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Chemical Composition and Hypolipidemic Effects of an Aromatic Water of *Ziziphora tenuior* L. in Cholesterol-fed Rabbits

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Abstract

Dyslipidemia is one of the major components of metabolic syndrome. The aims of this study were to determine whether aromatic water (AW) derived from *Ziziphora tenuior* L. (ZT) could improve lipid profile of cholesterol-fed rabbits. Control group received standard pellet and daily gavaged by 10 ml distilled water. Three other groups were fed 15% sunflower oil/0.47% cholesterol (HC) diet and concomitantly gavaged with 0, 1 and 3% v/w AW dissolved in 10 ml distilled water, respectively. HC diet increased the levels of total cholesterol (TC), triglycerides (TGs), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), very low density lipoprotein cholesterol (VLDL-C), atherogenic index (AI) and coronary heart disease index (CHD) in comparison to standard chew diet and none of treatments was able to restore the serum lipids to basal levels. However, inclusion of 3% AW of ZT declined the levels of TC, TGs, LDL-C, HDL-C, VLDL-C, AI and CHD more than those of 1% AW of ZT as compared with HC diet. The GC-MS analysis of hydrodistilled essential oil derived from ZT showed towenty-five known components. Pulegone, isomenthone, limonene, and 8-hydroxy-*delta*-4(5)-p-menthen-3-*one* were major compounds. In sum, AW of ZT and showed hypocholesterolemic and overt hypotriglyceridemic effects.

Keywords: *Ziziphora tenuior* L., Rabbit, Hypercholestremia, Hypertriglyceridemia, Pulegone.

INTRODUCTION

Metabolic syndrome (MetS) was considered as a leading risk factor for cardiovascular morbidity and mortality in the late 1990s and the early 21st century [1]. MetS represent an amalgamation of cardiometabolic risk determinants like obesity, glucose intolerance and insulin resistance, dyslipidemia, hypertension and more recently a growing list of clinical manifestations like polycystic ovarian syndrome, atherosclerosis, proinflammatory state, oxidative stress and non-alcoholic fatty liver disease has been associated to it [1]. In this context, atherogenic dyslipidemia is an integral component of MetS and a major contributor to the cardiovascular risks in patients [1].

Orthodox medicine offered different pharmacotherapeutic programs like common statin monotherapy to control and/or combat various features of dyslipidemia [2,3], however adverse effects of modern pharmacotherapeutic agents encouraged patients, pharmaceutical companies and researchers to seek more healthier drugs or alternative therapeutic programs. Non-drug approach to treating dyslipidemia suggests that canonical medicine still has many remedies and putative phytomedicines that need to be shortlisted.

Ziziphora tenuior L. (ZT) belongs to the Labiatae family which widely distributed in Iran [4]. In Iranian folk medicine, ZT (Kakuti in Persian) were used in maladies like fever, dysentery, metritis, pyometra, cholelithiasis, enteritis, flatulence, cough, bronchitis, common cold, infertility, rheumatoid arthritis, rickets, hemorrhage, and hypertension [5]. Ziziphora species are being used as aromatic herbs in Iranian culinary system. The leaves of ZT are used in commercial dairy drink "*doogh*" that produced in Iran because of their prokinetic and flavoring activity and its decoction has been used for different types of pain. *Ziziphora tenuior* L. and *Z. clinopodioides* Lam. are very popular as teapot herbs that sold in local markets and herbal stores in Iran [6]. Pulegone, menton, isomentone, neoisomenthone, x-pinene, piperitenone, and thymol are major components of Ziziphora species as reported previously [7].

The lack of scientific studies on the pharmacognostical properties of essential oil (EO) and/or aromatic water (AW) of ZT, the presence of hepatotoxic substances such as pulegone and its main metabolite, menthofuran, in EO of ZT, [8] and use of over-the-counter AW of ZT among Iranian led us to evaluate and consider lipid and lipoprotein profiles of cholesterol-fed rabbits as safety pharmacologic endpoints following intake of AW of ZT.

MATERIALS AND METHODS

Plant collection and authentication

The aerial parts of ZT were collected from wild ecosystem of Takhte Sartashtak (Kerman Province, Iran) at the flowering stage. Then they were air dried in the shade at room temperature and used for distillation. The *Ziziphora* species was authenticated by Dr Abbas Siami, Professor of Botany in Department of Biology, College of Science, Urmia University, Iran.

Preparation of essential oil

Dried aerial parts (1000 g) of plant was put into the distillation unit along with 300 ml water and the oil isolated by hydrodistillation method for 3.5 hours using a clevenger type apparatus to produce the oil with a yield of 0.9 (w/w%). The resulting oils were dried over anhydrous sodium sulfate and stored in sealed vials at low temperature before analysis. The resulting EO was diluted with distilled water to prepare 1% v/w.

Phytochemical analysis of essential oil

Qualitative and quantitative analyses of the oil were performed using GC-FID and GC-MS techniques. GC analysis was carried out using a Hewlett-Packard 5890 chromatography equipped with a FID detector and a HP-1 fused silica column (60m x 0.25mm, and film thickness of stationary phase 0.25µm). GC/MS analysis was carried out on a Hewlett-Packard 5793 connected to a mass detector HP 6890 using a HP-1 column (55m x 0.25mm, film thickness 0.25µm). To perform GC/FID and GC/MS, oven temperature programming was 40-250 °C with an increase of 3 °C/min. Injector and detector temperatures were 320 and 310 °C, respectively. Helium was the carrier gas and its flow was by the 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 of chemical compound

The identification of the compounds was based on a comparison of retention indices and mass spectra with those of authentic samples and with NIST MS library. The identification was also confirmed by comparison of the retention indices with data in the literature [9,10]. 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. Identification was based on sample retention data and comparison with authentic standards, computer matching using NIST MS library.

Experimental diets and animal subjects

Cholesterol (25 g) was dissolved in ethanol (175 ml) and mixed with sunflower oil (800 ml). The solution has been homogenized and mixed with standard pellet (Niro-Sahand Co. Tabriz, Iran) in the ratio of 15% v/w. The weekly re-pelleted 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.

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 received only standard pellet and daily gavaged by 10 ml distilled water served as negative control. Three other groups received above mentioned HC diet as follows: HC, HC+1% ZT and HC+3% ZT groups which received 0, 1 and 3% v/w EO dissolved in10 ml distilled water, respectively for four weeks. 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 reviewed and approved by the Laboratory Animal Care Committee of Urmia University, Western Azerbaijan, Iran.

Analytical procedures

The levels 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: VLDLC=TG/5 [11]. Atherogenic Index (AI) was calculated according to the following equation: AI=(TC-HDL-C)/HDL-C [12].

Statistical analysis

All data 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. 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. Results are reported as mean±SEM and statements of significance were based on $p<0.05$ unless otherwise noted.

RESULTS

Chemical composition of essential oil

The analysis of hydrodistilled EO derived from the aerial parts of ZT are given in Table 1. As presented in Table 1, the components are listed in order of their elution on the HP-1 column. In this regards, towenty-five known components representing about 95.5% of total composition of the EO while 4.4% of total composition was not identified and showed in Table 1 as "unknown" compounds. Pulegone (77.4%) was the main component identified, followed by isomentone (8.1%) and limonene (1.8%). Chromatogram of esential oil of ZT are shown in Figure 1. However, Figure 2 represents the mass spectrum of the major component, pulegone (1) in this EO compared with the mass spectra of the compound in NIST library which was used by GC-MS instrument. The compound has been assigned with high accuracy (upper than 97%). Comprehensive GC/MSa and GC/FID were used to analyze volatile compounds of ZT in this study.

Table 1. Volatile constituents identified in the essential oil of *Ziziphora tenuior* L. at the flowering stage

Compounds	RI*	Relative (%)
Isooctane		0.092
Heptane		0.005
3-Methylcyclopentanone	737	0.122
β-Pinene	895	0.255
β-Myrcene	914	0.067
Cymol	943	0.049
Unknown	947	0.238
1,8-Cineole	950	0.341
Limonene	954	1.839
cis-Limonene oxide	1031	0.110
trans-Limonene oxide	1035	0.274
D-Menthone	1045	0.196
Isomenthone	1055	8.120
cis-Isopulegone	1062	1.309
endo-Borneol	1064	0.145
4-Terpineol	1072	0.085
4,7-Menthano-5H-inden-5-*one*, octahydro	1089	0.431
Unknown	1102	1.137
Unknown	1105	0.205
Pulegone	1129	77.423
Unknown	1135	0.474
Unknown	1139	0.404
Unknown	1141	0.636
8-hydroxy-delta-4(5)-p-menthen-3-*one*	1158	1.340
p-Menth-4-*en*-3-*one*	1162	0.502
Piperitenone	1201	0.545
Unknown	1211	0.866
Menthofuranone	1339	0.101
Unknown	1380	0.483
Caryophyllene oxide	1428	0.110
Hexadecanoic acid	1781	0.311
9,12-Octadecadienoic acid	1897	0.713
9-Octadecenoic acid	1904	0.894
Octadecanoic acid	1922	0.180

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

Effects of essential oil extracted from Ziziphora tenuior L. on lipid metabolism

Body weight change and consumed calorie were not different in all groups of rabbits (data was not shown). Four weeks of HC diet feeding raised serum TC (35.8%), TGs (38.6%), LDL-C (35.8%), HDL-C (29.1%), VLDL-C (38.0%) and AI (17.2%) and CHD (12.8%) in comparison to NC group that fed normal standard chew diet ($p<0.05$; Table 2). All measured parameters have been increased in HC+1%ZT group in comparison to NC group that received normal chow diet ($p<0.05$; Table 2). In this context, the levels of TC (29.7%), TGs (27.7%), LDL-C (28.5%), HDL-C (22.7%), VLDL-C (27.7%), AI (17.2%) and CHD (12.8%) have increased in HC+1%ZT group compared with NC group ($p<0.05$; Table 2). However, 1% of AW of ZT could decrease TC (8.7%), TGs (15.0%), LDL-C (10.25%), HDL-C (8.3%), VLDL-C (14.2%) in comparison to HC group while the levels of AI and CHD remained unchanged in both HC and HC+1%ZT groups. The level of TGs is only showed significant decrease (15.0%) in HC+1%ZT group as compared with that of HC group ($p<0.05$; Table 2). The levels of TC (21.3%), LDL-C (21.8%), VLDL-C (23.5%), TGs (23.5%), HDL-C (15.0%), AI (14.2%), and CHD (10.5%) have been increased in HC+3% ZT group as compared with those of NC group. However, only TGs, TC, LDL-C, and VLDL-C showed significant differences in HC+3% ZT groups compared with NC group ($p<0.05$; Table 2). The inclusion of EO (3%) in HC diet decreased the levels of TC (18.4%), LDL-C (17.9%), VLDL-C (23.5%), TGs (19.8%), HDL-C (16.6%), AI (3.4%), and CHD (2.5%) in HC+3.0% ZT group as compared with HC group (Table 2). However, TGs, TC, and LDL-C showed significant differences in HC+3.0% ZT group compared with HC group ($p<0.05$; Table 2). A significant difference in the serum TGs, HDL-C, LDL-C, VLDL-C, TC, AI, and CHD levels of cholesterol-fed groups on 1, 2, 3 and 4 weeks was observed ($p_{ANOVA}<0.05$; Figure 3).

Table 2. The effects of *Ziziphora tenuior* L. on lipid profile and predictors of cardiovascular risk in cholesterol-fed rabbits

Group (n=5)	TG	HDL-C	VLDL-C	LDL-C	TC	AI	CHD
NC	65(2.5)†	17(0.6)†	13(0.5)†	25(0.7)†	59(1.0)†	2.4(0.1)†	3.4(0.1)†
HC	106(6.9)*	24(1.6)*	21(1.3)*	39(1.5)*	92(3.9)*	2.9(0.1)*	3.9(0.1)*
HC+1%ZT	90(3.8)*†	22(1.2)*	18(0.7)*	35(1.2)*	84(2.7) *	2.9(0.1)*	3.9(0.1)*
HC+3% ZT	85(2.8)*†	20(1.0)	17(0.5)*	32(0.9)*†	75(2.1) *†	2.8(0.1)	3.8(0.1)

Note: NC group received standard pellet and daily gavaged by 10 ml distilled water. Three other groups received high cholesterol (HC) diet as follows: HC, HC+1% ZT and HC+3% ZT groups which received 0, 1 and 3% v/w EO dissolved in 10 ml distilled water, respectively for four weeks. Values are mean±SEM (n=5);*Data in columns were significantly different at $p<0.05$ compared to NC; †Data in columns were significantly different at $p<0.05$ compared to HC diet fed control. AI: atherogenic index, CHD: coronary heart disease index.

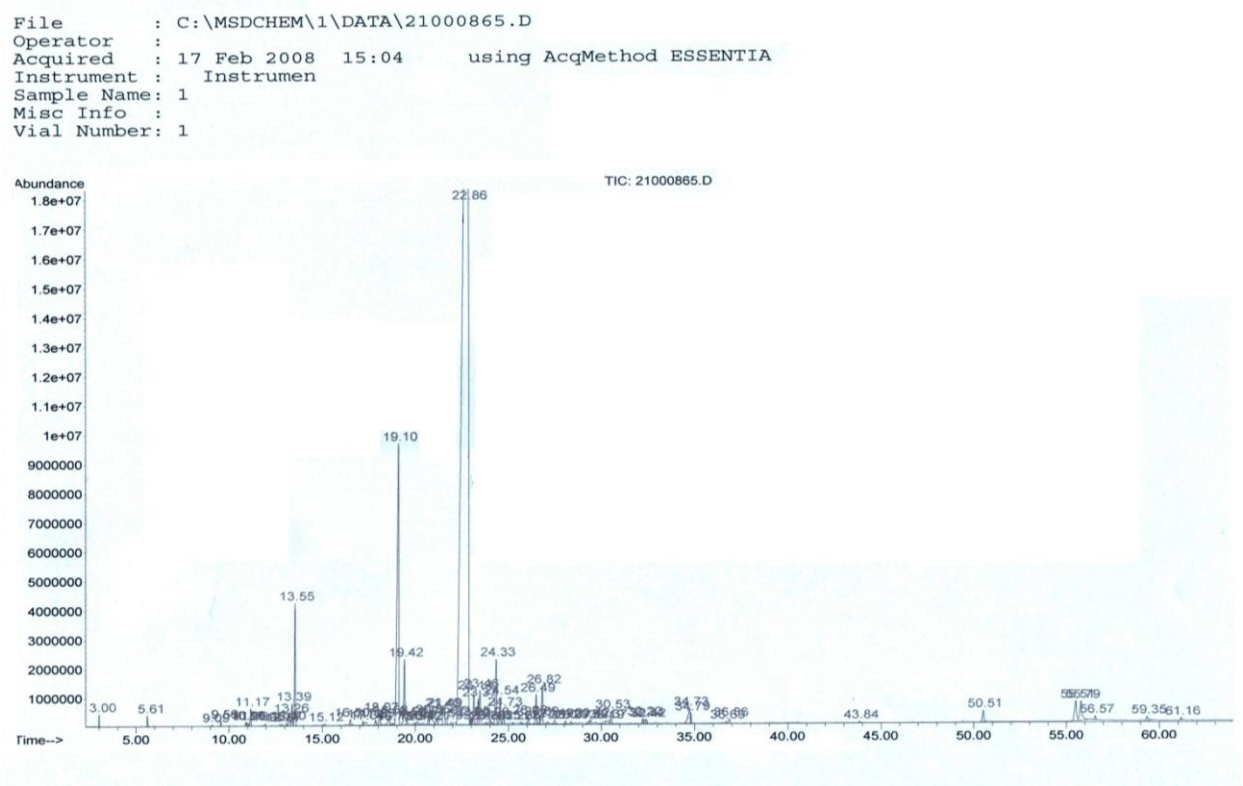

Figure 1. GC chromatogram of the essential oil extracted from aerial parts of *Ziziphora tenuior* L by hydrodistillation. The main compounds represented from left to right on the chromatogram are limonene, isomentone , cis-isopulegone, pulegone, and 8-hydroxy-*delta*-4(5)-p-menthene -3-*one* respectively.

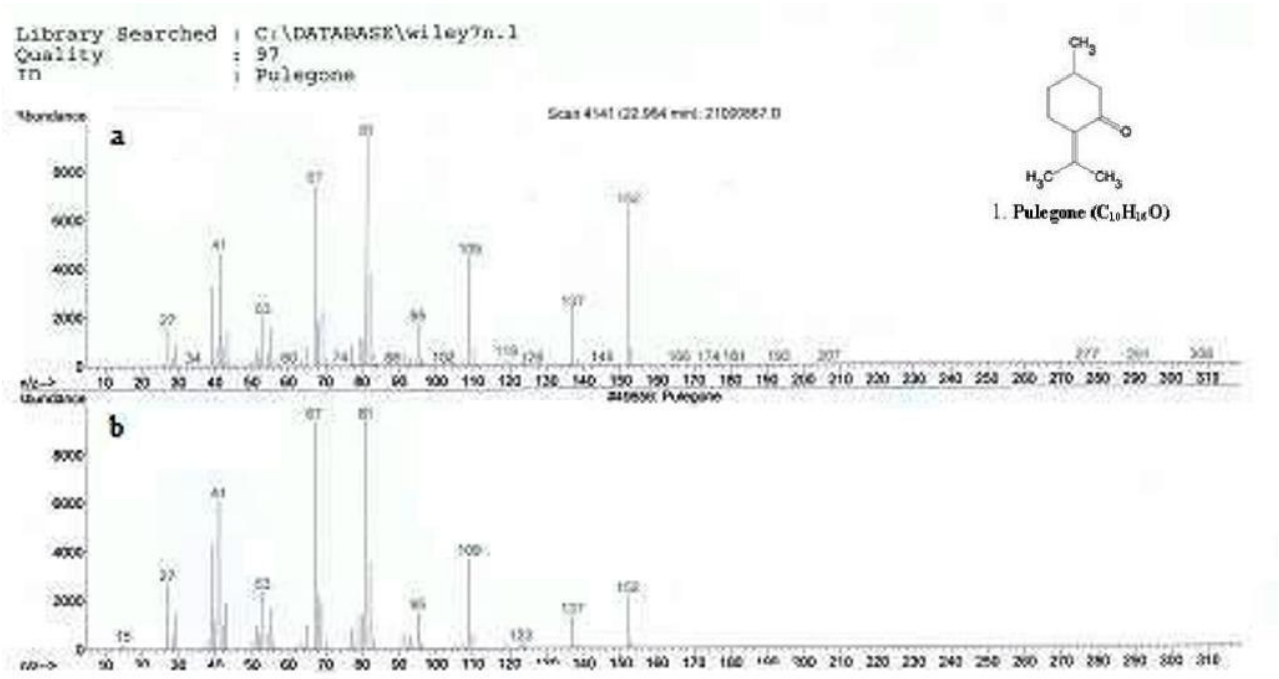

Figure 2. a: Mass spectrum of pulegone that detected in the essential oil extracted from aerial parts of *Ziziphora tenuior* L., b: mass spectrum of pulegone in NIST library which was used by GC-MS instrument.

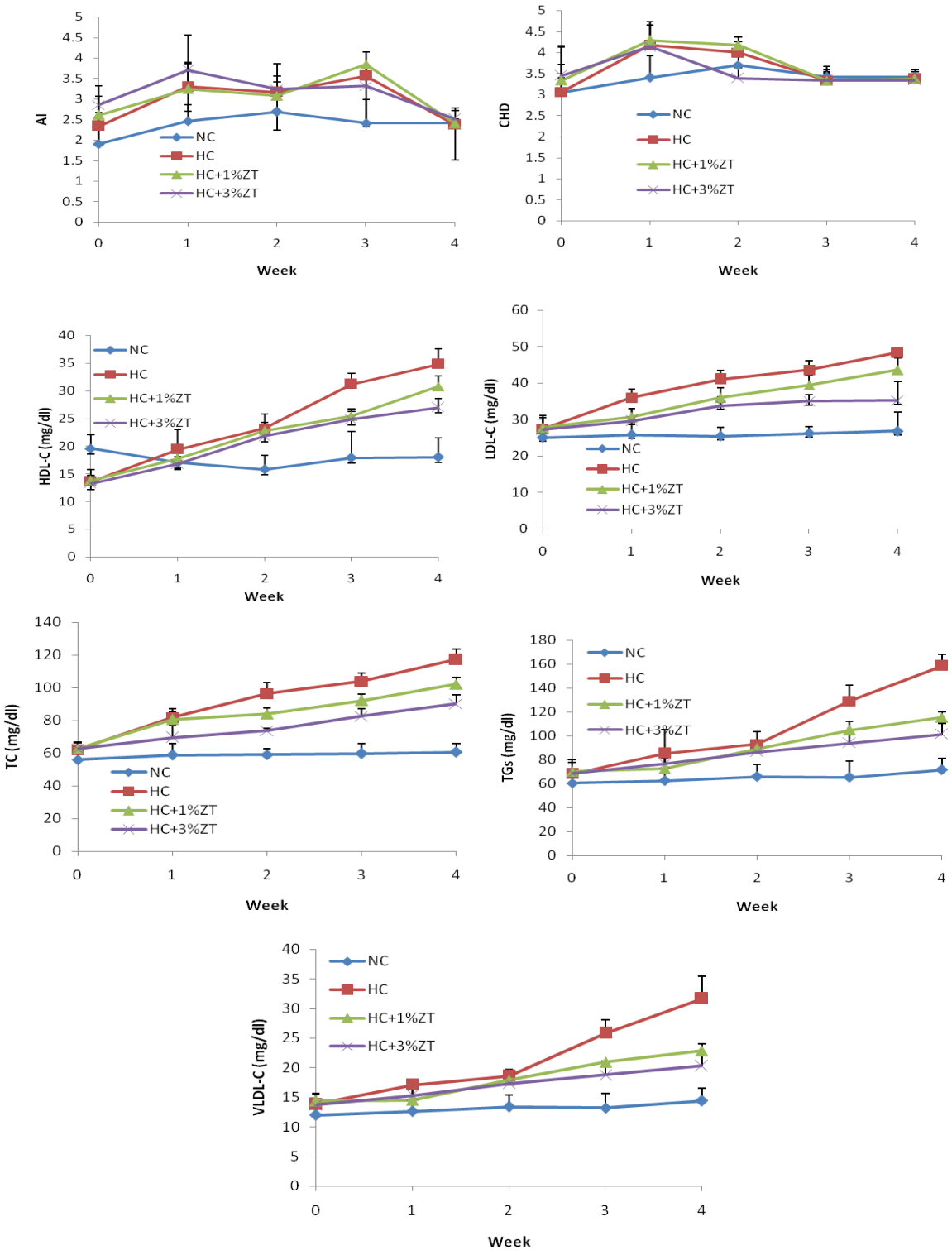

Figure 3. Lipid profiles at 0, 1, 2, 3 and 4 week of treatment with essential oil of *Ziziphora tenuior* L. Note: NC group received standard pellet and daily gavaged by 10 ml distilled water. Three other groups received high cholesterol (HC) diet as follows: HC, HC+1% ZT and HC+3% ZT groups which received 0, 1 and 3% v/w EO dissolved in 10 ml distilled water, respectively for four weeks.

DISCUSSION

In the present experiment, cholesterol load caused mild hyperlipidemic or pre-atherosclerotic condition in rabbits because 28 days of HC diet feeding raised both lipidemic indices like TC, TGs, LDL-C, HDL-C, VLDL-C and atherosclerotic indices like AI and CHD when compared with those of NC group that fed normal standard chew diet. This accrual impact of cholesterol load on lipid profile confirms that our laboratory's model is well translated and reliable for short-term investigations [13,14]. Our results showed that 15% sunflower oil/0.47% cholesterol (HC) diet could increase the levels of TC, TGs, LDL-C, HDL-C, VLDL-C, AI and CHD in comparison to standard chew diet. The cholesterol added to the chow diet induced hypercholesterolemia and hypertriglyceridemia in HC group relative to control animals and none of treatments was able to reduce the serum lipids to basal levels.

The increase of HDL-C in parallel to LDL-C and VLDL-C in HC group may be related to high content of unsaturated fatty acids (oleic-and linoleic acid) that found in sunflower oil [15]. Aguilera et al [15] have also shown that administration of sunflower oil to atherosclerotic rabbits provokes a significant progression of the atherosclerotic lesion development in male New Zealand rabbits that were fed for 50 days on a diet containing 3% lard and 1.3% cholesterol. To the best of our knowledge, this is the first report about inducing pre-atherosclerotic condition in rabbits fed a combination of sunflower oil and cholesterol diet. Van and Zilversmit [16] reported some kind of overt hypertriglyceridemia and hypercholesterolemia in rabbits fed a14% coconut oil/0.5% cholesterol (CNO/Chol) diet [16]. These authors concluded that the hypertriglyceridemia and the enhanced hypercholesterolemia in the CNO/Chol rabbit results primarily from increased hepatic secretion of VLDL-C and a modest decrease in VLDL-C triglyceride clearance capacity. Further studies are needed to explore the causes of hypertriglyceridemia and hypercholesterolemia that occurred in rabbits fed HC diet in the present study.

The impact of AW of ZT did not show an overt dose-dependent response since there were no significant differences between two HC+1%ZT and HC+3%ZT groups regarding their lipid profile. However, inclusion of 3% AW of ZT declined the levels of TC, TGs, LDL-C, HDL-C, VLDL-C and atherosclerotic indices like AI and CHD more than those of 1% AW of ZT as compared with the amounts of these parameters in cholesterol-fed group. In this sense, the decrement of TC, LDL-C and VLDL-C in 3% AW oil of ZT was significant compared to those of cholesterol-fed group.

We hypothesize that hypocholesterolaemic effects of AW of ZT may attribute to its ability to inhibit cholesterol absorption from the intestine, to upregulate LDL receptor and increased fecal excretion of cholesterol and bile acids, to decrease in cholesterol synthesis throughout inhibition of pivotal enzymatic reactions involving biosynthesis of cholesterol. These hypothetical mechanisms for hypocholesterolemic effects of AW of ZT must be investigated in future studies.

Both 1 and 3% doses of AW of ZT showed a significant hypotriglyceridemic effects compared with cholesterol-fed group. Several hypotheses would be proposed concerning hypotriglyceridemic effects of AW of ZT. For example, AW of ZT may elicit hypotriglyceridemic effects by inhibiting hepatic lipogenesis through reducing levels of sterol receptor element binding protein-1c (SREBP-1c), enhancing fatty oxidation in the liver and skeletal muscle through peroxisome proliferator-activated receptors (PPARs) activation, and upregulating flux of glucose to glycogen through downregulation of hepatocyte nuclear factor-4alpha (HNF-4alpha). The net result is the repartitioning of metabolic fuel from triglyceride storage toward oxidation, thereby reducing the substrate available for VLDL synthesis. These mentioned combined effects have been reported for use of omega-3 fatty acids as a valuable clinical tool for the treatment of hypertriglyceridemia [17].

The major known components found in EO of ZT were pulegone, isomenthone, limonene, and 8-hydroxy-delta-4(5)-p-menthen-3-one. The present study and a huge body of literature showed that pulegone is the major component of EO of *Ziziphora* spp [18-21]. Pulegone and its metabolite, menthofuran, has been known as hepatotoxin that lead to some kind of massive centrilobular necrosis, pulmonary oedema and internal haemorrhage [22,23]. These adverse effects have been not observed in internal organs of ZT-treated groups in the present study (data not shown) because of the low level of pulegone that found in the used AW. However higher levels of pulegone in comparison to the level of other lipid-lowering compounds found in AW of ZT like limonene [24] may need more investigations to find reasons why lipid profile has been improved after intake of AW of ZT.

It is well known that the hepatotoxicity and cardiomyopathy of blood lipid-lowering agents, such as statins and fibrates, limits their clinical application. It is interesting that administration of AW of ZT for 4 weeks obviously exerted a hypolipidemic effect. These preliminary data imply that AW of ZT probably is safe for use in regulating lipid although the further evaluation on preclinical safety needs to be carried out.

CONCLUSION

In summary, the present findings suggest that administration of AW of ZT in cholesterol-fed rabbits could prevent occurring dyslipidemia and atherosclerosis since both lipid profile and atherosclerotic indices like AI and CHD were positively improved. Further investigation needs to be performed in order to investigate the strict mechanism and therapeutic potential of AW of ZT and its major components in treating hyperlipidemia and the related cardiovascular diseases.

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