

Activity of Essential Oils and Individual Components against Acetyl- and Butyrylcholinesterase

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We have tested acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities of nineteen essential oils obtained from cultivated plants, namely one from *Anethum graveolens* L. (organic fertilizer), two from *Foeniculum vulgare* Mill. collected at fully-mature and flowering stages (organic fertilizer), two from *Melissa officinalis* L. (cultivated using organic and chemical fertilizers), two from *Mentha piperita* L. and *M. spicata* L. (organic fertilizer), two from *Lavandula officinalis* Chaix ex Villars (cultivated using organic and chemical fertilizers), two from *Ocimum basilicum* L. (green and purple-leaf varieties cultivated using only organic fertilizer), four from *Origanum onites* L., *O. vulgare* L., *O. munitiflorum* Hausskn., and *O. majorana* L. (cultivated using organic fertilizer), two from *Salvia sclarea* L. (organic and chemical fertilizers), one from *S. officinalis* L. (organic fertilizer), and one from *Satureja cuneifolia* Ten. (organic fertilizer) by a spectrophotometric method of Ellman using ELISA microplate-reader at 1 mg/ml concentration. In addition, a number of single components widely encountered in most of the essential oils [γ -terpinene, 4-allyl anisole, (–)-carvone, dihydrocarvone, (–)-phencone, cuminyl alcohol, cumol, 4-isopropyl benzaldehyde, *trans*-anethole, camphene, *iso*-borneol, (–)-borneol, L-bornyl acetate, 2-decanol, 2-heptanol, methyl-heptanol, farnesol, nerol, *iso*-pulegol, 1,8-cineole, citral, citronellal, citronellol, geraniol, linalool, α -pinene, β -pinene, piperitone, *iso*-menthone, menthofuran, linalyl oxide, linalyl ester, geranyl ester, carvacrol, thymol, menthol, vanilline, and eugenol] was also screened for the same activity in the same manner. Almost all of the essential oils showed a very high inhibitory activity (over 80%) against both enzymes, whereas the single components were not as active as the essential oils.

Key words: Essential Oil, Anticholinesterase, Monoterpenes, Alzheimer's Disease

Introduction

Alzheimer's disease (AD) is the most common cause of dementia, which is the loss of intellectual and social abilities severe enough to interfere with daily functioning (Cummings, 2004; Mattson, 2004). The most striking early symptom of the disease is loss of short-term memory (amnesia), which usually manifests as minor forgetfulness that becomes steadily more pronounced with illness progression, with relative preservation of older memories (Verghese et al., 2003). Inhibition of acetylcholinesterase (AChE), the key enzyme in the breakdown of acetylcholine, is considered as a promising strategy for the treatment of neurological disorders such as AD, senile dementia, ataxia, and myasthenia gravis (Orhan et al., 2006; Mukherjee et al. 2007).

On the other hand, a number of essential oils, e.g. from some *Salvia* species, *Foeniculum vulgare*, *Acorus calamus*, *Melaleuca alternifolia* (tea tree oil), *Citrus paradisi*, have been so far reported to be effective against AD (Perry et al., 1996; Miyazawa et al., 2001; Irie and Keung, 2003; Savelev et al., 2004; Miyazawa and Yamafuji, 2005; Joshi and Parle, 2006).

In our enduring studies on discovery of new cholinesterase inhibitors from Turkish medicinal plants and pure compounds (Orhan et al., 2004; Sener and Orhan, 2004; Orhan et al., 2007a, b), we have now focused on acetylcholinesterase and butyrylcholinesterase (BChE) inhibitory effects of nineteen essential oils obtained from plants cultivated using organic and/or chemical fertilizers, namely one from *Anethum graveolens* L. (organic

fertilizer), two from *Foeniculum vulgare* Mill. collected at fully-mature and flowering stages (organic fertilizer), two from *Melissa officinalis* L. (cultivated using organic and chemical fertilizers), two from *Mentha piperita* L. and *M. spicata* L. (organic fertilizer), two from *Lavandula officinalis* Chaix ex Villars (cultivated using organic and chemical fertilizers), two from *Ocimum basilicum* L. (green and purple-leaf varieties cultivated using only organic fertilizer), four from *Origanum onites* L., *O. vulgare* L., *O. munitiflorum* Hausskn., and *O. majorana* L. (cultivated using organic fertilizer), two from *Salvia sclarea* L. (organic and chemical fertilizers), one from *S. officinalis* L. (organic fertilizer), and one from *Satureja cuneifolia* Ten. (organic fertilizer); they were tested by a spectrophotometric method of Ellman using an ELISA microplate-reader at 1 mg/ml concentration. In addition, a number of single components widely encountered in most of the essential oil; namely γ -terpinene, 4-allyl anisole, (–)-carvone, dihydrocarvone, (–)-phencone, cuminyl alcohol, cumol, 4-isopropyl benzaldehyde, *trans*-anethole, camphene, *iso*-borneol, (–)-borneol, L-bornyl acetate, 2-decanol, 2-heptanol, methyl heptanol, farnesol, nerol, *iso*-pulegol, 1,8-cineole, citral, citronellal, citronellol, geraniol, linalool, α -pinene, β -pinene, piperitone, *iso*-menthone, menthofurane, linalyl oxide, linalyl ester, geranyl ester, carvacrol, thymol, menthol, vanilline, and eugenol, were also screened for the same activity in the same manner.

Materials and Methods

Plant material

The herbs of *Foeniculum vulgare* Mill. collected at fully-mature and flowering stages, *Anethum graveolens* L., *Melissa officinalis* L., *Mentha piperita* L. and *M. spicata* L., *Lavandula officinalis* Chaix ex Villars, *Ocimum basilicum* L. (green and purple-leaf varieties), *Origanum onites* L., *O. vulgare* L., *O. munitiflorum* Hausskn., and *O. majorana* L., *Salvia sclarea* L. and *S. officinalis* L. as well as *Satureja cuneifolia* Ten. were cultivated in the experimental farm belonging to Faculty of Agriculture, Selcuk University, Konya, Turkey under ecological conditions in 2006. The samples of *F. vulgare* were harvested at fully-mature and flowering stages of the plant during the harvest season in 2006 and used in the experiments.

Test compounds

The pure components tested in this study [γ -terpinene, 4-allyl anisole, (–)-carvone, dihydrocarvone, (–)-phencone, cuminyl alcohol, cumol, 4-isopropyl benzaldehyde, *trans*-anethole, camphene, *iso*-borneol, (–)-borneol, L-bornyl acetate, 2-decanol, 2-heptanol, methyl heptanol, farnesol, nerol, *iso*-pulegol, 1,8-cineole, citral, citronellal, citronellol, geraniol, linalool, α -pinene, β -pinene, piperitone, *iso*-menthone, menthofurane, linalyl oxide, linalyl ester, geranyl ester, carvacrol, thymol, menthol, vanilline, and eugenol] were purchased from Carl Roth Chemical Company (Karlsruhe, Germany).

Distillation of essential oils

An air-dried and powdered sample (approx. 100 g) of each plant material was subjected to hydrodistillation for 3 h using a Clevenger-type apparatus to produce the essential oils.

Determination of AChE and BChE inhibitory activities

AChE and BChE inhibitory activities of the essential oils along with their main constituents commercially available were determined by slightly modifying the spectrophotometric method developed by Ellman *et al.* (1961). Electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma, St. Louis, MO, USA) and horse serum BChE (EC 3.1.1.8, Sigma) were used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma) were employed as substrates of the reaction. 5,5'-Dithio-bis(2-nitrobenzoic)acid (DTNB; Sigma) was used for the measurement of the anticholinesterase activity. All the other reagents and conditions were same as described in our previous publications (Orhan *et al.*, 2004, 2007a, b). Galanthamine, the anticholinesterase alkaloid-type of drug firstly isolated from the bulbs of snowdrop (*Galanthus* sp.), was purchased from Sigma and used as reference.

Statistical analysis of data

Data obtained from *in vitro* experiments were expressed as mean standard error (\pm SEM). Statistical differences between the treatments and the control were evaluated by ANOVA test. $P < 0.05$ was considered to be significant (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Results and Discussion

The essential oils were screened for their AChE and BChE inhibitory activities *in vitro* by the Ellman method at 1 mg/ml. As seen from Table I, the essential oils appeared to be quite effective against both enzymes as compared to galanthamine, the reference compound. Amongst them, *A. graveolens* and *O. munitiflorum* showed 100% inhibition on AChE followed by *O. vulgare* (97.9%), *O. onites* (96.3%), *F. vulgare* collected at fully-mature stage (94.3%), *S. cuneifolia* (86.5%), *M. piperita*, *L. officinalis*, *M. spicata* cultivated using organic fertilizer (81.5, 81.2, and 80.9%, respectively), and *F. vulgare* collected at flowering stage (80.8%). Additionally, the essential oils of *S. officinalis* and *L. officinalis* cultivated using chemical fertilizer displayed a moderate inhibition towards AChE having 63.8 and 62.3% inhibition, respectively, whereas two essential oils obtained from *M. officinalis* and *S. sclarea* did not have any ability to inhibit AChE. On the other hand, most of the essential oils such as those from *S. cuneifolia*, *Origanum* species, *L. officinalis*, *M. spicata*, and *A. graveolens* were rather active against BChE exerting inhibition over 80%, while *M. officinalis*, *O. basilicum*, *F. vulgare* of fully-mature stage and *S. sclarea* essential oils had approx. 70% of BChE inhibition. In fact, except for *S. sclarea* (chemical fertilizer),

all of the essential oils were remarkably active towards BChE showing inhibition over 60%.

In terms of inhibition of the main components widely found in essential oils, they were not as active as the essential oils (Table II). For instance, only α -pinene (76.3%), carvacrol (70.3%), and menthofurane (55.3%) showed a notable inhibitory activity against AChE as compared to galanthamine (99.8%), whereas (–)-carvone, dihydrocarvone, citronellal, piperitone, *iso*-menthone, and menthol exhibited BChE inhibition percentages ranging between 60–80%. Besides, methyl heptanol, citronellol, menthofurane, geranyl ester, and carvacrol were found to have inhibitions at about 50%. However, none of them showed a stronger inhibitory activity than the essential oils. On the other hand, there has been not much activity difference between the same plant species cultivated using organic or chemical fertilizers. Only the essential oil of *L. officinalis* showed a better AChE inhibition when cultivated using organic fertilizer.

Several other studies have been carried out on the anticholinesterase activity of essential oils and hardly any on their main components since essential oils were shown to be complex mixtures of a number of components with a high percentage of monoterpenes, especially dominated by oxygenated monoterpenes. The lack of this information has prompted us to work on this basis. After hav-

Essential oil	Percentage of inhibition	
	AChE	BChE
<i>Anethum graveolens</i>	100.0 \pm 0.88	90.9 \pm 0.56***
<i>Foeniculum vulgare</i> (fully-mature)	94.3 \pm 0.12	71.6 \pm 0.85***
<i>Foeniculum vulgare</i> (flowering)	80.8 \pm 0.64***	65.4 \pm 0.78***
<i>Melissa officinalis</i> (organic fertilizer)	– ^b	73.3 \pm 0.67***
<i>Melissa officinalis</i> (chemical fertilizer)	–	66.5 \pm 1.07***
<i>Mentha piperita</i> (organic fertilizer)	81.5 \pm 0.98***	93.5 \pm 1.36***
<i>Mentha spicata</i> (organic fertilizer)	80.9 \pm 1.33***	91.0 \pm 0.86***
<i>Lavandula officinalis</i> (organic fertilizer)	81.2 \pm 0.39***	92.9 \pm 1.74***
<i>Lavandula officinalis</i> (chemical fertilizer)	62.3 \pm 0.66***	95.0 \pm 1.65***
<i>Ocimum basilicum</i> (green variety)	10.7 \pm 0.54	74.1 \pm 1.00***
<i>Ocimum basilicum</i> (purple variety)	33.3 \pm 0.99	79.1 \pm 1.56***
<i>Origanum onites</i> (organic fertilizer)	96.3 \pm 0.93***	92.9 \pm 1.36***
<i>Origanum vulgare</i> (organic fertilizer)	97.9 \pm 1.56	88.4 \pm 1.76***
<i>Origanum munitiflorum</i> (organic fertilizer)	100.0 \pm 1.07	94.7 \pm 1.25***
<i>Origanum majorana</i> (organic fertilizer)	94.6 \pm 0.86	94.4 \pm 1.66***
<i>Salvia sclarea</i> (organic fertilizer)	–	76.0 \pm 0.76***
<i>Salvia sclarea</i> (chemical fertilizer)	11.6 \pm 0.94	45.1 \pm 1.06***
<i>Salvia officinalis</i> (organic fertilizer)	63.8 \pm 1.32**	66.3 \pm 1.45***
<i>Satureja cuneifolia</i> (organic fertilizer)	86.5 \pm 0.95***	98.4 \pm 1.24***
Galanthamine	99.8 \pm 0.31	80.3 \pm 1.14

Table I. AChE and BChE inhibitory activities of the essential oils^a.

^a Values are expressed as mean \pm SEM ($n = 3$). $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

^b –, No inhibition.

Main constituent	Percentage of inhibition	
	AChE	BChE
γ -Terpinene	– ^b	–
4-Allyl anisole	–	–
(–)-Carvone	23.6 \pm 0.83	62.4 \pm 1.23
Dihydrocarvone	31.1 \pm 0.87***	66.5 \pm 1.05
(–)-Phencone	8.9 \pm 0.96	9.9 \pm 0.53
Cuminyl alcohol	–	18.1 \pm 1.02
Cumol	–	–
4-Isopropyl benzaldehyde	–	30.4 \pm 0.79***
<i>trans</i> -Anethole	–	–
Camphene	–	–
<i>iso</i> -Borneol	–	–
(–)-Borneol	–	12.1 \pm 1.12
L-Bornyl acetate	–	–
2-Decanol	–	42.0 \pm 1.28***
2-Heptanol	–	–
Methyl heptanol	–	53.7 \pm 0.95***
Farnesol	5.4 \pm 0.45	30.8 \pm 0.99***
Nerol	–	33.0 \pm 1.35***
<i>iso</i> -Pulegol	–	44.7 \pm 0.99***
1,8-Cineole	NT	48.2 \pm 0.87***
Citral	–	32.6 \pm 0.77
Citronellal	9.3 \pm 0.44	60.6 \pm 1.25***
Citronellol	15.3 \pm 0.88	55.4 \pm 1.55***
Geraniol	–	21.1 \pm 0.79
Linalool	–	15.7 \pm 0.24
α -Pinene	76.3 \pm 1.27***	23.5 \pm 1.08***
β -Pinene	–	31.3 \pm 1.05***
Piperitone	28.5 \pm 0.96	76.1 \pm 0.89**
<i>iso</i> -Menthone	13.9 \pm 1.34	64.7 \pm 0.67***
Menthofurane	55.3 \pm 1.00***	55.4 \pm 0.94***
Linalyl oxide	–	29.0 \pm 0.91***
Linalyl ester	–	35.8 \pm 1.01***
Geranyl ester	8.9 \pm 1.76	59.1 \pm 0.56***
Carvacrol	70.3 \pm 1.24***	54.6 \pm 0.89***
Thymol	28.4 \pm 0.64	27.6 \pm 0.99***
Menthol	–	70.0 \pm 0.71***
Vanilline	–	8.5 \pm 1.10
Eugenol	–	45.4 \pm 0.78***
Galanthamine	99.8 \pm 0.31	80.3 \pm 1.14

Table II. AChE and BChE inhibitory activities of the main constituents found in the essential oils^a.

^a Values are expressed as mean \pm SEM ($n = 3$).
 $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

^b –, No inhibition.

ing done the crucial literature survey on the topic, we have come across the following reports. In a recent study, the water and ethanol extracts of *F. vulgare* growing in Portugal as well as its essential oil were tested for their antioxidant and AChE inhibitory activities (Mata *et al.*, 2007). The results showed that only the essential oil had a notable degree of AChE inhibition. The essential oil composition of the plant in the mentioned study was also determined and (*E*)-anethole was found to be the major one constituting 70.2% of the oil, followed by its (*Z*)-isomer. However, the authors concluded that the activity was not due to (*E*)-anethole; it may result from another isomer

present in the oil as a second major component (18.6%). On the other hand, the methanolic extract of *F. vulgare* of Indian origin was also tested for its nootropic and anti-amnesic effects in mice (Joshi and Parle, 2006). The extract administered for eight successive days ameliorated the amnesic effect of scopolamine and increased step-down latency as well as AChE inhibition in mice significantly. Hence, *F. vulgare* was suggested to be employed in the treatment of AD, which is in good agreement with our data.

Relevantly, Turkish fennel essential oils seemed to have a quite similar chemical composition to their counterparts in the world (Isan *et al.*,

2002; Özcan *et al.*, 2006). On the other hand, the essential oils of *A. graveolens* obtained from different countries were reported to consist of carvone, camphor, limonene, dillapiol, α -pinene, and linalool as the main constituents (Singh *et al.*, 2005; Fatope *et al.*, 2006; Rafii and Shahverdi, 2007; Callan *et al.*, 2007). In one study, linalool and citral were revealed to be the reversible competitive inhibitors of AChE (Ryan and Bryan, 1988), whereas they exhibited no effect against AChE and insignificant inhibition on BChE, which might be resulted from the use of different enzyme sources, like insect and electric eel AChEs. In a similar study, the *in vitro* AChE inhibitory activity exerted by the main constituents of the essential oil obtained from *Thymus vulgaris* L. was examined, and specifically linalool, thymol, and carvacrol along with their derivatives thymoquinone and thymohydroquinone were tested for AChE inhibition (Jukic *et al.*, 2006). The AChE inhibitory potential decreased in the following order: thymohydroquinone > carvacrol > thymoquinone > essential oil > thymol > linalool. It is interesting to note that the AChE inhibitory effect exerted by carvacrol was ten times stronger than that exerted by its isomer thymol, although thymol and carvacrol have a very similar structure. Consistently, we have herein obtained matching results with this report on carvacrol and thymol as well.

As shown in Table I, the essential oils that we obtained from *M. spicata* and *M. piperita* were potent inhibitors of AChE and BChE. Mints, as well-proved, are very variable with respect to their essential oil compositions, chemotypes and chemical races (Karousou *et al.*, 2007). In a recent article (Ferreira *et al.*, 2006) the essential oils, ethanolic extracts, and decoctions of ten Portuguese plants were screened for their *in vitro* AChE inhibitory activities, and among them, *Melissa officinalis* and *Mentha suaveolens* essential oils were reported to have strong AChE inhibitory activity with 65.3 and 52.4% at 1 mg ml⁻¹. Conversely, in Salah and Jager's study (2005), the water and ethanolic extracts of *M. officinalis* of Lebanese origin were not effective against AChE. Although our results revealed that the essential oil of *M. officinalis* (lemon balm) did not show any activity at 1 mg ml⁻¹, this could clearly depend on dissimilarity in chemical compositions of both essential oils. These authors demonstrated as well that the essential oil of *S. officinalis* had 46.4% of inhibition on AChE at 0.5 mg ml⁻¹, which is virtually consist-

ent with our results. On the contrary, in that study two species of *Lavandula* (*L. angustifolia* and *L. pedunculata*), which, in the XVI century was believed to enhance intelligence (Cananagh and Wilkinson, 2002), were also tested and reported to display an anti-AChE effect below 50%, which may again be due to a discrepancy in the essential oil composition of the plants. Miyazawa *et al.* (1998) also elucidated the anticholinesterase activity of several *Mentha* essential oils of Japanese origin. The sesquiterpene alcohols viridiflorol and elemol, as the main components of these *Mentha* oil showed the most potent inhibition. But none of them showed a stronger inhibitory activity than the essential oils. Relevantly, the methanolic extract prepared from the flowers of *M. arvensis* inhibited AChE in a TLC assay in a dose-dependent manner, and the active compound was claimed to be linarin (acacetin-7-*O*- β -D-rutinoside) (Oinonen *et al.*, 2006). In our experiment, both green and purple varieties of *O. basilicum* had no capability to inhibit AChE whereas they remarkably inhibited BChE. However, the ethanolic extracts obtained from the leaves and stems of *O. americanum* were reported by Khattak *et al.* (2005) to show anticholinesterase property *in vitro*. Although this was a report on a species different from ours, the difference on the activity may doubtlessly depend on chemical compositions.

On the other hand in Adsersen *et al.*'s study (2006), the water and ethanolic extracts of eleven plants used in Danish folk medicine for their memory-enhancing properties including *M. spicata*, *M. officinalis*, *L. angustifolia*, and *O. vulgare*, were screened for their AChE inhibitory effects at 0.025, 0.05, and 0.1 mg ml⁻¹. However, none of these extracts appeared to be active at the concentrations tested, which were quite low as compared to our test concentration (1 mg ml⁻¹). This might be yet again due to phytochemical deviations between the extract and essential oil components. The extracts may also show an inhibition as the tested concentration rises up.

Although a number of studies have been so far carried out on the anticholinesterase effect of essential oils, a few reports have mentioned about the anticholinesterase activity of essential oil components. As a matter of fact, this is a very important focus in order to elucidate the compound(s) responsible for the activity of any essential oil. In a study by Perry *et al.* (2000), *in vitro* inhibition of human erythrocyte AChE by *Salvia lavandulifolia*

essential oil and its major monoterpenes including camphor, 1,8-cineole, α -pinene, β -pinene, bornyl acetate, as well as of some minor components (1% or less) like geraniol, linalool, γ -terpinene was determined. Consequently, α -pinene, 1,8-cineole, and camphor were found to be the competitive reversible inhibitors of AChE, and it was suggested that the inhibitory activity of this essential oil was primarily due to its main terpenoids, which showed a major synergistic effect.

In another study, Miyazawa and Yamafuji (2005) worked on the anti-AChE activity of bicyclic monoterpenoids commonly encountered in essential oils. The results pointed out that the bicyclic monoterpenoids containing an allylic methyl group showed strong inhibition. Besides, (+)- and (–)- α -pinene and (+)-3-carene were potent inhibitors of AChE as well, which was supportive of our data. In another study, a minor synergy was apparent in 1,8-cineole/ α -pinene and 1,8-cineole/caryophyllene oxide combinations at higher concentrations, and the anticholinesterase activity of 1,8-cineole was almost the same as that of the natural plant oil (Savelev *et al.*, 2003). Among all these main components, α -pinene was found to be active on AChE which is in accordance with our results. But, still, it cannot be considered as the main active component in the essential oils.

In conclusion, essential oils are complex mixtures of components that show usually higher activities than their isolated components; their final

activities are due to the combined effects of several minor components. Thus, they contain multifunctional components that exert their activities through different mechanisms (Koroch *et al.*, 2007). Although there have not been many studies on the anticholinesterase activity of single components of essential oils, the data reported so far suggests that not a single component is responsible for the activity. Although research in this scope aims to elucidate rationally active compound(s) of any plant extract regarding any desired biological activity, it is not always one compound that is responsible for these effects, which may depend on several compounds that act in a synergistic manner or on compounds which mutually regulate each other. A few previous studies confirmed that α -pinene and 1,8-cineole are monoterpenes highly active on AChE, however, there has been so far no study about their inhibitions on BChE.

In this study, we demonstrated AChE and BChE inhibitory activities of essential oils and main constituents found commonly in essential oils and concluded that the anticholinesterase activity of the essential oils tested in this study most likely depends on a number of oil components, mostly monoterpenes, rather than on a single component. To the best of our knowledge, this is the first report on the BChE inhibitory activity of these essential oils as well as on the anticholinesterase effect of the aforementioned main components except for 1,8-cineol, α -pinene, carvacrol, thymol, linalool, and citral.

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