Vol. 17, No. 3, Autumn, 2021, 59-67 *Original Article*
DOI: 10.22055/IVJ.2021.278447.2360
DOR: 20.1001.1.17356873.1400.17.3.6.1
Journal Homepage: http://www.ivj.ir/

The effect of *Carum carvil* oil on the level of heat shock proteins 70 and 72 in the liver and skeletal muscle tissues in diabetic rats

Faranak Moradi[1], Nasrin Kazemipour[2], Foad Noorbakhsh[3] and Saeed Nazifi[4*]

[1] MSc Student of Biochemistry, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
[2] Associate Professor, Department of Basic Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
[3] Assistant Professor, Department of Basic Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
[4] Professor, Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

Received: 25.03.2021 Accepted: 31.07.2021

Abstract

The metabolic abnormalities of diabetes lead to superoxide overproduction in vessels and myocardium resulting in diabetes complications. *Carum carvi* oil probably reduces oxidative stress and increases heat shock protein by hypoglycemic, hypolipidemic, and antioxidant effects. We aimed to study the effect of *Carum carvil* oil on HSP in the liver and skeletal muscle in diabetic rats. 50 male Sprague Dawley rats were randomly divided into 5 groups (control, drug control, drug carrier negative control, negative control, and treatment). Diabetes was induced in the negative controls and treatment groups with streptozotocin (40mg/kg body weight, single dose, IP). Control and negative control groups did not receive any therapy but treatment and drug control rats were gavaged with 10 mg/kg of *Carum carvil* oil daily during 30 days of the study. The weight of rats was measured on 3,7,21,30 days and fasting blood sugar was measured on 3 and 30 days. The liver and skeletal muscle tissues were removed at the end of the experiment. The level of HSP 70 and 72 was determined by an ELISA kit. The findings of HSP measurement demonstrated an increase in level of these protein in the treatment group but in the drug carrier negative control and negative control groups. HSP was decreased significantly due to the injection of STZ. Our results showed that the compounds in this oil (*Carum carvi*) can increase the concentration of HSP 70 and 72 in the liver and skeletal muscle tissues.

Key words: Diabetes mellitus, *Carum carvil* oil, Heat shock proteins 72 and 70, Liver, Skeletal muscle

Introduction

Diabetes mellitus is one of the most common metabolic disorders that is increasing rapidly, therefore, appropriate proceeding to prevent, control and treat is necessary (American Diabetes Association 2018). Diabetes is predominantly associated with chronic hyperglycaemia and glucosuria, and as a result of the disease progression, anatomical and metabolic disorders may occur. In diabetes, hyperglycemia and increased glycosylated proteins can be the source of free radicals production. Nowadays, oxidative stress is considered as one of the effective mechanisms in diabetes mellitus that affects the metabolism of carbohydrates, lipids, and proteins (Borcea et al. 1999). Lipid metabolism disorder lead into injury of various organs such as kidney, eye, nerve, heart and blood vessels (Uusitupa et al.

[*] **Corresponding Author**: Saeed Nazifi, Clinical Pathology Division, Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran, E-mail: nazifi@shirazu.ac.ir

1990). In diabetes, the defense mechanisms of damaged tissues and tissue vulnerability to stress increases (Borcea et al. 1999). In physiological abnormalities, the level of HSP expression is reduced (Craig and Gross 1991). However, their amount increases in response to stress-inducing stimuli such as elevated temperature, oxidative stress and infection (Su et al. 2006; Njemini et al. 2004; Kilgore et al. 1998; Delogu et al. 1997; Njemini, et al. 2004). The HSP is essential for the natural regeneration of skin ulcers, and in diabetic patients, ulcers usually heal later due to the impaired HSP performance (Atalay et al. 2009). Although HSP are mainly intracellular molecules, they can be moved and placed in the cell membrane when over-expressed in response to stress, and identified by the immune system, and then act as an autoantigen. The HSP can also be released from the cells into the circulation and had biological activity. Important HSPs based on molecular weight include: HSP10, HSP20, HSP25/27, HSP60, HSP70, HSP90, HSP100 (Madrigal-Matute et al. 2011; Bellini et al. 2017). In a study on type 2 diabetic patient, the expression of HSP60 was declined significantly in the myocardium and adipose tissue and this reduction may increase apoptosis. In muscular biopsy of type 2 diabetic patients compared with healthy people, the levels of mRNA of induced isoforms of HSP70 and 72 were significantly reduced (Kurucz et al. 2002). Njemini et al. in 2004 showed that there was a positive and direct relationship between the serum level of HSP 70 and inflammatory markers such as tumor necrosis factor, C reactive protein, fibrinogen and the number of blood monocytes (Njemini et al. 2004). Strokov et al. (2000) in type 2 diabetes rats, showed that the expression of HSP 70 in skeletal muscle was decrease due to insulin resistance (Strokov et al. 2000). It is said by Bruce et al. (2003) that insulin injections in diabetic rats increase the expression of HSP 70. Hunter-Lavin et al. (2004) reported that administration of folic acid supplementation significantly reduced the serum level of HSP 70 in type 2 diabetic patients (Hunter-Lavin et al. 2004). Serum HSP 70 could be a sensitive indicator for the severity of type 2 diabetes (Atalay et al. 2004). The expression of HSP 72 in the heart, liver, skeletal muscle, and kidney is limited in diabetic patients. HSP 47can bond to collagen type I to III, and increase the synthesis of type I collagen in subcutaneous tissues, and therefore are effective in diabetic wound healing (Macdonald and Bächinger 2001). HSP and insulin control can reduce amyloid congestion in the brain (Hooper et al. 2014). Many of the drugs used to treat diabetes have side effects and problems for the patient. So over the last few decades, the use of plants has increased for the treatment of various diseases, especially diabetes (Porte and Kahn 1991).Muda et al. in 1997 indicated that *Carum carvil* is effective in controlling type 2 diabetes (Modu et al. 1997).This plant has hypoglycemic and hypolipidemic (Ene et al. 2006; Eddouks et al. 2004; Iacobellis et al. 2005; Haidari et al. 2011), antimicrobial (Simic et al. 2008), anti-tumor (Kumar and Singh 2006), and antioxidant (Zheng et al. 1992) properties. The oil and different extracts of *Carum carvil* contain phenolic compounds with significant antioxidant activity (Johri 2011). and also has the ability to suppress hydroxyl and lipoperoxide radicals. As we are aware, there has been no study on the effect of *Carum carvil* oil on HSPs in liver and skeletal muscle in diabetic rats and, thus, the present study was conducted.

Materials and Methods

Animals

A total of 50 male Sprague–Dawley rats with 250±25 gr mean weight were housed in animal room of the veterinary medicine school of shiraz university at 22±2 °C with a 12:12 light–dark cycle and 50% moisture. they were given access to water and food *ad libitum*. The study protocol was reviewed

and approved by the Ethical Research Committee of Shiraz University (IACUC no: 4687/63).

Experimental protocol

The animals were randomly divided into 5 groups: control: 5 rats without any intervention; drug control:5 rats receiving 10 mg/kg *Carum carvil* oil; drug carrier negative control: 10 diabetic rats receiving sunflower oil 0.1 ml /100 g BW; negative control:15 diabetic rats receiving water and food and treatment:15 diabetic rats receiving 10 mg/kg BW *Carum carvil* oil dissolved in 0.1 ml sunflower oil.

Plant material

Preparation of *Carum carvil*

We used a cold pressing machine to make *Carum carvil* oil, and this process was carried out on seeds of *Carum carvil* which was obtained from 1kg of *Carum carvil*, 75 g oil.

Induction of diabetes

STZ powder (Sigma USA,40 mg/kg) was dissolved in 1 ml of 0.1M citrate buffer (pH=4.5); 0.1 ml/100 g BW of the solution was injected IP to induce diabetes. In non-diabetic rat, in order to remove the interfering factors, citrate buffer0.1 ml / 100 gr BW was injected.

Blood glucose is reduced sharply due to the destruction of beta cells, and can lead to animal death, 12 hours after STZ injection. As a result, after 12 hours of injection, the drinking cups were filled with 5% glucose solution for 12 hours.

Blood glucose level of all was measured by the glucometer, 72 hours after STZ injection. Rats with a blood glucose level above 250 mg/dl were considered as diabetic rats (Adeyemi et al. 2014).

Treatment with *Carum carvil* **oil**

The diabetic rats were treated with *Carum carvil* oil. Since calculated dosage based on mg/kg BW was very low and was not appropriate for gavage, 0.1 ml sunflower oil added to 10 mg *Carum carvil* oil. This mixture 0.1 ml/100 g BW administered during 30 days following diabetes induction. In order to eliminate the effect of sunflower oil, the drug carrier negative control received 0.1 ml of sun flower oil /100 g BW.

Determination of weight and blood glucose

At the end of each week, the weight of all rats was measured using a digital scale. Blood glucose was measured on the first, third and final days of the experiment.

Preparation of tissue samples

At the end of the experiment, the rats euthanized by ether and the extensor digitorun longus (EDL) and the soleus (SOL) muscles and the liver were excised, frozen in liquid nitrogen, and stored in −80°C until HSP determination.

Preparation of tissue extracts

Five-hundred (500) mg of the liver and 300 mg of the skeletal muscle tissues respectively were crushed and sodium phosphate buffer (0.1 M, pH=7.4) was added 5 times their weight and then homogenized with homogenizer The suspensions were centrifuged for 15 min at 15000 g and 4°C. The supernatants were transferred to 0.5 ml micro tubes and placed at -4°C.

Determination of protein concentration

The protein content of tissues was determined by Bradford method.

Determination of HSP

Tissue levels of HSP70 and HSP72 were measured by a quantitative sandwich enzyme immunoassay using commercial rat-specific kits (Shanghai Crystal Day Biotech, Shanghai, China). The sensitivity of HSP70 kit was 0.021 ng/ml. The intra-assay precision and inter-assay precision of HSP 70 kit were CV< 8% and CV< 10%, respectively. The sensitivity of HSP72 kit

was 0.023 ng/ml. The intra-assay precision and inter-assay precision of HSP72 kit were CV < 8% and CV < 10%, respectively.

Statistical analysis

Statistical analysis was accomplished using SPSS (Version 16.0; SPSS, Chicago, USA). Analysis of variance (ANOVA) followed by Tukey's multiple range tests was used to compare measured factors in all groups and determine statistical significance. P<0.05 was considered as statistically significant.

Results

Changes of weight

The effects of *Carum carvil* oil on the weight of the rats have been shown in Table 1. Three days after STZ injection, weight loss was observed in the diabetic groups. Seven days after treatment with *Carum carvil* oil, weight loss in drug carrier diabetic, diabetic control and treatment groups continued, and there was a significant difference between the weight of control and diabetic groups. This weight loss continued after fourteen days of treatment with *Carum carvil*, except the control groups and treatment. On the 21st day weight loss was observed in the treatment group, however in other groups were observed weight gain. At the end of the last week of weighing, weight gain was recorded in the drug carrier and treatment group, but weight reduced in negative control group.

Table 1. Mean±SEM of weight (gr) in the different groups in consecutive weeks.

Sampling group	First week	Second week	Third week	Fourth week
C	275±18.70^{a}	290.6±20.34^{a}	316.2±25.58^{a}	318.2±26.72^{a}
DC	287.8±10.68^{a}	308.2±17.10^{a}	324.8±21.23^{a}	335.6±18.70^{a}
DCNC	211.8±16.11^{b}	199.6±28.59^{b}	203.6±27.7^{b}	210.25±19.77^{b}
NC	208.22±12.08^{b}	185±17.01^{b}	193.17±13.73^{b}	189.83±13.34^{b}
T	202.62±13.73^{b}	222.68±10.85^{b}	186.83±10.57^{b}	204.2±12.93^{b}

Different superscript letters within each column show significant differences among experimental groups in each week (ANOVA, $p < 0.05$). C, healthy rats without treatment; DC, healthy rats were treated by *Carum carvil* oil; DCNC, diabetic rats and treated by sunflower oil; NC, diabetic rats and non-treated; T, diabetic rats and treated by *Carum carvil* oil.

HSP 70 of liver and muscle tissues

The results showed (Table 2) that concentration of Heat Shock Proteins 70 of skeletal muscle and liver tissues significantly decreased in diabetic rats, however the level of this protein was increased after treatment with *Carum carvil* oil in diabetic rats and was near to control group.

HSP 72 liver and muscle tissues

Based on the results of ELISA analysis, treatment with *Carum carvil* oil is same as HSP70 caused an increase in HSP72of liver and skeletal muscle tissues diabetic rats (Table 3). Induction of diabetes decreased the level of this protein in the negative control group. Gavages with *sunflower* oil had no obvious effect on HSP72 in the liver and skeletal muscle tissues of drug carrier negative control group.

Table 2. Mean± SEM concentration (ngr/dl) of HSP70 liver and muscle tissues.

Sampling group	HSP70 muscle	HSP70 liver
C	0.954±0.022^{a}	0.919±0.017^{a}
DC	1.054±0.060^{b}	1.002±0.051^{b}
DCNC	0.752±0.021^{c}	0.722±0.022^{c}
NC	0.744±0.023^{c}	0.713±0.021^{c}
T	0.814±0.023^{d}	0.790±0.023^{d}

Different superscript letters within each column show significant differences among experimental groups (ANOVA, $p < 0.05$). C, healthy rats without treatment; DC, healthy rats were treated by *Carum carvil* oil; DCNC, diabetic rats and treated by sunflower oil; NC, diabetic rats and non-treated; T, diabetic rats and treated by *Carum carvil* oil.

Table 3. Mean± SEM concentration (ngr/dl) of HSP 72 liver and muscle tissues.

Sampling group	HSP72 liver	HSP72 muscle
C	1.244±0.059[a]	1.539±0.060[a]
DC	1.355±0.058[b]	1.566±0.067[a]
DCNC	0.970±0.013[c]	1.229±0.351[b]
NC	0.977±0.023[c]	1.209±0.04[b]
T	1.104±0.039[d]	1.349±0.031[c]

Different superscript letters within each column show significant differences among experimental groups (ANOVA, $p < 0.05$). C, healthy rats without treatment; DC, healthy rats were treated by *Carum carvil* oil; DCNC, diabetic rats and treated by sunflower oil; NC, diabetic rats and non-treated; T, diabetic rats and treated by *Carum carvil* oil.

Discussion

Weight changes

No significant difference was observed between the weights of different groups on day 0. Three days after STZ injection, in treatment, negative control and drug carrier negative control groups, significant weight loss was observed compared to control. Many studies have argued that STZ injection causes muscle and weight loss in animals (Kim et al. 2014; Furman 2015). In diabetic groups, due to insulin deficiency, the body is not able to consume glucose, resulting in weight loss. Gluconeogenesis that occurs in diabetes can decrease lipid synthesis and fat storage. During gluconeogenesis, muscle protein is used as a source of glucose, and muscle loss is one of the causes of slimming in the animal (Shirwaikar et al. 2004). Seven days after treatment with *Carum carvil* oil, weight loss in drug carrier negative control, negative control, and treatment groups continued, and a significant difference between the weights of different groups was noticed. The weight loss after 14 days of treatment with *Carum carvil* oil continued except for drug control and control groups that weighed up, and there was still a significant difference between the weights of different groups. On the 21st day of the course of treatment, weight loss was observed and there was a significant difference between the weights of different groups. At the end of the last week, weight gain was observed in treatment group and the weight of the different groups was significantly different. However, there was no significant difference between the negative control, drug carrier negative control, and treatment groups as in all stages of the trial. But there was a difference between the negative control, treatment and drug carrier negative control groups with the control and drug control groups. The results are roughly equivalent to the results of previous studies on the weight gain of rat receiving the specified doses of *Carum carvil* oil. It prevents body muscle loss and gluconeogenesis, and thus increases body weight in diabetic rats (Ene et al. 2006).

Changes in the level of heat shock proteins

The amount of HSP70 and 72 in skeletal and liver muscles in negative control and drug carrier negative control groups had a significant decrease compared to the other groups. This significant decrease reflects the role of streptozocin in reducing the amount of HSP. Bitar et al. (1999) were the first researchers who discovered this effect of streptozocin, and suggested that reducing the expression of HSP70 in the tissues of diabetic animals is due to streptozocin. It can cause DNA alkalization and thus damage to the hereditary substance. In this condition, the ATP synthesis rate decreases due to NAD + depletion, and since the HSP 70 and 72 require ATP for their chaperone performance, their activity reduce by the injection of streptozocin (Kiang and Tsokos 1998).

HSPs are one of the most important and abundant proteins in the body whose expression in the normal body condition is normally low. In abnormal conditions such as stress, apoptosis, necrosis and any cell damage, their expression increases which is consistent with their protective, antioxidant, and anti-inflammatory properties (Atalay et

al. 2009). This protein family as chaperone molecules plays an important role in facilitating the folding of cellular proteins, preventing protein accumulation, repairing damaged proteins and their proper transfer, and thus increases the life span of the cell (Awasthi and Wagner 2005). In diabetes, a wide range of metabolic and functional disorders occurs. Due to stressful conditions in the body, the amount of free radicals and oxidative agent's increases, these harmful compounds damage the body's protein and lipid structures. On the other hand, insulin production is low due to the damage to the pancreatic beta cells, or despite the presence of enough insulin, the cells are not able to use insulin. In these conditions, an increase in blood glucose and the absence of treatment of Keto-acidosis occur (Hooper and Hooper 2009). Oglesbee et al. (2005) showed that diabetic ketoacidosis increased the level of extracellular HSP70 which was due to the systemic response of the body to stress, and its extracellular amount increased due to changes in blood glucose in diabetic patients. Reducing the activity of chaperones in diabetic patients is one of the main causes of complications of diabetes (Hooper et al. 2014). In fact, the vital role of HSPs in diabetes is due to their ability to cope with the degeneration of tissue proteins and facilitate cellular repair and defense mechanisms. Reducing glycation and increasing protein stability can dramatically reduce the risk of diabetes complications (Jafarnejad et al. 2008). Although the amount of HSPs increases under stressful conditions, it decreases in diabetes, and here's a paradox. In the past, it has been anticipated that another possible reason for lowering HSP70 concentrations in patients with diabetes is that after glycation, this protein is not known by monoclonal antibodies, and thus, the diagnostic kit is not capable of detecting and determining the concentration of this protein (Baynes 1991). Because of high blood glucose in diabetes, HSPs are bound to glucose and because of glycation, they lose their chaperone characteristics and this causes the HSPs to not protect the cell structure well. Consequently, the disease progresses. On the other hand, Kurucz et al. (2002) and Bruce et al. (2003) demonstrated that the expression of HSP 70 in human and animals´ tissues with type 2 diabetes and insulin resistance significantly decreased. Borcea et al. (1999) Showed that insulin injections in diabetic rats increased the expression of HSP 70. Patti et al. (2003) also found that the expression of HSP 70 in skeletal muscle in diabetic patients was lower than that of healthy subjects and had an inverse relationship with fasting blood glucose levels. Studies have been done on the effect of plants on HSP levels and the reduction of complications of diabetes. A human study showed that stevia plant had anti-diabetic properties, including the resurgence of damaged pancreatic beta cells, and compared to glibenclamide, it has no side effect (Misra et al. 2011). Barberry and saffron can reduce some of the complications of diabetes by reducing anti-HSP antibodies (Shemshian, et al. 2014; Zilaee, et al. 2014). The study of the amount of HSP in the liver and skeletal muscle of diabetic animals using *Carum carvil* oil indicates that their amount was significantly increased, but their levels were still lower than those of healthy ones. This finding suggests that *Carum carvil* oil can increase the amount of HSP70 and 72 in the liver and skeletal muscle tissue of diabetic rats. This was probably due to the good combinations of black cumin, as major combinations of the *Carum carvil* have antioxidant properties and decrease blood glucose levels (Johri 2011; Najda et al. 2008). Hence, presumably *Carum carvil* can reduce damage to heat shock proteins by reducing harmful oxidants. It also reduces blood sugar. In diabetes, HSPs bind to glucose and form a passive structure that loses its chaperone property (Jafarnejad et al. 2008). Cumin reduces blood glucose, thus prevents glycation of HSPs and avoids

lowering their chaperone activity. Except for cumin, research has been carried out on barberry and saffron plant. These plants reduce some of the complications of diabetes by reducing anti-HSP antibodies (Shemshian, et al. 2014; Zilaee, et al. 2014).

Our findings indicated that the amount of HSP70 and 72 in the liver and skeletal muscle tissues of the diabetic rats has decreased, but in the treatment group that daily consumed *Carum carvil* oil, not only no decrease in the amount of HSPs was observed, but also there was an increase in this family of proteins in the mentioned tissues.

Acknowledgments

The authors would like to thank the Research Council of Shiraz University and School of Veterinary Medicine, Shiraz University for the technical assistance.

Funding information

This study was supported by School of Veterinary Medicine, Shiraz University (grant number: 71-GR-VT-5) and Research Council of Shiraz University.

Conflict of interest

The authors declare that they have no conflict of interest.

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Received: 25.03.2021
Accepted: 31.07.2021

DOI: 10.22055/IVJ.2021.278447.2360
DOR: 20.1001.1.17356873.1400.17.3.6.1

اثر روغن زیره سیاه بر روی غلظت پروتئین‌های شوک گرمائی ۷۰ و ۷۲ در بافت‌های کبد و عضلات اسکلتی موش‌های صحرائی دیابتی

فرانک مرادی[۱]، نسرین کاظمی‌پور[۲]، فواد نوربخش[۳] و سعید نظیفی[۴*]

[۱] دانشجوی فوق لیسانس بیوشیمی، دانشکده دامپزشکی، دانشگاه شیراز، شیراز، ایران
[۲] دانشیار گروه علوم پایه، دانشکده دامپزشکی، دانشگاه شیراز، شیراز، ایران
[۳] استادیار گروه علوم پایه، دانشکده دامپزشکی، دانشگاه شیراز، شیراز، ایران
[۴] استاد گروه علوم درمانگاهی،دانشکده دامپزشکی، دانشگاه شیراز، شیراز، ایران

دریافت: ۱۴۰۰/۱/۵ پذیرش: ۱۴۰۰/۵/۹

چکیده

ناهنجاری‌های متابولیکی دیابت منجر به تولید بیش از حد سوپراکسید در عروق و میوکارد قلب و در نتیجه عوارض دیابت می‌شود. روغن زیره سیاه احتمالاً با اثرات افت قند خون، کاهش چربی خون و آنتی‌اکسیدانی باعث کاهش استرس اکسیداتیو و افزایش پروتئین شوک گرمایی می‌شود. هدف ما مطالعه‌ی اثرات روغن زیره‌ی سیاه بر پروتئین‌های شوک گرمائی در کبد و عضلات اسکلتی موش‌های دیابتی است. پنجاه سر موش صحرائی اسپراگ داولی نر به طور تصادفی به پنج گروه (شاهد، کنترل دارو، کنترل منفی حامل دارو، کنترل منفی و درمان) تقسیم شدند. دیابت در گروه‌های کنترل منفی و درمان با استرپتوزوتوسین ایجاد شد (۴۰ میلی‌گرم در کیلوگرم وزن بدن، تک دوز و داخل صفاقی). گروه‌های شاهد و کنترل منفی هیچ درمانی دریافت نکردند اما موش‌های صحرایی تحت درمان و کنترل دارو طی ۳۰ روز دوره‌ی مطالعه، روزانه با ۱۰ میلی‌گرم در کیلوگرم روغن زیره سیاه گاواژ شدند. وزن موش‌ها در روزهای ۳، ۷، ۲۱ و ۳۰ و قند خون ناشتا در روزهای ۳ و ۳۰ اندازه‌گیری شدند. در انتهای دوره‌ی آزمایش، بافت‌های کبد و عضلات اسکلتی جدا و برداشته شدند. میزان پروتئین‌های شوک گرمائی ۷۰ و ۷۲ با کیت‌های الیزا اندازه‌گیری شدند. نتایج اندازه‌گیری پروتئین‌های شوک گرمائی نشان دهنده افزایش سطح این پروتئین‌ها در گروه درمان بود. اما پروتئین‌های شوک گرمائی در گروه‌های کنترل منفی و کنترل منفی حامل دارو به دلیل تزریق استرپتوزوسین به طور معنی‌دار و قابل توجهی کاهش یافتند. نتایج ما نشان داد که ترکیبات موجود در این روغن (زیره سیاه) می‌تواند غلظت پروتئین‌های شوک گرمائی ۷۰ و ۷۲ را در بافت‌های کبد و عضلات اسکلتی افزایش دهد.

کلمات کلیدی: دیابت قندی، روغن زیره سیاه، پروتئین‌های شوک گرمائی ۷۰ و ۷۲، کبد، عضله اسکلتی

*نویسنده مسئول : سعید نظیفی، استاد گروه علوم درمانگاهی، دانشکده دامپزشکی، دانشگاه شیراز، شیراز، ایران

E-mail: nazifi@shirazu.ac.ir