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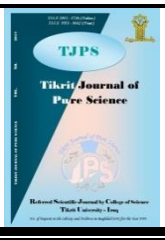

Biochemical and Histopathological study Of The Proteinaceous Compounds Separated From Aqueous Extract Of *Marus Albul L.* Fruit in alloxan diabetic mice

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ABSTRACT

This work was concerned with isolating and molecular weight determination of the proteinous compounds isolated from the cold and boiled aqueous extract of *Marus Albal L.* using different biochemical techniques . Also this study indicated the effect of the proteinaceous compounds Ac, Bc and Ab, Bb on some biochemical parameters including glucose, cholesterol and total lipids levels in blood serum and glycogen content in liver tissues in normal and diabetic mice . A single intraperitoneal injection of these isolated compounds with a dose of 75 mg / kg body weight was used .As well as pancreas were removed and stained with H and E of pancreatic sections. Results indicated that intraperitoneal injection of insulin and all the proteinaceous compounds Ac , Bc and Ab, Bb obtained by gel filtration chromatography from the plant used had hypoglycemic effect on serum glucose level in normal and diabetic mice. The rate of decrease was from (18.2) to (43.7) % in the normal and form (29.57) to (50.20) % in the diabetic mice respectively. While compounds (Bb) showed a negligible to an increasing effect (3.91) % in the normal and (7.99)% in the diabetic mice. Also the highest decrease was obtained for compound (Ac), this decrease were (16.8)% and (15.73) % for serum cholesterol levels in the normal and the diabetic mice respectively .As well as the highest decrease was obtained for compound (Ac) , this decrease were(23.62) % and (28.88) % for serum total lipids levels in the normal and the diabetic mice respectively. Also a decrease between (5.8 , 14.1) % and (6.4 , 12.9) % glycogen content in liver tissues was obtained when the normal and in the diabetic mice respectively were injected intraperitoneally by the proteinaceous compounds Ac, Bc and Ab , Bb from plants used. Finally alloxan induced diabetic mice group, the endocrine pancreas histologically showed decreased in the size and number of Langerhans islets with vacuolar degeneration and necrosis of almost all cell in the atrophied islets as compared with control group. The histomorphometic study of the pancreas of the treated group didn't show a significant change of the pancreatic tissue.

Introduction

Forages, plants have been the main source of drugs when administered empirically or otherwise in the cure of various diseases. Realizing the limitation of the therapy with modern synthetic drugs, human once again began to explore the nature's botany for the availability of useful drugs. Before the discovery of insulin in the early 1920s and later the development of oral hypoglycemic agents, patients with non-insulin requiring diabetes have been treated orally in folk medicine with variety of plant extracts [1].

Plants provide a vast resource of novel compounds with potential for the development of new antidiabetic drugs a worldwide more than 800

different plants have been described as traditional treatments for diabetes [2].

In general, there are many great hypoglycemic plants and the chemical structure of their active principle varies widely. Therefore some act by increasing the release of insulin and require a minimum of β-cells to exert their action. Other plant extracts or constituents act by modifying glucose metabolism and finally there are some that appear to correct the complications of diabetes [3].

Materials and methods

Plant material: *Marus albal L*. fruit was collected from the garden of the University of Mosul . Its classified according to plants taxonomy and plant classification [4]. But the fruit of plant indicted above which was used in the study, was collected , cleaned and kept in a nylon bags in a deep freeze until the time of use for the preparation of their extracts .

Animals used: Healthy male adult albino mice weighing (30-35) g was obtained from animal house, College of Education, University of Mosul were used in the experiments. They were housed under standard conditions, pelleted food and water were available *ad Libitum*. Animals described as fasted were deprived of food for at least 16 hours, but allowed free a access of tap water.

Preparation of cold crude aqueous extract: Cold crude aqueous extract was prepared by freezing and thawing the fruit (250 g) with liquid nitrogen several times to rupture the cell membrane distilled water (750 m1) was added and the crude homogenate was stirred for additional two hours then filtered through several layers of moselin (cheese-cloth). Finally the mixture was centrifuged at refrigerated centrifuge for 15 minute at 33520 xg. The filtrate (crude extract) after reduction its volume to about l/3 by lyophilization was kept for further investigation [5] .

Preparation of boiled crude aqueous extract: Boiled crude aqueous extract was prepared by freezing and thawing the fruit (250g) with liquid nitrogen several times to rupture the cell membrane distilled water (750m1) was added and heat for 30 minute than cold and the crude homogenate was stirred for additional two hours then filtered through several layers of moselin (cheese-cloth) . Finally the mixture was centrifuged at refrigerated centrifuge for 15 minute at 33520 xg. The filtrate (crude extract) after reduction its volume to about 1/3 by lyophilization was kept for further investigation [5].

Precipitation of the protein: The proteinaceous substance was separated from the crude aqueous extract by cold acetone precipitation technique [6].

Fractionation of the total protein: The isolated protein from the cold acetone precipitation technique was fractionated by gel filtration chromatography using a Sephadex G-50 gel on a (1.8 x 120) cm column. Final separation and apparent molecular weight estimation of the isolated components was accomplished on a similar column that using before. Distilled water used as eluent in both cases [7].

Intraperitoneal injection of the mice : Groups of healthy male adult mice (30-35) g weight were obtained from the animal house of the College Education, University of Mosul. The mice were fasted for (16) hours [8], divided randomly in to groups each containing (3) mice. Group one was kept as control and second group injection (10 iu/kg) with insulin (Actrapid 100iu /ml, Novo Nor disk A/S. Denmark), while other group injection with (75mg / kg) of the fractionated protein compounds (A.B). After two weeks of injection (one times daily) the blood samples were collected for analysis by the orbital sinus puncture technique under ether an aesthesia, using non-heparinzed microhernatocrit capillary types [9].

Induction of diabetes in mice: Healthy male adult albino mice, weight (30-35)g were selected and randomly divided into groups of third mice per group .They were fasted for 24 hours before induction of diabetes .They were then intraperitoneally injected with alloxan tetrahyclrate [7]. Which was dissolved in normal saline solution immediately before use at a dose of 180 mg / kg body weight [10] . Control animals were injected with normal saline only. Sine alloxan is capable of producing fatal hypoglycemic as a result of massive pancreatic insulin release. The animals were kept for the rest 24 hours on 5 % glucose solution in drink water, to improve the survival and to protect the animals from the profound hypoglycemic .The animals then allowed to take diet and water *ad libitum*. The diabetic state was monitored by periodic tests for glucoseuria (T-Tape®. Eli Lilly and Co. USA) . Mice with blood glucose levels more than 250 mg glucose/l00 ml were considered diabetic and used for the study. At the end of the period, third alloxan diabetic animals were randomly divided for each group for the present study [11].

Determination of parameters: Serum blood glucose and cholesterol levels were measured according to the enzymatic methods using Fortress/UK kit. [12].Total lipids levels were determined by the method of Chabrol and Chardonnel,1937. Glycogen content in the liver tissues was estimated by an throne method [13].

Statistical analyses: Results were expressed as mean ± SE. estimation of the significance of difference between control and proteinaceous compounds, insulin treated groups were analyzed by student 's T-test [14]. The percentage of glycemic variation after two hours of injection for treated groups was calculated by applying the formula:

% change of glycemia = (Gx - Gc / Gc)x 100

where Gc and Gx the values of control and glycemia after two hours [15].

Histopatholgical study: On the day of the experiment the mice were anesthized and the pancreas was removed and kept in 10% formaldehyde. Dehydration and clearing of tissues were formed automatically. The prepared 5-micron

thickness sections were stained with Hematoxylin and Eosin [16].

Results and discussion

Precipitation of the protein: precipitation of total protein from the aqueous extract of plant used in the research was accomplished by cold acetone as a precipitating organic agent [6], and not by saturated ammonium sulphate. Since the former can be easily removed by evaporation beside the fact that the precipitating power of both reagents were approximately similar. Moreover dialysis of the proteinous fraction to get rid of ammonium sulphate may remove some of low molecular weight proteins or peptides similar to that of insulin. The amount of total protein before and after precipitation by acetone and the efficiency of the precipitation were listed in table (1).

Table (I): Total amount of protein the aqueous extract, precipitated proteinous materials from plant used and the efficiency of precipitation .

Sample	Total amount of protein in the aqueous extract (g) in (250 g) plant weight	Total amount of precipitated proteinous materials as powder	Efficiency of precipitation %
Cold crude aq.ext	1.46	1.27	86.98
Boilied crude aq.ext	1.67	1.48	88.62

Fractionation total protein: Fractionation of total protein from plant resulting from acetone precipitation was obtained by get filtration using Sephadex G-50. Figure(1and 2) which showed that total protein isolated from cold aqueous extract of *Marus albal L*. containing mainly two components The first one (Ac) has a high molecular weight and the second component (Bc) has a low molecular weight. Also total protein isolated from boiled aqueous extract of *Marus albal L*. containing mainly two components. The first one (Ab) has a high molecular weight and the second component (Bb) has a low molecular weight. Fractionation of the total protein from plant showed approximately the same pattern and results were listed as elution volumes of (Ac, Ab) and (Bc, Bb) components.

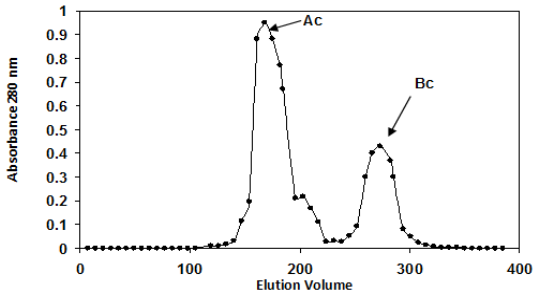

Figure (l): Elution profile of the proteinous materials isolated from cold aqueous extract of *Marus albal L*. on sephadex G-50. The dimensions of the column are (1.8x 120) cm. The Ac and Bc represent the elution volumes for the first (Ac) and the second (Bc) peaks respectively. The volume of each fraction is (7ml) at a flow rate (42 ml/hr).

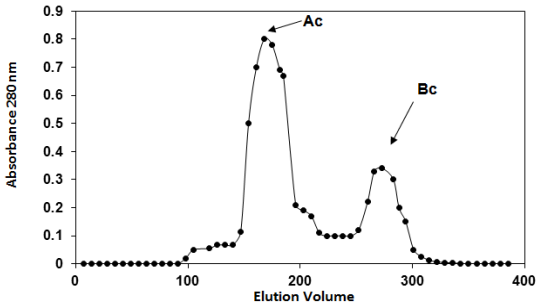

Figure (2): Elution profile of the proteinous materials isolated from boiled aqueous extract of *Marus albal L*.. on sephadex G-50. The dimensions of the column are (1.8x 120) cm. The Ac and Bc represent the elution volumes for the first (Ab) and the second (Bb) peaks respectively. The volume of each fraction is (7ml) at a flow rate (42 ml/hr).

Quantitative determination of total protein in each peak after gel filtration chromatography by a modified lowry method was performed and then the percentage of each compound was calculated and listed in table (2).

Table (2): percentage of protein in the aqueous extract of plant and the percentage of each compound in the proteinous materials

Samples	% of protein in the aqueous of the plant	% of compounds in each peak	
		A	B
Cold crude aq.ext	0.584	40	45
Boiled crude aq.ext	0.668	31.6	40

Molecular weight determinations: After complete separation of compounds Ac, Ab and Bc, Bb as indicated in materials and methods, apparent molecular weights estimation were obtained using column chromatography of the dimension (1.8 x 120) cm containing Sephadex G-50. A linear plot was obtained as shown in (Figure 2) the estimated apparent molecular weight of each compounds Ac, Bc and Ab, Bb were found to be 41550, 3602 and 29735, 2930 Dalton, respectively (Table 3).

Table (3): Elution volume and molecular weight of standard compounds and Ac, Bc and Ab, Bb compound.

Compounds	Molecular weight Dalton	Elution volume (ml)
Bovine serum albumin	67000	124
α-amylase	58000	134
Eggs albumin	45000	140
Pepsin	36000	165
Trypsin	23000	172
Insulin	5750	226
Oxytocin	1051	282
Tryptophan	204	333
Ac	41550	154
Bc	3602	247
Ab	29735	169
Bb	2930	256

These values were obtained from figure (3).

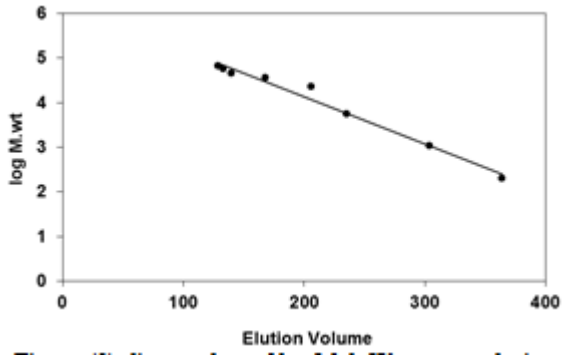

Figure (3): linear plots of log Mol. Wt. versus elution volume on a Sephadex G-50: Total volume of sample was (7 ml) at a flow rate of (42 ml/hr). Fraction volume of points 1,2,3,4,5,6,7,8 represent BSA, α-amylase, Egg albumin, Pepsin, Trypsin, Insulin, Oxytocin, Tryptophan respectively (see Table3).

Effect of compounds Ac, Bc and Ab, Bb on some metabolic parameters:

The result of treating normal and diabetic mice with insulin showed a decrease in serum blood glucose level which was in agreement with many studies in normal and diabetic individuals with Abed Al-Saadon [17]. The hypoglycemic effect of insulin may be due to the increase in the rate of entrance of various sugars and glucose into the cell through increasing the number of glucose transporters in the plasma membrane [18].

The results of compound Ac, Bc and Ab, Bb showed a significant ($P < 0.05$) decrease in blood glucose compared to the control group except compound (Bb) showed there was a negligible to an increasing effect (3.91) and (7.99) in normal and diabetic mice respectively. These results are in agreement with the previous work on the hypoglycemic activity of the proteinaceous compounds isolated from the aqueous extract of other local plants as Ahmad [19]. Which showed that the mechanism of action of the low, molecular weight protein isolated from different local plants was similar to insulin in its action .Also, a decrease in serum glucose level of mice treated with high molecular weight protein compound (compound Ac) was in agreement with the results obtained by other investigators Al-Chalabi and Al-Choka [20]. This suggested that the protein compound with high molecular weights which were isolated from the aqueous extract of plant might contain sequence of amino acid similar to insulin which binds to specific insulin receptors located on the plasma membrane. Binding might mediator facilitate the rate of uptake of glucose inside the cell leading to hypoglycemic activity or may caused an increased secretion of internal insulin by impairing langerhans cells in normal and diabetic mice.

The decrease in cholesterol level for compounds (Ac, Bc),(Ab,Bb) and insulin were mentioned in Table (4-5), these result were in agreement with Al-Bajari in normal and diabetic mice. This decrease in cholesterol level might be due to the in activation of the regulatory enzyme β-hydroxyl-β-methyl glutaryl-CoA (HMG-CoA) reductase responsible for cholesterol biosynthesis [7]. Also the decrease of cholesterol level when treated with insulin is in agreement with the results obtained on diabetic rats and rabbits Mahmood et al. [21]. This might be due to inhibiting intestinal acyl CoA cholesterol acyl transferase which is responsible for absorbing cholesterol form the intestine [22].

Table (4-5) indicates the effects of the same proteinaceous compounds Ac, Bc and Ab, Bb on serum total lipids levels which statistically showed a significant ($P < 0.05$) decrease in total lipids. These results are in agreement with results of decreasing the proteinaceolls compound of *Phaseolus vulgaris* and *Vigna sinensis* Fruits [23]. Whereas the proteinaceous compound of *Apium graveolens* [17].

This decrease might be due to the inhibiting of lipase enzyme and inhibiting lipolysis of stored lipids [11].

Finally, a decrease in glycogen content in liver tissues was obtained when the mice were injected intraperitoneally by the proteinaceous compounds Ac, Bc and Ac, Bb (table 4-5). This decrease is in agreement with the results obtained by other investigators for aqueous extract of different plants [24]. This decrease might stimulate the glycogen break down by the cascade process and reduce the level of glycogen [25].

Table (4) Effect of (Ac, Bc and Ab, Bb) compound and insulin after two hours given intraperitoneally injection in fasted normal mice on serum glucose, cholesterol, total lipids levels and glycogen content in liver tissues.

Group N=3	1	2	3	4	5	6
Sample	control	insulin	Ac	Bc	Ab	Bb
Dose	----	10iu/kg	75mg/ kg	75mg/ kg	75mg/ kg	75mg/ kg
Glucose mmol/l	5.88±0.12	1.86±0.14	3.31±0.21	4.82±0.19	3.87±0.31	6.11±0.22
% change	----	-68.36	-43.7	-18.02	-34.18	3.91
Cholesterol mmol/l	2.38±2.3	1.85±0.03	1.98±0.12	2.15±0.27	2.03±0.09	2.40±0.27
% change	----	-22.26	-16.8	-9.66	-14.70	0.84
Total lipids mg/dl	375.78±2.3	215.38±1.6	286.99±7.2	313.17±9.3	322.71±9.7	370.91±8.9
% change	----	-42.68	-23.62	-16.66	-14.12	-1.29
Glycogen mg/kg	1.20±0.11	1.29±0.13	1.03±0.09	1.07±0.11	1.11±0.12	1.29±0.11
% change	----	7.5	-14.1	-10.8	-7.5	-5.83

Blood samples and liver tissues were taken after two hours of group intraperitonal injection.
Glucose, Cholesterol, Total lipid, levels and glycogen content in liver tissue were expressed in MEAN ± SE
N= number of mice each group.
Significantly different from control $P < 0.05$.

Table (5) Effect of (Ac, Bc and Ab, Bb) compound and insulin after two hours given intraperitoneally injection in fasted diabetic mice on serum glucose, cholesterol, total lipids levels and glycogen content in liver tissues.

Group N=3	1	2	3	4	5	6
Sample	control	insulin	Ac	Bc	Ab	Bb
Dose	----	10iu/kg	75mg/ kg	75mg/ kg	75mg/ kg	75mg/ kg
Glucose mmol/l	16.77±0.19	3.53±0.07	8.35±0.9	11.81±0.12	8.97±0.31	18.11±0.33
% change	----	-78.95	-50.20	-29.57	-46.51	7.99
Cholesterol mmol/l	2.86±0.1	2.20±0.09	2.41±0.11	2.56±0.12	2.51±0.08	2.79±0.09
% change	----	-23.07	-15.73	-10.48	-12.23	-2.44
Total lipids mg/dl	560.11±2.3	363.75±2.1	398.33±9.2	497.19±7.7	422.51±11.1	555.31±11.0
% change	----	-35.05	-28.88	-11.23	-24.62	-0.85
Glycogen mg/kg	0.93±0.08	1.21±0.09	0.81±0.07	0.84±0.08	0.87±0.09	0.88±0.10
% change	----	30.10	-12.9	-9.6	-6.4	-6.4

Blood samples and liver tissues were taken after two hours of group intraperitonal injection.
Glucose, Cholesterol, Total lipid, levels and glycogen content in liver tissue were expressed in MEAN ± SE.
N= number of mice each group.
Significantly different from control $P < 0.05$

Effect of compounds Ac, Bc & Ab, Bb on Histopatholgy of the pancreas:

Pancreatic section of control group showed the normal structure of islet notice the normal cells shape surrounding by exocrine pancreas normal mice stained with HE (Fig 1). Pancreatic section of alloxan induced diabetic mice group, the endocrine pancreas histologically showed decreased in the size and number of Langerhans islets with vacuolar degeneration and necrosis of almost all cell in the atrophied islets as compared with control group (Fig.2) these results were agreed with results obtained by Al-Sabawy [26]. Also there was congestion of blood vessels in the interlobular space, thrombus of the blood vessel , degeneration and necrosis of acinar cells and oedema and thickening of blood vessel wall in the section of Pancreas alloxan diabetic mice and treated Ac, Ab, Bc and Bb (Fig.3,4,5and 6).

Histopathological study of diabetic untreated mice showed almost complete destruction of β-cells, which was due to the proper dose of alloxan used in this study. An inadequate dose will cause partial destruction of β-cell in islet [27]**.** The histopathological study of diabetic treated group did

not show a significant difference with the untreated group. This finding reveals that the hypoglycemic effect *Marus Albul L.* is not through the action of *Marus Albul* on the number of β- cells, and will support the theory that *Marus Albul L.* is hypoglycemic effect might be due to the action of substances like allyle propyl disulphide or diallyle disulphide [16],or due to an increase in the insulin response.

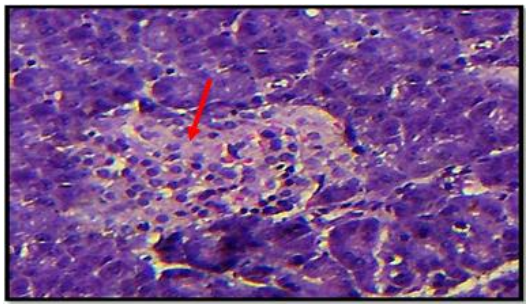

Fig.1 Section of pancreas of control group showed the normal structure of islet notice the normal cells shape surrounding by exocrine pancreas ( ). H & E (400X) .

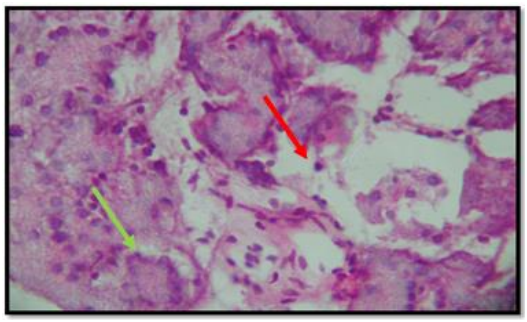

Fig.2 Section of mice pancreas of alloxan diabetic mice showed necrosis of islet cells ( ) and atrophied islets ( )H & E (400X).

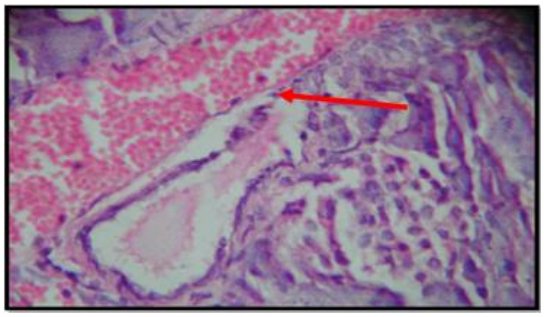

Fig.3 Section of pancreas alloxan diabetic mice after treated with Ac compound showed the thrombus of the blood vessel, H & E (400X).

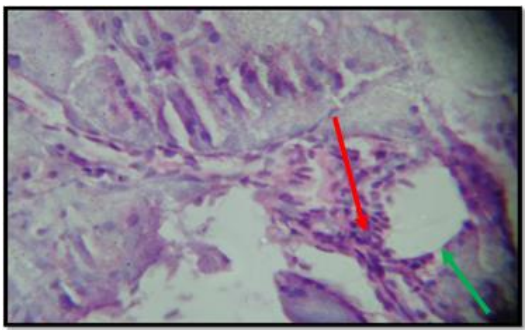

Fig.4 Section of pancreas alloxan diabetic mice after treated with Ab compound showed distortion of exocrine pancreas, notice the degeneration and necrosis of acinar cells ( ) and there is oedema ( ) .H & E (400X).

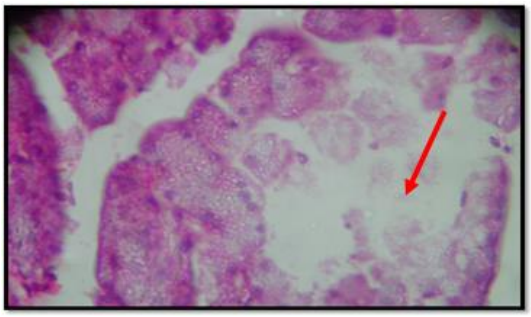

Fig.5 Section of pancreas alloxan diabetic mice after treated with Bc compound showed necrosis of islet cells ( ) H & E (400X).

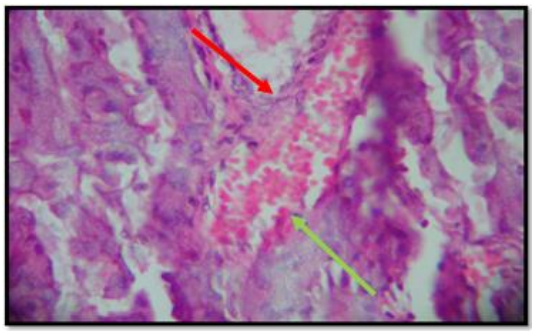

Fig.6 Section of pancreas alloxan diabetic mice after treated with Bb compound showed the thrombus of the blood vessel ( ), and showed thickening of blood vessel wall ( ), H & E (400X).

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دراسة كيموحيوية ونسجية للمركبات البروتينية المفصولة من المستخلص المائي لثمرة نبات التوت *Marus albaL.* في الفئران المصابة بداء السكر المستحدث بالالوكسان

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الملخص

تضمن البحث دراسة تاثير المركبات البروتينية Ac , Bc , Ab , Bc المفصولة بتقنية الترشيح الهلامي من المستخلصات المائة الباردة والمغلية لثمرة نبات التوت الابيض على بعض المتغيرات كيموحيوية (كلوكوز ,الكوليستيرول والدهون الكلية) في مصل دم الفئران السليمة والمصابة بداء السكر المستحدث بالالوكسان, وكذلك محتوى الكلايكوجين في كبد الفئران السليمة والمصابة. حيث تم في هذا البحث اعطاء جرعة واحدة من المركبات البروتينية عن طريق الحقن في التجويف البريتوني بمقدار 75 ملغم / كغم من وزن الجسم. فضلا عن ذلك تم استئصال البنكرياس لتحضير مقاطع نسيجية من البنكرياس صبغت بالهيماتوكسلين والايوسن. اظهرت نتائج حقن الانسولين وكل من المركبات البروتينية , Ac , Bc Ab , Bc في الفئران السليمة والمصابة الى انخفاض معنوي ملحوظ في مستوى الكلوكوز يتراوح بين (18.2) و(43.7) % للسليمة وبين (29.57) و (50.20) % للمصابة، بالمقارنة مع مجاميع السيطرة السليمة والمصابة على التوالي. ماعدا المركب البروتيني (Bb) العائد للمستخلص المائي المغلي لثمرة التوت، حيث ادى الى ارتفاع معنوي في مستوى الكلوكوز في الفئران السليمة والمصابة وبمقدار (3.91) و (7.99) % على التوالي. كما ابدى المركب (Ac) تاثير اكثر تخفيضا في مستوى الكوليستيرول من بقية المركبات مقداره (16.8) % في السليمة و (15.73) % في المصابة على التوالي. اما بالنسبة للدهون الكلية فقد ابدى المركب (Ac) اكثر انخفاضا بمقدار (23.62) % في الفئران السليمة و (28.88) % في الفئران المصابة . كما اشارت النتائج الى ان المركبات Ac , Bc , Ab , Bc والتي تم فصلها من النبات ادت الى حدوث انخفاض كلايكوجين الكبد بين (14.1) % و (5.83) % للفئران السليمة و بين (12.9) % و (6.4)% للفئران المصابة على التوالي. اخيرا فقد اظهرت الدراسة النسجية للفئران المحقونة بالالوكسان وجود نقصان في حجم وعدد جزر لانكرهانس فضلا عن نخر وتحطم واحتقان شديد للاوعية الدموية في خلايا البنكرياس مقارنة بالسيطرة السليمة. كما بينت النتائج عدم وجود اي تغير معنوي بين المجاميع المصابة المعالجة بالمركبات Ac , Bc , Ab , Bc مع المجموعة المصابة غير المعالجة بتلك المركبات في انسجة البنكرياس.