A non-insulin herbal treatment to improve liver tissue in diabetic rats through gavage of walnut oil enriched with a phytosterol

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Abstract: The present study was formulated in order to evaluate the effect of gavage of walnut oil enriched with different doses of a phytosterol, i.e. β-sitosterol (30, 45 and 60 mg kg⁻¹) on liver tissue in diabetic rats. Walnut oil was extracted through cold press method and analyses of the extracted oil were performed by gas chromatography (GC) and high-performance liquid chromatography (HPLC). Determination of liver tissue was carried out through staining via three protocols, i.e. hematoxylin and eosin (H&E stain), trichrome, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). The results of the present study revealed that gavage of the extracted oil from the walnut enriched with medium and high doses of β-sitosterol could improve liver tissue condition in the diabetic rats. These results indicate that the herbal treatment introduced in this study could be considered a potential non-insulin method to treat diabetic patients.

Subjects: Biochemistry; Laboratory Animal Science; Biology; Biotechnology

ABOUT THE AUTHOR

The authors have been mainly focusing on studies on biology, especially those highlighting the therapeutic effects of various extracts and oil on rats. The present study is in line with the authors’ previous studies where they have considered various aspects of natural solutions to solve various health disorders in animal models. Dr. Shiravi, Dr. Vaezi, and Dr. Hojati are experienced faculty members in Department of Biology, Damghan Branch, Islamic Azad University, Damghan, Iran and they have carried out extensive research on different aspects of animal biology. Dr. Sepehri from Golestan Neuroscience Research Center, Golestan University of Medical Sciences, Gorgan, Iran and Dr. Khor from Ischemic Disorders Research Center, Golestan University of Medical Sciences, Gorgan, Iran, are also experienced researchers in the field. Dr. Ghorbani has recently fulfilled her PhD in Islamic Azad University, Damghan, Iran at the Department of Biology.

PUBLIC INTEREST STATEMENT

The present study introduced a non-insulin herbal treatment to improve liver tissue in diabetic rats through gavage of walnut oil enriched with a phytosterol. The results of this study showed that the enriched walnut oil could be regarded as a potential substitute for the existing synthetic drugs to reduce the negative effect of diabetics. These results could be of great interest for public because they might gain a higher awareness of nutritional value of walnut and products produced from walnut, such as oil. Furthermore, they may use the results of this study to build up their knowledge in medicinal aspects of plant products to cope with various ailments, especially those whose treatment is very dependent on chemical and synthetic medications.
Keywords: walnut; phytosterol; liver tissue; diabetes mellitus

1. Introduction
Liver function is very dependent on insulin, and therefore, diabetes mellitus might increase the risk of hepatic diseases (Junejo, Rudrapal, Nainwal, & Zaman, 2017). Liver has a profound role in keeping glucose levels at a normal range during the fasting period after eating and has a well-established role in determining the pathogenesis in Diabetes Type II (Ahn et al., 2014). Glycogenosis and increase in glucose formation in liver is caused by the lack of insulin effect on liver (Philip, Mathias, Kumari, Gowda, & Shetty, 2014), and therefore, it is very important to take measures to improve liver tissue in diabetic patients.

Although insulin injection is the most common medication for diabetes mellitus, nutritional considerations have also gained attention in treating this disorder (Rocha et al., 2018). Before the discovery of insulin and common antidiabetics, diabetic patients were cured using medicinal plants and traditional medications. Positive effects of many of these plants on reduction of blood sugar and amelioration of consequences caused by diabetes mellitus have been shown. For instance, the positive effects of the extracts of Citrus maxima leaves (Feksa et al., 2018), Heracleum persicum extract (Alkan & Celik, 2018), Ilex paraguariensis extract (Rocha et al., 2018), polysaccharides from Suillellus luridus (Zhang et al., 2018), walnut powder extract (Liang, Chen, Cao, & Zhao, 2017), walnut leaves powder (Mollica et al., 2017), Pistachia lentiscus leaves extract (Cherbal et al., 2017), Callicarpa arborea extract (Junejo et al., 2017) on diabetes mellitus were reported.

Use of herbal medicine to render positive effects on various disorders was documented in different studies. For instance, Uysal et al. (2019) reported that methanol and ethyl acetate extracts of Heracleum sphondylium showed pronounced inhibition effects on cholinesterases, amylase, glucosidase, and tyrosinase using in silico docking studies. They also reported that the extracts did not cause mutation of bacterial strains and showed potent antimutagenic capacity. In another study, Trampetti et al. (2019) found that Cistanche phelypaea roots/flowers water extracts have high antioxidant activity and enzymatic inhibitory properties. Mahomoodally et al. (2018) also reported that essential oil from Aegle marmelos leaves possesses antioxidant, antibacterial and enzyme inhibitory effects. Furthermore, Stefanucci et al. (2018) Capparis spinose extracts showed enzyme inhibitory and antioxidant activities. Zengin et al. (2017) also stated that Ipomoea batatas leaf extracts have antioxidant activities, enzyme inhibitory activities and effects on inflammation pathways.

Walnut has positive effects on nervous system (Asadi Shekari et al., 2012), motor and cognitive functions (Willis, Shukitt-Hale, Cheng, & Joseph, 2009), learning and memory (Haider et al., 2011; Harandi et al., 2015), lipid metabolism (Abam, Oladipo, Atasie, & Obayomi, 2013). Moreover, it is known to possess antioxidant properties (Abam et al., 2013). Walnut oil is rich in polyunsaturated fatty acids (PUFA), which are very important from nutritional and medicinal perspectives. Consumption of walnut and products containing walnut may improve endothelial performance in type II diabetic patients and therefore, it can reduce the risk of cardiovascular disease (Rahimi, Kabiri, Asgary, & Setorki, 2011).

Phytosterols are similar to cholesterol in structure and bioactivity. These compounds have protective effects against chronic diseases such cardiovascular disease (Jones & AbuMweis, 2009), hepatic disorders (Plat et al., 2014), diabetes mellitus (Misawa et al., 2012), and cancer (Bradford & Awad, 2007). Phytosterols have cellular membrane structures and they play role in liquidity, permeability, and metabolism of cellular membrane (Priyadarsini & Nagini, 2012). Walnut contains phytosterols, especially β-sitosterol, which was shown to have anti-cancer properties (Hardman, 2014).
However, only few studies have been performed on protective effect of walnut oil enriched with phytosterols to improve liver tissue in diabetes mellitus. Therefore, the present study is formulated in order to evaluate the effect of gavage of walnut oil enriched with a phytosterol, i.e. β-sitosterol, to improve liver tissue in diabetic rats.

2. Materials and methods

2.1. Chemicals
Streptozotocin (STZ) was purchased from Sigma-Aldrich (Steinheim, Germany) and β-sitosterol was bought from Merck (Darmstadt, Germany). In addition, normal saline, chloroform, and formalin were purchased from Merck. All other materials were of analytical grade and obtained from Merck (Darmstadt, Germany).

2.2. Extraction and analyses of walnut oil
Walnut oil was extracted through cold press method in Golkaran Co. (Kashan, Iran). Analyses of the extracted oil were performed through gas chromatography (GC) and high-performance liquid chromatography (HPLC).

HPLC (Younglinm South Korea) with SP930D pump equipped with UV730D detector, manual injection system with ul20 loop and AUTOchrom software were used. Separation was performed using a 250 × 4.6 mm C18 column via a gradient of acetonitrile in water and 0.1% phosphoric acid at a flow rate of 1 mL/min at 30°C.

Fatty acid composition was measured by an Agilent 6890 (Santa Clara, CA, USA) equipped with a TR-CN100 Teknokroma column (100 m × 0.25 mm, 0.2 μm) at the flow rate of 5 ml/min. The injection and detection temperatures were 280°C and 320°C, respectively. The initial oven temperature was 165°C and was raised to 210°C at 40 min. After comparing the retention times of authentic standards and peak integrations, quantification of fatty acids was performed by comparing integrated areas.

Sterol profile was measured by a Young Lin Instrument Co., Ltd (Anyang, South Korea) equipped with a Restek, RSS column (30 m × 0.25 mm, 0.25 μm) at the flow rate of 2 ml/min. The injection and detection temperatures were 280°C and 300°C, respectively. The oven temperature was isothermal at 260°C. After comparing the retention times of authentic standards and peak integrations, sterol profile was obtained by comparing integrated areas.

2.3. Laboratory animals
Male rats (200 g) were purchased from Pasteur Institute (Amol, Iran) and kept at lab condition (temperature of 22 ± 1°C and moisture of 60%) at 12 h light and 12 h darkness. The rats were given free access to standard food and water. All the operations on animals were performed according to the ethical conduct on doing experiments with animals issued by the university and after getting permission from Ethics in Research Committee of the university.

2.4. Experimental
Diabetes mellitus was induced to rats by intraperitoneal injection of STZ at 65 mg/kg (Graham et al., 2011). After 72 h, blood sugar was measured in blood obtained from the tail area of the rats. To ensure the successful induction of diabetes mellitus, blood sampling was repeated a week later and the rats with >300 mg/dL sugar in their blood serum (16.6 mM) were considered diabetic. Overall, five treatments were taken into account as follows:

(1) Gavage of normal saline (control group)
(2) Gavage of pure walnut oil without fortification with β-sitosterol;
(3) Gavage of walnut oil with low dose of β-sitosterol (30 mg kg⁻¹);
(4) Gavage of walnut oil with medium dose of β-sitosterol (45 mg kg\(^{-1}\));
(5) Gavage of walnut oil with high dose of β-sitosterol (60 mg kg\(^{-1}\)).

Oil gavage was performed for 4 weeks at 0.5 ml/kg on a daily basis. For performing measurements, the rats were anesthetized using ether and were then killed and their livers were separated for histological tests. After separation, the livers were washed with physiologic serum and were then placed in 10% formalin for hydration and preparation for subsequent steps. After dehydration, the tissue cuts were prepared and staining was carried out via three protocols, i.e. hematoxylin and eosin (H&E stain), trichrome, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

2.5. Statistical analysis
Data analyses were performed through Analysis of Variance (ANOVA) and differences between means were determined by Tukey test. All the statistical operations were carried out in the Statistical Package for the Social Sciences (SPSS) version 21.0 (SPSS Inc., USA). Differences were considered significant at \( p < 0.05 \).

3. Results

3.1. Characterization of walnut oil
Fatty acid profile of walnut oil is shown in Table 1. As seen in this table, linoleic acid (C18:2c) is the most abundant fatty acid in walnut oil (around 60% of total fatty acids) followed by linolenic acid (C18:3), both of which are known as very important fatty acids. In addition, the table shows that PUFAs are the most abundant fatty acids in the oil, indicating the high nutritional and medicinal value of this oil.

3.2. Tocopherols
According to the results, γ-tocopherol (539.15 ppm) and α-tocopherol (16.57 ppm) accounted for the highest and lowest amounts of tocopherols in the extracted oil, respectively. Furthermore, δ-tocopherol was found to be 54.84 ppm in the extracted walnut oil. It is noteworthy that β-tocopherol was not detected in the extracted oil.

3.3. Sterol profile
Table 2 depicts sterol compounds in the extracted walnut oil. As seen in this table, the extracted oil does not contain cholesterol. In addition, β-sitosterol is the most abundant sterol compound (around 86%), which is indicative of high nutritional and health value of the extracted oil. After β-sitosterol, the highest sterol compounds in the extracted oil were \( \Delta^5 \)-avenasterol and campesterol.

3.4. Liver tissue
Figure 1 depicts the results of histological studies carried out through H&E and trichrome methods in control rats. As seen in this figure, in control rats, there is an extensive inflammation around central vein and inside sinusoids and there is evident sinusoidal dilatation and inflammation. Furthermore, there are several irregularities in Remak bundles in addition to accumulation of lymphocytes.

Figure 2 shows the results of histological studies carried out through H&E and trichrome methods in rats receiving gavage of pure walnut oil without fortification with β-sitosterol. As seen in this figure, there is a little inflammation around central vein but an extensive inflammation inside sinusoids and there are some sinusoidal dilatation and inflammation. Furthermore, there are a few irregularities in Remak bundles in addition to accumulation of lymphocytes.

Figure 3 shows the results of histological studies carried out through H&E and trichrome methods in rats receiving gavage of walnut oil with low dose of β-sitosterol. After gavage of fortified walnut oil with low concentration of β-sitosterol, inflammation around central vein and
### Table 1: Fatty acid profile of extracted oil from walnut

| Fatty acid | Percentage 1st | Mean ± SD 1st | Percentage 2nd | Mean ± SD 2nd | ppm 1st | ppm 2nd | ppm Mean ± SD |
|------------|----------------|---------------|----------------|---------------|---------|---------|---------------|
| C16:0      | 6.2            | 6.3           | 0.05 ± 0.04    | 6.2           | 6.3     | 0.05 ± 0.04| 52046.26     |
| C16:1      | 0.07           | 0.09          | 0.01 ± 0.01    | 0.07          | 0.09    | 0.01 ± 0.01| 58613.28     |
| C17:0      | 0.04           | 0.04          | 0.00 ± 0.00    | 0.04          | 0.04    | 0.00 ± 0.00| 7984.84      |
| C17:1      | 0.01           | 0.01          | 0.00 ± 0.00    | 0.01          | 0.01    | 0.00 ± 0.00| 40218.84     |
| C18:0      | 3.76           | 3.71          | 0.02 ± 0.00    | 3.76          | 3.71    | 0.02 ± 0.00| 29699.781    |
| C18:1t     | 0.01           | 0.02          | 0.005 ± 0.0015 | 0.01          | 0.02    | 0.005 ± 0.0015| 174109.12  |
| C18:1c     | 2.22           | 2.22          | 0.00 ± 0.0015  | 2.22          | 2.22    | 0.00 ± 0.0015| 1727758.84   |
| C18:2t     | 0.04           | 0.04          | 0.00 ± 0.0015  | 0.04          | 0.04    | 0.00 ± 0.0015| 313656.375   |
| C18:2c     | 59.15          | 59.15         | 0.02 ± 0.0013  | 59.15         | 59.15   | 0.02 ± 0.0013| 489876.615   |
| C18:3      | 8.46           | 8.39          | 0.03 ± 0.0152  | 8.46          | 8.39    | 0.03 ± 0.0152| 79003.56     |
| C20:0      | 0.02           | 0.02          | 0.00 ± 0.0015  | 0.02          | 0.02    | 0.00 ± 0.0015| 2000.00      |
| Others     | 9.86           | 9.86          | 0.02 ± 0.0015  | 9.86          | 9.86    | 0.02 ± 0.0015| 2000.00      |
| SFA        | 1443023 ± 50603.03 | 1443023 ± 50603.03 | 1443023 ± 50603.03 | 1443023 ± 50603.03 | 1443023 ± 50603.03 |
| MUFA       | 49159.8         | 52046.26      | 49159.8 ± 52046.26 | 49159.8         | 52046.26 |
| PUFA       | 67.5            | 67.5          | 0.015 ± 67.555 | 67.5          | 67.5    | 0.015 ± 67.555 | 49159.8         | 52046.26 |

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Table 2. Sterol profile of extracted oil from walnut

| Sterol            | Percentage | ppm         |
|-------------------|------------|-------------|
|                   | 1st | 2nd | Mean ± SD    | 1st | 2nd | Mean ± SD   |
| Cholesterol       | ND  | ND  | -            | ND  | ND  | -            |
| Campesterol       | 5.60| 5.84| 0.12 ± 5.72  | 64.476| 62.408| 1.034 ± 63.442|
| Stigmasterol      | 0.8 | 0.8 | 0.00 ± 0.8   | 9.254| 8.513| 0.3705 ± 8.8835|
| Clerosterol       | 0.61| 0.68| 0.035 ± 0.645| 6.995| 7.252| 0.1285 ± 7.1235|
| β-sitosterol      | 86.17| 85.53| 0.32 ± 85.85 | 991.380| 914.111| 38.6345 ± 952.74|
| Δ5-avenasterol    | 6.7 | 7   | 0.15 ± 6.85  | 77.078| 74.863| 1.1075 ± 75.970|
| 7-Δ Stigmastenol  | 0.12| 0.15| 0.015 ± 0.135| 1.311| 1.63| 0.1595 ± 1.4705|
| Total             | -   | -   | -            | 1150.496| 1068.777| 40.85 ± 1109.63|

*Not Detected.*
Figure 1. Microscopy of liver samples in control diabetic rats; (a) H&E (20 µm); (a) H&E (100 µm); (c) trichrome (20 µm); (d) trichrome (100 µm).

Figure 2. Microscopy of liver samples in diabetic rats receiving gavage of pure walnut oil without fortification with β-sitosterol; (a) H&E (20 µm); (b) H&E (100 µm); (c) trichrome (20 µm); (d) trichrome (100 µm).
inside sinusoids decreased whereas there was still a little dilatation and inflammation in sinusoids. In addition, although there are few irregularities in Remak’s codons, there is no accumulation of lymphocytes.

Figure 4 shows the results of histological studies carried out through H&E and trichrome methods in rats receiving gavage of walnut oil with medium dose of β-sitosterol (40 mg/kg). As seen in this figure, a considerable reduction in inflammation around central vein is detected. There is very little inflammation inside sinusoids and there are no sinusoidal dilatation and inflammation. Furthermore, there is no irregularity in Remak bundles. Moreover, no accumulation of lymphocytes is detected.

Figure 5 shows the results of histological studies carried out through H&E and trichrome methods in rats receiving gavage of walnut oil with high dose of β-sitosterol (60 mg/kg). As seen in this figure, there is no inflammation around central vein. Moreover, there are no inflammation inside sinusoids and no sinusoidal dilatation and inflammation. Furthermore, there is no irregularity in Remak bundles. Likewise, no accumulation of lymphocytes is detected.

The results obtained from staining through TUNEL procedure revealed that STZ-induced diabetic rats fed with walnut oil fortified with medium and high concentrations of β-sitosterol had very few apoptotic cells compared to control rats and rats receiving walnut oil without or with low concentration of β-sitosterol (Figure 6).

4. Discussion
Diabetes mellitus is a chronic disorder caused by defects in production, secretion, or function of insulin (Shaw, Sicree, & Zimmet, 2010). The key factor in outbreak of diabetes mellitus is
Figure 4. Microscopy of liver samples in diabetic rats receiving gavage of walnut oil with medium dose of β-sitosterol; (a) H&E (20 µm); (b) H&E (100 µm); (c) trichrome (20 µm); (d) trichrome (100 µm).

Figure 5. Microscopy of liver samples in diabetic rats receiving gavage of walnut oil with high dose of β-sitosterol; (a) H&E (20 µm); (b) H&E (100 µm); (c) trichrome (20 µm); (d) trichrome (100 µm).
malfunction of glucose-regulating endocrine organs or of genes involved in metabolism and transport of carbohydrates, proteins and lipids (Fatima, Rajasekhar, & Kumar, 2010). Chronic studies showed that hepatic disorders are the main cause of fatality in diabetic patients (Jin, Gu, Yu, & Li, 2005), and therefore, finding solutions to stop or reduce these disorders could be a promising way to save lives in diabetes mellitus. One way to do so is the modification of eating habits (Riediger et al., 2008).

Some plants, such as walnut, contain very low concentration of saturated fatty acids (SFA) but they have high levels of mono- and polyunsaturated fatty acids (MUFA and PUFA, respectively). Furthermore, they have high concentrations of tocopherols, phytosterols, polyphenolic antioxidants, and fiber (Bolling, McKay, & Blumberg, 2010). Diets rich in walnut could improve lipid condition in diabetes mellitus (Wu et al., 2010). The walnut oil extracted in the present study contained high levels of PUFAs and tocopherols and did not have cholesterol. It also contained high level of β-sitosterol, which indicates high nutritional value of the extracted oil.

The results of the present study revealed that fortification of the extracted oil with β-sitosterol improved liver tissue condition in STZ-induced diabetic rats. Fink et al. (2014) stated that quality and quantity of lipids in diets have substantial influence on liver lipid metabolites and blood
circulation. They also mentioned that high absorption of walnut oil resulted in lower triglycerides in liver while triglycerides in blood serum in fasting state increased in fat rats. They found that lower triglycerides in liver was concomitant with a significant change in fatty acids in liver and reduction in Stearoyl-CoA Desaturase (SCD) activity index, as well as yielding normal Δ6-Δ3 in liver. Histological analysis also showed that fortification of walnut oil with β-sitosterol resulted in considerable decrease in inflammation around central vein and inside sinusoids and minimized irregularities in Remak bundles and accumulation of lymphocytes in STZ-induced diabetic rats.

Treatment with β-sitosterol results in increased insulin in fasting state. Furthermore, β-sitosterol improves the results of oral glucose test and increases insulin secretion induced by glucose, which is similar to the effect of Glibenclamide, a hyperglycemic antidiabetic drug. Gupta, Sharma, Dobhal, Sharma, and Gupta (2011) showed that treating diabetic rats with β-sitosterol prevented the development of diabetes mellitus and reduced its adverse effects. They further stated that β-sitosterol increased glucose adsorption in adipocytes and stimulated adipogenesis in differentiating preadipocytes. In contrast, β-sitosterol induces lipolysis in adipocytes, which is not ameliorated with insulin and simultaneous incubation with epinephrine. Like insulin, β-sitosterol downregulates GLUT4 expression; however, it is to be determined whether increased glucose adsorption by β-sitosterol in adipocytes is related to GLUT4. Moreover, it should be determined whether lipolysis is related to downregulation of Akt and PI3K. Although β-sitosterol is considered an important potential factor in diabetes mellitus due to its effects on regulation of glucose adsorption, adipogenesis, and lipolysis in adipocytes (Chai et al., 2011), it should be found whether β-sitosterol has any role in insulin sensitivity and glucagon secretion (Bin Sayeed, Rezaul Karim, Sharmin, & Morshed, 2016).

Histopathological results on the diabetic rats revealed that there was inflammation in lobules of liver so that accumulation and infiltration of monocytes and proliferation of Kupffer cells, as well as separation of Kupffer cells from sinusoid membranes were detected in liver tissue. Furthermore, there were accumulation of Kupffer cells and monocytes around the central vein. The liver tissues in this study showed irregularities in Remak bundles and extension of central vein in lobule of liver in diabetic rats. These histopathological changes in liver caused by diabetes mellitus were reported in previous studies (e.g. Mahmoud & Sakr, 2013). It seems that walnut oil enriched with β-sitosterol prevents pathologic changes in diabetic rats so that there was no accumulation of monocytes and proliferation of Kupffer cells around central vein in lobules in livers of diabetic rats receiving enriched walnut oil.

5. Conclusion
The present study aimed to determine protective effect of β-sitosterol in the oil extracted from walnut through cold press method in STZ-induced diabetic rats. The results of this study showed that fortification of walnut oil, especially at medium and high concentrations, improved liver tissue by reducing inflammation around central vein and inside sinusoids and preventing irregularities in Remak bundles. Moreover, fortification of walnut oil with inflammation of lymphocytes considerably decreased inflammation of lymphocytes and prevented the formation of apoptotic cells. Therefore, use of walnut oil, especially the one fortified with β-sitosterol, is recommended in diets to prevent the consequences of diabetes mellitus and improve hepatic health.
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