Contents available at ScienceDirect

Diabetes Research and Clinical Practice

journal homepage: www.elsevier.com/locate/diabres

Synergistic improved efficacy of *Gymnadenia orchidis* root Salep and pumpkin seed on induced diabetic complications

Subiah H. Arzoo [a], *Krishna Chattopadhyay* [b], *Saheli Banerjee* [a], *Brajadulal Chattopadhyay* [a,*]

[a] *Department of Physics, Jadavpur University, Kolkata 700032, India*
[b] *Department of Chemistry, Jadavpur University, Kolkata 700032, India*

ARTICLE INFO

Article history:
Received 8 August 2018
Received in revised form
1 October 2018
Accepted 29 October 2018
Available online 10 November 2018

Keywords:
Diabetes
DNA damage
Lipid profile
Pumpkin seed
Salep

ABSTRACT

Objective: Diabetes mellitus occurs due to either deficiency of insulin or resistance to insulin. Synthetic drugs and insulin therapy against diabetes possess numerous drawbacks. Diabetic people are advised to choose low-glycemic food and herbal products to control diabetes. This study aims to examine the synergistic effects of aqueous root Salep of *Gymnadenia orchidis Lindl* and pumpkin seed powder on Streptozotocin induced diabetic mice.

Methods: Out of 6 groups, animals in 2 groups were kept as control and rest 4 groups were made diabetic by Streptozotocin. Animals in one diabetic group were supplemented with effective dose (200 mg/kg of body weight) of root Salep, one with pumpkin seed powder (5%) mixed food, and another with Salep and pumpkin seed food. Changes in various biochemical parameters, DNA damage and liver and kidney structures were noted after 21 days treatment.

Results: Salep with pumpkin seed supplementation significantly normalized the alterations of different biochemical parameters of diabetic mice. The DNA damage in blood cells of diabetic mice was recovered by this supplementation. Terpenoids of root Salep and antioxidants of pumpkin seed may play the active role against diabetes.

Conclusion: The root Salep and pumpkin seed synergistically prevent diabetic complications and could be better supplementation against type-2 diabetes.

1. Introduction

The World Health Organization (WHO) has addressed diabetes mellitus as the world's largest growing metabolic disorder characterized by hyperglycemia, due to deficiency either in the production of insulin or resistance to the action of insulin [1]. The diabetic condition leads to alteration in the metabolism of lipids, carbohydrates and proteins [2], cardiovascular disorder due to the functional disabilities of several organs [3] and end-stage renal disease [4]. It also affects visual impairment [5] and increases the risk of pregnancy complications, sexual dysfunction and incontinence [6], memory loss, Alzheimer's disease [7] etc. In diabetes mellitus, increased formation of reactive oxygen species (ROS)

* Corresponding author.
E-mail address: bdc_physics@yahoo.co.in (B. Chattopadhyay).
https://doi.org/10.1016/j.diabres.2018.10.025
0168-8227/

creates oxidative stress and damages the tissues [8]. Enzymatic antioxidants like SOD, GSH and GPx constitute a mutually supportive team of defense against ROS that have been found to decrease in diabetic induced patients.

With the increasing prevalence of over consumption and inactivity associated with the Western lifestyle, the risk of increase in chronic diseases and their associated metabolic disorders continues to be a matter of great concern. This has resulted in continued interest in both diet and lifestyle modifications in prevention and treatment. Consumption of low glycemic index (index $\leq$ 55) foods reduces the rate of glucose absorption, which, in turn, induces a lower rise in circulating insulin and related gastrointestinal hormones [9]. One of the foods low in glycemic index and beneficial for diabetes is pumpkin seeds (*Cucurbita maxima)* (index of 10). *Cucurbitaceae* (Cucurbit), an important family comprising one of the most genetically diverse groups of food plants. Pumpkin seeds are easily available locally and rich sources of unsaturated oil, energy and vitamin E, while the dominant fatty acids present in the oil were oleic 29% and linoleic 47% [10]. It is also richly endowed in macro elements (magnesium, phosphorus and calcium) and moderate amounts of micro elements (calcium, manganese, copper and zinc) and thus the seed can be used as a valuable food supplement [10].

The uses of synthetic drugs and insulin therapy in diabetes possess numerous drawbacks like insulin resistance [11], anorexia nervosa, brain atrophy, fatty liver and so on [12]. One of the medicinal plants (*Gymnadenia orchidis Lindl*) has the ability to reduce blood glucose level [13]. The plant belongs to the family *Orchidaceae* and is found in the Himalayan region from Pakistan to South–East Tibet at an altitude range of 2400–4000 m. This perennial herb has a tuberous root which is divided into 2 or 3 lobes. The roots of this plant when grinded and mixed with water form a thick 'Salep' which is traditionally used by the people of Bhootia community to get some relief against diabetes [13]. There is no toxic effect observed by using the root Salep even at higher doses [13]. So for alternative treatment, a study was designed to observe the synergistic effect of the root Salep and the pumpkin seed on diabetes and its associated complications.

2. Material and methods

2.1. Preparation of root Salep

The fibrous root of *Gymnadenia orchidis Lindl* was collected from the local market in Darjeeling, West Bengal, India [14]. The root Salep was prepared by suspending the powder root in double distilled water and used at an effective dose (200 mg/kg body weight) to the diabetic animals through oral supplementation [13].

2.2. Preparation of the low glycemic supplemented diet

The pumpkin (*Cucurbita maxima)* seed was procured from the local super market, dried and crushed into powder and then supplemented (5% w/w) to the normal protein diet (18% casein, 70% carbohydrate, 7% fat, 4% salt mixture and 1% vitamin mixture) [15] to make low glycemic supplemented food (18% casein, 65% carbohydrate, 7% fat, 4% salt mixture and 1% vitamin mixture, pumpkin seed powder 5%).

2.3. Animals and treatment

Adult female albino mice (n = 30, body weights 45–50 g) of BALB C strain was procured from the animal housing facility. The work was approved by the Institutional Animal Ethics Committee of Jadavpur University, Kolkata, India (Ref. No.: AEC/PHARM/1601/11/2016). The animals were acclimatized under standard conditions of temperature and humidity with 12 h light/dark cycles and fed with normal protein diet one week before the commencement of treatments. Some animals were injected intra-peritoneally with Streptozotocin (STZ) (25 mg/kg body weight dissolved in 0.1 M citrate buffer at pH 4.5) to induce diabetes [16]. Root Salep and low glycemic supplemented food were given to different groups of animals. The experiment was conducted on 6 groups of mice each containing 5 animals as shown in Table 1. Streptozotocin was injected to the 6 hr fasting mice at 3 p.m. in three consecutive days and fasting blood glucose level was checked after 3 days of last injection. The effective dose of root Salep was fed orally at 12 noon every day to the respective diabetic groups for 21 days. The diet was given in adequate amount twice daily.

2.4. Sample collection

Blood glucose was checked in every 7 days interval throughout the treatment period by pricking the tail of the animals. After 21 days of treatment, animals were kept in fasting condition overnight and sacrificed on the following morning with mild anesthesia. Blood samples were collected from the heart immediately after sacrifice and stored in both, with and without anticoagulant (heparin) containing containers. Serum was separated by centrifugation and stored at −20 °C for further analysis. Liver and kidney were dissected out and stored in vacuum desiccators at −20 °C to prevent auto oxidation.

2.5. Biochemical analysis

Blood glucose was estimated through Contour TS (Bayer Polychem India Ltd). The glycosylated haemoglobin (HbA1c) percentage was determined by using the kit supplied by Euro Diagnostic Systems Pvt. Ltd. Aspertate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT) activity in serum were measured by kit. The phosphatases activities in serum were assayed according to the method of Bergmeyer and Bernt [17]. Urea and creatinine were determined from serum of all the animals by using the standard kit supplied by ARKRAY Healthcare Pvt. Ltd., India. The lipid components; total cholesterol (TC) [18] and tryglyerides (TG) [19] were estimated in serum by using standard kits. Superoxidise (SOD) [20], catalase (CAT) [21], glutathione peroxidase (GPx) [22] and reduced glutathione (GSH) [23] contents of mice liver were determined accordingly. By the assay of thiobarbituaric acid reactive substances (TBARS) liver lipid peroxidation was measured [24]. The amount of MDA was calculated by taking the extinction coefficient of MDA to be

Table 1 – Experimental group with their initial fasting blood glucose, diet and treatment.

Name of the group	Initial fasting blood glucose levels	Diet and treatment
Normal control (NC)	<100 mg/dl	Normal protein diet
Low glycemic food supplemented control (LGFC)	<100 mg/dl	Low glycemic supplemented diet
Diabetic control (DC)	>120 mg/dl	Normal protein diet
Low-glycemic food supplemented diabetic (LGFD)	>120 mg/dl	Low glycemic supplemented diet
Root Salep supplemented diabetic (RSD)	>120 mg/dl	Normal protein diet + oral administration of effective dose of root Salep
Root Salep with low-glycemic food supplemented diabetic (RSLGFD)	>120 mg/dl	Low glycemic supplemented diet + oral administration of effective dose of root Salep

$1.56 \times 10^5\ M^{-1}\ cm^{-1}$. Glutathione-independent and glutathione-dependent superoxide and peroxide handling capacity (SPHC) were calculated from the ratio of CAT / SOD and GPx / SOD, respectively [25,26].

2.6. DNA content

The total DNA content in whole blood cells was measured by slightly modifying the protocol of DNA preparation by National Institute of Health [27] as described by Banerjee et al [28]. The concentration and purity of the DNA content was determined by spectrometer at A_{230}, A_{260} and A_{280}.

Concentration of DNA (μg/ml) = O.D at 260 nm × dilution factor × 50,

where 1 O.D = 50 μg DNA/ml.

DNA yield (μg) = Concentration (μg/ml) × sample volume (ml).

2.7. Comet assay

The procedure for comet assay was followed as described by Bandhopadhyaya et al. [29]. The photomicrograph of each slide was taken with Leica fluorescent microscope (Model 300 FX at 40× magnification). Measurements of total comet area, head area, tail length and mean DNA density etc. were done by using the Perceptive Comet Assay IV software version 4.3. The percentage of DNA damage and tail moment was calculated as described by Helma and Uhl [30]. A total of 100 cells, at least, were screened per animal the data were averaged.

Total DNA in comet = (Total comet Area) × (Mean DNA intensity)

Total DNA in comet head = (Total Head area) × (Mean DNA intensity)

$$\%\ \text{of DNA damage} = \frac{(\text{Total DNA in comet}) - (\ \text{Total DNA in comet head})}{(\text{Total DNA in Comet})} \times 100$$

Tail moment = (Tail length) × (Mean DNA intensity in tail)

2.8. Histological examination

Cleaned tissue of liver, kidney and pancreas were fixed by using Bouin's fluid. After fixation, the tissues were washed several times by different graded alcohol, embedded in paraffin and sliced by using rotary microtome. The paraffin sections were attached on the slides and washed by xylol before staining. The tissue section was then stained by using hematoxylin and eosin staining and examined under Leica fluorescent microscope (Model 300 FX at 10× magnification) [31].

2.9. Statistical analysis

Whole experimental set up was repeated twice and data were averaged over 10 animals and given as mean ± SD. Two-way analysis of variance (ANOVA) was used to identify the effects of two treatments – (a) Root Salep (RS), (b) Low glycemic food (LGF), as well as their interactions on the measured and calculated parameters of diabetic animals – LGFD, RSD, RSLGFD and LGFC. For two-way ANOVA, the effects were considered significant with $p < 0.05$. One way ANOVA was used to analyze the data for all the groups including NC and DC. Tukey's HSD test was used to analyze the level of significance for the differences between the means. Two levels of significance were used in the assessment, $p < 0.01$ and $p < 0.001$.

3. Results

The Fig. 1A summarizes the observed value of fasting blood glucose levels in all the groups over a period of 21 days. The initial blood glucose value of NC group was 86 ± 2.96 mg/dl. After STZ injection, the fasting glucose levels of all the animals in DC group were increased significantly ($p < 0.001$) to 215 ± 3.65 mg/dl. The fasting blood glucose levels were also decreased significantly in LGFD group (115 ± 3.74 mg/dl; $p < 0.01$) and in RSD group (90 ± 2.98 mg/dl; $p < 0.001$) and

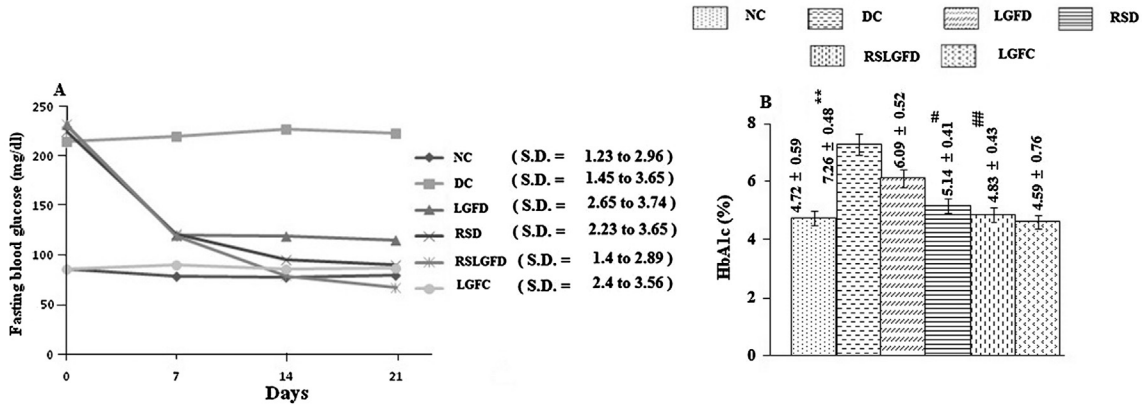

Fig. 1 – A fasting blood glucose level of the mice in different groups. N = 10, data presented as mean ± SD with significance levels (* is significant and ** is more significant in-comparison to NC, # is significant and ## is more significant in-comparison to DC.). B: Glycosylated haemoglobin percentage of the mice in different groups. N = 10, data presented as mean ± SD with significance levels (* is significant and ** is more significant in-comparison to NC, # is significant and ## is more significant in-comparison to DC.)

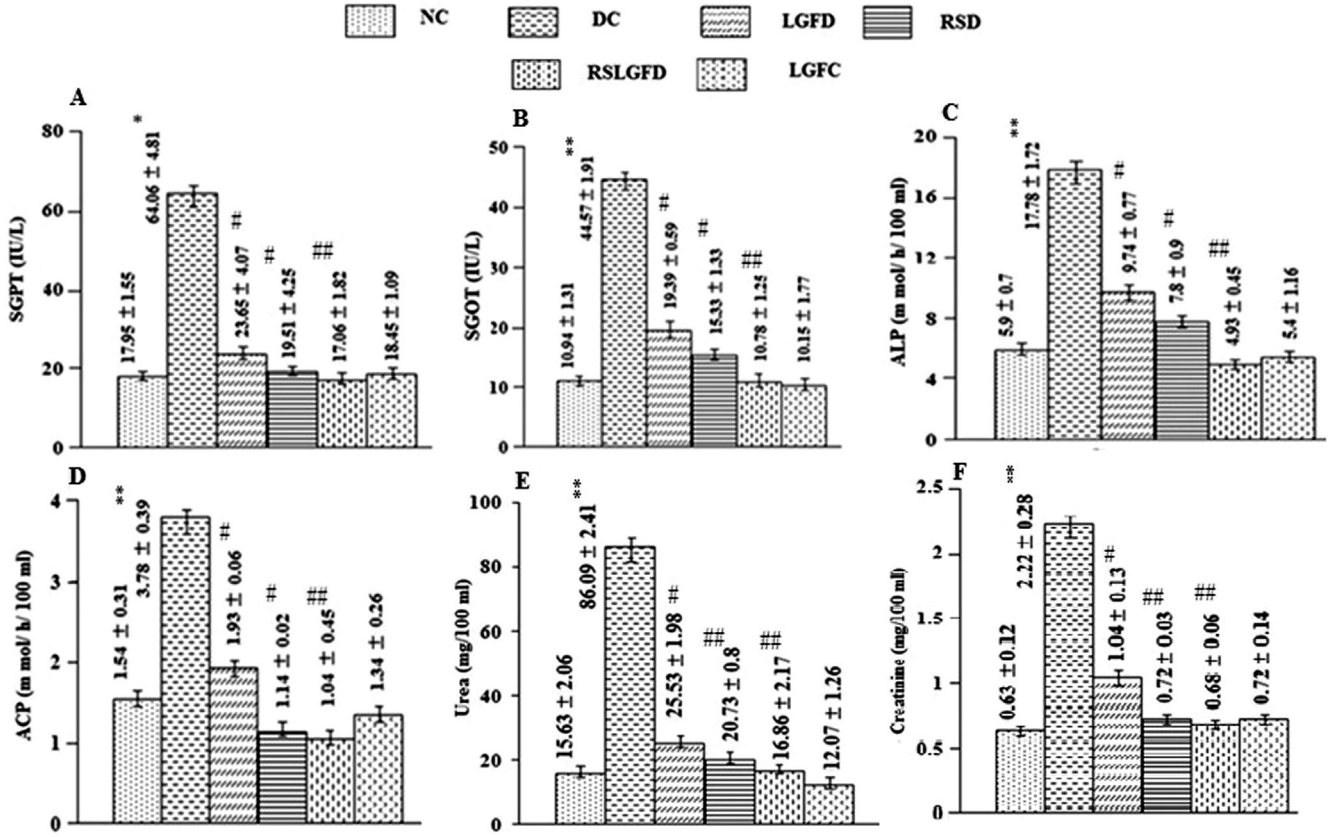

Fig. 2 – (A, B, C, D): Liver function enzymes of the mice in different groups. N = 10, data presented as mean ± SD with significance levels (* is significant and ** is more significant in-comparison to NC, # is significant and ## is more significant in-comparison to DC.). (E, F): Kidney function parameters of the mice in different groups. N = 10, data presented as mean ± SD with significance levels (* is significant and ** is more significant in-comparison to NC, # is significant and ## is more significant in-comparison to DC.)

RSLGFD group (67 ± 2.89 mg/dl; $p < 0.001$) in comparison to DC group.

The average HbA1c% was increased from 4.7 ± 0.6 of NC group to 7.3 ± 0.5 of DC group ($p < 0.001$) by Streptozotocin injection (Fig. 1B). The HbA1c% of the RSD group mice was reduced to 5.1 ± 0.4 ($p < 0.01$) in comparison to the DC group. More significant ($p < 0.001$) decrease of HbA1c% of RSLGFD group (4.8%) was noted in comparison to the DC group. The

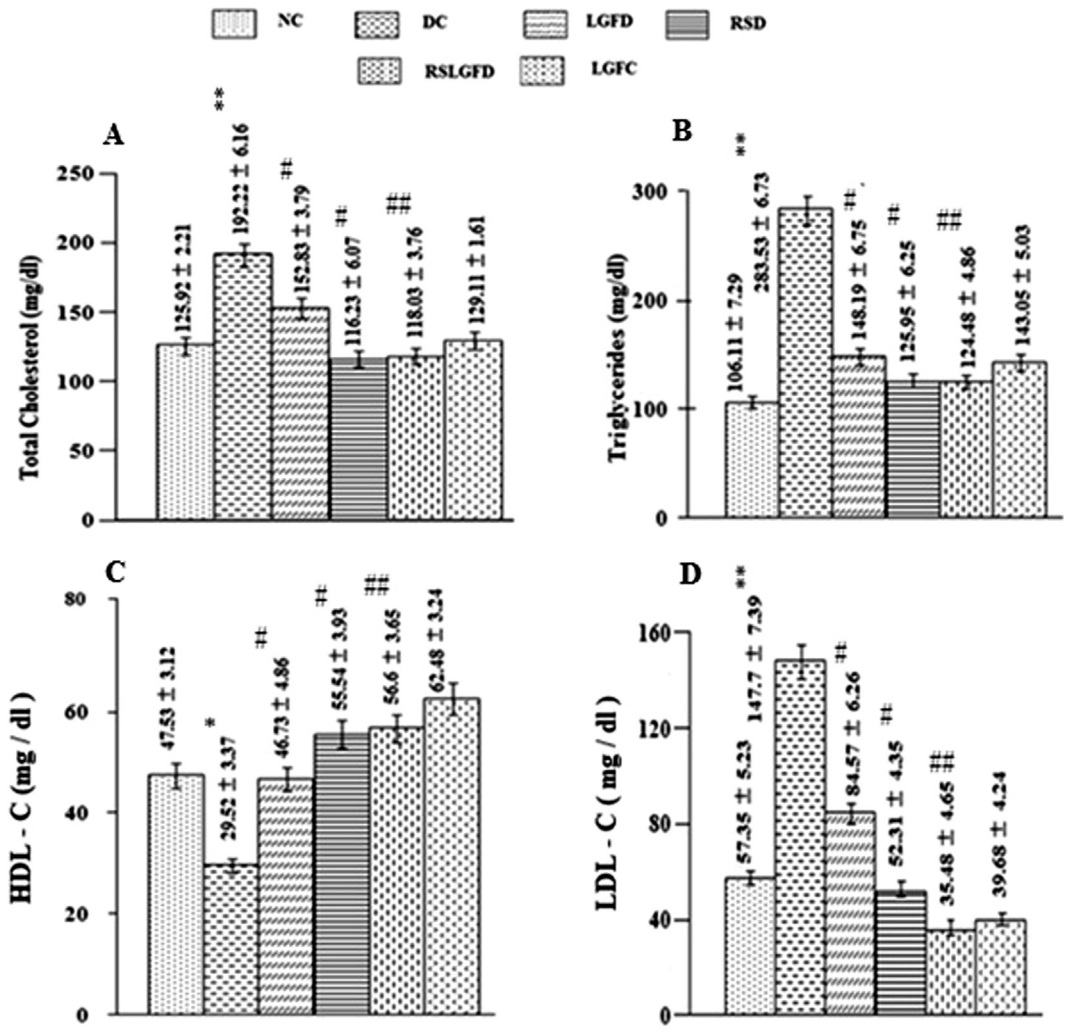

Fig. 3 – (A, B C, D): Lipid profiles of the mice in different groups. N = 10, data presented as mean ± SD with significance levels (* is significant and ** is more significant in-comparison to NC, # is significant and ## is more significant in-comparison to DC.)

reduction of HbA1c% in the LGFD (6.1%) group was insignificant with respect to the DC group. The measurement of HbA1c in NGSP and IFCC unit are given in supplementary data (Table S1). In diabetic animals, significant influence of LGF treatment was observed in HbA1c level. Though RS treatment showed only insignificant influence, its interaction with LGF treatment was found to influence the HbA1c of diabetic animals significantly.

The graphical presentations of the liver profiles (SGOT, SGPT, ALP and ACP levels) in serum of different groups showed significant ($p < 0.001$) increments of SGOT activity(Fig. 2B) and a SGPT activity (Fig. 2A) in the DC group with respect to NC group. Significant reductions of these two hepatic enzyme activities with respect to DC group were noted in the diabetic animals of LGFD ($p < 0.01$), RSD ($p < 0.01$) and RSLGFD ($p < 0.001$) in 21 days. Similar activities were observed for the other two (ALP and ACP) hepatic enzymes (Fig. 2C,D). Significant influences of RS and LGF as well as their interactions were observed in case of SGOT and ACP of diabetic animals. On the other hand, significant influences of RS and its interaction with the LGF were noticed in case of SGPT and ALP of diabetic animals.

Urea and creatinine levels of the DC group were significantly ($p < 0.001$) increased compared to the NC group (Fig. 2E, F). Significant ($p < 0.001$) reduction in the kidney function parameters of RSLGFD group was noted but the same in LGFD group was not significant as compared to the RSLGFD group. Creatinine levels of diabetic animals were significantly influenced by RS and LGF treatments. Their interactions were also significant in creatinine levels of diabetic animals. However, in case of urea, though RS treatment failed to produce any significant impact, its interaction with LGF treatment was found to be significant along with lone LGF treatment.

Total cholesterol, triglycerides and LDL-C in serum of the DC group were significantly ($p < 0.001$) increased compared to the NC group (Fig. 3A, B, D). Significant reduction in the lipid profile of the LGFD group ($p < 0.01$), RSD group ($p < 0.01$) and RSLGFD group ($p < 0.001$) were noticed after the treatment with root Salep, root Salep with pumpkin seed and pumpkin seed separately. Very good increment of HDL-C levels ($p < 0.001$) of the RSLGFD mice was observed in comparison to the DC mice (Fig. 3C). Though the total cholesterol and LDL-C of diabetic animals were significantly influenced by the

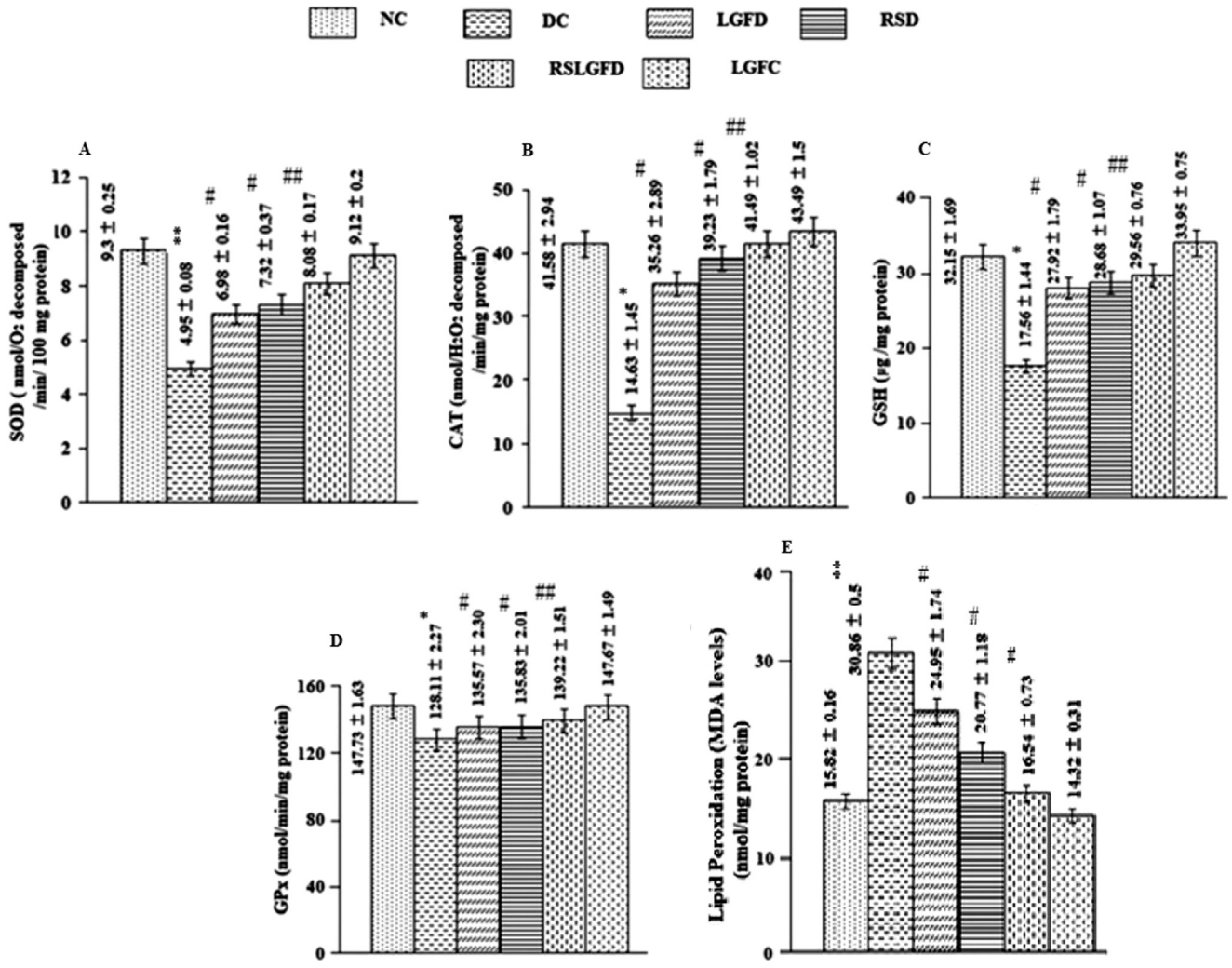

Fig. 4 – (A, B, C, D): Anti-oxidant enzymes activities of the mice in different groups. N = 10, data presented as mean ± SD with significance levels (* is significant and ** is more significant in-comparison to NC, # is significant and ## is more significant in-comparison to DC.). E: Lipid peroxidation of the mice in different groups. N = 10, data presented as mean ± SD with significance levels (* is significant and ** is more significant in-comparison to NC, # is significant and ## is more significant in-comparison to DC).

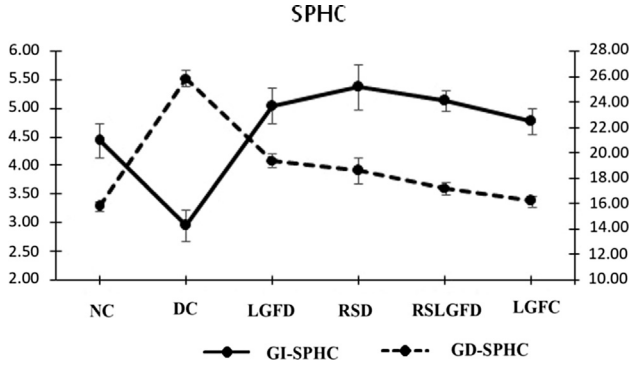

Fig. 5 – SPHC activities of the mice in different groups.

RS and LGF treatments as well as their interactions, the triglycerides of diabetic animals was significantly influenced by RS treatment only. Similarly, the HDL-C of those animals was also influenced by LGF treatment and its interaction with RS treatment, but the only RS treatment failed to influence it.

Activities of all the antioxidant enzymes (SOD, CAT, GSH and GPx) of liver tissues were decreased due to induction of diabetes (Fig. 4A–D). Significant ($p < 0.01$) decrements of CAT, glutathione and GPx activities and significant decrement ($p < 0.001$) of SOD activity were observed in serum of the DC group. Administration of the root Salep or supplementation of pumpkin seed powder in food significantly ($p < 0.01$) increased the GSH and GPx levels of the RSD and LGFD group respectively. There were significant ($p < 0.001$) increase in the SOD, GSH and GPx activities observed in the RSLGFD group. Oral root Salep administration along with the pumpkin seed powder (5%) supplemented food significantly ($p < 0.001$) decreased the lipid peroxidation caused by diabetes (Fig. 4E). The observed MDA level was significantly lowered in RSLGFD group than that of DC group. All the oxidative stress parameters of diabetic animals were significantly influenced by both RS and LGF treatments, as well as their interactions. The

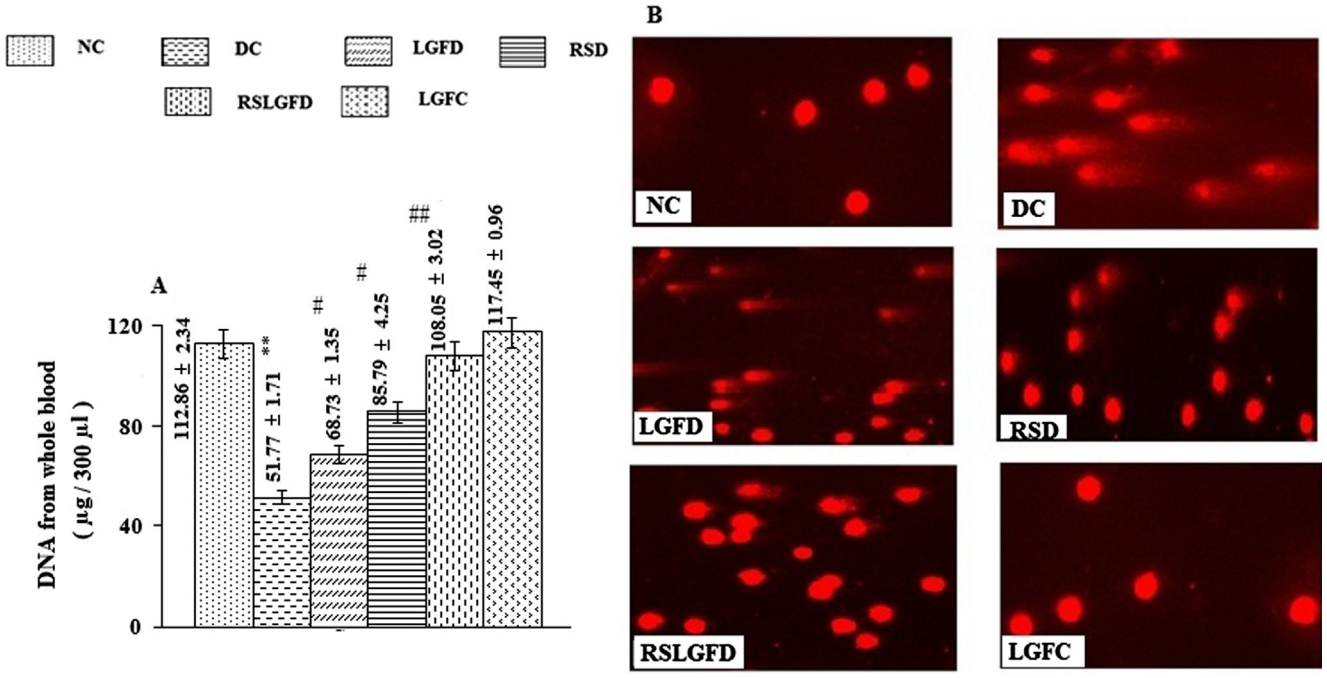

Fig. 6 – A: Total DNA content of the whole blood of the mice in different groups. N = 10, data presented as mean ± SD with significance levels (* is significant and ** is more significant in-comparison to NC, # is significant and ## is more significant in-comparison to DC.). B: Comet photographs of whole blood of the mice in different groups.

Table 2 – DNA damage and tail moment of mice in different groups.

Group	% DNA damage	Tail moment (Arbitrary units)
NC	8.77 ± 1.02**	68.69 ± 8.08**
DC	56.06 ± 1.96##	1348.46 ± 17.30##
DLGF	33.31 ± 1.76##	562.90 ± 29.81##
DRS	22.13 ± 1.26##	373.97 ± 21.43##
DRSLGF	14.46 ± 2.31	113.75 ± 18.17
NCLGF	7.97 ± 1.21	62.66 ± 9.53

At least 100 comet cells were taken and data presented as mean ± SD with significance levelswhere, * is significant ($p < 0.01$) and ** is more significant ($p < 0.001$) incomparison to NC, # is significant ($p < 0.01$) and ## is more significant ($p < 0.001$) in comparison to DC.

SPHC of diabetic animals demonstrated an interesting pattern (Fig. 5). Though the interaction between RS and LGF treatment was able to influence the SPHC of diabetic animals significantly, the RS treatment was able to influence the glutathione-independent SPHC only, while, LGF treatment was able to influence the glutathione-dependent SPHC only.

Streptozotocin treatment decreased 54% total DNA contents of whole blood cells of the DC group with respect to the NC group ($p < 0.001$) (Fig. 6A). Administration of the root Salep ($p < 0.01$) or supplementation of pumpkin seed powder with root Salep ($p < 0.001$) both showed an increase of total DNA content of whole blood cells compared to the DC group mice. Comet like pictures of whole blood DNA appeared due to diabetes (Fig. 6B). The shape of whole blood cells DNA of RSD and RSLGFD group animals was normalized to some extent. Diabetes more significantly ($p < 0.001$) increased the percentage of DNA damage (56.06%) in blood cells of DC group as compared to the NC group (8.77%) (Table 2). Root Salep supplementation reduced the DNA damage to 22.13% in RSD group. A better result was seen in the blood cells DNA damage when supplemented with root Salep along with the pumpkin seed powder in food (14.46%; $p < 0.001$) as observed in RSLGFD group. While the DNA contents and DNA damage of diabetic animals were significantly influenced by both LGF treatment and its interaction with RS treatment, the influence of RS treatment was only significant for DNA content.

The regular arrangement of hepatic cells and normal shape of the central vein of the liver were observed from the histological study of NC, LGFC and RSLGFD group animals (Fig. 7A, C, F). The hepatic cell arrangement was distorted and central vein dilated of the DC group (Fig. 7B). Slight improvement in LGFD group (Fig. 7E) and better improvement in RSD group (Fig. 7D) were observed. The normal arrangement of glomerulus, bowman capsule and proximal/ distal tubules of the kidney tissue was observed in NC, LGFC and RSLGFD groups (Fig. 7G, I, L). A slight improvement in LGFD group (Fig. 7K) and better improvement in RSD group (Fig. 7J) also noted. The histological examination of the pancreas of NC, RSD, LGFC and RSLGFD groups showed normal β-cell architecture whereas administration of STZ in DC group showed significant morphological changes with severe injury of pancreatic β-cell (Fig. 8). The root Salep showed better improvement in preservation of pancreatic β-cell than only pumpkin seed when used in induced diabetic animals. The synergistic effect of root Salep and pumpkin seed could be evaluated by the normal architecture of the pancreas as revealed by the histology of RSLGFD group in Fig. 8.

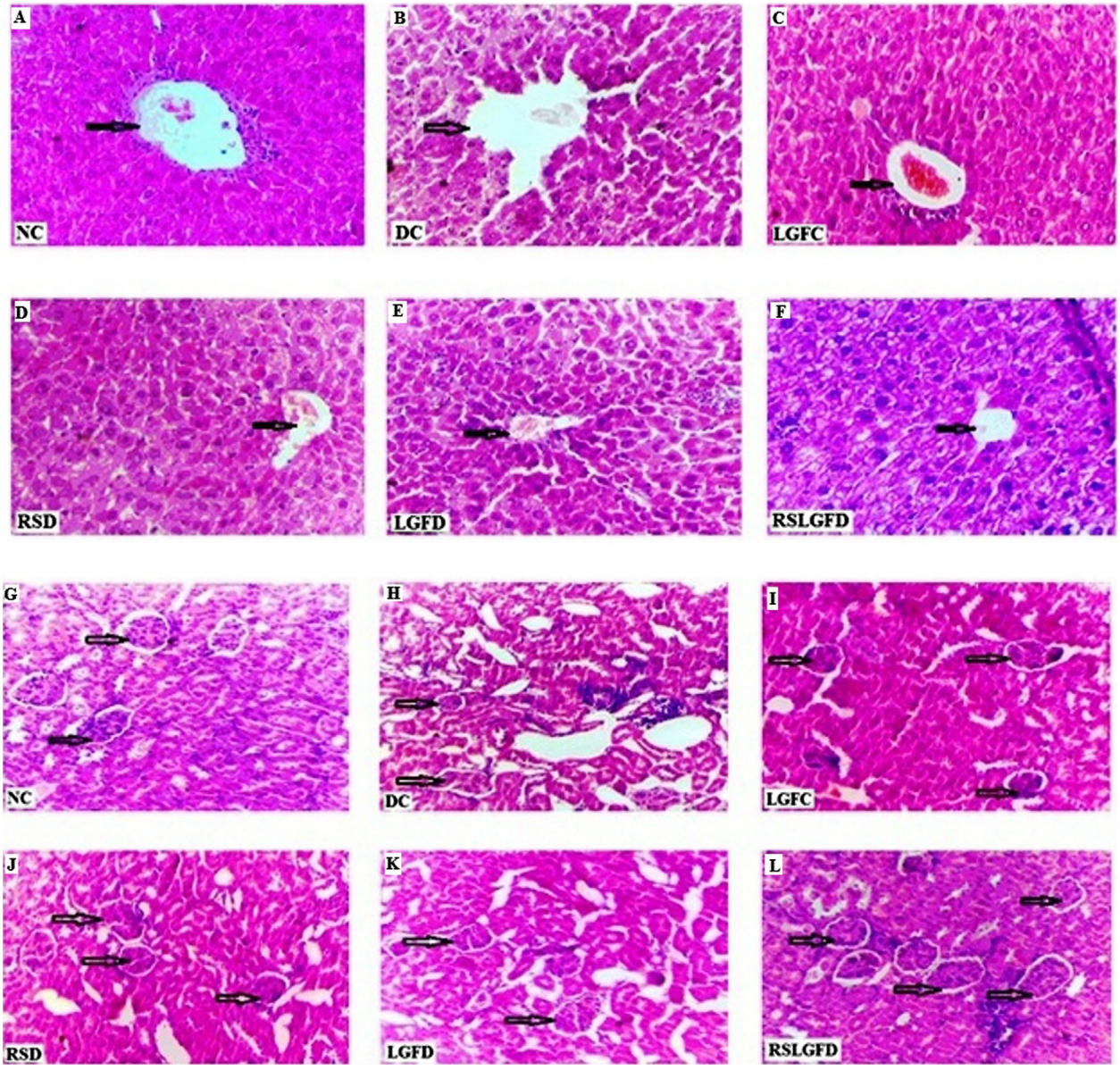

Fig. 7 – (A, B, C, D, E, F): Histological section of mice liver in different groups (arrowhead indicates the central vein). (G, H, I, J, K, L): Histological section of mice kidney in different groups (arrowhead indicates the glomerulus).

4. Discussion

Dietary intervention, particularly the use of low glycemic index and traditional therapeutic agent derived from natural sources, is the main stay in the management of diabetes mellitus and its associated complications. In this context, there has been a growing interest in recent times in identifying as many dietary sources as possible in reducing the effect as well as management of diabetes. Till now, there are rare reports about the medicine that can attenuate synchronously the damage on main organs of diabetics.

Single dose injection of STZ causes severe type 2 diabetes to the animals [16]. Recovery from hyperglycemia from Streptozotocin-induced diabetic mice within a short time was observed in the groups supplemented with root Salep or root Salep with pumpkin seed powder mixed food or pumpkin seed powder mixed food separately (Fig. 1A). Such an effect could be related to the partial regeneration or preservation of pancreatic β-cell mass by the action of Salep and pumpkin seed after Streptozotocin treatment as revealed by the histological examination of pancreas (Fig. 8). STZ administration produced diabetes status by selective destruction of pancreatic β-cells with changes in metabolic variables as well as kidney and liver functions [16].

The increase of hepatic enzyme activities in serum of untreated diabetic mice indicated that diabetes might induce hepatic dysfunction as observed in earlier findings [32]. Supplementation of the root Salep, pumpkin seed mixed food or both in different groups reduced significantly the hepatic enzymes activities in serum of diabetic mice (Fig. 2). Terpenoids present in *Gymnadenia* root Salep is an effective agent which can reduce the blood glucose level [13]. Our findings are thus in agreement

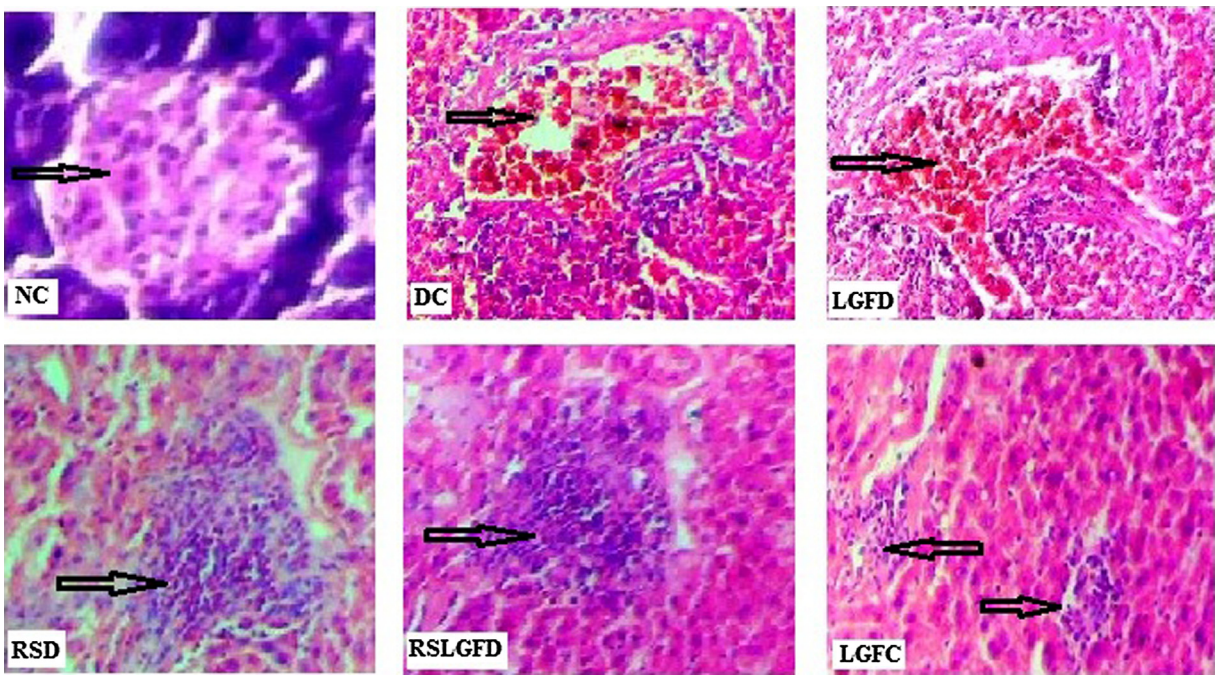

Fig. 8 – Histological section of mice pancreas in different groups (arrowhead indicates the Islets of Langerhan).

with the results of Nazaruk and Borzym-Kluczyk [33] where they have shown that triterpenes involve in glucose metabolism, prevent the development of insulin resistance due to which plasma glucose and insulin levels become normal in blood. Trigonelline (TRG) and nicotinic acid (NA) found in pumpkins also have anti-diabetic properties [34]. The presence of flavanoids, phenols or saponins in the seed extracts of *Cucurbita maxima* could explain its role as potential anti diabetic agents [35]. Also, different forms of tocopherol present in pumpkin seeds shows enhancement for the β cells in pancreas for insulin sensitivity [36]. It is known that the HbA1c% in diabetic mice reflects the state of erythrocytes influenced by the diet that has been fed in the previous 2–3 weeks [37]. So the lower level HbA1c% of RSLGFD group in comparison to the diabetic group suggest that the Root Salep and pumpkin seed synergistically inhibits glycation of the hemoglobin.

In the present study, there was an elevation in urea and creatinine levels in the diabetic group, suggestive of renal damage. We determined that STZ caused a significant damage to renal structures, including the glomeruli and tubules (Fig. 7G-L). Due to synergistic effect of roots Salep along with the pumpkin seed food, significant ($p < 0.001$) reductions of urea and creatinine levels were seen in RSLGFD group. This was in agreement with the previous studies where the diabetes induced kidney damage was reversed to a significant extent by the supplementation of *Gymnadenia orchidis Lindl* root Salep [13] or pumpkin seed and flax seed [38].

The high cholesterol level in liver and serum might be due to increased uptake of exogenous cholesterol and subsequent deposition and decreased cholesterol catabolism [39]. Administration of the root Salep normalized the lipid profile of diabetic animals but this result was significant ($p < 0.001$) with root Salep along with pumpkin seed supplementation. Linoleic acid in pumpkin seed possesses hypo-cholesterolemic property by inhibiting cholesterol absorption [39]. Thus the lowering levels of lipid profiles by the synergistic effect of *Gymnadenia orchidis Lindl* root Salep and pumpkin seed in diabetes induced mice can be explained similarly.

Enzymatic antioxidants like SOD, CAT, GSH and GPx constitute a mutually supportive team of defense against ROS and depletion activities of these antioxidant enzymes in diabetic liver not only impairs cell defense against toxic compounds, but also results in enhanced oxidative stress and tissue damage (Fig. 7A-F). Supplementation of root Salep along with pumpkin seed caused improvement in antioxidant status and reduction in lipid peroxidation (Fig. 4). Root and pumpkin seed powder can possibly reduce generation of free radicals by scavenging the peroxyl radicals. The level of MDA in diabetic mice was significantly ($p < 0.01$) increased due to the decreased activity of scavenging enzymes (CAT and SOD). A decrease in the activity of these enzymes can lead to the excessive availability of superoxide and peroxide radicals, which in turn generates hydroxyl radicals resulting in the initiation and propagation of lipid peroxidation [40]. When the SPHCs are considered, there was reduction in GI-SPHC and increment in GD-SPHC in diabetic animals (Fig. 5). Significant influences of RS treatment on improving the GI-SPHC and LGF treatment on lowering the GD-SPHC are actually compensating each other.

Diabetes causes a significant ($p < 0.001$) decrease in total DNA contents of whole blood cells of animals in DC group (52 μg/300 μl) compared to NC group (113 μg/300 μl) (Fig. 6A). The purity of the isolated DNA has been checked by the ratios of absorbance (A) of isolated DNA (A230/A260 = 0.41 and A260/A280 = 1.78), which is in agreement with previously published data [29]. Increased generation of ROS causes oxidative DNA damage resulting increased rate of cell death and thus decreased DNA content in diabetes induced blood cells [41].

There was only 8.77% DNA damage occurred in the blood cells of NC group mice, which is acceptable. But in DC group, DNA damage significantly ($p < 0.001$) increased to 56.06%. As DNA damage is directly proportional to the tail moment, the large value of the tail moments (19 times more in DC group compared to NC group) confirmed the higher degree of DNA damage in diabetes induced condition. The comet like photographs of mice blood nuclear DNA of DC group was a clear indication of DNA stand breakage compared to NC group which was significantly recovered in the group administered with root and supplemented with pumpkin seed. The analytical results of the Comet assay confirmed the protective effect of the root Salep and pumpkin seed against the diabetes induced genotoxicity.

We thus demand that there is a promising synergistic role of *Gymnadenia orchidis* root Salep and the pumpkin seed in attenuating the deleterious effect of diabetes and its associated complications for which they can be used as potential therapeutic supplementation in food against type-2 diabetes.

Acknowledgment

The financial support of the Research Society for the Studies of Diabetes in India (RSSDI) for the study is gratefully acknowledged. Miss Subiah H. Arzoo gratefully acknowledges the fellowship support from UGC, Government of India. The authors are also thankful for the experimental facilities provided by the Biophysical Laboratory of Physics Department of Jadavpur University.

Conflict of interest

There is no conflict of interest of any kind related to this manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diabres.2018.10.025.

REFERENCES

[1] World Health Organization: Global Reports on Diabetes. World Health Organization, http://www.who.int; 2016. 20 Avenue Appia, 1211 Geneva 27, Switzerland.
[2] Brand-Miller J, McMillan-Price J, Steinbeck K, Caterson I. Dietary glycemic index: health implications. Am Coll Nutr 2009;28:446S–9S.
[3] Sanne AEP, Huxley RR, Woodward M. Diabetes as risk factor for incident coronary heart disease in women compared with men: a systematic review and meta-analysis of 64 cohorts including 858,507 individuals and 28,203 coronary events. Diabetologia 2014;57:1542–51.
[4] Ghaderian SB, Hayati F, Shayanpour S, Mousavi BSS. Diabetes and end-stage renal disease; a review article on new concepts. J Renal Inj Prev 2015;4:28–33.
[5] National Eye Institute, Facts about Diabetic Eye Disease, 2015.
[6] Review Riaz S. Diabetes mellitus. Sci Res Essays 2009;4:367–73.
[7] Whitmer AR, Karter JA, Yaffe K, Quesenberry PC, Selby VJ. Hypoglycemic episodes and risk of dementia in older patients with type 2 diabetes mellitus. JAMA 2009;30:1565–72.
[8] Matough AF, Budin BS, Hamid AZ, Alwahaibi N, Mohamed J. The role of oxidative stress and antioxidants in diabetic complication. SQU Med J 2012;12:5–18.
[9] Brand-Miller JC, Stockmann K, Atkinson F, Petocz P, Denyer G. Glycemic index, postprandial glycemia, and the shape of the curve in healthy subjects: analysis of a database of more than 1000 foods. Am J Clin Nutr 2009;89:97–105.
[10] Karanja JK, Mugendi BJ, Khamis FM, Muchugi AN. Nutritional composition of the pumpkin (*cucurbita spp.*) seed cultivated from selected regions in Kenya. J Hortic Lett 2013;3:17–22.
[11] Lebovitz HE. Insulin: Potential negative consequences of early routine use in patients with type 2 diabetes. Diabetes Care 2011;2:5225–30.
[12] American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care 2010;33:562–9.
[13] Parvin T, Chattopadhyay K, Tamang T, Arzoo HS, Ghosh S, Chattopadhyay B. Therapeutic implications of *Gymnadenia Orchidis Lindl* root salep against induced-diabetes. AJPT 2018;8 (ISSN):2249–3387.
[14] Bhatt A, Joshi SK, Gairola S. *Dactylorhiza hatagirea (D. Don) Soo*-a west Himalayan orchid in peril. Curr Sci 2005;89:610–2.
[15] Hawk PB, Oser BL, Summerson WH. Carbohydrates, fats, and protein metabolism. In: Hawk PB, Oser BL, Summerson WH, editors. Practical physiological chemistry. New York: Blakiston Co; 1954.
[16] Deeds MC, Anderson JM, Armstrong AS, Gastineau DA, Hiddinga HJ, Jahangir A, et al. Single dose streptozotocin-induced diabetes: considerations for study design in islet transplantation models. Lab Anim 2011;45:131–40.
[17] Bergmeyer HU, Bernt E. Methods of enzymatic analysis. New York: Academic Press; 1974.
[18] Allain CC, Poon LS, Chan CS, Richmond WS, Fu PC. Enzymatic determination of total serum cholesterol. Clin Chem 1974;20:470–5.
[19] Werner M, Gabrielson DG, Estman J. Ultramicro determination of serum triglycerides by bioluminescent assay. Clin Chem 1981;27:268–71.
[20] Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 1971;44:276–87.
[21] Cohen G, Dembiec D, Marcus J. Measurement of catalase activity in tissue extracts. Anal Biochem 1970;34:30–8.
[22] Levander OA, Alfthan G, Arvilommi H, Gref CG, Huttunen JK, Kataja M, et al. Bioavailability of selenium to Finnish men as assessed by platelet glutathione peroxidase activity and other blood parameters. Am J Clin Nutr 1983;37:887–97.
[23] Davila JC, Davis PJ, Acosta D. Changes in glutathione and cellular energy as potential mechanisms of papervine – induced hepatotoxicity in vitro. Toxicol Appl Pharmacol 1991;108:28–36.
[24] Chatterjee SN, Agarwal S. Liposomes as membrane model for study of lipid peroxidation. Free Radic Biol Med 1998;4:51–72.
[25] Nayak P, Shrama SB, Chhowdary NVS. Alpha-tocopherol supplementation restricts aluminium- and ethanol-induced oxidative damage in rat brain but fails to protect against neurobehavioral damage. J Diet Suppl 2018;5:1–12.
[26] Nayak P, Shrama SB, Chhowdary NVS. Aluminum and ethanol induce alterations in superoxide and peroxide handling capacity (SPHC) in frontal and temporal cortex. Indian J Biochem Biophys 2013;50:402–10.
[27] National Institute of Health. Guideline for DNA preparation from blood. Genetic Branch of National Institutes of Health: Section of Cancer Genomics; 2004.
[28] Banerjee S, Chattopadhyay K, Chhabra KJ, Chattopadhyay B. Protein dependent fate of hepatic cells under nicotine

induced stress and curcumin ameliorated conditions. Eur J Pharmacol 2012;684:132–45.
[29] Bandyopadhyaya G, Sinha S, Chattopadhyay BD, Chakraborty A. Protective role of curcumin against nicotine-induced genotoxicity on rat liver under restricted dietary protein. Eur J Pharmacol 2008;588:151–7.
[30] Helma C, Uhl M. A public domain image-analysis program for the singlecell gel-electrophoresis (Comet) assay. Mutat Res 2000;466:9–15.
[31] Slaoui M, Fiette L. Histopathology procedures: From tissue sampling to histopathological evaluation. Methods Mol Biol 2011;691:69–82.
[32] Smith WB, Adams AL. Nonalcoholic fatty liver disease and diabetes mellitus: pathogenesis and treatment. Nat Rev Endocrinol 2011;7:456–65.
[33] Nazaruk J, Borzym-Kluczyk M. The role of triterpenes in the management of diabetes mellitus and its complications. Phytochem Rev 2015;14:675–90.
[34] Yoshinari O, Sato H, Igarashi K. Anti-diabetic effects of pumpkin and its components, trigonelline and nicotinic acid, on Goto-Kakizaki rats. Biosci Biotechnol Biochem 2009;73:1033–41.
[35] Sharma A, Sharma AK, Chand T, Khardiya M, Yadav KC. Antidiabetic and antihyperlipidemic activity of *Cucurbita maxima Duchense* (Pumpkin) seeds on streptozotocin induced diabetic rats. J Pharmacogn Phytochem 2013;1:108–16.
[36] Bharti SK, Kumar A, Sharma NK, Prakash BO, Jaiswal SK. Tocopherol from seeds of *Cucurbita pepo* against diabetes: Validation by in vivo experiments supported by computational docking. J Formos Med Assoc 2013;112:676–90.
[37] Gallagher JE, Roith LD, Bloomgarden Z. Review of hemoglobin A1c in the management of diabetes. J Diabetes 2009;1:9–17.
[38] Makni M, Fetoui H, Gargouri NK, Garoui M, Zeghal N. Antidiabetic effect of flax and pumpkin seed mixture powder: effect on hyperlipidemia and antioxidant status in alloxan diabetic rats. J Diabetes Complicat 2011;25:339–45.
[39] Micallef AM, Garg LM. Beyond blood lipids: phytosterols, statins and omega-3 polyunsaturated fatty acid therapy for hyperlipidemia. J Nutr Biochem 2009;20:927–39.
[40] Chattopadhyay K, Mandal S, Chattopadhyay BD, Ghosh S. Ameliorative effect of sesame lignans on nicotine toxicity in rats. Food Chem Toxicol 2010;48:3215–20.
[41] Jiang L, Zhang X, Zheng X, Ru A, Ni X, Wu Y, et al. Apoptosis, senescence, and autophagy in rat nucleus pulposus cells: Implications for diabetic inter-vertebral disc degeneration. J Orthop Res 2012;31(5):692–702.