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PHARMACOLOGICAL SCREENING OF TRADITIONAL SIDDHA POLYHERBAL FORMULATION ASHUWATHI CHOORANAM GIVEN FOR PCOS

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ABSTRACT

This *Siddha* drug "Ashuwathi Chooranam*"* is yet remained unexplored for its exact pharmacological values in terms of scientific research. To fill these scientific lacunae, the present work was undertaken to standardize Ashuwathi chooranam (AC) to validate through pharmacological screening in animal model. Group I: Control rats; Group II: Letrozole induced polycystic ovarian syndrome rats (PCO) where letrozole was given orally and daily in a dose of 1 mg/kg dissolved in 0.9% NaCl solution for 21 days; Group III: Clomiphene received group. IV. Polycystic ovarian syndrome rats subjected to Ashuwathi chooranam (100mg) V. Polycystic ovarian syndrome rats subjected to Ashuwathi chooranam (200mg) orally for three weeks after the induction of polycystic ovarian syndrome. Plasma levels of hormones, fasting glucose and fasting insulin were measured. Serum insulin level when measured in all group of animals were increased significantly in untreated PCOS rats (***p<0.001). Treatment of AC caused a decrease in insulin level as compared to PCOS group (p<0.001). Iincreasing the doses of Ashuwathi chooranam (200mg) all rats showed improved cyclicity and reverted back to normal cycle. AC at higher doses (100 mg and 200 mg) at 30 days of treatment, number of peripheral cysts significantly decreased and increased normal growing follicles; indicating normal ovulation due to functional ovary. The hormonal assay was done by ELISA method. It showed that there is considerable increase in the FSH and estradiol hormones and AC possess the antioxidant activity from DPPH assay. AC has magnificent ovulogenic activity over PCOS *(Soothaga vayu)* in female.

KEYWORDS: Pharmacological studies, Siddha, Ashuwathi chooranam, ovulation inducing activity, OGTT, hormonal and DPPH assay.

INTRODUCTION

World's knowledge and procedure of human reassurance and human well-being may be enhanced usefully and scientifically that obtained from the mine of Siddhas [1a]. Siddha Pharmacopeia, chemistry and metallurgical medicines seem to have been perfected in a wonderful manner to suit their own needs and aims; and Rasa-sastra is indeed admirable.[1b] Preparation of medicines requires an accurate knowledge of ingredients and identification is an essential condition for success.[1c] In Siddha the clinical features of PCOS are described in "Yugi Muni Vaithyya Kaaviyam" ,"Thirumoolar Karukidai Vaithyam" and in "Gnanvettiyan-1500" by Thiruvalluva Nayanaar discussed in detail about female reproductive problems such as PCOS, infertility, fibroids, etc., Prevention and cure are the basic aims of all systems of medicine whereas the Siddha system has in addition the transcedental motivation of what might called the immortality of the body.[2] According to Siddha, many formulations have been reported which help to restore the ovulation and minimize the PCO phenotypes. One of such effective formulation mentioned in this system is "Ashuwathi Chooranam*"*.[3] for treating '*Karpa Vayu'* (PCOS) successfully.[4] World Health Organisation (WHO) greatly recommends herbal based traditional medicine for its safety, simple accessibility and low cost and well known for its abundant source of phytosterols, phenol and other phytoconstituents.[5] Some principal herbal ingredients of this formulation which include *Withania somnifera*, *Zingiber officinale*, *Piper nigrum*, *Piper longum*, *Myristica fragrans*, *Glycyrizzha glabra*, *Syzygium aromaticum*, *Picrorrizha Scrophularia*, are known to possess various beneficial activities. *Hyoscymus niger* has a stimulatory effect on the ovarian

tissue, which may produce an estrogen-like activity that enhances repair of the endometrium and stops bleeding.[6] The evaluation of physicochemical, elemental analysis, instrumental and toxicological studies were done and it revealed the phytoconstituents and phytosterols polyphenols can have a role in modulation of steroid status in PCOS,[7] ash values were in accordance with AYUSH guidelines, heavy metals present below detectable limits, presence of nano size paricles,[8] and there were no mortality in rats showed the safety of drug.[9] This *Siddha* drug "Ashuwathi Chooranam*"*(AC) is yet remained unexplored for its exact pharmacological values in terms of scientific research. To fill these scientific lacunae, the present work was undertaken to standardize AC to validate through pharmacological screening in animal model.

MATERIALS AND METHODS

Preparation of the Drug

The drug Ashuwathi chooranam (AC) for Soothaga vayu as per siddha literature [10] and collected from surrounding Chennai district in Tamilnadu. The test drug was identified and authenticated by Gunapadam Experts, P.G Gunapadam branch, GSMC, Arumbakkam, Chennai-106. The raw materials were altogether purified separately as per siddha literature.

Purification of the Chooranam

The drugs that are purified by removing the sand, dust particles, and roasted are Withania somnifera, Piper nigram, Myristica fragrans, Picrorhiza scrophulariiflora, Hyoscymus niger; Zingiber officinale (dried ginger)-the outer skin were removed, the other drug was roasted slightly Piper longum, Myristica fragrans, Glycirriza glabra, Syzygium aromaticum -the flower buds were removed and fried slightly [11]. Ashuwathi Chooranam consists of *Amukkura (Withania somnifera), Chukku (Zingiber officinale) (dried ginger), Milagu (Piper nigrum) Thippili (Piper longum), Jaadhikaai (Myristica fragrans), Jaadhipathiri (Myristica fragrans), Adhimadhuram (Glycirriza glabra), Krambu (Syzygium aromaticum), Kadugurohini (Picrorhiza scrophulariiflora)* all the above ingredients are taken up to 35gms except *Krosaniomam (Hyoscymus niger)* taken 15.3gms and Sugar (*Saccarum officinarum)* is taken 127.5gms were prepared according to the method mentioned in the Siddha literature and following the AYUSH guidelines [12] and made into fine powder and stored in airtight container.

Pharmacological Studies

Ovulation inducing activity in female Wistar albino rat model

Method

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.[13]

The study tried to evaluate the effect of this exercise type on reproductive dysfunction in rats with polycystic ovarian syndrome.

Materials and Methods: Female white albino rats were allocated into three groups:

- Group I: Control rats
- Group II: Letrozole induced polycystic ovarian syndrome rats (PCO) where letrozole was given orally and daily in a dose of 1 mg/kg dissolved in 0.9% NaCl solution for 21 days.
- Group III: Polycystic ovarian syndrome rats subjected to AC (PCO+AC) of the lower limbs for three weeks after the induction of polycystic ovarian syndrome. After 6 weeks from the beginning of the study, final body weight, body mass and Lee indices were determined. Plasma levels of LH, free testosterone, estradiol, progesterone, prolactin, fasting glucose and fasting insulin were measured.

OGTT

- Women with polycystic ovarian syndrome (PCOS) are at increased risk for developing glucose intolerance leading to type 2 diabetes mellitus (DM) [14]. Hence, it was necessary to evaluate the efficacy of AC on glucose homeostasis. Thereby, Oral glucose tolerance test (OGTT) was performed in all groups of animals. PCOS rats exhibited high glucose tolerance compared to normal control rats (**$p<0.01$, ***$p<0.001$) at all the time points of OGTT profile. AC formulation treated PCOS rats in different doses (5 mg, 10 mg) demonstrated significantly reduced glucose intolerance and improved cellular glucose uptake upon increasing time period of dose (30 days) of OGTT profile.
- Glucose homeostasis is governed by insulin action. Thereby, we measured serum insulin level in all group of animals wherein serum insulin levels of untreated PCOS rats were increased significantly (***$p<0.001$). Treatment of fresh AC caused a decrease in insulin level as compared to PCOS group ($p<0.001$).[15]

Hormonal assay

Experimental design

- Group I Normal Control animals 1ml/kg of CMC solution.
- Group II rats were administered AC 100mg/kg for 10days,
- Group III rats were administered AC 200mg/kg for 10 days
- Group IV received Clomiphene 10mg/kg and served as standard. All the drugs were given orally.
- Group V Letrozole induced PCOS animals.

Biochemical assay

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

Estimation of serum luteinizing hormone (LH)

The method employed was Micro well 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 micro plate 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 micro plate 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.

Estimation of serum follicle stimulating hormone (FSH)

The method employed was Micro well 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 micro plate 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 micro plate 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.

Determination of serum progesterone levels

The method employed was Micro well 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 micro plate 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 micro plate 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.

Determination of serum Estradiol levels

The method employed was Micro well 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 micro plate 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 micro plate 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.[16]

Antioxidant activity

Free radical scavenging activity

DPPH ASSAY (2, 2-diphenyl -1-picrylhydrazyl)**.**

The radical scavenging activity of AC extracts was determined by using DPPH assay according to Chang et al. [2001]. The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517 nm. Ascorbic acid (10mg/ml DMSO) was used as reference.

Principle

1,1-diphenyl-2-picryl hydrazyl was a stable free radical with red colour which turns yellow when scavenged. The DPPH assay makes use of this character to show free radical scavenging activity. The scavenging reaction between the (DPPH) and an antioxidant (H-A) can be written as,

DPPH + [H-A] →DPPH-H + (A)

The antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates that the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

Reagent preparation

0.1mm DPPH solution was prepared by dissolving 4mg of DPPH in 100ml of ethanol.

Procedure

Different volumes (2.5µl - 40µl) of plant extracts were made up to a final volume of 40µl with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture is incubated in dark condition at room temperature for 20 minutes. After 20 minutes, absorbance of the mixture was read at 517nm. 3ml of DPPH was taken as control.[17]

Calculation

% Inhibition = $\frac{control-test}{control} X100$

RESULTS

Pharmacological Studies

The study tried to evaluate the outcome of this exercise type on reproductive dysfunction in rats with polycystic ovarian syndrome.

Experimental design: Female white albino rats were allocated into three groups:

Group I: Control rats; Group II: Letrozole induced polycystic ovarian syndrome rats (PCO) where letrozole was given orally and daily dose of 1 mg/kg dissolved in 0.9% NaCl solution for 21 days; Group III: Clomiphene received group. IV. Polycystic ovarian syndrome rats subjected to AC (100mg) V. Polycystic ovarian syndrome rats subjected to AC (200mg) orally for three weeks after the induction of polycystic ovarian syndrome. After 6 weeks from the beginning of the study, final body weight, body mass and Lee indices were determined. Plasma levels of Luteinizing hormone, free testosterone, estradiol, progesterone, prolactin, fasting glucose and fasting insulin were measured.

Table 1: Effect on Estrus cyclicity in letrazole induced PCOS rat model.

Groups	Normal Animal	Extended Proestrus	Extended Estrus	Extended Metaestrus	Extended Diestrus
Control	10/10	-	-	-	-
PCOS	-	2/10 >24 hr	-	2/10 >32 hr	6/10 >72 hr

Rats when induced PCOS exhibited arrested estrus cyclicity in late diestrus phase of cycle.

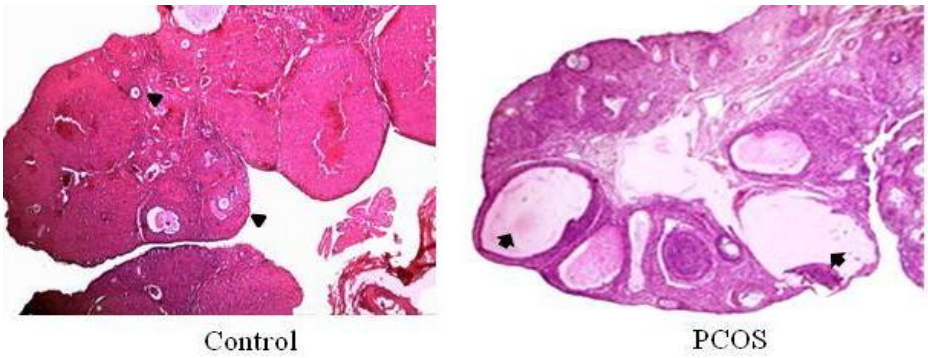

Fig. 1: Effect on Ovarian structure in letrozole induced PCOS rat model.

Development of PCOS in Rat Model

Rats treated with letrozole for induction of PCOS showed a significant increase in body weight and altered estrus cyclicity as compared to control. As shown in Figure .3. PCO animals exhibited an increase in body weight and glucose tolerance as compared to control and histology of ovary revealed many peripheral small atretic cysts Figure 2. Whereas no histological abnormalities were observed in control rat.

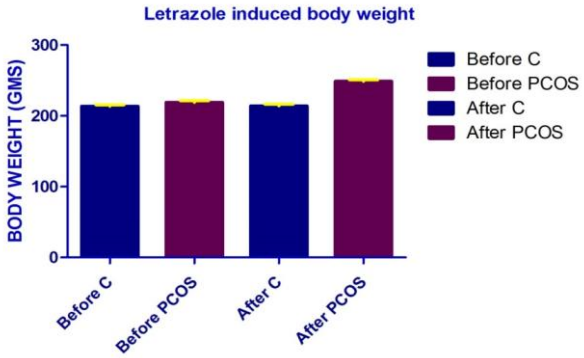

Fig. 2: Body weight of letrozole induced PCOS in wistar albino rats.

The results from the above experiments clearly demonstrate that Letrozole induced PCOS rats exhibited all the pathological characteristics similar to the clinical

manifestations found in PCOS women. The main aim of the current chapter was to evaluate the efficacy of AC in PCOS rodent model. Hence, dose and time dependent experiments were directed towards the understanding the most effective dose and minimum time required for management of PCOS phenotype.

Body weight

Obesity is a major feature in women with polycystic ovary syndrome (PCOS), and evidence suggests that obesity contributes to the pathogenesis of PCOS (Nestler 2000). Generally, excess abdominal adipose tissue (AT) initiates metabolic and endocrine aberrations that are central in the progression of PCOS [18]. PCOS rat model exhibited significant increase in body weight with abdominal fat as compared to normal rats. However, after treatment with AC, body weight reduction was not seen.

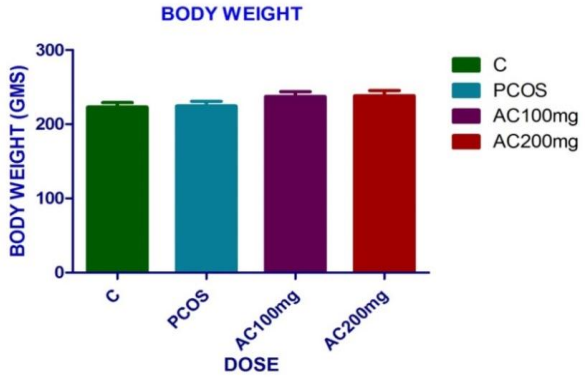

Fig. 3: Dose and time dependent effect of Ashuwathi Chooranam (AC) on Body weight.

Oral Glucose Tolerance Test (Ogtt)

Women with polycystic ovarian syndrome (PCOS) are at increased risk for developing glucose intolerance leading to type 2 diabetes mellitus (DM) (Salley et al. 2007). Hence, it was necessary to evaluate the efficacy of AC on glucose homeostasis. Thereby, Oral glucose tolerance test (OGTT) was performed in all groups of animals. PCOS rats exhibited high glucose tolerance compared to normal control rats (**p<0.01, ***p<0.001) at all the time points of OGTT profile. AC treated PCOS rats in different doses (100mg, 200mg) demonstrated significantly reduced glucose intolerance and improved cellular glucose uptake upon increasing time period of dose (30 days) of OGTT profile (Figure. 4.).

Glucose homeostasis is governed by insulin action. Thereby, we measured serum insulin level in all group of animals wherein serum insulin levels of untreated PCOS rats were increased significantly (***p<0.001). Treatment of AC caused a decrease in insulin level as compared to PCOS group (p<0.001).

Dose and time dependent effect of *Ashuwathi chooranam* on Oral Glucose Tolerance Test (OGTT Test)

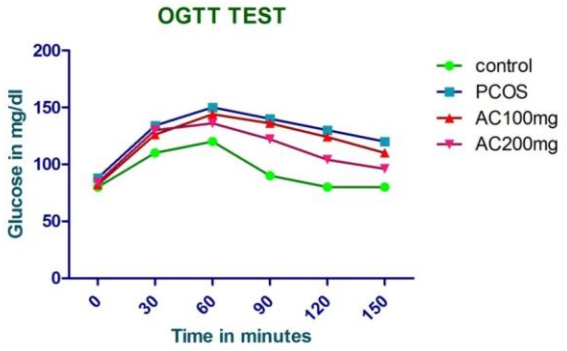

Fig. 4: Effect of Ashuwathi Chooranam on OGTT Test.

Table 2. *Ashuwathi chooranam* (A C) on Insulin status.

Groups	Insulin(µIU/ ml)
Control	7.34±1.67
PCOS	17.6±0.9
100 mg/30 days	5.2±0.4
200 mg/30 days	5.7±0.4

*N= 6 per group, All values are represented as Mean + SEM. ***P<0.001 as compared to Control Group; P<0.001 as compared to PCOS group. Normal insulin resistance: < 3; Moderate Insulin resistance: Between 3 – 5; Severe Insulin resistance: > 5*

Estrus Cyclicity

The primary clinical manifestations of polycystic ovary syndrome (PCOS) are irregular menstrual cycle and chronic anovulation, which is found to be associated with approximately 80% of PCOS women [19]. Hence, estrus cyclicity in PCOS rats was monitored, wherein PCOS rats exhibited arrested estrus cyclicity in late diestrus phase of cycle as compared to control rats. After treatment of AC at various doses (100mg) and various time periods of (30 days), estrus cyclicity was evaluated wherein 100mg for 30 days treated group of animals exhibited reversion to normal cycle in 80% of PCOS rats. But upon increasing the doses of AC (200mg) all rats showed improved cyclicity and reverted back to normal cycle.

Ovarian Histological study

Normal ovarian function relies upon the selection of a follicle that become dominant with appropriate signal FSH and ovulates with the help of LH surge during ovulation. This mechanism is disturbed in women with PCOS, resulting in multiple small cysts (or follicles), most of which contain potentially viable oocytes but within dysfunctional follicles.[20] PCOS rat model in current study also demonstrated peripheral empty follicular cysts as compared to control ovary with normal growing follicles. In dose dependent study, 100 mg dose/30 days of treatment exhibited normal growing

follicles but some cysts were present in ovary. AC at higher doses (100 mg and 200 mg) at 30 days of treatment, number of peripheral cysts significantly decreased and increased normal growing follicles with presence of corpus luteum was present; indicating normal ovulation due to functional ovary.

Interpretation

PCOS has many clinical manifestations, which includes oligomennorhea and hyper- androgenism, leading to metabolic dysfunction (Dickerson et al. 2010). Rat model created using letrozole exhibited an increase in ovarian androgens and thus leading to hyperandrogenism, which is a hallmark of PCOS. Also, significant weight gain was observed in letrozole treated PCO as compared to control rats, which could be attributed to deposition of abdominal fat[21] the model created show similar characteristics of PCOS shown by.[22a] It has been well documented that PCOS is positively correlated with insulin resistance.[19b] Apart from, systemic level changes, ovarian steroidogenesis were also altered leading to high testosterone level in PCO phenotype.[22b] which could be correlated to ovarian structural changes as seen in present study.[19c] Thereby, letrozole induced PCOS rat model demonstrated increased body weight, arrested cyclicity and impaired glucose intolerance with hyperandrogenim that are key features of PCOS phenotype.

Aim of current chapter was to understand the dose and time required by AC for the management of PCOS condition. Thereby, future studies were directed to evaluate minimum effective dose and time period for 30 days AC treatment which would manage PCOS phenotype and restore normal ovarian function.

Thereby, experiments were carried out with various doses (100mg, 200mg,) at different time points (30 days) with A C formulation.

Dose and time dependent effect demonstrated that treatment irrespective of time and dose could cause Dose and time dependent effect demonstrated that treatment irrespective of time and dose could cause a reversion to normo-glycemic condition from hyperglycemic condition as observed in PCO phenotype. AC treatment with higher dose (100mg, 200mg) for short period time (30 days of treatment). This could be attributed to the nutritionally rich phytosterols and phyto-phenols present in the plant [23], that helps to recover the syndrome and could be able to sensitize the insulin receptors for the glucose uptake. Also, it should be noted that AC is rich in fibers that could increase transit time for diet to be get absorbed which could modulate glucose homeostasis in PCO phenotype.

In this study, PCO rats demonstrated the formation of empty cysts with follicular fluid which is similar to ovarian structural changes that was reported by[22c] PCOS rats treated with fresh AC and formulation exhibited normal follicular growth which was evident from normal estrus cyclicity as seen in higher doses (100mg, 200 mg) rats also exhibited reversion in ovarian structure. However, it should consider that with increasing dose, phyto-components content is increased. These phyto-components present in AC could be active components which would alter the steroidogenesis and expression of steroidogenic protein, which alters the PCO condition.[24] However, at 30 days of treatment caused significant change with lower dose (100mg). The reversion of estrus cyclicity upon extracts treatment could be attributed to phytochemical components present in the AC that maintains steroid status, regaining back the fertility status.

The results of ovulation effect revealed the significant influence at the dose level of 25mg/kg and this marked effect was ensured with the histological evaluation of uterus of experimental rats also. Hence it may be concluded that the AC is an excellent traditional medicine in the treatment for anovulatory conditions like PCOS and the effect may be attributed to the elevation of the ovulation stimulatory hormones in animal models.

Table 3: Effect of *Ashuwathi Chooranam* (AC) on weight of uterus and ovary after 10 days treatment.

S. No	Group	Treatment and dose	Weight of uterus(mg)	Weight of ovary (g)
1.	Normal	2ml/kg 2% CMC	90.49±0.49	21.42±0.65
2.	Letrozole group	1.0ml/ kg	55.42±0.52	25.67±0.98
3.	Standard	Clomiphene 10mg/kg	73.49±0.55***	22.74±0.56**
4.	Test-I	AshuwathiChooranam 100mg/kg	56.97±0.70**	24.40±0.52**
5.	Test-II	AshuwathiChooranam 200mg/kg	64.39±0.63**	23.29±0.53**

N = 6. Values are expressed as Mean ± SEM. ^{ns}*P>0.0, *p<0.05;**p<0.01;p***<0.001 When compared to normal control.*

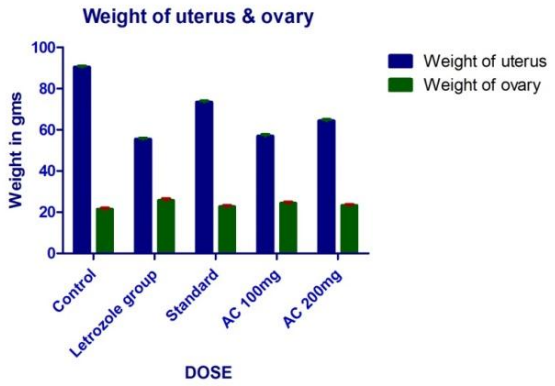

Fig. 5: Effect of AC on weight of Uterus and ovary.

Experimental design

1. Group I Normal Control animals 1ml/kg of CMC (Carboxymethyl Cellulose) solution.
2. Group II rats were administered Ashuwathi Chooranam 100mg/kg for 10days,
3. Group III rats were administered Ashuwathi Chooranam 200mg/kg for 10 days
4. 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.

Hormonal assay

Biochemical assay

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

Estimation of serum luteinizing hormone (LH)

The method employed was Micro well immunoassay (ELISA) using analytical grade reagents. 0.050ml of the serum was pipetted inside 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 with 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.

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.

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.

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. 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.

Table 4: Effect of *Ashuwathi Chooranam* (AC) on Serum Concentration of reproductive hormones of female Wistar albino rat.

S. No	Group	Treatment and dose	LH (IU/ml)	FSH (IU/ml)	Estrodial (pg/ml)	Progesterone (pg/ml)	Testosterone (pg/ml)
1.	Normal	2ml/kg 2% CMC	0.28±0.01	0.33±0.0	54.10±3.5	9.03±1.65	0.41±0.08
2.	Letrozole group	1mg	2.6±0.6	±	±	±	±
2.	Standard	Clomiphene 10mg/kg	0.26±0.03**	0.46±0.02**	36.02±18**	5.5±1.11**	0.86±0.11
3.	Test-I	100 mg /kg	0.66±0.02	0.73±0.02*	27.017±18	7.2±0.30**	0.72±0.2
4.	Test-II	200 mg /kg	0.44±0.02*	0.52±0.03**	32.68±2.1*	6.02±0.32*	0.58±0.05

*N = 6. Values are expressed as Mean±SEM. *p<0.05;**p<0.01*

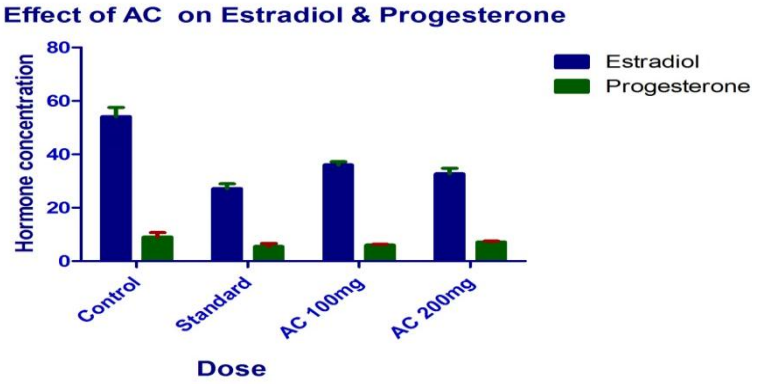

Fig. 6: Effect of Ashuwathi Chooranam on Serum Estrdiol and Progesterone in rats.

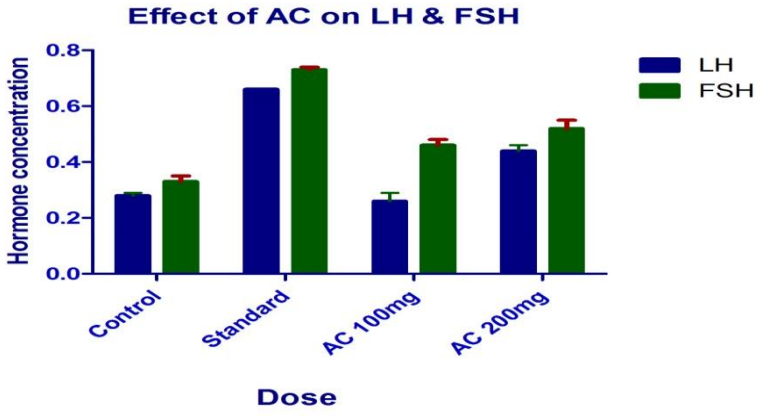

Fig. 7: Effect of Ashuwathi Chooranam (AC) on Serum LH and FSH in rats

Antioxidant Assay

Dpph Assay[25]

DPPH(1,1-diphenyl-2-picrylhydrazyl)is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most of the free radicals. The delocalisation so gives rise to the deep violet colour, characterised by an absorption band in ethanol solution centered at about 520nm. While the solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with loss of this violet colour (although there would be expected to occur with residual pale yellow colour from the picryl group still present).

Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction is

Z•+AH=ZH+A•

Where ZH is the reduced form and A• is free radical produced in this first step. Here the latter radical will then undergo advance reactions which control the overall stoichiometry, particularly the number of molecules present in DPPH reduced (decolourised) by one molecule of the reductant.

Chemicals

1. 1, 1 – diphenyl-2-picrylhydrazyl (DPPH)
2. Dimethylsilphoxide (DMSO)
3. BHT (standard)-1.6mg/ml in methanol
4. Samples of the desired concentration from 1 mg/ml– max of 5mg/ ml (in /DMSO)

Procedure

Aliquot 3.7ml of absolute methanol in all test tubes and 3.8ml of absolute methanol was added to blank. Add 100µl of BHT to tube marked as standard 100µl of respective samples to all other tubes marked as tests. 200µl of the DPPH reagent was added to all the test tubes including blank. Incubate all test tubes at room temperature in dark condition for 30minutes.The absorbance of all samples was read at 517nm.

Table 5: Procedure for DPPH Assay.

S.NO	REAGENTS	BLANK	STANDARD	TEST
1	Methanol	3.8ml	3.7ml	3.7ml
2	BHT	-	100µl	-
3	Sample	-	-	100µl
4	DPPH	200µl	200µl	200µl
Incubation at dark for30minutes				
O.D at 517 nm				

Calculation

(Absorbance at blank) - (Absorbance at test)

% Antioxidant activity = $\frac{100X}{\text{(Absorbance at blank)}}$

Table 6: Antioxidant activity.

Sample concentration (µg/ml)	Absorbance		Percentage of Inhibition	
	Drug	Standard	Drug	Standard
Control	0.5461	0.324	-	-
1.25	0.4742	0.262	13.1661	19.14
2.50	0.3863	0.198	29.2621	38.89
5	0.2545	0.113	53.3969	65.15
10	0.1912	0.084	64.9881	74.08
20	0.1001	0.041	81.6701	87.35

µg/ml: microgram per millilitre. Drug: AC (1.25-20µg/µl). Standard: Ascorbic acid (10mg/ml DMSO)

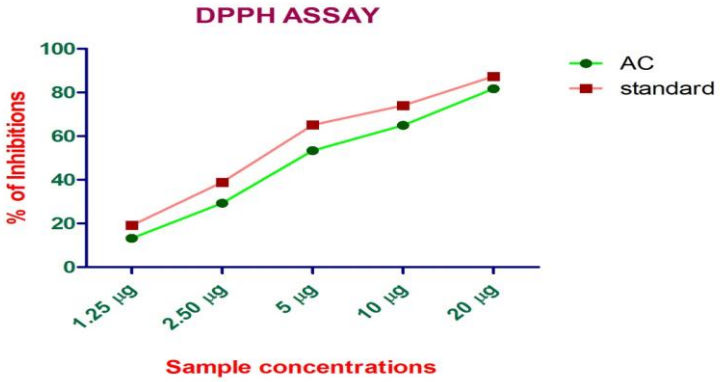

Fig. 8: DPPH ASSAY.

DISCUSSION

A study of the specifics and treatment prescribed in Siddha system will be of paramount value and nothing will be so fruitful as to go into the unexplored lore of medical genius of the glorious past [26]. Group I: Control rats; Group II: Letrozole induced polycystic ovarian syndrome rats (PCO) where letrozole was given orally and daily in a dose of 1 mg/kg dissolved in 0.9% NaCl solution for 21 days; Group III: Clomiphene received group. IV. Polycystic ovarian syndrome rats subjected Ashuwathi chooranam (AC) (100mg) V. Polycystic ovarian syndrome rats subjected to AC

(200mg) orally for three weeks after the induction of polycystic ovarian syndrome. After 6 weeks from the beginning of the study, final body weight, body mass and Lee indices were determined. Plasma levels of Luteinizing hormone, free testosterone, estradiol, progesterone, prolactin, fasting glucose and fasting insulin were measured. PCOS rat model exhibited significant increase in body weight with abdominal fat as compared to normal rats. However, after treatment with AC, body weight reduction was not seen. Serum insulin level when measured in all group of animals wherein serum insulin levels of untreated PCOS rats were increased significantly (***$p<0.001$). Treatment of AC caused a decrease in insulin level as compared to PCOS group ($p<0.001$). Iincreasing the doses of AC (200mg) all rats showed improved cyclicity and reverted back to normal cycle. In dose dependent study, 100 mg dose/30 days of treatment exhibited normal growing follicles but some cysts were present in ovary. AC at higher doses (100 mg and 200 mg) at 30 days of treatment, number of peripheral cysts significantly decreased and increased normal growing follicles with presence of corpus luteum was present; indicating normal ovulation due to functional ovary. In hormonal assay the 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. It showed that there is considerable increase in the FSH and estradiol hormones and Ashuwathi chooranam possess the antioxidant activity from DPPH assay.

CONCLUSION

In current study, treatment with both Ashuwathi chooranam (AC) formulation with higher dose (100mg, 200 mg) at longer period of time (30days) demonstrated more significant effect and it restored glucose sensitivity and normal insulin level. Along with the above changes, ovarian structure-function (in terms of hormone levels and presence of developing follicles) was seen upon the AC treatment. This could be attributed to the nutritionally rich phytosterols and phyto- phenols present in the plant that helps to sensitize the insulin receptors for the glucose uptake. Current study demonstrated altered *in utero* environment like decrease in progesterone with increased testosterone levels may result in failure of implantation and prenatal defects, which is implicated in PCOS phenotype as justified by other studies. In the pharmacological studies, Letrozole induced PCOS rat's model treated with two different forms of *Ashuwathi chooranam* formulation in various doses (100 mg, 200 mg) and time (30 days) dependent manner suggested maximum effect that reduced PCO like phenotype such as decreased in peripheral cysts with growing follicles, decreased glucose intolerance. *Ashuwathi chooranam* has magnificient ovulogenic activity over PCOS *(Soothaga vayu)* in female, which is a typical example of the intelligence of the Siddha literature to reach globally for the human well-being.

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REFERENCE

1. Dr. Ramamurthi Iyer T.G. ,The Gems of Siddha Medicine, Published by Sri Satguru Publications, First Floor, 40/5. Shakti Nagar, Delhi-110007, India. 1a p.no.20, 1b p.no.17, 261.
2. Johanna Schmidt, Polycystic ovarian syndrome: ovarian pathophysiology and consequences after the menopause, The Sahlgernska Academy, University of Gothenburg, Goteborg, Sweden, 2011; Nov 18: 978-91-628-8365-2.
3. Johanna Schmidt, Polycystic ovarian syndrome: ovarian pathophysiology and consequences after the menopause, The Sahlgernska Academy, University of Gothenburg, Goteborg, Sweden, Nov 18, 2011; 978-91-628-8365-2.
4. Ramachandran S P, Agathiyar Vaithya Kaviyam 1500, first edition, Reprinted, 2001; Thamarai Noolagam, 7 NGO colony, Vadapalani Chennai, 26: 293.
5. Ramakrishnan Get al. Physicochemical and phytochemical Standardisation of Thraachathi Chooranam-A Polyherbal Siddha Formulation, 7(6): 305-313.
6. Murugesa Mudhaliyar K.S, Gunapadam Mooligai Vaguppu, Indian Medicine by Homeopthy Dept, Reprinted in, Chennai, 106: 696 -700.
7. Devaki R et.al, Physicochemical and Phytochemical Analysis of Siddha Polyherbal Formulation Ashuwathi Chooranam for its Naturally Curing PCOS, 10.20959/wjpps2019-14872, 8(10).
8. Devaki R et.al, Sophisticated Instrumental Analysis of Polyherbal Siddha Formulation Ashuwathi Chooranam for Curing PCOS, 10.20959/wjpr201911-15904, 8(11).
9. Devaki R. et.al, Acute And 28 Days Repeated Dose Oral Toxicity Studies Of Classical Siddha Polyherbal Formulation Ashuwathi Chooranam In Wistar Rats As Per Oecd Guidelines, 10.20959/wjpps2109-14917, 8.
10. Agathiyar Vaithya Kaaviyam 1500, first part published by Tamarai noolagam, Edition, 2001; 293.
11. Egenaidi, Abdalla Ramadan, Effects of Libyan traditional plants on the reproductive system of male and female rats, University of Western Cape, http:/hdl.handle.net/ 11394/5412.
12. Good Clinical Practice Guidelines for Clinical Trials in Ayurveda, Siddha and Unani Medicine, Department of AYUSH, Ministry of Health & Family Welfare, Government of India, New Delhi.

13. Asolkar L.V, Kakkar K.K, Chakre O.J, Glossary of Indian medicinal plants with active principles, Publications & information directorate council of scientific & Industrial research New Delhi, 1992, p.no.414.
14. Galazis N,Iacovou C, Haoula Z, and Antiomo W. Metabolomic biomarkers of impaired glucose tolerance and type 2 diabetes mellitus with a potential risk for stratification in women with polycystic ovary syndrome. *Eur J Obstet Gynecol Reprod boil,* 2012; 160(2): 121-30.
15. Guha Bakshi, P.Sensarma & D.C.Pal, A Lexicon of Medicinal Plants in India, Vol.I Published by Naya Prokash, 1999; 206.
16. Tamura, M., Sasano, H., Suzuki, T., Fukaya, T., Funayama, Y., Takayama, K., Takaya, R., and Yajima, A. (1995). Expression of epidermal growth factors and epidermal growth factor receptor in normal cycling human ovaries. Molecular Human Reproduction *1*, 233-238.
17. Anna janeczko and Andrezej Skoczowski, Mammalian sex hormones in plants, Folia Histochemica et ctyobiologica, 2005; 43(2): 71-79.
18. Das J M et.al, Comparative study on serum macro and micro mineral profiles during oestrus in repeat breeding crossbred cattle with impaired and normal ovulation, May 1, 2009.
19. Leanza L, Zoratti M, Gulbins, E, and Szabo, I, Mitochondrial ion channels as oncological targets. Oncogene 33, Dec 4, 2014; 5569-5581.
20. Webber L J et.al Fromation and early development of follicles in the polycystic ovary, Lancet, 2003 Sep 27; 362(9389): 1017-21.
21. Carmina E, Bucchieri S, Esposito A, Del Puente A, Mansueto P, Orio F, Di Fede G, Rini G. Abdominal fat quantity and distribution in women with polycystic ovary syndrome and extent of its relation to insulin resistance. The Journal of Clinical Endocrinology & Metabolism, 2007; 92: 2500-2505.
22. Kafali H, Iriadam M, Ozardalı I, Demir N. Letrozole-induced polycystic ovaries in the rat: a new model for cystic ovarian disease. Archives of medical research, 2004; 35: 103-108.
23. Rajasekaran S, Ravi K, Sivagnanam K, Subramanian S. Beneficial effects of Aloe vera leaf gel extract on lipid profile status in rats with streptozotocin diabetes. Clinical and Experimental Pharmacology and Physiology, 2006; 33: 232-237.
24. Sharpe RL, Woodhouse A, Moon TW, Trudeau VL, MacLatchy DL. β-Sitosterol and 17β- estradiol alter gonadal steroidogenic acute regulatory protein (StAR) expression in goldfish, Carassius auratus. General and comparative endocrinology, 2007; 151: 34-41.
25. Philip Molyneux The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity, Songklanakarin J.Sci. Technol., 2004; 26(2): 211-219.
26. Dr. Ramamurthi Iyer T.G. The Gems of Siddha Medicine, Published by Sri Satguru Publications, First Floor, 40/5. Shakti Nagar, Delhi-110007, India, 527.