

## Research Article

# Activities of Three Erythrocyte Enzymes of Hyperglycemic Rats (*Rattus norvegicus*) Treated with *Allium sativa* Extract.

Chikezie, P.C<sup>1</sup> and Uwakwe A.A<sup>2</sup><sup>1</sup>Department of Biochemistry, Imo State University, Owerri, Nigeria.<sup>2</sup>Department of Biochemistry, University of Port-Harcourt, Port-Harcourt, Nigeria

**ABSTRACT: Background/Objective:** The present study sought to investigate erythrocyte glutathione S-transferases (GST), NADH-Methaemoglobin reductase (NADH-MR) and Na<sup>+</sup>/K<sup>+</sup>-ATPase activities of hypoglycemic rats treated with ethanol/water (1:2 v/v) extract of *A. sativa* as an agent of glycaemic control. **Materials and Methods:** Hyperglycemia was induced by a single intra-peritoneal injection of 0.1 mol/L alloxan monohydrate in phosphate buffer saline (PBS) solution (pH = 7.4); dosage = 140 mg/kg. At the end of the experimental time (*t* = 76 h), erythrocyte GST, NADH-MR and Na<sup>+</sup>/K<sup>+</sup>-ATPase activities as well as serum fasting blood sugar (FBS) levels were measured by spectrophotometric methods. **Results:** Serum FBS levels of control/normal (C/N) rats ranged between 72.93 ± 0.82–95.12 ± 0.92 mg/dL, whereas experimental rats without glycaemic control gave: 249.41 ± 1.03–256.11 ± 1.23 mg/dL. Hyperglycemic rats treated with ethanol/water (1:2 v/v) extract of *A. sativa* exhibited comparative reduced serum levels of FBS alongside with erythrocyte GST, NADH-MR and Na<sup>+</sup>/K<sup>+</sup>-ATPase activities. The average relative activities of the three enzymes and corresponding order of enzyme activity in hyperglycemic rats treated with ethanol/water (1:2 v/v) extract of *A. sativa* was: NADH-MR = 60.99% > GST = 47.81% > Na<sup>+</sup>/K<sup>+</sup>-ATPase = 46.81%. In the same order, relative activities of the three enzymes in rats without glycaemic control were: NADH-MR = 49.65% > GST = 23.69% > Na<sup>+</sup>/K<sup>+</sup>-ATPase = 17.02%. **Conclusion:** Erythrocyte GST, NADH-MR and Na<sup>+</sup>/K<sup>+</sup>-ATPase activities gave insights into the pathophysiology of the diabetic state and served as biomarkers for ascertaining therapeutic control in Type 1 diabetes mellitus.

**KEYWORDS:** Glutathione S-transferases, NADH-Methaemoglobin reductase, Na<sup>+</sup>/K<sup>+</sup>-ATPase, *Allium sativa*, hyperglycemia, diabetes mellitus.

## INTRODUCTION

Diabetic mellitus is an endocrine disorder characterized by insufficiency in circulating plasma levels of insulin (type 1, or insulin-dependent diabetes mellitus; IDDM) and peripheral resistance and insensitivity to insulin (type 2, or non-insulin-dependent diabetes mellitus; NIDDM). Unlike type 1 diabetes mellitus, type 2 is associated with hyperinsulinism. Primarily, overall physiologic distortions prompted by poor control of metabolism in the absence or insufficiency of insulin engender hyperglycemia and associated

metabolic disorders.<sup>[1,2]</sup> Striking consequential effects of prolonged hyperglycemia include changes in the structure and function of macromolecules,<sup>[3,4]</sup> auto-oxidation of glycated proteins, increased production of reactive oxygen species (ROS), decreased antioxidant defense, increased lipid peroxidation, and associated apoptosis or necrosis occasioned by membrane degeneration.<sup>[4,5]</sup> Notably, alterations/adjustments in most glycolytic and tricarboxylic acid cycle (TCA) enzyme activities are associated with diabetic states.<sup>[5,6]</sup> Activities of some enzymes (pyruvate kinase, pyruvate dehydrogenase, glycogen synthase, pyruvate carboxylase, fructose 1, 6-bisphosphate etc.) are regulated by insulin. Of note, these enzymes are all phosphoenzymes. Activation of enzyme activity in response to insulin stimulus is prompted by the cyclic adenosine monophosphate (cAMP) phosphodiesterase mediated pathway,<sup>[6]</sup> or through secondary metabolic events connected to insulin action.

### \*Correspondence

Chikezie, P.C

e-mail: p\_chikezie@yahoo.com

Phone: +2348038935327

DOI: 10.5530/pc.2014.2.5

Glutathione S-transferases (GSTs) are multi-gene and multifunctional antioxidant enzymes that comprise several classes of GST isozymes. These enzymes by virtue of their activities act as a subset of numerous cellular antioxidants defense systems against ROS species that are associated with many disease-causing electrophiles.<sup>[2,7,8]</sup> NADH-methaemoglobin reductase (NADH-MR) (EC: 1.6.2.2) transfers electrons from NADH + H<sup>+</sup> to cytochrome b<sub>5</sub> via its flavin adenine dinucleotide (FAD) prosthetic group.<sup>[9]</sup> This erythrocyte enzyme maintains hemoglobin in its ferrous (Fe<sup>2+</sup>) state.<sup>[10]</sup> Na<sup>+</sup>/K<sup>+</sup>-ATPase, also called the sodium pump, is a soluble conserved trimeric pump ( $\alpha$ -133 kDa;  $\beta$ -35 kDa;  $\gamma$ -10 kDa) involved in transmembrane cation regulation via ATP-dependent dual efflux/influx of sodium (Na<sup>+</sup>) and potassium (K<sup>+</sup>) ions in various cells.<sup>[11,12]</sup> The regulation of this pump activity is dependent on the phosphorylation of the  $\alpha$ -subunit of Na<sup>+</sup>/K<sup>+</sup>-ATPase.<sup>[11,13]</sup>

*Allium sativa* has been widely reported to exhibit the therapeutic benefits to numerous pathologic states whose etiology is linked to oxidative stressors and electrophiles<sup>[14]</sup> such as diabetes mellitus,<sup>[15–18]</sup> atherosclerosis,<sup>[19,20]</sup> hyperlipidemia<sup>[20,21]</sup> thrombosis,<sup>[22]</sup> hypertension.<sup>[23]</sup> Phytochemical and biochemical profiles of *A. sativa* have been reported elsewhere.<sup>[24]</sup> The present study was based on the premise that hyperglycemia is one of the various indicators and promoters of distortional haemostasis associated with diabetes mellitus. Therefore, we sought to investigate level of alterations in erythrocyte GST, NADH-MR and Na<sup>+</sup>/K<sup>+</sup>-ATPase activities of hypoglycemic rats treated with ethanol/water (1:2 v/v) extract of *A. sativa* as agent of glycaemic control.

## MATERIALS AND METHODS

**Collection of plant specimen:** Fresh samples of *A. sativa* were obtained in July, 2012 from a local market at Umoziri-Inyishi, Imo State, Nigeria. The plant specimen was identified and authenticated by Dr. F.N. Mbagwu at the Herbarium of the Department of Plant Science and Biotechnology, Imo State University, Owerri, Nigeria. A voucher specimen was deposited at the Herbarium for reference purposes.

**Preparation of extract:** Fresh bulbs of *A. sativa* were washed under a continuous stream of distilled water for 15 min and air-dried at room temperature for 5 h. The bulbs were chopped and further dried for 5 h in an oven at 60 °C and subsequently ground with a ceramic mortar and pestle. Twenty-five grams (25 g) of pulverized specimen

was suspended in 250 mL of ethanol/water mixture (1:2 v/v) in stoppered flasks and allowed to stand at 4°C for 24 h. The suspensions were filtered with Whatman No. 24 filter papers. The filtrate was concentrated in a rotary evaporator at 50°C and dried in a vacuum desiccator. The yield was calculated to be 3.4 % (w/w). The extract was finally suspended in phosphate buffered saline (PBS) solution (extract vehicle), osmotically equivalent to 100 g/L PBS (90.0 g NaCl, 17.0 Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O and 2.43 g NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O), and used in all the studies with doses expressed in mg/kg of body weight of the animals.

**Experimental animals:** Male rats *Rattus norvegicus* (8–10 weeks old) weighing 150–200 g were generous gift from Professor A.A. Uwakwe (Department of Biochemistry, University of Port Harcourt, Nigeria). The rats were maintained at room temperatures of 25±5°C, 30–55% of relative humidity on a 12-h light/12-h dark cycle, with access to water and food *ad libitum* for 2 weeks acclimatization period. The handling of the animals was in accordance with the standard principles of laboratory animal care of the United States National Institutes of Health (NIH, 1978).

**Induction of hyperglycemia and study design:** Hyperglycemia was induced by a single intra-peritoneal injection of 0.1 mol/L alloxan monohydrate in phosphate buffer saline (PBS) solution (pH =7.4) at a dosage of 140 mg/kg. The animals were considered hyperglycemic when their blood glucose concentrations exceeded 250 mg/dL 72 h after alloxan treatment, which was in conformity with our previous study.<sup>[24]</sup> The animals were deprived of food and water for an additional 16 h before commencement of treatment (control and test experiments) as described elsewhere.<sup>[24]</sup>

A total of twenty four (24) rats were divided into six (6) groups of four ( $n = 4$ ) each as follows:

- Group C1; Control-Normal (C/N): Normal rats received only PBS (Vehicle; 1.0 mL/kg/16 h, i. p.) for 64 h.
- Group C2; Control-Hyperglycemic (C/H): Hyperglycemic rats received PBS (Vehicle; 1.0 mL/kg/16 h, i. p.) for 64 h.
- Group T1; H<sub>[A. sativa] = 1.0 mg/kg</sub>: Hyperglycemic rats received *A. sativa* (1.0 mg/kg/16 h, i. p.) for 64 h.
- Group T2; H<sub>[A. sativa] = 2.0 mg/kg</sub>: Hyperglycemic rats received *A. sativa* (2.0 mg/kg/16 h, i. p.) for 64 h.
- Group T3; H<sub>[A. sativa] = 4.0 mg/kg</sub>: Hyperglycemic rats received *A. sativa* (4.0 mg/kg/16 h, i. p.) for 64 h.
- Group T5; H<sub>[Glibenclamide] = 5.0 mg/kg</sub>: Hyperglycemic rats received glibenclamide (5.0 mg/kg/16 h, i. p.) for 64 h.

**Measurement of fasting blood sugar:** After alloxan treatment, blood samples were drawn from the apical region of the tails of the rats i.e., at experimental  $t = 0$  h and by carotid artery puncture at experimental  $t = 76$  h for measurement of fasting blood sugar (FBS). Determination of serum level of FBS was by the glucose oxidase method according to the Randox® kit manufacturer's procedure (Randox® Laboratories Ltd. Ardmore, United Kingdom). Glibenclamide, a standard anti-diabetic agent is a product of Aventis Pharma. Ltd. Goa, India.

**Collection of blood and preparation of erythrocyte haemolysate:** At the end of the treatment, the animals were fasted for 12 h<sup>[15]</sup> and subsequently sacrificed according to United States National Institutes of Health approved protocols (NIH, 1978). A blood volume of 4.0 mL was obtained by carotid artery puncture using hypodermic syringe. The erythrocytes were separated from plasma by bench centrifugation for 10 min. The harvested erythrocytes were washed by previously described methods.<sup>[25, 26]</sup> Within 2 h of collection of the blood specimen, 1.0 mL of harvested erythrocyte was introduced into centrifuge test tubes containing 3.0 mL of buffer solution pH = 7.4: 250 mM tris (hydroxyl methyl) amino ethane-HCl (Tris-HCl)/140 mM NaCl/1.0 mM MgCl<sub>2</sub>/10 mM glucose). The erythrocytes suspension was further centrifuged at 1200 *g* for 10 min and repeated 3 times to remove platelets and leucocytes.<sup>[27]</sup> The pellet was re-suspended in 3.0 mL of phosphate-buffered saline (PBS) solution (pH = 7.4) and passed through a column (3.5 cm in a 30 mL syringe) of cellulose-microcrystalline cellulose (ratio *w/w* 1:1).<sup>[28]</sup> The eluted fraction was passed twice through a new column of cellulose-microcrystalline cellulose (ratio 1:1 *w/w*) to obtain an erythrocyte suspension sufficiently devoid of leucocytes and platelets. Finally, erythrocytes were re-suspended in 1.0 mL of this buffer and stored at 4 °C. The washed erythrocytes were lysed by freezing/thawing as described previously.<sup>[29, 30]</sup> The erythrocyte haemolysate was used for the determination of erythrocyte glutathione S-transferase (GST) and NADH-Methaemoglobin reductase (NADH-MR) activity.

**Erythrocyte haemolysate haemoglobin concentration:** A modified cyanomethaemoglobin reaction<sup>[26, 31]</sup> was used for the measurement of haemolysate haemoglobin concentration. A 0.05 mL portion of erythrocyte haemolysate was added to 4.95 mL of Drabkins reagent (100 mg NaCN and 300 mg K<sub>4</sub>Fe(CN)<sub>6</sub> per liter). The mixture was left to stand for 10 min at 25±5°C and the absorbance was read at  $\lambda_{\max} = 540$  nm against a blank. The absorbance was used to evaluate for haemolysate haemoglobin concentration by comparing the values with the standard.

**Erythrocyte glutathione S-transferase:** GST activity was measured by previously described methods<sup>[3, 32, 3]</sup> with minor modifications.<sup>[26]</sup> The reaction mixture contained 1.0 mL of 0.3 mM phosphate buffer (pH = 6.5), 0.1 mL of 30 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 1.7 mL of distilled water. After pre-incubating the reaction mixture at 37 °C for 5 min, the reaction was started by the addition of 0.1 mL of erythrocyte haemolysate and 0.1 mL of glutathione (GSH) as substrate. The absorbance was followed for 5 min at  $\lambda_{\max} = 340$  nm. The enzyme activity was expressed as erythrocyte GST activity in international unit per gram haemoglobin (IU/gHb) using an extinction coefficient ( $\Sigma$ ) of 9.6 mM<sup>-1</sup> cm<sup>-1</sup> in reaction in which 1 mole of GSH is oxidized (Equation 1).

**Erythrocyte NADH-methaemoglobin reductase:** NADH-MR activity was determined according to the method of Board.<sup>[33]</sup> Briefly, a mixture of 0.2 mL tris-HCl/EDTA buffer pH = 8.0, 0.2 mL NADH and 4.35 mL of distilled water was introduced into a test tube and incubated for 10 min at 30 °C. The content was transferred into a cuvette and the reaction started by adding 0.2 mL of K<sub>3</sub>Fe(CN)<sub>6</sub>/0.05 mL erythrocyte haemolysate. The increase in absorbance of the medium was measured at  $\lambda_{\max} = 340$  nm per min for 10 min at 30 °C against a blank solution. NADH-MR activity was expressed in international units per gram haemoglobin (IU/gHb) using an extinction coefficient ( $\Sigma$ ) of 6.22 mM<sup>-1</sup> cm<sup>-1</sup> in reaction in which 1 mole of NADH + H<sup>+</sup> is oxidized (Equation 1).

#### Calculation of GST and NADH-MR activities:

$$E_A = \frac{100}{[\text{Hb}]} \times \frac{\text{OD}}{\Sigma} \times \frac{V_C}{V_H} \quad (\text{Equation 1})$$

Where,

$E_A$  = Enzyme activity in IU/gHb

[Hb] = Haemolysate haemoglobin concentration (g/dL)

0. D/min = Change per min in absorbance at 340 nm.

$V_C$  = Cuvette volume (total assay volume) = 1.0 mL.

$V_H$  = Volume of haemolysate in the reaction system (0.05 mL).

**Erythrocyte ghost membrane preparation:** A modified standard procedure was used for erythrocyte ghost membrane preparation.<sup>[34, 35]</sup> Briefly, 10 mL of ice cold 5 mM Tris/0.1 mM Na<sub>2</sub>EDTA (pH = 7.6) were added to test

tubes containing buffy coat free-packed erythrocytes of test and control rats to achieve osmotic lysis. The resulting membranes were centrifuged at 20,000 *g* for 20 min at 4 °C. The membrane suspensions were washed 3 times in 0.017 M NaCl/5 mM Tris-HCl, pH = 7.6 and 3 times with 10 mM Tris-HCl (pH = 7.5). The haemoglobin-free membrane suspension was finally stored at -20 °C in 10 mM Tris-HCl buffer (pH = 7.5).

**Erythrocyte Na<sup>+</sup>/K<sup>+</sup>-ATPase:** The erythrocyte total ATPase activity was determined by incubating 50 µL of ghost membrane suspension (~200 µg of membrane protein) of test and control rats with 5 mM Tris-ATP, 25 mM KCl, 75 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 25 mM Tris-HCl (pH = 7.5) in 500 µL for 90 min at 37 °C in a shaking water bath. The reaction was stopped by adding tricarboxylic acid (TCA) to a final concentration of 5% (*w/v*). After centrifugation for 20 min at 1,500 *g*, an aliquot of the supernatant was used to measure total inorganic phosphate liberated.<sup>[36]</sup> This assay was repeated in the presence of 200 µM methyl digoxin, an inhibitor of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity. Total ATPase activity was expressed as micromole of inorganic phosphate liberated per milligram membrane protein per hour (µM pi/mg protein/h). The activity of Na<sup>+</sup>/K<sup>+</sup>-ATPase was subsequently determined by subtracting total ATPase activity in the presence of digoxin from enzyme activity in the absence of the inhibitor drug.

**Ghost erythrocyte membrane protein:** Membrane protein was measured by standard methods<sup>[37]</sup> after solubilizing aliquots of ghost membrane suspension with 0.2% sodium dodecyl sulfate (SDS). Bovine serum albumin (BSA) (50-300 µg), (Sigma Chemical Company, Saint Louis, Missouri, USA), was used as standard. Absorbance was measured with a Beckmann D700 spectrophotometer (Beckmann, USA) at λ<sub>max</sub> = 720 nm.

**Statistical analyses:** The data collected were analyzed by the analysis of variance procedure while treatment means were separated by the least significance difference (LSD) incorporated in the statistical analysis system (SAS) package of 9.1 version (2006). The correlation coefficients between the results were determined with Microsoft Office Excel, 2010 version.

## RESULTS

The serum FBS levels of C/N rats ranged between 72.93 ± 0.82 and 95.12 ± 0.92 mg/dL, whereas the experimental rats without glycemic control (C/H) was between 249.41

**Table 1: Serum fasting blood sugar levels of hyperglycemic rats with/without glycemic control.**

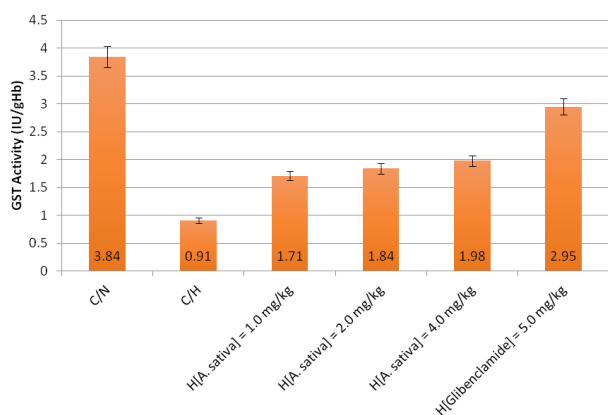
Group	[FBS] mg/dL	
	t = 0 h	t = 76 h
C/N	95.12±0.92 <sup>a</sup>	72.93±0.82 <sup>a</sup>
C/H	256.11±1.23 <sup>b</sup>	249.41±1.03 <sup>b</sup>
H <sub>[A. sativa] = 1.0 mg/kg</sub>	255.64±1.09 <sup>b,c</sup>	125.11±0.91 <sup>c</sup>
H <sub>[A. sativa] = 2.0 mg/kg</sub>	261.13±2.00 <sup>b,c,d</sup>	129.32±1.50 <sup>c,d</sup>
H <sub>[A. sativa] = 4.0 mg/kg</sub>	267.94±0.92 <sup>c,d,e</sup>	132.61±0.81 <sup>d,e</sup>
H <sub>[Glibenclamide] = 5.0 mg/kg</sub>	265.49±49 <sup>c,d,e,f</sup>	101.12±0.80 <sup>f</sup>

Means in the column with the same letters are not significantly different at *p* > 0.05 according to LSD.

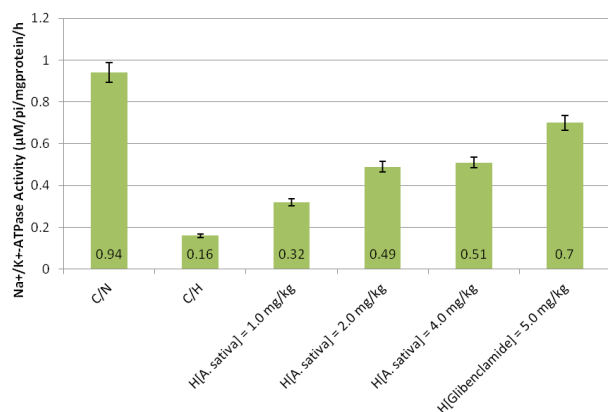
± 1.03 and 256.11 ± 1.23 mg/dL (Table 1). These values represented a decrease in serum FBS levels by 22.19 mg/dL and 6.7 mg/dL in C/N and C/H rats respectively within the experimental time of 76 h. At experimental time *t* = 0 h and *t* = 76 h, serum FBS levels of C/H was significantly (*p* < 0.05) higher than in C/N rats. It is apparent from Table 1 showed that hyperglycemic rats treated with ethanol/water (1:2 *v/v*) extract of *A. sativa* exhibited comparative reduced serum levels of FBS in a dose dependent manner. The serum FBS levels of hyperglycemic rats at *t* = 0 h were within the range of 255.64 ± 1.09 to 267.94 ± 0.92 mg/dL.

These values represent marginal variations in serum FBS levels amongst the three categories of *A. sativa* treated hyperglycemic rats (Group T1, Group T2 and Group T3) within the experimental time: 0 h ≤ *t* ≤ 76 h. Specifically, at *t* = 76 h, serum FBS<sub>[A. sativa] = 1.0 mg/kg</sub> = 125±0.91 mg/dL; FBS<sub>[A. sativa] = 2.0 mg/kg</sub> = 129.32±1.50 mg/dL and FBS<sub>[A. sativa] = 4.0 mg/kg</sub> = 132.61±0.81 mg/dL.; *p* < 0.05 compared to C/N rats. Furthermore, *t* = 76 h, the three groups of *A. sativa* treated hyperglycemic rats exhibited: H<sub>[A. sativa] = 1.0 mg/kg</sub> = 51.06%, H<sub>[A. sativa] = 2.0 mg/kg</sub> = 50.48% and H<sub>[A. sativa] = 4.0 mg/kg</sub> = 50.41% reduction in serum FBS levels compared to their corresponding FBS levels at *t* = 0 h. Similarly, compared to serum FBS levels at *t* = 0 h, H<sub>[Glibenclamide] = 5.0 mg/kg</sub> rats showed reduced serum FBS level by 61.91% at *t* = 76 h, representing a ratio of 1: 1.4 decrease in serum FBS levels compared to C/N rats; *p* < 0.05. At the end of the experiment, serum FBS levels of H<sub>[A. sativa] = 1.0 mg/kg</sub> was not significantly different (*p* > 0.05) from H<sub>[A. sativa] = 2.0 mg/kg</sub> rats. Likewise, FBS levels of H<sub>[A. sativa] = 2.0 mg/kg</sub> showed no significantly difference (*p* > 0.05) compared to H<sub>[A. sativa] = 4.0 mg/kg</sub> rats.

Within the experimental time, hyperglycemic rats with or without glycemic control exhibited decreased levels of erythrocyte GST activity. Specifically, erythrocyte GST



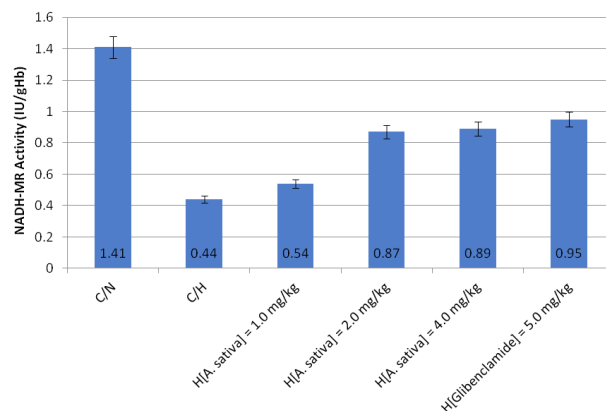
**Figure 1.** Erythrocyte glutathione S-transferase activity of hyperglycemic rats with/without glycemic control.



**Figure 3.** Erythrocyte Na<sup>+</sup>/K<sup>+</sup>-ATPase activity of hyperglycemic rats with/without glycemic control.

activity of C/H rats represented 23.69% of GST activity of C/N rats ( $p < 0.05$ ). Figure 1 showed a corresponding increase in erythrocyte GST activity of hyperglycemic rats treated with ethanol/water (1:2 *v/v*) extract of *A. sativa* in a dose dependent manner ( $H_{[A. sativa] = 1.0-4.0 \text{ mg/kg}}$ ) with no comparative significant difference ( $p > 0.05$ ). Furthermore, within the experimental time, the decreased levels of erythrocyte GST activity of rats treated with ethanol/water (1:2 *v/v*) extract of *A. sativa* were significantly different ( $p < 0.05$ ) from that of C/N rats. Also, erythrocyte of  $H_{[Glibenclamide] = 5.0 \text{ mg/kg}}$  rats showed 76.82% GST activity compared to the C/N rats ( $p < 0.05$ ).

Figure 2 showed that erythrocyte NADH-MR activity of rats without glycemic control (C/H rats) was not significantly different ( $p > 0.05$ ) from those with glycemic control ( $H_{[A. sativa] = 1.0-4.0 \text{ mg/kg}}$  and  $H_{[Glibenclamide] = 5.0 \text{ mg/kg}}$  rats). Similarly, erythrocyte NADH-MR activity of  $H_{[A. sativa] = 1.0-4.0 \text{ mg/kg}}$  rats was not significantly different ( $p > 0.05$ )



**Figure 2.** Erythrocyte NADH-Methaemoglobin reductase activity of hyperglycemic rats with/without glycemic control.

from  $H_{[Glibenclamide] = 5.0 \text{ mg/kg}}$  rats. Specifically, erythrocyte NADH-MR activity of  $H_{[Glibenclamide] = 5.0 \text{ mg/kg}}$  rats was 67.38 % compared to erythrocyte NADH-MR activity of C/N rats, whereas  $H_{[A. sativa] = 1.0-4.0 \text{ mg/kg}}$  rats NADH-MR activity showed relative enzyme activity between the range of 49.65–63.12 %. Erythrocyte NADH-MR activity of C/H rats was significantly ( $p < 0.05$ ) lower than C/N rats, representing 68.97 % reduction of NADH-MR activity in C/H rats.

Erythrocyte Na<sup>+</sup>/K<sup>+</sup>-ATPase activity of C/H rats was reduced by 82.98 % compared to C/N rats ( $p < 0.05$ ) (Figure 3). At experimental  $t = 76 \text{ h}$ , erythrocyte Na<sup>+</sup>/K<sup>+</sup>-ATPase activity  $H_{[A. sativa] = 1.0-4.0 \text{ mg/kg}}$  rats showed progressive increase in relative enzyme activity:  $H_{[A. sativa] = 1.0 \text{ mg/kg}} = 34.04\%$ ,  $H_{[A. sativa] = 2.0 \text{ mg/kg}} = 52.13\%$  and  $H_{[A. sativa] = 4.0 \text{ mg/kg}} = 54.26\%$ .  $H_{[Glibenclamide] = 5.0 \text{ mg/kg}}$  rats was 74.47 %. An overview of Figures 1, 2 and 3 shows that the average relative activities of the three enzymes and corresponding order of enzyme activity in hyperglycemic rats treated with ethanol/water (1:2 *v/v*) extract of *A. sativa* was: NADH-MR = 60.99 % > GST = 47.81% > Na<sup>+</sup>/K<sup>+</sup>-ATPase = 46.81 %. In the same order, relative activities of the three enzymes in rats without glycemic control were: NADH-MR = 49.65 % > GST = 23.69 % > Na<sup>+</sup>/K<sup>+</sup>-ATPase = 17.02 %. Furthermore, percentage decreases in GST, NADH-MR and Na<sup>+</sup>/K<sup>+</sup>-ATPase activities in  $H_{[A. sativa] = 1.0-4.0 \text{ mg/kg}}$  rats was related to the capacity of ethanol/water (1:2 *v/v*) extract of *A. sativa* to exert dose dependent glycemic control.

## DISCUSSION

The use of experimental animal models for study of type 1 diabetes mellitus has been widely reported.<sup>[38–41]</sup> The

cytotoxic action of diabetogenic agents is mediated by the formation of superoxide radicals and other related reactive oxygen species (ROS), causing massive destruction of the  $\beta$ -cells.<sup>[39,42,43]</sup> From the present study, experimental rats treated with the widely used diabetogenic agent—alloxan, in conformity with previous reports elsewhere,<sup>[24,39,42,43]</sup> showed evidence of hyperglycemia (Table 1). Hyperglycemia is the earliest and primary clinical presentation in diabetic states.<sup>[3,44]</sup> Studies on the application of nutraceuticals, sourced from spices and other edible plants and their products, for the treatment and management of diabetes mellitus have received attention of several research endeavours.<sup>[45]</sup> The present study showed evidence of the capacity of ethanol/water extract of *A. sativa* to reduce serum level of FBS in hyperglycemic rats, which compared reasonably with the standard anti-diabetic drug—glibenclamide (Table 1). The anti-diabetic properties of *A. sativa* extract have been previously reported.<sup>[15,24]</sup> The therapeutic action of *A. sativa* as it applies to its role in the treatment and management of diabetes mellitus is identical to the mode of action of other numerous anti-diabetic agents of plant origin such as *Coriandrum sativum*,<sup>[45]</sup> *Gongronema latifolium*,<sup>[46]</sup> *Allium cepa* Linn.<sup>[15]</sup> However, another mechanism of therapeutic action, which involves increased peripheral glucose consumption induced by *Eugenia Floccosa*,<sup>[47]</sup> *Berberis lycium*<sup>[48]</sup> and *Tinospora cordifolia* roots<sup>[49]</sup> has also been documented. The active principles of these plant extracts exhibited insulin-like effects by mimicry. However, within the experimental time, administration of the three experimental doses of ethanol/water (1:2 v/v) extract of *A. sativa* as an instrument of glycemic control did not restore normal serum level of FBS (72.93±0.82–95.12±0.92 mg/dL) in hyperglycemic rats with [FBS] > 250 mg/dL.

According to Raza *et al.*,<sup>[5]</sup> oxidative stress is an important factor in the etiology and pathogenesis of diabetes mellitus. Furthermore, Pasupathi *et al.*,<sup>[3]</sup> had observed a significant ( $p < 0.001$ ) decrease in reduced glutathione (GSH) concentration in diabetic erythrocytes compared to control participants. They further averred that a decreased level of GSH was an after effect of increased utilization of the coenzyme for scavenging ROS due to elevated oxidative stress associated with diabetes. Consequently, we observed decreased levels of erythrocyte GST activity in hyperglycemic rats, which was in conformity with previous studies,<sup>[3,44,45,50–52]</sup> since the co-substrate (GSH) required for GST antioxidant protective activity<sup>[1,53]</sup> may have been utilized for other non-enzymatic reductive pathways. Judging from the erythrocyte GST activity of rats without glycemic control, the higher relative levels of erythrocyte GST activity of hyperglycemic rats treated

with *A. sativa* extract in dose dependent pattern (Figure 1) was an obvious indication of the capability of the ethanolic plant extract to serve as an anti-diabetic agent comparable to the standard anti-diabetic drug glibenclamide. Erythrocyte GST activity has been proven to be a reliable biochemical index and the basis for diagnosis and monitoring of therapeutic events in the course of treatment and management of other pathologic/metabolic disorders whose etiologies and manifestations are linked to oxidative stress. Notable among which are: parasitic infections,<sup>[26,54]</sup> gout and rheumatoid arthritis,<sup>[55,56]</sup> haemoglobinopathies,<sup>[26]</sup> malignancy,<sup>[57]</sup> hypertension,<sup>[58]</sup> stroke<sup>[59]</sup> and atherosclerosis.<sup>[60]</sup> In a related perspective, the use of GT activity as a reliable biomarker in depicting the etiology of diabetes mellitus has been described.<sup>[2]</sup> It was posited that two isoforms of GST (GSTM1 and GSTT1) might be involved in the pathogenesis of Type 2 diabetes mellitus in a South Iranian population. In addition, investigations have shown that the GSTM1 gene may play a significant role in the aetiopathogenesis of diabetes mellitus and could serve as a useful biomarker in the prediction of diabetes mellitus susceptibility of the Turkish population.<sup>[7]</sup>

Poor glycemic control in diabetes and combination of oxidative, metabolic, and carbonyl stresses has been shown to cause restriction in supply but excessive demand for reducing equivalents.<sup>[61]</sup> Therefore, repressed NADH-MR activity in hyperglycemic rats could be linked to the substantial diversion and utilization of reducing equivalents to other reductive pathways in efforts to minimize oxidative stress, prompted by erythrocyte high ROS content. Thus, the decreased level of erythrocyte NADH-MR activity of hyperglycemic rats (Figure 2) is a reflection of a compromised erythrocyte antioxidant status associated with hyperglycemia.<sup>[61,62]</sup> Furthermore, in concordance with the present reports, Zerez *et al.*,<sup>[63]</sup> have stated that conditions that engender decreased erythrocyte NADH content resulted to decreased rate of methaemoglobin reduction in connection to impaired NADH-MR activity. This condition is responsible, in part, for relatively high methaemoglobin content in sickle erythrocytes and susceptibility to oxidative damage.<sup>[27]</sup> Based on the present observations, it is presumed that adjustments in diabetic erythrocyte methaemoglobin levels might provide early indication of diabetic antioxidant and oxidative stress status.

Studies suggest that insulin plays a stimulatory role in  $\text{Na}^+/\text{K}^+$ -ATPase activity through tyrosine phosphorylation process.<sup>[11]</sup> The relatively reduced levels of erythrocyte  $\text{Na}^+/\text{K}^+$ -ATPase activity in hyperglycemic rats

(Figure 3) was consistent with the findings of previous authors. Soulis-Liparota *et al.*,<sup>[64]</sup> reported reduced Na<sup>+</sup>/K<sup>+</sup>-ATPase activity streptozotocin-induced diabetic rats with nephropathy, whereas others<sup>[65,66]</sup> reported impairment in the enzyme activity in diabetic rats and mice with retinopathy. In a different study using human participants, it was noted that there was compromised erythrocyte Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in Type 1 diabetic patients from Lagos, Nigeria.<sup>[35]</sup> This finding was corroborated by another study in which a reduction of erythrocyte Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in Type 2 diabetic patients with hyperkalemia was noted.<sup>[67]</sup> It has been suggested that diabetes-induced Na<sup>+</sup>/K<sup>+</sup>-ATPase activity dysfunction could be implicated in the pathogenesis of human diabetic neuropathy and the electrophysiological abnormalities.<sup>[68]</sup>

The findings reported here were in concordance with previous studies that noted that hypercholesterolemia and free radical-induced mechanisms may be responsible for the inhibition of erythrocyte Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in patients with Type 2 diabetes mellitus.<sup>[69]</sup> According to the present study, decreased erythrocyte Na<sup>+</sup>/K<sup>+</sup>-ATPase activity of hyperglycemic rats was analogous to altered enzyme activity in peripheral neurons of individuals with diabetic neuropathy. Impaired Na<sup>+</sup>/K<sup>+</sup>-ATPase activity is induced by hyperglycemia with characteristic distortions in myo-inositol and phosphoinositol metabolism,<sup>[70]</sup> which normalizes with intensive insulin therapy that controls hyperglycemia.<sup>[71]</sup> Thus, decreased erythrocyte Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was an obvious confirmation of a connection between the capacity of erythrocyte to actively transport Na<sup>+</sup>/K<sup>+</sup> ions (antiport) and obligatory utilization of ATP for  $\alpha$ -subunit of Na<sup>+</sup>/K<sup>+</sup>-ATPase phosphorylation required for enzyme activity.<sup>[11,13,72]</sup> Hyperglycemia with associated depressed glucose utilization in diabetic states results in low intracellular ATP concentration, insufficient for the required obligatory phosphorylation of the enzyme. The dose dependent increase in erythrocyte Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in hyperglycemic rat treated with extract of *A. sativa* as an instrument of glycemic control was an indication of improved glucose utilization exemplified in hyperglycemic rats treated with the standard anti-diabetic drug. The role and mechanism of insulin in regulation of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity has been described elsewhere.<sup>[73]</sup> In another study, it was reported that hypercholesterolemia and free radical-induced mechanisms may be responsible for the inhibition of erythrocyte Na<sup>+</sup>/K<sup>+</sup>-ATPase activity patients with type 2 diabetes mellitus.<sup>[69]</sup>

The present study showed that erythrocyte GST, NADH-MR and Na<sup>+</sup>/K<sup>+</sup>-ATPase activities gave insights into the

pathophysiology of diabetic state and could serve as a biomarker for ascertaining therapeutic control in Type 1 diabetes mellitus.

## REFERENCES

1. Nowier SR, Kashmiry NK, Rasool HAA, Morad H, Ismail S. Association of Type 2 diabetes mellitus and glutathione S-transferase (GSTM1 and GSTT1) genetic polymorphism. *Res J Med Medical Sci.* 2009; 4(2):181–188.
2. Moasser E, Kazemi-Nezhad SR, Saadat M, Azarpira N. Study of the association between glutathione S-transferase (GSTM1, GSTT1, GSTP1) polymorphisms with type II diabetes mellitus in southern of Iran. *Mole Biol Reports.* 2012; 39(12):10187–10192.
3. Pasupathi P, Chandrasekar V, Kumar US. Evaluation of oxidative stress, antioxidant and thyroid hormone status in patients with diabetes mellitus. *J Med.* 2009; 10:60–66.
4. Velladath SU, Das A, Kumar RKN. Erythrocyte glutathione S-transferase activity in diabetics and its association with HbA<sub>1c</sub>. *Webmed Central Clin Biochem.* 2011; 2(7):WMC002004.
5. Raza H, Prabu SK, Robin MA, Avadhani NG. Elevated mitochondrial cytochrome P450 2E1 and glutathione S-transferase A4-4 in streptozotocin-induced diabetic rats: Tissue-specific variations and roles in oxidative stress. *Diabetes.* 2004; 53:185–194.
6. Grodsky GM. Chemistry and functions of hormones: III. Pancreas and gastrointestinal tract. In: *Harper's Review of Biochemistry.* Lange Medical Publications. Los Altos, California. 1983; 511–522.
7. Yalin S, Hatungil R, Tamer L, Ates NA, Dogruer N *et al.*, Glutathione S-transferase gene polymorphisms in Turkish patients with diabetes mellitus. *Cell Biochem Function.* 2007; 25(5):509–513.
8. Bid HK, Konwar R, Saxena M, Chaudhari P, Agrawal CG, Banerjee M. Association of glutathione S-transferase (GSTM1, T1 and P1) gene polymorphisms with type 2 diabetes mellitus in north Indian population. *J Postgraduate Med.* 2010; 56:176–81.
9. Yubisui T, Takeshita M. Reduction of methaemoglobin through flavin at the physiological concentration by NADPH-flavin reductase of human erythrocytes. *J Biochem.* 1980; 87(6):1715–1720.
10. Rockwood GA, Armstrong KR, Baskin SI. Species Comparison of Methaemoglobin Reductase. *Experi Biol Med.* 2003; 228:79–83.
11. Fe'raille E, Carranza ML, Gonin S, Be'guin P, Pedemonte C *et al.*, Insulin-induced stimulation of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in kidney proximal tubule cells depends on phosphorylation of the  $\alpha$ -subunit at Tyr-10. *Molecular Biol Cell.* 1999; 10:2847–2859.
12. Kaplan JH. Biochemistry of Na<sup>+</sup>/K<sup>+</sup>-ATPase. *Annual Rev Biochem.* 2002; 71:511–535.
13. Carranza, M.L., Fe'raille, E., and Favre, H. Protein kinase C-dependent phosphorylation of the Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ -subunit in rat kidney cortical tubules. *Am J Physiol.* 1996; 271:C136–C143.
14. Banerjee SK, Maulik SK. Effect of garlic on cardiovascular disorders: a review. *Nutri J.* 2002; 1:4.
15. El-Demerdash, F. M., Yousef, M. I., Abou El-Naga, N. I. Biochemical study on the hypoglycemic effects of onion and garlic in alloxan-induced diabetic rats. *Food Chem Toxicol.* 2005; 43:57–63.
16. Chauhan A, Sharma PK, Srivastava P, Kumar N, Duehe R. Plants having potential antidiabetic activity: a review. *Der Pharm Lett* 2010; 2(3):369–387.
17. Ayodhya S, Kusum S, Anjali S. Hypoglycemic activity of different extracts of various herbal plants Singh. *Int J Res Ayurveda Pharm.* 2010; 1(1):212–224.
18. Patel DK, Prasad SK, Kumar R, Hemalatha S. An overview on antidiabetic medicinal plants having insulin mimetic property. *Asian Pac J Trop Biomed.* 2012; 320–330.
19. Lau BHS, Adetumbia MA, Sanchez A. *Allium sativum* (garlic) and atherosclerosis: A review. *Nutri Res.* 1983; 3(1): [http://dx.doi.org/10.1016/S0271-5317\(83\)80128](http://dx.doi.org/10.1016/S0271-5317(83)80128).
20. Choudhary R. Beneficial effect of *Allium sativum* and *Allium tuberosum* on experimental hyperlipidemia and atherosclerosis. *Pak J Physiol.* 2008; 4(2):7–9.
21. Mahmoodi M, Islami MR, Karam AGR, Khaksari M, Sahebghadam LA, Hajizadeh MR, Mirzaee MR. Study of the effects of raw garlic consumption

- on the level of lipids and other blood biochemical factors in hyperlipidemic individuals. Pak J Pharmacol Sci. 2006; 19:295–298.
22. Fukao H, Yoshida H, Tazawa YI, Hada T. Antithrombotic effects of odorless garlic powder both in vitro and in vivo. Biosci Biotechnol Biochem. 2007; 71:84–90.
  23. Benavides GA, Squadrito GL, Mills RW, Patel HD, Isbell TS, Patel RP, Darley-Usmar VM, Doeller JE, Kraus DW. Hydrogen sulfide mediates the vasoactivity of garlic. PNAS. 2007; 104:17977–17982.
  24. Ibegbulem CO, Chikezie PC. Hypoglycemic properties of ethanolic extracts of *Gongronema latifolium*, *Aloe perryi*, *Viscum album* and *Allium sativum* administered to alloxan-induced diabetic albino rats (*Rattus norvegicus*). Pharmacog Commun. 2012; 3(2):12–16.
  25. Tsakiris S, Giannoulia-Karantana A, Simintzi I, Schulpis KH. The effect of aspartame metabolites on human erythrocyte membrane acetylcholinesterase activity. Pharmacol Res. 2005; 53:1–5.
  26. Chikezie, PC, Uwakwe AA, Monago CC. Glutathione S-transferase activity of three erythrocyte genotypes (HbAA, HbAS and HbSS) of male subjects/volunteers administered with Fansidar and Quinine. Afri J Biochem Res. 2009; 3(5):210–214.
  27. Chikezie PC. Methaemoglobin concentration and NADH-methaemoglobin reductase activity of three human erythrocyte genotypes. Asian J Biochem. 2011; 6(1):98–103.
  28. Kalra VK, Sikka SC, Sethi GS. Transport of amino acids in gamma-glutamyl transpeptidase-implanted human erythrocytes. J Biol Chem. 1981; 256:5567.
  29. Galbraith DA, Watts DC. Changes in some cytoplasmic enzymes from red cells fractionated into age groups by centrifugation in Ficoll™/Triosil™ gradients: Comparison of normal human and patients with Duchenne muscular dystrophy. Biochem J. 1980; 191:63–70.
  30. Kamber K, Poyiagi A, Delikonstantinos G. Modifications in the activities of membrane-bound enzymes during *in vivo* ageing of human and rabbit erythrocytes. Comp Biochem Physiol. 1984; B.77B:95–99.
  31. Baure JD. Laboratory investigation of hemoglobin. In: *Gradwohl's Clinical Laboratory Methods and Diagnosis*, (Eds) Sonnenwirth AC, Jarett L. St. Louis, MO: Mosby. 1980.
  32. Habig WH, Pabst MJ, William BJ. Glutathione S-transferases; the first enzymatic step in mercapturic acid formation. J Biol Chem. 1974; 249(6):130–137.
  33. Board P, Coggan M, Johnston P, Ross V, Suzuki T, Webb G. Genetic heterogeneity of the human glutathione transferases; a complex of gene families. Pharmacol Therap. 1990; 48:357–69.
  34. DeLuise M, Flier JS. Functionally abnormal Na<sup>+</sup>/K<sup>+</sup>-ATPase pump in erythrocytes of a morbidly obese patient. J Clin Invest. 1982; 69:38–44.
  35. Iwalokun BA, Iwalokun SO. Association between erythrocyte Na<sup>+</sup>/K<sup>+</sup>-ATPase activity and some blood lipids in type 1 diabetic patients from Lagos, Nigeria. BMC Endocrine Disorders. 2007; 7:7
  36. Fiske CH, Subbarow Y. The colorimetric determination of phosphorous. J Biol Chem. 1925; 66:375–400.
  37. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193:265–275.
  38. El-Missiry MA, El Gindy AM. Amelioration of alloxan induced diabetes mellitus and oxidative stress in rats by oil of *Eruca sativa* seeds. Annals Nutri Metabolism. 2000; 44:97–100.
  39. Szkudelski T. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res. 2001; 50(6):537–546.
  40. Gwarzo MY, Nwachuku VA, Lateef AO. Prevention of alloxan induced diabetes mellitus in rats by vitamin a dietary supplementation. Asian J Animal Sci. 2010; 4:190–196.
  41. Shahabuddin ME, Pouramir M, Moghadamnia AA, Lakzaei M, Mirhashemi SM, Motalebi M. Antihyperglycemic and antioxidant activity of *Viscum album* extract. Afri J Pharm Pharmacol. 2011; 5(3):432–436.
  42. Lankin VZ, Korchin VI, Konovalova GG, Lisina MO, Tikhaze AK, Akmaev IG. Role of antioxidant enzymes and antioxidant compound probucol in antiradical protection of pancreatic beta-cells during alloxan-induced diabetes. Bull Experi Biol Med. 2004; 137:20–23.
  43. Sharma US, Kumar A. Anti-diabetic effect of *Rubus ellipticus* fruits extracts in alloxan-induced diabetic rats. J Diabetol. 2011; 2:4.
  44. Choudhuri S, Dutta D, Chowdhury IH, Mitra B, Sen A, Mandal LK, Mukhopadhyay S, Bhattacharya B. Association of hyperglycemia mediated increased advanced glycation and erythrocyte antioxidant enzyme activity in different stages of diabetic retinopathy. Diabetes Res Clin Practice. 2013; 100(3):376–384.
  45. Rajeshwari CU, Andallu B. Oxidative stress in NIDDM patients: influence of coriander (*Coriandrum sativum*) seeds. Res J Pharmaceut, Biol Chem Sci. 2011; 2(1):31–41.
  46. Ugochukwu NH, Babady NE. Antihyperglycaemic effect aqueous and ethanolic extracts of *Gongronema latifolium* leaves on glucose and glycogen metabolism in livers of normal and streptozotocin induced diabetic rats. Life Sci. 2003; 73(15):1925–1938.
  47. Kala SMJ, Tresina PS, Mohan VR. Antioxidant, anti-hyperlipidemic and antidiabetic activity of *Eugenia floccosa* Bedd leaves in alloxan induced diabetic rats. Journal of Basic and Clinical Pharmacy. 2012; 3(001):235–240.
  48. Gulfranz M, Qadir G, Nosheen F, Parveen Z. Antihyperglycemic effects of *Berberis lycium* Royle in alloxan induced diabetic rats. Diabetologia Croatica 2007; 36–3:49–54.
  49. Stanely P, Prince M, Menon VP. Hypoglycaemic and other related actions of *Tinospora cordifolia* roots in alloxan-induced diabetic rats. J Ethnopharmacol. 2000; 70(1):9–15.
  50. Mrobie DJ, Glover DD, Tracy TS. Effects of gestational and overt diabetes on human placental cytochromes P450 and glutathione S-transferase. Drug Metabolism and Disposition. 1997; 26(4):367–371.
  51. Rathore N, Kale M, John S, Bhatnagar D. Lipid peroxidation and antioxidant enzymes in isoproterenol induced oxidative stress in rat erythrocytes. Indian J Physiol Pharmacol. 2000; 44:161–166.
  52. Surapanenin KM. Oxidant-antioxidant status in gestational diabetes patients. J Clin Diagnostic Res. 2007; 1(4):235–238.
  53. Bekris LM, Shephard C, Peterson M, Hoehna J, Van Yserloo B, Rutledge E, Farin F, Kavanagh TJ, Lernmark A. Glutathione S-transferase M1 and T1 polymorphisms and associations with Type 1 diabetes age-at-onset. Autoimmunity. 2005; 38(8):567–575.
  54. Sohail M, Kaul A, Raziuddin M, Adak T. Decreased glutathione S-transferase activity: Diagnostic and protective role in vivax malaria. Clin Biochem. 2007; 40(5-6):377–382.
  55. Hassan MQ, Hadi RA, Al-Rawi ZS, Padron VA, Stohs SJ. The glutathione defense system in the pathogenesis of rheumatoid arthritis. J Appl Toxicol. 2001; 21:69–73.
  56. Bohanec GP, Logar D, Tomsic M, Rozman B, Dolzan V. Genetic polymorphisms of glutathione S-transferases and disease activity of rheumatoid arthritis. Clin Experi Rheumatol. 2009; 27(2):229–236.
  57. Zafereo ME, Sturgis EM, Aleem S, Chaung K *et al.*, Glutathione S-transferase polymorphisms and risk of second primary malignancy after index squamous cell carcinoma of the head and neck. Cancer Prevention Res (Phila). 2009; 2(5):432–439.
  58. Lee BK, Lee SJ, Joo SJ, Cho KS, Kim NS, Kim HJ. Association of glutathione S-transferase genes (GSTM1 and GSTT1) polymorphisms with hypertension in lead-exposed workers. Molecular Cell Toxicol. 2012; 8:203–208.
  59. Turck N, Robin X, Walter N, Fouda C, Hainard A *et al.*, Blood Glutathione S-transferase-p as a time indicator of stroke onset. Plos One. 2012; 7(9):e43830.
  60. Yang Y, Yang Y, Xua Y, Lick SD, Awasthi YC *et al.*, Endothelial glutathione-S-transferase A4-4 protects against oxidative stress and modulates iNOS expression through NF- $\kappa$ B translocation. Toxicol Appl Pharmacol. 2008; 230(2):187–196.
  61. Coleman MD. Use of in vitro methaemoglobin generation to study antioxidant status in the diabetic erythrocyte. Biochemical Pharmacology. 2000; 60(10):1409–1416.
  62. Memişoğulları R, Türkeli M, Bakan E, Akçay F. Effect of Metformin or Gliclazide on lipid peroxidation and antioxidant levels in patients with diabetes mellitus. Turk J Med Sci. 2008; 38(6):545–548.
  63. Zerez CR, Lachant NA, Tanaka KR. Impaired erythrocyte methaemoglobin reduction in sickle cell disease: Dependence of methaemoglobin reduction on reduced nicotinamide adenine dinucleotide content. Blood. 1990; 76:1008–1014.
  64. Soulis-Liparota T, Cooper ME, Dunlop M, Jerums G. The relative roles of advanced glycation, oxidation and aldose reductase inhibition in the development of experimental diabetic nephropathy in the Sprague-Dawley rat. Diabetologia 1995; 38:1492–1493.
  65. Di Leo MA, Santini SA, Cercone S, Lepore D, Gentiloni Silveri N, Caputo S, Greco AV, Giardina B, Franconi F, Ghirlanda G. Chronic taurine supplementation ameliorates oxidative stress and Na<sup>+</sup>/K<sup>+</sup>-ATPase impairment in the retina of diabetic rats. Amino Acids. 2002; 23:401–406.
  66. Kowluru RA. Retinal metabolic abnormalities in diabetic mouse: comparison with diabetic rat. Curr Eye Res. 2002; 24:123–128.
  67. Mimura M, Makino H, Kanatsuka A, Yoshida S. Reduction of erythrocyte (Na<sup>+</sup>-K<sup>+</sup>) ATPase activities in non-insulin dependent diabetic patients with hyperkalemia. Metabolism. 1992; 41(4):426–430.



68. Raccach D, Fabreguettes C, Azulay JP, Vague P. Erythrocyte Na<sup>+</sup>-K<sup>+</sup>-ATPase activity, metabolic control, and neuropathy in IDDM patients. *Diabetes Care*. 1996; 19(6):564–568.
69. Konukoglu D, Kemerli GD, Sabuncu T, Hatemi H. Relation of erythrocyte Na<sup>+</sup>-K<sup>+</sup>-ATPase activity and cholesterol and oxidative stress in patients with Type 2 diabetes mellitus. *Clin Invest Med*. 2003; 26(6):279–284.
70. Greene DG, Lattimer SA, Sima AAF. Are disturbances of sorbitol, phosphoinositide, and Na<sup>+</sup>/K<sup>+</sup>-ATPase regulation involved in pathogenesis of diabetic neuropathy? *Diabetes*. 1988; 37:688–693.
71. Greene DA, DeJesus PV, Winegrad AI. Effects of insulin and dietary myo-inositol on impaired peripheral motor nerve conduction velocity in acute streptozotocin diabetes. *J. Clin Invest*. 1975; 55:1326–1336.
72. Mishra G, Routray R, Das SR, Behera HN. Alloxan diabetes in Swiss mice: Activity of Na<sup>+</sup>-K<sup>+</sup>-ATPase and succinic dehydrogenase. *Indian J Physiol Pharmacol*. 1995; 39(3):271–274.
73. Hatou S, Yamada M, Akune Y, Mochizuki H, Shiraishi A *et al.*, Role of insulin in regulation of Na<sup>+</sup>/K<sup>+</sup>-dependent ATPase activity and pump function in corneal endothelial cells. *Investigative Ophthalmol Visual Sci*. 2010; 51(8):3935–3942.