European Review for Medical and Pharmacological Sciences 2015; 19: 3107-3117

Herbal formulation, DIA-2 and Rosiglitazone ameliorates hyperglycemia and hepatic steatosis in type 2 diabetic rats

K.S. KESAVANARAYANAN[1,2], R.J. PRIYA[1,3], C. SELVAKKUMAR[1,4], S. KAVIMANI[5], D. PRATHIBA[6]

[1]Centre for Toxicology and Developmental Research, Sri Ramachandra University, Chennai, Tamilnadu, India
[2]Pharmacology and Toxicology Research Laboratory, Faculty of Pharmacy, Universiti Teknologi MARA (UiTM), Bandar Puncak Alam, Selangor Darul Ehsan, Malaysia
[3]College of Poultry Production and Management, Tamil Nadu Veterinary and Animal Sciences University, Hosur, Krishnagiri District, Tamil Nadu, India
[4]Department of Microbiology and Immunology, Faculty of Medicine, Misurata University, Misurata, Libya
[5]Department of Pharmacology, Division of Pharmacy, Mother Therasa Institute of Health Sciences, Pondicherry, India
[6]Department of Pathology, Sri Ramachandra University, Chennai, Tamilnadu, India

Abstract. – DIA-2 is a herbal mixture containing standardized extract of *Allium sativum* and *Lagerstroemia speciosa*. Recently we have reported the anti-diabetic effect of DIA-2 in high fat diet (HFD) and streptozotocin (STZ) induced type 2 diabetic (T2D) rats. The purpose of this study was to investigate and compare the effects of DIA-2 with Rosiglitazone (RG) on plasma biomarkers of hepatocellular injury, liver carbohydrate metabolizing enzymes, glycogen content, oxidant/antioxidant status and histopathological changes in T2D rats. ALT and ALP levels were significantly decreased after DIA-2 and RG treatment compared to T2D rats. Total protein and albumin remained unaltered in all the groups. Significant decrease in AST levels were observed after DIA-2 (125 mg/kg) and RG treatment. Hepatic hexokinase activity was significantly increased after RG and DIA-2 treatment and fructose-1, 6-bisphosphatase activity were inversely correlated with hexokinase activity. Hepatic gucose-6-phosphatase activity was significantly ($p < 0.05$) reduced after DIA-2 (62.5 mg/kg) and RG treatment. Lipid peroxides levels was significantly decreased in the liver of DIA-2 (62.5; $p < 0.01$ & 125 mg/kg; $p < 0.05$) treated animals. Hepatic glycogen content ($p < 0.05$) and antioxidant enzymes [SOD ($p < 0.01$; 62.5 mg/kg); GPx and GSH (125 mg/kg; $p < 0.01$)] were significantly increased after DIA-2 treatment. RG treatment on hepatic glycogen, GPx ($p < 0.01$) and SOD, GSH ($p < 0.05$) levels were significant when compared to T2D rats. These biochemical parameters were also correlated with histopathological evaluation. The above findings revealed that administration of DIA-2 could ameliorate the biochemical and histopathological changes in liver of T2D rats indicating the protective role of DIA-2 against HFD/STZ induced diabetes. In addition, DIA-2 and RG treatment resulted in amelioration of hepatic steatosis in T2D rats.

Key Words:
Allium sativum, Lagerstroemia speciosa, Rosiglitazone, DIA-2, High fat diet (HFD)/streptozotocin (STZ), Hepatic steatosis.

Abbreviations

ATP = Adenosine triphosphate; DTNB = 5, 5'-dithiobis-(2-nitrobenzoic acid); G-6-Pase = Glucose-6-phosphatase; G-6-P = Glucose-6-phosphate; F-1,6-BPase = Fructose 1,6-bisphosphatase; Pi = Inorganic phosphate; PMS = Phenazine methosulfate; T2DM = type-2 diabetes mellitus; TCA = Trichloroacetic acid.

Introduction

Diabetes mellitus is associated with increased oxidative stress (OS) that could lead to the development of diabetic complications[1]. Various clinical studies indicate that modern medicines control hyperglycemia but do not improve the altered oxidant/antioxidant levels during chronic hyperglycemia, suggesting their failure to manage the OS[2]. Medicinal plants have been widely used by humans as therapeutic agents for the management of metabolic disorders such as diabetes mellitus[3]. *Allium sativum* (ASE), *Lagerstroemia*

Corresponding Author: Kesavanarayanan, M. Pharm, Ph.D; e-mail: drkskesavan@gmail.com

speciosa (LSE) are one such medicinal plant, were used as a folk remedy for the management of diabetes mellitus in various regions of the world[4,5]. Herbal medicines are given as mixtures of one or more herbs rather than individual herb, considered to be synergistic in their therapeutic action due to their multiple therapeutic effects of individual herbs[6] and lack from undesirable side effects[7].

DIA-2 (1:1 w/w mixture of ASE and LSE) is one such herbal preparation prepared with the intention to combat hyperglycemia and hyperglycemia induced OS. During diabetes mellitus, the enzymes involved in glucose metabolism are markedly altered resulting in increased production of hepatic glucose, which leads to the condition known as hyperglycemia[8]. Liver is the major organ involved in the glucose homeostasis, metabolism and detoxification process[9]. Several authors[10,11] have also reported that altered glucose metabolism results in diabetic hyperglycemia induced OS and impaired tissue antioxidant status that is a causative mechanism involved in the pathogenesis of hepatocellular injury with concurrent increase in the activities of alanine aminotransferase (ALT); aspartate aminotransferase (AST); alkaline phosphatase (ALP) in the plasma. We have reported earlier the efficacy of DIA-2 in ameliorating hyperglycemia in high fat diet (HFD)/streptozotocin (STZ) induced diabetic rats[12]. The present study compares the effect of DIA-2 with rosiglitazone (RG) on the regulation of hepatic glucose metabolism enzymes and its ability to restore the altered liver oxidant/antioxidant status, plasma biomarkers of hepatocellular injury and histolopathological changes in the liver.

Materials and Methods

Plant Material, Chemicals and Biochemical Kits

Standardized aqueous extract of dried bulbs of ASE (1.1 % alliin w/w) and 40% methanolic extract of leaves of LSE (1.28% w/w corosolic acid) were procured commercially (M/s. Amsar Pvt. Ltd, Indore, India and M/s. K.Patel PhytoExtractions Pvt Ltd, Mumbai, India, respectively). Individual powder extracts were mixed in equal proportion (1:1) and triturated using a mortar and pestle to yield a consistent homogeneity. This mixture was named as DIA-2. All the chemicals were of analytical grade obtained from M/s. Himedia laboratories, Mumbai, India and M/s. Sisco Research Laboratories Pvt. Ltd, Mumbai, India. The diagnostic biochemical kits were purchased from M/s. Accurex Biomedical Pvt. Ltd., India. The histological stains were obtained from Merck Specialities Private Limited, Mumbai, India.

Experimental Group and Study Design

The rats were made diabetic as described earlier[12]. These diabetic rats were randomly divided into 5 groups (n = 6) namely, group-1: normoglycemic (0.5% carboxy methyl cellulose; 10 ml/kg b.w), group-2: Hyperglycemic (T2D rats; 0.5% carboxy methyl cellulose; 10 ml/kg b.w), group-3: Rosiglitazone (8 mg/kg b.w), group-4: DIA-2 (62.5 mg/kg b.w), group-5: DIA-2 (125 mg/kg b.w). The respective groups were kept on either normal pelleted diet (group-I) or high fat diet (group-2 to 5) throughout the study. Treatment was scheduled once a day *via* oral route for 14 consecutive days. Weekly body weight, fasting plasma glucose, fasting plasma insulin (FPI), total cholesterol (TC), triglycerides (TG) were measured[12]. The experimental study design was approved by the institutional animal ethics committee of Sri Ramachandra University.

Liver Function Test

After 14 days of treatment, blood was collected by sino-orbital puncture under general anaesthesia using dipotassium EDTA (K_2EDTA) as anticoagulant and the plasma separated was used for the determination of biomarkers (ALT, AST, ALP, total protein and albumin) of hepatocellular injury. The estimations were performed as per manufacturers instructions (Accurex Biomedical Pvt. Ltd. Mumbai, India) using star-21plus biochemistry auto analyser (Rapid Diagnostic Pvt. Ltd, Delhi, India).

Preparation of Liver Homogenate for Biochemical Estimations

After blood collection, all the animals from each group were sacrificed by cervical dislocation under general anaesthesia. The livers were harvested from all the experimental groups, washed in ice cold normal saline and stored at ultra low deep freezer –86°C until they are processed for biochemical estimation. 10 % liver homogenate was prepared with ice cold 10% KCl and centrifuged at 1000 rpm for 15 min. The supernatant was used as the source of enzyme. The total protein content was estimated[13].

Determination of Activities of Carbohydrate Metabolizing Enzymes

Activity of Glycolytic Enzymes

Hexokinase

Hexokinase activity was assayed as per the earlier methods[14]. Briefly, the incubation mixture containing 2.5 ml of tris HCl buffer (0.01 M, pH 8), 1 ml of glucose (0.005 M) as substrate, 0.5 ml of ATP (0.72 M), 0.1 ml of $MgCl_2$ (0.05 M), 0.1 ml of Sodium fluoride (0.5 M), 0.4 ml of KH_2PO_4 (0.02M) and 0.4 ml of KCl (0.1M) were pre-incubated at 37°C for 15 min. 0.1 ml of supernatant was added to the incubation mixture and incubated further at 37°C for 30 min. The reaction was immediately arrested by the addition of 10% TCA. The control reaction rate was correspondingly assessed by adding 0.1 ml of supernatant only after the arresting reaction and the protein precipitate was removed by centrifugation at 3500 rpm for 10 min. Aliquots of supernatant were taken and made up to 1 ml with distilled water followed by addition of 4 ml of anthrone. The absorbance was read at 630 nm using spectrophotometer (Multiskan, Thermo Scientific Inc., Waltham, MA, USA). The results are expressed in nanomoles of glucose consumed/min/mg of protein.

Activity of Gluconeogenic Enzymes

Glucose-6-phosphatase

Glucose-6 Phosphatase (G-6-Pase) was assayed as per the reported method[15]. Briefly, 0.3 ml of citrate buffer (0.1 M pH 6.5) was followed by the addition of 0.5 ml of Glucose-6-Phosphate (0.01 M) as substrate. To the test, 0.2 ml of supernatant was added and further incubated at 37°C for 1 h. The reaction was immediately arrested by the addition of 10% TCA. The control reaction rate was correspondingly assessed by adding 0.2 ml of supernatant only after the arresting step. The precipitate was removed by centrifugation at 3500 rpm for 10 min. Aliquots of supernatant were used to determine the activity glucose-6-phosphatase by measuring the amount of inorganic phosphate (Pi) liberated from glucose 6-phosphate according to earlier methods[16] at 640 nm using a spectrophotometer (Multiskan, Thermo Scientific Inc., Waltham, MA, USA). The activity of G-6-pase was expressed in nanomoles of Pi liberated/min/mg of protein.

Fructose-1, 6-bisphosphatase

Fructose 1, 6 diphosphatase (F-1,6-BPase) was assayed as per earlier methods[17]. Briefly, 1.2 ml of citrate buffer (0.1 M pH 6.5) was followed by the addition of 0.1 ml of fructose 1, 6 diphosphate (0.005 M) as substrate, 250 µl of $MgCl_2$ (0.1 M) ,0.1 ml of KCl and 0.25 ml of K_2EDTA (0.001 M) were added subsequently. To the test, 0.1 ml of supernatant was added and further incubated at 37°C for 15 min. The reaction was immediately arrested by the addition of 10% TCA. The control reaction rate was correspondingly assessed by adding 0.1 ml of supernatant only after the arresting step. The precipitate was removed by centrifugation at 3500 rpm for 10 min. Aliquots of supernatant were used to determine the activity fructose 1, 6 diphosphatase by measuring the amount of Pi liberated from fructose 1, 6 diphosphate according to earlier methods[16] at 640 nm using a spectrophotometer (Multiskan, Thermo Scientific Inc., Waltham, MA, USA). The activity of F-1, 6-BPase was expressed in nanomoles of Pi liberated/min/mg of protein.

Determination of Glycogen Content

The hepatic glycogen content was estimated as per the earlier methods[18]. Briefly, to 100 mg liver add 400 µl of 80% hot ethanol. This was centrifuged and washed for about 2-3 times. The residue obtained was dried in a sand bath for 2-3 min. To the dried residue, 2 ml of distilled water and 2.5 ml of 52% perchloric acid was added and incubated at 0ºC for 20 min. The precipitate was removed by centrifugation at 3500 rpm for 10 min. Aliquots of supernatant were made up to 1 ml with distilled water and 4 ml of anthrone was added and boiled for 5 min. The amount of glucose liberated was measured at 630 nm using spectrophotometer (Multiskan, Thermo Scientific Inc., Waltham, MA, USA). The glycogen content was expressed as nM of glucose/g of tissue.

Determination of Hepatic Oxidant/Antioxidant Status

Assay of Lipid Peroxides

The amount of lipid peroxides was estimated according to the earlier method[19]. Briefly, 0.2 ml of tissue homogenate was made up to 1 ml with normal saline, 0.5 ml of BHT (0.05%) and 3.5 ml TBA (0.8%) reagent were added and heated for 90 min in a boiling water bath. After cooling, the solution was centrifuged at 3500 rpm for 10 min and the precipitate obtained was discarded. The

absorbance of the supernatant was determined at 532 nm using spectrophotometer (Multiskan, Thermo Scientific Inc., Waltham, MA, USA). The levels of lipid peroxidation was expressed in terms of nanomoles of malondialdehyde (MDA) equivalents/g of tissue.

Super Oxide Dismutase

Super oxide dismutase was assayed by the earlier method[20]. Briefly, to 0.05 ml of supernatant, 0.3 ml of sodium pyrophosphate buffer (0.025 M), 0.025 ml of PMS (186 µM) and 0.075 ml of nitro blue tetrazolium [NBT] (300 µM) was added. The reaction was started by addition of 0.075 ml of NADH (780 µM). After incubation at 30°C for 90 seconds, the reaction was immediately stopped by addition of 0.25 ml glacial acetic acid. Then the reaction mixture was stirred vigorously and shaken with 2.0 ml of n-butanol. The mixture was allowed to stand for 10 min and centrifuged. The colour intensity in the butanol layer was read at 560 nm (Multiskan, Thermo Scientific Inc., Waltham, MA, USA). The activity of SOD was expressed as units/min/mg of protein. One unit of SOD activity is defined as the enzyme reaction, which give 50% inhibition of NBT reduction in one minute under the specified assay conditions.

Glutathione Peroxidase

The activity of glutathione peroxidase (GPx) was assayed as per the earlier methods[21]. Briefly, to a reaction mixture containing 200 µl of tris HCl buffer (0.4 mM), 200 µl K_2EDTA (0.4 mM), 100 µl of sodium azide (10 mM); 200 µl of supernatant was added and mixed well. Thereafter, 200 µl of reduced glutathione (2 mM) solution followed by 0.1 ml H_2O_2 (1 mM) were added. The overall reaction was arrested by adding 0.5 ml of 10% TCA. The non-enzymatic reaction rate was correspondingly assessed by replacing the enzyme sample by buffer. The precipitate was removed by centrifugation at 4000 rpm for 10 min. The remaining reduced glutathione in the supernatant was determined by adding 1.0 ml of DTNB (0.6 mM) and the absorbance was read at 412 nm using spectrophotometer (Multiskan, Thermo Scientific Inc., Waltham, MA, USA). The results are expressed in micromoles of GSH consumed/min/mg of protein.

Reduced Glutathione

The assay was performed as per the earlier methods[22]. Briefly, 0.25 ml of supernatant was added to equal volume of ice cold 5% TCA to precipitate the protein present in the tissue. The precipitate was removed by centrifugation at 4000 rpm for 10 min. To 1 ml aliquot of supernatant, 0.25 ml of phosphate buffer (pH 8.0) and 0.5 ml of DTNB (0.6 mM) was added and mixed well. The absorbance was read at 412 nm using spectrophotometer (Multiskan, Thermo Scientific Inc., Waltham, MA, USA). The results are expressed in nanomoles of GSH/ mg of protein.

Liver Pathology

At necropsy, the liver from all the experimental animals from the respective treatment groups were dissected and fixed in 10% neutral buffered formalin for 48 h. Thereafter, the liver tissues were processed for paraffin embedment and sectioned to 4 µm thickness using the microtome (Leica RM2125RT, Germany). The sections were stained with haematoxylin and eosin (H&E) stain for general histopathological evaluation. For demonstration of liver glycogen, liver tissues were stained with periodic acid Schiff (PAS) stain[23]. The H&E and PAS stained liver sections were examined under light microscope (Motic, B1 Series, Hong Kong, China). The percent of liver tissue staining for glycogen content by PAS was evaluated.

Statistical Analysis

The results were expressed as the mean ± SEM. The data's were analysed by the method of repeated measures and one-way analysis of variance (ANOVA) using GraphPad Prism software (version: 4.0). $p < 0.05$ was considered to be statistically significant.

Results

Liver Function Test

A significant ($p < 0.01$) increase in plasma levels of ALT, AST and ALP were observed in T2D rats when compared to normoglycemic rats (Table I). Whereas treatment with DIA-2 at the doses of 62.5 and 125 mg/kg b.w significantly ($p < 0.01$) decreased the levels of ALT and ALP respectively. DIA-2 at 125 mg/kg b.w showed significant protection of AST. RG treatment significantly ($p < 0.01$) reduced the plasma levels of ALT, AST and ALP when compared to T2D rats. No significant differences were observed in the plasma levels of total protein and albumin in all the experimental groups.

Table I. Effect of DIA-2 on plasma activities of ALT, ALP, AST, total protein and albumin levels.

Group	Treatment	ALT (U/l)	ALP (U/l)	AST (U/l)	Total Protein (g/dl)	Albumin (g/dl)
I	Normoglycemic	54.03 ± 4.55	348.62 ± 57.89	84.00 ± 3.88	6.15 ± 0.25	2.19 ± 0.15
II	Hyperglycemic (T2D)	425.08 ± 26.16$^{\#\#}$	925.55 ± 49.88$^{\#\#}$	354.78 ± 48.86$^{\#\#}$	5.80 ± 0.54	1.68 ± 0.20
III	Rosiglitazone (8mg/kg b.w)	277.83 ± 26.68**	583.36 ± 77.01**	172.92 ± 38.60**	5.98 ± 0.40	2.08 ± 0.04
IV	DIA-2 (62.5 mg/kg b.w)	227.02 ± 32.66**	588.83 ± 72.00**	221.05 ± 21.07	6.12 ± 0.19	1.99 ± 0.16
V	DIA-2 (125 mg/kg b.w)	248.37 ± 20.73**	424.08 ± 54.41**	144.60 ± 31.30**	6.04 ± 0.35	2.01 ± 0.12

Values expressed in mean ± SEM; n = 6; $^{\#\#}$& $^{\#}$- p value of 0.01 & 0.05 compared to the normoglycemic group; **& *- p value of 0.01 & 0.05 compared to the T2D rats.

Carbohydrate Metabolizing Enzymes

A significant ($p < 0.01$) decrease in hepatic hexokinase and significant ($p < 0.01$) increase in G-6-Pase and F-1, 6-BPase activities were observed in the liver of T2D rats when compared to normoglycemic rats (Table II). Treatment with DIA-2 significantly ($p < 0.01$) increased hexokinase and decreased F-1, 6-BPase activity respectively at both the dose levels. The activity of G-6-Pase was significantly ($p < 0.05$) decreased at 62.5 mg/kg b.w and no significant changes were found at 125 mg/kg b.w of DIA-2 treatment. RG treatment significantly ($p < 0.01$) increased hexokinase activities and significantly decreased G-6-Pase ($p < 0.05$) and F-1, 6-BPase activities ($p < 0.01$) respectively when compared to T2D rats.

Hepatic Glycogen Content

A significant ($p < 0.01$) decrease in hepatic glycogen content was observed in T2D rats when compared to normoglycemic rats (Table II). When compared to T2D rats, hepatic glycogen content were improved significantly after DIA-2 treatment ($p < 0.05$) at both the dose levels and after RG ($p < 0.01$) treatment.

Hepatic Oxidant/Antioxidant Status

A significant ($p < 0.01$) increase in the content of thiobarbituric acid reactive substances (TBARS) and significant ($p < 0.01$) decrease in SOD, GPx, GSH levels were observed in T2D rats when compared to normoglycemic rats (Table II). DIA-2 treatment significantly ($p < 0.01$ and $p < 0.05$) decreased the hepatic TBARS content at 62.5 and 125 mg/kg b.w respectively. RG treatment did not show any protection in ameliorating the altered TBARS content. The liver SOD levels were significantly ($p < 0.01$) increased at 62.5 mg/kg b.w and 125 mg/kg b.w did not show any significant protection on SOD activity. A significant increase in liver GPx ($p < 0.01$); GSH ($p < 0.05$) activity were observed after DIA-2 treatment at 125 mg/kg b.w and 62.5 mg/kg b.w did not show any significant protection on both the parameters when compared to T2D rats. RG treatment significantly increased

Table II. Effect of DIA-2 osf carbohydrate metabolising enzymes, glycogen content, and oxidant/antioxidant status of liver.

Parameter	NG	T2D	RG	DIA-2 62.5 mg/kg b.w	DIA-2 125 mg/kg b.w
Hexokinase	51.20 ± 0.67	21.71 ± 0.43$^{\#\#}$	49.81 ± 0.20**	42.85 ± 3.16**	38.66 ± 1.81**
Glucose-6-phosphatase	28.18 ± 1.98	59.46 ± 3.96$^{\#\#}$	33.42 ± 2.39*	33.22 ± 8.72*	40.82 ± 6.18
Fructose 1,6 bisphosphatase	29.07 ± 4.29	70.2 ± 4.42$^{\#\#}$	26.81 ± 9.80**	27.52 ± 2.07**	23.40 ± 3.59**
Glycogen	61.46 ± 5.06	18.02 ± 3.59$^{\#\#}$	61.18 ± 3.21**	48.16 ± 6.04*	49.48 ± 11.04*
TBARS	30.87 ± 3.02	70.17 ± 4.41$^{\#\#}$	57.37 ± 3.78	36.04 ± 4.82**	40.22 ± 10.29*
SOD	28.37 ± 2.37	7.03 ± 0.72$^{\#\#}$	20.54 ± 1.74*	26.86 ± 5.09**	12.31 ± 1.39
Glutathione peroxidase (GPx)	0.25 ± 0.03	0.03 ± 0.01$^{\#\#}$	0.16 ± 0.01**	0.08 ± 0.01	0.18 ± 0.01**
Reduced glutathione (GSH)	170.50 ± 6.33	60.90 ± 12.82$^{\#\#}$	134.01 ± 15.26*	127.95 ± 27.59	134.10 ± 17.46*

NG, Normoglycemic group; T2D, Type 2 diabetic rats; PCG, RG, rosiglitazone. Values expressed in mean ± SEM; n = 6; $^{\#\#}$& $^{\#}$- p value of 0.01 & 0.05 compared to the normoglycemic group; **& *- p value of 0.01 & 0.05 compared to the T2D rats.

the levels of GPx ($p < 0.01$), SOD and GSH ($p < 0.05$) in the liver when compared to T2D rats.

Liver Pathology

H&E stained sections of liver from the experimental groups were shown in Figure 1. The histopathological examinations of liver from normoglycemic animals revealed normal histology characterised by distinct hepatocytes with radiating hepatic cords around central vein (Figure 1A). The liver sections from T2D rats revealed hypertrophic hepatocytes with sinusoidal narrowing, nuclear indentations, karyolysis and mild neutrophilic infiltrations suggestive of centrilobular coagulative necrosis (Figure 1B) associated with the presence of multifocal periportal and midzonal macrovesicular steatosis (Figure1C) interspersed with activated stellate cells.

T2D rats treated with 125 mg/kg b.w of DIA-2 (Figure 1F) and RG (Figure 1D) exhibited normal liver architecture, similar to that of normoglycemic rats. While, mild neutrophilic infiltrations were observed in the liver of T2D rats treated with 62.5 mg/kg b.w of DIA-2 (Figure 1E).

Figure 2 represents the PAS-stained liver sections from the experimental groups. The PAS staining of liver sections from the normoglycemic rats revealed intense expression of intracytoplasmic glycogen (Figure 2A) in 90-95% of centrilobular and midzonal hepatocytes. However, the PAS staining of sections of liver from T2D rats (Figure 2B) and T2D rats treated with 62.5 mg/kg b.w of DIA-2 (Figure 2D) exhibited absence of PAS-positive hepatic glycogen expression respectively. DIA-2 treatment at 125 mg/kg b.w (Figure 2E) and RG (Figure 2C) treatment demonstrated mild (40-45%) and moderate (60-70%) PAS-positive hepatic glycogen expression respectively.

Discussion

In our earlier study, we have reported DIA-2 for its anti-diabetic effect using HFD/STZ induced T2D rats[12]. In an attempt to understand the biochemical mechanism of the antidiabetic effect of DIA-2, we investigated the key glycolytic and gluconeogenic enzymes along with glycogen content in the liver of T2D rats. The key biomarkers of hepatocellular injury and liver oxidant/antioxidant status and histopathological changes were also observed in the liver of DIA-2 treated animals.

The liver is an important insulin dependent tissue that plays a major role in the glucose homeostasis (*i.e.* glucose production and utilization) by strictly controlling the activities of the key enzymes involved in the pathways of carbohydrate metabolism (glycolysis and gluconeogenesis). Liver also controls the synthesis (glycogenesis) and degradation of glycogen (glycogenolysis) as a part of their glucose homeostasis mechanism. During T2DM due to lack of or resistance to insulin, increase in hepatic gluconeogenesis, glycogenolysis and decrease glycolytic, glycogenic activity were observed[24].

Hexokinase is the first key enzyme involved in the glycolytic pathway which phosphorylates glucose to glucose-6-phosphate (G-6-P). G-6-Pase, a key enzyme involved in the gluconeogenesis and glycogenolysis that catalyzes the last common step of both the processes *i.e*., the dephosphorylation G-6-P to glucose[25]. F-1, 6-BPase, a gluconeogenic enzyme that controls the production of hepatic glucose through gluconeogenesis process[26,27].

HFD)/low-dose STZ induced diabetes mellitus causes insulin resistance and subsequent insulin secretory defect in rats that closely reflects the metabolic disturbances that are characterised during T2DM in humans. Compared to normoglycemic animals, the T2D rats showed a significant reduction insulin level[12] and decrease in the activities of hexokinase and significant elevation in the activities of G-6-Pase, F-1, 6-BPase in the liver were observed. Similarly, the glycogen contents were decreased in T2D rats. Activation of hexokinase activity, inhibition of activities enzymes involved in gluconeogenesis and restoration of glycogen content have been proposed as potential targets for suppressing hepatic glucose levels during T2DM.

The use of herbal medicines to modulate the altered carbohydrate metabolism during diabetes is quite high and, therefore, finds its own importance in the management of this metabolic disorder. Unfortunately, there is an insufficient evidence to actively recommend the use of herbal medicines in improving the altered carbohydrate metabolism[4,28]. The individual ingredients of DIA-2 were reported earlier for their possible role on carbohydrate metabolism[29,30]. The combined effect of ASE and LSE (*i.e.* DIA-2) on hepatic carbohydrate metabolising enzymes, *viz*. hexokinase, G-6-Pase and F-1, 6-BPase and hepatic glycogen in T2D rat were investigated in the present study. Admin-

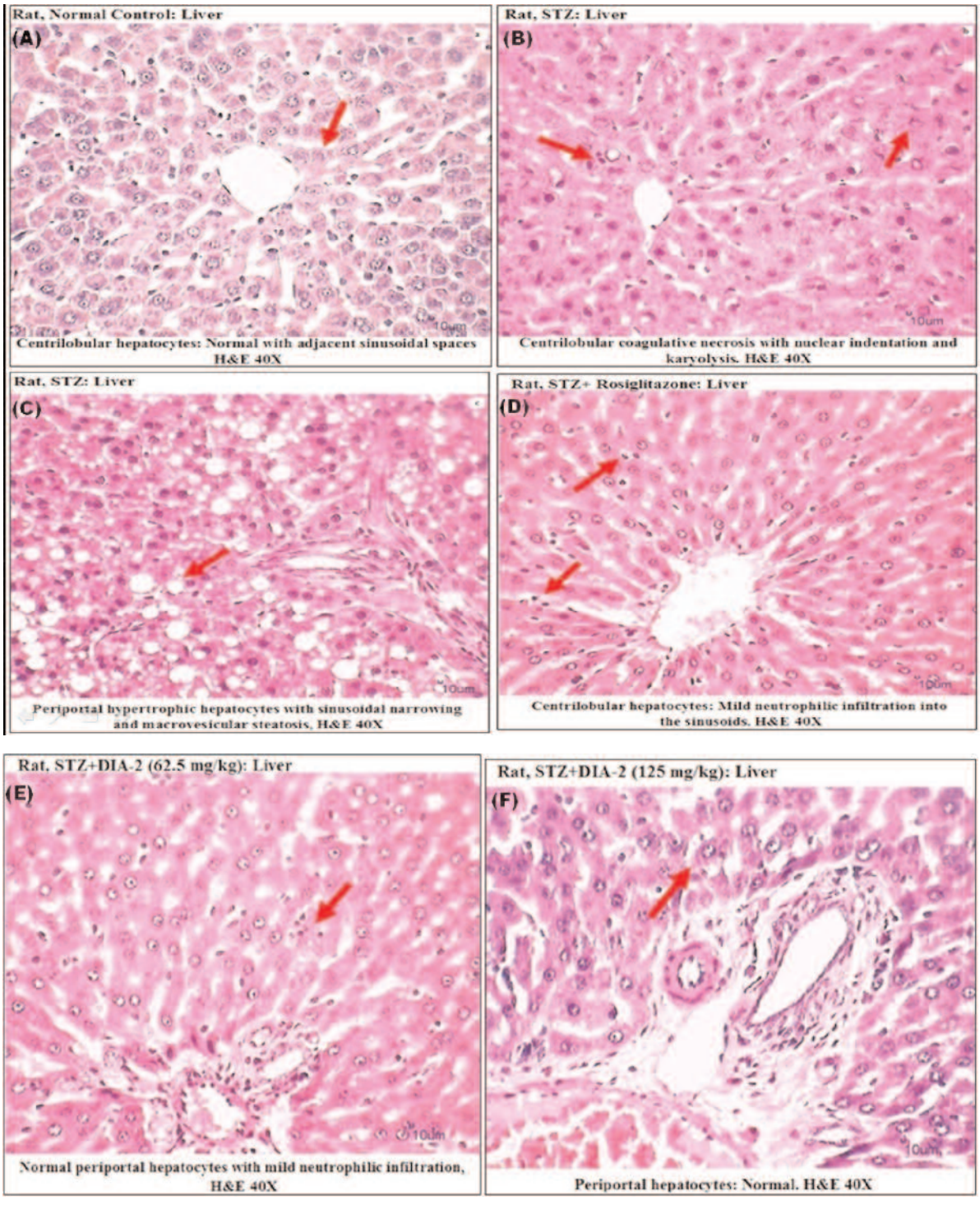

Figure 1. H&E stained liver sections. ***A,*** Normoglycemic group. ***B-C,*** Hyperglycemic (T2D). ***D,*** Hyperglycemic + rosiglitazone. ***E,*** Hyperglycemic + 62.5 mg DIA-2/kg b.w. ***F,*** Hyperglycemic + 125 mg DIA-2/kg b.w. (Original magnification: 400×).

istration of DIA-2 have enhanced the hepatic activity of hexokinase and decreased the G-6-Pase and F-1, 6-BPase activity. DIA-2 treatment has also restored the levels of glycogen when compared to T2D rats. S-allyl cysteine sulphoxide (SACS), a sulphur containing amino acid of ASE have reported to modulate the carbohydrate metabolism through activa-

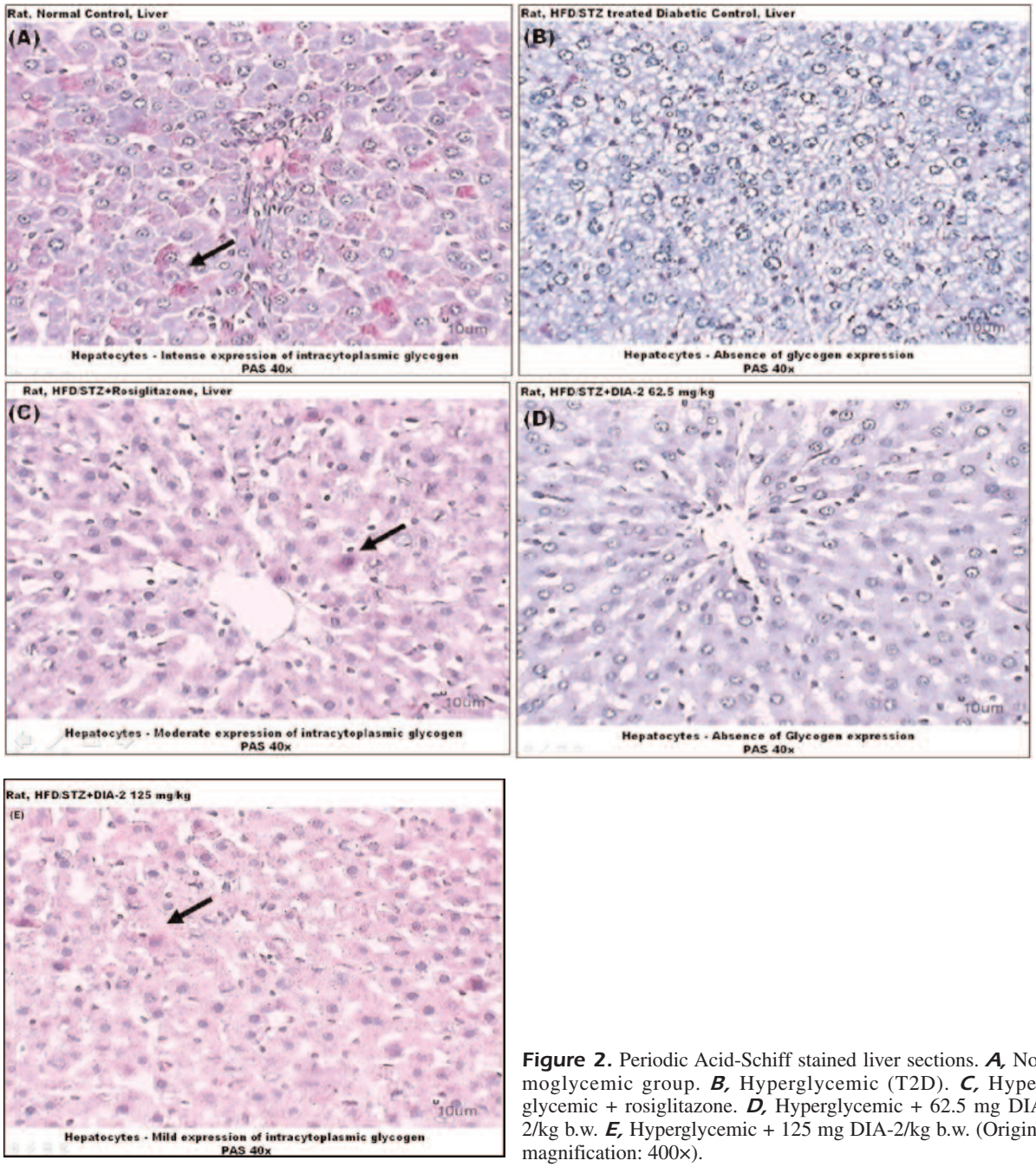

Figure 2. Periodic Acid-Schiff stained liver sections. ***A,*** Normoglycemic group. ***B,*** Hyperglycemic (T2D). ***C,*** Hyperglycemic + rosiglitazone. ***D,*** Hyperglycemic + 62.5 mg DIA-2/kg b.w. ***E,*** Hyperglycemic + 125 mg DIA-2/kg b.w. (Original magnification: 400×).

tion of glycolytic, inactivation of gluconeogenetic enzymes and stimulation of insulin secretion from β cells[31,32]. The pentacyclic triterpenoid, corosolic acid present in LSE have also been reported to inhibit the activity against glycogen phosphorylase, a rate limiting enzyme during glycogenolysis that break glycogen into glucose[33,34]. Recent report[35] reveals that polyphenols may also influence carbohydrate metabolism mainly by affecting glucose transport and insulin-receptor function, insulin release from β cells of the pancreas. Amelioration of carbohydrate metabolism by DIA-2 treatment could be attributed due to the presence of these phytoconstituents available with the individual ingredients of DIA-2. DIA-2 maintains glucose and lipid homeostasis[12] and could also significantly restore the altered car-

bohydrate metabolic parameters, suggesting its potential use as a therapeutic agent in the management of T2DM.

Oxidative stress is one of the causative factors in the development consequences of diabetes. In the present study, the activities of hepatic oxidant/antioxidant status were measured to ascertain the role of DIA-2 in the alleviation of altered antioxidant defence system. The levels of GSH and GPx were decreased in T2D rats when compared to normoglycemic animals. DIA-2 treated animals have protected their activities significantly. Conversely, the increased levels of TBARS in T2D rats were attenuated significantly in DIA-2 treated group. The inhibition of lipid peroxidation of DIA-2 might be due to the presence of organosulfur compounds available with ASE[32] and LSE have reported to inhibit lipid peroxidation[36,37].

Insulin and oral hypoglycemics agents are reported to cause liver damage[38]. Regular monitoring of liver enzymes is still recommended during the therapy with few anti-diabetic class of drugs. Liver enzymes such as ALT, AST and ALP are marker enzymes for liver function. Hyperglycemia is usually accompanied by an increase in the activities of the enzymes of the liver[39]. Administration of DIA-2 has led to a significant decrease in ALT, AST and ALP levels. The improvement in the hepatic maker enzymes level by DIA-2 treatment indicates that it does not cause hepatotoxicity that is likely caused by few classes of antidiabetic drugs. DIA-2 seems to posses hepatoprotective potential along with antidiabetic activity. The active constituent in the ingredients of DIA-2 could be responsible for antidiabetic and hepatoprotective activity. ASE was reported to decrease AST and ALT levels during hyperglycaemic conditions[40]. Improvement in the activities of liver marker enzymes level by DIA-2 treatment indicates that DIA-2 has hepatoprotective action along with antidiabetic activity.

During diabetes, the protein metabolism is altered and remains unaltered during diabetes associated obesity. Insulin plays a major role on protein metabolism in the liver[41]. Administration of STZ to HFD treated rats did not affect the protein metabolism in both T2D rats and DIA-2 treated T2D rats.

Experimental animal model of T2DM has provided considerable insight into the histological alterations during diabetic state. DIA-2 treatment preserved the normal hepatocyte architecture and suppressed the diabetes induced morphological alterations in the liver. Biochemical analysis of liver tissues and histopathological evaluation of PAS-stained liver sections revealed hepatocellular glycogen depletion in HFD/STZ induced T2D rats when compared to normoglycemic rats. After DIA-2 treatment the hepatic glycogen content was restored when compared to T2D rat as confirmed by biochemical and histopathological analysis. From these observations, it is evident that the DIA-2 treatment restored the glycogen levels in the liver of T2D rats.

T2DM is frequently accompanied by hepatic steatosis, which refers to a histopathological condition characterized by increased content of hepatocellular lipids and could be differentiated as macro or microvesicular steatosis according to the size of the lipids[42]. Hepatic steatosis could be induced by administration of HFD. In the present study histological analysis of liver tissue taken from T2D rats with HFD showed multifocal periportal and midzonal macrovesicular hepatic steatosis (Figure 1C) compared to normoglycemic animals (Figure 1A) fed with normal pelleted diet under our experimental conditions. The reduction of lipid accumulation in the liver could be considered as a new therapeutic target in T2DM and diet-induced hepatic steatosis. Administration of DIA-2 (Figure 1E and 1F) has shown to have protective effect on HFD induced hepatic steatosis in rats when compared to positive control, RG (Figure 1E). The possible mechanism by which RG ameliorates hepatic steatosis were reported earlier[43].

Conclusions

Our results suggested that DIA-2 maintains glucose homeostasis by controlling the carbohydrate metabolizing enzymes and also mitigates the hyperglycemia induced OS. DIA-2 treatment also mitigated the hepatic steatosis induced by HFD/STZ in the liver of diabetic rats. These findings suggested that DIA-2 could be beneficial in the therapeutic intervention of diabetes and its complications.

Acknowledgements

The authors wish to thank Prof. Dr Aishah Adam, Dean, Faculty of Pharmacy, Universiti Teknologi Mara (UiTM) Puncak Alam Campus for her support.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Kesavanarayanan KS, Sathiya S, Ranju V, Sunil AG, Ilavarasan R, Saravana Babu C, Kavimani S, Prathiba D. *In vitro* cytotoxic, antioxidative and alpha-glucosidase inhibitory potential of a herbal mixture comprised of *Allium sativum* and *Lagerstroemia speciosa*. Eur Rev Med Pharmacol Sci 2012; 16 Suppl 3: 58-68.
2) Waisundara VY, Hsu A, Huang D, Tan BK. *Scutellaria baicalensis* enhances the anti-diabetic activity of metformin in streptozotocin-induced diabetic Wistar rats. Am J Chin Med 2008; 36: 517-540.
3) John CM, Sandrasaigaran P, Tong CK, Adam A, Ramasamy R. Immunomodulatory activity of polyphenols derived from *Cassia auriculata* flowers in aged rats. Cell Immunol 2011; 271: 474-479.
4) Cefalu WT, Ye J, Wang ZQ. Efficacy of dietary supplementation with botanicals on carbohydrate metabolism in humans. Endocr Metab Immune Disord Drug Targets 2008; 8: 78-81.
5) Klein G, Kim J, Himmeldirk K, Cao Y, Chen X. Antidiabetes and Anti-obesity Activity of *Lagerstroemia speciosa*. Evid Based Complement Alternat Med 2007; 4: 401-407.
6) Li Y, Huang TH, Yamahara J. Salacia root, a unique Ayurvedic medicine, meets multiple targets in diabetes and obesity. Life Sci 2008; 82: 1045-1049.
7) Szentmihalyi K, Gere A, Szollosi-Varga I, Blazovics A, Jasztrab S, Lado K, Then M. Inhibition of lipid peroxidation of herbal extracts (obtained from plant drug mixtures of *Myrtilli folium*, *Phaseoli fructus sine seminibus* and *Salviae folium*) used in type 2 diabetes mellitus. Acta Biol Hung 2010; 61: 45-51.
8) Sivakumar S, Subramanian SP. D-pinitol attenuates the impaired activities of hepatic key enzymes in carbohydrate metabolism of streptozotocin-induced diabetic rats. Gen Physiol Biophys 2009; 28: 233-241.
9) Diehl-Jones WL, Askin DF. The neonatal liver, Part 1: embryology, anatomy, and physiology. Neonatal Netw 2002; 21: 5-12.
10) Maritim AC, Sanders RA, Watkins JB 3rd. Effects of alpha-lipoic acid on biomarkers of oxidative stress in streptozotocin-induced diabetic rats. J Nutr Biochem 2003; 14: 288-294.
11) Boone L, Meyer D, Cusick P, Ennulat D, Bolliger AP, Everds N, Meador V, Elliott G, Honor D, Bounous D and others. Selection and interpretation of clinical pathology indicators of hepatic injury in preclinical studies. Vet Clin Pathol 2005; 34: 182-188.
12) Kesavanarayanan KS, Sathiya S, Kalaivani P, Ranju V, Sunil AG, Saravana Babu C, Kavimani S, Prathiba D. DIA-2, a polyherbal formulation ameliorates hyperglycemia and protein-oxidation without increasing the body weight in type II diabetic rats. Eur Rev Med Pharmacol Sci 2013; 17: 356-369.
13) Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.
14) Brandstrup N, Kirk JE, Bruni C. The hexokinase and phosphoglucoisomerase activities of aortic and pulmonary artery tissue in individuals of various ages. J Gerontol 1957; 12: 166-171.
15) Koide H, Oda T. Pathological occurrence of glucose-6-phosphatase in serum in liver diseases. Clin Chim Acta 1959; 4: 554-561.
16) Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. Journal of Biological Chemistry 1925; 66: 375-400.
17) Gancedo JM, Gancedo C. Fructose-1,6-diphosphatase, phosphofructokinase and glucose-6-phosphate dehydrogenase from fermenting and non fermenting yeasts. Arch Mikrobiol 1971; 76: 132-138.
18) Morales MA, Jabbagy AJ, Terenizi HR. Mutations affecting accumulation of glycogen. Neurospora News Lett 1973; 20: 24-25.
19) Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-358.
20) Kakkar P, Das B, Viswanathan PN. A modified spectrophotometric assay of superoxide dismutase. Indian J Biochem Biophys 1984; 21: 130-132.
21) Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: biochemical role as a component of glutathione peroxidase. Science 1973; 179: 588-590.
22) Moron MS, Depierre JW, Mannervik B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim Biophys Acta 1979; 582: 67-78.
23) Bancroft JD, Stevens A. Theory and Practice of Histological Techniques. World Publishing Corporation, 1990.
24) Postic C, Dentin R, Girard J. Role of the liver in the control of carbohydrate and lipid homeostasis. Diabetes Metab 2004; 30: 398-408.
25) Chatelain F, Pegorier JP, Minassian C, Bruni N, Tarpin S, Girard J, Mithieux G. Development and regulation of glucose-6-phosphatase gene expression in rat liver, intestine, and kidney: in vivo and *in vitro* studies in cultured fetal hepatocytes. Diabetes 1998; 47: 882-889.
26) Hers HG. Mechanisms of blood glucose homeostasis. J Inherit Metab Dis 1990; 13: 395-410.
27) Wasserman DH, Connolly CC, Pagliassotti MJ. Regulation of hepatic lactate balance during exercise. Med Sci Sports Exerc 1991; 23: 912-919.
28) Prabhakar PK, Doble M. A target based therapeutic approach towards diabetes mellitus using medicinal plants. Curr Diabetes Rev 2008; 4: 291-308.
29) Chang ML, Johnson MA. Effect of garlic on carbohydrate metabolism and lipid synthesis in rats. J Nutr 1980; 110: 931-936.

30) Yamada K, Hosokawa M, Fujimoto S, Fujiwara H, Fujita Y, Harada N, Yamada C, Fukushima M, Ueda N, Kaneko T and others. Effect of corosolic acid on gluconeogenesis in rat liver. Diabetes Res Clin Pract 2008; 80: 48-55.

31) Sheela CG, Augusti KT. Antidiabetic effects of S-allyl cysteine sulphoxide isolated from garlic Allium sativum Linn. Indian J Exp Biol 1992; 30: 523-526.

32) Augusti KT, Sheela CG. Antiperoxide effect of S-allyl cysteine sulfoxide, an insulin secretagogue, in diabetic rats. Experientia 1996; 52: 115-120.

33) Zhang P, Hao J, Liu J, Lu Q, Sheng H, Zhang L, Sun H. Synthesis of 3-deoxypentacyclic triterpene derivatives as inhibitors of glycogen phosphorylase. J Nat Prod 2009; 72: 1414-1418.

34) Wen X, Xia J, Cheng K, Zhang L, Zhang P, Liu J, Ni P, Sun H. Pentacyclic triterpenes. Part 5: synthesis and SAR study of corosolic acid derivatives as inhibitors of glycogen phosphorylases. Bioorg Med Chem Lett 2007; 17: 5777-5782.

35) Hanhineva K, Torronen R, Bondia-Pons I, Pekkinen J, Kolehmainen M, Mykkanen H, Poutanen K. Impact of dietary polyphenols on carbohydrate metabolism. Int J Mol Sci 2010; 11: 1365-1402.

36) Priya TT, Sabu MC, Jolly CI. Free radical scavenging and anti-inflammatory properties of Lagerstroemia speciosa (L). Inflammopharmacology 2008; 16: 182-187.

37) Saumya SM, Basha PM. Antioxidant effect of Lagerstroemia speciosa Pers (banaba) leaf extract in streptozotocin-induced diabetic mice. Indian J Exp Biol 2011; 49: 125-131.

38) Chitturi S, George J. Hepatotoxicity of commonly used drugs: nonsteroidal anti-inflammatory drugs, antihypertensives, antidiabetic agents, anticonvulsants, lipid-lowering agents, psychotropic drugs. Semin Liver Dis 2002; 22: 169-183.

39) Mori DM, Baviera AM, de Oliveira Ramalho LT, Vendramini RC, Brunetti IL, Pepato MT. Temporal response pattern of biochemical analytes in experimental diabetes. Biotechnol Appl Biochem 2003; 38: 183-191.

40) Eidi A, Eidi M, Esmaeili E. Antidiabetic effect of garlic (Allium sativum L.) in normal and streptozotocin-induced diabetic rats. Phytomedicine 2006; 13: 624-629.

41) Abu-Lebdeh HS, Nair KS. Protein metabolism in diabetes mellitus. Baillieres Clin Endocrinol Metab 1996; 10: 589-601.

42) Roden M. Mechanisms of Disease: hepatic steatosis in type 2 diabetes--pathogenesis and clinical relevance. Nat Clin Pract Endocrinol Metab 2006; 2: 335-348.

43) Park CY, Park SW. Role of peroxisome proliferator-activated receptor gamma agonist in improving hepatic steatosis: Possible molecular mechanism. J Diabetes Investig 2012; 3: 93-95.