



Contents lists available at UGC-CARE

International Journal of Pharmaceutical Sciences and Drug Research

[ISSN: 0975-248X; CODEN (USA): IJPSPP]

Available online at www.ijpsdronline.com

Research Article

Effect of Nutraceutical Formulation Ovajal on DHT and Fructose-induced Polycystic Ovary Syndrome in a Rodent Model

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ARTICLE INFO

Article history:

Received: 22 January, 2022

Revised: 09 March, 2022

Accepted: 15 March, 2022

Published: 30 March, 2022

Keywords:

Clomiphene citrate,
DHT,
Fructose,
Polycystic ovarian syndrome,
Metformin,
Nutraceutical formulation,
Ovajal.

DOI:

10.25004/IJPSDR.2022.140216

ABSTRACT

Polycystic ovary syndrome is a most common female reproductive disorder, involving endocrine and metabolic disorders with unclear etiology. It may clinically be manifested in young women of reproductive age as oligo-ovulation, abnormal levels of reproductive hormones, clinical hyperandrogenism, hirsutism, male pattern baldness, acne, acanthosis nigricans, and polycystic ovaries, additionally having a long prodroma with detectable abnormalities that present as the metabolic syndrome like Insulin resistance, hyperinsulinemia, obesity, and dyslipidemia. Current treatment options are unable to manage PCOS and suffer from unwanted effects. The present study aimed to investigate the effect of nutraceutical formulation ovajal on dihydrotestosterone (DHT)-fructose-induced polycystic ovary syndrome (PCOS) in Sprague Dawley (S.D.) female rats. Prepubertal rats in the experimental group (except control) received coadministration of DHT (s.c.) and fructose(p.o.). Along with DHT+F other groups were treated with OV100 & OV200, clomiphene, and metformin, at a dose of 100 mg/kg p.o., 200 mg/kg p.o., 100 mg/kg p.o., & 200 mg/kg p.o., respectively for 90 days. Estrus cyclicity, OGTT, luteinizing hormone, follicle-stimulating hormone, insulin, testosterone, and estradiol serum levels were assessed. Ovary, uterus, abdominal fat, and subcutaneous fat were collected, weighed and an ovary histomorphology examination was done. Results showed that OV100 and OV200 reversed the DHT-fructose induced changes by significantly ($p < 0.05$) increasing serum FSH level, estradiol level and decreasing their body weight, ovary weight, uterine weight, serum luteinizing hormone level, testosterone level, oral glucose tolerance, irregular estrous cyclicity and no. of cystic follicles. However, the OV200 notably ameliorated the abnormalities of experimental PCOS. Our study findings demonstrate that ovajal formulation exerted preventive benefits in an experimental model of PCOS. Hence can be suggested in the management of PCOS.

INTRODUCTION

Polycystic Ovary Syndrome (PCOS) comprises metabolic and reproductive ailment among women of fertile age. According to the diagnostic criteria of the National Institute of Health (NIH), 4 to 10% of women of reproductive age are affected by PCOS all over the world. [1] PCOS suffering women of reproductive age show phenotypes such as hyperandrogenism, anovulation, amenorrhea, hirsutism, male pattern baldness, acanthosis nigricans, and polycystic ovaries, but then too PCOS

has a long list of other detectable abnormalities that exist as insulin resistance, hyperinsulinemia, obesity, dyslipidemia, hypertriglyceridemia, atherosclerosis, increased risk of development of type II diabetes with limited treatment options. [2] Different therapeutic regimes consisting of drugs such as metformin, clomiphene citrate, and aromatase inhibitors like letrozole have been recommended for PCOS treatment which is associated with various side effects such as multiple ovulations, lactic acidosis, vitamin B6 deficiency, increased pain

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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sensitivity, and vaginal bleeding have been reported.^[3] Nowadays focus is being laid on therapies that show minimal or no side effects. Herbal formulations containing several active constituents seem to be useful but are found to have varied effects in the women population.^[4] Although the etiology of PCOS is unclear, various research suggests that IR plays a role in metabolic and reproductive problems.^[5-7] Insulin is a key hormone for hyperandrogenism in the pathophysiology of PCOS through two different pathways: 1) Insulin stimulates androgen production in theca cells by releasing luteinizing hormone (LH), and increased androgen production causes hirsutism, acne, and infertility.^[8] 2) Insulin inhibits the synthesis of sex hormone-binding globulin (SHBG) in the liver, which is associated with hyperandrogenism.^[9] SHBG is a plasma protein that binds androgens and estrogens, so low SHBG levels in PCOS can lead to hyperandrogenism.^[10] Nutrition-related signaling pathways are important in the regulation of ovarian follicle growth and ovulation rates. Deficiencies in Myo-inositol, minerals, and vitamins especially vitamin D can cause PCOS pathogenesis complications.^[11-13] Thus, nutritional supplementation^[14,15] could help to alleviate PCOS complications like immature oocytes, IR, hyperandrogenism, and oxidative stress. Ovajal is a polynutraceutical formulation of astaxanthin, biotin, carnitine tartrate, CQ10, DHEA, folic acid, L-arginine, lycopene, myoinositol, sodium selenite, vitamin. B12, vitamin. B6, vitamin. C and zinc. Each of these ingredients has been reported to have major significance in the pathophysiology of PCOS. Astaxanthin has antioxidant potential reducing ovarian cell atresia.^[16] Myoinositol, biotin, vitamins, and lycopene improve menstrual cyclicity, acne, and hirsutism, reducing and regulating hormonal balances.^[17] DHEA improves ovarian and improves pregnancy rates.^[18] Selenium sodium shows antidiabetic, anti-apoptotic action, reducing oxidative stress and mitochondrial dysfunction.^[19] To sum up, these nutrients have regulatory roles in the insulin signaling pathway, androgen synthesis and regulation of ovarian follicle growth, ovulation rates, and antioxidants.^[20] Affording these sufficient nutrients supplementation, for growth and reproduction depends on the optimal nutrient composition, which could prevent, and normalize the PCOS state. In previous studies^[21-23], androgen DHT has been used to induce PCOS, reflecting human PCOS phenotypes in experimental rats. Unfortunately, this model fails to reproduce the hyperinsulinemia state as seen in PCOS women. Fructose^[24,25] when given concomitantly with DHT, could completely mimic the clinical manifestation of PCOS. Thus, on this basis, the current study was planned to examine the effect of ovajal nutraceutical formulation on the DHT-fructose-induced PCOS in female rats of prepubertal age.

MATERIALS AND METHODS

Materials

DHT and fructose crystalline powder were obtained from TCI Chemicals (Mumbai, India) and Suvchem Pvt. Ltd (Mumbai, India) respectively. ELISA kits of FSH, LH, estradiol, testosterone, and insulin were obtained from Shanghai Korean Bioassay BT (Wuhan, China). Oral glucose tolerance test (OGTT) was performed using a one-touch glucometer product of life scan medical devices PVT. LTD.

Experimental Animals

Sprague Dawley (SD) female rats of 3 weeks (21 days) of age were obtained from an in-house Animal Breeding Facility (Jai Research Foundation, Vapi). The animals were housed in the cage under well-controlled conditions of temperature ($22 \pm 3^\circ\text{C}$), humidity (30–70%), and 24 hrs (12 hrs. light: 12 hrs. dark cycle). Animals had free access to standard rat feed and purified R. O. water *ad libitum*. All the experimental procedures were completed according to guidelines prescribed by the Institutional Animal Ethical Committee of ROFEL, Shri G.M. Bilakhia college of Pharmacy (Ethic protocol No: ROFEL/IAEC/2021/0011).

Experimental Design

Acute oral toxicity study: Acute toxicity study of ovajal was carried out using OECD- 423 guidelines i.e., Toxic Class Method. According to Annex 2d and paragraph 23 of OECD guidelines 423, the dose was determined based on body weight. As mentioned in a guideline, three healthy female S.D. rats were orally administered ovajal were employed for the control and dose level of 2000mg/kg. All animals were monitored for changes in body weight, clinical symptoms, and mortality during the first four hours following the drug administration and then twice daily for the next 14 days.^[26]

DHT-F induced PCOS

S.D. rats were acclimatized for a week and randomly divided into six groups (n = 6). DHT was prepared freshly every day by suspending the drugs in sunflower oil whereas clomiphene citrate, metformin, and fructose 20% solution was prepared using distilled water. Ovajal was suspended in water and dose volume was given. Each group received subsequent treatment; Group I: served as control and received 0.5mL of sunflower oil. Group II: received coadministration of DHT (83 $\mu\text{g/day/kg}$. b. wt; s.c). + fructose (20%; p.o.). Group III: received cotreatment of ovajal (100 mg/ k.g. b. wt;p.o.); DHT(83 $\mu\text{g/day/kg}$.b.wt; s.c) and fructose (20%; p.o.)., Group IV: received cotreatment of ovajal (200 mg/ k.g. b. wt;p.o); DHT(83 $\mu\text{g/day/kg}$.b.wt; s.c) and fructose (20%; p.o.). Group V: received cotreatment of clomiphene citrate (100 mg/k.g. b.wt.; p. o); DHT (83 $\mu\text{g/day/kg}$.b. wt; s.c)

and fructose (20%; p.o.). Group VI: received cotreatment of metformin (200 mg /k.g. b.wt.; p. o); DHT (83 µg/day/kg.b. wt; s.c.) and fructose (20%; p.o.). Rats were dosed for 13 weeks. On the study terminal day, rats in the fasting state were anesthetized, and blood was drawn by cardiac puncture for reproductive hormone assessments. Rats were euthanized, and reproductive organs were collected and stored for histomorphology analysis.

EXPERIMENTAL PARAMETERS

Measurement of Bodyweight

Weekly, individual animal body weights were recorded till the end of the study.

Estrous Cyclicity

Vaginal smears were taken from the 11th week of age of the animals to the end of the experiment. Vaginal discharges were collected every day with a prefilled normal saline (NaCl, 0.9 %) plastic pipette, by inserting it into the rat vagina. Collected vaginal fluid was dropped on glass slides, mixed with crystal violet stain, and observed under a microscope, with 10x and 40x objective lenses. Cyclicity in rats was determined based on the presence/absence and proportion of epithelial cells, cornified cells, and leucocytes. Cyclicity was also compared by calculating the percentage of days spent in the diestrus stage. The calculation was done using the following: % days in diestrus = No. of days exhibiting diestrus stage/Total no. of days X 100.^[27]

Oral Glucose Tolerance Test

Animals were kept fasting for 18 hrs. before the test. The rats were given an oral meal of glucose at a dose of 2 gm/kg body weight. The blood was withdrawn at 0, 30, 60, 120, 180 and 240 minutes after the glucose meal using a glucometer strip for quantification. The pre-prandial blood glucose (FBG), postprandial blood glucose (PPBG), and AUC were determined for all the groups.^[28-30]

Measurement of Reproductive Hormones

Collected blood samples were kept ideal for clotting at room temperature, centrifuged for 15 mins at 1000×g at 2 to 8°C. The collected supernatant was stored at -20°C and assayed using an ELISA kit, for reproductive hormones levels.^[31]

Ovary, Uterine Weight, and Histomorphology Assessment

Animals both ovary and uterus were collected at terminal sacrifice, weighed, and stored in 10% formalin solution for histomorphology evaluation. Collected ovary and uterine horn were kept in 10% formalin solution overnight. For histoslides preparation they were desiccated, fixed in paraffin wax, and sectioned at 5-µm thickness, stained with hematoxylin and eosin (H & E) dye. Slides were

examined using light microscopy for histomorphology assessment.^[32]

Statistical Analysis

All data were subjected to one-way or two-way ANOVA followed by Tukey's multiple comparison test using a graph pad prism. $p < 0.05$ was considered a difference of significance.

RESULT AND DISCUSSION

Acute Oral Toxicity: The nutraceutical formulation ovajal was given at a limit dose of 2000 gm/kg. Animals were observed for the first 30 min, periodically for 24 hours, and further for 14 days. The observation includes mortality, morbidity, clinical signs, behavioral traits, and general awareness. There were no signs of toxicity observed on administration of ovajal at the single dose of 2000 gm/kg.

Effect of Ovajal on Body Weight of Animals.

The body weight of all experimental group rats showed a rise in their body weight. DHT-fructose PCOS group rats' weight increased significantly ($p < 0.05$) from 8 weeks to 13 weeks. Ovajal-treated groups demonstrated a significant ($p < 0.05$) decline in their body weight when compared to the DHT-fructose PCOS group as shown in Fig. 1.

Effect of Ovajal on Estrus Cyclicity of Animals

The pseudo-diestrus phase was prominently seen, indicating the disturbance of rat estrus cyclicity as seen in human PCOS. DHT+F (Fig. 2) group showed a rise in the ovary weight, with unevenness in their estrus cycle. Rats treated with ovajal showed a regular estrus cycle. % Days diestrus phase spent in both ovajal treatment groups is lesser when compared with the DHT+F group indicating regular cyclicity.

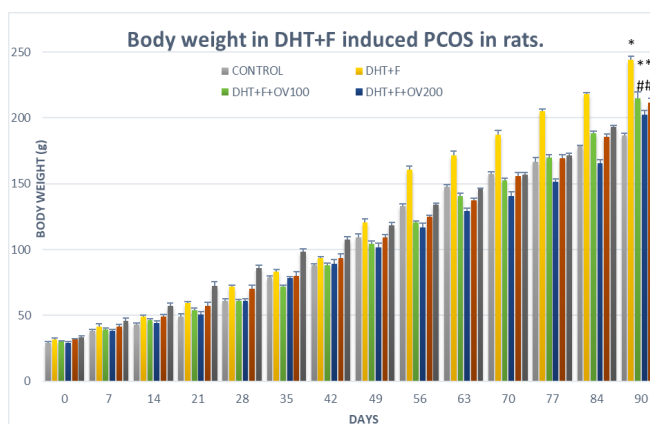


Fig. 1: Bodyweight of DHT-induced PCOS in rats.

All data are expressed as mean $\pm$ Sd ($n = 6$ in each group) and analyzed by two-way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$ when compared to control, # $p < 0.05$ when compared to DHT+F



Effect of Ovajal on Ovary, Uterus, the Subcutaneous and Abdominal Fat Weight of Animals

PCOS women generally suffer from obesity. DHT+F group showed elevation in their ovary weight and uterus weight significantly ($p < 0.05$) when assessed with the normal group. Ovajal-treated groups show a significant ($p < 0.05$) decrease in ovarian weight, but no significant impact was seen on uterine weight. Ovajal treatment group significant ($p < 0.05$) decrease in fat weight when compared with DHT+F. Significant variation was also observed in the metformin-treated group as shown in Fig. 3.

All data are expressed as mean $\pm$ Sd ($n=6$ in each group) and analyzed by one-way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$ when compared to control, # $p < 0.05$ when compared to DHT+F.

Effect of Ovajal on Reproductive Hormones of Animals

Reproductive and insulin hormones levels were analyzed using ELISA from blood serum which was stored earlier at -20°C in the deep freezer. Ovajal significantly ($p < 0.05$) suppressed the elevation of serum LH, T, Estradiol and insulin, glucose (AUC) except for FSH when compared DHT+F group as shown in Figs. 4 and 5.

All data are expressed as mean $\pm$ S.d. ($n=6$ in each group) and analyzed by one-way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$ when compared to control, # $p < 0.05$ when compared to DHT+F.

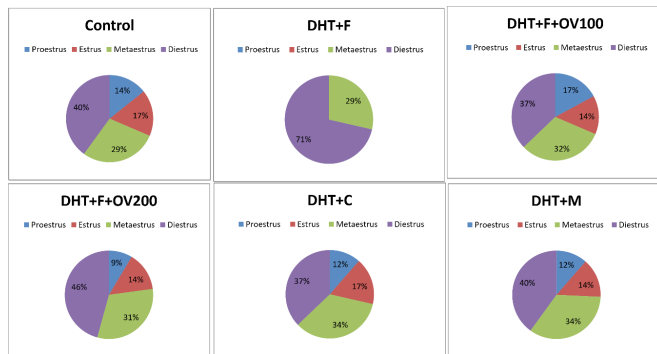


Fig. 2: % days spent in each phase of the estrous cycle by animals from different groups

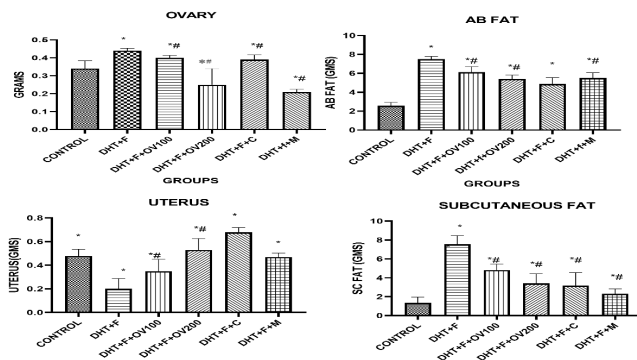


Fig. 3: Reproductive organ and body fats weights.

All data are expressed as mean $\pm$ S.d. ($n = 6$ in each group) and analyzed by one-way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$ when compared to control, # $p < 0.05$ when compared to DHT+F.

Effect of Ovajal on Histomorphology of Ovary

The normal histology structure of ovaries got disrupted with the co-administration of DHT and fructose as shown in Table. 1. Ovajal treated groups showed a decline in cyst follicle formation and atrophic changes when compared to the DHT+F group. Ovajal could have reduced or inhibited the action of potent androgen DHT on receptors which aggravated and nullified excess testosterone, a causative

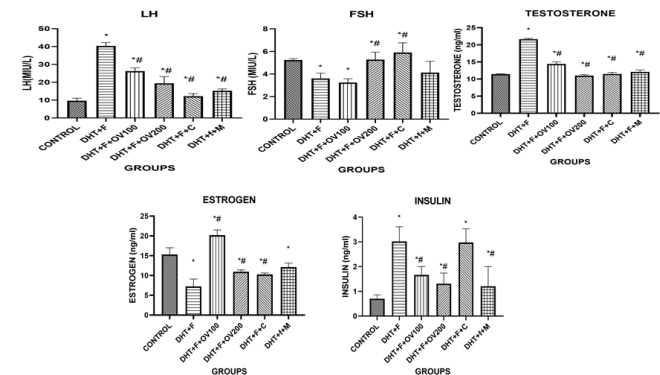


Fig 4: Reproductive Hormones levels in different groups.

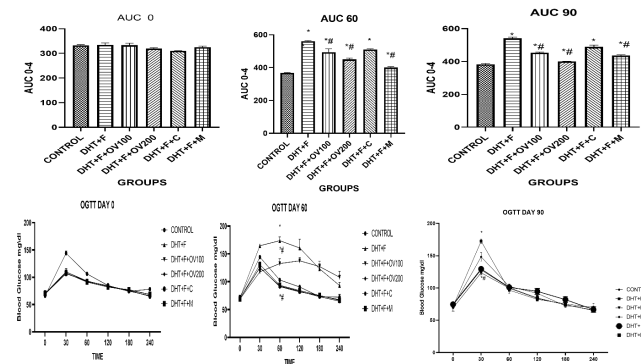


Fig 5: Oral glucose tolerance test and AUC in DHT groups.

All data are expressed as mean $\pm$ Sd ($n=6$ in each group). OGTT analyzed by two-way ANOVA while AUC analysed by one-way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$ when compared to control, # $p < 0.05$ when compared to DHT+F

Table 1: Histomorphological evaluation of ovaries in DHT+F induced PCOS rats:

Groups:	Corpus luteum (%)	Cystic follicle (%)	Atrophic changes (%)
G1: Control	85	12	0
G2: DHT+F	70	85	90
G3: DHT+F+C	79	68	44
G4: DHT+F+M	90	57	65
G5: DHT+F+OV100	76	45	59
G6: DHT+F+OV200	80	33	52

factor of PCOS. Fructose showed metabolic disturbance indicating glucose intolerance and reduced glucose uptake. The metabolic disturbance was reversed due to sodium selenite content which showed antidiabetic potential and reduces insulin resistance.^[33]

In our study, androgen dihydrotestosterone (DHT) and fructose were administered together to induce polycystic ovary syndrome in prepubertal female Wistar rats. This DHT+F induced PCOS model resembles human PCOS phenotypes. Regular examination of vaginal smears and the presence of pseudo diestrus phase confirmed the model's worth. When compared to control animals, the DHT+F group had a significant rise in their body weight, irregular cyclicity, serum LH, T, and insulin, glucose levels, and lower levels of serum estradiol and FSH levels as observed in PCOS.

Ovajal was able to normalize the elevation of body weight induced by DHT primarily regulating adiposity and fat distribution. PCOS women also suffer from obesity with hyperandrogenism. DHT and Fructose when administered together showed pronounced fat mass and central adiposity, relating connectivity with hyperinsulinemia, and insulin resistance associated with type 2 diabetes mellitus. The reduction in body weight in ovajal group rats can be correlated to a relative reduction in body fat mass, as seen in PCOS women when treated with metformin.

Androgen DHT+ fructose-induced rat PCOS model represents oligomenorrhea and a prevalence of the "pseudo diestrus" phase. The estrus cycle of the ovajal exposed rats showed a reduction in % diestrus cycle in the estrus stage and showed a longer cycle duration. Normal cyclicity mediated by OV200 can be correlated with clomiphene and metformin; Thus, showing a strong connection between nutraceutical components and their role in the pathogenesis of PCOS.^[34]

According to previous reports, exposure to DHT impacts the hormonal balance in the female rat, resulting in diminished development of the reproductive organs. Rats when exposed to DHT+F for 90 days, produced a significant ($p < 0.05$) decrease in the ovarian weight significant. Metformin and clomiphene both significantly ($p < 0.05$) lowered the ovary weight probably by normalizing folliculogenesis and steroidogenesis indicating a promising role in therapy in PCOS women. DHT increases abdominal fat depots, which as per the previous studies reported^[35,36] on the DHT model. Abdominal fat and subcutaneous fats were assessed and found to be highly significant in DHT+F showing a profound obese state, which is a prominent phenotype seen in PCOS Women. Ovajal-treated rats showed normal ovarian weight by nullifying the DHT effects.

PCOS is also linked to type 2 diabetes mellitus (T2DM) which begins with hyperglycemia and progresses to insulin resistance and hyperinsulinemia over time^[37]. In our study Insulin levels (Fig. 4) in the DHT+F group were seen as elevated showing a significant ($p < 0.05$)

difference when compared to the control group at different time points of 30-, 60-, and 120 mins. Ovajal significantly prevented the rise in glucose intolerance, and serum insulin levels indicating its beneficiary effect in preventing diabetic complications and insulin resistance (Fig. 5). Possible action reasonably due to selenium^[38-40] content in the formulation which already has insulin sensitizers class profile, whereas zinc plays a crucial role in insulin metabolism including synthesis, storage secretion, and functioning.

Hypersecretion of luteinizing hormone and testosterone concentration shows the establishment of hallmark features of PCOS responsible for interrupting the fertilization process. Ovajal, like metformin and clomiphene citrate, was able to normalize LH/FSH and testosterone levels respectively. Estrogen levels were higher due to repetitive administration of ovajal. In previous reports,^[41,42] ovajal components like melatonin cysteine, carnitine and inositol have shown a promising role in preventing hyperandrogenism and regularizing the LH/FSH levels. Thus, ovajal was similarly effective as metformin.

CONCLUSION

Current study results show the therapeutic potential of ovajal on experimental induced PCOS, possibly due to the combined effect of the different nutraceutical components which seems to have a pathogenesis role in maintaining the reproductive system through regulating estrus cyclicity, ovarian morphology, antiandrogen effect, and restoration of ovarian functional tissue. Thus, it can be concluded, that ovajal indicates its anti-PCOS potential and can be included in the management of PCOS. However, a clinical investigation is required to confirm its therapeutic potential.

ACKNOWLEDGEMENT

We acknowledge with much appreciation the crucial role of Mr. Uday Kapurkar, Head Pathology, Jai Research Foundation, Valvada, Gujarat to help generate histopathology slides.

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HOW TO CITE THIS ARTICLE: Kumar GS, Tirgar PR, Rachh PR. Effect of Nutraceutical Formulation Ovajal on DHT and Fructose-induced Polycystic Ovary Syndrome in a Rodent Model. *Int. J. Pharm. Sci. Drug Res.* 2022;14(2):256-261. DOI: 10.25004/IJPSDR.2022.140216