ANTIOXIDANT ACTIVITY OF CITRULLUS COLOCYNTHIS PULP EXTRACT IN THE RBC'S OF ALLOXAN-INDUCED DIABETIC RATS

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Background: Previous studies in our laboratory showed that Citrullus colocynthis pulp seedless extract have antihyperglycemic and insulinotropic effects in alloxan induced diabetes. Reactive oxygen species have been implicated in the mechanism of damage of red blood cells and anaemia in diabetic patients. So the current study was carried out to investigate the protective role of citrullus colocynthis against oxidative stress in the RBC's of alloxan induced diabetic rats. **Methods:** Rats were divided into four groups each of ten rats, the first group was normal non diabetic rats given normal saline orally and was named control group, the second group was diabetic rats given normal saline orally and were named normal saline treated-diabetic rats, the third and fourth group were diabetic rats treated with the pulp extract or glibenclamide (a positive control) orally. Evaluations were made for haematological parameters in the blood and for lipid peroxidation and oxidative stress enzymes activities in the RBC's of all experimental rats. **Results:** The diabetic rats had a significant decrease ($p<0.05$) in total erythrocytes count and Packed Cell Volume (PCV) and a normal Haemoglobin (Hb) value in the blood. They also showed decreased levels of Thiobarbituric Acid Reactive Substances (TBARS) and decreased activities of Superoxide Dismutase (SOD) and Catalase (CAT) in the RBC's hemolysate. On other hand, oral administration of citrullus colocynthis or glibenclamide alleviated these altered parameters in the treated rats, they resulted in a significant increase ($p<0.05$) in the in total erythrocytes count and PCV (Haematocrit) values in the blood and caused a significant decreased levels of TBARS and increased activities of SOD and CAT in the RBC's of those diabetic treated rats when compared to diabetic rats given normal saline. The effect was more profound in citrullus colocynthis treated diabetic rats. **Conclusion:** Citrullus colocynthis pulp extract possesses a potent antioxidant property against oxidative stress in the RBC's of alloxan induced diabetic rats.

Keywords: antioxidant, Citrullus colocynthis, alloxan, diabetes, Red Blood Cells

INTRODUCTION

Diabetes mellitus, a leading non communicable disease with multiple aetiologies, affects more than 100 million people worldwide and is considered as one of the five leading causes of death in the world.[1] The World Health Organization (WHO) reported that 300 million peoples would suffer from diabetes mellitus by the year 2025.[2] Diabetes mellitus is characterised by an increased concentration of blood glucose due to derangement in carbohydrates metabolism and defective secretion of insulin. These metabolic disturbances result in acute and long-term diabetic complications, which are responsible for premature death and disability.[3]

Chronic hyperglycaemia during diabetes causes gyration of body proteins that in turn leads to secondary complications affecting eyes, kidneys, nerves and arteries.[4] Along with hyperglycaemia and abnormalities in serum lipids,[5] diabetes is associated with microvascular and macrovascular complications which are the majors causes of morbidity and death in diabetic subjects.[6] Also, diabetes mellitus leads to many complications, such as increasing the risk of developing arterial disease by two to six folds.[7]

Increased oxidative stress is a widely accepted to be the main factor plays a role in the development and progression of diabetes and its complication. Hyperglycaemia-induced glucose oxidation initiates membrane lipid peroxidation which are vital for the maintenance and integrity of cell function and initiates an non-enzymatic glycation of proteins, which in turn lead to enhanced production of reactive oxygen species (ROS) or result in decreased efficiency of inhibitory and scavenging system.[8] The stress may then be amplified and propagated by an autocatalytic cycle of metabolic stress, tissue damage and cell death, leading to further increase in free radical production and depletion of antioxidants.[9]

The blood is a vital fluid, which contains the Red Blood Cells (RBC's), White Blood Cells (WBC's) and platelets suspended in the serum in homeostatic concentrations. The circulatory blood volume makes up about 8% of the weight of an average man. The blood cells take up about 45% of the blood, while plasma constitutes about 55%.[10] The Blood is important for pulmonary and tissue respiration, as a medium of endocrine and neurohumoral transmissions, biotransformation and metabolic excretion,[11] nutritional and immunological processes, as well as homeostatic responses.[12] Reactive oxygen species, have been implicated in the mechanism of damage of red blood cells in diabetic patients.[13–15] As a result, haematological complications develop which consist mainly of abnormalities in the function, morphology and metabolism of erythrocytes, leukocytes and platelets.[16] It

has been suggested that Red and white blood cells counts decrease in diabetics than in non-diabetic people.[17]

The search for an effective and safer hypoglycaemic agents with a protective effect from diabetic complications has continued to be a research topic of considerable interest.[18,19] The World Health Organization has recommended and encouraged the use of alternative therapy especially in countries where access to the conventional treatment of diabetes is inadequate.[20]

Plant medicines (phytotherapies) have a long history as treatments for diabetes. With a disturbing rise in the prevalence of this metabolic disease and associated healthcare costs, interest in alternative or complementary therapies has grown.[21] Over the two decades, data from controlled investigations in animal models and patients have validated the therapeutic value of numerous phytotherapies for diabetes. Phytotherapies and their combinations demonstrate multiple beneficial anti-diabetic mechanisms,[21] including modulation of carbohydrate metabolism, restoration of beta-cell integrity and function, insulin-releasing activity, improvements in glucose uptake/utilisation, antioxidant properties and a reduction in the risk of cardiovascular disease.

One of these anti-diabetic phytotherapies is Citrullus colocynthis. Citrullus colocynthis (Cucurbitaceae), commonly known as 'bitter apple', 'colosynth', 'vine-of-Sodom' and 'tumba' is a tropical plant that grows abundantly in the Arabian countries and widely in other parts of the world. In the traditional medicine, this plant has been used to treat constipation,[22] Diabetes,[23] oedema, fever, jaundice leukaemia, bacterial infections, cancer and used as an abortifacient.[24]

We have previously reported an efficient anti-hyperglycaemic, insulin releasing and hepato-nephroprotective effect of this plant following chronic administration in alloxan-induced diabetic rats,[25,26] thus the aim of our current study is to investigate the effect of chronic administration of this plant on some haematological parameters in alloxan-induced diabetic rats and to investigate the potential of this plant in alleviating the oxidative stress induced in the RBC's of those diabetic rats.

MATERIALS AND METHODS

Preparation of Citrullus colocynthis pulp extract: Fresh one kilogram of Citrullus colocynthis fruits was collected from Aseer area, South-Western region of Saudi Arabia. Mature black seeds were separated manually from the pulp of the fruits and then the pulp was dried and grinded with a grinder into a powder prepared for extraction. The pulp powder was extracted by one litre of water-ethanol mixture (80/20, v/v) for 6 hours, this step was repeated three times.[27] The filtrate was pooled and concentrated under vacuum at temperature (not exceeding 50 ºC) and dissolved in freshly prepared normal saline to a final concentration of 300 mg /ml for further use.

Experimental animals: Male albino Wistar rats (150–200 g) bred in the Central Animal House, Medical College at King Khalid University, were used in this study. The animals were fed on rat chow and water ad libitum. The animals were maintained in their respective groups for 30 days. All studies were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.[28]

Experimental induction of diabetes: Diabetes was induced in male Wistar albino rats by intraperitoneal administration of alloxan monohydrate (150 mg/kg body weight),[29] dissolved in normal saline. Since alloxan is capable of producing fatal hypoglycaemia as a result of massive pancreatic insulin release, rats were treated with 30% glucose solution orally at different time intervals after 6 h of alloxan induction and 5% glucose solution was kept in bottles in their cages for the next 24 h to prevent hypoglycaemia. After 10 days, rats with diabetes mellitus having glycosuria (indicated by Benedict's test) and hyperglycaemia, with blood glucose range of 250–375 mg/dl, were used for this study.

Experimental design: The rats were divided into four groups comprising six animals in each group (n=6). All treatments were given orally to experimental rats using cavage needle at a single dose daily. The rats were treated for 30 days as follows:

Group 1: Control rats, given only normal Saline (1 ml/kg).

Group 2: Non-treated diabetic group, given only normal Saline (1 ml/kg).

Group 3: Diabetic group treated with glibenclamide (600 mg/kg body wt/day).[30]

Group 4: Diabetic group treated orally with 1 ml/kg of Citrullus colocynthis pulp extract/kg (300 mg/kg body weight/day).

Physiological and biochemical analysis: After the treatment (24 h after the last administration), the animals were sacrificed by decapitation always between 8:00 and 10:00 AM and fresh blood was immediately collected into heparinised test tubes for routine haematological analysis. Some heparinised Blood were used for preparation of hemolysate for determination of TBARS, SOD and CAT activities. The following haematological parameters were determined in the plasma: Erythrocytes were counted on haemocytometer using a light microscope at 40×10 magnification. Blood samples were diluted to 200 times by physiological saline (0.9% sodium chloride solution) before counting. Haematocrit value were determined by the method of Strumia *et al.*[31] The haemoglobin concentration was determined by the cyanmethaemoglobin method.[32]

Preparation of hemolysate: After collecting blood samples in heparinised tubes, centrifugation was performed at 1000 g for 15 min to remove the buffy coat. The packed cells obtained at the bottom were washed thrice with phosphate buffer saline (0.9% NaCl in 0.01 M phosphate buffer, *p*H 7.4). A known amount of erythrocytes was lysed with hypotonic phosphate buffer. The hemolysate was obtained after removing the cell debris by centrifugation at 3000 g for 15 min and used for determination of Thiobarbituric Acid Reactive Substances (TBARS), and superoxide dismutase (SOD) and catalase (CAT) activities.

Measurement of TBARS levels SDO and CAT activity: Superoxide dismutase activity in the red blood cells hemolysate was measured by using commercial kits (Biovision, K335-100). The activity was expressed as U/mL. Catalase Activity (CAT) in the red blood cells hemolysate was determined by using commercial kit (biovision K773-100). CAT activity was expressed as U/ml. One unit of catalase is the amount of catalase decomposes 1.0 µmol of H_2O_2 per min at *p*H 4.5 at 25 °C.)

The concentration of Thiobarbituric Acid Reactive Substances (TBARS) in the RBCs hemolysate was determined by the method of Okhawa.[33] In brief, the reaction mixture contained 0.1 ml of hemolysate, 0.2 ml of sodium dodecyl sulfate, 1.5 ml of acetic acid and 1.5 ml of aqueous solution of thiobarbituric acid. The *p*H of 20% acetic acid was pre-adjusted with 1 M NaOH to 3.5. The mixture was made up to 4 ml with distilled water and heated at 95 °C for 1 h, in a water bath. After cooling, 1 ml of distilled water and 5 ml of mixture of n-butanol and pyridine (15:1) were added and mixture was shaken vigorously on a vortex mixer. The absorbance of the upper organic layer was read at 532 ηm. The values were expressed as mM/ml.

Statistical analysis: Data are expressed as Mean±SD. Student's *t*-test was used to determine the difference between groups. Statistical significance was considered at $p<0.05$.

RESULTS

Table-1 shows the results of total RBC count, haemoglobin concentration (Hb) and Haematocrit value (PCV) in all groups of rat. Diabetic rats administered Normal saline orally showed decreased values of erythrocyte counts and haematocrit while haemoglobin levels were normal when compared to normal rats received normal saline orally. On other hand oral administration of Citrullus colocynthis or glibenclamide significantly increased these parameters to their normal levels when compared to diabetic rats.

Table-2 shows the activity of Superoxide Dismutase (SOD) and Catalase (CAT) as well as Thiobarbituric Acid Reactive Substances (TBARS) concentration in RBC's of all groups of rats. Diabetic rats showed a significant decrease in the activity of these enzymes (SOD and CAT) and a significant increase in the level of TBARS. Rats treated with Citrullus colocynthis or glibenclamide showed a significant increase in the activity of SOD and CAT as well as a significant decrease in the levels TBARS in the RBC's of the treated rats when compared to diabetic rats.

Table-1: Mean values of some haematological parameters of the different experimental groups of rats

Parameter	Control	Diabetic	Diabetic+glibenclamide	Diabetic+C.c (300 mg/kg)
RBC's (x 10 6⁄µl)	6.13±0.14	3.38±0.22*	5.57±0.13*	5.98±0.18*
Hb (g/dl)	12.2±0.13	12.17±0.45	12.5±0.25	12.44±0.69
PCV %	39.9±0.78	29.9±0.82*	36.9±0.41*	38.03±1.45*

Values are given as Mean±SD for groups of six rats each. Values are statistically significant* at p<0.05. Diabetic rats were compared with control rats; Citrullus colocynthis treated-diabetic rats were compared with diabetic rats; glibenclamide treated-diabetic rats were compared with diabetic rats.

Table-2: TBARS levels, SDO and CAT activities in the RBC's of all experimental rats

Parameter	Control	Diabetic	Diabetic+glibenclamide	Diabetic+C.c (300 mg/kg)
TBARS (mM/ml)	30.1± 0.68	46.15±2.56*	36.95± 2.50*	30.23±1.24
SOD (U/ml)	10.22±0.52	4.62±0.56*	8.08±0.37*	8.83±0.54*
CAT (U/ml)	100.78± 1.77	51.85±2.56*	85.5±4.05*	94.23±3.78*

Values are given as mean ± SD for groups of six rats each. Values are statistically significant* at p<0.05. Diabetic rats were compared with control rats; Citrullus colocynthis treated-diabetic rats were compared with diabetic rats; glibenclamide treated-diabetic rats were compared with diabetic rats

DISCUSSION

Diabetes mellitus is a life threatening metabolic disorder and it is estimated that its annual incidence rate will continue to increase in the future worldwide. Hyperglycaemia, the primary clinical manifestation of diabetes mellitus, is associated with the development of micro and macro vascular diabetic complications.[6]

Alloxan induces damage and death of pancreatic islet-cells in several experimental animal models, thus causing diabetes mellitus and decreasing the secretion of insulin. The cytotoxic action of this diabetogenic agent is mediated by reactive oxygen species. Alloxan and the product of its reduction dial uric acid; establish a redox cycle with the formation of super oxide radicals. These radicals undergo dismutation to hydrogen peroxide. Therefore, highly reactive hydroxyl radicals are formed by the Fenton reaction.

The present study is the first study that shows that oral administration citrullus colocynthis (300

mg/kg) for 30 days ameliorated the disturbed haematological and oxidative stress parameters of diabetic rabbits induced by alloxan. It has been suggested that anaemia occurrence in Diabetes mellitus is due to the increased non-enzymatic glycosylation of RBC membrane proteins, which correlates with hyperglycemia.[34,35] Oxidation of these glycosylated membrane proteins and hyperglycaemia in Diabetes mellitus cause an increase in the production of lipid peroxides causing a haemolysis of RBC through many pathological consequences.[35,36] The major pathological consequences of free radical induced membrane lipid peroxidation include increased membrane rigidity, decreased cellular deformability, reduced erythrocyte survival, and lipid fluidity.[36]

In this study, the RBC's membrane TBARS level in all experimental rats was measured. Measurement of plasma TBARS help to assess the destruction of RBC's.[37] Elevated plasma TBARS observed in the diabetic rats can therefore be related to overproduction of lipid peroxidation byproducts in RBC's and can explain the lowered RBC's count founded in those animals. Packed Cell Volume measures the percentage by volume of packed RBC's in a whole blood sample after centrifugation. In our study PCV levels decrease in diabetic rats is a result of RBC's haemolysis. On the other hands, the levels of these parameters (RBC's and PCV) were significantly increased in diabetic rats treated with glibenclamide or the Citrullus colocynthis extract. Thus, increased RBC's count and PCV value in these rats appears to be due to the lowered lipid peroxidation level in RBC's membrane leading to a decrease susceptibility of RBC's to haemolysis.

Haemoglobin test measures the amount of HB in grams in 1 dl of whole blood and provides an estimate of oxygen carrying capacity of the RBC's. As the results indicate, haemoglobin levels didn't change in the RBC's of all experimental rats which indicates normal haemoglobin synthesis.

The increased lipid peroxidation in the RBC's is also due to an Inhibition or changing the activity of non enzymatic and enzymatic components of the oxidative system (reduced glutathione (GSH), Superoxide Dismutase (SOD) and Catalase (CAT) activities. The glutathione peroxidase system consists of several components, one of which is reduced glutathione (GSH).[38] The enzymatic antioxidant defence system including Superoxide Dismutases (SODs) and Catalases (CATs) which can decompose superoxide and hydrogen peroxide in the cells are the main defence against oxidative injuries. SOD catalyses the rapid removal of superoxide radical. Because the SOD enzyme generates H_2O_2, it works in collaboration with H_2O_2 removing enzymes. Catalase present in the peroxisomes of nearly all aerobic cells, serves to protect the cell from the toxic effects of hydrogen peroxide by catalysing its decomposition into molecular oxygen and water without the production of free radicals.

Reaction of alloxan that produced toxic ions in vivo resulted in decreased activity of enzymatic antioxidant system accompanied by increased lipid peroxidation, there was a decreased activity of SOD and CAT in erythrocytes of alloxan induced diabetic rats. Decrease in antioxidant enzymes is an indication that there might be generation of an active radical—a factor which is a key to alteration of these enzymes. The decrease in both enzyme activities could be the result of a reduced synthesis of these enzymes proteins as a result of higher accumulation of free radicals or there may be a drug-enzyme interaction resulting in the deactivation of these enzymes.[39] However, chronic oral administration of glibinclamide or citrullus colocynthis restored the altered levels of the enzymatic components of the antioxidant system to their normal levels. The effect was more profound in citrullus colocynthis treated rats indicating a potent antioxidant property of this plant.

CONCLUSION

Based on the results obtained in this study, it can be concluded that oral administration of citrullus colocynthis might alleviate the diabetes-induced disturbances of haematological parameters and can protect the RBC's from the oxidative stress produced from alloxan administration.

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