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Research Article
Modulatory Effect of *Achyranthes aspera* L., Seeds on Carbohydrates and Protein Bound Enzymes on High Fructose Fed Diet

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Abstract

Background and Objectives: High fructose feeding in rats not only induces insulin resistance, but it may also induce hyperinsulinemia, hyperglycemia and dyslipidemia. *Achyranthes aspera (A. aspera)* exhibited potent hypolipidimic activity. This study' investigated the effects of *A. aspera* on the content and characteristics of carbohydrate bound enzymes and protein bound carbohydrates of rats with high fructose fed diet (HFFD). **Materials and Methods:** The insulin resistance in fructose-fed rats is associated with the defects in insulin signalling pathways. Twenty four male wistar rats were randomly divided into four groups of six animals each. Normal Control group I, *A. aspera* supplemented control group II, *A. aspera* not supplemented group III and supplemented fructose-fed group IV. The estimation of carbohydrate bound enzymes and protein bound carbohydrates were studied. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) using SPSS software package 19.0 (significant value $p<0.05$). **Results:** Administration of *A. aspera* results in significant reduction of hexokinase D, glucose-6-phosphatase (G-6-P) and fructose-1, 6-bisphosphatase (F-1, 6-P) activities, hexose, hexosamine, sialic acid and fucose in plasma. **Conclusion:** It was concluded that *A. aspera* treatment was found to be effective in improving insulin sensitivity and metabolic alterations of carbohydrate and protein associated with consumption of HFFD.

Key words: Metabolic syndrome, pyruvate kinase, hexokinase D, hyperinsulinemia, insulin resistance

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes Mellitus (DM) is a clinically and genetically heterogeneous metabolic disease characterized by hyperglycemia and dysregulation of carbohydrate, protein and lipids metabolism due to deficiency in insulin secretion, insulin function or both of them[1]. Insulin resistance is the main defect associated with the metabolic syndrome and obesity is the critical factor that induces insulin resistance[2]. Excess consumption of fructose is an important contributor to the metabolic syndrome[3]. The liver is the primary site for fructose extraction and metabolism, therefore, chronic high fructose load impairs hepatic glucose metabolism[4].

Oxidative stress is one of the major causes for disease burden in diabetes and related metabolic disorders and antioxidant supplementation is proven to be beneficial[5]. Reactive Oxygen Species (ROS) and reactive nitrogen species cause DNA damage and peroxidation of membrane lipids. Superoxide (O^{2-}) radicals generated during glucose-oxidation reacts with Nitric Oxide (NO) released by endothelium and macrophages forming highly reactive peroxynitrite radical ($ONOO^{-}$) and eventually aggravating. The liver is the primary organ whose inflammatory responses leading to diabetic micro and macrovascular complications[6]. Chronic consumption of fructose may lead to promoting change in redox state by increasing ROS production, promoting oxidative stress[7]. Administration of fructose can trigger free radical production, thereby decreasing antioxidant status and causing oxidative damage to proteins and lipids in the liver[8]. Increased fructose consumption can lead to increase in blood lipids[9], development of insulin resistance[10], increase in inflammatory biomarkers, oxidative stress and risk on development of obesity.

Ayurveda and other traditional medicinal system describe a number of plants used as herbal drugs for the treatment of diabetes. Safe effective and inexpensive indigenous remedies are gaining popularity among the people of both urban and rural areas especially in India. *Achyranthes aspera* L. belonging to family Amarathaceae, is commonly found as a weed on way side throughout India. Zambare *et al.*[11] showed the ethanol extract of the whole plant posses beneficial effect on biochemical parameters in alloxan induced diabetic rats. This plant also posses various pharmacological properties like, astringent, digestive, diuretic, laxative, purgative and stomachic. The juice of the plant is used in the treatment of boils, diarrhea, dysentery, hemorrhoids, rheumatic pains, itches and skin eruptions[12]. *Achyranthes aspera* is having phytoactive constituents and reduction of lipid peroxidation and enhancement in free radical scavenging activity of the herbal seed crude powder[13]. The previous work shows that *A. aspera* has antihypolipidimic activity in high fructose fed diet (HFFD) rats[14].

The plant *A. aspera* was chosen to find its antidiabetic efficacy against alloxan induced diabetic. Earlier phytochemical studies reported that it contains saponins, alkaloids (betaine, achyranthine), amino acids, steroids (stigmasterol), triterpenoids (oleanolic acid and its glucoside), phenolic content (indole acetic acid oxidase) and flavonoids. It has also been reported to have antiarthritic and antirheumatic activity as per folklore practice[15]. Therefore the present study was designed to investigate the development of obesity in response to a HFFD and to estimate oxidative stress markers and antioxidant defense in rats.

MATERIALS AND METHODS

Animals: Twenty four female adult Wistar strain albino rats 6-7 weeks old, weighing 100-120 g were purchased from "Sri Venkateswara Enterprises", Bangalore, India. They were housed clean sterile polypropylene cages in animal room under the kept at constant environmental and nutritional conditions throughout the period of experiment. During the course of the experiments, the temperature was maintained between 27±2°C[16]. The rats were fed on a standard pellet diet during experimental period and water *ad libitum*. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

After one week of acclimatization the animals were divided into two batches. One batch was provided with a control diet containing starch as the source of carbohydrate and the other was fed a fructose-enriched diet for 45 days. They were fed either a control diet, containing 60% corn starch, 20% casein, 0.7% methionine, 5% groundnut oil, 10.6% wheat bran, 3.5% salt mixture and 0.2% vitamin mixture, or a high-fructose diet, which had the same composition as the control diet, except that corn starch was replaced with an equal amount of fructose. Group I (CON) received the control diet and tap water *ad libitum*. Group II (CON+*A. aspera*) were control rat supplemented with *A. aspera*, group III are HFFD rats and group IV (HFFD+*A. aspera*) were fed HFFD and supplemented with *A. aspera*. The total experimental duration was 45 days. *A. aspera* was given orally for the last 15 days of the experimental period. The following experimental groups, consisting of six rats each, were maintained as follows:

Experimental design: The rats were divided into four groups, each group consisting of six animals:

Group I : Normal control rats

Group II : Control rats treated with the crude powder of *A. aspera* seeds (100 mg kg^{-1} b.wt.,) twice daily for a last 15 days of the experimental period

Group III : High fructose fed rats (>60% fructose for 45 days)

Group IV : High fructose fed rats treated with the crude powder of *A. aspera* seeds (100 mg kg^{-1} b.wt.,) twice daily for a last 15 days of the experimental period

Chemicals: Fructose, bovine serum albumin, G-6-P, γ-glutamyl paranitroaniline, nicotinamide adenine dinucleotide (NAD^+, NADH) nicotinamide adenine dinucleotide phosphate ($NADP^+$, NADPH), reduced glutathione, oxidized glutathione, adenosine triphosphate, adenosine monophosphate and 1,2,4-Aminonapthol sulphonic acid were obtained from Sigma Chemical Company, ST. Louis, MO, USA.

All other chemicals and reagents used were of highest purity and of analytical grade marketed by Glaxo Laboratories, Mumbai, SD Fine Chemicals, Mumbai and Sisco Research Laboratories, Pvt. Ltd., India.

Collection of samples: At the end of the 45th day the rats were fasted overnight and killed by cervical decapitation under mild ether anesthesia. Blood was collected tube with heparin and serum was separated by centrifugation (for 15 min at 2000 rpm). Plasma was separated by centrifugation at 1000 rpm for 10 min. The liver and kidney were immediately removed and washed in ice cold saline to remove blood. The tissues were sliced and homogenized in 0.1 M Tris HCl buffer (pH 7.0). The homogenates were centrifuged at 1000 rpm for 10 min at 0°C in a cold centrifuge[17].

Analytical method: The activities of glucokinase, G-6-P and F-1, 6-P were assayed by the methods of Vidhya *et al.*[18], Ramesh and Pugalendi[19], Yogalakshmi *et al.*[20], respectively. Assays were carried in plasma for hexose[21], hexosamine[22], sialic acid[23] and fucose[24]. The levels of phosphoglucomutase[25], pyruvate kinase[26], glycogen phosphorylase[27] and glycogen[28] were determined in plasma.

Statistical analysis: Values or Mean±SD for six rats in the each group and statistically significant differences between mean values were determined by one way analysis of variance (ANOVA) followed by Tukey's test for multiple comparison values of $p<0.05$ was considered to be significant. Statistical Package for Social Studies (SPSS Inc., Chicago, IL)) 19.0 versions were used for this analysis[29].

RESULTS

Table 1 shows the effect of *A. aspera* on carbohydrate metabolic enzymes in tissues of normal and fructose fed rats. The hexokinase D increased and as well as, the activities of gluconeogenic enzymes such as G-6-P and F-1, 6-P is also attenuated in the liver and kidney of HFFD rats when compared with normal rats. A significant $p<0.05$ reduction of hexokinase D, G-6-P and F-1, 6-P activities were observed in the groups treated with *A. aspera* (Group IV) and may be due to the presence of the phytoactive constituents such as flavonoids and saponins, which were reported to present in these herbal seeds. G-6-P and F-1, 6-P levels when compared with HFFD rats (Group III). There are no significant changes of G-6-P and F-1, 6-P in group I and II.

Table 2 shows the activities of pyruvate kinase and hepatic glycogen significantly $p<0.05$ decreased and

Table 1: Effect of *A. aspera* seeds on the activities of carbohydrate metabolic enzymes in liver and kidney of normal and experimental rats

Groups	Glucokinase (hexokinase D) (Unit* h^{-1} mg^{-1} $protein^{-1}$) liver	Glucose-6-phosphatase (Unit** min^{-1} mg^{-1} $protein^{-1}$)		Fructose-1,6-phosphatase (Unit*** h^{-1} mg^{-1} $protein^{-1}$)	
		Liver	Kidney	Liver	Kidney
I (Normal control)	0.271±0.01[a]	0.172±0.02[a]	0.191±0.01[a]	0.425±0.03[a]	0.829±0.07[a]
II (Control+AAS)	0.273±0.01[a]	0.170±0.05[a]	0.189±0.01[a]	0.423±0.03[a]	0.823±0.05[a]
III (HFFD control)	0.91±0.001[b]	0.468±0.03[b]	0.286±0.02[b]	0.728±0.06[b]	1.628±0.12[b]
IV (HFFD+AAS)	0.262±0.02[c]	0.191±0.08[c]	0.199±0.02[c]	0.434±0.04[c]	0.834±0.04[c]

Values are Means±SD for six rats in each group. Values sharing a common superscript in a column are not significant with each other ($p<0.05$, Duncan's Multiple Range Test [DMRT]), *μmoles of glucose phosphorylated, **μmoles of inorganic phosphorous liberated, ***μmoles of inorganic phosphorous liberated

Table 2: Effect of *A. aspera* seeds on protein-bound carbohydrates in plasma of control and experimental animals

Groups	Phosphoglucomutase[α]	Pyruvate kinase[β]	Glycogen[γ]	Glycogen phosphorylase[δ]
I (Normal control)	1.46±0.21[a]	0.66±0.05[a]	26.32±2.11[a]	3.99±0.38[a]
II (Control+AS)	1.44±0.15[a]	0.67±0.04[a]	25.96±2.49[a]	3.98±0.38[a]
III (HFFD control)	3.62±0.32[b]	0.26±0.01[b]	14.05±1.19[b]	8.01±0.72[b]
IV (HFFD+AAS)	1.51±0.20[c]	0.58±0.04[c]	25.99±2.41[c]	4.09±0.32[c]

Values are Means±SD for six rats in each group. Values sharing a common superscript in a column are not significant with each other ($p<0.05$, Duncan's Multiple Range Test [DMRT]). α: μ M of Pi liberated h^{-1} g^{-1} protein, β: Units g^{-1} protein, γ: mg/l00 g wet tissue, δ: μ M of Pi liberated l h g^{-1} protein

Table 3: Effect of *A. aspera* seeds on protein-bound carbohydrates in plasma of control and experimental animals

Groups	Hexose (mg dL^{-1})	Hexosamine (mg dL^{-1})	Fucose (mg dL^{-1})	Sialic acid (mg dL^{-1})
I (Normal control)	96.67±9.1[a]	75.53±6.41[a]	31.61±2.96[a]	55.44±5.20[a]
II (Control+AAS)	95.74±8.21[a]	74.96±7.05[a]	30.87±2.71[a]	54.81±4.37[a]
III (HFFD control)	151.57±12.9[b]	118.9±8.14[b]	62.92±3.14[b]	89.17±5.34[b]
IV (HFFD+AAS)	97.71±8.17[c]	76.91±7.14[c]	32.01±3.11[c]	56.11±4.40[c]

Values are Means±SD for six rats in each group. Values sharing a common superscript in a column are not significant with each other [p<0.05, Duncan's Multiple Range Test (DMRT)]

phosphoglucomutase, glycogen phosphorylase significantly p<0.05 increased in fructose fed hypertensive rats 45 days. *Achyranthes aspera* administration to fructose fed rats significantly attenuated phosphoglucomutase, glycogen phosphorylase, altered pyruvate kinase and hepatic glycogen as compared to HFFD rats. *Achyranthes aspera* might have regulated the activation of the hexokinase, phosphoglucomutase, pyruvate kinase and glycogen. There is no significant alteration of hexokinase, phosphoglucomutase, pyruvate kinase and glycogen between groups I and II.

Table 3 gives the levels of hexose, hexosamine, sialic acid and fucose in plasma in control and experimental rats. Increased the levels hexose, hexosamine, sialic acid and fucose in plasma in HFFD rats (group III) is compared to normal control rats (group I). The results of the present study showed increased hexose, hexosamine, sialic acid and fucose concentration in the high fructose fed rats. This may be due to presence of saponins, alkolids and oleic acid. Administration of *A. aspera* having phyto active constituents involved to HFFD rats (Group IV) brought back the levels to normal levels (Group III).

DISCUSSION

The chronic administration of fructose for 45 days caused insulin resistance increased the levels of carbohydrate bound enzyme activities and alteration of protein bound carbohydrates. *Achyranthes aspera* administration improved insulin sensitivity and decreased carbohydrate bound enzyme activities and alteration of protein bound carbohydrates. In human beings and the rats, fructose is metabolized primarily in the liver, although both the small intestinal mucosa and kidney incorporate the enzymes necessary for the catabolism of fructose[30]. Preliminary catabolism of fructose and other sugars is phosphorylation. Fructose can be phosphorylated either by hexokinase, which occurs throughout the body and phosphorylates a number of 6 carbon sugars at carbon-6 and/or by fructokinase, which is predominantly found in the liver and phosphorylates at carbon-1. Fructokinase can also phosphorylate other ketoses, including galactoheptulose, sorbose, tagatose and xylulose. Increasing concentration of fructose in diets of rats and humans resulted in increase in activity of fructokinase[31], which is also observed in the HFFD rats in the present study.

Glucose-6- phosphatase (G-6-P) is a multi component enzyme that is tightly related with the endoplasmic reticular membrane and catalyzes the dephosphorylation of G-6-P, the terminal step of glycogenolysis and gluconeogenesis[32]. G-6-P is a key enzyme of glucose homeostasis since it catalyzes the ultimate reaction of both glycogenolysis and gluconeogenesis. Insulin decreases gluconeogenesis by decreasing the activities of key enzymes, such as G-6-P, fructose-1, 6-bisphosphatase (F-1, 6-P), phosphor enolpyruvate carboxykinase and pyruvate carboxykinase[33]. F-1, 6-P is one of the key enzymes of gluconeogenic pathway. It is present in liver and kidney but absent from heart, muscle and smooth muscle. In this study, the increased activities of G-6-P and F-1, 6-P in liver and kidney of fructose fed rats may be due to insulin deficiency. In *A. aspera* fructose rats, the activities of these two enzymes were significantly p<0.05 reduced, which is responsible for the improved glycemic control. This may be due to *A. aspera* role of alteration in the rate of gluconeogenic key regulatory enzymes have been effectively regulated and thus controlled the gluconeogenic pathway.

The changes in the enzyme activities and glycogen content in fructose fed rats are indicative in the liver at gluconeogenic state. The activities of the regulatory enzymes likes glucokinase, G-6-P, glycogen phosphorylase and hexokinase were altered during chronic fructose feeding leading to hepatic insulin resistance. Furthermore, fructose feeding has been shown to lead to a decrease in the ability of insulin to suppress activation of hepatic G-6-P activity. Phosphoglucomutase and G-6-P exhibited a common pattern. Both enzymes appeared to require a minimum supply of protein (about 20%). In fact, if carbohydrate or fat was substituted for up to 80% of the calories contributed by protein, the same levels of activity as in the livers of rats fed the 100% protein diet were observed. The specific activities remained approximately the same under the various dietary conditions, this being especially clear in the case of phosphoglucomutase. L-type pyruvate kinase (EC 2.7.1.40) is a key enzyme in the glycolytic pathway whose activity

fluctuates according to the dietary status in the liver. Reduction in the hepatic glycogen concentration has been reported in this model[34]. Fasting liver and soleus muscle glycogen were markedly reduced in fructose fed rats compared with control. Hyperglycemia per se can increase hepatic glycogen synthesis and contribute to the direct pathway to total glycogen synthesis in rats. From the results of this study, it was clearly understood that on administration of the *A. aspera* to the rats fed with the HFFD, the alteration in the rate of gluconeogenic key regulatory enzymes like glucokinase, G-6-P, glycogen phosphorylase and hexokinase have been effectively regulated and thus controlled the gluconeogenic pathway. It also looked at the levels of sugars and amino sugars bound to proteins. Glycosylation is an enzymatic process utilizing amino sugars during the post-translational covalent modification of proteins. Increase in glucose can increase the flux through the hexosamine biosynthetic pathway in tissues. Increase in the level of amino sugars and protein-bound sugars represent the liberation of these substances from tissues. The protein bound carbohydrates viz hexosamine and sialic acid increased in fructose fed rats whereas *A. aspera* supplementation shows decreased the levels. Studies reported that since fructose-fed rats showed hyperglycemia and the protein bound sugars may be altered in these rats. The glycoproteins levels were elevated in plasma of fructose-fed rats[35].

Sialic acid is one of the markers of acute and chronic phase responses[36]. It is a terminal component of the non-reducing end of carbohydrate chains of glycoproteins and glycolipids[37]. Elevated levels of sialic acid concentration are a risk factor for cardiovascular mortality in humans[38]. A significant $p<0.05$ increase in serum sialic acid concentration was observed in fructose fed rats which sialic acid is a component of cell membranes and elevated levels may indicate excessive cell membrane damage but more specifically to the cells of vascular tissue. Also, sialic acid can be used as a measurement of acute phase response because many of the proteins of the immune response are actually glycoproteins which have sialic acid as the terminal sugar of their oligosaccharide chain[39]. Treatment with *A. aspera* to HFFD rats resulted in significant $p<0.05$ decrease in serum sialic acid level. These results might be attributed to the Protection of cellular membranes through *A. aspera* that it may exert a direct effect on the membrane. It may prevent cell damage by stabilizing the membrane against free radical-induced injury and also may prevent mitochondrial injury, thus increasing energy production and decreasing the leakage of free radicals[40]. Hexosamine biosynthesis has been proposed to play a role in mediating the effects of chronic hyperglycemia. Excess flux through the pathway has been shown to result in insulin resistance in cultured cells, tissues and in intact animals[41]. Fucose the organic compound and fucose is a deoxyhexose that is present in a wide variety of organisms. Unlike most sugars, fucose occurs in nature as the L-form and lacks a hydroxyl group on the carbon at the 6-position. L-Fucose is used as a substrate to identify, differentiate and characterize enzymes such as the fucosidase(s), L-fucose isomerase(s) and L-fucose dehydrogenase(s). This may be due to administration of *A. aspera* having phyto active constituents.

CONCLUSION

The results indicate that feeding *A. aspera* can have significant hypoglycemic effects. *A. aspera* treatment was found to be effective in improving insulin sensitivity and metabolic alterations of carbohydrate and protein associated with consumption of HFFD.

SIGNIFICANCE STATEMENT

This study discovers the effects of *A. aspera* modulate insulin resistance that can be beneficial for HFFD rats. This study will help the researcher to uncover the critical areas of carbohydrate metabolizing enzymes and protein bound carbohydrates that many researchers were not able to explore. Thus a new theory on carbohydrate metabolism and treatment of diabetes mellitus *by A. aspera* modulation after a clear mechanistic study and clinical trial may be arrived at this study.

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