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RESEARCH ARTICLE

Antioxidant activities of Dhanwantaram Kashayam –an Ayurvedic poly herbal formulation alleviates diabetic complications in rats

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Abstract

Purpose Phytochemicals of ethno medicines are being developed as effective drugs with minimum or no toxic side effects. Dhanwantaram Kashayam (DK) is a polyherbal formulation used as a potent general health tonic and is found to have antioxidant activities, but there are no proper scientific studies on its possible benefits. In this study we investigated the antioxidant and antidiabetic properties of DK and trying to explore the possibility of employing DK for the treatment of diseases like diabetes mellitus caused by the imbalance in Reactive Oxygen Species (ROS).

Methods We investigated the effect of DK on normal and rat model of diabetes. Rats were fed with DK for 21 days. Fasting blood glucose level, haemoglobin, glycosylated hemoglobin, non enzymatic antioxidants like vitamin C, vitamin E, reduced glutathione, and MDA were evaluated.

Results DK was found to ameliorate the disease symptoms of diabetes. A significant decrease in non enzymatic antioxidants – vitamin C, vitamin E, reduced glutathione and an increase in MDA and fasting blood glucose level was observed in diabetic rats. Administration of DK resulted significant increase in non enzymatic antioxidant levels both in diabetic and normal rats and a decrease in MDA, fasting blood glucose, glycosylated hemoglobin and HOMA-IR index was observed.

Conclusion This study reveals the protective role of DK in diabetic condition through its antioxidant and antihyperglycemic activity. Our results strongly indicate the possibility of DK being developed as a potent antidiabetic drug. DK could be an alternative in the treatment of diseases with ROS imbalance to expensive and toxic synthetic 'medicines'.

Keywords Reactive oxygen species · Diabetes mellitus · Ayurveda · Kashayam · Antioxidants · MDA

Introduction

Oxidative stress is an important contributing factor in the pathogenesis of type 2 diabetes mellitus (DM) [1]. Several previous studies had already shown that oxidative stress is the major causative factors for diseases such as DM [2], cancer [3] etc. Hyperglycemia induced abnormal metabolism can result in the overproduction of reactive oxygen species (ROS) such as hydroxyl and superoxide radicals [4]. Balance between production and removal of ROS is necessary for proper functional integrity of cells. ROS formed from oxygen and nitrogen cause damages to the complex antioxidant system that exists in Mammals [5] and are implicated as causative agents for many diseases [6].

Changes in glucose metabolism due to diabetes are reported to induce cell damage through different metabolic pathways which includes increase in glycation of proteins [7]. Most of the diabetic complications are caused by the mitochondrial over production of reactive oxygen species (ROS) leading to oxidative stress. High glucose in diabetic condition due to insufficient insulin activity is linked to different metabolic abnormalities. Excessive amounts of ROS oxidizes biomolecules, such as, DNA, protein, carbohydrates and lipids after surpassing various endogenous anti-oxidative defensive mechanisms which leads to oxidative stress [8]. Oxidative stress and the resultant tissues damages lead to diabetes associated complications [9]. Glycosylation of haemoglobin (HbAIc) elevated levels of glycosylated hemoglobin (GHb) and reduced total hemoglobin concentrations are salient features of diabetes [10]. The measurement of GHb is one of the well established means of monitoring glycemic control in

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patients with DM [11]. The excess amount of glucose present in the blood during severe diabetes reacts with hemoglobin to form glycosylated hemoglobin [12] which alter the affinity for oxygen and lead to tissue hypoxia [13]. Oxidative stress along with anoxia complicates diabetic conditions [14].

Several research reports had demonstrated that ROS triggered disease condition can be corrected by sufficient supply of antioxidants [15]. Various experimental studies and clinical trials have revealed the possibility of preventing or treating diabetes complication using antioxidants [16]. Natural products are getting importance in clinical medicine due to their potential treatment efficacy with minimal side effects [16, 17]. Dhanwantaram Kashayam (DK) as quoted in the classical text Ashtanga Hridayam is an Ayurvedic polyherbal decoction and it consists of 40 herbal ingredients [18]. Most of these plant products have strong antioxidant capacity and are used in Indian traditional medicinal system, Ayurveda [19]. DK is used in Ayurveda as a growth stimulant in children and for nerve regeneration [20]. It has strong antioxidant activities [21] and its possible role in scavenging free radical in our body is already reported [22]. Favorable influences of polyphenols on the regulation of glucose homeostasis and diabetes pathogenesis are well reported [11].

Vit C and vit E are potent dietary antioxidants [23]. Vitamin C is reported to reduce glycosylation of proteins. A significant reduction in the fasting blood glucose (FBG) and postprandial blood glucose (PPBG) was reported after oral supplementation of vitamin C [24]. Vitamin E helps in preventing damage to the lipids by the oxygen free radicals [25] by halting the chain reaction of lipid per oxidation caused by the highly-reactive oxygen species [26]. GSH is another important non enzymatic antioxidant, protecting our body from the attack of reactive oxygen species [27]. The lipid peroxidation product MDA level is an important marker for increased oxidative stress [28].

Our aim in the present study is to evaluate the effect of DK in ameliorating the complications associated with diabetes and determine the correlation, if any between the radical scavenging capacity of DK and its antidiabetic activity. With this aim our present study mainly focused on the effect of DK administration on the levels of glycosylated hemoglobin and the non enzymatic antioxidant parameters like vit C, vit E, GSH and also the lipid peroxidation product MDA in Streptozotocin (STZ) induced diabetic rats. This study also investigated the hypoglycemic and antidiabetic effects of DK by evaluating the oral glucose tolerance (OGTT) and homeostasis model assessment (HOMA). We postulated that DK with its antioxidant properties will help in neutralising the imbalance occurred in the ROS production and removal and can be developed as an effective cure for diseases such as diabetes caused by such altered levels of reactive oxygen species.

Materials and methods

Chemicals

All chemicals and bio chemicals used in this work were of analytical grade and were obtained from Sisco Research Laboratories Inc. (Mumbai, India).

Experimental animals

48 Male rats of Wistar strain (200-220 g) maintained in the Departmental animal house (Room temperature 24 ± 2 °C with alternate 12 h light and dark cycles) were used for the study with each group having 6 rats. Rats were housed in hygienic polypropylene cages, provided with rodent food (VRK Nutritional Solutions, Maharashtra, India) and water ad libitum. All procedures involving animals were in accordance with the guidelines on the care of laboratory animals and their use for scientific purpose and permissions were obtained from the Institutional Animal Ethics Committee. Diabetes was induced by single intraperitoneal injection of freshly prepared STZ (Sisco Research Laboratories, Mumbai, India) at a dose of 40 mg/kg, prepared in 0.1 M citrate buffer, pH 4.5 [29]. Rats were immediately supplied with drinking water containing 5% glucose (*W*/*V*) for the first 24 h to encounter any initial hypoglycemia. Animals with blood glucose level > 300 mg/dl on the third day were classified as diabetic group. DK freshly diluted with sterile water (1:3) was administered orally at a dosage of 1 ml (Dose A), 1.5 ml (Dose B) and 2 ml/kg (Dose C) respectively twice a day on empty stomach or before food for 21 days. Dose B is equivalent to the dose administered to humans and two other doses; a lower and a higher dose than human equivalent were also used.

Animals groups & experimental design

Animals were divided into eight groups namely;

Experimental group I: Control (NC)
Experimental group II: Diabetic Control (DC)
Experimental group III: Diabetic + DK Dose A (DDKA)
Experimental group IV: Diabetic + DK Dose B (DDKB)
Experimental group V: Diabetic + DK Dose C (DDKC)
Experimental group VI: Control + DK Dose A (CDKA)
Experimental group VII: Control + DK Dose B (CDKB)
Experimental group VIII: Control + DK Dose C (CDKC)

On 21st day they were deprived of food overnight and sacrificed by cervical dislocation. Blood collected in centrifuge tubes. Tissues were collected in ice cold containers.

Biochemical parameters

Non-enzymatic antioxidants and lipid peroxidation product

Non enzymatic antioxidants and lipid peroxidation product MDA were evaluated by using standard protocols. Vit C was determined by the method of Omaye et al. [30], vit E by the method of Desai [31], GSH level was determined according to the method of Patterson and Lazarow [32] and the concentration of MDA was estimated by the method of Ohkawa et al. [33].

Determination of fasting blood sugar level

Fasting Blood glucose was measured using "Dr. Morepen Gluco One Blood glucose monitoring system" BG-03 model glucose strips (Morepen Laboratories Limited, Delhi).

Homeostasis model assessment (HOMA)

HOMA model, which represent both fasting plasma glucose (FPG) and insulin levels, was used as an index of insulin resistance (IR). This was calculated using the following formula [34]:

$$HOMA = [insulin\ (\mu U/ml) \times glucose\ (nM) \div 22.5]$$

as described previously.

Oral glucose tolerance tests

At the end of the study period, oral glucose tolerance test (OGTT) was conducted by the method described elsewhere [35]. Rats were devoid of food overnight for 12 h and fasting blood glucose was determined. Each rat was administered with a dose of 2 g/kg body mass of sterile 50% (*w*/*v*) D-glucose solution (Sigma, India) via orogastric gavage. Thereafter blood glucose concentrations were measured at T = 30, 60, 90 and 120 min using "Dr. Morepen Gluco One Blood glucose monitoring system" BG-03 model glucose strips (Morepen Laboratories Limited, Delhi). The blood samples were collected following a sterile pin prick of the distal tail vein.

Determination of haemoglobin and glycosylated haemoglobin

Haemoglobin (Merck, India) and glycosylated haemoglobin (Glyco-Tek Affinity column kits and reagents from Helena Laboratories (Beaumont, TX) levels were measured in plasma samples by using commercial kits.

Quantitation of the expression of L-gulonolactone oxidase (gulo) gene in rat liver

The mRNA expression of *gulo* gene was determined by real time PCR. Gene specific primers were designed in Primer-3 software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) using the mRNA sequence of the gene from NCBI nucleotide database; accession number: NM_022220 (https://www.ncbi.nlm.nih.gov/nuccore/NM_022220) and got custom synthesise by RFCL Science solutions, Bhopal, India. Primer sequences are given in Table 1. Total RNA from rat liver tissue was isolated using Trisol reagent (ThermoFisher Scientific) following the procedure supplied by the manufacturer. First-strand cDNA was generated from the extracted RNA using cDNA Synthesis Kit (Thermo Scientific, USA) as described before [36]. Gene amplification was performed in qPCR system (Biorad) using hot start SYBRgreen master mix (Origin Labs, Karunagappally, India) with initial denaturation at 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C for 10s, annealing for 20s, and extension at 72 °C for 30s with data collection at the end of extension phase. Sequences of primers used are given in Table 1. Threshold cycle (Ct) for each well was obtained using the software of the instrument and the melting-curve to ensure the nature of each amplicon was prepared after the cycling program. The β2-microglobulin gene was used as the endogenous control gene to normalise the relative expressions of the genes of interest calculated using $2^{-\Delta\Delta CT}$ method as practiced before [37].

Statistical analysis

Data were represented as Mean ± SD (Standard Deviation) of the values of 6 animals. The results were analysed by one - way ANOVA (analysis of variance) followed by Tukey Post hoc multiple comparison test. Values with $P < 0.001$ and 0.05 were considered as statistically significant. SPSS -21 version software for windows was used for this analysis.

Results

Effect of DK on fasting blood sugar level

Blood sugar level increased in DC rats ($P < 0.001$) as compared to NC rats (Table 2). Compared to DC rats all DK fed rats were having a significantly lower blood sugar ($P < 0.001$ and $P < 0.05$). In diabetic rats, on day 21, the blood glucose level increased by 367%. DK administration in all doses significantly decreased the blood sugar level of diabetic rats in a dose dependent manner. After 21 days the percentage decrease in blood glucose levels in DK fed diabetic rats were 76% with dose C, 54% with dose B and 17% with dose A.

Effect of DK on HOMA –IR index

Homeostatic model assessment of insulin resistance of normal and experimental rats is given in Fig. 1. HOMA-IR index was

Table 1 Sequences of primers used in gene expression studies

Gene name and accession number	Primer sequence	Annealing temperature	Accession number
β2-microglobulin	F- 5' GTCGTACCACTGGCATTGTG 3′ R- 5'CTCTCAGCTGTGGTGGTGAA 3′	58 °C	NM_031144
L-gulonolactone oxidase (Gulo)	F- 5' GTTTCACCTTCAGGAGACATCC 3′ R- 5' CTCCAGTAGGTAGAACCCGATG 3'	58 °C	NM_022220

Details of the primers used to amplify the L-gulonolactone oxidase and β2-microglobulin. Primers were designed for the genes using the mRNA sequence obtained from the nucleotide data base of NCBI. Suitable primers were picked using Primer-3 software

significantly higher in diabetic rats compared with normal control rats and remained more or less constant during the study period. Supplementation of different doses of DK to diabetic rats significantly ($p < 0.05$) decreased the HOMA-IR index in a dose dependent manner in comparison to the untreated diabetic rats. This decrease is due to the increase in the insulin level and resultant decrease in blood glucose level. Dose C brought the HOMA-IR index very near to the normal rats and the difference between them is not significant. Administration of DK to normal rats did not make any significant changes.

Effect of DK on oral glucose tolerance (OGT)

The results of oral glucose tolerance test (OGTT) of the control and experimental rats are shown in Fig. 2. OGTT revealed that, in normal control rats, maximum elevation in blood glucose level was at 60 min after glucose load and declined to near basal level at 120 min, whereas, in diabetic rats, the peak increase in blood glucose level was noticed even after 60 min and remained high even at 120 min. In both the DK fed normal as well as diabetic rats, blood glucose level rose to the maximum after 60 min of glucose load. Interestingly, supplementation of DK to diabetic rats elicited a significant decrease in blood glucose level at 90 min and beyond when compared with untreated diabetic rats. The effect of DK on glucose tolerance was dose dependent and dose C brought the glucose tolerance to the normal range. In DK fed normal rats, oral glucose tolerance values remained in the normal range.

Table 2 Fasting blood sugar levels in different rat groups

SL:No	Groups	Blood
1.	NC	115.83 ± 10.57^{a}
2.	DC	$542.23 \pm 49.48^{**}$
3.	DDKA	$453.96 \pm 41.59^{**b}$
4.	DDKB	$253.18 \pm 23.11^{**a}$
5.	DDKC	$182.45 \pm 16.65^{*a}$
6.	CDKA	131.20 ± 11.97^{a}
7.	CDKB	129.15 ± 11.79^{a}
8.	CDKC	114.80 ± 10.48^{a}

Effect of Dhanwantaram Kashayam (DK) on fasting blood sugar level (mg/dl) of diabetic rats on 21st day. NC -Normal Control, DC- Diabetic Control, DDKA -Diabetic + Dose A, DDKB - Diabetic + Dose B, DDKC - Diabetic + Dose C, CDKA -Normal + Dose A, CDKB -Normal + Dose B, CDKC - Normal + Dose C. Values are expressed as mean ± SD of six rats. '**' indicates values are significantly different from NC rats with $p < 0.001$. '*'indicates values are significantly different from NC rats with $p < 0.05$. 'a' indicates values are significantly different from DC rats with $p < 0.001$. 'b' indicates values are significantly different from DC rats with $p < 0.05$

Effect of DK on haemoglobin and glycosylated haemoglobin levels

Reduced haemoglobin (Fig. 3) and increased glycosylated haemoglobin (Fig. 4) levels were observed in diabetic control rats. Administration of DK reversed these conditions to a great extent. These changes were dose dependent and statistically significant ($p < 0.001$ and 0.05). In diabetic rats the haemoglobin

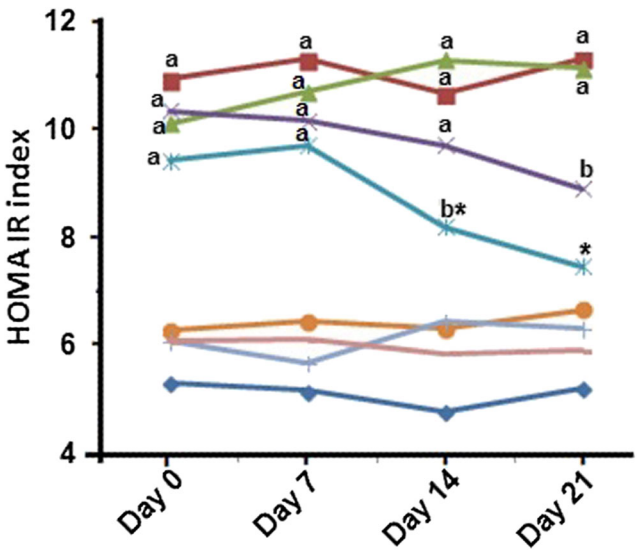

Fig. 1 HOMA-IR index in normal, diabetic control and diabetic/normal groups treated with different doses of Dhanwantaram Kashayam (DK). Values are expressed as mean of six rats. '*'indicates values are significantly different from NC rats with p<0.05. 'a' indicates values are significantly different from diabetic control (DC) rats with p<0.001. 'b' indicates values are significantly different from DC rats with p<0.05

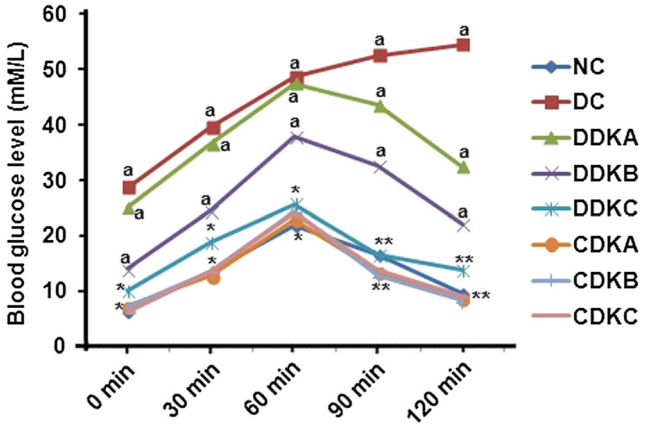

Fig. 2 Oral glucose tolerance (OGT) in normal, diabetic control, and diabetic/normal groups treated with different doses of Dhanwantaram Kashayam (DK). Values are expressed as mean of six rats. '**' indicates values are significantly different from normal control (NC) rats with p<0.001. '*'indicates values are significantly different from NC rats with p<0.05. 'a' indicates values are significantly different from diabetic control (DC) rats with p<0.001. 'b' indicates values are significantly different from DC rats with p<0.05

content was decreased by 63% and glycosylated haemoglobin level was increased by 84%. Administration of DK improved the levels of both Hb and HbAIc with haemoglobin level increasing in a dose dependent manner. In dose C fed rats, the haemoglobin level reached near normal (93% of normal value). Decrease in the glycosylated haemoglobin level due to DK was also dose dependent with dose C producing the highest decrease. In dose C fed rats the glycosylated haemoglobin level reached close to normal level (125% of normal value).

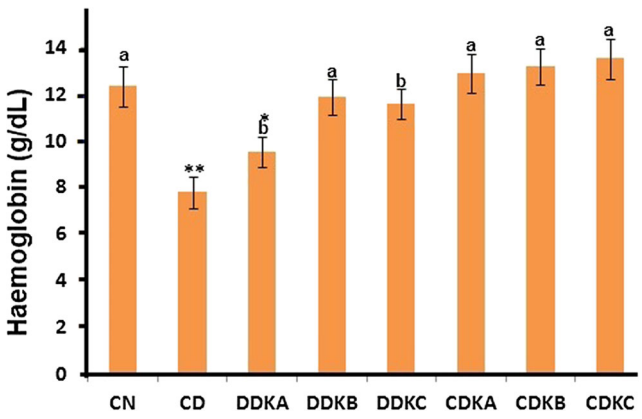

Fig. 3 Haemoglobin levels in the blood of normal, diabetic control, and diabetic/normal groups treated with different doses of Dhanwantaram Kashayam (DK). Values are expressed as mean ± SD of six rats. '**' indicates values are significantly different from normal control (NC) rats with p<0.001. '*'indicates values are significantly different from NC rats with p<0.05. 'a' indicates values are significantly different from diabetic control (DC) rats with p<0.001. 'b' indicates values are significantly different from DC rats with p<0.05

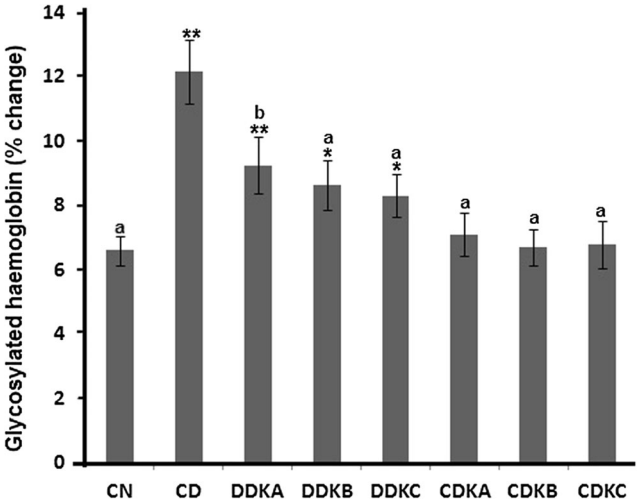

Fig. 4 Glycosylated haemoglobin levels in the blood of normal, diabetic control, and diabetic/normal groups treated with different doses of Dhanwantaram Kashayam (DK). Values are expressed as mean ± SD of six rats. '**' indicates values are significantly different from normal control (NC) rats withp<0.001. '*'indicates values are significantly different from NC rats with p<0.05. 'a' indicates values are significantly different from diabetic control (DC) rats with p<0.001. 'b' indicates values are significantly different from DC rats with p<0.05

Effect of DK on Vit C level

Vit C level decreased in diabetic rats compared to normal control (Fig. 5). Administration of DK caused significant ($p < 0.001$ and 0.05) increase in vit C level in all tissues of diabetic rats in a dose dependent manner, but the rate of increase varied in different tissues. The highest increase was observed in liver (185%) compared to diabetic rats which is highly significant ($p < 0.001$). In diabetic rats, the administration of DK helped to bring the vitamin C level to the normal level or higher in liver and kidney, but in heart, though there was an increase in vitamin C the increase was not up to the normal level for all the doses. Administration of DK to normal rats also caused an increase in vit C concentration, with the highest percentage increase observed in heart (54%) of dose A fed rat followed by kidney (48%) of dose B fed rats.

Effect of DK on vitamin C synthesis at mRNA level

To further investigate the molecular mechanism underlying the altered levels of Vit C, the expression of L-gulonolactone oxidase (gulo) gene at mRNA level was studied. As expected the expression of gulo gene was markedly decreased (66%) in the liver of diabetic rats (Fig. 6). There was no significant change in the expression in the normal control rats fed with DK. At the same time DK administration enhanced gulo expression significantly in the DK fed diabetic rats in a dose dependent manner with the highest increase in expression in the dose C fed rats. The percentage increase in

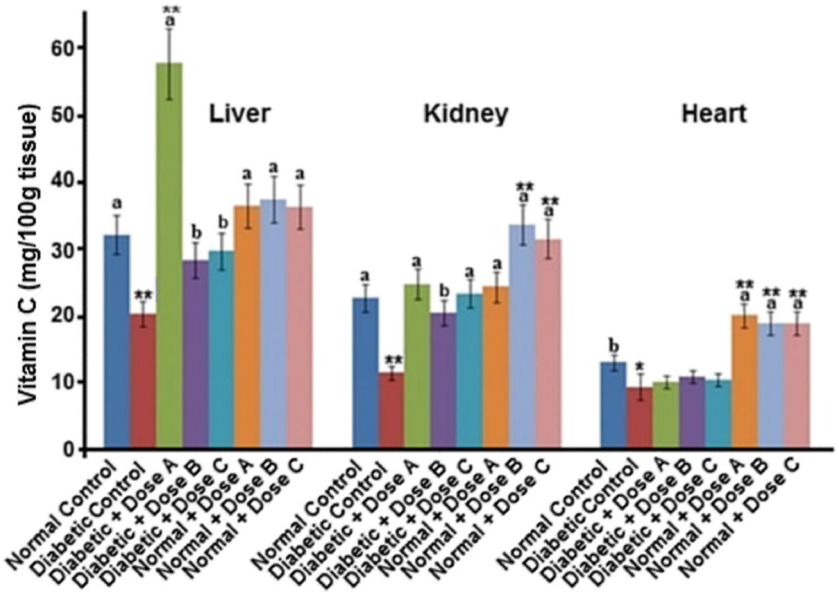

Fig. 5 Vitamin C levels in the various tissues of normal, diabetic control, and diabetic/normal groups treated with different doses of Dhanwantaram Kashayam (DK). Values are expressed as mean ± SD of six rats. '**' indicates values are significantly different from normal control (NC) rats with p<0.001. '*'indicates values are significantly different from NC rats with p<0.05. 'a' indicates values are significantly different from diabetic control (DC) rats with p<0.001. 'b' indicates values are significantly different from DC rats with p<0.05

expressions from that of diabetic rats were 18% with dose A, 62% in dose B and 84% in dose C fed rats.

Effect of DK on vitamin E

Vit E concentration was significantly decreased in diabetic rats ($P < 0.05$) as compared to NC rats (Table 3). Administration of DK increased Vit E concentration in all the diabetic groups with the highest increase in dose B fed rats. Vitamin E levels in all the DK administered diabetic rats were significantly higher ($p < 0.001$ and $p < 0.05$) than in diabetic control rats, with dose B producing the highest increase (56%) followed by dose A (40%) and dose C (23%). The increase in vitamin E level in diabetic rats fed with dose A and B is significantly higher than that in normal control as well as diabetic control rats. Dose C administration brought the vitamin E level in diabetic rats in par with that of normal control and this increase was significantly higher ($p < 0.05$) than in diabetic control rats. Normal rats administered DK also

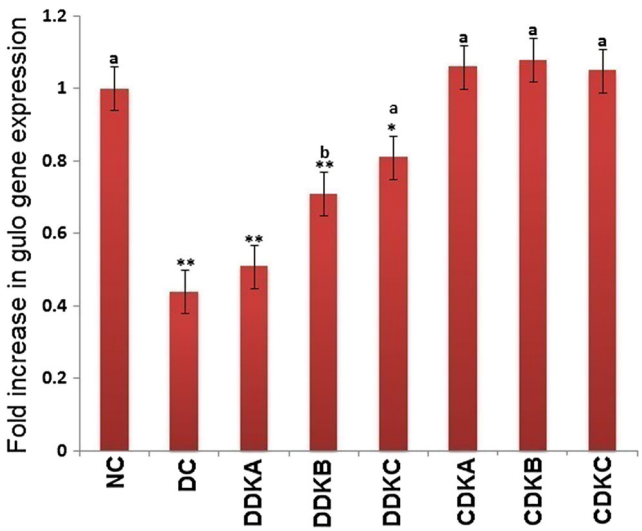

Fig. 6 Activity of gulo gene in the liver of normal, diabetic control, and diabetic/normal groups treated with different doses of Dhanwantaram Kashayam (DK). Values are expressed as mean ± SD of six rats. '**' indicates values are significantly different from normal control (NC) rats with p<0.001. '*'indicates values are significantly different from NC rats with p<0.05. 'a' indicates values are significantly different from diabetic control (DC) rats with p<0.001. 'b' indicates values are significantly different from DC rats with p<0.05

Table 3 Vitamin E levels in different rat groups

SL:No	Groups	Plasma
1.	NC	6.64 ± 0.61^{b}
2.	DC	$5.36 \pm 0.49^{*}$
3.	DDKA	7.48 ± 0.68^{a}
4.	DDKB	$8.38 \pm 0.77^{**a}$
5.	DDKC	6.64 ± 0.61^{b}
6.	CDKA	6.84 ± 0.53^{b}
7.	CDKB	7.26 ± 0.57^{b}
8.	CDKC	$7.97 \pm 0.73^{*a}$

Effect of DK (Dhanwantaram Kashayam) on non enzymatic antioxidant vitamin E (mg/dl) in diabetic rats on 21st day. NC -Normal Control, DC-Diabetic Control, DDKA -Diabetic + Dose A, DDKB - Diabetic + Dose B, DDKC - Diabetic + Dose C, CDKA -Normal + Dose A, CDKB - Normal + Dose B, CDKC - Normal + Dose C. Values are expressed as mean ± SD of six rats. '**' indicates values are significantly different from NC rats with $p < 0.001$. '*'indicates values are significantly different from NC rats with $p < 0.05$. 'a' indicates values are significantly different from DC rats with $p < 0.001$. 'b' indicates values are significantly different from DC rats with $p < 0.05$

had a higher vitamin E level with dose C fed animals have the highest increase followed by dose B and dose A in decreasing order. These increases in vitamin E due to DK administration in normal rats were not significant compared to untreated normal rats.

Effect of DK on GSH level

The level of GSH also decreased significantly ($P < 0.001$ and $P < 0.05$) in all the tissues of DC rats compared to NC rats (Table 4). Administration of DK significantly increased GSH level in both DC and NC rats in a dose dependent manner in all tissues and blood ($P < 0.001$ and $P < 0.05$). In diabetic rats fed dose B and C, the GSH level went even above the normal value in all tissues. The extent of increase in GSH levels due to DK administration in dose B and C in diabetic rats was between 81 to 132% with the highest difference in the liver of dose C fed diabetic rats. Similar trends were observed in normal rats fed DK, but the increase is not that drastic as observed in diabetic rats.

Effect of DK on MDA level

MDA level was significantly increased in diabetic rats ($P < 0.001$) as compared to normal control rats (Fig. 7). Highest increase was observed in Kidneys (110%), followed by heart (101%), liver (35%) and serum (30%). Administration of DK decreased MDA level in all tissues of diabetic rats in a dose dependent manner. DK in dose C fully mitigated the increase in MDA levels in liver, heart and serum of diabetic rats. A decrease in MDA level was also observed in the kidneys of DK administered diabetic rats, but even with dose C the MDA level remained 26% higher than in normal control rats. Administration of DK to normal rats decreased the levels of MDA in the liver, heart and serum, but in kidney the MDA level showed an increase.

Discussion

Diabetes is a major public health challenge in modern world. Persistent hyper glycaemia results in secondary complications such as hypertension, coronary artery disease, neuropathy, nephropathy, foot ulcers, sexual dysfunction etc. Irrespective of the extensive efforts to find a cure to DM, no permanent cure for this disease is available. The standard form of treatment is through the modifications of diet and life style combined with drug therapy with hypoglycaemics, such as sulphonylureas, α-glucosidase inhibitors and thiazolidenediones. Administration of exogenous insulin becomes necessary when blood glucose levels cannot be controlled by controlling diet, exercise or by hypoglycaemics. The inconvenience and the pain of taking these treatments are pushing the scientists all over the world to identify a permanent cure or a management program through dietary supplements [38]. Healthy diet rich in plant derived materials are found to delay the complications associated with DM or even prevent DM development [11]. DK is a health tonic reported to induce tissue repair and regeneration. In this study we investigated the effect of DK on relieving diabetic complications and found it as a promising health tonic in the management of diabetes. Several scientific reports have proved that plant derived drugs can significantly reduce high blood sugar, increase insulin synthesis and improve glucose tolerance. In this study we tested the possibility of attaining glucose homeostasis through the regulation of oxidative stress and insulin synthesis in diabetic rats administered different doses of DK.

The body weights of the diabetic rats were decreased by 20–30%. Total fluid and food intake and urine production significantly increased in diabetic rats. Decreased glucose

Table 4 Levels of GSH in different rat groups

Sl:No	Groups	Liver	Kidney	Heart	Blood
1.	NC	20.07 ± 1.83^{a}	8.99 ± 0.82^{b}	4.32 ± 0.40	0.43 ± 0.04^{a}
2.	CD	$11.31 \pm 1.03^{**}$	$6.62 \pm 0.60^{*}$	3.67 ± 0.34	$0.27 \pm 0.03^{**}$
3.	DDKA	22.35 ± 2.04^{a}	10.53 ± 0.96^{a}	4.83 ± 0.44^{b}	0.47 ± 0.04^{a}
4.	DDKB	24.50 ± 2.24^{a}	$14.63 \pm 1.34^{**a}$	$6.96 \pm 0.64^{**a}$	0.50 ± 0.45^{a}
5.	DDKC	$26.25 \pm 2.40^{*a}$	$13.28 \pm 1.21^{*a}$	$6.66 \pm 0.61^{**a}$	$0.59 \pm 0.54^{*a}$
6.	NDKA	20.86 ± 1.72^{a}	9.82 ± 0.90^{a}	$6.07 \pm 0.55^{**a}$	$0.55 \pm 0.05^{*a}$
7.	NDKB	$24.01 \pm 2.47^{*a}$	10.11 ± 0.92^{a}	$6.17 \pm 0.56^{**a}$	$0.67 \pm 0.62^{**a}$
8.	NDKC	23.52 ± 2.15^{a}	$11.21 \pm 1.02^{*a}$	$7.29 \pm 0.67^{**a}$	$0.59 \pm 0.05^{*a}$

Effect of DK (Dhanwantaram Kashayam) on non enzymatic antioxidant reduced Glutathione (mM/100 g tissue and mM/dl) of diabetic rats on 21st. NC - Normal Control, DC- Diabetic Control, DDKA -Diabetic + Dose A, DDKB - Diabetic + Dose B, DDKC - Diabetic + Dose C, CDKA -Normal + Dose A, CDKB -Normal + Dose B, CDKC - Normal + Dose C. Values are expressed as mean ± SD of six rats. '**' indicates values are significantly different from NC rats with $p < 0.001$. '*'indicates values are significantly different from NC rats with $p < 0.05$. 'a' indicates values are significantly different from DC rats with $p < 0.001$. 'b' indicates values are significantly different from DC rats with $p < 0.05$

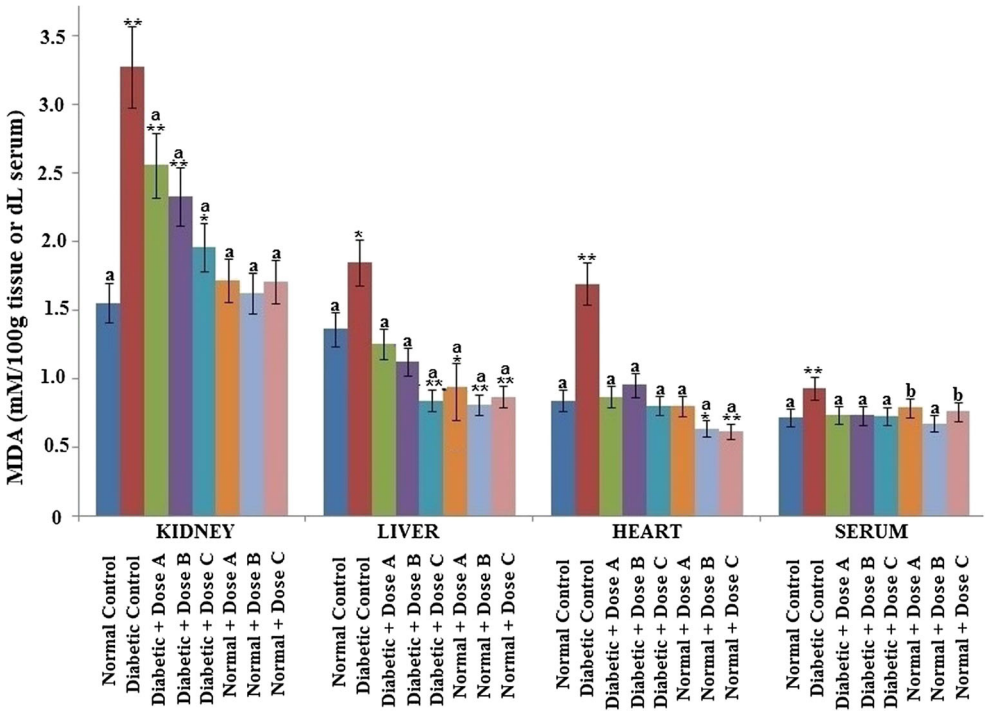

Fig. 7 MDA levels in the various tissues of normal, diabetic control, and diabetic/normal groups treated with different doses of Dhanwantaram Kashayam (DK). Values are expressed as mean ± SD of six rats. '**' indicates values are significantly different from normal control (NC) rats with $p<0.001$. '*'indicates values are significantly different from NC rats with $p<0.05$. 'a' indicates values are significantly different from diabetic control (DC) rats with $p<0.001$. 'b' indicates values are significantly different from DC rats with $p<0.05$

availability was reported to augment muscle wasting and tissue loss during diabetes [39]. In the present study, though food and water intake were increased in diabetic rats, the weight gain was considerably decreased. A significant inhibition in diuresis was observed in all the DK fed diabetic groups. Similarly weight loss was reverted in all the DK fed diabetic groups (results not shown). DK administered rats were relieved of hyperglycemia. This might be due to the increase in the production insulin or its effectiveness in the body. OGTT also revealed better management of glucose turnover. HOMA-IR index analysis revealed the positive change in insulin mediated glucose homeostasis. These positive changes show the improved management of hyper glycaemia in DK fed diabetic rats.

Significantly lower Hb and higher GHb levels were observed in diabetic rats. Administration of DK exerted beneficial effect of the levels of Hb and GHb in diabetic rats by increasing the Hb level and decreasing the glycosylated haemoglobin levels. The increased levels of HbAIc in diabetic state could be a consequence of abnormal carbohydrate metabolism. Decreased hyperglycemia could be the reason for this positive effect [7]. Increase in the Hb level and decreased glycosylation of Hb improve the oxygen status of the tissues and the metabolic process will come back to normal and would have resulted in the improved health as observe in the DK fed diabetic rats. DK administration restored all the above parameters to near normalcy which clearly indicate the normalisation of the disturbed carbohydrate metabolism in diabetic rats by exerting its antidiabetic effects.

Vit C is a natural water soluble antioxidant and has the capacity to act as a reducing agent in free radical mediated oxidation process [40]. Vit E is a potent fat soluble antioxidant generally found in several foods, fats and oils. It also has the ability to detoxify superoxide and H_2O_2 free radicals, and offers membrane stability [41]. The decreased level of vit C and vit E observed in diabetic rats is in agreement with the reports of Jayachandran et al. in 2018 [16]. In the present study we have observed an increase in vit C, vit E, and GSH in all rats fed DK irrespective of being diabetic or normal. We for the first time report the increased vit C and vit E level in Diabetic rats supplemented with DK. Biochemicals like vit C, vit E and GSH play an important role as antioxidants in protecting the cells from oxidative damage [27]. Previous reports points to the possibility of decreased GSH levels observed in experimental rats affecting the vit C and vit E levels [42]. In this study we observed a decrease in the non enzymatic antioxidant GSH in diabetic rats and on administration of DK the level of this non enzymatic antioxidants increased in both diabetic and normal control rats. Increased oxidative stress is a major causative factor in the development and progression of diabetes [43]. Decreased level of these non enzymatic parameters in DC rats may be due to the increased use for the detoxification of the over produced ROS. Increment of these parameters in diabetic and normal rats may be due to two reasons. One may be DK act as a powerful antioxidant in biological system. Because of this the concentration of the free radicals were decreased in diabetic and normal rats fed with DK. So, the turnover rates of the body's antioxidant

metabolites were decreased. Other may be due to direct stimulating effect of DK on the production of vitamin C, vitamin E and GSH. This result validates the claim that DK exert antioxidant property and protect the tissues from lipid peroxidation.

Rats synthesise vitamin C [44], and we attributed enhanced synthesis as the possible reason for the increased levels of vit C in DK fed rats. As expected vitamin C synthesis was decreased in diabetic rats as evidenced by the down regulated expression of the gulo gene and DK administration brought the expression level near to normalcy. Hence the increase in vitamin C level in the DK fed rats might be partially due to the increased synthesis and also the decreased requirement of it for detoxification of the excessively produced ROS as the components of the DK might be doing the job.

GSH is one of the main non enzymatic antioxidants present in tissues that help to counter- balance free radical mediated damage [45]. Previous studies had reported a decrease in the GSH level in diabetic rats and that GSH play a major role in the development and progression of diabetes [46, 47]. It has a direct scavenging activity, act as a co-substrate for Glutathione Peroxidase, an antioxidant enzyme and also have the ability to act as a cofactor. It helps in protecting the cell structure. This protection is extended by maintaining redox homeostasis or through its role in detoxification reactions [45]. Studies have already shown that GSH level was decreased in STZ induced diabetic rats as compared to normal control rats [46]. We observed same pattern of results in diabetic rats. Reduction in GSH in diabetic rats may be due to increased degradation of GSH by oxidative stress or the decrease in its synthesis [47]. Increased GSH in DK fed diabetic rats may be due to the reversal of the changes that led to its decreased levels by the antioxidant ingredients of DK.

Hyperglycemia observed in diabetes results in the generation of free radicals which may exhaust the natural antioxidant defense of the body. This may lead to oxidative damage to membranes and enhanced susceptibility to lipid peroxidation [39]. TBAR levels are found to be higher during diabetes [48]. Increased oxidative stress reported in this study is in agreement with previous reports [49]. Membrane lipids are easily damaged by the actions of reactive oxygen species [50]. As observed by Arabmoazzen et al [51], in our present study the lipid peroxidation product MDA was elevated in tissues and serum of the DC rats and decreased in DK fed normal and diabetic rats. The elevated level of lipid peroxidation indicates oxidative damage. Sruthi and Sindhu in 2012 had already reported that DK a formulation of 40 different plant ingredients is rich in phenolic content and has antioxidant activity [21]. Phenolic compounds are highly potent water soluble antioxidants in plants which have the ability to scavenge the free radicals in our body [11, 22].

Diabetes mellitus is normally monitored by measuring fasting blood sugar [52]. We for the first time are reporting the decrease in blood glucose in DK fed diabetic and normal rats. DK was effective in reducing blood glucose levels since it was able to attenuate the rising glucose level observed in the DK fed groups by approximately 76% with dose C. The decoction was not able to completely block the rising levels of blood glucose, but the fact that this study is for a short duration and DK administration for a longer duration may fully remove the diabetic complications needs to be considered. Suba et al. in 2004 had reported a similar result in diabetic rats fed with *Barlerialupulina* extract [53]. They suggested stimulation of pancreatic mechanism, protection or partial regeneration of pancreatic cells and enhanced secretion of insulin by the remaining protected β-cells in the islets of Langerhans as the possible mechanisms behind the decreased fasting blood glucose level in diabetic and normal rats fed with *Barlerialupulina* extract. Whereas in another study with the extract of *Solenostemon monostachys* leaves, Erah et al. in 1996 suggested the probable increase in peripheral utilization of glucose [54]. Decrease in glucose level observed in the present study may be due to the protection offered by the components of DK having antioxidant property protecting the remaining β-Cells of the pancreas or some components of DK helping in the regeneration of the pancreatic β-cells. Increase in the functional β-Cells ensures increased availability of insulin that in turn reduces blood glucose level.

The above findings help us to conclude that DK exhibits great potential as an antidiabetic agent by reducing the levels of blood glucose and HbAIc and also by decreasing oxidative stress. This shows the beneficial effects of DK in controlling the progression and complications of diabetes.

Conclusion

General well being of the diabetic rats was improved by DK administration. The levels of lipid peroxidation products, fasting blood sugar and HbAIc increased in diabetic rats whereas levels of non enzymatic antioxidants decreased. Administering DK to diabetic rats resulted in a reversal of the situation. From these results, it can be concluded that DK, a PHF used in Ayurveda exerts significant antioxidant activity. This herbal product could be developed as a promising natural and safe remedy for the prevention or delay of diseases caused by imbalance in the metabolism of ROS, especially diabetes.

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Compliance with ethical standards

Permission was obtained from Institutional Ethical Committee.

Conflict of interest Authors report no conflict of interest.

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