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The Extract of *Anastatica hirerochuntica* (Kaff Maryam) Flavonoids Creates a Novel Pharmacophore at the position of Inflammation in Iraqi Female with Type 2 Diabetes Mellitus.

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ABSTRACT

Type -2- diabetes mellitus is characterized by insulin resistance , pancreatic β- cells dysfunction , high levels of blood lipids and systemic inflammation caused by disorder in immune system. The aim of treating type-2-diabetic patients with aqueous flavonoids extract of *Anastatica hireochuntica* (quercetin and luteolin) is to overcome the inflammation , enhance immune function and maintain blood glucose and lipid profile within ranges near normal values. (Two hundred fifty) mg of aqueous flavonoids extract were given to 50 female with type-2- diabetes mellitus three times daily for six weeks, and biochemical parameters (glucose , lipid profile , erythropoietin , vitamin D , MDA and liver enzymes) were measured in sera of patients before and after treatment with polyphenols and also measured in the sera of healthy female as a control group. Glucose and lipid profile levels (except HDL) , MDA levels and (GOT , GPT , ALP) activities were depressed while erythropoietin and vitamin D were increased after treatment with flavonoid extract. The present study has reported for the first time the mechanism by which erythropoietin level was depressed in type-2- diabetic patients . Moreover , erythropoietin and vitamin D appear to be good biochemical markers for Iraqi patients with type-2- diabetic patients. Ultimately , this study suggested that flavonoids extracted from *Anastatica hireochuntica* exerts both immunomodulatory effect and antioxidant action.

Keywords: Eryrhropoitein , vitamin D , flavonoids , type- 2 – diabetes mellitus.

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INTRODUCTION

Among the plants used in medicine for the treatment of chronic diseases related to immunity and oxidative stress are *Anastatica hirerochuntica* (kaff maryam) , a small gray annual herb[1]. The widespread use of medical plants for health purposes has increased dramatically due to their importance to public health [2]. Novel potential bioactive flavonoids with antioxidant therapeutic properties were contained in this medical plant [3]. The presence of flavonoids in the plant is associated with the antioxidant and antidiabetic potentials. Many studies have indicated the beneficial role of flavonoids in the treatment of diabetes mellitus. Flavonoids which are a major class of polyphenols are sub-divided into many classes such as quecetin , rutin , diomin , catechins and luteolin [4]. These compounds have biochemical activity due to the effect of polyhydroxyl groups on inhibition of lipid peroxidation and prevention of several biological changes in living cells that lead to different disease such as type 2 diabetes mellitus and atherosclerosis [5]. Flavonoids not only down-regulate reactive oxygen species ROS formation, but also contribute to improved endothelial health through anti-inflammatory action [6]. Indeed , the findings presented that polyphenols from all plant sources are capable to regulate the immune function [7]. Type 2 Diabetes Mellitus (T2DM) is the most common form of the disease, affecting approximately 90% of the diabetic population , characterized by a combination of insulin resistance , dysfunction of beta cells in pancreas and elevation of lipids in blood [8]. A previous study has reported that inflammation and dysfunction of innate immune system is strongly included in pathophysiology of type 2 diabetes mellitus[9]. Erythropoietin is a 30.4 kDa Cytokine consisting of four glycosylated polypeptide chains . it act as a growth factor , controls immune modulation and vascular function [10] , regulate erythropoiesis and promotes angiogenesis [11]. It is now widely recognized that vitamin D have a wide range of biochemical function in cell differentiation. Moreover , both innate and adaptive immunity are regulated by vitamin D , this affective vitamin enhances insulin secretion and promotes B_cell survival [12] . Further , vitamin D deficiency may be associated with a range of chronic diseases including diabetes mellitus [13]. Liver enzymes activities (GPT , GOT and ALP) must be studied in the course of diabetes mellitus because of the direct effect of insulin to suppress hepatic glucose production[14]. This study highlights the influence of *Anastatica hirerochuntica* flavonoids extract on glucose, lipid profile , erythropoietin , vitamin D , MDA levels and liver enzymes (GOT, GPT and ALP) activities in females patients with T2DM, before and after oral supplementation with aqueous flavonoids extract.

MATERIALS AND METHODS

Patients Groups: Blood samples were collected from 50 T2DM female patients with age in range of 40-55 years who are suffering from this disease for two years and higher, these samples were collected from volunteers in several laboratories. Samples collection was performed in Baghdad Teaching hospital (Medical city)Patients groups are classified into three groups:-

B: Patients group before oral supplementation with aqueous *Anastatica hirerochuntica* flavonids extract.
A_1: Patients group after oral supplementation with aqueous *Anastatica hirerochuntica* flavonoids extract over 3 weeks.
A_2: Patients group after oral supplementation with aqueous *Anastatica hirerochuntica* flavonoids extract over 6 weeks.
C: healthy female volunteers are included in this study as control group, their ages are in range of 40-55 years, Non of the control has diabetic mellitus, atherosclerotic cardiovascular diseases CVD, renal disease, allergy and heavy smokers.

Specimens Collection , Preparation of aqueous flavonoids extract and test for flavonoids: About 10 mL of venous blood were obtained from the capital vein using disposable needles and seringes. In all cases, blood samples were collected in the morning after 12 hours fasting. Blood samples were left for 20 min at room temperature. After coagulation, the sera were separated by centrifugation at 3000 rpm for 5 min. The sera were stored and frozen at -20°C until analysis. *Anastatica hirerochuntica* whole plant were purchased from local market. The plant material was authenticated by taxonomist, the samples were washed with clean tap water. The dried material was powdered using electrical blender. 300g from powdered whole plant was added to 3 liters distilled water, mixed thoroughly and heated at 80°C for 60 min in a water bath, with continuous stirring. Aqueous extract was filtered through a 0.45 Milipore nylon filter. The flavonoids content of aqueous extract was estimated by Mg method [18]. An aliquot of extract solution was added to concentrated HCl, along with 250 mg of metal Mg. After boiling, a strong cherry-red color indicated the presence of high amounts of

flavonoids. Dried aqueous extract was then collected by spray-dried to give a very fine yellowish-brown color, water soluble powder identified by HPLC-UV. to give flavonoids. Extraction and purification were performed in a laboratory in the college of education for pure science (Ibn Al-Haitham) /Baghdad University.

***Anastatica hirerochuntica* flavonoids extract samples:** Flavonoid extract was in a form of capsules containing 250mg that was extracted from *A. hirerochuntica* plant, these capsules were firstly examined by central organization for standardization and quality control in Ministry of Sciences and technology , given to female patients with T2DM, one capsule three times daily during a period of six weeks, and parameters are measured after 3 weeks and 6 weeks of oral supplementation with aqueous flavonoids extract.

Biochemical assays: Fasting blood glucose , TC , TG were determined by enzymatic methods. HDL was measured by precipitation method using phosphotungestic acid in the presence of magnesium ion Mg^{2+} .LDL and VLDL . levels calculated manually according to Equations below . [15]

VLDL mg/dl = TG mg/dl /5.
LDL mg/dl = TC mg/dl – [HDL mg/dl VLDL mg/dl].

MDA was determined using thiobarbituric acid. Erythropoitein was defermined by a quantitative sandwich enzyme linked immunosorbent assay (ELISA) technique . Antibody specific for erythropoietin was precoated into the microplate accompanied with erythropoietin kit , standards and samples were pipetted into the wells and immobilized antiboody binds any erythropoitein present . After removing any unbound substances , a biotin conjugated antiboody specific for erythropoitein was added to the wells , after washing , avidin conjugated horse radish (H R P) was added to the wells and color develops in proportion to the amount of erythropoitein bond in the initial step . The color development was stopped and the intensity of the color was measured. Vitamin D was determined by routin high performance liquid chromatography (H P L C) analysis using column of silica gel as a stationary phase and hexane as a solvent. GOT activity was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-Dinitrophenyl hydrazine. GPT activity was measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-Dinitrophenyl hydrazine ALP activity was measured according to king methodusing commercially available kit supplied by Bio Merieux-France. Lastly, results were statistically expressed as mean±S.D, student T-test was applied for comparing the significance of difference between patient and control group, P refers to probability whereby: ($P<0.05$), ($P<0.001$) and ($P>0.05$) were respectively considered

RESULTS

Table.1 : Levels of glucose and lipid profile in patients and control groups before and after oral supplementation with flavonoids extract.

Parameter	Mean± S.D				P B/C	P A_1/B	P A_2/B
	C	B	A_1	A_2			
Glucose mg/dl	92±13.4	184±28.4	164±20.1	138±16.6	H.S	S	H.S
TC mg/dl	180±13.4	220±24.2	210±16.8	195±13.6	S	N.S	S
TG mg/dl	120±22.4	158±25.2	148±13.9	130±10.4	S	N.S	S
HDL mg/dl	44.6±22.6	38±8.1	40.1±9.2	42.0±8.8	S	N.S	N.S
LDL mg/dl	113.4±11.2	150.6±14.2	140.3±15.1	126.6±13.2	H.S	N.S	H.S
VLDL mg/dl	24±4.1	31.4±6.8	29.6±6.8	26.4±5.4	S	N.S	N.S

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Table. 2 : Levels of Erythropoitein and vitamin D in patients and control groups before and after oral supplementation with flavonoids extract

Parameter	Mean± S.D				P B/C	P A_1/B	P A_2/B
	C	B	A_1	A_2			
Erythropoitein mU/mL	10.84±2.7	2.81±0.73	4.37±1.03	8.21±2.1	H.S	S	H.S
Vitamin D ng/mL	37.24±9.3	17.43±2.6	30.39±4.1	33.41±4.9	S	H.S	H.S

Table .3 : Levels of MDA and liver enzymes in patients and control groups before and after oral supplementation with flavonoids extract.

Parameters	Mean± S.D				P B/C	P A_1/B	P A_2/B
	C	B	A_1	A_2			
MDA nmol/dl	0.42±0.05	1.68±1.76	1.29±0.13	0.61±0.01	H.S	S	H.S
GOT IU/L	7.1±3.1	13.2±6.1	11.9±4.1	9.2±5.3	H.S	S	H.S
GPT IU/L	6.9±2.9	15.2±4.5	12.4±3.8	8.4±4.4	H.S	S	H.S
ALP IU/L	24±4.1	52.1±8.2	42.1±7.2	28.1±5.2	H.S	S	H.S

Flavonoids action on glucose:

Results of table 1 have revealed that glucose level was high significantly increased in sera of group B (184±28.4) mg/dL compared with group C (92±13.4) mg/dL . On the other hand , glucose level was significantly decreased in sera of group A_1 (164±20.1) mg/dL compared with group B (184±28.4) mg/dL , while a high significant decrease was shown in group A_2 (138±16.6) mg/dL compared with group B (184±28.4) mg/dL.

Flavonoids action on lipid profile :

Results have reported that TC level was significantly increased in sera of group B (220±24.2) mg/dL compared with group C (180±13.4) mg/dL while it was non significantly and significantly decreased in sera of groups A_1 (210±16.8) mg/dL and A_2 (195±13.6) mg/dL respectively compared with group B (220±24.2) mg/dL , table 1 . Also , TG level was significantly increased in sera of group B (158±25.2) mg/dL compared with group C (120±22.4) mg/dL whereas it was non significantly and significantly decreased in sera of groups A_1 (148±13.9) mg/dL and A_2 (130±10.4) mg/dL compared with group B (158±25.2) mg/dL. Interestingly , HDL level was significantly decreased in in sera of group B (38±8.1) mg/dL compared with group C (44.6±12.6) mg/dL, conversely it was non significantly increased in groups A_1 (40.1±9.2) mg/dL and A_2 (42.0±8.8) mg/dL compared with group B (38±8.1) mg/dL. Results of table 1 have also represented that LDL level was high significantly increased in sera of group B (150.6± 14.2) mg/dL compared with group C (113.4±11.2) mg/dL but it was non significantly and high significantly decreased in groups A_1 (140.3±15.1) mg/dL and A_2 (126.6±13.2) mg/dL compared with B (150.6±14.2) mg/dL. Moreover , VLDL level was significantly increased in sera of group B (31.4±6.8) mg/dL compared with group C (24±4.1) mg/dL while it was non significantly decreased in sera of groups A_1 (29.6±6.8) mg/dL and A_2 (26.4±5.4) mg/dL compared with group B (31.4±6.8) mg/dL.

Flavonoids action on Erythropoietin:

Results of table 2 have reported that ERP level was highly significant decreased in sera of group B (2.81 ± 0.73) mU/mL compared with group C (10.84 ± 2.7) mU / mL. On the other hand , Erythropoietin level

was significantly and high significantly increased in sera of groups A_1(4.37 ± 1.03) mU / mL and A_2(8.21±2.1) mU/mL respectively compared with group B (2.81±0.73) mU/mL.

Flavonoids action on vitamin D

Results of table 3 have shown that vitamin D level was significantly decreased in sera of group B (17.43 ± 2.6) ng/mL compared with group C (37.24 ± 9.3) ng/mL , while , It was high significantly increased in sera of group A_1 (30.39 ± 4.1) ng/mL and A_2 (33.41 ± 4.9) ng /mL respectively compared with group B (17.43 ± 2.6) ng/ mL.

Effect of aqueous flavonoids extract on MDA:

Results of table (3) have indicated that MDA level was high significantly increased in sera of group B (1.68±1.76) nmol/dL compared with group C (0.42±0.05) nmol/dL while it was significantly and high significantly decreased in sera of groups A_1(1.29±0.13) nmol/dL and A_2 (0.61±0.01) nmol/dL respectively compared with group B (1.68±1.76) nmol/dL.

Flavonoids action on liver enzymes :

GOT was high significantly increased in sera of group B (13.2±6.1) IU/L compared with group C (7.1±3.1) IU/L while it was significantly and high significantly decreased in sera of groups A_1 (11.9±4.1) IU/L and A_2 (9.2±5.3) IU/L respectively compared with group B (7.1±3.1) IU/L. Furthermore , GPT was high significantly increased in sera of group B (15.2±4.5) IU/L compared with group C (6.9±2.9)IU/L , conversely it was significantly and high significantly decreased in sera of groups A_1 (12.4±3.8) IU/L and A_2 (8.4±4.4) IU/L compared with group B (6.9±2.9) IU/L. Similarly , ALP was high significantly increased in sera of group B (52.1±8.2) IU/L compared with group C (24±4.1) IU/L , also it was significantly and high significantly decreased in sera of groups A_1 (42.1±7.2) IU/L and A_2 (28.1±5.2) IU/L respectively compared with group B (52.1±8.2) IU/L.

DISCUSSION

The increased glucose level in sera was recorded in T2DM patients , table 1. It may have derived from glycogenolysis and/or gluconegenesis which could be causative reasons of hyperglycemia in different diabetic states [16]. A recent study has reported that quercetin has antioxidant properties reflected by regeneration of the pancreatic islets and probably increases insulin release [17]. Another recent study has suggested the antidiabetic properties of luteolin which is a free radical scavenger , a promoter of carbohydrate metabolism , and an immune system modulator. Moreover , luteolin decrease glycemia level (approximately 50%) , increase in insulin secretion a 2.5 – fold , inhibit alpha- glucosidase and alpha amylase. Also luteolin was found to influence insulin action and production of cytokines [7] . Our data are consistent with that reported by researchers who attributed the increases at TC levels in T2DM female diabetic to oxidative stress condition , table 1 , which had been occurred in diabetes and positively correlated with a long period of the disease, in addition to a defect in LDL receptors as result of glycation of receptors [18]. A recent study agrees with our results , it has indicated that quercetin exerts multiple effects, antioxidant , antiatherogenic , antihypertensive, anti- inflammatory[19]. Another study has revealed that *Anastatica hierochuntica* has a greater anti-hyper lipidemia effect [3] . The present study indicate that flavonoids extract showed a hypolipidmic agent , table 1 . On the other hand , the increase in TG levels in T2DM patients might be due to a variety of metabolic abnormalities such as effect on the activity of lipoprotein lipase LPL, this decrease in LPL activity led to increase of TG levels in T2DM patients [20]. After oral supplementation of flavonoids extract by diabetic patients oxidative stress condition was improved and free radical became less and TG level was decreased [21] , table 1. This improvement may be due to improved glycemic control and inhibition of lipid peroxidation process [22]. Indeed , The interpretation for our finding related to lipid profile could be explained basing on polyphenolic flavonoid lead to increasing the activity of some enzymes that associated with HDL-c, such as lecithin cholesterol acyl transferase LCAT and paraxonase PON, these enzymes play important roles in antioxidant properties [23], and catalyze hydrolysis of lipid peroxides, cholesteryl linoleate hydroperoxides and organo- phosphates in oxidized HDL [24]. Interestingly , luteolin may qualify as an antiatherogenic agent in LDL systems , which may have implications for strategies attenuating monocyte / macrophage dystunction- related atherosclerosis.[2]. Because the largest amount of TG are positioned on VLDL-c, therefore our results related to VLDL in table 1 could be explained just like our interpretation about TG levels. Quercetion and Luteolin that

could affect either VLDL secretion or VLDL-c removal in the blood circulation, flavonoids reduce the number of VLDL particles secreted by liver, increase the activities of enzymes that regulate lipid metabolism and inhibit lipid peroxidation [25,26]. Moreover, quercetin scavenges oxygen radicals , inhibits xanthine oxidase and inhibits lipid peroxidation in vitro [21]. Although autoimmunity was a well known component in type 1 diabetes mellitus , the assumption that pathogeneses of type 2 diabetes mellitus also encompasses autoimmune aspects is recently recognized on the basis of circulating auto antibodies against beta cells [12]. In this regard , a previous study has revealed that inflammation and the activation of the innate immune system is closely involved in the etiology of type-2-diabetes mellitus[8], another previous study linked between prevalence of infections in diabetes mellitus and defects in immunity [28]. Indeed , erythropoietin as a superior growth factor can regulate immunological responses [29] , enhance the clearance of apoptotic cells (the failure of apoptotic cell clearance is linked with autoimmune disorders) [30]. Erythropoietin has four glycosylated chains that include three N_ Linked and O_Linked acidic Oligosaccharide side chains . Both of the production and secretion of the mature Erythropoietin protein is dependent on N- and O- linked chain integrity in other words , EPO is fostered by carbohydrate chains [10]. At this point , a recent study has reported that diabetes mellitus may be accompanied by removal of both O- and N- linked oligosaccharides from the glycoprotein [31] . Moreover , the two disulfide bonds formed between cysteine control the biochemical function of EPO. The presence of disulfide bonds is an effective switch for EPO function [32] . In diabetic patients and other patients suffering from diseases accompanied by high level of oxidative stress and reactive oxygen species , degradation of disulfide bonds may be occurred because of direct attack of the hydroxyl anion on these bonds [33] . For reasons mentioned above , EPO was highly significant decrease in diabetic patients group compared with control group , table 2 . Polyphenols are potent antioxidant acting on immune system by shifting pro _ oxidant / antioxidant balance towards anti-oxidant . In this regard , a recent study has reported that polyphenols have a modulator effect on the immune system [7], flavonoids can modulate the innate response through their potent anti-oxidant and ant- inflammatory mechanisms , those vital compounds can regulate immunological responses by influencing T cells , B cells and macrophages in a way includes shifting the pro _ inflammatory status to anti _ inflammatory status [34] . Polyphenolic compounds generally inhibit the production of ROS by neutrophils. Interestingly the inverse relationship between polyphenols and inflammation could be explained on the basis of the aromatic nature of polyphenols which make them potential targets of pro-inflammatory oxidants , so oxidants react with polyphenolic tyrosine residue on proteins and create a novel pharmacophore at the site of inflammation [35] . Vitamin D deficiency seem to have a role in the pathophysiology of type -2- diabetes mellitus because it has an immunomodulatory and anti _ inflammatory function, reduce insulin resistance , increase insulin secretion and decrease autoimmune insulates , a novel study strongly supports our results related to vitamin D , table 2. It explains the association between vitamin D deficiency in diabetes mellitus in accordance with two facts : the first is the discovery of vitamin D receptors and 1- hydroxylase enzyme inside beta -cells the second is the presence of calcium linking protein vitamin D dependent in pancreatic tissue [36] . A previous study also indicates the connection between vitamin D receptors in pancreas and altered insulin secretion in diabetes mellitus [37]. The unexpected increasing of vitamin D level after treatment with polyphenols may be illustrated based on the basis of quercetin ability to stimulate beta-cells function [38]. Quercetin induces insulin secretion by direct activation of L_type calcium channels in pancreatic beta-cells [39]. Another recent study agrees with our results revealing that luteolin has a protective action on pancreas [40]. The crude extract of Kaff maryam *Anastatica hierochuntica* was evaluated in the term of antioxidant[2].Quercetin reduced MDA level (MDA is a potent indicator for lipid peroxidation) because of its strong antioxidant properties by increasing endogen antioxidant activities and by directly scavenging free radicals [19] , table 3. Another recent study has revealed that luteolin has shown to scavenge hydroxyl and peroxyl radicalsand contributes to defense against lipid peroxidation [41]. The higher levels of GOT and GPT and ALP may give rise to a high concentration of glucose. In other words, the gluconeogenic action of these enzymes play the role of providing new supplies of glucose from other sources such as amino acids [42, 43] ,following oral supplementation with aqueous flavonoids extract of *A. hirerochuntica* , GOT, GPT, and ALP activities significantly decrease , table 3 . So we concluded that *Anastatica hireochuntica* extract have hepatoprotective effect in females patients with T2DM.

CONCLUSIONS

- The present study have reported for the first time the mechanism by which erythropoietin level was depressed in type diabetes mellitus which involves two pivotal points : a) removal of both O- and N- linked oligosaccharides from the glycoprotein structure of erythropoietin. b) degradation of

disulfide bonds (the dynamic switch for erythropoietin function) because of the direct attack of the hydroxyl anion on these bonds.

- Erythropoietin and vitamin D appear to be good biochemical markers for Iraqi patient with type-2- diabetes mellitus. In accordance with this study , these parameters have a strong diagnostic power to this chronic disease.

- Flavonoids extracted from aqueous *Anastatica hierochuntica* exert both immunomodulatory effect (through the down regulation of inflammation) and antioxidative action (through free radical scavenging and inhibition of lipid peroxidation).

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