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THE EFFECT OF AQUEOUS EXTRACT OF ORIGANUM VULGARE LEAVES ON ADIPONECTIN AND C-CBL ASSOCIATED PROTEIN LEVELS IN ADIPOSE TISSUE OF STREPTOZOTOCIN-NICOTINAMIDE INDUCED DIABETIC RATS

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ABSTRACT

Increased c-Cbl associated protein (CAP) and adiponectin level in adipocytes can facilitate insulin dependent glucose uptake in adipocytes and skeletal myocytes. Peroxisome proliferator activated receptor γ (PPARγ) can increase the level of these proteins in adipocytes. Aqueous extract of Origanum Vulgare (OV) leaves contains Biochanin A, which is known as a PPARγ agonist. This study was designed to investigate the effect of aqueous extract of OV leaves on adiponectin and CAP levels in adipose tissue of diabetic rats. Adult male rats used as animal model in the present study. Animals were made diabetic by single intraperitoneal injection of Streptozotocin-Nicotinamide and treated by 20mg/kg of aqueous extract of OV leaves for 28 days. Fasting blood glucose (FBG) and insulin levels were tested. Furthermore, adiponectin and CAP levels in adipose tissue were examined by immunoblotting analysis. The presence of Biochanin A in aqueous extract of OV leaves was determined by high pressure liquid chromatography (HPLC) assay. In treated animals, FBG level reduced and insulin resistance index improved significantly compared with untreated diabetic rats ($p<0.01$). CAP and adiponectin levels in adipose tissue of treated animals increased significantly compared with diabetic and healthy control animals ($p<0.01$). HPLC analysis confirmed presence of Biochanin A in aqueous extract of OV leaves. Consumption of aqueous extract of OV leaves can increase adiponectin and CAP levels in adipocytes, which can lead to increased insulin dependent glucose uptake in adipocytes and skeletal myocytes.

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Introduction

Nowadays, high calorie diet and sedentary lifestyle are two of the most important dilemmas in developed countries. Obviously, they can lead to overweight and obesity, which are the main risk factors of metabolic syndrome and subsequently type 2 diabetes mellitus (T2DM). At the moment, more than 400 million people are affected by T2DM all over the world. In

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the other words, about 4% of world population is type 2 diabetic patients [1;2]. Due to high prevalence of this chronic disease, special look to prevention and treatment of T2DM are needed.

During the last decades, adipose tissue has been considered more than the storage depot for lipids. It plays an important role in carbohydrate and lipid metabolism. Actually, proteins which derived from adipocytes, can affect the carbohydrate and lipid metabolism in whole body [3]. Peroxisome proliferator activated receptor γ (PPARγ), a ligand inducible transcription factor which belongs to nuclear hormone receptor super family, mainly exists in adipocytes. Several studies have reported that PPARγ has crucial role in glucose homeostasis [4;5].

Adiponectin or adipocyte complement related protein of 30KDa (Acrp30) is a plasma protein which derived from adipocytes. Its receptors exist on hepatocytes and skeletal muscle cells [6;7]. Adiponectin stimulates glucose uptake via insulin dependent signaling pathway in skeletal muscle cells; it means that increased plasma level of adiponectin can be useful in reducing plasma glucose. It should be added that there is a PPARγ-responsive element (PPRE) in promoter region of adiponectin gene [8;9]. CAP (c-Cbl associated protein) is an adaptor protein which can help glucose uptake in an alternate insulin dependent signaling pathway in adipocytes and skeletal muscle cells. Previous studies indicated a PPRE region in promoter of CAP gene. In the other words, activation of PPARγ can lead to increased expression of CAP and adiponectin genes; subsequently CAP and adiponectin levels [10;11].According to above, it can be deduced that, PPARγ agonists can be effective in reducing the blood glucose.

During the last years researchers were trying to discover the new plants with therapeutic potentials. Origanum Vulgare (OV), belongs to Lamiceae family, has been used traditionally in diabetic patients. OV usually grows in temperate region of southwestern Europe and Mediterranean regions. Its characteristics are 20-80 cm tall, opposite leaves with 1-4 cm long, purple flowers with 3-4 mm long, preferred pH range between 6 and 8.

Researchers have indicated that aqueous extract of OV leaves contains Biochanin A (BCA), which is known as PPARγ agonist. A class of flavonoid phenolic compounds is isoflavonoids. They are known as phytoestrogenes; because they can activate estrogen receptors. Biochanin A is an O-methylated isoflavone and is found in soy bean and red clover. In previous studies, in addition to estrogen-like effect of BCA, its antidiabetic effect was illustrated. As a result, it can be stated that antidiabetic effects of aqueous extract of OV leaves can be due to presence of BCA [5;12-14].

The aim of present study was to evaluate the effect of aqueous extract of OV leaves supplementation on adiponectin and CAP levels in adipose tissue of Streptozotocin-Nicotinamide (STZ-NCD) induced diabetic rats. Also, body weight (BW), fasting blood glucose (FBG) and HOMA index was investigated. At the end, existence of BCA in aqueous extract of OV leaves was determined using high performance liquid chromatography (HPLC) assay.

Methods

Preparation of aqueous extract of Origanum Vulgare leaves

Plant materials (OV leaves) were collected from populations growing in Hamadan province, west of Iran. A voucher specimen 231 was dedicated in Department of Pharmacognosy, School of Pharmacy, Hamadan University of Medical Sciences, Hamadan, Iran. The leaves were dried at room temperature. Thereafter 20g of powdered leaves was boiled with 2000ml deionized water. In the next step, aqueous extract was filtrated by using a 0.2 mm Millipore filter. At the end, filtrate was freeze dried and stored at room temperature [12].

Animals

Thirty adult male rats, from Wistar strain and BW of 250-300 g, were purchased from central animal house of Hamadan University of Medical Sciences, Hamadan, Iran. Rats were maintained in 12-hour light/dark periods and temperature of 21-23˚C and relative humidity of 55%±10%. Animal's nourishment was performed by using standard chow and water [1;14;15]. The research protocol was designed according to the guideline which was approved by Ethics committee of Hamadan University of Medical Sciences.

Induction of Diabetes mellitus in Rats

Following the overnight fasting, the animals were made diabetic by a single intraperitoneal injection of 120mg/kg Nicotinamide (NCD, N3376, Sigma). In the next step, after 15 minutes, Streptozotocin was injected intraperitoneally at dose of 60mg/kg (STZ, S130, Sigma). For dissolving the Nicotinamide, normal physiological saline, and for dissolving Streptozotocine, 0.1 M citrate buffer (pH=4.7) were used. Seventy two hours after performing this protocol, blood samples were taken from tail vein of rats and FBG level was tested by using a glucometer (Glucocard, 01, Arkray, Japan). The animals which displayed FBG level higher than 126mg/dl were diagnosed diabetic. Unlike type 1 diabetes mellitus, in type 2 diabetes mellitus, pancreatic beta cells are not destroyed completely and insulin secretion usually continued; so that, to induce type 2 diabetes mellitus, NCD is used with STZ for preventing the complete destruction of pancreatic beta cells [14;16-18].

Study design

Seven days after induction of diabetes, twenty diabetic rats were divided into two groups; a group which received 20mg/kg/day aqueous extract of OV leaves and a control group. Also there was a healthy control group (n=10). The prepared aqueous extract was dissolved in deionized water and was used orally by using a gavage syringe; control groups received only deionized water by a gavage syringe. Treatment was continued for 28 days. BW and FBG were measured at the

beginning and the end of the treatment. After the end of the treatment, animals were anesthetized by using ether, then adipose tissue and blood samples were collected. Adipose tissue were immediately transferred into -80˚C freezer. Serum, for measuring insulin level, was separated from blood samples and stored at -20˚C [3;17;19].

Serum insulin level testing

For evaluating the insulin level in serum a rat insulin ELISA kit (ERINS, Thermo scientific) was used. Insulin resistance index (HOMA-IR) was measured according to this formula: Insulin (µU/ml)×FBG(mg/dl)/405 [1;20].

Protein extraction from adipose tissue

For extracting intra cellular proteins from adipose tissue, 0.25 g frozen adipose tissue was lysed by using RIPA lysis buffer system (Sc-24948, Santa Cruz). In the next step, homogenates were cleared by centrifugation at 4˚C (13000g, 25min); the particulate matter was removed and the supernatant was collected and kept at -20˚C. Protein concentration was determined by Bicinchononic acid assay (Sc-202389, Santa Cruz)[10].

Analysis of proteins by immunoblotting

Protein extracts of different samples, in equal amounts, were loaded onto 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After separation, the proteins were electroblotted to nitrocellulose membrane. In the next step, non-specific sites were blocked by using 5% skim milk which was dissolved in TBST (0.1% Tween 20 in Tris buffered saline, pH=7.6); this step was performed on a shaker for one hour. Thereafter, for immunoblotting, the membrane was incubated with primary antibodies against β-actin (1/1000, Sc-130657, Santa Cruz), adiponectin (1/500, Sc-25496, Santa Cruz) and CAP (1/500, Sc-25496, Santa Cruz); overnight with agitation at 4˚C. For prevention of non-specific binding of primary antibodies, the membrane was washed in TBST for 30 minutes on a shaker. In next step, the membrane was exposed to horse-radish peroxidase (HRP) conjugate secondary antibodies (1/200, Sc-2005, Santa Cruz) to detect the immunoblots, this step was lasted one hour at room temperature with agitation. After that, another washing protocol, like above, was performed to remove residual secondary antibodies. At the end, for detecting the protein bands, enhanced chemiluminescence (ECL) detection kit (Sc-2048, Santa Cruz) was used. Quantification of bands density was performed by using Image J software [16;21;22].

HPLC assay

To show the presence of BCA in aqueous extract of OV leaves a HPLC system was used. The HPLC system characteristics were as follows: isocratic pump (515; Waters, USA), UV detector (2487; Waters, USA), RP-C18 column (3.9×150 mm, 5µm; Waters, USA). Mobile phase consisted of water (pH=2.7, adjusted by sulfuric acid): acetonitrile (58:42, v/v %). The UV detection wavelength was set at 254 nm. The flow rate was 1ml/min [23]. Aqueous extract of OV leaves was dissolved in mobile phase and Biochanin A (BCA, D2016, Sigma) was dissolved in 75% DMSO (dimethyl sulfoxide).The calibration curve of BCA was linear within the concentration range of 1.56-50 ng/ml and limit of detection was 1.56ng/ml. The concentration of injected aqueous extract of OV leaves was 2mg/ml.

Statistics

The values are presented as Mean±SD. To compare the data between different groups, one-way ANOVA analysis, followed by post hoc Tukey's test was used. P value <0.05 was considered significant.

Results

The influence of aqueous extract of Origanum Vulgare leaves on body weight, FBG and insulin levels of STZ-NCD induced diabetic rats were evaluated in the present study. According to Table 1, BW increased considerably in animals which received 20 mg/kg aqueous extract of OV leaves compared with untreated diabetic rats (p= 0.0009); however, weight gain did not reach to that of healthy animals (p= 0.0008).

It can be inferred from Table 2 that FBG level, in animals which treated by 20mg/kg of OV leaves compared with that of untreated diabetic rats, decreased significantly (p= 0.0009), but it should be noted that FBG level in healthy animals was considerably lower compared with that of treated diabetic rats (p= 0.0009). Insulin level in diabetic animals (treated and untreated) was notably lower compared with that of healthy animals (p=0.0008). As is shown in Table 2, treatment with 20mg/kg of OV leaves improved insulin resistance index or HOMA-IR significantly, as compared with untreated diabetic rats (p=0.0008). At the end, there were notable differences between treated diabetic animals and healthy animals in HOMA-IR (p=0.009).

The influence of aqueous extract of OV leaves on adiponectin level in adipose tissue of STZ-NCD induced diabetic rats was illustrated in Fig.1. As is shown in Fig.1, aqueous extract of OV leaves increased adiponectin level in adipose tissue of diabetic animals significantly compared with that of healthy and untreated diabetic rats (p= 0.0008). According to Fig.2, it can be deduced that CAP level in adipose tissue of animals which received aqueous extract of OV leaves, is notably higher than that of healthy and diabetic control animals (p= 0.0009).

Results of HPLC assay are shown in Fig.3. According to chromatogram (A), which represents HPLC analysis of standard BCA, retention time of BCA was 7.6 minutes. Chromatogram (B), which indicates HPLC analysis of the aqueous extract of OV leaves, had a peak in 7.45 minutes. As shown in Chromatogram C, aqueous extract of OV leaves after adding 25ng/ml (50:50; v/v) of standard BCA showed a clean peak at 7.59 minutes, which is stronger than chromatogram B peak, supporting

the existence of BCA in aqueous extract of OV leaves. According to BCA standard curve, the concentration of BCA in the aqueous extract of OV leaves was 12.5- 30 ng/mg.

Discussion

Consistent with the previous studies, hypoglycemic effect of aqueous extract of OV leaves was confirmed by the results of our study. In the other side, some studies have illustrated that aqueous extract of OV leaves contains BCA. Also it should be considered that PPARγ plays an important role in glucose homeostasis [4;12;24]. As a result, probably, hypoglycemic effect of OV leaves arises from BCA.

According to above, present study was designed to investigate the effect of aqueous extract of OV leaves on two of the proteins in adipose tissue.

In adipose tissue, adiponectin and CAP are the proteins which can facilitate the insulin signaling pathway. Adiponectin is an adipose-derived plasma protein. In skeletal muscle cells, adiponectin facilitates interaction between insulin receptor and insulin receptor substrate (IRS) via an adaptor protein called APPL1. This interaction is the first step in insulin signaling pathway which will lead to insulin dependent glucose uptake [8;25]. CAP is an adaptor protein in adipose tissue which increases insulin dependent glucose uptake in an alternate insulin signaling pathway. This protein facilitates the translocation of GLUT 4 to cell surfaces of adipocytes; which is the last step in insulin dependent glucose uptake [11]. As a result, increased level of adiponectin and CAP in adipose tissue can be effective in insulin dependent glucose uptake in adipocytes and skeletal muscle cells; which is the most important therapeutic target in type 2 diabetic patients.

Wang et al. reported that PPARγ plays an important role in glucose homeostasis [5]. Maeda et al. indicated that there is a PPRE in promoter region of adiponectin gene and PPARγ agonists increase the level of adiponectin in adipocytes [24]. Lin et al. demonstrated a PPRE region in promoter region of CAP gene; in other words, activation of PPARγ leads to increased level of CAP in adipocytes [11]. Mueller et al. illustrated the existence of BCA in aqueous extract of OV leaves [13]. Lemhardi et al. reported that aqueous extract of OV leaves has hypoglycemic effect in diabetic rats [12]. Guo et al. notified that adiponectin and CAP are the proteins which can be useful in insulin resistance improvement [4].

Based on findings of the present study, it can be indicated that presence of BCA in aqueous extract of OV leaves, make it an appropriate agent in lowering the blood glucose in diabetic patiants. Although, it should be added that further studies are needed to suggest Origanum Vulgare as an effective drug in type 2 diabetic patients; the most effective dose and its side effects are important challenges. At the end, it should be considered that our study was the first one which designed to investigate the possible mechanism of hypoglycemic effect of aqueous extract of OV leaves.

Conclusion

Findings of the present study demonstrated that supplementation of aqueous extract of OV leaves in STZ-NCD induced diabetic rats, increased the levels of adiponectin and CAP in adipose tissue. Decreased FBG level and improved insulin resistance should be noted as important findings in this study that shows the useful effect of OV in diabetes. As a conclusion, OV can be a beneficial drug in T2DM, because it can improve insulin dependent glucose uptake, which is the main therapeutic target in type 2 diabetic patients.

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Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Table1. Effect of aqueous extract of Origanum Vulgare leaves on body weight of the studied animals

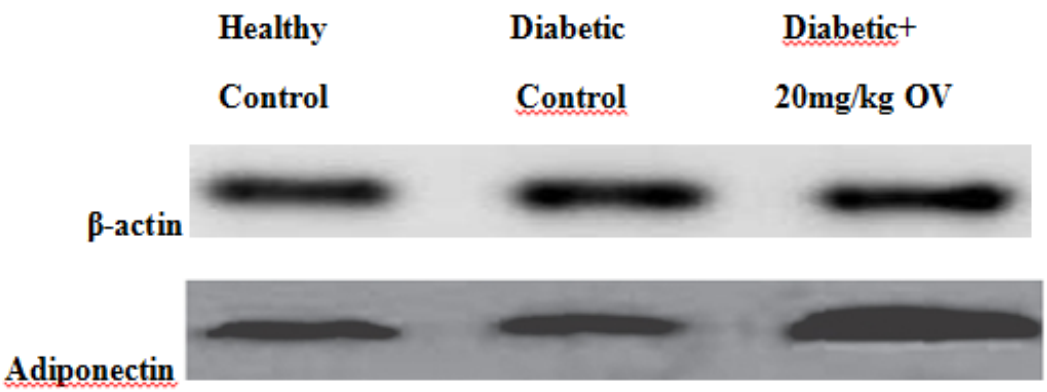
	Healthy Control (g)	**Diabetic Control (g)**	**Diabetic+ 20mg/kg OV (g)**
Pre treatment day 0	285.63±25.2	283.64±26.3	289.18±22.7
7 days after diabetes induction	289.51±27.3	261.64±25.2	263.42±24.3
28 days after treatment	350.34±29.7	230.17±20.1^{a*}	295.52±24.1^{a*,b*}

Mean±SD BW in healthy control (n=10) , diabetic control (n=10) and OV-treated rats (20mg/kg; n=10). a: compared with healthy control; b:compared with diabetic control. *:p<0.001.

Table2. Effect of aqueous extract of Origanum Vulgare leaves on FBG and insulin levels and HOMA index of the studied animals.

	Healthy Control	**Diabetic Control**	**Diabetic+ 20mg/kg OV**
Pre treatment day 0 FBG (mg/dl)	88.34±12.1	86.54±11.9	88.62±12.2
7 days after diabetes induction FBG(mg/dl)	89.13±12.3	200.71±20.7	198.89±22.4
28 days after treatment FBG(mg/dl)	87.15±13.2	214.56±24.7^{a*}	168.25±26.7^{a*,b*}
Insulin (µU/ml)	12.7±0.75	9.6±0.63^{a*}	9.8±0.83^{a*}
HOMA-IR	2.75±0.54	4.83±0.67^{a*}	3.9±0.73$^{a\#,b*}$

Mean±SD FBG, insulin and HOMA-IR in healthy control (n=10), diabetic control (n=10) and OV-treated rats (20 mg/kg; n=10). a :compared with healthy control; b:compared with diabetic control.#: p<0.01, *: p<0.001.



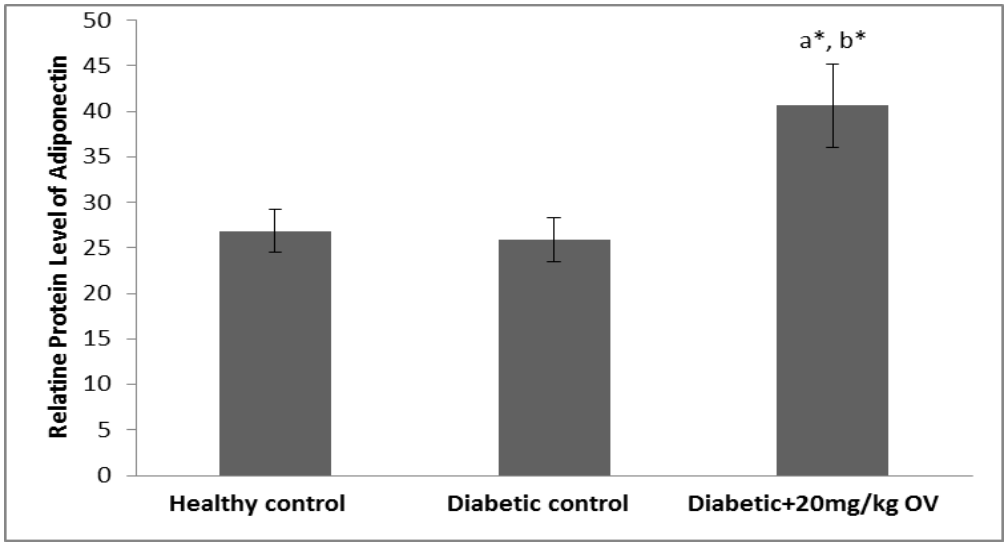
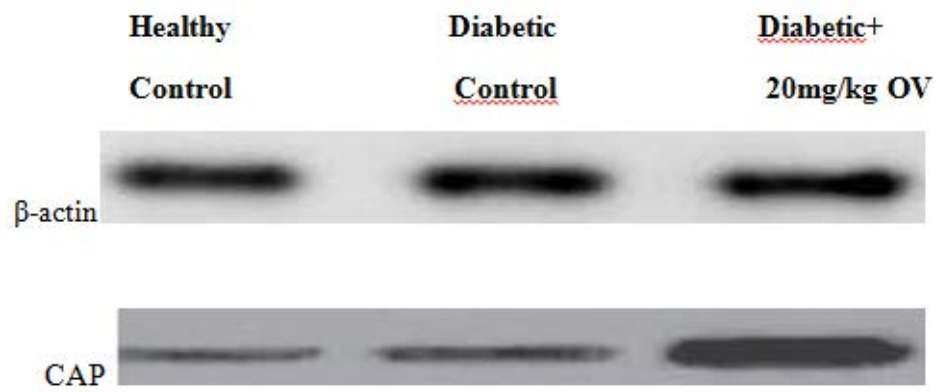


Fig.1. Results of western blot analysis of Adiponectin in adipose tissue. Protein levels are expressed in arbitrary units after densitometry analysis. Bars represent the mean±SD. a: compared with healthy control; b: compared with diabetic control. *: p<0.001.



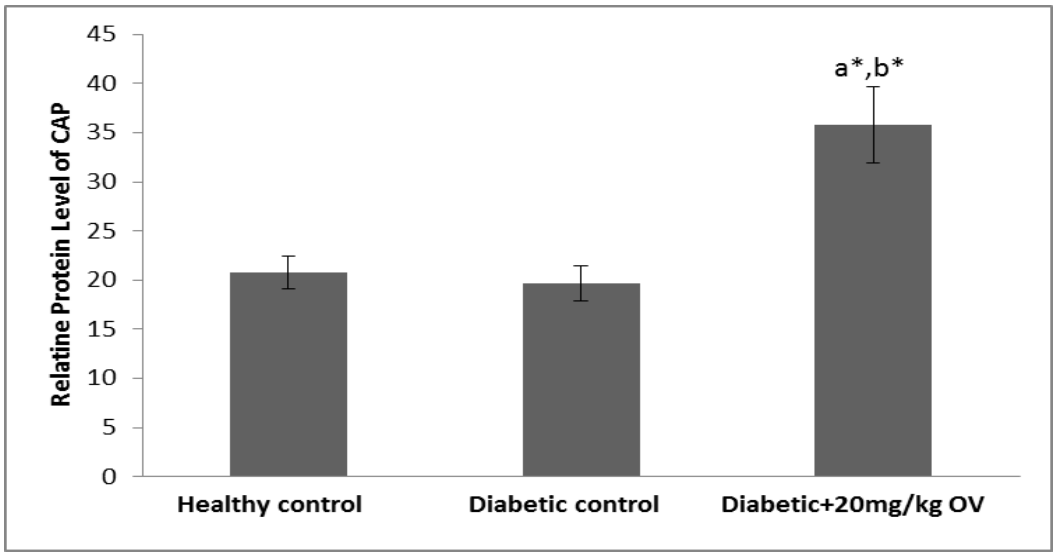
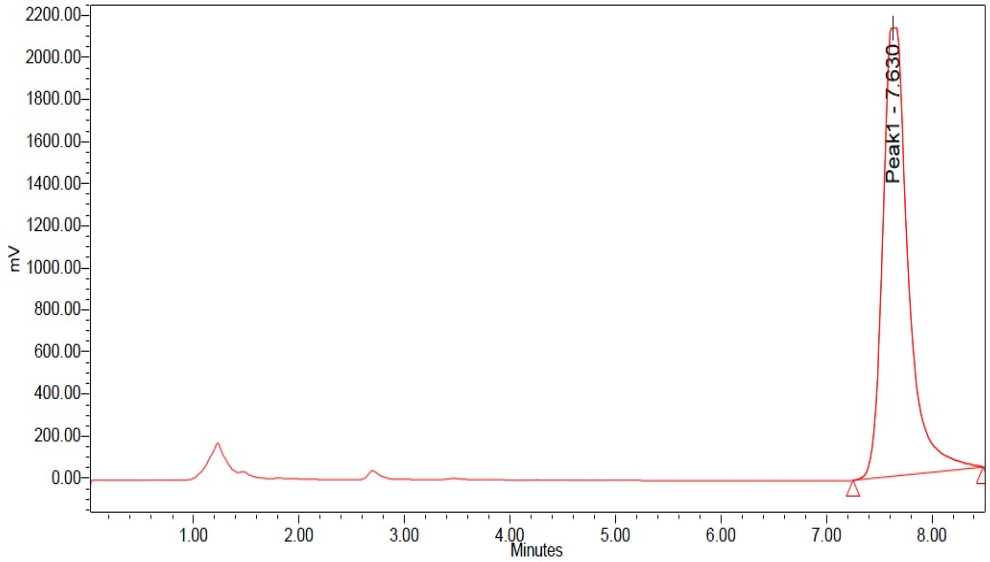


Fig.2. Results of western blot analysis of CAP in adipose tissue. Protein levels are expressed in arbitrary units after densitometry analysis. Bars represent the mean±SD. a: compared with healthy control; b: compared with diabetic control. *: p<0.001.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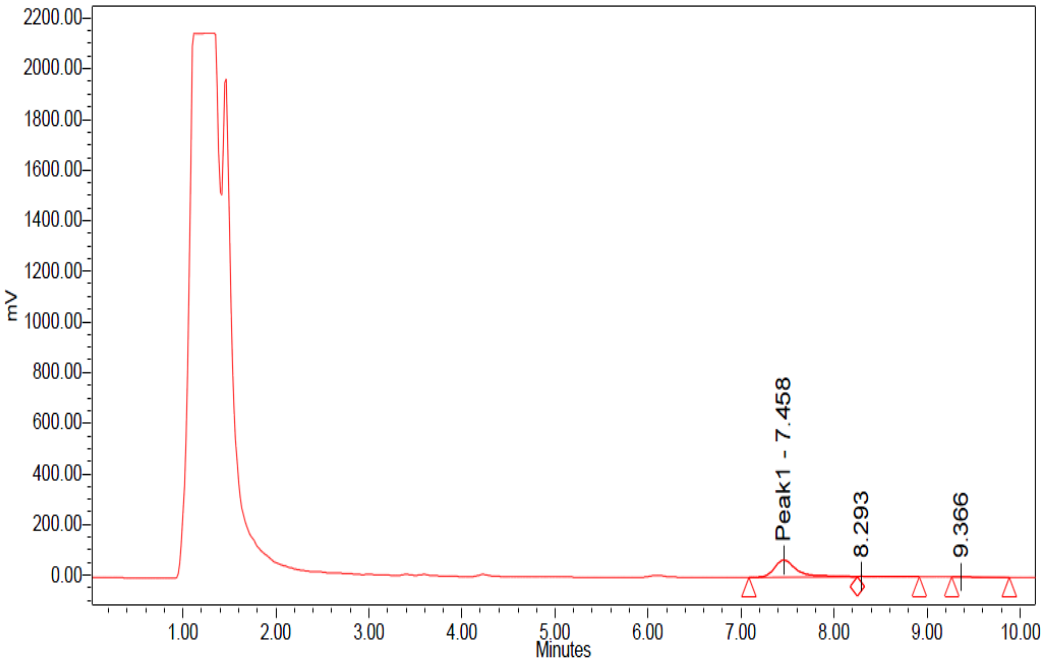
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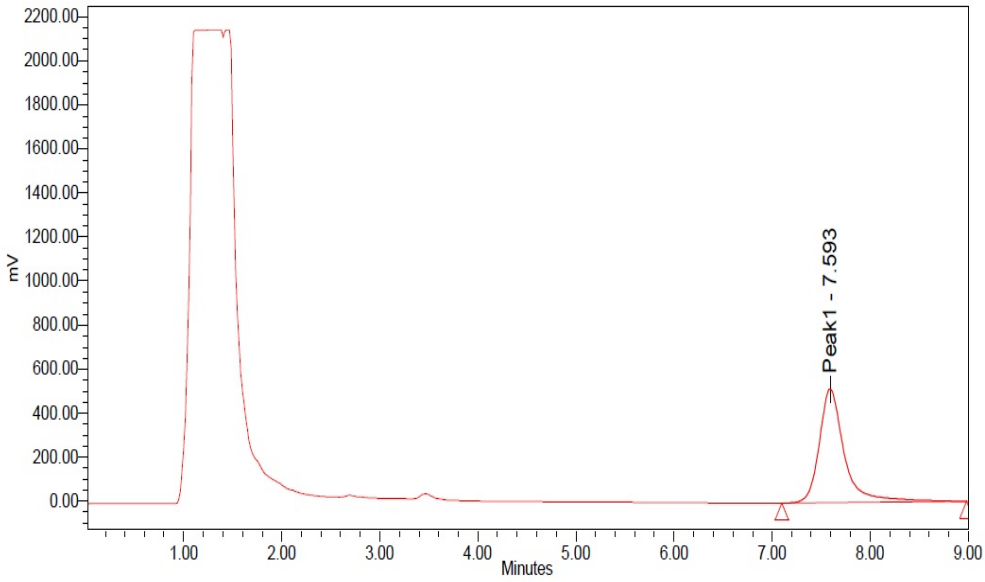

Fig.3. A) chromatogram of the Biochanin A standard; B) chromatogram of the aqueous extract of *Origanum Vulgare* leaves for the determination of Biochanin A. C) chromatogram of the aqueous extract of *Origanum Vulgare* leaves and Biochanin A standard with concentration of 25 ng/ml (50:50; v/v). The chromatographic conditions are given in the text.