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Some Biochemical Studies On Some Blood Constituents In Rats

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ABSTRACT

This study was designed to investigate the anti-diabetic effect of cucurbitacin extracts from *Citrullus colocynthis* in Streptozotocin-Nicotonamide (STZ-NIC) induced diabetes in rats. The diabetes was induced by single dose of STZ (65 mg/kg) in citrate buffer after 15 min of I/P injection of nicotinamide (110 mg/kg) in normal saline. After seven days of induction of diabetes, the diabetic animals were treated for further eight weeks with (100mg/kg) cucurbitacin extracts or (100mg/kg) metformin or combination of both. Blood glucose estimation was performed every week of the study. STZ-induced diabetic rats showed marked hyperglycemia all over the study period. The expression levels of GSI, insulin, GK and G6PDH were reduced. The glycated haemoglobin, ALT, AST and creatinine levels were significantly increased while the level of liver glycogen was decreased in diabetic rats. Supplementation with (100 mg/kg) cucurbitacin extracts for eight weeks significantly ameliorated the alterations in fasting blood glucose, liver glycogen, glycated haemoglobin, creatinine, ALT, AST and the expression levels of GSI, insulin, GK and G6PDH in diabetic rats. Thus, the present study suggested the potential of citrullus colocynth extract in diabetes as well as related complications.

Key words: Cucurbitacin , *Citrullus colocynthis*, streptozotocin , Nicotonamide, Glucokinase.

INTRODUCTION

Diabetes mellitus (DM) is a major public health problem, ranked seventh among the leading causes of death, and third when its fatal complications are taken into account. Traditional anti-diabetic plants might provide a useful source of new oral hypoglycemic compounds for development as pharmaceutical entities or as simple dietary adjuncts to existing therapies *(1)*. Type II diabetes mellitus developed by metabolic abnormalities such as impaired insulin secretion, increased hepatic glucose production and decreased insulin-stimulate of glucose uptake in peripheral *(2)*.

Citrullus colocynthis also known as bitter apple is a desert plant of the family Cucurbitaceae naturally adapted to arid environments, originally from tropical Asia and Africa, it is now widely distributed in the Saharo-Arabian phyto geographic region in Africa and the Mediterranean region. In folk medicine, *C. colocynthis* is widely used by rural inhabitants as a purgative, anti-diabetic, anti-neoplastic, anti-rheumatic, and anti-allergic agent. They are found mainly in plants belonging to the Cucurbitaceae family, but have also been found in several other families of the plant kingdom *(3)*. Previous studies proved that different *Citrillus colocynthis* seed extracts have an insulin tropic effect which could at least partially account for the antidiabetic activities of these fruits *(4)*. The present study was designed to investigate the anti-diabetic effect of cucurbitacin extract from citrullus colocynth.

MATERIALS AND METHODS

Chemicals Streptozotocin (STZ), Nicotinamide and Metformin were purchased from Sigma- Aldrich, Chemical Cp.(St. Louis, Mo, USA).

Plant material Fruits of Citrullus colocynth were collected during August and September from oasis in Eastern desert of Egypt. The plants material was identified and confirmed by anatomical examination in comparison with herbarium specimen retained in the faculty of Pharmacy, University of Assiut. Air dried powdered herb (500g) was separately extracted with 1 liter ethanol (95%, v/v) at room temperature. The ethanolic extract was concentrated under reduced pressure at 40°C *(5)*.

Animal management: One hundred twenty Male wistar rats weighting at the beginning of the experiment (150±20gm) were obtained from animal house of Zagazig University. Rats were acclimatized for a period of 7 days under standard environmental conditions of temperature, relative humidity and dark/light cycle. Animals stated as fasting were deprived of food and water for 16 hrs.

Induction of experimental diabetes: Experimental diabetes was induced by single intra-peritoneal injection of 60mg/kg of STZ, freshly dissolved in cold citrate buffer, pH 4.5 after 15 min of I /P injection of nicotinamide (110 mg/kg) prepared in normal saline. Rats with fasted blood glucose level greater than 200 mg/dl after one week of administration of STZ were used for this study *(6)*.

Experimental design

Rats were randomly divided into six groups, each of 20 rats as follow: Group I: Served as normal control. Group II: given orally100 mg/kg cucurbitacin. Group III: STZ- Diabetic. Group IV: treated with STZ-then given100 mg/kg cucurbitacin orally. Group V: treated with STZ-then given100 mg/kg Metformin. Group VI: treated with STZ- then given orally 100 mg/kg cucurbitacin+100 mg/kg metformin.

Sampling: At the end of the experiment, rats were scarified; Serum was separated for biochemical determination of blood glucose, creatinine, ALT, and AST using commercially available kits (Span diagnostics). Thirty mgs of rat's livers were separated as early as possible after scarifying and immerged in liquid nitrogen then kept at -80 for molecular analysis.

RNA Isolation and cDNA synthesis

RT-PCR was performed according to ***Meadus (7)***. Firstly RNA was extracted from the liver samples using IQeasy™ plus CTB RNA Extraction Mini Kit, iNtRON Biotechnology, Korea. cDNA strands were synthesized from RNA using Maxime RT Premix Kit, iNtRON Biotechnology, Korea. Two µl from cDNA was used as a template in a PCR performed for GLUT-2, Insulin, GK, G6PDH, GSI and GAPDH according to primers in table 1 in a condition of 95°C for 2 min. as an initial denaturation, followed by 95°C for 15sec. extension at 7°C for 30 sec with annealing 60°C for 30sec. and 40 cycles in GLUT-2, Insulin, GSI and GAPDH while annealing was 55 oC for 1 min. with 25 cycles for Gk and G6PDH. 10 ul of PCR products were analyzed on a 2% agarose gel stained with ethidium bromide in 1X Tris acetate EDTA buffer (TAE) pH 8.3-8.5. The electrophoretic picture was visualized and analyzed by image J program to detect the fold increase/ pixel.

The obtained data were analyzed and graphically represented using the statistical package for social science (SPSS, 18.0 software, 2011) for obtaining means and standard error. Duncan's test was used for making a multiple comparisons among the groups for testing the inter-grouping homogeneity *(8)*.

Table 1. Primer used in determination of the expression of glucose homeostasis related genes

Genes	Sequences 5'→3'	Product size (bp)	Accession No.
GLUT-2	F CAGCTGTCTCTGTGCTGCTTGT R GCCGTCATGCTCACATAACTCA	150	NM_012879
Insulin	F TCTTCTACACACCCATGTCCC R GGTGCAGCACTGATCCAC	149	NM_019130
GK	F TGGATGACAGAGCCAGGATGG R ACTTCTGAGCCTTCTGGGGTG	208	LUKB
G6PDH	F GACCTGCAGAGCTCCAATCAAC R CACGACCCTCAGTACCAAAGGG	214	RATG6PD3
GSI	F TCCACTGTGCCTGTGTCTTCA R AGAGAACTTCTTCACATTCAGTCCATT	119	XM_229128
GAPDH	F TGGTGGACCTCATGGCCTAC R CAGCAACTGAGGGCCTCTCT	105	XM_344448

RESULTS

Table 2. Effect of cucurbitacin extract and/or metformin on serum glucose, ALT, AST and creatinine levels in control and STZ - diabetic rats

	Duration	Normal control	100 mg/kg cucurbitacin	STZ-Diabetic	Diabetic +100mg/kg cucurbitacin	Diabetic +100 mg/kg Metformin	Diabetic + 100 mg/kg of both
Blood glucose (mg/dl)	1st week	78.52±4.81^{a}	76.31±6.02^{a}	312.96±5.46^{b}	294.12±5.35^{c}	282.72±3.01^{c}	278.15±6.26^{d}
	2nd week	85.11±7.03^{a}	77.21±5.11^{b}	328.54±4.89^{c}	246.12±7.88^{d}	274.64±3.62^{e}	226.16±2.17^{f}
	4th week	75.11±3.99^{a}	70.02±3.21^{b}	331.58±6.24^{c}	159.21±5.03^{d}	229.62±4.55^{e}	143.08±3.05^{f}
	8th week	77.8±5.80^{a}	68.91±2.66^{b}	360.1±4.51^{c}	112.02±8.77^{d}	175.24±4.12^{e}	99.05±6.14^{f}
ALT (U/L)	1st week	53.34±4.53^{a}	52.33±2.64^{a}	70.87±3.09^{b}	65.12±7.11^{b}	67.47±5.12^{b}	64.83±2.23^{cb}
	2nd week	52.05±3.02^{a}	50.05±2.72^{a}	73.07±4.22^{b}	60.09±4.02^{b}	62.19±4.03^{b}	58.22±3.01^{c}
	4th week	55.09±2.03^{a}	48.34±2.35^{b}	75.11±7.01^{c}	57.23±4.12^{a}	58.06±3.71^{a}	52.13±4.77^{a}
	8th week	54.42±5.02^{a}	47.01±2.33^{b}	82.09±3.09^{c}	51.09±.12^{d}	56.11±5.04^{a}	48.55±2.13^{b}
AST (U/L)	1st week	121.55±13.23^{a}	117.71±14.27^{a}	158.33±10.77^{b}	143.12±7.32^{c}	146.34±6.34^{c}	138.22±5.21^{d}
	2nd week	118.44±13.22^{a}	112.11±11.87^{a}	164.11±8.12^{b}	137.32±4.02^{c}	142.66±3.23^{d}	126.11±4.02^{e}
	4th week	116.15±8.44^{a}	107.54±9.43^{b}	166.24±7.67c	131.54±5.12^{d}	138.27±4.01^{e}	120.81±3.99^{f}
	8th week	119.04±7.21^{a}	102.66±7.81^{b}	173.21±6.88^{c}	123.11±6.23^{d}	129.43±5.12^{e}	116.25±2.16^{f}
Creatinine (mg/dl)	1st week	0.83±0.04^{a}	0.84±0.02^{a}	1.36±0.04^{c}	1.42±0.03^{d}	1.33±0.05^{e}	1.28±0.05^{f}
	2nd week	0.74±0.03^{a}	0.72±0.03^{b}	1.43±0.03^{c}	1.33±0.02^{d}	1.29±0.03^{e}	1.22±0.04^{f}
	4th week	0.77±0.05^{a}	0.73±0.04^{b}	1.49±0.04^{c}	1.21±0.04^{d}	1.22±0.05^{d}	1.02±0.04^{e}
	8th week	0.80±0.05^{a}	0.69±0.05^{b}	1.52±0.03^{c}	0.91±0.03^{d}	1.02±0.06^{e}	0.73±0.06^{f}

Means ± SE in the same rows carrying different superscripts are significantly different at $p < 0.05$

Table 2 showed that, fasting Blood Glucose, ALT, AST and serum creatinine were significantly increased in diabetic rats. Administration of cucurbitacin at a dose of 100 mg/kg body weight showed a highly significant effect compared with control rats in same duration. Combination between cucurbitacin and Metformin succeeded to nearly restore the control levels.

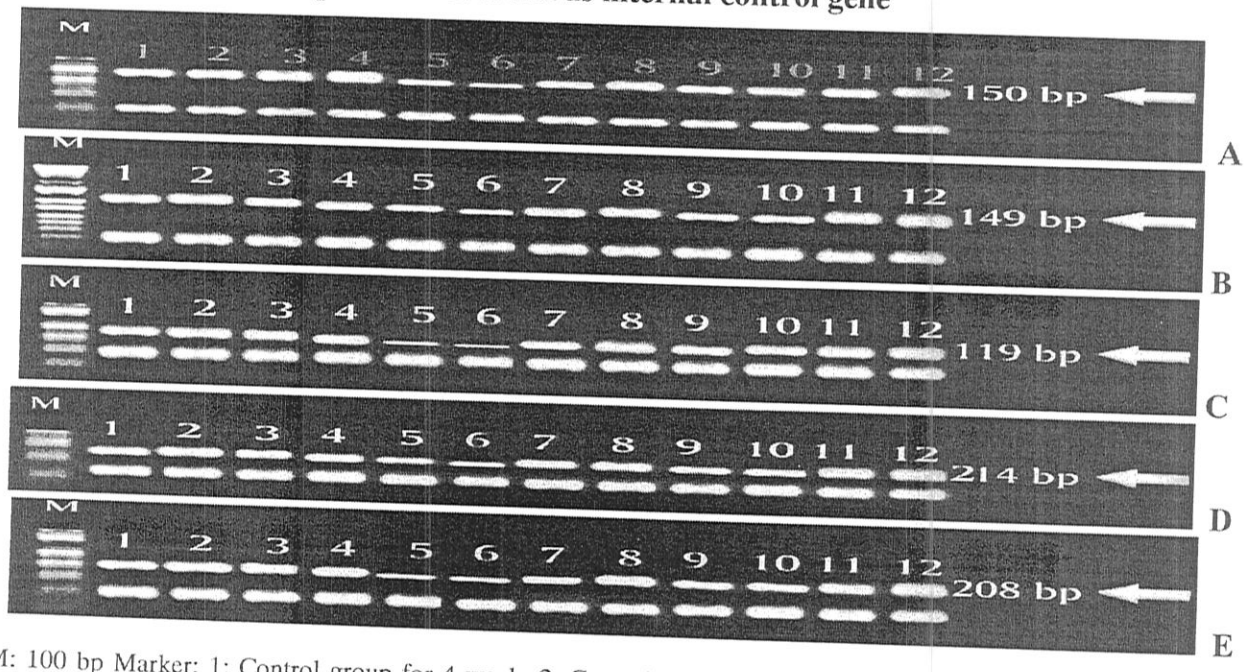
Table 3. Effect of cucurbitacin extract and/or metformin on glycated hemoglobin (HbA_1c), hepatic glucokinase activity (U/L) and hepatic glycogen level (mg/gm tissue), in control and STZ – diabetic

	Duration	Normal control	100 mg/kg cucurbitacin	STZ-Diabetic	Diabetic + 100mg/kg cucurbitacin	Diabetic +100 mg/kg Metformin	Diabetic + 100 mg/kg of both
Glycated hemoglobin (HbA1c)	4th week	5.38± 0.21^{a}	5.29± 0.23^{b}	8.53±0.41^{c}	6.23±0.22^{d}	6.52±0.16^{e}	6.13±0.21^{f}
	8th week	5.43±0.19^{a}	5.19±0.21^{b}	8.62±0.32^{d}	5.71±0.19^{e}	5.91±0.13^{e}	5.44±0.19^{f}
Hepatic glucokinase activity (U/L)	4th week	146.07 ± 10.35^{a}	159.56 ± 10.59^{b}	103.65 ±8.13^{c}	122.29 ± 9.21^{e}	116.45±8.12^{e}	151.76±9.96^{fh}
	8th week	163.11 ± 11.22^{a}	170.56 ± 13.42^{b}	92.32 ± 7.21^{c}	143.33 ± 8.33^{e}	134.33±9.02^{e}	173.56±7.55^{fh}
Hepatic glycogen level (mg/gm tissue)	4th week	32.84 ± 3.16^{a}	34.25 ± 2.56^{b}	20.07 ± 1.71^{c}	22.36 ± 1.17^{e}	26.36±2.25^{e}	29.36±2.15^{fh}
	8th week	35.74 ± 4.26^{a}	39.84 ± 3.86^{b}	16.84± 2.26^{c}	36.84± 3.26^{e}	31.44±2.15^{e}	42.84±4.46^{fh}

Means ± SE in the same rows carrying different superscripts are significantly different at $p < 0.05$

The results in table 3 showed that, the oral administration of cucurbitacin and/or metformin significantly restores the increase of glycated hemoglobin (HbA1c) that occurred in diabetic rats. Administration of cucurbitacin at a dose of 100 mg/kg body weight showed a highly significant effect compared with control rats in same duration. The hepatic glucokinase activity was significantly decreased in STZ-diabetic rats when compared with the control group. Administration of cucurbitacin and/or Metformin showed enhancement in the activity of hepatic glucokinase in the liver. The hepatic glycogen level was significantly decreased in STZ- diabetic rats when compared with the control group. Administration of cucurbitacin and/or Metformin showed enhancement in the hepatic glycogen level in the liver.

Fig. 1. The electrophotograph of GLUT-2 (A), Insulin (B), GSI (C), G6PDH (D) and GK (E) genes compared to GAPDH as internal control gene



M: 100 bp Marker; 1: Control group for 4 week; 2: Control group for 8 week; 3: Control group given orally 100 mg/kg cucurbitacin for 4 week; 4: Control group given orally 100 mg/kg cucurbitacin for 8 week; 5: STZ-Diabetic group for 4 week; 6: STZ-Diabetic group for 8 week; 7: STZ-Diabetic group given 100 mg/kg cucurbitacin for 4 week; 8: STZ-Diabetic group given 100 mg/kg cucurbitacin for 8 week; 9: STZ-Diabetic group given 100 mg/kg metformin for 4 week; 10: STZ-Diabetic group given 100 mg/kg metformin for 8 week; 11: STZ-Diabetic group given 100 mg/kg cucurbitacin 100 mg/kg metformin for 4 week; 12: STZ-Diabetic group given 100 mg/kg cucurbitacin 100 mg/kg metformin for 8 week.

Table 4. Analysis of PCR product of GLUT-2, insulin, GSI, G6PDH, GK and GAPDH genes after 4 and 8 weeks (Fold increase/ pixel)

	Duration	Control	100 mg/kg cucurbitacin	STZ-Diabetic	Diabetic + 100 mg/kg cucurbitacin	Diabetic +100 mg/kg Metformin	Diabetic + 100 mg/kg of both treatments
GLUT-2	4th week	152	164	72	74	70	141
	8th week	158	178	64	100	79	184
Insulin	4th week	152	164	102	122	120	144
	8th week	158	178	100	124	122	184
GSI	4th week	150	164	46	76	70	142
	8th week	158	178	42	100	78	186
G6PDH	4th week	154	166	104	122	120	146
	8th week	158	178	100	126	122	184
GK	4th week	154	164	46	72	64	142
	8th week	158	176	44	98	78	148
GAPDH	4th week	144	145	144	145	144	144
	8th week	144	145	144	145	144	144

Means in the same rows carrying different superscripts are significantly different at $p < 0.05$

The expression level of GLUT-2 gene was significantly increased in STZ- diabetic rats when compared with the control group. Administration of cucurbitacin and/or metformin showed enhancement in the expression of GLUT-2 gene in the liver. The expression levels of insulin, GSI, G6PDH, GK gene were significantly decreased in STZ-diabetic rats when compared with the control group. Administration of cucurbitacin and/or metformin showed enhancement in the expression of insulin gene in the liver. Combination between cucurbitacin and metformin succeeded to nearly restore the control levels.

DISCUSSION

DM is a chronic disease characterized by elevation of blood glucose due to an absolute or relative deficiency of circulating insulin levels *(9)*. Generally, the increases of hepatic glucose production, plus decreases in hepatic glycogen synthesis and glycolysis, are the major defects in type 2 diabetes that result in hyperglycemia *(10)*. The use of multiple medicinal plants has been increased several years ago *(11)*. Studies on the anti-diabetic potential of *Citrullus colocynthis* extracts were already briefly evoked in the introduction of this report. In the present study we used diabetic animal model to evaluate the possible hypoglycemic effect of cucurbitacins extract from *citrullus colocynthis* on the glycemic state in STZ- diabetic induced rats.

Although there are several reports demonstrated the hypoglycemic activity of aqueous and ethanol extract from *Citrullus colocynthis* in diabetic model, we find that these reports are not enough for explanation of the underlying mechanisms and the active principle responsible for the hypoglycemic effect. No reports had tested the effect of cucurbitacins extract on expression level of target genes of glucose homeostasis. Therefore our study was done to complete the research and to clarify the hypoglycemic activity of cucurbitacins extract.

STZ-induced hyperglycemia is a useful experimental model for studying the anti-hyperglycemic activity. Because of its structural features, STZ gets a selective entry into the β cells of the islets of Langerhans via the low affinity glucose transporter GLUT2 in its plasma membrane and causes destruction of β cells, which leads to a reduction in insulin release, which in turn results in a rise in blood glucose concentration *(12)*.

In our study, the fasting blood glucose level (FBG) was elevated in all diabetic groups when compared to control groups. FBG level in diabetic groups was 1.62 times more than that of control groups at the beginning of the experiment, the hypoglycemic effects of cucurbitacins was estimated as the reduction ratio of fasting blood glucose level in control groups to that of the diabetic treated groups with metformin and cucurbitacins. FBG was reduced by 58.6% in cucurbitacins treated group (Table 2). In the same direction, many data revealed the possible hypoglycemic effect of *Citrullus colocynthis* seed extract to its high polysaccharides contents *(13)*; others concluded that *Citrullus colocynthis* seed extract can lower blood glucose due to its insulin releasing activity *(14)*.

The level of HbA1C has been shown to be an important parameter of chronic glycaemic control in patients with DM, an elevated HbAlc almost always indicates DM *(15)*. The present data showed that, the high levels of HbAlc in diabetic rats were significantly lowered by the treatment with cucurbitacins extract of *citrullus colocynthis*. Decreased HbA1C levels in the treated diabetic rats could be due to an improvement in insulin secretion from the remnant pancreatic β-cells in diabetic rats, as manifested by increase in the gene expression of insulin detected in figure 1 and table 4, consequently, resulting in improvement in glycemic control *(16)*.

Oka, et al., (17) demonstrated that GLUT2 mRNA were increased in the liver of streptozotocin (STZ)-induced diabetic rats However, the expression of GLUT2 isoform in the liver of diabetic rats might be affected by many factors, such as high blood glucose concentrations, low insulin levels, and direct effects of STZ. In this study, The expression level of mRNA of GLUT2 gene was measured in hepatic tissue to study the ability of cucurbitacins to stimulate hepatic uptake of glucose, the liver contains glucose transporter 2 (GLUT-2), which is membrane bound and thus is not translocated by insulin. Cucurbitacins succeed to improve the expression level of GLUT2 in hepatic tissues of diabetic rats, which gives a recent explanation of hypoglycemia resulted from cucurbitacins (Figure 1). This is in agreement with recent experiments showing that GLUT-2 mRNA is increased in the presence of high glucose concentrations in cultured rat hepatocytes *(18)* and by experiments in vivo in which liver GLUT-2 mRNA does not decrease in response to hyperinsulinaemia in 24 h-fasted rats clamped at hyperglycaemic levels *(19)*.

Hepatic GK has a major effect on glucose homeostasis and is a potential target for pharmacological treatments of diabetes. Rats overexpressing GK in the liver had reduced blood glucose. The elevation of hepatic GK activity presented in this study and confirmed by increase in the mRNA level (table 3 and figure 1) can cause an increased utilization of the blood glucose for energy production or glycogen storage in the liver *(20)*. In the current study, The GK activity was determined in the hepatic tissues in all groups. The activity in the cucurbitacin and /or metformin treated groups is increased in both parts of the experiment, the GK activity in diabetic treated groups is compared to the control groups, in cucurbiacins group the activity is 1.7 and 1.2 times more than that in control at 4 weeks and 8 weeks respectively; in metformin treated groups the activity is 0.95 and 1.05 times (Table 3).

Glycogen is the primary intracellular storable form of glucose and its level usually related to insulin level in various tissues. Our results revealed that the hepatic glycogen contents and glycogen synthase mRNA expression in liver tissues of rats show variation between groups in the durations of the experiment. In cucurbitacins and/or metformin treated groups, there is a significant increased as compared to control groups, (Table 3). The observed decrease in hepatic glycogen in STZ-diabetic rats may be due to insufficient insulin and inactivation of the glycogen synthase system in the diabetic state *(14)* as DM impairs the normal capacity of the liver to synthesize glycogen *(21)*.

Glucose-6-phosphate dehydrogenase activity was decreased in diabetic state which can results in the diminishing of the pentose phosphate pathway and thereby the production of reducing equivalent such as NADH and NADPH *(22)*. Several studies have shown that G6PD activity is decreased in liver and other tissues of diabetic organisms *(23)*. In the current study, Glucose- 6-phosphate dehydrogenase mRNA expression in hepatic tissues was increased in the diabetic rats treated with cucurbitacins and/or metformin. The observed increasing in the activity of the gene expression of the enzyme may be due to the hypoglycemic effect of cucurbitacins proven in the present study.

The plasma levels of transaminases in our study were significantly increased in diabetic group while ameliorated with administration of cucurbitacin extract and/or metformin. This increase may reflects hepatocelluar damage associated with diabetes *(24)*.The activity of these enzymes was ameliorated after two months of treatment by cucurbitacin extract from *Citrullus colocynthis*, indicating that this cucurbitacins extract could, have the ability to repair liver tissue damage. These results are in agreement with other previous studies on the aqueous extract of the seed of *Citrullus colocynthis* *(25)*. Diabetes mellitus also causes renal damage due to abnormal glucose regulation, including elevated glucose, creatinine levels and glycosylated protein tissue levels, haemodynamic changes within the kidney

tissue and increased oxidative stress *(26)*. Plasma creatinine levels were higher in non-treated diabetic rats than in control group. The level of these substances had reduced after two months of treatment by cucurbitacin extracts from *Citrullus colocynthis*, which may indicates the ability of this plant extract to enhance renal function. These results are in agreement with other previous studies on the mesocarp extract of Balanites aegyptiaca *(27)*.

With these informations in mind, the present study provide novel experimental findings that, glucose tolerance is improved in STZ- diabetic rats, after the simultaneous oral administration of cucurbitacin extract from *Citrullus colocynthis*. Also it suggests that the external exposure to the plant extract may improve glucose homeostasis.

Conclusion

The present study showed that cucurbitacin extract of *Citrullus colocynthis* significantly reduced elevated blood glucose level in STZ-diabetic rats, since STZ effectively destroys pancreatic beta cells and causes persistent hyperglycemia, the mechanism of action of cucurbitacin extract might involve actions other than pancreatic beta cells insulin release or secretion. The anti-diabetic effect of the extract could be due to increased utilization of glucose by peripheral tissues, improved sensitivity of target tissues for insulin or it may be due to improved metabolic regulation of glucose.

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الملخص العربى

بعض الدراسات البيوكيمانية علي بعض مكونات الدم في الفئران

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يهدف البحث دراسة تأثير مادة الكاكوربيتاسين المستخلصة من نبات الحنظل على الفئران المصابة بالسكري .اشتملت الدراسة علي ١٢٠ من ذكور الجرذان قسمت إلى مجموعات ١ : مجموعة ضابطة ٢ : مجموعة ضابطة تم تجريعها ١٠٠ مليجرام / كجم من الكاكوربيتاسين .٣ : تم استحداث الداء السكري (مجموعة ضابطة) ٤ : تم استحداث الداء السكري و تجريعها ١٠٠ مليجرام / كجم من الكاكوربيتاسين.٥ : تم استحداث داء السكري تجريعها١٠٠ مليجرام / كجم من الميت فورمين .٦ : تم استحداث داء السكري ثم تم تجريعها١٠٠مليجرام / كجم من مادة الميت فورمين +١٠٠ مليجرام / كجم من الكاكوربيتاسين . أظهرت النتائج بعد تحليلها إحصائيا إلى نقص في مستوى السكر والكريتانين وناقلات الأسبرتات و الألنين في الدم وزيادة في نشاط ومعدل التعبير الجيني لإنزيم الجلوكوكينيز و الجلوكوز -٦- فوسفيت ديهيدروجينيز و ناقل الجلوكوز-٢ و لمستقبلات الأنسولين وإنزيم تخليق الجليكوجين في أنسجة الكبد