Acetylcholinesterase Inhibitory Activity and Chemical Composition of Commercial Essential Oils

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Commercially available essential oils extracted from *Artemisia dracunculus* L., *Inula graveolens* L., *Lavandula officinalis* Chaix, and *Ocimum sanctum* L. and the components of these oils were screened by the microplate assay method for determining their acetylcholinesterase (AChE) inhibitory activity. The composition profiles of the oils were characterized by gas chromatography−mass spectrometry (GC-MS) analysis, and the relationships between the oil components and the AChE inhibitory activity of the oils were outlined. The results showed that all of the oils, except that of *A. dracunculus* from Hungary, exhibited AChE inhibitory activity, and the *A. dracunculus* oil from France showed the most potent inhibitory activity [50% inhibition concentration (IC$_{50}$) = 0.058 mg/mL]. The AChE inhibitory activity of *I. graveolens* oil has not been reported to date, and this study is the first to reveal this activity in the oil. Among the essential oil components, five components, namely, 1,8-cineole, α-pinene, eugenol, α-terpineol, and terpinen-4-ol, showed AChE inhibitory activity, with IC$_{50}$ values of 0.015, 0.022, 0.48, 1.3, and 3.2 mg/mL, respectively. Eugenol, in particular, was found to be a potent AChE inhibitor along with determination of the IC$_{50}$ value, a finding that has been reported for the first time in this study. However, the ratio of the contribution of the active components, including a novel AChE inhibitor, to the observed AChE inhibitory activity of the essential oils was not very high. The results of this study raise concerns about the AChE inhibitory activity of widely produced and readily accessible commercial essential oils.

KEYWORDS: AChE inhibitory activity; essential oils; Asteraceae; Lamiaceae; GC-MS; contribution ratio

INTRODUCTION

Herbs have been extensively used in the liquor and confectionary industries, in perfume production, for medicinal purposes, and as flavoring agents in different industries (*1*). Many natural compounds extracted from plants exhibit important biological activities. Among these diverse natural compounds, essential oils extracted from aromatic plants are attracting special attention. For example, it is well-known that essential oils possess antimicrobial and antioxidant properties (*2*−*4*). It has been recently reported that extracts from some plants exhibit inhibitory activity against acetylcholinesterase (AChE) (*5*−*8*). AChE is the principal enzyme involved in the hydrolysis of acetylcholine; therefore, these AChE inhibitors are being developed for the symptomatic treatment of Alzheimer's disease (*9*, *10*). Alzheimer's disease is a progressive degenerative neurological disorder, which is principally characterized by impaired memory and disturbed behavior. The role of cholinergic neurotransmission in memory processing and storage is the basis for the widely accepted "cholinergic hypothesis" (*11*). One approach for the treatment of Alzheimer's disease is to increase the acetylcholine level in the brain (*12*). Therefore, essential oils

have gained importance because of their AChE inhibitory activity.

The results of previous studies indicate that several essential oils show significant AChE inhibitory activity. Perry et al. have demonstrated the inhibitory activity of the essential oil extracted from sage, which is traditionally used to enhance memory, both in vivo and in vitro (*13*, *14*). In addition, essential oils from food crops (*15*), medicinal plants (*16*, *17*), and tea tree oils (*18*, *19*) have been screened to identify the oils with high AChE inhibitory activity. Various components of essential oils have also been investigated for their effects on AChE. It has been found that the majority of AChE inhibitors are terpenoids (*19*−*22*). The *Artemisia* and *Inula* genera, which are important members of the Asteraceae family, are widely distributed in the northern hemisphere (*23*, *24*). These species have traditionally been used as ingredients of cosmetics and herbal remedies because of their biologically active compounds. The genus *Lavandula*, of the Lamiaceae family, consists of approximately 20 species with more than 100 varieties of lavender (*25*). The genus *Ocimum*, of the Labiatae family, includes at least 60 species and numerous varieties, and the plants of this species are an important source of essential oils (*26*). Moreover, plants of these species are extensively used in food industries such as those concerned with flavor and fragrance. The essential oils extracted from these

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plants are the most representative examples of the most widely produced and consumed essential oils. However, very few investigations have been conducted to determine the AChE inhibitory activity of these readily accessible commercial essential oils. Moreover, there are very few reports on the specific evaluation of the active components of essential oils. Therefore, in this study, we aimed to evaluate the AChE inhibitory activity of 10 commercial essential oils and their components by screening them using the microplate assay method. The composition profiles of the oils were characterized by gas chromatography−mass spectrometry (GC-MS) analysis, and the relationships between the chemical components and AChE inhibitory activity were outlined. We analyzed the oils extracted from *Artemisia dracunculus* L. (Asteraceae; 7 products), *Inula graveolens* L. (Asteraceae), *Lavandula officinalis* Chaix (Lamiaceae), and *Ocimum sanctum* L. (Lamiaceae).

MATERIALS AND METHODS

Essential Oils. Steam-distilled oils of *A. dracunculus* (lots 1 and 2, from Italy; lot 3, from the United States; lots 4−6, from Hungary; and lot 7, from France), *I. graveolens* (from France), *L. officinalis* (from France), and *O. sanctum* (from India) were purchased from an online shopping site or a department store in Shizuoka.

Chemicals. Anethole, (−)-borneol, 1,8-cineole, camphor, (±)-limonene, linalyl acetate, 4-methoxycinnamaldehyde, 4-terpineol, α-terpineol, and galantamine hydrobromide were purchased from Tokyo Chemical Industry, Tokyo, Japan. Bornyl acetate, estragole, and γ-terpinene were purchased from Acros Organics, Geel, Belgium. β-Caryophyllene and methyleugenol were purchased from Wako Pure Chemical Industries, Osaka, Japan. Camphene, α-humulene, α-pinene, β-pinene, tris(hydroxymethyl)aminomethane (Tris), 37% HCl, NaCl, MgCl$_2$·6H$_2$O, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), bovine serum albumin (BSA), AChE (type VI-S) from the electric eel *Electrophorus*, and acetylthiocholine iodide (ATCI) were purchased from Sigma-Aldrich Japan KK, Tokyo, Japan. Unless otherwise specified, 50 mM Tris-HCl buffer (pH 8.0) was used throughout the experiment. The lyophilized enzyme was dissolved in the buffer to obtain a 1000 U/mL stock solution. Further dilutions of the enzyme solution were prepared in a buffer solution containing 0.1% BSA. DTNB was dissolved in a buffer solution containing 0.1 M NaCl and 0.02 M MgCl$_2$. ATCI was dissolved in deionized water. All of the chemicals and solvents used were of analytical grade or high-performance liquid chromatography grade.

Chemical Composition Analysis of the Essential Oils. The samples were analyzed by GC-MS (GC, HP 6890 series, Hewlett-Packard, Palo Alto, CA; MS, HP 5972A series mass-selective detector, Hewlett-Packard) on an HP-5 fused-silica capillary column (internal diameter, 30 m × 0.32 mm; film thickness, 0.25 μm; J&W Scientific, Folsom, CA). The column temperature was initially maintained at 50 °C for 1 min, after which it was raised to 170 °C at a rate of 5 °C/min and from 170 to 280 °C at a rate of 10 °C/min, with a final hold time of 4 min.

Helium was used as the carrier gas (flow rate, 1.3 mL/min), and the column head pressure was maintained at 6894 Pa. The injector temperature was maintained at 250 °C, and the injection volume was 1.0 μL in the splitless mode. The electron-multiplier voltage for MS was 1988 V, and the interface temperature was maintained at 280 °C. The mass spectra of the oil components were obtained by electron impact ionization at a voltage of 70 eV and scanned in the *m/z* range of 45−400 atomic mass units at a rate of 1.5 scans per second; the ion source temperature was maintained at 250 °C. The retention indices of the compounds were calculated on the basis of the retention times of *n*-alkanes. The oil components were identified by comparing their gas chromatographic retention indices and mass spectra with those of the authentic standards. The percentage composition of each oil sample was computed from the peak areas on the total ion chromatogram by using the normalization method.

Microplate Assay for AChE Inhibitory Activity. AChE inhibitory activity was measured by using an assay described by Ellman et al. (*27*) along with the modifications described by Hammond and Forster (*28*). Briefly, 25 μL of the oil sample was dissolved in methanol along with

50 μL of the buffer and 25 μL of 0.22 U/mL AChE, and the mixture was incubated at 37 °C for 15 min. The time at which the first enzyme addition was performed was considered as time zero. In this case, because the further analyses were performed using an end-point reading, the kinetics of reactions were not considered to be important (*29*). After the 15 min incubation, 125 μL of 3 mM DTNB and 25 μL of 15 mM ATCI were added, and the final mixture was incubated at room temperature for 30 min. The absorbance of the mixture was measured at 412 nm by using a microplate reader (Varioskan Flash; Thermo Fisher Scientific, Yokohama, Japan). A control mixture was prepared by using 25 μL of methanol instead of the oil sample, with all other procedures similar to those used in the case of the sample mixture. The inhibition (percent) was calculated by using the equation

$$I\,(\%) = 100 - (A_{\text{sample}}/A_{\text{control}}) \times 100$$

where A_{sample} was the absorbance of the oil-sample-containing reaction mixture and A_{control} was the absorbance of the reaction-control mixture. The sample concentration showing 50% inhibition (IC$_{50}$) was calculated by plotting the inhibition percentages against the corresponding sample-solution concentrations. Galantamine was used as the positive control. The concentration ranges used for the microplate assay were 1.0×10^{-5}−100 or 1.0×10^{-6}−10 mg/mL for the essential oils, 3.7×10^{-8}−0.37 mg/mL for galantamine, and 1.0×10^{-5}−100 or 1.0×10^{-6}−10 mM for the chemicals.

Statistical Analysis. Data are expressed as mean ± standard deviation (SD) of the values obtained from multiple independent experiments.

RESULTS AND DISCUSSION

Chemical Composition of the Essential Oils. The results of the GC-MS analysis of the essential oils are presented in **Table 1**. The identified components accounted for 79−96% of the chemical constituents of the essential oils. We identified 10 components in the *A. dracunculus* (Asteraceae) oils. Estragole was the principal component (77−89%); we also identified limonene, but it was present at a very low percentage (0.27−4.9%). These results are in good agreement with those of the previous studies that have reported a large number of components in the oils extracted from other European tarragon species (*4, 23, 30*). Moreover, there were no geographical distribution related differences in the chemical composition of the essential oils. We identified nine components in the *I. graveolens* (Asteraceae) oil, and the principal components were bicyclic monoterpenes such as bornyl acetate (54%), borneol (20%), and camphene (4.9%). These results were consistent with those of a previous report on the composition of the commercially available *I. graveolens* oil obtained from Corsica (*24*). Although there were a large number of components in the *L. officinalis* (Lamiaceae) oil, the acyclic monoterpene linalool and its ester were dominant (70%). The percentages of the other components, namely, β-caryophyllene (4.5%), terpinene-4-ol (1.7%), and 1,8-cineole (1.2%), were quite less. It is known that plants of the genus *Lavandula* contain a high proportion of linalyl acetate and linalool (*25, 31*). The *O. sanctum* (Lamiaceae) oil consisted of only three components: eugenol (59%), β-caryophyllene (33%), and α-humulene (3.0%). These findings on the quantitative composition of the *O. sanctum* oil were in agreement with those reported in the studies that indicated that eugenol and β-caryophyllene were the principal components (*26, 32*).

Screening of AChE Inhibitory Activity of Essential Oils and Their Components. The IC$_{50}$ values of the essential oils and their components, which were measured by the microplate assay method and indicated their AChE inhibitory activity, are presented in **Table 2**. All of the essential oils, except 1, exhibited AChE inhibitory activity. The dose−response relationships are shown in **Figure 1**. In the assays, one essential oil

Table 1. Chemical Composition of the Commercial Essential Oil Components Identified by GC-MS

			% composition[c]									
			Asteraceae									Lamiaceae
			Artemisia dracunculus									
component	RI[a]	ID[b]	lot 1	lot 2	lot 3	lot 4	lot 5	lot 6	lot 7	Inula graveolens	Lavandula officinalis	Ocimum sanctum
α-pinene	944	MS, RI	0.79 ± 0.036	0.81 ± 0.055		1.1 ± 0.037	0.71 ± 0.038	0.76 ± 0.065	1.4 ± 0.066	0.21 ± 0.0050	≤0.10	≤0.10
camphene	956	MS, RI						≤0.10		4.9 ± 0.29	0.19 ± 0.023	
β-pinene	980	MS, RI			1.8 ± 0.19	0.12 ± 0.0030	≤0.10			0.64 ± 0.021	≤0.10	
limonene	1031	MS, RI	4.7 ± 0.092	4.7 ± 0.29	0.27 ± 0.032	3.7 ± 0.18	4.5 ± 0.027	4.7 ± 0.25	4.9 ± 0.26	0.77 ± 0.039	0.54 ± 0.10	0.11 ± 0.077
1,8-cineole	1033	MS, RI			≤0.10						1.2 ± 0.22	0.44 ± 0.30
linalool	1064	MS, RI									34 ± 1.3	≤0.10
camphor	1104	MS, RI									0.52 ± 0.058	
borneol	1147	MS, RI								20 ± 0.35	1.0 ± 0.075	
terpinen-4-ol	1171	MS, RI									1.7 ± 0.050	
α-terpineol	1182	MS, RI								1.6 ± 0.062	0.95 ± 0.17	
estragole	1197	MS, RI	82 ± 0.71	80 ± 0.71	89 ± 1.8	77 ± 1.2	83 ± 0.69	80 ± 1.3	77 ± 1.2			
linalyl acetate	1206	MS, RI									36 ± 1.2	
bornyl acetate	1266	MS, RI								54 ± 2.0		
eugenol	1296	MS, RI	≤0.10						≤0.10			59 ± 6.0
methyleugenol	1370	MS, RI	0.53 ± 0.086	0.44 ± 0.066		0.31 ± 0.049	0.46 ± 0.046	0.46 ± 0.068	0.73 ± 0.075			
β-caryophyllene	1415	MS, RI	0.44 ± 0.034	0.51 ± 0.050	0.41 ± 0.062	≤0.10	0.49 ± 0.041	0.52 ± 0.031	≤0.10	1.9 ± 0.13	4.5 ± 0.099	33 ± 9.1
α-humulene	1427	MS, RI										3.0 ± 0.23
γ-cadinene[d]	1460	DB								1.0 ± 0.063		
4-methoxycinnamaldehyde	1566	MS, RI	≤0.10		0.61 ± 0.13							
total			88 ± 1.0	86 ± 0.28	92 ± 1.4	82 ± 0.97	89 ± 0.57	86 ± 0.93	84 ± 0.88	85 ± 1.2	79 ± 1.0	96 ± 2.9

[a] RI, retention index. [b] ID, identification method: MS, reference mass spectrum; RI, reference retention index; DB, reference commercial library search (NIST'98). [c] Values are presented as the mean ± standard deviation ($n = 4$). [d] Tentatively identified.

(oil of *A. dracunculus*, lot 4) showed increased dose inhibition; however, even at its highest concentration, the oil did not show 50% inhibition. The AChE inhibitory activity of the essential oils, relative to that of the positive control galantamine, ranged from 0.00020 to 0.0066, with the highest activity shown by *A. dracunculus* oil, lot 7 ($IC_{50} = 0.058$ mg/mL). The observed inhibitory activity of this essential oil was equal to the reported inhibitory activities ($IC_{50} = 0.050-0.069$ mg/mL) of Spanish sage oil extracted from *Salvia lavandulaefolia* (*21*), Australian tea tree oil extracted from *Melaleuca alternifolia* (*19*), and *Rosmarinus officinalis* oil (*15*). With regard to the AChE-related properties of the genus *Artemisia*, the ethanolic extracts of European medicinal plants show binding affinity to the cholinergic receptor (*33*). The inhibitory activity of the *I. graveolens* oil was one-fifth that of the *A. dracunculus* (lot 7) oil, and its IC_{50} value was 0.27 mg/mL. This value is similar to the potencies of the essential oils extracted from fennel, mint, and pennyroyal ($IC_{50} = 0.25-0.36$ mg/mL) (*15*). Moreover, the AChE inhibitory activity of *I. graveolens* has not been reported thus far. In comparison with the inhibitory activities of the essential oils extracted from the members of Asteraceae, the two essential oils from the members of Lamiaceae that were studied by us showed moderate AChE inhibition. The IC_{50} values for the *L. officinalis* and *O. sanctum* oils were 0.82 and 1.6 mg/mL, respectively. Previous papers have indicated that essential oils and solvent extracts from the genera *Lavandula* (*17, 33, 34*) and *Ocimum* (*35*) show AChE and butyrylcholinesterase (BChE) inhibitory activities.

Among the essential oil components, five components showed AChE inhibitory activity (**Figure 2A**) and seven did not show 50% inhibition (**Figure 2B**). 1,8-Cineole and α-pinene showed AChE inhibitory activities that were 0.017-0.025-fold that of galantamine, whereas eugenol showed a moderate inhibitory activity. Previous studies have shown that 1,8-cineole

Table 2. Screening of Commercial Essential Oils and Their Components for Acetylcholinesterase Inhibitory Activity

sample	IC_{50} (mg/mL)
Asteraceae	
A. dracunculus lot 1	0.14 ± 0.050
lot 2	0.19 ± 0.10
lot 3	1.9 ± 0.10
lot 4	ND[a]
lot 5	1.1 ± 0.72
lot 6	0.59 ± 0.13
lot 7	0.058 ± 0.026
I. graveolens	0.27 ± 0.10
Lamiaceae	
L. officinalis	0.82 ± 0.60
O. sanctum	1.6 ± 0.69
chemicals	
1,8-cineole	0.015 ± 0.0030
α-pinene	0.022 ± 0.0030
eugenol	0.48 ± 0.16
α-terpineol	1.3 ± 0.060
terpinen-4-ol	3.2 ± 2.3
β-pinene	ND
4-methoxycinnamaldehyde	ND
camphor	ND
methyleugenol	ND
citral	ND
t-anethole	ND
linalool	ND
bornyl acetate	NA[c]
β-caryophyllene	NA
estragole	NA
limonene	NA
γ-terpinene	NA
galantamine[c]	$3.8 \times 10^{-4} \pm 1.6 \times 10^{-4}$

[a] ND, not determined, means the maximum level of inhibition below 50%. [b] NA, not active (10 mM tested). [c] Positive control.

and α-pinene are potent AChE inhibitors with IC_{50} values ranging from 0.049 to 0.10 mg/mL and from 0.086 to 0.090 mg/mL, respectively (*13, 21, 19*). In this study, eugenol exhibited inhibitory activity, with an IC_{50} value of 0.48 mg/mL; however, previous studies have reported contrasting results on the inhibitory activity of this compound. Orhan et al. demonstrated the AChE and BChE inhibitory activities of essential oils extracted from Turkish plants and the individual components of the oils by using a 96-well microplate method (*35*). In that study, eugenol showed 45% inhibition of BChE and no AChE inhibition at a nominal concentration of 0.1 mg/mL. In our study, although the dose–response curve of eugenol showed that the compound exhibited AChE inhibitory activity at higher concentrations of 1.6–16 mg/mL (**Figure 2A**), lower

concentrations of eugenol did not exhibit significant inhibitory activity. On the basis of the results of this study, we estimated that this compound would have shown only 10% inhibition at a concentration of 0.1 mg/mL. Therefore, the differences in the AChE inhibitory activity of eugenol may be attributed to the concentration ranges used in the assay. To date, there have been no reports on the evaluation of the AChE inhibitory activity of eugenol along with determination of the IC_{50} value, and this aspect was demonstrated for the first time in this study. α-Terpineol and terpinen-4-ol are considered to be weak inhibitors of AChE; the degree of inhibitory activities of these monoterpene alcohols in our study was consistent with those observed in previous studies (*13, 18*).

Relationships between Oil Components and AChE Inhibitory Activity. To evaluate the contribution of the above-mentioned active components in the observed AChE inhibitory activity of the essential oils, the data obtained from the GC-MS experiments were converted into galantamine equivalent concentrations by multiplying the concentrations of the components by the corresponding values for galantamine activity (**Table 3**). Eugenol had the highest contribution ratio among the active components, and it accounted for 25% of the observed inhibitory activity of the *O. sanctum* oil. The contribution ratio of 1,8-cineole accounted for over 6% of the inhibitory activity of the *L. officinalis* oil. The GC-MS analysis of the *O. sanctum* oil revealed the presence of eugenol, β-caryophyllene, and α-humulene, which together accounted for 95% of the constituents of the oil sample. Therefore, the AChE inhibitory activity of the *O. sanctum* oil may be due to the combined effects of the three identified components. In fact, synergistic and antagonistic interactions between several terpenoids in essential oils have been reported (*21*). 1,8-Cineole and α-pinene appear to be mildly synergistic, whereas 1,8-cineole and camphor apparently show antagonism. In contrast, the results from the analysis of
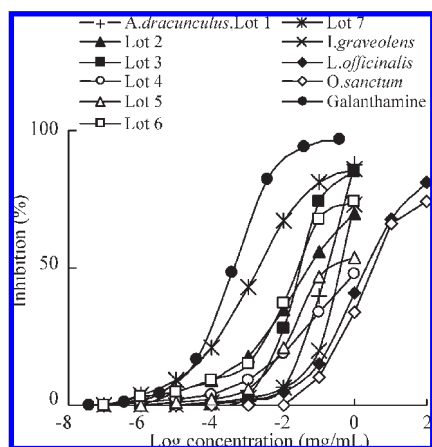

Figure 1. Acetylcholinesterase inhibitory activity of the commercial essential oils. Each point represents the mean of the values obtained from three independent experiments, each of which was performed in duplicate.
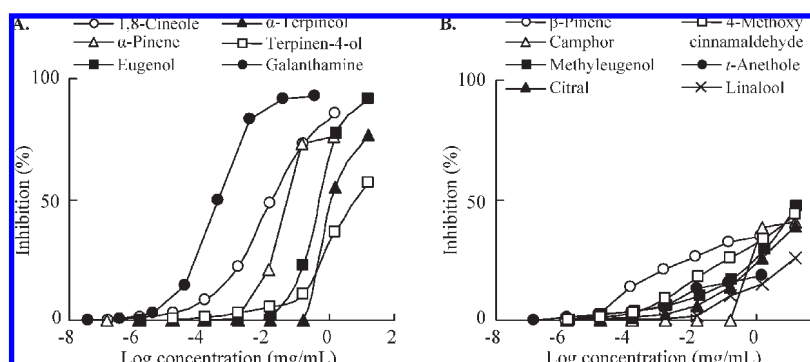

Figure 2. Acetylcholinesterase inhibitory activity of the commercial essential oil components. Each point represents the mean of the values obtained from three independent experiments, each of which was performed in duplicate.

Table 3. Percent Contribution of Active Components to the Inhibitory Activity of Commercial Essential Oils

oil	GEQ[a]	α-pinene		1,8-cineole		eugenol		α-terpineol		terpinen-4-ol		% Σ CR
		GEQ	CR[b]	GEQ	CR	GEQ	CR	GEQ	CR	GEQ	CR	
A. dracunculus lot 1	2.8	0.014	0.50									0.50
lot 2	2.0	0.014	0.69									0.69
lot 5	0.34	0.012	3.6									3.6
lot 6	0.64	0.013	2.0									2.0
lot 7	6.6	0.024	0.37									0.37
I. graveolens	1.4	0.0036	0.25					0.00047	0.033			0.29
L. officinalis	0.47			0.030	6.5			0.00020	0.043	0.00028	0.060	6.6
O. sanctum	0.23			0.011	4.8	0.047	20					25

[a] GEO, galantamine-equivalent concentration (μg/mL), which is calculated by multiplying the concentrations of the chemical components by the corresponding galantamine activity. [b] CR, contribution ratio (%).

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A. dracunculus oils showed that the activity of α-pinene, the primary component, does not greatly contribute to the overall activities of the essential oils. The GC-MS analysis of the seven oils of *A. dracunculus* revealed that all of the oils had almost similar chemical composition with almost the same components. However, there were significant differences among the AChE inhibitory activities of the seven different oils, with one being suspected to be active (lot 4) and another being identified as the most active (lot 7). Although it can be proposed that AChE inhibition occurs by the synergistic activity of terpenoids, we cannot exclude the possibility that a minor, thus far unidentified, constituent of the *A. dracunculus* oils was a more potent AChE inhibitor. The active components of *I. graveolens* also showed a low contribution ratio (0.29%).

In this study, we have used the microplate assay screening method and determined that nine commercial essential oils exhibited excellent AChE inhibitory activity. The oils of *A. dracunculus* (Asteraceae) from France (IC_{50} = 0.058 mg/mL) exhibited the highest AChE inhibitory activity. The AChE inhibitory activity of the *I. graveolens* oil has not been reported to date, and this is the first study to demonstrate this activity in the oil. Moreover, this study was the first to reveal the potent AChE inhibitory activity of eugenol, one of the components of the *O. sanctum* oil (Lamiaceae). Meanwhile, the individual components of the oils, including a novel AChE inhibitor, contributed up to 25% of the observed AChE inhibitory activity. Therefore, detailed information on the structure of the most active components may prove to be potentially helpful in developing these components as therapeutic remedies for Alzheimer's disease. The results of this study raise concern about the AChE inhibitory activity of widely produced and readily accessible commercial essential oils.

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