Research Article

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Volatile terpenoids as potential drug leads in Alzheimer’s disease

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Abstract: Alzheimer’s disease (AD) is by far the most prevalent of all known forms of dementia. Despite wide-spread research, the main causes of emergence and development of AD have not been fully recognized. Natural, low-molecular, lipophilic terpenoids constitute an interesting group of secondary plant metabolites, that exert biological activities of possible use in the prevention and treatment of AD. In order to identify secondary metabolites possessing both antioxidant activity and the potential to increase the level of acetylcholine, selected terpenoids have been screened for possible acetylcholinesterase inhibitory activity by use of two methods, namely Marston (chromatographic assay) and Ellman (spectrophotometric assay). In order to describe the interaction between terpenes and AChE active gorge, molecular docking simulations were performed. Additionally, all analyzed terpenes were also evaluated for their cytotoxic properties against two normal cell lines using MTT assay. The obtained results show that: carvone (6), pulegone (8) and γ-terpinene (7) possess desirable AChE inhibitory activity. MTT assay revealed low or lack of cytotoxicity of these metabolites. Thus, among the investigated terpenes, carvone (6), pulegone (8) and γ-terpinene (7) can be recognized as compounds with most promising activities in the development of multi-target directed ligands.

Keywords: terpenes, AChE inhibitor, molecular docking, MTT assay, acetylcholinesterase

1 Introduction

It has been more than a century since the first description of symptoms of neurodegenerative ailment, known today as Alzheimer’s disease (AD) was published. Pathophysiological changes in the brains of affected patients have been well studied and recognized, for example the deposits of amyloid plaques, neurofibrillary tangles, decrease in acetylcholine level, lost connections between neurons, hippocampus shrinking and others [1-3]. Many different hypotheses have been published, trying to explain the exact causes of AD. The most popular ones are cholinergic hypothesis, oxidative stress theory of AD, amyloid cascade hypothesis, mitochondrial cascade hypothesis and so on [4-6].

As the AD therapy is hypothesis driven, not causal the currently approved drugs only treat some of the symptoms of the ailment [7-8]. Taking into consideration a multifactorial character of AD origin there is a current trend in developing so called multi-target directed ligands, that may affect multiple processes responsible for onset and progress of AD. Many secondary plant metabolites have been identified as possible drug candidates to be used in the treatment of different types of dementia, including AD. These compounds are often used as components of larger molecules, that exhibit biological activities relevant for the treatment of AD. The following are some examples of secondary plant metabolites that have been reported as effective in development of multi-target directed ligands: genistein (antioxidant), curcumin (antioxidant), galantamine (AChE inhibitor) and scoparone (antioxidant) [9-11].

However, one of the major drawbacks of currently developed multi-target directed ligands is the size of molecules, which cause problems in their bioavailability. There is a need to search for compounds that, besides desirable biological effects, possess favorable
Volatile terpenoids are low-molecular, lipophilic compounds, that can easily cross biological barriers, e.g. the blood-brain barrier [12,13]. There have been many reports published, including clinical trials with AD demented patients, showing beneficial effects of essential oils for human health [14,15]. Essential oils as well as individual terpenes have been shown to exert, for example, antioxidant as well acetylcholinesterase inhibitory activity [16,17].

The aim of our scientific project was the identification of terpenoids possessing most desirable properties for possible use in the prevention and treatment of AD, based on current knowledge. Our previous reports discussed free radical scavenging activity of selected common terpenoids, such as synergistic and antagonistic effects observed for some of them [18]. In this paper these secondary plant metabolites were tested for acetylcholinesterase inhibitory activity. The assays based on two methods, namely Marston based on TLC and spectrophotometric Ellman technique, and additionally molecular docking simulations were used to check the possible inhibitory effect [19,20]. Additionally, in order to determine cytotoxicity of all tested terpenoids, MTT assay was used.

The obtained results, combined with our previous findings, helped to identify terpenoids characterized with the most desirable biological effects and relatively low cytotoxicity.

2 Experimental procedure

2.1 Reagents

Acetylcholinesterase, type V-S from Electrophorus electricus, albumin from bovine serum, 1-naphthyl acetate, Trizma® (2-amino-2-(hydroxymethyl)-1,3-propanediol) hydrochloride solution (1M, pH 7.8), acetylthiocholine iodide (≥99%), DTNB (5,5′-dithiobis(2-nitrobenzoic acid)), galantamine hydrobromide from Lycoris sp. (≥94%) and Fast Blue B salt 95% were purchased from Sigma Aldrich (USA). Terpenes: (-)-isopulegol (≥99%), (+)-α-pinene (≥99%), (+)-menthol (≥99%), p-cymene (≥99%), eucalyptol (≥99%), pulegone (97%), γ-terpinene (97%), (+)-inalool (≥97%), (+)-carvone (≥96%), α-terpinene (≥95%), (+)-citronellal (≥95%), (+)-terpinene-4-ol (≥95%), citral (≥95%), ocimene (≥90%), (+)-menthone (≥90%), farnesene (≥90%), α-phellandrene (≥90%) and β-myrcene (≥90%) were also obtained from Sigma-Aldrich. Structures of the terpenes with appropriate numbering are presented in Figure 1. Solvents ethanol, methanol and ethyl acetate, analytical purity grade, were obtained from Polish Reagents (Gliwice, Poland). For cytotoxicity evaluation Eagle’s Minimum Essential Medium (EMEM) was supplied from American Type Culture Collection (UK). Fetal bovine serum (FBS) was purchased from PAA Laboratories (Germany). Penicillin, streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), galantamine and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich (USA).

2.2 Acetylcholinesterase inhibitory assay on TLC plates according to Marston method

Thin-layer chromatography based assays are often used to screen complex mixtures for the presence of secondary metabolites exerting desired biological effect [21]. In our study TLC assay was used for two main reasons: (i) the optimized system would be used, in future experiments, for screening essential oils for biologically active terpenes and (ii) solubility issues related to the use of lipophilic terpenoids; many terpenes are insoluble in aqueous-buffer media used in AChE inhibitory assays, therefore an alternative approach aimed at preliminary identification of pharmacologically active terpenes was used.

The experiment, according to Marston method [20], was performed by means of chromatographic HPTLC silica gel 60 (F254) plates, (Merc, Germany). The experiment was started by preconditioning chromatographic plates. The plates (10 cm x 5 cm) were eluted with ethanol and activated for 40 minutes at 105°C. After preconditioning and activation, the analyzed compounds were applied onto the plates. Due to high volatility of terpenes, which caused issues during the application step, all the analyzed terpenes were diluted 10 times with either ethyl acetate (β-myrcene (5), ocimene (12), farnesene (18)) or ethanol (remaining terpenes), depending on solubility of individual compounds. In the first part of the assay, the amount corresponding to 0.1 mg of each of the analyzed compounds was applied onto the plates. Due to high volatility of terpenes, which caused issues during the application step, all the analyzed terpenes were diluted 10 times with either ethyl acetate (β-myrcene (5), ocimene (12), farnesene (18)) or ethanol (remaining terpenes), depending on solubility of individual compounds. In the first part of the assay, the amount corresponding to 0.1 mg of each of the analyzed compounds was applied onto the plates. Due to high volatility of terpenes, which caused issues during the application step, all the analyzed terpenes were diluted 10 times with either ethyl acetate (β-myrcene (5), ocimene (12), farnesene (18)) or ethanol (remaining terpenes), depending on solubility of individual compounds. In the first part of the assay, the amount corresponding to 0.1 mg of each of the analyzed compounds was applied onto the plates. Due to high volatility of terpenes, which caused issues during the application step, all the analyzed terpenes were diluted 10 times with either ethyl acetate (β-myrcene (5), ocimene (12), farnesene (18)) or ethanol (remaining terpenes), depending on solubility of individual compounds. In the first part of the assay, the amount corresponding to 0.1 mg of each of the analyzed compounds was applied onto the plates. Due to high volatility of terpenes, which caused issues during the application step, all the analyzed terpenes were diluted 10 times with either ethyl acetate (β-myrcene (5), ocimene (12), farnesene (18)) or ethanol (remaining terpenes), depending on solubility of individual compounds. In the first part of the assay, the amount corresponding to 0.1 mg of each of the analyzed compounds was applied onto the plates. Due to high volatility of terpenes, which caused issues during the application step, all the analyzed terpenes were diluted 10 times with either ethyl acetate (β-myrcene (5), ocimene (12), farnesene (18)) or ethanol (remaining terpenes), depending on solubility of individual compounds. In the first part of the assay, the amount corresponding to 0.1 mg of each of the analyzed compounds was applied onto the plates. Due to high volatility of terpenes, which caused issues during the application step, all the analyzed terpenes were diluted 10 times with either ethyl acetate (β-myrcene (5), ocimene (12), farnesene (18)) or ethanol (remaining terpenes), depending on solubility of individual compounds. In the first part of the assay, the amount corresponding to 0.1 mg of each of the analyzed compounds was applied onto the plates. Due to high volatility of terpenes, which caused issues during the application step, all the analyzed terpenes were diluted 10 times with either ethyl acetate (β-myrcene (5), ocimene (12), farnesene (18)) or ethanol (remaining terpenes), depending on solubility of individual compounds. In the first part of the assay, the amount corresponding to 0.1 mg of each of the analyzed compounds was applied onto the plates. Due to high volatility of terpenes, which caused issues during the application step, all the analyzed terpenes were diluted 10 times with either ethyl acetate (β-myrcene (5), ocimene (12), farnesene (18)) or ethanol (remaining terpenes), depending on solubility of individual compounds. In the first part of the assay, the amount corresponding to 0.1 mg of each of the analyzed compounds was applied onto the plates. Due to high volatility of terpenes, which caused issues during the application step, all the analyzed terpenes were diluted 10 times with either ethyl acetate (β-myrcene (5), ocimene (12), farnesene (18)) or ethanol (remaining terpenes), depending on solubility of individual compounds. In the first part of the assay, the amount corresponding to 0.1 mg of each of the analyzed compounds was applied onto the plates.
The next part of the experiment was focused on studying concentration-dependent activity of terpenes towards AChE in the TLC assay. The amount of the analyzed terpenes was applied onto a TLC plate corresponding to [mg]: 0.1; 0.05; 0.025; 0.01 and 0.001 of each of the compounds. The experiment was carried out according to the same procedure.

### 2.3 Spectrophotometric Ellman esterase assay

AChE inhibitory activity with Ellman reagent was performed spectrophotometrically using a UV-Vis spectrophotometer (Genesys 20, Thermo Fisher Scientific, Waltham, MA, USA) [19]. The experiment was performed according to [19] with some modifications. The samples were prepared by mixing 40 μL of AChE solution and appropriate amount of each terpene, in order to obtain the following concentrations of each terpene [mM]: 3.5; 7; 14. These concentrations were chosen based on the activity of individual terpenes and the amount of AChE required to perform the test (40 μL, which corresponds to 0.27 U of AChE). 0.27 U of AChE will hydrolase 0.27 μM of acetylcholine per minute (pH 8.0, 37°C), which is a relatively high amount of the enzyme considering the fact, that the concentration of acetylcholine found in the hippocampus of freely moving rats was found to be 0.40 ± 0.12 nM [22]. Some terpenes were also previously described to inhibit acetylcholinesterase activity at mM concentration [23].

After mixing AChE solution with each individual terpene, the samples were incubated for 15 minutes at room temperature. Thereafter, 80 μL of ATChI solution were added to the sample and it was incubated for 5 minutes at room temperature. Finally, 80 μL of DTNB solution were added and the sample was diluted with Trizma hydrochloride up to 1 mL. Absorbance changes were recorded starting at 10 up to 30 minutes at 412 nm. Each measurement was repeated three times for freshly prepared solution of each individual terpene. The final result is an average of the three replicates. Galantamine hydrobromide was used as a positive control. AChE inhibitory activity of all studied terpenes was calculated using the following equation:

\[
\text{Inhibition} \% = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100\%
\]
Where: $A_{\text{blank}}$ - absorbance of negative control (studied terpenes were substituted by Trizma buffer), $A_{\text{sample}}$ - absorbance of a studied terpene sample.

In order to compare the activity of all the terpenes and a reference standard, IC$_{50}$ values were determined. The parameter indicating the concentration of a test substance able to inhibit the hydrolysis of substrate, by 50%, was calculated from dose-effect curves by linear regression. Inhibition factor (IF) of each terpene was calculated, which is an activity measurement of each compound in comparison to activity of positive control compound, i.e. galantamine [24]. The IF value was calculated according with the following equation:

$$IF = \frac{IC_{50} \text{of reference inhibitor}}{IC_{50} \text{of studied terpene}}$$

### 2.4 Molecular docking procedure

Group of 18 terpenes and galantamine were docked to human acetylcholinesterase (huAChE) using AutoDock (V.4.0) and Molegro Virtual Docker (MVD, V.5.0.0., Molegro ApS Aarhus, Denmark). Crystal structure of huAChE was retrieved from protein data bank (PDB ID: 4EY6). In order to prepare the AChE model for docking procedure, co-factors were removed using Yasara 11.2.15 package (Yasara Bioscience, Graz, Austria). Additionally, non-polar hydrogen atoms were deleted. Three dimensional structures of ligands were prepared and optimized using Spartan 10 V.1.1.0 (Wavefunction, Inc. Irvine, CA, USA). The first and most significant step of docking simulation was a grid generation to select the AChE enzyme active site by selecting the AChE inhibitors binding sites. In this case, the binding site was a little extended compared to the binding site in complex with galantamine (PDB ID: 4EY6).

In the case of AutoDock program, molecular docking was performed using the empirical free energy function together with the Lamarckian genetic algorithm. The grid box dimensions were 58 x 60 x 58 Å. The other docking parameters were characteristic for standard procedure. The docking process was carried out using standard Autogrid4 and AutoDock4 syntax [25]. Clustering histogram analyses were performed after docking procedures. The program generated ten energetically lowest conformations of the terpene ligands and galantamine in the active site of the enzyme, from which the most desirable are cases with the lowest values, indicating large binding affinity towards AChE.

The docking space in Molegro Virtual Docker was defined as a sphere of 11 Å dimension in diameter. MVD generates a series of docking poses and arranges them using energy based criterion and the embedded scoring function (MolDockScore) [25]. Similarly to AutoDock, the used docking parameters followed standard procedure. The obtained results were analyzed according to ligand/ enzyme interactions. Due to the fact that water molecules can play an important role in protein-ligand interactions, they were included to the procedure.

### 2.5 In vitro cytotoxicity assay

The cell culture experiment was performed using two normal cell lines. Human skin fibroblast cell line (BJ cells) was obtained from American Type Culture Collection (ATCC, England, UK) and green monkey kidney cell line (GMK cells) was purchased from BIOMED Serum and Vaccine Production Plant (Lublin, Poland). Both cell lines were cultured in EMEM supplemented with 10% FBS, 100 U mL$^{-1}$ penicillin and 100 μg mL$^{-1}$ streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO$_2$ and 95% air.

To determine the cytotoxic activity of the investigated terpenes, the cells were seeded in 96-well plates in 100 μl of a complete growth medium (supplemented with 10% FBS) at a concentration of 1.5 x 10$^4$ cells/well (BJ cells) and 2.0 x 10$^4$ cells/well (GMK cells) and incubated for 24 hours at 37°C in a humidified atmosphere of 5% CO$_2$. After this time, all tested terpenes were firstly diluted in DMSO to obtain stock solution at 10 mg mL$^{-1}$ and the solutions were further diluted in culture medium supplemented with 2% FBS. Galantamine was used as a reference compound. It was dissolved in DMSO (10 mg mL$^{-1}$) and then diluted in culture medium supplemented with 2% FBS. Subsequently, the culture medium was replaced with 100 μL of the serial dilutions of the terpenes and reference compound. Additionally, untreated cells were used as a control of cytotoxicity and different concentrations of DMSO were used as a solvent control. The cell cultures were incubated at 37°C for 24 hours. The cytotoxic effect was estimated using MTT test as described previously [26,27]. The experiment was repeated in three separate measurements. The half maximal cytotoxic concentration (CC$_{50}$) was defined as the compound concentration (mM) required to reduce cell viability to 50%. The CC$_{50}$ values were calculated via 4-parameter nonlinear regression analyses (GraphPad Prism 5, version 5.04) and were presented as mean values ± standard deviation (SD).

Ethical approval: The conducted research is not related to either human or animals use.
2.6 Statistical analysis

All the assays were carried out in triplicates to validate reproducibility. The calculated parameters were determined with a relative uncertainty of less than 5% (GraphPad Prism 5, version 5.04).

3 Results

3.1 Acetylcholinesterase inhibitory activity of terpenes

The first part of the presented studies was focused on identification of most potent AChE inhibitors from among a group of 18 common terpenes, previously studied for free radical scavenging activity [17,18]. This is another step in the process of discovery of common terpenes acting as possible multi-target directed ligands, in the prevention and the treatment of AD. Galantamine, a known AChE inhibitor, was used as a positive control. Acetylcholinesterase inhibitory activity of terpenoids has been focused on numerous studies, however, most of them dealt with essential oils without taking into account the activity of individual terpenes [28]. It is for the first time, that both free radical scavenging and AChE inhibitory data, for common terpenoids, are used to identify terpene compounds most suitable to become candidates for development of multi-target directed ligands.

3.2 Preliminary Marston assay

In the first stage of our study thin-layer chromatography-bioassay test [19] was used to check all the analyzed terpenes for possible AChE inhibitory activity. First the potential biological activity was checked for the terpenes spotted on TLC plates, at the same concentration (volume corresponding to 0.1 mg of each of the analyzed compound). TLC-based test was chosen as the first, preliminary step in identification of AChE inhibitors, mostly due to the fact that numerous terpenes are insoluble in aqueous media, used in other screening tests.

Marston, the originator of the applied method, indicates that active substances appear as white spots against a colored background [20]. Upon completion of the assay, the following terpenes appeared as dark spots, when compared to the background: β-myrcene (5), p-cymene (13) and α-phellandrene (15). The remaining terpenes caused discoloration of the background testifying their possible AChE inhibitory activity.

Spots of the following terpenes (0.1 mg each of the terpenes) appeared after 5 minutes upon completion of the test: citronellal, α-terpinene (14), carvone (6), isopulegol (10), linalool and pulegone (8). After another 5 minutes, white spots of the following terpenes appeared against the background: citral (1), farnesene (18), γ-terpinene (7), eucalyptol (17), α-pinene (16), menthone (9), terpinene-4-ol (3) and ocimene (12).

In the next step, compounds that produced positive effect in the first stage of experiment were selected and applied onto TLC plate at different concentrations (Figure 2). α-Phellandrene (15) and β-myrcene (5), two compounds

Figure 2: Marston assay results obtained for selected terpenes.
producing darker spots, were also applied to observe the produced effect, at different concentrations.

Here we discuss results observed 10 minutes after derivatization step. Farnesene (18) was the only terpene producing noticeable AChE inhibitory effect when a volume corresponding to 0.001 mg was applied. Citral (1), citronellal (2), carvone (6) and ocimene (12) exhibited activity at 0.01 mg whereas for pulegone (8), 0.025 mg was the lowest amount at which any effect was visible.

### 3.3 Ellman assay

Terpenes pose many difficulties in analyzing their potential pharmacological activity, due to insolubility of many of them in aqueous media. Farnesene (18), α-terpinene (14), p-cymene (13), menthol (11), citronellal (2) and β-myrcene (5) formed emulsions, while performing the spectrophotometric test. For the twelve remaining substances the percentage of AChE inhibition IC$_{50}$ values and inhibition factors, were determined (Table 1). The activity of only these twelve compounds was considered in further studies. Galantamine was used as a standard. One of the values used for comparison of compounds’ activity is the percentage of enzyme inhibition. The comparison was made for terpenes at a lowest concentration (3.5 mM), at which all the analyzed compounds showed activity and the results were recorded 30 minutes after starting the reaction. The highest activity was observed for ocimene (12) (30min: 62.5%), carvone (6) (30min: 68.23%), menthone (9) (30min: 43.49%), γ-terpinene (7) (30min: 19.01%) and pulegone (8) (30min: 24.27%). In comparison, galantamine inhibited 46.63% activity of the enzyme after 30 minutes, at 0.1 mM. IC$_{50}$ values were further obtained for 12 terpenes and the standard. All analyzed terpenes exerted much lower activity compared to galantamine (IC$_{50}$ = 0.14 mM, Table 1). The most promising results were obtained for carvone (6) (IC$_{50}$ = 2.9 mM), ocimene (12) (IC$_{50}$ = 4.7 mM) and pulegone (8) (IC$_{50}$ = 9 mM). Additionally, in order to compare activity of analyzed substances, inhibitory factors (IF) are presented in Table 1. The highest activity, thus the highest IF values, were observed for carvone (6) and ocimene (12). An interesting phenomenon was observed for γ-terpinene (7) and terpinene-4-ol (3) for which inhibitory activity decreased with increasing concentration of the analyzed terpene. In these cases, a negative slope coefficient of dose-effect curves was achieved.

<table>
<thead>
<tr>
<th>Compound</th>
<th>AChE inhibition [%] ± SD 3.5 mM</th>
<th>IC$_{50}$ [mM]</th>
<th>Inhibition factor [IF]</th>
</tr>
</thead>
<tbody>
<tr>
<td>galantamine [0.1 mM]</td>
<td>46.63 ± 1.91</td>
<td>0.14 ± 0.005</td>
<td>---</td>
</tr>
<tr>
<td>carvone</td>
<td>68.23 ± 2.88</td>
<td>2.9 ± 0.12</td>
<td>0.05</td>
</tr>
<tr>
<td>citral</td>
<td>26.02 ± 0.99</td>
<td>72.0 ± 2.81</td>
<td>0.02</td>
</tr>
<tr>
<td>eucalyptol</td>
<td>2.78 ± 0.08</td>
<td>266.0 ± 11.87</td>
<td>0.001</td>
</tr>
<tr>
<td>isopulegol</td>
<td>6.25 ± 0.27</td>
<td>233.0 ± 10.08</td>
<td>0.001</td>
</tr>
<tr>
<td>linalool</td>
<td>21.23 ± 0.89</td>
<td>11.0 ± 0.42</td>
<td>0.013</td>
</tr>
<tr>
<td>menthone</td>
<td>43.49 ± 1.76</td>
<td>9.0 ± 0.39</td>
<td>0.016</td>
</tr>
<tr>
<td>ocimene</td>
<td>62.5 ± 2.66</td>
<td>4.7 ± 0.20</td>
<td>0.03</td>
</tr>
<tr>
<td>pulegone</td>
<td>24.27 ± 1.11</td>
<td>9.0 ± 0.41</td>
<td>0.016</td>
</tr>
<tr>
<td>terpinene-4-ol</td>
<td>15.5 ± 0.68</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>α-phellandrene</td>
<td>8.04 ± 0.27</td>
<td>27.0 ± 1.11</td>
<td>0.005</td>
</tr>
<tr>
<td>α-pinene</td>
<td>11.3 ± 0.48</td>
<td>102.0 ± 4.17</td>
<td>0.001</td>
</tr>
<tr>
<td>γ-terpinene</td>
<td>19.01 ± 0.90</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>farnesene</td>
<td>emulsion</td>
<td>emulsion</td>
<td>emulsion</td>
</tr>
<tr>
<td>α-terpinene</td>
<td>emulsion</td>
<td>emulsion</td>
<td>emulsion</td>
</tr>
<tr>
<td>p-cymene</td>
<td>emulsion</td>
<td>emulsion</td>
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<tr>
<td>menthol</td>
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<tr>
<td>citronellal</td>
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<tr>
<td>β-myrcene</td>
<td>emulsion</td>
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</tr>
</tbody>
</table>

* emulsification with increasing concentration of added terpene
3.4 Molecular docking results

In the case of AutoDock and MVD, the energetically lowest conformations, for studied docked terpenes (carvone (6), pulegone (8), γ-terpinene (7)), were selected. All of other poses were clustered in one space in the AChE binding site. Since molecular docking results did not indicate significant differences between procedure providing water molecules and without them, the analysis was based on results including the molecules.

The obtained docking results suggested that all selected ligands interact with the AChE active site. The analysis of residues in close contact (up to 4Å) are presented in Table 2.

Detailed interpretation of obtained docking results was performed for selected terpenes (carvone (6), pulegone (8), γ-terpinene (7)). Each ligand is located inside the enzyme binding site and interacts with the most important amino acids residues that create the AChE active site. Similarly to galantamine, a reference molecule, all studied terpenes were docked to overlapping sites that form the AChE active site. Figure 3 presents docking results for carvone (6), pulegone (8) and γ-terpinene (7) as examples of potential AChE inhibitors. As aforementioned, our studied terpenes exhibited interactions (H-bonds) with amino acids residues in close contact (up to 4Å).

The detailed analysis of molecular docking results obtained for selected ligands revealed significant interactions with amino acids residues of AChE. It is worth noting that carvone (6) interacts with Ser203 and His447 that create esteratic site, and Trp86, Tyr133, Tyr337 and Phe338 which create anionic site of AChE active gorge responsible for catalytic machinery and choline binding, respectively (Table 2, Figure 4b). Additionally, analysis of possible interactions between carvone (6) and amino acids of AChE, revealed putative H-bond interaction between the terpene and Tyr337 (2.92Å) creating anionic subsite. As aforementioned, docking results obtained

Table 2: Amino acid residues in close contact (Å) to selected ligands and hydrogen bonds between them. Results obtained for docking simulation with use of MVD with consideration of H2O molecules in crystal structure of AChE.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Amino acids residues</th>
<th>Hydrogen bonds (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galantamine</td>
<td>Tyr133, Ile451, Gly120, Glu202, Ala204, Ser203, Ser125, Trp86, Gly121, Gly122, Tyr124, Asp74, Gly448, Phe297, His447, Trp236, Phe295, Phe338, Tyr337, Tyr341</td>
<td>Tyr337 (2.99) Ser203 (2.77) Glu202 (2.77) H2O (3.19) H2O (3.01) H2O (2.94)</td>
</tr>
<tr>
<td>Carvone</td>
<td>Trp439, Tyr449, Pro446, Ile451, Gly448, Tyr133, Trp86, His447, Glu202, Tyr337, Tyr133, Ser203, Gly121, Ser125, Gly122</td>
<td>Tyr337 (2.92)</td>
</tr>
<tr>
<td>Pulegone</td>
<td>Tyr133, Ile451, Tyr119, Gly128, Gly120, Glu202, Trp86, Tyr449, Gly448, Ser125, Gly121, Ser203, Pro448, His447, Tyr337, Trp439</td>
<td>Glu202 (2.9) Glu202 (2.91)</td>
</tr>
<tr>
<td>γ-Terpinene</td>
<td>Tyr449, Tyr133, Ile451, Gly448, Pro446, Gly202, Gly120, His447, Ser203, Gly121, Tyr337, Trp86, Pro446, Trp439, Phe338, His447</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 3: Results of molecular docking for the most active terpenes and galantamine, a reference molecule. (a) Comparison of carvone (green) and galantamine (magenta) bind to AChE active site, (b) docking results obtained for the most active AChE inhibitors: carvone (green), pulegone (blue) and γ-terpinene (yellow).
Volatile terpenoids as potential drug leads in Alzheimer's disease

for galantamine and our docking terpene suggested that carvone (6) is mediated by Trp86, Tyr133, Tyr337, Phe338 (anionic subsite), His447, Ser203 (esteratic site), Gly121, Gly122 (oxyanion hole), Ile451, Gly448, Glu202, Gly120 and Ser125, which together create the most significant parts of AChE binding site (Figure 4).

As the results present (Table 2) pulegone (8) interacts with Tyr133, Trp86 and Tyr337 that create anionic site, and Ser203 and His447 being part of esteratic site of AChE. In contrast to carvone (6), this ligand did not exhibit interactions with Phe338 and Glu334, amino acids residues belonging to anionic and esteratic sites, respectively. Analysis towards interactions between the ligand and the analyzed enzyme revealed two H-bonds with Glu202 (2.90 and 2.91 Å). The detailed analysis of molecular docking results for galantamine and pulegone (8) suggested that our terpene is mediated by ten amino acid residues: Tyr133, Tyr337 (anionic subsite), Ser203, His447 (esteratic site), Gly121 (oxyanion hole), Ile451, Gly120, Gly202, Gly448 and Ser125, which play a key role in AChE enzyme functioning. In comparison with carvone (6) and pulegone (8), in the case of considered ligand, there was no H-bond interaction between γ-terpinene (7) and amino acids residues.

3.5 Cytotoxicity

The investigated terpenes and standard reference compound (galantamine) were evaluated for their cytotoxic activity towards two normal cell lines (BJ and GMK cells) after 24-hour incubation. Based on MTT assay results, the half maximal cytotoxic concentration (CC50) was determined for all compounds and summarized in Table 3. Referring to Kuete et al. [30] the terpenes were divided into four groups (based on CC50 value): strong (<0.1 mM), moderate (>0.1 mM - < 0.3 mM), low (>0.3 mM - <1 mM) and no (>1 mM) cytotoxicity. Among eighteen tested terpenes only two of them, citral (1) and farnesene (18), exhibited strong cytotoxicity towards BJ cells. The CC50 values of these monoterpenes ranged from 0.076 to 0.093 mM. Citronellal (2), ocimene (12), and α-pinene (16) possessed moderate cytotoxicity against BJ cells, while citral (1), ocimene (12), farnesene (18) and α-pinene (16) showed moderate cytotoxic activity against GMK cells. The other terpenes showed low or no cytotoxicity against both cell lines. Similar effects were obtained for reference compound (galantamine), which exhibited no cytotoxicity against both BJ and GMK cells.
The presented research is focused on the activity of selected, common terpenes towards acetylcholinesterase inhibition and their cytotoxicity. Due to the fact that substances exhibiting both free radical scavenging ability and AChE inhibitory activity are most desirable, particular attention was paid to terpenes revealing these two activities [17]. Additionally, cytotoxicity is just as important as inhibitory and antioxidant activities. Therefore, interpretation of obtained results provided all of the aforementioned important points. The obtained results revealed a few potential inhibitors. Taking into account the TLC bioassay, according to Marston’s method, the results indicated sufficient activity of citronellal (2), farnesene (18), ocimene (12), α-phellandrene (15), carvone (6) and pulegone (8), which revealed the highest activity 10 min after completing the derivatization (Figure 2). Among these substances farnesene (18) exhibited the most promising activity (limit of activity equals 1μg). The compounds exhibiting desirable AChE inhibitory effect in TLC-based bioassay turned out to be active also in the spectrophotometric test. Unfortunately, a few among the analyzed terpenes, which revealed promising activity with the use of Marston method, e.g. farnesene (18), were excluded from Ellman test due to their solubility. Taking into account all of the performed assays, the most promising AChE inhibitors are pulegone (8), carvone (6), and γ-terpinene (7).

Aside from results obtained for Ellman and Marston assays, essential information revealing terpene interactions with AChE binding site were obtained from molecular docking procedure. Significant feature of AChE structure is a deep, narrow and 20Å long hydrophobic gorge which penetrates more than halfway into the enzyme [31]. Another feature influencing potential enzyme-ligand interactions are two amino acid residues, Trp286 and Asp74, located at the entrance to the active site which can block the entry of substrates and the exit of products from the active site [32]. The residues, including Tyr72, Tyr124 and Tyr341, create a peripheral anionic site responsible for binding substrate transiently as the first step in catalytic pathway [33]. Therefore, taking into account the structure of AChE binding site the most suitable inhibitors should be characterized by a relatively simple structure that can easily penetrate into the AChE active gorge. Considering the active-gorge site, influential role is mostly played by two sites: esteratic created by catalytic triad of AChE including Ser203, His447 and Glu334, and anionic subsite created by Trp86, Tyr133, Tyr337 and Phe338, responsible for catalytic machinery and choline binding, respectively [31,33]. Additional hindrance in AChE structure is created by two amino acid residues of phenylalanine (Phe295 and Phe297) creating an acyl pocket responsible for substrate selectivity by preventing access of larger members of the choline ester series [33]. The oxyanion hole created by Gly121, Gly122 and Ala204, provides hydrogen bond donors that stabilize the tetrahedral transition state of substrate.

Considering the docking results, most important are interactions with amino acid residues building anionic and esteratic subsites.

As aforementioned, detail analysis of all performed studies determined selected group of terpenes. The most promising terpene, exhibiting the highest acetylcholinesterase inhibitory activity, as confirmed by both in vitro tests, turned out to be carvone (6). In

### Table 3: Cytotoxicity of monoterpenes on normal human skin fibroblast cell line (BJ cells) and green monkey kidney cell line (GMK cells) assessed using MTT test after 24-hour incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CC50 ± SD [mM]</th>
<th>BJ cells</th>
<th>GMK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galantamine</td>
<td>2.621 ± 0.013</td>
<td>&gt; 3.484</td>
<td></td>
</tr>
<tr>
<td>Carvone</td>
<td>1.35 ± 0.009</td>
<td>1.713 ± 0.012</td>
<td></td>
</tr>
<tr>
<td>Citral</td>
<td>0.076 ± 0.012</td>
<td>0.136 ± 0.012</td>
<td></td>
</tr>
<tr>
<td>Citronellal</td>
<td>0.267 ± 0.008</td>
<td>0.392 ± 0.016</td>
<td></td>
</tr>
<tr>
<td>Eucalyptol</td>
<td>1.184 ± 0.012</td>
<td>1.366 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>Farnesene</td>
<td>0.093 ± 0.011</td>
<td>0.141 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>Isopulegol</td>
<td>0.42 ± 0.012</td>
<td>0.522 ± 0.014</td>
<td></td>
</tr>
<tr>
<td>Linalool</td>
<td>0.599 ± 0.010</td>
<td>1.139 ± 0.012</td>
<td></td>
</tr>
<tr>
<td>Menthol</td>
<td>0.79 ± 0.017</td>
<td>1.619 ± 0.021</td>
<td></td>
</tr>
<tr>
<td>Menthone</td>
<td>1.984 ± 0.018</td>
<td>2.713 ± 0.011</td>
<td></td>
</tr>
<tr>
<td>Ocimene</td>
<td>0.183 ± 0.011</td>
<td>0.265 ± 0.019</td>
<td></td>
</tr>
<tr>
<td>p-Cymene</td>
<td>0.763 ± 0.017</td>
<td>1.225 ± 0.011</td>
<td></td>
</tr>
<tr>
<td>Pulegone</td>
<td>1.176 ± 0.008</td>
<td>1.711 ± 0.017</td>
<td></td>
</tr>
<tr>
<td>Terpinene-4-ol</td>
<td>0.752 ± 0.008</td>
<td>1.646 ± 0.011</td>
<td></td>
</tr>
<tr>
<td>α-Phellandrene</td>
<td>0.595 ± 0.016</td>
<td>1.051 ± 0.013</td>
<td></td>
</tr>
<tr>
<td>α-Pinene</td>
<td>0.116 ± 0.008</td>
<td>0.195 ± 0.010</td>
<td></td>
</tr>
<tr>
<td>α-Terpinene</td>
<td>0.591 ± 0.017</td>
<td>0.858 ± 0.013</td>
<td></td>
</tr>
<tr>
<td>β-Myrcene</td>
<td>0.963 ± 0.030</td>
<td>1.756 ± 0.021</td>
<td></td>
</tr>
<tr>
<td>γ-Terpinene</td>
<td>0.445 ± 0.022</td>
<td>0.682 ± 0.021</td>
<td></td>
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</tbody>
</table>

*CC50 - the compound concentration (mM) required to reduce cell viability to 50%
accordance with results demonstrated in Figure 2, and Table 1, the substance revealed high inhibitory activity both in Marston and Ellman assays. Carvone (6), out of all studied terpenes, exhibited the lowest IC₅₀ value (after 30 min) in the Ellman test. Previously published data showed carvone as a potential AChE inhibitor [23]. Apart from the in vitro studies, molecular docking simulation revealed that carvone (6) was docked in the most important part of AChE active site responsible for correct functioning of the enzyme (Figure 4b). Additionally, this ligand exhibited H-bond interaction with Tyr337. It is noteworthy to note that the ligand, similarly to galantamine, interacts with amino acid residues creating anionic and esteratic subsites, playing key role in the AChE functioning. Considering identification of potential multi-target directed ligands, important is the fact that carvone (6) has been characterized by many other properties such as anticancer, antimicrobial [34-37] or free radical scavenging activity [17]. Additionally, lack of cytotoxicity, towards both BJ and GMK cell lines, confirms relative safety of the terpene. However, there have been some data reported on pharmacological activity of some of these terpenes in the CNS. De Sousa et al. [38], revealed that both (S)-(+-)carvone and (R)-(+-)carvone may exhibit depressant effects. Additionally, it has been stressed that (R)-(+-)carvone potentiates pentobarbital sleeping time. Nogóceke et al. [39] suggests that both (S)-(+-)carvone and (R)-(+-)carvone reveal positive influence on decreasing spontaneous locomotor activity in sleep what may prove their sedative effect.

Acetylcholinesterase inhibitory activity of γ-terpinene (7) and pulegone (8) should also be emphasized. As seen in Table 1, both terpenes are characterized with satisfactory AChE inhibition equaling 19.01% and 24.27%, respectively. Detailed analysis of results obtained for molecular docking revealed favorable docking of the two studied terpenes in AChE active gorge. Each of the ligand is binding to the crucial part of AChE pocket site (Figure 3b). Similarly to aforementioned carvone (6), pulegone (8) exhibited lack of cytotoxicity towards both BJ and GMK cell lines. Slightly higher cytotoxicity, determined as low, in accordance with assumptive standards, was exhibited by γ-terpinene (7). Biochemical activities of pulegone (8), γ-terpinene (7) and essential oils, with high content of these substances, were a basis of several studies [23]. Beside AChE inhibitory activity, the substances were previously found to be potent free radical scavengers [17]. Nevertheless, considering pulegone (8) as potential AChE inhibitor, some additional points should be considered. In the case of pulegone (8), one of the most significant issues is its possible carcinogenic activity. According to NTP Technical Report on the toxicology and carcinogenesis studies of pulegone (8), the presented results explicitly revealed carcinogenic activity of the terpene in mice based on increased incidences of hepatocellular neoplasms. Additionally, according to the Opinion of the Scientific Committee on Food on pulegone and menthofuran [40], the aforementioned terpenes are substances revealing toxicity and procarcinogenic activity. In the case of γ-terpinene (7), there is no evidence of any significant toxicity, nor negative influence on the CNS nor procarcinogenic activity.

Some terpenes have been previously studied for their acetylcholinesterase inhibitory activity, as for example attested by several publications of López et al. [41-43] Houghton et al. discusses other examples of terpenes possessing AChE inhibitory activity [44]. López et al. underlined that particular attention should be paid to carvone (6) and γ-terpinene (7). γ-terpinene (7) is considered to be a competitive AChE inhibitor whereas carvone (6) showed non-competitive inhibition [41]. AChE docking experiments have been also performed for some terpenes, which included: fenchone, γ-terpinene (7), linalool (4), carvone (6), geraniol, anethole, estragole and camphor. These experiments revealed also promising inhibitory activity of these compounds towards AChE. Additionally, López et al. [42] indicated linalool (4) is as strong inhibitor as carvone (6) or γ-terpinene (7). The results presented in Table 1 also indicate satisfactory AChE inhibition of linalool (4). However, taking into account its weaker antioxidant activity, compared to carvone (6), pulegone (8) and γ-terpinene (7), linalool (4) was not considered in detail in our further research. The data presented in this manuscript confirmed previous reports regarding AChE inhibitory activity of selected terpenes. However, it is for the first time that candidates, possessing both antioxidant and AChE inhibitory activity, are indicated for the development of multi-target directed ligands. Additionally, docking experiments predicted interaction of these terpenes with the active sites of the enzyme. Low cytotoxicity of the recommended terpenes was confirmed in the cytotoxicity assays.

As already mentioned all the analyzed terpenoids exerted AChE inhibitory activity in mM level. This relatively high concentration is mostly due to the high amount of enzyme used in the Ellman assay. All of the analyzed terpenes exhibited activity lower than galantamine. This fact can be perceived as a negative feature of these terpenes but it is worth noting, that the main aim of the presented studies is finding a substance active towards multiple factors that might be responsible for the development of Alzheimer’s disease.
According to available literature, oxidative stress, similarly to low level of acetylcholine, is indicated as one of the most important causative factor of Alzheimer’s disease. Considering the issue of differences in activity of galantamine and terpenes, high antioxidant activity of investigated substances must be highlighted. In light of performed promising experiments, terpenes, contrary to galantamine, exhibit ability to scavenge free radicals and may prevent from deleterious effects of oxidative stress. The considered three terpenes revealed satisfactory free radical scavenging activity. The substances are able to scavenge the reactive molecules at percentage level equal 82% for carvone (6), 97% for γ-terpinene (7) and 95% for pulegone (8) after 15 min from reaction initiation [17]. In view of terpenes’ small molecules and their hydrophobic character, the substances should easily become bioavailable when used in in vivo assays. The compounds characterized with most desirable activity will be tested, in the future experiments, in in vivo models.

5 Conclusions

Taking into account the results obtained towards AChE inhibition, several substances among the investigated terpenes exhibited desirable activity, confirming previous reports. Interesting activity of carvone (6), pulegone (8) and γ-terpinene (7) was confirmed by all the applied assays. Additionally, all these compounds were characterized by potent antioxidant activity and low cytotoxicity. It can be concluded that carvone (6), pulegone (8) and γ-terpinene (7) constitute promising candidats for the development of multi-target directed ligands.

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References

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