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DIA-2, a polyherbal formulation ameliorates hyperglycemia and protein-oxidation without increasing the body weight in type II diabetic rats

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Abstract. – BACKGROUND: The dried bulbs of *Allium sativum* (Garlic) and leaves of *Lagerstroemia speciosa* (Banaba) are used as medicinal food for the treatment of diabetes and other ailments.

AIM: The present study was undertaken to ascertain whether the combination of both garlic and banaba extract produces synergistic therapeutic effect in diabetic state.

METHODS: In the *in vitro* studies, the effect of standardized aqueous extract of *Allium sativum* (ASE), methanolic extract of *Lagerstroemia speciosa* (LSE) and their mixture (1:1 ratio), DIA-2 on insulin stimulated glucose uptake in 3T3-L1 cells, erythrocyte sorbitol accumulation and protein glycation were evaluated. Impetus from the *in vitro* findings triggered to screen the anti-diabetic potential of DIA-2 in rat model of type II diabetes and associated oxidative stress. In the *in vivo* studies, acute oral toxicity of DIA-2 was determined following OECD-423 guidelines in female rats. Anti-diabetic activity of DIA-2 was investigated in high fat diet/low dose streptozotocin induced type II diabetes at four dose levels (62.5, 125, 250 and 500 mg/kg b.w) in rats.

RESULTS: Combination of ASE and LSE produced synergistic and a dose dependent increase in glucose uptake in 3T3 adipocyte cell lines when compared to the individual extracts. A similar effect was observed in the inhibition of sorbitol accumulation and protein glycation tests. DIA-2 restored the glucose and lipid level near to normal level without gain in body weight which is the most commonly encountered side effect with the use of conventional antidiabetic agents, particularly insulin, insulin secretagogues, sulfonylureas and thiazolidinediones. DIA-2 also decreased hepatic protein carbonyl content levels significantly in the diabetic rats.

CONCLUSIONS: The study concluded that DIA-2 posses potent anti-diabetic activity and anti-oxidant effects.

Key Words:
Garlic, *Banaba*, Glycation, Sorbitol, 3T3-L1 adipocytes, Diabetes.

Introduction

Four main molecular mechanisms have been implicated in hyperglycemia induced vascular damage namely, increased polyol pathway flux, advanced glycation end (AGE) products formation, hyperactivation of protein kinase C and hexosamine signaling flux. All these mechanisms are reported to be the consequence of overproduction of superoxide radical $(O_2^{\bullet})^-$ in hyperglycemic state[1]. The multifactorial pathogenicity of diabetes needs multi-modal therapeutic approach. Therapeutic approaches with synthetic medicines root to toxicity on long term administration. One of the most commonly encountered adverse effects with the use of antidiabetic agents, particularly insulin, insulin secretagogues, and thiazolidinediones is gain in body weight[2,3] and hepatotoxicity[4]. Only few agents control hyperglycemia without gain in body weight which includes pancreatic beta-cell hormone amylin analogs, glucagon-like peptide-1 (GLP-1), exendin, and their analogs[5]. Therapeutic treatments with medicinal plants either as single[6] or as combination[7] have better control on glycemic status and lipid levels without influence on body weight gain. On the other hand, inclusion of large numbers of ingredients in a formulation may condense the potency of each other, can arise in difficulties during industrial scale up process and may not be accepted by the concerned regulatory agency due to the difficulties in standardization. One of the common methods to minimize the number of ingredients in a formulation is to choose an active ingredient that performs more than one function[8].

Allium sativum (ASE) is being used in southern part of India as culinary and medicinal agent

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since time immemorial. It is widely recognized for its use in the management of cardiovascular and other metabolic diseases such as atherosclerosis, hyperlipidemia, thrombosis, hypertension and diabetes[9]. *Lagerstroemia speciosa* (LSE), more commonly known as banaba, is traditionally consumed in various forms by peoples Philippines for treatment of diabetes and obesity[10]. Both ASE and LSE are well known for their multiple therapeutic actions like antidiabetic[10,11]; α-glucosidase inhibition[12]; anti-adipogenic[13,14]; anti-oxidant[15,16] and antiglycation activity[15]. Though several reports were available for ASE and LSE individually, data on the anti-diabetic and toxic effects of their combination is not hitherto known. These spurt interest to develop a combination of ASE and LSE and to investigate their synergistic effects, if any. DIA-2 is one such formulation containing fixed proportion (1:1 w/w) of aqueous extract of ASE) and 40% methanolic extract of LSE. In the present study, the individual extracts of ASE, LSE and DIA-2 were subjected to 2-deoxy-d-^{3}[H] glucose uptake assay in 3T3-L1 cell lines to evaluate its anti-diabetic activity. Also its effect on sorbitol accumulation in erythrocytes and protein glycation were investigated by *in vitro* methods to ascertain DIA-2 influence on diabetic complications. The results of the *in vitro* assays triggered to investigate anti-diabetic effect of DIA-2 in a rat model of type 2 diabetes (T2D) and associated complications.

Materials and Methods

Drug and Chemicals

All cell culture solutions and supplements were purchased from Life Technologies Inc. (Gaithersburg, Maryland, USA). Dulbecco's Modified Eagle Medium (DMEM) was obtained from GIBCO, BRL (Gaithersburg, MD, USA). 2-Deoxy-D-^{3}[H] glucose (2-DOG) was obtained from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). Streptozotocin (STZ), insulin and sorbitol dehydrogenase (SDH) were obtained from Sigma Aldrich (Sigma Chemical Co, St Louis, MO, USA). Rosiglitazone (RG) was a kind gift from Dr. Reddy's Laboratories, Hyderabad, India. Biochemical kits were obtained from Merck, Mumbai, India. Rat Insulin ELISA kit was obtained from DRG Instruments GmbH, (Marbourg, Germany). All other chemicals were of analytical grade obtained from Himedia Laboratories Pvt Ltd (Mumbai, India) and Sisco Research Laboratories Pvt. Ltd (Mumbai, India).

Extracts of ASE, LSE and Formulation of DIA-2

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.

In Vitro Studies

Insulin Stimulated Glucose Uptake in Differentiated 3T3-L1 Adipocytes

3T3-L1 preadipocytes [obtained from American Type Culture Collection (ATCC-CL-173] were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal bovine serum (FBS) and supplemented with penicillin (120 units/ml), streptomycin (75 µg/ml), gentamycin (160 µg/ml) and amphotericin B (3 µg/ml) in 5% CO_2 environment. 3T3-L1 preadipocytes grown in 24 well plates were induced by the differentiation medium (combination of IBMX, DEX and insulin in DMEM medium with 10% FBS) to differentiate into adipocytes. The extent of differentiation was established by observing multinucleate of cells[17].

3T3-L1 adipocytes grown in 24-well plate were subjected to glucose uptake as per standard methods. After differentiation, the medium was replaced using DMEM with 10% FBS containing 1 mg/ml of insulin for 2 days followed by serum starvation for 5 h. After the induction, the cells were incubated with various concentrations of plant extracts for 24 hours and stimulated with Insulin (100 nM) for 20 min. After experimental incubation, cells were rinsed once with Krebs Ringer Phosphate HEPES (KRPH) solution (118 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 30 mM HEPES - pH 7.4) and were subsequently incubated for 20 min in KRPH solution containing 0.5 µCi/ml 2-Deoxy-D-^{3}[H]glucose (2-DOG). The uptake was terminated by aspiration of media. Cells were washed thrice with ice-cold KRPH solution and lysed in 0.1% Sodium dodecyl sulphate (SDS). The lysates were transferred to 96-well plate with glass fiber paper and air dried overnight. This plate was used to measure the cell-associated radioactivity by liquid scintillation counting. All the assays were performed in triplicates for concor-

dance. Results were expressed as % glucose uptake with respect to solvent control. Rosiglitazone (50 µM) was used as the positive control.

Erythrocyte Sorbitol Accumulation

Heparinized rat blood samples were collected and immediately centrifuged at 1500 rpm at 4°C for 10 min. The plasma was separated and erythrocytes were washed three times with cold isotonic saline and centrifuged at 1500 rpm for 15 min. 200 µl of washed erythrocytes was added to 1 ml of Hank's balanced salt solution [HBSS] (pH 7.4) containing various concentrations of ASE, LSE, DIA-2 or ascorbic acid (5-100 µg/ml). All samples were incubated at 37°C for 3 h at room temperature. The erythrocytes were centrifuged at 3000 rpm at 4°C for 10 min and the supernatant was removed; then the cells were washed three times with cold isotonic saline and centrifuged again. The protein was precipitated with 2 ml of cold 6% perchloric acid and the supernatant was neutralized with cold 1M K_2CO_3. The supernatant solution was stored at –20°C until analysis for sorbitol content. Sorbitol content was analyzed as described earlier[18,19] with minor modifications. In brief, the reaction mixture contained the appropriate protein-free supernatant, 50 mM glycine buffer (pH 9.4), 0.2 mM NAD^+, and 1.28 units of sorbitol dehydrogenase. The mixture was incubated at 37°C for 30 min, and the relative fluorescence due to NADH was measured by a fluorescence spectrometer (Perkin Elmer, LS-45, UK) at an excitation wavelength of 366 nm and an emission wavelength of 452 nm. The experiments were performed in triplicates.

Protein Glycation

Glycation reaction was initiated by addition of equal volumes of high concentration glucose solution and a known concentration of albumin solution. To prevent microbial contamination, sodium azide (0.1%) was added to the reaction mixture prepared in 0.01 M phosphate buffer (pH=7.4). Various concentrations (10-300 µg/ml) of ASE, LSE and DIA-2 were added in triplicates and incubated at room temperature. The samples were dialyzed in phosphate buffer (the dialyzed bag was prepared in 10 mM ethylene diamine tetracetic acid (EDTA) before this process) on day 7. The extent of albumin glycation was measured as thiobarbituric acid reactive substances (TBARS) content[20]. In brief, 1 ml 20% trichloroacetic acid (TCA) was added to above solution and then centrifuged for 10 minutes at 3000 rpm and the supernatant was discarded. 1 ml phosphate buffer with above specification and 0.5 ml 0.3 N oxalic acid were added to the precipitate and placed in boiling water bath for 30 min. The mixture was allowed to cool in room temperature, 0.5 ml 40% TCA was added to each sample. After centrifugation for 10 min at 3000 rpm, the supernatant was separated and 0.5 ml 5% M TBA was added to 1 ml of supernatant solution, then the whole was set in 40°C water bath for half an hour. At the end, the absorbance of the sample was measured in 443 nm (Perkin Elmer, l25, Norwalk, CA, USA).

In Vivo Study

Animals

Sprague-Dawley rats, weighing (120 to 150 g at the start of the study) were obtained from central animal facility, Sri Ramachandra University, Chennai, India. Animals were housed in colony cages (5-6/cage) and were kept under laboratory standard conditions with temperature (22±2°C), 12-h light/12-h dark cycle and relative humidity 40-60%. They had free access to Nutrilab rodent pellet feed (Tetragon Chemie Pvt Ltd, Bangalore, India) and purified water *ad libitum* prior to the dietary manipulation. The study protocol was approved by the Institutional Animal Ethics Committee (XIth IAEC/SRMC & RI/61/19/12/2006).

Acute Oral Toxicity

Acute oral toxicity study was performed according to the Organisation for Economic Co-operation and Development (OECD) test guideline 423: acute toxic class (ATC) method. Female Sprague Dawley rats (120-150 g) were used for the study and they were acclimatized for a period of 7 days before the start of the experiment. The experimental animals were divided into two groups (n=3/group). Group I served as control and received water as vehicle (10 ml/kg, p.o) and Group II received DIA-2 (2000 mg/kg, p.o) prepared in water. Mortality, clinical signs such as convulsion, abnormal respiration, piloerection, changes in skin and fur, locomotor activity, tremors, salivation, diarrhoea and lethargy were recorded at timely intervals for 24 h, with special attention given during first 4 h following drug administration and once a day for 14 days thereafter. Gross pathological changes were observed and recorded at the end of the study.

Antidiabetic activity

Induction of Type 2 Diabetes in Rats[21,22]

The rats were allocated to two dietary regimens consisting of 10 and 80 animals by feeding either

normal pellet diet (NPD) or high fat diet (HFD) *ad libitum*, respectively for the initial period of four weeks. The composition of the high fat diet is given in the Table I. After 4 weeks of dietary manipulation, HFD animals with ~3 fold total cholesterol (TC) and ~4 fold triglycerides (TG) levels increase were defined as hyperlipidemic, and were injected with low dose (35 mg/kg, i.p) of streptozotocin (STZ) (freshly prepared in ice cold citrate buffer pH 4.5). NPD animals were injected with citrate buffer (1ml/kg, i.p). 72 h after STZ injection, the rats with the fasting glucose level ≥ 250 mg/dl were considered diabetic and selected for the anti-diabetic study. The rats were kept on their respective diets until the end of the study.

Treatment and Groups

Treatment was scheduled once a day for 14 consecutive days. Group 1 kept on NPD while groups 2-7 on HFD throughout the study period.

Group 1: NPD + citrate buffer (1 ml/kg, i.p) + received 0.5% Carboxy Methyl Cellulose [CMC] (10 ml/kg, p.o); Normoglycemic (NG)
Group 2: HFD + STZ (35 mg/kg, i.p) + received 0.5% CMC (10 ml/kg, p.o); Hyperglycemic (DG)
Group 3: HFD + STZ + RG (8 mg/kg, p.o) (RG)
Group 4-7: HFD + STZ + DIA-2 at 62.5, 125, 250 and 500 mg/kg, p.o, respectively.

Weekly body weight, fasting plasma glucose (FPG), fasting plasma insulin (FPI), total cholesterol (TC) and triglycerides (TG) were measured in all the experimental animals to ascertain the role of DIA-2 in type II diabetic state.

Protein Carbonyl Content

Liver protein carbonyl content was determined as described earlier[23] with minor modifications.

Table I. Composition of high fat diet (HFD).

Ingredients	Percentage/100 g
Wheat	23.4
Yellow corn	23.4
Milk powder	19.5
Calcium chloride	1.0
Crude coconut oil	15.6
Pork lard	15.6
Vitamin B_{12}	0.5
Common salt	1.0

Protein carbonyl content was assayed by taking 50 µl of tissue homogenate followed by the addition of 800 µl of 2,4-dinitrophenylhydrazine (0.2%) to the test sample and 2N HCl to the control. This mixture was incubated in dark at room temperature for 1 h. Tubes were vortexed for every 15 min. Reaction was immediately arrested by the addition of 1 ml of 20% TCA. Tubes were incubated in ice for 5 min. The supernatant was removed by centrifugation at 10,000 × g for 10 min. The pellet was resuspended in 1ml of 10% TCA and the tubes were again placed in ice and incubated for 5min. Supernatant was removed by centrifugation at 10,000 × g for 10 min. Pellet was resuspended in 1 ml of ethanol:ethylacetate (1:1) and washed for 3 times. The pellet obtained was suspended in guanidine hydrochloride and centrifuged at 10,000 × g for 10 min at 4°C. Then, 1 ml of the supernatant was taken and the absorbance was read at 360 nm using spectrophotometer (Perkin Elmer, l25, Norwalk, CA, USA). Guanidine HCl reagent serves as blank. Results were expressed as µmoles/g tissue.

Statistical Analysis

Results were expressed as mean ± standard error mean (SEM). Mean difference in body weight, FPG, FPI, TC, TG and protein carbonyl content were analysed by one way ANOVA followed Dunnett's multiple comparison as post hoc test. A $p < 0.05$ was considered to be significant.

Results

In Vitro Studies

Effect of ASE, LSE and DIA-2 on Insulin-Stimulated Glucose Uptake in Differentiated 3T3-L1 Adipocytes

Figure 1 shows the effect of ASE, LSE and DIA-2 on insulin stimulated glucose uptake in differentiated 3T3-L1 adipocyte cells. Pretreatment with various concentrations (1 pg to 10 µg/ml) of ASE, LSE and DIA-2 were able to potentiate the glucose uptake in 3T3-L1 adipocytes in a concentration dependent manner up to 0.01 µg/ml; further increase in the concentration of drugs produced an opposite effect. ASE and LSE showed a maximum of 122.32±0.58% and 145.16±1.32% of glucose uptake, respectively, at a concentration of 0.01 µg/ml. DIA-2 was able to improve the glucose uptake at 10 fold lesser concentration (0.001 µg/ml) showing 151.51±0.9% of glucose uptake com-

pared to ASE and LSE. Rosiglitazone (RG) was used as positive control which showed 163.9±0.42% at a concentration of 50 µM.

Effect of ASE, LSE and DIA-2 on Erythrocyte Sorbitol Accumulation Inhibition

In diabetic state, the slow and steady activation of sorbitol pathway results in augmented accumulation of intracellular sorbitol which ultimately ends in diabetic complications. Prevention of intracellular sorbitol accumulation is a widely recommended concept to avert diabetes related complications. In the present study, ASE, LSE and DIA-2 showed inhibitory effect against sorbitol accumulation. The half maximal inhibitory concentration (IC_{50}) value of ASE and LSE were found to be 21.7 and 80.97 µg/ml respectively. Interesting, the IC_{50} value of DIA-2 was found to be 0.16 µg/ml, which was found to be synergistic when compared to the individual extracts. Ascorbic acid was used as positive control which showed an IC_{50} value of 9.50 µg/ml (Table II).

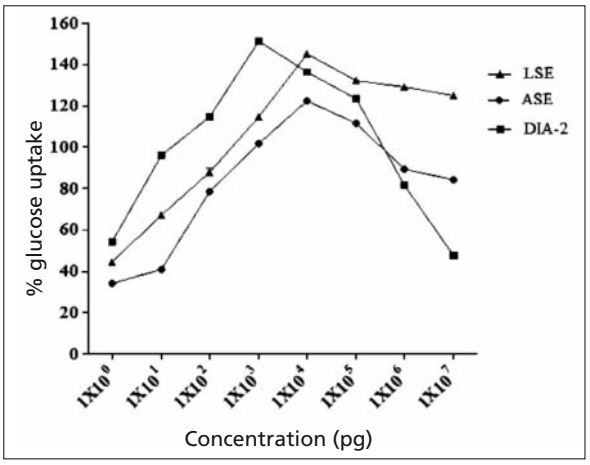

Figure 1. The differentiated 3T3-L1 adipocytes were incubated with ASE or LSE or DIA-2 (1 pg to 10 µg/ml) for 24 h. The cells were then stimulated with insulin (100 nM) for 20 min followed by rinsing with Krebs Ringer phosphate HEPES (KRPH) solution (118 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 30 mM HEPES – pH 7.4). The cells were subsequently pulsed for 20 min in KRPH solution containing 0.5 µ Ci/ml 2-DOG. The assay was terminated by aspirating the medium. The cells were then washed thrice with ice cold KRPH solution and lysed in 0.1% SDS. The lysate was transferred to a 96-well plate (Packard) with glass fiber paper and air dried overnight. The radioactivity of the samples was measured using a top count liquid scintillation counter. The amount of 2-DOG taken up by cells incubated without insulin is subtracted from the amount of radioactivity taken up by cells incubated with insulin and or extracts giving the true insulin-stimulated glucose uptake value. The results were expressed as % change in glucose uptake with respect to the solvent control (DMSO). Data are mean ± SEM (n = 3). *$p < 0.05$ as compared with untreated control group.

Effect of ASE, LSE and DIA-2 Protein Glycation

The nonenzymatic adduct, formation between the keto group of sugar and amino group of proteins, a process called protein glycation is one of the molecular basis of diabetic complications in hyperglycemic state. Inhibition of this process will be useful in the management of diabetic complications. In the present study, the effect of ASE, LSE, and DIA-2 against protein glycation was studied and their potency was compared with the standard antiglycation agent, aminoguanidine (AG). 7 days exposure to ASE, LSE and AG showed a maximum of 65.79±0.18, 69.39±0.32 and 55.01±0.91% of inhibition at 30 µg/ml, where as DIA-2 showed a synergistic effect with 66.14±0.19% inhibition at 10 µg/ml (Figure 2).

In Vivo Study

The results of *in vitro* studies triggered to evaluate the effect of DIA-2 in experimental rat model of type II diabetes and associated complications.

Acute Oral Toxicity of DIA-2

Administration of DIA-2 once orally at 2000 mg/kg, b.wt didn't produced mortality, morbidity or toxic signs during the 14 days follow up period. There were no significant changes in body weight (Table III) and gross pathology findings between the vehicle and DIA-2 administered rats during the course of the experiment. Hence, DIA-2 can be classified as category-5 in the Globally Harmonised System (GHS), the hazard category defined by: 2000 mg/kg < LD_{50} < 5000 mg/kg, which means that DIA-2 is a non-toxic regimen.

Effect of DIA-2 on Body Weight in Type II Diabetic Rats

Weekly changes in the body weight of the normoglycemic group (NG) and diabetic groups (DG) were recorded and shown in Figure 3. Four weeks of dietary manipulation with high fat diet increased the body weight significantly ($p < 0.05$) in DG groups (Group 2-7) when compared to the animals in NG group. Administration of low dose of STZ decreased (~6-8%) the body weight in the DG animals when compared to their day 0 weight during the course of the experiment. Except group V (125

Table II. Effect of ASE, LSE and DIA-2 on sorbitol accumulation in erythrocyte.

Test substance	Inhibition (%) [concentration in µg/ml]	IC_{50} (µg/ml)
ASE	36.96 ± 0.19 [25]	21.7
LSE	37.96 ± 0.18 [250]	80.97
DIA-2	54.67 ± 0.58 [100]	0.16
[b]AA	43.12 ± 0.35 [250]	9.50

Erythrocytes were separated from plasma of heparinized rat blood by centrifuging at 1500 g for 10 min. The cells were routinely washed three times with cold isotonic saline. 200 µl of erythrocyte were then incubated in the presence or absence of ASE, LSE, DIA-2 and AA dissolved in Hank's balanced salt solution (pH 7.4) containing 28 mM glucose at 37°C for 3 h. The erythrocytes were washed with cold saline by centrifuging at 1500 g for 10 min, precipitated by adding 6% of cold perchloric acid (2 ml),and centrifuged again at 1500 g for 10 min.The supernatant was neutralized with cold 1 M K_2CO_3 and used for sorbitol determination. The reaction mixture contained the appropriate supernatant, 50 mM glycine buffer (pH 9.4), 0.2 mM NAD^+, and 1.28 U SDH. The incubations were performed at 37°C for 30 min, and the relative fluorescence due to NADH was measured by a fluorescence spectrometer at an excitation wavelength of 366 nm and an emission wavelength of 452 nm. The IC_{50} value was defined as the concentration of the extract required to inhibit 50% of the sorbitol accumulation under the assay conditions specified. AA (5-100 µg/ml) was used as positive control. Data are ± S.E.M (n =3). *$p < 0.05$ as compared with control group.

mg/kg), there is no significant difference in body weight between the DIA-2 and DG groups. On the other hand, animals treated with DIA-2 showed no significant change in body weight when compared to their respective day 0 levels. However, RG treated animals showed significant ($p < 0.01$) increase when compared to the DG group rats.

Effect of DIA-2 on FPG, FPI, TC and TG Levels in type II Diabetic Rats

The effect of the DIA-2 on FPG level is shown in Figure 4. STZ administration resulted in significant ($p < 0.01$) elevation (~4 fold) in FPG levels in all groups when compared to citrate buffer administered NG animals. Vehicle treated DG animals demonstrated persistently higher FPG levels throughout the study. Treatment with DIA-2 decreased the glucose levels significantly ($p < 0.01$) at 62.5 and 125 mg/kg in a dose dependent manner and a non-significantly at 250 and 500 mg/kg during the course of the experiment. RG decreased glucose levels by 47.81% and 57.91% on day 7 and 14, respectively and the effect of DIA-2 (125 mg/kg; 47.84% and 60.04% on day 7 and 14, respectively) was very much comparable with that of RG.

The effect of DIA-2 on FPI is shown in Figure 5. Induction of diabetes decreased the plasma insulin by four fold ($p < 0.01$) in the vehicle treated diabetic rats when compared to the normoglycemic animals. Treatment with DIA-2 increased insulin levels by 18.52% ($p < 0.05$) and 39.26% ($p < 0.01$) at 62.5 and 125 mg/kg, respectively, when compared to the hyperglycemic group. RG restored by 11.11% as compared to hyperglycaemic group. However, DIA-2 at 250 and 500 mg did not show any significant change on insulin levels.

Experimental animals fed with high fat diet and subsequent injection of STZ showed elevated levels of TC and TG (~4 fold and ~6 fold, respectively). Fourteen days treatment with RG decreased TC ($p < 0.01$) and TG ($p < 0.05$) level in comparison to vehicle treated hyperglycemic rats. Administration of DIA-2, at all tested dose levels, decreased TC level significantly ($p < 0.01$) when compared to the vehicle treated hyperglycemic rats. After 14 days of treatment, DIA-2 at the dose level of 125 mg/kg brought down the TC (56.44±4.61 mg/dl) and TG (83.08±7.48 mg/dl) near equal to normoglycemic animals. DIA-2 didn't produce any significant effect on TG levels (Figures 6, 7).

Effect of DIA-2 on Liver Protein Carbonyl Content in Diabetic Rats

Influence of DIA-2 on liver protein carbonyl content in the experimental rats is shown in Fig-

Table III. Body weight changes on acute oral exposure of DIA-2.

Treatment	Day -1[a]	1[b]	1[c]	2	7	14
Vehicle	135.87 ± 2.49	127.70 ± 2.78	132.27 ± 3.21	136.37 ± 2.80	141.60 ± 2.25	145.27 ± 1.65
DIA-2	134.00 ± 2.00	123.27 ± 2.43	125.00 ± 2.05	130.00 ± 2.07	129.33 ± 4.07	139.00 ± 1.70

Changes in body weight were recorded [a]before 18 h fasting, [b]before 1 h of DIA-2 administration, [c]6 h after DIA-2 administration and thereafter on day 2, 7 and 14. Values are expressed as mean ± SEM (n=3).

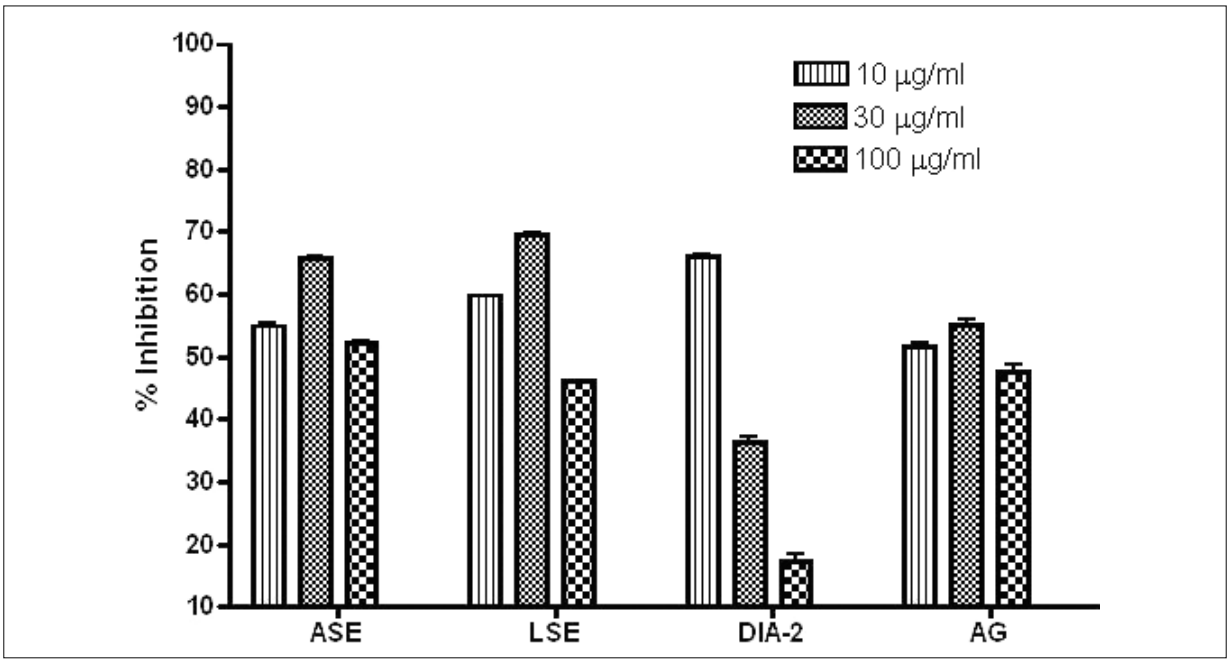

Figure 2. A reaction mixture consists of equal volume of 25 mM glucose and 25 mM fructose dissolved in 0.2 M phosphate buffer (pH 7.4) containing 0.02% sodium azide and 10 mg/ml BSA in 0.2 M phosphate buffer was incubated in presence or absence of ASE, LSE, DIA-2 and AG (10, 30,100 µg/ml) at 37°C for 7 days. After 7 days the reaction mixture was dialyzed extensive dialysis over night in the phosphate buffer to remove sugars from the reaction mixture. The proteins present in the reaction mixture were precipitated with 40% trichloroacetic acid. The glucose moiety of glycated albumin is converted to furfuraldehyde upon heating in boiling water bath after addition of 1 M oxalic acid to the precipitate. An adduct is formed by reacting 2-thiobarbituric acid with furfuraldehyde, results in a compound which is measured photometrically at 443 nm. The % of inhibition was calculated and expressed as mean ± SEM (n=3). AG was used as a positive control.

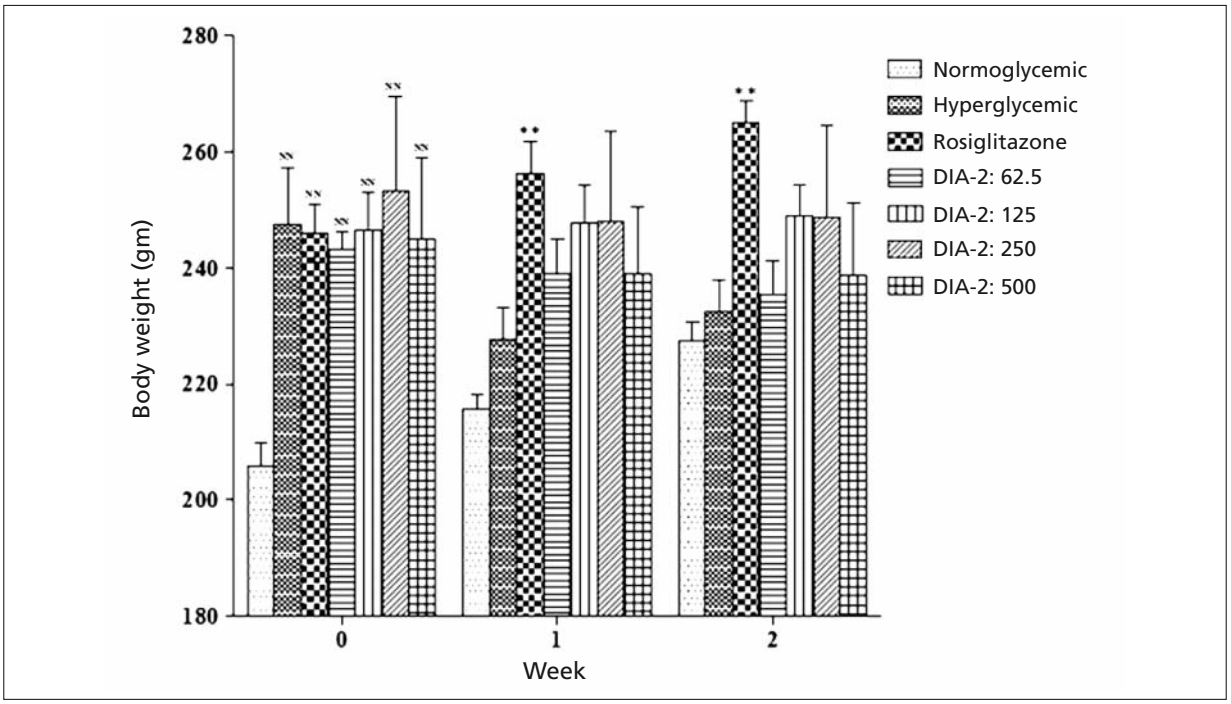

Figure 3. Effect of DIA-2 on body weights in the type II diabetic rats. Values are expressed as mean ± SEM (n=6); NN & N - *p* value of 0.01 & 0.05 compared to the normoglycemic group; **&* - *p* value of 0.01 & 0.05 compared to the hyperglycemic group.

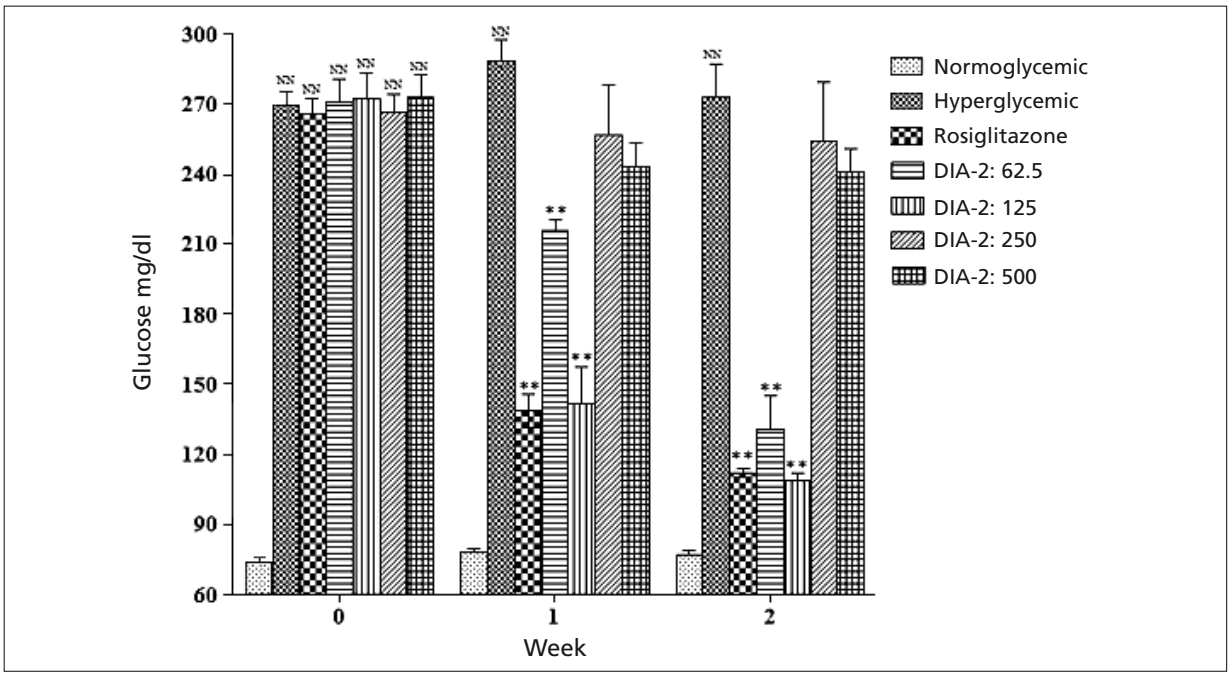

Figure 4. Effect of DIA-2 on FPG level in the type II diabetic rats. Values are expressed as mean ± SEM (n=6); NN & N - *p* value of 0.01 & 0.05 compared to the normoglycemic group; **&* - *p* value of 0.01 & 0.05 compared to the hyperglycemic group.

ure 8. Induction of type II diabetes increased the liver protein carbonyl content in the vehicle treated hyperglycemic rats. Treatment with DIA-2 at 62.5 and 125 mg/kg produced significant and dose dependent decrease in liver protein carbonyl content in the diabetic rats. However, DIA-2 at 250 and 500 mg/kg didn't produce any significant effect on protein carbonyl content. RG failed to decrease the protein carbonyl content in the diabetic rats.

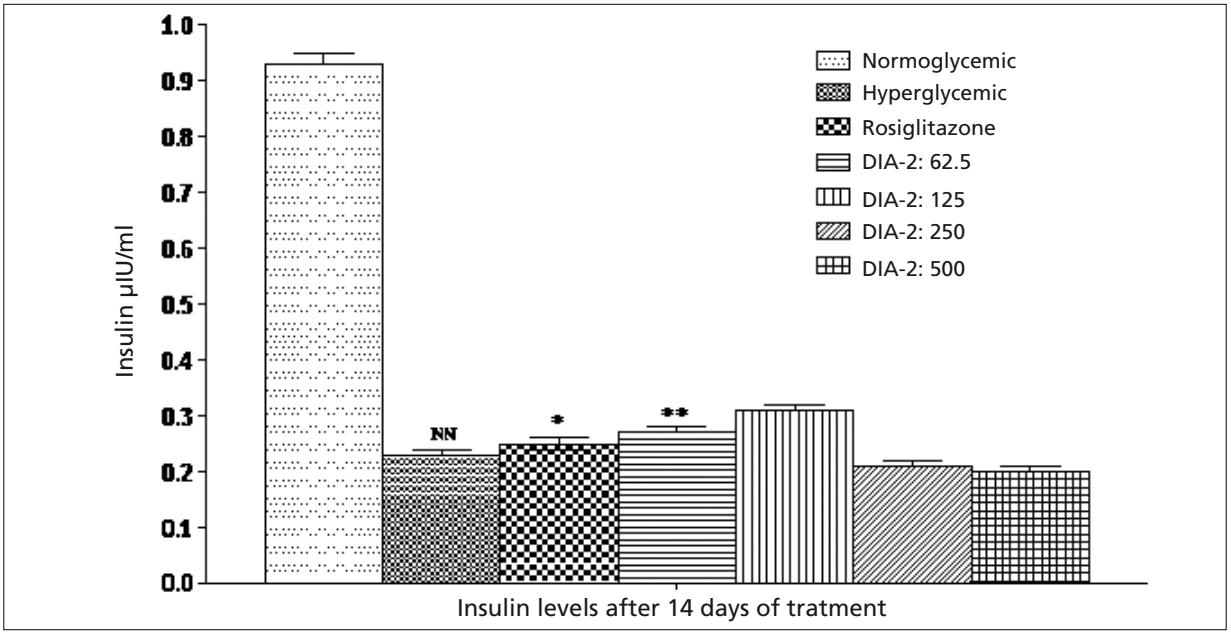

Figure 5. Effect of DIA-2 on FPI level in the type II diabetic rats. Values are expressed as mean ± SEM (n=6); NN & N - *p* value of 0.01 & 0.05 compared to the normoglycemic group; **&* - *p* value of 0.01 & 0.05 compared to the hyperglycemic group.

Discussion

In Vitro Studies

In the insulin resistance state, the glucose transporter-4 (GLUT-4) translocation is decreased. In the present investigation, ASE, LSE and DIA-2 were tested for their glucose uptake enhancing effect. 3T3-L1 differentiated adipocytes are the most preferred cell line to study insulin-stimulated glucose uptake assay by *in vitro* method. 3T3-L1 preadipocytes expresses non-insulin-sensitive glucose transporter GLUT-1, but when it gets differentiated it expresses the insulin-responsive glucose transporter GLUT-4. The maximum percentage of 2-DOG uptake into the differentiated 3T3-L1 adipocytes was found to be 145.16±1.32% on addition of 0.01 µg/ml of LSE compared to vehicle control. Corosolic acid, a triterpenoid compound[24] and Lagerstroemin, a potent ellagitannins present in banaba leaves are responsible for glucose transport stimulatory activity[14,25]. Similarly at 0.01 µg/ml ASE showed 122.32±0.58% of glucose uptake.

The pharmacologic properties of garlic are extremely complex and are mainly derived from the organo-sulfur compounds. Administration of fresh garlic juice and processed garlic has been reported to modulate GLUT-4 and peroxisome proliferator-activated receptors (PPARs) expression in heart tissues, through the up regulation of GLUT-4, PPARα and PPARδ. Both freshly crushed garlic and processed garlic provide cardioprotection, the former has additional cardioprotective properties presumably due to the presence of H_2S[26]. In the present status, it is assumed that the glucose uptake activity of ASE may be due to the presence of these organosulfur compounds. DIA-2 showed maximum of 151.51±0.9% glucose uptake at a 10 fold lower concentration (0.001 µg/ml), than that of ASE and LSE. PPARγ agonist, RG (50 µM), was used as positive control which showed 163.9±0.42% of glucose uptake compared to vehicle control. The ellagitannins present in banaba[8,12] and diallysulphide (DAS) present in garlic[7,13] were also responsible for anti-adipogenic effect. Most of the

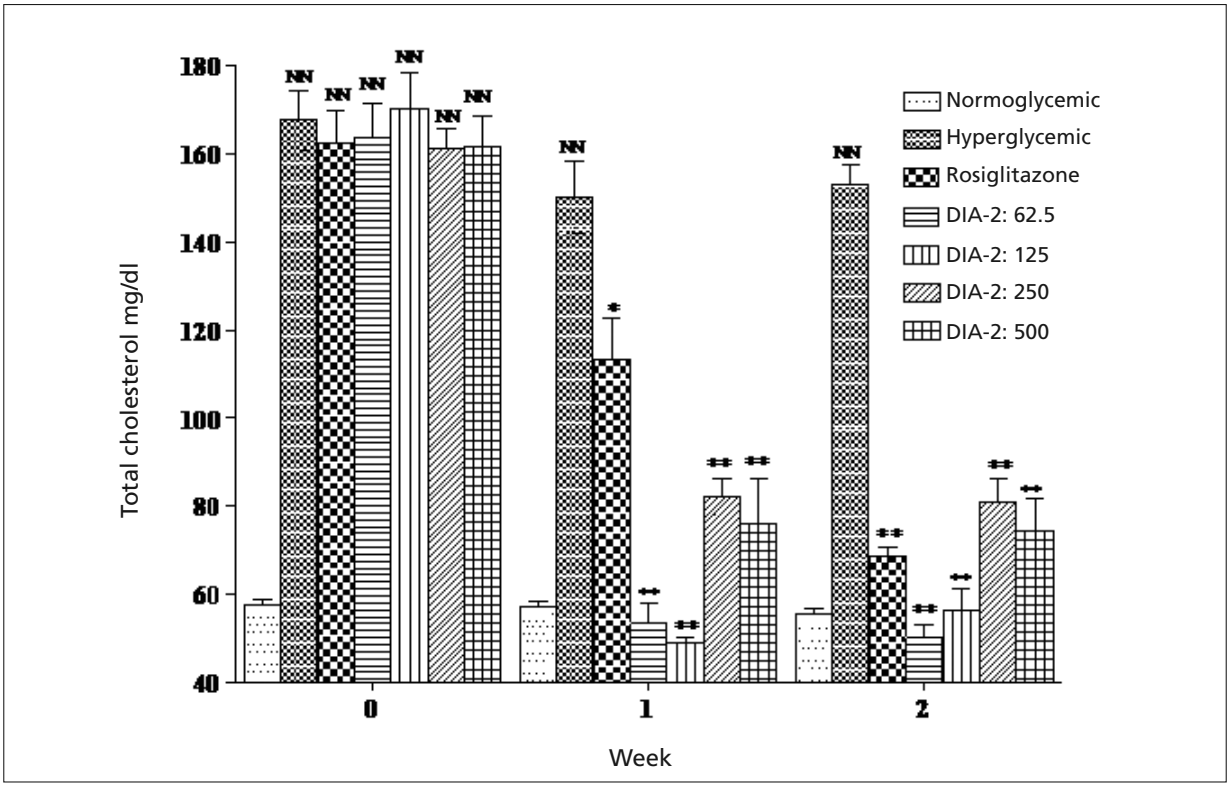

Figure 6. Effect of DIA-2 on TC level in the type II diabetic rats. Values are expressed as mean ± SEM (n=6); NN & N - *p* value of 0.01 & 0.05 compared to the normoglycemic group; **&* - *p* value of 0.01 & 0.05 compared to the hyperglycemic group.

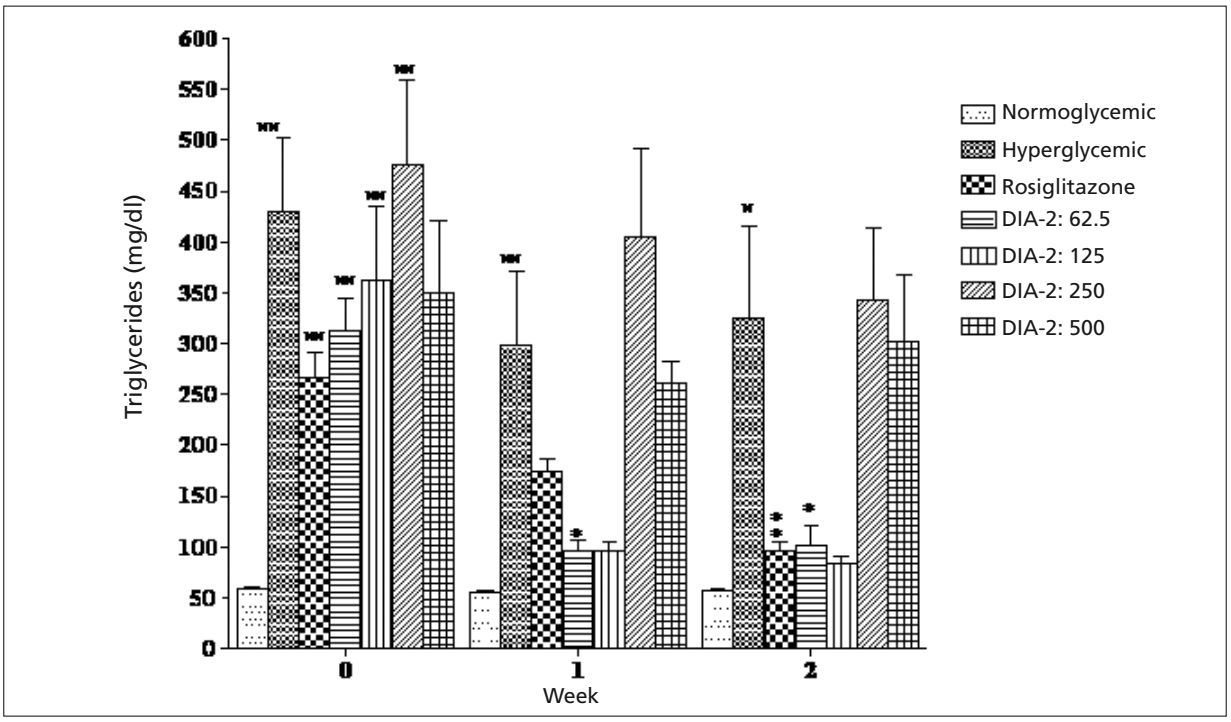

Figure 7. Effect of DIA-2 on TG level in the type II diabetic rats. Values are expressed as mean ± SEM (n=6); NN& N - *p* value of 0.01 & 0.05 compared to the normoglycemic group; **&* - *p* value of 0.01 & 0.05 compared to the hyperglycemic group.

antidiabetic drugs such as insulin or thiazolidinediones (TZD), except few, up regulate both glucose transport and lipid biosynthesis in adipocytes and causes gain in body weight. Thus, these drugs treat one of the key symptoms of type 2 diabetes, hyperglycemia, but exacerbate the condition of being overweight or obese. Therefore, while these drugs are beneficial over the short term, they may not serve the need for long term health. The most desirable situation would be the development of new types of antidiabetic drugs that are either hypoglycemic or antihyperglycemic without the side effect of promoting weight gain. Both LSE and ASE have better glucose uptake activity and anti-adipogenic effect. DIA-2 is a unique combination having both these properties, suggest that it may be useful for the management of hyperglycemia and obesity in type II diabetes

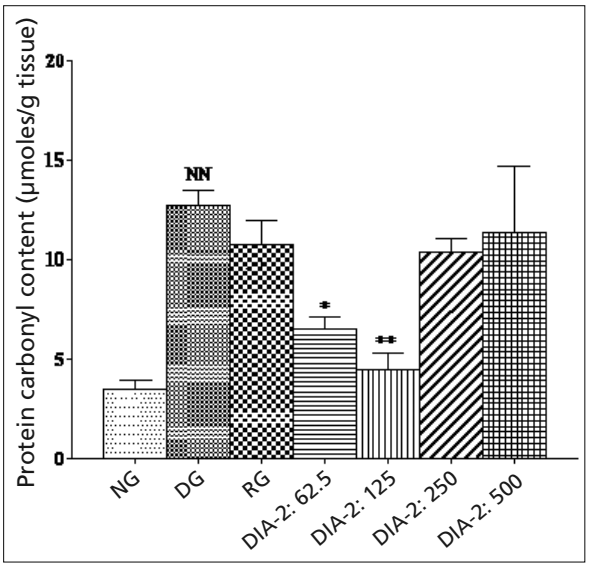

Figure 8. Effect of DIA-2 on protein carbonyl content in the liver of type II diabetic rats. Values are expressed as mean ± SEM (n=6); NN & N - *p* value of 0.01 & 0.05 compared to the normoglycemic group; **&* - *p* value of 0.01 & 0.05 compared to the hyperglycemic group.

In the diabetic state to maintain glucose homeostasis the excess glucose enters the polyol pathway, which includes enzymatic reduction of glucose to sorbitol by NADPH dependent aldolase reductase (AR) followed by oxidation of sorbitol to fructose by NAD dependent sorbitol dehydrogenase (SDH). The impermeability of sorbitol through the cell membrane causes osmosis in cell, one of the main causative factors in the long term complications. In non-diabetics, sorbitol is converted to fructose and is easily excreted from the cell. AR in-

hibitors like hydantoin derivatives (Sorbinil) and carboxylic acid derivatives (Epalrestat) are useful, but limited in use because of undesirable side effects. We here demonstrate the decreasing order of potency in terms of IC_{50} value: DIA-2 (0.16 µg/ml) < AA (9.50 µg/ml) < ASE (21.70 µg/ml) < LSE (81.00 µg/ml). It was found that tannoid principles of *Emblica officinalis* were potent inhibitors of rat lens AR and human recombinant AR[27]. The tannins present in LSE might have possible role in inhibition of AR. During diabetes AR levels are expressed intensely in insulin-independent tissues, over-expression of AR enhances production of reactive oxygen species (ROS), which cause membrane damage and cellular leakage. On the other hand, the organosulfur compound (DAS) present in garlic was reported to reduce the expression of AR and subsequently lowering the production of ROS[28]. DAS present in ASE may be responsible for reduced expression of AR. AA inhibits the enzymatic conversion of glucose to sorbitol by virtue of it structural similarity with glucose. Due to this structural similarity, AA competes with glucose for transport into cells. In the diabetic state the uptake of AA into cells appears to be impaired. When ascorbic acid (AA) is supplemented orally to diabetic patients it may help prevent many of the complications of diabetes[29]. DIA-2 showed enhanced inhibitory activity on sorbitol accumulation in erythrocytes at 0.16 µg/ml compared to its individual ingredients which might be due to multiple effects of tannins and organosulphur compound (DAS) present in LSE and ASE, respectively.

Glycation is a complex chemical reaction and occurs between reactive aldose or ketose sugars and protein bound free amino groups. An initial labile Schiff's base formed during the reaction undergoes Amadori rearrangement and yields a stable ketoamine derivative. Following dehydration and subsequent chemical modification, some Amadori products accumulate with time as advanced glycation end products (AGEs). The formation of AGEs has been proposed to play an important role in the pathogenesis of the long term complications of diabetes. AGE inhibitors inhibit the glycation cascade and offer a promising therapeutic approach for the prevention of diabetic complications. Unfortunately, clinical trials of these inhibitors in diabetic patients have been suspended due to their adverse effects. In the present study, the effect of ASE, LSE, DIA-2 and aminoguanidine (AG) on inhibition of AGEs formation was demonstrated by *in vitro* method using bovine serum albumin (BSA) as a model of protein because it is a key protein found abundantly in human plasma. The nature of the reducing sugars influences the rate and extent of the glycation. A mixture of fructose and glucose was included in our study, because fructose is present in tissues at a concentration comparable to that of glucose and reacts with protein approximately 10 times more rapidly than glucose. DIA-2 showed maximum % (66.14 ± 0.19) at 10 µg/ml, while ASE (65.79 ± 0.18), LSE (69.39 ± 0.32) and AG (55.01 ± 0.91) showed maximum % of inhibition at 30 µg/ml. S-allyl cysteine, present in garlic is responsible inhibitor of formation of AGE[15,30].

Non-enzymatic glycation plays a major role in the development of diabetic complications[33]. Transglycation is a deglycation process operating on the very first product of nonenzymatic glycation i.e. Schiff's bases. Glucose-cysteine is one of the transglycation product found at increased concentration during diabetes[34]. ASE is rich in S-allyl cysteine, S-ethylcysteine, N-acetylcysteine[15,30,35,36]; we assume that the presence of these cysteine residues may influence the formation of sugar-cysteine adduct than sugar-protein adduct, thereby preventing the formation of AGE end products. No studies on LSE pertaining to its role on glycation have been so far carried out, but it has been reported that polyphenols, condensed tannins and flavonoids can inhibit the glycation process. LSE is reported for its rich in presence of these bioactive principles may have potential role on inhibiting the glycation process[37]. Administration of organosulphur compound, diallyl tetrasulfide isolated from ASE was found to normalize the elevated liver protein carbonyl content in cadmium induced oxidative damage in rats[38]. Tannins have shown to be beneficial in protecting protein oxidation and glycation. LSE may have probable action on protein oxidation as well due to rich availability of tannins[39].

In Vivo Study

There were no mortality or morbidity or toxic signs observed following DIA-2 administration (2000 mg/kg). The major organ systems such as cranial, thoracic and abdominal cavities were examined visually in the euthanized animals and no gross histopathological alterations were found at necropsy. Hence, DIA-2 shall be considered as "Category 5 or Unclassified" in accordance to Globally Harmonized Classification System (GHS).

One of the classical symptoms of diabetes mellitus is weight loss. Induction of diabetes with STZ showed significant loss in body weight which may

be due to increased muscle wasting and proteolysis of muscle proteins. Treatment with RG increased the body weight in animals however DIA-2 treated animals showed non significant change in body; weight at all dose levels. Newer classes of antidiabetic drugs are beneficial only over a short term period; they are not optimal for long term use, since they promote weight gain and exacerbate the condition of being overweight or obese, one of the leading causes of type 2 diabetes. Reducing weight gain constitutes a way to delay the rate of incidence of type 2 diabetes. The most desirable situation would be the development of an antidiabetic drug without the side effect of promoting weight gain. We observed that the weight loss that occurred in STZ diabetic rats was attenuated by DIA-2 treatment. Hence, DIA-2 may be useful in the management of type-2 diabetes without increasing the body weight.

Administration of HFD and low dose of STZ was considered as an ideal model for pharmacological screening of drugs for type 2 diabetes mellitus (T2DM) since it resembles the clinical manifestation of type 2 diabetes in humans. Administration of low dose of STZ to rats fed with HFD for 4 weeks was characterized by hyperglycemia and light impaired insulin secretion[40-41]. The low dose of STZ partially destroys the pancreatic beta cells with deficiency in insulin secretion (see Figure 5) rather destroying it completely and its administration along with high fat diet induces insulin resistance in rats[22]. The DIA-2 restored 18.52% ($p < 0.05$) and 39.26% ($p < 0.01$) of insulin levels respectively in a dose dependent manner at 62.5 and 125 mg/kg when compared to the hyperglycemic group. Capacity of DIA-2 to increase insulin and decreased plasma glucose level demonstrates the insulin secretagogue and sensitizer effect.

Hypercholesteromia and hypertriglyceridemia is one of the common lipid metabolic abnormalities in diabetes resulting in insulin resistance and, thereby, reducing the capability of glucose utilization stimulated by insulin. This may be the reason for perpetuation of elevated glucose level (> 250 mg/dl) in hyperglycemic group throughout the study. The DIA-2 significantly decreased the plasma TC and TG levels in diabetic rats and, thereby, improving glucose metabolism and utilization.

The presence of organosulphur compounds and nonsulfur compounds such as saponins in ASE may work synergistically to produce various biological effects[31]. Corosolic acid, a triterpenoid compound present in LSE has been widely claimed for its antidiabetic, anti-adipogenic, antioxidant, anti-obesity and insulin-like properties. It has also been reported to have possible role on carbohydrate and lipid metabolism[32]. DIA-2 treatment has the ability to normalize the altered lipid and glucose metabolism and may hence have the potential to manage diabetes mellitus. However, the research on botanical based preparations is not easy to pursue. We have attempted to develop this unique herbal preparation with minimum number of standardized ingredients and investigated its ability to manage diabetes without gain in body weight with grounded scientific evidence.

Protein carbonylation is an irreversible oxidative damage which is an indicator of severe oxidative damage and disease – derived protein dysfunction. In the present study, induction of type II diabetes triggered fourfold increase in protein carbonylation in the liver tissues of diabetic rats. Administration of organosulphur compound, diallyl tetrasulfide isolated from ASE was found to normalize the elevated liver protein carbonyl content in cadmium induced oxidative damage in rats[38]. Tannins have shown to be beneficial in protecting protein oxidation and glycation. LSE may have probably had certain role in preventing protein oxidation. Interestingly, DIA-2 decreased protein carbonylation significantly which demonstrates its role on oxidative stress in diabetic state.

Regular examination of liver enzymes is still recommended with patients with certain class of prescription anti-diabetic drugs[4]. Within the tested dose levels (62.5, 125 and 250 mg/kg body weight), a repeated oral toxicity was conducted as per the OECD-407 test guideline in rats. The results (data not shown) revealed that repeated oral exposure of DIA-2 did not affect the liver enzymes in both the sexes of the rat up to the dose of 250 mg/kg body weight.

Conclusions

DIA-2 a combination of garlic and banaba extracts showed significant synergistic effect in the tested *in vitro* models of diabetes and associated complications. Further, the *in vivo* study demonstrate that oral administration of DIA-2 for 14 days evokes a beneficial effect on the hyperglycemic associated with hyperlipidemia and other diabetic complications. These findings show that consumption of DIA-2 could prevent or be helpful in reducing the complications of diabetes.

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