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TRIBULUS TERRESTRIS AMELIORATES PCOS PHENOTYPE BY MODULATING STEROIDOGENESIS AND FREE RADICAL LOAD IN LETROZOLE-INDUCED PCOS MICE

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ABSTRACT
Polycystic ovary syndrome (PCOS) is one of the most common endocrinological disorder affecting about 15% of reproductively active women. Prolonged usage of metformin (current treatment) has been associated with various side effects. *Tribulus terrestris* is used in folk medicine as a tonic, aphrodisiac, stomachic, anti-hypertensive and urinary disinfectant. Ethno-medically, it has been used in Ayurveda to treat impotence and sexual debility for centuries. Thus, the aim of this study was to investigate the effect of *T. terrestris* on PCOS-mice and the possible underlying mechanisms. PCOS was induced in adult (12 weeks) mice by letrozole for 21 days. PCOS-induced mice were subsequently supplemented with extract of *T. terrestris* and the effect was evaluated by analysis of ovarian histology, serum steroids concentration, oxidative stress and immunoblot analysis. Gas chromatography-mass spectroscopy (GC-MS) analysis was also performed to chemically characterize the plant extract. Supplementation of *T. terrestris* to PCOS-mice restored body mass, decreased androgen synthesis, number of cystic follicles, increased healthy antral follicles and induced ovulation. It also significantly reduced oxidative load in the PCOS-mice. Thus, *T. terrestris* restored normal ovarian activities by acting on key steroidogenic enzymes and decreasing the free radical load and can be considered as a potential therapeutic agent for PCOS in near future since it's easy to procure, cheap and has no potential side effects.

KEYWORDS: PCOS, Hyperandrogenism, *Tribulus terrestris*, Oxidative stress, Obesity.

Abbreviations
PCOS: Polycystic Ovary syndrome; HI: Hyperinsulinemia; HA: hyperandrogenism; IR: insulin resistance; FSH: follicle stimulating hormone; LH: luteinizing hormone; Tt: *Tribulus terrestris*; CMC: carboxymethylcellulose; SOD: superoxide dismutase; LPO: lipid peroxidation; TBARS: thiobarbituric acid reactive substances; ALT: Alanine transaminase; AST: aspartate transaminase; GC-MS: Gas chromatography-Mass spectrometry; JNU: Jawahar Lal Nehru University; StAR: steroidogenic acute regulatory protein; LH-R: luteinizing hormone receptor.

INTRODUCTION
Infertility due to polycystic ovary syndrome (PCOS) is a global menace. PCOS is a heterogenous disorder in women of reproductive age and accounts for up to 75% of anovulatory infertility. It can be diagnosed when two of the following three features are present in the women: 1) presence of more than 10 cystic follicles (diameter >2-8 mm) on ultrasound; 2) oligo or chronic anovulation; and 3) clinical and biochemical evidence of hyperandrogenism.[1]

Hyperandrogenism is considered as a core feature since approximately 70-80% of women with elevated androgen are diagnosed with PCOS.[2] It is also the main abnormality behind most clinical manifestations and is linked to abnormal follicular development and anovulation.[3] Insulin resistance (IR) followed by compensatory hyperinsulinemia (HI) is regarded as the second most important factor responsible for development of HA and other PCOS-like features.[4] Insulin, through its receptor, is shown to be responsible for inducing hyperandrogenism, formation of cystic follicles and development of anovulation. It is also suggested as one of the key factors contributing to oxidative stress.[5] Recent studies have shown disruption of the pathway that regulates synthesis and release of FSH which consequently affects ovarian functions crucial in development of PCOS. Given the pivotal role of hyperandrogenism, insulin resistance, obesity,

oxidative stress, and abnormal FSH in the pathogenesis of PCOS, these features may be considered as essential therapeutic targets.

Because the primary cause of PCOS is uncertain, treatment is mainly directed towards the symptoms of the disorder. Currently, reversing HA and IR or inducing ovulation in women with PCOS constitute the fundamental approach towards its management. Conventional drugs such as insulin sensitizers (metformin), anti-androgens (spironolactone), and ovulation inducers (clomiphene citrate) often induce ovulation and pregnancy in women,[6] but effective treatment to manage PCOS is still a challenge. Although these conventional drugs are effective, treatment is associated with considerable cost and is various side effects such as irregular menstruation, gastrointestinal disturbances, weight gain, and increased insulin resistance.[7] In this context, role of many medicinal plants such as *Panax ginseng, Curcuma longa, Gymnema sylvestre, Poliomintha longiflora* etc., has been extensively investigated and some of these have shown very promising results.[8]

Tribulus terrestris has been used since ancient times in traditional folk medicine, (both Indian and Chinese) as an aphrodisiac as well as to treat urinary infections, inflammation, oedema and various kinds of reproductive disorders.[9] It contains many compounds such as alkaloids, flavonoids oil, saponins, resins and nitrates[10] and can be used without significant side effects within the safe range "250-750 mg/day".[11] In traditional Chinese medicine and Ayurveda, it has been used as a herb to improve fertility in both men and women. In an earlier study, the treatment of *T. terrestris* was shown to induce ovulation in anovulatory infertile women by stimulating secretion of FSH and estradiol.[12] It is also an ovarian stimulant and can assist in the normalization of the menstrual and ovulatory cycles, making it a suitable therapeutic choice for women with PCOS.[13]

However, the mechanism by which it improves fertility in PCOS is completely lacking. Hence, the aim of the present study was to comprehensively examine the effect of *T. terrestris* fruit extract in regulating the reproductive features and oxidative stress in PCOS-mice to provide an alternative option for its treatment. Further, an attempt was also made to study phytochemical constituents of *T. terrestris* extract by GC-MS and find constituent compounds which may have potential to improve its core features.

2. MATERIAL AND METHODS

2.1. Animals

All the experiments conducted in the present study were approved by the Institutional Animal Ethical Committee of Banaras Hindu University, Varanasi, India (1802/GO/Re/S/15/CPC5EA). Healthy Swiss strain female mice (*Mus musculus*) weighing approximately 20-25 gm were maintained under standard housing conditions of light and temperature (22-24°C and 12h/12h light dark schedule) with feed and water *ad libitum*.

2.2. Induction of PCOS

PCOS was induced in adult mice (12 weeks) by letrozole as previously reported by Pandey *et al.*[14] Letrozole {6mg/kg bw dissolved in 0.5% aqueous solution of carboxymethylcellulose (CMC)} was given orally (by a canulla/oral gavage) to adult mice for 21 days. Control mice received vehicle (0.5% CMC) for the same duration.

2.3. Plant procurement and authentication Procedure

Fruits of *T. terrestris* were purchased from the local market of Varanasi and got identified by Prof. N.K. Dubey, Department of Botany, Banaras Hindu University, Varanasi, India (Voucher No. Zygo-2013-1).

2.4. Crude Extract Preparation

Crude Extract was prepared as previously reported by Kumar and Singh,[15] and stored in a desiccator at 4°C.

2.5. *In vivo* study

Mice were divided in three groups (n=6 each) (a) Vehicle treated-control (0.5% CMC); (b) PCOS and (c) PCOS-mice supplemented with *T. terrestris* (500mg/kg bw). PCOS was induced as described above (2.2). PCOS-mice were further supplemented with fruit extract of *T. terrestris*, orally, for 25 days. During the supplementation period, control and PCOS-mice were given the vehicle only. A fourth group was also included to carry out a comparative study between PCOS mice treated with *T. terrestris* (our drug of choice) and metformin (standard drug for treatment of PCOS). The dose and duration of metformin treatment was in accordance to Elia et al.[16] After completion of the treatment, mice were sacrificed by decapitation under a mild dose of anaesthesia. One ovary of each mouse was cleaned of the adhered fat, weighed and fixed in Bouin's fixative for 18 hours at room temperature, dehydrated in graded alcohol, cleared in xylene and embedded in paraffin wax. Sections were cut at 6µm and processed further for hematoxylin and eosin staining. Ovary of other side was stored at -20°C for immunoblot.

2.6. Hormone assay: testosterone (T), estradiol (E2) and progesterone (P4)

Serum steroids were measured using Diametra (Italy) ELISA kits for testosterone (DKO002) and estradiol (DKO003) and LDN kit for progesterone (FR E-2500). The assays were performed as per manufacturer's instructions.

2.7. Immunoblot

For immunoblot study, ovaries were pooled and homogenized to produce a 10% w/v homogenate. Briefly, pooled ovaries were homogenized in suspension buffer (0.01 M Tris pH 7.6, 0.001 M EDTA pH = 8.0, 0.1 M NaCl, 100 µg/ml PMSF) to produce 10% (w/v)

homogenate. Further extraction of protein and immunoblotting was performed in accordance to Singh and Krishna,[17] Equal amount of proteins (40 µg) as determined by Bradford method was loaded on to 10% SDS–PAGE for electrophoresis. Proteins were then transferred electrophoretically to a polyvinylidene fluoride mem- brane (Millipore India Pvt. Ltd.) overnight at 4 °C. Thereafter, the membranes were blocked with Tris-buffered saline (TBS; Tris 50 mM (pH 7.5), NaCl 150 mM, 0.02% Tween 20) containing 5% fat-free dry milk for 90 minutes and incubated with the desired primary antibodies for 3 hours at room temperature. Membranes were then washed thrice with PBS for 10 min each. Immunoreactive bands were detected by incubating the membranes with horseradish peroxidase tagged secondary antibody (at a dilution of 1:4000) for 120 minutes. Finally, the blot was washed three times with PBS and developed with enhanced chemiluminescence (ECL) detection system (BioRad, USA). Blot for each protein was repeated thrice. The densitometric analysis of the blots was performed by quantifying density of the bands by a computer assisted image analysis (Image J 1.38, NIH, USA). The densitometric data was presented as mean of the integrated relative density value ± SEM.

2.8. Activity of antioxidant enzymes and lipid peroxidation

Superoxide dismutase was estimated according to Das *et al.*[18] Briefly, 100 µl of sample mixed with 1.4 ml of reaction cocktail (1100 µl PBS, 80 µl L- Methionine, 80 µl HAC, 100 µl EDTA, 40 µl Triton-X) was incubated at room temperature for 10 min. 50 µl of PBS was added in blank instead of sample. 80 µl of 100 µM riboflavin was added to the desired tubes and incubated in CFL light in a SOD chamber. 1ml of Greiss reagent (1% naphthylethylenediamine + 1% sulphanilamide in 5% orthophosphoric acid, ratio 1:1) was added to stop the reaction. Absorbance was recorded against blank at 543 nm and expressed as unit/mg protein.

Catalase activity in the ovary was estimated according to Aebi[19] with minor modifications. In brief, 5µl of ethanol was added to 500µl of sample and allowed to stand for 30 minutes in an ice chamber. This mixture (450 µl) was dispensed in a tube containing 50µl of 10% Triton-X-100 and this preparation was used for catalase assay. To the above prepared sample (100 µl), 2.8ml of phosphate buffer was added. Optical density was measured at 240 nm and determined in terms of rate of H_2O_2 decomposition. The catalase activity was also expressed as unit/mg protein.

The product of LPO was determined by thiobarbituric acid reactive substances (TBARS) as described by Ohkawa et al.[20] In brief, 10% tissue homogenate was prepared in PBS (pH 7.4) and a reaction mixture was prepared by adding 200 µl of supernatant, 200 µl of BHT (butylated hydroxyl toluene), 100 µl of SDS, 1.5 µl acetic acid, 1.5 µl aqueous TBA, 200 µl of distilled water. The mixture was then heated at 95°C in waterbath for 60 min, cooled and centrifuged at 2000 rpm for 10 min. Absorbance of the supernatant was taken at 532 nm against blank and the results were expressed as nmoL/mg protein.

2.9. Estimation of ALT and AST levels in serum

Levels of ALT and AST enzymes in the serum were determined by commercial kits (Beacon LS-1795). Initial absorbance was recorded at 340nm and repeated every minute for a total of three minutes. Mean absorbance change per minute (ΔA/min) was calculated and expressed in U/L for both the enzymes.

2.10. Phytochemical Analysis

Absolute ethanolic extract was subjected to GC-MS for complete phytochemical analysis. Phytochemical analysis was done at AIRF, JNU, New Delhi, India as earlier described by Patel *et al.*[21] The Retention Time of all the obtained peaks was noted and the library provided was scanned for the compound type, molecular weight, common names and complete comparative analysis.

2.11. Fertility Test

For fertility test, six mice from each of the three groups (control, PCOS and PCOS mice-treated with *T.* terrestris) were housed with single male mice of proven fertility in separate cages. Females were checked every morning for the presence of vaginal plug (an indication of mating). Pregnant females were further allowed to deliver at term, and the litter number was recorded.

2.12. Statistical Analysis

Data is expressed as mean ± S.E.M. (n=6) and was analyzed using one way ANOVA followed by Duncan test using SPSS software (SPSS 16, Chicago, IL, USA). The data was considered significant if $p < 0.05$.

3. RESULTS

3.1. Effect of *in vivo* treatment of ethanolic fruit extract of *T. terrestris* on body and ovarian mass and accumulation of White Adipose Tissue (WAT)

PCOS-mice showed a significant ($p<0.05$) increase in the body mass and ovarian mass as compared with the vehicle-treated control mice. This increase in body mass of PCOS mice might be due to increased accumulation of adipose tissue in its abdominal region (Fig. 1b) The PCOS-mice supplemented with *T. terrestris* showed a marked decline in body and ovarian mass. Depletion of accumulated adipose tissue in abdominal region was also noted in *T. terrestris* supplemented PCOS-mice (Fig. 1c).

3.2. Effect of *in vivo* treatment of ethanolic fruit extract of *T. terrestris* on ovarian histology and follicular development

The ovaries of control mice showed the presence of numerous small and large antral follicles together with many healthy corpus luteum (Fig. 2A & 2D). The ovaries of PCOS-mice showed numerous large cystic and atretic follicles with a complete absence of corpus luteum (Fig.

2B & 2E). The cystic follicles contained only a thin layer of granulosa cells and cumulus cells incompletely surrounded oocytes with a large antral cavity. The ovaries of PCOS-mice treated with extract of *T. terrestris* showed presence of some healthy antral follicles with multi-layered granulosa cells, a few cystic follicles and newly formed corpus luteum (Fig. 2C & 2F). The average diameter of large antral follicles was also reduced in PCOS-mice supplemented with *T. terrestris* (Fig. 3). Variation in number of antral follicles, cystic follicles and corpus luteum are shown in Table 1.

3.3. Effect of *in vivo* treatment of ethanolic fruit extract of *T. terrestris* on circulating steroid concentrations and ovarian expression of steroidogenic markers (StAR, 3β-HSD, 17β-HSD, aromatase and LH-R)

Circulating T, E2 and P4 concentrations in control, PCOS-mice and PCOS-mice supplemented with *T. terrestris* are shown in Fig 4. The PCOS-mice showed significant increase in circulating T concentration, but a marked ($p<0.05$) decline in E2 and P4 concentrations as compared with the control mice. The PCOS-mice supplemented with *T. terrestris* showed significant ($p<0.05$) decrease in T level and increase in E2 level as compared with the PCOS-mice treated with the vehicle only.

Changes in the expression of LH-R, aromatase and ovarian steroidogenic markers (StAR, 3β-HSD, 17β-HSD) in control, PCOS-mice and PCOS-mice supplemented with *T. terrestris* were determined by western blot analyses followed by densitometry and results are shown in Fig. 5a and 5b respectively. The expression of LH-R protein increased significantly in the ovaries of PCOS-mice as compared to the control, but its expression increased further in the ovaries of PCOS-mice supplemented with *T. terrestris.* Unlike the other steroidogenic markers, the expression of aromatase protein decreased significantly ($p<0.05$) in the ovary of PCOS-mice as compared to the control, but increased significantly ($p<0.05$) in the ovaries of PCOS-mice upon supplementation with the extract of *T. terrestris*. The ovaries of PCOS-mice also showed significant ($p<0.05$) increase in the expression of StAR, 3β-HSD and 17β-HSD proteins as compared to the control. These steroidogenic markers decreased significantly ($p<0.05$) in the ovaries of PCOS-mice supplemented with *T. terrestris*.

3.4. Effect of *in vivo* treatment of *T. terrestris* extract on ovarian antioxidant enzyme activity and LPO product level

The activities of SOD and catalase were increased significantly in the ovaries of PCOS-mice as compared with the control. By contrast, the activities of these enzymes decreased significantly ($p<0.05$) in the ovaries of PCOS-mice supplemented with *T. terrestris* (Fig. 6A and 6B). In comparison to control, level of TBARS (LPO product) was significantly higher in PCOS-mice which decreased significantly in PCOS-mice supplemented with *T. terrestris* (Fig.7).

3.5. Effect of *in vivo* treatment of *T. terrestris* extract on ALT and AST enzyme levels

There was a significant ($p<0.05$) increase in serum AST in PCOS mice as compared with the control which declined marginally in PCOS-mice supplemented with *T. terrestris* (Figure 8A). Serum ALT was also found to be significantly higher in the PCOS mice which declined significantly in PCOS-mice supplemented with *T. terrestris* (Figure 8B). However, *T. terrestris* supplementation did not cause any increase in ALT and AST enzymes.

3.6. Fertility test

All letrozole treated PCOS-mice exhibited complete infertility since no pups were delivered at completion of term. However, PCOS-mice supplemented with *T. terrestris* showed partial resumption of fertility as one out of six PCOS treated with *T. terrestris* showed pregnancy for full term with a sex ratio of 1:1. Although, three PCOS mice treated with *T. terrestris* showed presence of corpus luteum in ovarian histology indicating ovulation. Details are given in Table 2.

3.7. Phytochemical analysis of *T. terrestris*

A total of 49 compounds were obtained by the GC-MS analysis of ethanolic extract of *T. terrestris.* (Fig.9 and Table 3). Amongst the listed components, β-Sitosterol, Vitamin E and phytol were considered as important compounds of interest.

3.8. Comparative study on effect of *T. terrestris* supplementation and metformin treatment to the PCOS mice

A study was carried out to compare the effect of *T. terrestris* and metformin (standard drug for PCOS) and the results are presented in Table 4.

Table 1: Variation in number of follicles on control mice, PCOS-mice and PCOS-mice supplemented with *T. terrestris*. Values are represented as mean ± S.E.M. *Values are significantly ($p<0.05$) different in comparison to control. * indicate significant variation as compared with PCOS mice.

Parameters	Control	PCOS	PCOS + *Tribulus terrestris*
Cystic Follicles	0	$12\pm0.21^{*}$	$2.2\pm0.12^{*a}$
Corpus Luteum	1.2±0.17	0^{*}	$2.21\pm0.08^{*a}$
Antral Follicles	3.4±0.21	0^{*}	$2.4\pm0.11^{*a}$

Table 2: Effect of *T. terrestris* supplementation on presence or absence of corpus luteum and fertility restoration in control, PCOS-mice and PCOS-mice supplemented with *T. terrestris*. Data is represented as Mean ± SEM.

Groups	Number of females/group	Vaginal plug	Number of females pregnant	Litter number	Corpus luteum
Control	6	+	5	8.0±0.38	+
PCOS	6	_	0	0	_
PCOS + *T. terrestris*	6	+	1	4	+

+,present; -,absent

Table 3: Phytocomponents identified in extract of *Tribulus terrestris*.

Peak	Retention Time	Area%	Analyte
1	9.253	1.85	Cyclopentane-1, 2-diol
2	10.054	1.29	1H-Benzocycloheptene,
3	10.924	0.21	1-tetradecanol
4	11.775	0.21	1,2,3-propanetricarboxylic acid, 2-hydroxy-,
5	12.045	0.24	benzene, 1,2,4-trimethoxy-5-(1-propenyl)-, (z)-
6	12.333	0.10	boric acid (h3bo3), tris(1-methylethyl) ester
7	12.381	0.13	5a-methyl-3,8-dimethylene-2-oxododecahydr
8	13.177	0.70	1h-indene, 2,3,3a,4,7,7a-hexahydro-2,2,4,4,7,7-hexamethyl-
9	13.696	0.33	2,6,10-trimethyl,14-ethylene-14-pentadecne
10	13.786	0.44	tetradecanoic acid, trimethylsilyl ester
11	14.148	0.16	2,6,10-trimethyl,14-ethylene-14-pentadecne
12	14.304	0.23	nonadecane
13	14.584	0.53	Hexadecanoic acid, methyl ester
14	14.800	0.33	n-Pentadecanoic acid, trimethylsilyl ester
15	15.017	8.90	pentadecanoic acid
16	15.258	4.08	hexadecanoic acid, ethyl ester
17	15.773	10.98	Hexadecanoic acid, trimethylsilyl ester
18	16.260	1.85	9,12-octadecadienoic acid (z,z)-, methyl ester
19	16.306	0.39	9-Octadecenoic acid (Z)-, methyl ester
20	16.444	0.33	Phytol
21	16.730	22.99	9,12-octadecadienoic acid (z,z)-
22	16.885	16.22	ethyl (9z,12z)-9,12-octadecadienoate
23	17.134	1.13	octadecanoic acid, ethyl ester
24	17.342	11.44	linolsaeure, trimethylsilylester
25	17.582	1.48	Octadecanoic acid, trimethylsilyl ester
26	17.737	0.34	ethyl (9z,12z)-9,12-octadecadienoate
27	18.158	0.98	pentacosane
28	18.987	0.30	ethyl 9-hexadecenoate
29	19.323	0.39	pentacosane
30	20.176	0.36	undecanal
31	20.488	0.39	4-cyanobenzoic acid, undec-10-enyl ester
32	20.746	1.23	Pentacosane
33	22.024	1.55	Ethyl 13-docosenoate (ethyl erucate)
34	22.488	0.30	2,7,7-trimethyl-4-(4-methylsulfanyl-phenyl)-5
35	22.813	0.49	cis-13-Docosenoic acid, trimethylsilyl ester
36	23.438	0.63	Cyclopropane, 1,1-dichloro-2,2,3,3-tetramethyl-
37	24.036	1.11	Pentacosane
38	24.628	1.01	9-Octadecenoic acid
39	24.958	0.52	9-octadecenoic acid, 2.3.-dihydroxypropyl ester
40	25.313	0.39	2-methyltetracosane
41	25.778	0.29	Squalene
42	26.683	0.66	TETRACONTANE
43	30.147	0.27	TETRACONTANE
44	30.530	0.29	Stigmast-5-en-3-ol, oleate
45	31.223	0.51	Vitamin E
46	34.522	0.43	Chondrillasterol
47	35.394	0.23	Stigmasterol trimethylsilyl ether
48	36.260	0.53	Stigmast-5-en-3-ol, (3.beta.)/ β-sitosterol
49	37.207	037	beta-Sitosterol trimethylsilyl ether

Table 4: Comparative study on effect of *T. terrestris* (drug of our choice) and metformin (standard drug for PCOS) supplementation on reproductive phenotype of PCOS-mice. Data is represented as mean ± S.E.M. *Values are significantly (p<0.05) different in comparison to control. * indicate significant variation as compared with PCOS mice.

Groups	Control	PCOS	PCOS + *T. terrestris*	PCOS +metformin
Body weight(g)	27.87 ± 1.23	33.65 ± 0.98*	29.72 ± 1.62*a	22.76 ± 1.70*a
Ovary weight (mg)	6.89 ± 0.87	7.98 ± 0.76*	7.01 ± 1.08*a	6.98 ± 1.91*a
Serum testosterone (ng/ml)	0.1 ± 0.07	1.27 ± 0.3*	0.17 ± 0.12*a	0.15 ± 0.61*a
Serum estradiol (pg/ml)	75.23 ± 1.43	58.61 ± 1.70*	68.92 ± 1.50*a	69.91 ± 1.80*a
Serum progesterone (ng/ml)	68.81 ± 1.87	41.27± 0.91*	43.29 ± 2.10	44.12± 1.71
Number of cystic follicles	0	12 ± 0.21*	2.2 ± 0.12 *a	2.7 ± 0.98*a
Number of Antral follicles	3.4 ± 0.21	0*	2.4 ± 0.11*a	1.98 ± 0.60*a

Figure Legends

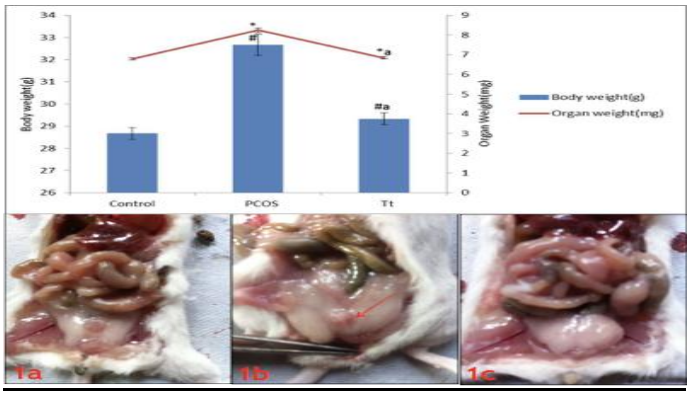

Figure 1: Changes in body mass, ovarian mass and WAT accumulation in control mice, PCOS mice and PCOS mice supplemented with *T. terrestris* (Tt). Values are represented as mean ± S.E.M., n=6. *#Values differ significantly (p<0.05) as compared to control. *#a indicate significant variation as compared with PCOS mice. (1a) abdominal adipose tissue mass in control mice. (1b) Increased abdominal adipose tissue deposition in PCOS-mice. (1c) Depleted abdominal adipose tissue in PCOS-mice supplemented with *T. terrestris*. Arrow indicates deposited adipose tissue mass.

Figure 2.

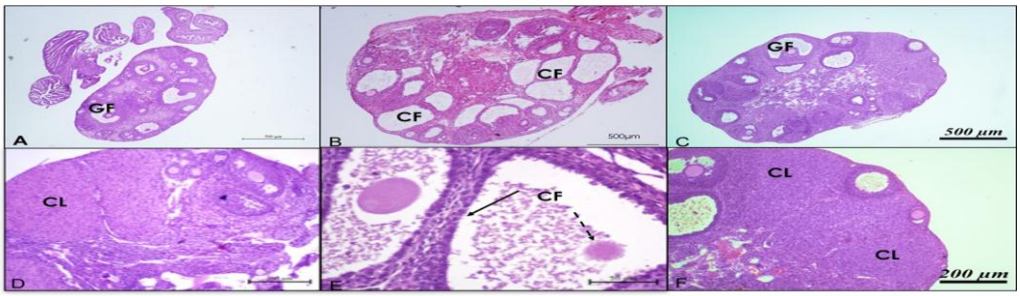

Figure 2: Histological changes in the ovary of control mice, PCOS-mice and PCOS- mice supplemented with *T. terrestris.* (A & D) Section of control ovary showing corpora lutea and healthy antral follicles at various stages of development. (B & E) Section of PCOS ovary showing numerous fluid filled cystic follicles (CF) and a complete absence of corps luteum. (C & F) Section of PCOS-ovary supplemented with *Tribulus terrestris* showing corpus luteum(CL), healthy antral follicles(AF), Graafian follicles(GF) and decrease in number of cystic follicles.

Note: Reattainment of corpus luteum in *T. terrestris* supplemented mice.

Arrow indicates thinning of granulosa cell layers. Broken arrow indicates degenerated oocyte.

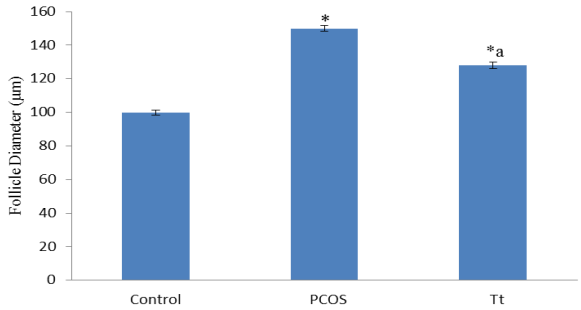

Figure 3: **Variation in follicle diameter in control mice, PCOS-mice and PCOS-mice supplemented with *T. terrestris* (Tt). Values are represented as mean ± S.E.M. *Values are significantly (p<0.05) different in comparison to control. *a indicate significant variation as compared with PCOS mice.**

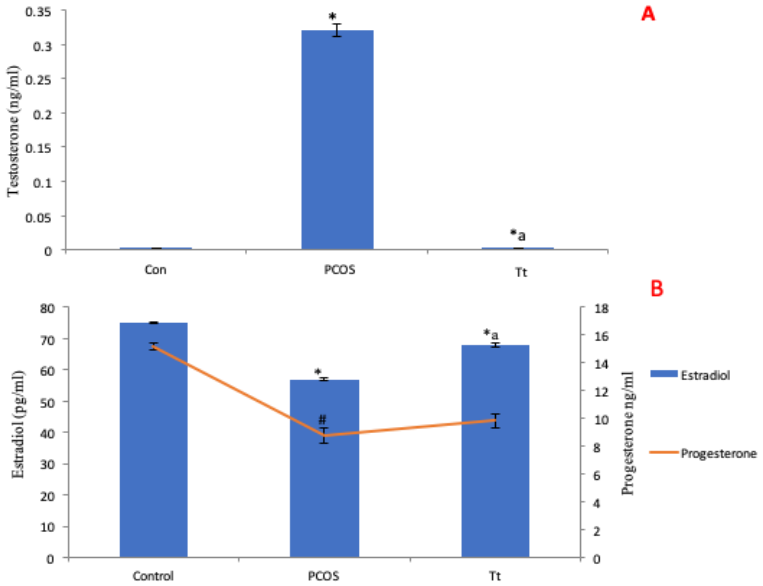

Figure 4: **Variation in serum testosterone (A), estrogen and progesterone (B) production by the ovaries of control, PCOS and *T. terrestris* (Tt) supplemented mice. Values are represented as ± S.E.M. *#Values are significantly (p<0.05) different versus control. *#a denote significant variation as compared with PCOS mice.**

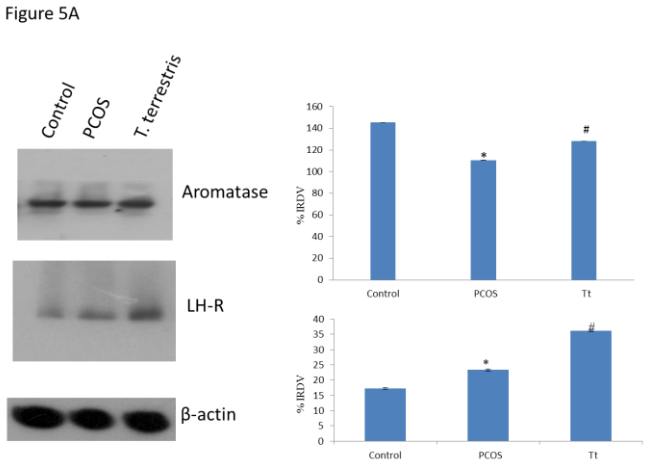

Figure 5a: Immunoblot analyses of aromatase and LH-R proteins in the ovary of control, PCOS-mice, PCOS-mice supplemented with *T. terrestris*. Values are represented as mean ± S.E.M. *Values of band intensity differ significantly (p<0.05) as compared to the control. # indicate significant variation as compared with PCOS mice. Bar diagram represents densitometric analysis of the immunoblots. IRDV= Integrated relative density value.

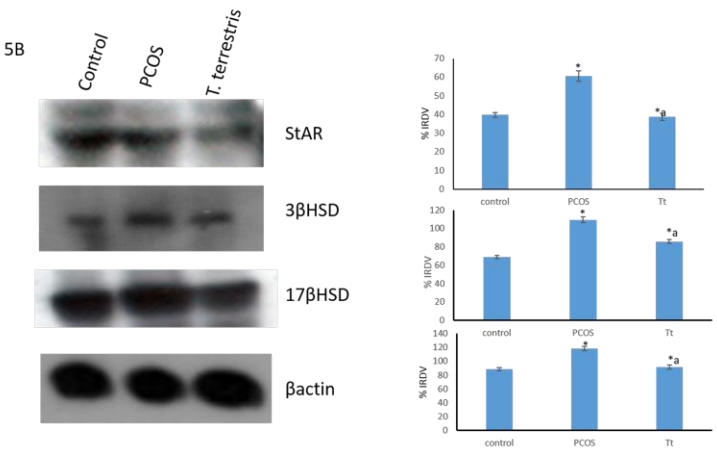

Figure 5b: Immunoblot analyses of StAR, 3 β-HSD and 17 β-HSD proteins in the ovary of control, PCOS-mice, PCOS-mice supplemented with *T. terrestris*. Values are represented as mean ± S.E.M. *Values of band intensity differ significantly (p<0.05) as compared to the control. # indicate significant variation as compared with PCOS mice. Bar diagram represents densitometric analysis of the immunoblots. IRDV= Integrated relative density value.

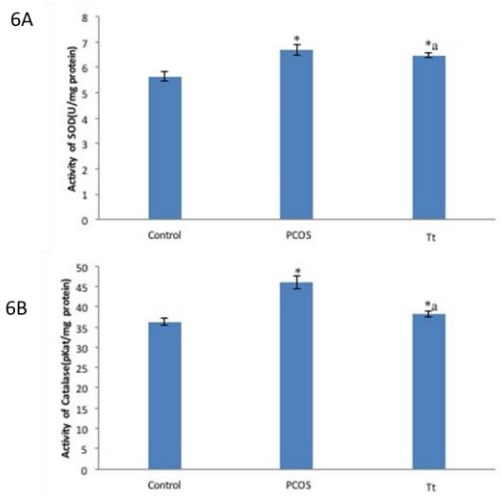

Figure 6: Ovarian activity of superoxide dismutase (6A) and catalase (6B) of control mice, PCOS-mice and PCOS-mice supplemented with *T. terrestris* (Tt). Values are represented as mean ± S.E.M. *Values are significantly (p<0.05) increased in comparison to control. * denote significant variation as compared with PCOS mice.

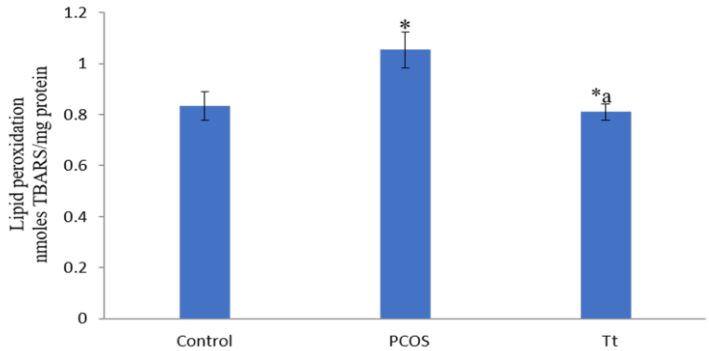

Figure 7: Variation in level of lipid peroxidation (LPO) product of control mice, PCOS-mice and PCOS-mice supplemented with *T. terrestris* (Tt). Values are represented as mean ± S.E.M. *Values are significantly (p<0.05) increased in comparison to control. *a indicate significant variation as compared with PCOS mice.

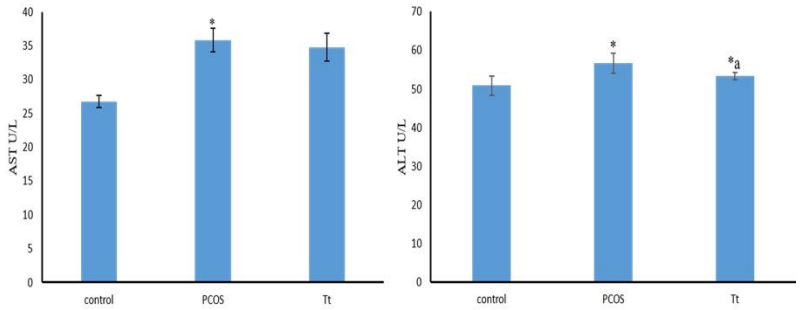

Figure 8A & 8B: Effect of letrozole treatment and *T. terrestris*(Tt) supplementation on serum aspartate transaminase (AST) and serum alanine transaminase (ALT) levels in serum. Values are represented as mean ± S.E.M. *Values are significantly ($p<0.05$) increased in comparison to control. *a indicate significant variation as compared with PCOS mice.

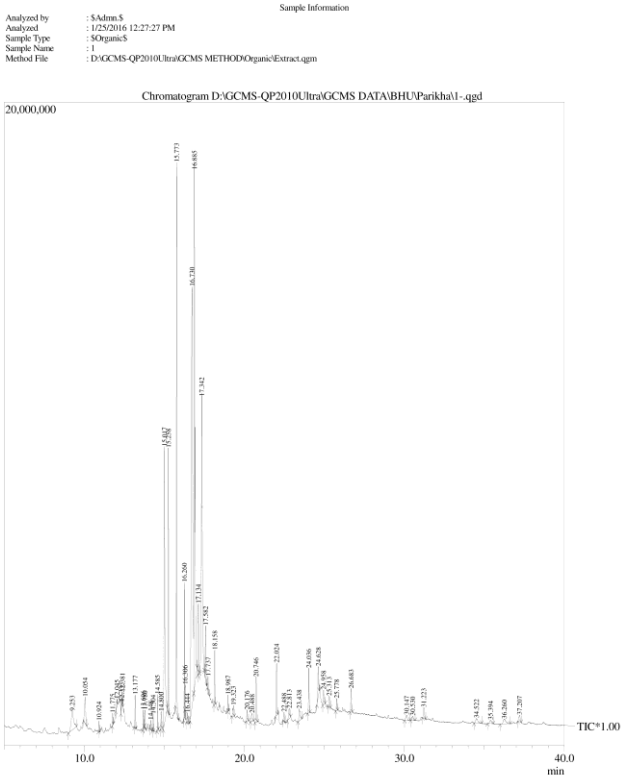

Figure 9: A gas chromatography mass spectrometry (GC-MS) chromatograph of *Tribulus terrestris.*

DISCUSSION

The primary aim of this study was to examine the efficacy of the fruit extract of *Tribulus terrestris* in regulating PCOS-like symptoms in letrozole induced PCOS-mice. The letrozole-treated mice developed several PCOS-like features such as gain in body mass, presence of numerous cystic follicles, anovulation, increased level of testosterone and alterations in oxidative stress markers, as shown previously in letrozole-treated rats.[14] Previous reports suggest that letrozole-induced PCOS condition depicts human PCOS.[22] This study showed that treatment of PCOS-mice with *T. terrestris* extract reversed PCOS-like features and induced ovulation. About sixteen percent of fertility restoration was observed in PCOS-mice when supplemented with *T. terrestris*, suggesting a partial resumption in fertility.

The letrozole-treated PCOS-mice showed a significant increase in the body mass which is attributed to

accumulation of white adipose tissue in the abdominal region. PCOS-mice showed a significant increase in the body mass coinciding with a recent report that the rats treated with letrozole showed increase in the body mass.[14] Obesity plays an important role in elevated oxidative stress, which further contributes to insulin resistance.[23] The important finding of this study is that the PCOS-mice supplemented with *T. terrestris* resulted in significant decline in accumulation of white adipose tissue. This feature of *T. terrestris* might be attributed to abundant presence of flavonoids. Flavonoid mediated improvement in insulin resistance and glucose tolerance has been earlier reported by Song et al.[24]

In the present study, PCOS-mice showed presence of numerous cystic follicles with large antral cavity, similar to ovarian histology of PCOS women.[25] The PCOS-mice supplemented with *T. terrestris* extract demonstrated a sharp reduction in number of cystic follicles. The antral follicles in *T. terrestris* supplemented PCOS-mice exhibited morphologically normal features. Some of these antral follicles ovulated in response to treatment with extract of *T. terrestris* as indicated by presence of corpus luteum in the treated ovary. This is consistent with the earlier studies in which the PCOS-rats treated with *T. terrestris* revealed significant decrease in cystic follicles and resumption of ovulation.[12,26] The phytosterols of *T. terrestris* could be responsible for alteration in steroidogenesis consequently leading to ovulation.[26]

Androgen excess has been implicated in the development of abnormal follicular development and anovulation in women with PCOS.[3] In this study, the PCOS-mice supplemented with *T. terrestris* showed normalized serum testosterone and estradiol levels. Serum estradiol was significantly increased by administration of *T. terrestris*, confirming its earlier reported phyto-estrogenic activity. Significant decrease in aromatase enzyme level in the ovary of PCOS-mice as compared to the control in the present study further confirmed that the inhibition of aromatase enzyme activity results in the accumulation of androgen. This study showed a significant decline in circulating progesterone level in the PCOS mice as compared to the control mice which might be due to complete lack of corpus luteum in the ovary of the PCOS mice. A positive correlation between circulating testosterone levels and ovarian expression of steroidogenic markers StAR, 3β-HSD and 17β-HSD in the ovaries of control mice, PCOS-mice and PCOS-mice supplemented with *T. terrestris* was also noted. Administration of *T. terrestris* extract caused reduction in testosterone production by suppressing expression of the steroidogenic marker proteins (StAR, 3β-HSD and 17β-HSD). 3β-HSD and 17β-HSD are important enzymes in steroidogenic pathways and play a vital role in biosynthesis and conversion of androgens. Decrease in circulating testosterone in the treated PCOS mice elucidate modulation of these steroidogenic enzymes by *T. terrestris*.

A marked alteration in oxidant-anti-oxidant profile has been reported in women with PCOS.[27] Increased oxidative stress in PCOS-mice was evident by increased level TBARS in the ovary. PCOS-mice supplemented with *T. terrestris* showed significant decline in oxidative stress markers. *T. terrestris* contains saponins which possess hypoglycemic properties and also inhibit oxidative stress.[28] Phytosterols such as β-sitosterol have also been determined as potential antioxidant agents. Although results of studies about antioxidant levels are contradictory, it is possible to infer that an imbalance between oxidants and antioxidants occurs in PCOS.[29] Serum ALT and serum AST are reliable markers of liver injury. No significant rise in the enzymes levels after supplementation with *T. terrestris* justify the notion that *T. terrestris* if used within the range is non-toxic, indicating its safe use. The GC-MS analyses of ethanolic extract of *T. terrestris* showed presence of a number of phyto compounds shown in Table 3. The extract was dominated by presence of a variety of phyto-sterols such as stigmasterol, β-sitosterol and chondrillasterol etc. In addition to plant sterols, *T. terrestris* extract was also found to be rich in antioxidants (vitamin E, phytol), flavonoids and saponins (protodioscin) which possesses antioxidant and hypoglycemic properties respectively.

CONCLUSION

This is the first exemplified study on recuperative effects of *Tribulus terrestris* on PCOS-mice. Exogenous supplementation with ethanolic extract of *T. terrestris* to PCOS-mice restored body mass, androgen synthesis and oxidant-antioxidant balance. It also caused significant reduction in number of cystic follicles and development of healthy antral follicles. Ovulation in *T. terrestris* supplemented PCOS-mice is the highlight of this study suggesting that *Tribulus terrestris* may emerge as a potential therapeutic agent for PCOS in near future. The results presented in this study clearly suggest that the effect of ethanolic extract of *T. terrestris* may be attributed to its multiple pharmacological activities such as estrogenic, insulin-sensitiser, anti-hyperlipidemic, anti-oxidative and anti-hyperandrogenic.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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