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The effect of essential oils of dietary wormwood (*Artemisia princeps*), with and without added vitamin E, on oxidative stress and some genes involved in cholesterol metabolism

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Abstract

Wormwood (*Artemisia princeps*) due to the abundance of antioxidant in its essential oils (EO), has been used as a traditional drug and health food in Korea. Oxidative stress plays an important role in the etiology of atherosclerosis thus antioxidative chemicals improves hepatic lipid metabolism partly by reducing oxysterol formation. The antioxidant activity was assessed using two methods, human low-density lipoprotein (LDL) oxidation and the anti-DPPH free radical assays. It was found that the antioxidant activity of EO with vitamin E higher than EO alone. To study mechanisms accounting for the antiatherosclerotic properties of this wormwood EO, we examined the expression of key genes in cholesterol metabolism such as the LDL receptor, the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and sterol regulatory element binding proteins. The induction was increased up to twofold at 0.05 mg/mL of EO treatment in HepG2 cells for 24 h. When EO (0.2 mg/mL) was co-incubated with vitamin E, interestingly, the LDL receptor was dramatically induced by 5–6-folds. HMG-CoA reductase did not change. However, treatment with the higher concentration resulted in cytotoxicity. Our data suggest that wormwood EO with vitamin E may be anti-atherogenic due to their inhibition of LDL oxidation and upregulation of the LDL receptor.

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Keywords: Antioxidant; Vitamin E; Cholesterol; Oxidative stress; Toxicity

1. Introduction

Wormwood (*Artemisia princeps*) is a popular aromatic herb that has been used as a flavoring agent and aromatic tonic. Wormwood is oriental medicinal plant widespread in Korea and China. The medicinal plant wormwood and the essential oils in wormwood, contain bioactive compounds such as phenolic compounds, alkaloids, vitamin A, B1, B2, C and various minerals (Hoffmann and Herrmann, 1982; Lee et al., 1999; Kim, 2003). Some studies suggest that the coumarine derivatives in wormwood protect liver function (Kimura et al., 1985; Gilani and Janbaz, 1993).

In this study, we selected wormwood essential oils (EO), with a high content in bioactive compounds (Kim, 2003),

Abbreviations: ANOVA, one-way analysis of variance; CYP7A1, cholesterol 7 α -hydroxylase; CYP27A1, sterol 27-hydroxylase; DEPC, diethylpyrocarbonate; DMEM, dulbecco's minimum essential medium; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ECL, chemiluminescent; EO, essential oils; FBS, fetal bovine serum; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low-density lipoproteins; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazole-2-yl)2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PEST, penicillin and streptomycin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SREBPs, sterol regulatory element binding proteins; TBARS, thiobarbituric acid relative substances; T-EDTA, trypsin-ethylenediaminetetraacetic acid.

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to investigate mechanisms of their potential atherosclerosis protective effects in the presence or absence of vitamin E, the main antioxidant which protects fatty acids from oxidation (Lowry et al., 1951). The oxidation of low-density lipoproteins (LDL) may play an important role in the development of atherosclerosis (Palinski et al., 1989; Witztum and Steinberg, 1991; Jialal and Devaraj, 1996; Shaikh et al., 1988). Animal and cell culture studies suggest that α -tocopherol (vitamin E) can prevent atherosclerosis (Brigelius-Flohé et al., 2002; Hathcock et al., 2005) and vitamin E in lipoprotein (mainly LDL) has been assumed to play a central role in reducing atherosclerosis by preventing lipid peroxidation (Brigelius-Flohé et al., 2002).

LDL is the major transport protein for lipoproteins containing apolipoprotein B and E, especially the cholesterol-rich LDL in human plasma. After binding to the receptors, LDL is internalized via receptor-mediated endocytosis and degraded in lysosomes (Brown and Goldstein, 1986) and the cholesterol is liberated for use in the synthesis of steroid hormones and new plasma membranes. Upregulation of liver LDL receptor expression is effective in treating hypercholesterolemia (Kong et al., 2006). Regulation of hepatic LDL receptor expression is thus of primary importance in controlling the plasma cholesterol and further suggests a reduced risk of cardiovascular disease.

Liver is the major gateway in human for LDL cholesterol exit. Cholesterol degradation to bile acids in the liver can be initiated by either cholesterol 7α -hydroxylase (CYP7A1) of the classic (neutral) pathway, or by mitochondrial sterol 27 -hydroxylase (CYP27A1) of the alternative (or acidic) pathway and they would be excreted from the body when not reabsorbed (Chiang, 2004).

Cholesterol synthesis is catalyzed by a group of microsomal enzymes including 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and reductase known to commit rate-limiting steps. They are regulated at the transcriptional level and their transcriptional regulation is controlled by a family of transcription factors known as sterol regulatory element binding proteins (SREBPs). Three forms of SREBP have been characterized, SREBP-1a, -1c, and -2. SREBP-1c retains some ability to stimulate cholesterol synthesis (Shimano et al., 1997). SREBP-1a and SREBP-2 were elevated by higher potential for cholesterol synthesis. The SREBPs also regulate the LDL receptor, which supplies cholesterol through receptor-mediated endocytosis.

The most potent cholesterol-lowering drugs, the HMG-CoA reductase inhibitors, exploit the feedback system by blocking cholesterol synthesis, thereby reducing the liver's content of cholesterol and increasing its production of LDL receptors (Brown and Goldstein, 1986; Goldstein et al., 1995).

In the first part of this study, we tested effects of LDL oxidation and free radical-scavenging activity by EO in the presence or absence of synthetic vitamin E (all-*rac*- α -tocopherol) and investigated the mechanisms of the prevention of atherosclerosis offered by wormwood EO in

the presence or absence of vitamin E. The transcription levels of the key genes were measured by real time RT-PCR and the amount of LDL receptor protein were measured by western blotting. The work was carried out on human liver HepG2 cells.

2. Materials and methods

2.1. Chemicals

Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), liquid gentamicin reagent solution, penicillin and streptomycin (PEST), and trypsin-ethylenediaminetetraacetic acid (T-EDTA) were purchased from Join Bio-Innovation (Seoul, Korea). Chemiluminescent (ECL) western blotting detection reagents and Hyperfilm™ ECL were obtained from Amersham-Pharmacia Korea (Seoul, Korea). Anti-Rabbit IgG, and H&L Chain Specific (Coat) Peroxidase Conjugate were purchased Calbiochem (Darmstadt, Germany). PowerScript reverse transcriptase was obtained from Clontech (Palo Alto, CA USA). The oligo(dT)₁₅ primer and random hexamers were obtained from Promega (Madison, WI USA). iQ™ SYBR® Green Supermix was obtained from Bio-Rad (Bio-Rad, Hercules, CA USA). All other reagents used were purchased from Sigma Chemical (St. Louis, MO USA). HepG2 cells were obtained from the Korean Cell Line Bank (Seoul, Korea).

2.2. Isolation of the essential oils

The EO in fresh plant wormwood was obtained by steam distillation. 100 g of fresh plant material was cut into small pieces and placed in a distillation flask with approximately five times water. The distillation chamber was heated in a liquid paraffin bath at about 120 °C and allowed to boil until the distillation was completed. The distillate was collected in a separating funnel in which the aqueous portion was separated from the EO. The water (lower) layer was slowly drawn off until only the oil layer remained. This procedure was repeated until at least 20 mL of oil had been recovered. Remaining moisture in the separations was removed by MgSO₄ and then was filtered. Ethyl ether was added to the samples and the ethyl ether was removed under a rotary vacuum evaporator. Through this process only a very small amount of essential oil was obtained and the EO was determined by gas chromatograph–mass spectrometer (GC/MS). Analysis was carried out on Hewlett–Packard gas chromatograph (GC-5890A, ES Inc., Cleveland, OH, USA) equipped with a flame ionization detector and a DB-5 column (60 m × 0.32 mm × 0.25 μ m film thickness). Nitrogen was used as a carrier gas, and the temperature of the injector and detector was 230 °C. Initial oven temperature was 50 °C for 5 min, and then increased by 4 °C/min to 200 °C. The mass spectrometer was a Jeol JMS-700 (Jeol USA Inc., Peabody, MA, USA), and Wiley 138 library (Gerstel, Inc., Helethorpe, MD, USA) was used to identify each peak.

2.3. Cell culture

HepG2 cells were seeded in 6-well Falcon plates at 1×10^6 cells/mL in DMEM supplemented with 10% FBS, 1% liquid gentamicin reagent solution, and 1% PEST. The cells were cultured at 37 °C in a humid atmosphere containing 5% CO₂ until 60–80% confluent and were then used for the real-time RT-PCR. For the cell viability assay, HepG2 cells were seeded in 24-well Falcon plates at 1×10^4 cells/mL and grown for 48 h. The culture medium was replaced on alternate days, and the cells were kept in medium free of serum and antibiotics.

2.4. Treatments

In the cultures used in the real-time RT-PCR assays, HepG2 cells were incubated in fresh DMEM with or without experimental additives. Cells were exposed to wormwood EO (0, 0.025, 0.05, 0.1, and 0.2 mg/mL) or vitamin E (0, 0.05, and 0.1 mM) for 24 h. They were also exposed to

wormwood EO (0.2 mg/mL) with vitamin E (0.05 or 0.1 mM) simultaneously for 24 h. Cells were treated with 0.5 or 0.6 mg/mL wormwood EO for 12 h and for 24 h (0.5 mg/mL) for DNA isolation.

In experiments examining the effects of wormwood EO on cell viability using the MTT[3-(4,5-dimethylthiazole-2-yl)2,5-diphenyltetrazolium bromide] assay, HepG2 cells were treated with 0–1 mg/mL wormwood EO in DMEM medium for 24 h. Vitamin E was applied as DL-all-rac- α -tocopherol (Sigma Chemical, St. Louis, MO USA; synthetic form; ~95%, determined by HPLC) and were dissolved in 100% ethanol to prepare stock solutions (0.1 M). We used two controls. The control for cells treated with EO only was untreated cells and control for vitamin E and wormwood EO with vitamin E (0.05 or 0.1 mM) was the cells treated with ethanol only (0.05% and 0.1% final concentration, respectively). The stock vitamin E (0.1 mM) was added to DMEM or DMEM including EO (added stock ethanol solution; 1/2000 and 1/1000 of the original DMEM, respectively).

2.5. Cytotoxicity test

Cell viability was measured using mitochondrial dehydrogenase activity assays with the substrate MTT according to the method as previously described (Chung et al., 2005).

DNA isolation was performed according to the TRI REAGENT (Sigma, St. Louis, MO USA) protocol. The extract DNA content was measured spectrophotometrically. Isolated DNA samples were electrophoresed on a 1.2% agarose gels at 90 V for 1 h and visualized with ethidium bromide. The DNA fragmentation was detected with UV light.

2.6. LDL isolation

Fresh human blood was collected at the Korea University Hospital. LDL was isolated from the serum according to the method previously described (Lee et al., 2003). The protein content of isolated LDL was determined using Bio-Rad protein kit (Bio-Rad, Hercules, CA USA) with bovine serum albumin (Sigma, St. Louis, MO USA) as a standard.

2.7. LDL-oxidation

The stock LDL fraction was dialyzed against the degassed PBS (pH 7.4) in the dark for 24 h. The dialysis solution was changed at least four times. Then, the dialyzed LDL was diluted to 600 mg protein/L with 0.01 M sodium phosphate buffer (pH 7.4). For the control incubation tubes, 30 μ L LDL (600 mg/L) was mixed with 5 μ L of 50 μ M CuSO₄ solution and 15 μ L of 0.01 M sodium phosphate buffer (pH 7.4), and incubated at 37 °C for up to 18 h. For the experimental incubation tubes, 30 μ L LDL (600 mg/L) was pre-incubated with 0.2 mg/mL wormwood EO in the presence or absence of vitamin E (0.05, 0.1 mM) for 5 min. Then, 5 μ L of 50 μ M CuSO₄ solution were added to initiate the oxidation, followed by incubation at 37 °C for up to 18 h. The oxidation was then stopped by addition of 2.5 μ L of 27 mM EDTA and cooled at 4 °C. The degree of LDL-oxidation was monitored by measuring the production of thiobarbituric acid relative substances (TBARS). In brief, the LDL-incubation tubes were immediately treated with 100 μ L ice cold 10% trichloroacetic acid to precipitate protein and were incubated for 15 min on ice. The incubation mixture was then centrifuged at 2200g for 15 min at 4 °C. Hundred microliters supernatant was placed into a new labeled screw top 1.5 mL tube and an equal volume of 0.67% (w/v) thiobarbituric acid was added. The mixture was then heated at 95 °C for 25 min, and then cooled on ice. TBARS were then determined by measuring the absorbance at 532 nm. The calibration was done using a malondialdehyde (MDA) standard solution prepared from 1,1,3,3-tetramethoxypropane. The value of TBARS was expressed as μ mol MDA/mg LDL protein.

2.8. DPPH free radical scavenging activity

The free radical scavenging capacity of wormwood EO in the presence or absence of vitamin E were analyzed using the 1,1-diphenyl-2-pic-

rylhydrazyl (DPPH), as previously described (Singh and Rajini, 2004) with minor modification. The percentage inhibition of DPPH was calculated using the following equation: Radical scavenging activity (%) = $(A_{\text{sample}(517 \text{ nm})}/A_{\text{control}(517 \text{ nm})}) \times 100$, where $A_{\text{sample}(517 \text{ nm})}$ is the absorbance of the sample and $A_{\text{control}(517 \text{ nm})}$ is the absorbance of the control at 517 nm.

2.9. Isolation of total RNA and real-time RT-PCR

Total RNA was extracted from cells using a TRI Reagent kit from Sigma (St. Louis, MO USA) according to the manufacturer's protocol and suspended in diethylpyrocarbonate (DEPC)-treated water. For cDNA synthesis, 2 μ g of total RNA was reverse transcribed using the PowerScript Reverse Transcriptase (Clontech) according to the Clontech Laboratories Protocol, using a combination of oilgo(dT)₁₅ primer and random hexamers. Real time RT-PCT was performed. PCR primers were designed using published nucleotide sequences for LDL receptor, HMG-CoA reductase, SREBP-1a, SREBP-1c, SREBP-2 from Skarits et al. (2003), and the sequences for 18 s rRNA are from Hasumi et al. (2003) and CYP7A1 is from Andreou and Prokipcak (1998).

The real-time PCR was performed by using 12.5 μ L iQ SYBR Green Supermix from Bio-Rad (Hercules, CA USA), 0.5 μ L of each primer (15 μ M), 1 μ L of cDNA and 10.5 μ L sterile water. The final volume of the reaction mixture was 25 μ L. Real-time PCR using the LDL receptor, HMG-CoA reductase, SREBP-1a, SREBP-1c, SREBP-2 templates was performed in one cycle of 3 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 62 °C and 30 s at 72 °C. A final extension was carried out for 5 min at 72 °C. Real time PCR using the template 18 s rRNA and CYP7A1 was performed similarly, except different annealing temperature (18 s, 30 s of 60 °C; CYP7A1, 30 s of 57 °C) were used. Following amplification, a melting curve of amplified DNA was analyzed at temperatures between 54 and 95 °C with a heating rate of 0.2 °C per second. All real-time PCR were performed in iCycler iQ (Bio-Rad, Hercules, CA USA). During the primer extension step, the increase in the fluorescence from the amplified DNA was recorded by using the SYBR Green optic channel set at a wavelength of 490 nm. Data were collected and viewed using the iCycler iQ optical system software version 3.1 (Bio-Rad, Hercules, CA USA).

The primers were LDL receptor: F, 5'-CAATGTCTCCCAAGCTCTG, and R, 5'-TCTGTCTCGAGGGGTAGCTG; HMG-CoA reductase: F, 5'-TACCATGTCAGGGGTACGTC, and R, 5'-CAAGCCTAGAGACATAATCATC; SREBP-1a: F, 5'-CTGCTGACCGACATCGAAGAC, and R, 5'-GATGCTCAGTGGCACTGACTCTTC; SREBP-1c: F, 5'-CGGAGCCATGGATTGCACTTTC, and R, 5'-GATGCTCAGTGGCACTGACTCTTC; SREBP-2: F, 5'-CCCTTCAGTGCAACG-GTCATTAC, and R, 5'-TGCCATTGGCCGTTTGTGTGTC; CYP7A1: F, 5'-GCATCATAGCTCTTTACCCAC, and R, 5'-GGTGTTCGCA-GCAGTCCTGTAAT. The 18 s rRNA (F, 5'-CGGCTACCACATCCAAGGAA and R, 5'-GCTGGAATTACCGCGGCTGC) transcripts were used as internal controls.

2.10. Western blotting

The cells were lysed in a buffer containing 10 mM Tris-HCl pH 7.4, 0.1 M EDTA, 10 mM NaCl, 0.5% Triton X-100 and one protease inhibitor Cocktail tablet at 4 °C. The lysate was clarified by centrifugation at 14,000 rpm for 10 min at 4 °C. The protein concentration was determined using a Bio-Rad protein kit (Bio-Rad, Hercules, CA USA) with bovine serum albumin (Sigma, St. Louis, MO USA) as a standard. Equal amounts of protein were boiled in a sample buffer (5% β -mercaptoethanol) for 5 min. Samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were blotted onto a nitrocellulose membrane [(0.45 μ M), Schleicher & Schuell BioScience PROTRAN Nitrocellulose Transfer Membrane]. Nonspecific protein binding sites were blocked by incubation in phosphate buffered saline (PBS) pH 7.4; 0.1% Tween 20% and 5% skimmed milk. To examine LDL-receptor expression the samples were incubated with an anti-LDL receptor

antibody 1/2000 (The antibody was a gift from Dr. Allen Cooper). After washing several times with PBS-0.1% Tween 20, the membrane was incubated with 1/5000 Anti-Rabbit IgG, H&L Chain Specific (Goat) Peroxidase Conjugate secondary antibody (Calbiochem, Darmstadt, Germany). Immunoreactive bands were detected by a ECL western blotting detection reagents (Amersham-Pharmacia Korea, Seoul, Korea) and exposed to high performance chemiluminescence film for 10 s. Protein immunoblots were scanned by 690 Bio-Rad Densitometer using the Multi-Analyst program (Bio-Rad, Hercules, CA USA). The density of each band was quantified using Sigmagel software (Jandel Scientific, San Rafael, CA USA).

2.11. Statistical analyses

Each experiment was repeated at least three times. One-way analysis of variance (ANOVA) followed by Tukey's test was used to compare the results from different treatments. The Student's *t* test was used for comparisons between two groups. Statistical significance was indicated by *p*-values < 0.05. Data are reported as the mean \pm SD.

3. Results

3.1. Chemical composition of the essential oil

EO content of wormwood was 46.2 ± 2.2 mg/100 g. The chemical composition of wormwood EO was analyzed by GC-MS and the result are shown in Table 1. Eighteen compounds were identified and the main constituents were 1,8-cineole (20.1%), *trans*-caryophyllene (16.3%), 1-limonene (12.6%), phenol (7.5%), and camphene (7.5%).

3.2. Cytotoxicity by high concentration essential oil in wormwood

Clear cytotoxic effects were observed in cell viability and DNA fragmentation in high concentration EO treated-cells (Fig. 1a and b). No toxic effects were found when the oils were treated up to 0.4 mg/mL EO in HepG 2 cells for

24 h but the oil from wormwood was significantly toxic in 0.5 mg/mL-treated cells in the concentration dependent manner (Fig. 1a). After treatment of 1 mg/mL EO from wormwood, we observed about a 90% decrease of cell survival (Fig. 1a). Cells treated with EO (0.5 or 0.6 mg/mL) displayed the characteristic formation of a DNA ladder due to endonuclease activity during apoptosis (Fig. 1b, lanes 3–5). EO-induced cytotoxicity involves oxidative stress (Bakkali et al., 2005).

Thus, to avoid any possible cytotoxic effect, the concentration of 0.025–0.2 mg/mL wormwood EO was selected in following experiments.

The oxidative modification of LDL is considered to be a key step in the development of atherosclerosis, and antioxidants are possibly able to prevent atherosclerosis because inhibition of LDL oxidation by the antioxidant should lead to an inhibition of early atherogenic events (Steinberg et al., 1989; Chu and Liu, 2005).

Thus, we tested antioxidant activity of EO in the presence or absence of vitamin E by DPPH free radical scavenging activity and human LDL oxidation. Radical scavenging activity of 0.2 mg/mL wormwood EO in the presence of vitamin E (0.05, 0.1 mM) was significantly higher than those treated with oil or vitamin E alone (Fig. 2a). The present study examined the protective effect of 0.2 mg/mL wormwood EO in the presence or absence of vitamin E against Cu²⁺-mediated LDL oxidation. TBARS was used as an index of LDL oxidation. As shown in Fig. 2b, all the samples tested could inhibit LDL oxidation up to 18 h and the inhibitory effect of 0.2 mg/mL wormwood EO in the presence of vitamin E were more effective than EO alone at 3 h and 18 h (Fig. 2b). Incubation of LDL with 0.2 mg/mL wormwood EO in the presence of vitamin E (0.05, 0.1 mM) showed an additive protection against LDL lipid oxidation than vitamin E alone at 3 h but inhibition of LDL oxidation was not affected by wormwood EO in the presence of vitamin E (0.05, 0.1 mM) compared to vitamin E alone at 8 h. Wormwood EO in the presence of vitamin E (0.05, 0.1 mM) resulted in lower protection against LDL oxidation than vitamin E alone for 18 h. Inhibitory effect of LDL oxidation by wormwood EO in the presence of vitamin E was reduced in a time-dependent manner.

3.3. Effect of wormwood EO and wormwood EO with vitamin E on the key genes in cholesterol metabolism (LDL receptor, HMG-CoA reductase, SREBP-1a, SREBP-2 and SREBP-1c)

Treatment with 0.025, 0.05 and 0.1 mg/mL concentration wormwood EO, respectively, resulted in increased LDL receptor mRNA levels (Fig. 3a) but 0.2 mg/mL wormwood EO had no effect on LDL receptor mRNA levels when compared with untreated cells.

While we failed to induce LDL receptor mRNA at 0.2 mg/mL, we found that a concentration of 0.2 mg/mL with 0.05 or 0.1 mM vitamin E significantly increased

Table 1
Identification of chemical components in the essential oils of wormwood (*Artemisia princeps*)

No.	Retention time (min)	Compounds	Retention indices	%
1	3.56	1,8-Cineole	116	20.1
2	5.12	<i>Cis</i> -sabinene hydrate	192	5.0
3	6.35	(-)- β -Pinene	274	2.5
4	7.46	Camphor	345	0.6
5	9.30	1-Limonene	449	12.6
6	10.13	Camphene	492	7.5
7	12.46	Endobornyl acetate	644	0.8
8	14.52	α -Cubebene	770	0.1
9	15.31	Benzene	809	0.2
10	15.46	Tetradecane	824	0.1
11	16.23	<i>Trans</i> -caryophyllene	861	16.3
12	17.20	α -Humulene	917	1.8
13	17.56	β -Bisabolene	953	0.3
14	18.20	Naphthalene	977	5.5
15	20.02	Phenol	1079	7.5
16	21.31	(-)-Caryophyllene oxide	1168	3.0
17	24.10	Heptadecane	1326	0.2
18	26.38	Octadecane	1474	0.5

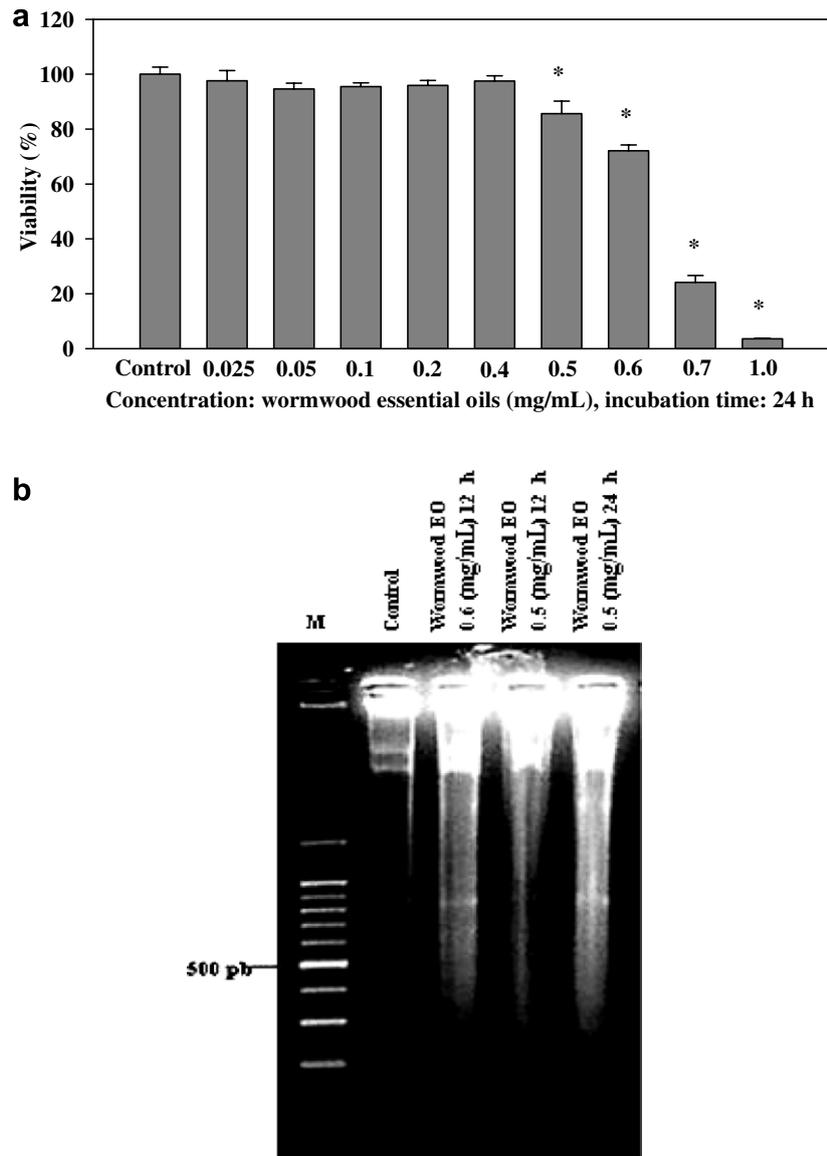


Fig. 1. Effect of essential oil in dietary wormwood on the cell viability (a) and DNA fragmentation (b). EO indicates essential oil in dietary wormwood (*Artemisia princeps*). * $p < 0.05$ vs. control. Data are the means \pm standard deviation ($n = 4$).

expression when compared with 0.2 mg/mL wormwood EO or vitamin E (0.05 or 0.1 mM)-treated cells alone (Fig. 3a) ($p < 0.05$). The concentration of 0.2 mg/mL wormwood EO with 0.05 or 0.1 mM vitamin E did cause a large increase in expression of LDL receptor mRNA (5.0, 5.4-fold higher than the control). Since wormwood EO with vitamin E was successful to inducing LDL-receptor mRNA in HepG2 cells (Fig. 3a), we investigated their effect on LDL-receptor protein. When the intensities of the bands were quantified and were analyzed by SigmaGel software (Jandel Scientific, San Rafael, CA), LDL-receptor protein levels in treated cells were all upregulated compared with untreated cells (Fig. 3b). Interestingly, a similar result in protein levels was obtained for 0.2 mg/mL wormwood EO with vitamin E-treated cells (Fig. 3b) compare with results of mRNA expression. The treatment of cells with wormwood EO in the presence of vitamin E consis-

tently resulted in LDL receptor protein levels that were significantly higher than in the treatments with vitamin E or the EO alone (Fig. 3b). 0.2 mg/mL wormwood EO in the presence of 0.1 mM vitamin E appears to be more effective than 0.2 mg/mL wormwood EO plus 0.05 mM vitamin E in increasing the LDL-receptor protein (Fig. 3b). These results suggested that the essential oil and vitamin E should have positive synergistic effects on the overexpression of the LDL-receptor.

No such effect was observed on HMG-CoA reductase mRNA, which remained at or close to control levels in all treatments containing wormwood EO in the presence or absence of vitamin E (Fig. 3a).

HepG 2 cells treated with 0.2 mg/mL wormwood EO for 24 h had similar levels of SREBP-1a, SREBP-2 and SREBP-1c mRNA when compared with untreated cells (Fig. 4a). However, SREBP-1c mRNA levels in HepG2

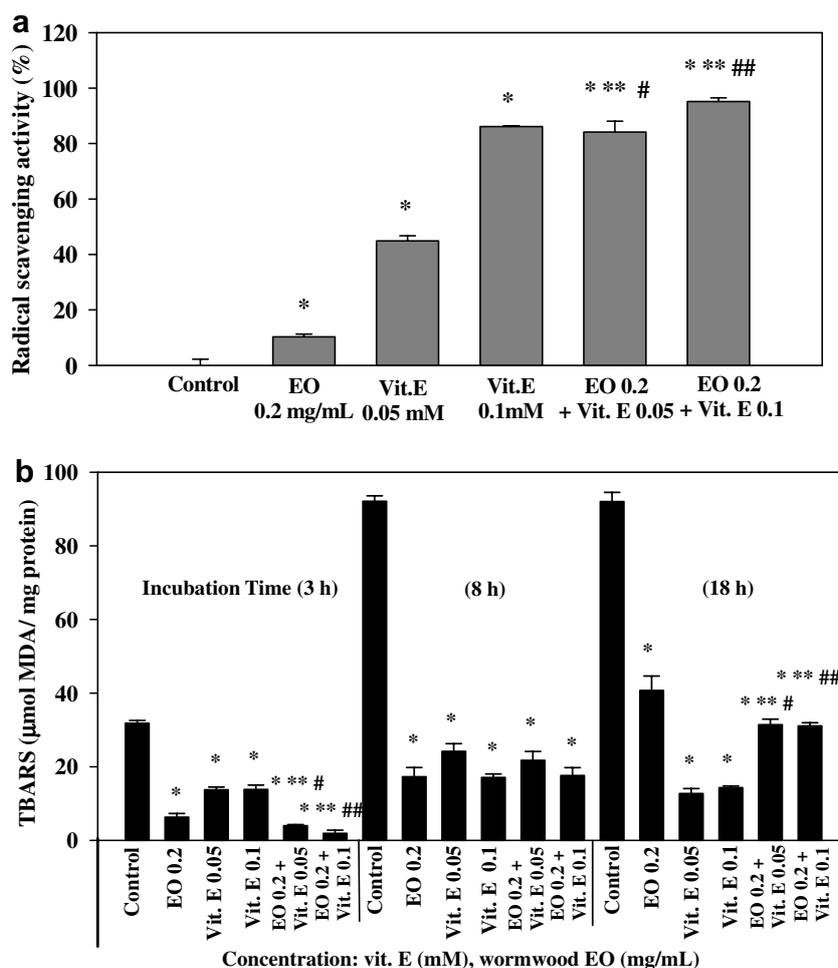


Fig. 2. Effect of essential oil in dietary wormwood in the presence or absence of vitamin E on radical scavenging activity (a), and on Cu^{2+} -mediated oxidation of human low-density lipoprotein (b). EO indicates essential oil in dietary wormwood (*Artemisia princeps*). * $p < 0.05$ vs. control; ** $p < 0.05$ vs. 0.2 mg/mL EO; # $p < 0.05$ vs. 0.05 mM vitamin E; ## $p < 0.05$ vs. 0.1 mM vitamin E. Data are the means \pm standard deviation ($n = 3$).

cells treated with 0.2 mg/mL wormwood EO plus vitamin E decreased (Fig. 4).

Our data suggests that wormwood EO with vitamin E may reduce more plasma cholesterol levels by the synergistic effects than wormwood EO may reduce alone. Cholesterol biosynthesis may not be significantly affected.

4. Discussion

Cytotoxicity was only seen at EO concentrations >0.4 mg/mL. We have shown that treatment of HepG2 with EO produces cytotoxicity as demonstrated by the increase of DNA fragmentation and an associated loss in cell viability. At a very high toxic concentration of wormwood EO, this oil appears to reduce LDL receptor and CYP7A1 mRNA levels (data not shown). Soderberg et al. (1996) and Sivropoulou et al. (1996) reported that the cytotoxicity of the essential oils against human cell. A clear cytotoxic effect was observed in *Artemisia herba alba* EO-treated cells and the oil clearly induced damage to mitochondrial DNA (Bakkali et al., 2005). The production by high concentrations of highly damaging agents such as

OH^{\cdot} and $\text{O}_2^{\cdot-}$ radicals and H_2O_2 , provides an explanation for the observed cytotoxic effects (Bakkali et al., 2005) and this result is in agreement with our results in high concentration EO-treated cells. We also suggested that atherosclerosis associated with oxidative stress and the down-regulation of LDL receptor may be one of the mechanisms in progression of atherosclerosis. Thus, it appears that at lower and probably more physiological concentrations of wormwood EO, humans may be protected against atherosclerosis. Thus, to avoid cytotoxicity from CA, concentration up to 0.2 mg/mL EO was chosen for most experiments.

The purpose of the present work was to investigate the effect of wormwood EO and its' synergetic effect in the presence of vitamin E on the expression of key genes in cholesterol metabolism using the human liver HepG2 cell line. In the early event of atherogenesis LDL oxidation is assumed to represent a characteristic mechanism (Parthasarathy et al., 1999; Chisolm and Steinberg, 2000). Daily antioxidants from several nutrients such as vitamin E, beta-carotene, phenols or flavonoids can inhibit the oxidative modification of low-density lipoproteins due to reduc-

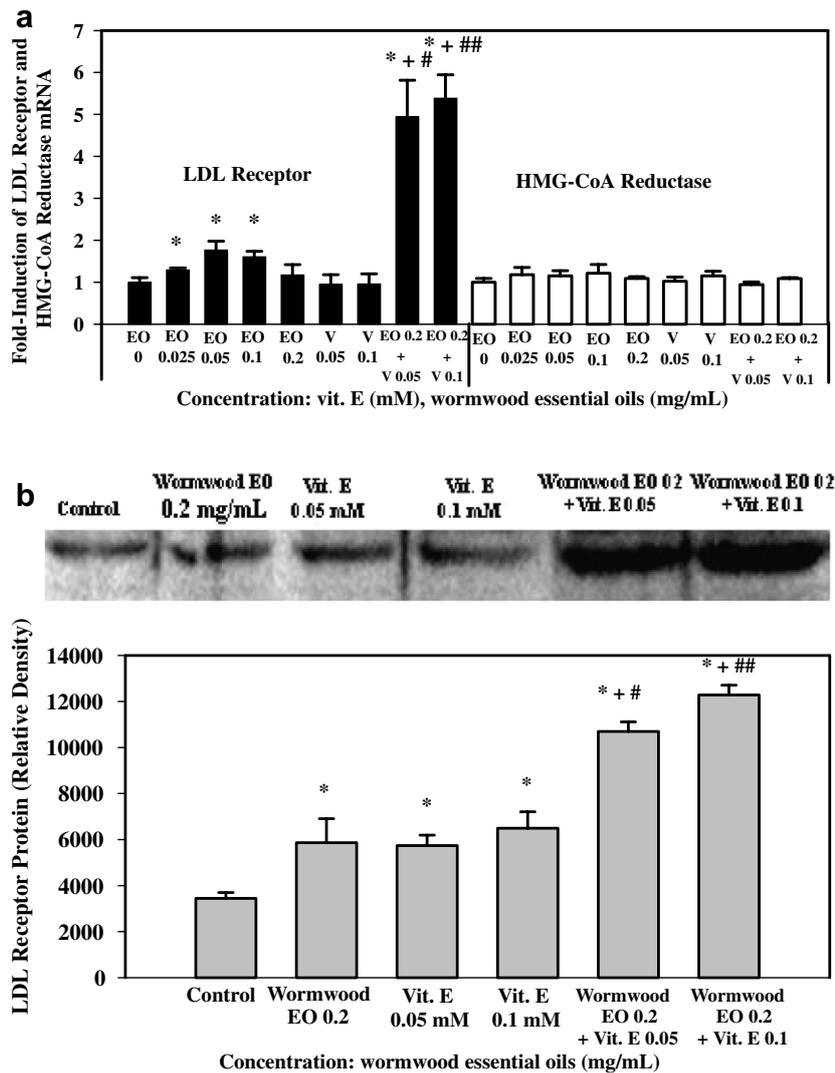


Fig. 3. Effect of essential oil in dietary wormwood in the presence or absence of vitamin E on the expression of LDL-R and HMG-CoA reductase mRNA expression (a) and on the expression of LDL-R protein (b). EO and V indicate essential oil in dietary wormwood (*Artemisia princeps*) and vitamin E, respectively. Cellular proteins were separated and identified using SDS-PAGE and western blotting (b). The density of each band of LDL-receptor protein was quantified using Sigmagel software (Jandel Scientific, San Rafael, CA). * $p < 0.05$ vs. control; + $p < 0.05$ vs. 0.2 mg/mL EO; # $p < 0.05$ vs. 0.05 mM vitamin E; ## $p < 0.05$ vs. 0.1 mM vitamin E. The data are the means \pm standard deviation ($n = 3$). The LDL receptor and HMG-CoA reductase mRNA levels in each sample were normalized to the quantity of 18 s rRNA (18 s). Fold-induction of target gene mRNA in real-time PCR result: d Threshold cycle (dCt) = (Ct of target gene mRNA) - (Ct of 18 s rRNA); ddCt = (dCt of mRNA in treated cells) - (dCt of mRNA in untreated cells); fold-induction = 2^{-ddCt} .

ing free radical production. This protective effect could possibly retard atherogenesis and in consequence avoid coronary problems (Mashima et al., 2001; Niki and Noguchi, 2002; Chu and Liu, 2005). So a sufficient maintenance of LDL with antioxidants should protect LDL from oxidation and in turn inhibit development of atherosclerosis.

The present results clearly showed that the essential oil mixed with vitamin E strongly increases DPPH free radical scavenging activity. Hypothetically, essential oil with vitamin E may function as a primary antioxidant by directly inhibiting the formation of oxidized LDL by synergistic effect. Our hypothesis was tested through protective effect of wormwood EO in the presence or absent of vitamin E against Cu^{2+} -mediated LDL oxidation. Wormwood EO

and vitamin E could prevent Cu^{2+} -mediated LDL oxidation. When comparing the inhibitory effects of LDL oxidation of 0.2 mg/mL wormwood EO in the presence of vitamin E (0.05, 0.1 mM) and vitamin E alone, the TBARS production of vitamin E plus EO significantly reduced after 3 h, showed no significant difference after 8 h but showed a significant increase after 18 h compared with vitamin E alone. These data suggested that the inhibitory effect of EO with regards to LDL-oxidation may be related to the content of antioxidants such as phenolic compounds in EO. The antioxidants in EO with added vitamin E may offer lipid stability of EO and LDL at the presence of CuSO_4 as an oxidation initiator for 3 h. However, the inhibitory effect of EO plus vitamin E with regards to

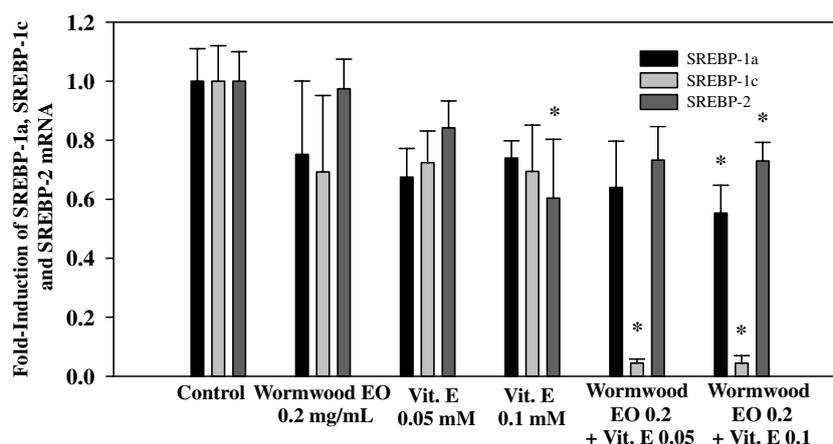


Fig. 4. Effect of essential oil in dietary wormwood in the presence or absence of vitamin E on the expression of SREBP-1a, SREBP-1c and SREBP-2 mRNA. EO indicates essential oil in dietary wormwood (*Artemisia princeps*). * $p < 0.05$ vs. control. Data are the means \pm standard deviation ($n = 3$). The SREBP-1a, SREBP-1c and SREBP-2 mRNA levels in each sample were normalized to the quantity of 18 s rRNA (18 s). Fold-induction of target gene mRNA in real-time PCR result: d Threshold cycle (dCt) = (Ct of target gene mRNA)-(Ct of 18 s mRNA); ddCt = (dCt of mRNA in treated cells) – (dCt of mRNA in untreated cells); fold-induction = 2^{-ddCt} .

LDL-oxidation may be not strong enough to control lipid oxidation by oxidant up to 8 h. The inhibitory effect of vitamin E on lipid oxidation rapidly decreased during incubation for 18 h, causing the lipid oxidation that occurred in wormwood EO treated vitamin E. α -tocopherol is well known for its' ability to protect LDL from oxidation and particularly for its' synergistic effects (Esterbauer et al., 1991; Thomas et al., 1995; Upstone et al., 1999; Schneider and Elstner, 2000; Kaikkonen et al., 2000; Raitakari et al., 2000). The flavonoids have been shown to possess good antioxidative capacity from *in vitro* and *in vivo* studies (Harborne and Williams, 2000; Pietta, 2000) and essential oils are known on the high antioxidative properties due to include flavonoids and other antioxidants (Graßmann et al., 2000, 2001). Essential oils are complex mixtures deriving from secondary metabolism within the plant. They are extracted from specific plant organs such as leaves and peels, and possess various pharmacological activities. It appears that the observed radical scavenging properties of the essential oil might contribute positive effects to the defense of the plant. Due to the high antioxidant content of the essential oil, potent antioxidant capacity against *in vitro* LDL oxidation may be recognized for its' potential prevention of atherosclerosis.

In our results, essential oil and essential oil mixed with vitamin E could increase the synthesis of hepatic LDL receptors for an enhanced uptake of plasma cholesterol into the hepatocytes (Brown and Goldstein, 1986; Chu and Liu, 2005). The up-regulation of LDL receptors increases the uptake of cholesterol into the hepatocytes through receptor-mediated endocytosis (Brown and Goldstein, 1986; Chu and Liu, 2005) and the result could enhance clearance of excessive plasma cholesterol in the blood. Similarly, Chu and Liu (2005) reported increased LDL receptor in antioxidant-treated HepG 2 cells. Cranberries have inhibited *in vitro* LDL oxidation due to the

high antioxidant activities and cranberry extracts could significantly elevate synthesis of hepatic LDL receptors (Chu and Liu, 2005) and the up-regulation of LDL receptors has been widely used as therapeutic strategy (Brown and Goldstein, 1986). Our results suggest consumption of wormwood EO mixed with vitamin E may play an important role in the prevention of atherosclerosis.

We have shown that only 0.2 mg/mL EO plus 0.1 mM vitamin E (not 0.05 vitamin E) resulted in down regulation of the SREBP 1a and 2 mRNA level and a treatment of essential oil mixed with 0.05 or 0.1 mM vitamin E completely down-regulated the SREBP-1c mRNA level. SREBP-1c enhances fatty acid synthesis and mRNA levels for SREBP isoforms 1a and 1c are decreased in unsaturated fatty acids-treated cells (Hannah et al., 2001). Analogous results for the explanation of our results were obtained by Smith et al. (1990), Brown and Goldstein (1999) and Brown et al. (2000). When cholesterol accumulates in the cells, sterol-sensitive cleavage is inhibited, less mature SREBPs are released and transcription declines, thereby preventing excessive accumulation of cholesterol in the cells (Smith et al., 1990; Brown and Goldstein, 1999; Brown et al., 2000). Three isoforms of SREBPs, SREBP-1a, SREBP-1c, and SREBP-2, are expressed in the liver and SREBP-1c plays a crucial role in the dietary regulation of most hepatic lipogenic genes, whereas SREBP-2 is a key regulator of cholesterol (Shimano et al., 1997; Horton et al., 1998).

In conclusion, suppressed LDL oxidation may play an important role in the initiation and progression of atherosclerosis. Furthermore, the observed significant increase in the LDL receptor, both in mRNA and protein levels, may result in a higher net removal of LDL particles from the circulation. The low SREBP-1c mRNA levels in the human liver cells may also be effective in lowering plasma triglyceride levels in humans. Wormwood EO acts in synergy with

vitamin E in the inhibition of LDL oxidation and the gene expression in relation to cholesterol metabolism. Our results suggest that wormwood EO with vitamin E may generate a hypocholesterolemic effect through multiple mechanisms. Future animal studies are needed to determine whether dietary feeding of EO with vitamin E can reduce plasma triglyceride and cholesterol levels as observed mechanism in HepG2 cells studies.

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