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Evaluation of Protein Thiols and Liver Glycogen Content on Streptozotocin Induced Diabetic Rats Treated with Aqueous Extract of *Bixa orellana* Leaves

R. Vinutha Bhat[1], E. G. Padmanabha Udupa[1], U. Anushree[1], Samreen M. Sheik[1] and B. Shivananda Nayak[2*]

[1]Department of Biochemistry, Kasturba Medical College, Manipal, India.
[2]Department of Preclinical Sciences, Faculty of Medical Sciences, St. Augustine, Trinidad and Tobago.

Authors' contributions

This work was carried out in collaboration between all authors. Authors RVB and BSN designed the study, wrote the protocol and supervised the work. Authors UA and SMS carried out all laboratories work and performed the statistical analysis. Author EGPU managed the analyses of the study. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Background: Diabetes mellitus is a common metabolic disorder causing hyperglycemia affecting the world wide population. In recent years most of the plant extracts with their potential antioxidant property are used in the treatment of diabetes mellitus. The aim of our study is to determine protein thiols and glycogen content to evaluate the hypoglycemic activity of the aqueous leaf extract of *Bixa orellana* using diabetes induced rats.

**Corresponding author: E-mail: Shiv25@gmail.com;*

Methods: The leaves of *B. orellana* were obtained, cleaned and shade dried to make the extract. Wistar male rats weighing about 200-300 g were used and all the animals were provided with wheat gluten feed pellets and water. Diabetes was induced using Streptozotocin. The experimental group consisted 6 animals were treated with aqueous extract of 60 mg/kg body weight. The diabetic and normal control group animals were treated with 0.9% of saline.
Results: There was decrease in protein thiol in diabetic control group when compared to normal control and increase in liver glycogen in the diabetic control group. P value between the group's normal control and diabetic control for protein thiols and glycogen was found to be 0.004 and 0.005 which were highly significant. When Normal group was compared with Test group there was statistical significance difference in which aqueous extract treated group had an increase in serum protein thiol ($P<0.002$) and a decrease in glycogen content ($P <0.001$). When Diabetic control group was compared with Test group there was no statistical significance difference in which aqueous extract treated group and had an increase in serum protein thiol ($P>0.05$) and decrease in glycogen content ($P >0.05$).
Conclusions: The study showed a significant increase in the protein thiol and decrease in liver glycogen in the test group of streptozotocin induced diabetic rats treated with aqueous extracts of *B. orellana* leaves.

Keywords: Diabetes mellitus; protein thiol; glycogen; Bixa orellana.

1. INTRODUCTION

Diabetes mellitus (DM) is a common metabolic disorder causing hyperglycemia affecting the world wide population of about 170 million people [1]. It was found to be ineluctably connected with increased oxidative stress both in diabetic humans and hyperglycemic animals [2]. Most of the plant extracts contain fatty acids, sterols, flavonoids, alkaloids, and vitamins which add to the potential antioxidant property and are used in the treatment of diabetes mellitus like *Ocimum sanctum* L. (OS), *Murraya koenigii* L. (MK*), Catharanthus roseus* L. (CR) and *Azadirachta indica* A. Juss. (AI) [3,4]. Literature surveys showed that the various parts especially seeds and leaves of the plant *Bixa orellana* (annatto) are known to have hypoglycemic effects [5,6].

Thiols bound to albumin in its sulfhydryl group at cysteine 34 position contribute majorly to the protein bound thiols. They play significant role in detoxification reactions, apoptosis of the cell along with free radical defense mechanisms. Various renal, cardiovascular, neurological disorders, metabolic disorders like diabetes mellitus, Liver cirrhosis due to alcohol all contribute to decrease in the serum thiols [7]. Uncontrolled diabetes mellitus leads to raise in oxygen free radicals from autoxidation of glucose [8] and glycosylation of scavenging enzymes and depletion of low molecular antioxidant causing oxidative stress which has been implicated in the pathogenesis of DM and its complications [9].

The pancreas will respond to hyperglycemia, by releasing insulin that promotes the liver and muscles to take up glucose from the blood, lowering blood glucose levels and stimulating glycogenesis. In case of diabetes, the glycogen metabolism is altered due to the lack of insulin.

More than 80% of the diabetic subjects are known to have glycogen accumulation in the liver and the reason behind this is said to be long standing insulin deficiency facilitating the synthase activity along with increased gluconeogenesis [10,11].

Bixa. orellana is a bushy shrub ranging from 4-10 m in height. They possess glossy ovate evergreen leaves with reddish veins. Pink flowers and brownish to maroon two valved fruit is a characteristic feature of the plant [9].

Several bioactive compounds in Bixa such as sesquiterpenes are known to have antifungal activity against *Candida albicans* and also it is shown to have an antibacterial activity against *Escherichia coli* [12]. Studies done in dogs reveal that seeds of *Bixa orellana* are known to be effective against skin diseases [13].

Literature surveys showed that the seeds and leaves of the plant *Bixa orellana* (annatto) are known to have hypoglycemic effects [5,6]. Therefore in this study we attempted to study the effect of aqueous extract of leaves of *Bixa orellana* on antioxidant and hypoglycemic activity in diabetes mellitus. For this reason we have

analysed the protein thils and glycogen content of the rats treated with this *B. orellana* extract.

2. MATERIALS AND METHODS

2.1 Preparation of the Different Extracts

The plant was authenticated by the retired professor of botany (Poorna Prajna College, Udupi, India). The leaves (54 gm) of *B. orellana* were obtained from the Udupi district, cleaned and shade dried. The dried leaves were powdered and the final yield of 53 g obtained was stored in an air tight container.

2.1.1 Aqueous extract

Finely powdered leaves were soaked in water for 1-2 hour. Refluxed for 2-3 hour by reflux condensation method and decanted when hot. Concentrated the contents under reduced pressure (evaporate water). Kept in a water bath and heated till the syrupy consistency was obtained. Preserved it in an air tight container and this container was stored in a desiccator at room temperature.

The application for the research proposal was submitted to the Institutional Animal Ethics Committee. The Committee has scrutinized the project and approved it with the ethics approval number of IAEC/KMC/34/2015 on the letter dated May 22, 2015.

2.2 Experimental Animals

Male wistar rats weighing about 200-300g were used for the study. They were housed in Central Animal House, Manipal, India. Solid bottom shoe box type cages constructed of durable plastic and contact bedding material of wheat husk were used. All the animals were provided with wheat gluten feed pellets and water was provided in separate compartments of the cage.

2.3 Induction of Diabetes

Rats were fatsted for about 8 hours before inducing the diabetes. Diabetes inducing drug Streptozotocin of 60 mg/kg body weight and the volume (dissolved in 0.1 M citrate buffer of pH 4.5) is 1 ml/kg body weight was used to induce the diabetes. Normal control group was injected with 0.9% of saline.

After 7 days of stabilization period following the induction of the diabetes on the 8th day about 1-1.5 ml of blood was drawn into a grey vacutainer containing sodium fluoride and potassium oxalate as anticoagulant. Blood was collected intraorbitally using mucap capillary and tested for serum glucose to confirm the diabetes. The diabetes induced groups were randomly assigned into different groups with 6 animals in each group. Day of confirmation of diabetes was considered as day 1 for further course of treatment as per the group. Estimation of protein thiols was done on day 30. After assaying all the parameters the animal was sacrificed and liver. Glycogen was estimated. Treatment schedule is given below.

2.4 Treatment

After the confirmation of diabetes, 8th day was considered as day 1 of treatment. Table 1 shows the grouping of animals and the treatment given to them.

Group 1 (Normal Control (NC), n=6): treated with vehicle

Group 2 (Diabetic control (DC), n=6: treated with vehicle

Group 3 (Standard drug (SD), n=6: treated with pioglitazone orally at a dose of 200 mg/kg body weight

Group 4 (Test (T), n=14: Treated with aqueous extract orally at a dose of 200 m/ kg body weight.

2.4.1 Estimation of protein thiols (mg/dl)

Spectrophotometric method using Dinitro-benzene (DTNB)-Ellman's method.

Principle: Ellman's reagent or 5,5'-dithiobis (2-nitrobenzoate) [DTNB] is a symmetrical aryl disulfide which readily undergoes the thiol-disulfide interchange reaction in the presence of a free thiol. The TNB dianion has a relatively intense absorbance at 412 nm compared to both disulfides. Because the stoichiometry of protein thiol to TNB formed is 1:1, TNB formation can be used to assess the number of thiols present. In the absence of denaturants, only accessible thiols will react, whereas in the presence of chaotropic agents the total number of reduced Cys residues present can be measured. Reduction of the protein followed by the treatment with chaotropes and DTNB can yield the total number of cysteines (Cys-SH plus Cys-s-s-Cys).

Reagents: Buffer: 0.2 M Na_2HPO_4 containing 2 Mm Na_2–EDTA, pH: 7.4:

Phosphate Buffer saline (PBS) pH: 7.4:

10 mM DTNB 5, 5'-Dithio-bis (2-nitrobenzoic acid) in 0.2 Na_2HPO_4 (MW: 396.35; 3.96 mg/ml):

GSH standard; 1mM: 0.03 g of GSH in 90 ml of PBS and q.s 100 ml with PBS.

Procedure: Pre incubate PBS, DTNB, 0.2 M Na_2HPO_4 in 2 Mm EDTA at 37℃ before the assay.

Take 3 appendorf tubes for Reagent Blank (RB), Test (T) and Sample Blank (SB).

Pipette successively as follows:

Reagent	RB	SB	T
0.2 M Na_2HPO_4 with 2 Mm EDTA(µl)	900	920	900
PBS (µl)	100	-	-
10 mM DTNB (µl)	20	-	20
Sample (µl)	-	100	100

The contents were mixed in a Vortex and incubated for 5.0 min at room temperature (or 37℃).

Two minutes gap was maintined between RB and T and read each tube after 5.0 minutes at 412 nm against air as blank.

The reaction takes about 5 minutes to go to completion, during which there is an increase in the absorbance. After reaching a maximum the absorbance may decrease at slow and steady rate.

A calibration curve was produced using GSH dissolved in PBS. The protein thiol concentration in plasma/Serum was determined from the standard curve using the corrected absorbance values for the test sample.

2.5 Estimation of Glycogen

The rats liver tissue was isolated to estimate glycogen.

A direct method by Morris et al. was used to analyse liver glycogen.

Principle: The organic anthrone reagent was used for the qualitative estimation of carbohydrates (glycogen). The mechanism of reaction between the carbohydrates and the anthrone reagent is that the sulfuric acid in the reagent causes dehydration of the sugar to furfural derivatives which then condenses with anthrone to form a blue colored compound which is estimated spectrophotometrically [14].

Reagents: KOH 30%, Ethanol (95%), Anthrone reagent (200 mg anthrone / 100 ml of conc. sulfuric acid 95%)-prepared fresh.

Standard Glucose: Stock: 10 mg / 100 ml. Working: 10 ml made upto 100 ml with double distilled water.

Procedure: 1 g of liver tissue was taken and to this 3.0 ml of 30% KOH is added.

Weight of the tissue was determined by difference between the weight of the beaker containing tissue and the empty beaker. Tubes were kept in a boiling water bath for 20 minutes till no tissue was left undigested. Contents were cooled at room temperature.

The contents were made upto 50.0 ml in a volumetric flask with double distilled water and mixed. The above solution (300 µg) was taken and made upto 100 ml with double distilled water (DDW) as test solution.

Blank	Standard	Test
DDW 5 ml	Glucose 5 ml	Test solution 5 ml
Keep in ice chest		
10 ml anthrone reagent		
Mix, keep in boiling water bath 10 minutes, cooled		
Read at 620 nm		

Calculation:

µg of glycogen in aliquot= 100*U/1.11*S

U = OD of unknown
S = OD of standard (glucose)
1.11 = Morris conversion of glucose to glycogen

2.6 Statistical Analysis

All data obtained were analyzed using SPSS (commonly used Software version 17.0). The significance of difference among the groups was assessed using Kruskal Wallis test followed by Man Whitney 'U' test. Significant value was set to$p$ <0.05. Comparisons of data within the group at different intervals were assessed by Wilcoxoll Signed Rank Sum test.

3. RESULTS

The results of the present study confirms that the administration of aqueous extract of *B. orellana* possess antidiabetic activity against streptozotocin induced diabetic rats. Table 1 depicts P value for protein thiol and glycogen between NC, SD and Test group and is found to be 0.003 which is highly significant for protein thiol. Increase in protein thiol concentration was seen in the test group treated with aqueous extract (Table 1). P value for glycogen was found to be 0.002 which is highly significant. Liver glycogen in the test group was less compared to that of NC (Table 1).

There was decrease in protein thiol in diabetic control group when compared to normal control and increase in liver glycogen in the diabetic control group. The P value between normal control and diabetic control for protein thiols and liver glycogen were found to be 0.004 and 0.005 respectively and which are highly significant.

When Normal group was compared with Test group there was statistical significance difference in which aqueous extract treated group had an increase in serum protein thiol (P<0.002) and decrease in glycogen content (P <0.001).

When Diabetic control group was compared with Test group there was no statistical significance difference in which aqueous extract treated group and had an increase in serum protein thiol (P>0.05) and decrease in glycogen content (P >0.05).

There is statistical significant decrease in liver glycogen and increase in protein thiol in the group treated with aqueous extract compared to that of the normal control. Table 2 shows the blood glucose concentration of animals treated with *B. orellana* and the standard drug.

4. DISCUSSION

In the present study we demonstrated that streptozotocin induced diabetes mellitus in rats causes hyperglycemia, renal function deficits and oxidative damage in liver and pancreas.

Measurement of protein thiol is a good reflection of free radical generation since the conformation of albumin is altered showing SH groups to be

Table 1. Descriptive – protein thiol and glycogen

	N	Mean	Standard deviation	H	P
Protein thiol (nmol/L)					
NC	6	0.326	0.059		
SD	6	0.638	0.098	11.827	0.003 hs
Test	9	0.667	0.184		
DC	6	0.331	0.228		
Glycogen					
NC	6	73.317	5.075		
SD	6	42.567	10.865	12.453	0.002 hs
Test	9	42.193	10.467		
DC	6	72.438	7.9501		

p< 0.05 considered significant. NC-Normal control, SD-Standard Drug, Test-Aqueous extract of B. orellana, DC= Diabetic control H=Kruskal Wallis test

Table 2. Blood Glucose of the animals treated with aqueous extract of *B. orellana* and the standard drug

Group	N	Mean	Std. deviation	H	p
Aqueous extract					
Before	14	88.973	14.636		
Day 1	14	135.295	5.788	34.97	<0.01 VHS
Day 15	9	116.083	10.398		
Day 30	9	99.567	11.083		
Standard drug pioglitazone					
Before	14	93.933	8.969		
Day 1	14	132.730	5.053	15.73	<0.01 VHS
Day 15	9	108.070	10.940		
Day 30	9	106.100	9.943		

oxidized. Decrease in blood glucose due to aqueous extracts *of B. orellana* might have caused the decrease in free radical formations and hence the protein thiols. The major source of thiols in plasma is albumin and glycation modifies thiol groups to form disulfide bonds and intermolecular aggregates. Hypoglycemic plants like *Camellia sinensis*, *Annona muricata* are known to reduce oxidative stress of pancreatic β-cells [15]. The antioxidants in the extract decreased the glycemic excursion and hence increase in the protein thiol concentration as a result of decreased oxidative protein damage during the course of treatment [16]. A major modification of protein structure due to glycation is the loss of protein thiol groups which is the direct reflection of excess free radical generation.

Estimation of Liver glycogen level can be considered as the best marker for assessing the hypoglycemic activity of any drug. *B. orellana* might enhance glucose utilization by peripheral tissues and increase the glycogen stores in the liver. Treatment with methanolic extract of Rhinacanthus nasutus als showed an increase in the glycogen content in liver tissue of diabetic rats. The alcoholic extract of Inula racemose decreased blood glucose and enhanced liver glycogen in rats. Administration of streptozotocin causes increase in liver glycogen content due to increased glycogenolysis which is due to insulin deficiency so the normal capacity of the liver to synthesize glycogen is impaired. A significant decrease in liver glycogen by administration of *B. orellana* extract may be due to an increase in level of insulin by it. In patients with noninsulin dependent diabetes mellitus increased gluconeogenesis is given as an explanation for the preservation of liver glycogen [17]. Liver glycogen changes may require higher doses of the extract and are not observed at lower doses.

5. CONCLUSION

The study showed a significant increase in the protein thiol and decrease in liver glycogen in the test group of streptozotocin induced diabetic rats treated with aqueous extracts of *B. orellana* leaves. The parallel effects of the aqueous extract with antioxidant properties along with the hypoglycemic effects had added upto the increase` in protein thiol. The decrease in liver glycogen is due to decreased rate of gluconeogenesis and increase in insulin caused by the hypoglycemic property of the above studied plant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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