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BENEFICIAL EFFECT OF SINDHU VALLATHI MEZHUGU (SVM) IN ESTRADIOL VALERATE INDUCED POLYCYSTIC OVARIAN SYNDROME IN FEMALE WISTAR RATS

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ABSTRACT

Objective: To assess beneficial effect of Hydroalcoholic extract of Sindhu Vallathi Mezhugu (SVM) in Estradiol valerate induced poly cystic ovary syndrome in female wistar rats. Methods: Estradiol valerate (EV) 4mg was administered intramuscular, vaginal smear were examined daily in all animals. Followed by doses of Sindhu Vallthi Mezhugu (SVM) extract 200mg/kg, 400mg/kg per orally for 15 days. Results: The administration of Estradiol valerate led to abnormally in serum sex steroid profile. Sindhu Vallathi Mezhugu (SVM) was able to successfully number of cystic follicles reduces and found numerous healthy follicles development. Conclusion: Sindhu Vallathi Mezhugu showed beneficial effects in Estradiol valerate (EV) induced PCOS in female wistar rats. It effect was comparable to that of clomiphene citrate, most widely used treatment for ovulation induction in PCOS condition.

Key Words: PCOS, Sindhu Vallathi Mezhugu, Estradiol valerate, Clomiphene citrate.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is probably one of the most frequently observed ovulatory disorders that arrests approximately 10-15% women of reproductive age. It is also known as Stein-Leventhal syndrome. [1] Increase due to changes in lifestyle and stress. Long term consequences lead to cancer, type-II diabetes mellitus, hypertension, cardiovascular disorder. The etiology of PCOS is not clearly understood, but oxidative stress, insulin resistance, lipid imbalance and genetics are some of the contributing factors.[2] The exact pathophysiology of PCOS are uncertain, evidence suggests that an excess of ovarian androgen production, either genetically or due to extra ovarian factors such as hyperinsulinemia or disturbances of the hypothalamic-pituitary-ovarian axis is the main cause in the pathogenesis of PCOS Estradiol valerate is utilized to create PCOS by prompting hormone variations from the normal. EV, which is presented as a prodrug, is an ester derived from 17β -estradiol. [3]

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Exposure to a single intramuscular infusion, of estradiol valerate (EV) in rat can cause irregularity in reproductive cycles with the lack of ovulation and polycystic ovaries presenting with high number of atretic follicles and cysts. These ovarian changes are similar to those of PCOS in women. 4mg/kg body weight of EV injection can develop PCOS with the appearance of anovulation, and cystic appearance in ovary and also shown alterations in the sex steroids and androgen levels. [5]

Siddha system of medicine is the ancient, holistic system of medicine. Siddha system of treatment has many formulatory medicines for both internal Oral medication and external medicine. These formulations are made of herbal, mineral, metallic, and animal products. SVM is a herb mineral formulatory preparation internally used. [6]

2. MATERIALS AND METHODS

2.1 EXPERIMENTAL MODEL

For the study of poly cystic ovary syndrome an experimental model is selected in such a way that it would satisfy the following condition. The animal should develop

cyst rapidly. Pathological changes in the site of induction should result from PCOS formation. The symptom should be ameliorated or prevented by a drug treatment effective in human beings. The drug tested should be administered orally. [7] Drug dosage should approximate the optimum therapeutic range for human, scaled the test animal weight.

2.2 Selection grouping and Acclimatization of Laboratory Animal

Female albino rats (180-220gm) are produced from animal experimental laboratory, and used throughout the study. They are housed in micro nylon boxes in a control environment and 12 hours dark\ light cycle with standard laboratory diet and water *ad libitum*. The study is conducted after obtaining institutional animal ethical committee clearance. [8] As per the standard practice the rats are segregated based on their gender and quarantined for 15 days before the commencement of the experiment. They are fed on healthy and maintained in hygienic environment in our animal house.

2.3 Technique for inducing PCOS

Technique for induction of PCOS in animals by chemically induced (using Estradiol valerate oil, Letrozole, Androgen, Prenatal Androgen, Dehydroepiandrosterone, etc) have been used in experimental studies of PCOS activity.

2.3.1 Induction of PCOS in the Animals

In the present study, Estradiol valerate (EV) induced PCOS is used to evaluate the treatment of PCOS. Thirty adults virgin Wistar rats of approximately 10-12 weeks of age, weighing between 180-220gm and with regular 4-5day estrus cycles as assessed by vaginal smear, were used for the study. [9] Five of the rats were kept as controls, and the others were each given intramuscular injection of 4 mg EV in an oily solution per rat Vaginal smears were examined daily in all animals. Cessation of cyclicity, which was shown by the persistent cornification of vaginal smears, was used as a criterion for selection into the PCOS group.

2.4 Vaginal Smears

The stage of cyclicity was determined by microscopic analysis of the predominant cell type in vaginal smears. [10] Estrous cyclicity was monitored by vaginal smears obtained between 800 and 1200 hours, and it was assessed by analysis at the light microscopy level of the relative proportion of leukocytes, epithelial and cornified cells found in daily vaginal lavages, which characteristically change during different stages of the estrous cycle. The rat estrous cycle (estrus, diestrus1, diestrus2, and proestrus) usually lasts about 4 days, in controls or PCOS rats**.**

2.4.1 Vaginal cytology

Vaginal smears were obtained daily. The rats were held at the thorax, ventral side upper, whilst providing lumbar support. Vaginal secretions were collected using cotton-tipped with a drop of physiological saline. After about 1-2 inches of the swab was inserted into the vagina of the female rats and the end was rotated through 2-3 revolutions (which allowed the cotton tip to pick an adequate load of cells), the swab was then gently withdrawn and the tip of the cotton rolled along the length of a glass slide. [11] The dried smear was fixed by dipping it in a container of 70% alcohol. The slides were then stained with 0.5% methylene blue solution, rinsed in tap water and microscope.

2.5 Treatment Protocol

Thirty animals of Wistar rat were randomly selected and divided into five groups (n=6) and housed as such (6 rats per cage). [12] All the animals in four groups were injected with Estradiol valerate by intra muscularly and the remaining one group is normal control group. The rats were allowed to establish PCOS for 30 days. [13] After 30 days, groups in G4 & G5 were dosed orally by gavage for 15 days, where as rats in the standard group was dosed for 5 days.

Group 1 served as the normal control.
Group 2 served as the PCOS control. Group 1 and 2 receives normal diet and water.
Group 3 served as the positive control, was treated with injection Clomiphene citrate at 20 mg/kg body weight, Intra peritoneally. [14]
Group 4 served as the treatment control, treated with Hydroalcoholic extract of (SVM) at 200 mg/kg body weight, through orally.
Group 5 served as treatment control which was treated with Hydroalcoholic extract of (SVM) at 400 mg/kg of body weight, through orally**. [**15**]** On 16th day, Six animals from each group (Control and PCO) were randomly selected and an anaesthetist with ether. Blood samples were collected by retro orbital puncture, and the serum were used for hormonal assays (FSH, LH, estradiol, progesterone and testosterone). The ovaries were excised and weighed, and histopathological examination was conducted on the ovaries. [16]

2.6 Phytochemical analysis

The extract of SVM was subjected to preliminary screening of phytochemical such as alkaloids, flavonoids, glycosides, steroids, phenol, tannin, saponins, sugar.

2.7 Serum hormonal assays

Serum testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), progesterone and estradiol were measured using an enzyme immunoassay kit for the quantitative determination of the corresponding hormones.

2.8 Organ weight:

On the 16th day, some body organs of rats in all treatment groups except the Positive group were excised and weighed; organs from rats in the Positive group were instead excised and weighed on the 6th day. [17]

2.9 Histopathological examination:

The excised ovaries were fixed in Bouin's solution. They were dehydrated in an ascending series of alcohol, cleared in xylene and embedded in paraffin wax that melted at 60℃. Serial sections were mounted on 3-aminopropyl triethsilane-coated slides and dried for 24 hours at 37ºC. [18] The sections on the slides were deparaffinised, hydrated and stained with Mayer Êshematoxylin and eosin dyes; they were then dried and mounted for histology. The ovaries were viewed at 40x magnification using the Scope photo 3.0 imaging device (Scope Tek DCM 200 (USB 2.0, Hangzhou Scope Tek Opto-Electric Co Ltd). The diameter and thickness of the cystic follicles were measured. The cystic follicles were defined by thickened and fibrotic cortex with a prominent outer theca and internal layer. [19]

2.9.1 Histology

The ovaries from toxic controls (EV-treated), standard & treated groups were removed, cleaned of adherent connective fat tissue, and tissue samples were fixed in 10% formaldehyde buffer for histological examinations. Ovaries were imbedded in paraffin, cut in 8-µm sections, and stained with hematoxylin and eosin examined by light microscopy [hematoxylin and eosin staining) Ovaries were examined for evidence of polycystic morphology, as described previously. [20]

Statistics

The results are expressed as Mean ± SEM. Data was evaluated using ONE WAY ANOVA followed by Newman – Keul's multiple range test. Probability values less than ($p< 0.01$) were considered significant. [21]

3. RESULTS

3.1 Effect of Hydroalcoholic extract of (SVM) on testosterone in EV induced PCOS rats

There was no significant rise in testosterone levels after exposure of rats to estradiol valerate ($p<0.01$) for 30 days. [22] Treatment with SVM at two doses 200mg/kg and 400mg/kg for 15days doesn't show any significant changes in testosterone levels. Similar results were observed after clomiphene treatment. [23]

3.2 Effect of Hydroalcoholic extract of (SVM) on ovarian morphology:

Ovaries of toxic control (Estradiol valerate) group exhibited more cystic follicles compared with other groups but these were not evident in extract control group. Both the 200mg/kg & 400mg/kg showed normal follicle at different stage of development. There was evident of atretic follicles present in 200mg/kg. The group that received 400 mg/kg showed numerous healthy developing follicles. [24]

3.2.1 Effect of Hydroalcoholic extract of (SVM) on follicular diameter & thickness:

The follicular diameter & thickness of the cysts in PCOS treated group were increased whereas it was reduced in standard & extract treated groups. [25]

3.2.2 Effect of Hydroalcoholic extract of (SVM) on ovarian weight:

The ovarian weight of EV control group showed a significant decrease ($p<0.01$), when compared with other groups, whereas in treatment control group 200mg/kg & 400mg/kg it was restored to near normal values. [26]

3.3 HISTOPATHOLOGICAL EXAMINATION

Section of ovary from normal rat showing the presence of Antral follicle Corpus luteum, oocyte surrounded by Granulosa cells and theca layer.

Section of ovary from PCOS rat exhibiting many cystic degenerating follicles and atretic follicle with degenerated granulosa layer

Section of ovary from PCOS rat treated with clomiphene citrate showing the presence of normal follicle with clear antrum, Oocyte in granulosa layer. [27]

Section of ovary from PCOS rat treated with low dose of SVM 200mg/kg shows mild degenerative follicle and absence of cystic, atretic follicle Section of ovary from PCOS rat treated with high dose of SVM 400mg/kg shows the presence of developing regenerating follicle and carpus luteum, Oocytes with in Granulosa layer. [28]

Table 1: Effect of on serum hormone in Estradiol valerate induced PCOS

GROUP	LH	FSH	ESTRODIAL	TSN	PRGSN
G1	14.13±3.16	13.59±4.18	75.17±2.05	0.48±8.65	18.45±7.03
G2	18.11±6.27	7.91±6.35	32.18±1.06	0.68±3.16	11.23±1.15
G3	12.09±8.12	14.85±7.02	72.49±6.05	0.33±6.85	15.89±6.28
G4	17.62±2.03	11.12±8.22	49.65±3.04	0.60±5.95	17.34±3.08
G5	14.12±4.08	12.10±1.07	59.28±1.08	0.50±6.45	17.46±5.19

Table 2: Effect of SVM on ovarian morphology of PCOS rats

GROUP	Atretic follicle	Cystic follicle	Cystic follicle diameter	Cystic follicle thickness
G1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
G2	5.02±0.56	11.82±0.23	87.13±0.18	43.07±0.37
G3	2.05±0.48	4.81±0.08	76.06±0.05	33.26±0.18
G4	4.57±0.46	0.00±0.00	0.00±0.00	0.00±0.00
G5	0.07±0.48	0.00±0.00	0.00±0.00	0.00±0.00

Figure 1: Effect of on serum hormone in Estradiol valerate induced PCOS

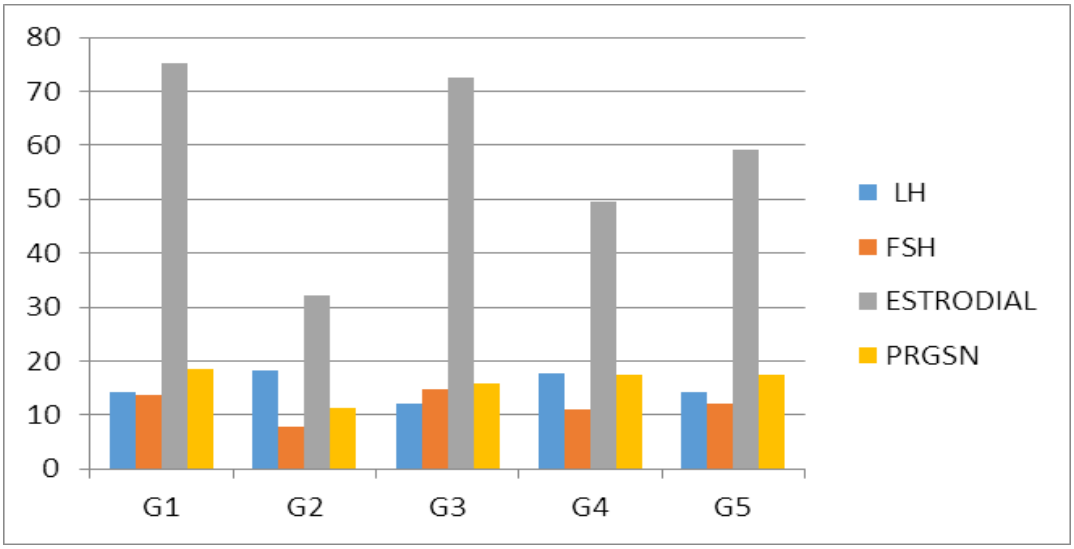
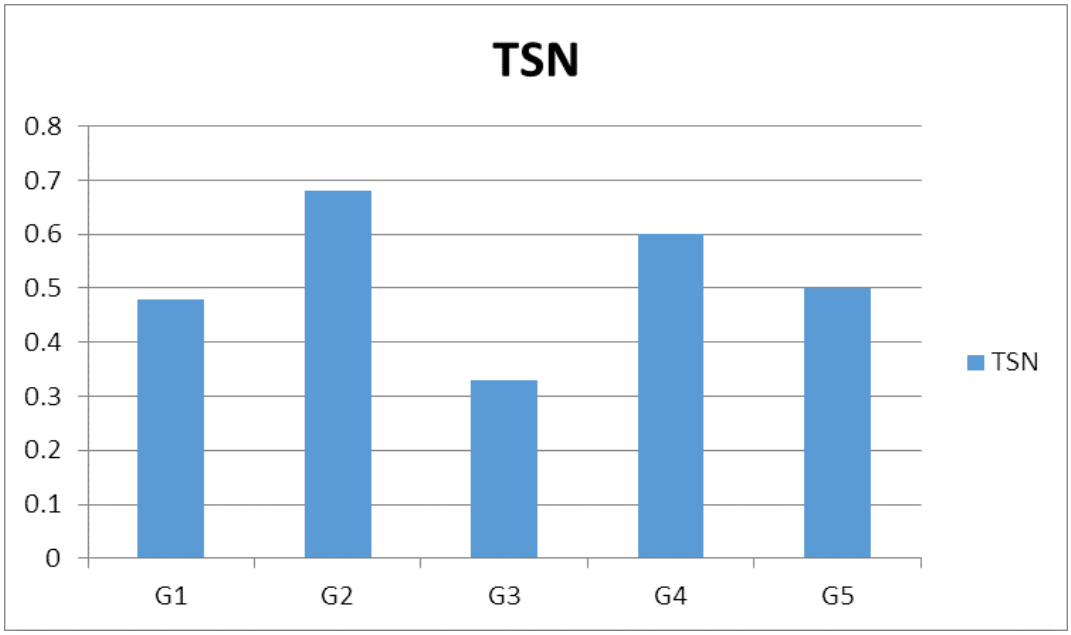
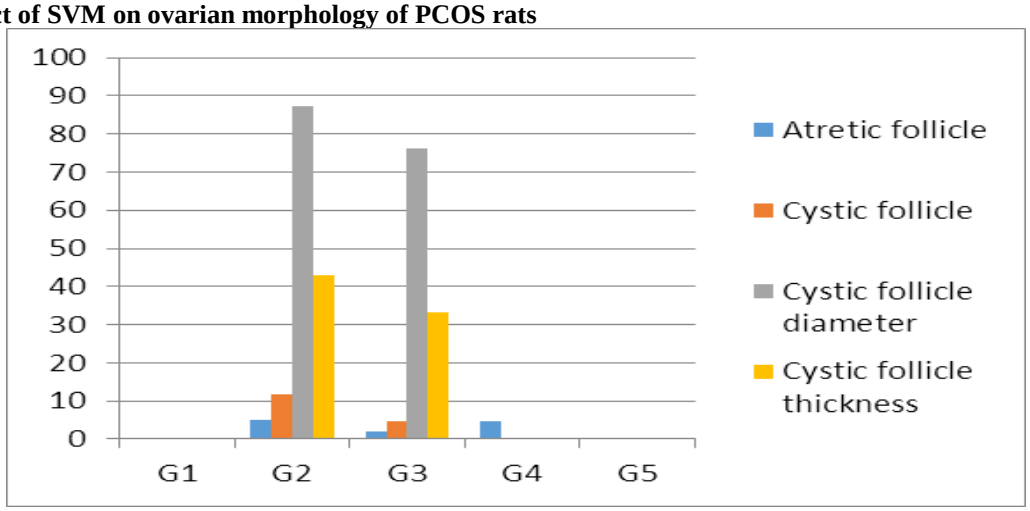


Figure 2: Effect of on TSN in Estradiol valerate induced PCOS



G1- Normal, G2-Toxic, G3-Standard,
G4-Low dose (SVM), G5-High dose (SVM)
All values expressed as means ± SEM for 6 animals in each group.
**a- Values are significantly different from Normal control (G1) at P<0.001
**b- Values are significantly different from PCOS control (G2) at P<0.001
*b- Values are significantly different from PCOS control (G2) at P<0.01

Figure 3: Effect of SVM on ovarian morphology of PCOS rats



G1- Normal, G2-Toxic, G3-Standard,
G4-Low dose (SVM), G5-High dose (SVM)
All values expressed as means ± SEM for 6 animals in each group.
**b- Values are significantly different from PCOS control (G2) at P<0.001
*b- Values are significantly different from PCOS control (G2) at P<0.01.

Figure 4: G1: Normal Control (10ml/kg Normal saline)	**Figure 5: G2 Toxic Control (Estradiol valerate 4mg/kg)**

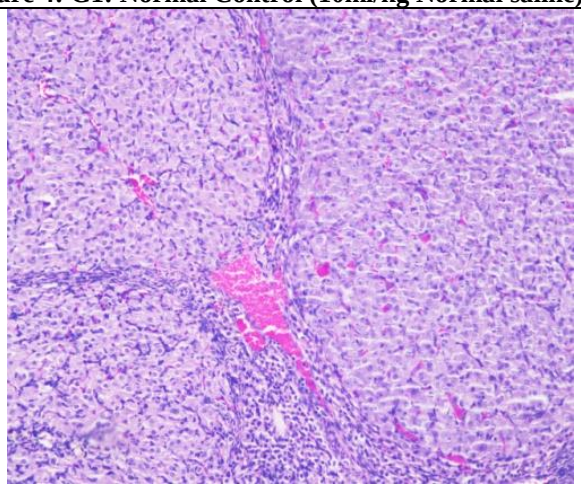
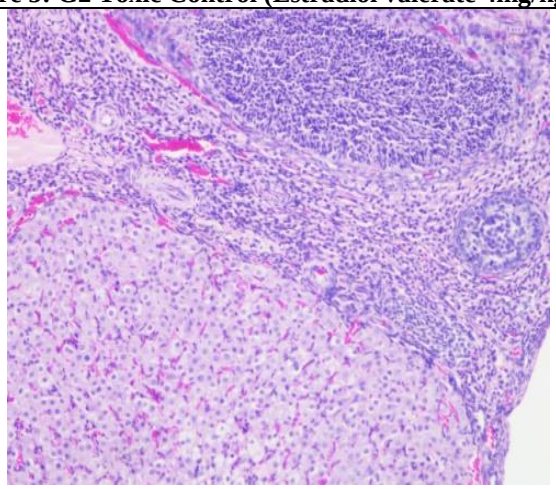

	

Figure 6: G3 Standard Control (Clomiphene Citrate 20mg/ml)	**Figure 7: G4: Treatment Control Low Dose SVM Extract 200mg/k g)**

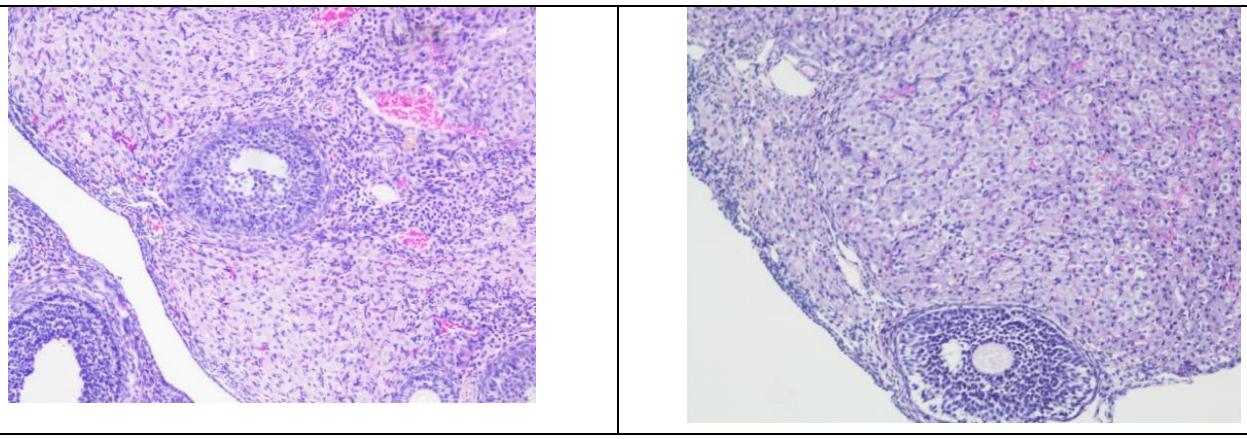
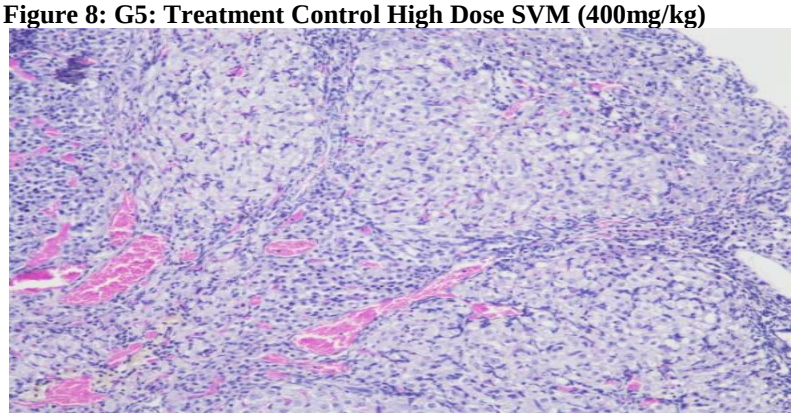


Figure 8: G5: Treatment Control High Dose SVM (400mg/kg)



4. DISCUSSION

PCOS has been considered a progressive multi glandular endocrinopathy where the delicate balance of the hypothalamic–pituitary–adrenal– ovarian axis is disturbed, resulting in a failure of the cyclic reproductive mechanism. A total loss of the cyclic reproductive changes appears to follow a phase of irregular rhythmicity in rats experimental PCOS. The precise cause of polycystic ovarian syndrome is unknown; however, it is considered to be a complex multi-genetic disorder characterized by disordered gonadotropin release, dysregulation of steroid genesis, insulin insensitivity, chronic anovulation, menstrual irregularities, clinical or biochemical hyperandrogenism, and ultrasound data of polycystic ovaries. Although many models can be used to study PCOS, induction of PCOS by Estradiol valerate can also be considered as one of the best models for studying PCOS. Hence, in terms of exhibiting the majority of reproductive and endocrine symptoms associated with PCOS, rodent PCOS models appear to closely parallel the human condition. [29]

This study investigated the effect of Hydroalcoholic extract of *SVM extract* on the serum levels of LH, FSH, estradiol, testosterone & progesterone in EV induced PCOS. After 30 days of PCO induction, animals were analysed both harmonically & histologically, on 16th day after treatment with hydroalcoholic extract, animals were also analysed irrespective of their estrus cycle. In PCOS condition, normal gonadotropin-ovarian axis is disturbed results in hormonal imbalance reflected by the higher levels of LH, lower FSH levels and reversal of LH: FSH ratio. An elevated LH/FSH ratio and anovulation are typical findings in women with PCOS. [30] The mechanism for this LH hyper secretion is not entirely clear, but recent data suggest that in anovulatory PCOS condition, the predominant reason for high serum LH concentrations is abnormal negative feedback on LH secretion mediated by either estradiol or progesterone. The extract treated groups shows better reduction in this LH/ FSH ratio indicate that extract could reverse PCOS condition.

Oestrogen similar to other steroids become altered in PCO. Normally, testosterone and androstenedione are converted to estradiol and estrone, respectively, with the help of cytochrome P450 aromatase, which plays an important role in ovary's hormonal balance. The level of estradiol is very minimum in PCOS rats since the metabolic conversion is very slow. Repetitive administration of SVM led to significant rise in estradiol. Similarly, the reduction in the level of progesterone in the PCOS-induced animals could be responsible for the Persisten to estrus phase. Elevation in the concentration of serum progesterone by SVM may

be responsible for the reversal of the luteal phase dysfunction and restoration of normalcy of the estrous cycle. [31] Our study showed that SVM extract induced an increase in serum estradiol implies that medicine causes marked improvement in endocrine function and recovery of ovulary functions in the rats. Hyper androgenism (as a result of high testosterone levels) which is evident in human PCOS. was not present in this animal model of EV induced PCOS.

Therefore, no effect of the extract on androgen levels was observed using this model of PCOS induction. Ovarian weight in PCOS induced rats was more than the normal rats which is in accordance with earlier findings. Treatment with SVM prevented further increase in ovarian weight & returned to normaly. The biochemical results are also supported by histopathological observation of light microscopy. The histomorphometry of PCOS was a suitable measurement for describing the cystic status because differences were observed in the morphological characteristic and in the presence or absence of follicular cysts. It is reported that the histopathological study of PCOS induced rats shows the formation of poly cysts in the ovary. [32] Ovaries exhibited increased follicle atresia and multiple cysts with thin granulose cell layers and thickened theca cell layers. After treatment with extract of SVM PCOS condition was reversed, number of cystic follicles reduces & found numerous healthy follicles at different stage of development. This indicate that treatment group shows marked recovery of ovarian tissue and the animals may probably be preparing for ovulation.

Furthermore, treatment with SVM brought back feedback inhibition of gonadotropin (LH & FSH) along with corresponding increase in estradiol &progesterone. All these together emphasize the ability of extract in attenuating clinical, biochemical, histological features of PCOS. The presence of flavonoids in SVM might account for pharmacological effect. Again, since PCOS condition has been reported to reduce the level of antioxidant enzyme/molecule apart from that flavonoids reported to posses playing an antioxidant role in PCOS rats. Antioxidants play an important role in protecting the human body against damage from reactive oxygen species. **SVM** containing phenolic compounds, in particular flavonoids have been reported to exhibit strong antioxidant properties. These phytochemicals may be responsible for acclaimed folklore use of medicine in management of gynecological problem.

CONCLUSION:

SVM showed many beneficial effects similar to clomiphene citrate treating PCOS condition and inducing ovulation. It restored the hormone, antioxidant as well as ovarian morphology in Estradiol valerate induced PCOS animals. These effects may be described to its multiple pharmacological activities like estrogenic, antioxidant effects which could be useful in PCOS condition. This drug used for treating clinical and pathological abnormalities in PCOS condition.

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