Pharmaceutical Composition of Nanotea Powder for the Prevention of Diabetic Nephropathy

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Abstract--- The chronic loss of kidney function in patients with long standing poorly controlled diabetes mellitus and end-stage kidney failure globally is diabetic nephrotherapy (DN) which is also called "diabetic kidney disease." Numerous shifts in the processing units of the kidneys, i.e. the nephrons, accompany this. In chronic Type 1 and Type 2 diabetes mellitus, this type of disease is seen. Various research studies have been carried out in both fundamental science as well as clinical therapy which have increased the understanding and pathophysiology of diabetic nephrotherapy (DN) and expanded the therapeutic agents for the treatemnt. The present invention was designed to provide a novel nanotea powder formulation comprising of constituents that bear different mechanism of actions for the management of diabetic mellitus and diabetic nephropathy. Nanotea powder composed of Epigallocatechingallate, Rutin and Stevioside was formulated using lyophilization (freeze drying) method following which pharmaceutical characteristics of powder were evaluated. The disease was caused by a single dose of streptozotocin (STZ) (50mg / kg, i.p) in the overnight-fasted adult wistar rats weighing 180-210 g. After 8 weeks of STZ administration, diabetic nephropathy grew. Thereafter, Diabetic rats were treated with formulated nanotea powder by oral route for 15days. An array of biochemical estimations were performed such as serum glucose level, serum albumin, serum creatinine, blood urea nitrogen, serum total cholesterol, serum high density lipoprotein (HDL) and serum triglycerides, urine albumin and urine creatinine to measure the extent of pathological damage.

Keywords--- Diabetic nephrotherapy (DN), diabetes mellitus, nanotea powder, lyophilization, streptozotocin (STZ), pathological damage

I. INTRODUCTION

Diabetic nephropathy or diabetic kidney disease is a condition characterized by the occurrence of urinary albumin discharge abnormal quantities of urine, diabetic glomerular lesions, and GFR deficiency in diabetic patients [1]. In 2015, “The International Diabetic Federation” estimated that the prevalence of diabetes in the population of about 440 million people aged between 20-79 years was 8.8% [2][3]. It is estimated that by 2035 this will rise to over 550 million people [4]. The combination of diabetes with chronic tissue complications is one of the most significant medical characteristics. A short-term hyperglycemia rise does not lead to major medical complications. The duration and intensity of hyperglycemia is the main factor for organ damage. Nephromegaly and modified Doppler are early morphological indicators of renal damage but proteinuria and Glomerular Filtration Rate (GFR) are better determined for damage [5]. In the first 10 to 20 years after the initiation of diabetes, the average incidence of diabetic nephropathy is relatively high i.e., 3% per year [6].

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Diabetes is the most common source of diabetic nephropathy (DN) which accounts for up to 50% of people who develop end-stage renal disease (ESRD) [7][8][9]. Progression leads to considerable morbidity and mortality necessitating dialysis or renal transplantation [10] with obvious enhanced cost of health care. However the prognosis of ESRD patients is inadequate with 6% to 20% annual mortality rate for all patients on dialysis. DN progression may be delayed adequately by controlling the metabolic and hemodynamic abnormalities. Current treatment options include “angiotensin converting enzyme inhibitor (ACEI)” or “angiotensin receptor blocker (ARB)”, “Glucagon-like peptide-1 receptor agonists (GLP-1 agonists)”, “dipeptidyl peptidase 4 inhibitors (DPP-4 inhibitors)”, and “Sodium-glucose co-transporter-2 inhibitors”. SGLT2 inhibitors reduce “glucose levels, blood pressure, heart failure incidences and high potassium levels” but increase the genital infections and slightly reduce kidney functions. However, their effects on “mortality rate, hypoglycaemia, acute kidney injury, urinary tract infection, end-stage kidney disease, bone fractures and diabetic ketoacidosis” are uncertain. It is evidence also indicating that while DPP 4 and GLP 1 inhibitors are decreased, their impact on heart attack and stroke risk have been questionable, migraine, hypoglycaemia, pancreatitis and pancreatic cancer were inhibited by the upper respiratory system. A single streptozotocin injection (50 mg / kg i.p.), which resulted in hyperglycemia, has been selected for the analysis, with rats displaying a blood glucose level greater than 240mg / dl. Enhanced level of blood urea nitrogen, lipid profile, serum creatinine level, urine albumin level and renal TBARS level was observed. Additionally reduced urine creatinine level, serum albumin level and renal GSH level in comparison to normal untreated animals was also indicated. Administration of nanotea powder (100 mg/kg p.o for 15 days) significantly reversed the avert biochemical changes induced by STZ. Histopathological examination of renal sections of STZ treated rats indicated shrinking of the glomerular tufts, increase of Bowman’s space and dilation of proximal and distal convoluted tubules along with relatively higher number of mesangial cells. However, administration of nanotea powder to the diabetic rats normalized the glomerular capillaries, bowman’s capsule, proximal and distal tubules also improved in size and thickness. Hence it may be concluded that the formulated nanotea powder may be an excellent therapeutic option for diabetic nephropathy.

II. MATERIALS AND METHODOLOGY:

1. Drugs and reagents:

The reagents used in this study were of analytical grade and Epigallocatechingallate from (Sigma-Aldrich Corporation), Rutin and Stevioside from (Yucca Enterprises) were. Streptozotocin (STZ) from (Oswal-Scientific store, India), “DTNB (5, 5-dithiobis-2-nitrobenzoic acid)”, “Folin-Ciocalteu Phenol reagent”, “Bovin serum albumin”, “n-butanol, Pyridine”, “reduced glutathione (GSH)” from “Loba Chemicals (Mumbai, India)”. Trichloroacetic acid was purchased from Nice Chemicals Pvt. Ltd. (Chochin, India) and “Glucose estimation Kit (GOD-POD)” was purchased from “Reckon diagnostic Pvt. Ltd”. “Thiobarbituric acid, Blood urea nitrogen (BUN)” (Berthelot method) kit purchased from “Magus chemical & scientific equipment India”, total cholesterol (CHOD-PAP), triglycerides (GOD-PAP) and HDL kits from (PTA) kits were purchased from “Reckon diagnostic Pvt. Ltd” and serum creatinine “Reckon diagnostic Pvt. Ltd” and serum albumin estimation kits from “Recombigen Laboratories Pvt. Ltd” were employed for various biochemical estimation. Streptozotocin was diluted with citrate
buffer (0.1M, adjusted pH 4.5 with citric acid) and administered intraperitoneally (i.p.).

2. Preparation and characterization of nanotea powder:

The nanotea powder bearing Epigallocatechingallate (8.9 mg), Rutin (1.89 mg), and Stevioside (3.45 mg) was prepared by using the lyophilization (freeze drying) method.

3. Preparation of Effervescent Solution:

The effervescent nanotea powder was prepared by effervescent granulation technology. In brief, 300 mg “sodium carbonate” and 1000 mg “spray dried lactose monohydrate” (FlowLac) was dissolved in a mixture containing 3ml distilled water and 300 μL of “ammonium hydroxide” (28-30%). Subsequently 250 mg citric acid was added to the above mixture. The suspension was lyophilized to obtain the dry powder. The particle size of nanopowder was estimated to be below 200 nm.

4. Induction of diabetic nephropathy:

Single injection of Streptozotocin (STZ) at a dose (50mg/kg, i.p.) dissolved in ice cold citrate buffer (pH 4.5) was injected intraperitoneally to overnight fasted rats, following which 5% sucrose was supplemented for 24 hrs to prevent the animals from fatal hyperglycemia. The blood glucose level was assessed one week after STZ injection. Animals more than 240mg/dl with blood glucose were regarded as diabetic and included in the study. After 4 weeks of STZ administration physiological changes start and after 8 weeks of STZ administration diabetic nephropathy develop. After the 6th week of STZ administration, drug treatment was started for 2 weeks. At the initiation and end of the study various biochemical parameters pertaining to DN were evaluated in urine and serum using different treatment groups. For the estimation of “glucose, albumin, creatinine, cholesterol, triglycerides, and blood urea nitrogen (BUN) levels”, serum was added into the solution. For estimation of urinary parameters, rats were placed in the metabolic cages and urine was collected for 24h for the analysis of creatinine and albumin levels. The estimations of above mentioned parameters were performed using commercially available kits as per manufacturer’s protocol. Further at the end of the experiments the rats were sacrificed, kidneys were removed. Histopathological examination of the isolated left kidney was carried out using standardized hematoxylin-eosin staining techniques.

5. Biochemical Estimations:

i) Collection of samples: At the end of experimental protocol, the sample of blood was collected by the retro orbital sinus puncture just before sacrificing the animals under light ether anesthesia and the blood was kept at room temperature at 37°C for 15 min after which it was centrifuged at 4000 rpm for 15 min and serum was separated. The serum samples were stored at 4-6 C for the estimation of biochemical parameters such as “serum glucose level, serum blood urea nitrogen serum and urine albumin, serum and urine creatinine, serum total cholesterol, serum triglycerides, serum HDL” and animals were sacrificed after the blood sample collection by cervical dislocation. The two kidneys were quickly found and removed. Removed right kidney has been excised into small parts with a 0.1 M Sorenson buffer for phosphates (10% w/v, pH 7.4) prepared for the renal homogenates. The homogenated test tubes were held for 30 minutes in ice water and centrifuged at 4 °C (10,000 rpm, 10 min).
The supernatant of homogenates was segregated from the thiobarbituric acid reactive level (TBARS) of total protein contents and glutathione level (GSH) was used for estimating it.

a) *Estimation of serum glucose:*

The amount of glucose was measured using a commercially available kit with the aid of the glucose peroxidase (GOD-POD) process (Tietz, 1982). At 505 nm of spectrophotometric content (UV-1700 Spectrophotometer, Shimadzu, Japan), the absorption of tests and standard samples was noted against blank and was expressed in mg / dl.

*b) Assessment of Diabetic Nephropathy:*

Through measuring serum BUN, creatinine and albumin, the level of diabetic nephropathy was measured biochemically.

b) *Estimation of blood urea nitrogen:*

The Berthelot test used the commercially available package was used to measure Blood Urea Nitrogen (BUN). Test and standard sampling absorption were observed at 578 nm (UV-1700 Spectrophotometer, Shimadzu, January) against blank spectrophotometric and were expressed as mg / dl.

c) *Estimation of serum creatinine:*

The alkaline picrate process used a commercially available package measured serum creatinine concentration. The absorptiveness was measured against distilled water and expressed as mg / dl at 510 nm (UV-1700 Spectrophotometer, Shimadzu, Japan).

d) *Estimation of serum albumin:*

Total albumin was measured with the commercially available package using bromocresol green (BCG) teething method (Doumas, 1978). Immediately on a spectrometer 630 nm (UV-1700 Spectrophotometer, Shimadzu, Japan) the ability to perform test and regular samples was noted blankly and expressed in mg / dl.

6. *Assessment of Renal Hypertrophy and Renal Fibrosis:*

(a) *Estimation of kidney weight/body weight (%):*

The left and right kidneys were separated and the renal fascia had been removed. Weight / weight of the kidney (percent) according to the following formula has been determined.

“Kidney weight/body weight (%) = left kidney weight (g) + right kidney weight (g)/body weight (g) × 100”.

(b) *Assessment of lipid profile:*

(a) *Estimation of serum total cholesterol:*

Use of commercially available kits to calculate total cholesterol using cholesterol oxidase peroxidase (CHOD-PAP) method. At 505 nm spectrophotometrically (UV-1700 Spectrophotometer, Shimadzu, Japan), test absorption and standard specimens are measured as mg / dl against blank (UV-1700).
(b) Estimation of Serum Triglyceride:

Serum triglyceride was measured using commercially available kits for glycerophosphate oxidase peroxidase (GOD-PAP). At 505 nm spectrofotometric (UV-1700 Spectrophotometer, Shimadzu, Japan), the absorption of tests and regular samples was noted against blank and expressed as mg / dl.

(c) Estimation of serum high density lipoprotein (HDL):

The rate of HDL has been measured using the commercially available kit using a phosphotungstic acid (PTA) approach. At 505 spectrophotometric nm (UV-1700 Spectrophotometer, Shimadzu, Japan), the absorption of tests and standard samples is observed and expressed as mg / dl against the flat head.

7. Assessment of Renal Oxidative Stress:

a) Estimation of thiobarbituric acid reactive substances (TBARS) level:

A lipid peroxidation index was measured as a thiobarbituric acid reactive (TBARS) material. The absorbance has taken the form of nanomoles / mg of protein at an altitude of 532 nm (UV-1700 Spectrophotometer, Shimadzu, Japan).

b) Estimation of reduced glutathione (GSH) levels in the kidney:

A method estimated the GSH level in the kidney. At 412 nm (UV-1700 Spectrophotometer, Shimadzu, Japan), spectrometric absorbance was observed and results showed as micromoles / mg of protein.

8. Histopathological evaluation:

a) Hematoxylin and eosin staining method:

Isolated kidney samples stored in a fixing solution and fixed to maintain the integrity of the cells. (10% formalin). Then the nerve parts are washed and then impregnated with alcohol in molten paraffin and held for 24 hours. In addition, the sections have been decreased into 4 μm thickness and hematoxyl and eosin stained. The micrographs of the corresponding stained-kidney areas were then taken using light (100 magnification). The microscope was taken.

b) Statistical analysis:

The data were analyzed on the prism program for GraphPad and all values were indicated as mean ± SEM (n=8). Evidence from various biochemical parameters was evaluated statistically by single-way ANOVA, followed by a multiple comparison test performed by Bonferroni. The p < 0.05 was statistically significant.

III. RESULTS:

1. Assessment of Diabetic Nephropathy:

a) Effect on serum glucose level:

Upon administering STZ (50 mg / kg, i.p.) the glucose level of the diabetic group was increased compared with the normal control group. When STZ rats were treated with the formulated nanotea powder (100 mg/kg p.o. for 2
weeks) a significant decline in blood glucose level was indicated in comparison to diabetic control rats. Further similar effects were observed in marketed formulation group and no significant change in blood glucose level was seen in blank group in comparison to STZ group (Figure 1).

**Fig.1. Effect of various pharmacological interventions on serum glucose level (mg/dl).**

b) *Effect on serum albumin level:*

Albuminuria (increase in albumin levels in urine) with a decrease in its serum level is diagnosed with diabetic nephropathy. In the study, the levels of serum alBumin decreased significantly from the normal control group throughout our diabetic rats. Administration of nanotea powder (100 mg/kg *p.o.*for 2 weeks) significantly elevated the serum albumin level of diabetic rats. Further similar effects were observed in marketed formulation group and no significant change in serum albumin level was observed in blank group in comparision to STZ control group (Figure 2).

**Fig.2. Effect of various pharmacological interventions on serum albumin level (mg/dl).**
c) **Effect on urine albumin level:**

Our study showed that the level of urinary albumin in rats was significantly increased in comparison with normal control groups. Nanotea powder administration (100 mg / kg p.o. for two weeks) significantly lowered the amount of diabetic rats’ urine albumin. Further similar effects were observed in marketed formulation group and no significant change in urine albumin level was observed in blank group in comparison to STZ control group (Figure3).

![Fig.3. Effect of various pharmacological interventions on urine albumin level (mg/dl).](image)


d) **Effect on serum blood urea nitrogen level:**

![Fig.4. Effect of various pharmacological interventions on serum blood urea nitrogen level (mg/dl).](image)

e) **Effect on serum creatinine content:**

Marked rise in serum creatinine level of diabetic rats was observed after the administration of STZ in comparison to the normal control group. Treatment with formulated nanotea powder significantly reduced the serum
creatinine content in STZ animals. Further similar effects were observed in marketed formulation group and no significant change in serum creatinine level was observed in blank group in comparison to STZ control group (Figure 5).

![Fig.5. Effect of various pharmacological interventions on serum creatinine level (mg/dl).](image)

f) Effect on urine creatinine content:

The urine creatinine level in diabetic rats significantly decreased after the administration of STZ in comparison to the normal control group. Treatment with nanotea powder (100 mg/kg p.o. for 2 weeks) significantly elevated the urine creatinine content in STZ treated animals. Further, similar effects were observed in marketed formulation group and no significant change in urine creatinine level was observed in blank group in comparison to STZ control group (Figure 6).

![Fig.6. Effect of various pharmacological interventions on urine creatinine level (mg/24 h).](image)
2. **Assessment of lipid profile:**

a) *Effect on total serum cholesterol:*

A marked elevation was indicated in total serum cholesterol level in STZ treated rats as compared to normal untreated group. Furthermore, there was a significant fall in serum cholesterol level of rats treated with nanotea powder (100 mg/kg p.o. for 2 weeks) in comparison to STZ – treated rats. Further similar effects were observed in marketed formulation group and no significant change serum cholesterol level was observed in blank group in comparison to STZ control group (Figure 7).

![Fig.7. Effect of various pharmacological interventions on serum total cholesterol level (mg/dl).](image)

b) *Effect on Serum triglyceride:*

The triglycerides level of diabetic group was accentuated after the administration of STZ (50 mg/kg, i.p.) when compared to normal control group. Drug treatment with nanotea powder (100 mg/kg p.o. for 2 weeks) to diabetic group resulted in significant decrease in triglyceride level as compared to diabetic control group. Further similar effects were observed in marketed formulation group and no significant change triglyceride level was observed in blank group in comparison to STZ control group (Figure 8).

![Fig.8. Effect of various pharmacological interventions on serum triglyceride level (mg/dl).](image)
Fig. 8. Effect of various pharmacological interventions on serum triglycerides level (mg/dl).

c) Effect on serum high density lipoprotein (HDL):

A significant decrease in HDL level was noted in diabetic rats treated with STZ (50 mg/kg, i.p.) as compared to normal control animals. However, administration of nanotea powder (100 mg/kg p.o. for 2 weeks) to diabetic rats produced a marked rise in HDL level. Further similar effects were observed in marketed formulation group and no significant change in HDL level was observed in blank group in comparison to STZ control group (Figure 9).

Fig. 9. Effect of various pharmacological interventions on serum high density lipoprotein level (mg/dl).

3. Assessment of renal hypertrophy:

a) Ratio of kidney weight and body weight:

In comparison with regular control in diabetic rats, a significant increase in kidney weight and body weight ratio was observed. STZ Rats, however, treated with nanotea dust, showed a significant decrease in kidney weight / body weight ratio (100 mg/kg p.o. for 2 weeks). Similar effects were seen for the group in which the kidney / body weight ratio was promoted and no significant changes in the group in white compared with the STZ control group were observed (Figure 10).

Fig. 10. Effect of various pharmacological interventions on kidney weight/ Body weight (%).
4. **Assessment of oxidative stress in kidney:**

a) *Effect on renal thiobarbituric acid reactive substance (TBARS) and renal reduced glutathione (GSH):*

   The level of renal TBARS in diabetic rats was significantly accentuated and that of GSH was markedly attenuated upon administration of STZ as compared to the normal control group indicating oxidative stress. Moreover treatment with nanotea powder significantly reversed this effect. Further similar effects were observed in marketed formulation group and no significant change in renal TBARS level was observed in blank group in comparison to STZ control group (Figure 11 and 12).

![Graph showing effect of various pharmacological interventions on renal TBARS level](image)

**Fig.11.** Effect of various pharmacological interventions on renal TBARS level (nmol/mg of protein).

![Graph showing effect of various pharmacological interventions on renal glutathione (GSH) level](image)

**Fig.12.** Effect of various pharmacological interventions on renal glutathione (GSH) level (nM/mg of protein).
5. **Assessment of histopathology in kidney tissue:**

Histopathological examination of kidney tissue was done by using hematoxylin-eosin staining method. The observations of the renal section reveal that the normal control group shows normal appearance of the glomerular capillary wall thickness with both proximal convoluted tubules (PCT) and distal convoluted tubules (DCT), no mesangial expansion, nodular glomerulosclerosis was observed. On the other hand, renal section of STZ treated rats (50mg/kg, i.p.) indicated shrinking of the glomerular tufts, increase of bowman’s space and dilation of proximal and distal convoluted tubules and relatively higher number of mesangial cells. The renal section of diabetic rats after treatment with nanotea powder (p.o. for 15 days) shows that glomerular capillaries retain their normal size and appearance. The Bowman’s capsule, proximal and distal tubules also improve in size and thickness (Figure 13).

![Fig.13](image)

**Fig.13.** Effect of formulated nanotea powder (100mg/kg/day) on kidney histopathology (H&E staining)(Optical microscope; X 40): A) Control group: Normal appearance of glomerular capillaries (black) with normal epithelium, mesangial cells (red) and normal basement membrane (green). B) STZ treated diabetic rats: Glomerular capillaries are widened, irregular, mesangial matrix deposition. C) Kidney tissue after treatment with Blank formulation (100mg/kg/day): Glomerular capillaries are widened, irregular, mesangial matrix deposition. D) Kidney tissue after treatment with Green tea(100mg/kg/day): Glomerular capillaries retain their normal size and appearance and mesangial cell number is relatively higher than normal. E) Kidney tissue after treated with Nanoteapowder(100mg/kg/day): Histological features improved and normal structure of glomerular capillaries was observed.
IV. CONCLUSION:

The present invention provides a novel effervescent nanotea powdered dosage form for the prevention of diabetic nephropathy. Currently most of the treatments available in the market are in tablet or capsule form. Due to these dosage forms, these drugs cause dysphasia (difficulty in swallowing) and phagophobia (fear of swallowing), in patients and also show difficulty in absorption which cause discomfort and distress. The treatment available in the market shows many of side effects including “fluid retention, weight gain, increased risk for bladder cancer, and urinary tract infections, diarrhea, drop in red blood cell count, upper respiratory tract infections hypotension, hypoglycemia, increase in cholesterol and hypersensitivity reactions”.

There is no such effervescent nano tea powder dosage form containing epigallocatechingallate, rutin and stevioside available in the market for the treatment of diabetic nephropathy which makes our invention a unique dosage form. Due to presence of effervescence and nanostructure of drugs, this dosage form avoids all the systemic side effects as compared to other oral formulations available in the market. With the support of the results obtained during studies, it has been confirmed that this formulation is has glucose lowering and renoprotective effects. Hence it may be concluded that the novel formulated nanotea powder may be an excellent therapeutic option for diabetic nephropathy.

REFERENCES