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Research Article

Hepatic Gene Expression, Antioxidant Enzymes and Anti-diabetic Effect of *Nigella sativa* in Diabetic Rats

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Abstract

Background and Objective: Role of black cumin seeds (*Nigella sativa*) in enhancing gene expressions of catalase (CAT), glutathione-s-transferase (GST) and B-cell CLL/lymphoma 2 (Bcl-2) in diabetic rats has not investigated so far. Furthermore, sensitive technique is required to confirm its overexpression to superoxide dismutase (SOD), glutathione peroxidase (GPX) and insulin like growth factor-1 (IGF-1). This study was aimed to investigate the effect of black seed on gene expression of CAT, GST and Bcl-2 and also examine its expression to SOD, GPX and IGF-1 by high sensitive RT-PCR in diabetic rats. **Materials and Methods:** Eight non-diabetic albino rats assigned as control (group I). Sixteen rats rendered diabetic by streptozotocin (STZ; 60 mg kg^{-1} b.wt.). Diabetic rats divided into group II and group III (eight rats in each). Rats in group II were untreated diabetic group while rats of group III were diabetic and treated with Black-cumin seed (2 g kg^{-1} b.wt.) for 6 weeks. **Results:** Diabetes increased blood glucose level than control while black seed reduced the higher blood glucose in diabetic rats significantly. Except for IGF-1, diabetes induced significant increases in gene expressions of Bcl2, CAT, SOD, GPX and GST compare to control. Black seed increased expression of Bcl2, CAT, SOD, GPX, GST and IGF-1 genes compare to both control and diabetic untreated rats. **Conclusion:** The current study observed that the *Nigella sativa* showed overexpression to CAT, GST and Bcl2 genes and also to SOD, GPX and IGF-1 by higher quantitative PCR in STZ diabetic rats.

Key words: Diabetes mellitus, *Nigella sativa*, Bcl-2, blood glucose, black cumin seeds, gene expression, diabetic rats, glutathione peroxidase

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The levels of Reactive Oxygen Species (ROS) regulated by a variety of cellular defense mechanisms consisting of enzymatic and non-enzymatic antioxidants[1-7]. High levels and/or inadequate removal of ROS may cause severe metabolic imbalance and oxidative damage to biological macromolecules named oxidative stress as noticed in clinical[8] and experimental STZ diabetes[9]. The ROS interacts with lipid bilayer of cell membrane causing lipid peroxidation[10]. The enzymatic antioxidants, SOD, CAT, GPX and GST are responsible for removal of these radicals and maintain acellular protection against oxidative stress[1]. Dietary supplementation of black cumin seed reduced blood glucose level in STZ diabetic rats[11]. The authors did not interpret the mechanism of hypoglycemic effects that induced by black cumin seeds. However, other work[12] explained that, the hypoglycemic effect of black cumin seed (*Nigella sativa*) attributed to amelioration of β-cell ultra-structure thus leading to increased insulin levels. Authors did not measured mRNA expression of antioxidant enzymes. Molecular explanations of the role of hypoglycemic effect of Black cumin seeds (*Nigella sativa*) investigated in regarding to different genes in brain[13] and kidneys[14]. Authors measured the activities of antioxidants and neglected the measurement of expression of their genes. Previous study[3] demonstrated that, black cumin seed stimulate the expression of GPX and SOD without mentioning its effects on the rest of antioxidant enzymes (CAT and GST). In addition, the measurement of gene expression performed by densitometry analysis of the expressed bands of RT-PCR of lower sensitivity. Thereby, technique that is more sensitive needed to quantify the mRNA expression of antioxidant enzymes as conducted in the current study (real time RT-PCR). The significant values of gene expression of Bcl2 as antidiabetic biomarkers demonstrated only for curcumin[9] not for black cumin seed. Furthermore, IGF-1 may probably be involved in metabolic abnormality and complications associated with diabetes[15] and its gene expression have been stimulated as described earlier[3] by non-sensitive RT-PCR. Beside the documented role of black cumin seed in enhancing the expression of SOD and GPX, does it has any role in gene expression of CAT and GST. Is there any role of black cumin seed in regulation of B-cell CLL/lymphoma 2 (Bcl-2) in liver tissues of diabetic rats. Is the stimulatory effect of black cumin seed on gene expression of IGF-1 and subsequent hypoglycemia differed if measured by a higher sensitive real time PCR technique than those performed earlier? Scientific answers needed for these questions. Therefore, the present study was aimed to evaluate the effects of black cumin seed on gene expression of selected antioxidant enzymes and B-cell CLL/lymphoma 2 (Bcl-2) in streptozotocin-induced diabetes in rats.

MATERIALS AND METHODS

Chemicals: Streptozotocin, agarose and ethidium bromide were purchased from Sigma Chemical Co., MO, USA. The glucotest strips supplied by Roche, USA. The DNA ladder purchased from MBI, fermentas, USA. Primers were purchased from metabion international Ag, Germany. One-step RT-PCR kits purchased from Qiagen, Germany. All other chemicals were of the highest analytical grade.

Plant materials: Black cumin seeds (*Nigella sativa*) purchased from a local market in Al-Ahsa, Saudi Arabia and identified by botanists of the faculty of Agriculture, King Faisal University, Saudi Arabia. Black cumin seeds analyzed and their ingredients presented in Table 1.

Preparation of plant suspension: The whole black cumin seeds crushed in a blender and 12.5 g of the seeds added to 100 mL distilled water at room temperature to prepare a crude suspension a few minutes before experiment[16]. The black cumin seed used orally by gastric gavages in a dose of 2 g kg^{-1} b.wt., daily for 6 weeks[17-19].

Experimental animals: Thirty adult albino rats weighing between 190±10 g maintained as performed by national guidelines and protocols, approved by the University Scientific Research Ethics Committee, King Faisal University, Saudi Arabia (DSR # 160017). They housed in clean and disinfected cages. Commercial basal diet and water provided *ad libitum.* Rats subjected to natural photoperiod of 12 h light:dark cycle throughout the experimental period (6 weeks). All rats received basal diet for 2 weeks before the start of the experiment for adaptation and maintain normal growth and behavior.

Induction of experimental diabetes: Eight rats kept non-diabetic and not injected with STZ. Diabetes induced by

Table 1: Proximate analysis of ingredients of black cumin seed used in the diets of the experiment, dry matter basis (El-Bahr and El-Sabagh[3])

Ingredients	Moisture (%)	CP (%)	EE (%)	CF (%)	NFE* (%)	Ash (%)
Black cumin seed	5.8	19.54	34.4	6.1	36.16	3.8

CP: Crude protein, EE: Ether extract, CF: Crude fiber, *NFE: Nitrogen free extract, calculated by differences

intraperitoneal injection of a freshly prepared solution of STZ (60 mg kg^{-1} b.wt.) in 0.1 M cold citrate buffer (pH 4.5) to the overnight fasted 22 rats[20]. Because of the instability of STZ in aqueous media, the solution made using cold citrate buffer (pH 4.5) immediately before administration. Control rats injected with citrate buffer alone. The rats allowed drinking 5% glucose solution overnight to overcome the drug-induced hypoglycemia. After 72 h, fasting blood glucose levels monitored. Animals having blood glucose levels 145 mg dL^{-1} (n = 6) were excluded from the experiment because they considered as non-diabetic non-responsive animals[21]. Animals having blood glucose values above 250 mg dL^{-1} (n = 16) on the 3rd day after STZ injection were considered as diabetic rats[21]. Then the treatment started on the 3rd day after STZ injection and it considered as 1st day of treatment.

Experimental design: Eight healthy adult non-diabetic albino rats assigned as control group (group 1). Sixteen diabetic animals divided into 2 groups, group II and group 3 (8 rats in each). Diabetic rats of group 2 were untreated diabetic group while rats of group 3 were diabetic and treated with oral black cumin seed (2 g kg^{-1} b.wt.) for 6 weeks by gastric gavages[3,17-19]. All animals kept on ordinary ration and received water *ad libitum*.

Samples collection and estimation of blood glucose level: Blood samples collected from the fasted rats of three groups prior to the treatment with black cumin seed and three times per weeks after oral administration of the treatments up to 6 weeks. Blood samples collected by snipping tail with sharp razor and blood glucose level measured immediately by glucose strips (haemo-glucotest). At the end of the experiment, liver samples collected from all groups, washed by normal saline solution, dried by towel, flash frozen in liquid nitrogen and subsequently frozen at -80°C until the time of RT-PCR analysis.

RT-PCR of hepatic antioxidant enzymes, CAT, GST and Bcl-2: The primers[22] of CAT, β-actin genes[23] and Bcl-2[24] taken from literature (Table 2). The primers checked for their Tm values, hairpin loops, dimers, cross-dimers and number of repeats and runs using net primer (Oligoanalyzer 3.1). β-actin gene was used as an internal standard (house keeping gene). Frozen liver samples (approximately 1 g per sample) were immediately added to lysis buffer (Qiagen, Germany) and homogenized using homogenizer (Tissue Ruptor, Qiagen GmbH, Germany). Reverse transcriptase polymerase chain reaction (RT-PCR) performed with Qiagen one-step RT-PCR kit (Qiagen, Germany) according to the manufacturer's instructions. The RNA quality and integrity assured by gel visualization and spectrophotometric analysis (OD 260/280) and quantified at 260 nm. The master mix was prepared according to the manufacture instruction. The RT-PCR reactions and conditions executed as described earlier[9]. The PCR products were then resolved on 1.5% agarose gels. The bands identified based on the product size using a 5000 bp DNA ladder; documented using a gel documentation system (Gel Doc™ XR System, Bio-Rad) and the prints scanned. The scanned images quantified densitometrically with the aid of NIH image program (http//rsb.info.nih.gov/nih-imag/). The results were normalized to the levels obtained for the β-actin gene by taking a ratio of the value obtained for the gene of interest to that of β-actin and then relative to the control.

Total RNA isolation and real time PCR analysis of SOD, CAT, GPX, G-ST, Bcl-2 and IGF-1: The RNA extraction and precipitation from liver tissues done as described earlier[5]. The cDNA was prepared from RNA samples according to Revers Transcription System Kit (Promega, Madison, USA) by using

Table 2: Details giving primer sequences and expected product size for the genes amplified

cDNA	Sequence	Annealing temperature	Number of cycles	RT-PCR product size
β-actin F	5'-AGC CAT GTA CGT AGC CAT CC-3'	55	40	230*
β-actin R	5'-CTC TCA GCT GTG GTG GTG AA-3'			
IGF1F	5'-CTG GGT GTC CAA ATG TAA CT-3'	52	40	170*
IGF1R	5'-GTA TCT TTA TTG GAG GTG CG-3'			
SODF	5'-AGG ATT AAC TGA AGG CGA GCA T-3'	55	40	410*
SODR	5'-TCT ACA GTT AGC AGG CCA GCA G-3'			
CATF	5'-ACG AGA TGG CAC ACT TTG ACA G-3'	55	40	341
CATR	5'-TGG GTT TCT CTT CTG GCT ATG G-3'			
GPxF	5'-AAG GTG CTG CTC ATT GAG AAT G-3'	57	40	406*
GPxR	5'-CGT CTG GAC CTA CCA GGA ACT T-3'			
Bcl-2F	5'-TCC ATT ATA AGC TGT CAC AGA GG-3'	55	40	350
Bcl-2R	5'-GAA GAG TTC CTC CAC CAC C-3'			
GSTF	5'-GCT GGA GTG GAG TTT GAA GAA-3'	55	40	575
GSTR	5'-GTC CTG ACC ACG TCA ACA TAG-3'			

*El-Bahr and El-Sabagh[3]

Bio-Rad Thermal Cycler (T100TM, Foster city, California, USA)[5]. Real time RT-PCR performed using QuantiFastTM SYBR Green PCR Master Mix kit (QIAGEN, Hilden; Germany) as described earlier[5]. For each examined gene, duplicate samples from each cDNA analyzed by real time RT-PCR using the Bio-Rad CFX Manager 3.0 Software of the C1000 Touch thermal cycler-CFX96 real time PCR (BIO-RAD, Foster city, California, USA). The β-actin mRNA fragment used as house keeping gene to normalize the expression data. The primer sequences are the same of conventional PCR technique as described above (Table 2).

Statistical analysis: All data presented as Mean±Standard error of mean by using student-t test. All tests performed using computer package of the statistical analysis system[25]. The relative gene expression of target genes in comparison to the β-actin reference gene calculated using the Bio-Rad CFX Manager 3.0 software of the C1000 Touch thermal cycler-CFX96 real time PCR (BIO-RAD, Foster city, California, USA).

RESULTS

After injection of STZ, the mean values of blood glucose levels in untreated diabetic rats were remained above 330 mg dL^{-1} during the entire period of the study, which were significantly ($p<0.05$) higher than those of the non diabetic normal control rats during the experimental period (Table 3). Dietary supplementation of black cumin seed to diabetic rats induced a significant ($p<0.05$) decrease in blood glucose levels compared to diabetic untreated rats but still significantly higher ($p<0.05$) than that of control non diabetic values throughout the whole experimental period (6 weeks, Table 3).

The RT-PCR data as showed in Fig. 1 indicated that, experimental diabetes by STZ up regulated ($p<0.05$) the gene expression of CAT, GST and Bcl2 compared to control non-diabetic rats. Overexpression of these genes induced when diabetic rats treated with black cumin seed compared to control non-diabetic and diabetic untreated rats (Fig. 1).

The real time PCR results revealed that, experimental diabetes induced significant ($p<0.05$) increase in the gene

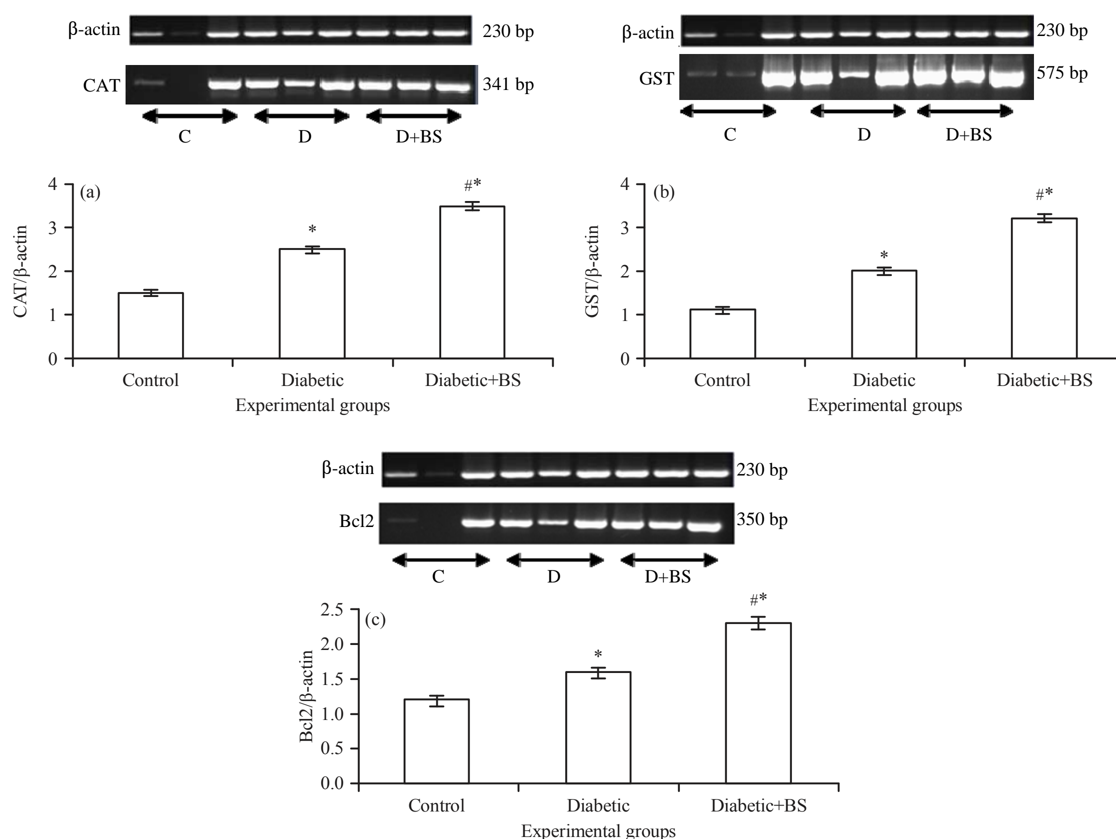

Fig. 1(a-c): RT-PCR analysis of (a) CAT, (b) GST and (c) Bcl2 in liver tissues of control non-diabetic (C), experimentally diabetic (D) and Black cumin seed (BS) treated diabetic rats

Values expressed as Mean±SEM for 8 rats, *Values are significant different ($p<0.05$) compared to control, #Values are significant different ($p<0.05$) compared to diabetic group control

Table 3: Effect of oral administration of black cumin seed for 6 weeks on blood glucose concentration (mg dL^{-1}) in streptozotocin diabetic rats

	Blood glucose concentration (mg dL^{-1})			
Groups	At the end of 1st week	At the end of 2nd week	At the end of 4th week	At the end of 6th week
1	100 ± 2.1^{c}	96 ± 1.2^{c}	92 ± 1.1^{c}	90 ± 2.1^{c}
2	366 ± 2.1^{a}	330 ± 1.6^{a}	340 ± 1.5^{a}	350 ± 1.8^{a}
3	255 ± 2.1^{b}	250 ± 3.0^{b}	247 ± 4.1^{b}	240 ± 5.1^{b}

1: Non-diabetic, 2: Diabetic, 3: Diabetic treated with black cumin seed, values are Mean±SD of 8 rats, Means within the same column with different letters are significantly differed ($p\leq0.05$), a: Highest value, b: Decreased value, c: Lowest value

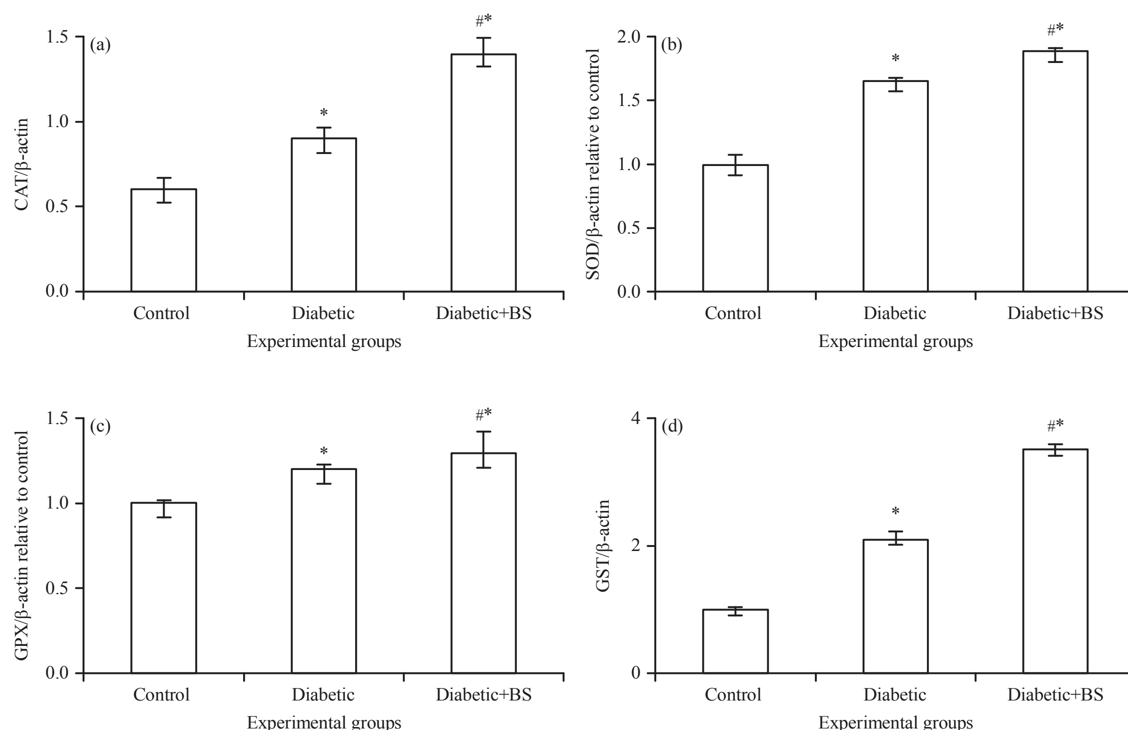

Fig. 2(a-d): Real time PCR analysis of (a) CAT, (b) SOD, (c) GPX and (d) GST in liver tissues of control non diabetic (C), experimentally diabetic (D) and Black cumin seed (BS) treated diabetic rats

Values expressed as Mean±SEM for 8 rats, *Values are significant different ($p<0.05$) compared to control, #Values are significant different ($p<0.05$) compared to diabetic group control

expression of CAT (Fig. 2a), SOD (Fig. 2b), GPX (Fig. 2c), GST (Fig. 2d) and Bcl2 (Fig. 3b). Overexpression of these genes induced when diabetic rats treated with black cumin seed compared to control non-diabetic and diabetic untreated rats (Fig. 2, 3b). The real time PCR data as showed in Fig 3a indicated that, experimental diabetes by STZ did not significantly affected ($p>0.05$) the gene expression of IGF-1 compared to control non diabetic rats. The expression of this gene increased significantly when diabetic rats has been treated with black cumin seed compared to control non-diabetic and diabetic untreated rats (Fig. 3a). In addition, the real time PCR data as showed in Fig 3b indicated that, experimental diabetes by STZ induced a significant increase ($p<0.05$) in gene expression of Bcl2 compared to control non diabetic rats. Overexpression of this gene induced when diabetic rats treated with black cumin seed compared to control non-diabetic and diabetic untreated rats (Fig. 3b).

DISCUSSION

The present findings revealed that, injection of STZ (60 mg kg^{-1} b.wt.) induced significant hyperglycemia in rats. This dose caused damage to β-cells of the islets of langerhans and emergence of clinical diabetes within 2-4 days as results of autoimmune process[8]. Similar to the present findings, previous articles[5,9,11-14,26,27] reported that, STZ induced

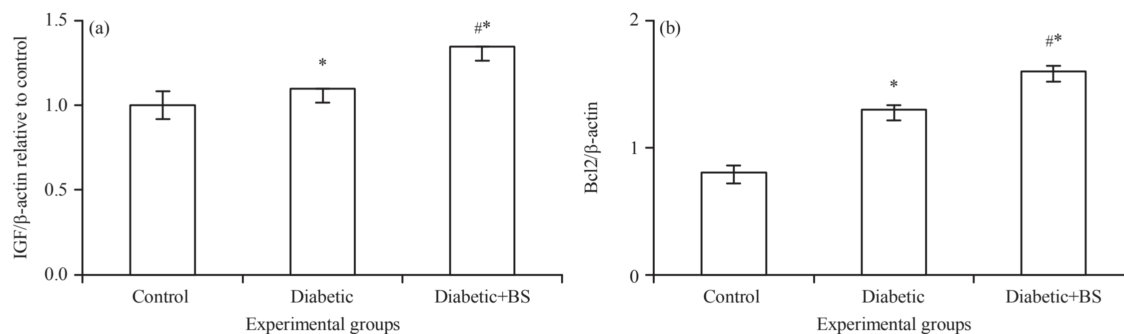

Fig. 3(a-b): Real time PCR analysis of (a) IGF-1 and(b) Bcl2 in liver tissues of control non-diabetic (C), experimentally diabetic (D) and black cumin seed (BS) treated diabetic rats

Values expressed as Mean±SEM for 8 rats, *Values are significant different ($p<0.05$) compared to control, #Values are significant different ($p<0.05$) compared to diabetic group control

hyperglycemia and oxidative stress in rats. Hyperglycemia may be attributing to enhancement of gluconeogenesis because of absence of insulin[28] due to oxidative stress of β-cells of pancreas and release of reactive oxygen species[29]. The hypoglycemic effect of black cumin seed reported in this work was in agreement with previous reports in alloxan induced diabetic rabbits[30] and rats[31,32]. In addition, the same effect reported in STZ induced diabetic rats[2,3,11,13,14] and in human subjects[33]. Conversely, no significant changes have reported in fasting blood glucose level when *Nigella sativa* administered to normal and streptozotocin-induced diabetic rats[34,35]. The antidiabetic effect of black cumin seed was due to an insulin-like stimulation of glucose uptake by muscle and adipose tissue[36] or inhibition of intestinal glucose absorption[19]. Other works attributed the antidiabetic effect of black cumin seed into improvement of insulin resistance in diabetic rats[37], improved extra pancreatic actions of insulin[38] and antioxidant chemical constituents of black cumin seed[13,14,39,40]. The liver is the main source of circulating[41] IGF-1, thus the liver tissues used for investigation of the expression of this gene in the current study. The current findings indicating a non-significant stimulation of STZ to of IGF-1 gene expression confirmed the earlier findings of our laboratory[3]. However, in the current work the expression of IGF-1 gene in STZ diabetic rats estimated by using higher sensitive relative quantitative PCR than those used earlier[3]. Moreover, the significant increase in expression of IGF-1 in liver tissues of diabetic rats treated with black seed compare to control non-diabetic and diabetic untreated rats confirmed the earlier results of our laboratory[3] but the PCR technique used was of higher sensitivity, because of, IGF-1 induced glucose uptake and improved the insulin sensitivity[42,43], increased its expression as reported in the current study augmented this effect in diabetic rats treated with black cumin seed. Conversely, previous findings[15,44] indicated a significant down regulation of IGF-1 in diabetic rats after acute or chronic administration of alloxan. This contradiction might be due to using different diabetogenic factor, experimental time and different RT-PCR protocols. The Bcl-2 reduced the generation of ROS through binding to cytochrome c or prevented its entry to the cytosol[45]. An increase in the expression of Bcl-2 exhibit elevated expression of antioxidant enzymes and higher levels of cellular GSH[46]. The significant increase in the expression of Bcl-2 in diabetic rats compared with control non-diabetic rats was to counteract the oxidative stress arisen by diabetes. This finding agrees with those reported earlier in STZ diabetic rats[9]. The significant values of gene expression of Bcl2 as antidiabetic biomarkers demonstrated only for curcumin[9]. However, the current work suggests the overexpression of Bcl2 gene in STZ diabetic rats treated with black cumin seed compare to control and diabetic untreated rats. The significant up regulation of Bcl2 gene expression in diabetic rats treated with black cumin seed compare to diabetic untreated rats may attribute to maintaining the anti-apoptotic and antioxidant effect of Bcl2 in diabetic rats[9]. Parallel to the present results, studies form other laboratories[47] and our laboratory[3] indicated that, STZ induced up regulation of gene expression of SOD and GPX in rats. Conversely, no differences have detected in the mRNA expression concentrations of SOD and GPX between control and diabetic rats[22]. Other work[48] reported that, diabetes decreased the activities and protein expression of SOD and GPX enzymes. Earlier work[3] in our laboratory explored only the effect of STZ on the gene expression of SOD and GPX in diabetic rats without mentioning its effect on CAT and GST. The current study suggests that STZ induced up-regulation not only to SOD and GPX but also to CAT and GST. Regarding the significant increase in gene expression of SOD and GPX in diabetic rats treated with black seed, the current study

confirmed the earlier work in our laboratory[3] but using more advanced relative quantitative PCR than those used earlier[3]. Earlier works[3] in our laboratory did not investigate the effect of black cumin seed on gene expression of other antioxidant enzymes such as CAT and GST in STZ diabetic rats making the redox cycle incomplete. These enzymes are essential and of critical roles for removal of released reactive oxygen species making the earlier data[3] was insufficient to answers many questions that arisen by scientific researchers. Here, the current study suggested that, black cumin seed induced overexpression of gene expression of CAT and GST in STZ diabetic rats drawing a complete picture for the mechanism of action of its antidiabetic effect in STZ diabetic rats. The current study corrected the weak points of the earlier published article[3]; in addition providing a new evidence about the mechanism of action of antidiabetic effect of black cumin seed representing in overexpression to CAT, GST and Bcl2 genes and confirmed its overexpression to other antioxidants genes such as SOD, GPX and IGF-1 by higher sensitive quantitative PCR. These findings will open new prospective toward the role of black cumin seed (*Nigella sativa*) in treatment of diabetes and diabetic complications.

CONCLUSION

This study suggested new evidence about the mechanism of action of antidiabetic effect of black cumin seed representing in overexpression to CAT, GST and Bcl2 genes and confirmed its overexpression to other antioxidants genes such as SOD, GPX and IGF-1 by higher sensitive quantitative PCR.

SIGNIFICANCE STATEMENT

This study suggests new evidence about the mechanism of action of antidiabetic effect of black cumin seed representing in overexpression to catalase (CAT), glutathione-s-transferase (GST) and B-cell CLL/lymphoma 2 (Bcl2) genes and confirmed its overexpression to other antioxidants genes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and insulin like growth factor-1 (IGF-1) by higher sensitive quantitative PCR. This study will help the researchers to explore important aspect of the potential molecular mechanisms of black cumin seed (*Nigella sativa*) in STZ diabetic rats, thereby aiding in further researches into the treatment of diabetes. Thus, a new theory on black cumin seed (*Nigella sativa*) and diabetes perhaps arrived at.

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