JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Hypolipidemic and Antioxidant Activity of Mountain Celery (*Cryptotaenia japonica* Hassk) Seed Essential Oils

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Mountain celery seed essential oils (MC-E) contained 109 compounds, including mainly nine kinds of monoterpenoids, 31 kinds of of sesquiterpenoids, and 22 kinds of alcohols. A successive gel column adsorption with solvent fractionation yielded four fractionates. The pentane fractionate revealed potent hypolipidemic but poor antioxidant activities. The ether fractionate exhibited strong hypolipidemic activity in addition to excellent 1,1-diphenyl-2-picrylhydrazyl free radical- and superoxide anion-scavenging capabilities. The third acetone fractionate only showed moderate superoxide anion-scavenging activity. Finally, the fourth methanol fractionate having a rather high content of γ-selinene, 2-methylpropanal, and *Z*-9-octadecenamide uniquely revealed very strong superoxide anion-scavenging capability. All MC diets except the MC-E-added diet simultaneously exhibited both significant hypolipidemic and high-density lipoprotein-cholesterol (HDL-C)-elevating capabilities. However, all diets totally failed to affect the hepatic phospholipid levels. Conclusively, the MC-E can be fractionated by such a separation technology to produce products uniquely possessing hypolipidemic and HDL-C-elevating activities.

KEYWORDS: ***Cryptotaenia japonica*; Hassk; sesquiterpenoids; terpenoids; antioxidant; hypolipidaemic capabilities**

INTRODUCTION

Mountain celery (MC) *Cryptotaenia japonica* Hassk *Umbelliferae* is one kind of perennial plant. It has a common name, "duckling celery", often synonymously recognized as "su-celery", "fragrant mountain celery", "mountain light celery", or "stalk celery". MC is a popular Asian vegetable species widely distributed in Shikoku, Kyu-shu, and Okinawa of Japan and the middle of Taiwan. MC has long been familiar to the Asian folks because of its hypotensive, hypolipidemic, and antiobese effects. Its relative species *Apium graveolens* L. was reported to contain two categories of phthalides, that is, dihydrophthalides and alkylidene phthalides. The former consists of 3-isobutylidene-3a,4-dihydrophthalide, and 3-isovalidene-3a,4-dihydrophthalide, whereas the latter comprises 3-isobutylidene phthalide and 3-isovalidene phthalide (*1*). Early in 1969, profound terpenoids were identified in parsley (*2*). On finishing the identification of 13 different alcoholic substances (*3*), Wilson later isolated a number of constituents including two epoxides, five ketones, five esters, three acids, and three phthalides, including 3-*n*-butylphthalide, sedanolide, and 3-*n*-butylhexahydrophthalide from the leaf and stalk essential oils (*4*). In addition, Bjeldanes and Kimet et al. (*5*) and Uhlig et al. (*6*) independently identified several novel phthalides. The presence of monoterpenoids in parsley stalks and leaves was first identified by Macleod and Ames (*7*). The main component was 3-butyl-4,5-dihydrophthalide. Moreover, phthalides and psoralens were also found as the main components in its volatiles (*8*). Alternatively, in the essential oils obtained from three Egyptian local indigenous celeries, Saleh et al. (*9*) identified a huge number of volatiles including α-pinene, β-pinene, myrcene, limonene, *cis*-β-ocimene, γ-terpinene, *cis*-alloocimene, *E*-farnesene, humulene, apiol, β-selinene, senkyunolid, and neocnidilide. In addition, Le and Elliott demonstrated 3-*n*-butylphthalide to be an effective hypotensive as well as a hyocholesterolemic (*10*).

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10.1021/jf703593v CCC: $40.75
Published on Web 05/13/2008

Essential oils of celery seeds apparently revealed very potent enzyme-inducing capabilities and proliferation-enhancing effects on liver, gastric, and enteromucosa (*11*). Zheng et al. (*12*) identified five anticarcinoma components in celeries including *d*-limonene, *p*-mentha-2,8-dien-1-ol, *p*-mentha-8(9)-en-1,2-diol, 3-*n*-butylphthalide, and sedanolide. These compounds possess prominent glutathione S-transferase (GST) activating activities in female mice; hence, conventional celeries have been considered to be associated with detoxification capability. For a long time, in folkloric medication, the aqueous extract of parsley has been used as a hyperlipidaemic agent (*13*). In addition, Momin et al. (*14*) indicated the *n*-hexane extracts of celery seeds containing β-selinene, 3-*n*-butyl-4,5-dihydrophthalide (BDHP), and 5-allyl-2-methoxyphenol to be potently effective antiparasitics against the fourth-instar *Aedes aegyptii*. In vitro, BDHP also was revealed to be 100% lethal to nematoda *Panagrellus redvivus* and *Caenorhabditis elegans.* Biochemically, BDHP was also confirmed to be an effective inhibitor for enzymes topoisomerase-I and topoisomerase-II required for the growth of *Candida albicans* and *Candida kruseii*.

To study the antioxidative and hypolipidemic bioactivities of MC seed essential oils (MC-E), we performed serial isolation and fractionation processes. The chemical constituents in each fraction were analyzed using gas chromatography/mass spectrometry (GC/MS). In parallel to the antioxidant assay, the hypolipidemic bioactivity of the pentane and ether fractionates was examined with a Syrian hamster model. Thus, MC was confirmed to be potentially one kind of useful vegetable regarding antioxidative and hypolipidemic activities.

MATERIALS AND METHODS

Plant Material. Fresh MC (*C. japonica* Hassk) seeds were purchased from a local farm located in Nan-Tou, a middle Taiwan County. The seeds were stored immediately in the dark at −20 °C before treatment.

Chemicals and Reagents. *E*-Nerolidol, heptanal, phenylacetaldehyde, ethyl formate, ethyl acetate, valencene, geranyl acetate, nerol, geraniol, thymol, *p*-cresol, dodecyl acetate, acetaldehyde, acetal, and α-terpinolene were manufactured by Aldrich (United Kingdom). α-Terpinene, linoleic acid, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), ferrous chloride ($FeCl_2$), ferrozine, nitroblue tetrazolium (NBT), dihydronicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), ethylene diaminetetraacetate (EDTA) (disodium salt), H_2O_2 (30%), peroxidase, phenol red, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ascorbic acid, and citric acid were products of Sigma Chemical Co. (United States). The authentic compounds β-myrcene, *d*-limonene, octanal, linalool, and palmitic acid were provided by Merck Co. (Germany). All were of reagent grade.

Isolation of Essential Oils. Celery seeds were ground and mashed with a mini grinder. The pulverized particles were passed through a stainless sieve (#35). The screened particles (100 g; smaller than #35) were transferred into a 5 L steam distillator, added with 1 L of purified water. The steam distillation was conducted for 4 h while an adapted receiver collected the distillates. The supernatant oily layer was cautiously separated, added with 3 g of anhydrous sodium sulfate, and shaken vigorously. The dehydrated oily distillate was filtered through Whatman #1 filter paper (**Figure 1**). Triplet experiments were performed. The mean value and the standard deviations were calculated. The yellowish essential oils were combined and stored at −20 °C for further tests.

Fractionation of Essential Oils. A silica gel column (i.d. 2 cm × 50 cm) was set up by compactly and evenly filling it with 40 g of silica gel (76−230 mesh, Merck). One gram of the product essential oils was transferred onto the silica gel column. The elution was performed sequentially each with 250 mL of *n*-pentane, diethyl ether, acetone, and finally with methanol. The eluents were respectively collected and concentrated in a rotary evaporator to almost dryness.

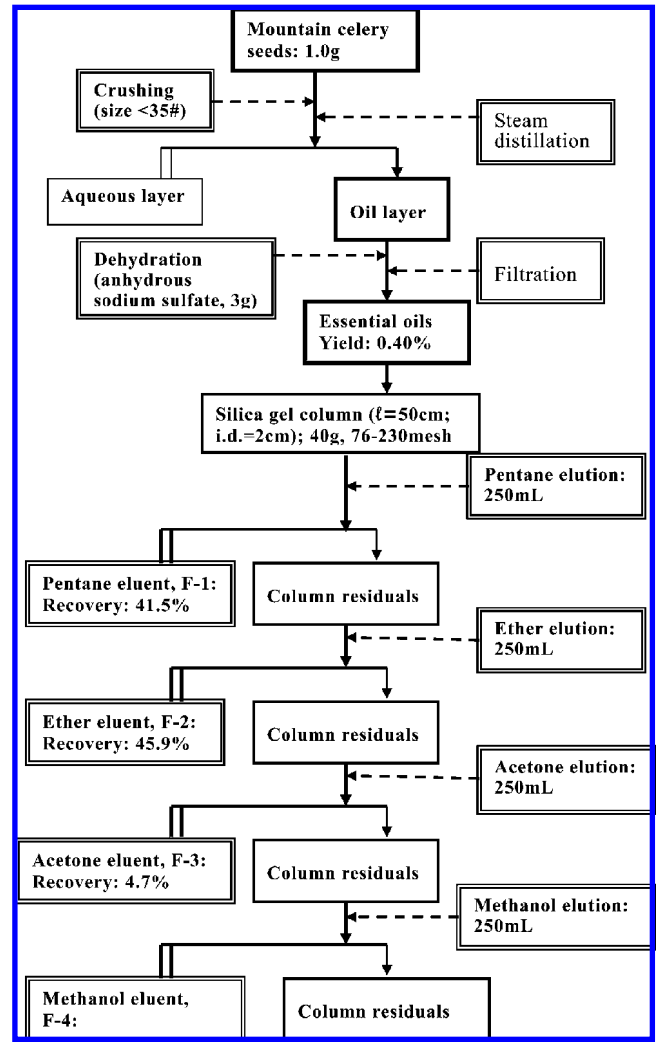

Figure 1. Flowchart for fractionation of the essential oils of MC seeds.

The concentrate was transferred into a smaller evaporator dish to continue the evaporation under nitrogen blowing until dry. The fractions were designated, respectively, as F-1 (pentane eluent), F-2 (diethyl ether eluent), F-3 (acetone eluent), and F-4 (methanol eluent) (**Figure 1**). The fractionated dried products were stored at −20 °C for further tests. The products obtained were diluted with the original eluting solvent immediately before examination to the desired concentrations required in each determination.

Chemical Characterization. *Instruments.* The HP6890 series GC coupled with 5973 Network Mass Selective Detector was used for quantification and determination of the chemical components in the essential oils of the MC seeds, respectively.

GC/MS Analysis. A capillary column type DB-1 (i.d. 0.25 mm × 60 m; membrane thickness, 0.25 μm) was used. Helium was used as the carrier gas and operated at a flow rate of 1 mL/min. The ionization potential used was 70 eV. The temperature of the ion source was set at 230 °C. The flux ratio was set at 50:1. Initially, the temperature was set at 40 °C for 10 min, then programmed at 2 °C/min up to 240 °C, and held at this temperature for 20 min.

Quantification of Volatile Constituents. Aliquots (0.5 μL) of the essential oils were, respectively, measured with a GC microsyringe from fractionates F1 to F4 and analyzed with GC/MS.

Quantification of each constituent in fractions F1−F4 was calculated from the integrated diagrams obtained by eq 1

$$Q = A \times Y \quad (1)$$

where Q = the quantity of each volatile constituent in fractions F1−F4, A = the percent peak area in the gas chromatograms occupied by each constituent,and Y = the recovery yield of essential oils.

Identification of Constituents in Essential Oils. A reference mixture of *n*-alkanes (C_5-C_{25}) was used to calculate the retention indices (RI) from the retention time (t_R) for each component. By referring to the documented data, each exact constituent was deduced. Alternatively, by comparing the GC/MSD spectra, each component was qualitatively searched by mapping and confirmed. As for the structural analysis for the volatiles, the database provided by Schonburg and Dielmann (*15*), Wiley MS Chemstation Libraries, NBS Computer Data Base, and the authentic patterns from the cited were referred.

Scavenging Capability for DPPH Free Radicals. The method described by Shimada et al. (*16*) was adopted. To each 1 mL of aliquot from fractions F1 to F4 (0.1−2 mg/mL) were added methanol (4 mL) and DPPH (Merck) (1 mL, 0.2 mM). The mixture was agitated thoroughly and left to stand in the dark for 30 min.The absorbance was measured at 517 nm using a Hitachi U-2001 Spectrophotometer. BHA and *t*-ascorbic acid were, respectively, used as the positive controls, with citric acid as the negative control.

Chelating Capability for Ferrous Ions. Dinis et al. (*17*) were followed to determine the ferrous ion chelating capability. Aliquots (1 mL) of fractions F1−F4 (0.1−2 mg/mL) were mixed with methanol (3.7 mL) and $FeCl_3-4H_2O$ (0.1 mL; 2 mM), agitated thoroughly, and left to stand for 30 s. The mixture was added to ferrozine (Merck) (0.1 mL; 5 mM). After vigorously mixed, the mixture was left to stand at ambient temperature for 10 min. The absorbance was measured at 562 nm using a Hitachi U-2001 Spectrophotometer. Solutions of EDTA (0.5−5 mg/mL) and citric acid (0.5−5 mg/mL) were used as the positive controls.

Scavenging Capability for Superoxide Anions. The method of Gülcin et al. (*18*) was followed. Briefly, each milliliter of aliquot of fractions F1−F4 (0.1−2 mg/mL) was added with NBT solution (1 mL, 50 μM NBT in 16 mM Tris-HCl buffer, pH 8.0) and NADH solution (1 mL, 78 μM NADH in 16 mM Tris-HCl buffer, pH 8.0). The mixture was thoroughly mixed and added with PMS solution (1 mL; 10 μM PMS in 16 mM Tris-HCl buffer, pH 8.0). After mixed thoroughly, the mixture was incubated at 25 °C for 5 min. The absorbance was measured at 560 nm using a Hitachi U-2001 Spectrophotometer. *t*-Ascorbic acid was used as the positive control. The percent inhibition was calculated according to eq 2

$$\% \text{ inhibition} = [(A_o - A_1)/A_o] \times 100 \quad (2)$$

where A_o is the absorbance of positive control *t*-ascorbic acid and A_1 is the absorbance of samples obtained from fractionates F1−F4.

Scavenging Capability for H_2O_2. The testing procedures were carried out by following the method of Rinkus and Taylor (*19*) with a slight modification. Aliquots (1 mL) of fractions F1−F4 (0.1−2 mg/mL) were added with 0.4 mL of H_2O_2 (4 mM). The mixture was left to stand at ambient temperature for 20 min (S_m). In parallel, HRPase was freshly prepared in 0.2 M potassium phosphate (pH 6.2) to a final concentration of 0.5 mg/mL. Phenol red was dissolved in 0.2 M potassium phosphate solution (pH 6.) to a final concentration of 7.5 mM. The ratio was adjusted to HRPase:phenol red = 1:2 on mixing (HP). To S_m, 0.6 mL of HP was added. The mixture was left to stand for 10 min and then iced cooled for another 10 min at ambient temperature. The optical density was measured at 610 nm using a Hitachi U-2001 Spectrophotometer. Ascorbic acid was used as a reference control.

Animal Experiment. All studies performed with animal models were approved by the Hungkuang University Supervising Ethic Committee in accordance with the Helsinki Declaration of 1975.

Animals and Diets. Because of the limitation of the yields, animal experiments were performed using only the two representative fractionates F-1 and F-2; 64 male Syrian hamsters, aged 6−7 weeks, were purchased from the National Laboratory Animal Centre. During the first week, the hamsters were caged and fed with common market feeds only. The acclimation period was conducted in an animal room maintained at 24 ± 1 °C with a relative humidity of 40−60%. The light cycle was alternated every 12 h (The light was on from 6:00 am to 19:00 pm, and the dark period was from 19:00 pm to 6:00 am). Then, the hamsters were randomly grouped by body weight into eight groups, two hamsters in each stainless cage and eight in each group, based on the diets: All diet formulas were based on AIN-76 (*20*) (**Table 1**). The control group N was fed on the regular diet; the high lipid diet group H contained 5 g of corn oil and 5 g of lard per formula (**Table 1**). Other groups were, respectively, fed with diet EA made of diet H plus 0.01 g of native essential oils (E); diet EB containing diet H plus 0.05 g of E; diet F1A by mixing diet H plus 0.01 g of pentane fractionate (F-1); diet F1B formulated with diet H plus 0.05 g of F-1; diet F2A consisting of diet H plus 0.01 g of ether fractionate F-2; and diet F2B, a mixed diet of diet H plus 0.05 g of F-2 per formula (**Table 1**). Water and diet consumptions were ad libidum. Body weights and amounts of diet uptake were recorded every 2 days until the end of the experiment. After 8 weeks of feeding, the hamsters were first fasted for 12 h before they were anesthetized with CO_2 before authenized. Blood was bled from the abdominal aorta. Blood and livers obtained were stored at −70 °C for further lipid analysis. The whole blood sample was centrifuged at 1800*g* for 10 min. The supernatant sera were collected. Livers were excised and rinsed with ice-cold saline (150 mM). After the adhering saline and water drops were wiped off, weights of livers were taken. The sera and the livers thus obtained were stored at −70 °C for further lipid analysis (*21*).

Table 1. Ingredients of Experimental Diets[a]

ingredients	N	H	EA	EB	F1A	F1B	F2A	F2B
casein	20	20	20	20	20	20	20	20
sucrose	15	15	15	15	15	15	15	15
corn starch	50	45	45	45	45	45	45	45
corn oil	2.5	5.0	5.0	5.0	5.	5.0	5.0	5.0
lard	2.5	5.0	5.0	5.0	5.0	5.0	5.0	5.0
M_P[b]	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
V_P[b]	1	1	1	1	1	1	1	1
choline	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
methionine	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
α-cellulose	5	5	5	5	5	5	5	5
E/F-1 or F-2	0	0	0.01	0.05	0.01	0.05	0.01	0.05

[a] E, native essential oils of MC. F1 and F2 denote essential oils eluted by pentane and ether, respectively. Data of ingredient formulated were in absolute weight g. [a]N, normal formula; H, high lipid diet; EA, diet H added with 0.01 g of E; EB, diet H added with 0.05 g of E; F1A, diet H added with 0.01 g of F-1; F1B, diet H added with 0.05 g of F-1; F2A, diet H added with 0.01 g of F-2; and F2B, diet H added with 0.05 g of F-2. [b] M_P (mineral premix): $CaHPO_4 \cdot 2H_2O$, NaCl, $K_3C_6H_5O_7$, K_2SO_4, MgO_3, MnO_3, Fe-citrate, $ZnCO_3$, $CuCO_3$, KI, $NaSeO_3$, and $K_2SO_4 \cdot Cr_2(SO_4)_3 \cdot 24H_2O$. V_P (vitamin premix): thiamin hydrochloride, pyridoxine hydrochloride, riboflavin, nicotinic acid, vitamin B_{12}, retinyl palmitate, vitamin D_3, vitamin E, and vitamin K.

Determination of Serum Lipoproteins. Sera collected from the above were assayed for levels of total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG) according to Richmond (*22*), which had been previously described by Lin et al. (*23*). Briefly, the enzymatic CHOD-PAP method using a Teco Diagnostics kit was used for the determination of serum total cholesterol. A reference cholesterol control (10 μL) supplied by the manufacturer was treated in the same manner for calibration.

For the determination of LDL-C, the amount of final colored product produced was measured spectrophotometrically at 600 nm. Alternatively, the enzyme GDP-PAP triglyceride kit (Teco Diagnostics) was used to determine the serum TG content by following the instructions given by the manufacturer (also refer to Lin et al.) (*23*).

The extraction of hepatic phospholipids contents was carried out according to Folch et al. (*24*). The following procedures were described elsewhere by Lin et al. (*23*). The method of Bartlett (*25*) was followed for its determination (also refer to Lin et al.) (*23*). The color reaction proceeded with perchloric acid (70%)−ammonium molybdate (2.5%)−ascorbic acid (10%) method of Bartlett (*25*). The absorbance was measured at 820 nm. The concentration of hepatic phospholipids was calculated from the calibration curve established using a standard sample supplied by the manufacturer.

Statistics. Data obtained in the same group were analyzed by Student's *t* test with computer statistical software SPSS 10.0 (SPSS, Chicago, United States). Statistical Analysis System (2000) software was used to analyze the variances, and Duncan's multiple range tests

Table 2. Volatile Components in the Native Essential Oils of MC Seeds and Its Pentane, Ether, Acetone, and Methanol Eluents[a]

compounds	RI	composition (%) MC-E[c]	F-1	F-2	F-3	F-4	compounds	RI	composition (%) MC-E[c]	F-1	F-2	F-3	F-4
							monoterpenoid hydrocarbons						
α-pinene	940[b]	0.8	0.43	ND	ND	ND	α-terpiene	1021	0.16	0.01	ND	ND	ND
camphene	945	trace	0.06	ND	ND	ND	d-limonene	1024[b]	0.25	0.24	ND	ND	ND
sabinene	968	trace	0.02	ND	ND	ND	*E*-β-ocimene	1060	ND	0.03	ND	ND	ND
β-pinene	981	10.34	5.86	ND	ND	ND	α-terpinolene	1092[b]	0.29	0.04	ND	ND	ND
β-myrcene	986	1.35	0.46	ND	ND	ND	subtotal		13.19	7.15	0	0	0
							sesquiterpenoid hydrocarbons						
α-cubebene	1355	0.36	0.37	ND	ND	ND	*Z*-α-bisabolene	1492	1.66	0.76	1.37	ND	ND
α-copaene	1368[b]	0.47	0.21	ND	ND	ND	ar-curcumeme	1496	ND	ND	0.18	ND	ND
α-ylangene	1369	0.15	0.32	ND	ND	ND	α-selinene	1499	8.9	28.48	0.41	ND	ND
β-cubebene	1382	ND	1.78	ND	ND	ND	valencene	1499[b]	1.42	0.47	3.81	ND	ND
β-elemene	1384	1.71	2.31	ND	ND	ND	β-bisabolene	1501	trace	ND	0.55	0.14	ND
β-sesquiphellandrene	1385[b]	ND	ND	ND	ND	1.33	α-zingberene	1502	trace	0.28	trace	ND	ND
β-patchoulene	1392	trace	0.84	ND	ND	ND	cuparene	1505[b]	1.8	ND	3.4	ND	ND
E-caryophyllene	1419	0.75	1.15	0.23	ND	ND	γ-selinene	1516[b]	ND	ND	ND	ND	12.59
γ-elemene	1425	5.08	8.23	ND	ND	ND	δ-cadinene	1520	2.93	2.43	0.22	ND	ND
E-β-farnesene	1430	17.3	23.39	ND	ND	1.32	aromadendrene	1527[b]	ND	ND	ND	ND	0.05
α-guaiaene	1431	0.69	2.45	ND	ND	ND	*E*-γ-bisabolene	1538[b]	0.64	0.89	0.34	ND	ND
alloaromadendrene	1444	ND	ND	ND	0.2	ND	selina-3,7-[11]diene	1540[b]	1.34	1.79	ND	ND	ND
α-humulene	1453[b]	0.53	trace	2.5	ND	ND	ledene	1540[b]	ND	ND	ND	ND	0.05
α-amorphene	1481	0.23	trace	ND	ND	ND	widdrene	1550	0.12	ND	0.84	ND	ND
α- farnesene	1482	1.77	0.9	0.15	ND	ND	germacrene B	1556	5.93	6.62	0.18	ND	ND
germacrene D	1483	2	ND	0.9	ND	ND	subtotal		60.84	86.97	15.32	0.34	15.34
β-selinene	1487	5.06	3.3	0.24	ND	ND							
							alcohols						
1,4 -butanediol	<500[b]	ND	ND	ND	0.03	6.37	spathulenol	1565[b]	trace	ND	1.47	ND	ND
ethanol	500	trace	ND	1.57	1.9	ND	ledol	1620	ND	ND	1.78	ND	ND
α-pentanol	678[b]	ND	ND	ND	0.15	ND	veridiflorol	1621[b]	0.54	ND	1.72	ND	ND
diacetone alcohol	780[b]	ND	ND	ND	0.09	1.27	farnesol	1636[b]	ND	ND	ND	ND	0.14
3-pentanol	820[b]	ND	ND	ND	14.61	ND	α-bisabolol	1681[b]	1.77	ND	13.86	ND	ND
2-butoxyethanol	874[b]	ND	ND	ND	12.61	ND	valerenol	1689[b]	ND	ND	1.98	ND	ND
linalool	1108[b]	0.49	ND	1.49	ND	ND	α-copaene-8-ol	1720[b]	ND	ND	ND	0.01	ND
nerol	1231	ND	ND	1.68	ND	ND	α-cedrol	1824[b]	ND	ND	ND	0.03	ND
geraniol	1234	0.47	ND	trace	ND	ND	α-*E*-sesquicyclogeraniol	2320[b]	ND	ND	ND	ND	12.01
E-nerolidol	1554	ND	ND	1.78	ND	ND	subtotal		3.27	0	27.33	29.43	19.79
							phenol						
ρ-cresol	1120	0.89	ND	2.31	ND	ND	2,4-bis[1,1-dimethylethyl]phenol	1435[b]	ND	ND	ND	4.6	6.96
thymol	1274[b]	2.79	ND	0.64	ND	ND	subtotal		3.68	0	2.95	4.6	6.96
							aldehydes						
acetaldehyde (?)	<500	ND	ND	1.48	7.38	ND	octanal	993[b]	ND	ND	0.97	ND	ND
2-methyl-propanal	568[b]	ND	ND	ND	ND	5.34	phenylacetaldehyde	1030	trace	0.09	0.26	ND	ND
acetal	675[b]	ND	ND	0.51	0.03	ND	subtotal		0.13	0.09	3.69	7.41	5.34
heptanal	879[b]	0.13	ND	0.47	ND	ND							
							ketones						
2-butanone	575[b]	ND	ND	ND	1.25	ND	4-hydroxy-3-methylacetophenone	1278[b]	ND	ND	20.09	ND	ND
4-methylhept-4-en-3-one	884[b]	ND	ND	ND	ND	0.08	zierone	1501[b]	ND	ND	ND	ND	0.93
6-methylhept-5-en-2-one	967	ND	ND	0.55	0.51	0.04	subtotal		0.19	0	20.8	1.76	1.05
2-nonanone	1082	0.19	ND	0.16	ND	ND							
							esters						
ethyl formate	554[b]	ND	ND	1.66	11.2	ND	geranyl acetate	1361[b]	0.29	ND	0.99	ND	ND
ethyl acetate	590[b]	trace	ND	5.36	14.37	ND	decyl acetate	1397	ND	ND	0.67	ND	ND
n-butyl acetate	760	ND	ND	ND	0.5	ND	subtotal		0.29	0	8.68	26.07	0
							acids						
formic acid	582[b]	ND	ND	0.58	1.65	ND	linoleic acid	2009	0.02	ND	1.21	ND	ND
acetic acid	657[b]	trace	ND	1.26	6.86	ND	subtotal		0.06	0	3.67	8.51	0
palmitic acid	1947	0.04	trace	0.62	ND	ND							
							miscellaneous compounds						
toluene	776	ND	trace	0.68	ND	ND	hexadecane	1589	ND	0.04	ND	ND	ND
octane	793	ND	trace	0.15	ND	ND	isoaromodendrene epoxide	1599[b]	ND	ND	3.52	ND	ND
undecane	1102	ND	0.11	ND	ND	ND	8-heptadecene	1665[b]	ND	0.43	ND	ND	ND
1-dodecene	1139[b]	ND	ND	ND	0.54	ND	1-octadecene	1711[b]	ND	ND	ND	1.26	0.74
2,5-diethylthiophene	116[b]	5.42	ND	3.9	0.04	ND	octadecane	1801	trace	0.42	trace	ND	ND
tridecane	1301	3.2	3.6	ND	ND	ND	neophytadiene	1826	0.05	0.11	ND	ND	ND
1-tetradecene	1332[b]	ND	ND	ND	1.33	ND	hexadecanenitrile	1859[b]	ND	ND	4.21	ND	ND
tetradecane	1395	0.85	0.93	ND	ND	ND	hexadecanamide	2045[b]	ND	ND	ND	0.76	ND
α-calacorene	1450[b]	ND	ND	ND	1.4	0.04	heptadecanenitrile	2122[b]	ND	ND	0.44	ND	ND
denderalasin	1491[b]	ND	ND	ND	0.35	1.57	*Z*-9-octadecenamide	2298[b]	ND	ND	1.57	4.6	38.61
pentadecane	1501	8.37	0.11	1.38	0.66	ND	1-nonadecene (?)	2301	ND	ND	ND	1.42	ND
1-hexadecene	1522[b]	ND	ND	ND	1.9	ND	subtotal		18.25	5.75	17.53	14.26	41.09
caryphyllene oxide	1565	0.36	ND	0.57	ND	0.13	total		99.9	99.96	99.97	92.38	89.57
aromodendrene oxide	1582[b]	ND	ND	1.11	ND	ND							

[a] RI, Kovat's GC retention index calculated with C_5−C_{25} *n*-alkanes as references; ND, not detected; and trace, <0.01. [b] These volatiles were identified by comparing with the standard or authentic sample, while others were identified by referring to the computer mass libraries to compare the mass spectrum with the published data of known chemical structures. [c] MC-E, seed essential oils of MC; F-1, pentane fractionate; F-2, ether fractionate; F-3, acetone fractionate; and F-4, methanol fractionate.

were used to test their significances of difference between paired means. Significance of difference was judged by a confidence level of $p < 0.05$.

RESULTS

The overall yield of MC-E obtainable was 0.40%, which is only a moderately low content when compared with other essential oil-producing common herbs. The combined yield of the pentane and the ether fractionates (87.4%) evidenced that the majority of which was nonpolar in nature (**Figure 1**). The more polar acetone and methanolic fractionates yielded only 4.7 and 6.0%, respectively, resulting in an efficient overall recovery of 98.1% (**Figure 1**). GC and GC/MS analyses apparently indicated a total of 109 volatile compounds present in MC-E, including nine monoterpenoids, 32 sesquitepenoids, 19 alcohols, three phenols, six aldehydes, six ketones, five esters, four acids, and 25 other miscellaneous compounds (**Table 2**). The contents of categories monoterpenoids and sesquitenoids were 13.19 and 7.15% and 60.84 and 86.97%, respectively, in MC-E and pentane fractionate F-1 (**Table 2**). However, these two categories were almost undetected in other fractionates, indicating a successful differential fractionation. The major compounds prominently present in MC-E were (in order of decreasing content %): *E*-β-farnescene (17.3), β-pinene (10.34), α-selinene (8.9), pentadecane (8.37), germacrene B (5.93), 2,5-diethylthiophene (5.42), γ-elemene (5.08), β-selinene (5.06), tridecane (3.2), thymol (2.79), cuparene (1.8), α-farnescene (1.77), α-bisabolol (1.77), β-elemene (1.71), *Z*-α-bisabolene (1.66), valencene (1.42), β-myrcene (1.35), and selina-3,7-[11]-diene (1.34) (**Table 2**). Most of these above compounds also appeared as the major constituents in pentane fractionate F-1, evidencing that in addition to having confirmed the nonpolarity of these compounds, such a separation and concentration technology can be useful in mass production design. Moreover, the compound 8-heptadecene (0.43%) was only uniquely identifiable in pentane fractionate F-1. As a contrast, the ether fractionate F-2 contained relatively more polar compounds; the more important constituents included (in order of decreasing content %) the following: 4-hydroxy-3-methylacetophenone (20.09), α-bisabolol (13.86), ethyl acetate (5.36), hexadecanenitrile (4.21), valencene (3.81), iosaromadendrene epoxide (3.52), α-humulene (2.5), valerenol (1.98), *E*-nerolidol (1.78), ledol (1.78), veridiflorol (1.72), nerol (1.68), ethyl formate (1.66), *Z*-9-octadecenamide (1.57), ethanol (1.57), linalool (1.49), acetaldehyde (1.48), spathulenol (1.47), pentadecane (1.38), *Z*-α-bisabolene (1.37), acetic acid (1.26), linoleic acid (1.21), aromadendrene oxide (1.11), and geranyl acetate (0.99) (**Table 2**). Similarly, some special constituents were only concentrated to appear in the ether fraction F-2, which involved α-humulene, widdrene, *E*-nerolidol, spathulenol, ledol, veridiflorol, farnesol, α-bisbolol, valerenol, thymol, 4-hydroxy-3-methylacetophenone, geranyl acetate, decyl acetate, aromadendrene oxide, isoaromadendrene epoxide, and hexadecanenitrile. In the acetone fractionate F-3, a total of 32 constituents were identified. The relatively higher polar constituents were found in the acetone eluent, which included acetic acid, alcohols, phenolics, and *Z*-9-octadecenamide. The final methanolic eluent F-4 consisted of only 19 constituents including 2,4-bis[1,1-dimethylethyl]phenol, *Z*-9-octadecenamide, γ-selinene, and α-*E*-sesquicyclogeraniol, etc. (**Table 2**).

Interestingly, only a minute amount of aldehydes occurred in MC-E. The main aldehyde compounds in ether fractionate F-2 were acetaldehyde, acetal, heptanal, octanal, and phenylacetaldehyde. Phenylacetaldehyde was the only aldehydic component detected in F-1. Likewise, 2-methylpropanal was only found in methanolic fractionate F-4 (**Table 2**.). Worth noting, the four compounds acetaldehyde, 2-methylpropanal, acetal, and octanal that initially had not been detected in the MC-E were identified in significantly amounts on fractionation with silica gel column, again implying the advantage of the specific separation and concentration effect of such a technology. Similar technology can be applied to the concentration of compounds such as camphene, sabinene, β-cubebene, β-sesquiphellandrene, alloaromadendrene, ar-cucumeme, β-bisabolene, α-zingberene, aromadendrene, ledene, 1,4 butanediol, α-pentanol, 2-butoxyethanol, nerol, 2,4-bis[1,1-dimethylethyl]phenol, *E*-lirolidol, valerenol, α-copaene-8-ol, α-cedrol, and α-*E*-sesquicyclogeraniol (**Table 2**). Similar to aldehydes, ketones and acids presented in very few quantities in the MC-E (**Table 2**). In further antioxidative studies, MC-E originally showed only moderately low ferrous ion chelating capability (29.2%) even at a rather high dose of 2 mg/mL. On fractionation, the antioxidative capability of the ether fractionate at the same dosage was apparently increased to 57.6% (**Figure 2A**). Obviously, the improved bioactivity can be attributed to the increased alcoholic content in ether fractionate (**Table 2**). Similarly, such a phenomenon was seen in acetone and methanol fractionates (**Figure 2A**). Interestingly, many alcoholic compounds that initially appeared in the ether fractionate were totally absent in the acetone and methaolic fractionates. The compounds concerned included linalool, *p*-cresol, nerol, geraniol, thymol, *E*-nerolidol, spathulenol, ledol, veridiflorol, α-bisabolol, and valerenol, etc. (**Table 2**). Speculatively, these compounds could play important roles in ferrous ion chelation. As can be seen, the ether fractionate exhibited excellent DPPH scavenging capability at a dosage of 0.5 mg/mL, revealing comparable bioactivity to the well-known reference compound BHA, a far better effect than ascorbic acid and the methanolic fractionate (**Figure 2B**). As a contrast, MC-E revealed only 65.5% of activity as compared with 95.3% of BHA (**Figure 2B**). Again, in the above-mentioned, the more concentrated and unique alcoholic constituents in the ether fractionate (**Table 2**) can be considered to play a very important role in DPPH scavenging. At a dose of 0.5 mg/mL, the methanolic fractionate exhibited 82.2% of superoxide anions scavenging capability as compared to 91.5% by BHA and 55.3% by the native essential oils, better than those of ether fractionate, the reference compound BHT, and the native essential oils in this regard (**Figure 2C**). As the methanolic fractionate contained relatively higher contents of the sesquiterpenoid γ-selinene (12.59%), 2,4-bis[1,1-dimethylethyl]phenol (6.96%), 2-methyl-propanal (5.34%), zierone (0.93%), denderalasin (1.57%), and extremely high content of *Z*-9-octadecenamide (38.61%); hence, its extraordinary superoxide anion scavenging capability could be ascribed to these special compounds. Worth noted, all fractions as well the native essential oils were ineffective in scavenging of H_2O_2 (**Figure 2D**).

In animal experiments, all diets did not show any significant difference in feed efficiency, organ weight, or organ to body weight ratios (**Table 3**), implicating that the MC seed diets would not induce any complication with fatty liver. Moreover, the serum TG, total cholesterol (TC), and LDL-C levels were significantly suppressed in groups having ingested native essential oils, EA and EB; in groups having taken pentane fractionate, F1A and F1B; and in groups having consumed ether fractionate, F2A and F2B. Greater degrees of significance were with groups F1A and F2A when compared to the high lipid

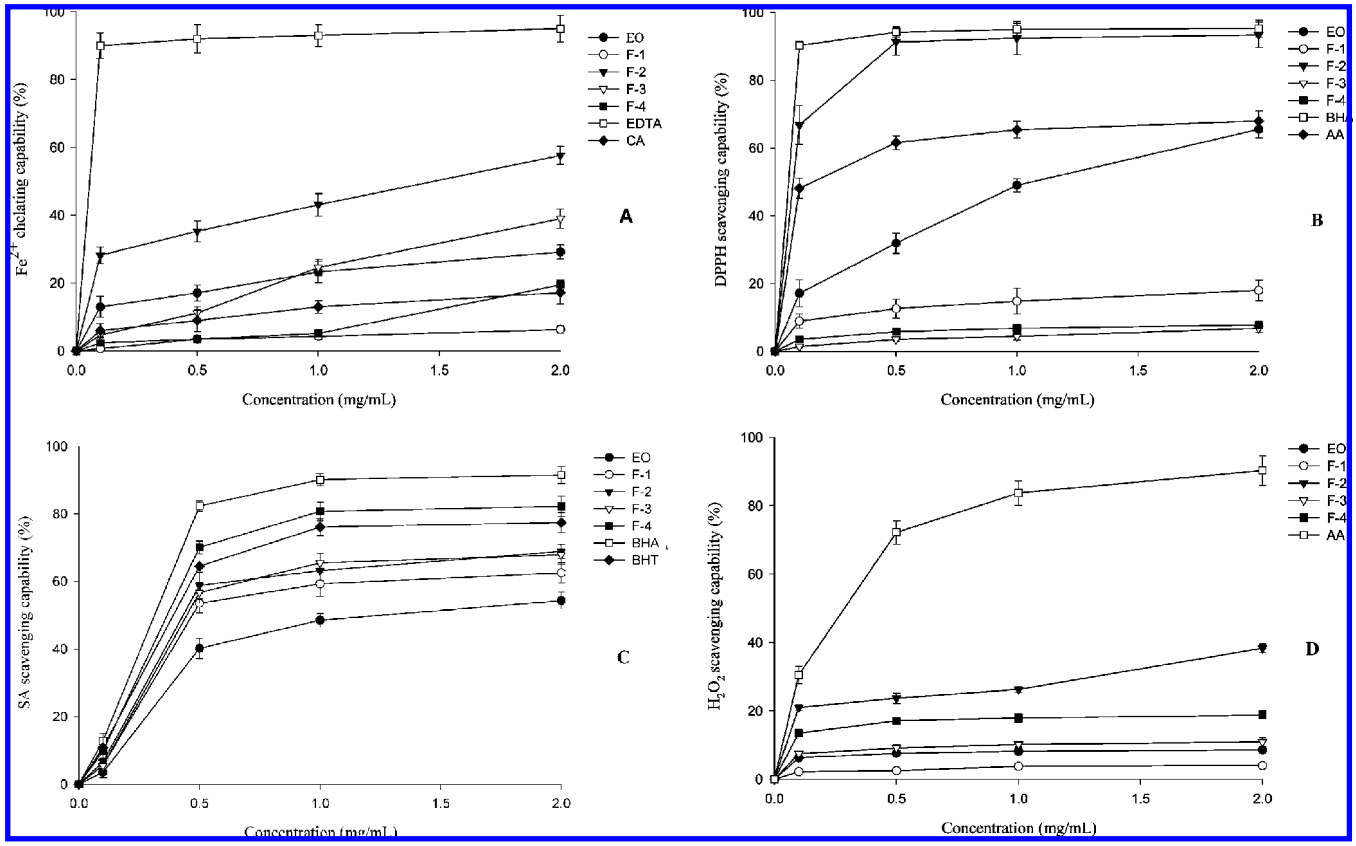

Figure 2. Comparison of different antioxidative behaviors among different preparations. Comparison of the ferrous ion chelating capability (A). Comparison of the scavenging capability on DPPH free radicals (B). Comparison of the scavenging capability on superoxide anions (C). Comparison of the scavenging capability on hydrogen peroxides (D). EO, essential oils; F-1, the pentane fractionate; F-2, the ether fractionate; F-3, the acetone fractionate; F-4, the methanol fractionate; CA, citric acid; AA, ascorbic acid; and SA, superoxide anion.

Table 3. Effects of Native Essential Oils of MC Seeds and Its Pentane and Ether Fractions on the Nutritional and Biochemical Parameters Related to Serum and Hepatic Levels in Male Hamsters[a]

growth parameters	N	H	EA	EB	F1A	F1B	F2A	F2B
food intake	7.60 ± 0.18	7.73 ± 0.10	7.66 ± 0.18	7.71 ± 0.21	7.71 ± 0.10	7.64 ± 0.18	7.72 ± 0.19	7.76 ± 0.18
body gain (g)	13.3 ± 3.75	14.2 ± 2.95	15.7 ± 3.35	14.6 ± 2.45	12.7 ± 2.8	12.8 ± 3.35	14.9 ± 3.15	15.3 ± 3.45
feed efficiency	1.60 ± 0.04	1.63 ± 0.02	1.61 ± 0.04	1.62 ± 0.04	1.62 ± 0.02	1.61 ± 0.03	1.62 ± 0.04	1.63 ± 0.03
				organ wt gain				
liver	3.77 ± 0.11 a	3.99 ± 0.17 bc	3.88 ± 0.19 bc	3.94 ± 0.23 abc	3.79 ± 0.19 ab	3.89 ± 0.23 abc	3.80 ± 0.19 ab	3.89 ± 0.19 abc
kidney	0.44 ± 0.02	0.42 ± 0.04	0.43 ± 0.03	0.45 ± 0.03	0.45 ± 0.04	0.44 ± 0.03	0.44 ± 0.04	0.43 ± 0.02
				blood				
TG	196.3 ± 9.5 c	255.9 ± 10.4 a	211.3 ± 11.9 b	212.4 ± 10.6 b	200.1 ± 10.6 c	217.6 ± 9.0 b	196.4 ± 8.0 c	212.6 ± 11.7 b
TC	133.5 ± 8.1 e	176.8 ± 10.1 a	140.4 ± 7.9 cde	146.3 ± 7.9 bc	138.4 ± 6.0 cde	144.5 ± 6.2 bcd	135.9 ± 8.4 de	152.3 ± 9.5 b
LDL-C	28.9 ± 2.2 cd	42.3 ± 2.9 a	31.7 ± 2.6 bc	35.5 ± 3.0 b	28.6 ± 3.2 cd	30.8 ± 2.6 cd	27.6 ± 2.5 bd	34.2 ± 4.2 b
HDL-C	186.8 ± 9.3 b	163.7 ± 8.5 a	162.0 ± 10.1 a	158.5 ± 11.0 a	182.6 ± 11.9 b	186.1 ± 9.4 b	183.5 ± 9.9 b	191.6 ± 10.2 b
LDL/HDL-C	0.16 ± 0.02 e	0.26 ± 0.02 a	0.20 ± 0.02 c	0.22 ± 0.03 b	0.16 ± 0.02 e	0.17 ± 0.01 de	0.15 ± 0.02 e	0.18 ± 0.02 cd
				liver (mg/g)				
TG	23.5 ± 3.8 ab	25.1 ± 2.8 a	22.1 ± 1.9 ab	22.3 ± 2.4 ab	20.7 ± 4.5 b	21.6 ± 2.7 b	20.7 ± 3.4 b	21.4 ± 1.6 b
TC	16.8 ± 2.7 bc	18.7 ± 1.8 a	17.2 ± 1.6 ab	17.3 ± 1.7 ab	15.7 ± 1.7 bc	16.1 ± 1.8 bc	15.2 ± 1.5 c	16.5 ± 1.9 bc
phospholipid	75.9 ± 1.58	75.1 ± 3.5	76.1 ± 2.7	76.0 ± 3.2	76.8 ± 3.0	76.6 ± 2.9	76.8 ± 3.2	76.6 ± 2.9

[a] N, normal regular diet; H, high lipid diet; EA, diet H added with 0.01 g of E; EB, diet H added with 0.05 g of E; F1A, diet H added with 0.01 g of F-1; F1B, diet H added with 0.05 g of F-1; F2A, diet H added with 0.01 g of F-2; and F2B, diet H added with 0.05 g of F-2. Feed efficiency = [wt gain (g)/total diet intake (g)] × 100%; fed for 10 weeks. Relative organ weight = [organ wt (g)/ body wt (g)] × 100%. LDL-C/HDL-C, ratio of low density lipoprotein-cholesterol/high density lipoprotein-cholesterol. Values in the same row with different superscripts are significantly different ($p < 0.05$). E, native seed essential oils of MC. F1 and F2 denote essential oil fractionates obtained by elution with pentane and ether, respectively. The ingredients formulated were expressed in absolute weight g.

diet group H (**Table 3**). The better effect revealed by lower consumption of ether fractionate F2 than those by higher dosages (F2B) regarding the TG, TC, and LDL-C levels indicates that the MC seeds still retained more or less lipogenic effect. Alternatively, Xu et al. isolated *Z*-9-octadecenamide (**Table 2**) and *Z*-12-octadecadienamide from *Mylabris phalerate* Pallas and indicated that these two compounds exhibited potential inhibitory effect on rat liver microsomal acyl-CoA: cholesterol acyltransferase (ACAT), hACAT-1, and hACAT-2 (*26*), which may interpret at least a part, if not the entire, the hypolipidaemic effect of all essential oil-related groups (**Table 3**).

As can be seen, the optimum dosages to exert hypolipidemic effect were 0.01 g/diet of pentane and ether fractionates, respectively (**Table 3**). HDL-C levels were significantly

increased in groups that consumed pentane fractionate (F1A and F1B) and ether fractionate (F2A and F2B). In contrast, the two groups that administered native essential oils were totally unaffected (**Table 3**), which might be due to too low a concentration of the active constituents present in native essential oils. However, when considering the ratio of LDL-C/HDL-C, all groups showed significantly reduced parameters (**Table 3**). As well-known, reduction in ratios of LDL-C/HDL-C can be very beneficial to the prevention of atherosclerosis (*27*). A similar effect was found for the hepatic TG and TC levels (**Table 3**). In addition, all meals did not reveal any effect on the hepatic phospholipid levels (**Table 3**), implicating that the biosynthetic pathways of phospholipids were not involved on MC-E ingestion.

DISCUSSION

The MC-E contains 109 compounds; the major categories are sesquiterpenoids and alcohols. In view of antioxidative bioactivity, ether fractionate exhibits only moderate ferrous chelating and poorly low hydrogen peroxide scavenging capabilities, alternatively possessing excellently high potential DPPH- and superoxide-scavenging bioactivities. Although the methanolic fractionate showed a slightly better superoxide anion scavenging capability than the ether fractionate, it possesses uniquely the most potent superoxide scavenging activity. Pentane fractionate is more potent with respect to hypolipidemic activity. However, all MC-containing diets totally had no effect on hepatic phospholipid levels. Conclusively, the MC seeds are potent antioxidative and hypolipidemic vegetable sources. Such a separation technology can be applied to produce some extraordinary hypolidemics and antioxidants.

ABBREVIATIONS USED

BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; EDTA, ethylene diaminetetraacetate (disodium salt); DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LDL-C/HDL-C, ratio of low-density lipoprotein cholesterol/high-density lipoprotein cholesterol; NADH, dihydronicotinamide adenine dinucleotide; NBT, nitroblue tetrazolium; PMS, phenazine methosulfate; TC, total cholesterol; TG, triglycerides.

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Received for review December 11, 2007. Revised manuscript received March 16, 2008. Accepted March 16, 2008.

JF703593V