the AST and ALT levels ($p<0.05$) (Figure 5, Table S1).

3.3.2 Effect of MLENN on TC, Lipids, and Protein

After induction of PCOS, TC, LDL-C, and TP levels increased ($p<0.05$) in comparison to other experimental groups. Following the treatment with MLENN (200 mg/kg and 400 mg/kg) and clomiphene citrate lowered the TC, LDL-C, and TP levels were lowered ($p<0.05$). However, the level of HDL-C decreased in

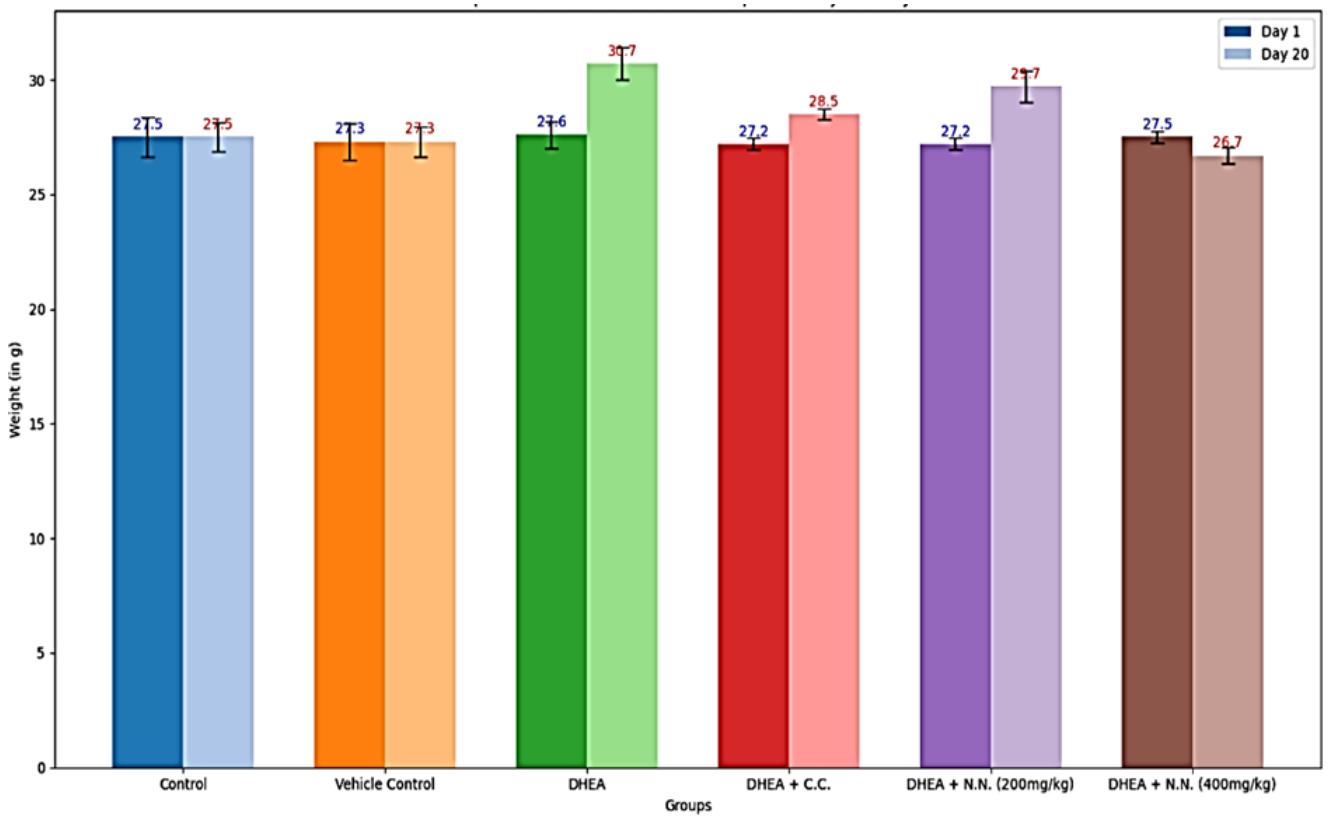


Figure 2. Effect of different treatment groups on the body weight of mice. No significant changes in body weight were found after administration of doses ($p<0.05$). Values are expressed in mean ± SEM.

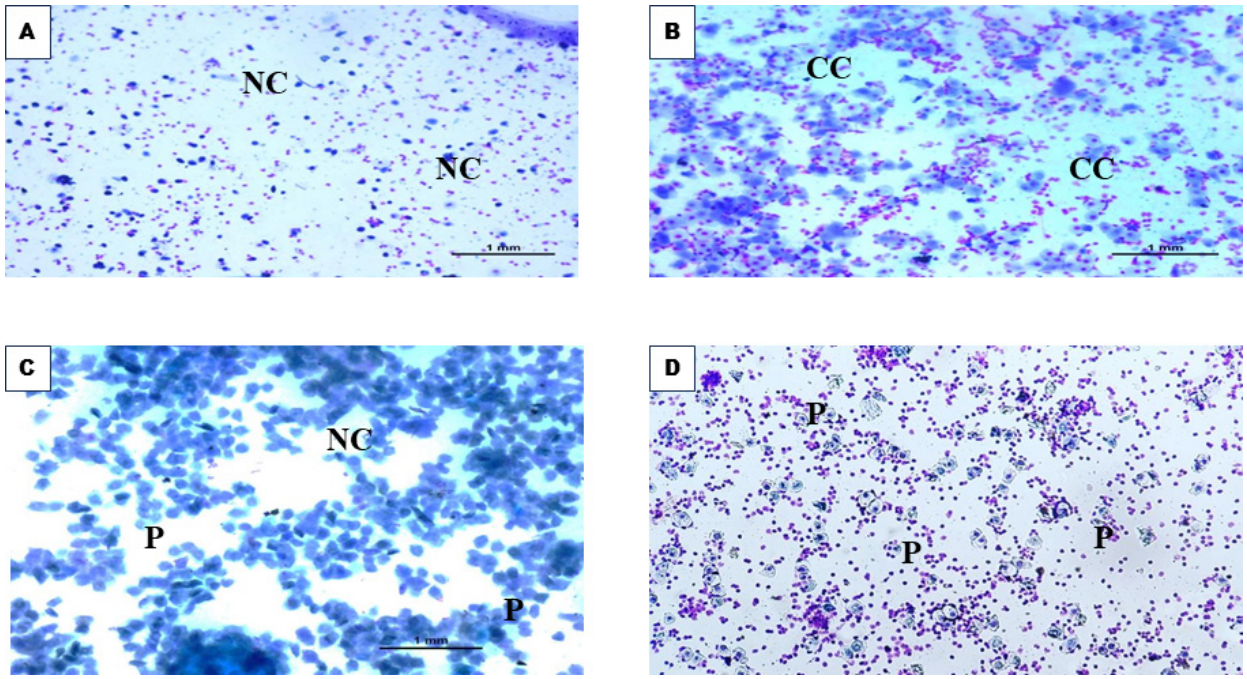


Figure 3. Exfoliative cytology during the oestrous cycle of female albino mice (control) (A) Proestrus phase consists of nucleated epithelial cells (B) Estrus phase shows anucleated cornified squamous epithelial cells (C) Metaestrus phase shows cornified and nucleated cornified squamous epithelial cells and polymorphonuclear leukocytes (D) Diestrus phase shows polymorphonuclear leukocytes (Giemsa's stain, Magnification × 40). Cornified cells (CC), polymorphonuclear leucocytes (P), nucleated epithelial cells (NC).

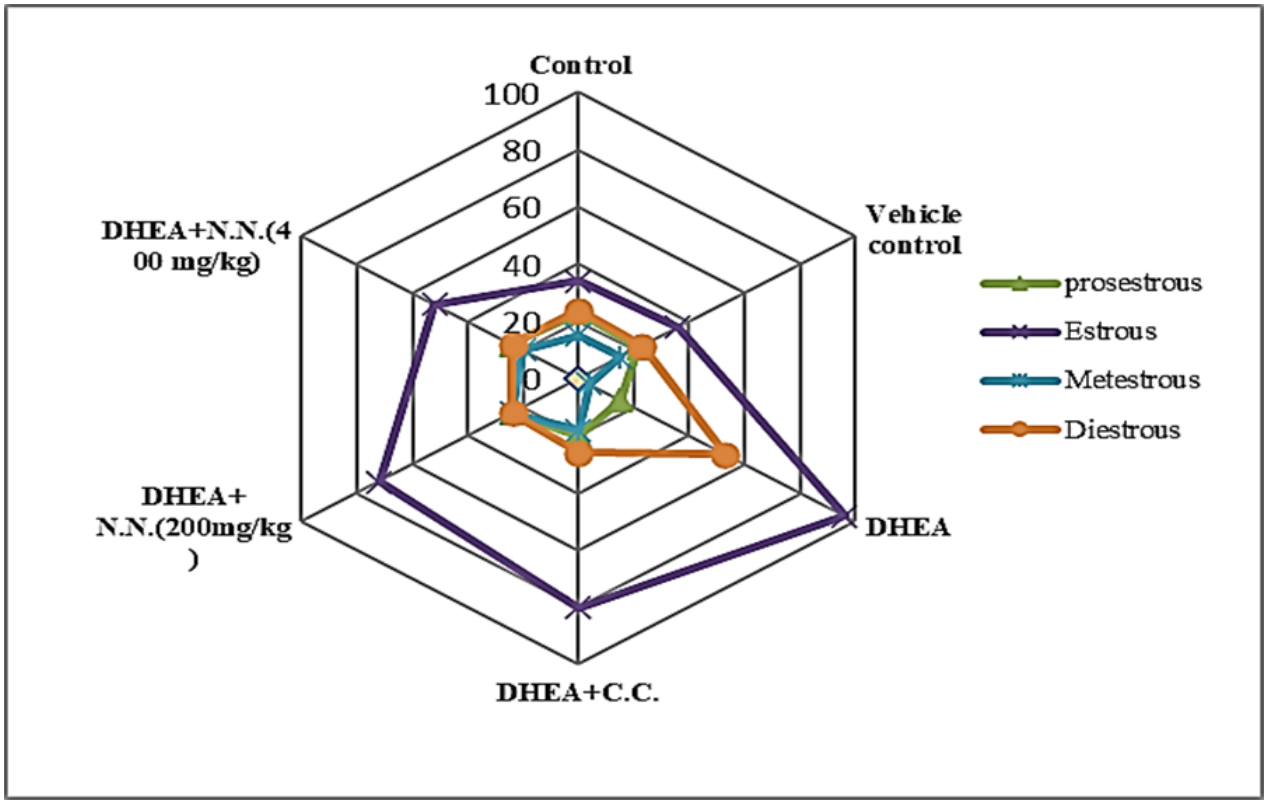


Figure 4. Stage-wise cell number variation of the oestrous cycle in the case of different treatments.

the DHEA-induced PCOS group ($p<0.05$), while it significantly increased when treated with clomiphene citrate and MLENN (200 mg/kg and 400 mg/kg) ($p<0.05$) (Figure 5, Table S1).

3.4 Effect of MLENN on Hormonal Levels

3.4.1 Serum LH and FSH Levels

According to the findings, the DHEA-induced PCOS group had a significantly higher mean serum concentration of LH than the control group ($p<0.05$). The treatment of PCOS animals with clomiphene citrate and doses of 200mg/kg and 400 mg/kg of the MLENN decreased LH levels ($p<0.05$). The level of FSH decreased in the PCOS group when compared to the control group and increased when treated with clomiphene citrate and MLENN (200 mg/kg and 400 mg/kg) ($p<0.05$). Furthermore, there were no significant differences in the LH/FSH ratio among the groups (Figure 6, Table S2).

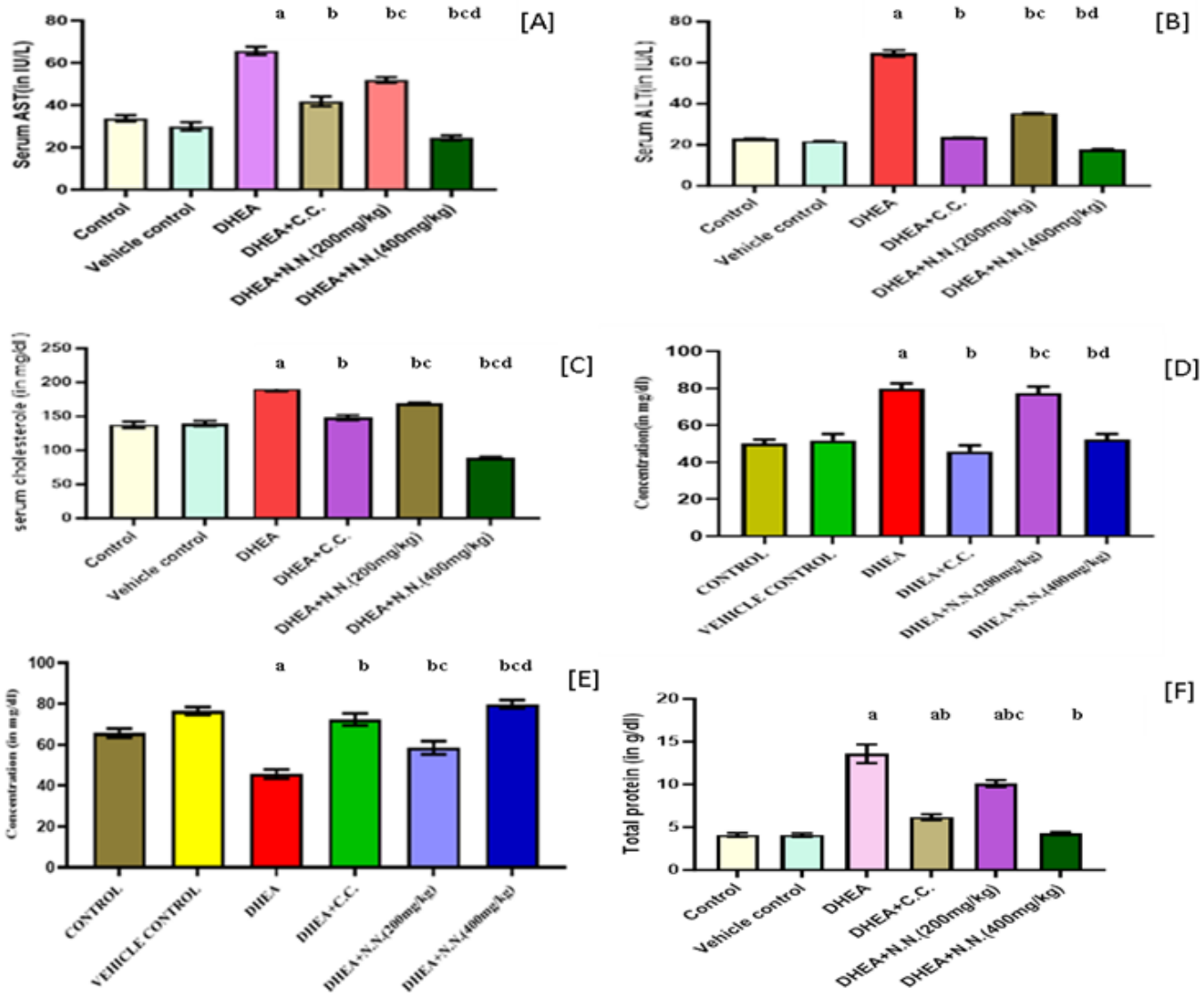


Figure 5. Effect of different treatment groups on (A) Aspartate Transaminase (AST), (B) Alanine Transaminase (ALT), (C) Total Cholesterol (TC), (D) High Density Lipid (HDL-C), € Low Density Lipid (LDL-C) and (F) Total Protein (TP) of mice. In the figure 'a' stands for a significantly different from control ($p<0.05$), 'b' significantly different from the DHEA group ($p<0.05$), 'c' significantly different from the DHEA +C.C. group ($p<0.05$), 'd' significantly different from the DHEA +N.N. (200mg/kg) group ($p<0.05$).

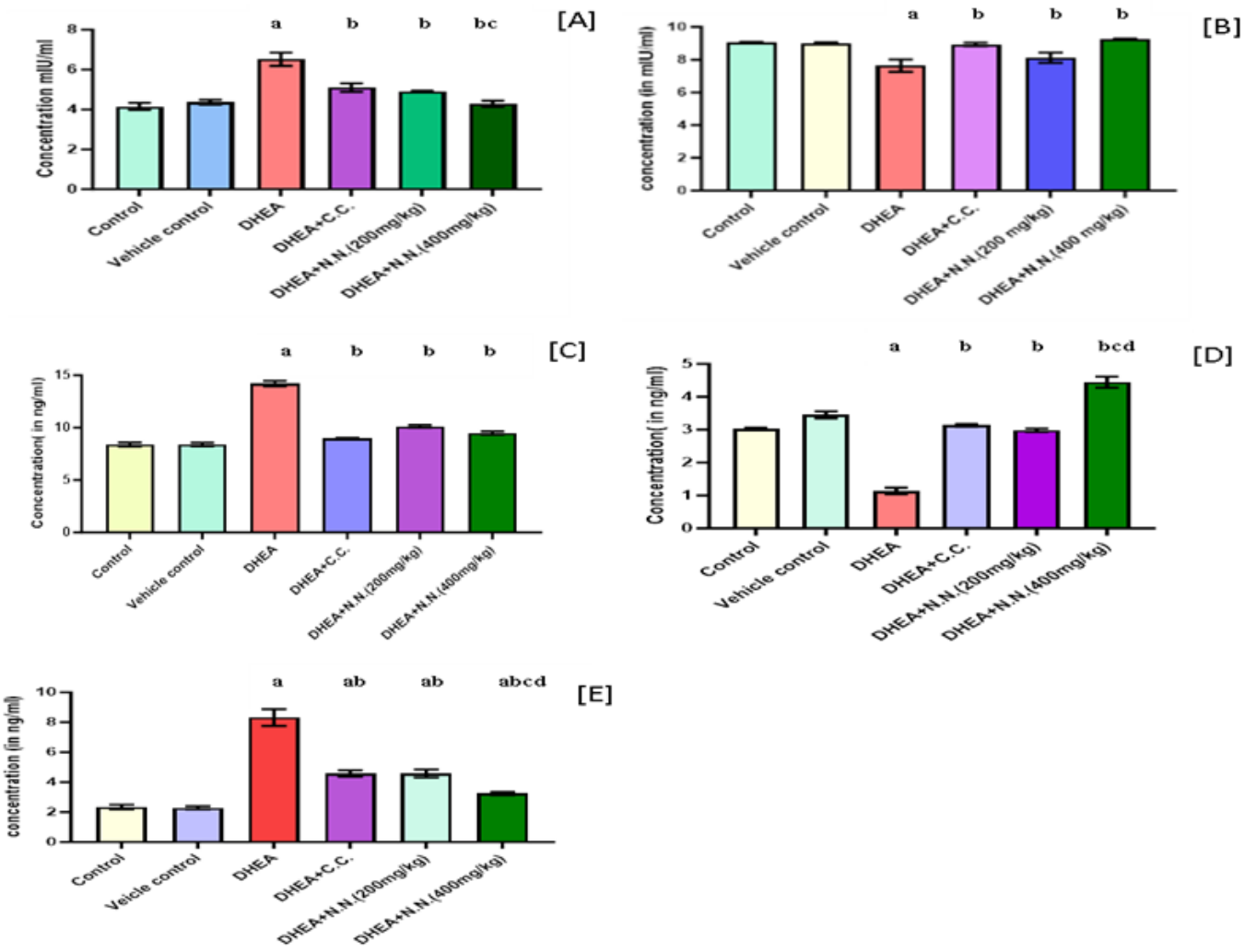


Figure 6. Showing the effect of different treatment groups on the serum (A) LH, (B) FSH, (C) Estrogen, (D) Progesterone and (E) Testosterone of mice. In the figure 'a' stands for a significantly different from control ($p<0.05$), 'b' shows significant difference from the DHEA group ($p<0.05$), 'c' significantly different from the DHEA +C.C. group ($p<0.05$), 'd' displays significant difference from the DHEA +N.N. (200mg/kg) group ($p<0.05$).

3.4.2 Serum Testosterone Level

The serum concentration of testosterone levels was elevated in the DHEA-induced PCOS group ($p<0.05$), and after treatment with clomiphene citrate and a dose of 200 and 400 mg/kg MLENN, there was a reduction in the level of serum Testosterone ($p<0.05$) (Figure 6, Table S2).

3.4.3 Serum Estrogen and Progesterone Levels

DHEA DHEA-induced PCOS group had significantly lower progesterone level and higher estrogen level when compared to the control group ($p<0.05$). The concentration of progesterone was considerably higher, and lowered estrogen level was lowered when treated with clomiphene citrate and MLENN (200 mg/kg and 400 mg/kg) ($p<0.05$) (Figure 6, Table S2).

3.5 Chi-Square Test, Probability Histogram, and Chao-Talalay Index Evaluation

The Chi-square test conducted to inspect the changes in TC-lipid profile and Sexual hormones by use of DHEA+N.N of 200 mg/kg and 400 mg/kg dose showed that such experimental variation is significant only on changes in TC-lipid profile ($p<0.001$) but not on sexual hormones ($p>0.05$). The probability histogram also depicted similar results. By Chao-Talalay Index evaluation, it was observed that both 200 mg/kg and

400 mg/kg sets are suitable for changing TC-lipid profile if compared to the set DHEA+C.C (Figure 7). However, for sexual hormones, these sets did not show a significant synergistic effect more than DHEA+C.C (Figure 8).

3.6 Effect of MLENN on Oxidative Stress Markers

Inducing PCOS reduced SOD, CAT and GPX levels ($p<0.05$) when compared to the control group. However, after treatment with clomiphene citrate, the level of SOD, CAT and GPX increased significantly ($p<0.05$). Treating the PCOS mice with a dose of 200 mg/kg of MLENN increased the level of SOD, GPX and CAT ($p<0.05$). In addition, SOD, CAT and GPX levels were statistically significantly higher ($p<0.05$) in DHEA+N.N. (400 mg/kg b.w./day) than in all other experimental groups (Figure 9, Table S3).

3.7. Histomorphological Studies

3.7.1 *Effect of MLENN on Histomorphological Changes in the Ovary of DHEA-Induced PCOS Mice after 20 Days of Treatment*

The normal histoarchitecture of the ovary, consisting of the outer cortex and inner medulla, was observed in the control and vehicle control groups. The cortex,

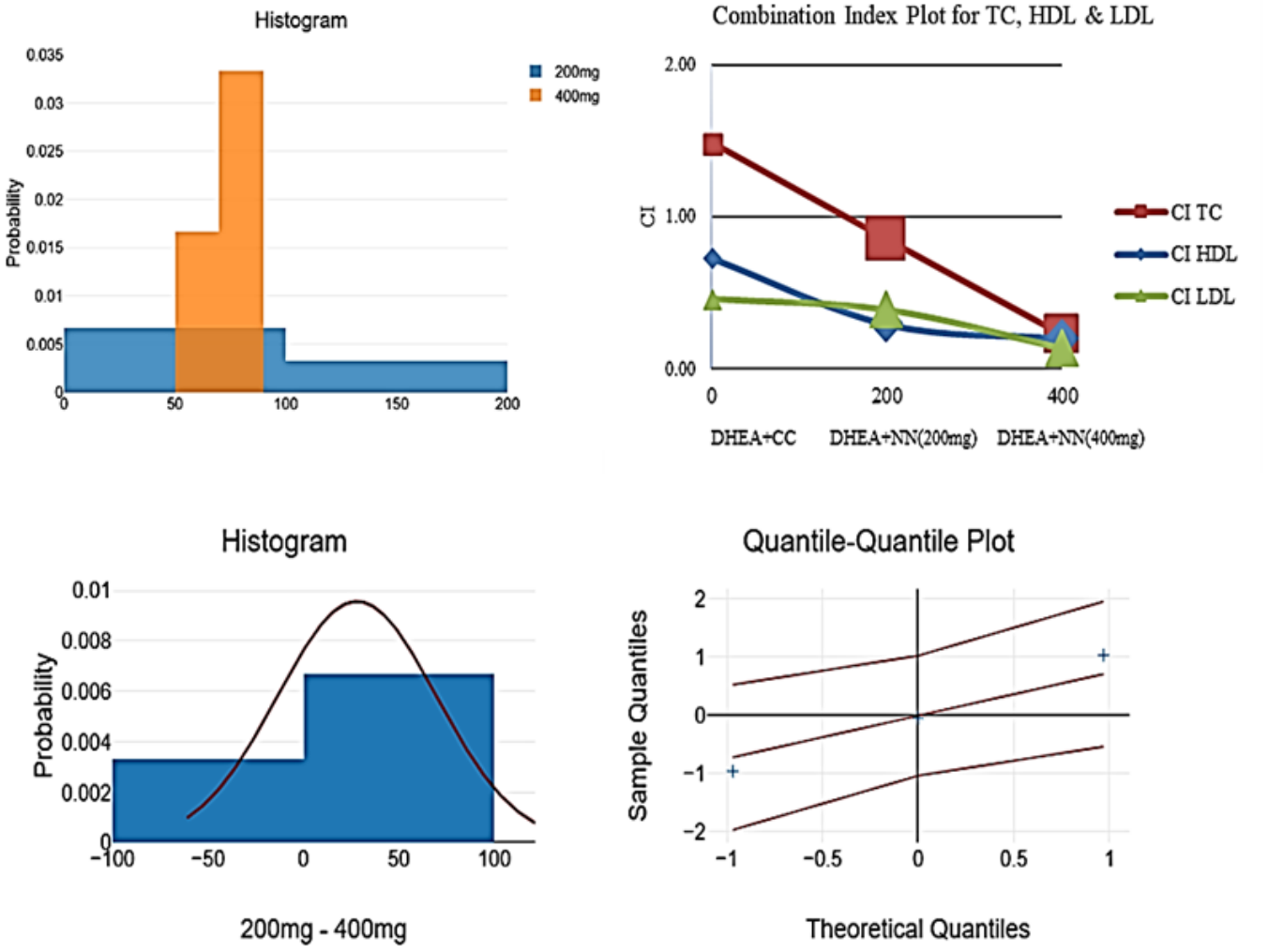


Figure 7. Histogram and theoretical quartiles resupprotings have significant results from the Chi-square test for the use of 200 mg and 400 mg of DHEA+MLENN on subjects in terms of changes in Cholesterol-Lipid profile. All *N. nimmoniana* experimental sets are showing a C.I. value <1, showing a high probability of their use to get a synergistic effect.

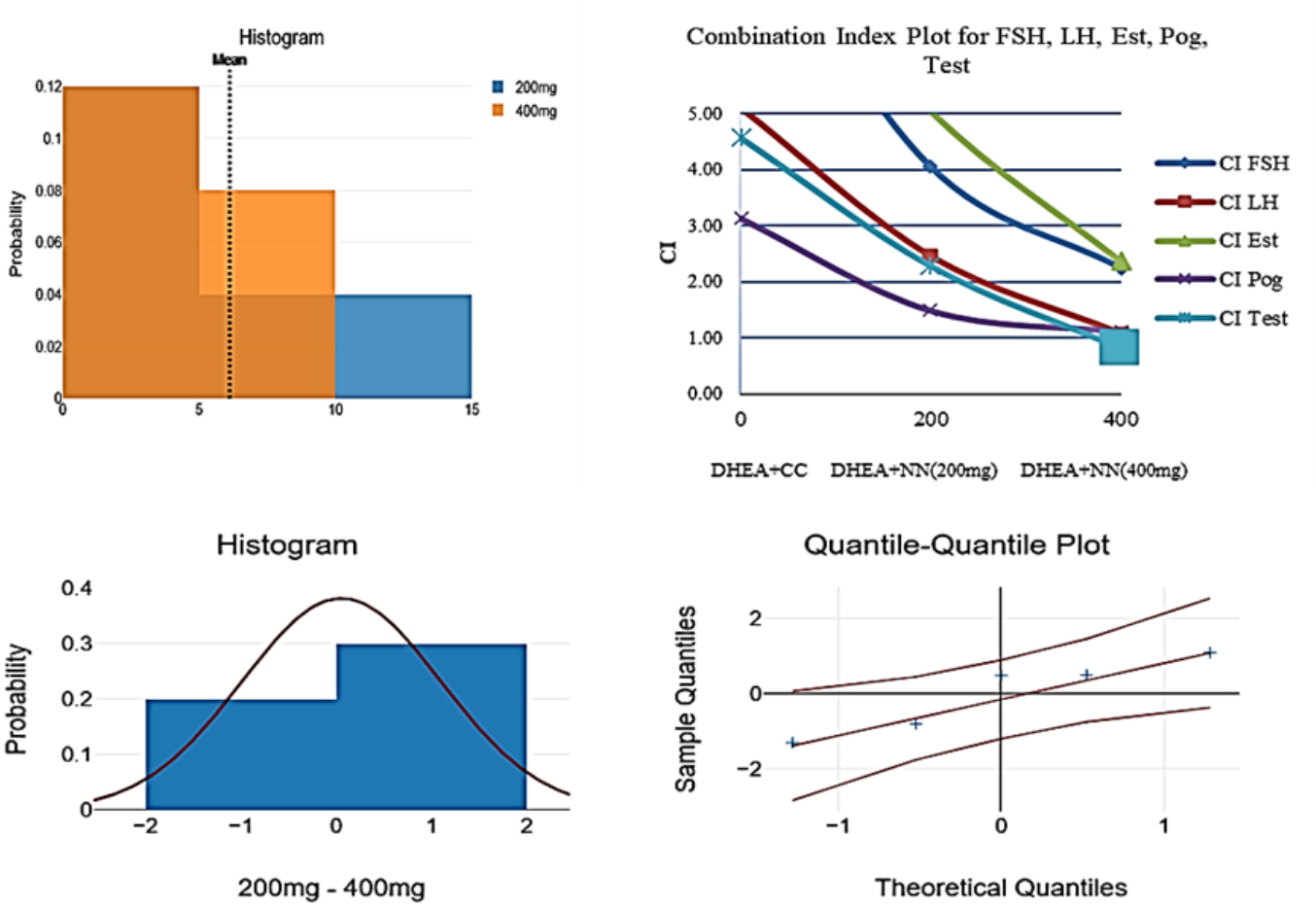


Figure 8. Histogram and theoretical quartiles resupportings non-significant results from the Chi-square test for the use of 200 mg/kg and 400 mg/kg of DHEA+MLENN on subjects in terms of changes in sexual hormones. All *N.nimmoniana* experimental sets are showing C.I. value >1 except DHEA+N.N (400 mg/kg) against testosterone, showing a low probability of their use to get a synergistic effect.

with its exterior germinal epithelium, which included cuboidal or columnar cells, and the ovarian arteries and veins, made up the majority of the medulla's circulatory networks. Compared to the medulla, the cortex's stroma was more densely packed.

According to the histopathological observation, subcutaneous injections of DHEA were efficient in inducing PCOS in mice and alterations were observed in DHEA-induced PCOS (6 mg/kg bw/day) mice ovarian tissues. The number of morphologically viable large antral follicles was significantly reduced in DHEA-induced PCOS animals. These follicles were characterised by a cavity filled with fluid, with the oocyte located at the edge of a collective of epithelial cells. The ovaries of DHEA-induced PCOS (6 mg/kg bw) mice showed weak granulosa cell layers, large subcapsular cysts, and an entirely unhealthy corpus luteum. The thicker theca cell layer resulted in thicker walls for the cystic follicles. In DHEA-induced PCOS mice, signs of follicular cell degeneration, atrophied granulosa cells, atretic follicles, and cell debris accumulation in the antrum were observed (Figure 10).

3.7.1.1 Ovarian Follicular Count

The ovaries of the DHEA-induced PCOS group contained numerous enlarged follicles. PCOS group showed reduced preantral follicles, antral follicles and corpus luteum, yet a higher number of cystic follicles compared to the control group. MLENN (200 and 400 mg/kg bw) administration in DHEA-treated animals restored the decreased count of preantral follicles, antral follicles and corpus luteum while simultaneously decreasing cystic follicle numbers ($p<0.05$) (Table 1).

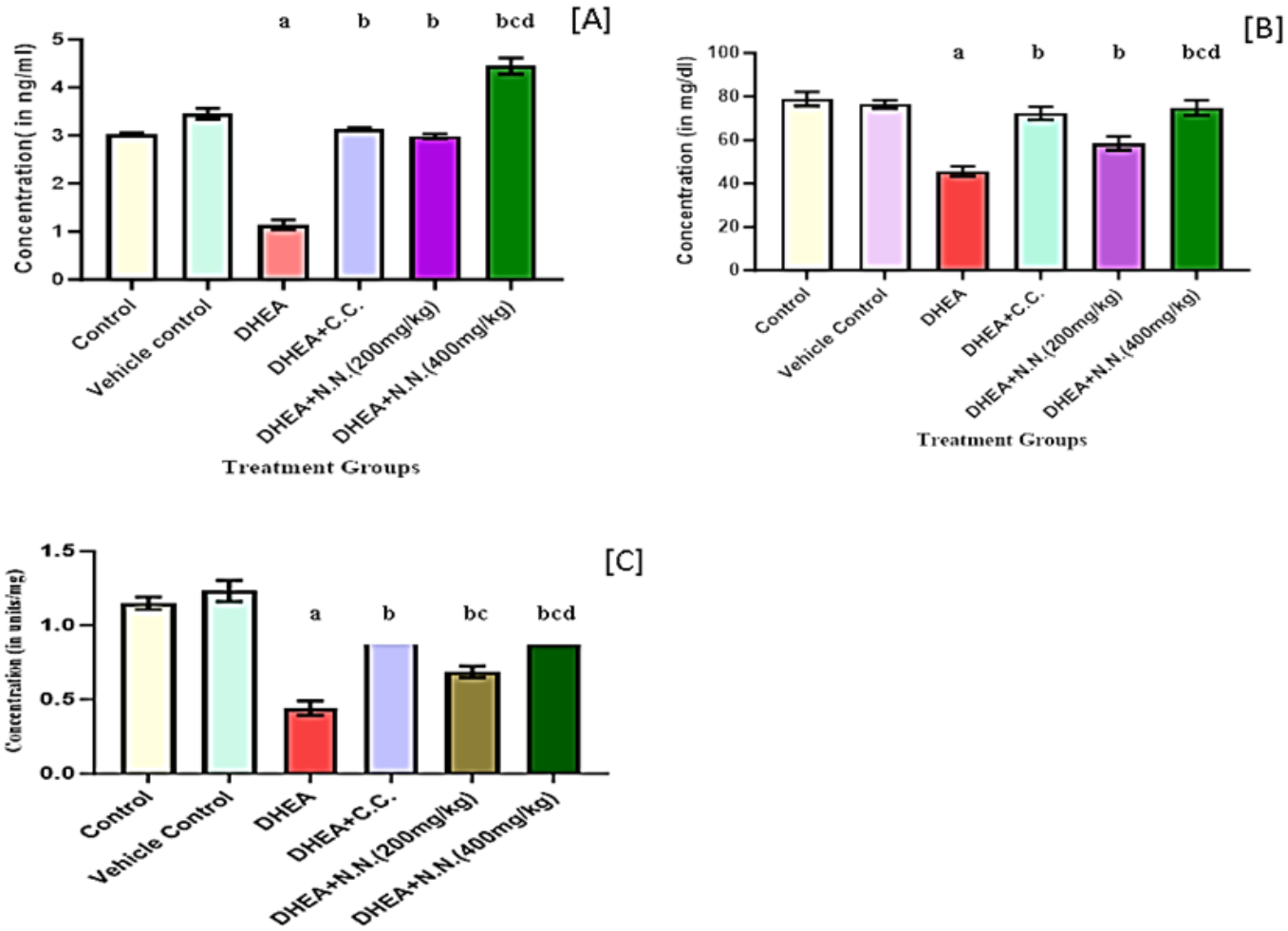


Figure 9. Graphs show (A) Impact of various treatment groups on SOD levels in mice, (B) Effect of different treatments on GPX activity in mice, and (C) Influence of treatment groups on CAT activity in mice. In the figure, 'a' display a significant difference from the control group ($p<0.05$), 'b' denotes a significant difference from the DHEA group ($p<0.05$), 'c' represents a significant difference from the DHEA + C.C. group ($p<0.05$), and 'd' signifies a significant difference from the DHEA + N.N. (200 mg/kg) group ($p<0.05$).

3.7.2 Effect of MLENN on histomorphological Changes in the Uterus of DHEA-Induced PCOS Mice after 20 Days of Treatment

In the control group, the epithelial lining was characterised by a simple columnar structure, predominantly comprising non-ciliated secretory cells, accompanied by a smaller number of ciliated cells. Beneath this layer, the stroma featured a highly cellular lamina propria containing a single tubular uterine gland. The perimetrium consisted of loose connective tissue and a thin mesothelial layer, while the myometrium was composed of three distinct layers of muscle with varying fibre orientations. Compared to the control group, the DHEA-treated group exhibited a markedly thickened and hyperplastic endometrium. The underlying stromal region showed reduced cellularity, and the luminal epithelium appeared more pronounced and invaginated. In the DHEA group, there was an increase in the number of eosinophils, lymphocytes, and macrophages, multiple cell layer development, greater vascularity in the endometrial stroma, and hyperplasia in both the glandular and luminal epithelium. Moreover, as cross-sections of uterine horns showed, the number of uterine glands reduced along with numerous glandular dilations, epithelial hyperplasia, and a less regular cellular disposition compared to controls. In comparison with the

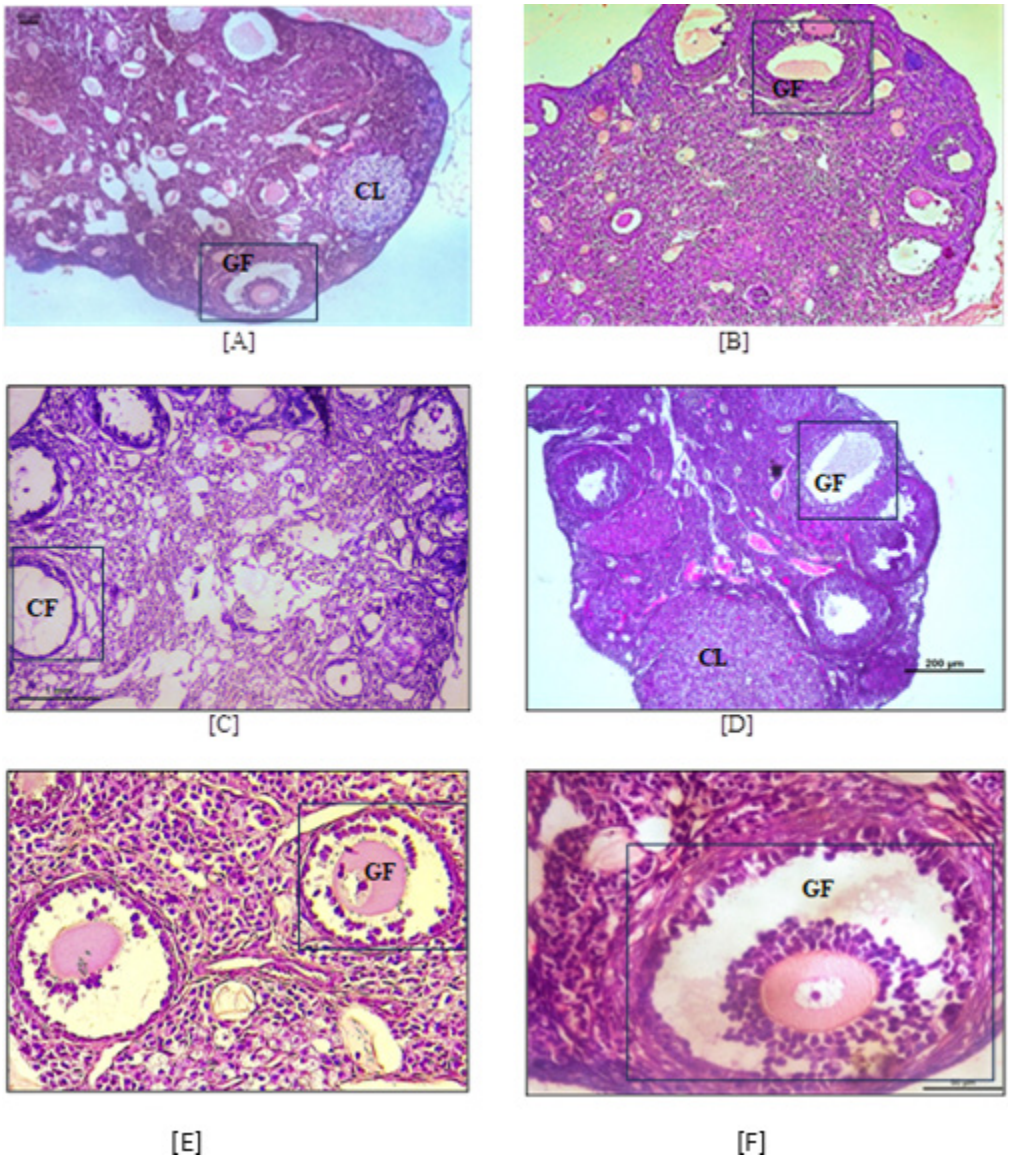


Figure 10. Photomicrographs show histopathological slides at 100X magnification of the mouse ovary after treatment for 20 days. A, B, C, D and E stand for control, vehicle control, DHEA-induced PCOS, DHEA +C.C., DHEA +N.N. (200 mg/kg) and DHEA +N.N. (400 mg/kg) group, respectively (GF stands for graafian follicle, CL stands for corpus luteum, CF stands for cystic follicle, scale bar 200 µm).

Table 1. Comparison of the number of ovarian follicles and corpus luteum after treatment of PCOS in mice with MLENN. Values are expressed in mean ± SEM

Treatment groups	Primary follicle	Preantral follicle	Antral follicle	Cystic	Corpus luteum
Control	19.37±1.15	17.87±2.29	10.0±0.65	0	10.93±0.54
DHEA	30.41±3.54^{a}	33.2±2.35^{a}	2.80±0.42^{a}	5.26±0.04^{a}	2.60±0.21^{a}
DHEA+C.C.	16.3±1.12^{a,b}	20.5±1.13^{a,b}	7.0±0.94^{a,b}	2.0±0.61^{a,b}	5.5±0.24^{a,b}
DHEA+N.N. (200mg/kg)	15.2±0.94^{a,b}	26.0±0.12^{a,b}	6.0±0.12^{a,b}	4.3±0.12^{a}	5.4±0.21^{a,b}
DHEA+N.N. (400mg/kg)	17.4±0.13^{a,b,c}	22.0±0.94^{a,b,c}	8.12±0.14^{a,b}	2.12±0.29^{a,b,c}	6.0±0.29^{a,b}

Here, a, b, c stands for a - significantly different from control ($p<0.05$), b - significantly different from the DHEA group ($p<0.05$), c - significantly different from the DHEA +C.C. group ($p<0.05$), d significantly different from the DHEA +N.N. (200 mg/kg) group ($p<0.05$).

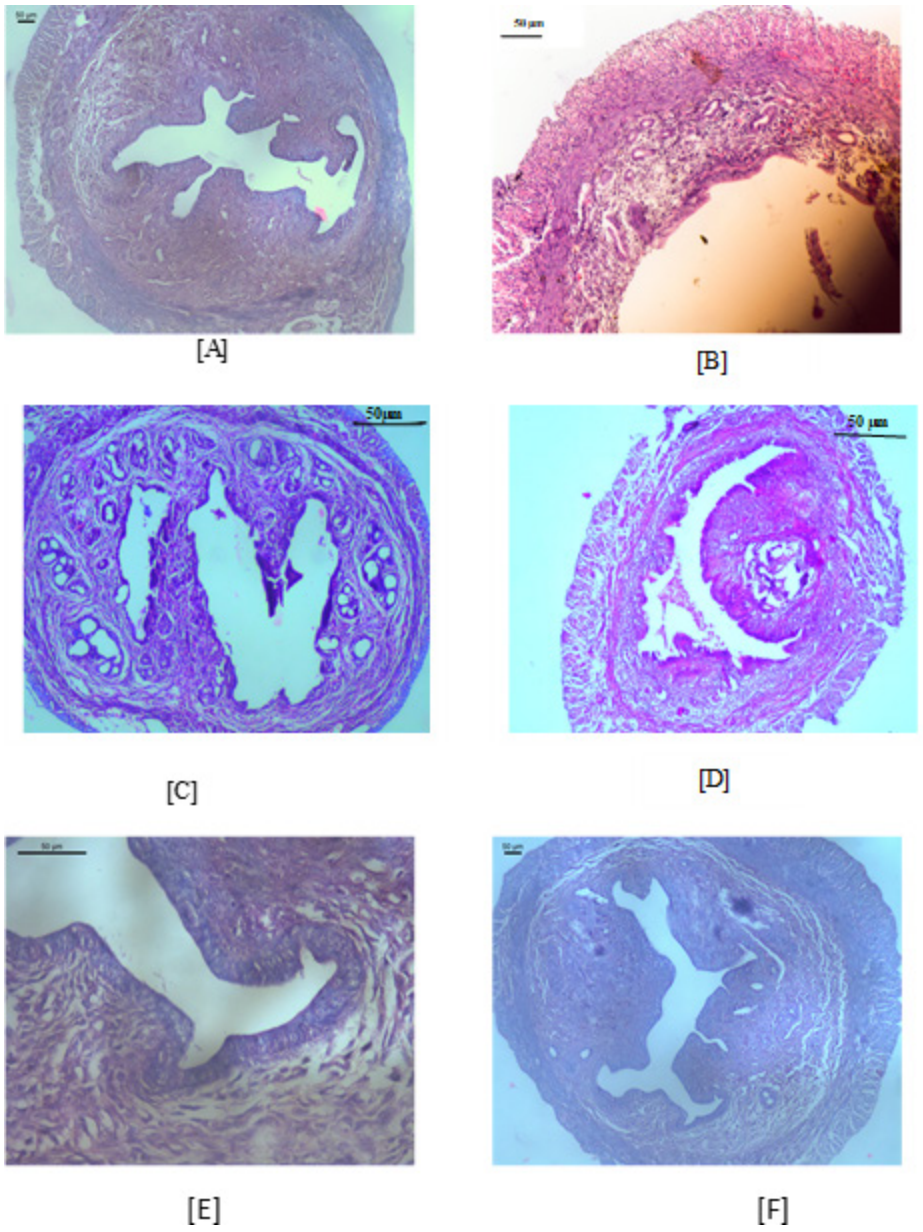


Figure 11. Photomicrographs (100X magnification) of the mice uterus after treatment for 20 days: (A) control, (B) vehicle control, (C) DHEA-induced PCOS, (D) DHEA +C.C., (E) DHEA +N. N. (200 mg/kg) and (F) DHEA +N. N. (400 mg/kg) group, respectively (scale bar 50µm).

DHEA and control groups, both the DHEA+C.C. and DHEA+MLENN (200 mg/kg b.w.) treatment groups showed a reduction in the number and size of uterine glands. This was accompanied by diminished infiltration of inflammatory stromal cells and a noticeable decrease in hyperplasia of the luminal and glandular epithelium. DHEA+MLENN (400mg/kg b.w.) had a more control-like general uterine histological architecture (Figure 11).

3.8 Scanning Electron Microscopic Analyses of Ovarian Tissue of DHEA-Induced PCOS Mice After 20 Days of Treatment

Many follicles in the early stages of development were visible in the ovarian cortex, located directly beneath the surface epithelium. Deeper into the ovarian cortex, larger follicles were found. Simplified squamous epithelium covered the ovarian surface in the control

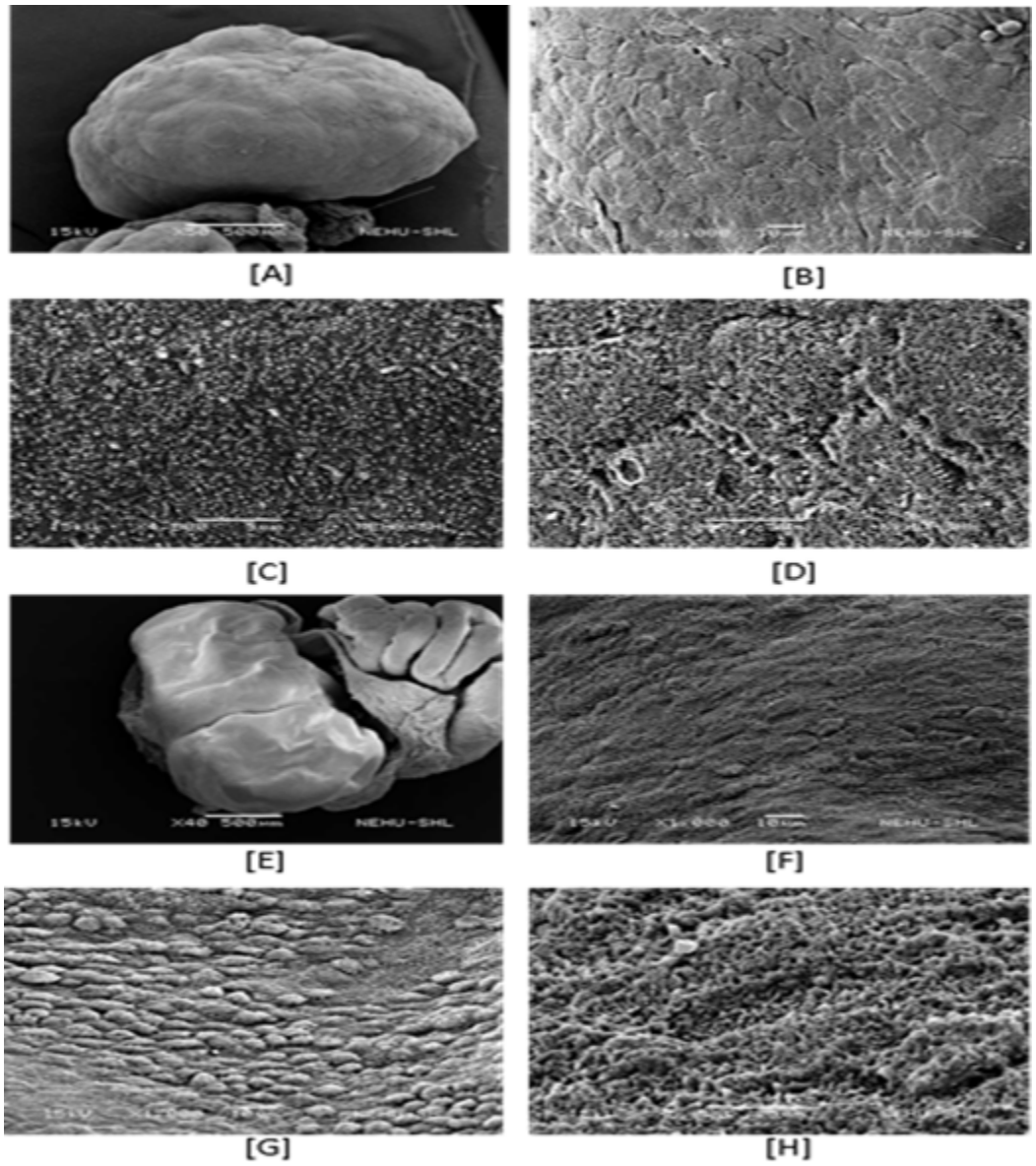


Figure 12. Photomicrographs represent ovarian histology of mice using Scanning Electron Microscopy of (A-D) Normal Control and (E-H) Vehicle control.

group's Ovaries (Figure 12). Normal structure was present in both primordial and primary follicles. Many smooth endoplasmic reticulum cisternae, many lipid droplets, and numerous mitochondria were seen in the interstitial cells. A significant proportion of the follicles in the ultrathin sections of the ovaries from the DHEA group showed signs of degenerative alterations in addition to those with normal structures (Figure 12 A-D). There were instances in which the granulosa cells of a follicle split off from one another, and their connections became less frequent. Distinct gaps were observed either between the oocyte and the zona pellucida or between the zona pellucida and the inner granulosa cells, following a consistent pattern.

GER cisternae were observed in the cytoplasm of secondary follicle granulosa cells. Additionally, apoptotic oocytes with nuclear fragmentation and noticeably thicker zona pellucida were among the several follicles in the DHEA group that displayed atresia symptoms. In the zona pellucida, granulosa cell microvilli retract or disappear. Concurrently, the oocyte's microvilli have decreased in quantity, with the majority ending just inside the zona pellucida. Additionally, shrinking granulosa cells with vacuolar development and lipid droplets in

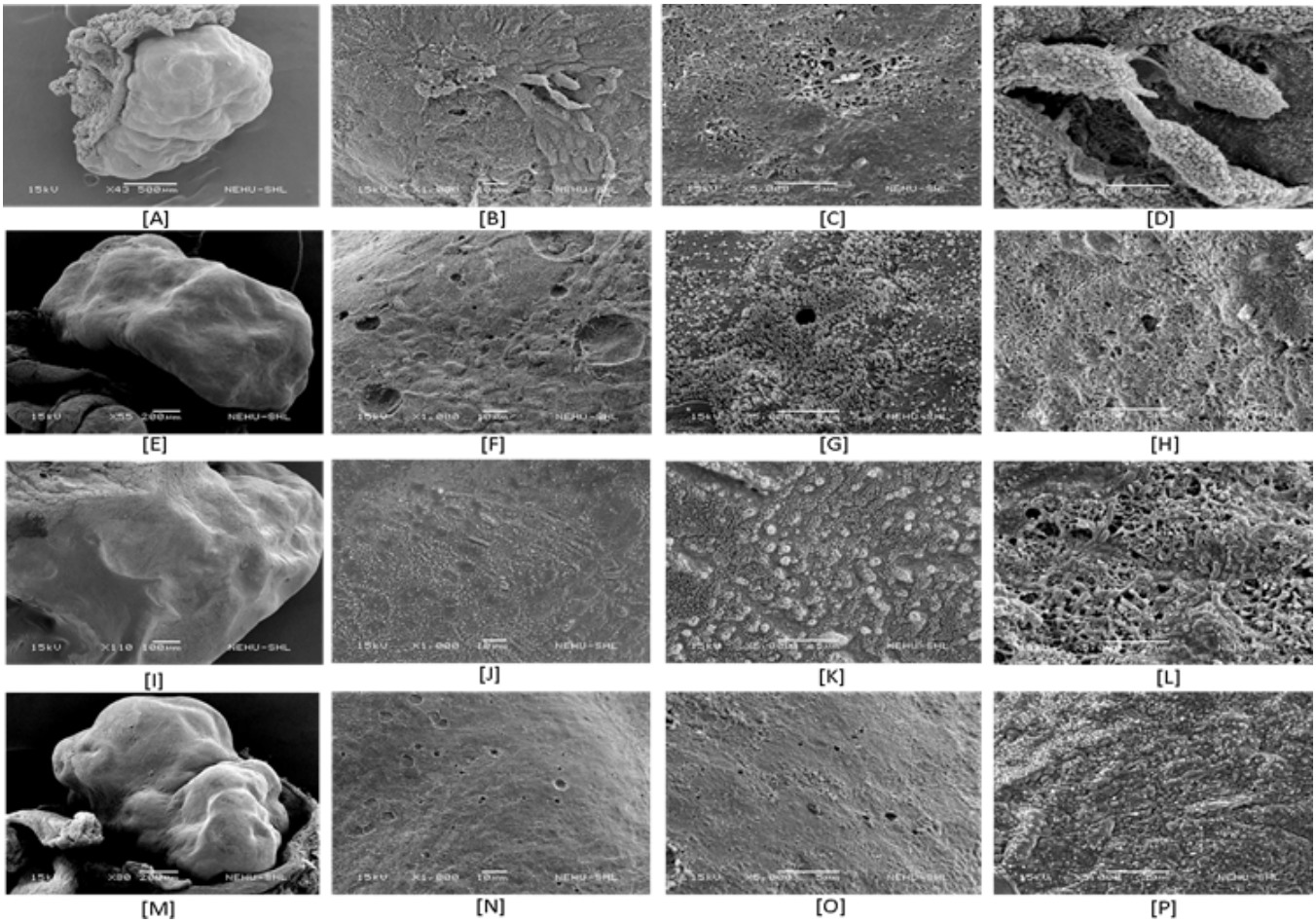


Figure 13. Photomicrographs show ovarian histology of mice using Scanning Electron Microscopy of (A-D) DHEA, (E-H) DHEA + C.C, (I-L) DHEA+MLENN (200 mg/kg b.w.), and (M-P) DHEA+MLENN (400 mg/kg b.w.).

very dark staining were seen. The layers of cystic follicle granulosa cells have become thinner. The thickened theca layer encircled the flattened granulosa cells on the outside. Numerous lipid droplets were seen to contain cholesterol crystals of different sizes that were either fusiform or polygonal in shape. Follicles at various developmental stages in the plant treatment group (400 mg/kg b.w.) often displayed normal histological structures (Figure 13 M-P). When compared to the DHEA group, the treatment group had fewer cystic follicles, and their granulosa cell layer was noticeably thinner (Figures 13 A-L, M-P). However, the theca layer was thinner, and the interstitial cells exhibited a typical fine structure, resembling those found in the control group.

4. Discussion

Polycystic Ovary Syndrome (PCOS) is a prevalent endocrine and metabolic condition that primarily affects women during their reproductive years. It is typically marked by elevated androgen levels, persistent anovulation, and the presence of multiple cysts in the ovaries. The complexity of its pathophysiology necessitates the exploration of alternative therapeutic approaches that offer improved efficacy with fewer side effects.

In this context, the current study evaluated the therapeutic efficacy of MLENN in a DHEA-induced mouse model of PCOS. The selection of DHEA was based on its well-established use as a potent inducer of PCOS in experimental mouse models[46]. Previous studies have demonstrated that administering DHEA to prepubertal female mice for 15-35 days induces PCOS-like symptoms. A 20-day exposure period is most frequently employed due to its consistency in reproducing PCOS phenotypes[26], and thus was adopted in this study.

The experimental design incorporated a positive control to provide a comparative basis for evaluating

the efficacy of MLENN. Pharmacological agents such as metformin and flutamide have been widely recognised for their therapeutic potential in managing PCOS symptoms and improving Assisted Reproductive Technology (ART) outcomes[47,48]. In addition, clomiphene citrate, a non-steroidal ovulation-inducing agent, has long been utilised for the treatment of anovulatory infertility linked to PCOS[49,50]. Given its extensive clinical use and well-documented mode of action, clomiphene citrate was chosen as the positive control to benchmark the therapeutic effects of MLENN.

Extracts from *N. nimmoniana* have previously demonstrated significant antioxidant activity, attributed largely to its bioactive constituents, including camptothecin[51]. Given its recognised pharmacological potential, this study aimed to investigate the efficacy of MLENN in mitigating the pathophysiological features of PCOS in a DHEA-induced mouse model. DHEA administration resulted in hallmark PCOS characteristics such as elevated serum androgens, disrupted oestrous cyclicity, and morphological abnormalities in ovarian and uterine tissues, consistent with the Rotterdam diagnostic criteria[52].

Body weight serves as a crucial clinical indicator of PCOS progression. Treatment with MLENN at 400 mg/kg b.w. resulted in a significant reduction in body weight compared to both the DHEA-only and DHEA+C.C. groups. While reductions in ovarian and uterine weights were observed, they did not differ significantly from the control group, supporting earlier findings that associate PCOS with increased body and organ weights[53-56]. Moreover, DHEA treatment led to an oestrous cycle dominated by the estrus phase (>80% estrus-stage cells), indicative of disrupted cyclicity. MLENN treatment reduced the estrus-stage percentage to approximately 60% (200 mg/kg) and below 40% (400 mg/kg), suggesting partial restoration of normal reproductive cycling.

PCOS is also characterised by metabolic disturbances, including elevated hepatic enzymes. In this study, DHEA significantly increased AST and ALT levels, reflecting hepatic stress known complication linked to insulin resistance and obesity in PCOS[57]. MLENN administration at 400 mg/kg effectively reduced these enzyme levels, occasionally below those of the control group, indicating a hepatoprotective effect. These results are consistent with the hepatoprotective profiles of other plant-based interventions, such as *Chlorella vulgaris*[58-60].

The lipid and protein profile analyses demonstrated a marked reduction in total cholesterol following MLENN treatment — 11.02% at 200 mg/kg and 53.41% at 400 mg/kg — compared to the DHEA group. While changes in LDL and HDL levels were not statistically significant, total protein levels declined by 25.57% and 68.61% for 200 and 400 mg/kg doses, respectively. These changes may reflect improved metabolic regulation. Comparable lipid-lowering and protein-modulating effects have been observed in PCOS models treated with plant extracts such as *Cuminum cyminum*[61,62].

Hormonal assays confirmed that DHEA induced a significant elevation in LH and testosterone, alongside decreased FSH and progesterone levels — hallmarks of hyperandrogenism and endocrine imbalance in PCOS[63]. MLENN at 400 mg/kg restored these hormone levels to near-control values, suggesting a reversal of the hormonal disruptions. Despite the widespread clinical use of pharmaceutical agents like clomiphene for PCOS, their side effects underscore the importance of safer, plant-based alternatives[64,65]. MLENN treatment improved levels of estrogen, testosterone, FSH, and progesterone, thereby supporting both follicular development and luteal function[66,67].

While both MLENN doses significantly improved total cholesterol levels ($p<0.001$), their effects on sex hormones were not statistically superior to the DHEA+C.C. group ($p>0.05$). According to the Chao-Talalay Index[68], both doses were effective in lipid modulation, with the higher dose (400 mg/kg) demonstrating superior lipid-regulating efficacy. However, the 200 mg/kg dose exhibited broader systemic effects, suggesting its suitability as a more balanced therapeutic intervention.

Mitochondrial dysfunction and oxidative stress play a pivotal role in PCOS pathogenesis. In this study, MLENN treatment, particularly at 400 mg/kg, significantly enhanced the activity of antioxidant enzymes such as SOD, GPX, and catalase, indicating a reduction in oxidative stress[69].

Histopathological analyses provided further evidence of MLENN's therapeutic potential. DHEA-treated ovaries exhibited classical PCOS-associated histological alterations, including disrupted follicular

architecture, granulosa cell degeneration, and theca layer thickening. MLENN treatment improved follicular development, increased oocyte count, and normalised granulosa layers. These outcomes are in agreement with studies involving *Trigonella foenum-graecum* and *Phoenix dactylifera*, which demonstrated similar restorative effects in PCOS models[70,71]. MLENN treatment also led to a reduction in both large and small follicular cysts, alongside an increase in corpus luteum count, further indicating restoration of ovulatory function. These findings align with those observed in studies involving *Crocus sativus* petal hydroalcoholic extract[72], *Nigella sativa* seed extract[73], and *Cinnamomum verum*[26].

Scanning electron microscopy provided additional insight into cellular-level recovery. In the DHEA group, granulosa cell disorganisation, zona pellucida disruptions, and oocyte atresia were prevalent. MLENN at 400 mg/kg restored normal follicular morphology, including well-organised granulosa layers and reduced signs of cellular degeneration, suggesting protection at the ultrastructural level[74,75].

In the uterus, DHEA induced epithelial hyperplasia, inflammatory infiltration, and increased stromal vascularity. These pathological changes were significantly alleviated in MLENN-treated groups, particularly at 400 mg/kg, which restored near-normal histoarchitecture. Similar uterine improvements have been reported with treatments involving liquorice and coconut palm extracts[76,77].

5. Limitations

While the current study demonstrates the beneficial therapeutic effects of *N. nimmoniana* in a PCOS-induced mouse model, several limitations must be considered. Firstly, the use of animal models, although valuable for preliminary evaluation, may not fully recapitulate the complex endocrine and metabolic disturbances observed in human PCOS. Moreover, the present study mainly focused on the short-term effects of treatment, while the long-term safety, effectiveness, and possible toxicity of *N. nimmoniana* have yet to be thoroughly investigated. The lack of in-depth mechanistic research restricts our knowledge of the molecular pathways underlying its effects. To overcome these limitations, future research should focus on using standardised extract formulations, conducting thorough molecular analyses, and pursuing clinical validation.

6. Conclusion

This present study rigorously investigated the therapeutic potential of the MLENN within a DHEA-induced mouse model, a well-established experimental system for mimicking PCOS pathophysiology. MLENN effectively mitigated hallmark features of PCOS, including hormonal imbalances, disrupted oestrous cyclicity, metabolic disturbances, and oxidative stress. Histological and ultrastructural improvements in ovarian and uterine tissues further validate the restorative capacity of MLENN. These findings collectively underscore the substantial potential of *N. nimmoniana* as a natural, multi-targeted intervention for the management of PCOS, warranting further investigation into its bioactive compounds and their precise mechanisms of action. Considering the promising therapeutic potential and endangered status of *N. nimmoniana*, its conservation is of critical importance. Sustainable harvesting practices, combined with ex-situ cultivation and habitat conservation, are crucial for ensuring the long-term availability of this pharmacologically valuable medicinal species for future research and therapeutic development.

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8. Author Contribution

Conceptualisation, R.A.; methodology, R.A. and N.T.; software, R.A. and R.S.; validation, R.A. and S.S.; formal analysis, R.A. and N.T.; investigation, R.A. and J.C.K.; resources, R.A.; data curation, R.A. and N.T.; writing – original draft preparation, R.A.; writing – review and editing, R.A. and N.T.; visualisation, J.C.K. and R.S.; supervision, J.C.K. All authors have read and agreed to the published version of the manuscript.

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