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Pharmacological Evaluation of Ovulation Inducing Potential of Siddha Herbomineral Formulation *Arputha Mathirai* on Estradiol Valerate Induced Poly Cystic Ovarian Syndrome Wistar Albino Rat Model

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Authors' contributions

This work was carried out in collaboration between both authors. The study was conducted and the draft was written by author GDB. It was then reviewed, edited, and approved by author NA who is the guide of the author and Head of the Department (HOD). Both authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study is to examine the potential of Arputha Mathirai, a tablet-based Siddha herbomineral formulation, in promoting ovulation in Wistar Albino rats with polycystic ovary syndrome (PCOS) induced by Estradiol Valerate (EV).

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Place of Study: The study took place at C.L. Baid Metha College of Pharmacy located in Thorapakkam, Chennai - 600 092, Tamil Nadu.
Methodology: Arputha Mathirai, the Siddha herbomineral formulation, was prepared in accordance with Good Manufacturing Practices (GMP) guidelines. Prior to conducting the study, approval was obtained from the Institutional Animal Ethics Committee (IAEC). The research adhered to ethical principles and guidelines established by the committee responsible for overseeing and regulating animal experimentation, ensuring proper control and supervision.
Female Wistar Albino rats were selected as the preferred rodent species for the study. Polycystic ovary syndrome (PCOS) was induced in the animals by administering subcutaneous injection of 100µg Estardiol valerate (EV). The reproductive cycles of the rats were synchronized to ensure consistency.
The rats were divided into four groups, each containing six rats, as follows:

- Group I: Normal Control animals received 1 ml/kg of Sodium Carboxymethyl Cellulose (CMC) solution.
- Group II: Rats were orally administered Arputha Mathirai at a dosage of 100 mg/kg for 10 days.
- Group III: Rats were orally administered Arputha Mathirai at a dosage of 200 mg/kg for 10 days.
- Group IV: Received Clomiphene at a dosage of 10 mg/kg and served as the standard group.

At the end of the study, blood samples were collected from the rats through retro-orbital and cardiac puncture. The levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, and progesterone were estimated using ELISA method. Ovaries from the experimental rats were dissected out for histopathological studies.
Results: The results consistently showed that the higher dosage of the drug (group III) had a more pronounced effect in normalizing hormone levels compared to the lower dosage group. However, it is important to note that the lower dosage group also exhibited effectiveness. While the results in both groups did not closely match those of the standard group (group IV), the drug Arputha Mathirai demonstrated significant potential in the experimental groups, supporting its efficacy in inducing ovulation.
Furthermore, the histopathological analysis confirmed the drug's potential in inducing ovulation, particularly in the higher dosage group (group III) compared to the lower dosage group (group II).
Overall, the findings suggest that Arputha Mathirai has promising ovulation-inducing activity, with the higher dosage showing more pronounced effects.
Conclusion: It is hypothesized that the bioactive phytocompounds present in Arputha Mathirai may exert their effects at various stages to restore hormone levels and reverse the pathological condition associated with PCOS. These natural compounds have the potential to regulate hormone levels and promote ovulation. However, it is important to acknowledge that there is a scarcity of published evidence regarding the specific ability of these phytochemicals to address the underlying causes of the condition. Further research is needed to explore the mechanisms by which these compounds act and their potential in addressing the root causes of PCOS.

Keywords: Siddha; Arputha Mathirai; PCOS; ovulation inducing activity; animal study; Wistar albino rats; estradiol valerate.

1. INTRODUCTION

“Polycystic ovary syndrome (PCOS) is the most common endocrinologic condition in women, affecting from 8% to 13% of reproductive-aged women. Research has exposed the still-large gap between the available evidence and its translation to improved diagnostic timing and evidence-based treatments. The pathogenesis of PCOS is complex and multifactorial, including genetic, environmental, and transgenerational components” [1]. Hyperandrogenism, anovulation, the presence of numerous ovarian cysts, abnormalities in the menstrual cycle, and fluctuating levels of gonadotropins are all symptoms of PCOS” [2].

Many allopathic and ayurvedic drugs are being prescribed by medical practitioners to address the secondary symptoms of PCOS. Although allopathic drugs like metformin, clomiphene citrate, tamoxifen, flutamide, oral contraceptive

pills, myo-inositol etc., assist in overcoming infertility and improving PCOS symptoms, they pose several side effects like hepatotoxicity, hot flushes, nausea, vomiting, impotency, blurred vision and osteoporosis in PCOS patients. "Hence, phytomedicine from herbal plants are being constantly explored for treating PCOS" [3].

"In recent times, there has been a growing trend in utilizing these medicinal preparations for addressing Poly Cystic Ovarian Syndrome (PCOS). Herbal remedies for PCOS have received attention as a form of lifestyle management in traditional medicine, in which the menstrual cycle and normal serum hormones levels can be recovered" [4]. "Different approaches to manage PCOS include the traditional system of drugs, herbal remedies, herbs, and phyto-ingredients that have proven their potential in treating PCOS" [5]. "In Siddha system of medicine, various herbal and herbo-mineral formulations have been used for the management of PCOS traditionally along with lifestyle changes and physical activity" [6]. "An attractive appeal of herbal medicine is the prospect to simultaneously target multiple pathophysiological mechanisms" [7].

"The etiology of PCOS is unknown, likely reflecting multiple pathophysiological mechanisms, and an accepted animal model of the disease has not been established. A recent review concluded that multiple models may be needed, depending on whether the goal is to investigate ovarian morphology or a particular PCOS-related disorder. In both mechanistic and treatment studies, we and others have used a model in which PCO is induced by a single intramuscular (i.m.) injection of estradiol valerate (EV) in 8-week-old rats. The rats cease ovulating and develop characteristics of human PCOS, including large cystic follicles in the ovaries and altered concentrations of luteinizing hormone" [8].

"New drug development process must continue through several stages in order to make a medicine that is safe, effective, and has approved all regulatory requirements" [9].

"The process of developing a novel drug is time consuming and costly. To increase the chances of successfully completing a clinical trial leading to the approval of a new drug, the choice of appropriate preclinical models is of utmost importance. Identifying a safe, potent, and efficacious drug requires thorough preclinical testing, which evaluates aspects of pharmacodynamics, pharmacokinetics, and toxicology in in vitro and in vivo settings. Nevertheless, merely a small fraction of investigational new drugs tested in clinical trials after passing preclinical evaluation eventually lead to a marketed product. Hence, there is a need for optimising current standard preclinical approaches to better mimic the complexity of human disease mechanisms" [10].

The Siddha system of Medicine offers distinct combinations of medicines that provide solutions for managing PCOS. Among these formulations, one specific herbomineral formulation is *Arputha Mathirai* (AM), which is mentioned in the Siddha literature, *Koshayee Anuboga Vaithiya Bramma Ragasiyam*. This formulation includes 7 herbal and two mineral drugs [11].

This preclinical evaluation was conducted to assess the ovulation-inducing activity of the mentioned formulation AM, in Wistar Albino rats with polycystic ovary syndrome (PCOS) induced by Estradiol Valerate (EV). Hence, the objective of this study was to evaluate the potential of the formulation to induce ovulation in Wistar Albino rats with EV-induced PCOS.

2. MATERIALS AND METHODS

2.1 Study Drug

2.1.1 Source and authentication of drugs

The herbal and mineral raw drugs were procured from reputed indigenous raw drug store, which were then identified and verified by the Botanist of Government Siddha Medical College, Chennai (Voucher number GSMC/MB- 566 – 571 & 614) and the Head of the Department of Gunapadam, Government Siddha Medical College, Chennai, Tamil Nadu, respectively, with the aim of ensuring authenticity.

2.1.2 Ingredients of *Arputha Mathirai* (AM) [11]

Cuminum cyminum L. (Cumin)- 70 g

Piper nigrum L. (Black Pepper)- 70 g

Zingiber officinale Roscoe. (Dried Ginger)-70 g

Piper longum L. (Long Pepper)- 70 g

Allium sativum L. (Garlic)- 70 g

Ferula asafoetida L. (Asafetida)- 70 g

Purified rock salt- 70 g

Purified Sulphur - 70 g

Citrus limon Linn. (Lemon juice)- Sufficient Quantity

2.1.3 Preparation of AM

2.1.3.1 Purification of raw drugs

All the herbal and mineral raw drugs were purified individually according to the purification methods mentioned in the classical Siddha texts, "*Sikitcha Ratna Deepam Ennum Vaidhiya Nool"* and "*Gunapadam Thathu Jeeva Vaguppu"* respectively, as mentioned below [12,13].

a) Cumin

Unwanted soil particles and dusts were removed, winnowed and sun dried.

b) Black Pepper

Soaked in sour buttermilk for 3 hours (1 *saamam*) and sun dried.

c) Dried Ginger

One part of dried ginger was bleached with 2 parts of lime stone (*kal sunnambu*) for 3 hours (1 *saamam*), washed, dried and the outer skin was peeled.

d) Long Pepper

Soaked in the leaf juice of *Plumbago indica* (*Kodiveli*) for 24 minutes (1 *naazhigai*) and then sun dried.

e) Garlic

Outer dry papery skin and the tip were removed and washed.

f) Asafetida

Roasted in coal fire and then powdered.

g) Rock Salt

Soaked in vinegar (*kaadi*) for 3 days and then sun dried.

h) Sulphur

Crude Sulphur (*Gandhagam*) was melted in an iron ladle containing little butter and the melted content was poured into a container containing juice of banana stem. This was repeated for 10 times.

i) Lemon Juice

Lemons were washed, pat dry, juice was extracted, and the seeds were removed.

2.1.3.2 Preparation of sample

The herbomineral siddha formulation *Arputha Mathirai* (AM) was prepared as per Siddha text [11].

- All the purified ingredients except garlic and lemon juice were taken in the equal quantities (70 g), grounded individually, manually in an iron mortar with pestle, into a very fine powder, and sieved using a sieving cloth individually.
- Then all the powdered single drugs were mixed together.
- Garlic (70 g) was grounded and made into a very fine paste in a stone mortar, and mixed and grounded with the above powder.
- The above mixture was grounded together in a stone mortar for about 12 hours with adequate lemon juice and then rolled into pills of 500 mg (*sundai alavu*) [14] size.
- The rolled pills were dried in shade and stored in a clean, dry, air tight container.

2.2 Ethical Approval and Experimental Animals

2.2.1 Ethical approval

Preclinical evaluations were commenced, after obtaining approval from the Institutional Animal Ethics Committee (IAEC) at C.L. Baid Metha College of Pharmacy in Thorapakkam, Chennai, Tamil Nadu (Postal Code: 600 092). All experiments were carried out in full adherence to ethical principles and guidelines established by the committee responsible for overseeing and regulating animal experimentation, ensuring proper control and supervision. (IAEC NO:05/ 31/PO/Re/S/01/CPCSEA/dated 06/04/2022).

2.2.2 Selection of animals species

Sexually matured Female Wistar albino rats (150 - 200 g) were obtained from Mass Biotech, Chennai. All the animals were kept under standard environmental condition (22±3°C). The animals had free access to water and standard pellet diet (Sai Meera Foods, Bangalore).

The preferred rodent species was female Wistar Albino rat, although other rodent species may be used. Healthy young adult animals were commonly used. All females were nulliparous and non-pregnant. Each animal, at the commencement of its inducing, were between 6 to 8 weeks old. The weight of the animals were 150 – 200 g, and the weight of the animals fell in an interval within ± 20 % of the mean weight of any previously dosed animals.

2.2.3 Housing and feeding conditions

The temperature in the experimental animal room was maintained between 22ºC ± 3ºC. Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim was 50-60%. Lighting was artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets were used with an unlimited supply of drinking water. Animals were group-caged by dose, but the number of animals per cage did not interfere with clear observations of each animal.

2.2.4 Preparation of animals

The animals were randomly selected and marked to permit individual identification, and kept in their cages for at least 7 days prior to inducing PCOS to allow for acclimatization to the laboratory conditions.

2.2.5 Induction of PCOS

PCOS was induced in the animals by subcutaneous injection of 100 µg Estardiol valerate (EV).

2.2.6 Examination of oestrous cycle

Before starting drug treatment, the reproductive cycles of the rats were synchronized by the following method. 100 µg estradiol valerate dissolved in 2 ml olive oil was injected subcutaneously. All rats after a 24 h period, received intramuscular injections of 50 µg progesterone dissolved in olive oil. After few hours, vaginal smears were obtained by vaginal lavage to monitor ovulation and oestrous cycle. Vaginal smears were prepared by washing vaginal opening with 0.9% w/v of sodium chloride with a glass dropper and placed in a clean glass slide and viewed under light microscope at 40X magnification. Examination of vaginal smears showed that all the animals were in the estrous stage.

2.3 Evaluation of Ovulation Inducing Activity

All the animals were weighed daily after drug administration for 10 days. The suitable sensitive rats were divided into four groups of six each as follows.

2.3.1 Experimental design

- Group I: Normal Control animals received 1 ml/kg of Sodium Carboxymethyl Cellulose (CMC) solution for 10 days.
- Group II: Rats were administered Arputha Mathirai 100 mg/kg for 10 days,
- Group III: Rats were administered Arputha Mathirai 200 mg/kg for 10 days
- Group IV: Received Clomiphene 10 mg/kg and served as standard. All the drugs were given orally.

2.3.2 Sample collection

At the end of the study, prior to sacrificing the animals, an overnight fasting period was implemented while ensuring they had access to water. The animals were then sacrificed using an excessive amount of anesthesia. Blood samples were collected through retro-orbital and cardiac puncture methods. Hormone estimation was subsequently performed using the Cobase 411 immunoassay analyzer.

After that 2 ml of blood was collected by retro orbital puncture. Blood samples were centrifuged for 15 minutes at 4000 rpm and the separated serum samples were frozen at -20°C and kept for later estimation of LH, FSH, Estradiol and progesterone by ELISA method.

2.3.3 Hormonal assay

2.3.3.1 Estimation of serum luteinizing hormone (LH)

“The method employed was Microwell Enzyme Linked Immunosorbent Assay (ELISA) using analytical grade reagents. 0.05 ml of the serum was pipetted into the assigned wells. 0.001 ml of LH-Enzyme reagent was added to all the wells. The microplate was swirled for 20-30 seconds and covered. This mixture was allowed to incubate for 60 minutes at room temperature. After which, the contents were discarded by decantation. 350 µl of wash buffer was added and decanted for 3 times. 100 µl of working substrate solution was added to all the wells and was allowed to incubate for fifteen minutes. 50 µl of stop solution was added to all the wells and

gently mixed for 20 seconds. The optical density was read at 450 nm in a microplate reader within 30 mins. The mean absorbance values for each set of standards, controls, and test samples were calculated and a standard curve was constructed by plotting a mean absorbance obtained from each of the reference standard against its concentration from the standard curve" [15].

2.3.3.2 Estimation of serum follicle stimulating hormone (FSH)

The method employed was Microwell immunoassay (ELISA) using analytical grade reagents. 0.05 ml of the serum was pipetted into the assigned wells. 0.001 ml of FSH-Enzyme reagent was added to all the wells. The microplate was swirled for 20-30 seconds and covered. This mixture was allowed to incubate for 60 minutes at room temperature. After which, the contents were discarded by decantation. 350 µl of wash buffer was added and decanted for 3 times. 100 µl of working substrate solution was added to all the wells and was allowed to incubate for fifteen minutes. 50 µl of stop solution was added to all the wells and gently mixed for 20 seconds. The optical density was read at 450 nm in a microplate reader within 30 mins. The mean absorbance values for each set of standards, controls, and test samples were calculated and a standard curve was constructed by plotting a mean absorbance obtained from each of the reference standard against its concentration from the standard curve.

2.3.3.3 Determination of serum progesterone levels

"The method employed was Microwell immunoassay (ELISA) using analytical grade reagents. 0.025 ml of the serum was pipetted into the assigned wells. 0.05 ml of progesterone enzyme reagent was added to all the wells. The microplate was swirled for 20 seconds to mix, 0.05 ml progesterone biotin reagent was added to all the wells, the mixture was swirled for 20 seconds to mix and covered, this mixture was allowed to incubate for 60 minutes at room temperature. After which, the contents were discarded by decantation. 350 µl of wash buffer was added and decanted for 3 times. 100 µl of working substrate solution was added to all the wells and was allowed to incubate for twenty minutes. 50 µl of stop solution was added to all the wells and gently mixed for 20 seconds. The optical density was read at 450 nm in a microplate reader within 30 mins. The mean absorbance values for each set of standards, controls, and test samples were calculated and a standard curve was constructed by plotting a mean absorbance obtained from each of the reference standard against its concentration from the standard curve" [15].

2.3.3.4 Determination of serum estradiol levels

"The method employed was Microwell immunoassay (ELISA) using analytical grade reagents. 0.025 ml of the serum reference was pipetted into the assigned wells. 0.05 ml of Estradiol Biotin reagent was added to all the well. The microplate was swirled for 20 seconds to mix, the mixture was incubated at room temperature for 30 mins, 0.05 ml Estradiol enzyme reagent was added to all the wells, the mixture was swirled for 20 seconds to mix and covered. This mixture was allowed to incubate for 90 minutes at room temperature, after which, the contents were discarded by decantation. 350 µl of wash buffer was added and decanted for 3 times. 100 µl of working substrate solution was added to all the wells and was allowed to incubate for twenty minutes. 50 µl of stop solution was added to all the wells and gently mixed for 20 seconds. The optical density was read at 450 nm in a microplate reader within 30 mins. The mean absorbance values for each set of standards, controls, and test samples were calculated and a standard curve was constructed by plotting a mean absorbance obtained from each of the reference standard against its concentration from the standard curve" [15].

2.3.4 Histopathology

Ovaries from the experimental rats were dissected out and fixed in 10% buffered neutral formal saline and processed. After fixation, tissues were embedded in paraffin. Fixed tissues were cut at 10 µm and stained with haematoxylin and eosin. The sections were examined under light microscope for histological changes.

3. RESULTS

All the results were inferred, tabulated and depicted in figures as hereunder.

3.1 Serum Hormone Levels

The high dose of the drug (group III) demonstrated a greater potential in normalizing hormone levels compared to the low dose group.

Table 1. Effect of *Arputha Mathirai* on serum concentration of reproductive hormones of female Wister Albino Rat

S. No	Group	Treatment and Dose	*LH (IU/ml)	*FSH (IU/ml)	* Estradiol (pg/ml)	*Progesterone One (pg/ml)
1.	Normal	2 ml/kg 2% CMC	0.28±0.12	0.33±0.24	54.10±3.5	9.03±1.65
2.	Low	100 mg /kg	0.23±0.07	0.43±0.14	36.11±1.3	5.7±1.22
3.	High	200 mg /kg	0.47±0.04	0.55±0.18	31.64±2.1	6.5±0.32
4.	STD	Clomiphene 10 mg/kg	0.66±0.27	0.73±0.21	26.16±18	7.5±0.32

**Mean value ± Standard Error*

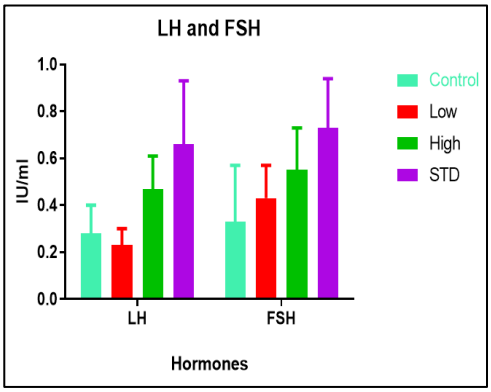

Fig. 1. Effect of *Arputha Mathirai* on Serum LH and FSH

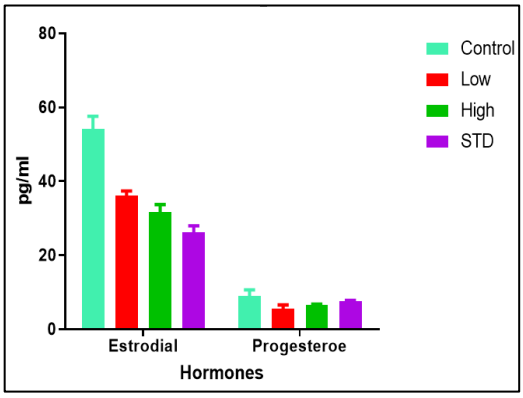

Fig. 2. Effect of *Arputha Mathirai* on Serum Estradiol and Progeterone

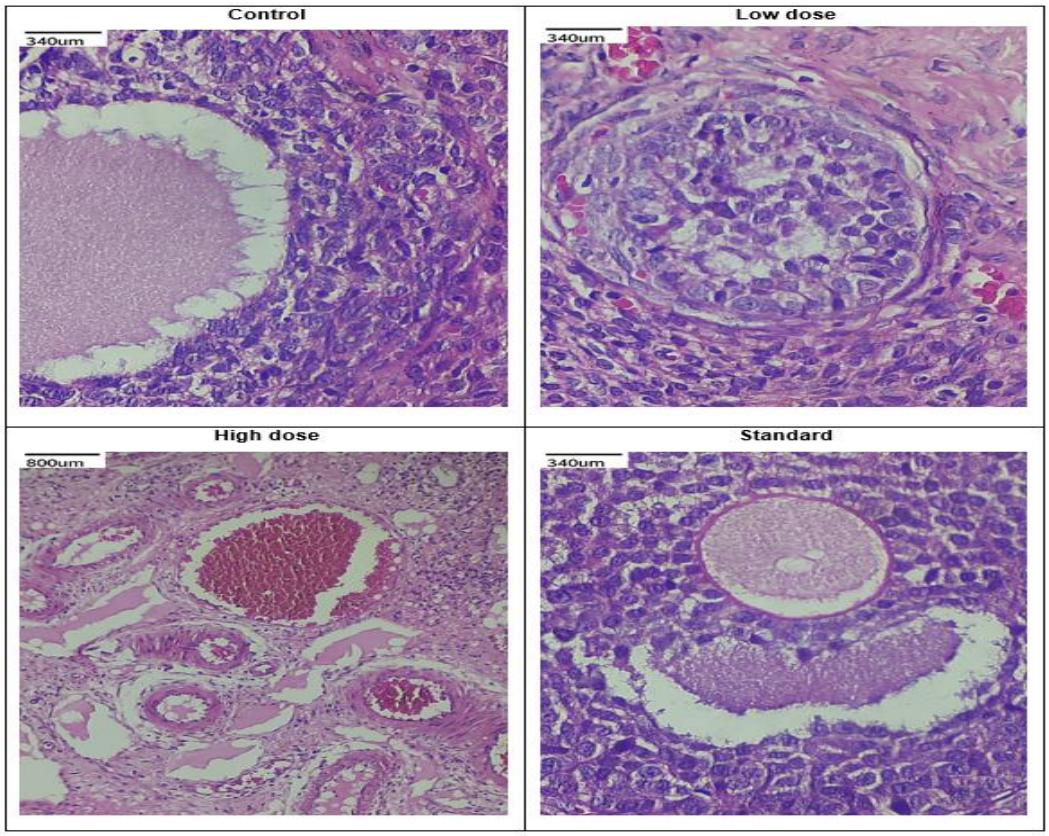

Fig. 3. Histopathology of ovary in all the group

3.2 Histopathology Results

The histopathology examination of samples from group I demonstrated a noteworthy increase in the number of follicles at various stages and corpus luteum. Treatment with AM at doses of 100 mg/kg and 200 mg/kg in group II & III respectively, reduced the follicle count compared to the control group to a significant level, and the histology of the corpus luteum was restored to a similar state as that of the normal control rats. The histopathology report of group IV showed the normal appearance of graafian and antral follicles, as well as the presence of corpora lutea (CL), atretic follicles (AF), and interstitial tissue (IT) that appeared normal.

4. DISCUSSION

The serum LH levels in group II and group III were measured to be 0.23+0.07 IU/ml and 0.47+0.04 IU/ml respectively. In comparison, the control group I had a level of 0.28+0.12 IU/ml, while the standard group IV showed a level of 0.66+0.27 IU/ml. The results clearly demonstrate a significant difference in the serum LH level in the high-dose group (group III), suggesting that the drug is effective at a high dosage of 200 mg/kg, in normalizing the serum LH levels.

Group II and III exhibited serum FSH levels of 0.43+0.14 IU/ml and 0.55+0.18 IU/ml, respectively. These levels were significantly higher compared to the control group, which had a level of 0.33+0.24 IU/ml. The standard group showed a serum FSH level of 0.73+0.21 IU/ml. These findings further confirm the efficacy of the drug in significantly normalizing the serum FSH levels.

When analyzing the serum estradiol levels, it was observed that group II and group III had levels of 36.11±1.3 pg/ml and 31.64+2.1 pg/ml, respectively. In comparison, the normal control group had a level of 54.10+3.5 pg/ml, while the standard group exhibited a level of 26.16+18 pg/ml. These results further validate the effectiveness of the drug in both groups, suggesting a potential impact in the high-dose group as well.

The serum progesterone levels in group II and III were found to be 5.7+1.22 pg/ml and 6.5+0.32 pg/ml, respectively. In comparison, group I and IV had levels of 9.03+1.65 pg/ml and 7.5+0.32 pg/ml, respectively. The significant difference observed in both the low and high dose groups confirms the efficacy of the drug in reducing serum progesterone levels.

The aforementioned findings consistently indicated that the high dose of the drug (group III) demonstrated a greater potential in normalizing hormone levels compared to the low dose group. However, it is important to acknowledge that the low dose group also exhibited efficacy. Despite the fact that the results in both groups did not closely resemble those of the standard group IV, the drug AM demonstrated significant potential in the test groups, providing support for its efficacy in inducing ovulation.

Prior research studies have extensively examined the specific ingredients present in *Arputha Mathirai* and their individual therapeutic effects [16]. Some of these studies have investigated the ovulation-inducing properties of asafetida [17], the hypolipidemic effects of cumin and black pepper [18-20] the anti-diabetic, anti-atherosclerotic, anti-thrombotic, and antihyperlipidemic activities of garlic as well as its role as an oxidative stress marker [21,22], and the emmenagogue properties of asafetida [23,24]. These studies have collectively established the therapeutic significance of these specific ingredients within the formulation [16].

The current physicochemical, phytochemical, and HPTLC analysis of AM yielded valuable evidence regarding the presence of certain compounds. These compounds are believed to be active components that have the potential to intervene in the pathological mechanisms associated with PCOS, thereby offering a potential means of managing the condition [16].

The standardization process implemented in this study was successful in confirming the presence of bioactive phytocomponents in the formulation. The presence of these bioactive compounds enhances the therapeutic potential of *Arputha Mathirai* (AM) as an intervention for PCOS [16].

Additionally the histopathological report also proved the potential ovulation inducing activity of the drug in the experimental groups, particularly with specific to high dose group II when compared to the low dose group.

The serum hormone levels and histopathological findings from the aforementioned study provide clear evidence that the tablet Arputha Mathirai effectively manages serum hormone levels and the condition of PCOS in animals.

5. CONCLUSION

Our hypothesis suggests that the natural bioactive compounds present in *Arputha Mathirai* functions at different stages to restore hormone levels and revert the pathological condition back to normal. Identifying the specific phytochemical component and its underlying mechanism is crucial in understanding how it contributes to the reversal of the pathogenesis of polycystic ovary syndrome (PCOS), since there is a lack of published evidence regarding the ability of these phytochemicals to repair the underlying causes of the condition.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Institutional Animal Ethics committee approval has been taken to carry out this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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