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Full Length Research Paper

Effects of Clove Oil on Liver and Antioxidant Status of Streptozotocin-Induced Diabetic Rats

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This study was design To evaluate the hepatoprotective and antioxidant effect of low and high doses of clove oil on diabetic rats. Forty female albino rats were equally divided into four groups. The first group served as control (G1). The other 3 groups were intraperitoneal injected with 45 mg/kg body weight of streptozotocin (STZ) to induce diabetes. One diabetic group (G2) kept untreated and served as positive control. The other diabetic groups (G3 and G4) were treated with 300 mg/kg and 600 mg/kg of clove oil respectively. Results revealed that the STZ-induced diabetic group exhibited high significant increase in the activities of the liver enzymes (alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase) as well as reduction in the activities of catalase, superoxide dismutase and glutathione reductase compared to the control group. Diabetic groups treated with different doses of clove oil showed a decrease in the activities of liver enzymes in and increase in antioxidant enzymes activities compared to untreated diabetic group. Histopathological examination of liver tissues of diabetic rats indicated sever changes. However, their changes were overcome by clove oil treatment and the majority of the cells tend to be normal. Clove oil supplementation may act as antioxidant agents and could be an excellent adjuvant support in therapy of diabetes mellitus and its complications.

Keywords: Diabetes, albino rats, clove oil, streptozotocin, diabetes mellitus, liver enzymes, antioxidant and histopathology.

INTRODUCTION

Diabetes mellitus is a metabolic disorder of multiple etiologies that has reached pandemic proportions worldwide. Persistent hyperglycemia damages pancreatic β-cells resulting in impaired insulin secretion and action thus affecting glucose utilization in peripheral tissues (Gayathri and Kannabiran, 2008; Sheweita et al., 2016). The chronic hyperglycemia of diabetes can lead to oxidative stress and serious complications such as heart diseases, high blood pressure, blindness, kidney disease, liver disease, nervous system disease, and endocrine system disorders (Al-Attar, 2010; Shanmugam et al., 2011; Vijayakumar et al., 2006). Insulin therapy is used for management of diabetes mellitus but there are some disadvantages, such as insulin antibodies, insulin hypoglycemia allergy, autoimmunity, lipodystrophy and other postponed complications like vascular complications and morphological changes in kidney (Al-Attar and Zari, 2007). There are several new synthetic antidiabetic agents produce severe side effects, such as hypoglycemic coma, hepatic and renal disturbances (Shanmugam et al., 2011). Drugs extracted from plants are often considered to be free of side effects and less toxic than synthetic ones (Ozsoy-Sacan et al., 2006). The

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antidiabetic effects of these plants is the capability to restoring the function of pancreatic β-cells by making a rise in insulin production or by facilitation of metabolites in insulin dependent processes or by inhibiting the intestinal absorption of glucose (Narasimhulu et al., 2014). *Syzygium aromaticum* (cloves) are the aromatic dried flower buds of a tree belonging to the family *Myrtaceae* (Alma et al., 2007). The essential oil isolated from clove is widely used due to their medicinal properties (Ramadan et al., 2013). Clove considered one of the richest plants of phenolic compounds and has great potential for pharmaceutical, food, cosmetic and agricultural uses. Meanwhile flavonoids are able to counteract the damaging effects of oxidative stress and decrease xenobiotic-induced liver toxicity in animals, cooperating with natural systems like endogenous protecting antioxidant enzymes, clove shows antioxidant properties and its extracts could be used as food antioxidants (Kadarian et al., 2002; Cortés-Rojas et al., 2014).This study aims to investigate the effect of different doses of clove oil on the activities of liver enzymes and the status of antioxidant defense system in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Experimental Animals

Forty female *albino* rats (Wistar strain), weighing 230-273 g, were obtained from the Animal Experimental Unit of King Fahad Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. The rats were kept in special cages at (24± 3 °C) and humidity (60%) under 12 hour cycles of dark and light. Rats were supplied with standard pellet chow with free access to water for one week before the experiment for acclimatization. Animal handling was performed in accordance with the guidelines provided and approved by the Experimental Animal Laboratory Committee of the King Abdulaziz University, Faculty of Science.

Chemicals

Streptozotocin was purchased from Sigma-Aldrich Chemical Co (St. Louis, MO). Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were purchased from Dimension Company (DAD BEHRING Company, USA).Superoxide dismutase (SOD) was purchased from Cayman Chemical Company, (Ann Arbor, MI, USA). Clove oil was purchased from local market in Jeddah.

METHODS

Induction of Diabetes Mellitus by Streptozotocin

The experimental animals were fasted for 10 hours followed by a single intraperitoneal injection of freshly prepared STZ (Sigma, USA) at the dose of 45mg/kg body weight dissolved in 50 mM citrate buffer (pH 4.5) according to (Al-Attar, 2010). Animals were given free access to food and water. After 4 days, the fasting blood glucose levels taken from tail were measured by using a portable glucometer (Accu-Chek, Roch, Germany) in order to confirm the diabetes. Animals with blood glucose levels more than 250 mg/dl were considered diabetic and used for the experiment according to Almohammadi *et al.* (2013).

Experimental design

Forty female *albino* rats (Wistar strain) were randomly divided into four groups (n=10/group) as follows: **Group one (G1):** normal control rats. Rats of this group receive normal commercial chow diet. **Group two (G2)**: non treated diabetic rats. Rats of this group was ip injected with 45mg/kg body weight of STZ. **Group three (G3):** diabetic rats treated with 300 mg/kg clove oil daily for six weeks. **Group four (G4):** diabetic rats treated with 600 mg/kg clove oil daily for six weeks. Rats were weighed at the start and at the end of the experimental period.

Biochemical assay

Measurement of serum insulin and blood glucose levels

At the end of the experimental period, rats were fasted for 10 hours, water was not constrained, and then blood samples were withdrawn under diethyl ether anesthesia from retro-orbital from the inner canthus of eye using capillary tubes (Micro Hematocrit Capillaries, Mucaps). Serum was separated and stored at -80° C until biochemical analysis. Serum was used for the measurement of glucose, insulin and the activities of ALT, AST and ALP.

Tissue preparation and measurement of antioxidant and lipid peroxidation parameters

After samples collection, rats were sacrificed under ether anesthesia and the liver was removed immediately for the homogenate and histological study. 0.5g of the liver was homogenized in 5ml of ice cold distilled water. The

homogenates were centrifuged at 3000 rpm for 15 minutes at 4ºC using cold centrifuge. The supernatants were collected and aliquoted and stored at -20°C pending assay. The homogenate was used for detecting catalase (CAT) and glutathione reductase (GR) activities. To detect superoxide dismutase (SOD) in the tissue the same procedure was followed except the liver tissue was homogenated in 5 ml of HEPS buffer (Fayed, 2013).Cayman's assay kits were used for determination of SOD.

Catalase activity was assayed in tissue liver homogenate by kinetic assay according to (Lubinsky and Bewley, 1979). This reaction can be followed spectrophotometrically by measuring the decrease in the absorbance at 240 nm for 3 minutes due to the decomposition of H_2O_2. Catalase activity was calculated using molar extinction coefficient for hydrogen peroxide (0.041 $\mu mol^{-1}cm^{-1}$) by applying the equation of John (1992). One unit of the enzyme activity is defined as the amount of enzyme that catalyzes the decomposition of one µmol of H_2O_2 / min /mg protein. Glutathione reductase was assayed in tissue liver homogenate by kinetic assay. This reduction can be followed spectrophotometrically by measuring the decrease in the absorbance at 340 nm for 3 minutes due to the oxidation of NADPH to $NADP^+$ (Erden and Bor, 1984). GR activity was calculated using molar extinction coefficient for NADPH (6.22 $\mu mol^{-1}cm^{-1}$) by applying the equation of John (1992). Malondialdehyde (MDA) was determined in liver tissue homogenate by colorimetric assay according to Ruiz-Larrea et al. (1994) and calculated using coefficient of MDA 1.56 × 105 / M / cm according to Buege and Aust (1978).

Histopathological Examination

Theliver was excised and placed in 10% neutral buffered formalin. The fixed tissues were then trimmed, washed with ice saline and dehydrated in ascending grades of isopropyl alcohol and cleared in xylene. The wax impregnated tissues were embedded in paraffin blocks using the same grade wax, the paraffin blocks were cut with rotary microtome at 3-5µ thickness. The sections were floated on a tissue floatation bath at 40°C and taken on glass slides. The sections were then melted in an incubator at 60°C and after 5 minutes the sections were allowed to cool and stained with Hematoxylen and Eosin according to Bancroft and Cook (1998) examined microscopically.

Statistical analysis

All values were expressed as mean ± standard error$(\overline{X} \pm SE)$. Statistical analyses were performed with one-way analysis of variance (ANOVA) test and independent sample *t*-test using MegaStat Excel (version 10.3, Butler University). Differences were considered significant when probability value less than 0.05

RESULTS

Effect of Clove Oil on Serum Levels of Glucose and Insulin

After six weeks of experiment, fasting blood glucose levels were obtained from all groups. In (Table 1) glucose levels in diabetic rats (G2) showed a very highly significant increase (P<0.0001) with respect to control group (G1). On the other hand, glucose levels in diabetic groups treated with 300 mg\kg (G3) and 600 mg\kg (G4) of clove oil showed highly significant decrease (P<0.0001) when comparison with diabetic group (G2). The levels of glucose improved significantly in diabetic groups after six weeks of treatment with clove oil. In concomitant to glucose results, insulin levels showed very highly significant reduction (P<0.0001) in diabetic group (G2) as compare to control group (G1). While the levels of insulin in both treated diabetic groups G3 and G4 were found to be restored to normal level as comparison to diabetic group (G2).

Effect of Clove Oil on the Activities of Liver Enzymes in Serum

Table 2 demonstrated the effect of clove oil on the activities of ALT, AST and ALP. There was a very highly significant increase (P<0.0001) in ALT serum level in diabetic group (G2) as compared to control group (G1). In diabetic groups G3 and G4 treated with 300 mg\kg and 600 mg\kg of clove oil respectively there were very highly significant decrease (P<0.0001) in ALT levels as compared to untreated diabetic group (G2). Serum levels of AST in diabetic group (G2) were significantly higher (P<0.0001) than control group (G1). AST levels in diabetic rats treated with 300 mg\kg (G3) and 600 mg\kg (G4) of clove oil were significantly (P<0.0001) lower than diabetic group (G2). Serum levels of ALP in diabetic group (G2) showed significant elevation (P<0.0001) as compared to control group (G1). Serum ALP levels were significantly decrease (P<0.0001) in both treated diabetic groups G3 and G4 as compared to diabetic group (G2)

Effect of Clove Oil on the Activity of Lipid Peroxidation and Antioxidant Enzymes in Liver Tissue

The biochemical changes of lipid peroxidation and antioxidant enzymes were studied on liver Tissue of rats (Table 3). There was a very highly significant increase (p<0.0001) in MAD level in diabetic group (G2) as compared to control group. After treatment diabetic groups (G3 and G4) with clove oil there was very highly

Table 1. Effect of Clove Oil on Serum Glucose and Insulin.($\overline{X}$ ± SE)

Groups / Parameters	G1	G2	G3	G4
Glucose mg\dl	90.5±3.0	a *** 450±3.2	b *** 163.2±2.7	b *** 161.5±3.1
Insulin ng\ml	1.68± 0.07	a *** 0.98±0.05	b ** 1.39±0.08	b *** 1.55±0.12

G1= control group, G2= diabetic group, G3= diabetic group treated with 300mg\kg of clove oil.
G4= diabetic group treated with 600mg\kg of clove oil.
*P≤0.05 significant, **P≤0.001 highly significant and ***P=0.000 very highly significant.
P value > 0.05 = NS: non significant,.
[a]**:** Significant difference between G2 and G1.
[b]**:** Significant difference between diabetic treated groups (G3&G4) and G2.

Table 2. Effect of Clove Oil on the Activities of ALT, AST and ALP in Serum ($\overline{X}$ ± SE).

Groups / Parameters	G1	G2	G3	G4
ALT U\L	28.40±0.98	a *** 130.3±1.53	b *** 88.20±2.81	b *** 77.12±1.91
AST U\L	78±1.62	a *** 139.6±1.23	b *** 116±1.26	b *** 106.80±1.91
ALP U\L	85.31±2.15	a *** 130.7±2.15	b *** 93±1.10	b *** 92±1.42

G1= control group, G2= diabetic group, G3= diabetic group treated with 300mg\kg of clove oil.
G4= diabetic group treated with 600mg\kg of clove oil.
*P≤0.05 significant, **P≤0.001 highly significant and ***P=0.000 very highly significant.
P value > 0.05 = NS: non significant,.
[a]**:** Significant difference between G2 and G1.
[b]**:** Significant difference between diabetic treated groups (G3&G4) and G2.

significant decrease (p<0.0001) in MAD levels as compared to untreated diabetic group. The results of CAT, SOD and GR activities are shown in Table 3. There was a very highly significant decrease (P<0.0001) in catalase activity in diabetic group (G2) as compared to control group (G1). While, there were a very highly significant increase (P<0.0001) in treated diabetic groups (G3 and G4) as compared to untreated diabetic group. A very highly significant decrease (P<0.0001) was found in SOD activity in diabetic group (G2) as compared to control group (G1). No significant change was found in SOD activity in treated diabetic group (G3) whereas a highly significant increase (P<0.0001) in treated diabetic group (G4) as compared to untreated diabetic group (G2). Diabetic group (G2) showed a highly significant decrease (P<0.0001) in GR activity as compared to control group. There were significant increase (P<0.01) in diabetic groups (G3)treated with 300mg\kg and (G4) after treated with 600mg\kg of clove oil as compared to untreated diabetic group (G2).

Histopathological Investigation of LiverTissues

A section of the liver tissue from the control group has showed the normal histological structure of hepatic lobule and central vein without alterations as shown in Figure 1(A). Additionally, the hepatocytes were arranged in the form of branching cords and appeared irregular polygonal or polyhedral shaped cells typically with single, central, large vesicular nucleus. Some binucleated cells were occasionally observed, occupying a central position of the hepatocytes. The cord separated by blood sinusoids and radiated from the central vein. In contrast, the liver tissue of the diabetic group, exhibited in Figure 1(B), has showed several alterations, including activation of Kupffer cells, formation of degenerated areas of destroyed hepatocytes that lost their normal characters and were fused together, presence of some vacuoles, markeddilation and congestion of hepatic sinusoids. Interestingly, liver tissue in diabetic groups treated with clove oil, displayed in Figures 2 exhibited apparent normal histological structure.

Table 3. Effect of Clove Oil on lipid peroxidation and antioxidant enzymesin Liver Tissues. ($\overline{X}$ ± SE)

Groups / Parameters	G1	G2	G3	G4
MAD (Nmoles\ g of tissue)	1.79±0.17	a *** 3.01±0.13	NS 2.96±0.23	b * 2.70±0.11
CAT (ɱmoles\min\mg of protein)	3.62±0.25	a *** 1.44±0.07	b *** 2.84±0.18	b *** 2.96±0.14
SOD (U\ml)	1.89±0.10	a *** 0.53±0.06	NS 0.72±0.09	b ** 1.06±0.19
GR (Nmoles\min\mg of protein)	2.58±0.10	a ** 2.09±0.08	b ** 2.62±0.17	b ** 2.47±0.08

G1= control group, G2= diabetic group, G3= diabetic group treated with 300mg\kg of clove oil.
G4= diabetic group treated with 600mg\kg of clove oil.
*P≤0.05 significant, **P≤0.001 highly significant and ***P=0.000 very highly significant.
P value > 0.05 = NS: non significant,.
[a]: Significant difference between G2 and G1.
[b]: Significant difference between diabetic treated groups (G3&G4) and G2.

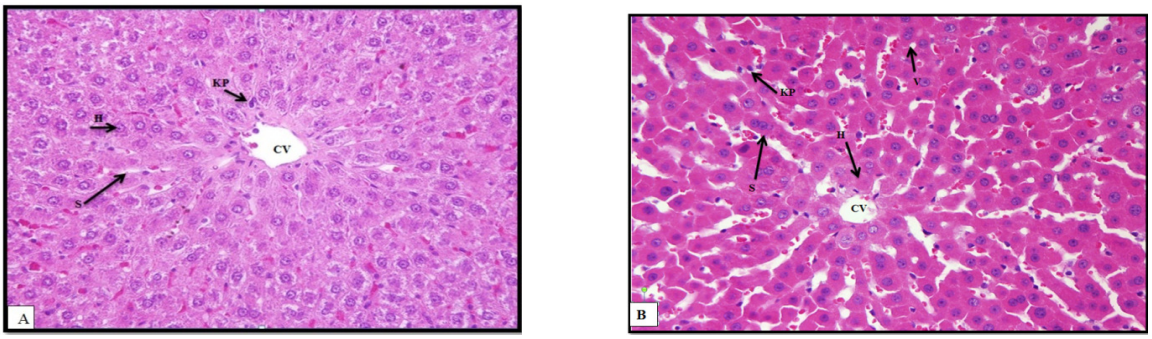

Figure 1. Photomicrographs of a section in the liver tissue of the A) control group showing normal structure, central vein (CV), normal arrangement of hepatic cords, normal blood sinusoids (S), hepatocytes (H) and Kupffer cells (KP), and B) diabetic group showing central vein (CV), congestion and dilation blood sinusoids (S), activation of Kupffer cells (KP), degeneration of some hepatocytes (H) and hepatocyte vacuolization (V) (H&E X 400).

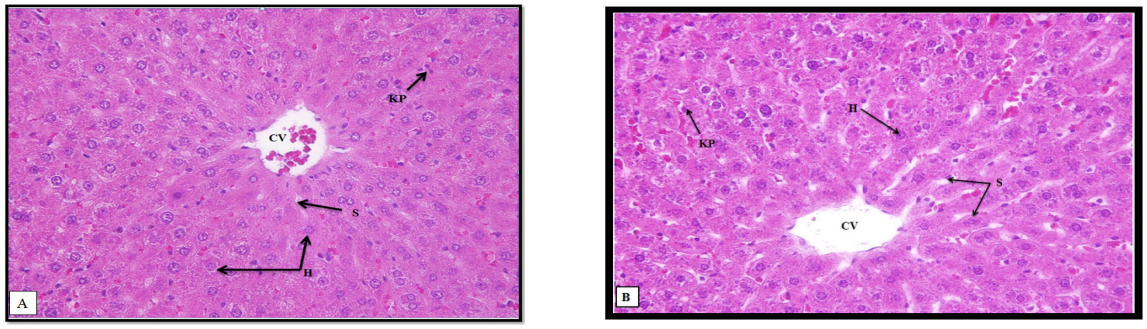

Figure 2. Photomicrographs of a section in the liver tissue of the A) diabetic group treated with 300mg/kg of clove oil and B) diabetic group treated with 600mg/kg of clove oil showing less activation of Kupffer cells (KP) and absence of widen sinusoids (H&E X 400)

DISCUSSION

In the present study, a significant increase in the glucose level and remarkable decrease in serum insulin levels was found in STZ-induced diabetic rats. Similarly, in previous studies STZ induced diabetic rats caused an increase in serum glucose level accompanied by a decrease in serum insulin levels (Aydin et al., 2012). Daniel et al. (2015) reported that fasting hyperglycemia caused decrease in serum insulin levels may due to the damage caused by STZ of β cells in the islets of Langerhans. Treatment of diabetic rats with different doses of clove oil exhibited remarkable improvement in glucose concentration and insulin levels when compared with untreated diabetic group (Basha and Sankaranarayanan, 2015). Chaudhry et al. (2013) explained glucose lowering effect of clove by stimulation of functioning pancreatic β-cells, to increase the release of insulin, or may be due to regeneration of β-cells.

Liver as an insulin-dependent tissue plays a vital role in the metabolism of glucose and other substances. The damage of liver cells cause a leakage of the contents out of the tissue into the blood stream (Ozsoy-Sacan et al., 2006) Aminotransferases, such as alanine ALT and AST serve as a marker of hepatocyte injury by measuring the concentration of intracellular hepatic enzymes which leaked into the circulation (Hadi Salih, 2013). Agbaje *et al.*(2010) stated that ALT is the enzyme produced inside the hepatocytes, recording rises in case of liver cells inflammation or cell death. In the current study, there were very highly significant increases in serum levels of ALT, AST and ALP in STZ- induced diabetic rats which indicate hepatocellular injury. A similar results was reported by Schmatz *et al*. (2012) who found that STZ-induced diabetic rats in a dose level of 55 mg/kg had elevation in serum levels of ALT and AST. Srinivasan *et al.*(2013) revealed that the activity of ALT, AST and ALP enzymes increase in rats injected with STZ. The outflow of liver enzyme markers into the blood stream could be an indication of hepatic damage. Elevation of ALT activity is more associated with necrotic state while the increase of AST activity is an index of hepatocellular injury in rats (Adefegha et al., 2014). The increase of the activity of ALP in serum is a marker of leakage of the enzyme from the liver cytosol into the blood circulation which gives an indication on the hepatotoxic effect of streptozotocin (Mansour et al., 2002). In the current study a decrease in serum levels of liver enzymes concentration (ALT, AST and ALP) was registered in diabetic groups after treated with clove oil (300 and 600 mg/kg) as compared to untreated diabetic group. Several studies demonstrated that there was a decrease in liver enzymes in diabetic rats treated with clove oil when compared with untreated diabetic rats (Al-Attar and Zari, 2007; Hassanen, 2010). The decrease in ALP activity in diabetic rats giving clove oil show that clove oil prevented liver damage (Hassanen, 2010). Abozid and EL-Sayed (2013) revealed that hepatoprotective action of clove may be due to flavonoids and polyphenolic compounds.

Hyperglycemia induces oxidative stress may be accompanying with elevation of lipid peroxidation which lead to the development of diabetic complications (Kota et al., 2012). Oxidative stress cause damage of cellular membranes and cause changes in the functional and structural integrity of subcellular organelles which may cause various complications in diabetis (Guven et al., 2006). Schmatz et al.(2012)stated that the increase of oxidative stress in the cell as a result of the depletion of antioxidant defense systems increase lipid peroxidation in diabetic conditions. In the present study, STZ-induced diabetic rats showed a very highly significant decrease in the activity of CAT, SOD and GR enzymes and elevation in malondialdehyde activity. Corroborating to our results, Schmatz et al. (2012) demonstrated that the activity of the antioxidant enzymes, such as SOD and CAT are reduced in tissues of diabetic rats. Adefegha *et al.*(2014) stated that antioxidant enzymes superoxide dismutase, catalase and glutathione reductase can inhibit free radical production and scavenging initiating radicals chelating the transition metal catalysts, breaking chain reactions, reducing concentration. Maritim et al.(2003) reviewed in detail that diabetes has multiple effects on the protein levels and activity of these enzymes, which further augment oxidative stress by causing a suppressed defense response. One possible mechanism for the reduction of SOD and CAT activities may be due to the excess of free radicals and by non-enzymatic glycation due to hyperglycemia (Schmatz et al., 2012). In addition, Yilmaz *et al*. (2004) exhibited that rat injected with STZ has been shown a marked decrease in SOD and CAT enzyme activities in the hepatic tissues. After treating the diabetic rats with (300 and 600 mg/kg) of clove oil, the results of our study showed an increase in the activity of antioxidant enzymes. In agreement with our study, Shukri et al.(2010) reported that clove supplements restored the antioxidant enzyme levels significantly. In addition, Saad et al.(2015) observed that plants rich with phenolic compounds can act as singlet oxygen scavengers ,reducing agents and hydrogen atom donators with stabilization of the generated free radicals forming stable compounds that do not proliferate or start oxidation. In particular, SOD enzyme catalyzes the reduction of the superoxide radical to hydrogen peroxide keeping the intracellular steady state concentrations of superoxide radical low. Simultaneously, CAT enzyme decomposes the produced H_2O_2thus, protects the tissue from highly reactive hydroxyl radicals (Saad *et al*., 2015). Meanwhile, GR regenerates glutathione that is used as a hydrogen donor by glutathione peroxidase during the elimination of hydrogen peroxide.

In the current study, the histological results showed that liver tissues in STZ-induced diabetic rats had several alterations including activation of Kupffer cells, several hepatocytes were fused together forming degenerated

areas of destroyed cells that lost their normal characters, presence of some vacuoles and markeddilation and congestion of hepatic sinusoids. A similar result reported by Sheweita *et al.*(2016) who reported that, STZ- induced diabetic rats revealed severe pathological changes including congestion and dilation of hepatic sinusoids. Moreover, Posuwan *et al.* (2013) reported that in diabetic group, cell degeneration was presented with foamy macrophage and sinusoid dilation. The damage effect of STZ could be attributed to the increase production of highly reactive intermediates of STZ, which are normally detoxified by endogenous GSH, but when present in excess can deplete GSH stores, allowing the reactive intermediate to react with and destroy hepatic cells (Khattab et al., 2013). Moreover, STZ stimulates H_2O_2 generation, which cause DNA fragmentation and increase oxidative stress in liver and pancreas cells (Bolkent et al., 2008). In the present study, liver tissues in diabetic rat treated with (300 and 600 mg/kg) of clove oil showed no histopathological alteration except Kupffer cells activation in few sections. Hassanen (2010) reported that the liver of diabetic rat treated with clove oil showed no change with apparent normal hepatocytes and the treatment with essential oil recovered the membrane damage by decreasing lipid peroxidation and improving antioxidant status. In addition, Shukri *et al.*(2010) reported that diabetic rats treated with clove had significantly reduced necrotic cells compared to diabetic rats by 21%. That indicated faster regeneration of new liver cells in diabetic rats in the presence of clove. Eugenol sustains the liver function to near-normal by suppressing lipid peroxidation and the release of cytokines. Thus, it is clear that the mechanism of eugenol protection might result from the diminished generation of ROS and reduction in inflammatory cell infiltration and generation of cytokines from Kupffer cells (Yogalakshmi et al., 2010).

CONCLUSION

This study together with existing reports demonstrates that the administration of clove oil has hepatoprotective and antioxidant effect. There for; clove oil supplementation may act as antioxidant agents and could be excellent adjuvant support in therapy of diabetes mellitus and its complications.

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