

Chemical composition and effect of an essential oil of *Salix aegyptiaca* (musk willow) in hypercholesterolemic rabbit model

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Abstract: The essential oils (EO) of *Salix aegyptiaca* L., Salicaceae (SA), leaves were extracted using the hydrodistillation method and their chemical composition was further determined by GC-MS. 1,4-Dimethoxybenzene was the main isolated compound. Other major isolated constituents were phenylethyl alcohol, carvone, citronellol, methyleugenol, eugenol, *n*-tetradecane and 4'-methoxyacetophenone. Twenty rabbits were equally divided into four groups: Normal control (NC) which fed a standard diet and three cholesterol-fed groups: HC, HC+1.0% SA and HC+3.0% SA groups which received 0.0, 1.0 and 3.0% EO, respectively for four weeks. The serum lipid and lipoprotein profiles and atherogenic index (AI) were measured weekly. The high cholesterol diet significantly raised the TC, LDL-C, VLDL-C, HDL-C, TG and AI level compared with NC group. HC+1.0% SA and HC+3.0% SA groups showed similar results compared with HC group. It can be concluded that the EO of SA leaves could not prevent dyslipidemia that occurred in rabbits following inclusion of cholesterol in diet in both dose- and time-dependent manners.

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Introduction

Several dietary constituents of plant origin are effective cholesterol-lowering agents (Ostlund, 2004; Karimi et al., 2010). Consequently, plant foods and manufactured products rich in these phytochemicals are being promoted to consumers as cardioprotective and beneficial to overall health but sometimes plants or plant products are harmful. Some plant products such as coffee (Terpstra et al., 2000), mistletoe (*Viscum album*, Ben et al., 2006) and marijuana seed (*Cannabis sativa*, Hayatgheybi & Karimi, 2007) were reported to have hyperlipidemic and/or hypercholesterolemic effects. The level of serum lipids serves as a marker for the risk of cardiovascular disease (Castelli et al., 1992), which is a major cause of death worldwide (WHO, 2003). Therefore, it is important to understand how diet affects these risk markers. For example, two chief diterpenes, cafestol and kahweol, were reported as potent cholesterol-raising compounds in boiled coffee and coffee oil in the human diet (Urgert & Katan, 1997).

Salix aegyptiaca L. (musk willow) is a dioecious plant belongs to the family of Salicaceae traditionally

has been cultivated in Turkey, Iran, Turkmenistan and Afghanistan. There are many industrial and traditional systems that are involved in cultivating musk willow (Bidmeshk in Persian) in different regions of Iran. The aqueous extract and essential oil (EO) of *Salix aegyptiaca* L. (SA) are being used in confectionary and flavorful syrups. In Iranian traditional medicine, SA has been employed as laxative, cardioprotective, nervonic, sedative, hypnotic, somnolent, aphrodisiac, orexiogenic, carminative, gastroprotectant, anthelmintic and vermifuge. The decoction of leaves or barks of SA have been used as an anthelmintic and vermifuge remedy. The decoction of SA's leaves in honey still is used as a nervonic functional food. The decoction of leaves of SA plus sugar has been used among Iranian and Turkish people for maladies like depression, neuropathic pain and rheumatoid arthritis.

The *Salix* family is famous due to its endogenous salicylate compounds e.g., salicylic acid and acetyl salicylic acid (ASA, Aspirin®). This class of compounds exert anti-inflammatory effects throughout the inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) leading to the inhibition of prostaglandin

synthesis (Yu et al., 2002; Mahdi et al., 2006). The anti-inflammatory and anti-nociceptive properties of extracts of willow family may be related to its phytochemicals such as salicin, myricetin, kaempferol, quercetin, rutin and luteolin (Qin & Sun, 2005; Nahrstedt et al., 2007). These compounds have immunomodulatory and anti-inflammatory activities by inhibiting pro-inflammatory cytokine production and their receptors (Qin & Sun, 2005; Nahrstedt et al., 2007). The considerable myricetin, rutin and catechin content of musk willow extracts could potentially contribute to the anti-inflammatory functions of willow extracts (Enayat & Banerjee, 2009).

According to Unani medicine, SA has warm humor nature and ethnic herbalists prescribed it for cholelithiasis, cholecystitis, arthritis and rheumatism. The EO of SA is febrifuge and is dubbed among Iranian people for its calming effect on heart and possibly its antihypertensive effect. Hence, in this study, we investigate the effect of Musk willow EO on lipid profile in cholesterol-fed rabbits.

Material and Methods

Plant collection and authentication

The leaves of SA were purchased from the Ghamsar botanical garden at Kashan, Iran and air dried in the shade. It was authenticated by Dr Abbas Siami, Professor of Botany in Department of Biology, College of Science, Urmia University, Iran.

Extraction

Fresh SA leaves (150 kg) were changed into the distillation unit along with 600 L fresh water. The unit was heated by steam water. The process was followed to collect of 450 L of distilled SA water. Liquid-liquid extraction procedure: 5 mL of each extractor solvent (*n*-hexane, dichloromethane and chloroform) mixed with 500 mL of SA water that was saturated by sodium chloride, then shook for 15 min and extracted in an ultrasonic batch for 30 min. This procedure was repeated three times and all recovered fractions were collected. The extracted oil was dried over anhydrous magnesium sulfate and stored at 4 °C before analysis.

Essential oil chemical analysis

GC analysis was performed using a Hewlett-Packard chromatograph 5890 series equipped with FID detector and a HP-1 fused silica column (30 m × 0.25 mm and film thickness 0.25 μm). GC/MS analysis was carried out on a Hewlett-Packard 5973 connected with a mass detector HP 6890 using a HP-1 column (30 m × 0.25 mm and film thickness 0.25 μm). Oven temperature

programming was 40-250 °C with an increase of 3 °C/min for both GC/FID and GC/MS. Injector and detector temperatures were 320 and 310 °C, respectively. The carrier gas was helium and flew at a rate of 1 mL/min. The mass spectrometer was operated at 70 eV with the mass range, 40-350 amu and scan time 1 s. Identification was based on sample retention data and comparison with authentic standards, computer matching using NIST MS library. The identification was also confirmed by comparison of the retention indices with data in the literature (Adams, 1996; Shibamoto, 1987). The percentages of compounds were calculated by the area normalization method without considering response factors. The retention indices were calculated for all volatile constituents using a homologous series of *n*-alkanes.

Preparation of essential oil

The EO (3% v/w) was obtained from dried powdered leaves of SA by steam distillation for 3 h, using a Clevenger apparatus. The resulting EO was diluted with distilled water to prepare 1% v/w.

Diet preparation

Cholesterol (25 g) was dissolved in 175 mL ethanol and mixed with 800 mL sunflower oil. The resulting solution vigorously has been homogenized and immediately mixed with standard pellet (Niro-Sahand Co. Tabriz, Iran) in the ratio of 15% v/w. The repelleted feed was air-dried under UV-illumination for two days and stored at -5 °C until use. The resulting "high-cholesterol (HC) diet" contained in 0.47% cholesterol.

Animals

Adult weight- and age-matched healthy male Albino rabbits (n=20), were maintained in an air-conditioned room (26±1 °C) and were divided into groups of five each. Group NC served as negative control that received only standard pellet and daily gavaged by 10 mL distilled water. Group HC served as positive control that received cholesterol enriched pellet and daily gavaged by 10 mL distilled water. Group HC+1% SA received cholesterol enriched pellet and daily gavaged by 10 mL EO 1% v/w of musk willow. Group HC+3% SA received cholesterol enriched pellet and daily gavaged by 10 mL EO 3% v/w of musk willow. Blood samples were collected through auricular vein on day 0 (week 0) and then at weeks 1, 2, 3, and 4. Sera were separated by centrifugation at 1400×g at 4 °C for 15 min, and stored at -20 °C until analysis. The study was approved by the Laboratory Animal Care Committee of Urmia University, Western Azerbaijan, Iran.

Analytical procedures

The concentrations of total cholesterol (TC), LDL-C and TGs in the serum were enzymatically determined with a commercial diagnostic kit (ELI TECH Diagnostic, French). Plasma lipoprotein fractions HDL-C were determined by immunoinhibition method (ELI TECH Diagnostic, French). Very low density lipoprotein-cholesterol (VLDL-C) was calculated by formula: $VLDL-C = TG/5$ (Friedewald et al., 1972). Atherogenic Index (AI) was calculated according to the following equation: $AI = (TC - HDL-C) / HDL-C$ (Lee & Niemann, 1996).

Statistical analysis

All data are reported as mean \pm SEM. All parameters were analyzed using ANOVA for a split-plot in time design, with diet as the whole-plot factor and time (weeks) sampled as the subplot factor. This procedure allowed testing for the effects of diet and week and their interaction. When significant differences were found, *post hoc* comparisons were made between control and all other (treated) groups with a Duncan's multiple range tests. All data were analyzed using the General Linear Models Procedure of SPSS ver.16. Statements of significance were based on $p < 0.05$ unless otherwise noted.

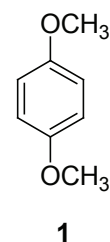
Results

Comprehensive GC/MS and GC/FID was used to analyze volatile components of SA leaves in this study. Figure 1 shows GC of the EO extracted from SA leaves. The chemical constituents of EO are displayed in Table 1. The main component that isolated from EO of SA in the presence of different solvents that employed in this study was 1,4-dimethoxybenzene (DMB, **1**) (Table 1, Figure 1).

In this experiment, calories consumed and mean body weight gains were statistically similar throughout study in all groups (results not shown). Serum TC,

LDL-C, VLDL-C, HDL-C, TGs concentrations and AI levels increased significantly after 28 days of cholesterol feeding ($p < 0.05$; Table 2). Concurrent administration of EO (1% and 3%) of SA with cholesterol did not positively modify lipid and lipoprotein profiles of HC+1%SA and HC+3%SA groups in comparison to HC group (Table 2). No significant differences were observed in the serum levels of TC, LDL-C, HDL-C and TGs as well as AI between HC+1% SA and HC+3% SA groups ($p > 0.05$; Table 2). After four weeks, the 1% and 3% *S. aegyptiaca*-treated groups displayed approximately 64 and 37% of increment in VLDL-C level compared to NC rabbits, respectively ($p < 0.001$; Table 2). Also, VLDL-C level were similar in HC+1% SA and HC+3% SA groups in comparison to HC group (Table 2). A significant difference in the serum VLDL-C levels of cholesterol-fed groups on 1, 2, 3 and 4 weeks was observed ($p_{ANOVA} < 0.001$; Figure 2).

After four weeks, the 1% and 3% *S. aegyptiaca*-treated groups displayed approximately 28% of increment in LDL-C level compared to NC rabbits ($p < 0.05$; Table 2). Also, LDL-C level were similar in HC+1% SA and HC+3% SA groups in comparison to HC group (Table 2). A significant difference in the serum LDL-C levels of cholesterol-fed groups on 1, 2, 3 and 4 weeks was observed ($p_{ANOVA} < 0.001$; Figure 2).



Inclusion of cholesterol in diet caused a significant increase ($\sim 35\%$; $p < 0.05$) in the HDL-C level compared to NC group while, administration of EO of SA could not positively improve this parameter in both of the HC+1% SA and HC+3% SA groups in comparison to HC group (Table 2). A significant difference in the

Table 1. Comparative chemical composition (%) of the essential oil of *Salix aegyptiaca* L. (Egyptian willow) in the presence of different solvents.

Compound (%)	Dichloromethane	Chloroform	Hexane	RI*
Phenylethyl alcohol	10.9	13.7	0.6	1002
1,4-Dimethoxybenzene	56.6	54.4	61.5	1050
Carvone	5.4	-	6.0	1118
Citronellol	5.0	-	8.0	1121
4'-Methoxyacetophenone	3.0	3.0	0.9	1204
Eugenol	1.9	6.0	2.0	1229
Methyleugenol	2.5	21.0	3.0	1287

*RI: retention indices in elution from HP-1 column.

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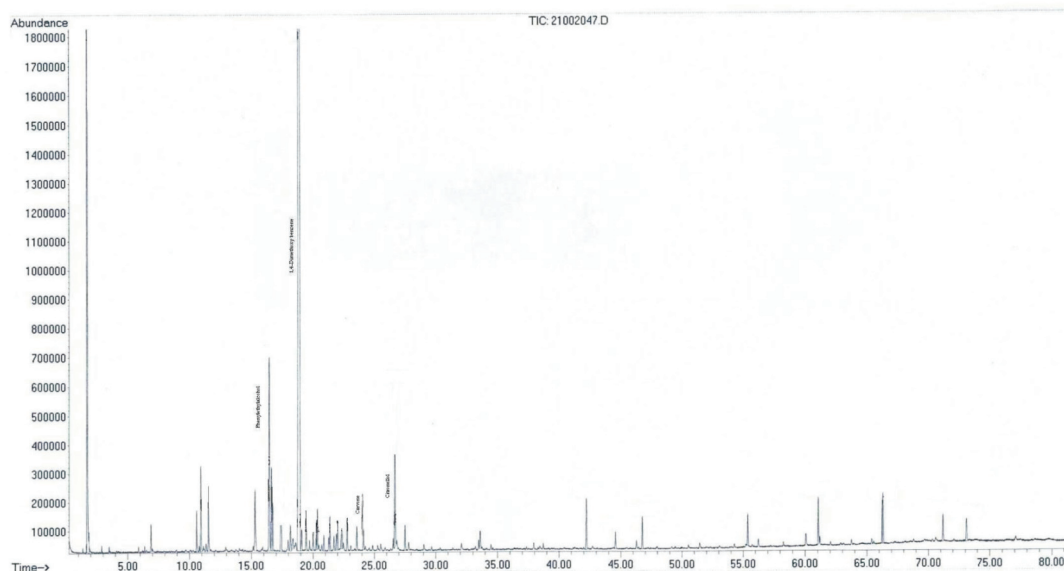


Figure 1. GC chromatogram of the essential oil extracted from *Salix aegyptiaca* L. leaves by dichloromethane. The main compounds represented from left to right on the chromatogram are phenylethylalcohol, 1,4 dimethoxybenzene, carvone and citronellol, respectively.

Table 2. Effect of essential oil of Egyptian willow on lipid profile and atherogenic index (AI) in cholesterol fed rabbits.

Group	TC	LDL-C	VLDL-C	HDL-C	TG	AI
NC	59.04±1.028†	25.84±0.76†	13.12±0.510†	17.64±0.637†	62.20±2.588†	2.37±0.094†
HC	92.32±3.996*	39.28±1.534*	21.39±1.381*	24.44±1.642*	106.08±7.093*	2.94±0.118*
HC+1%SA	92.92±3.981*	39.62±1.561*	21.56±1.398*	24.12±1.607*	107.92±6.841*	3.03±0.130*
HC+3%SA	92.96±3.985*	39.00±1.400*	18.05±0.761*	23.28±1.546*	107.36±6.943*	3.12±0.133*

Values are mean±SEM (n=5);*Data in columns were significantly different at $p<0.05$ compared to NC: Normal control; †Data in columns were significantly different at $p<0.05$ compared to HC: Hypercholesterolemic control. HC+1% SA and HC+3% SA groups received 1 and 3% essential oil of Egyptian willow plus atherogenic diet, respectively.

serum HDL-C levels of cholesterol-fed groups on 1, 2, 3 and 4 weeks was observed ($p_{ANOVA}<0.001$; Figure 2). A significant increment (~55%; $p<0.05$) in the circulating TC was detected in all cholesterol-fed groups and EO of SA could not prevent of this accrual effect in both levels of 1 and 3 percent in *S. aegyptiaca*-treated groups. The levels of TC in cholesterol-fed groups were changed in parallel to the levels of its composed fractions: HDL-C and LDL-C (Table 2). A significant difference in the serum TC levels of cholesterol-fed groups on 1, 2, 3 and 4 weeks was observed ($p_{ANOVA}<0.001$; Figure 2).

In comparison to NC group, the TGs also increased to a significant level ($p<0.001$) by 75% in cholesterol-fed groups. A significant difference in the serum TG levels of cholesterol-fed groups on 1, 2, 3 and 4 weeks was observed ($p_{ANOVA}<0.001$; Figure 2). With respect to NC group, AI also showed an increase of 24.0,

27.8 and 31.6 percent in HC, HC+1% SA and HC+3% SA groups, respectively ($p<0.05$; Table 2). The concurrent administration of EO of SA did not improve AI in rabbits and increased it by 3% percent in HC+3% SA compared to HC+1% SA groups ($p>0.05$; Table 2).

Discussion

The essential oil of *Salix aegyptiaca* L. did not improve lipid profile and possibly it was mildly atherogenic because AI increased following intake of SA despite the existence of lipid-lowering agents like eugenol and citronellol in EO of SA (Holmes & DiTullio, 1962; Germán et al., 1998). A possible explanation for lack of the anti-atherogenic effect of EO of SA related to its sedative, hypnotic and somnolent effects that lead to a sedentary behavior followed by increasing blood

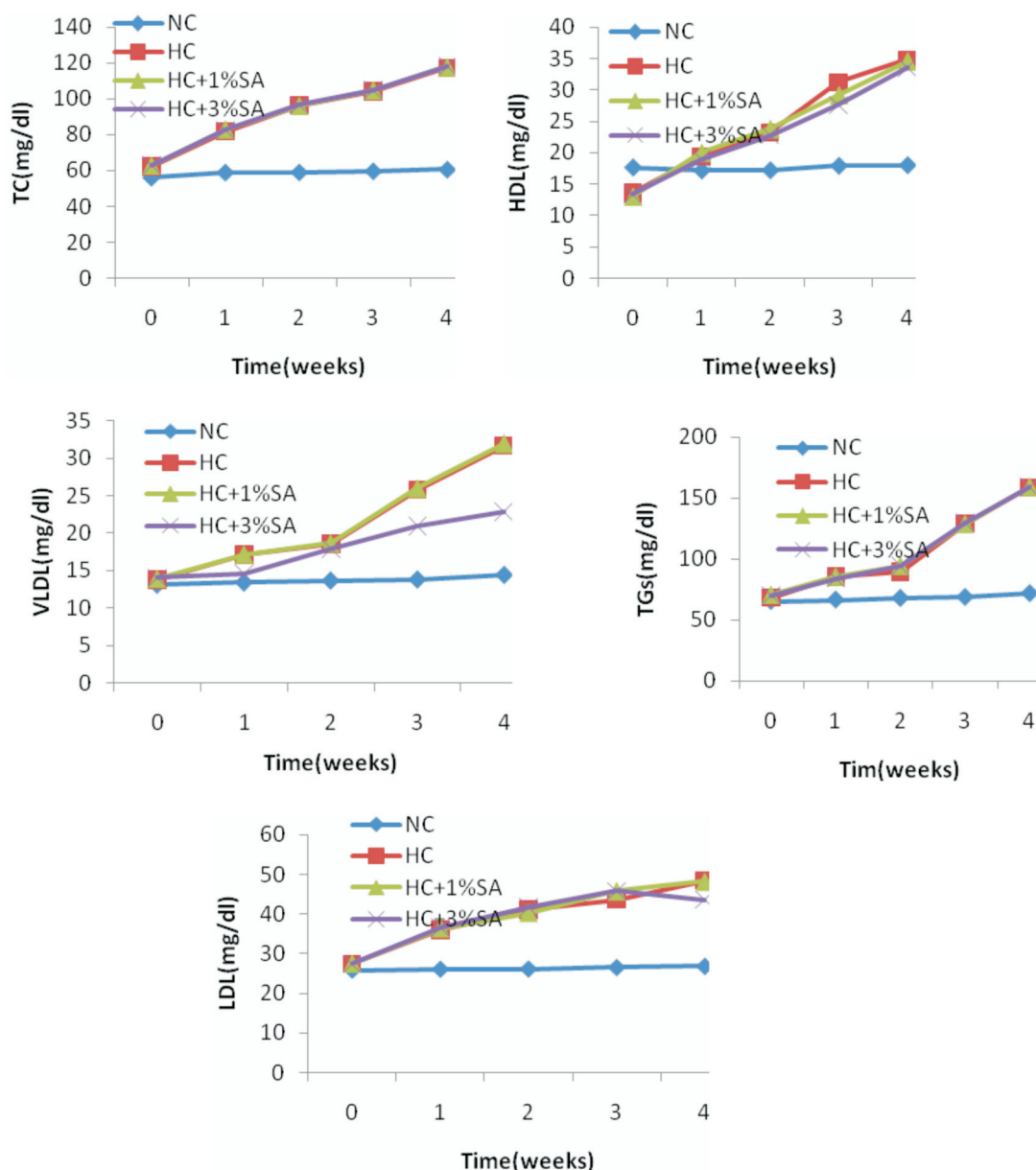


Figure 2. Lipid profiles at 0, 1, 2, 3 and 4 week of treatment with 1.0 and 3.0% essential oil of *Salix aegyptiaca* L. in HC+1% SA and HC+3% SA, respectively compare with Normal Control (NC) and Hypercholesterolemic Control (HC).

cholesterol and disturbing of lipid profile (Wijndaele et al., 2009).

We found higher amounts of DMB, citronellol, carvone and methyleugenol in EO of SA that isolated by *n*-hexane in comparison to the values reported by Babakhanlo et al., 1999. The amounts of dodecane, phenylethyl alcohol, tetradecane and eugenol were higher in our studied variety compared with the amounts that found in the EOs of SA from Research Institute of Forests and Rangelands, Iran (Babakhanlo et al., 1999). 1,4-Dimethoxybenzene also known as “hydroquinone dimethyl ether” is the para form of dimethoxybenzene, a

volatile aromatic ether with a sweet floral odor. It occurs naturally in willow (*Salix* spp.; Dötterl et al., 2005) and Zucchini (*Cucurbita pepo*; Mena Granero et al., 2005). The remarkable behavioral change that has been reported following intake of DMB in mice was somnolence as a general depressed activity (Sigma Aldrich, 2004). DMB known as poison by intraperitoneal route, moderately toxic by ingestion and as a skin irritant (Sigma Aldrich, 2004). DMB was identified as the major psychoactive chemical in musk willow in the present study.

Carvone, as a monoterpene was found naturally in caraway (*Carum carvi*) and dill (*Anethum graveolens*;

De Carvalho et al., 2006). Carvone, as a major volatile compound in different parts of *Anethum graveolens*, was reported in higher amounts (28-60%; Hajhashemi & Abbasi, 2008; Leung & Foster, 1996) compared with 6% in *A. aegyptiaca* in our study. Terpenoid compounds of EO of SA such as carvone may have potential of cholesterol-raising effect in long term intake. In this sense, cafestol and kahweol are lipid-raising diterpenes present in unfiltered coffee (Boekschoten, 2004). Another most active monoterpene of EO of SA, citronellal, also was shown to inhibit *in vitro* synthesis of cholesterol from mevalonate (Holmes & DiTullio, 1962). However, this monoterpene, when administered orally to rats (0.5 mg/kg.) for four weeks, had no effect on liver and serum cholesterol level (Holmes & DiTullio, 1962). Eugenol and eugenol acetate are two main components of EO extracted from *Melissa officinalis* leaves (Aliabadi et al., 2009). The myorelaxant, antispasmodic, antioxidant and hypolipidemic effects of eugenol have been reported (Germán et al., 1998; Lima et al., 2000; Lahlou et al., 2004). Eugenol as an antioxidant could inhibit LDL-C oxidation, thereby preventing atherosclerosis (Rajalakshmi et al., 2000; Teissedre & Waterhouse, 2000; Trevisan et al., 2006; Steinberg, 1995; Ito et al., 2005). LDL-C oxidation was increased in rabbit fed with HC diet for eight weeks (Özsoy & Pabuçcuolu, 2007). Low level of eugenol in the EO of SA seems to be insufficient to improve lipid profile e.g., level of cholesterol in the present study.

The result of this study support that EO of SA has not therapeutic and/or prophylactic effects against incoming dyslipidemia in rabbits. The EO of SA contains several serum lipid improving phytochemical ingredients such as eugenol and citronellol (Holmes & DiTullio, 1962; Germán et al., 1998). However, the other chemical ingredients in EO of SA might participate in the phenomena that hasten atherogenesis. In Iranian ethnomedicine, SA has been prescribed for ailments like cholecystitis and cholelithiasis may be due to inhibition of bile acid synthesis or antimicrobial effects of its active components like phenylethyl alcohol. Phenylethyl alcohol showed antibacterial activity against a wide range of bacteria such as *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Mycobacterium smegmatis* and *Mycobacterium phlei* (Fraud et al., 2003).

With respect to the lipid profile, our results suggested a time-dependent increase in the plasma TC level in all cholesterol-fed groups. This result is consistent with previous report that the TC increases with age (Chatterjea & Shinde, 2002). However, this increase in TC in *S. aegyptiaca*-treated groups could be mainly due to the lack of protective effects of the EO of SA and not due to age, since the TC was also increased as early as the first week of EO administration. A significant increase in plasma level of HDL-C was also observed.

This increase in HDL can solely be responsible for the observed increase in TC since the EO might be affecting the HDL metabolism in the liver (Eder & Gidez, 1982). In this experiment, 0.47% cholesterol load to the animals could generate hypercholesterolemia. Studies in both animals and humans have demonstrated that prolonged high cholesterol concentration in the circulating blood positively correlates with developing atherosclerosis (Pratico, 2001; Kurosawa et al., 2005). These changes are associated with the phenomenon that excessive load of cholesterol to the liver, above the acceptable level of its normal physiological limit, causes the liver to be unable in metabolizing the lipids, thus resulting in high cholesterol return in the circulating blood (Kushi et al., 1996). In this study, however, the treatment of high cholesterol fed animals concomitantly with EO of SA showed not protective effect against hypercholesterolemia and hypertriglyceridemia. Atherosclerosis is an inflammatory disease (Ross, 1999). The *Salix* spp. were dubbed for their anti-inflammatory effects both in traditional and orthodox medicines (Mahdi et al., 2006). However, the EO of SA in the current study did not prevent from cholesterol-induced atherogenesis because AI has not been improved following intake of EO of SA. It is widely known that both the liver and heart are at risk in patients with hypercholesterolemia. Hypercholesterolemic diet feeding induces oxidative stress to injure the liver and heart (Suanarunsawat et al., 2010).

Conclusion and perspectives

Administration of essential oil of *Salix aegyptiaca* L. in hypercholesterolemic rabbits could not prevent from occurring of dyslipidemia. More studies are needed to determine the mechanism(s) of effect of musk willow and its active ingredients on cholesterol and triglyceride metabolism in normal physiology. Because of the mixture of bioactive components present in the essential oil of musk willow, it is possible that more than one mechanism underlying this accrual effect on atherogenic index is involved. The investigation of the effects of essential oil of musk willow and its major compound, 1,4-dimethoxybenzene, in normocholesterolemic condition is our future endeavor.

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