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Phenolic Content and Antioxidant Capacity of Essential Oil Obtained from Sawdust of *Chamaecyparis obtusa* by Microwave-Assisted Hydrodistillation

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Summary

Reactive oxygen species and free radicals play a major role in food deterioration. Current research is directed towards finding naturally occurring antioxidants of plant origin. In the present study, the chemical composition analysis of the essential oil obtained from sawdust of *Chamaecyparis obtusa* (COEO) was conducted by gas chromatography coupled with mass spectroscopy (GC-MS). Further, the phenolic content and antioxidant capacity of the COEO were investigated using different radical scavenging assays. The COEO obtained from the dried sawdust material using a microwave-assisted hydrodistillation technique resulted in the determination of 46 different compounds by GC-MS analysis, representing 98.94 % of total oil content. The COEO was characterised by the presence of mono- and sesquiterpene hydrocarbons, oxygenated mono- and sesquiterpenes, steroids, diterpenes and indole derivatives. At the highest tested concentration range, the COEO showed antioxidant capacity, *i.e.* the inhibition of DPPH, nitric oxide, superoxide and hydroxyl radicals of 80.16, 82.93, 72.99 and 71.62 %, respectively. Moreover, the COEO displayed concentration-dependent reducing power ability and remarkable inhibitory effect on ferric ion-induced lipid peroxidation in bovine brain extract. In addition, the COEO yielded (6.13±0.05) mg of gallic acid per g of dry mass. The present study confirms that the *C. obtusa* essential oil has potent antioxidant, lipid peroxidation inhibition and radical scavenging abilities; therefore, it might be used as a natural antioxidant to prevent food deterioration.

Key words: Chamaecyparis obtusa, essential oil, microwave extraction, reactive oxygen species, free radicals, antioxidants

Introduction

In living organisms there is an equilibrium between natural antioxidant defense mechanism and reactive oxygen species (ROS), which are produced in the body by various exogenous and endogenous redox cycling processes as by-products (*1*). When the equilibrium is disturbed, the ROS can induce oxidative damage to various biomolecules, including carbohydrates, lipids, proteins, DNA and RNA in the human body, which is associated with cellular damage, tissue injury and genetic mutation (*2*). This oxidative damage may accelerate ageing and cause cancer, cardiovascular disorders, inflammation and neurodegenerative disorders (*3*,*4*).

It has been established that lipid peroxidation is a major factor of food deterioration during processing and storage which affects food quality severely, causing qualitative deterioration of food commodities because of chemical spoilage, rancidity, and altered physical prop-

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erties including colour and texture alterations (*4*). Consequently, to protect food and consumers from oxidative damage caused by ROS, synthetic antioxidants such as butylated hydroxyanisole (BHA), propyl gallate, and butylated hydroxytoluene (BHT) have been added to foodstuffs (*5*). However, due to toxicity and potential health hazards of synthetic antioxidants as well as their limited use, interest in finding naturally occurring antioxidant compounds for use in foods and/or medicines has considerably increased (*6*). There is epidemiological evidence correlating higher intake of dietary antioxidant components with the ability to lower the rate of human morbidity or mortality (*7*).

Recently, there has been an increasing interest in the therapeutic efficacy and preservative properties of natural plant antioxidants to prevent oxidative reactions both in foodstuffs and in biological systems. The plant-based essential oils have provoked interest as sources of natural products because of their safety, wide consumers' acceptability, and the likelihood of their exploitation for potential versatile functional uses (*7–9*). The plant-based volatile oils and non-volatile secondary metabolites have wide applications in dietary supplements, food flavouring and preservation, folk medicine and fragrance industry (*10,11*). Several reports have confirmed the antioxidant capacity of plant-based essential oils *in vitro* and *in vivo* (*12,13*).

The essential oils derived from plants have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases and the preservation of food from toxic effects of oxidants (*6*). Moreover, Ishino *et al.* (*14*) propose an efficient way to prevent the development of harmful compounds and rancidity, which are the result of lipid peroxidation of food. Essential oils are complex mixtures of biologically active volatile substances. Conventional methods of essential oil extraction such as tumbling and shaking, oil infusion, cold expression, steam distillation and Soxhlet extraction are time consuming, less efficient, and require higher amounts of solvents for extraction purposes. Moreover, these extraction methods can cause the loss of some volatile components, thermal or hydrolytic degradation of unsaturated or ester compounds, and leave toxic solvent residue in the sample. These shortcomings have led to the consideration of the use of a new green technique for the essential oil extraction, such as the application of microwaves, which typically uses less energy and is free from harmful effects of organic solvents (*15*). This unique heating mechanism can significantly reduce the extraction time as compared to conventional extraction methods (*16*). In microwave-assisted hydrodistillation technique, the moisture content in the substrate sample serves as the target for microwave heating. When the moisture inside the plant cell is heated due to the microwave effect, it evaporates and generates a remarkable pressure on the cell wall caused by swelling of the plant cell (*17*). The internal pressure on the cell wall causes stretching and ultimately rupture of the cell, which facilitates leaching out of the active constituents from the ruptured glandular and oleiferous vesicles to the surrounding solvent, thus improving the yield of phytoconstituents. This technique has a great advantage over conventional extraction of essential oils from nuts or the hard parts of plants, without any disruption of tissue prior to the extraction of oil (*17*).

Chamaecyparis obtusa, native to Northeast Asia where it is commonly known as hinoki, is a conifer of the cypress family (Cupressaceae), and it is rich in a variety of active pharmaceutical ingredients such as flavonoids and other essential components (*18,19*). The oil extracted from the leaves and twigs of *C. obtusa* has been commercially used as a functional additive in the production of soap, toothpaste and cosmetics due to its strong fragrance (*18*). The essential oil from *C. obtusa* leaf has an antimicrobial activity against various fungal pathogens and pathogenic bacteria as well as allelopathic and insecticidal potentials (*20–22*).

Sawdust is a by-product of cutting, grinding, drilling or pulverizing wood. The sawdust processing could be a new practical tool for the production of naturally occurring safe and biologically effective products from a cheap and easily accessible waste material. In addition, smaller particle size and smooth texture properties of sawdust make it a better candidate for the extraction of bioactive compounds, as compared to the whole wood, wooden chips or timber, which are harder or more rigid. Moreover, by recycling sawdust, other useful products such as saccharides, turpentine, *etc*. can be obtained (*23*). In the United States, 98 % of the bark, sawdust, and wood trimmings from sawmill operations, and the black liquor produced in the pulping process are currently used as fuel or to produce other fibre products (*24*).

Literature survey has confirmed that there are few reports available on the antioxidant and free radical scavenging capacities of essential oil obtained from sawdust of *Chamaecyparis obtusa* (COEO) extracted by the microwave-assisted hydrodistillation technique. However, other essential oils isolated from sawdust of different plant materials have shown numerous biological activities (*25,26*). Also, the essential oil obtained from sawdust of *Cedrus deodara* has been found to have potential use in aromatherapy (*24*). In addition, owing to the complexity of the oxidation-reduction processes, it is obvious that no single testing method can provide a comprehensive picture of the antioxidant profile of a studied sample. Hence, in the present study, a multi-method approach for the assessment of antioxidant capacity of the test sample was applied.

In this study, a microwave-assisted hydrodistillation method was conducted to isolate the essential oil from sawdust of *Chamaecyparis obtusa*. The objective of the present study is to evaluate the antioxidant potential and free radical scavenging capacity of COEO using various *in vitro* models including DPPH, nitric oxide, superoxide and hydroxyl radical scavenging, as well as to perform a lipid peroxidation assay, followed by determination of the reducing power ability and total phenolic content of the extracted oil. Furthermore, the chemical composition of COEO was investigated using gas chromatography-mass spectrometry (GC-MS) analysis.

Materials and Methods

Chemicals and instrument

The chemicals and reagents used in this study included: bovine brain extract, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-deoxy-2-ribose, ferric chloride, Folin-Ciocalteu reagent, gallic acid, Griess reagent, nitroblue

tetrazolium (NBT), phenazine methosulphate (PMS), potassium ferricyanide, sodium nitroprusside (SNP) and trichloroacetic acid (TCA), as well as standard antioxidant compounds: ascorbic acid, BHA and α-tocopherol. All chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Spectrophotometric measurements were done using a 96-well microplate ELISA reader (Infinite M200, Tecan, Mannedorf, Switzerland).

Plant material and extraction

The wooden sawdust of *Chamaecyparis obtusa* (Siebold & Zucc.) Endl. was purchased from a local timber company specialized in processing timber materials of *C. obtusa*, and a voucher specimen number was deposited in the library of Yeungnam University, Gyeongsan, Korea. The sawdust was dried in shade at room temperature. The dried sawdust sample (200 g) was immersed in water (2 L) in 10-litre glass bottle, and then the bottle was subjected to hydrodistillation using a microwave-assisted extraction apparatus (KMD Engineering, Yangju-si, Korea) for a period of 2 h. The commercial microwave apparatus was fitted with automatic thermocontrol system having oven power capacity of 40 W, operating at the frequency of $15 \cdot 10^{12}$ Hz (Fig. 1). The distillate was collected and mixed with dichloromethane, shaken and kept in a separating funnel. For essential oil extraction, the lower layer of dichloromethane containing the essential oil was collected and evaporated using rotary evaporator at room temperature. Eventually, the oil was dried over anhydrous sodium sulphate (Na_2SO_4) and preserved in a sealed vial at 4 °C until analysis.

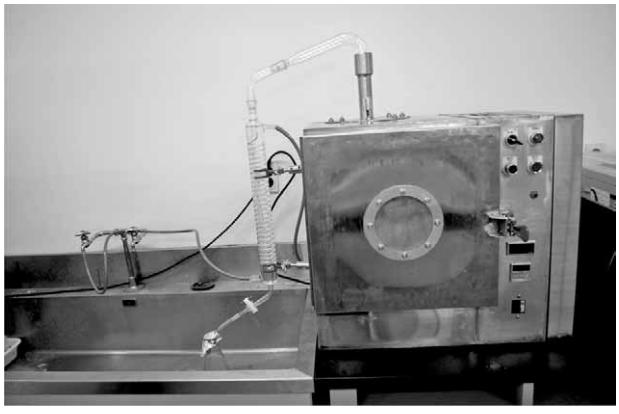

Fig. 1. Microwave apparatus used for the isolation of essential oil (COEO) obtained from the sawdust of *Chamaecyparis obtusa*

Analysis of COEO by gas chromatography-mass spectrometry

The detailed chemical composition of the COEO was analyzed using a JMS-700 mass spectrometer (JEOL USA, Inc., Peabody, MA, USA) equipped with an Agilent 6890N GC DB-5 MS (Agilent Technologies, Santa Clara, CA, USA) fused silica capillary column (30×0.25 m i.d., film thickness 0.25 μm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The temperature of the injector and MS transfer line was set at 280 and 250 °C, respectively. The initial oven temperature of 50 °C was maintained for 2 min, and then increased to 250 °C at a rate of 10 °C/min, followed by holding at 250 °C for 10 min. Diluted samples (1/100, by volume, in methanol) of 1.0 μL were injected manually in the splitless mode. The content of the oil constituents was expressed as relative percentages by peak area normalization. Identification of the COEO components was based on GC retention time on a DB-5 capillary column relative to computer matching of electron ionization mass spectra using Wiley and NIST libraries (Wiley-Blackwell, Hoboken, NJ, USA) for the GC-MS system.

Determination of the scavenging capacity against DPPH radical

The antioxidant capacity based on the scavenging of stable DPPH free radical was determined by the adopted method with minor modifications (*27*). A volume of 50 μL of COEO of various concentrations from 100 to 500 μg/mL was added to 0.004 % methanolic solution of DPPH (1:1 ratio) in a 96-well microplate. The mixture was incubated at 37 °C in the dark for 30 min with shaking at 150 rpm. Absorbance was recorded at 517 nm using the Infinite M200 ELISA reader against a blank sample. All the tests were performed in triplicate. The capacities of scavenging ascorbic acid and α-tocopherol were also measured as standard compounds in the concentration range of 25–150 μg/mL. The percent inhibition (X) capacity was calculated using Eq. 1:

$$X=(A_{control}-A_{test})/(A_{control})\cdot 100$$

where $A_{control}$ is the absorbance of the control reaction and A_{test} represents the absorbance of a test reaction.

Determination of nitric oxide radical scavenging capacity

Nitric oxide radical is an essential molecule required for several physiological processes including neural signal transmission, immune response, vasodilatation and regulation of blood pressure (*28*). The chronic emergence of nitric oxide radical is linked to various carcinomas and inflammatory conditions including arthritis, ulcerative colitis, juvenile diabetes, and multiple sclerosis (*29*). The nitric oxide radical has a strong NO^+ character which can alter the structure and function of many cellular components (*30*). Under aerobic conditions, nitric oxide molecule reacts with oxygen to produce intermediates such as NO_2, N_2O_4 and N_3O_4, as well as the stable products nitrate, nitrite and peroxynitrite when it reacts with superoxide. These products are highly genotoxic, causing deamination of purines, pyrimidines and denaturation of enzymes such as DNA ligase and DNA alkyltransferase (*31*). In this assay, SNP in aqueous solution at physiological pH generates nitric oxide, which interacts with oxygen to produce nitrate ions that can be estimated using the Griess reagent (1 % sulphanilamide, 2 % phosphoric acid and 0.1 % naphthyl ethylenediamine dihydrochloride).

In aqueous solution at physiological pH, SNP automatically generates nitric oxide, which reacts with oxygen to generate nitrite ions that can be measured by Griess reagent. Therefore, the application of free radical scavengers results in the reduced production of nitric oxide. In this assay, the solution of SNP (10 mM) in phosphate-buffered saline (PBS, pH=7.4) was mixed with different concentrations of COEO (100–500 μg/mL), and the mixtures were incubated at 37 °C for 60 min in the light. Half of the quantity of aliquots was taken and mixed with equal quantity of Griess reagent, and the mixture was incubated at 25 °C for 30 min in the dark. The absorbance of pink colour chromophore, generated during diazotization of nitric ions with sulphanilamide and subsequent coupling with naphthyl ethylenediamine dihydrochloride, was read at 546 nm against a blank sample (*32*). All the tests were performed in triplicate. Ascorbic acid and α-tocopherol were used as the reference compounds in the concentration range of 20–100 μg/mL. The percentage of inhibition (X) was calculated according to Eq. 1.

Determination of superoxide radical scavenging capacity

In the human body, superoxide radicals, highly toxic species, are generated by various metabolic and physiological processes. Although relatively weak oxidants such as superoxide radical show only restricted chemical reactivity, they can produce more reactive species, including singlet oxygen and hydroxyl radicals, which induce oxidative damage in lipids, proteins, DNA, *etc.* (*3*). Also, the superoxide radicals are produced by a number of autooxidation reactions in enzymatic systems and electron transfer reactions in non-enzymatic systems that univalently reduce molecular oxygen. Normally, the superoxide radical is formed first, and its deleterious effect can be magnified because it can generate more harmful free radicals (*33*). Superoxide radicals are derived from dissolved oxygen in PMS-NADH system and they reduce NBT. In this method, superoxide radicals reduce the yellow dye (NBT^{2+}) to produce the blue formazan, the absorbance of which is measured at 560 nm. Therefore, many antioxidants acting as scavengers of superoxide radical are able to inhibit the formation of blue formazan (*34*). The decrease of absorbance at 560 nm in the presence of antioxidants indicates the consumption of superoxide radical in the reaction mixture.

Superoxide radical (O^{2-}) scavenging capacity of COEO was measured by the reduction of NBT according to a previously reported method with a slight modification (*35*). The nonenzymatic phenazine methosulphate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce NBT to a purple colour formazan. In this assay, the reaction mixture (150 μL) contained phosphate buffer (0.2 M, pH=7.4), NADH (73 μM), NBT (50 μM), PMS (15 μM) and various concentrations (50–250 μg/mL) of COEO solution. After incubation for 60 min at room temperature, the absorbance of the reaction mixture was measured at 560 nm against an appropriate blank to determine the quantity of generated formazan. All tests in this assay were performed three times. Ascorbic acid and α-tocopherol were used as positive controls. The percentage of inhibition (X) was calculated using Eq. 1.

Determination of hydroxyl radical scavenging capacity

A previously described method with a minor modification was adopted for determining the hydroxyl radical (OH^-) scavenging capacity of COEO (*36*). The assay is based on the quantification of the degradation product of 2-deoxyribose sugar by condensation with TBA. Hydroxyl radical is generated by the Fenton reaction using Fe^{3+}-ascorbate/EDTA/H_2O_2 system. The reaction mixture in a final volume of 240 μL contained 2-deoxy-2-ribose (3 mM), KH_2PO_4/KOH buffer (20 mM, pH=7.4), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (2 mM), ascorbic acid (0.1 mM) and various concentrations (100–500 μg/mL) of COEO or standard compounds. After incubation for 45 min at 37 °C, 40 μL of 2.8 % TCA, and 40 μL of TBA (0.5 % in 0.025 M NaOH solution containing 0.02 % BHA) were added to the reaction mixture, and the mixture was incubated at 95 °C for 15 min in order to develop the pink colour. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests in this assay were performed three times. Ascorbic acid and BHA were used as positive controls. The percentage of inhibition (X) was calculated using Eq. 1.

Lipid peroxidation assay

Lipid peroxidation is the chemical reaction that affects the stability and shelf life of food, and is considered as a main factor contributing to oxidative damage *in vivo*. Currently, antioxidants are normally used as food additives to prolong the shelf life of fats and oils during processing and storage (*37*). *In vitro* lipid peroxidation was assessed by means of an assay system that determines the production of malondialdehyde (MDA) and related compounds in bovine brain extract (*3*). MDA is one of the major degradative products of lipid peroxidation and serves as a marker for oxidative stress. Thiobarbituric acid reactive species (TBARS), the by-products of lipid peroxidation that occur in nonpolar region of the biological membranes, are involved in the free radical-induced cellular damage that leads to many diseases in humans (*3*).

The Fe^{3+}/ascorbic acid-dependent nonenzymatic lipid peroxidation in bovine brain extract was performed according to the method described previously with a minor modification (*38*). The reaction mixture, in the presence or absence of different concentrations of COEO (50–250 μg/mL) or reference compounds, BHA and α-tocopherol, containing 50 μL of bovine brain phospholipids (5 mg/mL), 1 mM $FeCl_3$ and 1 mM ascorbic acid in 20 mM phosphate buffer with a final volume of 330 μL, was incubated at 37 °C for 1 h. The OH^- generated in the reaction initiated the lipid peroxidation, resulting in MDA production, which was measured by thiobarbituric acid (TBA) reaction using an ELISA reader. All tests were performed three times. The percentage of inhibition (X) was calculated according to Eq. 1.

Reducing power assay

This method was based on the reduction of the Fe(III)/ferricyanide complex to the ferrous form by the donation of one electron from the antioxidant. The ferrous ion (Fe^{2+}) is monitored by measuring the formation of Perl's Prussian blue at a wavelength of 700 nm.

The Fe^{3+} reducing power of the COEO was determined by the previously described method with a minor modification (*39*). Aliquots (50 μL) of different concentrations of COEO (5–25 μg/mL) were mixed with 50 μL of phosphate buffer (0.2 M, pH=6.6) and 50 μL of potassium ferricyanide (1 %), followed by incubation at 50 °C for 20 min in the dark. After incubation, 50 μL of TCA (10 %) were added to terminate the reaction and the mixture was subjected to centrifugation at 3000 rpm for 10 min. For final reaction mixture, the supernatant (50 μL) was mixed with 50 μL of distilled water and 10 μL of $FeCl_3$ solution (0.1 %). The reaction mixture was incubated for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. A higher absorbance of the reaction mixture indicated greater reducing power ability, confirming quantitative increase in the reduction of ferric ions to ferrous ions in the reaction mixture and *vice versa*. All tests were run in triplicate. Ascorbic acid and α-tocopherol as positive controls were also tested in the reducing power assay.

Determination of total phenolic content

The antioxidant capacity of plants is mainly contributed by the active compounds of essential oil and phenolic fraction present in them. Phenolic compounds are the main agents that can donate hydrogen atom to free radicals and thus break the chain reaction of lipid peroxidation and prevent polyunsaturated fatty acids from oxidative deterioration (*40*). The oxidation causes rancidity of unpreserved food rich in unsaturated fatty acids. This high potential of phenolic compounds to scavenge free radicals may be explained by their polyhydroxyl groups (*41*). In addition, phenolic compounds contribute directly to antioxidative action and inhibition of lipid peroxidation (*40*).

In this work, total phenolic content was determined using the Folin-Ciocalteu reaction according to the method as described by Kujala *et al.* (*42*) with a minor modification. An aliquot (50 μL) of COEO (100 μg/mL) was mixed with 50 μL of 5 % Folin-Ciocalteu reagent and the reaction mixture was incubated at 25 °C for 5 min in the dark, followed by the addition of 100 μL of 20 % Na_2CO_3 solution. After incubation at room temperature for 20 min, the absorbance of the developed blue colour chromophore was measured at 730 nm against an appropriate blank solution. The total phenolic content was evaluated from a standard calibration curve of gallic acid using the concentration range of 5–50 μg/mL and the results were expressed as mg of gallic acid equivalents per g of dry mass. All tests were run in triplicate.

Statistical analysis

All data are expressed as the mean value±standard deviation (S.D.) of three independent replicates. One-way analysis of variance followed by Duncan's test was performed to test the significance of differences between mean values obtained among the treatments at the 5 % level of significance using the SAS statistical software v. 9.1 (SAS Institute Inc., Cary, NC, USA).

Results and Discussion

GC-MS analysis of COEO

In this study, the GC-MS analysis of the COEO led to the identification of 46 different components, representing 98.94 % of the total oil. The identified compounds are listed in Table 1 according to their elution order on a DB-5 MS-fused silica capillary column. The COEO was characterized by a high fraction (in %) of oxygenated sesquiterpenes (44.17), followed by sesquiterpene hydrocarbons (37.45), steroids (4.24), diterpenes (2.30), indole derivatives (2.17), monoterpene hydrocarbons (1.52), aromatic ketones (0.66), oxygenated monoterpenes (0.54) and other compounds (5.89). The oil had a total yield of 3.79 %.

The major individual components detected in the oil were (in %): juniper camphor 12.47, fonenol 12.40, δ-9--capnellene-3-β-ol-8-one 10.09, α-patchoulene 8.80, α-muurolene 6.17, (+)longicyclene 5.55, α-amorphene 4.12, δ-cadinene 3.70, and 17-β-hydroxy-de-A-estra-5,7,9,14-tetraene 3.27.

In recent years, phenolic compounds, terpenes and their oxygenated phytoconstituents have been reported to have enormous antioxidant and free radical scavenging capacity (*6*). Previously, the essential oils containing monoterpene alcohols (thymol, terpineol) and sesquiterpenes (α-patchoulene, farnesol), which were also present in the COEO, have been shown to exert potent antioxidant and free radical scavenging activities in various antioxidant models (*43*). In general, the antioxidant compounds of essential oils are terpenes, which are phenolic in na-

Table 1. GC-MS analysis of the chemical composition of essential oil obtained by microwave-assisted hydrodistillation of sawdust of *Chamaecyparis obtusa*

No.	SI	RT/min	Compound[a]	Composition[b]/%	Identification method[c]
1.	472	6.63	deoxybenzoin	0.11	EI-MS
2.	836	6.78	1-borneol	0.07	EI-MS
3.	835	6.95	1,4-terpineol	0.16	EI-MS
4.	706	7.01	thymol	0.09	EI-MS
5.	817	7.13	linalyl propionate	0.12	EI-MS

Table 1 – continued

No.	SI	RT/min	Compound[a]	Composition[b]/%	Identification method[c]
6.	810	7.41	verbenone	0.05	EI-MS
7.	765	8.49	endo-bornyl acetate	0.06	EI-MS
8.	684	9.36	alloocimene	0.14	EI-MS
9.	744	9.43	α-copaene	0.08	EI-MS
10.	796	9.83	γ-cadinene	0.27	EI-MS
11.	789	10.01	α-guaiene	0.92	EI-MS
12.	769	10.88	α-humulene	0.09	EI-MS
13.	764	11.13	α-amorphene	4.12	EI-MS
14.	764	11.29	aromadendrene	0.91	EI-MS
15.	803	11.43	α-muurolene	6.17	EI-MS
16.	725	11.63	α-patchoulene	8.80	EI-MS
17.	657	11.69	δ-cadinene	3.70	EI-MS
18.	697	11.89	torreyol	1.36	EI-MS
19.	550	11.96	α-calacorene	1.40	EI-MS
20.	693	12.09	*cis*-farnesol	0.20	EI-MS
21.	664	12.21	β-carboline	0.72	EI-MS
22.	691	12.46	diethylpthalate	1.98	EI-MS
23.	564	12.84	γ-gurjunene	1.46	EI-MS
24.	573	12.89	4,5,9,10-dehydroisolongi-folene	0.56	EI-MS
25.	629	12.99	δ-cadinol	2.57	EI-MS
26.	603	13.16	fonenol	12.40	EI-MS
27.	625	13.17	(+)longicyclene	5.55	EI-MS
28.	443	13.22	17-β-hydroxy-de-A-estra--5,7,9,14-tetraene	3.27	EI-MS
29.	633	13.31	juniper camphor	12.47	EI-MS
30.	447	13.42	calamene	1.06	EI-MS
31.	744	13.52	azunol	2.64	EI-MS
32.	549	13.84	1S-*cis*-calamenene	2.37	EI-MS
33.	515	14.19	germacrone	0.83	EI-MS
34.	534	14.31	megastigmatrienone	0.55	EI-MS
35.	408	14.84	gelatonin	1.45	EI-MS
36.	453	15.00	δ-9-capnellene-3-β-ol-8-one	10.09	EI-MS
37.	368	15.42	2,2,4-trimethylfuro[6,7-c]--1,3,8H-azulene	1.38	EI-MS
38.	372	15.65	carbamazepine	0.77	EI-MS
39.	403	15.95	D-norandrostan-16-ol	0.97	EI-MS
40.	405	16.02	13,17-diepoxy-14,15-bis-norlabdane	1.24	EI-MS
41.	381	16.05	6-deoxycryptosporin	1.54	EI-MS
42.	398	16.09	13-hydroxystevane	1.06	EI-MS
43.	343	16.35	sclareolide	0.83	EI-MS
44.	383	16.44	2-methyl-2-hydroxyethyl--aminobenzothiazole	1.17	EI-MS
45.	346	16.60	cinchonine	0.43	EI-MS
46.	379	16.67	dihydroartemisinin	0.78	EI-MS

SI=library search purity value, RT=retention time; [a]compounds listed in order of elution from a DB-5 capillary column, [b]percentage based on GC-MS peak area normalization, [c]identification based on computer matching of electron ionization mass spectra using Wiley and NIST libraries for the GC-MS system

ture, and it would seem rational that their antioxidant mode of action might be related to that of other compounds (*44*). Interestingly, the COEO was also found to contain melatonin, a molecule which acts as a direct free radical scavenger (*45*). Moreover, it stimulates the synthesis of antioxidant enzymes and lowers the molecular damage under elevated oxidative stress conditions (*46*, *47*). In addition, the melatonin has a capacity to augment the effectiveness of mitochondrial electron transport chain, thus lowering the electron leakage and reducing free radical generation (*48*). These findings correlate with and support the phenomena that COEO could be a potent natural antioxidant.

Antioxidant capacity assays of COEO

DPPH radical scavenging assay

The DPPH radical is a stable radical with a maximum absorbance at 517 nm that can readily undergo reduction by the antioxidant molecule. Because of the easiness and convenience of this reaction, it has now widespread use in the assessment of free radical scavenging capacity of both hydrophilic and lipophilic compounds (*49*). The radical scavenging capacity of COEO, ascorbic acid and α-tocopherol is presented in Fig. 2, and expressed as a percentage reduction of the initial DPPH radical absorption by the tested compounds. In this assay, the COEO at the concentration of 500 μg/mL showed high scavenging of DPPH radical ($p<0.05$) with a percentage inhibition of 80.16 % ($\gamma(IC_{50})$=120 μg/mL). Consequently, the more rapidly the absorbance decreases, the more potent antioxidant capacity of COEO is observed in terms of hydrogen atom or electron-donating ability. Also, the reference compounds, ascorbic acid and α-tocopherol, at the concentration of 150 μg/mL, showed high inhibitory effect against DPPH radical, about 81.69 and 84.09 %, respectively. The COEO, ascorbic acid and α-tocopherol had the concentration-dependent DPPH radical scavenging capacity (Fig. 2). Previously, the antioxidant capacity of the leaf essential oil of *Metasequoia glyptostroboides* and various plant-based essential oils has been confirmed using a DPPH model (*12*,*50*,*51*) and our newly characterized COEO was also verified for the high DPPH scavenging capacity.

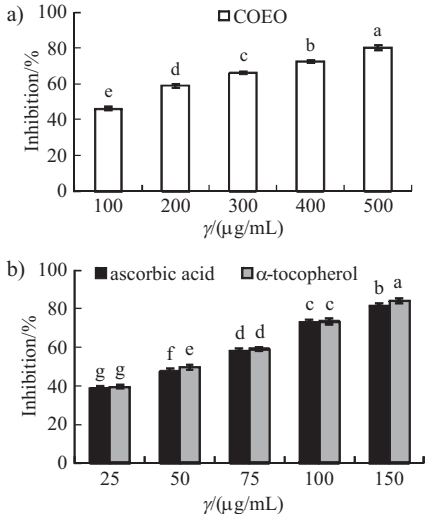

Fig. 2. DPPH radical scavenging activity of: a) the essential oil obtained from the sawdust of *Chamaecyparis obtuse* (COEO), and b) standard antioxidant compounds, ascorbic acid and α-tocopherol. Different letters in each column indicate the significant differences of the mean values ($p<0.05$)

Nitric oxide radical scavenging assay

Our study showed that COEO in reaction mixture decreased the levels of nitrite by competing with oxygen to react with nitric oxide radical, and that it has a possible protective effect against oxidative damage. As shown in Fig. 3, the nitric oxide radical scavenging capacity of COEO was concentration-dependent with the most efficient inhibition (82.93 %) at the concentration of 500 μg/mL ($\gamma(IC_{50})$=205 μg/mL). Also, the reference compounds ascorbic acid and α-tocopherol showed a concentration-dependent inhibitory effect against nitric oxide radical (Fig. 3). The results obtained for all the tested compounds (oil and standards) were statistically significant ($p<0.05$). The nitric oxide radical scavenging capacities of various plant-based essential oils have been reported previously (*51*,*52*), and our data designate that COEO could be an important natural nitric oxide radical scavenger.

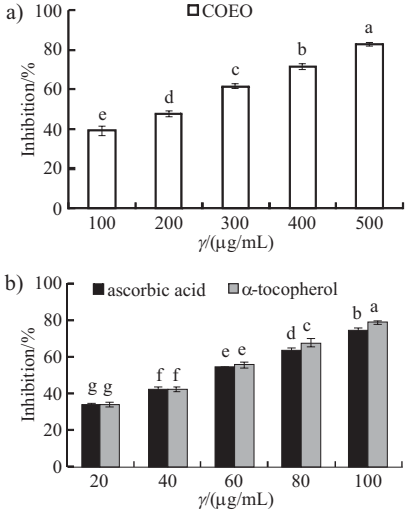

Fig. 3. Nitric oxide radical scavenging activity of: a) the essential oil obtained from the sawdust of *Chamaecyparis obtusa* (COEO), and b) standard antioxidant compounds, ascorbic acid and α--tocopherol. Different letters in each column indicate the significant differences of the mean values ($p<0.05$)

Superoxide radical scavenging assay

In the superoxide radical scavenging capacity assay, measured by the PMS/NADH superoxide generating system, the COEO demonstrated a concentration-dependent inhibition of the superoxide radical (Fig. 4). At the concentration of 250 μg/mL, the COEO had a high superoxide radical scavenging capacity of 72.99 % ($\gamma(IC_{50})$= 135 μg/mL), a value statistically equivalent to the superoxide radical scavenging capacity of ascorbic acid and α-tocopherol. Based on these findings, it can be concluded that COEO scavenged superoxide radicals by combining with superoxide radical ions to form stable radicals, thus terminating the radical chain reaction (*53*).

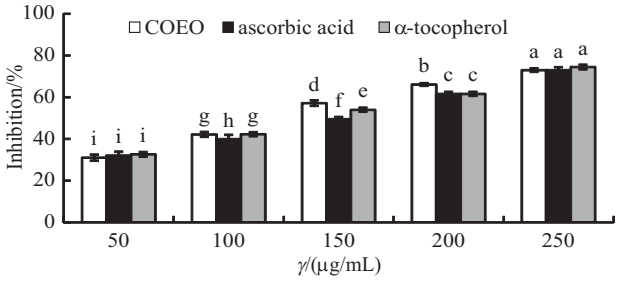

Fig. 4. Superoxide radical scavenging activity of the essential oil obtained from the sawdust of *Chamaecyparis obtusa* (COEO), and standard compounds, ascorbic acid and α-tocopherol. Different letters in each column indicate the significant differences of the mean values ($p<0.05$)

Our findings are supported by previous reports on essential oils that scavenged the superoxide radical effectively (*51,52*).

Hydroxyl radical scavenging assay

Hydroxyl radical is a major active oxygen species which causes lipid peroxidation and cellular damage (*3*). The assay showed the ability of the COEO and standard compounds ascorbic acid and BHA to inhibit the hydroxyl radical-mediated deoxyribose degradation in an *in vitro* system consisting of Fe^{3+}-EDTA/ascorbate and H_2O_2. In this system, the hydroxyl radicals are produced by incubating ferric-EDTA with ascorbic acid and H_2O_2 at pH=7.4 and by making it react with 2-deoxy-2-ribose sugar to generate MDA. This compound forms a pink chromogen upon heating with TBA at low pH. The hydroxyl radical scavenging effect of COEO in comparison with reference standards ascorbic acid and BHA is shown in Fig. 5. The results of this assay suggest that COEO at

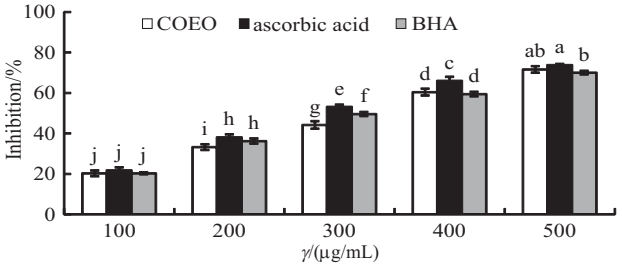

Fig. 5. Hydroxyl radical scavenging activity of the essential oil obtained from the sawdust of *Chamaecyparis obtusa* (COEO), and standard compounds, ascorbic acid and butylated hydroxyanisole (BHA). Different letters in each column indicate the significant differences of the mean values ($p<0.05$)

the concentration of 500 μg/mL was highly effective in inhibiting the oxidative DNA damage with the percentage inhibition of 71.62 % ($\gamma(IC_{50})$=135 μg/mL), whereas ascorbic acid and BHA inhibited the oxidative DNA damage by 73.79 and 70.02 %, respectively. The results also showed that COEO and the reference standards had a significant dose-dependent antioxidant capacity ($p<0.05$). The results obtained in this assay are in strong agreement with the previously reported findings on scavenging the hydroxyl radical (*51,54*).

Lipid peroxidation inhibition

The lipid peroxidation inhibitory capacity of COEO in Fe^{3+}/ascorbate system due to the inhibition of the formation of MDA compared to the standards α-tocopherol and BHA is shown in Fig. 6. In this assay, the inhibitory effect of COEO, α-tocopherol and BHA at the

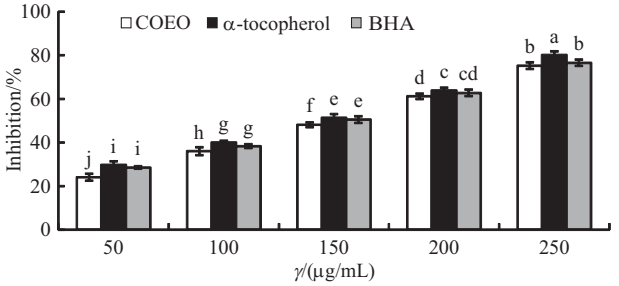

Fig. 6. Lipid peroxidation inhibitory effect of the essential oil obtained from the sawdust of *Chamaecyparis obtusa* (COEO) and standard compounds, α-tocopherol and butylated hydroxyanisole (BHA). Different letters in each column indicate the significant differences of the mean values ($p<0.05$)

concentration of 250 μg/mL on the formation of MDA was found to be 75.24 ($\gamma(IC_{50})$=135 μg/mL), 80.83 and 76.59 %, respectively. The results were significantly concentration-dependent ($p<0.05$). These findings suggest that COEO is a potent scavenger of TBARS. The hydroxyl radical scavenging capacity of COEO could reduce the amount of hydroxyl radicals, therefore, the lower amount of hydroxyl radicals might explain the inhibition of lipid peroxidation by COEO (*51*).

Reducing power of COEO

In this assay, the reducing power ability of COEO was significantly concentration-dependent (Fig. 7). As the concentration increased from 5 to 25 μg/mL, there was an increase in the absorbance values for all the tested samples. The increase of the absorbance of the reaction mixture indicates an increase in the reducing power ability. It is believed that antioxidant capacity and reducing power are correlated (*55*). Reductones inhibit lipid peroxidation by donating a hydrogen atom

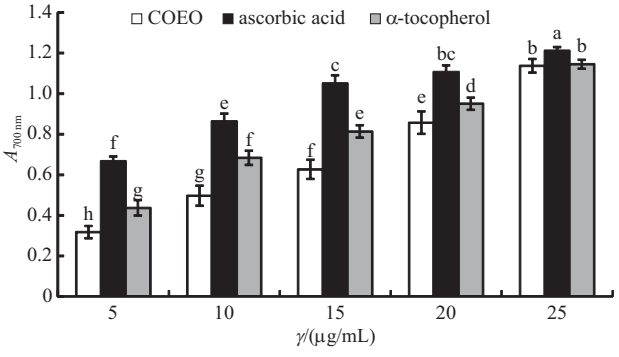

Fig. 7. Reducing power ability of the essential oil obtained from the sawdust of *Chamaecyparis obtusa* (COEO) and standard compounds, ascorbic acid and α-tocopherol. Different letters in each column indicate the significant differences of the mean values ($p<0.05$)

and thereby terminating the free radical chain reaction (*55*). Indeed, numerous essential oils containing terpene hydrocarbons and oxygenated terpenes exhibit antioxidant capacity through their reductive capacity in a Fe^{3+}/Fe^{2+} system (*56*). The data presented here indicate that the marked reducing power of COEO seems to be attributed to its antioxidant capacity.

Total phenolic content of COEO

The influence of phenolic content on the antioxidant capacity of plant-derived compounds has been demonstrated previously (*42*). Interestingly, the COEO contained (6.13±0.05) mg of gallic acid equivalents per g of dry mass of the sample of total phenols. It is reported that polyphenolic compounds of fruits and vegetables have remarkable antimutagenic and anticarcinogenic traits as well as health-promoting effects in humans (*57*).

Conclusion

In this study, a microwave-assisted hydrodistillation technique was employed. The analyses of the essential oil from *Chamaecyparis obtusa* (COEO) by GC-MS showed the presence of terpenes and phenolics including juniper camphor, fonenol, δ-9-capnellene-3-β-ol-8-one, α-patchoulene, α-muurolene, (+)longicyclene, α-amorphene, 1-borneol, 1,4-terpineol, thymol, *cis*-farnesol and δ-cadinene as the important phytoconstituents which exhibited high antioxidant and free radical scavenging capacities. It was confirmed in this study that the COEO exhibited different antioxidative values depending on the concentration and the measured antioxidant parameters. In DPPH, nitric oxide and superoxide scavenging assays, the COEO at the used concentrations showed almost similar antioxidant effect compared to the standard compounds. Also, the COEO had almost similar inhibitory effect in lipid peroxidation assay when compared with the BHA standard. However, COEO showed higher antioxidant capacity (71.62 %) in hydroxyl radical scavenging assay at the concentration of 250 μg/mL, when compared to the BHA standard (70.02 %). These *in vitro* assays indicate that COEO, containing bioactive compounds, can be a significant source of natural antioxidants, which might be helpful in preventing the progress of various oxidative stress-induced diseases caused by the overproduction of free radicals. Based on our results, the use of cheap and easily accessible waste material as by-products such as sawdust from *Chamaecyparis obtusa* could be considered as a new source for the production of naturally occurring safe and effective biological products. The present investigation provided evidence required for the utilization of newly characterized COEO for its possible use as a source of a cost-effective natural antioxidant. Moreover, the COEO, as a possible dietary supplement, is also expected to be used in stabilizing food against oxidative deterioration.

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