

## CHRONIC TREATMENT OF DIABECON IN THE REGULATION OF ALLOXAN INDUCED HYPERGLYCEMIA AND OXIDATIVE STRESS IN DIFFERENT TISSUES OF ADOLESCENT DIABETIC RATS

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### ABSTRACT

**Objective:** Diabetes mellitus in the youngsters is a rising concern that has become a serious metabolic disorder. The present study was aimed to assess the effects of chronic treatment of an ayurvedic drug, Diabecon on different body organs (liver, kidney, heart and testis) of premature diabetic male rats.

**Methods:** Four weeks old rats were made diabetic by alloxan and then they were routinely treated with Diabecon (1 or 2 gm/kg) for next eight weeks. Fasting serum glucose, body weight, tissue lipid peroxidation (LPO), activities of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GSH) were measured as oxidative stress biomarkers. *In vitro* 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) free radical scavenging efficacies were also tested.

**Results:** Diabetic rats showed hyperglycaemia, raised tissue LPO with decreased SOD, CAT and GSH in all tested tissues, while animals kept on test drug exhibited improvement in the most of the studied indices. Interestingly, the overall protective effects were found to be tissue specific. However, both the test doses were found to be more or less equally effective. *In vitro* DPPH and NO free radical scavenging activities and enhanced cellular antioxidant studies suggested that the drug may be directly involved in free radical removal and in up-regulation of the antioxidant synthesis.

**Conclusion:** Thus, the present results not only affirmed the diabetes ameliorating effects of Diabecon in adolescent male rats but also supported the role of antioxidants in its mechanism of action.

**Keywords:** Polyherbal drug, Adolescent diabetic rats, Antioxidant, Reduced glutathione, Testis, Serum glucose

### INTRODUCTION

The number of adolescent diabetic population is increasing rapidly in the world[1]. Recent epidemiological studies indicated two-to-six fold increase in the prevalence of child hood and adolescent diabetes in last two decades, leading to increased rate of mortality and morbidity[2]. Thus, it is thought to be a serious metabolic syndrome of the century[1,2]. Moreover, people having positive family history of diabetes mellitus (DM) and/or cardiac dysfunctions are known to be at higher risk of these metabolic disorders[3]. The major reasons for the increasing prevalence of DM may be changing life style, energy rich dietary habits, physical inactivity and stress[2,4]. Other than these factors, increase in oxidative stress plays an essential role in the onset and progression of DM[4,5]. Hyperglycaemia, the main symptoms of DM also induces generation of reactive oxygen species (ROS) which in turn cause lipid peroxidation (LPO), DNA- damage and non-specific protein oxidation[1,5]. At the same time, ROS plays a vital role in the development of secondary complications of DM such as fatty liver, cardiovascular abnormalities, nephropathy, neuropathy, testicular dysfunctions etc[3,5-8]. Free radical mediated degradation of bio-membranes produces malondialdehyde (MDA), an important LPO product that has been extensively used as oxidative damage marker[6-8]. To get rid of diabetes and it's related problems, different classes of antidiabetic drugs have been used, though, very few proved to be satisfactorily effective[3,8]. As synthetic drugs also impose undesirable effects[1,4,6], herbal therapy is believed to be harmless and convenient for long term administration. Although, several antidiabetic monoherbal and polyherbal formulations are available, later one is believed to be superior, as these served in multidimensional way[6,8,9]. Therefore, in the present investigation, a poly herbal formulation, Diabecon was taken as test drug. It is a mixture of forty-two herbs[10] and its antidiabetic and antioxidative[10,11] properties have been studied.

However, with reference to adolescent animals, no scientific findings have been established so far. Here, owing to the unusual increase in the incidence of DM among youngsters [12,13] four weeks old male rats were used as diabetic model. To the best of our knowledge, antidiabetic and protective effects of Diabecon on immature animals were not studied till date. Moreover, in the present study, we evaluated the effects of the test drug on different body organs. Since, with respect to sexually immature diabetes, experimental data are meagre[10] and some earlier reports also stated that the early onset of DM may cause male infertility and testicular oxidative stress[2,12,13], we also intended to find out effects of the test drug on antioxidative status of testis, as these tissues are more susceptible for oxidative stress[5,13].

### MATERIALS AND METHODS

#### Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich, St. Louis, MO, USA. While 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and Carboxy methyl cellulose (CMC) were from Hi-media; Diethylene triamine penta acetic acid (DTPA), Tris-(hydroxymethyl)-ammonium methane, sodiumdodecyl sulfate (SDS), thiobarbituric acid (TBA), ethylene-diaminetetra-acetic acid (EDTA), pyrogallol, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) were purchased from E. Merck Ltd. (Mumbai, India). All other chemicals were of reagent grade and obtained from Loba chemie (Mumbai). Diabecon was purchased from registered local market store.

#### Drug preparation

Diabecon (Himalaya Drug Company, Bangalore, Batch no. 37200283B) is a polyherbal formulation of different well known antidiabetic herbs, extracts and bhasma. Aqueous suspension of

Diabecon was prepared in 0.5% Carboxy methyl cellulose (CMC), a suspending agent and drug was administered at constant volume 0.1 ml/ rat p.o./day, for 8-weeks.

#### Animals

Healthy colony bred adolescent Wistar male rats were housed in polypropylene cages under constant temp. (27±1°C) and photo schedule (14 h light + 10 h dark). They were provided standard rat feed (Golden feeds, New Delhi, India) ad libitum and had free access to drinking water. Standard ethical guidelines of the Committee for the Purpose of Control & Supervision on Experiments on Animals (CPCSEA), Ministry of Environmental and forests, Govt. Of India were followed after the approval of departmental Ethical committee (Reg. No. 779) for handling and maintenance for experimental animals.

#### DPPH assay

In this assay the methanolic stock solutions of different concentrations of all drugs were prepared. One ml of extract was mixed with 0.5 ml of 0.15 mM DPPH in dark and incubated for 30 min in dark at 20°C. OD was taken at 517 nm and the % scavenging of free radicals was calculated assuming 0% scavenging of positive control (added methanol in place of drug). Ascorbic acid was used as standard antioxidant[6,7].

#### NO assay

Standard curve was prepared using sodium nitropruside as a NO donor *in vitro*. For assay 0.5 ml sodium nitropruside (10 mM in 0.2 M PBS at pH 7.4) was added with 0.5 ml of different concentrations of drugs and incubated for 150 min at 20°C in dark and then the NO scavenging was measured in % with respect to control, ascorbic acid was used as standard[6,7].

#### Chronic drug dose study

Twenty eight healthy rats were divided into four groups of seven mice each and acclimatized for one week. Animals of groups 2-4 were rendered diabetic by single intra-peritoneal injection of alloxan (100 mg/kg, in normal saline), whereas group 1 (control) animals were injected with normal saline. Hyperglycaemia was confirmed after 72 hours of alloxan treatment (Glucochek glucometer, Aspen Diagonstic, Delhi,India). Then, animals of group 3<sup>rd</sup> and 4<sup>th</sup> were treated with Diabecon 1 and 2 gm/kg/po respectively; while, group 1 and 2 were administered with an equivalent amount of 0.5% CMC in distilled water (the vehicle) for next eight weeks. Dose was given at a fixed time (10:00-11:00 AM) of the day to avoid circadian variation, if any. Body weight was measured. On the last day overnight fasted animals were sacrificed by cervical dislocation, blood and tissues were collected and processed for different biochemical estimations.

#### Biochemical estimation

At the last day, over night fasted animals were killed after being exposed to mild anaesthesia. Blood from each animal was collected to get clear serum. After exsanguinations liver, kidney, heart and testis were removed quickly, freed from blood clots and processed for the estimation of LPO, SOD, CAT and GSH activities. For the evaluation of LPO all tissues were homogenised separately in 0.1 M

ice cold phosphate buffer saline (pH 7.4) at 10% w/v concentration. This homogenate then centrifuged at 15,000 X g at 4°C for 30 min and the supernatant was used for all biochemical tests.

In brief, LPO was evaluated by the reaction of TBA with MDA formed due to peroxidation of lipids. The amount of MDA was measured by taking OD at 532 nm (extinction coefficient,  $\epsilon = 1.56 \times 10^5$ ) using a Shimadzu UV-1600 spectrometer (Shimadzu corp. Kyoto, Japan). LPO concentration was expressed as nano-moles per litre of MDA per hour per milligram of protein[14]. Activity of SOD in all tissue homogenates was estimated by measuring the % inhibition of the pyrogallol auto-oxidation by the enzyme[15].

One unit of enzyme is defined as the enzyme activity that inhibits the auto-oxidation of pyrogallol by 50% and was finally expressed as units/mg protein. Catalase activity was estimated as  $\mu\text{M}$  of H<sub>2</sub>O<sub>2</sub> decomposed/min/mg protein[16]. Tissue glutathione content (reduced sulphhydryl groups) was measured with Ellman's reagent read at 412 nm (extinction coefficient,  $\epsilon = 1.36 \times 10^4$ ) as earlier followed in our laboratory[17]. Protein content was determined by following the method of Lowery et al[18] using BSA as standard as routinely done in our lab. Serum glucose concentration was measured by glucose oxidase method using commercially available kit[6,7].

#### Statistical analysis

Data are expressed as mean  $\pm$  SE. Statistical analysis was done by using ANOVA followed by student's t-test and P-values of 0.05 or less were considered the level of significance.

#### RESULTS

In both DPPH and NO free radical scavenging *in vitro* assays test drug showed immense antioxidative activity in concentration dependent manner. Besides exhibiting reduced radical scavenging activity as compared to standard, Diabecon significantly inhibited the free radicals in all the studied concentrations. Very high inhibition was observed at 100 and 50  $\mu\text{g/ml}$  of the drug (83.7% and 82.3% respectively), which appeared to be comparable with that of standard, ascorbic acid (87.9% and 87.6% respectively, Table 1).

In case of LPO, in alloxan induced diabetic rats (in *in vivo* studies), a significant increase was observed in all tissues (310.4%, 12.8%, 31.5%, 93% for liver, kidney, testis and heart respectively;  $P < 0.001$  for all). A similar pattern was observed in serum glucose level. Here also a significant increase in serum glucose ( $P < 0.0001$ , 285.1%) was noticed in case of diabetic group.

For liver, kidney, heart and testis there was a significant decrease in SOD (56%, 33%, 50.8% and 38.7%, respectively), CAT (46.4%, 44%, 63.8% and 67.1%, respectively) and GSH (46.8%, 53.1%, 37.9% and 135.7%, respectively) while, with respect to body weight no significant change (+1.2%) was observed.

Rats receiving drug treatments showed significant improvement in serum glucose ( $P < 0.0001$  in both drug doses). However, HD group showed significantly ( $p < 0.01$ ) more decrease in glucose (67.2%) than that of LD group (56.3%) (Figure 1). Similarly, Diabecon treatment also showed increase in body weight in both LD (34%) and HD (27.5%) groups as compared to their initial body weight.

**Table 1: 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) free radical scavenging activities (in %) of test drug at different drug concentrations as compared to a standard, ascorbic acids**

	Concentration of test drug			
	12.5 $\mu\text{g/ml}$	25 $\mu\text{g/ml}$	50 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$
DPPH assay				
Diabecon	60.72 $\pm$ 0.67 <sup>c</sup>	69.51 $\pm$ 0.48 <sup>c</sup>	76.44 $\pm$ 0.53 <sup>c</sup>	83.73 $\pm$ 0.37 <sup>b</sup>
Ascorbic acid	79.23 $\pm$ 0.28	81.50 $\pm$ 0.82	85.78 $\pm$ 0.69	87.92 $\pm$ 0.66
NO assay				
Diabecon	62.18 $\pm$ 0.67 <sup>b</sup>	66.08 $\pm$ 0.78 <sup>c</sup>	82.32 $\pm$ 0.91 <sup>a</sup>	79.86 $\pm$ 0.74 <sup>c</sup>
Ascorbic acid	70.34 $\pm$ 0.71	78.56 $\pm$ 0.58	87.56 $\pm$ 0.74	91.55 $\pm$ 0.81

Values are in means  $\pm$  SEM (n= 3). <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$  and <sup>c</sup> $p < 0.001$  less effective than equivalent concentration of ascorbic acid.

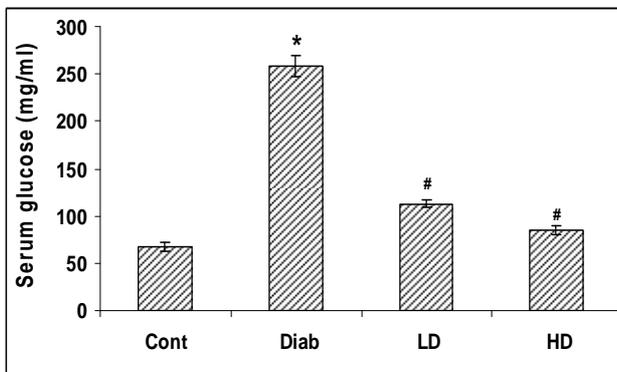
**Table 2: Effects of 8-week treatment with Diabecon on body weight (gm) in different groups of alloxan induced diabetic rats**

Groups	0 <sup>th</sup> day	15 <sup>th</sup> day	30 <sup>th</sup> day	45 <sup>th</sup> day	60 <sup>th</sup> day
Control	90.71± 1.17	102.77± 2.79 <sup>a</sup>	108.08± 0.82 <sup>b</sup>	116.77± 2.78 <sup>b</sup>	122.22± 3.07 <sup>b</sup>
Diabetic	92.71± 1.08	87.22± 2.41	94.97± 0.54	93.94± 1.22	92.81± 1.44
Diabetic+ Drug (1 g/kg)	92.57± 1.13	97.0± 2.28	106.4± 1.02 <sup>b</sup>	116.44± 1.53 <sup>b</sup>	123.97± 2.58 <sup>b</sup>
Diabetic+ Drug (2 g/kg)	92.28± 0.94	97.48± 1.12 <sup>a</sup>	111.77± 1.50 <sup>b</sup>	110.44± 3.12 <sup>b</sup>	117.65± 2.79 <sup>b</sup>

Data are in means ± SE (n=7). <sup>a</sup>P<0.01 and <sup>b</sup>P<0.001 as compared to their initial body weight. In both drug doses a significant decrease in tissue LPO was observed. For liver LPO a significant decrease (P<0.001 for both) was observed. Yet, there was a difference in percent decreases by LD and HD (63.2% and 51.9%, respectively). A significant increase (P<0.001) in SOD activity was also found in both the drug treated groups. Here again LD seemed to be more efficient (124%) than HD (111.8%). While, in case of liver CAT and GSH, a significant improvement in LD (P<0.001 for both, 63.3% and 89.9%, respectively) and HD (P<0.05, 54% and P<0.001, 109.4%, respectively) was noticed.

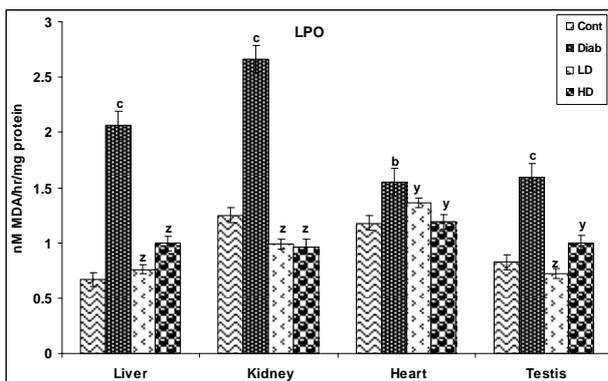
For kidney and heart, a significant decrease in LPO was noticed in case of both HD (62.9% and 12.3%, respectively) and LD (63.8% and 23.2%, respectively) of the test drug, whereas, significantly increased activities were found in case of SOD (79.1% and 148.7% respectively for HD; 42.6% and 133.67% respectively for LD), CAT (26.4% and 158% respectively, for HD; 35.7% and 156.7% respectively, for LD) and GSH (121.7% and 68.4% for HD; 97.7% and 67.5% for LD) respectively.

Moreover, in case of testicular LPO both drug doses, LD and HD significantly lowered (P<0.001, 54.8% and P<0.01, 37.2%, respectively) protected, the tissue. Similarly, in both LD and HD groups, the activities of SOD, CAT and GSH were improved significantly (for SOD 52% and 57.1% respectively, for CAT 135.6% and 192% respectively and for GSH 57.6% and 55.7% respectively).



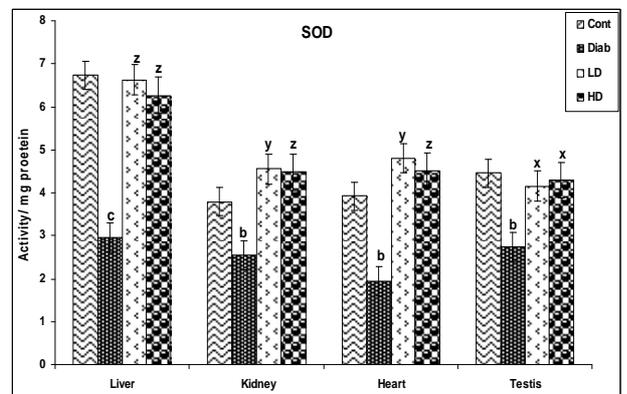
Each bar represents the mean± SEM (n=7). Cont (Control), Diab. (Diabetic), LD (Diabetic with 1 gm/kg of drug) and HD (Diabetic with 2 gm/kg of drug). \*P<0.0001 increase as compared to the respective control value. # P<0.0001 decrease as compared to the value of diabetic group

**Fig. 1: Effects of Diabecon for 8-weeks on fasting glucose (mg/dl) in adolescent male rats.**



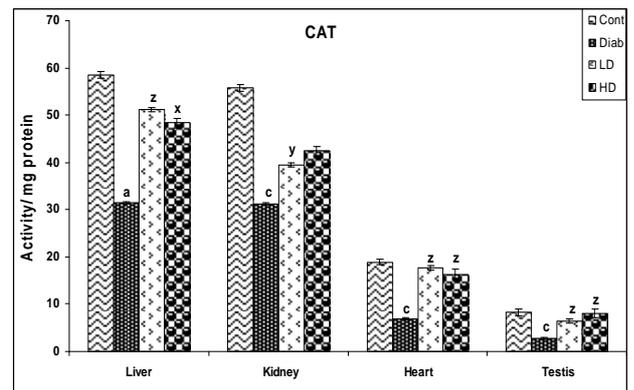
Each bar represents the mean ± SEM (n=7). Cont (Control), Diab. (Diabetic), LD (Diabetic with 1 gm/kg of drug) and HD (Diabetic with 2 gm/kg of drug). <sup>b</sup>P<0.001 & <sup>c</sup>P<0.0001 as compared to control value. <sup>y</sup>P<0.01 and <sup>z</sup>P<0.001 as compared to the value of diabetic group.

**Fig. 2: Effects of Diabecon treatment for 8-weeks on liver, kidney, heart and testis lipid peroxidation (LPO, nmol of MDA formed h<sup>-1</sup> mg protein<sup>-1</sup>) in adolescent male rats.**



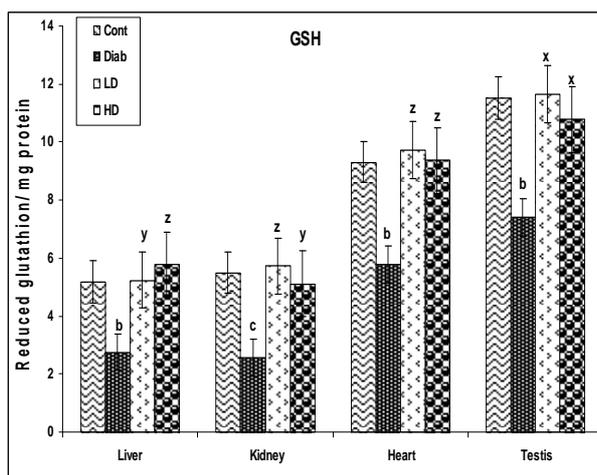
Each bar represents the mean± SEM (n=7). Cont (Control), Diab. (Diabetic), LD (Diabetic with 1 gm/kg of drug) and HD (Diabetic with 2 gm/kg of drug). <sup>b</sup>P<0.001 & <sup>c</sup>P<0.0001 increase as compared to the control value. <sup>x</sup>P<0.05, <sup>y</sup>P<0.01 and <sup>z</sup>P<0.001 as compared to the value of diabetic group.

**Fig. 3 Effects of Diabecon treatment for 8-weeks on activity of liver, kidney, heart and testis superoxide dismutase (SOD, units/mg protein) in adolescent male rats.**



Each bar represents the mean± SEM (n=7). Cont (Control), Diab. (Diabetic), LD (Diabetic with 1 gm/kg of drug) and HD (Diabetic with 2 gm/kg of drug). <sup>a</sup>P<0.05, <sup>b</sup>P<0.01 & <sup>c</sup>P<0.001 increase as compared to control value. <sup>y</sup>P<0.01 and <sup>z</sup>P<0.001 as compared to the value of diabetic group

**Fig. 4: Effects of Diabecon treatment for 8-weeks on activity of liver, kidney, heart and testis catalase (CAT, µM H2O2 decomposed/ min/mg protein) in adolescent male rats.**



Each bar represents the mean  $\pm$  SEM (n=7). Cont (Control), Diab. (Diabetic), LD (Diabetic with 1 gm/kg of drug) and HD (Diabetic with 2 gm/kg of drug). <sup>b</sup>P<0.001 & <sup>c</sup>P<0.0001 increase as compared to control value. <sup>y</sup>P<0.01 and <sup>z</sup>P<0.001 as compared to the value of diabetic group.

**Fig. 5: Effects of Diabecon treatment for 8-weeks on liver, kidney, heart and testis reduced glutathione (GSH,  $\mu$ M reduced glutathione/mg protein) content in adolescent male rats.**

## DISCUSSION

Results of the present study indicated antihyperglycemic and antioxidative potential of Diabecon, in adolescent male rats. With respect to tissue LPO and activity of SOD, CAT and GSH the test drug showed significant protective effects which were observed to be organ specific. Although, some dose specific alterations were also found in all tested parameters, these were not consistent in all organs, thus the overall effects appeared to be similar. Results from *in vitro* assays also supported free radical inhibition/scavenging potential of test drug at different concentrations, that can be compared with other polyherbal drugs [7, 9].

Generally, *in vitro* free radical scavenging effects are studied to see antioxidative efficiency of the test formulation [6,7,19]. Here, DPPH was used as stable synthetic free radical which exhibits different colours in its oxidized and reduced states [6,7,9]. *In vitro* NO scavenging efficacy also revealed positive results. The later observations supported the possible antidiabetic effects of the test drug, because NO free radical has been reported to cause pancreatic  $\beta$ -cell destruction in physiological systems [20,21]. It is also believed that drugs having better DPPH and NO scavenging efficacy may serve as strong physiological antioxidant [10,11,19].

Oxidative stress imperatively participates in development and progression of various pathological conditions by depleting antioxidant defences and generating excess free radicals [2,4], which result in tissue damage [6,9,14]. Alloxan is an oxygenated pyrimidine compound that accelerates toxic ROS generation and the pancreatic beta-cell toxicity leads the resultant diabetogenicity [7, 11]. In our study also, in diabetic rats it showed severe oxidative stress resulting in significant hyperglycaemia and tissue damage in the tested body organs [2,5,8]. Decrease in final body weight than normal, in accordance with the earlier findings [8,20], also supported the induction of diabetes in the experimental animals. To counteract oxidative stress our body has been equipped with efficient enzymatic and non-enzymatic antioxidants which continuously remove excessively formed free radicals. In all tissues SOD and CAT serve as main antioxidative enzymes [2,5,8]. However, with respect to testicular antioxidants, tissue possesses limited activity of CAT, as it holds sufficient GPx and GSH that serve to remove excess  $H_2O_2$  [5,22]. During diabetes mellitus, the activities of these antioxidants goes down [6-8,21]. Our findings revealed significantly increased activities of SOD and CAT by the test drug in test organs. Their enhanced antioxidative machinery/activity is supported by

earlier reports, where some phytochemicals have been reported to up-regulate the synthesis of SOD and CAT [1,6-8,20-22]. Normal activities of these antioxidants in drug treated groups revealed its protective effects in different tissues.

Glutathione serves as chief non-enzymatic antioxidant, primarily a cofactor in the GPx mediated destruction of hydroperoxides, ultimately protects against oxidative damage [20,23]. In this study, we noted marked reduction in GSH levels in alloxan treated rats, which reflected its higher consumption in the oxidative stress [5,6,11]. However, in drug treated groups GSH level was found to be restored in all tested organs. Further more, the decreased LPO can be explained by increased cellular antioxidants in drug treated animals. Earlier, one review report indicated the antidiabetic activity of Diabecon [11]. However, the present one is the first study that reveals the antidiabetic and antioxidative actions of the test drug in adolescent rats. Moreover, despite the fact that the diabetic complications directly influence hepatic, renal and cardiac tissues [3,5,8,23], to the best of our knowledge this is the first report which revealed the protective effects of the test drug in all these tissues. More interestingly, this report also reveals the beneficial effects of test drug on immature testis. As testes of young animals are relatively rich in poly unsaturated fatty acids and have less developed antioxidant system, they are more vulnerable to oxidative stress [4,5,24]. Significant increased SOD and CAT activity and GSH level in testis of drug treated groups suggested that drug might enhance their turnover rate that again elucidated by decreased tissue LPO. The mechanism of action of the drug can be explained by the presence of multiple active compounds of forty two known antidiabetic herbs in the formulation [10,11]. Diabecon may exert  $\beta$ -cells protective/proliferative activities, that helps to maintain/enhance insulin secretion, as reported for its individual herbs [6,7,24,25]. Some of the herbs have been reported to have gluconeogenesis or glycogenolysis inhibiting potential, which reduce blood glucose level [4,19,21,23]. In the present investigation a clear negative correlation was observed in level of cellular antioxidative machinery and oxidative stress. Therefore, it seems that the test drug might have induced synthesis of these antioxidant proteins and/or acted through scavenging free radicals, formed in various biochemical processes. This possibility is further consolidated by the presence of rich amounts of polyphenols and flavonoids in test drug, which are antioxidative in nature [10,25-28].

Reviewing all these results, it can be concluded that the test drug not only potentially improves and prevents diabetic condition, but can also protect the tissues from its secondary complications like oxidative stress. It could be stated that the drug may prove to be safe for long term use, in children/ adolescent diabetic patients too. Of course, more studies with other animals and clinical sets are required to confirm these findings.

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