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OVULATION INDUCING AND FOLLICULOGENETIC ACTIVITY OF *NAVACHARA CHUNNAM* IN FEMALE WISTAR ALBINO RATS

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ABSTRACT

The ovulation inducing activity of Navachara Chunnam was carried out in rats. Two doses of Navachara Chunnam (NC – 25 and NC – 50 mg/kg body weight) were selected for experiment. Before the experimental study, oestrous cycle of all the experimental rats were synchronized by the administration of 100µg estradiol on the first day followed by the administration of 50 µg progesterone after 24 hours. Oestrous cycle of all the rats was confirmed by the vaginal smears. Then the animals divided in to four groups. First group served as a normal group, while the second and third group treated with Navachara Chunnam 25mg/kg and 50mg/kg body weight respectively. The fourth group received Clomiphene citrate (10mg/kg) and considered as standard. From the result it was observed that Navachara Chunnam at the dose of 50 mg/kg showed the significant increased level of FSH ($p<0.05$) and decreases the plasma concentration of testosterone level. From histopathological study of rat ovarian tissues revealed that administration of NC 50 mg/kg in rats favours the increased number of primary follicles, matured graffian follicle and also corpus luteum when compared with standard and normal group. Hence, from the present experimental study with the rat showed that the successful ovulation-inducing activity as well as folliculogenesis activity of Navachara Chunnam at the dose level of 50 mg/kg as dose dependent manner can be due to the inhibition of testosterone and stimulation of FSH.

KEY WORDS

Clomiphene citrate, Folliculogenesis, FSH, Navachara Chunnam, Ovulation inducing activity, Testosterone.

INTRODUCTION

The W.H.O defines Reproductive health as "it is a state in which the reproductive development is accomplished in a condition of complete physical, mental and social well-being and is not only the absence (or) disorders of the reproductive process" [1,2]. One of the most common causes of female infertility among reproductive aged women is said to arise from ovulatory dysfunction. PCOS (Polycystic Ovarian Syndrome) accounts for about 75% of female infertility [3].

Polycystic ovarian syndrome is characterized by symptoms such as hirsutism and acne (Predominant androgenisation), obesity and infertility in case of bilateral PCO. Histologically the ovaries are found to be enlarged with thickened tunica albuginea and hyperplasic theca interna cells [4].

Stein and Leventhal first described the syndrome as a triad of amenorrhoea, bilateral PCO and obesity in 1935 and hence the name. Secondary to ovulatory disturbance, menstrual irregularity is a significant acute clinical problem in PCOS. Chronic anovulation if untreated is associated with increased risk of endometrial carcinoma [5].

In majority of the female, the ovulation does not occur systemically during every menstrual cycle. Chemical or hormonal imbalance is the most common cause for this anovulation problem. However, it is possible to stimulate the ovulation successfully with suitable medicine in the Siddha system of medicine.

High LH concentration is reported to be the second cause of female infertility since it interfere with normal ovarian cycle and is mainly associated with reduced rate of pregnancy and increased miscarriage rates [6].

The most frequently prescribed drug for anovulation is Clomiphene citrate. Even though this is the drug of choice, many adverse effects are reported. Among them hot flushes, ovarian enlargement, multiple cysts formation due to hyper stimulation of ovaries which may result in rupture and internal haemorrhage and multiple pregnancies, resulting from multiple ovulation are common [7].

In these present circumstances, the need of the day is to explore an efficient and highly therapeutic remedy for PCOS with no or less adverse effect. Many such remedies are available in our traditional Siddha system of medicine. From the resource, "*Anuboga Vaidya Navaneetham*" quotes a hopeful medicine-*Navachara Chunnam* for the same. But this activity is not validated scientifically till now. Hence, in this present study, a detailed investigation to explore and evaluate the ovulatory inducing activity of *Navachara Chunnam* was done scientifically.

MATERIALS AND METHODS

Preparation of trial drug

The trial drug *Navachara Chunnam* was formulated based on the Siddha classical text 'Anuboga Vaidya Navaneetham' [8]. The ingredients are *Navacharam* (Ammonium chloride impura), leaves of *Oomathai (Datura innoxia).* The raw material was obtained from country drug shop, Chennai and the plant was collected from in and around Chennai. Both materials were identified and authenticated by the botanist and the experts of *Gunapadam* (Pharmacology) at Govt Siddha Medical College, Arumbakkam, Chennai. After identification, the samples of raw materials have been preserved in the laboratory of the department for future reference.

Purification of Navacharam (Ammonium chloride impura)

After authentication, the raw material was dissolved in hot water and filtered the solution at room temperature. Then this solution was kept under direct sunlight until the water gets evaporated. The precipitated salt was taken out and kept in a glass container [9].

Preparation of Chunnam:

Place the Purified *Navacharam (Ammonium chloride impura)* on a sand plate and heat up with Datura leaf juice (*Surukku koduththal*). Then the leaves of Datura innoxia ground with mortar and pestle to make a herbal paste covering (*Kavasam)* with the thickness of 0.5 inch which was applied to the processed *Navacharam* which was dried under sunlight. Then this dried material was kept in a limestone pan with lid (*Moosai*) which was made by grinding the limestone with Datura innoxia leaf juice. This limestone pan was tightly closed with lid and sealed by five layers of moistened mud cloth. After this process, the pan was kept under sunlight until it completely dried. Then the said pan was subjected to calcinations process (*Pudam*) with cow dung cakes of weight six times more than that of *Kavasam*. After complete burning process, the pan was allowed to cool and the contents were taken out after the breaking of limestone pan. The contents were ground in the *Kalvam* (Stone mortar with pestle) and labelled as *Navachara Chunnam*.

Animal Selection

Mice of either sex weighing 25 – 30 gm for acute toxicity study and female albino rats of wistar strain weighing about 95gms to 135 gms for ovulation inducing activity were used. Pregnant animals were excluded. Animals were fed on conventional diets and water *ad libitum* and they were maintained under standard conditions of humidity, temperature (20- 24°C) and light (12 h light: 12 h dark cycle). Experimental animals were set aside in polycarbonate cages with laced steel roofs. The animals were acclimatized for one week under laboratory conditions. The study was conducted at the Vel's University, Chennai after obtaining Institutional Animals Ethical Committee clearance bearing the number XIII/ VELS/ PCOL/ 57/ 2000/ CPCSEA/ IAEC/ 08.08.2012.

Acute toxicity study:

Acute oral toxicity test for the *Navachara Chunnam* was carried out as per OECD guidelines 425[10]. As with other sequential test designs, care was taken to ensure that animals are available in the appropriate size and age range for the entire study. The test substance is administrated in a single dose by gavages using a stomach tube or a suitable incubation cannula. The fasted body weight of each animal is determined and the dose is calculated according to the body weight. After the substance has been administrated food was with held for further two hours in mice. The animals were observed continuously for the first four hours and then each hour for the next 24 hours and at 6 hourly intervals for the following 48 hours after administering the test drug, to observe any death or changes in general behaviour and other physiological activities and recorded.

Drug and stock solution

The *Navachara Chunnam* was accurately weighed using electronic balance and suspended in 2% carboxy methyl cellulose (CMC) solution to so as to get 200mg/kg of main stock solution and this was used in this study. All the chemicals and standard drugs were procured from authorized suppliers.

Ovulation inducing activity

To carry out ovulation inducing activity, twenty four Virgin female wistar rats weighing about 95-130 gm of 2 month old were randomly selected from the animal house at Vel's University, Chennai. Before entering into the ovulation inducing study, synchronization of the reproductive cycles was done in the experimental rats by the following method [11]. 100µg estradiol was taken and dissolved in 2 ml olive oil which was administered subcutaneously. After the period of 24 hours, all the animals are administered intramuscular injections of 50 µg progesterone dissolved in olive oil. Few hours later, vaginal smears of all the rats were collected by vaginal lavage for the analyzation of ovulation and oestrous cycle [12, 13]. 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. Assessment of vaginal smears confirmed that all the animals were in the estrous stage. [14]

Then the rats were grouped into four of six each.

Group I – Considered as normal Control given 2ml/kg of CMC solution only for 10 days.

Group II – Considered as NC – 25 drug treated group which were administered 25 mg/kg of *Navachara Chunnam* for 10days,

Group III – Considered as NC-50 drug treated group and were received 50mg/kg of *Navachara Chunnam* for 10 days

Group IV- Served as a standard group received Clomiphene citrate 10mg/kg for 10 days. All the drugs were given orally. Body weight of all the animals were weighed daily after drug administration for 10 days.

At the end of experiment, 2ml of blood was collected from all the animals by retro orbital puncture. Blood samples were centrifuged at 4000 rpm for 15 minutes and the serum was separated. This samples were frozen at -20°C and LH, FSH, estradiol, progesterone and testosterone level were estimated by ELISA method. After that, the animals were sacrificed under ether anaesthesia and the uteri along with oviduct were removed and weighed. The oviduct was dissected out from the rats, suspended in normal saline and placed on a microscopic slide with a cover slip to count the number of ova and recorded.

Histological analysis

The ovary was separated from the uterus and placed in formalin fixative for 20-24 hours. Then these fixed tissue samples were placed in ascending concentrations of alcohol and embedded in paraffin. Tissues were sliced with 5-7 µm thickness and stained with haematoxylin and eosin, and then monitored and analyzed with a light microscope. For the evaluation of folliculogenesis activity of trial drug, all tissue blocks were serially sliced. Follicle identification was based on the detection of a nucleus. The numbers of follicles (primordial, primary, etc.) were counted. Follicle recognition criterion on the slides was based on the type of epithelial cells surrounding them. For example, primordial follicles have squamous cells whereas primary follicles are surrounded by cuboidal cells. The numbers of follicles per slide were randomly counted [15].

STATISTICAL ANALYSIS

Statistical significance of data was assessed by analysis of variance (one-way ANOVA) followed by a comparison between different groups using Dunnet's test. Values are expressed as Mean±SEM, where the values are $^{ns}P>0.05$ compared to normal control, *p<0.05; **p<0.01 Vs Normal control; $^{a}p<0.01$ Vs Standard.

RESULTS

From the acute toxicity study, it was confirmed that the *Navachara Chunnam* considered being safe at the dose range of 500mg/kg p.o. In this level no mortality and changes in body weight, physiological and behavioural changes were observed.

Effect of *Navachara Chunnam* on mean uterus and ovary weight

Navachara Chunnam and the standard drug Clomiphene citrate had no significant effect on the uterus and ovary weight of the rats which is summarized in **Table No.1 and Graph No.1.**

Effect of *Navachara Chunnam* on Serum Concentration of reproductive hormones of female rats

The effect of the administration of trial drug *Navachara Chunnam* and standard drug Clomiphene citrate on serum concentration of reproductive hormones are presented in Table No.2 and Graph No.2, Graph No.3 and Graph No.4. LH, FSH, Estrogen, Progesterone and Testosterone were analyzed. The result of Table No.2 showed that the administration of *Navachara Chunnam* in the dose of 25 mg and 50 mg caused no significant effect on LH which was nearly similar to the normal and standard group. There was significant increase in FSH level (p<0.05) in standard drug Clomiphene citrate. Animals are pre treated with *Navachara Chunnam* 50 mg showed the significant increased the level of FSH (p<0.05) in dose - dependent manner. *Navachara Chunnam* in the dose of 25 mg was also an increase in the level of FSH though not statistically significant.

In accordance with the results related to estrogen and testosterone also showed a significant decrease after the administration of *Navachara Chunnam* when compared with

standard and normal control group. Both trial drug treated groups and standard group produce little decreasing effect on progesterone level which was insignificant.

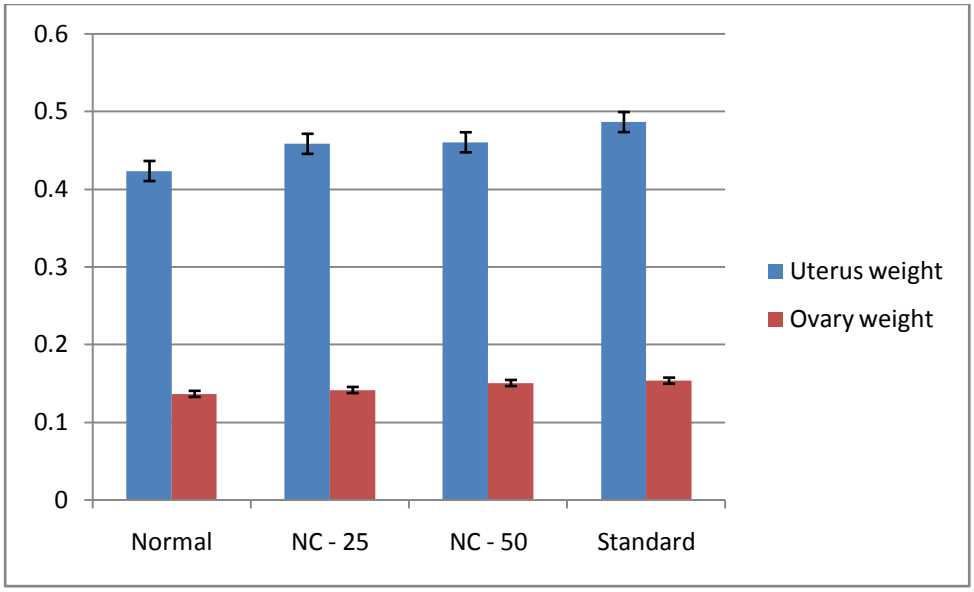
Histopathological study of ovary tissue

Histological studies of ovarian tissues of normal group, standard group, NC – 25 and NC -50 trial drug treated groups were presented in **Fig No.1, Fig No.2, Fig No.3 and Fig No.4** respectively. The ovarian tissue of the normal group showed the normal histological features with presence of few primordial follicles, matured graffian follicle. (Fig.1). Standard group and both doses of *Navachara Chunnam* (NC – 25 mg and NC-50 mg) showed some well defined histological features with increased number of primary follicles, matured graffian follicles and also corpus luteum when compared with normal rat. These were more pronounced in rat ovary that received NC – 50 mg and Clomiphene citrate. (**Fig.2 and Fig.4**)

Table No.1: Effect of *Navachara Chunnam* on weight of uterus and ovary after 10 days treatment

S.No	Group	Treatment and dose	Weight of uterus(g %)	Weight of ovary (g %)
1.	Normal	2ml/kg 2% CMC	0.423±0.02	0.136±0.02
2.	Test-I	*Navachara Chunnam* 25mg/kg	0.458±0.01	0.141±0.02
3.	Test-II	*Navachara Chunnam* 50mg/kg	0.460±0.04	0.15±0.01
4.	Standard	Clomiphene 10mg/kg	0.486±0.01	0.153±0.01

Values are expressed as Mean±SEM (n= 6) one way ANOVA followed by Dunnet's multiple comparison test.
Where the values are $^{ns}P>0.05$ compared to normal control.

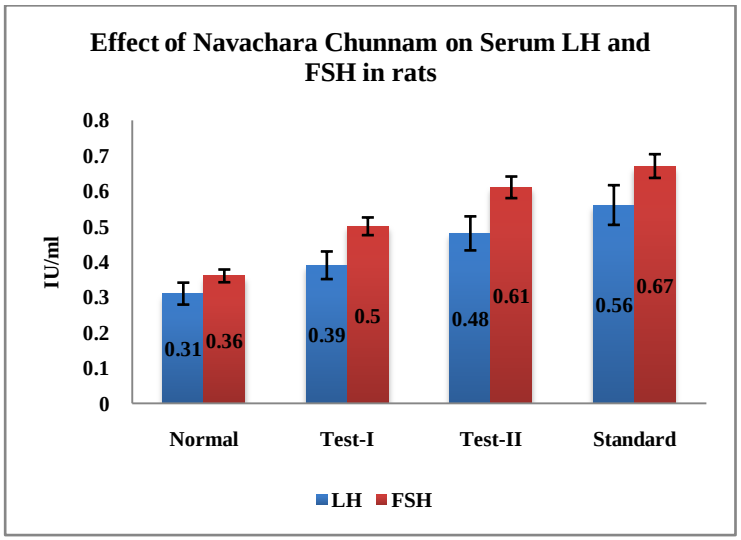


Graph No.1. Showing the effect of NC on weight of uterus and ovary

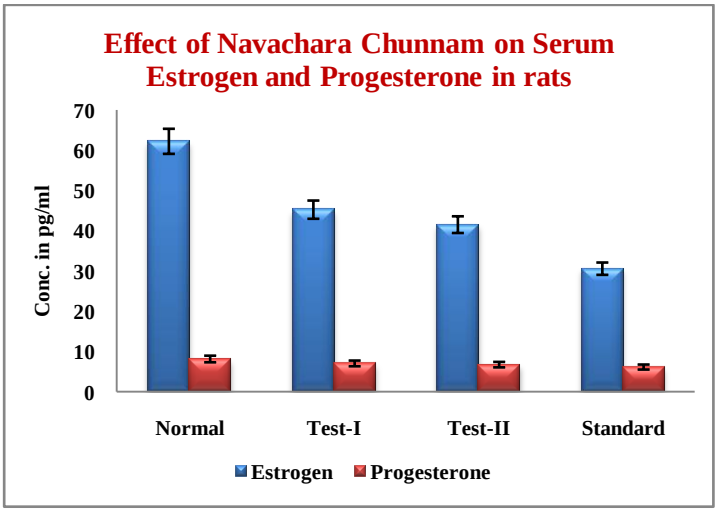
Table No.2: Effect of *Navachara Chunnam* on Serum Concentration of reproductive hormones of female rats after 10 days treatment

Group	Treatment and dose	LH (IU/ml)	FSH (IU/ml)	Estrogen (pg/ml)	Progesterone (pg/ml)	Testosterone (ng/ml)
Normal	2ml/kg 2% CMC	0.31±0.06	0.36±0.04	62.24±3.2	8.2±1.12	1.1±0.10
Test-I	*NC*-25mg/kg	0.39±0.08	0.50±0.06	45.27±2.2^{**,a}	7.1±1.00	0.6±0.05^{**,a}
Test-II	*NC*- 50mg/kg	0.48±0.08	0.61±0.08*	41.55±1.4^{**,a}	6.8±0.82	0.4±0.03**
Standard	Clomiphene 10mg/kg	0.56±0.14	0.67±0.10*	30.62±1.0**	6.2±0.61	0.3±0.02**

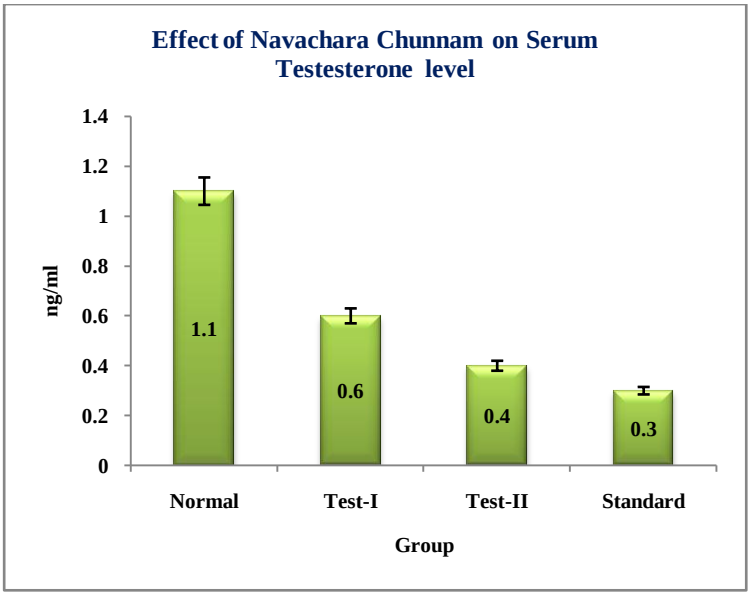
Values are expressed as Mean±SEM (n= 6) one way ANOVA followed by Dunnet's multiple comparison test.
Where the values are *p<0.05;**p<0.01 Vs Normal control; ^{a}p<0.01 Vs Standard.



Graph No.2. Showing the effect of NC on Serum LH and FSH in rats



Graph No.3. Showing the effect of NC on Serum estrogen and progesterone in rats



Graph No.4. Showing the effect of NC on serum testosterone level in rats

Histopathological study of ovary tissue

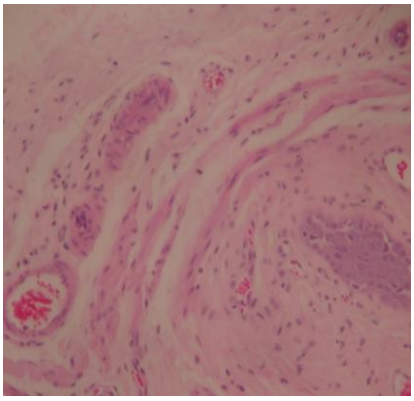


Fig No. 1- showing the ovary of normal rat

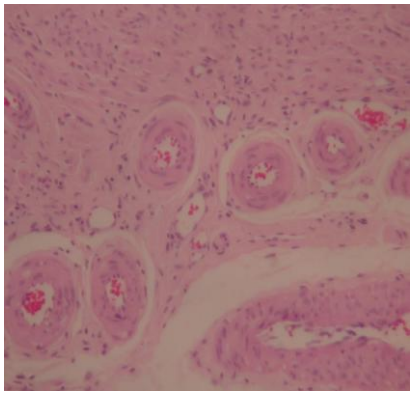


Fig No. 2- showing the ovary of rat treated with Clomiphene citrate (Standard)

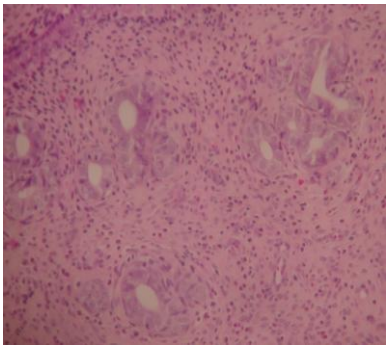


Fig No.3- showing the ovary of rat treated with NC – 25 mg.

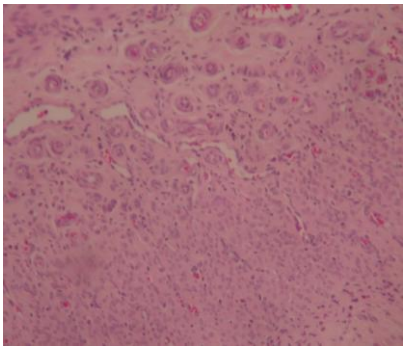


Fig No.4-showing the ovary of rat treated with NC – 50 mg.

DISCUSSION

Test drug *Navachara Chunnam* 25mg and *Navachara Chunnam* 50mg decrease serum testosterone level significantly ($p<0.01$), which is one of the requirement to manage PCOS as the serum concentration of testosterone is the primary reason for hyperandrogenism (hirsutism, hair loss appearing as thinning hair on the top of the head, acne, oily skin, obesity, depression and deepening of voice.) in PCOS.

Navachara Chunnam 50mg significantly increases the serum FSH in animal model ($p< 0.05$) which is primary step for inducing ovulation. The early stages of follicular growth are primarily driven by intraovarian factors, whereas maturation to the state required for ovulation, including the resumption of meiosis in the oocyte, requires the combined stimulus of FSH and Luteinizing hormone (LH) [16]. Gonadotropic hormones especially the FSH causes accelerated growth of 6 to 12 primary follicle each month. The early growth of the primary follicle up to the antral stage is stimulated mainly by FSH alone. After a week or more of growth before ovulation occurs, one of the follicles begins to outgrow all the others; The reminder begin to involute (process called atresia) and these follicle are said to become atretic. This process of atresia is important one, because it allows only one of the follicle to grow large enough to ovulate [17]. Different dosages of the *Navachara Chunnam* slightly increased the number of atretic follicles; a greater increase was observed at 50 mg/kg.

Treatment with doses of 25 and 50 mg/kg of *Navachara Chunnam* significantly increased the number of primary follicles, however it was statistically significant only in the 50mg/kg group.

The treatment with *Navachara Chunnam* caused an alteration in the amount of FSH, which was statistically significant. *Navachara Chunnam* in the first stage of ovarian folliculogenesis strongly stimulates the maturation of primordial follicles. This effect was more pronounced at the 50mg/kg dose of the *Navachara Chunnam*, in which it acted as a stimulant, causing progression of ovarian folliculogenesis to the stage of primary follicle formation. However, at the next stage, *Navachara Chunnam* caused an increase in the number of growing follicles. The *Navachara Chunnam* also caused an increase in the number of atretic follicles, which confirmed the repressing effect of the *Navachara Chunnam* on the natural growth of follicles

CONCLUSION

The results of ovulation effect of *Navachara Chunnam* revealed the significant influence at the dose level of 50mg/kg and this marked effect was ensured with the histological evaluation of ovary of experimental rats also. Hence it may be concluded that the *Navachara Chunnam* 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.

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