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Research article

The therapeutic effect of *Piper longum* Linn. fruits in testosterone induced polycystic ovarian syndrome in female wistar rats

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ABSTRACT

The regulation of polycystic ovarian syndrome (PCOS) is intricate but often disappointing. The intend of existing study is to investigate effects hydro alcoholic extract of *Piper longum* L. (HEPL) fruits in testosterone persuaded Polycystic ovarian syndrome. Female wistar arts were separated into 9 groups (n=6). Normal Control received Sesame oil as a vehicle control once daily. Testosterone (2mg/kg/s.c.) was administered for 31days to induce PCOS induction in all remained group. PCOS persuaded animals were given with Metformin (300mg/kg/oral route), Clomiphene citrate, (150mg/kg/oral), HEPL groups (200, 400 and 800 mg/kg), Combination group (*Piper longum* 800 mg/kg and Metformin 300mg/kg) and Combination group (*Piper longum* 800 mg/kg and Clomiphene citrate,150mg/kg) up to 59 days using vehicle as a Distilled water. Body weights, estrus cyclicity, biochemical, hormonal and histological orders were measured. Testosterone persuaded PCOS marked by uneven estrus cyclicity, enhanced triglycerides, cholesterol, LDL glycaemia, testosterone and LH (p<0.01-0.001) but there were low HDL and FSH (p< 0.05, 0.001) levels were analyzed. To study insulin resistance, Oral glucose tolerance test was performed. Multiple cysts had been observed with PCOS rats as compared with the control group. Also, HEPL established the estrus periodicity. These biochemical, hormonal and structural alterations were abridged by *Piper longum* L. Moreover, *Piper longum* significantly decreased Cystic follicles count, testosterone and LH (p<0.001) levels but proliferated FSH (p<0.001) concentrations. The hydroalcoholic extract of fruits of *Piper longum* L. showed significant amelioration of Testosterone induced PCOS.

Keywords: Piper longum L., Testosterone percuaded, Clomiphene citrate, Cystic follicles

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INTRODUCTION

N Polycystic ovary syndrome (PCOS) is a frequent holocrine complication that affects girls of accouchement generation.[1-4] The occurrence of PCOS extend between 2.5 and 7.5%. The widespread manifestations of PCOS include uneven menstrual cycles, weight put on, sterility, inflated androgen levels as well as numbers of ovarian cysts. PCOS is a complex condition characterized by chronic absence of ovulation of polycystic ovary, clinical and biochemical hyperandrogenism, hirutism, amenorrhoea and infertility which are used to recognize PCOS. [5-6] they have also been found to have low grade inflammation, contributing to insulin resistance. PCOS directly impacts fertility but has serious health complications if left untreated. [7] In PCOS estrogen level declined, how-ever, the progesterone amount inflated and LH/FSH relative amount become thrice of the ordinary level. Androgens are made up by theca cells.[8] Piper longum Linn., Piperaceae, is growing in warmer regions of India, Western ghats, Central Himalaya to Asam, Bengal as small aromatic plant. Leaves are smooth, entire, 7-ribed, narrow pointed, sessile, 6-10cm long, deeply cordate with big lobes at base, dark green and shinning, stalked. Flowers are dioecious, minute, inflorescence a spike, 3-4 cm long.

Roots contain parenchyma, simple or compound starch, grains, lignified and striated stone cell and pith is absent. Fruits are small, avoid, sunken in fleshy spike, 2.5-4 cm long, blackish green and shinning. Fruits mainly contains sesamin, sylvatine, a lignon dihydrostimasterol, piperine, N-isobutyl-decatrans-2-trans-4-dienamide, terpinolene, zingiberone, p-cymene, p-methoxyacetophenone, dehydrocarveol, phenylethyl alcohol also been reported. The leaves contains hentriacontane, triacentanol and β-sitosterol. [9]

Piper longum Linn., has been found to show activities like Insulin resistance contributes to anovulation, hyperandrogenism and

cardiovascular risks [10-11] , also known to possess action against Parkison's disease [12] , obesity [13] , glioma [14] , hyperlipidemia [15] , diabetes [16] , hormone induced melanoma [17] and neuronal damage. [18] Clomiphene citrate is an Insulin sensitizers as well as exogenous gonadotropins, like Metformin. It is useful to deplete insulin resistance that showed consequences to decrease ovarian androgen secretion along with resultant improvement in menstrual cyclicity. [19] Allopathic drugs are showing unpleasant effects like arthritis, muscle or joint ache [20] lactic acidosis [21] and psychological disturbances, it is challenging to researchers to search for new drugs. Many excellent herbal drugs are endorsed traditionally for cure and prevention of PCOS [23-26], however, lots of herbs are still to be studied for their mechanism. [27]

MATERIALS AND METHODS

Authentication of plant material

Fruits of *Piper longum* Linn Were collected from Pirangut area in Pune, Maharashtra, India, in during October (2018) after the flowering season. Taxonomic identification and authentication were carried out by Dr. C. R. Jadhav, Botanist, Botanical Survey of India, Koregoan Road, and Pune. A voucher specimen (BSI/WRC/ IDEN.CER./2029/H3) of the plant was deposited in our laboratory, Maratha Vidya Prasarak Samajs College of Pharmacy, Nashik.

Collection of Plant material

Fruits were dried and grinded to coarse powder with the help of mixer which was then kept in tight container. Using Soxhlet Extraction, powder was extracted thrice with 1000ml of diluted alcohol (70:30-Alcohol: Water) for 6 hours at 55°C. Using a rotary vacuum evaporator, the collected filtrate was concentrated. After complete extraction process, the extract was stored in sterile bottles under refrigeration conditions.

Animals

Female Wistar rats weighing about 200-250 gram were obtained from Crystal Biological Solutions, Handewadi, Pune, Maharashtra, India. Before the commencement of study, all animals were acclimatized for 1 week. Standard commercial normal pellet diet (NPD) and water were provided ad libitum for the animals during the course of an experiment. The animals were nurtured under monitored temperature (22 °C ±2°C) and relative humidity (55% ±5%) with 12:12 hr light and dark cycle. The study was approved by IAEC (Protocol No: MVPCP/IAEC/2019/05) of Maratha Vidya Prasarak Samajs College of Pharmacy, Nashik constituted under CPCSEA, Government of India. Ethical guidelines were strictly followed according to established public guidelines.

Experimental Groups and Study Design

Female wistar rats were categorised in nine sets containing (n = 6) animals. (Table 1) Estrous cycle regularity was checked for 2 consecutive estrous cycles by vaginal smear method. Only females with regular estrous cycle were kept in the study. Daily vaginal smear samples were collected (8 10 a.m.) [28], stained with Giemsa stain and studied microscopically. After the treatment duration, on 59 th day the animals will be sacrificed and the following parameters will be evaluated.

Table 1.Experimental design

Groups	Testosterone (mg/kg/sc) Day 0-31	Drug Treatment (mg/kg/po) Day 32-59
Group I : Normal Control (NC)	-	-
Group II: Disease control (DC)	2.5	-
Group III: Metformin (Met)	2.5	300
Group IV: Clomiphene citrate (C.C.)	2.5	150
Group V: HEPL 200 mg/kg	2.5	200
Group VI:HEPL 400 mg/kg	2.5	400
Group VII: HEPL 800 mg/kg	2.5	800
Group VIII: Metformin + HEPL 800 mg/kg	2.5	300 + HEPL 800
Group IX: Clomiphene citrate + HEPL 800 mg/kg	2.5	150+ HEPL 800

PARAMETRS TO BE ASSESSED

Physical parameters

The body weight of all animals was recorded at the beginning as well as at weekly interval basis throughout the study.

Vaginal smear test

A cotton bud dipped in normal saline was inserted gently in the vaginal opening of the female rats and a swab was obtained. The cotton bud was rolled on a clean grease free slide to make a smear and allowed to air dry. Few drops of methanol were added to fix the cells in the smear. The slide was air dried. Giemsa stain was added to the slide to cover the smear. The slide was kept covered in petridish for 5 minutes. After 5 minutes distilled water was added to the giemsa and gently rocked. A green scum appeared on top of the slide. The slide was stained for 10 minutes in dilute giemsa. The stained slide was drained and then washed in gentle stream of tap water. The washed slide was air dried and observed under the microscope in 40x objective. [29]

Biochemical parameters

Biochemical parameters were determined with the help of diagnostic kits (Pathozyme Pvt Ltd, Maharashtra, India). Insulin resistance was analyzed using Oral glucose tolerance test. Hormonal concentrations were assessed by Enzyme Linked Immunoassay (ELISA) AM 2100 (Alere) using Krishjan Biosystems Kit.[30]

Oral glucose tolerance test (OGTT)

OGTT was conducted on 18th and 45 th day for all rats in the experiments. [31] Glucose (2 g/kg) was given orally nightly starved rats and blood samples were taken after every 30 min for 2 hrs. The blood was centrifuged at 3000 rpm for 10 min to separate the serum. Glucose was assessed by means of Accu - Check Instant Glucometer.

Histopathology

Ovaries along with uterus were removed from each animal at the end of study, weighed as well as fixed in 10% formalin solution. Ascending grades of ethanol were used to dehydrate the specimen, xylene to clear and paraffin to embed. The sections of 3-5 µ thickness were cut and stained with hematoxylin-eosin stain and

observed under 40x. Histopathology examination of all the organs were carried out and noted down. [32]

Statistical evaluation

The outcomes were expressed as mean ± SEM. The statistical differences of the data was calculated by One-way analysis of variance (ANOVA) followed by Dunnet's post hoc test.

RESULTS

Effect of Different treatment on Body weight and relative weights of Ovaries and Uterus

Administration of testosterone showed no noticeable rise in body weights when evaluated against normal control. At the end of treatment body weights were considerably lowered in Metformin and HEPL 400 mg/kg against DC (Figure 1 A and 1 B). The relative organ weights of ovaries were considerably increased in all groups (except CC, HEPL (800mg/kg) and Met + HEPL (800mg/kg)) against normal control. The relative weight of ovaries were considerably decreased in CC, HEPL (800mg/kg) and Met + HEPL (800mg/kg) when compared with DC (Figure2 A). The relative organ weight of uterus was considerably increased in all groups (except CC+ HEPL (800 mg/kg)) against Normal control. The relative organ weight of uterus was considerably rised in Met, CC, HEPL (800mg/kg) and CC+ HEPL (800mg/kg) against DC (Figure 2 B).

Figure 1: Effects of Metformin, Clomiphene citrate, Hydroalcoholic extract of Piper longum L -A

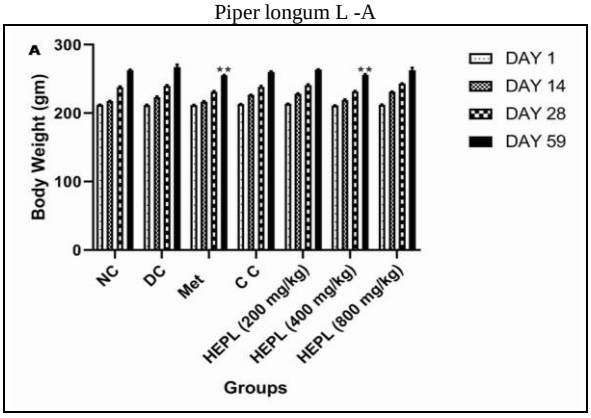


Figure 1 is the combination groups of standard drugs with Hydroalcoholic extract of Piper longum L. on body weight (A) and (B) in Testosterone induced PCOS rat. (NC-Normal Control, DC-Disease control, Met- Metformin, CC-Clomiphene citrate, HEPL (200mg/kg)- Hydro alcoholic extract of Piper longum L. 200mg/kg, HEPL (400mg/kg) - Hydro alcoholic extract of Piper longum L. 400mg/kg, HEPL (800mg/kg) - Hydro alcoholic extract of Piper longum L. 800mg/kg, Met +HEPL (800mg/kg) : Combination of Metformin and Hydro alcoholic extract of Piper longum L. (800mg/kg), CC+HEPL (800 mg/kg)- Combination of Clomiphene citrate and Hydro alcoholic extract of Piper longum L. (800mg/kg). Values are Means ± SEM, (n = 6 animals per group). There was no significant change in body weight of all animals when compared with normal control and disease control groups.

Figure 1 B

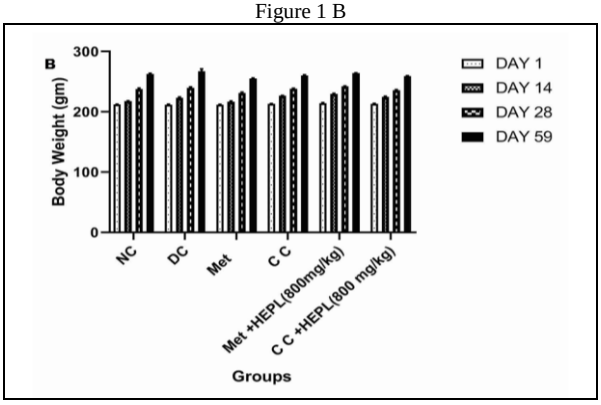


Figure 2: Effects of Metformin, Clomiphene citrate, HEPL and combination groups of standard drugs with Hydroalcoholic extract of *Piper longum* L - A

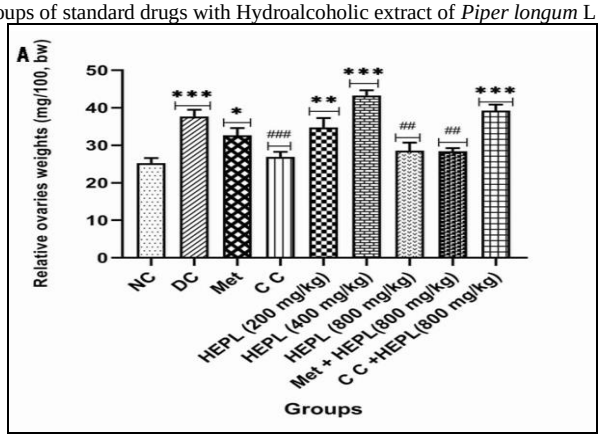
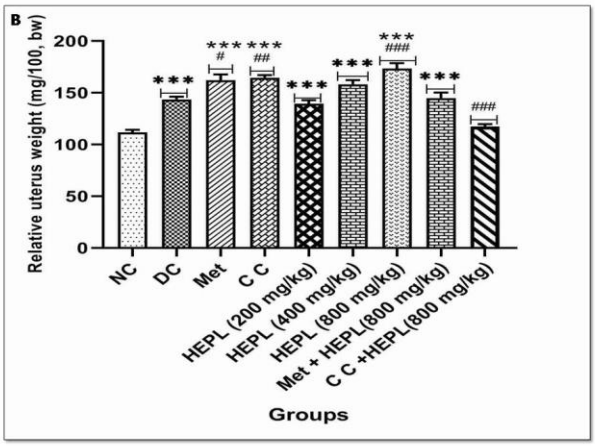


Figure 2: B



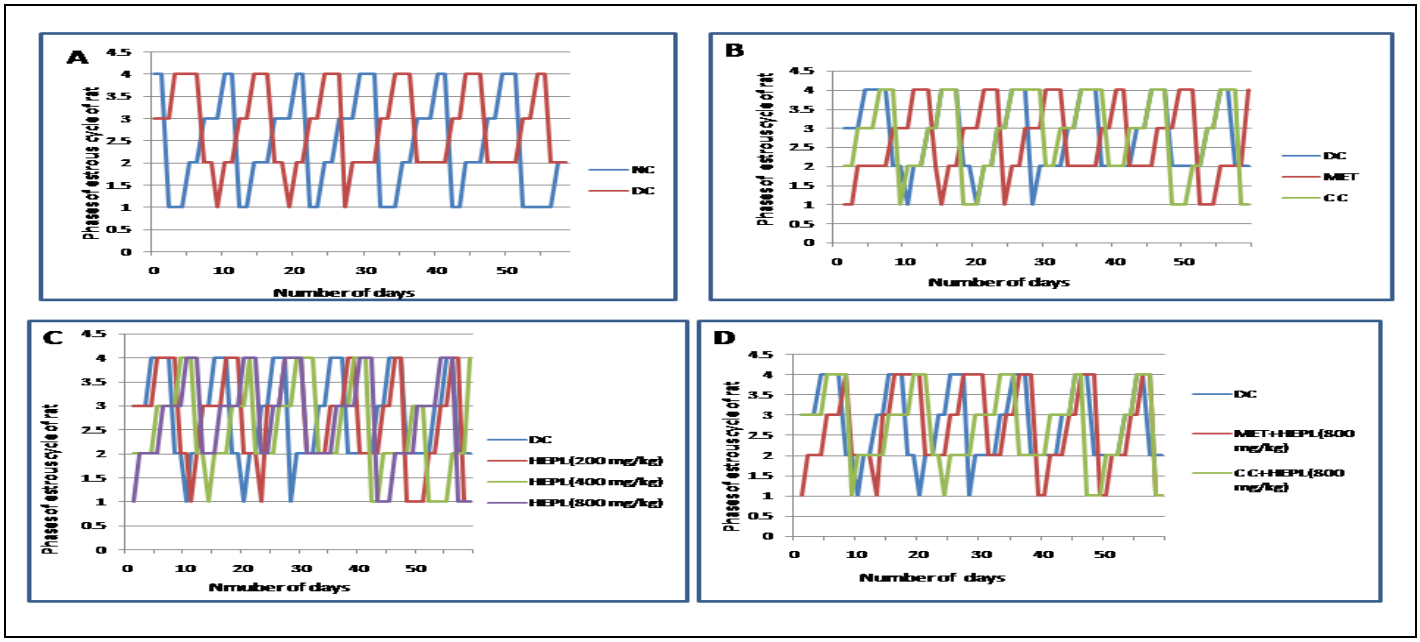
On ovaries (A) and Uterus (B) weight in Testosterone induced PCOS rat. (NC-Normal Control, DC-Disease control, Met-Metformin, CC-Clomiphene citrate, HEPL (200mg/kg)- Hydro alcoholic extract of *Piper longum* L. 200mg/kg, HEPL (400mg/kg)- Hydro alcoholic extract of *Piper longum* L. 400mg/kg, HEPL (800mg/kg)- Hydro alcoholic extract of *Piper longum* L. 800mg/kg, Met +HEPL (800mg/kg) : Combination of Metformin and Hydro alcoholic extract of *Piper longum* L. (800mg/kg), CC+HEPL (800 mg/kg)- Combination of Clomiphene citrate and Hydro alcoholic extract of *Piper longum* L. (800mg/kg). Values are Means ± SEM, (n = 6 animals per group). ***p <0.001: significantly different

compared with Normal control. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$: significantly different compared with Disease control.

Effects of Testosterone on Estrous cyclicity

Estrous cycle of rats is of 4 to 5 days which shows estrous (1), metaestrous (2), diestrous (3) and proestrous (4) (Figure 3 A). In the control group, animal showed regular phases of estrous cycle while disease control group showed irregular phases of estrous cycle during 31 days PCOS induction period (Figure 3 A). After 31 days treatment of testosterone, causes irregularity in reproductive cycle. From 32 to 59 days, disease control group showed irregular estrous cycle. Disease control showed lack of estrous phase along with constant metaestrous and proestrous phases. During study period of 32-59 days, all standard treatment groups, hydro alcoholic extract of Piper longum L. and combination groups caused asymmetric progress in the estrous cycle and presence of estrous phase decreased the duration of metaestrous and proestrous phase against DC (Figure 3 B, C and D).

Figure 3: Effect of Testosterone on estrous cycle of groups during PCOS induction 1-Estrous
2- Metaestrous
3-Diestrous
4-Proestrous



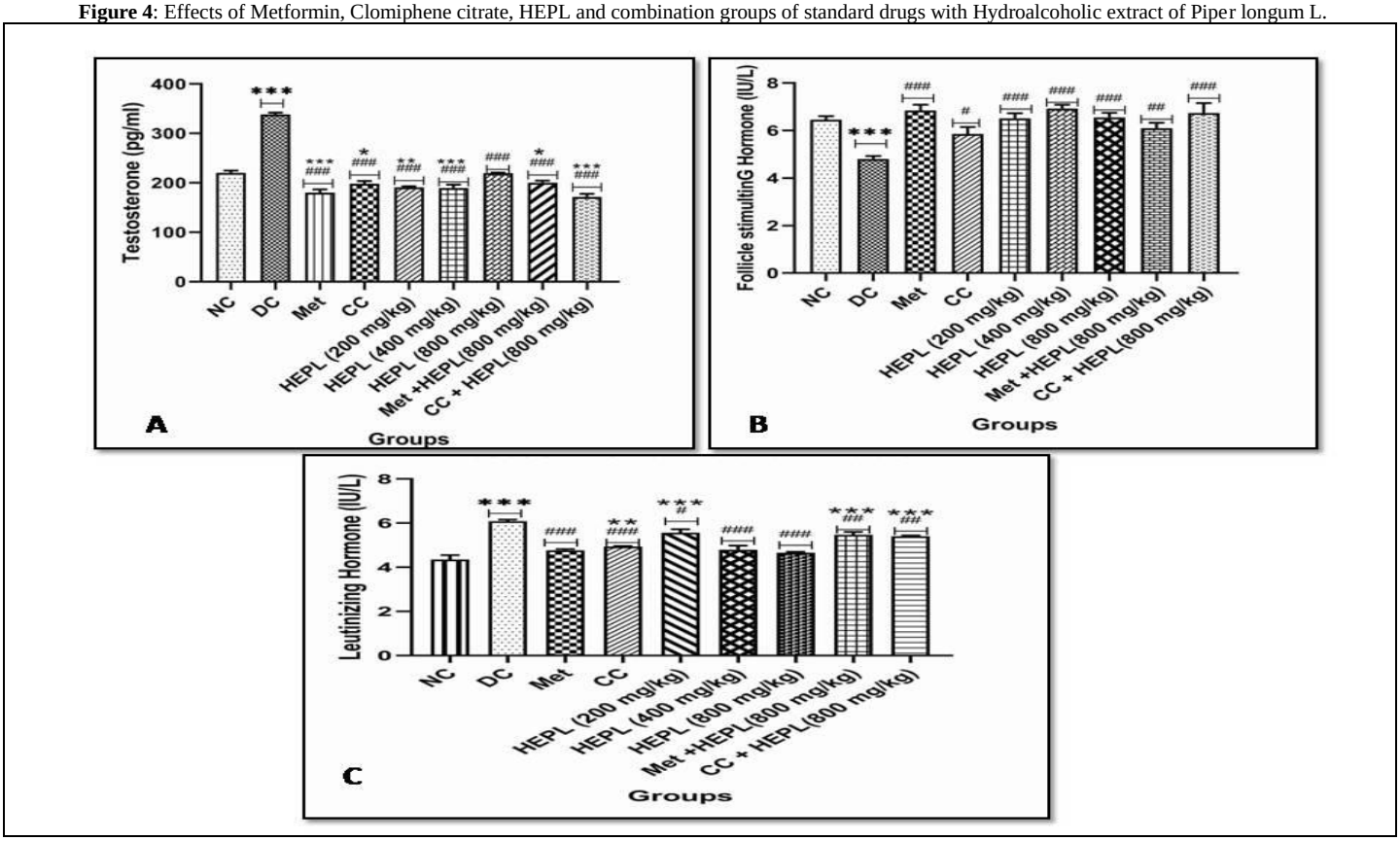
(NC-Normal Control, DC-Disease control, Met- Metformin, CC-Clomiphene citrate, HEPL 200- Hydro alcoholic extract of Piper longum L. 200mg/kg, HEPL 400- Hydro alcoholic extract of Piper longum L. 400mg/kg, HEPL 800- Hydro alcoholic extract of Piper longum L. 800mg/kg, Met + HEPL 800mg/kg: Combination of Metformin and Hydro alcoholic extract of Piper longum L. 800mg/kg, CC+HEPL 800 mg/kg- Combination of Clomiphene citrate and Hydro alcoholic extract of Piper longum L. 800mg/kg).

Hormonal parameters

On PCOS induction, testosterone and LH concentrations were increased considerably while FSH concentration was decreased as against DC. Amount of Testosterone was increased considerably ($p<0.05$, 0.001) in all treatment groups (Figure 4 A) except HEPL (800mg/kg) against Normal control. All treatment groups showed remarkable decreased ($p<0.001$) in concentration of Testosterone against DC (Figure 4 A).

Serum level of FSH was considerably decreased ($p< 0.001$) in disease control and non significant in all treatment groups against Normal control (Figure 4 B). All treatment groups (except CC) showed considerable increased ($p< 0.05$-0.001) in concentration of FSH against DC (Figure 4 B). Serum level of LH was considerably increased ($p < 0.01$-0.001) in all treatment groups (except Met, HEPL (400 mg/kg and 800mg/kg) against Normal control (Figure 4 C). All treatment groups showed considerable decreased ($p < 0.05$-0.001) in level of LH against DC (Figure 4 C). On Testosterone (A), Follicle stimulating hormone (B) and Luteinizing hormone (C) in Testosterone induced PCOS rat. (NC-Normal Control, DC-Disease control, Met- Metformin, CC-Clomiphene citrate, HEPL (200mg/kg)- Hydro alcoholic extract of Piper longum L. 200mg/kg, HEPL (400mg/kg)- Hydro alcoholic extract of Piper longum L. 400mg/kg, HEPL (800mg/kg)- Hydro alcoholic extract of Piper longum L. 800mg/kg, Met +HEPL (800mg/kg) : Combination of Metformin and Hydro alcoholic extract of Piper longum L. (800mg/kg), CC+HEPL (800 mg/kg)- Combination of Clomiphene citrate and Hydro alcoholic extract of Piper longum L. (800mg/kg). Values are Means ± SEM, (n = 6 animals per group).*$p<0.05$,**$p<0.01$,***$p<0.001$: significantly different compared with Normal control. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$: significantly different compared with Disease control.

Figure 4: Effects of Metformin, Clomiphene citrate, HEPL and combination groups of standard drugs with Hydroalcoholic extract of Piper longum L.



Biochemical parameters

Effect of triglyceride on Lipid Profile

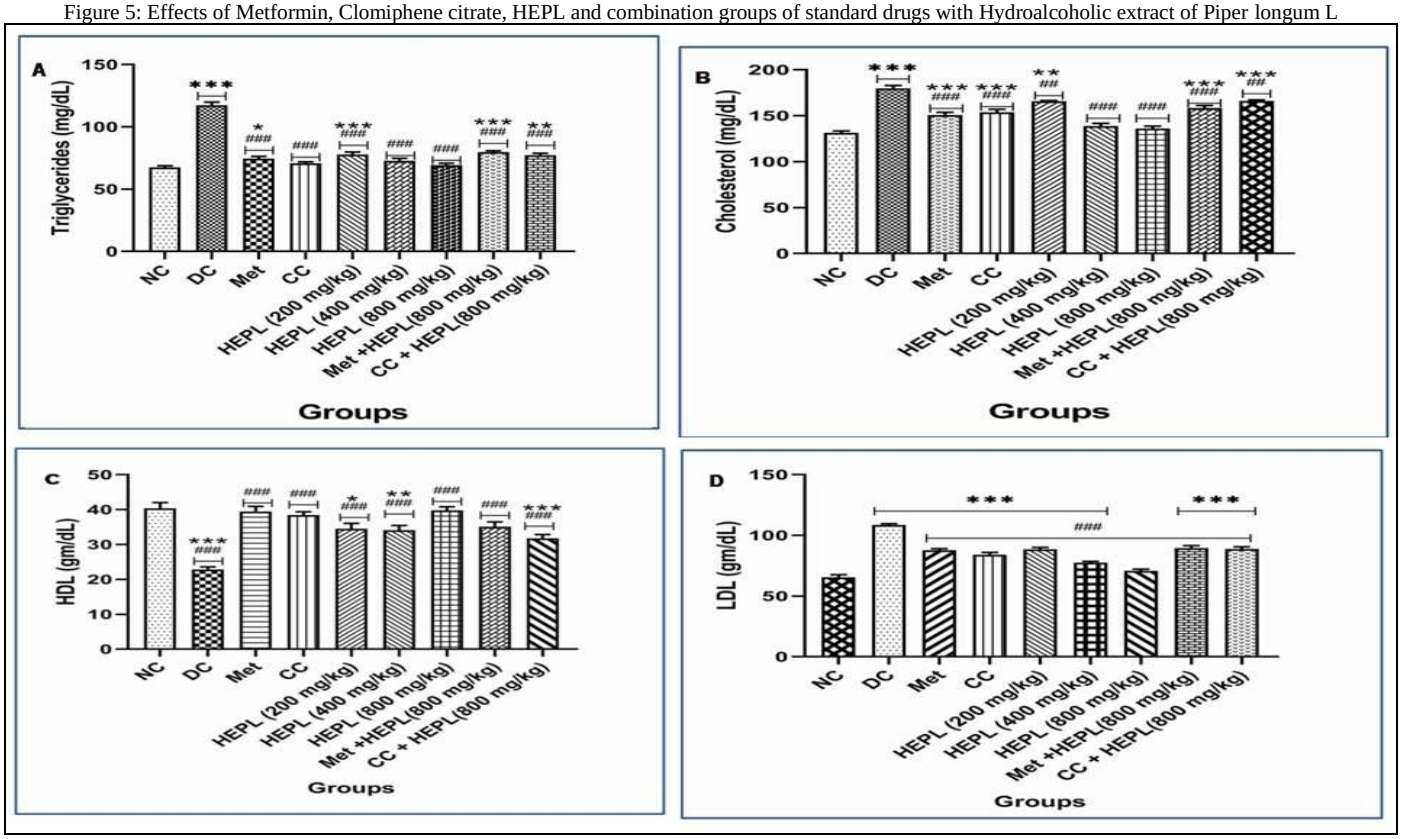
Serum level of Triglyceride (TG) was increased considerably (p< 0.05- 0.001) in all sets (Figure 5 A) against Normal control. All sets showed decreased (p<0.001) in concentration of TG against DC (Figure 4 A). Serum level of Cholesterol was increased considerably (p< 0.01- 0.001) in all sets (Figure 5 A) (except HEPL 400mg/kg and 800mg/kg) against Normal control. All sets showed decreased (p<0.01-0.001) in concentration of Cholesterol against DC (Figure 5 B). Serum level of HDL was decreased considerably (p< 0.05- 0.001) in all sets (Figure 5 C) (except Met, CC, HEPL (800mg/kg) and Met + HEPL (800mg/kg)) against Normal control. All sets showed decreased (p<0.01-0.001) in concentration of HDL against DC (Figure 5 C). Serum level of LDL was increased considerably (p< 0.001) in all sets (Figure 5 D) (except HEPL (800mg/kg)) against Normal control. All sets showed decreased (p< 0.001) in concentration of LDL against DC (Figure 5 D). Serum level of Glucose was increased significantly (p< 0.05, 0.001) in all treatment groups (Figure 5 D) (except Met, CC and DC) against Normal control. All sets showed decreased (p< 0.001) in concentration of Glucose against DC (Figure 5 E).

Figure 5 shows on Triglyceride (A), Cholesterol (B) HDL (C), LDL (D) and Glucose (E) in Testosterone induced PCOS rat. (NC-Normal Control, DC-Disease control, Met- Metformin, CC-Clomiphene citrate, HEPL (200mg/kg)- Hydro alcoholic extract of Piper longum L. 200mg/kg, HEPL (400mg/kg)- Hydro alcoholic extract of Piper longum L. 400mg/kg, HEPL (800mg/kg)- Hydro alcoholic extract of Piper longum L. 800mg/kg, Met +HEPL (800mg/kg) : Combination of Metformin and Hydro alcoholic extract of Piper longum L. (800mg/kg), CC+HEPL (800 mg/kg)- Combination of Clomiphene citrate and Hydro alcoholic extract of Piper longum L. (800mg/kg). Values are Means ± SEM, (n = 6 animals per group).*p<0.05,**p<0.01,***p <0.001: significantly different compared with Normal control. $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001: significantly different compared with Disease control.

Oral glucose tolerance test

Figure 6 on Oral glucose tolerance test on day 18 (A) and 45 (B). (NC-Normal Control, DC-Disease control, Met- Metformin, CC-Clomiphene citrate, HEPL (200mg/kg)- Hydro alcoholic extract of Piper longum L. 200mg/kg, HEPL (400mg/kg)- Hydro alcoholic extract of Piper longum L. 400mg/kg, HEPL (800mg/kg)- Hydro alcoholic extract of Piper longum L. 800mg/kg, Met +HEPL (800mg/kg) : Combination of Metformin and Hydro alcoholic extract of Piper longum L. (800mg/kg), CC+HEPL (800 mg/kg)- Combination of Clomiphene citrate and Hydro alcoholic extract of Piper longum L. (800mg/kg). Values are Means ± SEM, (n = 6 animals per group). ***p <0.001: significantly different compared with Normal control. $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001: significantly different compared with Disease control.

Figure 5: Effects of Metformin, Clomiphene citrate, HEPL and combination groups of standard drugs with Hydroalcoholic extract of Piper longum L



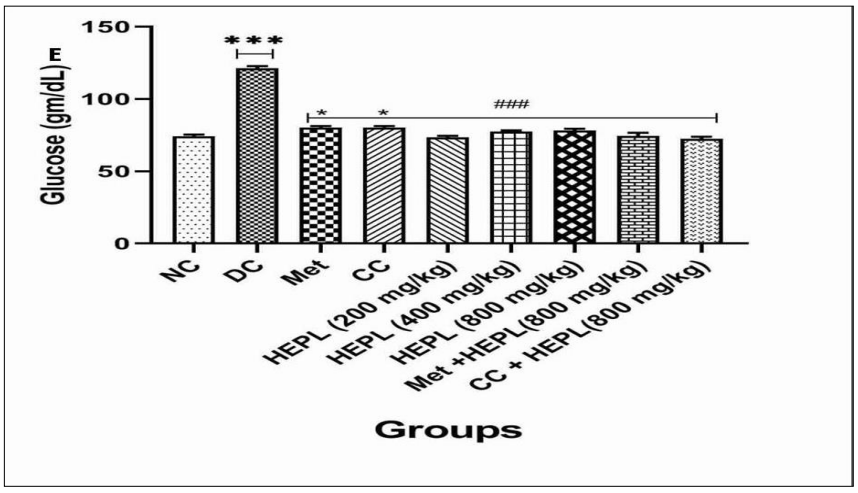


Figure 6: Effects of Metformin, Clomiphene citrate, HEPL and combination groups of standard drugs with Hydroalcoholic extract of Piper longum L.

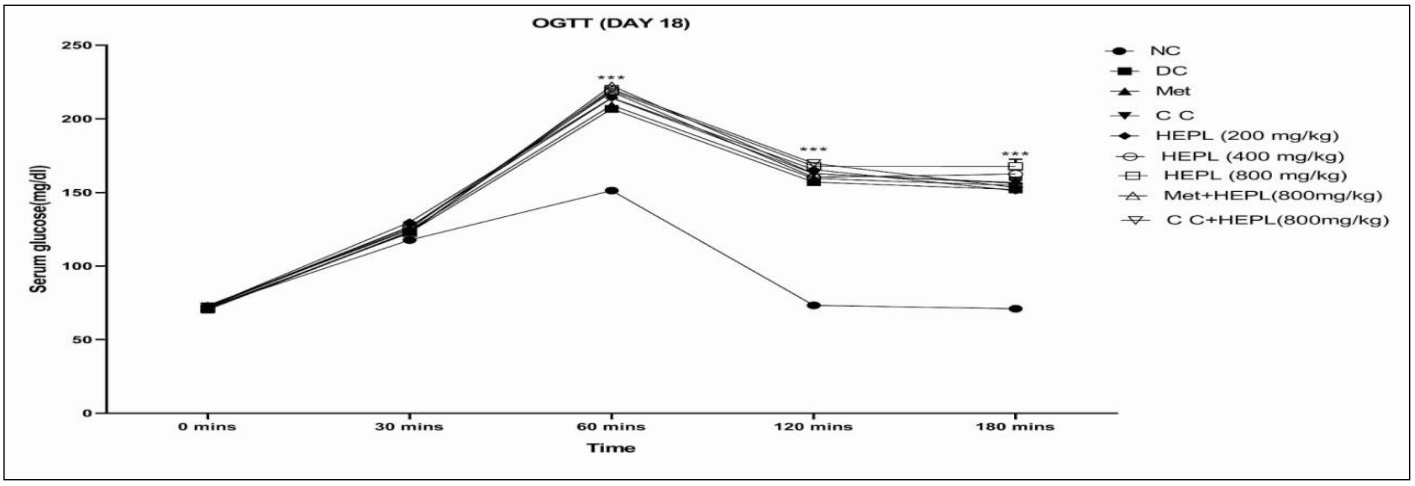
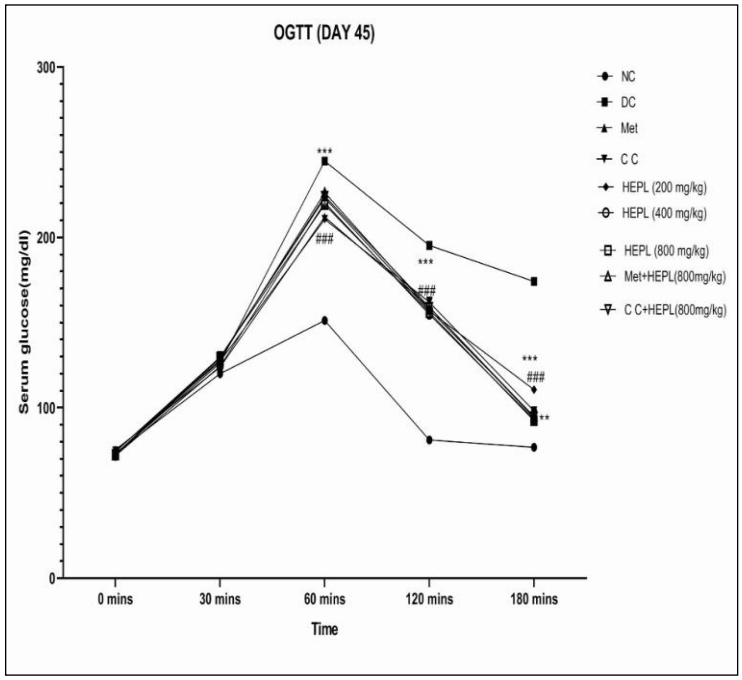


Figure 7: Effect of Metformin, Clomiphene citrate, HEPL and combination groups of standard drugs with Hydro alcoholic extract of Piper longum L



Histopathology

Microscopic examination of ovaries from 9 groups was carried out. Control rats were having normal corpus luteum and no cyst. All groups with PCOS induction using testosterone showed abnormalities in less or more severe scale. In Testosterone induced animals, microscopic examination of ovary showed cyst in many ovarian follicles as well as smaller number of corpus luteum.

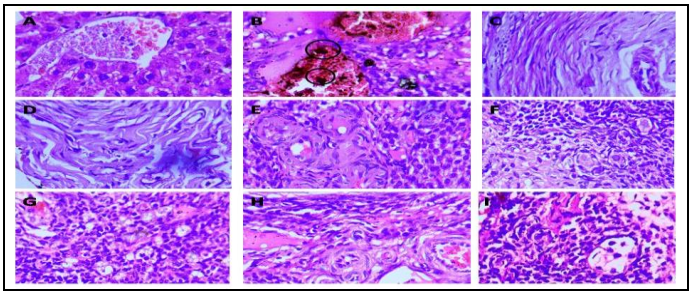


Figure 7 on ovarian follicular growth. A (NC): Normal follicular development; B(DC): Testosterone treatment causes formation of small cysts in ovaries as well as atretic follicles; C (Met), D(CC),E (HEPL 200 mg/kg), F(HEPL 400mg/kg), G (HEPL 800 mg/kg), H (Met +HEPL 800 mg/kg), I (CC+ HEPL 800 mg/kg)

DISCUSSION

In the present study, subcutaneous administration of Testosterone results in elevation in body weight and causes asymmetric estrous cycle which suggests the development of polycystic ovary syndrome in rats is because of increased LH and androgen levels. A considerable reduction in body weight was seen in Metformin and HEPL (400mg/kg) against DC. After treatment with Piper longum L. extract it was exhibited, a significant depletion

After loading glucose, serum glucose level were measured after every 30 min was considerably ($p<0.001$) increased in all sets (Figure 6 A) on day 18 against Normal control. The basal reading of glucose amount did not show the difference. Normally, after glucose loading, concentration of serum glucose showed considerable spike and retains the normal level within two to three hours but it was not seen in disease as well as treatment groups. It showed the build out of insulin resistance state. All treatment groups showed considerably decreased ($p<0.01$-0.001) in glucose levels as well as developed resistance to testosterone when compared to disease control group. (Figure-6-B)

in ovarian weight and elevated uterus weight may show regular follicle formation as well as uterotonic effects of plant.

On 31 days treatment with the testosterone showed lack of estrous phase along with extended metaestrous and proestrous phases in the DC and other treated sets. All treated sets causes enhancement in the estrous cyclicity and presence of estrous phase with shrinkage of metaestrous and proestrous phase against DC. The hydro alcoholic extract of Piper longum L. and its combination groups with Metformin and Clomiphene citrate in diseases induced rats restored the estrous cyclicity probably by stimulating the conversion of androgens to estrogen, by circulating improved estradiol concentration, by decreasing LH and Testosterone level and inducing ovulation.

PCOS rats showed enhanced testosterone and LH levels but depleted FSH levels when compared with Normal control group. The current results were matched with some researchers [33-34] and extra confirmation of the PCOS condition. Hydro alcoholic extract of Piper longum L. and combination groups showed diminished hyperandrogenism in rats as revealed by considerable depletion in testosterone and LH levels and considerably enhanced FSH amount that leads to follicular progress as well as induction of ovulation. Our study reveals, a fall in estrogen concentration in DC rats is associated with numerous cysts development along with less corpus luteum in ovary. [35] However, after treatment of Piper longum L. results in increased FSH level indicate a advantageous consequence on ovarian function. As per previous studies, Metformin exhibited regularization in Testosterone, LH and FSH which suggest that drug enhanced ovarian parameters and induced ovulation in PCOS rats.

PCOS is mostly connected to hyperlipidemia**.** In DC set, a considerably depleted level of HDL while enhanced triglycerides, cholesterol, LDL and Glucose levels were found, against Normal control. After treatment of Piper longum L. these biochemical parameters were improved. Metformin treated set showed a considerable decrease in serum glucose level against DC. After

treatment, with Piper Longum L. and combination sets depletion in serum glucose levels was seen. Hence, this suggest that Piper Longum L. reduces glucose resistance by ameliorate insulin secretion.

After 31 days testosterone dosing glucose tolerance is significantly increased. So, Piper longum L. extract and combination sets cause insulin sensitization. PCOS treatment normalizes glucose intolerance. In treated sets normal ovarian follicles were observed along with a few cysts against disease control. Dose dependent results were observed in pathological evaluation. Combination sets showed pathological changes equivalent to intermediate dose.

CONCLUSION

The hydro alcoholic extract of fruits of Piper longum L. showed significant amelioration of Testosterone induced PCOS at 800 mg/kg dose. Additional research has to be done to confirm the precise mechanism of action, extricate to control the serum hormone levels and improvement of fertility rate.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

No conflict of interest

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