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Research Article

Anti-hyperglycaemic Activity of *Tribulus terrestris* Fruit Extract Restores Metabolic Imbalance in Letrozole Induced -PCOS Mice

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ABSTRACT

Prevalence of obesity in women with PCOS varies from 50-80% and whether obesity leads to PCOS or vice versa is still a matter of debate. Thus, the aim of this study was to evaluate the efficacy of *Tribulus terrestris* on improving metabolic abnormalities associated with PCOS. PCOS was induced in mice by oral administration of letrozole for 21 days. PCOS induced mice treated with fruit extract of *T. terrestris* for 25 days restored body mass and showed a marked improvement in histo-morphological features of the ovary, pancreas and adipose tissue by decreasing expression of prohibitin, by decreasing serum glucose, insulin and lipid concentrations and by acting on key metabolic factors (pAKT, GLUT8 and IR). These findings also showed ameliorating effect of *T. terrestris* on insulin resistance as indicated by HOMA-IR and QUICKI values in the treated PCOS mice. Thus, the extract of *T. terrestris* might be considered as a potential therapeutic choice for a large majority of women dealing with PCOS.

Keywords: PCOS, *Tribulus terrestris*, Obesity, HOMA-IR, MetS

INTRODUCTION

Polycystic ovary syndrome (PCOS) is an increasingly prevalent and multifaceted gynaecological disorder and accounts for approximately 75% of anovulatory infertility[1]. Initially, the syndrome was mainly considered as a reproductive disorder, but recent research has laid an insight on metabolical implications of the syndrome. Based on the current studies, hyperandrogenism (HA), insulin resistance (IR), obesity, hyperglycaemia, abnormal release of GnRH/LH and oxidative stress are leading abnormalities responsible for progression and pathogenesis of PCOS. Thus, PCOS is considered as an important reproductive and metabolic disorder[2]. Insulin resistance in peripheral tissues like adipose tissue, ovaries, skeletal muscle is an important facet and under rigorous investigation. Majority of PCOS patients (approximately 50-70%) are associated with insulin resistance[3] leading to an increased free circulating insulin levels. Insulin mediated increase in steroidogenic enzymes like steroidogenic acute regulatory protein (StAR), Side chain cleavage enzyme (SCC), 3β- HSD and androgen receptor (AR) is also well documented in case of PCOS. These patients also show a strong correlation between hyperandrogenism and hyperinsulinemia. Indeed, hyperinsulinemia due to insulin resistance occurs in approximately 80% of women with PCOS and central obesity, and in 30%–40% of lean women with PCOS[4]. Women with obesity most commonly display the abdominal phenotype. Abdominal obesity is reckoned to cause ovarian and adrenal hyperandrogenism[5] which in turn leads to escalated abdominal fat deposition.

The PCOS women with insulin resistance show hyperglycaemia, dyslipidaemia, type 2 diabetes mellitus, and increased evidence of inflammation[6]. Apart from hyperandrogenism, increased insulin concentration also predisposes women with obesity towards development of glucose intolerance, type 2 diabetes mellitus (T2DM), dyslipidaemia and cardiovascular abnormalities. Women with PCOS also carry an increased risk of development of non-insulin dependent diabetes mellitus (NIDDM), in conjunction to reproductive dysfunctions. Hyperinsulinemia is therefore a debatable yet a principal feature of PCOS[2].

The common treatments of PCOS are based on managing the clinical symptoms of this disease including reversing hyperandrogenism and insulin resistance or inducing ovulation in women with PCOS. Use of insulin sensitizers like metformin and rosiglitazone are known to improve hyperandrogenism and restoration of normal ovulatory cycles in addition to improving hyperinsulinemia[7]. Although, these pharmaceutical approaches showed marked improvements in PCOS-like symptoms, however such treatments are associated with substantial cost and various side effects, such as irregular menstruation, gastrointestinal disorders, weight gain, low pregnancy rates and increased insulin resistance[8]. Thus, an effective treatment to manage PCOS is still a challenge. Many women treated with metformin develop gastrointestinal side effects such as bloating, cramping, diarrhoea,

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Table 1: Details of primary antibodies used in Western blot study.

Antibody	Species raised in: Monoclonal/polyclonal	Source	Concentration used
AKT	Rabbit; Polyclonal	Genscript	1:500
pAKT	Rabbit; Polyclonal	Genscript	1:500
Insulin receptor(IR)	Rabbit; Polyclonal	Santa cruz Biotechnology Inc.	1:1000
GLUT 8	Rabbit; Polyclonal	Santa cruz Biotechnology Inc.	1:800
Prohibitin	Rabbit; Polyclonal	Genscript	1:500
β-actin	Mouse; Monoclonal	Sigma A2228, 128K4813	1:2000

Table 2: Variation in biometry and blood biochemistry control mice, PCOS-mice and PCOS-mice supplemented with fruit extract of *T. terrestris*. Values are represented as mean ± S.E.M. *Values are significantly ($p<0.05$) different in comparison to control. *a indicates inter-group variation.

Biometry and blood biochemistry	Control	PCOS	. PCOS + *T. terrestris*
Final body weight (g)	26±0.6	32±0.32*	27±0.9*a
Total visceral fat (mg)	1.1±0.9	2.8±0.5*	1.4±0.4*a
Gonadal (Peri-ovarian) fat (mg)	0.44±0.4	0.85±0.3*	0.32±0.2*a
Serum Testosterone, ng/ml	0.1±0.02	1.23±0.4*	0.18±0.2*a
Adipocyte morphometry (µm)	58±0.8	88±0.8*	69±0.8*a

flatulence and nausea[9]. Lactic acidosis is a rare but a potential lifethreatening side effect of metformin[10]. In lieu of these side effects, alternative medicine is emerging as an efficient therapeutic choice to manage PCOS as they are source of important bioactive secondary metabolites. Herbal remedies are generally low costing, gentle on body and can be used for prolonged period and with fewer side effects. Recent reports have suggested *Tribulus terrestris* as an effective female fertility tonic, and an ovarian stimulant making it a suitable therapeutic choice for women with PCOS. It is a perennial herb commonly known as gokhshuru or puncture vine distributed mainly in subtropical regions of the world including India. The fruit of the plant is generally used for medicinal purposes. In males, it enhances libido and sperm production. Recent studies showed that *T. terrestris* can be useful in improving female reproductive dysfunctions. It is effective in inducing ovulation, normalizing reproductive cyclicity and treating endocrine disorders in females[11]. Our recent study reported that *T. terrestris* has potential in improving reproductive features of PCOS. But, before *T. terrestris* can be proposed as an effective treatment option for PCOS, it is essential to comprehensively investigate its role in insulin resistance and the metabolic aspects of the syndrome. Thus, the aim of the present study was to examine the role of *T. terrestris* in regulating insulin resistance in letrozole treated PCOS mice.

MATERIAL AND METHODS

Animals and diet

Twelve-week-old Swiss female mice were used in this study. All the animals were housed in a standard light and temperature controlled (22-24°C and 12h/12h light dark) room with free access to feed and water. All the experiments carried out in the present study were approved by the institutional animal ethical committee of Banaras Hindu University, Varanasi, India. (1802/GO/Re/S/15/CPCSEA).

Induction of PCOS & Treatment

The PCOS mice model was developed with adult female mice weighing 20-25 gram using letrozole. Mice were divided into two groups: control (n=5) received an oral dose of 0.5% aqueous solution of carboxymethylcellulose (CMC) and the experimental group (n=14) received letrozole (6 mg/kg bw) orally for 21 days. PCOS was confirmed using the Rotterdam criteria, 2003. PCOS group was further divided into two groups: PCOS untreated (control/vehicle treated) and PCOS group supplemented with 500mg/kg bw ethanolic fruit extract of *Tribulus terrestris*, orally for 25 days. At the end of the treatment, mice were sacrificed by decapitation under mild dose of anaesthesia. Tissues of interest were dissected out and fixed in Bouins' fixative for 24 hours at room temperature, dehydrated in graded ethanol series, and subsequently cleared in xylene. Paraffin sections were cut at 6µm and processed for hematoxylin and eosin staining. Rest were stored at -20 °C for Western Blot studies.

Authentication procedure and crude extract preparation

Fruits of *Tribulus terrestris* were purchased from the local market of Varanasi and got identified by Prof. N.K. Dubey, Department of Botany, Banaras Hindu University, Varanasi (Voucher No. Zygo-2013-1). Extract of *Tribulus terrestris* was prepared as reported earlier by Kumar and Singh[12]. In brief, the dried powder of fruits of *T. terrestris* was extracted by maceration with 70 % ethanol (1:4 ratio) for 48 h at room temperature. The filtrates were pooled and the solvent was removed under vacuum at 45 °C with a rotary evaporator. Percentage yield of the obtained crude extract was estimated as 2.48% and the extract was stored at 4°C for further use. GC-MS and chemical characterisation of the prepared extract was performed to evaluate individual constituents present.

Serum hormone assay: testosterone, and insulin

Serum testosterone and serum insulin were measured using commercial kits from Diametra (Italy) (DCM002-10) Millipore (EZRMI-13K) respectively. The assays were performed as per manufacturer's instructions.

Oral glucose tolerance test (OGTT)

Fasting blood glucose was measured after 6 hour of fast (blood collected from the tail vein), according to the

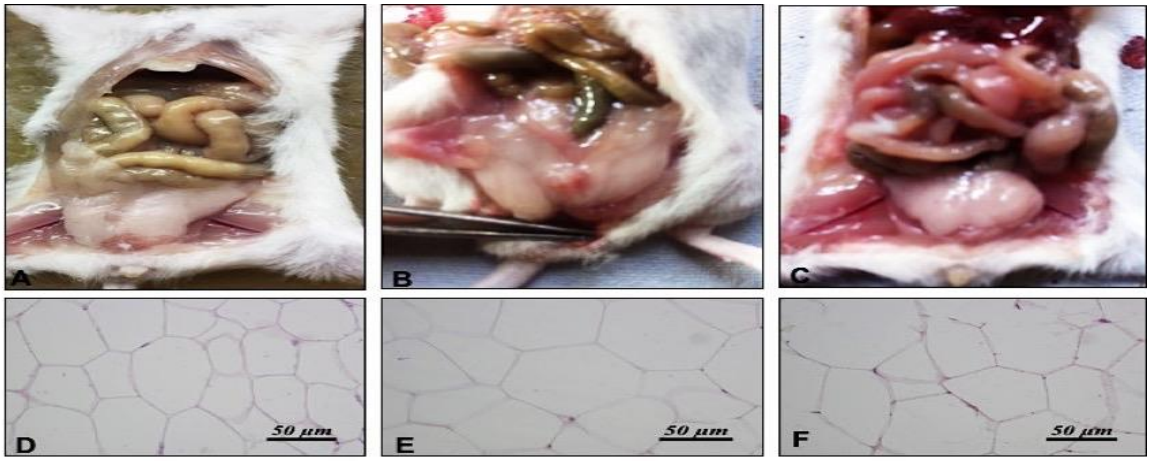

Figure 1: Abdominal ventral view of deposited fat pads in control mice, PCOS mice and PCOS mice supplemented with *T. terrestris*. (1A) abdominal adipose tissue mass in control mice. (1B) Increased abdominal adipose tissue deposition in PCOS-mice. (1C) Depleted abdominal adipose tissue in PCOS-mice supplemented with *T. terrestris*. (1D) HE stained photomicrograph of adipocytes of control mice. (1E) adipocytes of PCOS mice showing increased diameter. (1F) *T. terrestris* supplemented mice show slightly less hypertrophied adipocytes. Scale = 50µm

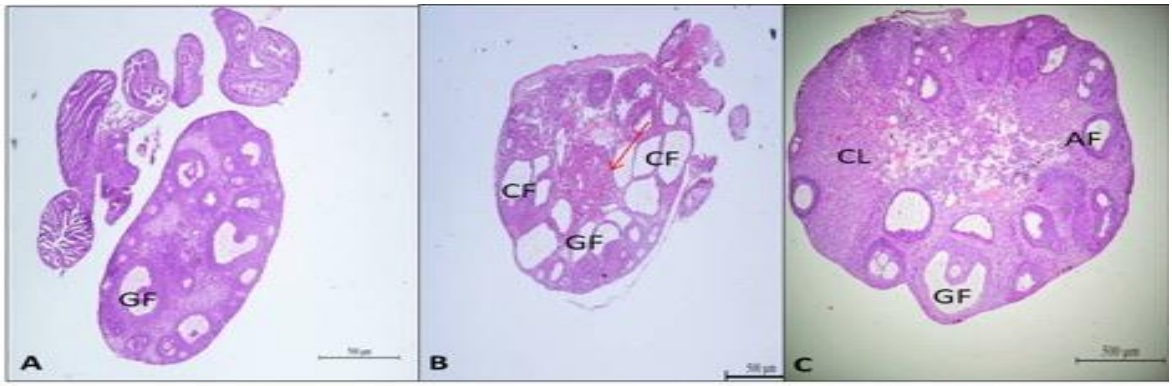

Figure 2: Changes in histo-morphology of the ovary of control mice, PCOS-mice and PCOS- mice supplemented with *T. terrestris*. (A). Section of control ovary shows healthy follicles at different stages of development. (B) Section of PCOS ovary showing numerous large fluid filled cystic follicles (CF) with a complete absence of corps luteum. (C) Section of PCOS-ovary supplemented with *Tribulus terrestris* showing corpus luteum(CL), healthy antral follicles(AF), Graafian follicle (GF) and a significant decrease in number of cystic follicles. Arrow indicates thinning of granulosa cell layers.

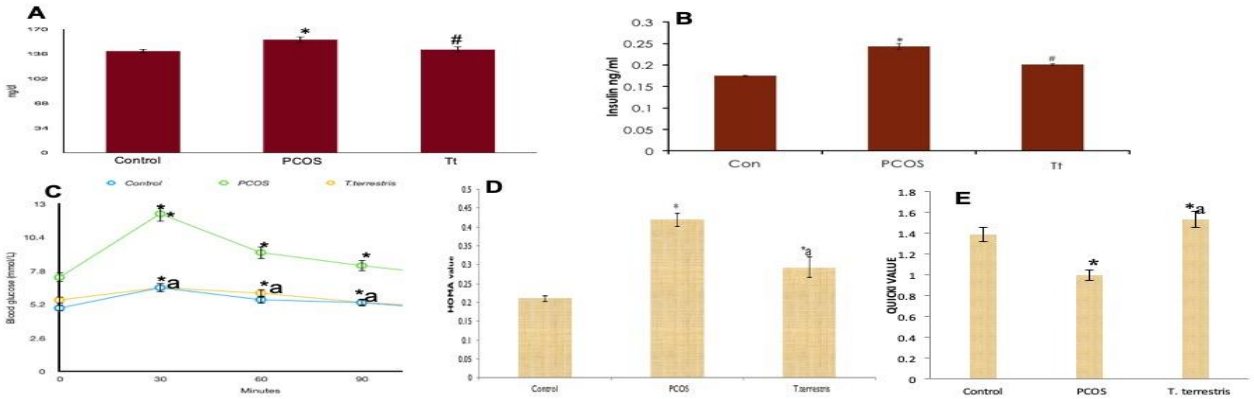

Figure 3: Changes in serum glucose (A), insulin (B), oral glucose tolerance (C), HOMA (D) and QUICKI (E) values of control mice, PCOS mice and PCOS mice supplemented with *T. terrestris* (Tt). Values are represented as ± S.E.M. $^{*\#}$Values are significantly ($p<0.05$) different versus control. $^{*\#a}$ indicate inter-group variation.

manufacturer's recommendation using (Accu-Chek glucometer). A sugar bolus (2g/kg) was then administered to the mice by an oral gavage and subsequent blood glucose was measured at time intervals of 15, 30, 60 and

120 minutes post glucose administration.

HOMA-IR and QUICKI

The HOMA-IR (homeostasis model assessment of insulin resistance) index was calculated as (fasting serum glucose * fasting serum insulin/ 22.5) to assess insulin resistance[13]. Quantitative insulin check index (QUICKI) was calculated according to the formula by Katz[14] to assess insulin sensitivity.

Biochemical Analysis

Glucose estimation was done by a commercialised kit (Autospan) as per manufacturer's instructions. Total cholesterol, HDL-c and triglycerides were assayed by commercial kits provided by Autospan. The low-density lipoprotein-cholesterol (LDL-c) was obtained via the Friedwald's formula[15].

Immunoblot

For immunoblot analyses, ovaries were pooled and homogenized to produce 10% w/v homogenate. The entire experiment was carried out in accordance to Singh and Krishna[16]. Details of all the antibodies used are provided in Table 1.

Statistical Analysis

Data is expressed as mean ± SEM. Data was analysed using one way ANOVA followed by Duncans test using SPSS software (SPSS 16, Chicago, IL, USA). The data was considered significant if $p < 0.05$.

RESULTS

Effect of in vivo supplementation of fruit extract of T. terrestris to PCOS-induced mice on changes in

Body mass, peri-ovarian adipose tissue mass and adipocyte morphometry

PCOS-mice showed a significant ($p<0.05$) increase in the body mass and peri-ovarian fat mass as compared with the vehicle-treated control mice (Table 2). The increase in body weight was due to an increase in deposition of adipose tissue in the abdominal region (Figure 1b). The PCOS-mice supplemented with extract of *T. terrestris* showed restoration in body mass, peri-ovarian adipose tissue mass (Table 2) and also signs of depleted fat deposition (Figure 1c).

Ovarian and adipocyte histology

Induction of PCOS-like features was confirmed upon observing histological changes in the ovaries of PCOS mice. PCOS-mice treated with the extract of *T. terrestris* showed presence of some healthy antral follicles with multi-layered granulosa cells, few cystic follicles and newly formed corpus luteum (Fig. 2C) indicating stimulatory effect of the extract on ovarian follicular development and ovulation. Adipocytes of the PCOS mice showed a marked increase in the diameter (indicating hypertrophy) which decreased significantly in mice supplemented with extract of *T. terrestris* (Figure 1).

Serum testosterone, glucose and insulin level

The PCOS mice showed a significant increase in serum glucose (Figure 3A) and insulin (Figure 3B) levels as compared to control. PCOS mice supplemented with extract of *T. terrestris* showed a significant decline in both glucose and insulin level as compared to the untreated PCOS mice. Serum testosterone was also significantly increased in the PCOS mice as compared to the control which then decreased significantly in mice supplemented with *T. terrestris* (Table 2).

Oral glucose tolerance test(OGTT)

In the OGTT, the plasma glucose level increased gradually to attain a maximum level 15 min after the oral administration of glucose in all the three groups, but this maximum was highest in the PCOS group as compared with control and the supplemented group ($p<0.0001$). The time-course of glucose clearance in PCOS mice was significantly delayed compared to control and *T. terrestris* supplemented mice, remaining elevated for 120 min ($p<0.001$) after glucose administration, thus qualifying for characterization as glucose intolerance (Figure 3C).

HOMA-IR and QUICKI

Both, fasting glucose and insulin were increased in the PCOS mice as compared to the control. The HOMA-IR value was also higher in the PCOS mice as compared to the control which then decreased significantly in *T. terrestris* supplemented mice (Fig. 3D). QUICKI values were decreased significantly in the PCOS mice which then increased significantly in *T. terrestris* supplemented mice (Fig. 3E).

Serum lipid profile

A significant increase in serum cholesterol (Figure 4A), triglyceride (Figure 4B) and LDL-c level (Figure 4D) was observed in the PCOS mice which decreased significantly in *T. terrestris* supplemented mice. HDL-c was found to be decreased significantly in the PCOS mice which increased significantly in *T. terrestris* supplemented mice (Figure 4C).

Ovarian expression of IR, GLUT8, pAKT and prohibitin

Densitometric analysis of Western blots exhibited a significant decrease in ovarian expression of GLUT-8, pAKT and insulin receptor in the ovaries of PCOS-mice as compared to the control Ovarian expression of GLUT-8,pAKT and IR protein then increased significantly in mice supplemented with extract of *T. terrestris*. Ovarian expression of prohibitin also increased significantly in the PCOS mice which then decreased significantly in the *T. terrestris* supplemented mice (Figure 5).

DISCUSSION

A majority of women with PCOS are treated with metformin and thiazolidinedione, both of which efficiently improve insulin resistance and enhance chances of ovulation simultaneously. However, side effects associated with these drugs motivated us to focus our present investigation on medicinal plants. Thus, the present study was carried out to elucidate the significance of fruit extract of *Tribulus terrestris* in improving the metabolic dysfunctions, particularly insulin resistance in the PCOS mice.

Similar to the insulin sensitising agents, supplementation of extract of *T. terrestris* also reversed the features of insulin resistance in the PCOS mice. The mechanism by which *T. terrestris* improves insulin resistance is not clearly explicated. The letrozole induced PCOS mice showed a significant increase in body mass due to an

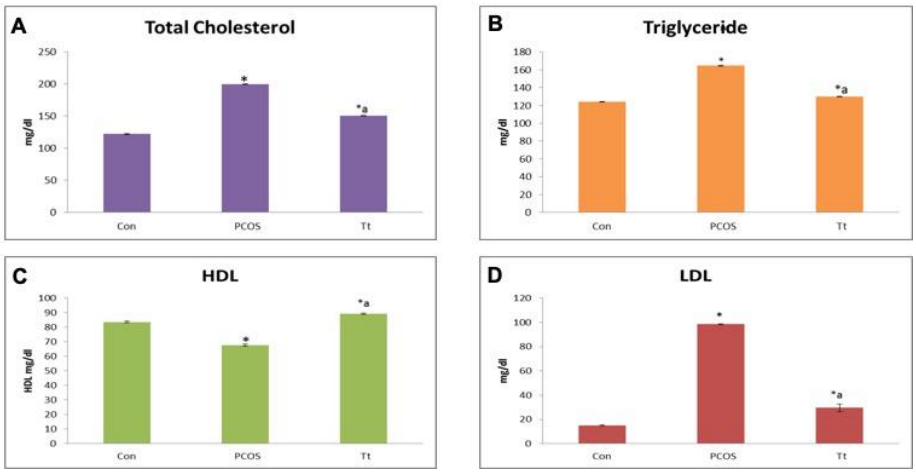

Figure 4: Variation in serum cholesterol (A), triglycerides (B), HDL-c (C) and LDL-c in values of control mice, PCOS mice and PCOS mice supplemented with *T. terrestris* (Tt). Values are represented as ± S.E.M. *#Values are significantly ($p<0.05$) different versus control. *#a indicate inter-group variation.

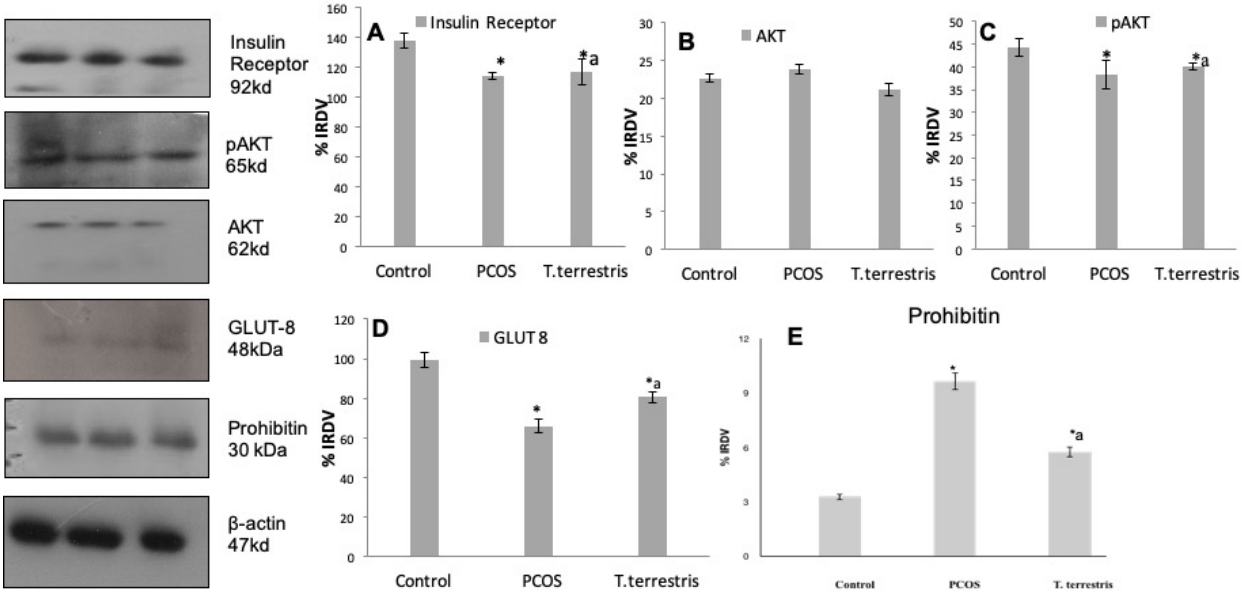

Figure 5: Immunoblot analyses of Insulin receptor(IR), AKT, pAKT, prohibitin and GLUT-8 in the ovary of control, PCOS-mice and PCOS-mice supplemented with *T. terrestris*. Values are represented as mean ± S.E.M. *Values of band intensity differ significantly ($p<0.05$) as compared to the control. #Values denote intergroup variation. Bar diagram represents densitometric analysis of the immunoblots. IRDV= Integrated relative density value.

increased accumulation of White Adipose tissue (WAT) as compared to the control mice which then decreased significantly in mice supplemented with *T. terrestris.* This decrease in body mass in *T. terrestris* supplemented mice is due to decrease in white adipose tissue deposition in the abdominal region. A significant increase in the gonadal fat (peri-ovarian) was also found in the PCOS mice which decreased significantly in PCOS-mice supplemented with *T. terrestris*. It has earlier been reported that an increase in accumulation of fat in the abdominal area might induce adipocyte dysfunction[17] and insulin resistance like state[18]. It has also been shown that an increase in adiposity with adipocyte hypertrophy results in an altered level of synthesis and secretion of adipokines[19].

It is earlier reported that letrozole-induced PCOS mice resemble many metabolic features of human PCOS like an increase in body weight, body fat[20], elevated glucose, cholesterol and triglycerides levels[21]. In consistence with a recent study in the rat, our study also showed PCOS-like features in mice treated with letrozole such as an increase in body mass, development of numerous cystic follicles, increased androgen synthesis, and anovulation[22]. The letrozole induced PCOS mice also showed metabolic abnormalities such as insulin resistance, hyperinsulinemia, glucose intolerance, hyperlipidaemia etc.

In the present study, supplementation of fruit extract of *T. terrestris* effectively improved insulin sensitivity and other PCOS-like features. Insulin resistance associated hyperinsulinemia may be a promoter of increased androgen synthesis leading to development of cystic follicles and anovulation. The letrozole treated PCOS mice showed significant increase in circulating concentrations

of insulin (hyperinsulinemia) and glucose (hyperglycaemia) as compared with the vehicle treated control. Hyperinsulinemia is considered to be a promoter of hyperandrogenism and anovulation by supressing SHBG and up-regulating ovarian LH-receptors[23]. Inhibition of aromatase enzyme by letrozole might be responsible for an increase in ~~total~~ serum testosterone in the PCOS mice. This increased testosterone concentration in addition with increased adipose tissue deposition creates a vicious cycle which might play an important role in sustainability of PCOS[24]. Supplementation of *T. terrestris* extract to the PCOS mice resulted in an improved hormonal profile and decreased adipose tissue deposition.

HOMA-IR is a non-invasive method of predicting insulin resistance in mice[25]. PCOS-mice displayed an elevated HOMA-IR index which is compatible with the presence of insulin resistance in these animals. Decrease in HOMA-IR value in *T. terrestris* supplemented PCOS mice provide an insight on insulin sensitising action of the plant. Similar to HOMA-IR, QUICKI is also a non-invasive mathematical model for assessing insulin sensitivity. Further, decreased QUICKI values in PCOS-mice provide an insight on decreased insulin sensitivity which was later enhanced in *T. terrestris* supplemented- PCOS mice.

In this study, ethanolic extract of *T. terrestris* improved PCOS-like features because of its inherent hypoglycaemic activity. Hypoglycaemic activity of *T. terrestris* is attributed to presence of saponins. The hypoglycaemic effect of *T. terrestris* found in this study is in concurrence with earlier reports[26]. It has ~~been~~ earlier been reported that *T. terrestris* regulates glucose homeostasis by influencing genes associated with insulin sensitivity and glucose uptake. Women with PCOS and insulin resistance underlie abnormalities of lipid metabolism[27]. In consistence with the study, the present study also showed significant increase in circulating concentrations of total cholesterol, triglycerides, and LDL-c, but decreased level of HDL in PCOS mice as compared to the control mice. It was earlier suggested that insulin resistance associated hyperinsulinemia may be responsible for dyslipidaemia in PCOS[28,29]. In the present study, administration of *T. terrestris* decreased cholesterol synthesis, which clearly showed benefit to the PCOS mice as it improves lipid profile in total. Lipid profile improving and serum lowering effect of *T. terrestris* on cholesterol, LDL and triglycerides is also in consistence with earlier studies[30]. Since, cholesterol is a substrate for ovarian steroidogenesis, increased availability of cholesterol might be the reason behind increased production of androgen in PCOS.

This study also showed a significant decrease in expression of insulin receptor (as observed in insulin resistant state) which co-related significantly with decreased expression of GLUT-8 and glucose concentration in the ovary of the PCOS mice. This finding suggested that inadequate uptake of glucose by the ovary due to an insulin resistant state may be responsible for abnormal follicular development and consequently anovulation in the PCOS mice since it is well established that a sufficient level of glucose is essential of proper ovarian function. In a recent study, relatively high concentration of glucose was reported in healthy follicles as compared to atretic follicles, with highest level obtained in pre-ovulatory follicles. It is also well documented that signalling molecule AKT undergoes changes during insulin resistant conditions and other metabolic disorders. This study also showed significantly decreased concentration of phosphorylated AKT pointing to impaired intracellular insulin signalling in the ovary of the PCOS mice. The decreased pAKT causes decreased expression of GLUT-8 and consequently decreased uptake of glucose by the ovary in the PCOS-mice which was later restored upon *T. terrestris* supplementation. These observations thus suggest that insulin resistance associated impaired insulin signalling lead to decreased uptake of glucose in the ovary, which in turn may be responsible for abnormal follicular development and anovulation.

A recent study demonstrated the potential involvement of prohibitin in the etiology of PCOS[31]. Similar to which, this study also showed a significant increased expression of prohibitin in the ovary of PCOS mice as compared with the control. Till now, prohibitin has been known to regulate important ovarian activities such as apoptosis, proliferation, differentiation and steroidogenesis[32]. However, scanty reports on its involvement in metabolic pathway and disorders are available. Since, its expression is reported to increase in PCOS rat with chronic hyperandrogenism, this suggests that up-regulation of prohibitin may be associated with etiology of PCOS. Interestingly, PCOS mice treated with extract of *T. terrestris* showed a significant decrease in the ovarian expression of prohibitin. This is also the first study to report such finding. Thus, this study also confirms the potential importance of prohibitin in PCOS which is less explored.

In conclusion, exogenous supplementation of ethanolic extract of *T. terrestris* to PCOS-mice restored body mass, serum glucose profile and its metabolism. It probably increases insulin release from the pancreas, thus exerting its anti-diabetic effect. Decreased HOMA-IR values in *T. terrestris*-supplemented mice resulted in improved glucose profile and insulin sensitivity. Hence, *T. terrestris* can be considered as a good therapeutic choice for women with PCOS also showing signs of metabolic syndrome and those facing side effects of metformin.

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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ABBREVIATIONS

PCOS: Polycystic Ovary syndrome; HI: Hyperinsulinemia; SHBG: serum hormone binding globulin; HA: hyperandrogenism; IR: insulin resistance;

FSH: follicle stimulating hormone; LH: luteinizing hormone; Tt: *Tribulus terrestris*; CMC: carboxy methyl cellulose; WAT: white adipose tissue; HOMA-IR: Homeostatic Model Assessment of Insulin resistance; QUICKI: Quantitative insulin check index; MetS: metabolic syndrome;

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