

Full Length Research Paper

***In vivo*, hypolipidemic and antioxidant effects of *Citrullus colocynthis* pulp extract in alloxan-induced diabetic rats**

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The study was designed to evaluate the effect of *Citrullus colocynthis* pulp hydro-ethanol extract on alloxan-induced hyperlipidemia in diabetic rats. 24 albino rats with body weights of 150 to 200 g were divided into one control and three experimental groups. Group 1 was the normal control rats, orally administered physiological saline. Groups 2, 3 and 4 were diabetic rats orally administered physiological saline (diabetic, untreated), glibenclamide (diabetic, GL) or *C. colocynthis* extract (300 mg/kg) (diabetic, CC), respectively. All treatments were administered orally, on a daily basis, for a period of 30 days. At the end of the experimental period, animals in all the four groups were fasted for 12 h and then sacrificed. Blood samples and liver tissues were taken for the determination of total cholesterol, triglycerides, free fatty acids and phospholipids. Portions of the liver were homogenized and used for determination of lipid peroxidation biomarkers [thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (HPx)], and for biological antioxidant levels [reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT)]. The results show significant decreases in the levels of total cholesterol, triglycerides, free fatty acids and phospholipids in serum and liver of *C. colocynthis* treated diabetic rats when compared to diabetic untreated rats. Also, relative to diabetic untreated rats, oral administration of *C. colocynthis* to diabetic rats significantly reduced elevated levels of liver TBARS and HPx to near normal levels and caused significant increases in GSH, SOD and CAT levels, an effect that is comparable to but more pronounced than that of glibenclamide. These results indicate that *C. colocynthis* pulp hydro-ethanol extract possesses potent hypolipidemic and antioxidant actions in alloxan induced diabetic rats.

Key words: *Citrullus colocynthis*, alloxan diabetes, hypolipidemia, antioxidant.

INTRODUCTION

Diabetes mellitus (DM) is a syndrome characterized by defects in insulin secretion, insulin action or both, resulting in impaired metabolism of glucose and other energy-yielding fuels such as lipids and protein (Latha and Pari, 2004). Thus, DM is by far the most common cause of chronic hyperglycemia, and is also associated with marked alterations in the concentration of lipids in both serum and tissues. Impaired carbohydrate utilization leads to increased lipolysis, resulting in hyperlipidemia

(Morel and Chisolm, 1989). The chronic hyperglycemia of diabetes is associated with long term damage, dysfunction and failure of various organs (Lyra et al., 2006). The liver, an insulin-dependent tissue that plays a pivotal role in glucose and lipid homeostasis, is severely affected by DM. The liver participates in the uptake, oxidation, and metabolic conversion of free fatty acids, and synthesis of cholesterol, phospholipids, and triglycerides. In DM, fatty acids are increasingly taken up

by the liver, and after esterification with glycerol phosphate, they are deposited as triglycerides. As a result, diabetic liver steatosis develops (Diniz et al, 2006).

Hyperlipidemia, in part to peroxidation of membrane lipids, is associated with microvascular complications which constitute the major cause of morbidity and mortality in diabetic patients (Nagappa et al., 2003). Increased lipid peroxidation impairs membrane function by decreasing membrane fluidity, and by changing the activity of membrane-bound enzymes and receptors (Hunt et al. 1990). In the process of lipid peroxidation, the fatty acid side chains of the membrane lipids, especially those containing one or more carbon-carbon double bonds, are oxidized by the action of free radicals to unstable hydroperoxides. In the presence of metal catalyst, these hydroperoxides decompose to form a complex mixture of hydrocarbons and cytotoxic aldehydes, such as thiobarbituric acid reactive substances (TBARS) including malondialdehyde (MDA) (Halliwell and Gutteridge, 1984). Under normal conditions, the level of lipid peroxidation and free radicals production is controlled by various cellular defense mechanisms (scavenging systems) including non-enzymatic [reduced glutathione (GSH)] and enzymatic systems [superoxide dismutase (SOD) and catalase (CAT)] (Kajimoto and Kaneto, 2004). Normal levels of these antioxidant defense enzymes is sufficient for the eradication of free radicals, thus preventing tissue injury and maintaining normal functions of organs.

It has been suggested that a decreased tissue antioxidant status is an important factor in tissue damage (Ravi et al., 2004). In DM, increased formation of reactive oxygen species (ROS) is one of the leading factors in diabetic complications (Ravi et al., 2004). Indeed, Kakkar et al. (1995) claim that all other factors stem from a single hyperglycemia-induced process of overproduction of superoxide. Hyperglycemia initiates non-enzymatic glycation of proteins accompanied by increased ROS activity that contributes to the bimolecular damage in DM (Cam et al., 2003). Oxidative stress may 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 (Bailey and Day, 1989).

In spite of the universal acceptance of insulin as one of the most important therapeutic drugs for the management of DM and its complications, efforts are ongoing to find substitute therapies. Aside from classical, chemically prepared antihyperglycemics, the use of traditional medicinal plants with hypoglycemic effect is gaining popularity, worldwide. More than 400 traditional plant treatments for DM have been reported, but only a small number of these herbal remedies have received scientific and medical evaluation (Ivorra et al., 1989). *C. colocynthis* (Cucurbitaceae), commonly known as "bitter apple", "colosynth", "vine-of-Sodom", and "tumba" is a tropical

plant that grows abundantly mainly in the Arabian countries but also in some other parts of the world. In traditional medicine, this plant has been used to treat constipation (Alkofahi et al., 1996), DM (Ziyyat and Legssyer, 1997), edema, fever, jaundice, leukemia, bacterial infections, cancer and used as an abortifacient (Madari and Jacobs, 2004).

In our laboratory, we previously reported the antihyperglycemic, insulin releasing and hepato-nephro protective effects of *C. colocynthis* pulp extract in alloxan induced diabetic rats (Dallak et al., 2009a and b). Lipid metabolism and the antioxidant system are known to be impaired in diabetes mellitus. This study was therefore undertaken to assess possible hypolipidemic and antioxidant promoting effects of a water ethanol extract of *C. colocynthis* pulp in alloxan-induced diabetic rats.

MATERIALS AND METHODS

Preparation of *C. colocynthis* pulp extract

One kilogram of fresh *C. colocynthis* fruits was collected from the Aseer area, southwestern Saudi Arabia. Mature black seeds were separated manually from the pulp of the fruits and then the pulp was dried and pulverized with a grinder (Moulinex) into a powder in preparation for extraction. The powder was extracted in 1 L of water-ethanol mixture (80/20, v/v) for 6 h. This step was repeated three times (Dallak et al., 2009b). The filtrate was pooled and concentrated under vacuum at tepid temperatures (not exceeding 50°C) 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 to 200 g) bred in the Central Animal House, Medical College, 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 60 days. All experiments were conducted in accordance with the National Institute of Health's Guide for the care and use of laboratory animals (NIH guide for the care and use of laboratory animals, Revised DHEW Publication, 1996). The design of the experiments was approved by the Physiology Department Research Committee of King Khalid University. Diabetes was induced in male Wistar albino rats by intraperitoneal administration of alloxan monohydrate (150 mg/kg body weight) (Ananthi et al., 2003), dissolved in normal saline. Since alloxan is capable of producing fatal hypoglycemia 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 hypoglycemia. After 10 days, rats with glycosuria (indicated by Benedict's test) and hyperglycemia (blood glucose range of 250 to 375 mg/dl) were classified as diabetic used in the study. The rats were divided into four groups of six animals in each group (n = 6). All treatments were given orally using gavage needles in single daily doses. The rats were treated for 30 days as follows: Group 1: normal control rats; given only normal saline (1 ml/kg); Group 2: diabetic control group; given only normal Saline (1 ml/kg); Group 3: diabetic GL group; treated with glibenclamide (600 mg/kg body wt /day) (22); Group 4: diabetic CC group; treated orally with 1 ml/kg of *C. colocynthis*

Table 1. Lipid levels in the serum of control and experimental groups of rat.

Parameter	Control	Non-treated diabetic	Diabetic + glibenclamide	Diabetic + C.c (300 mg/kg)
Cholesterol (mg/dl)	80.7 ± 4.01	102 ± 6.01*	92 ± 5.1*	82 ± 4.5*
Triglycerides (mg/dl)	45 ± 3.78	69.5 ± 5.22*	55 ± 5.01*	49.6 ± 4.87*
Free Fatty Acids (μM/L)	2701 ± 352	3422 ± 713.3*	3054 ± 622.1*	3030 ± 422*
Phospholipids (mg/dl)	83 ± 5.6	101.2 ± 7.6*	88.7 ± 5.43*	87 ± 4.99*

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

pulp extract (300 mg /kg body weight /day).

Collection of serum and lipid extraction

At the end of day 30, the rats were subjected to overnight fasting and then sacrificed by cervical dislocation. Blood samples were collected directly into plain tubes, allowed to clot at room temperature and then centrifuged at 4000 rpm for 10 min to obtain serum which was used for lipid profile estimation. The livers were dissected out immediately after the rats were killed, washed in ice-cold saline and patted dry. Lipids were extracted from tissues using chloroform-methanol (2:1 by volume) by the method of Folch et al. (1957). Dried lipid extracts were re-suspended in 1 ml of saline solution (9 g/L NaCl, 1% triton X - 100) and used for the estimation of lipid profile.

All the following parameters were assayed in serum and liver tissue; total cholesterol and triglycerides were estimated by the methods of Zlatkis et al. (1953) and Foster and Dunn (1973), respectively; free fatty acids and phospholipids were analyzed by the method of Falholt et al. (1973) and Zilversmit and Davis (1950), respectively.

Biochemical assays in liver homogenate

Liver portions were sliced into tiny pieces and homogenized in appropriate buffer (pH 7.00) in cold conditions to give 20% homogenate (w/v). The homogenate was centrifuged at 1000 rpm for 10 min in a cold centrifuge at 4°C. The supernatant was separated and used for estimation of TBARS, HPx, GSH, SOD, and CAT. The concentration of TBARS in liver homogenates was determined by the method of Okhawa (1979). In brief, the reaction mixture contained 0.1 ml of tissue homogenate, 0.2 ml of sodium dodecyl sulfate, 1.5 ml of acetic acid and 1.5 ml of aqueous solution of thiobarbituric acid (TBA). The pH 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 a mixture of n-butanol and pyridine (15:1) were added and the mixture was shaken vigorously on a vortex mixer. The absorbance of the upper organic layer was read at 532 nm and values were expressed as mmol/100 g of tissue.

The estimation of lipid HPx in liver homogenates was done by the method of Jiang (1992) in which 0.1 ml of tissue homogenate (supernatant) was treated with 0.9 ml of Fox reagent (88 mg of butylated hydroxytoluene, 7.6 mg of Xylenol orange, and 9.8 mg of ammonium iron sulfate added to 90 ml of methanol and 10 ml of 250 mM sulfuric acid) and incubated at 37°C for 30 min. The color developed was read at 560 nm and values were expressed as mmol/100 g of tissue.

The GSH content of liver homogenates was measured at 412 nm using the method of Sedlak and Lindsay (1968). The homogenate was precipitated with 50% trichloroacetic acid and then centrifuged at 1000 rpm for 5 min. The reaction mixture contained 0.5 ml of the supernatant, 2.0 ml of Tris-EDTA buffer (0.2 M; pH 8.9) and 0.1 ml of 0.01 M 5,5'-dithio-bis-2-nitrobenzoic acid. The solution was kept at room temperature for 5 min, and then read at 412 nm on the spectrophotometer. The values were expressed as mmol/100 g of tissue.

Catalase activity in liver homogenates was assayed using commercially available catalase activity assay kit (Biovision, K773-100). One unit of catalase is the amount of catalase that decomposes 1.0 μmol of H₂O₂ per min at pH 4.5 at 25°C.

Estimation SOD activity

The activity of SOD in liver homogenates was assayed using commercially available sod activity assay kit (Biovision, K335-100). One unit of SOD is that which causes 50% inhibition of the rate reduction of superoxide anions to produce a water-soluble formazan dye under the conditions of the assay.

Statistical analysis

Results were expressed as the mean value ± SEM. Statistical differences between groups were assessed by Student's t test. Values of $p < 0.05$ were considered significantly different.

RESULTS

The effects of *C. colocynthis* pulp extract on serum and liver lipids of the control and experimental rats are summarized in Tables 1 and 2, respectively. Serum and liver homogenate levels of cholesterol, free fatty acids, triglycerides, and phospholipids were significantly increased in diabetic control rats when compared to normal controls. Administration of glibenclamide and *C. colocynthis* pulp extract to diabetic rats significantly reduced these high lipid levels in serum and liver bringing them close to the corresponding values in the normal controls rats.

Table 3 shows the concentrations of TBARS, HPx and GSH in liver homogenates of the normal control and experimental groups of rats. The levels of TBARS and HPx in the diabetic control rats were significantly higher

Table 2. Lipid Levels in the liver homogenate of control and experimental groups of rat.

Parameter	Control	Non-treated diabetic	Diabetic + glibenclamide	Diabetic + C.c (300 mg/kg)
Cholesterol (mg/100 g wet tissue)	344 ± 2.31	530 ± 4.3*	452 ± 4.01*	422 ± 3.6*
Triglycerides (mg/100 g wet tissue)	349 ± 5.1	665 ± 9.45*	505 ± 6.1*	401 ± 5.7*
Free fatty acids (mg/100 g wet tissue)	604 ± 3.87	932 ± 8.54*	792 ± 6.34*	702 ± 3.21*
Phospholipids (g/100 g wet tissue)	1.54 ± 0.01	2.85 ± 0.31*	2.01 ± 0.18*	1.76 ± 2.67*

Values are given as mean ± SEM for groups of the six rats each. Values are statistically significant* at $p < 0.05$.

Diabetic rats were compared with control rats; *C. colocynthis* (C.c) treated-diabetic rats were compared with diabetic rats; glibenclamide treated-diabetic rats were compared with diabetic rats.

Table 3. Levels of TBARS, HPx, and GSH in liver homogenate of control and experimental groups of rats.

Parameter	Control	Non-treated diabetic	Diabetic + glibenclamide	Diabetic + C.c (300 mg/kg)
TBARS (mmol/100g of tissue)	0.85 ± 0.01	2.1 ± 0.10*	1.43 ± 0.30*	1.02 ± 0.21*
Hydroperoxides (mmol/100g of tissue)	70.21 ± 0.83	99.01 ± 1.31*	88.6 ± 0.6*	81.11 ± 0.44*
Glutathione (mmol/100 g of tissue)	47.1 ± 1.55	20.65 ± 0.88*	38.0 ± 1.44*	40.87 ± 4.0*

Values are given as mean ± SEM for groups of six rats each. Values are statistically significant* at $p < 0.05$. Diabetic rats were compared with control rats; *C. colocynthis* (C.c) treated-diabetic rats were compared with diabetic rats; glibenclamide treated-diabetic rats were compared with diabetic rats.

than those of the normal control rats, whereas in diabetic rats treated with *C. colocynthis* pulp extract or glibenclamide, these values were restored nearly to the corresponding values in normal controls. Also, the mean concentration of GSH in liver homogenates of diabetic control rats was significantly reduced, whereas administration of *C. colocynthis* pulp extracts or Glibenclamide resulted in a significant increase in GSH level bringing it to near normal levels. The effect was more pronounced in rats treated with *C. colocynthis*.

Table 4 shows the activities of SOD and CAT in liver homogenates of control normal and experimental rats. SOD and CAT activities were significantly reduced in diabetic control rats when compared to control normal rats; however, their levels returned to near normal levels in diabetic rats treated with glibenclamide or *C. colocynthis* pulp extract.

DISCUSSION

Diabetes mellitus remains the most common chronic disorder of carbohydrate, fat and protein metabolism. It is characterized by chronic and persistent hyperglycemia, degenerative vascular changes and neuropathy due to completely or partially depleted insulin secretion or increased insulin resistance (Scheen, 1997). Apart from hyperglycemia, diabetes mellitus (especially Type 1) is accompanied by hypercholesterolemia, hyperlipidemia and hepatic steatosis (Brixova, 1981). The hypercholesterolemia is a consequence of accelerated fatty acid

oxidation to acetyl CoA which is the primary substrate for cholesterol synthesis. Similarly, the hyperlipidemia associated with diabetes mellitus results from accelerated *de novo* hepatic biosynthesis and release of very low density lipoprotein (VLDLc) without a corresponding increase in its rate of clearance from the blood by lipoprotein lipase whose activity is dependent on high insulin: glucagon ratio (Brixova, 1981).

Traditional medicine, especially in developing countries uses medicinal plants as a readily available means of maintaining good health and treating diseases, including DM. (Bhattaram et al., 2002). This trend is on the increase with the recent extraction and development of several drugs and chemotherapeutics from these medicinal plants (Davis and Granne, 2001). In our laboratories, we earlier reported that the pulp extract of *C. colocynthis* exerted antihyperglycemic and insulin-releasing effects in alloxan-induced diabetic rats (Dallak et al., 2009a and b). In this study, we demonstrated a hypolipidemic effect of *C. colocynthis* extract in the same alloxan-diabetic animal model. Glibenclamide, which was used as a positive control in this study, is a prototype of the second generation sulfonylurea class of oral hypoglycemic agents. Glibenclamide is known to mediate its hypoglycemic effect by stimulating insulin release from pancreatic β cells, reducing hepatic insulin clearance, stimulating the release of somatostatin and suppressing the secretion of glucagon (Blumenthal, 1977).

Our data in Tables 1 and 2 show significant reductions in levels of free fatty acids, triglycerides, total cholesterol and phospholipids in the serum and liver of diabetic rats

Table 4. Enzymatic activities of SOD and CAT in liver homogenate of control and experimental groups of rats.

Parameter	Control	Non-treated diabetic	Diabetic + glibenclamide	Diabetic + C.c (300 mg/kg)
SOD (U/mg of protein)	12.0±1.20	3.2±2.1*	10.32±5.6*	10.16±4.4*
CAT (U/mg of protein)	76.3± 6.1	34.3±1.55*	65.45±7.4*	63.72±7.0*

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

treated with *Citrullus colocynthis*, when compared to non treated diabetic rats, an effect that is more pronounced than that of glibenclamide. A number of mechanisms can account for the observed hypolipidemic effect of the extract. It could be due to depressed hepatic gluconeogenesis; a positive relationship between gluconeogenesis and lipogenesis is well documented (Harris and Crabb, 1982). We previously reported that the pulp extract of *C. colocynthis* has a regulatory effect on gluconeogenetic enzymes (Dallak et al., 2009b). In our previous study, the activities of the key enzymes of gluconeogenesis (glucose 6 phosphate and fructose 1, 6 biphosphatase) were found to be decreased in livers of alloxan diabetic rats treated with *C. colocynthis* relative to the activities in non-treated diabetic rats. This effect was explained by an increase in insulin secretion from the remaining beta cells (Dallak et al., 2009a and b). Thus, *C. colocynthis* extract may induce hypolipidemia through this common pathway.

Lipid peroxide mediated tissue damage has been observed in the development of type I and type II DM (Feillet et al., 1999). In this study, we investigated *C. colocynthis* extract-induced changes on the antioxidant defense system by estimating TBARS and HPx levels in serum and liver of extract treated rats. Our results (Table 3) show statistically significant increases in levels of these lipid peroxidation biomarkers in the livers of diabetic control rats. In alloxan-diabetic rats, glucose auto-oxidation generates these free radicals and can cause lipid peroxidation (Kakkar et al. (1985); Pari and Latha, (2002); Venkateswaran et al. (2002). In our study, liver TBARS and HPx levels were significantly lowered in *C. colocynthis* extract-treated groups compared to diabetic normal rats, indicating reduced lipid peroxidation mediated tissue damage. This result is expected, being a direct consequence of the hypoglycemic effect of the extract.

In vivo, GSH acts as an antioxidant and its level is reduced in diabetes mellitus (Lyra et al., 2006). We observed significantly decreased GSH levels in diabetic rat livers. Decreased GSH levels represent increased utilization due to increased oxidative stress (Anuradha et al., 1993). Increased GSH content in livers of rats treated with *C. colocynthis* may be one important factor responsible for inhibition of lipid peroxidation, which could

explain the decrease in the levels of TBARS and HPx.

SOD and CAT are the two major scavenging enzymes that remove toxic free radicals *in vivo*. SOD is an antioxidant enzyme, which reduces superoxide radicals to H₂O₂ and oxygen. Catalase catalyzes the dismutation of H₂O₂. Previous studies had reported that SOD and CAT activities are decreased in diabetes mellitus (Vucic et al. (1997); Searle and Wilson (1980), an effect that was attributed to accumulation of O²⁻ and H₂O₂ (Searle and Wilson, 1980). Oral administration of *C. colocynthis* extract in our study caused significant increases in the levels of these enzymes, either by interfering with their biosynthesis or due to the presence of a free radical scavenging activity in the extract. Thus, the extract could exert a beneficial action against pathological alterations caused by the presence of O₂, H₂O₂ and OH⁻.

In conclusion, this study provides evidence that in alloxan induced diabetic rats, *C. colocynthis* pulp extract has a potent hypolipidemic effect, reducing the lipid content of both serum and liver. The extract also possesses an antioxidant protective effect on the liver of these rats as evidenced by the decrease in the levels of lipid peroxidation markers and the increase in the enzymatic and non enzymatic components of the oxidative system in the liver.

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