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Nephroprotective role of nanoencapsulated *Tinospora cordifolia* (Willd.) using polylactic acid nanoparticles in streptozotocin-induced diabetic nephropathy rats

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Abstract

Currently, the field of nanomedicine, which uses active compounds from medicinal plants, has emerged as a therapy for diabetic nephropathy. From this study, the renoprotective effect of TC-loaded PLA Nanoparticles (TC-PLA NPs) on streptozotocin (STZ)-induced diabetic nephropathy rats was investigated. The results showed that the nephroprotective effect of TC-PLA NPs reduces the blood glucose level, regulates the renal parameters, decreases the cytokine levels and reduces the mRNA expressions level of different genes related to diabetic nephropathy.

1 | INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease which results in deficiency of insulin secretion, insulin action or both [1]. DM causes microvascular and macrovascular complication such as, retinopathy, nephropathy, neuropathy, coronary heart disease, stroke and peripheral vascular diseases [2]. About one third of patients with diabetes develop diabetic nephropathy. The diabetic population is predicted to increase markedly by 2050, leading to a drastic rise in the number of end stage renal disease (ESRD) cases [3, 4]. The emergence of kidney disease is also influenced by genetic factors, impaired glucose metabolism and glomerular haemodynamic alteration, among which oxidative stress is a common mechanism [5]. Diabetic nephropathy (DN) is a chronic renal disease which leads to proteinuria, microalbuminuria, reduction in glomerular filtration, and basement thickness of the glomerular membrane and hypertrophy. The end-stage renal disease leads to tubular atrophy and interstitial fibrosis with gradual decrease in renal function [1, 2]. Hence it is necessary to explore therapeutic solutions for DN based on the different gene expression level correlated with nephropathy. Current survey many drugs are available for the treatment of DN, which causes adverse side effects and expensive therapy. Hence, as an alternative medicine based on traditional systems like Ayurvedic, Siddha and Unani and so forth is implemented to treat the disease. *Tinospora cordifolia* (TC) (Willd.) Miers ex

Hook.f. and Thoms. is an enormous, climbing shrub belonging to the family *Menispermaceae* and order Ranunculales [6]. This plant is commonly known as guduchi, giloy, amruthaballi and shindilakodi and is extensively distributed in India and other tropical regions. The stem and root of TC are mainly used in Ayurvedic and ancestry medicine. It is known for its general tonic, anticancer, antiarthritic, antihyperglycemic and anti-inflammatory properties [7]. Numerous biodegradable polymeric nanoparticles are used for the delivery of molecules and drugs [8]. Polylactic acid (PLA) have been notably used for targeted delivery of drugs associated with diabetes, kidney tissue engineering and other malignant diseases [8, 9]. PLA has been used in haemodialysis membrane because of its good haemocompatibility profile [10]. In this study, PLA polymer is used for the release of compounds which acts as drug carriers. Among many biodegradable polymers, PLA has been substantially used for the encapsulation of therapeutic agents relevant to its high biodegradability, low toxicity, biocompatibility, bioavailability and controlled drug release. Based on our previous study, the TC-PLA NPs are synthesized and their efficiency on anti-diabetic activity were studied [11], as an alternative medicine.

The hypothesis behind this study was to analyse the renoprotective effect of TC-PLA NPs on streptozotocin (STZ)-induced diabetic nephropathy rats. We have therefore investigated the therapeutic effect of TC-PLA NPs on DN rats based on blood glucose level, body weight, urine and serum renal

parameters such as protein, albumin, urea, creatinine level along with different gene expression levels based on the AGE-RAGE signaling pathway for diabetic nephropathy complications.

2 | MATERIALS AND METHODS

2.1 | Synthesis of TC-PLA NPs

TC-PLA NPs were synthesized using a double emulsion solvent evaporation method and based on the optimization of the Full Factorial Design. About 50 mg of the PLA and 5 mg of the dried TC stem extract were sonicated (Sonics and Materials, INC.53, Church Hill Road, New town, CT, USA, Model-VCX500) together at 40% amplitude in 2 ml of Dichloromethane (DCM) solution for 30 s at room temperature. Four millilitre (1%) of polyvinyl alcohol (PVA) solution was added again and sonicated to form an emulsion. Furthermore, the emulsion was diluted with a PVA solution (0.1%) to make up the volume to 80 ml and the organic solvent dichloromethane present in the emulsion was evaporated by stirring for 3 h. After stirring the solution was centrifuged at 16,500 rpm for 10 min at 10°C and washed thrice with distilled water. The resulting nanoparticles were lyophilized using lyophilizer (Lark Innovative Fine Teknowledge, India) and stored at 2–8°C till further use. In our previous study, synthesis and characterization of TC-PLA nanoparticles were investigated, and FT-IR, LC-MS/MS, XRD and HR-SEM analyses were carried out [8].

2.2 | Experimental animals

Wistar Albino rats; females (200 g) were selected for the study. They were obtained from the animal house, VIT, Vellore.

The animal experimental procedures were reviewed and approved by Institutional Animal Ethical Committee (VIT/IAEC/14/Nov4/07). Animals were conducted based on the guidelines prescribed by the Committee for the Purpose of Supervision and Control of Experiments on Animals (CPSCEA), Government of India, Chennai, Tamil Nadu and IAEC. Animals were maintained in standard laboratory conditions at 27°C: 12 h (light and dark cycle). The rats were fed with standard food pellet and water *ad libtium*.

2.3 | Induction of diabetes nephropathy

The rats were supplied with 10% fructose in drinking water for 2 weeks and after fructose induction, the rats fasted overnight and were administered with (40 mg/kg b. wt.) freshly prepared STZ in 0.1 M citrate buffer (pH 4.5). About 48 h after STZ administration, blood samples were collected from tail vein and blood glucose level was defined by the GOD-POD method. Body weight and blood glucose levels were monitored regularly. By the end of fourth week, blood from diabetic rats with ≥300 mg/dl blood glucose levels, was collected [12].

2.4 | Experimental design

The diabetic nephropathy rats were divided into three groups, each consisting of six animals and the treatment as follows.

Group I: Normal control (Rats treated with citrate buffer)
Group II: Negative control (Diabetic nephropathy rats)
Group III: Diabetic nephropathy rats treated with TC-PLA NPs 10 mg/kg b. wt.

Dose for treatment-Group III was selected based on the previous study [11]. After 4 weeks of treatment, animals were kept for fasting (12 h) and urine samples were collected using metabolic cages. Animals were euthanized, and blood samples were collected through a cardiac puncture, serum was separated by centrifugation at 5000 rpm for 20 min and preserved at −20°C for biochemical assays. The kidney tissues were dissected from the sacrificed rats, washed in saline to remove the blood stain and stored at −80°C for further use.

2.5 | Body weight

Body weight of rats are noted and measured throughout the study period.

2.6 | Renal functional parameters in urine

Renal functional parameters such as urine volume, total protein, albumin, urea and creatinine were determined using commercially available kits Arkray®, and procedures are followed as per manufacturer's protocols.

2.7 | Renal functional parameters in serum markers

Serum profile markers such as total protein, albumin, urea, BUN and creatinine were evaluated in blood serum using readymade kit Arkray® as per manufacturer's protocol.

2.8 | Kidney cytokine profiles

Proinflammatory cytokine analysis of TNF-α and IL-6 was performed using Rat-ELISA Kit RayBio®, USA, and protocols werre followed as per the user manual provided.

2.9 | RNA extraction and cDNA conversion

RNA from the kidney tissue was extracted using Trizol reagent (TaKaRa Bio., India). The isolated RNA is reverse transcribed to cDNA using the cDNA reverse transcriptase kit (TaKaRa Bio., Japan) as per protocol provided in manual.

2.10 | Real-time PCR

Different gene expression level in diabetic nephropathy rats was measured by real-time PCR using the oligonucleotide primers shown in (Table 1). Amplification was carried out using a 20-μL reaction mixture containing 2 μL of cDNA, 200 ng of each primer and 2X SYBR Green Master mix (TaKaRa) in CFX BIO RAD RT-PCR system. The PCR programme undergoes initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 15 s and extension at 72°C for 20 s. The RT-PCR product was analysed, and the expression was calculated using the comparative C_t method.

2.11 | Histopathological examination

Kidney tissues were fixed in 10% formalin buffer. Following the fixation, tissues were sliced using an automated tissue processor and embedded in wax. Kidney tissue sections were cut to a thickness of 5 μm using a Leica microtome and stained using haematoxylin and eosin stain. The slides thus obtained were subjected to histopathological analysis.

2.12 | Statistical analysis

All data are represented as mean ± standard error mean (SEM) analysed using GraphPad Prism® 7 software (GraphPad Software, Inc., La Jolla, CA, USA). The groups were compared by one-way ANOVA with Tukey's test.

3 | RESULTS AND DISCUSSION

Diabetic nephropathy is a microvascular complication of diabetes. Kidney plays a key role in eliminating harmful waste products from the body to balance the essential minerals, fluids and electrolytes at physiological conditions. The high blood glucose concentration will damage the cells and blood vessels of the kidney [13]. In the rat model diabetic nephropathy condition is studied by STZ induction. The intraperitoneal induction of STZ in rats leads to hyperglycemia and destruction of beta cells in the pancreas [14]. STZ induces destruction of pancreatic beta cell, through reactive oxygen species (ROS).

Increased level of ROS increases advanced glycation end products (AGEs) and activation of NF-κB, which increase proinflammatory cytokine levels. This mechanism is extensively directed towards inflammation and fibrosis thus progressing to diabetic nephropathy [15]. In our study at 4 weeks after STZ induction, iabetic nephropathy in rats was confirmed. Blood glucose level and body weight are noted throughout the study period. The reduction in blood glucose concentration may lower the prevalence of DN [16]. Figure 1a inferred that blood glucose level increased significantly in the STZ administration of DN rats Group II ($p < 0.001$) when compared to Group I ($p < 0.001$), and TC-PLA NPs treated DN rats showed significant decrease in blood glucose level seen in Group III when compared to Group II ($p < 0.001$). Reduction in body weight in Group II was observed in DN rats ($p < 0.001$). Treatment with TC-PLA NPs in DN rats restored the decreased body weight significantly (Figure 1b).

Renal parameters were monitored for 4 weeks and renal damage in rats were determined through biochemical parameters such as 24-h urine volume, protein, albumin excretion rate and creatinine levels. This study showed the significant increase in urine volume, protein, albumin, urea and creatinine level in STZ-induced rats after 4 weeks which confirms the establishment of diabetic nephropathy. Compared with Group I rats, the water intake and urine volume remarkably increased ($p < 0.001$) in the Group II DN rats. Upon treatment with TC-PLA NPs, the water intake was reduced (Figure 2a) and urine volume in Group III were lower than Group II DN rats ($p < 0.001$) (Figure 2b).

Urine functional parameters such as urine protein excretion (Figure 3a), urine albumin excretion rate (UAER) (Figure 3b), and urine urea level (Figure 3c) were significantly increased ($p < 0.001$) in Group II, when compared to Group I, whereas in Group III animals treated with 10 mg/kg (b)wt. of TC-PLA NPs, the urine functional parameters were significantly decreased ($p < 0.001$) when compared to Group II. Urine creatinine level in Group II DN rats ($p < 0.001$) was significantly reduced when compared to Group I and there was a notable increase ($P < 0.001$) in creatinine level in TC PLA NPs treated Group III animals when compared to Group II (Figure 3d). Treatment of DN rats with TC-PLA NPs effectively reduced the levels of urine volume, protein, albumin, urea creatinine and increased clearance from kidney. Diabetic nephropathy development leads to elevated levels of BUN and serum creatinine that causes interstitial atrophy and glomerular

TABLE 1 Primer sequence used for gene expression analysis in diabetic nephropathy study

Gene	ID	Forward (5′-3′)	Reverse (5′-3′)
TGF-β1	NM_021578.2	CCCCTACATTTGGAGCCTGG	TTGCGACCCACGTAGTAGAC
Collagen Type IV	XM_006226910.3	TTCAGTCGGCTCACAGTCAC	AACGATGGCAAGGAAAACAC
VEGF	NM_053653.1	TGCAATGCATGAACACCAGC	GTTTAGACATGCACCGGCAG
AGER	L33413.1	ACAGAAACCGGTGATGAAGG	CTCTCCTCGAGTCTGGGTTG
EGR-1	NM_012551.2	CTATACTGGCCGCTTCTCCC	TTGGAGGGTTGGTCATGCTC
Beta-actin	NM_031144.3	AGCCATGTACGTAGCCATCC	ACCCTCATAGATGGGCACAG
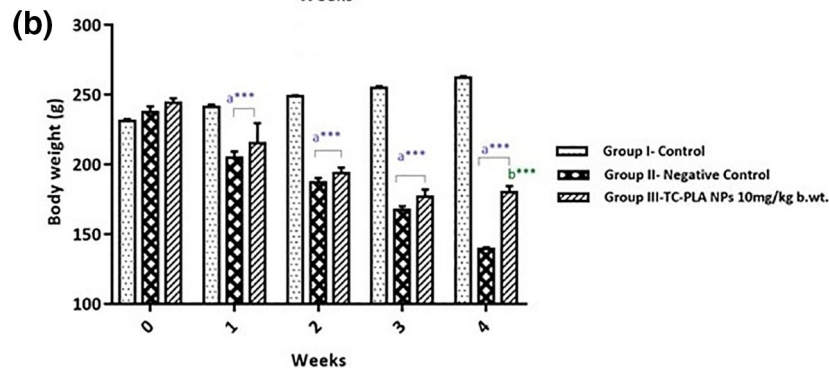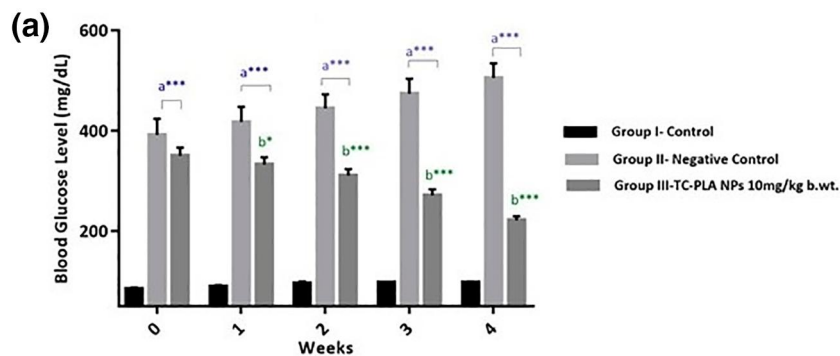
FIGURE 1 (a) Effects of TC-PLA NPs on blood glucose level of diabetic nephropathy rats. (b) Effects of TC-PLA NPs on body weight of diabetic nephropathy rats
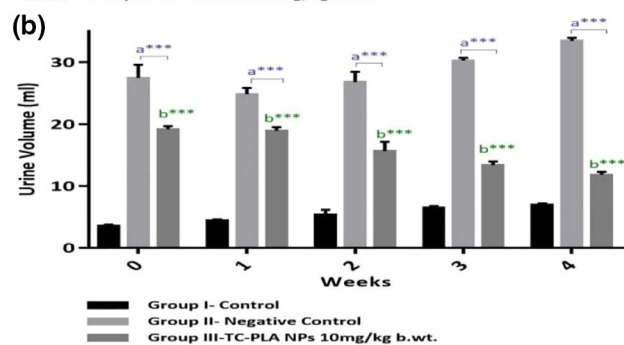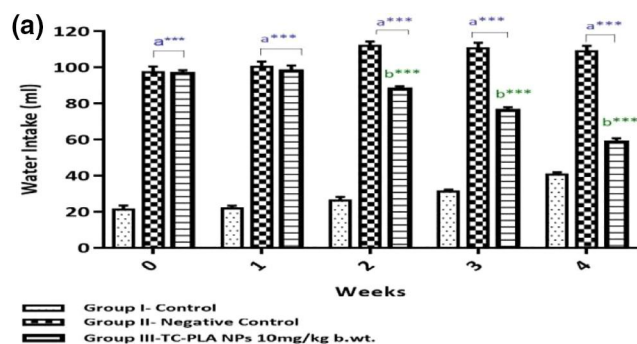
Values are Mean ± SEM, n=6, *P<0.5,**P<0.01,***P<0.001
Statistical analysis-ANOVA followed by Tukey's multiple comparison test.
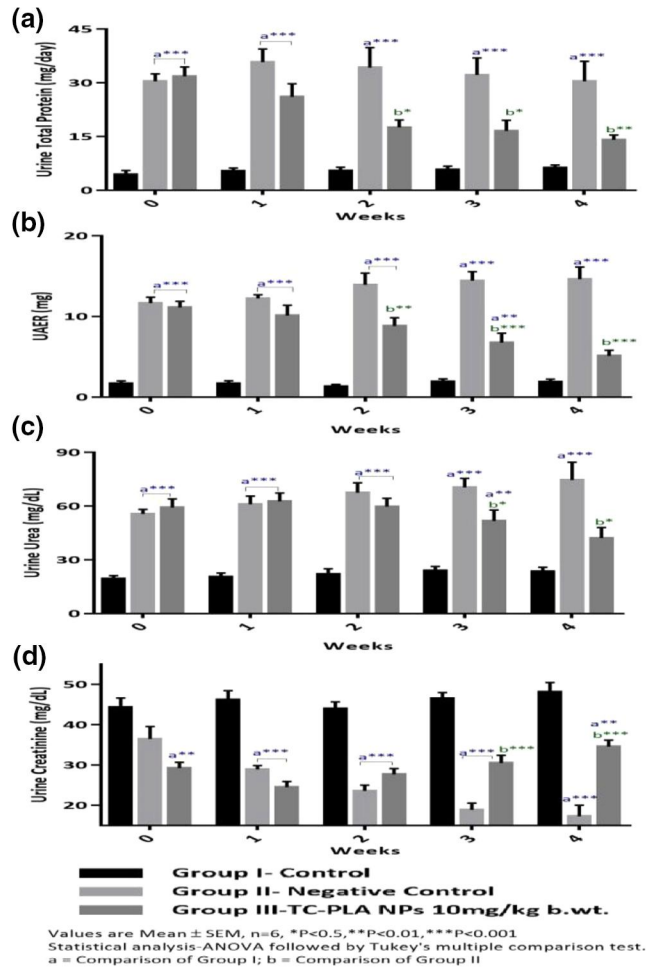a = Comparison of Group I; b = Comparison of Group II

FIGURE 2 (a) Effects of TC-PLA NPs on water intake level of diabetic nephropathy rats. (b)Effects of TC-PLA NPs on urine volume of diabetic nephropathy rats

changes [17]. A refinement in the abnormal condition of the kidney is an evidence of enhancement in DN [18]. Serum profile markers are used to measure the kidney function. From Table 2, it is inferred that total protein and albumin levels were significantly decreased in Group II ($p < 0.001$) DN rats when compared to Group I, but there was a remarkable increase in TC-PLA NPs treated Group III animals ($p < 0.001$) compared to Group II. Other renal markers such as urea, BUN and creatinine levels also increased significantly in Group II ($p < 0.001$) DN rats compared to Group I, whereas the group treated with TC-PLA NPs showed a significant decrease ($p < 0.001$) in urea, BUN and creatinine levels when compared to Group I. However, creatinine clearance (CrCl) was observed to decrease significantly in Group II ($p < 0.001$) when compared to Group I and increased significantly in Group III ($p < 0.001$). The serum parameters show reduction in serum protein, albumin, urea, BUN and creatinine level. These results prove the improvement in renal function and attenuation of nephropathy in DN rats through treatment of TC-PLA NPs.
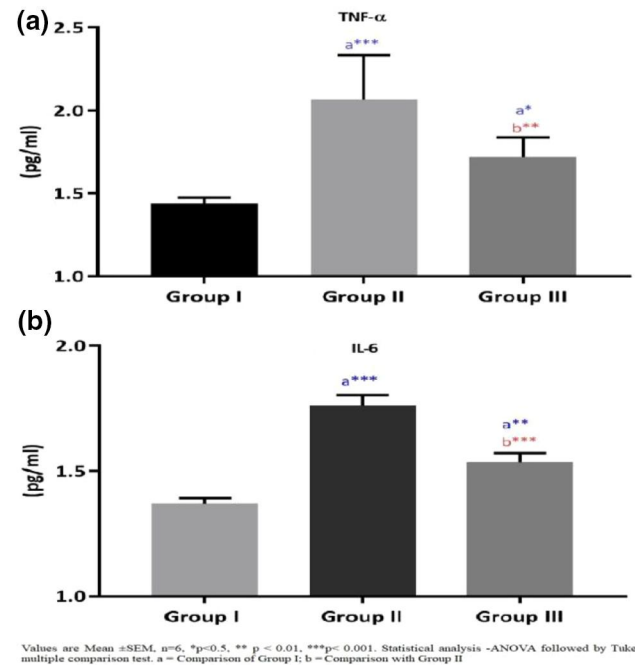
Role of inflammatory cytokines is the prime factor to be measured in diabetic nephropathy conditions. Cytokines are low molecular weight polypeptides that possess autocrine, paracrine, and juxtacrine effects with characteristic features [19]. Cytokines are produced by a wide range of cells and immune cells like B cells, T cells and macrophages. It is classified into interleukins, interferons, tumour necrosis factor and lymphokines [20]. IL-6 plays a crucial part in diabetic nephropathy and is associated with the expansion of mesangial cell leading to the accumulation of extra cellular matrix (ECM), glomerular basement membrane thickening. TNF-alpha is produced by T cells, macrophages and intrinsic renal cells. It is a pleiotropic inflammatory cytokine, involved in the oxidative stress and damage of glomerular capillary wall membrane [21]. From the results its inferred that TNF-alpha and IL-6 levels were increased significantly in Group II ($p < 0.01$) compared to Group I and there was significant reduction in Group III

$(p < 0.01)$ when compared to Group II (Figure 4a,b). In this study, the inflammatory cytokine level in reduced significantly in DN rats treated with TC-PLA NPs which protects the nephropathy condition by reducing the ROS and inflammatory level.

High blood glucose level led to increase in formation of AGEs, develops the reactive oxygen species formation, contributing to the progression of diabetic nephropathy. AGE-RAGE signaling pathway plays an important role in pathogenesis of diabetic nephropathy. Based on the pathway for our study, we selected different genes such as TGF-β1, Collagen Type IV, VEGF, EGR-1 and AGER. TGF-β1 and collagen type 4 which are the key regulators of ECM production casuing the expansion of the mesangial matrix in DN condition [22–24]. VEGF is a critical regulator of abnormal angiogenesis and its glomerular expression is involved in pathogenesis of DN, in nephrotic condition this gene level will over express [25]. EGR-1 is expressed in various types of cells in the kidney, such as glomerular mesangial cells, renal tubular fibroblasts, endothelial and epithelial cells. The ERG-1 expression changes are

FIGURE 3 (a) Effects of TC-PLA NPs on urine total protein of diabetic nephropathy rats. (b) Effects of TC-PLA NPs on Urine Albumin Excretion Rate of diabetic nephropathy rats. (c) Effects of TC-PLA NPs on the urine urea level of diabetic nephropathy rats. (d) Effects of TC-PLA NPs on urine creatinine level of diabetic nephropathy rats

FIGURE 4 (a) Effects of TC-PLA NPs on TNF-α level in diabetic nephropathy rats. (b) Effects of TC-PLA NPs on IL-6 level in diabetic nephropathy rats

TABLE 2 Effects of TC-PLA NPs on renal functional parameters in serum of DN rats

Parameters	Group I-Normal Control	Group II-Negative Control	Group-III TC-PLA NPs10 mg/kg (b)wt.
Total protein (g/dl)	6.79 ± 0.61	2.49 ± 0.34 a[***]	5.73 ± 0.47 b[***]
Albumin (g/dl)	4.67 ± 0.56	2.01 ± 0.12 a[***]	4.25 ± 0.15 b[***]
Urea (mg/dl)	62.77 ± 14.15	201.11 ± 12.66 a[***]	138.33 ± 6 b[**]
BUN (mg/dl)	15.82 ± 1.47	51.62 ± 3.20 a[***]	29.05 ± 1.37 b[***]
Creatinine (mg/dl)	0.33 ± 0.05	3.72 ± 0.41 a[***]	1.24 ± 0.19 b[***]
CrCl (ml/min)	2.96 ± 0.48	0.76 ± 0.06 a[***]	1.40 ± 0.17 b[**]

Note: Values are Mean ± SEM, $n = 6$, *$p < 0.5$, **$p < 0.01$, ***$p < 0.001$. Statistical analysis, ANOVA followed by Tukey's multiple comparison test. a = Comparison of Group I; b = Comparison with Group II.
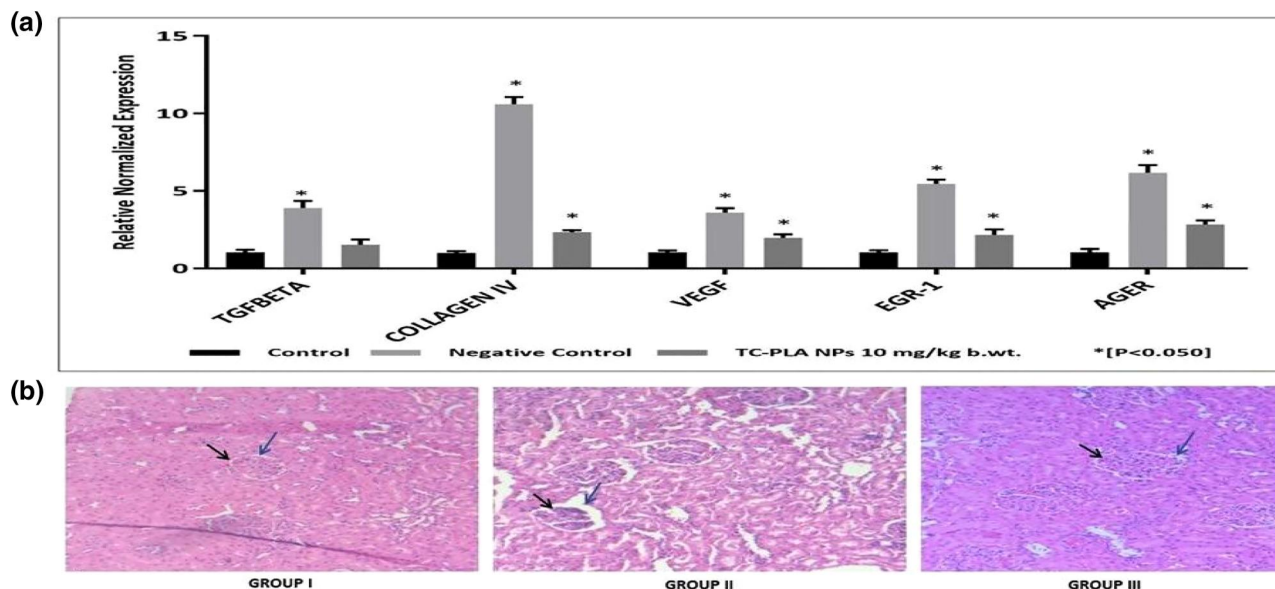
FIGURE 5 (a) Effects of TC-PLA NPs on different gene expression level of DN rats. (b) Histological examinations of kidney tissue. Tissues were stained with H&E-100×. Black arrow denotes glomerular, and blue arrow denotes Bowman's space

associated with renal fibrosis which stimulates proliferation of mesangial cell and extracellular matrix synthesis. This factor can cause diabetic nephropathy and yet research on the role of EGR-1 in DN have tends to focus more on vascular dysfunction [26]. AGER causes vascular cell derangement characteristics of diabetes and is mainly mediated by their interaction with receptor of AGE (RAGE), leading to oxidative stress in DN, where the expression of this gene will be elevated [27]. The expression of different genes such as TGF-β1, Collagen Type IV, VEGF, EGR-1 and AGER was evaluated through real-time PCR and normalized using a reference gene beta-actin. In TGF-β1, Collagen Type IV, VEGF, EGR-1 and AGER, significant upregulation was observed in Group II DN rats. On the other hand, on administration of TC-PLA NPs in group III animals, the gene expression level was downregulated when compared to Group II (Figure 5a). TC-PLA nanoparticles possess antioxidant potential and anti-inflammatory activities and have an impact on AGE-RAGE signalling pathway. Therefore, the integral role of TC-PLA NPs in the management of diabetes nephropathy may be attributed to the activity of the reported phytoconstituents, which may impart the therapeutic potential through enhanced antioxidant function and reduced the inflammatory rates either alone or via bioactive compound synergism. Thus, TC-PLA NPs validate the therapeutic effect on diabetic nephropathy. Histopathological examination of Group I showed normal kidney morphology. In Group II Diabetic nephropathy, the kidney sections of the rats exhibited severe glomerular interstitial inflammation and expansion in Bowman's space. TC-PLA NPs treated Group III possess normal kidney morphology and glomerular membrane with mild inflammation (Figure 5b).

4 | CONCLUSION

It is evident from the study that the nephroprotective effect of TC-PLA NPs lowers the blood glucose level, stabilizes the renal parameters, reduces inflammatory cytokines level and regulates gene expression level in diabetic nephropathy rats.

CONFLICT OF INTEREST
No.

PERMISSION STATEMENT TO REPRODUCE THE MATERIALS FROM THE OTHER SOURCES
None.

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