

Effect of 8 Weeks Treadmill Running with or without *Pistachia Atlantica* Liquid Extraction on Liver ABCG8 Gene Expression and Cholesterol Level in Female Rat

Saleh Rahmati Ahmadabad¹, Zarbakht Ansari Pirsaraei², Abbas Ghanbari Niaki^{1*},
Hamid Deldar², Navabeh Zare Kookandeh¹, Fariba Shahandeh¹

¹ Department of Physical Education and Sport Science, University of Mazandaran, Baboulsar, Iran
² Animal Science Department, Sari Agriculture Sciences and Natural Resources University, Sari, Iran

Received 17 December 2011

Accepted 25 January 2012

Abstract

Introduction: Reverse cholesterol transport (RCT) is a term used to describe the efflux of excess cellular cholesterol. ABCG8 is a member of the ABCG family that play a critical role in this process. It has been reported that several factors (nutrients, fasting and physical stress) could affect on gene expression. Thus, the current study was conducted to investigate the effect of endurance exercise with or without Baneh extraction on the expression of liver ABCG8 in female rat.

Material and Methods: In this study twenty wistar rats (six to eight weeks old, 125-135 g weight) were used. Animals were randomly assigned into training (n = 10) and control (n = 10) groups and further divided into saline-control (SC), saline-training (ST), and Baneh-control (BC), and Baneh-training (BT). Training groups was given exercise on a motor-driven treadmill at 25 m/min (0% grade) for 60 min/day, 5 days/week for eight weeks. Animals were fed as the oral with Baneh extraction and saline for four week. 72 hours after the last training session and after four hours fasting, rats were sacrificed and liver total RNA isolated, after PCR procedure, ABCG8 gene expression was detected by Real-time PCR method. Statistical analysis were performed using a one way analysis of variance and significance was accepted at $P < 0.05$.

Results: Results demonstrated that independently Baneh extraction reduce ABCG8 gene expression in liver ($P < 0.013$). Exercise training increase ABCG8 relative gene expression than BC ($P < 0.016$) and BT ($P < 0.011$) groups; however exercise don't have independent significant effect on ABCG8 fold change at the end of the program ($P < 0.27$).

Discussion and Conclusion: Our findings show that hepatic cholesterol in saline-control group significantly was lower than Baneh groups ($P < 0.001$) and there wasn't significant change between other groups.

Key Words: Endurance exercise, Reverse cholesterol transport, Cellular cholesterol efflux

Introduction

Reverse cholesterol transport (RCT) is process that, excess cholesterol from peripheral tissues is returned to the liver where they are broken down and excreted [1, 2]. This process prevents the sticking of macrophages with cholesterol to the lining cell and arterials [3, 4]. This process of reverse cholesterol transport mediates by the ABC transporters. ABC family based on Sequence and arrangement are divided to A-G categories [5]. ABCG is subfamily of ABC super family which has been considering as lipid transporter. ABCG has sever sub members such as ; ABCG 1, ABCG 2, ABCG 3, ABCG 4,

ABCG 5, ABCG 8, ABCG 11, ABCG 12, ABCG 26[6-9]. All the ABCG except G2 plays an important role in the reverse cholesterol transport process [10]. Recently it has been shown that ABCG1 acting as one of element of RCT in macrophage [11-13]. In addition to ABCG1, some studies focused on the action of ABCG8. Four mammalian ABCG members, namely the homodimers ABCG1:ABCG1 and ABCG4:ABCG4 and the heterodimer ABCG5:ABCG8, have been shown to have a role in transporting sterols across membranes [14]. This protein has important in the transport of various materials such as amino acids, organic particles, peptides, sugars, Lipopolysaccharides, many drugs and proteins [5, 15]. It has been reported that ABCG8 is expressed in different tissues such as liver and small intestine

* Corresponding author E-mail:
ghanbara@umz.ac.ir

[16]. Over the last few decades in most countries the rate of curing people using alternative therapy, particularly dietary supplements and herbal, to improve the types of diseases has increased. Herbal medicine has fewer side effects and now also recommended by the World Health Organization. Experiences of traditional Chinese medicine used to control body weight are gained. More than two thousand years ago, doctors began to recognize and treat the obesity, over one hundred traditional Chinese herb used for weight loss [17]. The effect of dietary supplement on healthy men have shown that walnuts oil, has properties that decreases blood total cholesterol [18]. The Effect of silymarin on animals that fed with high-fat diets shown that this plant has been a positive effect on plasma lipoproteins profile [19]. It has been reported that several factors (nutrients, fasting, physical stress) could effect on ABCG gene expression. Sobolova et al reported that silymarin has a positive feedback on ABC Transporters [20]. In recent years some research has been done on the ABC family. Ghanbari-Niaki et al had shown that endurance exercise (six weeks at 25 meters per minute to 90 minutes a day, five days a week) increasing ABCA1 gene expression in rat liver. Also The plasma levels of high density lipoprotein cholesterol (HDL), Pre β HDL and lecithin cholesterol asyltransferas (LCAT) significantly increased [21]. Khabazian et al showed that 12 weeks of aerobic exercise (at 25 meters per minute to 60 minutes a day, five days a week) increase mRNA expressions of ABCA1 in rat's small intestine [22]. it has been shown that eight weeks of low-intensity exercise (walking), significantly up-regulated ATP-binding cassette transporters A1 and GI (ABCA1 and ABCG1, respectively) [23] However, no research has examined the effect of exercise on ABCG8 gene expression, also knowledge about the effect of *Pistachia atlantica* on tissue ABCG8 gene expression is lacking. *Pistacia atlantica (Baneh)* are plants of *Anacardiaceae* family that is rich in antioxidants and unsaturated fatty acid. High levels of polyunsaturated fatty acids found in nuts can be caused by loss of appetite and its role in weight loss and plasma lipids is evident. It is Shown that this plant leave contains anti-oxidative compounds that reduce the amount of free radicals [24]. The total amount of essential oil obtained from *Pistachia atlantica* is higher than any other species of the genus *Pistachia* [25]. Also *Pistachia* has

anti-inflammatory effect. In this research, the possibility of exercising and *Pistachia atlantica (Baneh)* effect on relative gene expression of ABCG8 was studied.

Material and Methods

Plant material

The ripped fruit samples of *Pistachia atlantica* (Baneh) were collected from the fields of Maibod in the Yazd province of Iran, and were stored at -18°C until use. Plant Material was identified by herbarium collection in our department.

Preparation of the extracts

The extracts were prepared by maceration (72h) of the coarsely powdered hull and kernel of *Pistachia atlantica*, with 150 ml tap water for 45 min at room temperature and filtering twice through No. 4 filter. The volume of the filtered solution was increased to 100 ml with tap water so that 1ml was equivalent to 100 mg of starting material [26]. The freshly prepared extracts were used immediately after cooling in the experiments. The use of distilled water for the extraction was omitted, since the herbalists recommend using these traditional medicines after boiling in tap water. After training 100 mg/kg liquid extraction of Baneh was assigned to the Baneh groups as oral and the same amount of saline was fed to saline groups.

Animals

All experiments involving the animals were conducted according to the policy of the Iranian convention for the protection of vertebrate animals used for experimental and other scientific purposes; and the protocol was approved by the Ethics Committee of the Sciences, University of Mazandaran (UMZ) and Babol University of Medical Sciences (BUMS, Mazandaran, Iran). Twenty Wistar female rats (6-8 weeks old 125-135 g) were acquired from Pasteur's Institute (Amol, Mazandaran) and maintained in the Central Animal House of Faculty of Physical Education and Sports Science of UMZ. Five rats were housed per cage (46-L volume) with a 12-hour: 12-hour light-dark cycle. Temperature and humidity were maintained at $22^{\circ}\text{C} \pm 1.4^{\circ}\text{C}$ and $55.6\% \pm 4.0\%$, respectively. Temperature was maintained at $22^{\circ}\text{C} \pm 1.4^{\circ}\text{C}$. Diets (a pellet form) and water were provided ad libitum. Animals were randomly assigned into control (n = 10) and training (n = 10) groups. Rats were further

divided into saline-control (SC), saline-training (ST), and Baneh-control (BC), and Baneh-training (BT). The control group remained sedentary, whereas the training group underwent a moderate running exercise program.

Exercise training protocol

At first, the animals were familiarized with the rat treadmill apparatus, each day and for 4 days (the 14-lane motorized-driven treadmill was designed by the primary author (UMZ, Babolsar, Mazandaran, Iran). The exercise group was trained for 8 weeks using the same training methods previously described [21, 22]. The rats were run at 25 m/min for 60 minutes, 5 d/wk. The animals were killed 72 hours after the last exercise session. Food but not water was removed from the rat cages 4 hours before the sacrifices.

Tissue biopsies

Seventy-two hours after the last training session, rats were anesthetized with intra peritoneal administration of a mixture of ketamine (30– 50 mg / kg body weight) and xylazine (3– 5 mg / kg body weight). Liver were excised, cleaned, divided into two pieces, washed in ice-cold saline, and immediately frozen in liquid nitrogen and stored at $- 80^{\circ} \text{C}$ until RNA extraction.

RNA isolation, cDNA synthesis and Real-time PCR

Total RNA was extracted from 80 to 100 mg of tissue using RNA purification kits (Accu Zol, Bioneer company). Complementary DNA (cDNA) was extended from oligo-(dt)₁₈ primers (0.25 μg per reaction) using cDNA synthesis kit (AccuPower RT PreMix) according to the manufacturer's instructions. Real-time PCR was performed on light Cycler apparatus (Corbet). Real-time quantitative PCR was performed using QuantiFast SYBR Green PCR Kit (Cat. No. 204052; Qiagen, GmbH, Germany) in using 15 μl reaction containing 0.5 μl single-strand cDNA, 7.5 μl Master Mix, 1 μl of the each forward and reverse primers (5 pmol/ μl) and 5 μl dH₂O in a final reaction volume of 15 μl . ABCG8 sense primer; 5'-CGTCAGATTTCCAATGACTTCCG-3' and antisense primer were 5'-TCCGTCCTCCAGTTCATAGTACA-3' (AF351785, 241 bp), respectively [27]. The β -actin sense and antisense primers were; 5'-

TATCGGCAATGAGCGGTTCC-3' and 5'-CACTGTGTTGGCATAGAGG-3' (NM_031144, 145 bp), were used as a normalizer gene respectively [28].

Liver cholesterol levels

Liver cholesterol was measured by ELISA method (Greiner, Bahlingen kit made in Germany) and 5 mg/dl Sensitivity.

Statistical analysis

The data were analysed using the comparative threshold cycle method (CT). Briefly, Δ -CT value calculated by taking the CT of the ABCG8 gene and subtracting it from CT of β -actin. The $\Delta\Delta$ -CT was calculated by subtracting the Δ -CT(sample) values from the Δ -CT(control). The relative quantification was then calculated by the expression $2^{-\Delta\Delta\text{CT}}$ [29]. The Kolmogorov-Smirnov test was used to determine the normality of distribution, and variables were found to be normally distributed. All results are expressed as means \pm SEM. Statistical analysis were performed using a one way analysis of variance. Least significant difference post hoc test was used in the event of a significant ($P < .05$) F ratio. All statistical analysis was performed with SPSS (Version 13; SPSS, Chicago, IL).

Results

Data analysis revealed significant difference in liver ABCG8 relative gene expression between groups ($F=3.581$, $P<0.039$) (Fig.1). Therefore using a suitable post hoc test, data were showed that liver relative expression of ABCG8 mRNA was higher in ST group compared with BC ($P<0.016$) and BT ($P<0.011$) groups at the end of program (Fig.1). On the other hand, a lower ABCG8 relative expression were found in Baneh groups ($P<0.013$) (Fig.1) and exercise no significant effect as independent factor on ABCG8 gene expression ($P<0.27$). Our findings show that hepatic cholesterol in SC group significantly was lower than BC and BT groups ($P<0.001$) (Fig. 2) and there wasn't significant change between other groups.

Discussion and Conclusion

To our knowledge, this is the first study to demonstrate alterations of liver ABCG8 relative gene expression and cholesterol levels in response to a treadmill running and Baneh crud extraction

regime on female rat. The major findings of the present study were a higher ABCG8 relative mRNA gene expression in saline trained liver than Baneh-control and Baneh-trained groups. Other finding was a lower ABCG8 mRNA expression in Baneh groups compared with saline animals. ABCG8 is expressed in different tissues such as liver and small intestine [16, 30] Graf et al by Northern blot analysis method showed that the expression of ABCG5 and ABCG8 overlapped in the liver and small intestine and, to a lesser extent, in the colon, where both ABCG5 and ABCG8 are expressed at the apical membrane [31]. In this study we detected ABCG8 relative gene expression in liver tissue by Real time PCR method that is concurrent with previous reported in tissues ABCG8 gene expression. In the present study, exercise increase ABCG8 relative mRNA gene expression in liver tissue (Fig.1). ABCG8, ABCG1 and ABCA1, play a critical role in process of reverse cholesterol [4, 11]. In recent year several studies was made on ABCA1 transporter [21, 22]. They found that exercise increases liver and intestine ABCA1 gene expression. Also, it has been shown that eight weeks of low-intensity exercise (walking), significantly up-regulated ATP-binding cassette transporters A1 and GI (ABCA1 and ABCG1, respectively) [23]. All the Four of the five mammalian ABCG members, ABCG1, ABCG4, ABCG5, ABCG8, have been shown to have a role in transporting sterols across

membranes [14]. Therefore its possible increase in ABCG1 is consistent with our ABCG8 finding in this study after treadmill running program. It has been shown that a high-fat diet suppresses ABCA1, ABCG4 and ABCG8 gene expression [32]. Previous research using GC and GC-MC methods showed that pistachios are rich in essential oils [33]. Baneh is oil seed and is enrich fatty acids. Analysis of the *Pistachia atlantica* var. *Mutica* essential oil by GC-MS method, shown that it composed by α -pinene (70%), β -pinene (1.94%), 3-carene (0.2%), carveol (2.18%), epoxy-pinene (2.15%), limonene oxide (9%), myrtenol (5.31%), limonene (0.62%), citral (5.72%), α -phellandrene (0.2%), and β -myrcene (0.3%). The total amount of essential oil obtained was 22% v/w which is higher than any other species of the genus *Pistacia* [25]. Important point of this research is reduction of ABCG8 gene expression by Baneh in liver that probably is due to Baneh high fatty acid (Fig.1). Also the previous study shown that the lymphatic absorption of cholesterol in rats were fed by high-fat diet sesame oil, has declined [34]. In the high-fat regime many chylomicron formed and cholesterol accumulation in liver is due to this [35]. Our findings show that hepatic cholesterol in saline-control group was significantly lower than Baneh groups, that consistent with the results were fed by high-fat diet sesame oil.

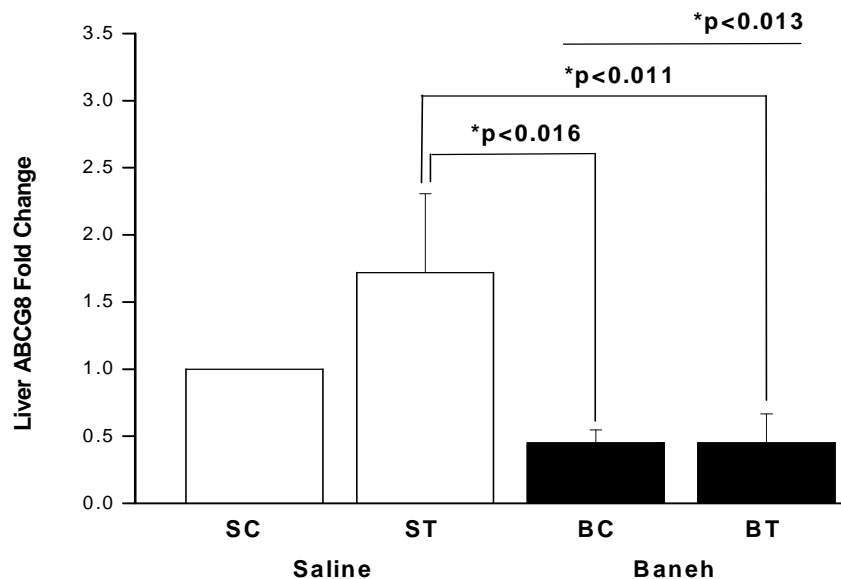


Figure1: Real-time PCR of liver ABCG8 relative mRNA expression in saline- control (SC), saline-training (ST), Baneh-control (BC), and Baneh-training (BT) wild-type female rats. Wild-type female rats Data expressed as mean \pm SEM. Each column is for each group and 5 rat per group

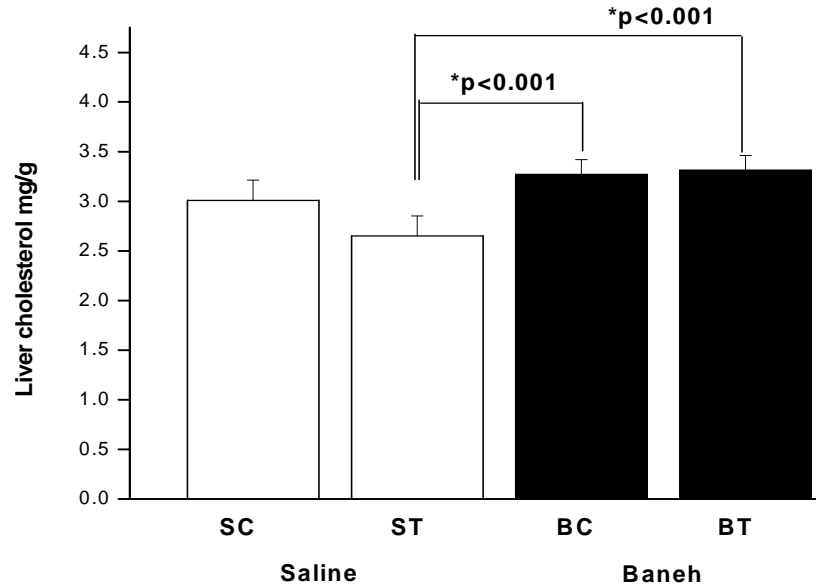


Figure2: Liver cholesterol levels in saline-control (SC), saline-training (ST), Baneh-control (BC), and Baneh-training (BT) wild-type female rats. Wild-type female rats Data expressed as mean \pm SEM. Each column is for each group and 5 rat per group

In summary, this investigation was about effect of eight weeks endurance training and *Pistachia atlantica* extraction on expression of ABCG8 transporter in female rats and this study confirms that ABCG8 transporter expresses in liver tissue. The main findings of the present study were higher and significant ABCG8 mRNA fold changes in liver tissue following treadmill running program in female rat. Others main finding was lower and significant ABCG8 gene expression in Baneh animals compare to Saline groups. Our study shows that, *Pistachia atlantica* (Baneh) can probably reduce ABCG8 relative gene expression in liver tissue due to its essential fatty acids. On the other hand, exercise increases the ABCG8 relative gene expression. Also probably *Pistachia atlantica* extraction can increase intestinal cholesterol synthesis that provided sufficient Cholesterol for the formation of chylomicrons. Lymphatic absorption of cholesterol by high-fat sesame oil has declined that probably reduces reverse cholesterol transporter and probably its reason of increase in hepatic cholesterol.

Acknowledgements

The authors hearty acknowledge the support of Dr R. Nekouie (Department of exercise Physiology, University of Kerman, Iran). Gratefully thanks

from students of Mazandaran lab (Department of Animal Science, Sari Agricultural Sciences and Natural Resources University, Sari, Iran). We also thank Dr. R. Fathi (Department of exercise Physiology, University of Mazandaran, Iran) for helpful comments and guidance.

References

- 1-Fielding CJ, Fielding PE (2001). Cellular cholesterol efflux. *Biochim Biophys Acta* 1533:175-89.
- 2-Yancey PG, Bortnick AE, Kellner-Weibel G, de la Llera-Moya M, Phillips MC, Rothblat GH (2003). Importance of different pathways of cellular cholesterol efflux. *Arterioscler Thromb Vasc Biol* 23:712-719.
- 3-Hayden MR, Clee SM, Brooks-Wilson A, Genest J, JR, Attie A, Kastelein JJ (2000). Cholesterol efflux regulatory protein, Tangier disease and familial high-density lipoprotein deficiency. *Curr Opin Lipidol* 11:117-122.
- 4-van der Velde AE (2010). Reverse cholesterol transport: from classical view to new insights. *World J Gastroenterol* 16:5908-5915.
- 5-Woodward OM, Kottgen A, Kottgen M (2011). ABCG transporters and disease. *FEBS J* 278:3215-3225.
- 6-Choi H, Jin JY, Choi S, Hwang JU, Kim YY, Suh MC, et al. (2011). An ABCG/WBC-type ABC transporter is essential for transport of sporopollenin precursors for exine formation in developing pollen. *Plant J* 65:181-193.

- 7-McFarlane HE, Shin JJ, Bird DA, Samuels AL (2010). Arabidopsis ABCG transporters, which are required for export of diverse cuticular lipids, dimerize in different combinations. *Plant Cell* 22:3066-3075.
- 8-Ozvegy C, Litman T, Szakacs G, Nagy Z, Bates S, Varadi A, et al (2001). Functional characterization of the human multidrug transporter, ABCG2, expressed in insect cells. *Biochem Biophys Res Commun* 285:111-117.
- 9-Xu J, Liu Y, Yang Y, Bates S, Zhang JT (2004). Characterization of oligomeric human half-ABC transporter ATP-binding cassette G2. *J Biol Chem* 279:19781-19789.
- 10-Kusuhara H, Sugiyama Y (2007). ATP-binding cassette, subfamily G (ABCG family). *Pflugers Arch* 453:735-744.
- 11-Wang N, Lan D, Chen W, Matsuura F, Tall AR (2004). ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc Natl Acad Sci USA* 101:9774-9779.
- 12-Wang N, Yvan-Charvet L, Lutjohann D, Mulder M, Vanmierlo T, Kim TW, et al (2008). ATP-binding cassette transporters G1 and G4 mediate cholesterol and desmosterol efflux to HDL and regulate sterol accumulation in the brain. *FASEB J* 22:1073-1082.
- 13-Out R, Hoekstra M, Habets K, Meurs I, de Waard V, Hildebrand RB, et al (2008). Combined deletion of macrophage ABCA1 and ABCG1 leads to massive lipid accumulation in tissue macrophages and distinct atherosclerosis at relatively low plasma cholesterol levels. *Arterioscler Thromb Vasc Biol* 28:258-264.
- 14-Tarr PT, Tarling EJ, Bojanic DD, Edwards PA, Baldan A (2009). Emerging new paradigms for ABCG transporters. *Biochim Biophys Acta* 1791:584-593.
- 15-Higgins CF (1992). ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* 8:67-113.
- 16-Kushwaha RS, Rosillo A, McGill HC, (2005). Expression levels of genes for ATP-binding cassette transporters and sterol 27-hydroxylase in liver and intestine of baboons with high and low cholesterolemic responses to dietary lipids. *J Med Primatol* 34:122-9.
- 17-Tian WX, Li LC, Wu XD, Chen CC (2004). Weight reduction by Chinese medicinal herbs may be related to inhibition of fatty acid synthase. *Life Sci* 74:2389-2399.
- 18-Sabate J, Fraser GE, Burke K, Knutsen SF, Bennett H, Lindsted KD (1993). Effects of walnuts on serum lipid levels and blood pressure in normal men. *N Engl J Med* 328:603-607.
- 19-Vecera R, Zacharova A, Orolin J, Skottova N, Anzenbacher P (2011). The effect of silymarin on expression of selected ABC transporters in the rat. *Veterinari Medicina* 56:59-62.
- 20-Sobolova L, Skottova N, Vecera R, Urbanek K (2006). Effect of silymarin and its polyphenolic fraction on cholesterol absorption in rats. *Pharmacol Res* 53:104-112.
- 21-Ghanbari-Niaki A, Khabazian BM, Hossaini-Kakhak SA, Rahbarizadeh F, Hedayati M (2007). Treadmill exercise enhances ABCA1 expression in rat liver. *Biochem Biophys Res Commun* 361:841-846.
- 22-Khabazian BM, Ghanbari-Niaki A, Safarzadeh-Golpordesari A, Ebrahimi M, Rahbarizadeh F, Abednazari H (2009). Endurance training enhances ABCA1 expression in rat small intestine. *Eur J Appl Physiol* 107:351-358.
- 23-Butcher LR, Thomas A, Backx K, Roberts A, Webb R, Morris K (2008). Low-intensity exercise exerts beneficial effects on plasma lipids via PPARgamma. *Med Sci Sports Exerc* 40:1263-1270.
- 24-Benhammou N, Bekkara F, Panovska T (2008). Antioxidant and antimicrobial activities of the *Pistacia lentiscus* and *Pistacia atlantica* extracts. *Pharmacy and Pharmacology* 2:22-28.
- 25-Delazar A, Reid RG, Sarker SD (2004). GC-MS Analysis of the Essential Oil from the Oleoresin of *Pistacia atlantica* var. *mutica* *Chemistry of Natural Compounds* 40:24-27.
- 26-Hamdan II, Afifi FU (2004). Studies on the in vitro and in vivo hypoglycemic activities of some medicinal plants used in treatment of diabetes in Jordanian traditional medicine. *J Ethnopharmacol* 93:117-121.
- 27-Yu H, Pandit B, Klett E, Lee MH, Lu K, Helou K, et al. (2003). The rat STSL locus: characterization, chromosomal assignment, and genetic variations in sitosterolemic hypertensive rats. *BMC Cardiovasc Disord* 3:4.
- 28-Gao X, Yuan S (2010). High density lipoproteins-based therapies for cardiovascular disease. *J Cardiovasc Dis Res* 1:99-103.
- 29-Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods* 25:402-408.
- 30-Ji-Young L, Timothy PC (2005). Dietary fatty acids regulate the expression of ABCG5 and ABCG8 in hamsters. *Nutrition Research* 25:167-175.
- 31-Graf GA, Li WP, Gerard RD, Gelissen I, White A, Cohen JC, et al (2002). Coexpression of ATP-binding cassette proteins ABCG5 and ABCG8 permits their transport to the apical surface. *J Clin Invest* 110:659-669.
- 32-de Vogel-van den Bosch HM, de Wit NJ, Hooiveld GJ, Vermeulen H, van der Veen JN, Houten SM, et al. (2008). A cholesterol-free, high-fat diet suppresses gene expression of cholesterol transporters in murine small intestine. *Am J Physiol Gastrointest Liver Physiol* 294:1171-1180.
- 33-Gourine N, Bombarda I, Yousfi M, Gaydou EM (2010). Chemotypes of *Pistacia atlantica* leaf essential oils from Algeria. *Nat Prod Commun* 5:115-120.

34-Satchithanandam S, Reicks M, Calvert RJ, Cassidy MM, Kritchevsky D (1993). Coconut oil and sesame oil affect lymphatic absorption of cholesterol and fatty acids in rats. *J Nutr* 123:1852-1858.

35-Biddinger S, Almind K, Miyazaki M, Kokkotou E,

Ntambi J, Kahn C (2005). Effects of diet and genetic background on sterol regulatory element-binding protein-1c, stearoyl-CoA desaturase 1, and the development of the metabolic syndrome. *Diab54*:1314-1323.