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Some Genes Expression In Streptozotocin Induced Diabetic Rats In Response To Mushroom Extract Treatment.

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ABSTRACT

The present study was designed to evaluate pleurotus ostreatus (PO) phenolic compounds and its biochemical effects on the DNA damage, expression level of some genes related to vascular complications and oxidative stress in STZ induced diabetic rats. Thirty two adult male albino rats were divided into 4 groups. Group I Normal Control, Group II non diabetic treated with PO, Group III STZ induced diabetic rats, Group IV STZ induced diabetic rats treated with PO. The levels of phenolic compounds, blood glucose, tissue malondialdehyde (MDA), reduced glutathione (GSH), glutathione peroxidase (GPx), DNA fragmentation, and m-RNA expression levels of Nrf2 and VEGF genes were determined. A high performance liquid chromatography analysis detected five phenolic compounds, including Gallic acid, p-Coumaric acid, Ferulic acid, Fumaric acid and Chlorogenic acid. Our results revealed that the methanolic extract of PO significantly decreased blood glucose level, tissue MDA and DNA fragmentation with significant increase in GSH content and GPx activity and gene expression of Nrf2 and VEGF. In conclusion PO might be considered a potential protectant that reduce the oxidative stress associated with the diabetes complications in rats.

Keywords: pleurotus ostreatus, Diabetic complications, Phenolic content, Nrf2, VEGF.

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INTRODUCTION

Diabetes Mellitus (DM) is a major metabolic disorder associated with significant morbidity and mortality increasing rapidly in the whole world [1]. DM is characterized by chronic hyperglycemia (CHG) accompanied by development of major biochemical and functional abnormalities including metabolic alternations and changes in antioxidant status [2]. CHG involved in the development of diabetic complications through 5 major damaging metabolic pathways (I) increased Polyol pathway, (II) increased formation of AGEs, (III) increased expression of the AGEs receptors, (IV) activation of Protein Kinase, (V) Over activity of the Hexosamine pathway triggered by the production of oxygen species (ROS), oxidative stress and the inhibition of glyceraldehyde-3-phpsphate dehydrogenase (GAPDH) enzyme activity, lead to cell death in vessels and nerves as in nephropathy, neuropathy, and cardiovascular diseases [3].

Nuclear factor E2-related factor 2 (Nrf2) is a pivotal transcription factor that regulates various antioxidant genes such as NAD(P)H quinone oxidoreductase, glutathione S-transferase, heme oxygenase-1, ɣ-glutamylcysteine synthetase and many other proteins that detoxify xenobiotics and neutralize ROS to promote cell survival and maintain cellular redox homeostasis [4]. Vascular endothelial growth factor (VEGF) is a pleiotropic protein critical for endothelial cell differentiation, proliferation and migration, as well as for vascular maintenance and remodeling [1]. CHG stimulates VEGF synthesis and secretion, and triggers a series of interconnected metabolic and hemodynamic effects that lead to DM microvascular complications [5].

Through different types of oral hypoglycemic agents are available along with insulin for the treatment of DM, there is increasing demand to use natural products with antidiabetic activity. As continuous use of the synthetic antidiabetic drugs causes side effects and toxicity [6]**.** Therefore, because of their effectiveness, limited side effects, and relatively low cost, Medicinal mushrooms have been studied for treatment of diabetes, as they considered as functional food contain high amounts of proteins, carbohydrates, fibers, minerals, vitamins and with low fat content. Regarding their medicinal value they are effective as antitumor, antibacterial, antiviral, antidiabetic, antioxidant, hematological agents, and in immuno-modulating treatments [7].

Pleurotus is a genus of edible mushrooms, comprises about 40 species and they are commonly referred to as Oyster mushroom. It grow widely in tropical and subtropical areas and easily artificially cultivated especially due to its high nutritional value and various bioactive compounds [8].

The present study was designed to evaluate PO antioxidant power and its biochemical effects on the DNA damage, expression level of some genes related to vascular complications and oxidative stress in STZ induced diabetic rats.

MATERIALS AND METHODS

PO was obtained from the Agricultural Exp. Station, Faculty of Agriculture, Cairo University, Streptozotocin (STZ) was purchased from Sigma (St. Louis, MO, USA), Glucose, Total protein, alanine amino transferase (ALT), aspartate amino transferase (AST), Creatinine and Urea kits were obtained from Spectrum (Cairo, Egypt), Total RNA extraction kit (Vivantis, Cat.No.GF-TR-100), RT-PCR Kit (Vivantis, Product No. RTPL 12), Real time PCR (Luminaris Color HiGreen Low ROX qPCR Master kit (Thermo Scientific, Cat. No. #K0371), all other chemicals were of pure analytical grade.

Preparation of PO extract

PO mushroom was collected and kept on ice until transportation to the laboratory. The methanolic extract of mushroom was prepared according to [9] and stored at 4° C refrigerator for until further use.

Determination of PO Phenolic compounds

Methanolic extract of PO was analyzed qualitatively for various phenolic constituents using HPLC (WinChrome Chromatography Ver.13) as per standard procedures. HPLC was equipped with a binary pump (LC 1110; GBC scientific Equipment, Hampshire, USA) and C18 column (Kromasil C18, Sigma Aldrich, CA, USA; 5µm, 150x6.4 mm). The injection volume was 20 µl with isocratic procedure where the mobile phase was methanol-

acetonitrile (85:18) and the flow rate 1 ml/ min. The ultraviolet detector (LC 1200; GBC Scientific Equipment) with a wave length 254 nm was used to detect and quantitate phenolic compounds where individual standard curves were prepared using different concentrations of standards used for calculation of each sample. Data were quantified and analyzed using Win Chrome Chromatography Ver. 1.3 software.

Experimental animals

A total number of 32 adult, male, albino rats weighing (180± 30g) were obtained from Faculty of Science, Cairo University. The animals were allowed to free access to water and a standard chow diet manufactured in accordance to the recommendations of the American Institute of Nutrition (AIN-93M) [10]. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Cairo University (CU-IIF7318).

Rats were randomly divided into 4 equal groups (8 rats for each). **Group I (NC)** normal control, orally received normal saline by a stomach tube. **Group II (PO)** non diabetic treated with PO, orally received 400mg/kg B.W of PO for 6 consecutive weeks [11]. The remaining 16 rats were fasted overnight and subjected to diabetic regimen by intraperitoneal injection of STZ at a two doses: initial dose of 32 mg/kg B.W followed by a booster dose of 18 mg/kg B.W which was 24 hours later [12]. 24 hours after the booster dose, blood glucose was estimated, rats with blood glucose level (200-350 mg/dl) were considered diabetic and grouped accordingly: **Group III (DC)** STZ induced diabetic rats kept on a basal diet only and given saline orally (diabetic control). **Group IV (STZ+PO)** STZ induced diabetic rats treated with PO in a daily dose of 400mg/kg B.W orally for 6 consecutive weeks [11].

Blood and tissue sampling

Blood samples were collected every 2 weeks for glucose determination. At the end of the experimental period (6 weeks) Blood samples were divided into two portions. The first portion was collected on S. Fluoride for plasma glucose determination. The second portion was collected without anticoagulant as serum for biochemical analysis. Then the rats were humanly scarified under anesthesia and the liver, heart and kidney were rapidly excised, washed with ice cold saline (0.9% NaCl) and—stored at -20Ċ till homogenization and determination of oxidative stress parameters (MDA, GSH contents, GPx) and DNA Fragmentation. Tissue samples for RNA extraction were kept in the lysis buffer of GF-1 Total RNA Extraction Kit at -20 C°till the subsequent procedures.

Blood biochemical analysis

The P. glucose was determined using Liquizyme Kit (Spectrum, Cat.No.250001), S. T. protein Biuret reagent Kit (Spectrum, Cat.No.310001), AST Colorimetric Kit (Spectrum, Cat.No.260001), ALT Colorimetric Kit (Spectrum, Cat.No.264001), Creatinine Colorimetric Kit (Spectrum, Cat.No.235001), and Urea Liquizyme (UV) Kit (Spectrum, Cat.No.319001) all according to the manufacturer's protocol.

Tissue biochemical analysis

Tissue total protein was determined according to [13], MDA [14], GSH [15], GPx [16] and DNA fragmentation [17].

Determination of the relative m-RNA level of Nrf2 and VEGF

RNA was extracted from Liver, Kidney and Heart tissues using a T. RNA extraction kit (Vivantis, Cat.No.GF-TR-100) according to the manufacturer's protocol. The concentration of RNA in each solution was determined at 260 nm by a Nanodrop (ND-1000). The ratio of A260 to A280 values is a measure of the RNA purity. Samples with a ratio of 1.8-2 were selected. First Reverse transcriptase reaction was done using Viva 2-steps RT-PCR Kit (Vivantis, Product No. RTPL 12) according to the manufacturer's protocol.

The expression levels of Nrf2 and VEGF mRNA in rats' liver, kidney and heart were determined by Luminaris Color HiGreen Low ROX qPCR Master kit (Thermo Scientific, Cat. No. #K0371) according to the manufacturer's protocol. Primers used were as follows: Nrf2 Forward: 5'- CCA GCA CAT CCA GAC AGA CAC-3'

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and Reserve: 5'-GAT ATC CAG GGC AAG CGA CTC-3'. VEGF Forward: 5'- ATCATGCGGATCAAACCTCACC-3'and Reserve: 5' GGTCTGCATTCACATCTGCTATGC -3'. B-actin Forward: 5'- TGT CAC CAA CTG GGA CGA TA-3' and Reserve: 5'- GGG GTG TTG AAG GTC TCA AA-3. Real time PCR for the target genes (VEGF and Nrf2) and B-actin as internal control. All the primer sets were designed using primer 3 software. Two-steps cycling protocol was adjusted as follows; UDG pre- treatment at 50°C for 2 minutes , Initial denaturation at 95°C for 10 minutes then 40 cycles of denaturation at 95°C for 20 seconds and annealing/extension at 60°C for 45 seconds. Fluorescent data were acquired during each extension phase. The fold change compared to control samples was calculated using CT, ΔCT, ΔΔCT by Mxpro software Stratgene [18].

Statistical analysis

The data was analyzed using the statistical package for social science (SPSS Inc., Version 22, Chicago, Illinois, USA). All results are expressed as mean ± SE. Comparison among groups was made by Student's t-test (unpaired), One-way analysis of variance (ANOVA). Duncan's test was used for testing the inter-grouping homogeneity. Statistical significance was set $p<0.05$.

RESULTS

Phenolic compounds

The individual profile of phenolic content of PO obtained by HPLC was represented in Table (1) and Figure (1) depicts a typical HPLC chromatogram of the phenolic acids (Gallic acid, p-Coumaric acid, Ferulic acid, Fumaric acid and Chlorogenic acid) recorded at 254 nm. Phenolics occurring in Po mushroom were identified by comparison of the absorption spectrum and the retention time with the corresponding standards.

Table (1): HPLC analysis of Phenolic acid and flavonoid composition of PO

Compound	Content (µg/g DW)
p-Coumaric acid	60
Ferulic acid	40
Chlorogenic acid	30
Fumaric acid	37
Vanillic acid	Not detected
Gallic acid	70
Caffeic acid	Not detected

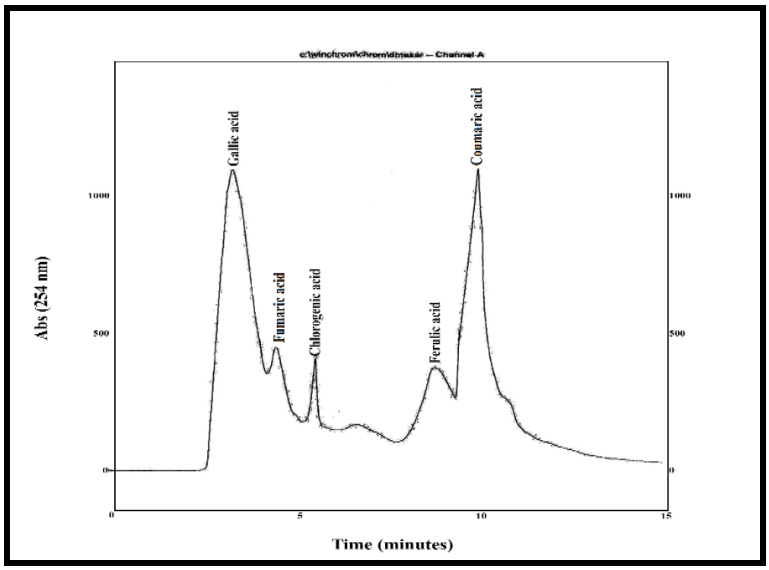

Figure (1): Chromatogram of phenolic compounds analysis in PO by HPLC.

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Biochemical effects of PO extract

Treatment of STZ diabetic rats by PO extract significantly decreased glucose concentration from the 2nd week of treatment till the end of experiment (6th) compared with their diabetic control group ($p<0.05$) (Figure 2A).

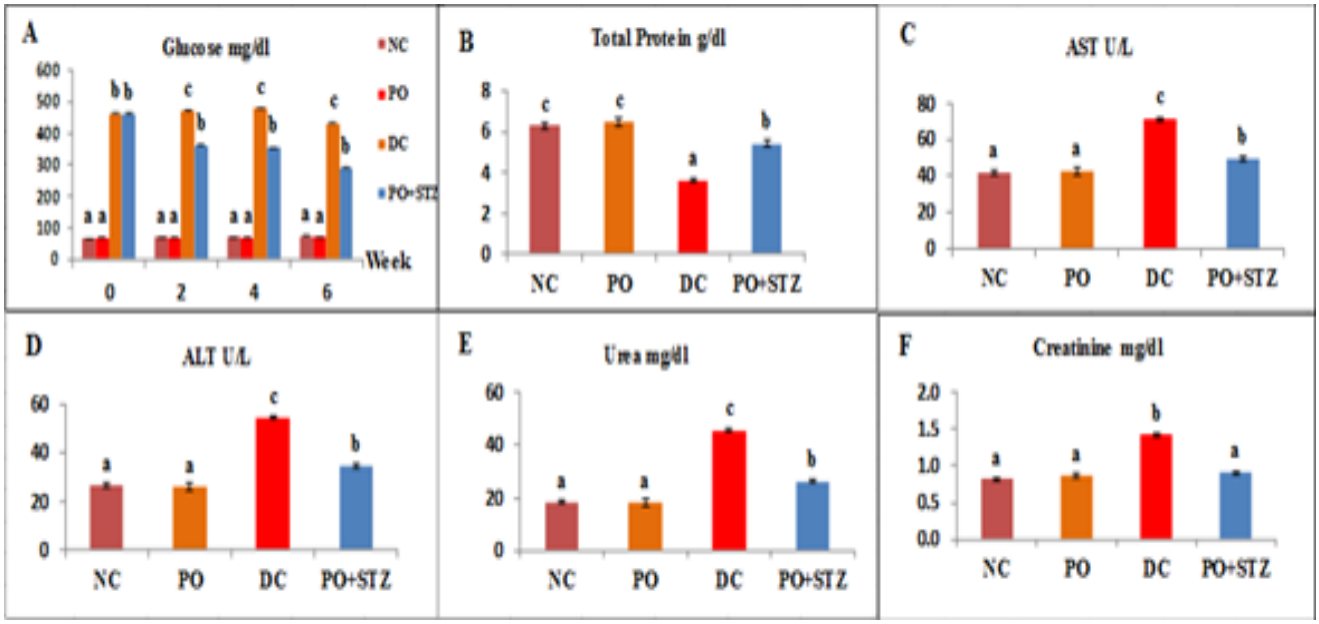

Figure (2): Effect of PO on (A): glucose, (B): T. protein, (C): AST, (D): ALT, (E): Urea, (F): Creatinine. Values are expressed as mean ± SE (n= 6); NC, normal control; PO, P. ostreatus, DC, diabetic control; PO+STZ, P. ostreatus+ streptozotocin.

Different small letters indicate significant difference between groups at $p<0.05$.

Also PO extract significantly restored the altered total proteins concentrations to approach the normal control values ($p<0.05$) (Figure 2B), while significantly decreased serum AST and ALT activities, serum urea and creatinine levels compared to the diabetic control group at the end of the experiment (6th week) ($p<0.05$) (Figure 2C, D, E, F).

Oxidative stress parameters

Treatment of STZ diabetic rats by the PO extract significantly lowered the liver, kidney and heart MDA concentrations ($p<0.05$) (Figure 3A (i), (ii) and (iii)) with a significant increase in GSH content ($p<0.05$) (Figure 3B (i), (ii) and (iii)) and GPx activity at the end of the experiment (6th week) compared with their diabetic control group ($p<0.05$) (Figure 3C (i), (ii) and (iii)).

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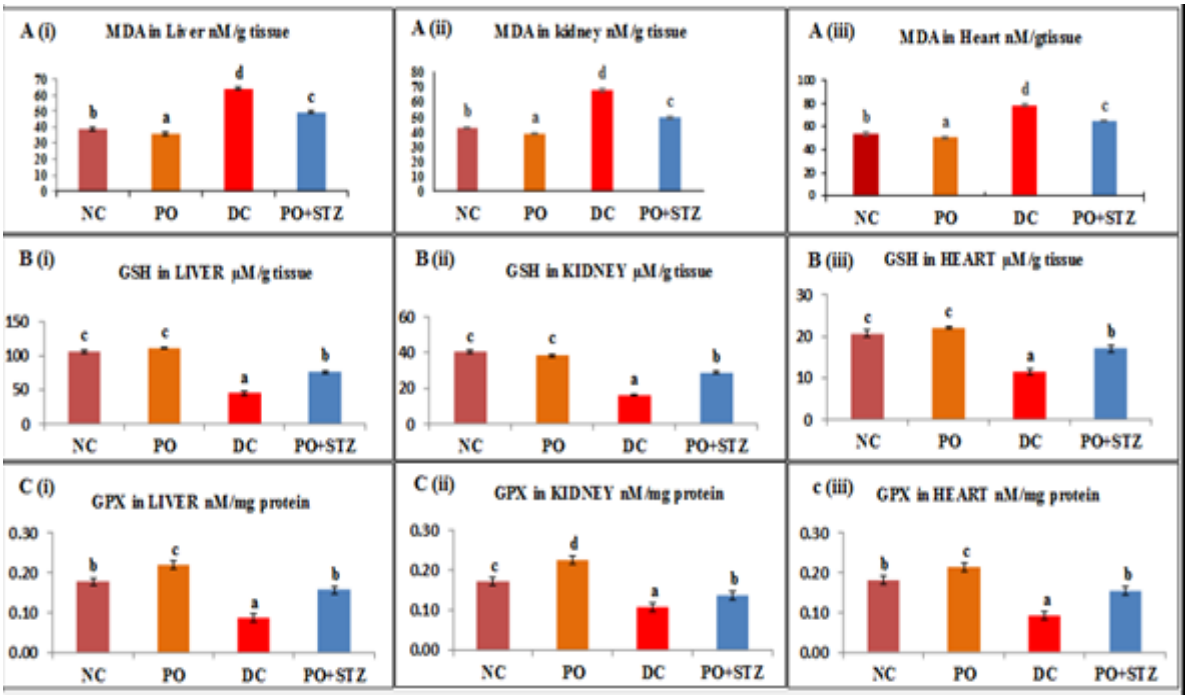

Figure (3): Effect of PO on oxidative stress parameters: (A): MDA in A(i): Liver, A(ii): Kidney, A(iii): Heart (B): GSH in B(i) Liver, B(ii) Kidney, B(iii) Heart (C): GPx in C (i) Liver, C(ii) Kidney, C(iii) Heart

Values are expressed as mean ± SE (n= 6); NC, normal control; PO, P. ostreatus, DC, diabetic control; PO+STZ, P. ostreatus+ streptozotocin.

Different small letters indicate significant difference between groups at $p<0.05$.

DNA fragmentation

Treatment of STZ diabetic rats by the PO extract significantly decreased the liver, Kidney and heart DNA Fragmentation at the end of the experiment (6^{th} week) compared with their diabetic control group ($p<0.05$) (Figure 4 A and B).

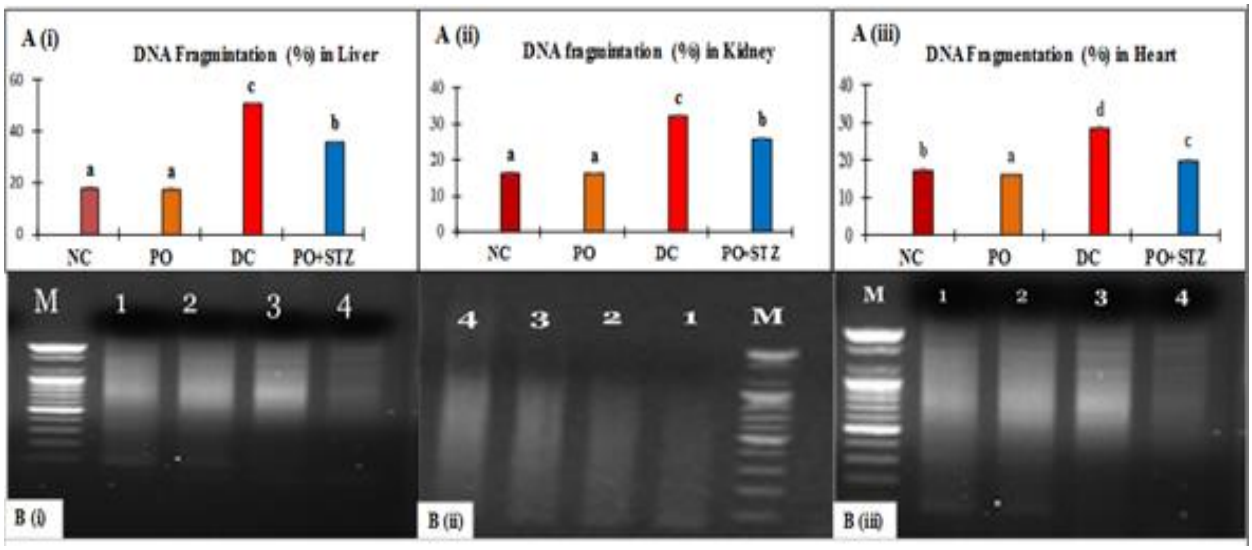

Figure (4):Effect of PO on DNA Fragmentation. A (i): Liver, A (ii): Kidney, A (iii): Heart tissue homogenates in STZ treated rats after six weeks.

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Values are expressed as mean ± SE (n= 6); NC, normal control; PO, P. ostreatus, DC, diabetic control; PO+STZ, P. ostreatus+ streptozotocin.

Different small letters indicate significant difference between groups at p<0.05.

(B): Effect of PO on DNA Fragmentation on 2% agaros/ethydium bromide gel.

B (i) Liver, A (ii): Kidney, A (iii): Heart Lane M: 100bp DNA lader; lane 1;NC; lane 2: PO; lane 3: DC; lane 4: PO+STZ.

Expression level of mRNA of Nrf2 in Liver Kidney and Heart

The gained results showed a significant decrease in the liver, Kidney and Heart expression level of mRNA of Nrf2 in STZ treated rats. While treatment with PO extract resulted in significant increase in the liver, Kidney and heart expression level of mRNA of Nrf2 compared to diabetic control group (p<0.05) (Figure5A).

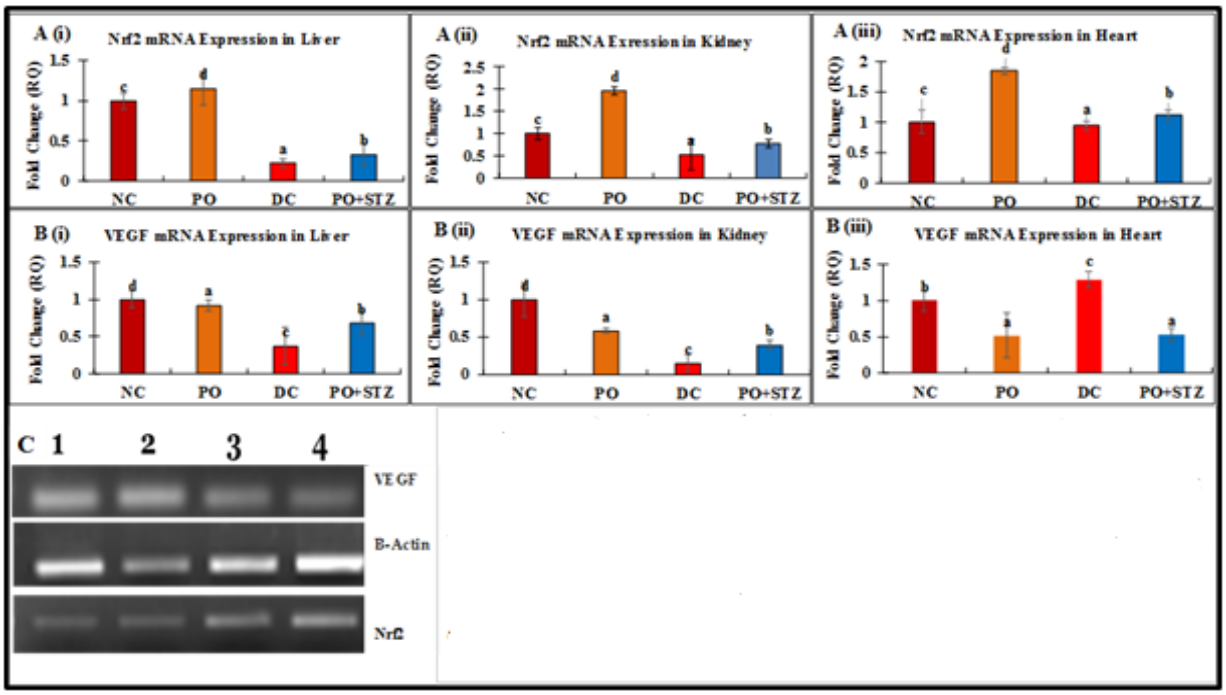

Figure (5): Effect of PO on Expression level of mRNA on Nrf2 in A (i): Liver, A (ii): Kidney, A (iii): Heart tissue homogenates. (B) Effect of PO on Expression level of mRNA on VEGF in A (i): Liver, A (ii): Kidney, A (iii): Heart tissue homogenates.

Values are expressed as mean ± SE (n= 6); NC, normal control; PO, P. ostreatus, DC, diabetic control; PO+STZ, P. ostreatus+ streptozotocin.

Different small letters indicate significant difference between groups at p<0.05.

(C): agaros/ethydium bromide gel for the studied genes; 1: NC; 2:PO; 3:DC; 4: PO+STZ.

Expression level of mRNA of VEGF in Liver Kidney and Heart

The gained results showed a significant increase in the Heart accompanied with significant decrease in the Liver and Kidney expression level of mRNA of VEGF in STZ treated rats.

Treatment of STZ diabetic rats with Po extract significantly decreased the Heart and significantly increased the Liver and Kidney expression level of mRNA of VEGF compared to diabetic control group ($p<0.05$) (Figure 5B).

DISCUSSION

Diabetes is a major public health problem worldwide, associated with serious complications and premature death due to continuous damage, dysfunction, and failure of various organs. To prevent diabetic acute problems and to reduce the risk of long term complications an agent with multiple strategies against diabetes is considered to be more effective. Mushrooms are functional foods and a potent source of biologically active compounds effective for both the control of blood glucose levels and the modification of the course of diabetic complications. In the present study phenolic content of pleurotus ostreatus and biochemical effects on oxidative stress markers, DNA damage and expression level of Nrf2 and VEGF in STZ induced diabetic rats were examined. Our results revealed that PO methanolic extract has high levels of phenolic compounds which could be used as an important indicator of antioxidant capacity. The experimental data are comparable to those reported by [19, 20]. Mushrooms phenolic compounds have been found to be an excellent antioxidant and synergist that is not mutagenic [21].

Treatment of STZ diabetic rats by PO significantly decreased glucose level, the hypoglycemic effect of PO may be attributed to several mechanisms including, firstly suppression of intestinal digestion and absorption of glucose through inhibition of hydrolyzing enzymes α-amylase and α-glucosidase in a dose dependent manner. Also viscosity of mushrooms polysaccharide acting as barrier to the digestive enzyme action increasing gastric emptying time and preventing rapid glucose increase [22]. Secondly inhibition of gluconeogenesis [23]. they found that Chitosan attenuated gluconeogenesis related signals for phosphoenolpyruvate carboxykinase (PEPCK) and AMP-activated kinase (AMPK) in liver of STZ-induced diabetic rats. Thirdly stimulation of insulin synthesis or secretion from β-cells and improvement of insulin sensitivity [11], demonstrated that pleurotus polysaccharides elevate serum insulin levels, improve insulin sensitivity through regulating peroxisome proliferator-activated receptor (PPAR) gamma-mediated lipid metabolism [24]., protect β-cells from oxidative stress [25] and stimulate the insulin release from the pancreatic islets of β-cells [26]. Fourthly modulation of genes related to glucose metabolism [27]. Xiong found that β-glucans from Pleurotus species upregulating the expression of GLUT4 and the adiponectin genes and down-regulating the expression NF-kB that controls the regulation of genes that encode proteins involved in immune and inflammatory responses, including interleukin-6 through the activation of the AMPK-signaling pathway.

Concerning the ameliorative effect of PO against hypoproteinemia, increased ALT, and AST activities, urea and creatinine induced by STZ, our results agree with many studies recorded by several authors including Refaie et al. [28]. who found that administration of PO polysaccharopeptides improve the capacity of hepatocytes to synthesis and export proteins, stimulate the humoral immune response and the liver antioxidant defense system. Also Gaafar et al., postulated the mechanism by which mushroom could suppress renal toxicity is by blocking oxidative injury in the kidney and restore the antioxidant enzymatic profile that interfere with inducible nitric oxide synthase activity, also attenuation of protein catabolism , and diminished activity of urea cycle [29]

It is known that diabetes is usually associated with deterioration in the antioxidant defense mechanisms, increasing the oxidative stress and ROS which attack the cell membrane, nucleus and genetic parts leading to possible protein and DNA modifications [6]. An increased oxidative stress have an important causal role in B-cell failure and the development of insulin resistance which is the main cause of type II DM [30]. On the other hand mushrooms are important source of strong antioxidants and have potent free radical scavenging activities due to antioxidant compounds found in fruit bodies, mycelium and broth confirmed to be phenolics, flavonoids, glycosides, polysaccharides, tocopherols, ergothioneine, carotenoids, vitamins and minerals [31]. This is what appeared in our results that the liver, kidney and Heart of STZ induced diabetic rats showed the highest level of MDA and DNA fragmentation with the lowest levels of GSH content and GPx activity compared with STZ induced diabetic rats treated with PO. Potential antioxidant therapy should include either natural free radical scavenging antioxidant enzymes or agents which are capable of augmenting the activity of the antioxidant enzymes [32]. In this respect mushrooms have been found to exert powerful antioxidant activity due to several non-enzymatic antioxidant agents, such as Vit A, Vit C, Vit E, and GSH

content [33], antioxidative polysaccharides and low molecular weight polysaccharopeptides and polysaccharoprotein complex (PSPC) [34]. In addition to many phenolic compounds such as polyketides, terpenes, steroids variegatic acid, gallic acid, chlorogenic acid, naringenin, hesperetin, and biochanin-A isolated from PO [19], which have considerable antioxidant effects including scavenging ability, reducing power and chelating ability which may serve as secondary antioxidant because they reduce the redox potential thereby stabilizing the oxidized form of the metal ions [35]. According to Liu [36] additive and synergistic effects of phytochemicals in fruits and vegetables' are responsible for their potent bioactive properties and their benefit may be attributed to the complex mixture of phytochemicals present in whole foods. This explains why no single antioxidant can replace the combination of natural phytochemicals to achieve health benefits. ROS are important secondary messengers for signaling pathways associated with apoptosis, proliferation, damage and inflammation. Their adverse effects were considered to play a leading role in the onset and progression of diabetes and diabetic complication diseases. Therefore, the downregulation of ROS generation may have a pivotal role in controlling diabetes and its complications [3]. A recurrent theme in oxidant signaling and antioxidant defense reactive cysteine thiol-based redox signaling. The Nrf2 is an emerging regulator of cellular resistance to oxidants. Nrf2 regulate cellular redox homeostasis under both stressed and non-stressed conditions [4]. Also Nrf2 has a key role in preserving a healthy endothelial phenotype and maintaining the functional integrity of the vasculature [37]. Vascular endothelial growth factor (VEGF) is an important element in tissue neogenesis and vascular healing, and has an initiatory destructive role in microvascular diabetic complications as it directly influence glomerular permeability lead to glomerular proteinuria with diminished vascular wound healing [1]. The expression levels of Nrf2 and VEGF in liver, kidney and heart were calculated quantitatively in comparison to B-actin as a house keeping gene. The expression levels of Nrf2 in liver, kidney and heart were highest in diabetic rats treated with PO while the expression levels of VEGF in liver and kidney were highest in diabetic rats treated with PO compared with STZ induced diabetic rats. Our observations are similar to that of many reports which attribute the ameliorative effects of PO on expression levels of Nrf2 and VEGF to the proved antioxidant properties of mushrooms [38]. The antioxidant activities of mushrooms are previously regarded to their contents of many natural bioactive components of antioxidant properties like, phenols [39], profilin-like protein, glyceraldehyde-3-phosphate dehydrogenase-like protein, and catalase-like protein [40]**,** phenolic compounds as Gallic acid, p-Coumaric acid, Ferulic acid, Fumaric acid and Chlorogenic acid [19], polyketides, steroids, Statins [41]**,** poly saccharides as B-glucan [27] and Pleuran [8].

CONCLUSION

PO is a widely distributed and cultivated mushroom with medical significance. It has a broad spectrum of biological activities and potentials in the prevention and treatment of diseases. Due to the high contents of mineral salts and phenolic compounds, it is of great dietary importance. Its activity is especially confirmed in decreasing blood glucose levels and in improving antioxidant status. Additionally, it has antiatherogenic, antioxidant and antineoplastic properties.

Availability of data and material

There are no restrictions to the availability of any materials and data upon request.

Fund

This study received no fund or grant.

Authors' contributions Prof Dr Said Zaki proposed the study design; Prof Dr. Adel El-Behairy and Dr Hanan Ogaly evaluated all the parameters. Dr Marwa Ibrahim performed the molecular assays. All authors drafted and revised the final manuscript.

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