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# Inhibitory effect of Turkish *Rosmarinus officinalis* L. on acetylcholinesterase and butyrylcholinesterase enzymes

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**Abstract**

In the current study, we have tested acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity of the petroleum ether, ethyl acetate, chloroform, and methanol extracts, rosmarinic acid as well as the essential oil obtained from *Rosmarinus officinalis* L. growing in Turkey by a spectrophotometric method of Ellman using ELISA microplate-reader at 0.2, 0.5, and 1.0 mg/mL concentrations. In addition, quantification of rosmarinic acid, a common phenolic acid found in rosemary, was carried out by reversed-phase HPLC in the methanolic extract of the plant, which was found to have $12.21 \pm 0.95\%$ ($122.1 \pm 9.5$ mg/g extract) of rosmarinic acid. Rosmarinic acid was also tested for its AChE and BChE inhibitory effect and found to cause 85.8% of inhibition against AChE at only 1.0 mg/mL. Besides, the essential oil was analyzed by GC–MS technique, which was shown to be dominated by 1,8-cineol (44.42%) and followed by α-pinene (12.57%).


*Keywords:* *Rosmarinus officinalis* L.; Essential oil; Acetylcholinesterase; Butyrylcholinesterase; Rosmarinic acid

## 1. Introduction

The most common form of dementia is known as Alzheimer's disease (AD), which is the loss of intellectual and social abilities severe enough to interfere with daily functioning (Cummings, 2004; Mattson, 2004). Although symptoms can vary widely, the first problem many people notice is forgetfulness severe enough to affect their work, lifelong hobbies or social life (Verghese et al., 2003). Inhibition of acetylcholinesterase (AChE), the key enzyme in the breakdown of acetylcholine, is considered one of the treatment strategies against several neurological disorders such as AD, senile dementia, ataxia, and myasthenia gravis (Mukherjee, Kumar, Mal, & Houghton, 2007; Orhan, Orhan, & Şener, 2006). Within the past few years, some synthetic compounds (tacrine, rivastigmine, donepezil, and galanthamine) have become available for clinical use; however, none of them have ability to stop the disease. Therefore, there is still a great demand in order to find new drug candidates for AD treatment.

On the other hand, *Rosmarinus officinalis* L. (Lamiaceae), also known as rosemary, is an aromatic evergreen shrub widely distributed throughout the Mediterranean region. *R. officinalis* L. has a very old reputation for improving memory and has been used as a symbol of remembrance in Europe (Moss, Cook, Wesnes, & Duckett, 2003).

As part of our continuing studies into discovery of new cholinesterase inhibitors from Turkish medicinal plants and pure compounds (Orhan et al., 2007; Orhan, Şener, Choudhary, & Khalid, 2004; Orhan et al., in press), we have now focused on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory effects of Turkish rosemary, *R. officinalis* L. (RO). For this intention, we have tested *in vitro* anticholinesterase action of the essential oil and

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petroleum ether (RO–PE), chloroform (RO–$CHCl_3$), ethyl acetate (RO–EtOAc), and methanol (RO–MeOH) extracts obtained from RO by the spectrophotometric method of Ellman using ELISA microplate-reader at 0.2, 0.5, and 1.0 mg/mL concentrations. Furthermore, rosmarinic acid quantification was performed in RO–MeOH using reversed phase-HPLC and tested for its AChE and BChE inhibitory activities in the same manner, while chemical composition of the essential oil was determined by capillary GC–MS.

## 2. Materials and methods

### 2.1. Plant material

The herbs of RO were collected from the vicinity of Edremit town of Balikesir province (Turkey) in July, 2007. Voucher specimen of the plant is deposited in the Herbarium of Faculty of Pharmacy, Ankara University, Ankara, Turkey (Code no. AEF 23852).

### 2.2. Preparation of the extracts

The dried and powdered material of RO was weighed accurately (31.96 g) and successively extracted with petroleum ether (PE) (1 × 1 L), chloroform ($CHCl_3$) (2 × 1 L), ethyl acetate (EtOAc) (1 × 1 L), and methanol (MeOH) (5 × 1 L). The yields of the extracts are given as follows: RO–PE: 1.18 g; RO–$CHCl_3$: 2.40 g; RO–EtOAc: 0.78 g; RO–MeOH: 3.68 g.

### 2.3. Chemicals for HPLC analysis

Chromatographic grade-double distilled water, HPLC grade methanol (Merck-1, 06007), 2-propanol (Merck-101040), analytical grade *ortho*-phosphoric acid 85% (Merck-563) and rosmarinic acid (Fluka; 44699) were purchased from their respective manufacturers and used in the HPLC analysis.

### 2.4. HPLC apparatus

The method development was performed with a LC system consisting of an HP Agilent 1100 series quaternary pump with a degasser and photodiode array detector. The methanol extract of the plant was injected to a HP Agilent 1100 Autosamplers with thermostatted column compartment on a Phenomenex-Hyperclone ODS $C_{18}$ column (5, 250, and 4.6 mm) at 30 °C. The system was controlled and data analysis was performed with Agilent ChemStation software. All the calculations concerning the quantitative analysis were performed with external standardization by measurement of the peak areas.

### 2.5. Analytical conditions for HPLC

HPLC analysis was performed by a gradient elution with flow rate of 1.0 mL $min^{-1}$.The mobile phase was delivered from three separate containers with gradient elution program. The first container was *o*-phosphoric acid 0.085% in water (solution A) and the second container was *o*-phosphoric acid 0.085% in methanol (solution B), while the third one was *o*-phosphoric acid 0.085% in 2-propanol (solution C). All solvents were filtered through a 0.45 m Millipore filter prior to use and degassed in an ultrasonic bath. Quantification was effected by measuring at 330 nm for rosmarinic acid using photo-diode array detector. The chromatographic run time was 20 min, where the column void volume was 1.60 min. Throughout the study, suitability of the chromatographic system was monitored by calculating the capacity factor ($k'$), the resolution ($R$), the selectivity () and peak asymmetry ($T$). Excellent linearity was obtained between peak areas and concentrations of 0.201–100.5 μg $mL^{-1}$ with $r^2 = 0.9999$ for rosmarinic acid.

### 2.6. Distillation of essential oil

Air-dried and powdered sample (100 g) of RO was subjected to hydrodistillation for three hours using a Clevenger-type apparatus to produce the essential oil yielding 2.39% of essential oil (v/w).

### 2.7. GC–MS conditions for essential oil analysis

GC analysis for the essential oil of RO was performed on an Agilent 6890N Network GC system, under the following conditions: column, HP Innowax Capillary; 60.0 m × 0.25 mm × 0.25 μm; oven temperature program, the column held initially at 60 °C for 10 min after injection, then increased to 220 °C with 4 °C/min heating ramp for 10 min and increased to 240 °C with 1 °C/min heating ramp without hold. The rest of the conditions were as follows; injector temperature: 250 °C; detector (FID) temperature: 250 °C; carrier gas: He; inlet pressure: 20.93 psi; linear gas velocity: 21 cm/s; split ratio: 60:1; and injected volume: 1.0 μL.

The oil was also analyzed by GC–MS using Agilent 6890N Network GC system combined with Agilent 5973 Network mass selective detector. The GC conditions were given in following order. Column: HP Innowax Capillary (60.0 m × 0.25 mm × 0.25 μm); oven temperature program: column held initially at 60 °C for 10 min after injection, then increased to 220 °C with 4 °C/min heating ramp for 10 min and increased to 240 °C with 1 °C/min heating ramp without hold; injector temperature, 250 °C; carrier gas, helium; inlet pressure, 20.96 psi; linear gas velocity, 28 cm/s; column flow, 1.2 mL/min; split ratio, 40:1; injected volume, 1.0 μL. MS conditions were regulated as follows; ionization energy: 70 eV; ion source temperature: 280 °C; interface temperature: 250 °C; and mass range: 34–450 atomic mass units.

Identification of the components in the rosemary oil was assigned by comparison of their retention times and mass spectra with corresponding data (Adams, 2001; Yeşil-Çeliktaş et al., 2007) and by comparison of their mass

spectra with Wiley, Nist, and Başer essential oil libraries. Percentages of the components were calculated from the GC peak areas using the normalization method.

### 2.8. Determination of AChE and BChE inhibitory activities

AChE and BChE inhibitory activities of the essential oil along with the extracts obtained from RO were determined by slightly modifying the spectrophotometric method developed by Ellman, Courtney, Andres, and Featherstone (1961). Electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma) and horse serum BChE (EC 3.1.1.8, Sigma) were used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma, St. Louis, MO, USA) were employed as substrates of the reaction. 5,5′-Dithio-bis(2-nitrobenzoic)acid (DTNB, Sigma, St. Louis, MO, USA) 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; 2007). All of the experiments were done in triplicate. Galanthamine purchased from Sigma (St. Louis, MO, USA) was the reference in this study.

### 2.9. Statistical analysis of data

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

## 3. Results

### 3.1. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities

At 0.2, 0.5, and 1.0 mg/mL concentrations; the essential oil of RO as well as its PE, $CHCl_3$, EtOAc, and MeOH extracts along with rosmarinic acid was tested for their AChE and BChE inhibitory activities *in vitro* by spectrophotometric Ellman method. As seen in Table 1, none of the extracts had an ability to inhibit AChE at 1 mg/mL, whereas only the essential oil was active against this enzyme having 63.7 ± 1.2% of inhibition. Besides, the extracts were not able to inhibit AChE and BChE at 0.2 and 0.5 mg/mL causing much less inhibition below 50%, which is not considered to be important. Rosmarinic acid also inhibited AChE moderately (47.3 ± 1.05%) at only 1.0 mg/mL. On the other hand, the essential oil had a remarkable anti-BChE effect (74.0 ± 0.79%), while RO–MeOH and rosmarinic acid were highly active against BChE at inhibition rates of 83.9 ± 0.97% and 85.5 ± 1.31%, respectively. Fascinatingly, RO–PE displayed a moderate inhibition towards BChE (54.2 ± 1.55%), however, RO-EtOAc showed a trivial inhibitory effect on BChE with 34.2 ± 0.85% of inhibition.

Table 1
AChE and BChE inhibitory activities of RO essential oil, extracts, and rosmarinic acid

| Extracts | Percentage of inhibition (1 mg/ml) | |
|---|---|---|
| | AChE | BChE |
| RO–PE | 8.5 ± 0.56[a] | 54.2 ± 1.55*** |
| RO–EtOAc | –[b] | 34.2 ± 0.85 |
| RO–$CHCl_3$ | – | – |
| RO–MeOH | – | 83.9 ± 0.97*** |
| RO–Essential oil | 63.7 ± 1.23*** | 74.0 ± 0.79*** |
| Rosmarinic acid | 47.3 ± 1.05 | 85.8 ± 1.31*** |
| Galanthamine | 99.8 ± 0.31 | 80.3 ± 1.14 |

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
[a] Values were expressed as mean ± SEM ($n = 3$), $P > 0.05$.
[b] – = No inhibition.

### 3.2. Quantification of rosmarinic acid by reversed-phase HPLC

Quantity of rosmarinic acid was established in RO–MeOH extract using reversed-phase HPLC by the method developed by Kan, Gökbulut, Kartal, Konuklugil, and Yilmaz (2007). Excellent linearity was obtained between peak areas and concentrations of 0.201–100.5 μg/mL with $r^2 = 0.9999$ for rosmarinic acid. Limits of detection (LOD) were established at a signal-to-noise ratio (S/N) of 3. Limits of quantification (LOQ) were established at signal-to-noise ratio (S/N) of 10. LOD and LOQ were experimentally verified by six injections of rosmarinic acid at the LOD and LOQ concentrations. The LOD and LOQ were calculated as 0.060 μg/mL and 0.201 μg/mL, respectively, for rosmarinic acid. Finally, RO–MeOH was found to contain 12.21 ± 0.95% (122.1 ± 9.5 mg/g extract) of rosmarinic acid.

### 3.3. Essential oil composition

The essential oil composition of RO was established by capillary GC–MS and twenty-eight components were identified representing 99.26% of total oil. Table 2 shows the identified compounds and their percentages obtained by GC–MS as well as the retention indices listed in order of their elution from the HP Innowax capillary column.

According to GC–MS data; 1,8-cineole was the main component amounting for 44.42% of total oil, followed by α-pinene (12.57%), and borneol (8.52%), which indicated that 64.08% of the oil consisted of oxygen-containing monoterpenes.

## 4. Discussion

Rosemary, used also as an aromatic tea and in aromatherapy, has been examined for its influence on mood and cognition and concluded that its essential oil produced a significant enhancement of performance and overall quality of memory in healthy adults (Moss et al., 2003). Most recently, two articles have reported on AChE inhibitory

Table 2
Composition of the essential oil (%) of RO

| | RI[a] | Compounds | Area %[b] |
|---|---|---|---|
| 1 | 994 | Tricyclene | 0.12 |
| 2 | 1008 | α-Pinene | 12.57 |
| 3 | 1036 | α-Fenchene | 0.15 |
| 4 | 1045 | Camphene | 4.43 |
| 5 | 1090 | β-Pinene | 5.18 |
| 6 | 1130 | δ-3-Carene | 0.19 |
| 7 | 1145 | Myrcene | 1.81 |
| 8 | 1160 | α-Terpinene | 0.77 |
| 9 | 1182 | Limonene | 2.45 |
| 10 | 1196 | 1,8-Cineole | 44.42 |
| 11 | 1213 | (*Z*)-β-Ocimene | 1.00 |
| 12 | 1223 | γ-Terpinene | 1.53 |
| 13 | 1229 | (*E*)-β-Ocimene | 0.30 |
| 14 | 1235 | 3-Octanone | 0.53 |
| 15 | 1247 | *p*-Cymene | 1.15 |
| 16 | 1257 | Terpinolene | 0.58 |
| 17 | 1406 | *p*-Cymenene | tr |
| 18 | 1426 | 1-Octen-3-ol | 0.79 |
| 19 | 1438 | *Trans*-Sabinene hydrate | 0.17 |
| 20 | 1486 | Camphor | 1.37 |
| 21 | 1515 | Linalool | 1.14 |
| 22 | 1554 | Bornyl acetate | 3.76 |
| 23 | 1572 | β-Caryophyllene | 1.24 |
| 24 | 1578 | Terpinen-4-ol | 1.45 |
| 25 | 1652 | δ-Terpineol | 0.53 |
| 26 | 1679 | α-Terpineol | 2.84 |
| 27 | 1695 | Borneol | 8.52 |
| 28 | 1829 | *p*-Cymen-8-ol | tr |
| 29 | 1834 | (*E*)-Geranyl acetone | 0.05 |
| 30 | 1973 | Caryophyllene oxide | 0.16 |
| 31 | 1992 | Methyl eugenol | 0.06 |
| 32 | 2142 | Isothymol | tr |
| 33 | 2147 | Eugenol | tr |
| 34 | 2178 | Thymol | tr |
| 35 | 2189 | Isocarvacrol | tr |
| 36 | 2196 | α-Bisabolol | tr |
| 37 | 2346 | Caryophylladienol-I | tr |
| Monoterpene hydrocarbons | 32.4 | | |
| Oxygen containing monoterpenes | 64.08 | | |
| Sesquiterpene hydrocarbons | 1.24 | | |
| Oxygen containing sesquiterpenes | 0.16 | | |
| Aromatic hydrocarbons | 0.06 | | |
| Hydrocarbons | 1.32 | | |
| Total identified | 99.26 | | |

[a] Retention Index relative to *n*-alkanes on the INNOWAX column.
[b] tr = trace (<0.05%).

activity of the essential oil of RO. Rosemary essential oil was previously found to exhibit a moderate inhibition on AChE, which is in accordance with our data (Perry, Court, Bidet, Court, & Perry, 1996). In Adsersen, Gauguin, Gudiksen, and Jager (2006), the aqueous and methanolic extracts of eleven plants used in Danish folk medicine for improvement of memory and cognition were screened for their AChE inhibitory activity. Among those plants, the aqueous and methanol extracts of RO were shown to have 12% and 17% of inhibition at 0.1 mg/mL concentration, respectively. In our study, the methanolic extract as well as the other extracts of RO was ineffective at 0.2 mg/mL. Similarly, in another study, the ethanol and water extracts of RO of Portuguese origin along with its essential oil were tested against AChE and the extracts displayed insignificant inhibition on this enzyme as compared to the essential oil (Mata et al., 2007). In the same study, where five plants (*Foeniculum vulgare*, *Mentha spicata*, *M. pulegium*, *R. officinalis*, and *Thymus serpyllum*) were screened totally, the conclusion was that for each plant, the inhibition capacity was as follows; essential oils > ethanol extracts > water extracts, which were in accordance with our data. Savelev, Okello, Perry, Wilkins, and Perry (2003) reported that some of the components identified in rosemary oil by GC–MS, such as 1,8-cineole, camphor, and 4-terpineol, inhibited the enzyme notably, the first one being the most potent. However, these compounds were present in small amounts in the rosemary oil of Portuguese origin, while verbenone, the major one, constituted 35.4% of the oil. The authors also tested verbenone alone in the same manner and its $IC_{50}$ value (163 ± 0.2 μg/mL) led to the suggestion that the activity exhibited by this essential oil is probably due to the summation of activities of several components. This is the typical condition happening with essential oils as well as plant extracts since they are chemically complex mixtures. In our case, we also obtained rather similar results with the essential oil and the extracts of RO of Turkish origin regarding their AChE inhibitory activities, nevertheless, observed better results with the same extracts and essential oil against BChE. The essential oil had even a higher inhibition towards BChE than AChE, while the PE extract also showed a moderate activity. The most striking outcome was that the MeOH extract and rosmarinic acid, its major phenolic acid found by our HPLC analysis (Fig. 1), gave almost the same inhibition rates on BChE, which made us thought that rosmarinic acid is most likely the active compound responsible for potent anti-BChE activity of the methanolic extract of the plant. Although, the aim of the researchers working in this scope is to elucidate rationally active compound(s) of any plant extract regarding any desired biological activity, it is not necessarily always to be only one compound that is responsible for these effects, which may as well be depend on several compounds that act in a synergistic manner or on compounds which regulate one another. The latter conclusion is applicable for our result on anticholinesterase activity of RO essential oil. Some previous studies confirmed that 1,8-cineole and α-pinene are highly active monoterpenes on AChE (Miyazawa, Watanabe, & Kameoka, 1997; Perry, Bollen, Perry, & Ballard, 2003; Perry, Houghton, Theobald, Jenner, & Perry, 2000). Savelev et al. (2003), synergystic or antagonistic interaction among 1,8-cineole, camphor, α-pinene, β-pinene, borneol, caryophyllene oxide, linalool, and bornyl acetate was investigated and minor synergy was observed between 1,8-cineole and α-pinene, while camphor and 1,8-cineole was found to cause antagonistic effect, which overall led to conclusion

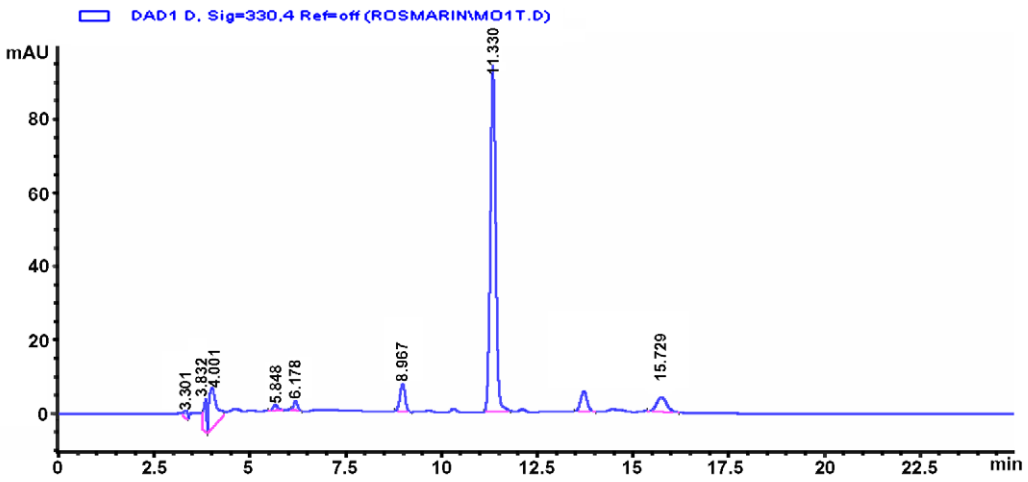


Fig. 1. HPLC chromotogram of the methanolic extract of RO.

that AChE inhibitory activity of the *Salvia lavandulaefolia* essential oil is produced by an interaction between its component terpenes. These results also seem to be in good accordance with our study, where 1,8-cineole was the most principal component in our rosemary oil, which was followed by α-pinene, borneol, and β-pinene. Therefore, we could also think existence of a synergistic interaction between 1,8-cineole and α-pinene in rosemary essential oil.

On the other hand, chemical variations encountered in essential oil of the same species of different origin were mentioned to be due to same factors in the past (Sacchetti et al., 2005). In an essential oil study carried out on RO collected from different localities (Çanakkale, Izmir, and Mersin) at several time intervals in Turkey during the year of 2006, it was also pointed out that composition of the essential oil depended mostly on climate. For instance; Mersin sample had 1,8-cineole as the major component whereas Çanakkale sample contained camphor in major amount (Yeşil-Çeliktaş et al., 2007), whereas ours dominated by 1,8-cineole (44.42%) (Table 1). Relevantly, the essential oil of RO growing in Iran was reported to contain piperitone in majority (23.7%) followed by α-pinene (14.7%), 1,8-cineole (7.43%), camphor (4.97%), and camphene (3.33%), which differs from ours (Gachkar et al., 2007). The major components; α-pinene, borneol, camphene, camphor, verbenone, and bornyl acetate, were also found to exist in Sardinian RO oil (Angioni et al., 2004). Therefore, it could be clearly said that the activity is quite correspondent to essential oil composition.

## 5. Conclusion

In this study, we demonstrated AChE and BChE inhibitory activities of the essential oil and extracts of RO growing in Turkey, established chemical composition of the essential oil which had quite notable inhibitions towards both AChE and BChE, and determined rosmarinic acid amount in the methanolic extract of RO that showed a remarkable BChE-inhibitory effect. 1,8-Cineole and α-pinene were found as the two major monoterpenes in our rosemary oil and we conclude that anticholinesterase activity of RO essential oil most likely depends on a synergistic mechanism between a number of oil components, whereas rosmarinic acid seem to be responsible for strong anti-BChE effect of RO–MeOH.

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