

Full Length Research Paper

Zataria multiflora essential oil reduces diabetic damages in streptozotocin-induced diabetic rats

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In this study the *Zataria multiflora* essential oil (EO) was prepared and its anti-diabetic effects were analyzed in the streptozotocin (STZ)-induced diabetic rats. The yield of EO was 3% and carvactol (53%), p-cymene (17%), and thymol (11%) were detected as the main EO components. The antioxidant and nitric oxide scavenging activities were estimated as 650 ± 50 and 54 ± 2 μg ascorbic acid equivalents per ml of EO respectively. The effects of the EO on the certain biochemical markers for pancreas and liver damages were investigated. As observed in this study the level of plasma glucose, alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were significantly elevated in STZ-induced diabetic rats but total protein and insulin concentration reduced considerably. On the other hand, orally administration of the *Z. multiflora* EO was accompanied with a markedly reduction in plasma glucose, ALP, AST, and ALT, and a significant increases of total protein and insulin. The obtained results indicated that *Z. multiflora* had protective effects in diabetic rats and reduced diabetic damages probably by oxygen and nitrogen radical scavenging.

Key words: *Zataria multiflora*, diabetes mellitus, insulin, protein, alkaline phosphatase, aminotransferases.

INTRODUCTION

Diabetes mellitus is a metabolic syndrome characterized by hyperglycemia and hypoinsulinemia. Hyperglycemia itself can induce oxygen and nitrogen species production by four mechanisms including; increased polyol pathway flux, increased hexosamine pathway flux, increased advanced glycation end products, and protein kinase C activation. In addition, hyperglycemia can lead to mitochondrial electron transport chain overloading, which results more superoxide ion. Exaggerated production of these reactive species in diabetes can lead to very serious problems including; cardiovascular disease, liver and kidneys failure, blindness, and nerve injury (Brownlee, 2001, 2005; Neyenwe et al., 2011). Thus antioxidant therapy is one of the strategies for diabetes treatment. Many herbal extracts or derivative with high antioxidant activity are useful for treatment of diabetes and other metabolic syndrome (Ameenah, 2006; Grover et al., 2002; Samad et al., 2009). Essential oils or extract derived from these herbal medicines contain terpenes, terpenoids, phenolic, and aliphatic compounds with different biological activity. Essential oils derived from

these plants have antioxidant, antibacterial, antiviral, antiparasitic, anti-insectal as well as anti-inflammation and other pharmacological activity (Bakkale et al., 2008). *Zataria multiflora*, a thyme-like plant that grows broadly in central and southern parts of Iran is one of these plants. Essential oil of the plant are rich in monoterpenes and aromatic compounds and has antibacterial, antiviral, and antifungal activity (Saleem et al., 2004; Hadian et al., 2011; Saharkhiz et al., 2010). The biological activity of this EO on the diabetic models is less studied. For this reason in this research, the anti-diabetic properties of the EO in the streptozotocin (STZ)-induced diabetic rat was investigated.

MATERIALS AND METHODS

Chemicals

Potassium persulfate, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), sodium nitrite, ascorbic acid, naphthylthyl-enediamine, sulfanilamide, streptozotocin, α -ketoglutarate,

L-alanine, L-aspartate, and nicotinamide adenine dinucleotide (NADH) were purchased from Sigma and Fluka companies. Lactate dehydrogenase and malate dehydrogenase were purchased from sigma. All other chemicals and reagents used were of the highest commercially available purity.

Plant materials essential oil preparation

Z. multiflora aerial parts were obtained from the wild plants in Shiraz Mountain, Fars Province, Iran. The taxonomic identification of plant was confirmed by a senior plant taxonomist, Ahmad Reza Khosravi (Department of Biology, Shiraz University, Shiraz, Iran). The voucher specimen (no.24984) was deposited at the Herbarium of the Department of Biology, Shiraz University. The leaves of plant materials were separated from the stem and dried in the shade. For essential oil extraction, plant materials was hydro distilled for 2.5 h using an all-glass Clevenger-type apparatus according to the method outlined by British pharmacopoeia (1998). The essence was collected and stored at -20°C until usage.

Essential oil analysis and identification

The analysis of EO was carried out on a thermoquest-finnigan trace GC-MS instrument equipped with a DB-5 fused silica column (60 m × 0.25 mm i.d., film thickness 0.25 mm). The oven temperature was programmed to increase from 60 to 250°C at a rate of 4°C per min and finally held for 10 min; transfer line temperature was 250°C. Helium was used as the carrier gas at a flow rate of 1.1ml/min with a split ratio equal to 1/50. The quadruple mass spectrometer was scanned over the 35 to 465 with an ionizing voltage of 70 eV and ionization current of 150 mA. GC-flame ionisation detector (FID) analyses of the oil were conducted using a Thermoquest-Finnigan instrument equipped with a DB-5 fused silica column (60 m × 0.25 mm i.d., film thickness 0.25 mm). Nitrogen was used as the carrier gas at the constant flow of 1.1ml/min; the split ratio was the same as for GC/MS. The oven temperature was raised from 60 to 250°C at a rate of 4°C / min and held for 10 min. The injector and detector (FID) temperatures were kept at 250 and 280°C, respectively. Semi-quantitative data were obtained from FID area percentages without the use of correction factors. For EO identification, retention indices (RI) were calculated by using retention times of n-alkanes (C6-C24) that were injected after the oil at the same temperature and conditions. Compounds were identified by comparison of their RI with those reported in literature (Adams, 2007).

Total antioxidant determination

For total antioxidant capacity, 1.0 ml of diluted ABTS radical solution (7 mM ABTS and 2.5 mM potassium persulfate) was added to 10 µl of different concentration of EO compounds (5, 10, 15 and 20 µl/ml in DMSO) or ascorbic acid (10.56, 5.28, 2.64, 1.32, 0.66 and 0.33 µg/ml). After mixing, the absorbance was read at 734 nm. Results were expressed as µg ascorbic acid equivalent per ml EO (Re et al., 1999).

Nitric oxide radical scavenging activity

For nitric oxide scavenging assay, one volume of different concentration of ascorbic acid (10.56, 5.28, 2.64, 1.32, 0.66 and 0.33 µg/ml) or one volume of different concentration of EO (5, 10, 15 and 20 µl/ml in DMSO) were incubated with sodium nitrite (4.2 µg/ml in sodium citrate pH 5) at 37°C for 12 h. After incubation,

the concentration of nitrite was measured by the griess reagent (Miranda et al., 2001). Results were expressed as µg ascorbic acid equivalent per ml of EO.

Cell viability assay

The murine macrophages cell line (1×10^4 cells per well) in 96-well plates were incubated with different concentration of EO (1, 10, 100, 1000 nI/ml, final concentration) at 37°C in 5% CO₂ for 24 h. After incubation, 10 µl of MTT solution (5 mg/ml) was added to each well. After 4h at 37°C, 100 µl of lysis buffer (10% SDS in 0.01 M HCl) was added and incubated for 12 h. The absorbance of wells was read at 540 nm (Green et al., 1984).

Preparation of diabetic rats and experimental design

Male Sprague-dawley rats (220±20 g) received free access to 23% protein rat chow and water for at least 3 days. Diabetes was induced in the rats by a single intraperitoneal injection of STZ (50 mg kg/body weight) dissolved in 10 mM sodium citrate (pH 4.0), while non-diabetic control group was injected with the buffer. Treatment with essential oils was started 3 days after SZT injection. Rats were divided into four groups as the following: (1) non diabetic rats were orally administered vehicle (DMSO) for 28 days (NC); (2) non diabetic rats were orally administered essential oils (50 µl/kg body weight of 100 µl/ml EO in DMSO) for 28 days (NC+EO); (3) diabetics rats were orally administered vehicle (DMSO) for 28 days (DC); (4) diabetics rats were orally administered essential oils (50 µl/kg body weight of 100 µl/mL EO in DMSO) for 28 days (DC+EO).

Biochemical assays

Rats of all the groups were anaesthetized by ether. The blood of each animal were taken by puncturing retro-orbital plexus and allowed to clot at room temperature. Serum was separated by centrifugation and analyzed for assorted biochemical parameters. Glucose was measured by glucose oxidase method with glucose as the standard. Glucose is oxidized by glucose oxidase to gluconic acid and hydrogen peroxidase, which is converted to water and oxygen by peroxidase. Aminophenazone, takes up oxygen and together with phenol forms a pink colored chromogen, which can be measured at 515 nm (Barham and Trinder, 1972). Plasma insulin was determined using ELISA rat/mouse insulin kit according to the kit procedure with rat insulin as standard (Millipore, EZRMI-13, USA). Total protein was determined by Bradford method. ALP activity was assayed as µM phenol released from phenyl phosphate per min. Phenol reacts with 4-amnioantipyridine in the presence of potassium ferricyanide to a colored complex, which can be measured at 510 nm (King and Armstrong, 1988). AST activity was assayed in a coupled reaction with malate dehydrogenase in the presence of NADH. The rate of decrease in absorbance of the reaction mixture at 340 nm is directly proportional to the AST activity. One unit of AST oxidizes one uM NADH per min (King and Armstrong, 1988). ALT activity was assayed in a coupled reaction with lactate dehydrogenase in the presence of NADH. The rate of decrease in absorbance of the reaction mixture at 340 nm is directly proportional to the ALT activity. One unit of ALT oxidizes 1 µM NADH per min (King and Armstrong, 1988).

Statistical analysis

Data were expressed as means ± SD. The statistical significance of differences between means was determined by one-way

Table 1. Chemical components identified by GC-MS in the EO of *Z. multiflora* Bioss.

Component	RI	Percentage	Component	RI	Percentage
Alpha-thujene	929	1.1	alpha-terpinolene	1090	0.5
Alpha-pinene	939	2.0	linalool	1098	0.0
Camphene	954	0.1	terpin-4-ol	1184	0.0
Beta-pinene	982	0.3	dihydrocarvone	1216	0.0
Myrcene	989	2.3	carvacrol methyl ether	1243	2.0
Alpha-phellandrene	1007	0.2	thymol	1292	11
Delta-3-carene	1014	0.1	carvacrol	1319	53
Alpha-terpiene	1019	1.3	carvacrol acetate	1373	0.7
P-cymene	1028	17	beta-caryophyllene	1435	1.2
Limonene	1031	0.0	alpha-humulene	1453	0.0
1,8-cineol	1035	0.3	allo-aromadendrene	1467	0.1
Z-beta-ocimeme	1044	0.1	bicyclogermacrene	1506	0.1
Gama-terpienne	1060	0.7	caryophyllene oxide	1598	0.1
Trans-sabinene hydrate	1079	0.1			

analysis of variance (ANOVA) using Duncan's multiple range test ($p < 0.05$).

RESULTS

Chemical composition, antioxidant capacity and nitric oxide scavenging

GC/MS analysis of the EO indicated that the main components of the EO from *Z. multiflora* were carvacrol (53%), p-cymene (17%), thymol (11%), myrcene (2.3%), α -pinene (2%), carvacrol methyl ether (2%), α -terpiene (1.3%), β -caryophyllene (1.2%), and α -thujene (1.1%). The antioxidant capacity was estimated as 650 ± 50 μ g ascorbic acid equivalents per ml of EO and the NO scavenging activity was estimated as 54 ± 2 μ g ascorbic acid equivalents per ml of EO (Table 1).

Effects of EO on the glucose level

The concentration of glucose in the plasma of the non-diabetic control rat was 109.9 ± 5 mg/dl. In the non-diabetic rats administered EO, this value was 105.8 ± 2.7 mg/dl, but in the diabetic control rats, it was 260 ± 6 mg/dl. In diabetic rats administered EO, this value was 121.5 ± 6 mg/dl. Thus, *Z. multiflora* EO had no effects on the glucose level in the healthy rats but in the diabetic rat, the level of glucose was reduced to the non-diabetic control level ($p = 1.75 \times 10^{-3}$, $n=8$) (Figure 1).

Effects of EO on the insulin level

The concentration of insulin in the plasma of the non-

diabetic control rat was 16 ± 0.8 ng/ml. In the non-diabetic rats administered EO, this value was 15.25 ± 0.98 ng/ml, but in the diabetic control rats, it was 7 ± 0.81 ng/ml. In the diabetic rats administered EO this value was 12.2 ± 0.95 ng/ml. Thus, *Z. multiflora* EO had no effects on the insulin level in the healthy rats but in the diabetic rat the level of insulin increased to the non-diabetic control level ($p = 1.3 \times 10^{-8}$, $n=8$) (Figure 2).

Effects of EO on the total protein level

The concentration of total protein in the plasma of non-diabetic control rat was 7.75 ± 0.28 mg/dl. In the non-diabetic rats administered EO, this value was 7.67 ± 0.69 mg/dl, but in the diabetic control rats, it was 4.47 ± 0.41 mg/dl. In the diabetic rats administered EO this value was 6.25 ± 0.64 mg/dl. Thus, *Z. multiflora* EO had no effects on the total protein level in the healthy rats but in the diabetic rat the level of total protein increased to the non-diabetic control level ($p = 4 \times 10^{-6}$, $n=8$) (Figure 3).

Effects of EO on the alkaline phosphatase

The concentration of alkaline phosphatase in the plasma of non-diabetic control rat was 76.2 ± 4.5 μ L. In the non-diabetic rats administered EO, this value was 79.5 ± 3.3 μ /L, but in the diabetic control rats it was 144 ± 5.3 μ /L. In the diabetic rats administered EO, this value was 94.5 ± 4.2 μ /L. Thus, *Z. multiflora* EO had no effects on the alkaline phosphatase level in the healthy rats but in the diabetic rat, the level of alkaline phosphatase reduced to the non diabetic control level ($p = 1.63 \times 10^{-10}$, $n=8$) (Figure 4).

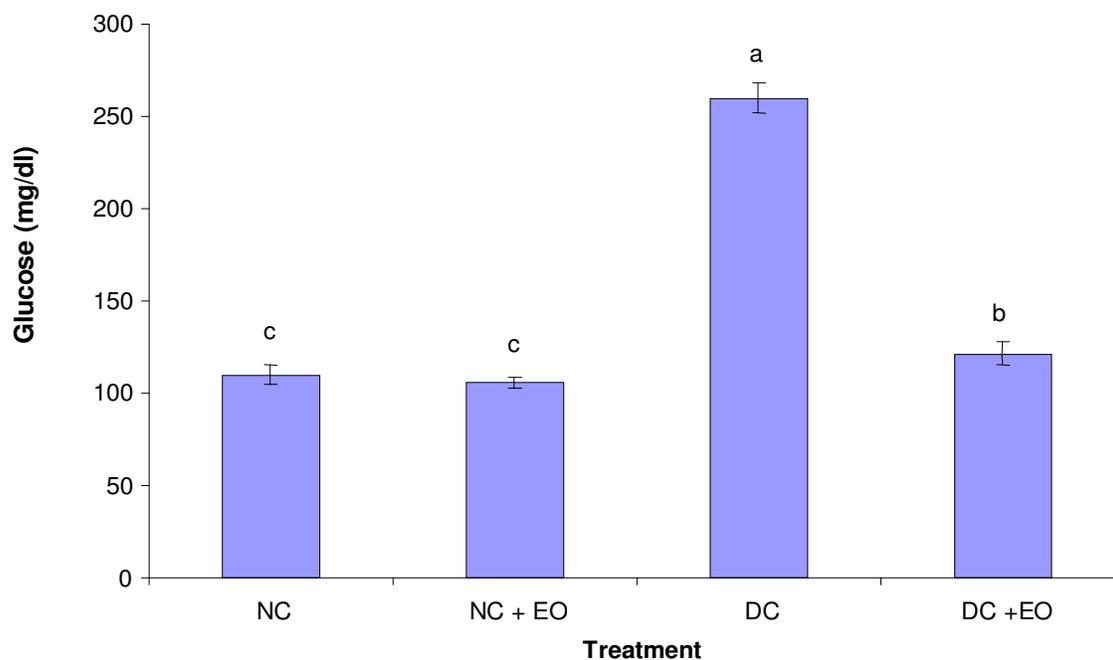


Figure 1. Effects of *Z. multiflora* EO on the plasma glucose (mg/dl) in the Streptozotocin- induced diabetic rats. Diabetic rats were prepared and administered with EO for 28 days. Plasma was collected and glucose level was assayed by glucose oxidase method. In diabetic rats, plasma glucose increased significantly, however, administration of EO reduced glucose to the control non-diabetic rats level. NC, nondiabetic control; NC + EO, nondiabetic control administered EO; DC, diabetic control; DC + EO, diabetic control administered EO. Means with the same letter within an assay are not significantly different as determined by Duncan's multiple range test analysis ($p < 0.05$).

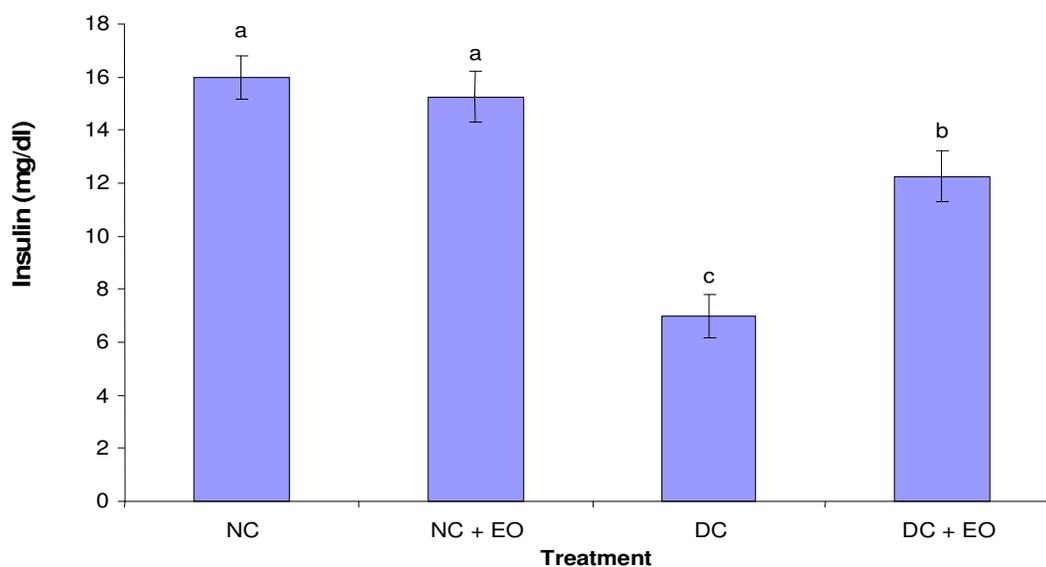


Figure 2. Effects of *Z. multiflora* EO on the insulin (ng/ml) in the Streptozotocin- induced diabetic rats. Diabetic rats were prepared and administered with EO for 28 days. Plasma was collected and insulin level was assayed by ELISA kit with rat insulin as standard. In diabetic rats plasma, insulin decreased significantly, however, administration of EO increased insulin. NC, nondiabetic control; NC + EO, nondiabetic control administered EO; DC, diabetic control; DC + EO, diabetic control administered EO. Means with the same letter within an assay are not significantly different as determined by Duncan's multiple range test analysis ($p < 0.05$).

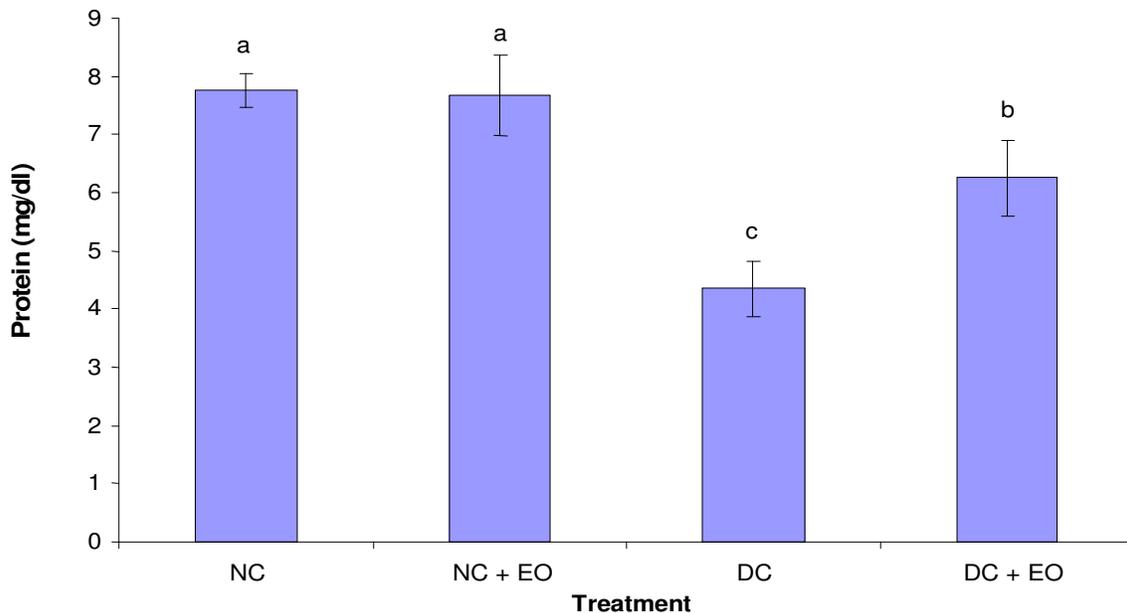


Figure 3. Effects of *Z. multiflora* EO on the Protein (mg/dl) the Streptozotocin- induced diabetic rats. Diabetic rats were prepared and administered with EO for 28 days. Plasma was collected and protein level was assayed by Bradford method with bovine serum albumin as standard. In diabetic rats plasma, protein decreased significantly, however, administration of EO increased protein to the control non-diabetic rats level. NC, nondiabetic control; NC + EO, nondiabetic control administered EO; DC, diabetic control; DC + EO, diabetic control administered EO. Means with the same letter within an assay are not significantly different as determined by Duncan's multiple range test analysis ($p < 0.05$).

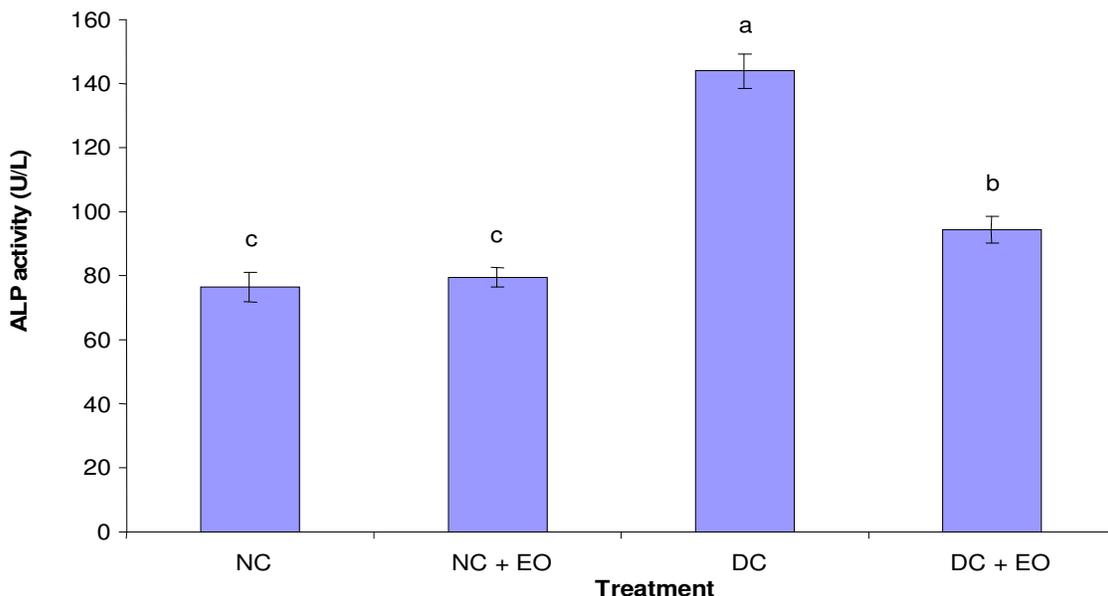


Figure 4. Effects of *Z. multiflora* EO on the ALP (U/L) in the Streptozotocin- induced diabetic rats. Diabetic rats were prepared and administered with EO for 28 days. Plasma was collected and ALP activity was assayed as μM phenol released from phenyl phosphate per min. In the diabetic rats, ALP activity increased significantly, however, administration of EO reduced ALP activity to the control non-diabetic rats' level. NC, nondiabetic control; NC + EO, nondiabetic control administered EO; DC, diabetic control; DC + EO, diabetic control administered EO. Means with the same letter within an assay are not significantly different as determined by Duncan's multiple range test analysis ($p < 0.05$).

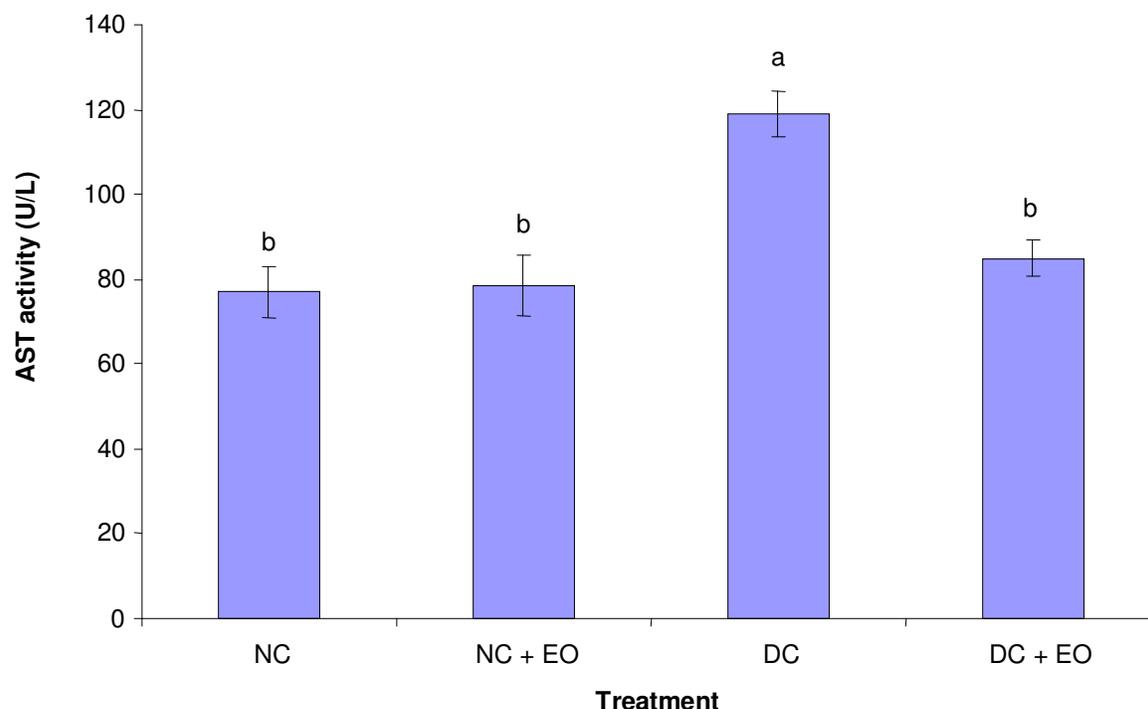


Figure 5. Effects of *Z. multiflora* EO on the AST (U/L) in the Streptozotocin- induced diabetic rats. Diabetic rats were prepared and administered with EO for 28 days. Plasma was collected and AST activity was assayed as oxidization of one μM NADH per min. In the diabetic rats, AST activity increased significantly, however, administration of EO reduced AST activity to control non-diabetic rats' level. NC, non-diabetic control; NC + EO, non-diabetic control administered EO; DC, diabetic control; DC + EO, diabetic control administered EO. Means with the same letter within an assay are not significantly different as determined by Duncan's multiple range test analysis ($p < 0.05$).

Effects of EO on the aspartate aminotransferase

The concentration of aspartate aminotransferase in the plasma of non-diabetic control rat was $77 \pm 6.2 \mu\text{L}$. In the non-diabetic rats administered EO, this value was $78.5 \pm 7.3 \mu\text{L}$, but in the diabetic control rats, it was $118.7 \pm 5.3 \mu\text{L}$. In diabetic rats administered EO, this value was $85 \pm 4.1 \mu\text{L}$. Thus *Z. multiflora* EO had no effects on the aspartate aminotransferase level in the healthy rats but in the diabetic rat, the level of aspartate aminotransferase reduced to the non-diabetic control level ($p = 9.05 \times 10^{-7}$, $n=8$) (Figure 5).

Effects of EO on the alanine aminotransferase

The concentration of alanine aminotransferase in the plasma of the non-diabetic control rat was $36 \pm 3.3 \mu\text{L}$. In the non-diabetic rats administered EO, this value was $37.5 \pm 4.5 \mu\text{L}$ but in the diabetic control rats, it was $66 \pm 3.65 \mu\text{L}$. In the diabetic rats administered EO, this value was $44 \pm 4.5 \mu\text{L}$. Thus, *Z. multiflora* EO had no effects on the alanine aminotransferase level in the healthy rats but in the diabetic rat, the level of alanine

aminotransferase reduced to the non diabetic control level ($p = 6.22 \times 10^{-7}$, $n=8$) (Figure 6).

DISCUSSION

In this work, the effects of *Z. multiflora* EO were investigated for anti-diabetic properties in the STZ-induced diabetic rats. The main components of this EO were carvacrol (53%), p-cymene (17%), thymol (11%), myrcene (2.3%), α -pinene (2%), carvacrol methyl ether (2%), α -terpiene (1.3%), β -caryophyllene (1.2%), and α -thujene (1.1%). The antioxidant activity and NO scavenging activity were estimated as 650 ± 50 and $54 \pm 2 \mu\text{g}$ ascorbic acid equivalents per ml of EO respectively. Thus, the antioxidant activity was higher than NO scavenging activity. These activities are the results of a synergism between all the molecules in the essence, but not the effects of one or two main components with high quantity. On the other hand the effects of EO are quality dependent but not quantity dependent (Bakkali et al., 2008).

In addition, the antidiabetic effects of this EO was investigated in the STZ-induced diabetic rats using

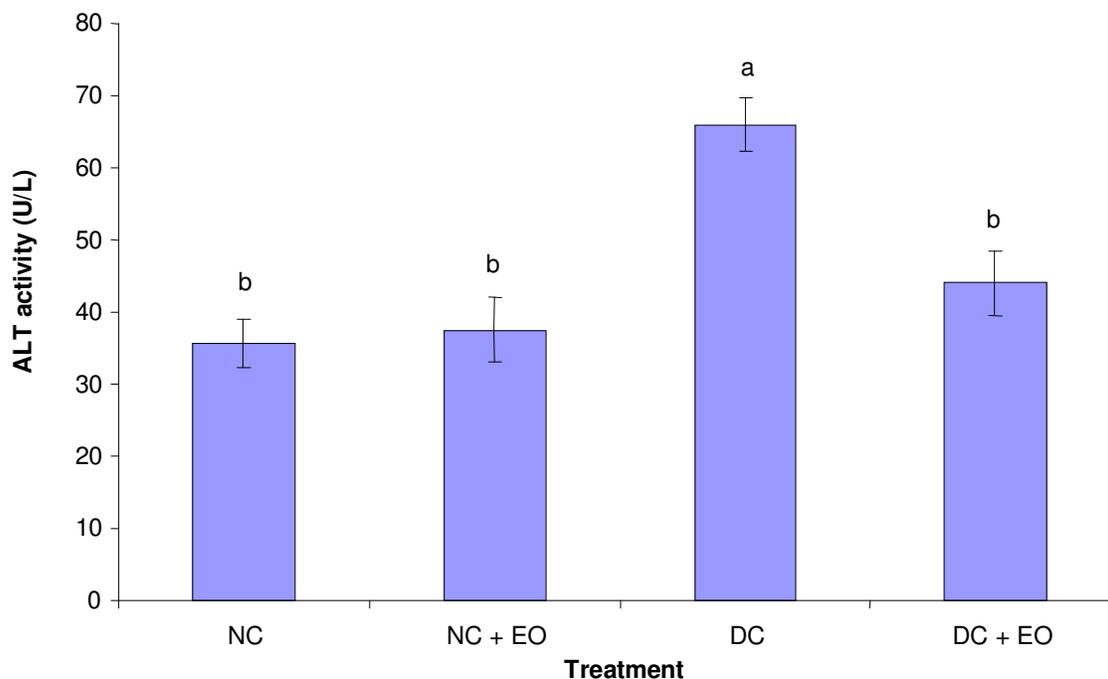


Figure 6. Effects of *Z. multiflora* EO on the ALT (U/L) in the Streptozotocin- induced diabetic rats. Diabetic rats were prepared and administered with EO for 28 days. Plasma was collected and ALT activity was assayed as oxidation of one μM NADH per min. In diabetic rats, ALT activity increased significantly, however, administration of EO reduced ALT activity to control non-diabetic rats level. NC, nondiabetic control; NC + EO, nondiabetic control administered EO; DC, diabetic control; DC + EO, diabetic control administered EO. Means with the same letter within an assay are not significantly different as determined by Duncan's multiple range test analysis ($p < 0.05$).

analysis of certain biochemical markers for pancreas (hyperglycemia and hypoinsulinemia) and liver (alkaline phosphatase and aminotransferase) damages. STZ is a toxic glucose analogue that preferentially accumulates in pancreatic beta cells through glucose transporter 2. In β cells, STZ is converted to glucose and methylnitrosourea which alkylates biological macromolecules, fragments DNA and destroys the β cells through necrosis and causes a state of insulin dependent diabetes which is characterized by hypoinsulinemia and hyperglycemia (Lenzen, 2008). In addition, STZ has deleterious effects on the liver and kidney (Laguens et al., 1980). Hyperglycemia itself induced oxidative stress can lead to more complicated damages to β cells and other tissues (Brownlee, 2001, 2005). Thus, antioxidant therapy is one of the major strategies for diabetes treatment (Johanson et al., 2005; Maritim et al., 2003). As these results indicated *Z. multiflora* as an antioxidant source, increased insulin level and reduced plasma glucose was firstly, due to the protective effects of *Z. multiflora* on the pancreas β cells against the deleterious effects of STZ and ROS produced by STZ. In addition, the components of EO might be stimulating normal β cells for insulin production (Pushparaj et al., 2001; Prabhu et al., 2008;

Cam et al., 2003).

Furthermore, these results indicated the reduction of alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase in STZ-induced diabetic rats after *Z. multiflora* administration for 28 days. The mechanism of STZ on the liver damage and hepatic cell disruption was not clearly recognized. However it seemed it has the same mechanism as β cells which can alkylate protein and DNA and cause cellular necrosis and cell destruction and the release of alkaline phosphatase and aminotransferase (Zafar et al., 2009). These results indicated that the levels of plasma alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase in diabetic rats increased but after EO administration, the level of these biochemical markers decreased to non-diabetic control. In addition, these results indicated a reduction in the total protein in the diabetic rats. Oral administration of EO restored protein concentration to the control level. Probably oxidative stress and at the same time production of nitric oxide can produce peroxynitrite, which led to tyrosine nitrosation (including ribosomal protein) and consequently reduced protein production (Maritim et al., 2003; Brownlee, 2005). Previous work by Kaleem et al. (2008) on the effects of

Annona squamosa, Singh et al. (2009) on the effects of *Cynodon dactylon*, Abdollahi et al. (2010) on the effects of *Momordica charantia*, and Zhu et al. (2010) on the effects of *Chinese propolis* and *Brazilian propolis* in diabetic rats indicated that glucose level, ALP, ALT, and AST decreased, while insulin increased significantly. Furthermore, the study of Guvena et al. (2006) indicated that melatonin as an antioxidant, improved the morphological and histopathological changes of the liver in the STZ-induced diabetic rats. Additionally, the study of Fu et al. (2010) on the molecular mechanism of genistein in diabetic mice indicated that genistein modulated b cell activation through cAMP/PKA-dependent ERK1/2 signaling pathway and improved hyperglycemia and insulin levels in STZ-induced diabetic mice. In conclusion, this study demonstrated that *Z. multiflora* EO significantly prevented the progression of STZ induced diabetics rats and reduced hepatic and pancreas injury probably with a mechanism similar to maltonin and genistein. In addition, *Z. multiflora* had protective effects in diabetic rats and reduced diabetic damages probably by oxygen and nitrogen radical scavenging.

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