



Comparative Study of Native or Nano Quercetin on Epigenetic Modification and Nephropathy Biomarkers Post Challenges in Diabetic Hamsters

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study is to examine the regulatory effects of quercetin nanoparticles (QNPs) compared with native quercetin either with or without metformin against the abnormal molecular and biochemical pathways that eventually culminate in the pathologic condition clinically known as diabetic nephropathy (DN) in hamsters

Methodology: After synthesis and characterization of the QNPs, seventy male adult hamsters are divided as following; G1:healthy control, G2:untreated DN hamsters, G3:DN+metformin G4:DN+native quercetin, G5:DN+QNPs, G6:DN+native quercetin+ metformin and G7: DN + QNPs + metformin.

Results: Our results illustrated that the effect of QNPs + metformin is the most effective in IRS-1 and GLUT-4 gene expression that correlated with inhibition of HDACs which in turn improved diabetic and nephropathy biomarkers, this effectiveness followed by G6 then G3 and finally G4 after G5. These results were confirmed by immunohistochemical examinations. Levels of antioxidant and inflammatory biomarkers also reflected the protective effect of our treatments.

Conclusion: All tested treatments significantly exhibit renal improvement.

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1. INTRODUCTION

Diabetic nephropathy (DN) is pathologically characterized by renal glomerular hypertrophy, expansion of mesangial and tubular compartments, accumulation of extracellular matrix in multiple renal cells and inflammatory cell infiltration. Clinically, DN is identified by rising creatinine levels and aberrant glomerular filtration rates (GFR). Signal transduction mediated by key factors associated with diabetes, such as high glucose, advanced glycation end products (AGEs), oxidized lipids and proinflammatory cytokines [1]. This microvascular complication is due to persistent hyperglycemia which due to the metabolic abnormality in diabetes mellitus that explained by the defect in glucose uptake due to defective regulation of glucose transporter-4 (GLUT-4) protein. The defect in translocation of GLUT-4 protein takes place due to the inhibition of tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1). This follows serine phosphorylation of IRS-1 which inhibits binding and activation of phosphatidylinositol 3-kinase (PI3K) and initiation of downstream signaling events which inhibits downstream signaling and insulin action [2]. Recent research has demonstrated a role for epigenetic histone modifications in diabetes and its complications. HDACs have been found to play important roles in the regulation of several key genes linked to diabetes. In general, The HDACs regulate this acetylation of histone and transcriptional factors that control glucose homeostasis so; play a central role in the regulation of glucose metabolism. Also, HDACs play a regulatory role in physiological insulin signaling. Thus, HDAC inhibitors increase GLUT-4 translocation and insulin-induced glucose uptake in skeletal muscle [3].

The treatment of diabetes mellitus is based on insulin and/or oral hypoglycemic drugs. These drugs act by various mechanisms to control the blood glucose level; however many side-effects have been reported. Therefore, there is considerable interest in the field of medicinal plants as phytotherapy due to their natural origin and less side effects [4]. Flavonoids have been devoted more attention by the scientific community due to their structural diversity, abundance in nature, powerful pharmacological activity with low adverse reaction [4]. Quercetin (3,3,4,5,7-pentahydroxyflavone) is classified as a

flavonol, one of the six subcategories of flavonoid compounds, and is the major polyphenolic flavonoid found in various vegetables and fruits, such as berries, broccoli, capers, tomato, dill, apples and onions which insoluble in cold water, and sparingly soluble in hot water [5]. Quercetin is considered as peroxy radical scavenger and has higher antioxidant activity compared to other well-known anti-oxidant molecules due to the number and positions of the free hydroxyl groups in its structure. It also exhibits a broad range of pharmacological activities such as anti-inflammatory, anti-oxidant, immunomodulatory, anti-ulcer and vasodilator effects [6]. However, native quercetin itself achieved less satisfaction because of its poor solubility, low bioavailability and short half-life [7]. The nanomaterials can easily cross through biological membranes because of their ultra-small size, and therefore can be highly absorbed by the digestive system [8-9].

Therefore, the present study was conducted to examine the regulatory effects of quercetin nanoparticles compared with native quercetin against the abnormal molecular and biochemical pathways that eventually culminate in the pathologic condition clinically known as diabetic nephropathy in hamsters.

2. MATERIALS AND METHODS

2.1 Synthesis and Characterization of Quercetin Nanoparticles

Quercetin nanoparticles were synthesized by adding ethanol to distilled water (volume ratio 1:35(v/v), fixed flow rate 10 ml/min) using magnetic stirring (1000 rpm). The commercial quercetin was dissolved in prepared ethanol solvent at concentration of 5 mg/ml (w/v), then grinded using mechanical ball mill for 8 hours. The prepared solution was filled and secured onto a syringe pump. At a fixed flow rate (10ml/minute), the solution was quickly injected into the anti-solvent (deionized water) under magnetic stirring. The nanoparticles of quercetin were filtered and dried under vacuum, then characterized by high resolution transmission electron microscope (HR-TEM) analysis [10].

2.2 Animal Trial

Seventy healthy adult male syrian hamsters weighing 180 ± 10 g were used after 7 days of

acclimatization period. The animals were randomly divided into 7 groups (10 hamsters in each group)

- G1: healthy control,
- G2 untreated DN hamsters,
- G3: DN+ metformin (100 mg/kg body weight/day),
- G4: DN+ native quercetin (20mg/kg body weight/day),
- G5: DN+ QNPs (20mg/kg body weight/day),
- G6: DN+ native quercetin (20mg/kg body weight/day + metformin (50 mg/kg body weight/day),
- G7: DN+ QNPs (20 mg/kg body weight/day) + metformin (50 mg/kg body weight/day).

To induce DN, hamsters were given a High-fat/High-fructose (H-F/H-Fr) diet for first 2 weeks [11,12]. then streptozotocin (STZ) was injected only once at dose 35 mg/kg through abdominal for induction of type 2 DM [12], then the animals were allowed to continue feeding on H-F/H-Fr diet for 3 weeks for incidence of DN. The incidence of DN was screened after 3 weeks of STZ induction by measuring blood and urine creatinine concentration from overnight fasted animals (9 hours) to measured glomerular filtration rate (GFR) .

Then the animals were treated with tested doses of native or nano quercetin with and without

metformin, as mentioned before, with continue feeding on of H-F/H-Fr diet for 8 weeks (Fig. 1) till the end of the study.

2.3 Assessment of Nephropathy Biomarkers

Nephropathy biomarkers included serum and urine creatinine [13], as well as glomerular filtration rate (GFR) at zero-time (after induction of DN), after 4 weeks and after 8 weeks (the end of experiment) [14].

2.4 Assessment of Real-time Quantitative Polymerase Chain Reaction (qRT-PCR)

Extraction of total RNA from anterior thigh muscle tissue using total RNA Purification Kit was done using Trisol reagent (Invitrogen, CA). Nanodrop method was used to quantify the concentration of RNA and cDNA to be sure that the concentrations are pure enough to conduct real time PCR [15]. IRS-1 and GLUT-4 genes' expressions were measured in four replicates with 18S rRNA expression level was used as an internal reference. Data were normalized using the $2^{-\Delta\Delta Ct}$ method [16]. The sequence of the designed primers was presented in Table (1)

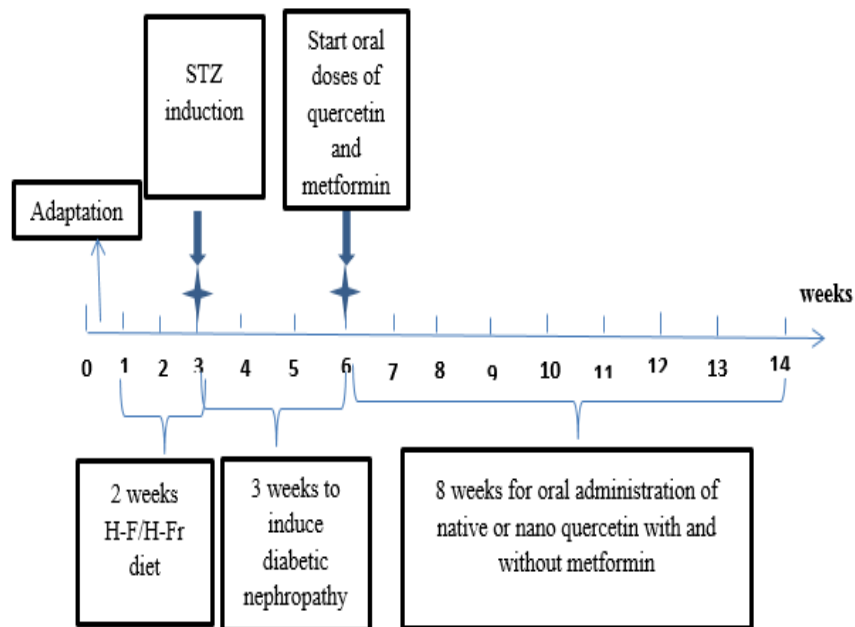


Fig. 1. Schematic experimental design of the study

Table 1. Forward and reverse sequences of the primers used for IRS-1, GLUT-4 and 18s rRNA

Gene	Forward primer (5' ----- 3')	Reverse primer (5' ----- 3')
IRS-1	TGCACTGTGACACCAGAATAAT	TTGGAATGAACAATGTGGGCG
GLUT-4	TCATTGTCGGCATGGGTTTC	CGGCAAATAGAAGGAAGACGTA
18S rRNA	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT

The approximate primer melting temperature (T^m) for the primers can be calculated using the following equation based on the nucleotide content of the primer(s) $T^m = 4 (G + C) + 2 (A + T)$

2.5 IRS-1 and GLUT-4 Immunohistochemical Analysis

Immunohistochemistry examination was performed on anterior thigh of skeletal muscle [15].

2.6 Determination of Histone Deacetylase (HDAC) Enzyme Activity

The activity of HDAC was measured in blood using a colorimetric assay kit (BioVision, kit number K331-100) [17].

2.7 Biochemical Assays

- Diabetic biomarkers include serum glucose [18], insulin level [19]., Insulin resistance was estimated by calculation of homeostasis model assessment (HOMA-IR) [20], β -cells function [21] and Glycated hemoglobin (HbA1c) in whole blood [22].
- Diabetic nephropathy biomarkers include serum urea [23], uric acid [24], renal advanced glycation end products (AGE) [25] and renal aldose reductase (AR) activity [26].
- Inflammatory biomarkers include serum interleukin-6 (IL-6) [27] and tumor necrosis factor- α (TNF- α) [28].
- Antioxidant and oxidative stress biomarkers include renal Superoxide dismutase (SOD) and Glutathione peroxidase (GPX) enzyme activities [29,30] as well as renal malondialdehyde concentration (MDA) [31] and renal nitric oxide (NO) [32].

2.8 Statistical Analysis

Data were statistically analyzed by Statistical Package for Social Science (SPSS) version 17.0 statistical packages. Values were presented as mean \pm Standard Deviation (SD). Statistical differences between groups were performed using one way ANOVA, the mean difference was significant at the ($P=0.05$) [33].

3. RESULTS

3.1 Characterization of QNPs Using HR-TEM

From the Fig. (2), it can be noted that the mean diameter of QNPs was 91 ± 0.8 nm and had a nano rod and smooth surface.

3.2 Effects of Tested Treatment on Nephropathy Biomarkers

Considering the results of GFR at zero time (Table 2) before consuming oral doses of tested treatment, it is clear that induction of DN in hamsters resulted in significant reduction in the values of GFR compared with G1 at ($P=0.05$). While after 8 weeks of treatment, the results of GFR was a significant ($P=0.05$) decrease in DN untreated group (G2) by 86.18% when compared with healthy control hamsters. In contrast, DN hamsters in G7 which consumed oral doses of QNPs with metformin, showed a significant increment in GFR levels by 291.49% compared with G2. Also, a statistically significant increase in GFR was observed in all treated groups. In the same way, the results of DN untreated hamsters show a significant increase ($P = 0.05$) in serum creatinine and decrease in urine creatinine levels as compared with G1. However, DN hamsters in all treated groups caused significant improvement in these biomarkers. QNPs + metformin group (G7) showed most improvement when compared with the untreated group.

3.3 Effects of Tested Treatment on Muscular IRS-1 and GLUT-4 Gene Expression and Immunohistochemical Measurements

Regarding IRS-1 and GLUT-4 gene expression, Table (3) illustrated that there was a highly significant decrease in the expression of these genes in DN untreated group. Consumption of oral doses of QNPs with metformin (G7) showed an increase in IRS-1 and GLUT-4 gene expression by 618.18% and 2533.33%,

respectively followed by G6 then G3 as compared to G2. Results of IRS-1 and GLUT-4 gene expression were significantly confirmed by immunohistochemical analysis as shown in Figs (3,4), where DN received phytotherapy with metformin in G6 and G7 achieved a highly expression in both of IRS-1 and GLUT-4 (score 3, +++).

3.4 Effects of Tested Treatment on Histone Deacetylase Enzyme Activity

The results presented in Table (4) show the activity of HDACs enzymes in G2 was found to be significantly ($P=0.05$) elevated (by 106.8%) following DN induction. However, in all treated groups the inhibition of HDAC was significantly ($P=0.05$) achieved as were compared to the untreated one. The most effective treatment was G7 (37.5% inhibition), closely followed by G6 at 28.58%, then G3 by trailed with 22.45% and finally, G4 by 8.52% after the 17.43% reduction in these enzymes' levels in G5.

3.5 Effects of Tested Treatment on Diabetic Biomarkers

As shown in Table (5), induction of DN found to cause extremely statistically significant ($P=0.05$) elevation in blood glucose with corresponding hypoinsulinemia in DN group compared to the healthy control group. On the other hand, all treated groups showed a reversal effect on hyperglycemia and hypoinsulinemia. Remarkably, the greatest improvement in glucose and insulin level was found in hamsters consumed QNPs plus metformin (101.47 ± 2.6 mg/dl and $10.66 \pm 0.17 \mu\text{IU/m}$) followed by group that given native quercetin plus metformin then treatment with metformin alone. Also, it is clear that administration of QNPs show more effect

than native quercetin in DN treated hamsters which indicates the superiority of using nanoformulation over native candidate. In the same way our results revealed that oral treatment with native or nano quercetin with metformin brought back HOMA-IR, HbA1c and β -cells function to near normal levels.

3.6 Effects of Tested Treatment on Diabetic Nephropathy Biomarkers

DN untreated group exerted a marked increment in blood levels of urea and uric acid, renal AGEs and renal AR activity by 366.16%, 283.41%, 184.45% and 174.91%, respectively compared to G2. Whereas, there was a significant reduction in diabetic nephropathy biomarkers in all treated groups with tested dose. Noticeably, these levels were reduced by the administration of quercetin in native or nano form with metformin (G6, G7) to almost in range values (Table 6).

3.7 Effects of Tested Treatment on Inflammatory Biomarkers

Our results in Table 7 confirmed that epigenetic alteration that correlated with defect in renal function was also accompanied by inflammation. The level of proinflammatory cytokines; IL-6 and TNF- α were increased in DN untreated group by 129.87% and 616.7%, respectively as compared to healthy group. While treated groups with oral doses of native or nano quercetin with or without metformin cause diminished in these inflammatory biomarkers. The overwhelming consensus of the superiority of QNPs + metformin to the other treatments were soundly confirmed by their effects on these inflammatory biomarkers.

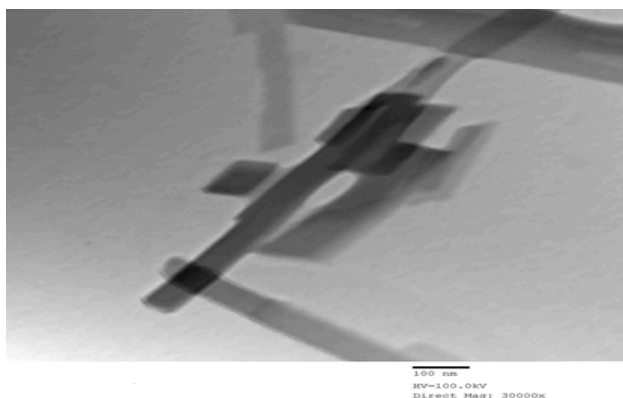


Fig. 2. HR-TEM of QNPs showing the morphology and average diameter (91 ± 0.8 nm)

Table 2. Oral effects of tested treatment on nephropathy biomarkers

	GFR at zero time (ml/g/day)	GFR after 4 weeks (ml/g/day)	GFR after 8 weeks (ml/g/day)	Serum creatinine (mg/dl)	Urine creatinine (g/day)
G1	a 3.65±0.20	a 3.59±0.15	a 3.40±0.17	g 0.45±0.1	a 1.23±0.025
G2	b 2.68±0.12	g 1.28±0.098	g 0.47±0.021	a 3.89±0.04	g 0.26±0.019
G3	b 2.68±0.25	d 2.59±0.093	d 1.53±0.039	d 1.57±0.08	d 0.74±0.025
G4	b 2.75±0.14	f 1.44±0.048	f 0.81±0.044	b 2.71±0.09	f 0.45±0.019
G5	b 2.64±0.27	e 1.87±0.097	e 1.07±0.09	c 2.13±0.06	e 0.60±0.02
G6	b 2.70±0.17	c 3.30±0.053	c 1.75±0.026	e 1.29±0.03	c 0.80±0.015
G7	b 2.69±0.22	b 3.43±0.063	b 1.84±0.042	f 1.18±0.02	b 0.96±0.017
LSD	0.26	0.12	0.10	0.108	0.013

* There was no significant difference between means have the same letter in the same column (P =.05). Where the letter (a,b,c,...) refer to the descending order of values; a the Highest value and g the lowest one

Table 3. Oral effects of tested treatment on muscular IRS-1 and GLUT-4 gene expression

	Relative gene expression of muscular IRS-1 (IRS-1 /18S rRNA)	Relative gene expression of muscular GLUT-4 (GLUT-4/ 18S rRNA)
G1	a 1.04±0.07	a 1.06±0.08
G2	g 0.11±0.02	g 0.03±0.003
G3	d 0.50±0.03	d 0.48±0.02
G4	f 0.28±0.02	f 0.16±0.02
G5	e 0.37±0.02	e 0.32±0.02
G6	c 0.61±0.03	c 0.54±0.02
G7	b 0.79±0.03	b 0.79±0.03
LSD	0.041	0.041

* There was no significant difference between means have the same letter in the same column (P =.05). Where the letter (a,b,c,...) refer to the descending order of values; a the Highest value and g the lowest one

Table 4. Oral effects of tested treatment on Histone Deacetylase enzyme activity

	HDAC activity (µmol/ ml)
G1	g 90.53±3.57
G2	a 187.24±2.94
G3	d 145.19±2.62
G4	b 170.72±1.54
G5	c 154.61±3.13
G6	e 133.72± 3.50
G7	f 116.99±2.76
LSD	3.79

* There was no significant difference between means have the same letter in the same column (P =.05). Where the letter (a,b,c,...) refer to the descending order of values; a the Highest value and g the lowest one

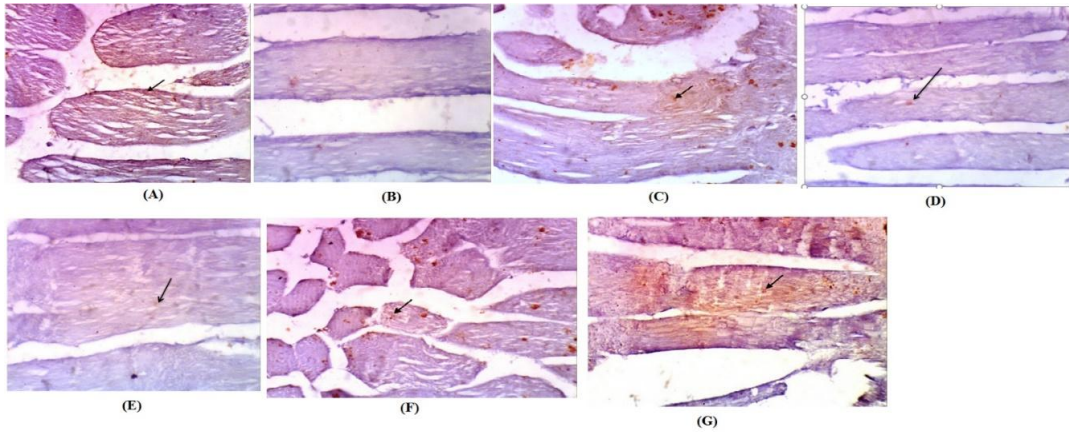


Fig. 3. Immunohistochemical staining of IRS-1 in skeletal muscles (X400)

- (A) : G1 showing strong positive expression of IRS-1
- (B) : G2 showing no expression of IRS-1
- (C) : G3 showing moderate positive expression of IRS-1 (score 2,++)
- (D) :G4 showing a weak positive expression of IRS-1 (score 1,+)
- (E) : G5 showing moderate positive expression of IRS-1 (score 2,++)
- (F) : G6 showing strong positive expression of IRS-1 (score 3,+++)
- (G) : G7 showing strong positive expression of IRS-1(score 3,+++)

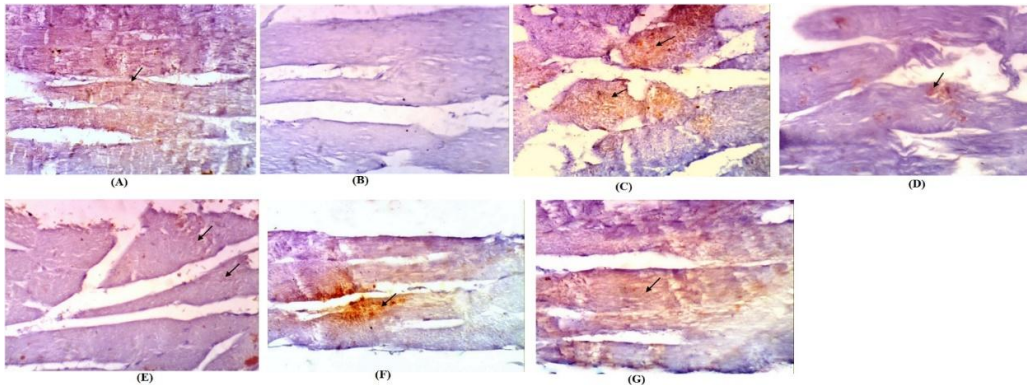


Fig. 3. Immunohistochemical staining of GLUT-4 in skeletal muscles (X400)

- (A): G1 showing strong positive expression of GLUT-4
- (B): G2 showing no expression of GLUT-4
- (C): G3 showing moderate positive expression of GLUT-4 (score2,++)
- (D): G4 showing a weak positive expression of GLUT-4 (score1,+)
- (E): G5 showing moderate positive expression of GLUT-4 (score2,++)
- (F): G6 showing strong positive expression of GLUT-4 (score3,+++)
- (G): G7 showing strong positive expression of GLUT-4 (score3,+++)

3.8 Effects of Tested Treatment on Antioxidant and Oxidative Stress Biomarkers

Antioxidant status was evaluated in the present study by determination of enzymatic biomarker SOD and GPX activity in kidney as target tissue, and is presented in Table (8) DN untreated group showed a statistically significant (P=.05)

decrease level of renal SOD and GPx activities by 58.61 % and 59.11 %, respectively whereas increase in oxidative stress status presented by increased renal MDA as lipid peroxidation biomarker and NO by 342.07 % and 123.38 %, respectively when compared with healthy group. Concisely, the results indicated increased antioxidant status and reduced oxidative stress status with consumption of oral doses of native

or nano quercetin with and without metformin by abolishing the accumulation of renal MDA and NO, accompanied by restoration in the activities of the antioxidant enzyme of renal SOD and GPx as compared with their corresponding untreated group. Remarkably, the greatest increment in renal tissue antioxidant enzyme activities was

found in hamsters consumed QNPs plus metformin followed by group that given native quercetin plus metformin then treatment with metformin alone. Also, it is clear that administration of QNPs show more effect than native quercetin in DN treated hamsters.

Table 5. Oral effects of tested treatment on diabetic biomarkers

	Glucose (mg/dl)	Insulin (μ lU/ml)	HOMA-IR	β -cells function	Glycated hemoglobin (HbA1c)(%)
G1	g 79.28 \pm 1.33	a 13.11 \pm 0.19	f 2.56 \pm 0.05	a 56.08 \pm 1.25	g 5.74 \pm 0.076
G2	a 301.07 \pm 6.74	g 6.05 \pm 0.18	a 4.50 \pm 0.12	g 3.74 \pm 0.33	a 28.38 \pm 0.48
G3	d 123.10 \pm 1.34	d 9.65 \pm 0.09	c 2.93 \pm 0.04	d 24.73 \pm 0.40	d 10.85 \pm 0.082
G4	b 162.90 \pm 2.55	f 7.24 \pm 0.13	c 2.91 \pm 0.06	f 12.50 \pm 0.41	b 15.54 \pm 0.17
G5	c 148.5 \pm 4.35	e 8.38 \pm 0.14	b 3.07 \pm 0.11	e 16.82 \pm 0.65	c 13.79 \pm 0.055
G6	e 114.45 \pm 1.79	c 9.85 \pm 0.07	d 2.78 \pm 0.04	c 27.5 \pm 0.57	e 9.72 \pm 0.06
G7	f 101.47 \pm 2.6	b 10.66 \pm 0.17	e 2.62 \pm 0.06	b 34.35 \pm 1.38	f 9.11 \pm 0.18
LSD	4.48	0.59	0.091	1.055	0.274

* There was no significant difference between means have the same letter in the same column ($P = .05$) Where the letter (a,b,c,...) refer to the descending order of values; a the Highest value and g the lowest one

Table 6. Oral effects of tested treatment on diabetic nephropathy

	Urea (mg/dl)	Uric acid (mg/dl)	Renal AGEs (μ g/g. tissue)	Renal AR activity (U/g. tissue)
G1	g 15.34 \pm 1.04	g 1.99 \pm 0.23	g 39.51 \pm 0.63	g 2.75 \pm 0.036
G2	a 71.51 \pm 0.26	a 7.63 \pm 0.13	a 112.39 \pm 1.17	a 7.56 \pm 0.23
G3	d 40.97 \pm 0.37	d 3.96 \pm 0.072	d 65.31 \pm 0.80	d 4.87 \pm 0.04
G4	b 45.01 \pm 0.61	b 5.34 \pm 0.18	b 80.69 \pm 1.07	b 6.66 \pm 0.19
G5	c 42.80 \pm 0.33	c 4.43 \pm 0.059	c 73.02 \pm 1.07	c 5.47 \pm 0.06
G6	e 37.43 \pm 0.26	e 3.75 \pm 0.072	e 58.82 \pm 0.84	e 3.96 \pm 0.05
G7	f 33.67 \pm 0.35	f 3.30 \pm 0.108	f 50.59 \pm 0.82	f 3.42 \pm 0.03
LSD	0.68	0.147	1.202	0.153

* There was no significant difference between means have the same letter in the same column ($P = .05$). Where the letter (a,b,c,...) refer to the descending order of values; a the Highest value and g the lowest one

4. DISCUSSION

DN, a major metabolic disorder, is one of the most prevalent chronic diseases around the globe which posing grave challenges to public health worldwide. Therefore, researchers direct their effort to find an ideal antidiabetic agent from a natural source which has both hypoglycemic

and antioxidant activities to cure diabetes and its complication. Traditional antidiabetic drugs, including insulin and synthetic oral hypoglycemic agents as metformin tend to pose a lot of clinical challenges. On the other hand, some natural herbal remedy found to offer nearly same effects without so many side effects [1].

Researchers have demonstrated the pivotal role of quercetin in prevention of STZ-induced β -cell dysfunction/death, this effect could be due to its antioxidant activity and its ability to scavenge free radicals [10]. However, the hypoglycemic effect of native quercetin still conflicting due to its low aqueous solubility, poor bioavailability and extensive excretion [7]. Our present study represents the diversion of native quercetin into nanoparticles for oral use to overcome its poor solubility/bioavailability and in turn enhances its therapeutic efficacy.

As shown in present results of GFR, at zero time before any treatment, signs of renal glomerular injury was observed in DN hamsters, which could be justified by reduced GFR with azotemia as well as changes within the glomerular filtration

barrier, the decrease in GFR was due to increase blood creatinine which inversely proportional with GFR level as well as due to decrease urine creatinine level. On the other hand, after 4 and 8 weeks of consuming the oral doses of native or nano quercetin with and without metformin, it might be able to ameliorate renal function and GFR by decreasing blood creatinine levels especially in the group consumed QNPs with metformin as the dissolution and solubility of QNPs was enhanced compared with native one. Our results confirmed the result of Gomes et al., who illustrated the protective effects of oral dose of quercetin on kidney function in DN mice, in which the DN mice exhibited impairment of renal clearance compared with healthy control animals while DN mice treated with quercetin, clearance returned nearly to baseline levels. They

Table 7. Oral effects of tested treatment on inflammatory biomarkers

	IL-6 (pg/ml)	TNF- α (pg/ml)
G1	g 115.42 \pm 2.8	g 8.43 \pm 0.32
G2	a 265.32 \pm 3.2	a 60.42 \pm 0.61
G3	d 175.33 \pm 1.86	d 31.49 \pm 0.44
G4	b 224.94 \pm 1.84	b 50.58 \pm 0.55
G5	c 198.50 \pm 1.65	c 40.21 \pm 0.67
G6	e 154.71 \pm 3.98	e 19.29 \pm 0.33
G7	f 139.62 \pm 2.68	f 9.75 \pm 0.33
LSD	3.42	0.623

* There was no significant difference between means have the same letter in the same column ($P = .05$). Where the letter (a,b,c,...) refer to the descending order of values; a the Highest value and g the lowest one

Table 8. Oral effects of tested treatment on antioxidant and oxidative stress biomarkers

	Renal tissue SOD activity (U/g.tissue)	Renal tissue GPx activity (U /g.tissue)	Renal tissue NO (μ mol/g.tissue)	Renal tissue MDA (nmol/g.tissue)
G1	a 15.56 \pm 0.18	a 7.90 \pm 0.04	g 3.85 \pm 0.12	g 16.55 \pm 0.13
G2	g 6.44 \pm 0.17	g 3.23 \pm 0.14	a 17.02 \pm 0.18	a 36.97 \pm 0.81
G3	d 11.01 \pm 0.16	d 5.21 \pm 0.077	d 8.81 \pm 0.16	d 25.82 \pm 0.75
G4	f 8.65 \pm 0.21	f 3.93 \pm 0.11	b 12.35 \pm 0.18	b 30.74 \pm 0.40
G5	e 9.81 \pm 0.15	e 4.61 \pm 0.036	c 9.85 \pm 0.11	c 29.13 \pm 0.71
G6	c 12.77 \pm 0.26	c 5.83 \pm 0.076	e 6.80 \pm 0.41	e 21.05 \pm 0.76
G7	b 13.41 \pm 0.23	b 6.58 \pm 0.044	f 6.21 \pm 0.32	f 18.87 \pm 0.54
LSD	0.258	0.108	0.306	0.812

* There was no significant difference between means have the same letter in the same column ($P = .05$). Where the letter (a,b,c,...) refer to the descending order of values; a the Highest value and g the lowest one

explained that; this bioflavonoid exhibited marked beneficial effects on renal function as indicated by the significant decrease of blood creatinine and restoration of the clearance of creatinine [34].

In our finding, the quercetin treated groups either native or nano with metformin showed a significant increase in IRS-1 and GLUT-4 expression level. However, the effects of QNPs have more effect than native quercetin that due to its high solubility and long circulation time than its original form. These results demonstrate that the flavonoid quercetin enhances glucose uptake and ameliorates hyperglycemic effect via GLUT-4 expression. Thus, quercetin supplementation potentially can lower the complications by controlling hyperglycemia and by inducing expression of GLUT-4 via mRNA expression and translocation to the plasma membrane, which may be very useful in the management of DM and DN. The expression of GLUT-4 in the skeletal muscles is regulated by metabolism and insulin, thus any dysfunctioning of pancreatic β -cells as the case occur in diabetes causes impaired expression of GLUT-4. In addition, quercetin improves insulin sensitivity by upregulating expressions of IR and glucose transporter as well as by promoting metabolism of glucose in peripheral tissues [35]. The IRS-1 and GLUT-4 immunohistochemistry data were in accordance with the protein expression results as previously described, that in agreement with Cai et al., [15] who illustrated that the immunohistochemical staining of IRS-1 and GLUT-4 in skeletal tissues were completely stained in healthy group, with exception of the diabetic untreated group, in which IRS-1 was weakly visible staining and weakly expressed.

In the present study, the untreated DN group showed up-regulation of HDACs while consuming QNPs with metformin was simultaneously effective at inhibiting HDAC activity. The changes in the enzyme activity correlated with the levels of blood glucose and insulin as observed in G7 where the most pronounced inhibition of HDACs activity mirrored the most increment in IRS-1 and GLUT-4 and hence the most reduction in blood glucose and HbA1c levels in addition to the highest insulin and improve other diabetic biomarkers. This study showed that quercetin either native or nano form possess an inhibitory effect on HDACs activity thereby offering a justification to its established antidiabetic effect. Therefore, their epigenetic modifying abilities were suggested as

the main mechanism behind their anti-diabetic effect.

The mechanism for increasing HDACs enzyme activity in diabetic animals was explained by the potent oxidative stress inducer H_2O_2 that increase HDAC activity, which may be an underlying mechanism in the pathogenesis of DN. Also, HDAC is regarded as a contributor to podocyte injury in diabetic animals and could suppress autophagy related with podocyte injury in DN, suggesting that HDAC is important to accelerate DN in epigenetic and nonepigenetic mechanisms. Also, a pathogenic role for aberrant HDAC activity in DN, by promoting histone acetylation and repression of genes of various signaling pathways, such as pro-inflammatory, pro-fibrotic, and antioxidant pathways [3].

In addition, impaired blood levels of urea and creatinine in untreated group indicate that the kidney is damaged or not properly functioning, which are correlated with the continually increment in blood sugar level. These observations are clearly indications of the development of DN as it is characterized by progressive renal insufficiency; and hyperglycemia is thought to be mediating these injuries through induction of metabolic and biochemical changes due to the increased ROS, NO production, oxidation and glycation of proteins as well as impaired body antioxidant system as it is cleared from our results. Also, the increased levels of glucose in DN lead to significant increase in the activity of renal AR in addition to allow excess formation of AGEs which leads to the generation of free radicals and hence damaging kidney tissues. Improvement of these biochemical variables in DN hamsters by quercetin supplementation suggests that quercetin plays a role, either directly or indirectly, in providing protection against DN or delay in its development. Our results show that quercetin prevented the development of DN by significantly lowering blood urea and uric acids in DN animals through increased their clearance by the kidney. In addition, quercetin decreased the early stage of glycation as evidenced by the low levels of HbA1c in DN treated groups Which illustrated before in diabetic biomarkers (Table 5). As quercetin strongly inhibited the Post-Amadori glycation suggesting that quercetin target almost all the stages of AGE production formation so improved blood glucose level and enhanced antioxidant system and subsequently lowered the activity of aldose reductase. On the other hand, the use of QNPs exerted not just similar, but

superior effects than their native counterparts as shortly demonstrated thereafter.

Quercetin inhibits AGEs formation by trapping methylglyoxal (MGO) and glyoxal (GO) which are reactive dicarbonyl compounds responsible for albumin glycation and hence precursors of AGEs that have been associated with diabetes-related long-term complication. The ability of quercetin to trap MGO and GO is directly and efficiently under physiological conditions [36]. In addition, the protective effects and treating action of oral dose of quercetin (20 mg/kg/day) on sorbitol level in diabetic rats for 8 weeks, due to reduction of aldose reductase activity, an enzyme which catalyzes the conversion of glucose to sorbitol. Quercetin as aldose reductase inhibitor are currently the most commonly used oral agents for their good penetrations through cellular membranes and fast metabolism of sorbitol by sorbitol dehydrogenase [37].

formation of AGEs leading to increased oxidative stress resulting in increased production of inflammatory cytokines. DN is associated with prolonged inflammation of the kidney tissues and this may reflect the increased blood levels of some inflammatory cytokines such as IL-6 and TNF- α . A decrease in endogenous TNF- α production in the presence of quercetin indicates that flavonoids have the capacity to modulate the immune response and have potential anti-inflammatory activity. Also, the previously expressed dominance of QNPs were shown to be more effective than native one in improving these biomarkers. Quercetin-induced suppression of TNF- α can result in the stimulation of anti-inflammatory cytokines via inhibiting the activation of NF- κ B, and therefore, one can anticipate that quercetin could be widely used as an anti-TNF- α therapy [38]. The inflammatory molecules, including TNF- α and IL-6 are upregulated in insulin-resistant state. TNF- α and IL-6 impair insulin signaling by suppressing the expressions of proteins such as IRS-1 and GLUT-4 and this theory explained by that TNF- α and IL-6 affect intracellular insulin signaling in fat, skeletal muscle, endothelial cells and other insulin-responsive tissues by inhibiting kinase activities in the insulin-signaling pathway. TNF- α reduces insulin-stimulated receptor tyrosine kinase activity at low concentrations and can also decrease the expression of IRS-1 and GLUT-4 at higher concentrations as well as its ability to augment the phosphorylation of serine or threonine residue in IRS-1. Altered IRS phosphorylation on serine or threonine reduces

the IRS phosphorylation of tyrosine residues through protein kinase C and NF- κ B, thus impairing its ability to bind to the insulin receptor and initiate downstream signaling. Circulating IL-6 levels are also increased in insulin-resistant states and type2DM. Thus, TNF- α and IL-6 play an important role in insulin resistance and the vascular inflammation process through its multiple actions [39].

Also, Hyperglycemia lead to increased oxidative stress and decrease in the activities of antioxidant enzymes as SOD and GPx in the kidney tissues of diabetic animals as a result of production of H₂O₂ or by glycation and AGEs formation. In the present study, the improvement of antioxidant profiles within kidney tissue by our treatments especially QNPs plus metformin by prevented damage caused by accumulation of ROS due to the anti-free radical activity of quercetin as well as the action of drug metformin on enhancing utilization of glucose.

Quercetin has a free radical scavenger, transfer electrons, chelate metals and superoxide radical inhibitor properties. Beneficial effects of quercetin are attributed to its antioxidant effects as well as protective effects on β -cells [10]. The oxidative stress and metabolic dysregulation of free fatty acids in DN condition alleviated by quercetin administration in high fat diet/STZ-induced diabetic rats. Oxidative stress status was assessed by measuring the MDA formation in diabetic control while MDA formation was suppressed by quercetin as assessed by increasing SOD activity and blocking MDA [35].

5. CONCLUSION

From the present study, our results shed light on the potential use of QNPs act as a modulator tool for alleviating the high risk of DM complications due to their abilities to modulate numerous biochemical pathways. QNPs exhibited anti-diabetic effect through a number of mechanisms; improving oxidative stress status and inflammatory biomarkers. Furthermore, nano-phytochemical treatment improves complication of diabetic disease through improving diabetic biomarkers, gene expression of IRS-1 and GLUT-4, epigenetic alteration as well as correct the abnormalities in kidney function tests in DN animals. The present study illustrated that using nanotechnology in DN development by enhancing the biochemical applications and therapeutic index of bioactive molecules by improving intracellular penetration and

bioavailability. In simple words the findings of this research work demonstrate the bio-powerful effects of consuming QNPs for improving the quality of life towards the chronic complex diseases such as DN.

CONSENT

It's not applicable

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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