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***Research Article** (Open Access)*

EFFECT OF *TERMINALIA CHEBULA* RETZ. ON THE MANAGEMENT OF PANCREATIC IMPAIRMENTS AND INSULIN RESISTANCE IN LETROZOLE-INDUCED PCOS RAT MODEL

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ABSTRACT
PCOS is a common endocrine disorder which is defined by the presence of polycystic ovaries, chronic anovulation, and clinical or biochemical hyperandrogenism. While hyperandrogenism was once considered as the primary feature of PCOS, recent evidence suggests that anomalies in insulin actions or insulin signalling pathways have a crucial role in the development of this syndrome. Deficiency in PCOS-related glucose-stimulated insulin secretion has been associated with an increased risk of developing diabetes mellitus, even in the absence of obesity. And so, most of the women affected with PCOS is known to experience reduced glucose tolerance and are at a higher risk of developing T2DM. Therefore, this study focused on examining alterations in insulin secretion and glucose metabolism in pancreatic tissue of the experimental animals. Therefore, this study aims to explore the effectiveness of *T. chebula* fruit extract in combating insulin resistance by improving the anatomical and physiological structure of the pancreatic β-cells in the islets of Langerhans and restoring the normal blood glucose levels. The mRNA expression profile of genes related to steroidogenesis (CYP17A1), insulin regulation and glucose metabolism (INSR, PPAR-γ), and oxidative stress induction (PI3K) in letrozole-induced PCOS animals were also examined to further confirm their influence in the pathophysiology of PCOS.

Keywords: *PCOS, Terminalia chebula* Retz*, Insulin Resistance, T2DM, Pancreatic β-cells*

INTRODUCTION

PCOS is a prevalent endocrine disorder characterised by polycystic ovaries, chronic anovulation, clinical or biochemical hyperandrogenism. Where hyperandrogenism has historically been thought to be the predominant feature of PCOS, there is growing evidence over the last few decades determine abnormalities in insulin actions or insulin signalling pathways as a key for the development of this syndrome (Lascar *et al*., 2019). Moreover, a deficiency in PCOS-related glucose-stimulated insulin secretion has been associated with a higher risk of developing diabetes mellitus, even in the absence of obesity (Rodgers *et al*., 2019). Depending on variables including weight and family history, the prevalence of type 2 diabetes mellitus (T2DM) and impaired glucose tolerance (IGT) in PCOS patients ranges from 4–10% and 23–35% respectively (Rubin *et al.*, 2017; Long *et al*., 2022) and also the corresponding prevalence in thin PCOS patients are 10-15% and 1-2% (Moghetti and Tosi 2013). Most PCOS women have reduced glucose tolerance and possess greater risk of developing type 2 diabetes mellitus (T2DM) (Lakhani *et al*., 2004; Orio *et al*., 2016). Recent clinical evidence indicates that insulin-sensitizing medications, such as combined oral contraceptive pills, metformin, antiandrogens, medication can improve the insulin resistance indices and lowers fasting glucose levels (Anwar and Shikalgar, 2017). According to a recent meta-analysis of randomised controlled trials (RCTs), combined metformin-antiandrogen is known to be more efficient in treating PCOS when compared to antiandrogenic therapy alone (Ortiz-Flores *et al*., 2018). Since up to 70–80% of women with PCOS show clinical signs of hyperandrogenism, understanding the role of insulin resistance or insulin actions which is identified as the significant cause of hyperandrogenemia in PCOS women is becoming more and more crucial. After evaluating a number of therapeutic plants, researchers determined that one of the most highly regarded medicinal plants is *Terminalia chebula (T. chebula)*

Retz. (Combretaceae) which is known by several names, including "King of medicine," "Black myrobalan," "Ink tree," or "Chebulic myrobalan" (Poudel *et al*., 2023). Since ancient times this plant demonstrated a number of therapeutic properties like antioxidant and free radical scavenging, hepatoprotective, cardioprotective, hypolipidemic, hypocholesterolemic, cytoprotective, antidiabetic, reno protective, anti-inflammatory, and anticarcinogenic due to the presence of many phytochemicals, including polyphenols, terpenes, anthocyanins, flavonoids, alkaloids, and glycosides fruit of *T.chebula* has been used as a traditional home medicine for a variety of human ailments due to its many health benefits (Bag *et al*., 2013). On considering these pharmaceutical benefits, this study aims to explore the effectiveness of *T. chebula* in in mitigating the consequences of type 2 diabetic mellitus state in letrozole induced PCOS rat.

MATERIALS AND METHODS

Fruit extract preparation

The fruits of *Terminalia chebula* Retz. were procured from Top Sengattupatti, Thuraiyur (Tk), Tiruchirappalli (Dt), Tamilnadu - 621011. A twig of *T. chebula* tree and the collected fruits were used for identification and verification at the Botanical Survey of India, Coimbatore, Tamil Nadu, India (Certified No. BSI/SRC/5/23/2022/Tech/678). The collected Terminalia chebula Retz. fruits were shade-dried and minced into fine powder using an electronic mixer. About 500 mL of ethanol and 50 g fruit powder was used for the fruit extract preparation through soxhlet extraction. After that the extract was filtered and kept in rotating evaporator for vapourization at 75^{o}C reduced pressure. The extract was stored at 4^{o}C for until further studies (Vignesh *et al*., 2023)**.**

Animals

Female albino Wistar rats (*Rattus norvrgicus*) of about 150 -180 g of were procured from the Veterinary and Animal Science College in Mannuthy, Kerala for this study. This work was ethically approved by CPCSEA – Animal ethical committee (Approval No: IAEC/BDU/P 25/2018/Dt.07.08.2018). The animals were feed ad libitum with standard laboratory feed purchased from Sai Durga Feeds and Foods Chennai and maintained in polypropylene cages with a 12h light: 12h dark cycle under regulated environment (25 °C).

Study design

Thirty female rats were used for the investigation, and five animals each were placed in each of the six experimental groups. Group I was the control group; they were not given any medical attention. As the negative control, Group II (L) was administered letrozole alone. Oral Metformin, a conventional PCOS medication, was administered to Group III (L+Met) at a dose of 20 mg/kg/day following PCOS induction. Oral administration of the ethanolic fruit extract from *T. chebula* at doses of 100 mg/kg.bw, 200 mg/kg.bw, and 400 mg/kg.bw was given to the remaining three experimental groups (Groups IV through VI) for a period of 28 days following PCOS induction.

PCOS Induction

Letrozole, an aromatase inhibitory drug obtained from Sigma Aldrich, was given to all experimental groups, with the exception of the control group, for 21 days at a dose of 1 mg/kg BW by dissolving in 0.5% carboxymethyl cellulose (CMC) (Kafali *et al*., 2004).

Oral Glucose Tolerance Test (OGTT)

The OGTT was performed in all experimental animals after a 12-hour fasting interval previous to the commencement of the experiment, after effective induction of PCOS, and after treatment with metformin and *T. chebula*. The blood samples were taken through tail vein puncture and the fasting rats' basal glucose levels were assessed with a glucometer and recorded as a 0-minute measurement. Following an overnight fast, glucose (2 g/kg) was orally administered to all experimental rats at a concentration of 200 mg/kg BW, and blood samples were collected at 30, 60, 90, and 120-minute intervals to measure glucose levels in the blood using the glucometer (Control D, HAIDEN technology Pvt, New Delhi, India) with appropriate strips (Rakic *et al.*, 2023).

Sample collection and preparation

After the experimental period, the experimental animals were sacrificed using through excessive CO_2 inhalation and the pancreas was removed weighed and stored -20^{o}C until further studies.

Antioxidant evaluation

The antioxidant level of pancreatic tissue was analysed by adopting the standard protocols as follows: Superoxide dismutase (SOD) (Marklund and Marklund, 1974), reduced glutathione (GSH) (Moron *et al.*, 1979), Glutathione peroxidase (GPX) (Kreheľová *et al*., 2021), Catalase (CAT) (Sinha, 1972), Lipid peroxidation (MDA) (Ohkawa *et al.*, 1979).

Histology

Pancreatic tissues removed from the experimental animals were preserved in formalin (10%), and then dried using different doses of xylene and ethyl alcohol. Thin sections of pancreatic tissue samples at 5 nm thickness were cut using a rotary microtome (Leica, USA), placed on a glass slide, and dried. Then the slides were deparaffinized, rehydrated, and stained with H&E (Himedia, USA), The pathology of the histological sections was examined using a light microscope (Magnus, India).

mRNA expression studies

Total RNA was isolated from the pancreatic tissue of the experimental groups through Trizol reagent (Himedia, India) and the mRNA concentration was measured at 260/280 nm using a Multiskan skyhigh Microplate Spectrophotometer (ThermoFisher Scientific, USA). Then the isolated RNA was reverse-transcribed for cDNA conversion using the Invitrogen Super Script III First-Strand Synthesis System. Using the primer sequence specified in Table 1, the relative expressions of CYP17A1, PPAR-γ, PI3K, and INSR were examined in quantitative PCR (Roche, USA). These expressions were normalised to β -actin.

Table 1: Oligonucleotide sequence of specified primers for RT-PCR

Gene name	Forward primer	Reverse primer
INSR	CAGTTTGTGGAACGGTGCTG	TGGTAGGGTCATCGGGTTCT
PPAR- γ	CATGTGCAGCCAAGACTCTG	AAAGCTGTCTGTGTCCAGGT
PI3K	GGGAGCCCCAGAAAAGCAGA	AGTTCTCCAGCTCCATGCCC
CYP17A1	AGGCTAACGTTGACTCCAGC	AGCTCCGAAGGGCAAGTAAC
β-Actin	GCGAGTACAACCTTCTTGCAG	CATACCCACCATCACACCCTG

Statistical Analysis

The software GraphPad Prism version 9.5.1 was used to analyse the data, and the results were shown as Mean ± SEM. The student "t" test was used to determine the statistical significances.

RESULTS

Oral Glucose Tolerance Test (OGTT)

In the OGTT (Figure 1), the initial glucose level of all the experimental animals were significantly lower. This level got raised rapidly after administration of glucose and then steadily dropped over time. After 21 days of the successful induction of PCOS through letrozole administration, the OGTT results showed elevated blood glucose levels that persisted for 120 minutes after glucose administration. This indicated that the glucose metabolism in the animals was affected through PCOS induction and further confirmed the onset of diabetes in the PCOS-induced animals, leading to the development of type 2 diabetes mellitus (T2DM). However, the OGTT assessment done at the end of the experiment following the administration of metformin and *T. chebula* fruit extract at varied concentrations showed an improvement in glucose metabolism that eventually returned to the normal range over time.

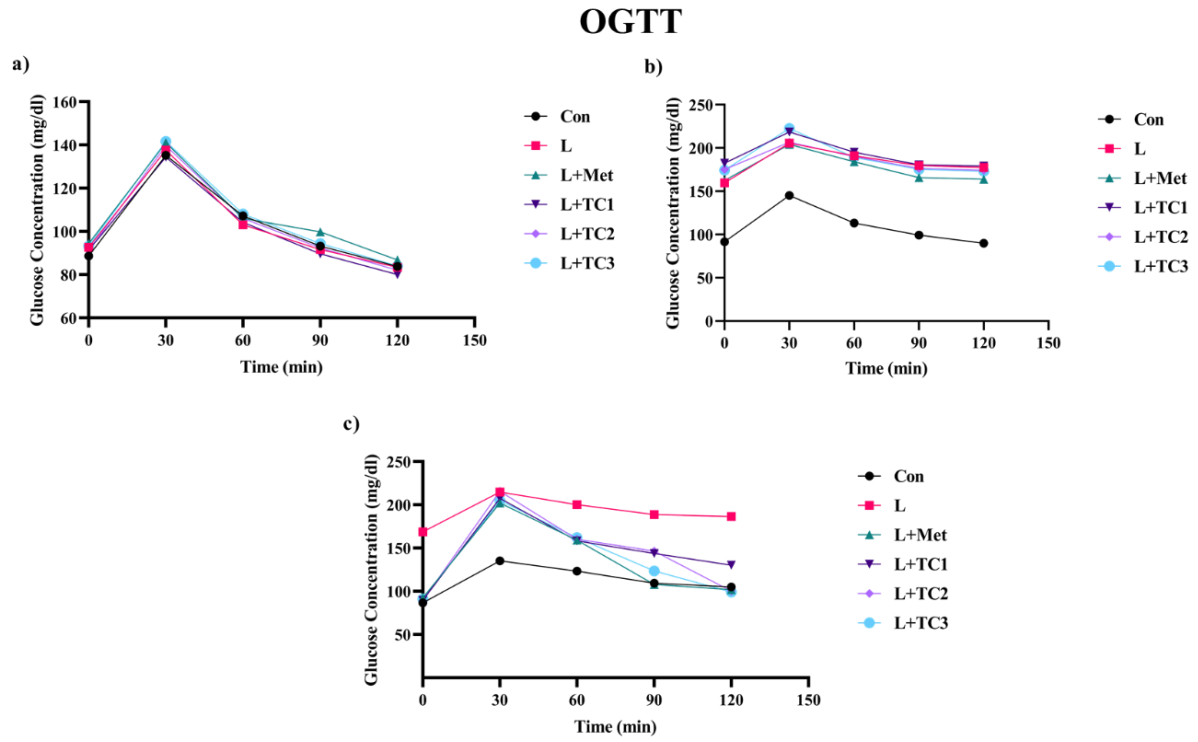

Figure 1: Line graph represents the OGTT among the experimental animals. a) OGTT values taken before PCOS induction; b) after PCOS induction; c) OGTT values of PCOS animals after treated with metformin and *T. chebula* fruit extract.

Pancreas weight

The animals which were induced for PCOS condition and without any treatment had significant decrease in the weight of pancreas compared to the control, metformin and *T. chebula* treated groups (Figure 2).

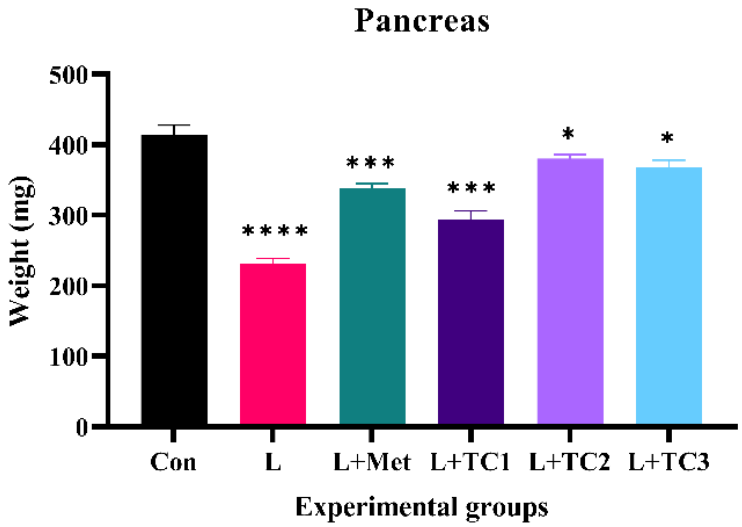

Figure 2. The bar graphs representing the weight of Pancreatic tissue of the experimental groups. the values are given in mean ± SEM, n = 5, *p < 0.05; **p < 0.01; ***p < 0.001; ns – nonsignificant.

Histopathology

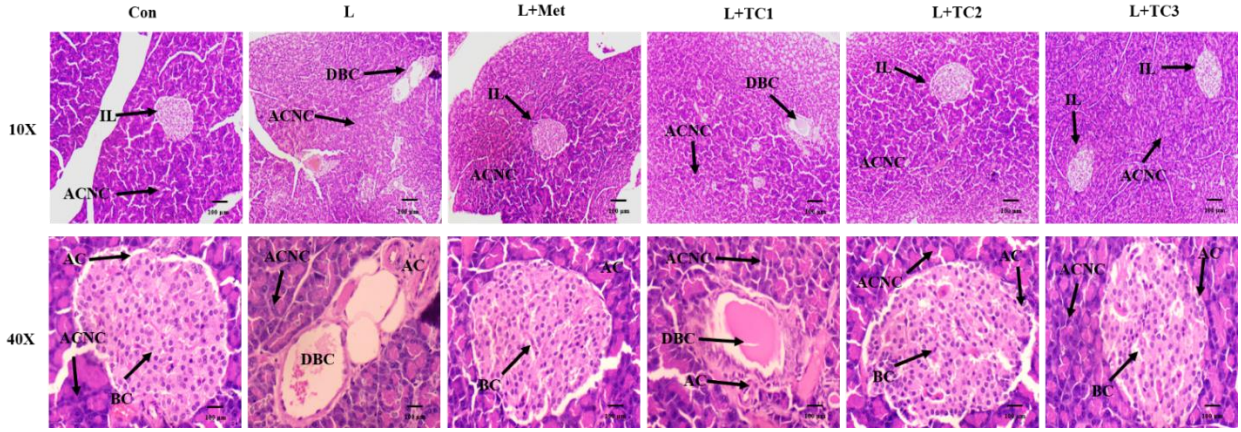

Figure 3. Hematoxylin-Eosin stained paraffin section of Pancreatic tissue, IL (the islets of Langerhans), ACNC (Acinar cells), DBC (Destroyed β cells), BC(β-cells), AC(α-cells).

The control group's (Con) pancreas, as shown a typical histology of both the pancreatic acini and pancreatic islets of Langerhans. The islets were composed of an extensive mantle of α- and δ-endocrine cells surrounding a focal core of β-cells. The untreated letrozole-induced PCOS group(L) has the considerable decrease in β-cell count in their pancreatic islets with α- and δ-endocrine cells as the more prevalent cell types. Additionally, the destruction in the pancreatic β-cells of the islets and significant dilatation of the pancreatic ducts accompanied by papillary hyperplasia of the epithelial lining were also evidenced in the sections of their pancreatic tissue. But when the PCOS induced rats were administered with metformin (L+Met) and the *T. chebula* fruit extract (L+TC1, TC2 and TC3), the β-cells count in the islets of Langerhans were significantly improved in dose dependent manner (Figure 3).

Anti-oxidant assay

Superoxide dismutase

The pancreatic tissue of the untreated PCOS rats had significantly reduced levels of SOD in comparison to the control group. This reduced level was found to be slightly elevated when the PCOS rats were administered with 200 and 400 mg/kg.bw of *T. chebula* **(Table 2**).

Reduced glutathione

Comparing the levels of GSH in the pancreatic tissues of the PCOS group experimental animals to those of the other experimental groups, a very modest decrease in GSH level was seen, which is typically suggestive of less significant changes in the neutralisation of GSH free radical **(Table 2**).

Glutathione peroxidase

In the experimental groups, there was a greater association between the levels of GSH and GPx. And so, GPx level in the letrozole induced PCOS rats was also declined when compared to the other experimental groups. This reduced level got raised up upon treatment with metformin and varied concentration of *T. chebula* fruit extract **(Table 2**).

Catalase

In comparison to the control and *T. chebula* treated groups, the rats in the letrozole-induced PCOS group had significantly lower levels of catalase activity in their pancreatic tissues. This suggests that the animals with PCOS may have a compromised antioxidant defence system **(Table 2**).

Lipid peroxidation

The levels of LPO in the pancreatic tissues were considerably greater in the PCOS group compared to the control group, which had enhanced MDA activity, confirming the belief that PCOS is related with hyperlipidaemia (Table 2). This increase in lipid peroxidation free radical production was restored back to normal when the experimental animals were given medium and high doses (200 & 400 mg/kg.bw of the *T. chebula* fruit extract, depending on the concentration of the supplied dosage **(Table 2**).

Table 2: Anti-oxidant assay of pancreatic tissue

Experimental Groups	SOD (U/mg protein)	GSH (U/mg protein)	GPx (U/mg protein)	CAT (U/mg protein)	LPx (µ mol/mg protein)
Con	14.2 ±0.72	7.9 ±1.47	95±8.88	65±13.22	21±3.60
L	4.5 ±0.62 ****	2.07±0.80**	48.5±8.32**	30.4±6.91*	38.4±9.05*
L+Met	10.7 ±1.12 *	5.67±0.41 ns	79±13.89 ns	57±4.58 ns	28.4±6.60 ns
L+TC1	6.6 ±1.44 **	3.50±0.70**	60±15.62*	38±10*	35.7±4.96*
L+TC2	12.53 ±1.74 ns	7.44±0.60 ns	92.7±13.82 ns	69±11 ns	22.7±2.04 ns
L+TC3	13.13 ±2.57 ns	6.98±1.08 ns	86±4.58 ns	61.7±8.54 ns	25.4±3.85 ns

Table 2. ROS enzyme levels in the pancreatic tissue of experimental groups. SOD – Superoxide dismutase; GSH – Reduced glutathione; GPx – Glutathione peroxidase, CAT - Catalase, and LPx – Lipid peroxidase. Values are represented as mean ± SEM, n = 3, *p < 0.05; **p < 0.01; ***p < 0.001; ns – not significant.

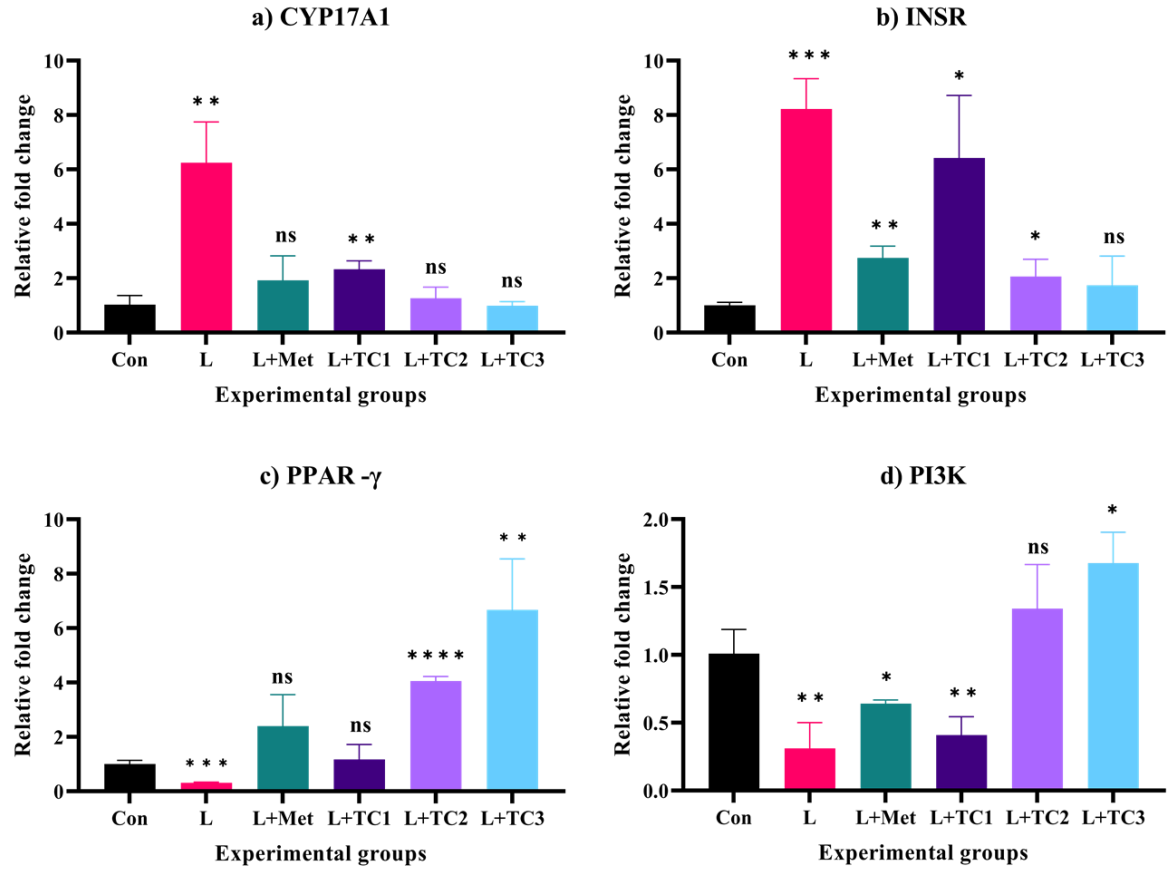

Figure 4. The relative mRNA expression of pancreatic tissue a) CYP17A1, b) INSR, c) PPAR –γ, and d) PI3K. The data were presented as mean ± SEM, n = 3, *p < 0.05; **p < 0.01; ***p < 0.001; ns – non-significant.

mRNA expression

On comparing the mRNA expression of the CYP17A1, INSR, PPAR- γ and PI3K in pancreatic tissue among experimental groups, the expression of CYP17A1, and INSR of the letrozole-induced PCOS group indicated significant increases whereas a decrease in the levels of PPAR-γ and PI3K was observed. Treatment with metformin and *T. chebula* fruit extract at the concentration of 200 & 400 mg/kg.bw (L+TC2, L+TC3) almost reversed their expression level from untreated PCOS group and brought to normal (Figure 4) and demonstrated the prospective efficacy of *T. chebula* in reducing insulin resistance associated with PCOS by interfering the molecular mechanism.

DISCUSSION

OGTT is regarded as the gold standard for the diagnosis of T2DM, though, since it is a standardised test that is simple to administer and the sole way to identify impaired glucose tolerance (IGT), which is crucial for women with PCOS (Andersen *et al*., 2018). In fact, women with IGT have a significantly higher chance of developing T2DM than the women with normal glucose tolerance, therefore early lifestyle adjustment and/or pharmacological intervention can be very beneficial to this at-risk group (Livadas *et al*., 2022). The histopathological findings of pancreatic tissue in letrozole induced PCOS rats demonstrated disturbance in the morphology of islets of Langerhans by the presence of damaged β-cells along with the increased α- and δ- cells (Saad *et al*., 2015). This impairment in the β-cells leads to a consequent decrease in insulin production and increase in the production of glucagon and somatostatin (Vrbikova *et al*., 2009). Fortunately, when these PCOS induced animals were treated with metformin and the *T. chebula*, the morphology of islets of Langerhans are found to be more intact with the healthy β-cells which may attribute to the secretion of insulin and blockage of α-glucosidase from α- cells and act as an pancreatic protectant. Human insulin receptors (INSRs) are membrane-spanning glycoproteins found on all cells that correspond to the tyrosine kinase receptor family (Chakraborty *et al*., 2021). The portion of the genome spanning exon 17-21, which encodes the INSR gene's protein tyrosine kinase domain, is critical for insulin action. Anything that alters the quantity or functionality of INSR can impair insulin's capacity to function appropriately, resulting in insulin resistance and type 2 diabetes (T2D). Insulin resistance affects more than half of PCOS patients, and they are at the increased risk of developing T2D in about 5- to 8-fold than the normal obese women (Moka *et al*., 2023). It has also been suggested that the inflammatory state linked to PCOS involves peroxisome proliferator-activated receptors (PPAR- γ), a subset of nuclear hormone receptor transcription factors. This is explained by their involvement in inflammation and the body's reaction to DNA damage, as well as in the metabolism of fat, glucose, and lipids which predisposes to the emergence of both obesity and insulin resistance, both of which are frequently linked to PCOS (Goldrat *et al*., 2018). The PI3K/Akt pathway is a crucial component of the intricate and interconnected insulin signalling network, which governs how insulin affects anabolism in all living things. Insulin receptor substrate (IRS) recruits and activates PI3K in response to insulin binding to the insulin receptor, producing PIP3. This in turn encourages the recruitment and activation of Akt, which sends signals to downstream pathways that regulate glucose, lipid metabolism, and cell survival. During the induction of PCOS, the elevated insulin levels interact with luteinizing hormone (LH) on insulin receptors of follicular theca cells by activating the PI3K/Akt cascade and ultimately initiate intracellular post-receptor signalling which is regulated by CYP17A1 and leads to an increased androgen synthesis (Baptiste *et al*., 2010).

CONCLUSION

In conclusion, this study evaluated the oral glucose tolerance among the experimental animals at varied stages of the experiment and the alterations in the levels oxidative stress-related enzyme in rats that had PCOS brought on by letrozole. These changes could be explained by oxidative damage, inflammation, and hyperinsulinemia that may lead to the onset of T2DM, which could be caused due to impairment of pancreas and its function. On the other hand, the treatment of metformin and *T. chebula* fruit extract were known to reduce the levels of the oxidative stress related enzyme while the function and histomorphology of the pancreas also restored with healthy β-cells production. According to all these evidences that supports the pharmaceutical potential of *T. chebula* in regulating

the level of blood glucose and reduces the complications of T2DM, and can be addressed as an alternative conventional therapy for treating PCOS associated T2DM.

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Conflict of Interest
The authors declare that there is no conflict of interest.

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