

Research Article



Studying the Testosterone Lowering Potential of *Vitex negundo* (Nirgundi) Seed Extract in Hyperandrogenic Female Rats Induced with Polycystic Ovaries

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ABSTRACT

Polycystic ovarian syndrome (PCOS) is a complex endocrine disorder characterised by high testosterone levels, chronic anovulation & metabolic disturbances. This study was conducted to assess testosterone lowering potential of *Vitex negundo* L (Nirgundi) in hyperandrogenised female Sprague Dawley rats induced with PCOS. Finasteride was used as the standard drug. 24 female Sprague Dawley rats were divided into 4 groups. Apart from Normal group, animals in Group II to IV received testosterone injections (Aquaviron 25mg/ml) intramuscularly to induce hyperandrogenism. After excessive testosterone levels were confirmed, its vaginal cytology was studied to confirm PCOS-like condition. Thereafter, the animals received nirgundi extract & standard drug for one month after which they were sacrificed & their ovaries were removed for histopathological evaluation. Blood tests were performed to evaluate serum testosterone levels, serum glucose, total cholesterol, HDL cholesterol and triglycerides levels. Serum testosterone levels and serum glucose were significantly reduced in Nirgundi which was comparable to Standard group. Histopathology reports showed least follicular cysts & lesions being found in nirgundi group. Nirgundi has promising potential as an anti-androgen from this research study.

Keywords: PCOS, hyperandrogenism, testosterone, nirgundi (*vitex negundo*), finasteride.

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is a condition characterized by accumulation of numerous cysts (fluid-filled sacs) on the ovaries associated with high male hormone levels, chronic anovulation (absent ovulation) and numerous metabolic disturbances.

Classic symptoms include excess facial and body hair (hirsutism), acne, obesity, irregular menstrual cycles, and occasionally infertility. It affects approximately 5 to 10% of women of reproductive age and is one of the most common causes of anovulatory infertility.

Menstrual disruption typically manifests in PCOS, ranging from oligomenorrhea (infrequent menstruation) to amenorrhea (absence of menstruation).

The world's oldest known system of medicine is attracting a legion of new patients suffering from PCOS.

The best known treatment at present is by using allopathic medicines such as clomiphene citrate, metformin, letrozole, tamoxifene and troglitazone.¹⁻³

All these allopathic medicines have mild to severe side effects including hot flushes, arthritis, joint or muscle pain and psychological side effects such as irritability, mood swings, depression and bloating.^{4,5}

Due to the adverse side effects caused by the allopathic medicines, alternative medicines are gaining importance.

Acupuncture, naturopathy and herbal medicines are some worth mentioning. The least known side effects come from herbal medicines.

Vitex negundo belonging to family Verbenaceae is a large aromatic shrub distributed throughout the greater part of India up to an altitude of 1500 meter in the Himalayas.

The shrub is very common in many part of the country and often occurs gregariously. It is abundant along the banks, rivers in moist situations, and open waste lands. It is widely planted as hedge plant along the roads and between fields.

The shrub can be reproduced readily from cutting and as it produces roots, suckers it is useful for planting against soil erosion.⁶ The traditional systems of Siddha and Ayurvedic medicine use this plant alone or in combination with other medicinal plants for the treatment of various diseases. It contains various chemical classes such as alkaloids, flavanoids and carbohydrates.

Traditionally, it has the folk claims like antimicrobial, anticancer, insecticidal, rheumatism, tranquillizer, tonic, febrifuge, expectorant and diuretic properties.⁷ Almost all parts of the herb are useful as a drug but the leaves, bark, roots and seeds are most important and sold as drugs.

The seeds have been reported to possess activities like anticancer, antioxidant, antifungal, anti-inflammatory, analgesic, immunostimulatory and estrogen-like activity.⁸⁻¹⁷

The goal when using nirgundi is to reduce circulating androgens, optimize ovarian functions and support optimal endocrine function.

This research study aims at exploring the testosterone lowering potential of nirgundi in comparison to conventional medicines in order to provide an insight into



how herbal medicines could contribute to the future of treating disorder such as PCOS.

MATERIALS AND METHODS

Collection

Vitex negundo Linn. (Chaste tree) seeds were collected from a local market in Borivali, Mumbai, Maharashtra in the month of June 2013.

Authentication of the collected material was carried out at Blatter Herbarium St. Xavier's College, Mumbai by Dr. Rajendra D. Shinde, and its identity was confirmed to be *Vitex negundo* Linn. (Chaste tree), family Verbenaceae, with herbarium accession no. 2888.

Processing of Nirgundi Seeds

Nirgundi seeds were garbled and unwanted plant parts, were remove. Seeds were then allowed to dry at room temperature.

Dried seeds were ground to powder in mixer and sieved through 20# to get seed powder.

Extraction

Extraction of seeds of *Vitex negundo* was carried in soxhlet extractor using ethanol and water (70:30) as solvent. 100gm of dried and powdered seeds were weighed and packed in filter paper, then extracted with solvent (500ml) at a temperature not exceeding 80°C in a soxhlet apparatus for 72 hrs.

After completion of extraction the solvent was evaporated in evaporating dish on the water bath to obtain dry extract.

The study employed nirgundi seed extract for evaluating its testosterone lowering potential which was conducted on 24 female Sprague Dawley rats for one month. Finasteride, a Type II 5 α -reductase inhibitor used to lower testosterone levels in PCOS women was used as standard drug.

The rats were purchased from Bharat Serum and Vaccines Pvt. Ltd, Thane; each weighing between 150-170g. The experimental protocol was approved by the Institutional Animal Ethics Committee under Protocol No. 131405. The experiment was carried out as per CPCSEA guidelines.

The following drugs were used to conduct the study:

Test drug: Hydro-alcoholic extract of *Vitex negundo* seeds 400 mg/kg

Standard drug: Finast 5mg tablet containing finasteride 20 mg/kg

(Dr. Reddy's Laboratory Ltd.)

Hormone drug: Aquaviron Testosterone 25mg/ml suspension (Piramal Healthcare Ltd.)

Animal Model

24 Female Sprague Dawley rats (avg. weight 160gms) were brought in the animal house and were provided with optimum feed and water on everyday basis.

Rats were thereby randomly divided into four groups:-

Group I

served to be Normal group receiving only animal feed and water orally.

Group II

received 0.1 ml Aquaviron testosterone suspension intramuscularly.

Group III

received testosterone suspension intramuscularly & hydroalcoholic nirgundi extract 400 mg/kg orally.

Group IV

received testosterone suspension intramuscularly & Standard drug. Finasteride 20 mg/kg suspended in water orally.

Nirgundi seed extract was administered in a suspension form as it was insoluble in water by using sodium CMC. Testosterone suspension was given intra-muscularly to animals in Groups II, III, IV prior to administering the plant drugs as discussed hereafter. Animals were allowed to get acclimatized to the animal house for an 8 day period after which the ones belonging to Groups II, III, IV were given 0.1 ml Aquaviron testosterone suspension intramuscularly (0.05ml Aquaviron on both thigh muscles) which means each female rat received 2.5mg of androgen hormone on Day 1. Animals in the Normal group however continued to receive only normal feed and water. After a gap of 6 days i.e on Day 7, animals belonging to Groups II, III, IV were again administered the same dose of Aquaviron and were kept under observation. During the 6 day gap, animals were observed for any signs of hypertension for eg. Nose bleeds, restlessness etc. since exogenously given androgens can cause hypertension. After another gap of 7 days, blood was withdrawn and collected from one animal in each group via the retro-orbital plexus and used for serum testosterone testing using Radio Immuno Assay technique (RIA). Vaginal smears were obtained. Once the excessive Testosterone levels in blood were confirmed it was essential to confirm PCOS like condition at ovarian level. This can be verified by considering the nature of estrous cycle in female rats i.e. the reproductive cycle in female rats which is divided into four phases: Proestrus phase, Estrus phase, Metestrus phase, Diestrus phase. Characteristics of each phase can be studied from the vaginal smears obtained from vaginal fluid of the rat.

Vaginal Smears^{18,19}

The vaginal smear obtained from one rat out of each group was observed under the microscope (45X) and



their vaginal cytology was studied. A typical polycystic ovarian condition in female rats renders anovulation in them i.e. absence of ovulation for a persistent period. This anovulatory phase can be detected from presence of cornified squamous epithelium which is typically anucleated in the vaginal smear of rats, in a persistent

manner. Pathologically speaking, it is the diestrus phase that remains persistent during the anovulatory condition and remains dominant throughout the estrous cycle. Presences of excessive Leucocytes and Cornified Squamous Epithelium according to the **Table: 1 & 2** conforms diestrus phase in rats.

Table 1: Criteria for Stage Determination.

Stage	Typical Cell Numbers					Reliability
	Leucocytes	Nucliated Epithelials	Cornified	Non-Nucleated Epithelials	Total	
Oestrus	-	-/+	+++	+/-	+++	+++
Metooestrus	+++	-	-/+	+	+++	++
Di-oestrus	++	+/-	+/-	+/-	+/**	+++
Pro-oestrus	-	++	-/+	-	+/**	++

- None or very few ; + Low ; ++ Moderate ; +++ High

Table 2: Stage Determination in various groups of animals.

Groups	Type of Cells				Total
	Leucocytes	Nucliated Epithelials	Cornified Squamous Epithelium	Non-Nucleated Epithelials	
Nirgundi	++	-	+	+	++
Testosterone	+++	-	+	-	++
Finast standard	++	-	+	+	++

Table 3: Determination of Serum Glucose, Total Cholesterol, HDL Cholesterol and Triglycerides.

Group Name	Glucose (mg/dl)	Total Cholesterol (mg/dl)	HDL Cholesterol (mg/dl)	Triglycerides (mg/dl)
Normal	106.927 ± 10.24	36.3305 ± 6.835	24.7045 ± 1.325	147.6285 ± 17.87
Nirgundi	63.7015 ± 5.269***	38.523 ± 3.563**	36.691 ± 1.397**	91.271 ± 17.32***
Testosterone	159.1865 ± 8.886 a	55.797 ± 1.397 a	23.161 ± 1.544 b	231.8895 ± 39.31 c
Standard	67.4695 ± 3.615***	31.474 ± 0.1800***	32.867 ± 1.985 ns	78.7495 ± 37.65***

All values expressed as mean ± SEM, n = 6: One-way ANOVA followed by Bonferonni's test is applied for statistical analysis. All treated groups were compared with toxicant group which was the Testosterone group. Testosterone group was compared with Normal group with 'a' significant at p < 0.001, 'b' is not significant and with 'c' significant at p < 0.05. *** Significant at p < 0.001, ** Significant at p < 0.01, * Significant at p < 0.05, ns: not significant.

Table 4: Determination of Morphological parameters.

Group Name	Body weight (grams)	Ovary weight (grams)	Ovary volume (ml)
Normal	160.5	0.1625	0.015
Nirgundi	150	0.1	0.0275
Testosterone	159.75	0.125	0.0325
Finast standard	166	0.125	0.03

Table 5: Histopathological Studies.

Group	Group Name	Microscopic Observations
Group 1	Normal	N1: Normal follicles, Corpus Lutea Normal
		N2: Normal follicles, Corpus Lutea Normal
Group 2	Testosterone	T1: Increased number of primordial follicles (2+), follicular cysts (3+), Hemorrhage (2+)
		T2: follicular cysts (3+), Hemorrhage (2+)
Group 3	Standard (finastat)	S1: Follicular Cysts (+), Primordial follicles (+)
		S2: Follicular Cysts (+)
Group 4	Nirgundi	V1: Follicular Cysts (+), Primordial follicles (+)
		V2: Follicular Cysts (+), Hemorrhage (+)

Once the excessive androgen levels and polycystic ovarian condition was confirmed, the one month study began of administering the plant drug and finasteride to animals from Group III and Group IV on everyday basis. Group I – the Normal group and Group II – the Hyperandrogenised group however continued to receive normal feed and water as mentioned before.

After completion of four weeks, blood was withdrawn from each animal via the retro-orbital plexus and collected in two parts – one part in vacuutainers – to be sent to Metropolis Labs for testosterone analysis and the other part collected in appendroffs for serum glucose and HDL cholesterol analysis. Animals were then sacrificed under deep ether anaesthesia and their bodies were dissected for removal of ovaries. Blood collected in vacuutainers was sent to Metropolis Labs for serum testosterone analysis. Ovaries were sent for histopathological studies at Dr. Jairaj's Diagnostic Centre, Kharghar, Navi Mumbai.

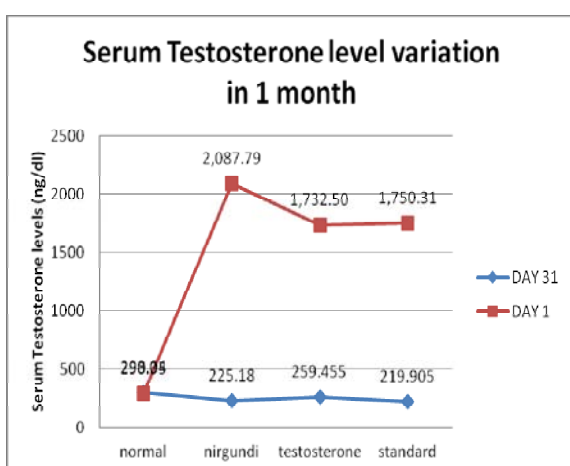
Statistical Analysis

Experimental results were grouped according to the treatment, and the arithmetic average was calculated for each group from the values for each individual with that group. This average was expressed as the mean \pm the standard error of the mean (SEM) for six determinations. Experimental data were analyzed statistically by one-way analysis of variance (ANOVA). Bonferroni's multiple comparison test was used to determine significant differences between means. The p value corresponding to the test statistic value was reported to denote the degree of significance. PRISM software was used for statistical analysis.

RESULTS AND DISCUSSION

RESULTS

Serum Testosterone Analysis



All values expressed as mean \pm SEM, n = 6; One-way ANOVA followed by Bonferroni's test is applied for statistical analysis. All treated groups were compared with toxicant group. *** Significant at p < 0.001, * significant at p < 0.05, ns: not significant.

Figure 1: Serum Testosterone Levels Before and After One Month.

The Serum Testosterone levels after the one month trial were found to be as follows:

Where values from Day 1st represent serum Testosterone levels before administration of the test drugs and values from Day 31st represent Testosterone levels after one month long treatment with the test drugs (**Figure 1**). The testosterone levels in Testosterone group, consisting of hyperandrogenised rats receiving no drug, were found to have reduced drastically within a month which was lower than the levels in Normal group. The testosterone levels in other Groups lied within the normal testosterone levels (40-600ng/dl) comparable with levels in the Normal group

Other Blood Tests

Blood collected was then centrifuged under at 10000 rpm at a temperature of 20°C for a period of 20 minutes.

Serum separated after centrifugation was divided into four parts and assayed for Serum Glucose, Total Cholesterol, HDL Cholesterol and Triglycerides (**Table: 3**).

Morphological Parameters

Determination of Body Weight

Rats were monitored every week for body weight. At the end of 7 weeks the body weight of each rat was recorded (Final Body Weight). Weight was measured by placing animal on weighing scale. Body weight per animal per cage per week was noted accurately (**Table: 4**).

Determination of Ovary Weight

After the rats were dissected the ovaries were removed, first washed in 0.9% saline solution thoroughly for removal of any uterine tissue and cleansing them. Then the ovary weight was measured by placing the ovaries on the weighing balance (**Table: 4**).

Determination of Ovary Volume

The volume of ovaries was found out by attaching a thread to the ovaries with the help of a needle and then dipping them in the plethysmometer and readings were noted for volume changes (**Table 4**).

The weight of the ovaries from all the groups was found to be less than the normal group. The volume of the ovaries from all the groups was found to be more than normal group.

Histopathological Studies

The histopathology of ovaries was described on basis of nature of its follicles:

PF: Primordial follicles (Early stage follicle)

FC: Follicular cysts

Hm: Haemorrhage

Four step grading system of **minimal** (+), **mild** (2+), **moderate** (3+) and **severe** (4+) were used to rank microscopic findings for comparison among groups.

The section of ovary of animals from Normal group showed normal Follicles and corpus luteas. In group 2 there were increased primordial follicles, hemorrhage and follicular cysts indicative of induction of Poly Cystic ovary (PCO). In group 4 the severity was less as compared to group 2 and well comparable to group 3 (Table 5) & (Figure 2).

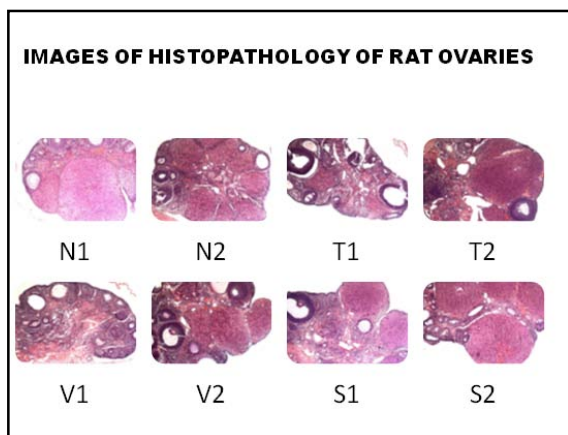


Figure 2: Histopathology of Ovaries.

DISCUSSION

The one month animal study on hyperandrogenised female rats was conducted in order to draw results with respect to the androgen levels after treatment with respective drugs and its effect on other biochemical parameters like Serum Glucose, Total Blood Serum Cholesterol, Serum Triglycerides Level and HDL Cholesterol.

Hydroalcoholic extract of seeds of *Vitex negundo* has potential of lowering excessive androgen levels when ingested on daily basis.

Also it may possess activity at ovarian levels, as was derived from the histopathological studies. Its anti-androgenic activity may be linked to the presence of flavanoids and phytosterols like beta-sitosterol that possess a steroidal nucleus which may participate in steroid metabolizing pathways and exert this action. The probable mechanism of action for antiandrogenic action of the extract may be due to the inhibition of 5 α -reductase.

It is essential to study this activity in further details by conducting trials longer than a month in order to optimize the dose.

Along with reducing androgen levels in hyperandrogenised female rats, *Vitex negundo* was also found to and balancing blood glucose, total Cholesterol and triglycerides levels and also increase HDL Cholesterol level at the same time.

CONCLUSION

Vitex negundo has anti-androgenic potential which may be due to the presence of phytosterols and flavonoids it also showed considerable potential in balancing

metabolic parameters. Also it may possess activity at ovarian levels.

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