Inhibition of Acetylcholinesterase Activity by Essential Oils of *Mentha* Species

Mitsuo Miyazawa,*,† Hitomi Watanabe,† Kazuyasu Umemoto,‡ and Hiromu Kameoka†

Department of Applied Chemistry, Faculty of Science and Engineering, Kinki University, Kowakae, Higashiosaka, Osaka 577-8502, Japan, and Laboratory of Chemistry, Nagoya Gakuin University, Kamishinano-cho, Seto, Aichi 480-1298, Japan

Inhibition of acetylcholinesterase (AChE) activity by several species of *Mentha* oils was investigated. AChE activity was measured by a colorimetric method. *Mentha aquatica* (water mint) containing sesquiterpene alcohols showed the most effective inhibition (IC_{50} of 26 μg/mL). In addition, some *Mentha* species such as *M. aquatica* (Akasaka-hakka II), *M. gentilis* (Fukuyama-hakka), *M. gentilis* (Akita-hakka), and *M. arvensis* (Nihon-hakka) showed potent inhibitory activity, and their IC_{50} values were 28−32 μg/mL. Viridiflorol and elemol showed the most potent inhibition in terpenoids as the main components of *Mentha* oils. But none of them showed stronger inhibitory activity than essential oils.

Keywords: *Acetylcholinesterase; Mentha species; essential oils; inhibitory activity; sesquiterpene alcohol; viridiflorol; elemol*

INTRODUCTION

Several *Mentha* oils are used as flavors, for example, in confectioneries, chewing gums, candies, and liqueurs. Also, these are utilized in medical supplies such as toothpaste, facial lotions, and gastrointestinal spasmolytics. The *Mentha* oils using flavors and medical supplies have exceedingly small toxicity for the human body. Peppermint oil, one of the constituents of chewing gum, has shown anti-allergic effects (Arakawa et al., 1992). It is interest to search for bioactive compounds from *Mentha* oils.

Acetylcholinesterase (AChE) inhibitors have been used in the treatment of Alzheimer's disease. Some of these have been found in plants. For example, galantamine, amaryllidaceae alkaloids, have shown effective results for Alzheimer's disease and safety of treatment (Thomsen and Kewitzet, 1990; Thomsen et al., 1991; Bickel et al., 1991; Bores et al., 1996). Testing of the inhibitory effect on AChE in erythrocytes has been proposed as a guide to the efficacy and safety of putative therapies.

We investigated the inhibition of AChE activity by several essential oils from *Mentha* species. These oils contained many kinds of terpenoids, in particularly *p*-menthanes such as limonene, menthol, menthone, and pulegone. Recently, we reported anti-AChE activity by 17 kinds of *p*-menthans (Miyazawa et al., 1997). We suggested that the terpenoids are useful compounds. Terpenoids and the plants containing them may be of value against disease in the body. In the present paper, we report the inhibition of AChE from bovine erythrocytes by essential oils of *Mentha* species and a comparison between *Mentha* oils and terpenoids as main components in the oils.

* To whom correspondence should be addressed (telephone +81-6-721-2332; fax +81-6-727-4301; e-mail miyazawa@apch.kindai.ac.jp).

† Kinki University.

‡ Nagoya Gakuin University.

MATERIALS AND METHODS

General Procedure. Electron impact mass spectra (EI-MS) were obtained by gas chromatography−mass spectrometry (GC-MS). GC-MS was performed on a HP 5972A mass-selective detector (70 eV; ion source 180 °C) interfaced with a HP 5890E Series II Plus gas chromatograph fitted with a capillary column [TC−WAX, 60 m × 0.25 mm i.d. (GL Sciences Inc.)]. Chromatographic conditions were as follows: column temperature, raised from 80 to 220 °C at 2 °C min^{-1}; injector temperature, 250 °C; detector temperature, 280 °C; carrier gas, He at 30 cm s^{-1}.

Materials. AChE from bovine erythrocytes was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide (ATC) were purchased from Tokyo Chemical Industry Co., Ltd. (TCI).

Essential Oils. Essential oils were extracted by the methods in the literature (Nagasawa et al., 1974a,b, 1975, 1976a,b; Umemoto et al., 1978, 1980, 1981, 1988; Umemoto, 1987, 1992, 1994, 1996).

Terpenoids. Piperitenone oxide (GC purity 92%) were obtained from essential oil. Menthyl acetate, neomenthyl acetate, and linalyl acetate were obtained by reaction with acetic anhydride and pyridine. Other terpenoids were purchased from TCI, Nagaoka Perfumery Co., Ltd., Taiyo Perfume Co., Ltd., and Aldrich Chemical Co., Inc. (Milwaukee, WI). Terpenoids that needed to be refined were chromatographed on a Si-60 column (Merck, Darmstadt, Germany).

Preparatory Solutions. AChE (0.04 units/mL) and ATC (75 mM) was dissolved in 0.1 M phosphate buffer (pH 8.0), respectively. DTNB (0.01 M) was made up in 10 mL of 0.1 M phosphate buffer (pH 7.0) containing 15 mg of $NaHCO_3$. Essential oils of *Mentha* species were dissolved in ethanol. The final ethanol concentrations in all assays were maintained at 5% (v/v), including controls.

Inhibition of AChE Activity. Inhibition of AChE was assessed by the colorimetric method of Ellman (1961). Inhibitor solution (50 μL) and AChE (0.5 mL) were mixed in a test tube, and the tube was set on the incubator (25 °C). DTNB (100 μL) and buffer (2.4 mL) were added to the tube. The tube

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Table 1. Composition of Essential Oil of *Mentha* Species

	Mentha species	main components
1	*M. aquatica* (Akasaka-hakka I)	menthol (24%), (−)-methyl acetate (17%), (−)-menthone (17%), (+)-menthofuran (17%), (+)-pulegone (11%)
2	*M. aquatica* (Akasaka-hakka II)	(−)-menthone (33%), (+)-neomenthol (21%), (+)-neomenthyl acetate (16%), (+)-menthofuran (11%), (−)-limonene (7%)
3	*M. aquatica* (water mint)	elemol (22%), viridiflorol (13%), (+)-menthofurane (21%), 1,8-cineole (10%), β-caryophyllene (7%), germacrene D (7%)
4	*M. arvensis* (Nihon-hakka)	(−)-menthol (66%), (−)-menthyl acetate (15%), (−)-menthone (8%)
5	*M. arvensis* (Tosa-hakka)	(−)-piperitone oxide (33%), (+)-piperitenone oxide (26%), (−)-menthol (59%), (−)-menthone (22%)
6	*M. citrata* (bergamot mint)	(−)-linalyl acetate (51%), (−)-linalool (39%)
7	*M. gentilis* (Akita-hakka)	(+)-pulegone (48%), (+)-piperitenone oxide (29%), menthone (5%)
8	*M. gentilis* (Chigiri-Yanagiha-hakka)	(−)-menthol (59%), (−)-menthone (22%)
9	*M. gentilis* (Ezo-hakka)	(−)-menthone (44%), (−)-linalool (39%)
10	*M. gentilis* (ginger mint)	(−)-linalool (45%), γ-terpinene (9%), β-pinene (8%), β-caryopyllene (7%), germacrene D (4%)
11	*M. gentilis* (Haruyama-hakka)	(+)−1,2-epoxyneomenthyl acetate (50%), menthol (19%), menthyl acetate (12%)
12	*M. gentilis* (Hattoriryokuchi-hakka)	(−)-methyl acetate (19%), (+)-pulegone (18%), (−)-menthol (15%), (+)-piperitone (14%), (−)-menthone (12%), piperitenone (7%)
13	*M. gentilis* (Hokkai MG5)	(−)-menthol (69%), (−)-menthone (18%), isomenthone (3%), germacrene D (3%)
14	*M. gentilis* (Fukuyama-hakka)	(−)-menthone (36%), (+)-pulegone (35%), (+)-neomenthol (11%)
15	*M. gentilis* (Manyou)	(−)-menthol (96%), menthyl acetate (3%)
16	*M. gentilis* (Nankai-hakka)	(−)-menthone (32%), (+)-piperitone (14%), (−)-menthol (16%), (+)-pulegone (11%), menthyl acetate (10%), piperitenone (4%)
17	*M. gentilis* (Okinawa-hakka)	(+)-pulegone (84%), piperitenone (4%)
18	*M. gentilis* (Scotch spearmint)	(−)-carvone (73%), (−)-limonene (16%)
19	*M. gentilis* (Seto-hakka)	(+)-pulegone (81%), (−)-borneol (6%)
20	*M. gentilis* (Shuubi)	(−)-menthol (65%), (−)-menthone (14%), isomenthone (6%)
21	*M. gentilis* (Yahata-hakka)	(−)-menthone (30%), (+)-neomenthol (22%), (+)-pulegone (13%), (+)-neomenthyl acetate (13%), borneol (5%)
22	*M. japonica* (Hime-hakka)	(−)-menthone (57%), (+)-pulegone (29%), limonene (4%)
23	*M. piperita* (black mint)	(−)-menthol (32%), (−)-menthone (31%), 1,8-cineole (7%), germacrene D (4%)
24	*M. pulegium* (pennyroyal mint)	(+)-pulegone (48%), menthone (41%)
25	*M. requienii* (corsican mint)	(+)-pulegone (83%), (−)-menthone (6%), isomenthol (4%)
26	*M. rotundifolia* (apple mint)	(+)-piperitenone oxide (46%), germacrene D (21%), β-caryophyllene (6%)
27	*M. rotundifolia* (pineapple mint)	germacrene D (43%), (+)-piperitenone oxide (17%), β-farnesene (8%)
28	*M. spicata* (native spearmint)	(−)-carvone (62%), (−)-limonene (7%), dihydrocarvone (6%), 1,8-cineole (5%)
29	*M. spicata* (Mesidai-Ke-hakka)	(−)-carvone (69%), 1,8-cineole (11%), (−)-dihydrocarvone (9%), limonene (8%)
30	*M. spicata* (self-pollinated Oranda-hakka)	(+)-piperitenone oxide (57%), 1,8-cineole (13%), β-myrcene (7%)
31	*M. spicata* (Soren-hakka)	(+)-linalool (98%), 1,8-cineole (2%)

was preincubated at 25 °C for 5 min. The reaction was started by adding ATC (40 μL), and the mixture was incubated at 25 °C for 20 min. The absorbance at 412 nm was measured spectrophotometrically (Spectronic 20D, Milton Roy Co., Rochester, NY), and all test and control (without essential oils) assays were corrected by blanks for nonenzymic hydrolysis. Each assay was run in triplicate, at a minimum.

RESULTS AND DISCUSSION

We examined 31 kinds of essential oils from *Mentha* species (Table 1). As shown in Table 2, *Mentha aquatica* [water mint (**3**)] showed the strongest inhibition of 31 kinds of essential oils. This oil (**3**) contained the sesquiterpene alcohols elemol and viridiflorol as main components.

The essential oils containing piperitenone oxide, *M. arvensis* [Tosa-hakka (**5**)], *M. gentilis* [Akita-hakka (**7**)] and *M. spicata* [self-pollinated Oranda-hakka (**30**)] showed comparatively strong inhibition of AChE activity. IC_{50} values of **5**, **7**, and **30** were less than 50 μg/mL. Two oils belonging to *M. rotundifolia* [apple mint (**26**) and pineapple mint (**27**)], although containing piperitenone oxide, showed almost no inhibition of AChE activity. The strong inhibitors (**5**, **7**, and **30**) also contained pulegone, menthol, and 1,8-cineole, respectively. The oils (**26** and **27**) contained mainly terpene hydrocarbons excluding piperitenone oxide.

The essential oils of *M. citrata* [bergamot mint (**6**)], *M. gentilis* [Ezo-hakka (**9**)], and *M. gentilis* [ginger mint (**10**)] contained linalool (40%), but IC_{50} values of oils **6** and **9** were 4 times potent as **10**. This difference seems to arise from the constituents other than linalool. The oils of **6** and **9** contained menthone (44%) and linalyl acetate (51%), respectively. On the other hand, **10** contained terpene hydrocarbons (30%).

In a previous study, we have reported inhibition of AChE activity by 17 monoterpenoids with the *p*-menthane skeleton (Miyazawa, 1997). Pulegone, menthone, menthol, and limonene, out of 17 *p*-menthanes, are contained as major components in essential oils of *Mentha* species. But inhibition of AChE activity by these oils was stronger than by the terpenoids. In addition, we investigated other main terpenoids in essential oils (Table 1). As shown in Table 3, viridiflorol, elemol, and 1,8-cineole showed potent inhibition. But these terpenoids were not as potent as the essential oil of **3** (Figure 1). The oil **3** contained elemol (22%), menthofuran (21%), viridiflorol (13%), and 1,8-cineole (10%). Inhibition AChE of **3** at 50 μg/mL was shown as 64.5%. The concentration of each main component in **3** (50 μg/mL) was as follows: elemol (11 μg/mL), menthofuran (10.5 μg/mL), viridiflorol (6.5 μg/mL), and 1,8-cineole (5 μg/mL). At this concentration, these components showed slight inhibitory activity; elemol (19%), menthofuran (8%), viridiflorol (26.5%) and 1,8-cineole (8.5%). Other oils also showed stronger inhibition than their major components. In particular, *M. gentilis* [Manyou (**15**), Okinawa-hakka (**17**), and Seto-hakka (**19**)] and *M. spicata* [Soren-hakka (**31**)] contained menthol (96%), pulegone (84%), pulegone (81%), and linalool (98%), respectively. But the inhibitory activity of **19** proved more than 3 times as potent as that of menthol, and **15** and **17** had 2 times the inhibitory effect as pulegone. The oil **31** at 41 μg/mL showed the same inhibitory activity as linalool at 164 μg/mL (37%).

Table 2. Inhibition of AChE by Essential Oils from *Mentha* Species

	Mentha species	IC_{50} (μg/mL)[a] or % inhibitory activity[b]
3	*M. aquatica* (water mint)	26
2	*M. aquatica* (Akasaka-hakka II)	28
14	*M. gentilis* (Fukuyama-hakka)	30
7	*M. gentilis* (Akita-hakka)	30
4	*M. arvensis* (Nihon-hakka)	32
16	*M. gentilis* (Nankai-hakka)	36
30	*M. spicata* (self-pollinated Oranda-hakka)	37
6	*M. citrata* (bergamot mint)	38
9	*M. gentilis* (Ezo-hakka)	40
21	*M. gentilis* (Yahata-hakka)	40
19	*M. gentilis* (Seto-hakka)	42
5	*M. arvensis* (Tosa-hakka)	49
17	*M. gentilis* (Okinawa-hakka)	52
15	*M. gentilis* (Manyou)	54
12	*M. gentilis* (Hattoriryokuchi-hakka)	56
11	*M. gentilis* (Haruyama-hakka)	56
29	*M. spicata* (Mesidai-Ke-hakka)	57
18	*M. gentilis* (Scotchspearmint)	58
8	*M. gentilis* (Chigiri-Yanagiha-hakka)	58
1	*M. aquatica* (Akasaka-hakka I)	64
23	*M. piperita* (black mint)	74
13	*M. gentilis* (Hokkai MG5)	88
28	*M. spicata* (native spearmint)	88
22	*M. japonica* (Hime-hakka)	120
31	*M. spicata* (Soren-hakka)	130
25	*M. requienii* (corsican mint)	130
20	*M. gentilis* (Shuubi)	154
10	*M. gentilis* (ginger mint)	164
27	*M. rotundifolia* (pineapple mint)	43%[b]
26	*M. rotundifolia* (apple mint)	39%[b]
24	*M. pulegium* (pennyroyal mint)	38%[b]

[a] Concentration of essential oils (treatment) required for 50% enzyme inhibition as calculated from the dose−response curve. [b] The % AChE inhibition values (164 μg/mL) were calculated as compared to control (without *Mentha* species) enzyme activity (assumed 0% inhibition).

Table 3. Inhibition of AChE Activity by Terpenoids

terpenoids	IC_{50} (μg/mL)[a] or % inhibitory activity[b]
viridiflorol	25
elemol	34
1,8-cineole	41
(+)-piperitenone oxide	64
piperitenone	110
(−)-piperitone	136
(+)-pulegone[c]	136
(−)-menthyl acetate	35% (41 μg/mL)[b]
(−)-linalyl acetate	38% (82 μg/mL)[b]
(+)-menthofuran	33% (82 μg/mL)[b]
(−)-carvone[c]	43% (164 μg/mL)[b]
(−)-menthone[c]	39% (164 μg/mL)[b]
(−)-borneol	38% (164 μg/mL)[b]
(−)-menthol[c]	38% (164 μg/mL)[b]
(−)-linalool	37% (164 μg/mL)[b]
(−)-limonene[c]	27% (164 μg/mL)[b]

[a] Concentration of compounds (treatment) required for 50% enzyme inhibition as calculated from the dose−response curve. [b] The percent inhibition values (41, 82, and 164 μg/mL) were calculated as compared to control (without terpenoids) enzyme activity (assumed 0% inhibition). [c] Data from previously report (Miyazawa et al., 1997).

As a result mentioned above, the essential oils containing piperitenone oxide and linalool showed effective inhibition of AChE, in the presence of polar compounds. And, anti-AChE activity by the oils themselves of *Mentha* species showed more potent inhibition than their major constituents. We suggested that inhibitory activity of essential oils were caused not by one strong inhibitor but by synergistic activity. As compared with inhibitory activity of **3** at 50 μg/mL

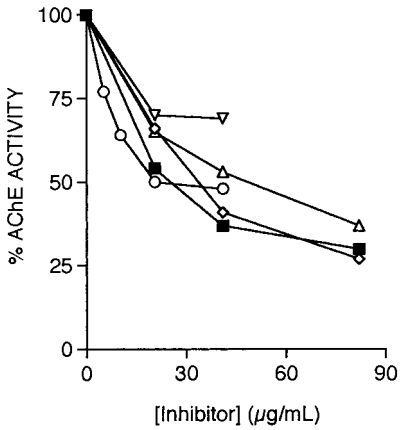

Figure 1. Effect of essential oils from *Mentha* species on AChE activity. The percentage of enzyme activity values for the inhibitors were calculated as compared to control activity, assumed to be 100%: (■) *Mentha aquatica* (water mint) (**3**); (◇) elemol; (▽) menthofuran; (○) viridiflorol; (△) 1,8-cineole.

Table 4. Inhibition of AChE by *Mentha aquatica* (water mint), Main Components, and Mixture

	% inibitory activity[a]		
compound	20.5 μg/mL[b]	41 μg/mL[b]	82 μg/mL[b]
Mentha aquatica (water mint)	46	63	70
elemol (22%)	7.5	15	30
menthofuran (21%)	6.5	13	26
viridiflorol (13%)	12	23.5	36.6
1,8-cineole (10%)	3.5	7	14
sum of % inhibitory activity in components	29.5	58.5	106.6
mixture elemol−viridiflorol−menthofuran−1,8-cineole (22%:13%:21%:10%)	19	38	52

[a] The percent AChE inhibition values were calculated as compared to control (without compounds) enzyme activity (assumed to be 0% inhibition). [b] The concentration of *Mentha aquatica* (water mint) oil and mixture. Indicative of each inhibitory activity at the following concentrations: elemol (4.5, 9, and 18 μg/mL), menthofuran (4.3, 8.6, and 19.2 μg/mL), viridiflorol (2.7, 5.3, and 10.7 μg/mL), and 1,8-cineole (2.05, 4.1, and 8.2 μg/mL).

(64.5%), the sum of inhibitory activities of each main component (62%) was almost the same. We considered the cause of inhibitory activity by **3** as synergistic effect of main components and tested the inhibitory activity of a 22:21:13:10 mixture of elmol−menthofuran−viridiflorol−1,8-cineole. As shown in Table 4, inhibitory activity of the oil **3** was not in accordance with that of the mixture. But inhibitory activity of the mixture affected that of elemol and viridiflorol. Inhibitory activity of *Mentha* oil can be expected from the synergistic effect by combination of constituents including minor components. Therefore, we will continue to investigate more synergistic inhibitory activity of *Mentha* oils.

The essential oils of Labiatae have an antimicrobial activity effect (Farag et al., 1989). The oils have been used as flavors for prevention of fungal growth. The plants are safe, widely cultivated, and contain naturally occurring compounds. Additionally, the plants have been used in folk medicine for germicidal activity and narcotic drugs for centuries. Also, essential oils have been available as medicines, such as for spasmolytic activity (Gamez et al., 1990) and countering the effects of periodontal disease (Oota et al., 1994). From our studies, we suggested that the *Mentha* oils can be applied as an AChE antagonist. Further investigation

will be undertaken to determine the unknown power of the essential oils.

ABBREVIATIONS USED

AChE, acetylcholinesterase; ATC, acetylthiocholine iodide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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