

Modulation of aquaporins expressions and its impact on alleviating diabetic complications in rats: role of *Citrullus colocynthis*

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ABSTRACT

Background: Aquaporins (AQPs) belong to a class of small, integral membrane proteins that enable water transfer across the plasma membranes of cells as a reaction to osmotic gradients. There are 13 known types of AQPs in humans (AQP0 to AQP12), which are expressed in various organs such as the kidney, brain, liver, lungs, and salivary glands. In patient with diabetes mellitus, a reduction in salivary secretion is noted in concurrence with low salivary AQP5 levels. The *Citrullus colocynthis* (CC) extracts were reported to have beneficial effects on glucose homeostasis and body weight maintenance, in either alloxan- or streptozotocin (STZ)-induced diabetic rats through exerting insulinotropic effect. **Aims and Objective:** To check the changes in AQPs expression and to understand the antidiabetic effect of CC as a medicinal herb in STZ-induced diabetic rats. **Materials and Methods:** Forty-two male *Wistar* rats were assigned equally into six groups. The first and second groups were kept as control groups: the first remained without treatment, but the second received CC orally. Diabetes was induced by a single intraperitoneal (i.p) injection of STZ (60 mg/kg b.w.) in the remaining four groups. Three diabetic groups were treated with insulin or CC or both together for 4 weeks. The remaining diabetic group was left untreated as control diabetic group. Blood and organs were taken for biochemical, gene expression, and immunohistochemistry examinations. **Result:** Diabetic rats displayed increases in blood glucose, lipid profile, liver, and kidney parameters, which were restored significantly to normal with insulin or CC or both. At molecular level, diabetic rats showed significant downregulation in mRNA expressions of antioxidant enzymes, insulin receptor substrate 1 and 2, pyruvate kinase, GLUT-2 in liver and of AQP1, AQP5, AQP7, and AQP8 expressions in kidney, salivary gland, and pancreas, respectively, and normalized significantly by insulin or CC or in combination administrations. Expressions of phosphoenol pyruvate carboxykinase in liver and of AQP2 and AQP3 in kidney were upregulated in diabetic rats and returned to normal completely by insulin alone or CC or their combination. Values were statistically significant at $p < 0.05$. Immunohistochemical examinations revealed a downregulation of insulin and GLUT-2 in pancreas and upregulation of AQP2 in kidney of diabetic rats, but treatments with CC augmented insulin effect and restored their expression to normal levels. **Conclusion:** Current results concluded that AQPs could be a promising target for drug development. Moreover,

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Citrullus colocynthis extract acts as antidiabetic medicinal herb, which has been confirmed by its molecular and immunohistochemical effects.

KEY WORDS: Diabetes mellitus; Streptozotocin; Aquaporins; *Citrullus colocynthis*; Polyuria, Antioxidants

INTRODUCTION

Diabetes mellitus (DM) is a worldwide epidemic with 382 million people affected by the disease, which is expected to rise to 582 million by 2035.^[1] Type 1 DM is defined as a metabolic disease characterized by chronic hyperglycemia and abnormal lipid and protein metabolism resulting from absolute insulin deficiency.^[2] The long-term damage to and dysfunction and failure of various tissues, particularly eyes, kidneys, nerves, heart, liver, and blood vessels are linked to the chronic hyperglycemic condition of diabetes.^[3] Oxidative stress is significantly increased in diabetes because prolonged hyperglycemia increases the generation of reactive oxygen species (ROS) and the depletion of the endogenous antioxidant system.^[3] Polyuria is a common complaint presented in most diabetic patients, which is believed to be the result of osmotic diuresis produced by hyperglycemia.^[4] Sustained hyperglycemia increases the glucose in the glomerular filtrate, and glucose that has not been reabsorbed through the proximal tubule appears in the urine as glucosuria.^[5] Glucosuria induces an elevation in urinary osmotic pressure, which reduces renal water reabsorption, leading to polyuria. Several researches report that urine concentration and metabolic disorder presented under diabetic condition is caused by aquaporins (AQPs) family.^[4]

AQPs belong to a class of small, integral membrane proteins that enable water transfer across the plasma membranes of cells as a reaction to osmotic gradients.^[6] To date, there are 13 known types of aquaporins in humans (AQP0 to AQP12), which are expressed in various organs such as the kidney, brain, liver, lungs, and salivary glands.^[7] Eight AQPs are expressed in the kidney of which five have been shown to play a role in body water balance: AQP1, AQP2, AQP3, AQP4, and AQP7.^[8] AQP5 is highly expressed in salivary glands and plays a critical role by contributing to salivary secretion.^[9] Xerostomia, commonly known as dry mouth, can result owing to diabetes. In patients with diabetes mellitus, a reduction in salivary secretion is noted in concurrence with low salivary AQP5 levels.^[10] Aquaglyceroporin (AQP) 7, one of the aquaglyceroporins, which is a subfamily of aquaporins, is permeable not only to water but also to small solutes such as glycerol and urea. AQP7 is expressed in pancreatic β -cells and recently described as being involved in the regulation of insulin secretion and release by allowing the entry or exit of glycerol and by acting directly or indirectly at a distal downstream site in the insulin exocytosis pathway.^[11]

Increased interest in alternative medicine and natural therapies is owing to their lesser side effects compared with serious complications associated with oral hypoglycemic agents and insulin.^[12] The *Citrullus colocynthis* (CC), also known as bitter apple, is traditionally used in Mediterranean countries as

infusions to treat diabetes and hemorrhoids and acts as purgative and antiinflammatory agents.^[13] CC extracts were reported to exhibit beneficial effects on glucose homeostasis and body weight maintenance, in either alloxan- or streptozotocin (STZ)-induced diabetic rats through exerting insulinotropic effect.^[14]

Therefore, this study was designed to examine, first, whether experimental DM is associated with changes in the expression of AQP levels in kidney, salivary gland, and pancreas and whether the changes in AQPs expression are associated with compensation of diabetes complications such as polyuria, dehydration, and xerostomia; and second, to determine the possible protective effect of CC extract on biochemical alterations, molecular changes, and immunohistochemical modulations in STZ-induced diabetic rats.

MATERIALS AND METHODS

Materials

Male Wistar rats were purchased from King Fahd Institute for Scientific Research, King Abdul Aziz University, Saudi Arabia. STZ was obtained from Sigma Aldrich, USA. Oligo dT, chloroform, ethanol, and aquaporin primers were from Wako Pure chemicals, Osaka, Japan. TriZol reagent was from Invitrogen, Carlsbad, CA. Commercial diagnostic kits from Human Diagnostics (Wiesbaden, Germany) were used for the determination of plasma creatinine and urea, and those from United Diagnostic Industry (Dammam, Saudi Arabia) were used for the determination of cholesterol, triglycerides (TGs), and high-density lipoprotein (HDL).

Animals

Forty-two male Wistar rats, 6 weeks old, weighing (150–200 g) were housed under conditions of controlled temperature ($25 \pm 2^\circ\text{C}$) with a 12-h/12-h day–night cycle in Medical Laboratory Department, College of Applied Medical Science, Turabah, Taif University. Animals gained free access to food and water ad libitum. The experiment was conducted on January 2016. All procedures were approved by the Animal Care Committee of Taif University for the project #4162/1436/1.

Preparation of Aqueous Extract of *Citrullus colocynthis* Seeds

C. colocynthis fruits were obtained from the local market. The black seeds of *C. colocynthis* were separated manually from the pulp of the dried fruit, were grounded into powder, and then defatted with hexane for 3 h. The remaining powder was infused for 1 h at 50°C and extraction under reflux for a further

15 min. The preparation was filtered and centrifuged, and the supernatant clear brownish liquid thus obtained was being conserved at +4°C until use.^[15]

Induction of Experimental Diabetes

Twenty-eight adult Wistar rats weighing 250–300 g (75–90 days old) were used for inducing diabetes. Diabetes was induced by a single intraperitoneal (i.p) injection of a freshly prepared solution of STZ (60 mg/kg b. w.) in 0.1 M cold citrate buffer (pH 4.5) to the overnight fasted rats.^[16] Control rats (n = 14) were injected with an equivalent amounts of citrate buffer alone. After injection, rats were allowed to drink 5% glucose solution overnight to counteract hypoglycemic shock. After 3 days, fasting blood glucose levels were checked from tail blood sample (Accu-Chek Aviva BG Meter; Roche Diagnostics, Indianapolis, IN). Rats showing blood glucose values above 250 mg/dL on the third day after STZ injection were considered as diabetic rats. Then, treatments were started on the third day after STZ injection, and it was considered as the first day of treatment.

Experimental Design

The experimental rats were divided into six groups (seven rats per group): (1) normal control group (N group), gained free access to food and water; (2) *C. colocynthis* control group (CC group), was administered orally aqueous extract of CC (300 mg/kg b.w.) using an intragastric tube daily for 4 successive weeks^[17]; (3) diabetic group (D group), did not receive any medication with free access to food and water; (4) diabetic insulin-treated group (D + insulin group), was injected subcutaneously with insulin (1 U/kg b.w.) (NPH Novolin N; Novo Nordisk, Bagsvaerd, Denmark) daily for 4 successive weeks^[18]; (5) diabetic CC-treated group (D + CC group), received orally aqueous extract of CC seeds daily by an intragastric tube for 4 successive weeks at a daily dose of 300 mg/kg b.w.^[19]; (6) diabetic insulin- and CC-treated group (D + insulin + CC group), received orally aqueous extract of CC seeds (300 mg/kg b.w.) by an intragastric tube^[19] and injected subcutaneously with insulin (1 U/kg b.w.)^[18] daily for 4 successive weeks. Random blood glucose levels were measured from tail blood every week to assure diabetic incidence. At the end of the experiment, rats were fasted overnight and anesthetized with diethyl ether inhalation; blood was collected from the inner canthus of the eyes to get serum. After that animals were killed, small parts of liver, kidney, pancreas, and salivary gland were immediately immersed in TriZol reagent and kept at -70°C until RNA extraction. Other parts of pancreas and kidney were preserved in 10% buffered neutral formalin for immunohistochemical examination.

Biochemical Analysis

Serum concentrations of glucose, TGs, total cholesterol (TC), HDL, creatinine, urea, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured spectrophotometrically using specific commercial kits (HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) according to manufacturer instructions.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from liver, kidney, pancreas, and salivary gland tissue samples (approximately 50 mg per sample) of experimental rats. Samples were flash frozen in liquid nitrogen and subsequently stored at -70°C in 1 mL Trizol (QIAGEN, Inc., Valencia, CA). Frozen samples were homogenized using a Polytron 300 D homogenizer (Brinkman Instruments, Westbury, NY). Then, 0.3 mL chloroform was added to the homogenate. The mixtures were shook for 30 s, followed by centrifugation at 4°C and 12,500 rpm for 20 min. The supernatant was transferred to a new set of tubes, and an equal volume of isopropanol was added to the samples, shook for 15 s, and centrifuged at 4°C and 12,500 rpm for 15 min. The RNA pellets were washed with 70% ethanol, briefly dried up, and then dissolved in diethylpyrocarbonate (DEPC) water. RNA concentration and purity were determined spectrophotometrically at 260 nm. The prepared RNA integrity was checked by electrophoresis. The ratio of the 260/280 optical density of all RNA samples was 1.7–1.9.

For cDNA synthesis, a mixture of 3 µg total RNA and 0.5 ng oligo dT primer (QIAGEN) in a total volume of 11 µL sterilized DEPC water was incubated in the Bio-Rad T100TM Thermal Cycler at 65°C for 10 min for denaturation. Then, 2 µL of 10 × RT-buffer, 2 µL of 10 mM dNTPs, and 100 U Moloney murine leukemia virus (M-MuLV) reverse transcriptase (SibEnzyme, Ltd., Ak, Novosibirsk, Russia) were added, and the total volume was made up to 20 µL by DEPC water. The mixture was then reincubated in the Thermal Cycler at 37°C for 1 hour, at 90°C for 10 min, and then preserved at -20°C until used.

Semi-Quantitative RT-PCR Analysis

For semi-quantitative RT-PCR analysis, specific primers for examined genes (Table 1) were designed using Oligo-4 computer program and synthesized by Macrogen (Macrogen Company, Seoul, Korea). PCR was conducted in a final volume of 25 µL consisting of 1 µL cDNA, 1 µL of 10 pM of each primer (forward and reverse), and 12.5 µL PCR master mix (Promega Corporation, Madison, WI); the volume was brought up to 25 µL using sterilized, deionized water. PCR was carried out using Bio-Rad T100TM Thermal Cycler machine with the cycle sequence at 94°C for 5 min of one cycle, followed by variable cycles (stated in Table 1) each of which consisted of denaturation at 94°C for 1 min, annealing at the specific temperature corresponding to each primer (Table 1), and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. As a reference, expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was examined (Table 1). PCR products were electrophorized on 1.5% agarose gel stained with ethidium bromide in Tris-borate-EDTA buffer. PCR products were visualized under UV light and photographed using gel documentation system. The intensities of the bands were quantified densitometrically using Image J software version 1.47 (<http://imagej.en.softonic.com/>).

Immunohistochemical Examinations

Pancreatic tissue and kidney sections were deparaffinized and treated with 3% H₂O₂ for 10 min to inactivate the peroxidases.

Table 1: PCR Conditions of Examined Genes

mRNA expression	Forward primer (5'-3')	Reverse primer (5'-3')	PCR cycles and annealing
PEPCK (236 bp)	TTTACTGGGAAGGCATCGAT	TCGTAGACAAGGGGGCAC	30 cycles, 52°C 1 min
PK (229 bp)	ATTGCTGTGACTGGATCTGC	CCCGCATGATGTTGGTATAG	35 cycles, 52°C 1 min
GLUT-2 (530 bp)	AAGGATCAAAGCCATGTTGG	GGAGACCTTCTGCTCAGTGG	35 cycles, 56°C 1 min
IRS-1 (337 bp)	GCCAATCTTCATCCAGTTGC	CATCGTGAAGAAGGCATAGG	35 cycles, 53.5°C 1 min
IRS-2 (151 bp)	CTACCCACTGAGCCCAAGAG	CCAGGGATGAAGCAGGACTA	35 cycles, 56.5°C 1 min
GPx (406 bp)	AAGGTGCTGCTCATTGAGAATG	CGTCTGGACCTACCAGGAACTT	35 cycles, 57°C 1 min
SOD (410 bp)	AGGATTAACCTGAAGGCGAGCAT	TCTACAGTTAGCAGGCCAGCAG	35 cycles, 55°C 1 min
GST (575 bp)	GCTGGAGTGGAGTTTGAAGAA	GTCCTGACCACGTCAACATAG	35 cycles, 55°C 1 min
AQP1 (232 bp)	CTTACCTCCAGGACCCTTCC	TAGTCACTCCACACGTGCTC	35 cycles, 55.5°C 1 min
AQP2 (198 bp)	GCTGTCAATGCTCTCCACAA	GGAGCAACCGGTGAAATAGA	37 cycles, 55°C 1 min
AQP3 (214 bp)	AGCAGATCTGAGTGGGCAGT	CTTGGGCTTAAGAGGGGAAC	35 cycles, 55.5°C 1 min
AQP4 (191 bp)	CGGTTTCATGGAAACCTCACT	CATGCTGGCTCCGGTATAAT	37 cycles, 55°C 1 min
AQP5 (170 bp)	TCTGGGTAGGCGCTATTGTG	CAGCTCGATGGTCTTCTTCC	31 cycles, 57°C 1 min
AQP7 (212 bp)	ATCCTTGTGTTGCGTTCTTGG	GCGTGAATTAAGCCAGGTA	37 cycles, 55°C 1 min
AQP8 (222 bp)	TGGAACCTGGAACCTCTTGG	AGTACGCATGGACTGGGTTC	37 cycles, 55°C 1 min
GAPDH (309 bp)	AGATCCACAACGGATACATT	TCCCTCAAGATTGTCAGCAA	25 cycles, 52°C 1 min

PCR cycle of respective genes are shown, while temperature and time of denaturation and elongation steps of each PCR cycle were 94°C, 30 s, and 72°C, 60 s, respectively.

Subsequently, the tissue samples were heated in 10 mM citrate buffer at 121°C for 30 min for antigen retrieval and blocked in 5% normal serum for 20 min, and pancreas was incubated with a rabbit polyclonal anti-insulin primary antibody (1:100; sc 9168; Santa Cruz Biotechnology, Inc., Dallas, TX) or GLUT-2 antibody (1:100; sc-9117; Santa Cruz Biotechnology, Inc.). Kidney was incubated with a rabbit polyclonal anti-aquaporin 2 primary antibody (1:100; sc-28629; Santa Cruz Biotechnology, Inc.) in phosphate-buffered saline (PBS) overnight at 4°C. After three extensive washes with PBS, the sections were incubated with a goat antirabbit IgG biotin-conjugated secondary antibody (1:2,000; sc 2040; Santa Cruz Biotechnology, Inc.) for 20 min at 32°C. After further incubation with horseradish peroxidase-labeled streptavidin, antibody binding was visualized using diaminobenzidine, and the sections were counter-stained with hematoxylin.^[20]

Statistical Analysis

Results were shown as means $\pm$ standard error of means (SEM). Data were analyzed using analysis of variance (ANOVA) and post hoc descriptive tests by SPSS software version 11.5 for Windows with $p < 0.05$ regarded as statistically significant. Regression analysis was performed using the same software.

RESULTS

Effect of *Citrullus colocynthis* and Insulin Administration on Biochemical Parameters in Diabetic Rats

As shown in Table 2, diabetic rats showed significant increase ($p < 0.05$) in serum levels of glucose (413.7 ± 10.4), cholesterol (85.7 ± 1.7), TGs (115.3 ± 1.8), AST (101.3 ± 1.2), ALT (59.3 ± 1.5), urea (75.3 ± 2.3), and creatinine (0.73

Table 2: Effect of *Citrullus colocynthis* and insulin administration on biochemical parameters in diabetic rats

Group parameters	Control	CC	Diabetic	Diabetic + insulin	Diabetic + CC	Diabetic + insulin + CC
Glucose (mg/dL)	97.67 $\pm$ 3.4	88.33 $\pm$ 2.3	413.7 $\pm$ 10.4*	161 $\pm$ 17.1**	268.3 $\pm$ 24.6**	85 $\pm$ 6.4**
Cholesterol (mg/dL)	36.4 $\pm$ 1.8	38 $\pm$ 3.5	85.7 $\pm$ 1.7*	52 $\pm$ 3.2**	56.33 $\pm$ 9**	47 $\pm$ 4.7**
Triglycerides (mg/dL)	39 $\pm$ 8.4	46.3 $\pm$ 4.2	115.3 $\pm$ 1.8*	74 $\pm$ 2.1**	51 $\pm$ 3.6**	86 $\pm$ 1.7
HDL (mg/dL)	35.5 $\pm$ 2.1	57.7 $\pm$ 4.1	19 $\pm$ 2.1*	36 $\pm$ 1.7**	46.3 $\pm$ 5.8**	44.3 $\pm$ 2.4**
ALT (U/L)	19.3 $\pm$ 1.5	18.7 $\pm$ 0.9	59.3 $\pm$ 1.5*	25 $\pm$ 1.2**	30.3 $\pm$ 0.9**	20 $\pm$ 1.2**
AST (U/L)	66.7 $\pm$ 2	69.6 $\pm$ 0.8	101.3 $\pm$ 1.2*	72.3 $\pm$ 1.5**	73.7 $\pm$ 1.8**	62.3 $\pm$ 2.02**
Urea (mg/dL)	37.7 $\pm$ 3.8	50.3 $\pm$ 5.0	75.3 $\pm$ 2.3*	54.7 $\pm$ 9.6**	47 $\pm$ 2.1**	40.7 $\pm$ 5.5**
Creatinine (mg/dL)	0.43 $\pm$ 0.03	0.5 $\pm$ 0.06	0.73 $\pm$ 0.03*	0.43 $\pm$ 0.03**	0.43 $\pm$ 0.03**	0.46 $\pm$ 0.06**

Values are means $\pm$ SEM for five different rats per each treatment.

Values are statistically significant at * $p < 0.05$ vs. control; ** $p < 0.05$ vs. diabetic rats.

± 0.03) compared with control group. These increases in all the parameters were significantly normalized after administration of either insulin or CC alone or in combination ($p < 0.05$). However, HDL decreased significantly ($p < 0.05$) in diabetic rats (19 ± 2.1) and normalized after administration of insulin (36 ± 1.7) or CC (46.3 ± 5.8) or both insulin and CC (44.3 ± 2.4) to its normal level.

Effect of *Citrullus colocynthis* and Insulin Administration on mRNA Expression of Antioxidants in Liver and Kidney of Diabetic Rats

Diabetic rats showed significant downregulation ($p < 0.05$) in glutathione peroxidase (GPx), glutathione transferase (GST), and superoxide dismutase (SOD) mRNA expressions in liver and kidney compared with control. The treatment of diabetic rats with insulin, CC, and their combination significantly upregulated GPx expression up to normal mRNA expression level (Figure 1A). Administration of CC alone to diabetic rats was not able to restore expression of both GST and SOD, but groups treated with insulin or the combination (insulin + CC) showed normalized expressions of both GST and SOD, as shown in Figure 1B,C respectively. Renal expression of GPx, GST, and SOD in diabetic and treated groups showed the same pattern of expression in liver (data not shown).

Effect of *Citrullus colocynthis* and Insulin Administration on mRNA Expression of IRS-1, IRS-2, and GLUT-2 in Liver of Diabetic Rats

As a result of decreased insulin in the blood of STZ diabetic rats, mRNA expressions of insulin receptor substrate (IRS)-1 and -2 and GLUT-2 were downregulated significantly ($p < 0.05$) compared with the control group (Figure 2). Treatment of diabetic rats with insulin, CC, and their combination restored mRNA expressions of IRS-1, IRS-2, and GLUT-2 to normal level, as exhibited in Figure 2A–C, respectively.

Effect of *Citrullus colocynthis* and Insulin Administration on mRNA Expression of PEPCK and PK in Liver of Diabetic Rats

In diabetic rats, there was an increase in gluconeogenesis and a decrease in glycolysis. Phosphoenol pyruvate carboxykinase (PEPCK), a key enzyme of gluconeogenesis in the liver, was upregulated significantly ($p < 0.05$) in diabetic rats compared with control group. CC administration to diabetic rats showed partial downregulation in mRNA expression of PEPCK compared with diabetic rats. However, diabetic group treated with insulin alone or coadministered with CC were completely normalized significantly (Figure 3A). In contrast to PEPCK expression, pyruvate kinase (PK), the rate-limiting enzymes of glycolysis, was downregulated significantly ($p < 0.05$) in diabetic rats in comparison with control group. Treatment of diabetic rats with CC partially recovered hepatic mRNA expression of PK compared with diabetic group. Administration of insulin alone or in combination with CC to diabetic rats upregulated PK expression toward the normal expression level, as seen in Figure 3B.

Effect of *Citrullus colocynthis* and Insulin Administration on mRNA Expression of AQP1 in Kidney of Diabetic Rats

As shown in Figure 4A, diabetes significantly downregulated AQP1 expressions in renal tissues compared with control. Administration of insulin or CC or coadministration of both to diabetic rats normalized mRNA expression of AQP1. In contrast, diabetes upregulated AQP2 and AQP3 expressions significantly in kidney compared with control group. Administration of insulin or CC or both returned mRNA expression of both AQP2 and AQP3 to normal levels compared with diabetic rats, as seen in Figure 4B,C respectively ($p < 0.05$). AQP4 expression showed no significant change between different groups (Figure 4D).

Effect of *Citrullus colocynthis* and Insulin Administration on mRNA Expression of AQP5 in Salivary Gland of Diabetic Rats

Diabetic rats showed significant downregulation in mRNA expression of AQP5 in salivary gland when compared with normal group. However, treatment of diabetic group with insulin or CC or both restored AQP5 expression in salivary gland to normal expression level as observed in Figure 5 ($p < 0.05$).

Effect of *Citrullus colocynthis* and Insulin Administration on mRNA Expression of Aquaporin-7 and -8 in Pancreas of Diabetic Rats

As presented in Figure 6, induction of diabetes with STZ induced a significant downregulation in mRNA expressions of AQP7 and AQP8 in pancreas compared with normal group. However, treatment of diabetic rats with insulin or CC or both normalized AQP7 and AQP8 expressions in pancreas compared with diabetic group (Figure 6A,B, respectively).

Effect of *Citrullus colocynthis* and Insulin Administration on Immunohistochemical Expression of Insulin, GLUT-2, and AQP2 in Pancreas and Kidney of Diabetic Rats

The pancreas of the healthy control rats and CC administered rats showed normal tissue architecture with normal insulin expression in the pancreatic β cells (Figure 7A,B). On the other hand, the pancreatic tissue from the type 2 DM (T2DM) rats exhibited a reduction in the expression of insulin, with atrophy of the pancreatic β cells (Figure 7C). Pancreatic tissue of STZ-administered rat treated with insulin showed the restoration of normal expression of insulin in the pancreatic β cells (Figure 7D). Pancreatic tissue of STZ-administered rat treated with CC showed the moderate expression of insulin in the pancreatic β cells (Figure 7E). Pancreas of T2DM treated with CC and insulin showed severe expression of insulin in the pancreatic β cells (Figure 7F). Meanwhile, the pancreas of the healthy control rats presented normal GLUT-2 expression in the pancreatic tissue (Figure 8A). Pancreatic tissue of CC-administered rats showed moderate expression of GLUT-2 in the β cells (Figure 8B). Pancreatic tissue of STZ-administered rat showed a reduction in the expression of GLUT-2 in the β cells (Figure 8C). Pancreatic tissue of STZ-administered rat treated with insulin showed restoration of normal expression of GLUT-2 in the pancreatic β

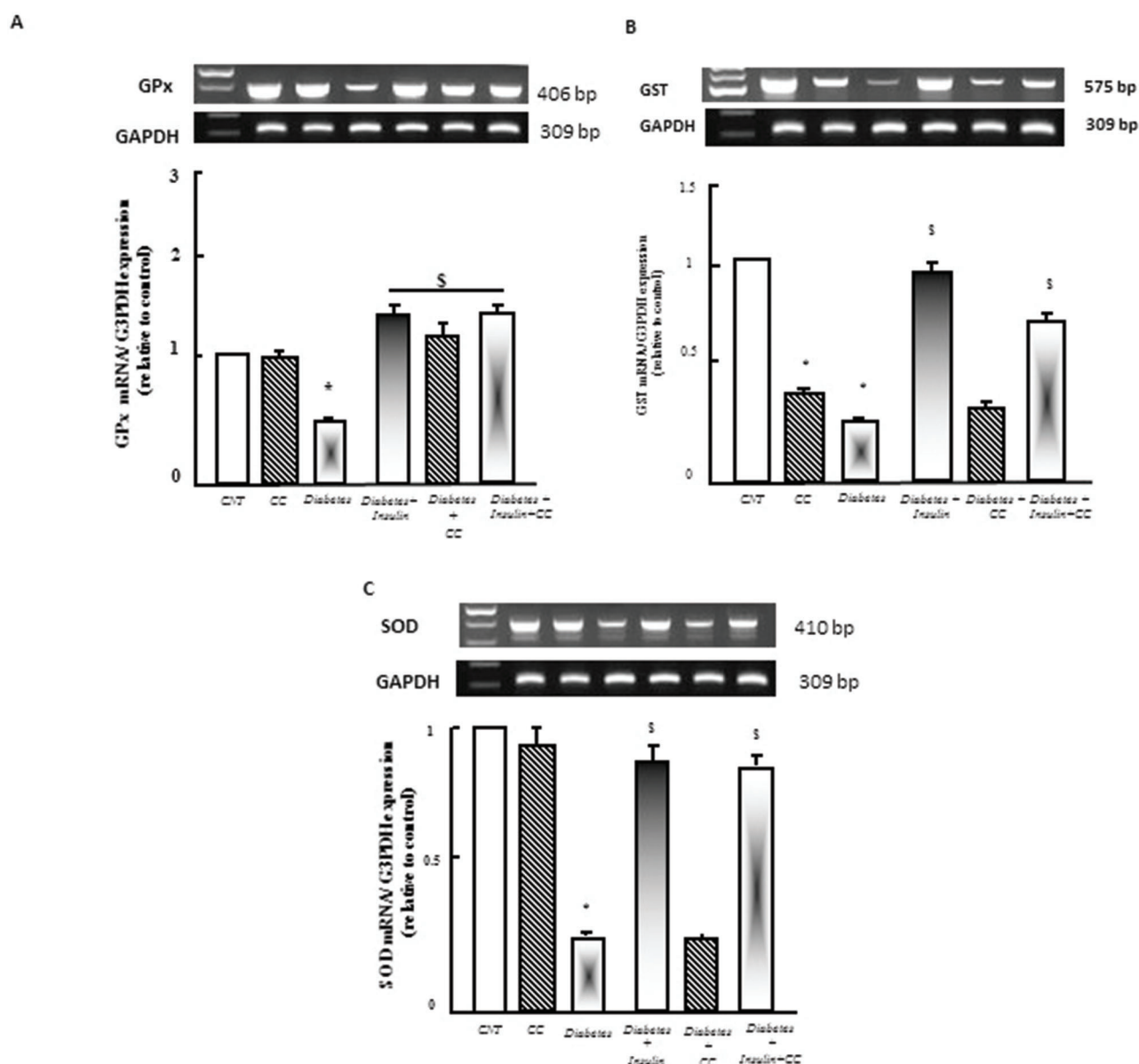


Figure 1: Effect of *Citrullus Colocynthis* and insulin administration on mRNA expression of GPx (A), GST (B), and SOD (C) in liver of diabetic rats. RNA was extracted and reverse transcribed (3 µg), and RT-PCR analysis was carried out for GPx, (A) GST (B), and SOD (C) expression as described in Materials and Methods. Densitometric analysis was carried for 3 different experiments. Data are means $\pm$ SEM for three independent experiments. Values are statistically significant at * $p < 0.05$ vs. control; \$ $p < 0.05$ vs. diabetic rats.

cells (Figure 8D). Pancreatic tissue of STZ-administered rat treated with CC showed the normal expression of GLUT-2 in the pancreatic β cells (Figure 8E). Pancreas of T2DM treated with CC and insulin restored normal expression of GLUT-2 in the pancreatic β cells (Figure 8F). On the other hand, kidney of a healthy control rat showed absence of expression of AQP2 in the renal tissue (Figure 9A). Kidney of CC-administered rat showed

no expression of AQP2 (Figure 9B). Kidney of STZ-administered rat showed severe expression of AQP2 in the renal tissue (Figure 9C). Kidney of STZ-administered rat treated with insulin showed moderate expression of AQP2 in the renal tissue (Figure 9D) and that treated with CC showed moderate expression of AQP2 (Figure 9E). Kidney of the T2DM treated with CC and insulin showed mild expression of AQP2 (Figure 9F).

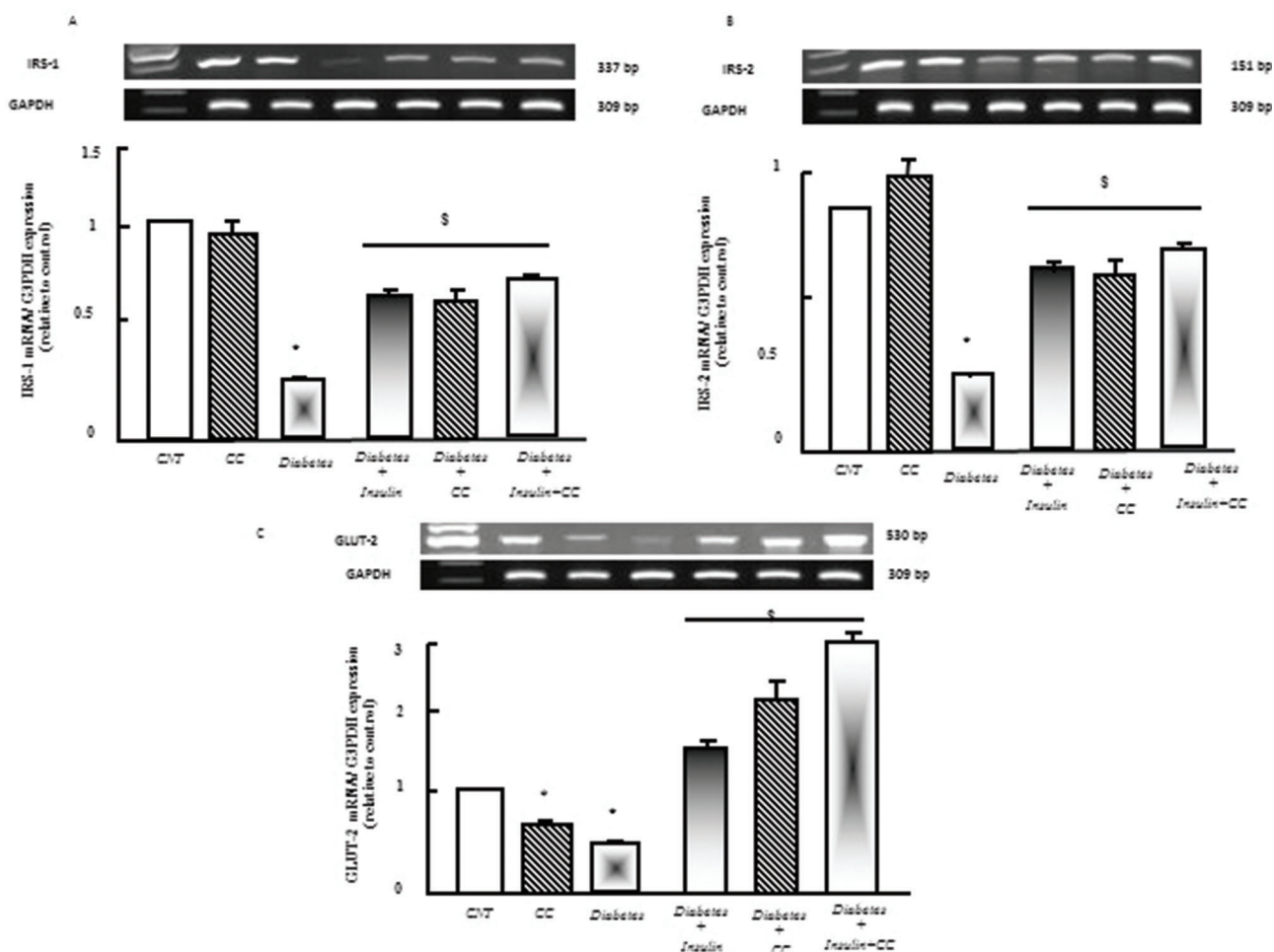


Figure 2: Effect of *Citrullus Colocynthis* and insulin administration on mRNA expression of IRS-1 (A), IRS-2 (B), and GLUT-2 (C) in liver of diabetic rats. RNA was extracted and reverse transcribed (3 μ g), and RT-PCR analysis was carried out for IRS-1 (A), IRS-2 (B), and GLUT-2 (C) expression as described in Materials and Methods. Densitometric analysis was carried for 3 different experiments. Data are means $\pm$ SEM for three independent experiments. Values are statistically significant at * $p < 0.05$ vs. control; \$ $p < 0.05$ vs. diabetic rats.

DISCUSSION

Diabetes mellitus is one of the fastest growing metabolic diseases. The treatment requires lifelong use of chemical drugs, which produce multiple side-effects in addition to high cost; hence, the search for more effective and safer antidiabetic drugs continues. *Citrullus colocynthis* has been widely used as antidiabetic drug in different countries.^[21] Regardless of the popularity of the traditional medicinal uses of CC for diabetes-related symptoms, very few studies have been reported to determine the molecular mechanism and genes expression involved in the remedial effects of the herb. Accordingly, this study was designed to evaluate the antihyperglycemic effects of CC extracts in STZ diabetic rats. This study indicated that oral administration of CC extract at 300 mg/kg decreased serum glucose in STZ diabetic rats, as shown in Table 2. The

hypoglycemic effect of extract could be attributed to the presence of saponins and glycosidic components of the plant.^[22] This is in parallel with number of studies that have shown that CC seed extract exhibits a hypoglycemic effect in patients with types 1 and 2 diabetes and in experimental animals with diabetes.^[23] The possible mechanism of action may be correlated with insulinotropic effect of CC fruits.^[14]

The findings of this study pointed out a significant increase in the hepatic transaminases of diabetic rats, which can be interpreted owing to the liver cell destruction, which is also confirmed with changes in the membrane permeability indicating severe hepatocellular damages.^[24] Increased levels of urea and creatinine in diabetic rats suggested impaired renal function.^[25] Administration of CC and insulin to diabetic rats significantly reduced levels of ALT, AST, creatinine, and urea, indicating that CC possesses hepatorenal protective action during diabetes.^[26]

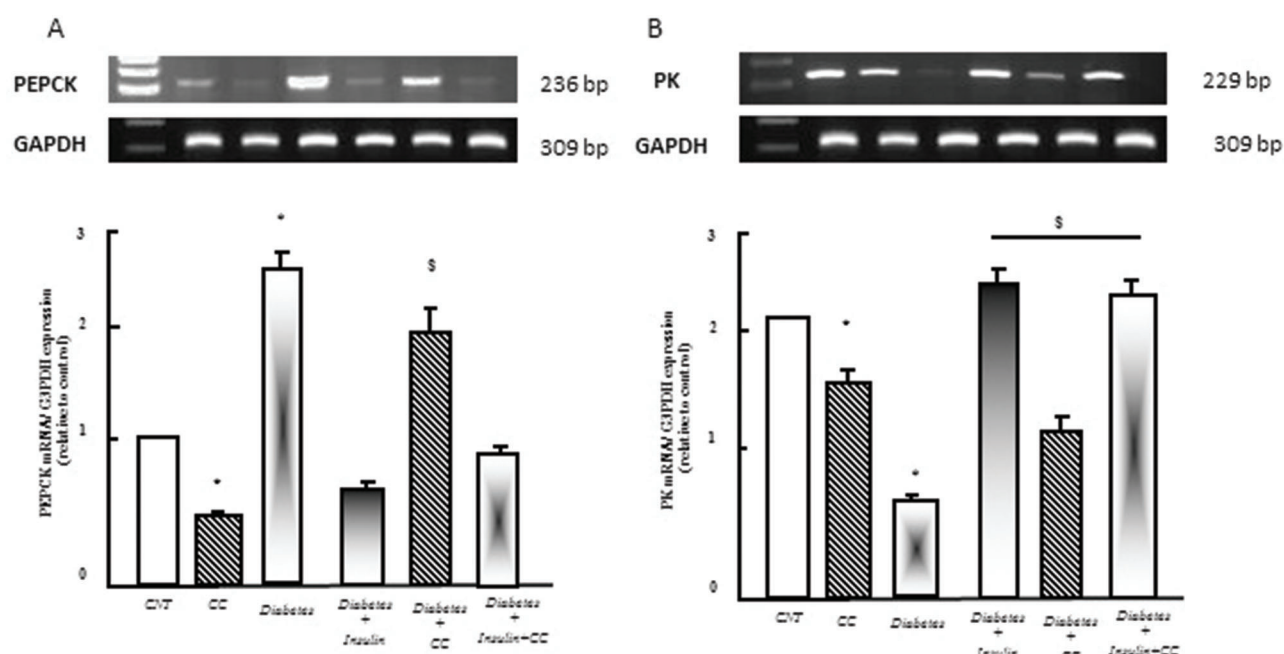


Figure 3: Effect of *Citrullus Colocynthis* and insulin administration on mRNA expression of PEPCK (A) and PK (B) in liver of diabetic rats. RNA was extracted and reverse transcribed (3 µg), and RT-PCR analysis was carried out for PEPCK (A) and PK (B) expression as described in Materials and Methods. Densitometric analysis was carried for 3 different experiments. Data are means ± SEM for 3 independent experiments. Values are statistically significant at * $p < 0.05$ vs. control; \$ $p < 0.05$ vs. diabetic rats.

Hyperlipidemia is a recognized consequence of DM and is one of the major cardiovascular risk factors. Our data are in agreement with previous observation that STZ-induced diabetic rats showed a significant elevation of cholesterol and TG levels in addition to a reduction in HDL-cholesterol level. It is known that HDL participates in the transport of cholesterol from peripheral tissues to the liver, thus inhibiting the genesis of atherosclerosis.^[27] Treatment with CC seed extract (300 mg/kg) and insulin for 28 days was sufficient to produce a significant decrease in serum TC and TG levels, suggesting that the polyphenolic components of CC have a hypolipidemic effect. The marked elevation in the level of HDL suggests a cardioprotective effect of CC treatment.^[28]

An important consequence of diabetic hyperglycemia is the enhanced oxidative stress resulting from an imbalance between the production and neutralization of ROS by antioxidant defense system. This imbalance impairs the cellular functions, leading to various pathological conditions and an alteration in the activity and expression of antioxidant enzymes, such as GST, GPx, and SOD and disturbed reduced glutathione.^[29] The therapeutic effects of several medicinal and food plants, used in traditional medicine, are usually attributed to their polyphenolic compounds.^[30] CC extracts are rich source of antioxidants (e.g., polyphenol and plant sterol).^[31] Our results showed that diabetes induction led to marked and significant decreases in hepatic and renal expressions of antioxidant enzymes (SOD, GST, and GPx). However, CC seed extract and insulin significantly restored expressions of SOD, GST, and GPx in

diabetic rats. These findings are in agreement with previous literatures that CC (300 mg/kg b.w.) might be involved in the restoration of the antioxidant defense system in rats with diabetes by regulating the expression of antioxidant.^[32]

PK is the most sensitive indicator of the glycolytic pathway in the diabetic state.^[33] PEPCK, a rate-limiting enzyme in gluconeogenesis, is negatively regulated by insulin and is highly resistant in hyperinsulinemic diabetic models.^[34] Observed hyperglycemia in current study is attributed to an increase in the level of gluconeogenesis and a decrease in glycolysis, which is manifested by upregulation in PEPCK and downregulation in PK expressions in diabetic STZ rats, respectively.^[35] The results of this study demonstrated that treatments with CC or insulin or both recover the suppressed mRNA levels of PK and normalized PEPCK mRNA in diabetic liver. CC seed extract appears, therefore, to be able to exert in vivo opposite effects on genes involved in liver glycolytic and gluconeogenic pathways. Moreover, this study showed a significant downregulation of insulin signaling components, IRS-1 and IRS-2, in diabetic rats. Downregulation of pathway components indicated a strong suppression of insulin signaling and deterioration of insulin sensitivity. Immunohistochemically, Figure 7 showed downregulation of insulin expression in pancreatic β-cell and recovered by insulin or CC or both. Herein, we have also reported that CC alone or in combination with insulin recovered expression of classical markers of insulin signaling cascade in the hepatic tissues of diabetic rats through increased expression of both insulin receptors, IRS-1 and IRS-2 (Figure 2). This is quite rational

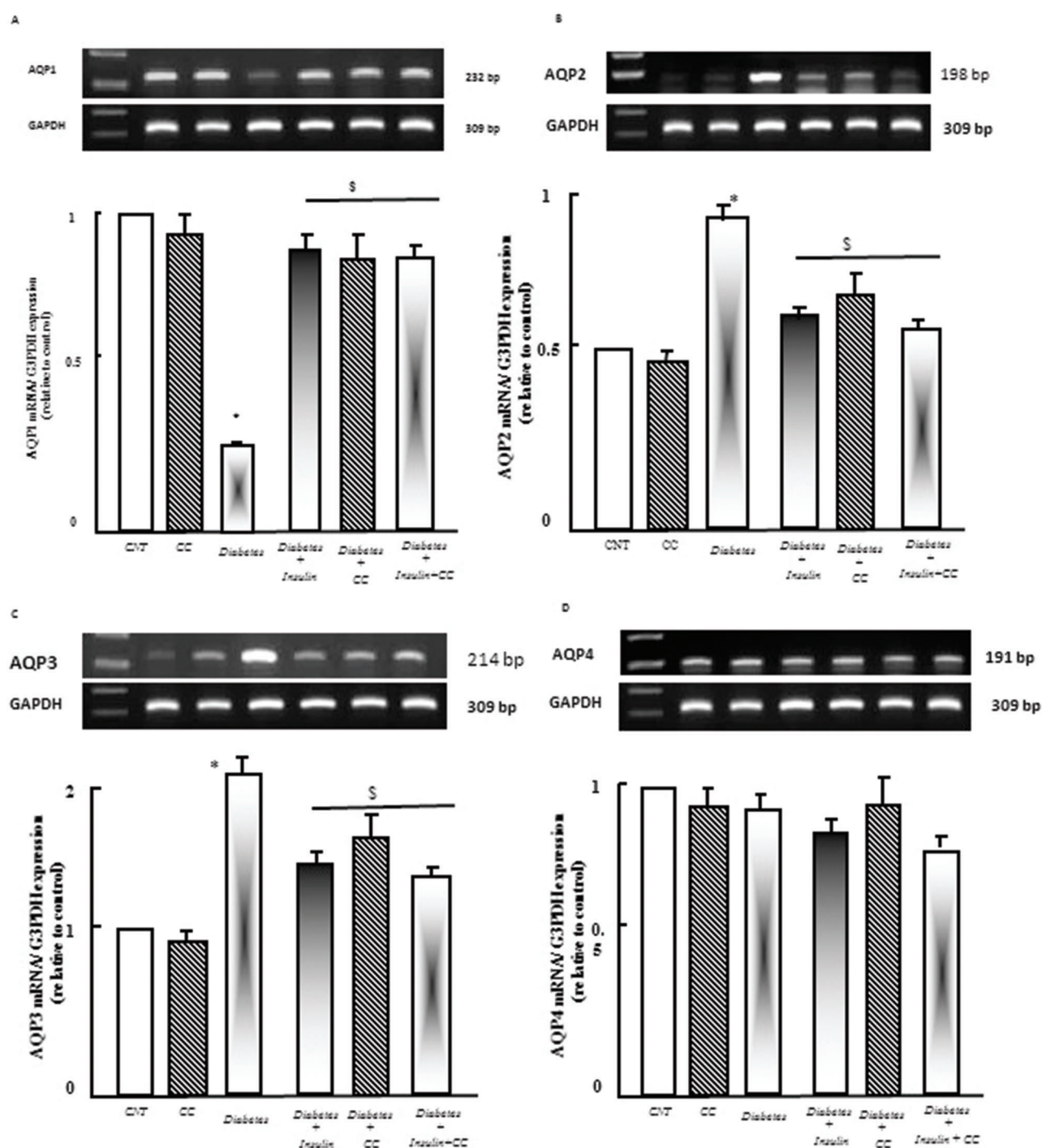


Figure 4: Effect of *Citrullus Colocynthis* and insulin administration on mRNA expression of AQP1 (A), AQP2 (B), AQP3 (C), and AQP4 (D) in kidney of diabetic rats. RNA was extracted and reverse transcribed (3 µg) and RT-PCR analysis was carried out for AQP1 (A), AQP2 (B), AQP3 (C), and AQP4 (D) expression as described in Materials and Methods. Densitometric analysis was carried for 3 different experiments. Data are means $\pm$ SEM for 3 independent experiments. Values are statistically significant at $*p < 0.05$ vs. control; S $p < 0.05$ vs. diabetic rats.

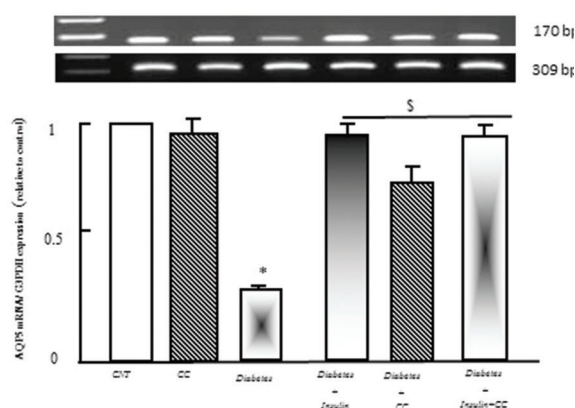


Figure 5: Effect of *Citrullus Colocynthis* and insulin administration on mRNA expression of AQP 5 in salivary gland of diabetic rats. RNA was extracted and reverse transcribed (3 mg), and RT-PCR analysis was carried out for AQP 5 expression as described in Materials and Methods. Densitometric analysis was carried for three different experiments. Data are means $\pm$ SEM for three independent experiments. Values are statistically significant at * $p < 0.05$ vs. control; \$ $p < 0.05$ vs. diabetic rats.

because the deficiency of insulin in the diabetic state would decrease the translocation of GLUT-2 and GLUT-4 from the vesicles to cell membranes. Our results showed downregulation in GLUT-2 expression in liver of diabetic rats and in β cells by immunohistochemical when compared with control. GLUT-2 downregulation, and consequently decreased uptake of glucose,

is essentially one of the main reasons of hyperglycemia in the diabetic state. Restoration of glucose transport gene levels enhance the uptake of glucose in liver and, thus, help to combat hyperglycemic conditions.^[36] This study revealed that the ability of CC to improve cellular glucose uptake was associated with upregulation of GLUT-2 expressions in liver and pancreas, which was owing to restoration of β -cell mass.

The AQPs, water channel proteins, are extensively dispersed in many organs and tissues. Their principal function is to enable water transport across cell plasma membranes, while some AQPs also transfer glycerol, hence called aquaglyceroporins.^[37] AQP1 is dominantly expressed in the renal cortex, including the proximal tubules, while AQP2, AQP3, and AQP4 are mainly expressed in the renal inner medulla, where the collecting tubules are dominantly present.^[38] Vasopressin regulates urinary volume via AQP2 and AQP3, which are expressed in the renal collecting duct. AQP1 and AQP4 expressions are not regulated by vasopressin.^[39] Vasopressin binds to the V2 receptor on the basolateral membrane of the renal collecting duct principal cells, which in turn stimulates gene transcription of AQP2 and induces the translocation of AQP2 from the intracellular vesicle to the luminal membrane.^[40] Secretion of vasopressin is increased in diabetes and mediates compensatory upregulation in AQP2 and AQP3 in response to the severe water loss induced by glucosuria after STZ administration.^[41] In this study, an increase in renal expression of AQP2 and AQP3 was detected in STZ diabetic rat, which serves as a compensatory mechanism to alleviate dehydration in diabetes mellitus as

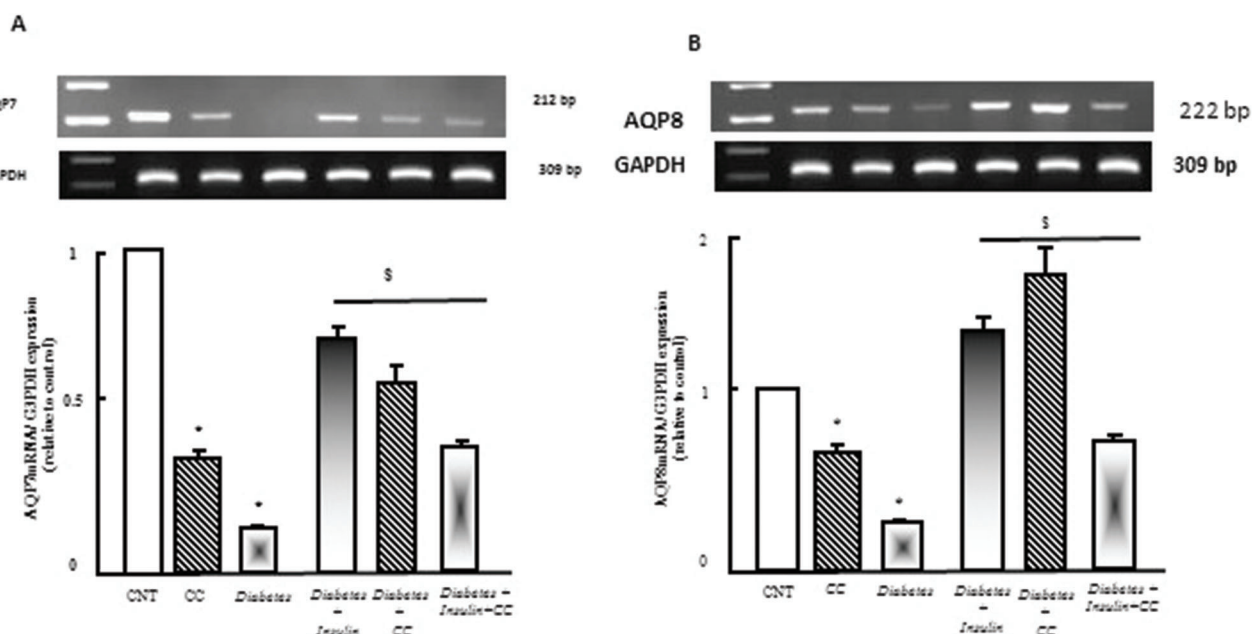


Figure 6: Effect of *Citrullus Colocynthis* and insulin administration on mRNA expression of AQP 7 (A) and AQP8 (B) in pancreas of diabetic rats. RNA was extracted and reverse transcribed (3 mg), and RT-PCR analysis was carried out for AQP 7 (A) and AQP8 (B) expression as described in Materials and Methods. Densitometric analysis was carried for three different experiments. Data are means $\pm$ SEM for three independent experiments. Values are statistically significant at * $p < 0.05$ vs. control; \$ $p < 0.05$ vs. diabetic rats.

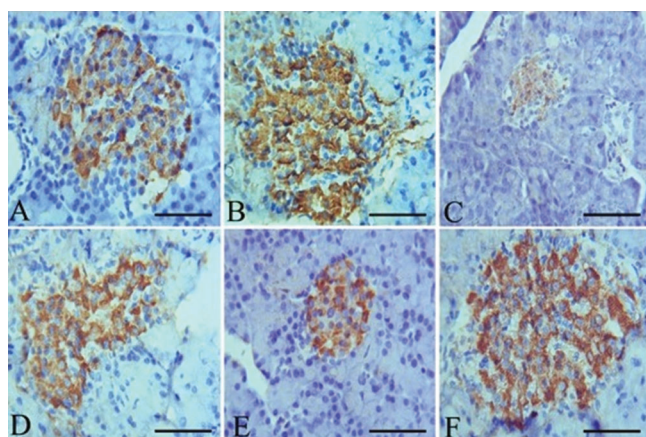


Figure 7: A: Pancreatic tissue of a healthy control rat showing a normal architecture with normal expression of insulin in the β cells. B: Pancreatic tissue of CC-administered rat showing normal expression of insulin in the β cells. C: Pancreatic tissue of streptozotocin-administered rat showing a reduction in the expression of insulin in the β cells, with atrophy of the β cells. D: Pancreatic tissue of streptozotocin administered rat treated with insulin showing the restoration of normal expression of insulin in the pancreatic β cells. E: Pancreatic tissue of streptozotocin administered rat treated with CC showing the moderate expression of insulin in the pancreatic β cells. F: the pancreas of the T2DM treated with CC and insulin showing severe expression of insulin in the pancreatic β cells. Scale bar=50 μ m.

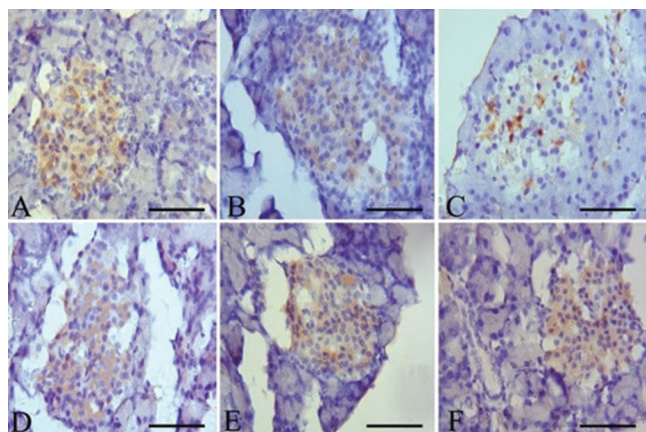


Figure 8: A: Pancreatic tissue of a healthy control rat showing normal expression of GLUT-2 in the β cells. B: Pancreatic tissue of CC-administered rat showing moderate expression of GLUT-2 in the β cells. C: Pancreatic tissue of streptozotocin-administered rat showing a reduction in the expression of GLUT-2 in the β cells. D: Pancreatic tissue of streptozotocin-administered rat treated with insulin showing the restoration of normal expression of GLUT-2 in the pancreatic β cells. E: Pancreatic tissue of streptozotocin-administered rat treated with CC showing the normal expression of GLUT-2 in the pancreatic β cells. F: the pancreas of the T2DM treated with CC and insulin showing normal GLUT-2 expression in β cells. Scale bar=50 μ m

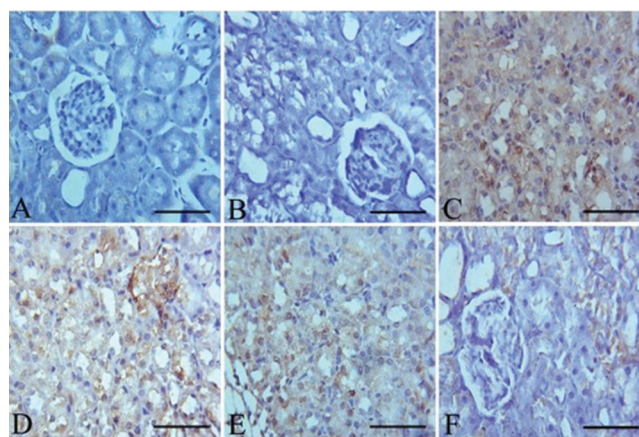


Figure 9: A: Kidney of a healthy control rat showing absence of expression of AQP2 in the renal tissue. B: Kidney of CC-administered rat showing no expression of AQP2. C: Kidney of streptozotocin-administered rat showing severe expression of AQP2 in the renal tissue. D: Kidney of streptozotocin-administered rat treated with insulin showing moderate expression of AQP2 in the renal tissue. E: Kidney of streptozotocin-administered rat treated with CC showing moderate AQP2 expression. F: Kidney of the T2DM treated with CC and insulin showing mild expression of AQP2. Scale bar=50 μ m.

a result of polyuria. CC administration alone or in combination with insulin normalizes their expression toward control level (Figures 4 and 9). Moreover, AQP4 did not show any significant change in different groups, in contrast to AQP1 that showed downregulation in diabetic rats^[42] and renormalized by CC administration alone or in combination with insulin (Figure 4).

Several AQPs have been identified in salivary glands.^[9] Among those, AQP5 plays a critical role by contributing to salivary flow. In the current study, a decrease in mRNA expression of AQP5 in salivary glands of STZ diabetic rats compared with normal was recorded. Orally administered CC alone or in combination with insulin recovers AQP5 expression toward normal (Figure 5). Diabetes represents a common cause of xerostomia. In patient with diabetes mellitus, a reduction in salivary secretion happens in concurrence with low salivary AQP5 levels.^[43] Salivary AQP5 levels correlate with salivary secretion in both healthy and disease states, suggesting that changes in salivary AQP5 levels can be used as an indicator of salivary flow rate.^[10]

Aquaglyceroporins represent a subfamily of aquaporins permeable not only to water but also to small solutes like glycerol and urea. AQP7 is expressed in rat and mouse pancreatic islet β -cells.^[44] AQP7 controls the cellular glycerol content and appears to play a key role in regulating proinsulin biosynthesis and insulin secretion.^[45] AQP7 is likely to play a dual role in the regulation of insulin release, by allowing the entry or exit of glycerol and by acting directly or indirectly at a distal downstream site in the insulin exocytosis pathway.^[11] There were previous studies explained role of on AQP7 in

T2DM and obesity; little is known about its role in type 1 diabetes. In this work, AQP7 expression in pancreas showed highly significant decrease in diabetic rats compared with normal and restored to normal expression in groups treated with CC alone or in combination with insulin (Figure 5). AQP8 showed the same pattern as observed in AQP7 (Figure 5). So, AQP7 regulation could become an attractive drug-target pathway for therapeutic diabetes strategies. Further quantitative studies are needed to identify the various mechanisms by which CC act as antidiabetic agent.

CONCLUSION

This study concluded that, first, changes in AQPs expression in kidney, salivary gland, and pancreas of diabetic rats are associated with compensation of diabetes complications such as polyuria, dehydration, and xerostomia. So, AQPs could be a promising target for drug development. Second, oral administration of CC was found to exhibit antihyperglycemic, antihyperlipidemic, antioxidant, hepatoprotective, and renoprotective activity in STZ-induced diabetic rats by acting on molecular and immunohistochemical levels in addition its effect on AQPs.

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