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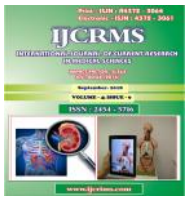

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Investigation of Ovulation Induction Potential of Siddha Formulation *Kalingathi Ennai* by Hormone induced Polycystic Ovarian Syndrome in Rats

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Abstract

Polycystic ovary syndrome (PCOS) is the commonest cause (70%) of an ovulatory infertility. The primary abnormality seems to be an excess of androgen production within the ovary that leads to the recruitment of large numbers of small preovulatory follicles, which fail to respond to normal concentrations of follicle stimulating hormone. In recent time's siddha system of traditional medicine gaining much attention for management of PCOS. Further there is some evidence of positive effects in women with PCOS for towards herbal medicines. It was noted from the siddha literatures that several siddha formulation has potential of preventing the progression of PCOS one such novel drug is *Kalingathi Ennai* (KE). The main aim of the present study is to evaluate the ovulation induction potential of the formulation KE in hormone induced PCOS in female rats. In the present study PCOS was induced in wistar rats by injecting estradiol and progesterone. Result of the investigation has cleared suggested that treatment with KE at the dose of 100 and 200 mg/kg has significantly increased the level of reproductive hormones such as luteinizing hormone (LH) and follicle stimulating hormone (FSH). Further it was also noted that the level of estrodial and progesterone has shown marked decrease in treatment group when compare to that of the hormone alone induced group. Similar results were observed in rates treated with standard drug clomiphene at the dose of 10mg/kg. It was concluded from the data's of the present study the ovulation induction potential of the siddha formulation KE may be due to some of the bioactive components present in it. Further investigation at clinical level need to be carried out to justify the present pre-clinical data before exploring the KE for the treatment of PCOS.

Keywords: *Polycystic ovary syndrome*, Siddha formulation, Kalingathi Ennai, LH, FSH, Progesterone, Estrodiol.

1. Introduction

PCOS disorders of anovulation account for about 30% of infertility and often present with irregular periods (oligomenorrhoea) or an absence of periods (amenorrhoea). Many of the treatments are simple and effective, so couples may need only limited contact with doctors. This makes it easier for a couple to maintain a private loving relationship than in the stressful, more technological environment of assisted conception. However, not all causes of anovulation are amenable to treatment by ovulation induction. Anovulation can sometimes be treated with medical or surgical induction, but it is the cause of the anovulation that will determine whether ovulation induction is possible. *Hyperprolactinemia* is usually caused by a pituitary microadenoma. This leads to a reduction in the production of pituitary FSH and LH. Although the commonest presentation is secondary amenorrhoea, some women may present with galactorrhoea[1].

PCOS is recognized as the most common endocrinopathy of women. Increased androgen synthesis, disrupted folliculogenesis, and insulin resistance lie at the patho-physiological core of PCOS. Current therapy for such a syndrome is use of insulin sensitizers. Large randomized clinical trials of metformin as the insulin-sensitizing drug, however, suggested that it produces many side effects after prolonged usage [2].

Pharmaceutical treatment for menstrual irregularity includes the oral contraceptive pill (OCP) and ovulation induction with clomiphene citrate (clomiphene) [3,4] depending on fertility needs. Women with PCOS are however likely to exhibit contraindications for the OCP [5] and whilst induction of ovulation with clomiphene has demonstrated success, pregnancy rates remain inexplicably low [6]. Up to thirty 30% of women, particularly overweight women with PCOS, fail to respond to clomiphene therapy [7,9]. Management for hyperandrogenism includes anti-androgens and hypoglycaemic pharmaceuticals such as metformin [9]. Metfomin has demonstrated effectiveness for improving insulin sensitivity and hyperandrogenism, however use of metformin is associated with the high incidence of adverse effects including nausea, vomiting and gastro-intestinal disturbances.

Herbal medicines are complex interventions with the potential for synergistic and antagonistic interactions between compounds [10]. Effects within the body may also exhibit complexity by simultaneous interactions with various body systems, both biochemically and by altering organ function [11].Traditional systems of medicine often contain information on treatments which have been used for centuries. These can be sources of new drug discoveries [12]. One of the difficulties in this area is that the paradigm of medicine was different and so the terminology used in ancient manuscripts is different from what we understand today. Ancient practitioners often had a holistic approach towards the human body as regards health and sickness. But if the medicines used by ancient healers were effective, it should be explicable by a rational mechanism of action as well. Siddha system of traditional medicine has several holistic approaches for the clinical management of PCOS in women's since several years. The main aim of the present study is to evaluate the ovulation induction potential of the formulation KE in hormone induced PCOS in female rats.

2. Materials and Methods

2.1. Source of raw drugs

The Required raw materials were procured from a well reputed indigenous drug shop from, Chennai, Tamil Nadu, India .All raw drugs were authenticated by respective authorities before utilizing the same for the preparing the formulation.

2.2. Ingredients

The siddha formulation *Kalingathi Ennai* comprises of the following ingredients

- Varithumatikaisaru (*Citrulluscolcocynthis)*
- Vengayasaru (*Allium cepa)*
- Elumitchampalasaru (*Citrus limon)*

- Chitramanakkuennai (*Castor oil)*
- Injirasam (*Zingiber officinale)*
- Seeragam (*Cuminum cyminum)*
- Dhaniya (*Coriandrum sativum)*
- Sathakuppai (*Anethum graveolens)*
- Lavangam (*Syzygium aromaticum)*
- Lavangapattai (*Cinnamom umverum)*
- Manjal (*Curcuma longa)*
- Elam (*Elettaria cardamomum)*

2.3. Formulation of Trial drug *KalingathiEnnai* [13]

The above mentioned juices and oil are mixed together in a mud pot and boiled. During boiling the above given purified herbal ingredients are made into powder form and mixed together. Finally, oil prepared in the way as mentioned in the literature is safely kept.

2.4. Animal

Healthy adult Wistar albino rats were used for the study. The animals were housed in poly propylene cages and were kept in well ventilated with 100% fresh air. A 12 light / dark cycle were maintained .Room temperature was maintained between 22 $\pm$ 2^0 Cand relative humidity 50–65%. They were provided with food (Sai feeds, Bangalore, India) and water *ad libitum*. All the animals were acclimatized to the laboratory for 7 days prior to the start of the study. The experimental protocol was approved by The Institutional Animal Ethics Committee of C.L.BaidMetha College of Pharmacy, Chennai, Tamil Nadu, India.XLVIII/02/CLBMCP/2016.

2.5. Synchronization of animals reproductive cycle [14,15]

Before starting drug treatment, the reproductive cycles of the rats were synchronized by the following method. 100µg estradiol dissolved in 2 ml olive oil was injected subcutaneously. All rats after a 24 hr 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 oestrous stage.

2.6. Grouping and Experimental design

- Group I Normal Control animals 1ml/kg of CMC solution.
- Group II rats were administered Kalingathi ennai100mg/kg for 10days,
- Group III rats were administered Kalingathiennai200mg/kg for 10 days
- Group IV received Clomiphene 10mg/kg and served as standard.

All the drugs were given orally. After that 2ml 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 and Estradiol by ELISA method.

2.7.Biochemical assay [16]

The method employed was Microwell Enzyme Linked Immunosorbent Assay (ELISA) using analytical grade reagents.

2.7.1.Estimation of serum luteinizing hormone (LH)

The method employed was Microwell immunoassay (ELISA) using analytical grade reagents. 0.050ml of the serum was pipetted into the assigned wells. 0.001ml 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. 350µl of wash buffer was added and decanted for 3 times. 100µl of working substrate solution was added to all the wells and was allowed to incubate for fifteen minutes. 50µl of stop solution was added to all the wells and gently mixed for 20 seconds. The optical density was read at 450nm in a microplate reader within 30mins. 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.

2.7.2.Estimation of serum follicle stimulating hormone (FSH)

The method employed was Microwell immunoassay (ELISA) using analytical grade reagents. 0.050ml of the serum was pipetted into the assigned wells. 0.001ml 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. 350µl of wash buffer was added and decanted for 3 times. 100µl of working substrate solution was added to all the wells and was allowed to incubate for fifteen minutes. 50µl of stop solution was added to all the wells and gently mixed for 20 seconds. The optical density was read at 450nm in a microplate reader within 30mins.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

2.7.3.Determination of serum progesterone levels

The method employed was Microwell immunoassay (ELISA) using analytical grade reagents. 0.025ml of the serum was pipetted into the assigned wells. 0.050ml of progesterone Enzyme reagent was added to all the wells. The microplate was swirled for 20 seconds to mix, 0.050ml 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. 350µl of wash buffer was added and decanted for 3 times. 100µl of working substrate solution was added to all the wells and was allowed to incubate for twenty minutes. 50µl of stop solution was added to all the wells and gently mixed for 20 seconds. The optical density was read at 450nm in a microplate reader within 30mins. 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.

2.7.4.Determination of serum Estradiol levels

The method employed was Microwell immunoassay (ELISA) using analytical grade reagents. 0.025ml of the serum reference was pipetted into the assigned wells. 0.050ml of Estradiol Biotin reagent was added to all the wells. The microplate was swirled for 20 seconds to mix, the mixture was incubated at room temperature for 30mins,0.050ml 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. 350µl of wash buffer was added and decanted for 3 times. 100µl of working substrate solution was added to all the wells and was allowed to incubate for twenty minutes. 50µl of stop solution was added to all the wells and gently mixed for 20 seconds. The optical density was read at 450nm in a microplate reader within 30mins. 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.

3. Results

3.1. Effect of *Kalingathi Ennai* on Serum concentration of reproductive hormones

Results analysis of the present study has revealed an evidence based data's on serum hormone concentration of the experimental animals. PCOS induced rats has shown an elevated level of estradiol and progesterone .Further depletion in the concentration of LH and FSH were observed in these rats. Treatment with KE at the dose of

100 and 200 mg/kg has significantly increased the level of reproductive hormones such as luteinizing hormone (LH) and follicle stimulating hormone (FSH). Further it was also noted that the level of estrodial and progesterone has shown marked decrease in treatment group when compare to that of the hormone alone induced group. Similar results were observed in rates treated with standard drug clomiphene at the dose of 10mg/kg. As shown in table 1.

Table 1: Effect of *Kalingathi Ennai* on Serum concentration of reproductive hormones

S. No	Group	Treatment and dose	LH (IU/ml)	FSH (IU/ml)	Estrodiol (pg/ml)	Progesterone (pg/ml)
1.	Normal	2ml/kg 2% CMC	0.28±0.12	0.33±0.24	54.10±3.5	9.03±1.65
2.	Low dose	KGE 100 mg /kg	0.36±0.09	0.46±0.20	38.32±2.6	6.6±1.14
3.	High dose	KGE 200 mg /kg	0.43±0.14	0.55±0.16	32.68±2.1	6.4±0.46
4.	Standard	Clomiphene 10mg/kg	0.56±0.27	0.63±0.21	27.17±1.8	5.9±0.86

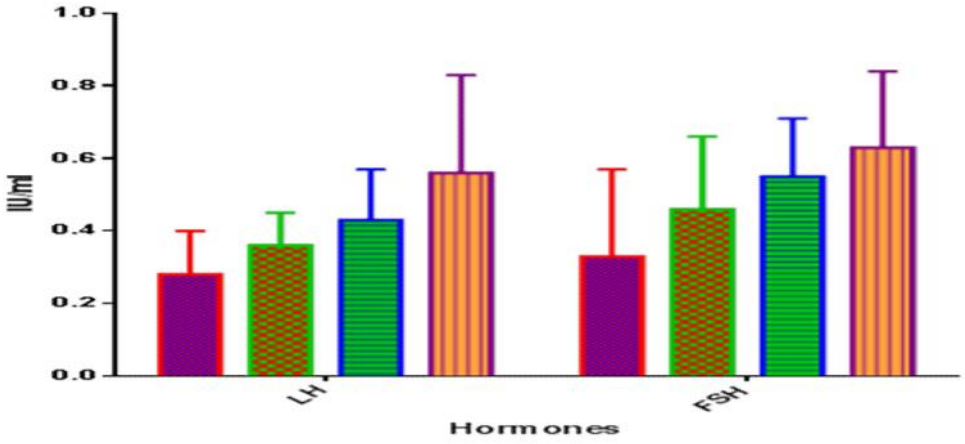

Figure 1: Representation of Serum concentration of LH and FSH level on *Kalingathi Ennai* and standard drug treated rats

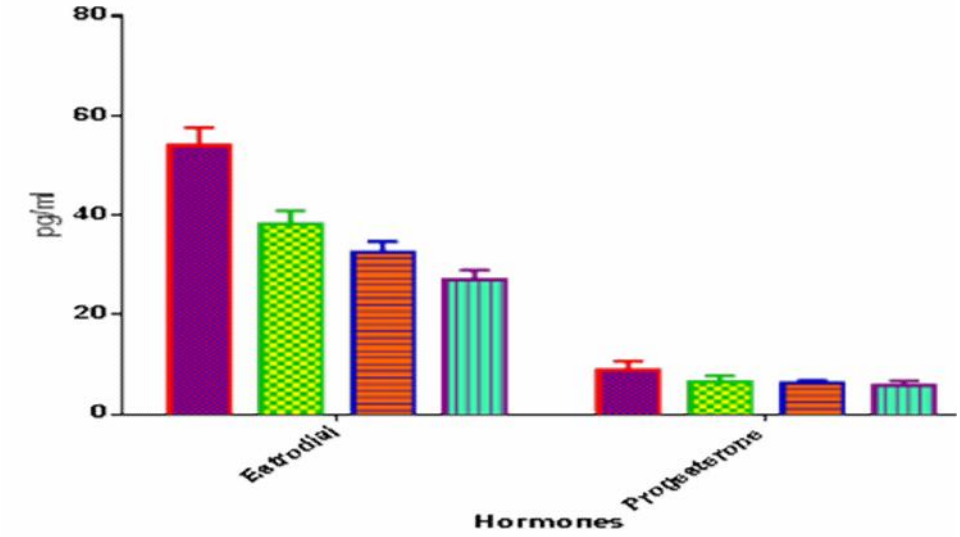

Figure 2: Representation of Serum concentration of estradiol and progesterone level on *Kalingathi Ennai* and standard drug treated rats

4. Discussion

Polycystic ovary syndrome (PCOS) is the most common female reproductive endocrine disorder [17] estimated to affect up to one in five women [18]. PCOS has broad health implications including adverse metabolic (obesity, type two diabetes, cardio vascular disease), reproductive (infertility, miscarriage, pregnancy and neonatal complications) [19,20], and psychological (anxiety, depression and stress) [21,22] risks. The pathogenesis is underpinned by insulin resistance [23] which affects up to 75% of lean women and up to 95% of obese women (compared to 62% in BMI matched non-POCS controls) [24]. Prevention and reduction of being overweight with lifestyle interventions is first-line evidence-based treatment [25], however the strength of evidence for lifestyle is limited by high attrition in randomised controlled trials (RCTs) [26,27] and, as in the general population, engagement and adherence to lifestyle intervention is impacted by psychosocial and physical barriers in women with established obesity [28]. Additional treatments for PCOS symptoms include pharmaceutical agents [29] (contraceptive pills (OCP) and hypoglycaemics), but contraindications for the OCP are common in overweight women [30] and hypoglycaemic agents are associated with significant rates of unpleasant side effects, potentially worsening women's quality of life. Women with PCOS have been shown to seek out alternative [31] and adjunct treatments including complementary medicines (CM) to improve their health, fertility and wellbeing [32].

PCOS has many clinical manifestations, including oligomenorrhea and hyper-androgenism, leading to metabolic dysfunction [33]. In the present study, we investigated the biochemical and clinical characters of PCOS in a rat model. In PCOS, excess production of androgens interferes with the process of follicular maturation and selection of dominant follicles during ova formation. It also promotes early stages of follicular growth in primate ovary leading to the syndrome's insulin resistance and fat distribution. In our study, PCO rats demonstrated the formation of empty cysts filled with follicular fluid similar to reported ovarian histology. In all these ways, the rat model behaves similarly to the human system indicating that it adequately mimics the human PC ovary.

Complementary medicine (CM) use by women has increased during the past ten years with rates of use ranging between 26% and 91% [34-38]. One of the popular types of CM is herbal medicine [39]. Herbal medicines are known to contain pharmacologically active constituents with physiological effects on female endocrinology and have been positively associated with reduced incidences of breast cancer, osteoporosis and cardiovascular disease [40-45].

PCOS is a life-long condition and although the exact cause is yet to be identified, it is believed to have epigenetic origins, influenced by the uterine environment and behaviouralfactors. Being overweight exacerbates all aspects of PCOS due to underlying metabolic disturbances [46]. Signs and symptoms are mediated by hormonal disorder including elevated androgens and fasting insulin, and abnormal relative ratio of the gonadotropins luteinising hormone (LH) and follicle stimulating hormone (FSH) . Endocrine imbalances occur within the framework of disordered ovarian folliculogenesis, chronic anovulation, clinical signs of hyperandrogenism and metabolic syndrome [47]. Results analysis of the present study has revealed an evidence based data's on serum hormone concentration of the experimental animals. PCOS induced rats has shown an elevated level of estradiol and progesterone. Further depletion in the concentration of LH and FSH were observed in these rats. Treatment with KE at the dose of 100 and 200 mg/kg has significantly increased the level of reproductive hormones such as luteinizing hormone (LH) and follicle stimulating hormone (FSH). Further it was also noted that the level of estrodial and progesterone has shown marked decrease in treatment group when compare to that of the hormone alone induced group. Similar results were observed in rates treated with standard drug clomiphene at the dose of 10mg/kg. Results of this current investigation contribute to the evidence for siddha system of ancient traditional medicine for PCOS. Traditional siddha herbal

formulations are another form of ingestible complementary medicine of interest to women with PCOS.

5. Conclusion

Polycystic ovary syndrome (PCOS) is a complex, common reproductive and endocrine disorder affecting up to 17.8% of reproductive aged women. Medical management places strong emphasis on a multidisciplinary approach as pharmaceutical treatments appear to be only moderately effective in treating individual symptoms. Conventional pharmaceutical management is limited by the prevalence. Herbal remedies from siddha origin has potential of alleviating the symptoms in PCOS since several years. Results of the present study also justifies the same which was evident by increase in the level of LH, FSH and decrease in the level of progesterone and estradiol in rats treated with siddha drug *KalingathiEnnai*. In conclusion, the present study indicates that *KalingathiEnnai* has potential efficacy in the prevention and maintenance of PCOS.

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