European Journal of Life Sciences

Volume: 3 • Issue: 2 • August 2024 • ISSN: 2822-5333

ANADOLU UNIVERSITY
Faculty of Pharmacy

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Faculty of Pharmacy

European Journal of Life Sciences

Volume: 3 • Issue: 2 • August 2024

ISSN: 2822-5333

European Journal of Life Sciences

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Publication Type
International peer-reviewed journal

Publication Frequency
Triannually

Language
English

Website
https://dergipark.org.tr/ejls

Publisher
Anadolu University Faculty of Pharmacy

Publisher Address
Anadolu University, Faculty of Pharmacy
Yunus Emre Campus, 26470 Tepebaşı/Eskişehir
Phone: +90 222 335 05 80
E-mail: ejls@anadolu.edu.tr
Web: https://eczacilik.anadolu.edu.tr

Publishing Services
Akdema Informatics, Publishing, and Consultancy Trade LLC
Address: Kızılay Mahallesi, Gazi Mustafa Kemal Bulvarı No: 23/8, 06420 Çankaya/Ankara
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Research Article

Phytochemical quality analysis of commercial preparations containing *Echinacea purpurea*

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https://doi.org/10.55971/EJLS.1505892

Received: 27.06.2024
Accepted: 30.07.2024
Available online: 30.08.2024

ABSTRACT

Echinacea species have been medicinally important plants from the past to the present. *Echinacea* is used in many diseases, especially cold and wound treatment. The root, flower, and leaf parts of the plant, especially the flower, contain medicinally important components such as chlorogenic acid, caffeic acid, echinacoside and cichoric acid. Today, the medicinal use of the *Echinacea* can be achieved with its flowers and herbal teas sold in herbal markets. In addition, *Echinacea* preparations available in pharmacies are used especially for immune boosting and to prevent colds and flu. In the present study, it was aimed to analyze 5 different *Echinacea* preparations by the HPLC method. According to the HPLC results of *Echinacea* preparations in different forms (syrup, capsule, etc.), none of the 4 expected standards could be detected in the 2 samples stated to contain *Echinacea*, while 0.1% caffeic acid was detected in EP-1 and 0.4% cichoric acid, 0.6% chlorogenic acid, 0.2% caffeic acid were detected in EP-3. In the sample purchased directly as a dried herb, all four of the required standards were determined to be following the ranges specified in the European Pharmacopoeia.

Keywords: *Echinacea*, HPLC, echinacoside, caffeic acid, market preparation

1. INTRODUCTION

Echinacea sp. grows and originates in West America, three of them are valuable as medicinal plants and used for a long time. These species are *Echinacea purpurea* L. Moench. (roots and aerial parts), *Echinacea angustifolia* D.C. (roots) and *Echinacea pallida* (Nutt.) Nutt. (roots) [1]. *E. purpurea* is a valuable medicinal plant that belongs to Asteraceae (Compositae) family and is known as 'purple coneflower', 'red sunflower' and 'rudbeckia' [2]. All three species contain polysaccharides, alkamides, flavonoids and caffeoyl conjugates such as cichoric acid, echinacoside and caffeic acid in different concentrations [3]. There are 4 different *Echinacea* monographs in the European Pharmacopoeia: *Echinacea purpurea* root, *Echinacea purpurea* aerial part, *Echinacea angustifolia* root, *Echinacea pallida* root. The materials used in this study are mostly samples containing *E purpurea*. According to European Pharmacopoeia 8.0, *E. purpurea* roots should contain a minimum of 0.5%, aerial parts should contain a minimum of 0.1% caftaric acid and cichoric acid, total [4].

Recently, natural alternatives for prevention from colds and flu have become very popular. There are more than one plant species that may be used for prophylactic treatment of cold, such as *Echinacea* species [5]. Since 1600, Americans traditionally used these species for many reasons such as cough, dyspepsia, sore throat, toothache, tonsillitis and

snake bite [6]. There are lots of beneficial effects of *Echinacea* sp. supported by *in vitro* trials such as immunomodulatory, antianxiety, cytotoxicity, anti-inflammatory, antioxidant, antiviral, antifungal, antiosteoporotic and antimicrobial effects [7-11]. There are recent studies that demonstrates the antiviral effect of *Echinacea purpurea* on SARS-CoV-2 variants [12]. The mechanism of action of *Echinacea* sp. on cold and influenza is in vogue and still unknown. About that, a study demonstrated that endotoxin-free *E. purpurea* extract activates the production of interleukin-6, interleukin-12, tumor necrosis factor and nitric oxide which also means it stimulates the immune system, *in vitro* [13]. Moreover, a meta-analysis of 14 studies supported that *Echinacea* sp. has beneficial effects on increasing the incidence and duration of common cold [3]. Caffeic acid derivatives, polysaccharides, alkamides and glycoproteins are believed to be the responsible for the immunostimulatory effect of *Echinacea* sp [8]. Another meta-analysis investigated the capacity of *Echinacea* sp. on reducing the antibiotic usage by preventing respiratory infections. The study resulted that especially alcoholic extract of the leaves of *Echinacea purpurea* lowered the risk of recurrent infections, complications from respiratory tract infections and the necessity for antibiotic treatment, resulting in a 80% reduction in total antibiotic therapy days. It is a safe opportunity for preventing recurrent infections [14]. A study stated that water is the optimal solvent for extracting a polysaccharide-containing complex (PSC) which may be a responsible compound for its immunostimulant activity [15]. Ethnobotanical usage of *Echinacea* species for cold is reported [16], hence obviously there is missing knowledge about the mechanism of action. Prophylaxis is an important step for influenza, because of that reason, commercial products (supplements) that contain *Echinacea* sp. are on the market and herbalists, recently. They promise to contain a significant amount of *Echinacea* extract or marker compounds for preventing colds, however, the results may not meet the promises for any commercial product. The most important factor that determines the quality of plants is that they meet certain phytochemical standards. The quality of a commercial health product directly affects public health [1].

The aim of this study is to analyze the phytochemical properties of samples from five different commercial products that contain *E. purpurea* in the herbalists and compare the phytochemical profiles with marker compounds by using the High Performance Liquid Chromatography (HPLC) method. Thus, quality control assessment of five different supplements from market and herbalists was studied in Türkiye.

2. MATERIALS AND METHODS

2.1. Material

Market preparations containing *E. purpurea* roots and aerial parts were purchased commercially from different pharmacies (Istanbul, Türkiye). Five different samples, three of which were capsules, one was syrup, and the other was directly dried *Echinacea* root, are coded from EP-1 to EP-5.

2.2. Extraction

100 g of ground *Echinacea* roots were weighed and macerated with ethanol for 3 days. Market preparations in powder and liquid form were directly treated with the same solvent. At the end of three days, the extracts were filtered through Whatman no:1 filter paper and concentrated with a rotary evaporator. Filter papers were soaked with ethanol before the process. The process was repeated three times in total [17].

2.3. HPLC Analyses

The prepared extracts were studied at the concentration of 10 mg/mL and were analyzed by filtering through a 0.22-micron membrane filter after dissolving in ethanol. HPLC analyses were carried out with the UV detector connected to the Agilent 1100 HPLC system. While the C18 column (100 x 4.6mm, 5 µm) was used as the stationary phase, A: Water: Formic acid (100:0.1, *v/v*), B: Acetonitrile was used as the mobile phase. It was studied as a linear gradient flow from 10% B to 78% B concentration between 0-18 minutes. Between 18-21 minutes in the flow, it returned from 90% B to 10% B (initial conditions). The flow rate was set at 1.2 mL/min. Retention times (tR) of caffeic acid, chlorogenic acid, cichoric acid and echinacoside were identified

by matching those of the standard analyzed under the same conditions. The peaks were analyzed at 330 nm and the calibration curve was obtained by working with 5 different standard concentrations (0.1-0.5 mg/mL). The injection volume was set to 5 µL and the column temperature was set to 26°C [18].

3. RESULTS AND DISCUSSION

The amount (*w/w*) of caffeic acid, chlorogenic acid, echinacoside and cichoric acid in the EP-1 sample was calculated by the HPLC method. HPLC chromatograms of caffeic acid, chlorogenic acid, echinacoside, cichoric acid and EP-1 are shown in Figures 1-5. In EP-1 HPLC analysis, it was confirmed that the peak at tR: 6.942 belonged to caffeic acid. Based on the calibration graphs, the amount of caffeic acid in EP-1 was calculated quantitatively as 0.1% (*w/w*).

EP-2 content was also analyzed by the HPLC method. The HPLC chromatogram of EP-2 is shown in Figure 6. However, no peaks belonging to the required standard substances could be detected in the HPLC chromatogram (Figure 6).

The content of EP-3 was studied with the HPLC method. The HPLC chromatogram of EP-3 is shown in Figure 7. In the HPLC analysis performed on the sample, it was confirmed that the peak at tR: 6.72 belonged to caffeic acid, the peak at tR: 4.497 belonged to chlorogenic acid, and the peak at tR: 13.579 belonged to cichoric acid. Based on the standard calibration graphs, the amount of caffeic acid in EP-3 was calculated quantitatively as 0.2% (*w/w*), the amount of chlorogenic acid as 0.6%, and the amount of cichoric acid as 0.4%, respectively.

EP-4 content was analyzed by the HPLC method. The HPLC chromatogram of EP-4 is shown in Figure 8. However, no peaks belonging to the required standard substances could be detected in the HPLC chromatogram (Figure 8).

The HPLC chromatogram of EP-5 is shown in Figure 9. In the HPLC analysis performed on the samples, it was confirmed that the peak at tR: 6.72 belonged to caffeic acid, the peak at tR: 4.800 belonged to chlorogenic acid, the peak at tR: 9.5492 belonged to echinacoside, and the peak at tR: 14.967 belonged to cichoric acid, respectively. Based on the standard calibration charts, the amount of caffeic acid in EP-5 was calculated quantitatively as 0.3% (*w/w*), the amount of chlorogenic acid as 0.6%, the amount of cichoric acid as 0.1% and the amount of echinacoside as 0.2% (Figure 9). A comparative table of contents of all samples is included in Table 1.

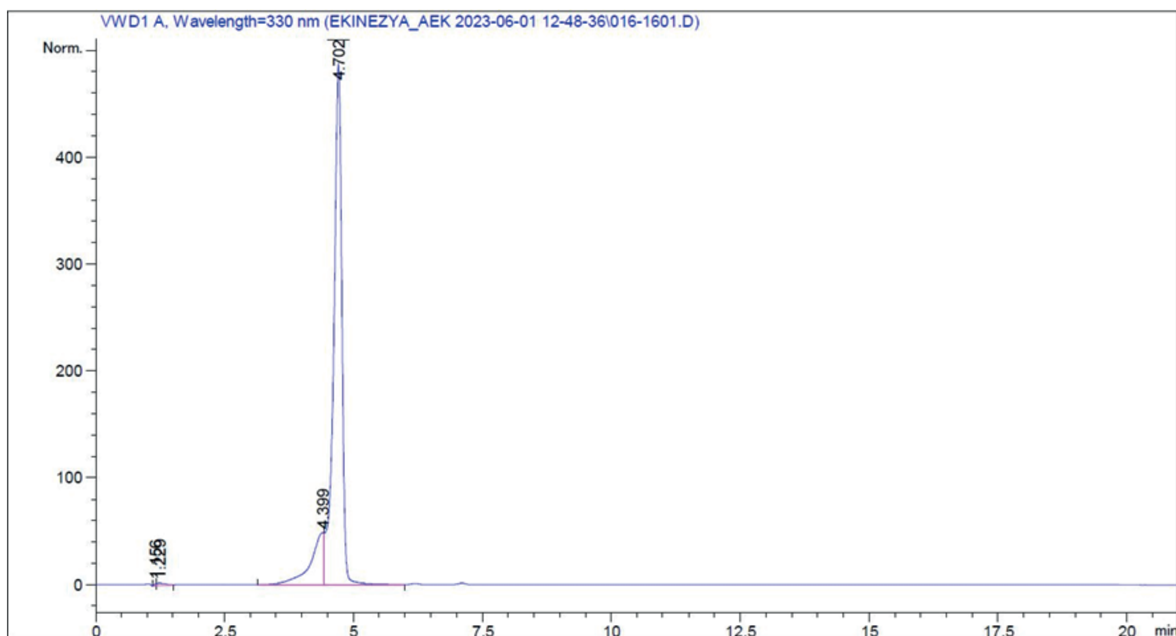

Figure 1. HPLC chromatogram of chlorogenic acid (tR: 4.702)

Table 1. Phytochemical analysis results of market preparations (*w/w*)

	Cichoric acid	Chlorogenic acid	Echinacoside	Caffeic acid
EP-1	-	-	-	%0.1
EP-2	-	-	-	-
EP-3	%0.4	%0.6	-	%0.2
EP-4	-	-	-	-
EP-5	%0.1	%0.6	%0.2	%0.3

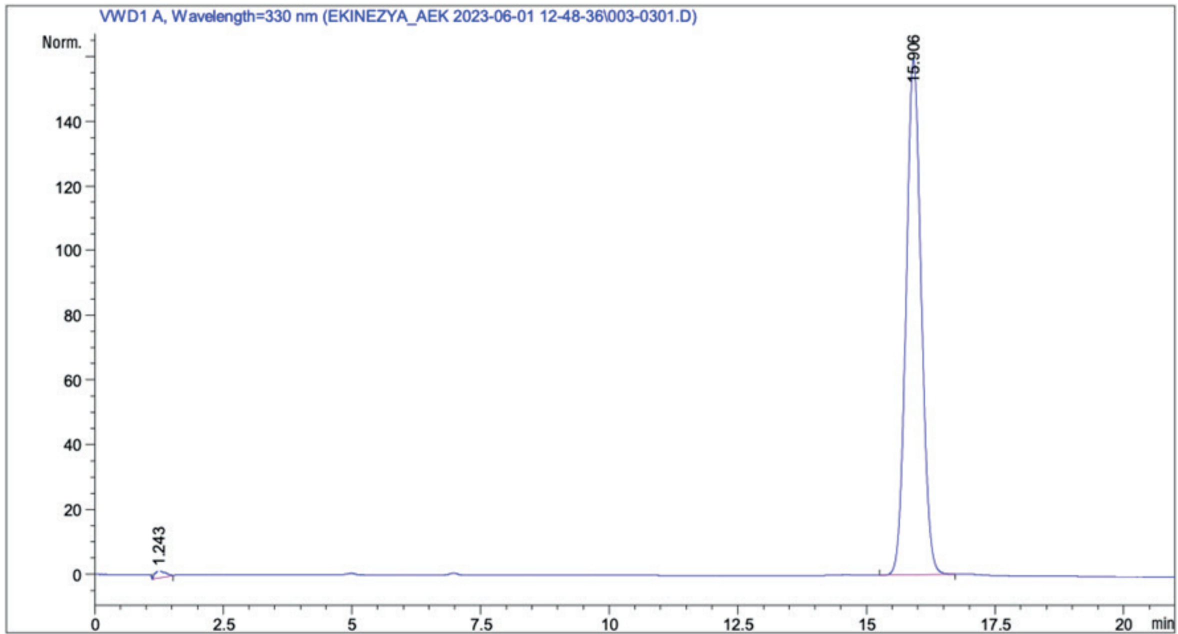

Figure 2. HPLC chromatogram of cichoric acid (tR: 15.906)

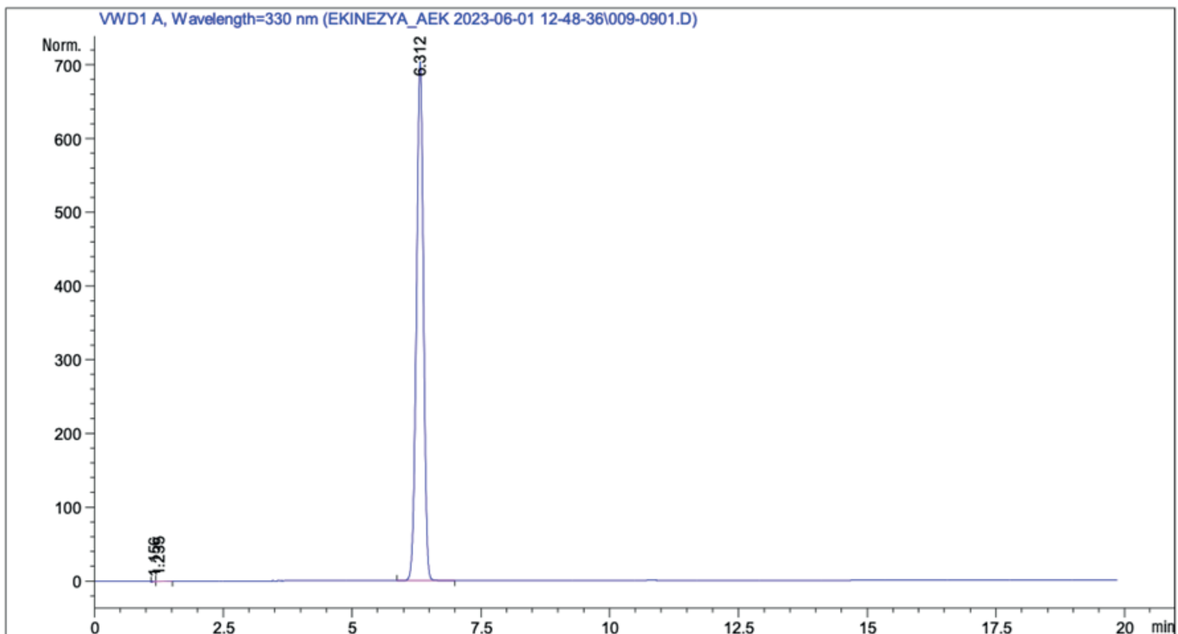

Figure 3. HPLC chromatogram of caffeic acid (tR: 6.312)

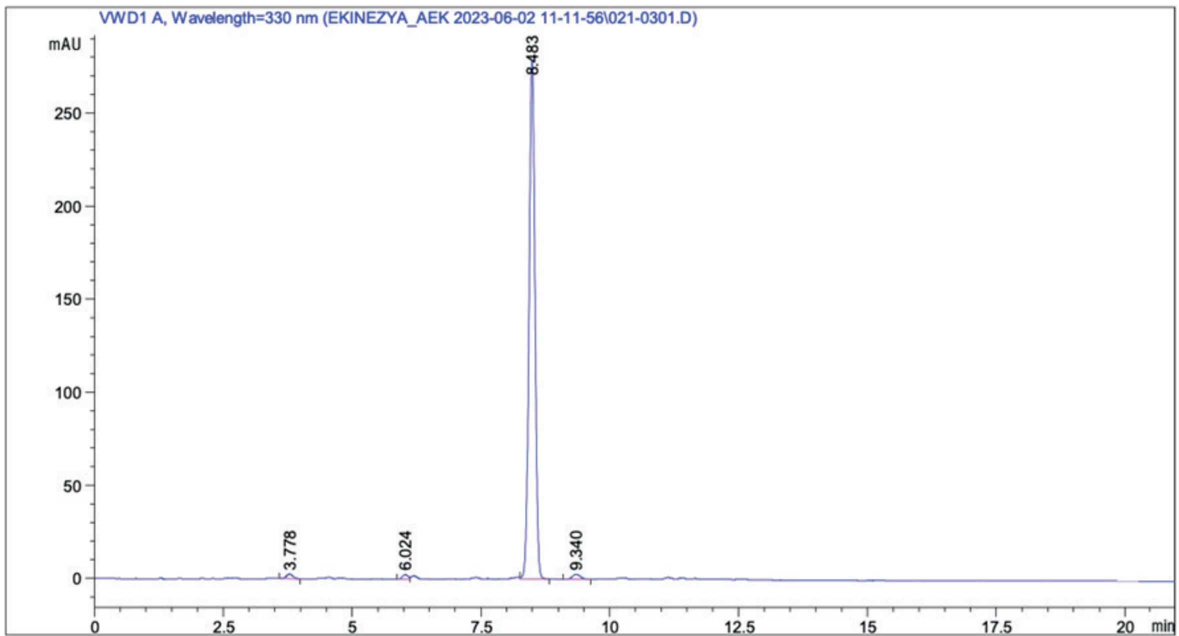

Figure 4. HPLC chromatogram of echinacoside (tR: 8.483)

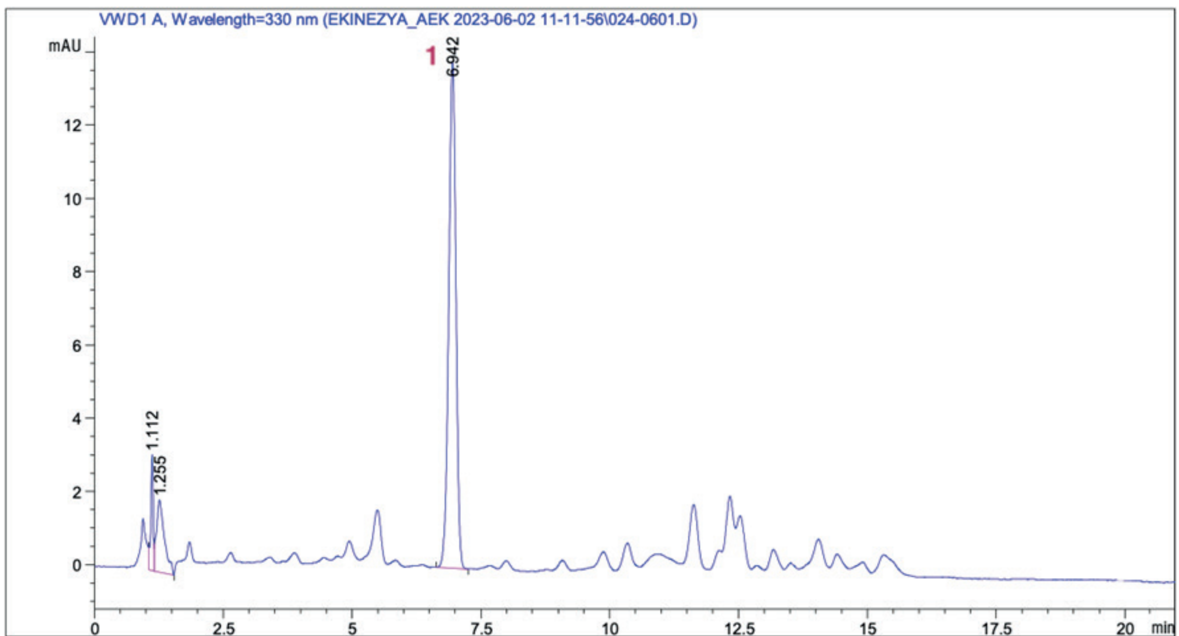

Figure 5. HPLC chromatogram of EP-1 (1. Caffeic acid tR: 6.942)

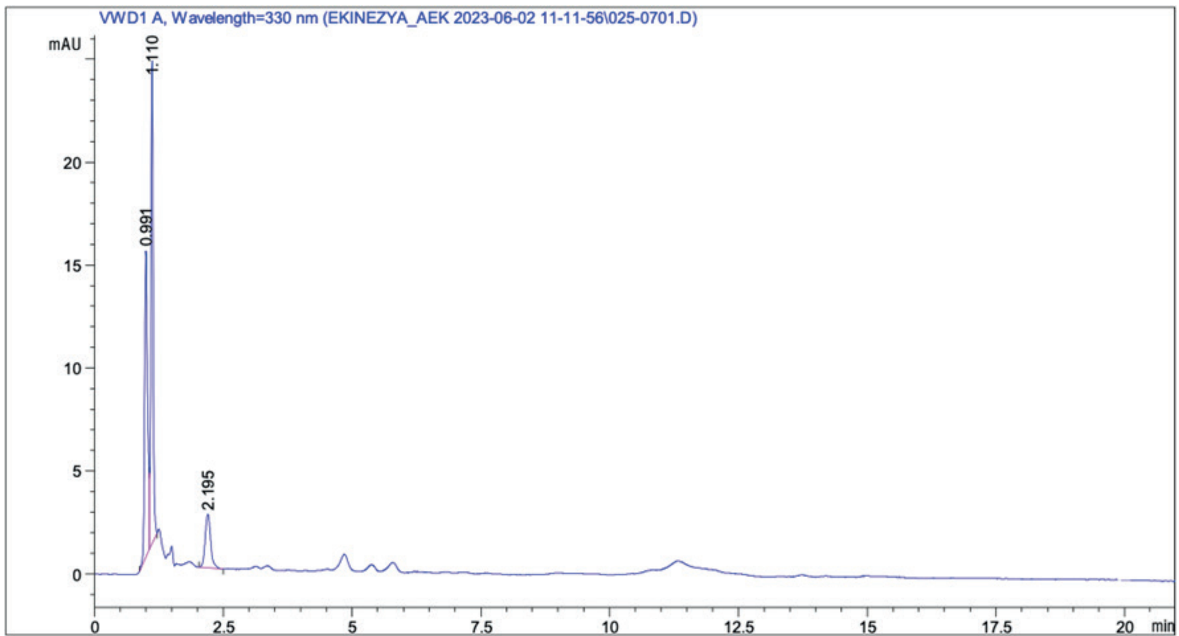

Figure 6. HPLC chromatogram of EP-2

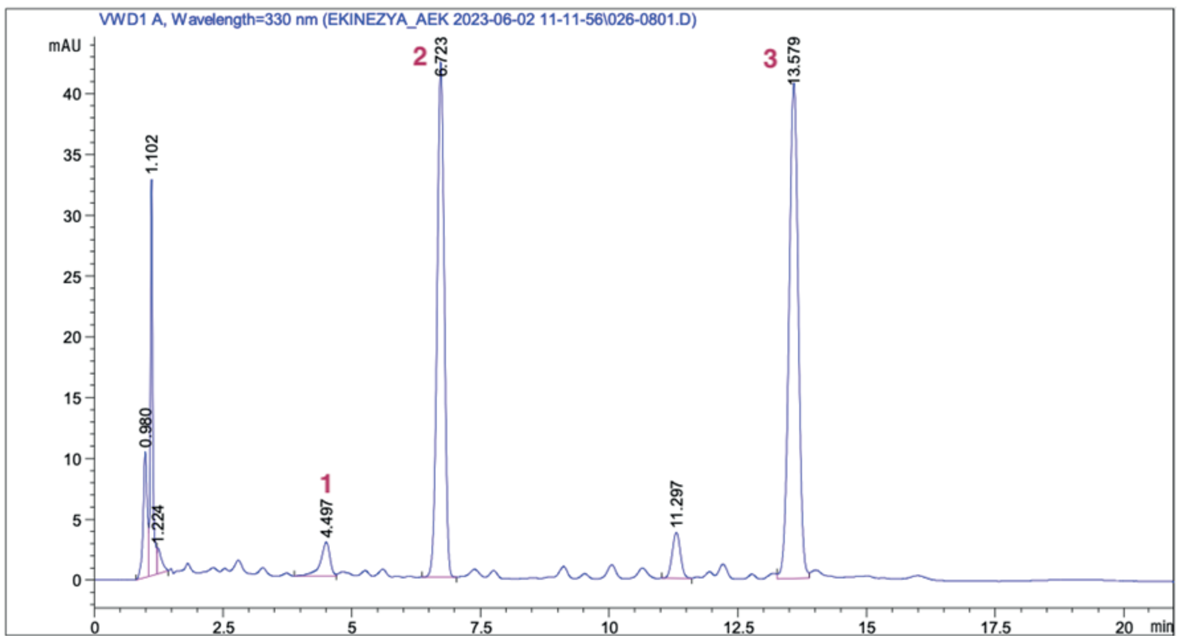

Figure 7. HPLC chromatogram of EP-3 (1. Chlorogenic acid tR: 4.497; 2. Caffeic acid tR: 6.723; 3. Cichoric acid tR: 13.579)

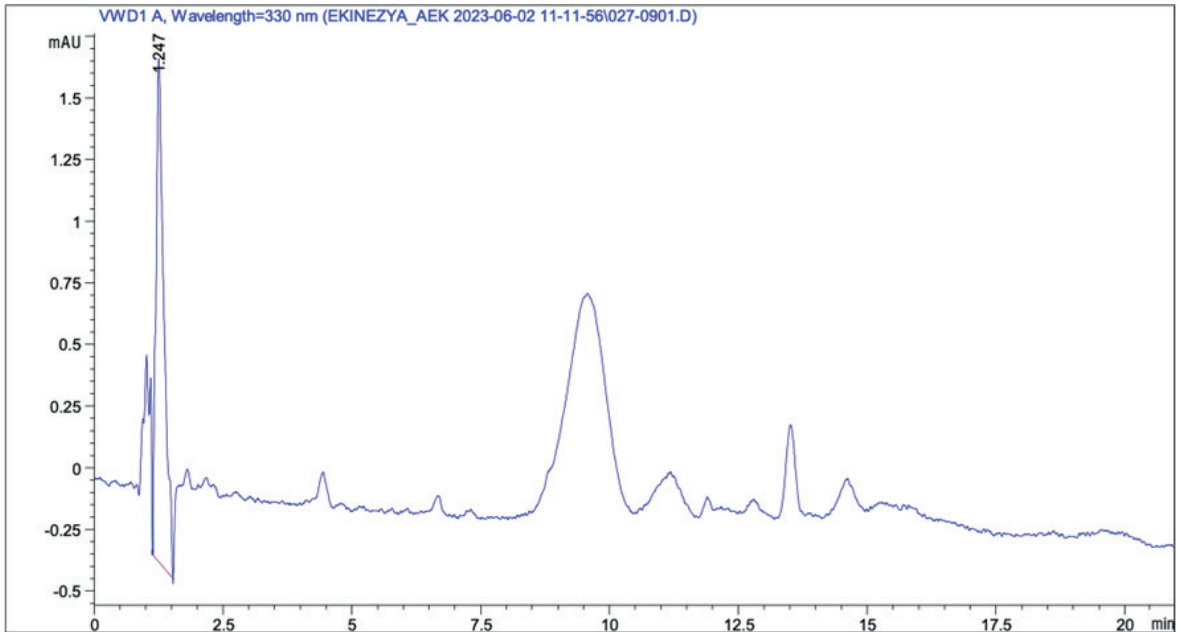

Figure 8. HPLC chromatogram of EP-4

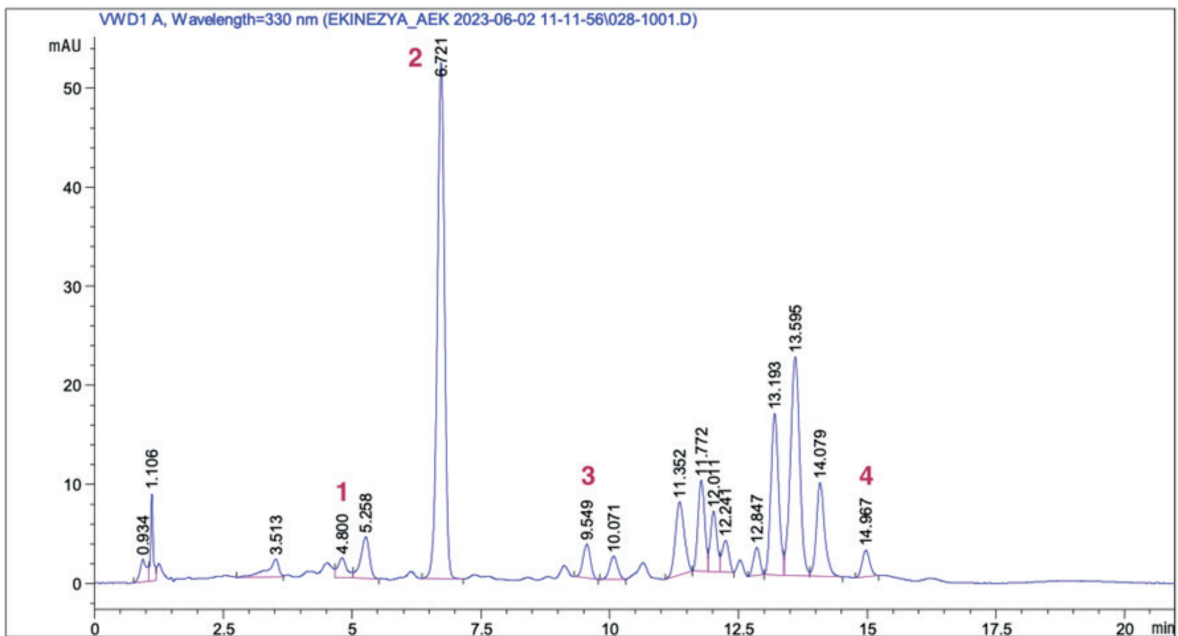

Figure 9. HPLC chromatogram of EP-5 (1. Chlorogenic acid tR: 4.800; 2. Caffeic acid tR: 6.721; 3. Echinacoside tR: 9.549; 4. Cichoric acid tR: 14.967)

According to European Pharmacopoeia, the total amount of caftaric acid and cichoric acid required for dry drugs in the roots of *E. purpurea* is at least 0.5%. It is expected to be at least 0.1% in the aerial parts. At least 0.2% echinacoside is required for *E. angustifolia* and *E. pallida*. The preparation called EP-1 used in the study, in capsule form, contains 265 mg *E. purpurea* (aerial part) powder and 65 mg *Echinacea* root extract. According to this present study, 0.1% caffeic acid was detected in EP-1. The preparation named EP-2 contains 100 mg *E. purpurea* in 2 capsules (recommended daily dose). Additionally,

it contains zinc, vitamin C, beta glucan and rosehip (*Rosa canina* L.). EP-3 preparation contains 150 mg *E. purpurea*. Additionally, it contains beta glucan, vitamin C and zinc in capsule form. EP-4 is in syrup form and contains 300 mg *Echinacea* extract and contains beta glucan, propolis, vitamin C and zinc. EP-5 is the aerial part of dried *Echinacea* sp.

When the phytochemical quality of the market preparations is evaluated according to the HPLC analysis, it was seen that the products of different companies vary greatly in quality and generally do not meet the Pharmacopoeia standards. No information can be found about the growing conditions, specific species and harvest times of *Echinacea* used in production.

Since EP-3 contains *E. purpurea*, it is not expected to contain echinacoside. Other phytochemicals (cichoric acid: 0.4%, chlorogenic acid: 0.6%, caffeic acid: 0.2%) are at a level that can provide the desired therapeutic effect from the preparation.

None of the expected phytochemicals is found in the analyses performed on EP-2 and EP-4. In EP-1, only low concentrations (%0.1) of caffeic acid were detected. These preparations, which must contain cichoric acid and caftaric acid, are not expected to give the promised immunostimulant effect or to provide results in the treatment of colds. This may be due to adulteration, the manufacturer's use of poor-quality herbal materials or inadequate production and storage conditions.

EP-5 is unprocessed, dried *Echinacea* spp. aerial part. It is not known which species it belongs to. Since it contains echinacoside (0.2%), it can be assumed that it is a medicinal species other than *E. purpurea*. It has a better profile than other preparations in terms of the phytochemicals it contains. This may be because the plant is not exposed to errors that may occur in production conditions due to the non-processing of the plant. Additionally, some preparations (EP-2,3,4) were found to contain different components. The possibility that the presence of these components may affect the results should be considered.

In 1998, a study was conducted to distinguish caffeic acid derivatives and lipophilic compounds in the tincture of the *Echinacea* species. According to the results of the previous study, it was found that *E. angustifolia* and *E. pallida* roots contain 0.3-1.7% echinacoside. It was also determined that *E. purpurea* contains cichoric acid and caftaric acid while it does not contain echinacoside. Cichoric acid is mostly found in all flowers of *Echinacea* species (1.2-3.1%) and the root of *E. purpurea* (0.6-2.1%) [19].

In a previous study, phytochemical analysis of *Echinacea*-containing products sold in Denmark was carried out. In this study, the root, leaf and flower parts of *E. purpurea* were analyzed by HPLC. According to the study results, the amount of cichoric acid was found as 24 mg/g in the roots, 42.4 mg/g in the leaves and 26.7 mg/g in the flowers, respectively. In studies conducted on the amount of alkamide, it was found as 1.20 mg/g in the root and 0.81 mg/g in the flower parts. In a study conducted on 13 different preparations (in tincture, capsule and tablet forms), it was explained that the amount of cichoric acid and alkamide was in a very variable range and even could not be found in some preparations [18].

The presence and amount of phytochemicals in plants may depend on many factors. Examples of these factors are seasonal conditions, soil type or the harvesting time of the plant. According to a previous study, the amount of phenolic acid in *E. purpurea* increases starting from spring until July. A limited increase may be seen starting from autumn. Huge differences may be encountered depending on genetic and climatic changes [20]. Factors affecting the quality include air conditions during the growing season such as rainfall, nutrition, attack by insects or microorganisms and handling of plant material during harvest and storage. Conditions during the processing of plant material are very important. Since cichoric acid is sensitive to heat, ultraviolet rays, enzymatic and oxidative degradation may occur during processing [18].

4. CONCLUSION

In conclusion, herbal products are used unconsciously without the control of a physician or pharmacist. This situation can cause major health and financial problems. As seen in the study, the contents of

herbal preparations used as food supplements for prophylactic or therapeutic purposes do not meet the desired conditions. For more reliable and healthier herbal supplements, standardization should be ensured for both raw materials and production conditions. Quality control and inspection of products must be ensured. It is important to support the results obtained in this study with a wider range of further studies in order to determine the safety levels of food supplements.

Acknowledgements

Part of the manuscript will be presented at the International Congress on Natural Products Research (ICNPR), 13-17 July 2024, Krakow, Poland.

Ethical approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Author contribution

Conceptualization, A.E.K. and R.B.; Methodology, A.E.K., R.B. and D.K.; Software, A.E.K.; Validation, A.E.K., R.B. and D.Y.; Formal analysis, A.E.K. and R.B.; Investigation, R.B.; Resources, A.E.K.; Data curation, R.B.; Writing—original draft preparation, A.E.K. and R.B.; Writing—review and editing, A.E.K. and D.K.; Visualization, A.E.K.; Supervision, A.E.K.. All authors have read and agreed to the published version of the manuscript.

Source of funding

This research received no grant from any funding agency/sector.

Conflict of interest

The authors declare that there is no conflict of interest.

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European Journal of Life Sciences 2024; 3: 55-65

Research Article

Investigation of dual AChE/MAO inhibitory activities of new morpholine and piperazine structured compounds

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https://doi.org/10.55971/EJLS.1497639

Received: 07.06.2024
Accepted: 01.08.2024
Available online: 30.08.2024

ABSTRACT

In this study, a series of new compounds containing piperazine and morpholine rings were synthesized. Characterization studies of the obtained compounds were carried out with the help of HRMS, ^{1}H-NMR and ^{13}C-NMR spectroscopic methods. Acetylcholinesterase (AChE) / Monoamine oxidase B (MAO-B) inhibitory potentials of the compounds were investigated using *in silico* and *in vitro* methods. Compound **3a** was the compound with the highest inhibitory potential against AChE and MAO-B enzymes, with IC_{50}=0.065±0.002 µM and IC_{50}=0.072±0.003 µM values, respectively. Compounds **3a** and **3b** interacted with crucial amino acid residues of the hMAO-B (PDB ID: 2V5Z) and AChE (PDB ID: 4EY7) enzymes in the docking studies. Compounds **3a** and **3b** had the highest affinity for the AChE and MAO-B enzymes.

Keywords: AChE, MAO-B, Piperazine, Morpholine, Docking

1. INTRODUCTION

One of the most serious health issues is Alzheimer's disease (AD). Today, it is one of the leading causes of dementia and directly affects the lives of many people. For this reason, radical and updated treatments are needed for the treatment of AD. There are currently different treatment approaches for AD [1–4].

FDA-approved Acetylcholinesterase (AChE) inhibitors such as donepezil and tacrine are actively used in the treatment of AD. In addition, Monoamine oxidase B (MAO-B) inhibitors are known to degrade reactive oxygen species (ROS) and hydrogen peroxide levels. Compounds that have more effects than only inhibiting the AChE, butyrylcholinesterase (BChE) or MAO-B enzyme have been the subject of numerous investigations [5–9]. In the healthy human brain, AChE activity suppresses BChE activity. The most important feature that distinguishes BChE from AChE is its kinetic responses to ACh concentrations. At low ACh concentrations, BChE is less effective in AChE hydrolysis, but when high ACh concentrations inhibit AChE, BChE begins to show more activity [10]. Numerous benefits can be obtained by using a single chemical to inhibit multiple enzymes, according to studies. Achieving the ideal acetylcholine level, preventing the formation of β amyloid plaque, and other similar activities make

compounds that simultaneously inhibit MAO-B and ChE enzymes important in the search for new compounds to treat neurodegenerative diseases like AD [11–16]. These compounds are also expected to have a neuroprotective effect.

Morpholine and piperazine are 6-membered ring systems containing heteroatoms. It is known that both rings play an active role in both AChE inhibition and MAO-B inhibition. Because of its non-planar, flexible shape, piperazine forms hydrogen bonds with target enzymes. The structure of many pharmacologically active compounds from various indication groups includes piperazine. Since piperazine's hydrophobic nature helps the structure it is a part of across the blood-brain barrier, it is widely employed in studies on the treatment of AD, Parkinson's disease and other neurodegenerative illnesses [12,14,17,18].

Many AChE/MAO-B dual enzyme inhibitors developed today have heterocyclic rings in their structure. And in this direction, five new compounds containing piperazine and morpholine rings were synthesized in this study. The synthesized compounds were subjected to characterization tests. Then, *in silico* and *in vitro* studies of obtained compounds were carried out.

2. MATERIALS AND METHODS

2.1. Chemistry

Every reagent that was acquired from a commercial provider was utilized without any additional purification. The melting points of the compounds were determined with a device (MP90, Mettler-Toledo, OH, USA). The results were given without correction. NMR (nuclear magnetic resonance) spectroscopy was recorded on ^{1}H-NMR Bruker DPX 300 FT-NMR spectrometer; ^{13}C-NMR, Bruker DPX 75 MHz spectrometer (Bruker Bioscience, Billerica, MA, USA). Mass spectra were recorded on a LCMS-IT-TOF (Shimadzu, Kyoto, Japan) using ESI.

2.1.1. Synthesis of 2-chloro-N-(4-morpholinophenyl)acetamide (1)

First, 4-morpholinoaniline (1.78 g, 0.010 mol) was dissolved in 20 mL dimethylformamide (DMF). Then, triethylamine (TEA) (1.02 g, 0.010 mol) was added to the solution and placed in an ice bath. Finally, chloroacetyl chloride (1.12 g, 0.010 mol) was added dropwise to the mixture. After 1 hour of mixing, the precipitated product was filtered and separated from the medium. The obtained product was crystallized from ethanol.

2.1.2. Synthesis of sodium 4-methylpiperazine-1-carbodithioate derivatives (2a-e)

Piperazine derivatives (0.005 mol) and NaOH (0.20 g, 0.005 mol) were dissolved in absolute ethanol. Then, carbon disulfide (0.38 g, 0.005 mol) was added dropwise to the solution placed in an ice bath. After two hours of mixing, the precipitated product was filtered.

2.1.3. Synthesis of target compounds (3a-e)

In acetone, 2-chloro-*N*-(4-morpholinophenyl) acetamide (**1**) (0.38 g, 0.0015 mol) and sodium 4-methylpiperazine-1-carbodithioate derivatives (**2a-e**) (0.0015 mol) were mixed for four hours. Once the reaction was finished, acetone was removed with less pressure. After the precipitated product was dried, it was rinsed with water to remove any remaining salt and recrystallized from EtOH.

*2-((4-Morpholinophenyl)amino)-2-oxoethyl 4-methylpiperazine-1-carbodithioate (**3a**)*

Yield: 81%, M.p.: 166.2-166.6°C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 2.33 (3H, s), 2.59 (4H, brs), 3.01-3.04 (4H, m), 3.70-3.73 (4H, m), 4.01 (2H, br.s.), 4.21-4.26 (4H, m), 6.88 (2H, d, *J*= 9.08 Hz), 7.43 (2H, d, *J*= 9.03 Hz), 10.10 (1H, s). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 41.9, 45.0, 49.3, 54.0, 66.6, 115.9, 120.6, 131.7, 147.7, 164.9, 195.4. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{18}H_{26}N_4O_2S_2$: 395.1570; found 395.1560.

*2-((4-Morpholinophenyl)amino)-2-oxoethyl 4-ethylpiperazine-1-carbodithioate (**3b**)*

Yield: 78%, M.p.: 160.0-160.3°C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.02 (3H, t, *J*=7.17 Hz), 2.34-2.41 (2H, m), 2.47 (4H, brs), 3.01-3.04 (4H, m), 3.70-3.73 (4H, m), 3.94 (2H, brs), 4.20 (4H, s), 6.88 (2H, d, *J*= 9.09 Hz), 7.43 (2H, d, *J*= 9.04 Hz), 10.07 (1H, s). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 12.3, 41.8, 49.3, 50.1, 51.5, 52.2, 66.6, 115.9, 120.6, 131.8, 147.7, 165.0, 194.9. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{19}H_{28}N_4O_2S_2$: 409.1726; found 409.1723.

*2-((4-Morpholinophenyl)amino)-2-oxoethyl 4-(4-fluorophenyl)piperazine-1-carbodithioate (**3c**)*

Yield: 79%, M.p.: 187.9-188.3°C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 3.01-3.04 (4H, m), 3.23 (4H, brs), 3.70-3.73 (4H, m), 4.10 (2H, brs), 4.23 (2H, s), 4.35 (2H, brs), 6.89 (2H, d, *J*= 8.98 Hz), 6.94-7.01 (2H, m), 7.04-7.11 (2H, m), 7.44 (2H, d, *J*= 8.93 Hz), 10.09 (1H, s). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 41.8, 49.0, 49.2, 49.3, 49.9, 51.1, 53.2, 66.6, 115.8, 115.9, 116.1, 117.9, 118.0, 120.6, 131.7, 147.4, 147.8, 155.2, 158.3, 165.0, 195.2. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{23}H_{27}N_4O_2FS_2$: 475.1632; found 475.1636.

*2-((4-Morpholinophenyl)amino)-2-oxoethyl 4-(4-(trifluoromethyl)phenyl)piperazine-1-carbodithioate (**3d**)*

Yield: 77%, M.p.: 188.9-190.7°C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 3.01-3.04 (4H, m), 3.51 (4H, br.s.), 3.70-3.73 (4H, m), 4.13 (2H, brs), 4.24 (2H, s), 4.35 (2H, brs), 6.89 (2H, d, *J*= 8.94 Hz), 7.03 (2H, d, *J*= 8.77 Hz), 7.44 (2H, d, *J*= 8.67 Hz), 7.54 (2H, d, *J*= 8.76 Hz), 10.10 (1H, s). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 41.7, 46.2, 49.3, 50.8, 66.6, 114.1, 115.9, 120.6, 126.7, 131.7, 147.8, 152.6, 165.0, 195.2. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{24}H_{27}N_4O_4F_3S_2$: 525.1600; found 525.1607.

*2-((4-Morpholinophenyl)amino)-2-oxoethyl 4-(4-nitrophenyl)piperazine-1-carbodithioate (**3e**)*

Yield: 79%, M.p.: 104.3-105.3°C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 3.01-3.04 (4H, m), 3.51 (4H, brs), 3.70-3.73 (8H, m), 4.15 (2H, brs), 4.25 (2H, s), 4.35 (2H, brs), 6.89 (2H, d, *J*=8.94 Hz), 6.94 (2H, d, *J*=9.44 Hz), 7.44 (2H, d, *J*=8.85 Hz), 8.10 (2H, d, *J*=9.23 Hz), 10.10 (1H, s). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 41.7, 45.1, 49.3, 50.5, 66.6, 112.3, 115.9, 120.6, 126.3, 131.7, 137.3, 147.8, 154.2, 164.9, 195.2. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{23}H_{27}N_5O_4S_2$: 502.1577; found 502.1572.

2.2. MAO Enzymes Inhibition Assay

Using the available fluorometric method, the *in vitro* MAO inhibition test was carried out and the percentages and IC_{50} values of the compounds obtained were computed in accordance with the previously published research group description [19–22].

2.3. Cholinesterase Enzymes Inhibition Assay

In vitro inhibitory potencies of compounds **3a**-**3e** against to AChE and BChE were investigated as previously published [18,23–28].

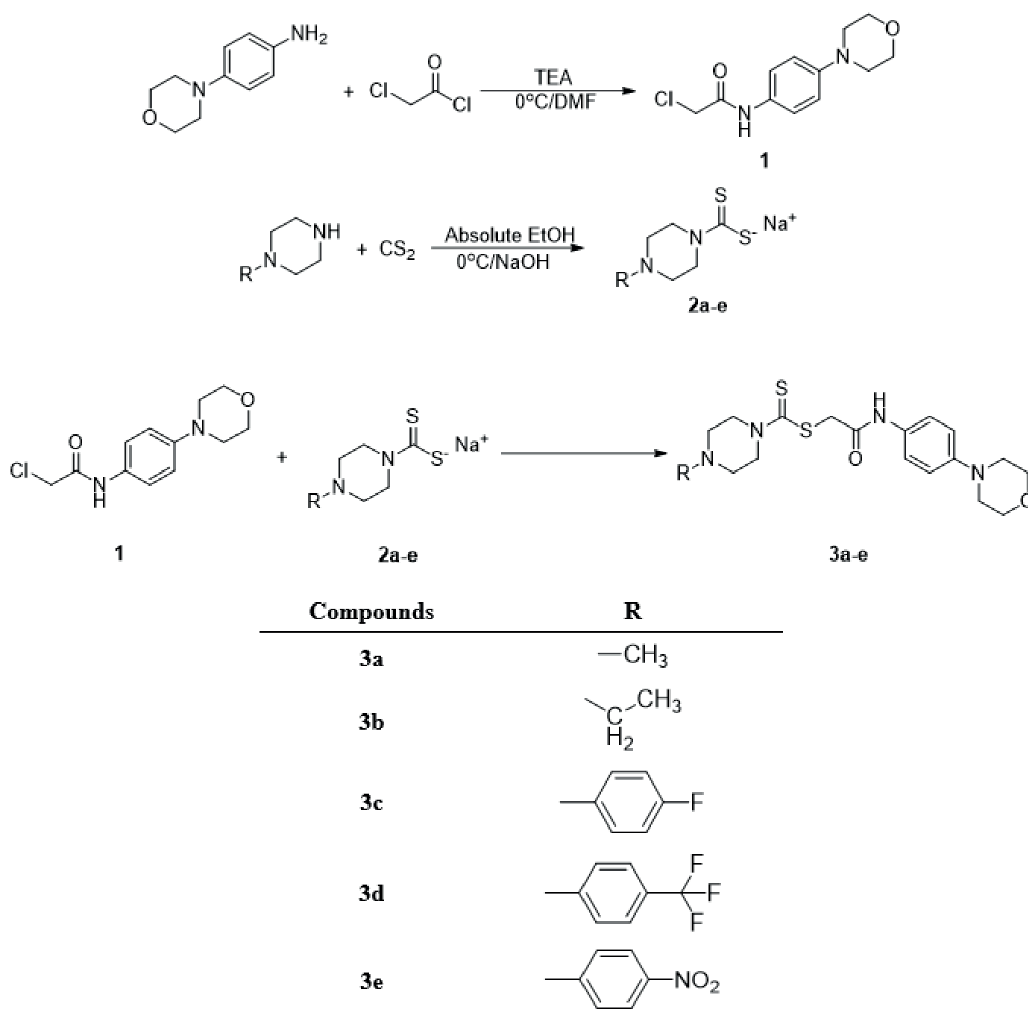
2.4. Molecular Docking Study

Molecular docking investigations were carried out as previously published [9,19,27]. Similar programs were used during the studies [28–31].

3. RESULTS AND DISCUSSION

3.1. Chemistry

As depicted in Scheme 1, the compounds **3a-3e** were synthesized. First, 2-chloro-*N*-(4-morpholinophenyl) acetamide (**1**) was obtained by acetylation of 4-morpholinoaniline. Then, dithiocarbamate salts (**2a-2e**) were obtained from piperazine derivatives with the help of carbon disulfide and NaOH. The target compounds (**3a-3e**) were obtained as a result of the reaction of 2-chloro-*N*-(4-morpholinophenyl) acetamide (**1**) and sodium 4-methylpiperazine-1-carbodithioate derivatives (**2a-2e**).



Scheme 1. Synthesis pathway for obtained compounds **(3a-3e)**

Table 1. IC_{50} Values of synthesized compounds, moclobemide and selegiline against MAO enzymes

Compound	MAO-A IC_{50} (µM)	MAO-B IC_{50} (µM)
3a	0.209±0.009	0.072±0.003
3b	0.371±0.017	0.109±0.004
3c	>100	0.167±0.007
3d	>1000	>100
3e	>100	0.212±0.006
Moclobemide	6.0613±0.2625	-
Selegiline	-	0.0374±0.0016

3.2. MAO Enzymes Inhibition Assay

MAO enzyme inhibition test results are presented in Table 1. In order to compare the enzyme inhibition potential of the compounds; moclobemide was chosen as the reference inhibitor molecule for the MAO-A enzyme and selegiline was chosen as the reference inhibitor molecule for the MAO-B enzyme. The compounds show selectivity towards the MAO-B enzyme. Compounds **3a** and **3b** were the compounds with the closest inhibitory potential to selegiline with their IC_{50} values (**3a** IC_{50}=0.072±0.003 µM, **3b** IC_{50}=0.109±0.004 µM) against the MAO-B enzyme.

3.3. Cholinesterase Enzymes Inhibition Assay

Cholinesterase enzyme inhibition test results are presented in Table 2. In order to compare the enzyme inhibition potential of the compounds; donepezil was chosen as the reference inhibitor molecule for the AChE enzyme and tacrine was chosen as the reference inhibitor molecule for the BChE enzyme.

Table 2. IC_{50} Values of synthesized compounds, donepezil and tacrine against AChE/BChE enzymes

Compound	AChE IC_{50} (µM)	BChE IC_{50} (µM)
3a	0.065±0.002	>1000
3b	0.084±0.003	>1000
3c	0.139±0.006	>1000
3d	0.285±0.013	>1000
3e	0.194±0.008	>1000
Donepezil	0.0201±0.0014	-
Tacrine	-	0.0064±0.0002

The compounds show selectivity towards the AChE enzyme. Compounds **3a** and **3b** were the compounds with the closest inhibitory potential to donepezil with their IC_{50} values (**3a** IC_{50}=0.065±0.002 µM, **3b** IC_{50}=0.084±0.003 µM) against the AChE enzyme.

3.4. Molecular Docking Study

The 2D and 3D binding model of compound **3a** with AChE enzyme (PDB ID:4EY7) is presented in Figure 1 and Figure 2, respectively. When the relevant models are examined, it is seen that compound **3a** has pi-pi interactions with Tyr337, His447 and H bonds with Tyr124. Figure 3 and Figure 4 show the 2D and 3D binding models of compound **3b** with the AChE enzyme (PDB ID:4EY7), respectively. Compound **3b** has a salt bridge with Asp74 and pi-pi interactions with Trp286 when the relevant models are looked at. As a result of these observations, it appears that both compounds interact with the catalytic active site of the AChE. While this interaction is provided through the phenyl ring in compound **3a**, it is provided through the piperazine ring in compound **3b**. In addition, the phenyl ring of compound **3b** interacted with the peripheral anionic region of the AChE, just like donepezil.

The 2D and 3D binding model of compound **3a** with hMAO-B enzyme (PDB ID:2V5Z) is presented in Figure 5 and Figure 6, respectively. When the relevant models are examined, it is seen that compound **3a** has pi-cation interactions with Tyr435. The 2D and 3D binding model of compound **3b** with hMAO-B enzyme (PDB ID:2V5Z) is presented in Figure 7 and Figure 8, respectively. When the relevant models are examined, it is seen that compound **3b** has pi-pi interactions with Tyr326. These interactions are provided by the piperazine ring in compound **3a** and the phenyl ring in compound **3b**.

4. CONCLUSION

Within the scope of this study, five piperazine/morpholine derivative compounds were designed and synthesized. Then, characterization studies of the obtained compounds were carried out. The biological activities of the obtained compounds were investigated by *in silico* and *in vitro* methods. The results of *in silico* and *in vitro* studies are in agreement with each other. In the docking studies, compound **3a** and compound **3b** showed interactions against AChE enzyme (PDB ID: 4EY7) and hMAO-B enzyme (PDB ID: 2V5Z) crystals. In *in vitro* activity studies, compound **3a** and compound **3b** have the highest affinity for AChE and MAO-B. When the molecular docking results were examined, interactions were observed with amino acids Asp74, Tyr124, Trp286, Tyr337 and His447, which are known to positively affect AChE activity. Among these interactions, Trp286 can be identified as the key interaction for AChE activity. A similar interaction is observed between the reference drug donepezil and Trp286 [32,33]. It is known that amino acids Tyr435 and Tyr326 are vital in the catalytic activity and selectivity against the MAO-B enzyme [34,35]. It is pleasing in this respect that compound **3a** interacts with Tyr435 and compound **3b** interacts with Tyr326. When the results of *in vitro* and *in silico* activity studies were evaluated, the compounds with the highest inhibitory potential against MAO-B enzyme and AChE enzyme were compound **3a** and compound **3b**. As a result, five new compounds were successfully synthesized, characterization studies were carried out and activity studies were started. *In silico* and *in vitro* activity studies were also successfully completed. And it is seen that all the studies were carried out in harmony and were successfully concluded. In the light of these results, it was observed that piperazine/morpholine derivatives could be potential dual AChE/MAO-B enzyme inhibitors.

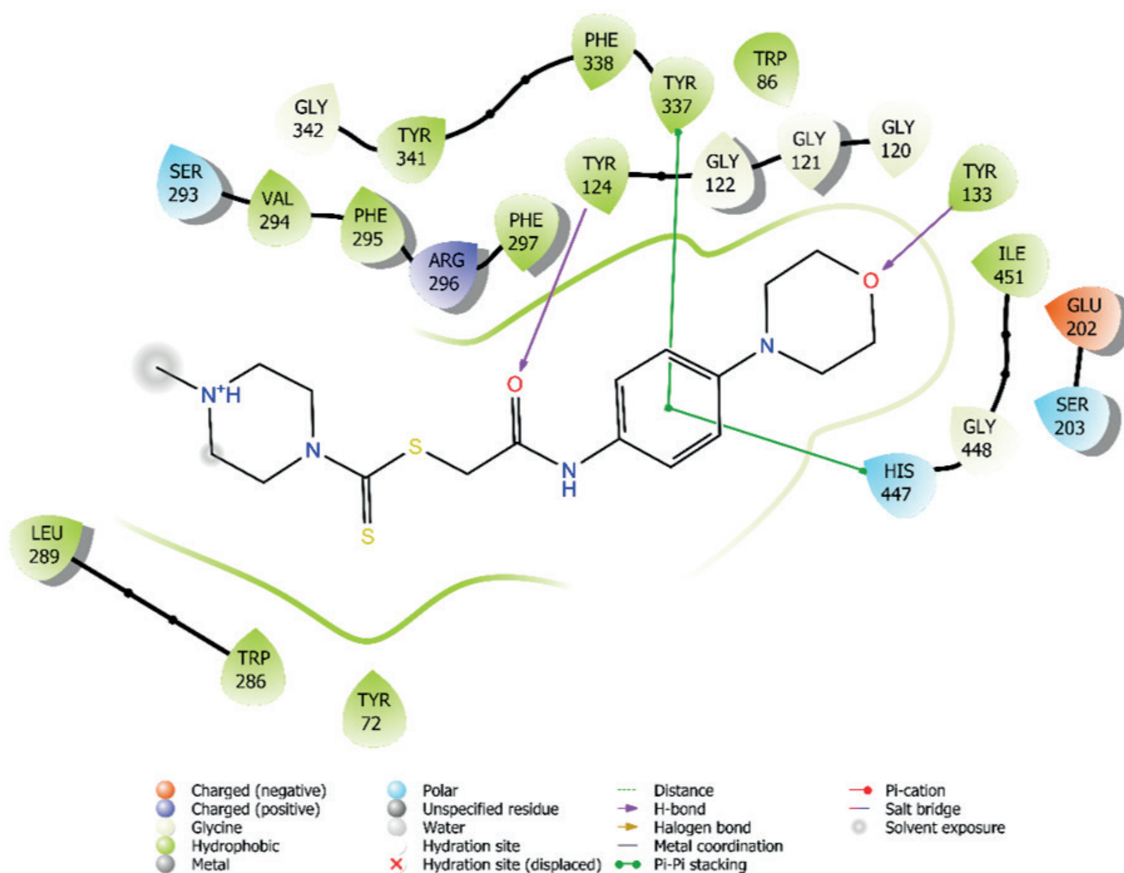


Figure 1. 2D pose of compound **3a** with AChE enzyme (PDB ID: 4EY7)

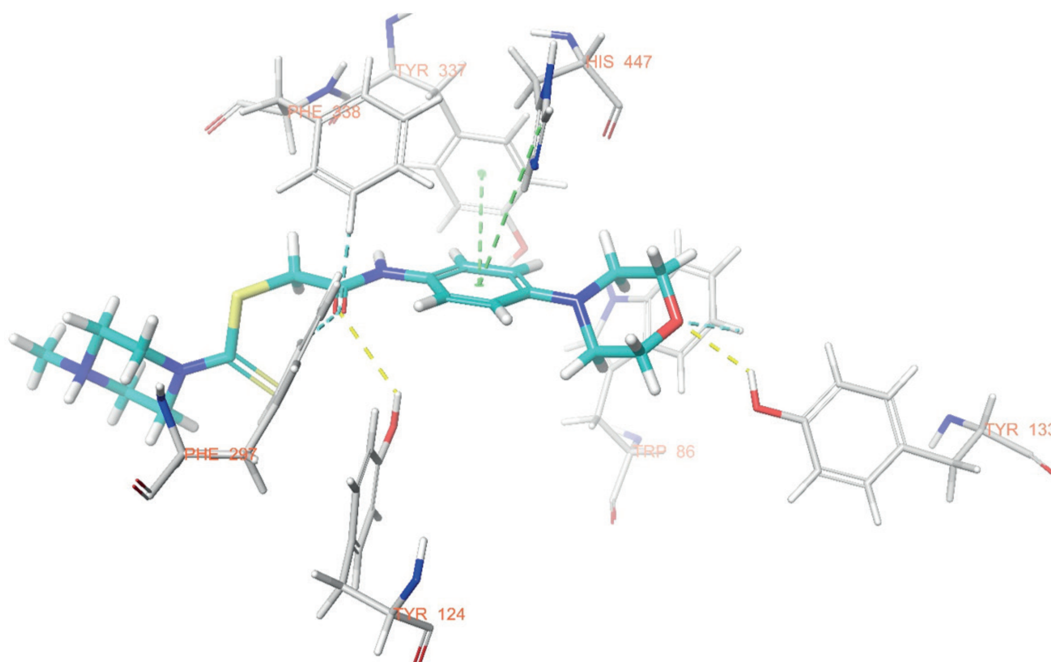


Figure 2. 3D pose of compound **3a** with AChE enzyme (PDB ID: 4EY7)

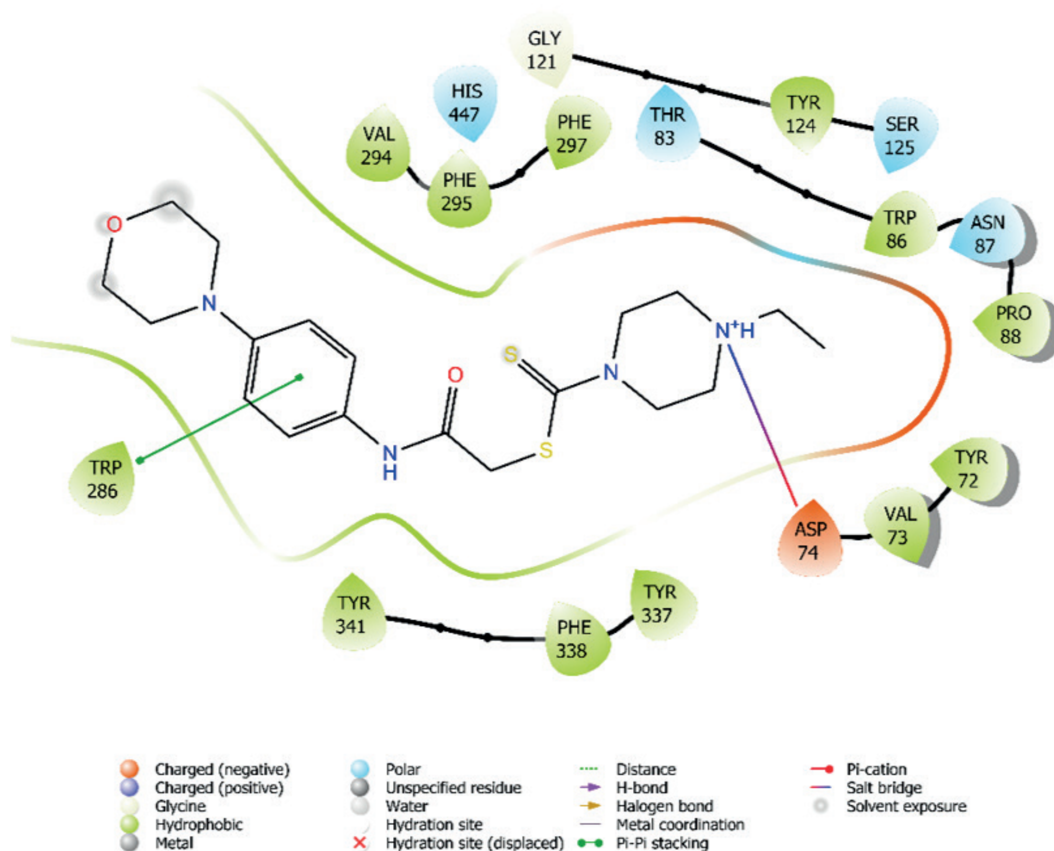


Figure 3. 2D pose of compound **3b** with AChE enzyme (PDB ID: 4EY7)

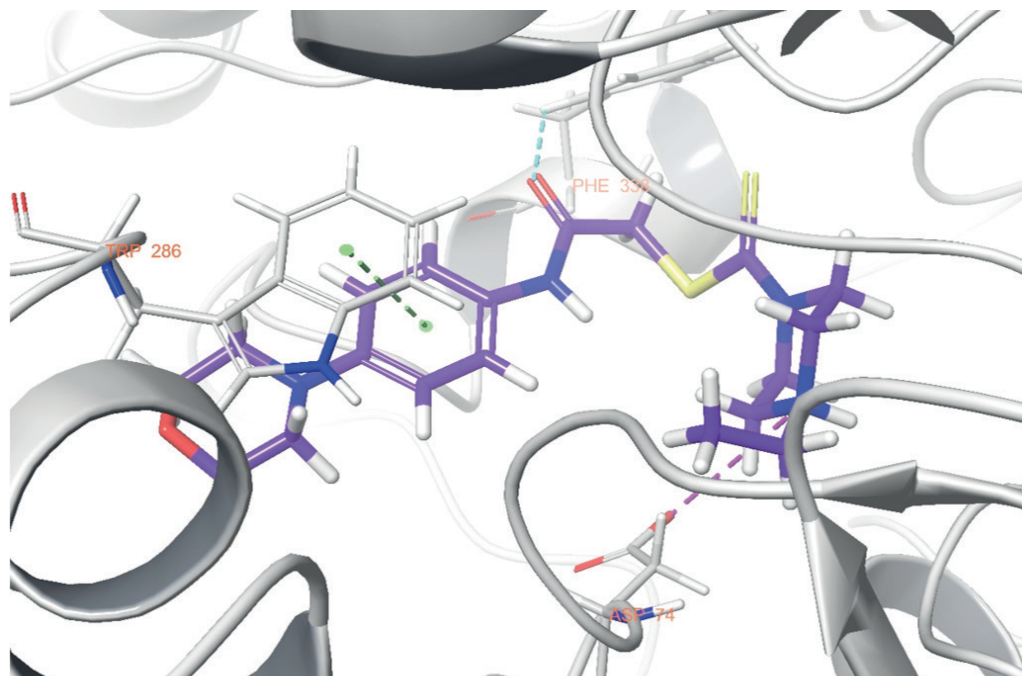


Figure 4. 3D pose of compound **3b** with AChE enzyme (PDB ID: 4EY7)

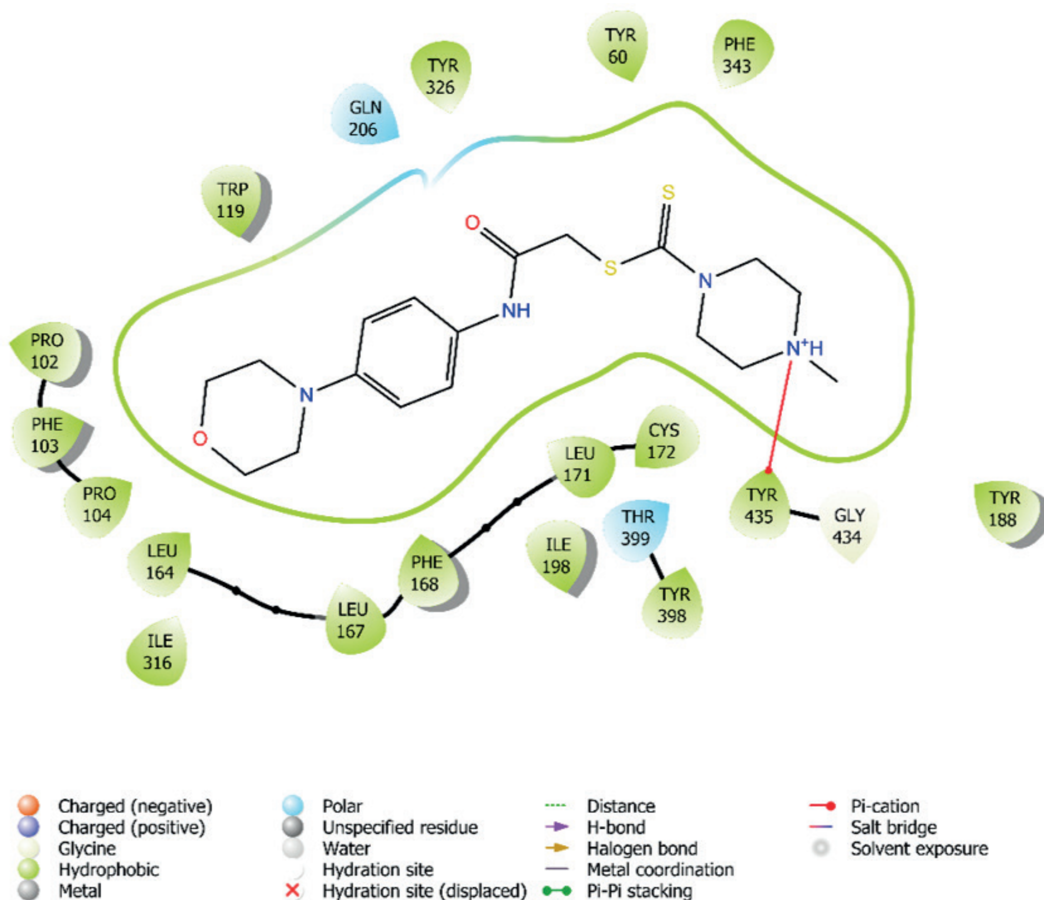


Figure 5. 2D pose of compound **3a** with hMAO-B enzyme (PDB ID: 2V5Z)

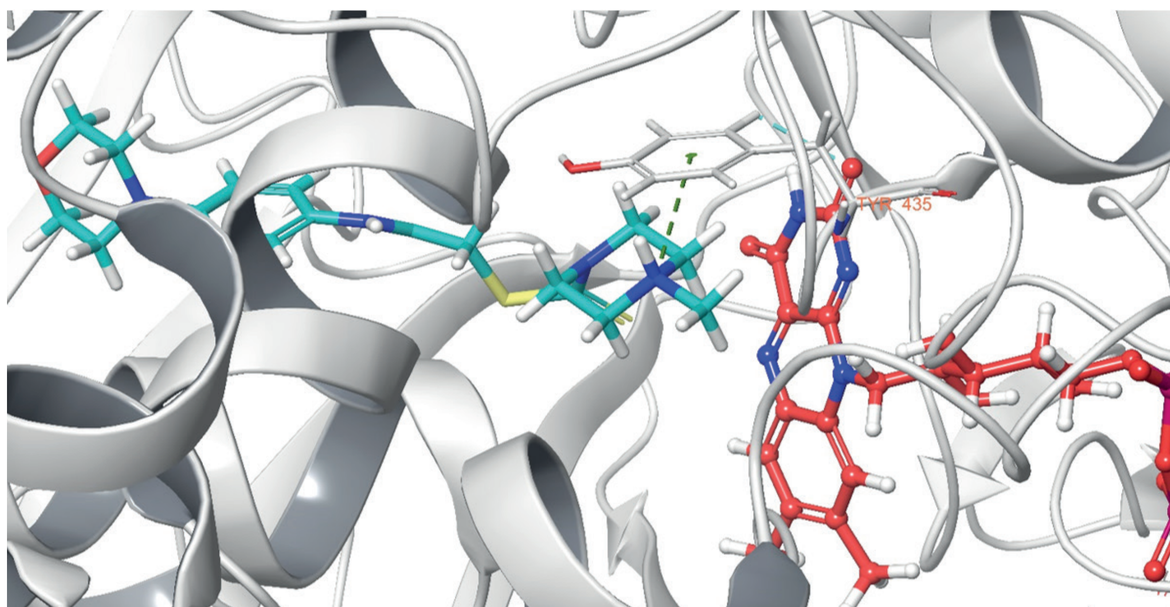


Figure 6. 3D pose of compound **3a** with hMAO-B enzyme (PDB ID: 2V5Z)

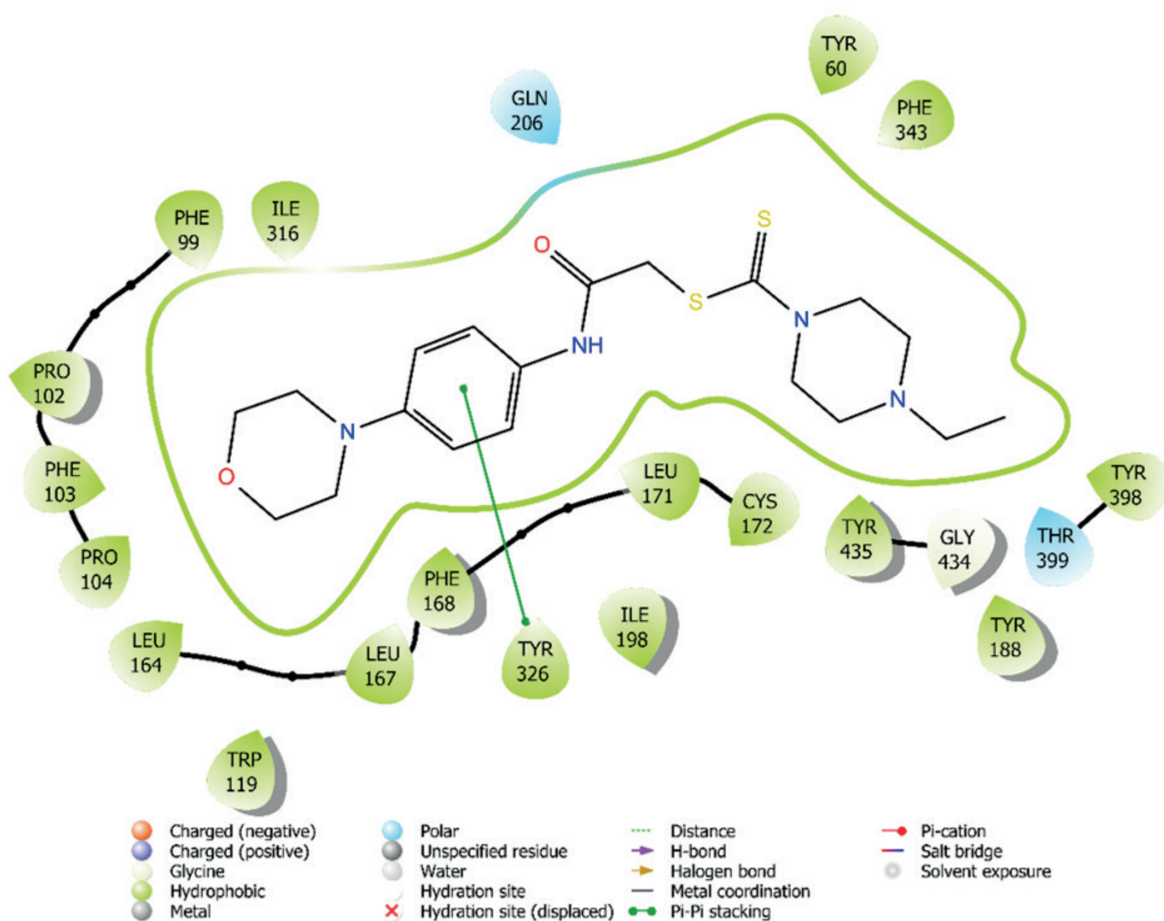


Figure 7. 2D pose of compound **3b** with hMAO-B enzyme (PDB ID: 2V5Z)

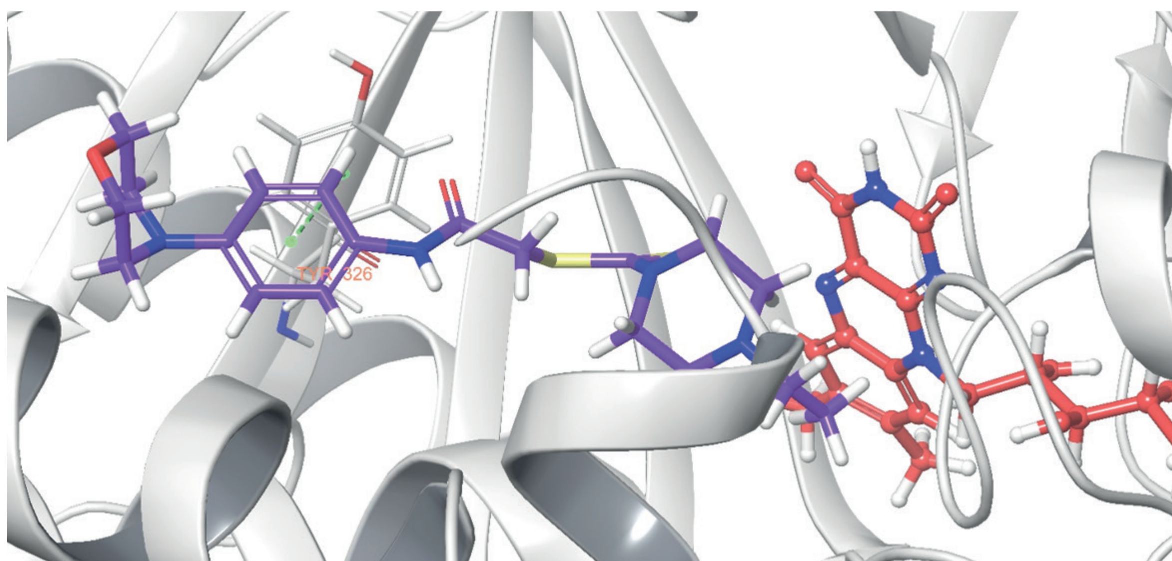


Figure 8. 3D pose of compound **3b** with hMAO-B enzyme (PDB ID: 2V5Z)

Acknowledgements

As the authors of this study, we thank Anadolu University Faculty of Pharmacy Central Laboratory for their support and contributions.

Ethical approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Author contribution

Conceptualization, B.K. and Z.A.K.; Methodology, B.K., D.O. and B.N.S.Ö.; Software, D.O.; Validation, Z.A.K.; Formal analysis, B.K.; Investigation, D.O. and B.K.; Resources, B.K., D.O. and B.N.S.Ö.; Data curation, B.K. and Z.A.K.; Writing—original draft preparation, B.K., D.O. and B.N.S.Ö.; Writing—review and editing, B.K. and Z.A.K.; Visualization, D.O.; Supervision, Z.A.K. All authors have read and agreed to the published version of the manuscript.

Source of funding

This research received no grant from any funding agency/sector.

Conflict of interest

The authors declare that there is no conflict of interest.

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European Journal of Life Sciences 2024; 3: 66-71

Research Article

Synthesis of new 1,3,4-thiadiazole derivatives, investigation of their AChE effects

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https://doi.org/10.55971/EJLS.1497661

Received:	07.06.2024
Accepted:	13.08.2024
Available online:	30.08.2024

ABSTRACT

Alzheimer's disease (AD) is the most common cause of dementia and one of the most prevalent neurodegenerative diseases. It begins with mild cognitive impairment and progressively affects all aspects of the patient's life functions. Alzheimer's disease is more commonly seen in the elderly and has a progressive incidence. With the global increase in the elderly population, Alzheimer's disease poses a significant threat. Additionally, current medications do not prevent AD, highlighting the need for new drug molecules to be used in AD treatment. Although 1,3,4-thiadiazoles have many biological activities such as anticancer and antiviral, their activities on acetylcholinesterase (AChE) are also being investigated. For this purpose, three new 1,3,4-thiadiazole compounds were synthesized in this study. The structure determinations of these compounds were carried out using ^{1}H-NMR and HRMS spectrophotometric methods. Activity studies were conducted *in vitro* using the modified Ellman method. As a result of the activity tests, compound **3b** showed the closest effect to donepezil with an IC_{50}= 0.096±0.004 µM.

Keywords: Acetylcholinesterase, Alzheimer's Disease, Molecular docking, 1,3,4-Thiadiazole

1. INTRODUCTION

Alzheimer's disease (AD) is an irreversible neurodegenerative disease that starts with memory loss and affects cognitive skills [1-2]. It is the most common cause of dementia and is an age-related disease [3-4]. Given the aging of the world's population, AD has an increasing prevalence [5].

Among the pathologic causes of AD is the cholinergic hypothesis [6-7]. Behavioral and cognitive impairment in AD is due to low acetylcholine levels in different regions of the Central Nervous System (CNS) [8]. Acetylcholinesterase (AChE) inhibitors are targeted to increase cholinergic levels in the brain by inhibiting the biological activity of AChE [9]. AChE inhibitors have therefore been one of the key strategies in developing anti-AD drugs [3-10].

Thiadiazoles are five-membered heterocyclic rings containing hydrogen, sulfur, carbon and nitrogen. It has an important position in heterocyclic chemistry because it contains both electron-withdrawing (S) and electron-donating (-C=N) groups [11]. The presence of a sulfur atom in the thiadiazole increases the compound's liposolubility and, consequently,

its pharmacokinetics [12]. This ring system exists in nature in four isomeric forms: 1,2,5-thiadiazole, 1,3,4-thiadiazole, 1,2,3-thiadiazole, 1,2,4-thiadiazole [13]. 1,3,4-Thiadiazoles show many biological activities such as antiviral, antibacterial, anticonvulsant, anti-inflammatory, and antioxidant [14-16]. They also exhibit antitumor activity [12]. In addition to showing many biological activities, they also have distinctive physicochemical properties, which is the reason why they are preferred in new drug production studies [17].

In this study, 3 compounds containing 1,3,4-thiadiazole core were synthesized. The structures of these compounds were determined and their AChE inhibitory effects were investigated.

2. MATERIALS AND METHODS

2.1. Chemistry

In the synthesis studies described, the control of the reactions was carried out with TLC applications. Samples were taken from the test flasks at certain time intervals. Then, the ethanol solutions of the starting materials used in the syntheses were applied to the silica gel F_{60}-coated aluminum plates previously saturated with appropriate solvent mixtures. Entrainment in the mobile phases was ensured and ultraviolet light (254 nm and 366 nm) was used to detect the stains. Petroleum ether: Ethyl acetate (4 : 1) was used as the appropriate mobile phase for the control of these syntheses. The melting points (M.p) of the synthesized compounds were determined by filling ½ cm of the powdered substance into capillary tubes with one end open. Electrothermal melting point determination device was used. The values found were recorded and not corrected. Spectroscopic methods were used for structure determination of the synthesized compounds. ^{1}H-NMR was performed using Bruker DPX 300 FT-NMR spectrometer. LCMS-IT-TOF (Shimadzu, Kyoto, Japan) was used for High Resolution Mass Spectra (HRMS).

2.1.1. General synthesis of N-substituted-hydrazinecarbothioamides (1a-c)

The hydrazine hydrate (0.04 mol) was reacted with an isothiocyanate derivative (0.02 mol) in ethanol at 80°C for 4 hours under reflux. After the reaction, the precipitated product was filtered and washed with ethanol.

2.1.2. General synthesis of 5-(substituted-amino)-1,3,4-thiadiazole-2-thiols (2a-c)

Compound 1a-c was reacted in ethanol with carbon disulfide (0.019 mol) and sodium hydroxide (0.019 mol) under reflux for 8 hours. After the reaction was completed, the solution was cooled and acidified to pH 4-5 with hydrochloric acid, then crystallized from ethanol.

2.1.3. Synthesis of target compounds (3a-c)

Compounds **2**(**a-c**) and 2-bromo-4′-(trifluoromethyl) acetophenone (0.0007 mol) were reacted in acetone with potassium carbonate (0.0007 mol). After the reaction, which occurred at room temperature, the mixture was filtered and washed with ethanol.

*2-((5-(Phenylamino)-1,3,4-thiadiazol-2-yl)thio)-1-(4-trifluoromethyl)phenylethan-1-one (**3a**)*

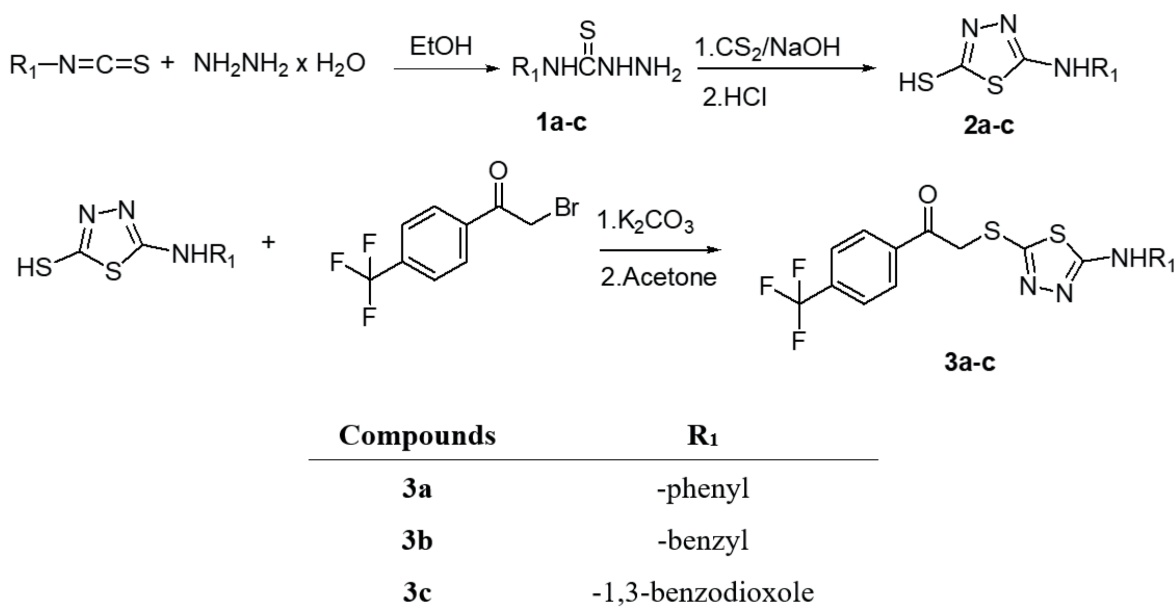
Yield: 80%, M.p: 171.2°C-172.8°C, ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 4.99 (2H, s, -$COCH_2$), 6.96-7.01 (1H, m), 7.28-7.32 (2H, m), 7.52-7.55 (2H, m), 7.93-7.96 (2H, m), 8.21-8.25 (2H, m), 10.38 (1H, brs). HRMS (*m/z*): $[M+H]^+$ calcd for $C_{17}H_{12}OF_3N_3S_2$: 396.0447; found 396.0465.

*2-((5-(Benzylamino)-1,3,4-thiadiazol-2-yl)thio)-1-(4-trifluoromethyl)phenylethan-1-one (**3b**)*

Yield: 77%, M.p: 162.7°C-165.1°C, ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 4.42-4.44 (2H, m, -$NHCH_2$), 4.86 (2H, s, -$COCH_2$), 7.32-7.34 (5H, m), 7.90-7.94 (2H, m), 8.17-8.20 (2H, m), 8.34 (1H, brs). HRMS (*m/z*): $[M+H]^+$ calcd for $C_{18}H_{14}OF_3N_3S_2$: 410.0603; found 410.0598.

*2-((5-((1,3-Benzodioxol-5-yl)methylamino)-1,3,4-thiadiazol-2-yl)thio)-1-(4-trifluoromethyl) phenylethan-1-one (**3c**)*

Yield: 79%, M.p: 182.6°C-183.9°C, ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 4.31-4.33 (2H, s, -$NHCH_2$), 4.86 (2H, s, -$COCH_3$), 5.98 (2H, s), 6.78-6.89 (3H, m), 7.91-7.94 (2H, m), 8.18-8.21 (2H, m). HRMS (*m/z*): $[M+H]^+$ calcd for $C_{19}H_{14}O_3F_3N_3S_2$: 454.0401; found 454.0503.



Compounds	R1
3a	-phenyl
3b	-benzyl
3c	-1,3-benzodioxole

Scheme 1. Synthesis pathway for obtained compounds **(3a-3c)**

2.2.Cholinesterase Enzymes Inhibition Assay

All synthesized compounds **(3a-c)** were subjected to the modified Ellman's method, previously prepared by our team, to evaluate their potency in inhibiting ChE enzymes [18].

2.3. Molecular Docking Study

Molecular docking studies of the synthesized compounds were performed using AChE (PDB: 4EY7 [19]) crystals. Docking studies were performed using standard procedures with the Schrödinger Suite 2020 Update 2 program [20]. The docking process was carried out with single precision (SP) using the LigPrep 3.8 [21] and Glide 7.1 [22] interfaces.

Table 1. The IC_{50} (µM) values of the obtained compounds against AChE and BChE enzymes

Compound	AChE IC_{50} (µM)	BChE IC_{50} (µM)
3a	>100	>1000
3b	0.096±0.004	>1000
3c	0.302±0.014	>1000
Donepezil	0.0201±0.0014	-
Tacrine	-	0.0064±0.0002

3. RESULTS AND DISCUSSION

3.1. Chemistry

The preparation of compounds **3a-3c** is as shown in Scheme 1. 1,3,4-Thiadiazole derivatives were obtained and their interactions with AChE were investigated by docking studies. The structure-determination of the obtained compounds was elucidated using spectroscopic methods. When the ^{1}H-NMR results were examined, it was seen that the protons of the aromatic rings were H, 2H, 3H between 6.80 ppm-7.94 ppm. The protons of methyl attached to the carbonyl group were 3.33 ppm-3.83 ppm 2H. The protons of methyl attached to the amino group in compound **3b** were shown to be 4.44 ppm 2H and 4.27 ppm 2H in compound **3c**. Mass spectra were performed using high resolution liquid chromatography and all compounds were recorded in excess of their molecular weights in the mass spectra obtained using electron sputtering method.

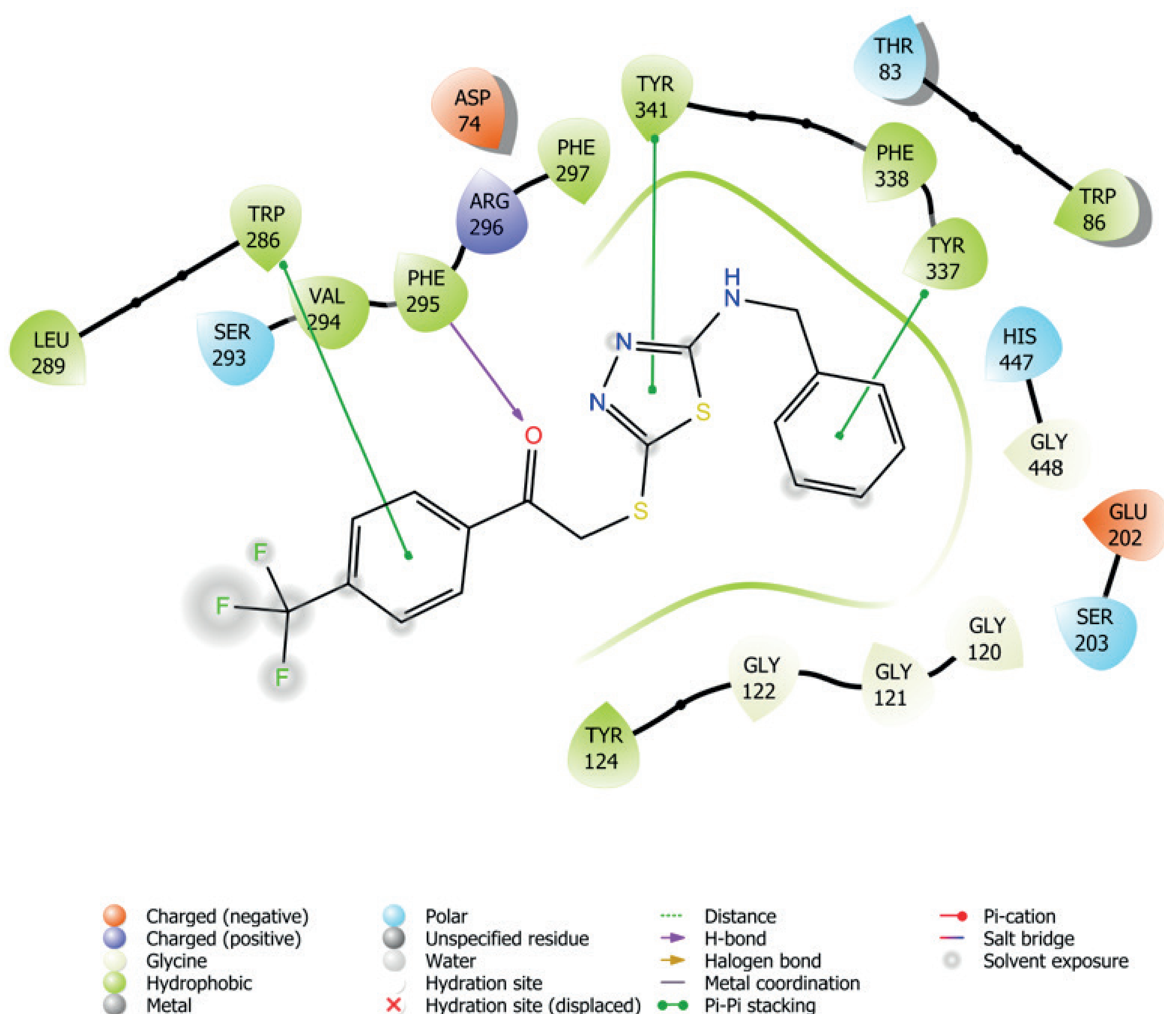


Figure 1. Two-dimensional interaction mode of compound **3b** in the active site of acetylcholinesterase enzyme (PDB: 4EY7)

3.2. Cholinesterase Enzymes Inhibition Assay

The synthesized compounds **(3a-c)** were assessed for their *in vitro* AChE and BChE inhibitory potencies using the modified Ellman's spectrophotometric technique. The results are shown in Table 1. The compound that showed the closest activity to donepezil was **3b**, with an IC_{50} value of 0.096±0.004 µM.

3.3. Molecular Docking Study

Molecular docking studies of the synthesized compounds were carried out using AChE (PDB: 4EY7 [13]) crystals and the interaction of compound **3b** with the active site of the AChE enzyme is best observed. Figure 1 shows the 2D localization of 3b in enzyme active site and Figure 2 shows the 3D localization of **3b** in the enzyme active site. The bonds formed by compound **3b** at the enzyme active site have been studied and can be summarized as follows. It forms a π-π interaction between the 1,3,4-thiadizole ring and the phenyl ring of Tyr341. The benzyl ring attached to the amino group formed a π-π interaction with the phenyl ring of Tyr337. The other phenyl ring in the structure formed a π-π interaction with the indole ring of Trp286. The hydroxyl group in the structure formed an H-bond with the amino acid Phe295.

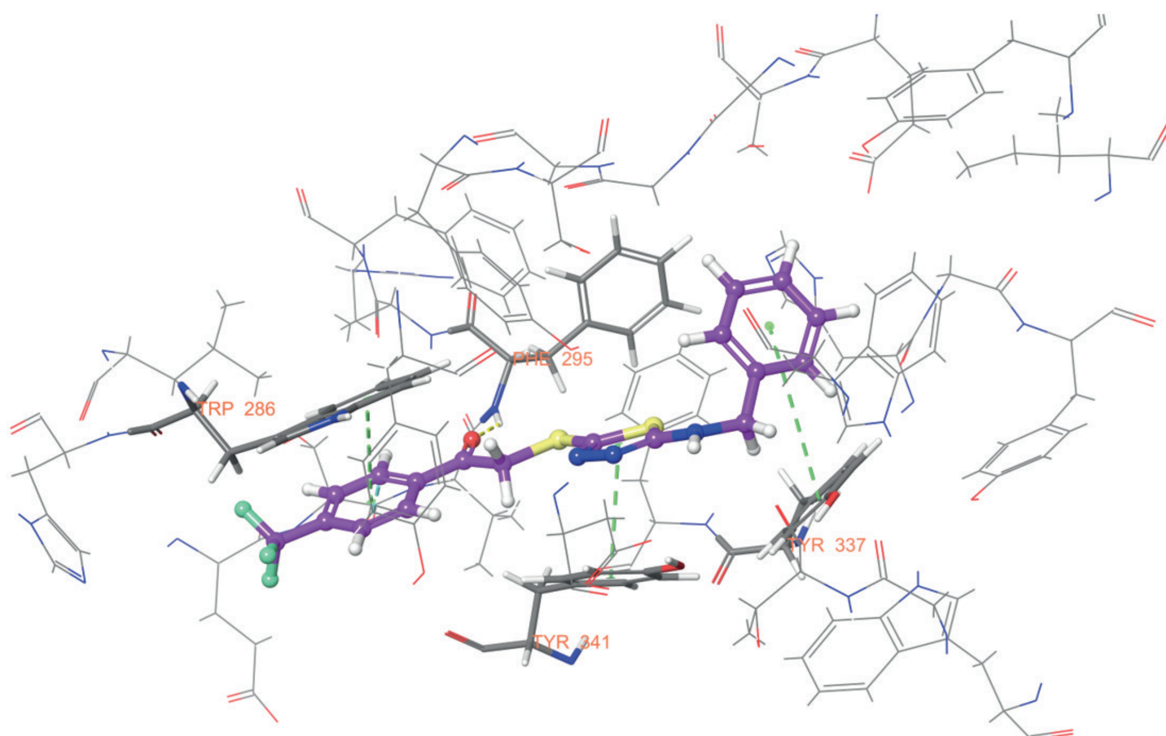


Figure 2. Three-dimensional interaction mode of compound **3b** in the active site of acetylcholinesterase enzyme (PDB: 4EY7)

4. CONCLUSION

When the studies carried out so far are examined, it has been revealed that 1,3,4-thiadiazole ring has many activities. Heterocyclic rings with electron acceptor and donor groups are used in many drug synthesis studies. 1,3,4-Thiadiazole ring is known to be used in many drug development studies. Therefore, in this study, 3 compounds bearing 1,3,4-thiadiazole ring were synthesized. ^{1}H-NMR and HRMS studies were carried out for structure determination of these synthesized compounds. 1,3,4-thiadiazole containing compounds have been shown to act as AChE inhibitors in previous studies. The molecular docking results with the AChE enzyme were then examined for each of the synthesized compounds. As a result of the docking study with AChE, it was observed that **3b** gave the best results. As a result of the activity study with the AChE enzyme, compound **3b** was found to have an IC_{50} value of 0.096±0.004 μM.

Acknowledgements

As the authors of this study, we thank Anadolu University Faculty of Pharmacy Central Laboratory for their support and contributions.

Ethical approval

Not applicable, as this article does not involve any studies with human or animal subjects.

Author contribution

Conceptualization, D.O. and A.N.C.; Methodology, D.O.; Software, D.O.; Formal analysis, B.N.S.Ö.; Resources, A.N.C.; Data curation, A.N.C., D.O.; Writing—original draft preparation, A.N.C.; Writing—review and editing, A.N.C.; Supervision, Z.A.K. All authors have read and agreed to the published version of the manuscript.

Source of funding

This research received no grant from any funding agency/sector.

Conflict of interest

The authors declare that there is no conflict of interest.

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Research Article

Synthesis of some benzothiazole-piperazine derivatives, investigation by *in vitro* and molecular modelling for hMAO inhibitory activities

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https://doi.org/10.55971/EJLS.1497558

Received: 07.06.2024
Accepted: 14.08.2024
Available online: 30.08.2024

ABSTRACT

Monoamine oxidase (MAO) is an enzyme that helps regulate the functions of intracellular amines, as well as chemicals such as dopamine, serotonin and norepinephrine, in the brain and its tissues. Active substances that are inhibitors of monoamine oxidases (MAOs) are used in the treatment of anxiety, depression and Alzheimer's disease. Previous studies have shown that compounds containing piperazine rings show MAO-A inhibitory activity. Based on these studies, 4 compounds containing piperazine and benzothiazole rings were designed, and the structures of the compounds were elucidated using spectroscopic methods such as HRMS and ^{1}H-NMR. hMAO-A and hMAO-B inhibitory activity was examined by *in vitro* methods. An *in silico* procedure was applied to investigate the residues and binding modes that interact with the docking of compounds **3a-d** to the active site of the hMAO-A (PDB ID: 2Z5X) enzyme identified in the previous study. Compound **3b** was found to be the most effective agent among the synthesized compounds with an IC_{50} value of 0.104±0.004 µM against the MAO-A enzyme.

Keywords: Enzyme Inhibition, MAO-A, Molecular Docking, Piperazine

1. INTRODUCTION

Monoamine oxidase (MAO) is an enzyme that plays a role in the oxidative deamination of intracellular amines as well as neurotransmitters such as dopamine, serotonin and norepinephrine, and helps regulate the concentrations of these chemicals in the brain and tissues outside the brain [1,2]. MAOs (MAO-A, MAO-B), which have 2 different isoforms with 70% homology, are located in the outer mitochondrial membranes of cells. The most common places in the body are the brain and liver [3].

While MAO-A is involved in the metabolization of neurotransmitters such as serotonin and adrenaline, MAO-B is involved in the metabolization of neuromodulatory neurotransmitters such as phenylethylamine [4]. MAOs have had different therapeutic uses due to their affinity for different substrates. While Monoamine oxidase-A inhibitors are used as antidepressants in the treatment of depression, Monoamine oxidase-B inhibitors are mostly used in Parkinson's and Alzheimer's disease [5-7].

The piperazine ring is a heterocyclic compound that exhibits a wide range of biological activities. It is found in the structure of compounds used in the treatment of anxiety disorders, such as the active ingredient buspirone [8-10]. When previous studies were examined, MAO inhibition activity was observed in many compounds containing phenylpiperazine and benzothiazole rings [11-13]. Although MAO-A inhibitors such as iproniazid, isocarboxazid, moclobemide and transylpromine have effective results in the treatment of depression, their clinical use has been limited due to side effects such as food-drug interactions or drug-drug interactions. Therefore, the emergence and discovery of new pharmacological groups have become important [14]. It is known that the diseases mentioned in the above text are not simpler diseases such as flu and cold, which have side effects and can be solved easily. Side effects that make life functions and quality of life unbearable and emotional states that can lead to suicide and end of life have led to more studies and efforts on these critical diseases.

In this study, four compounds containing piperazine and benzothiazole rings were synthesized, and their molecular structures were elucidated by various methods. Molecular docking studies and biological evaluation of their human MAO-A and MAO-B inhibition were carried out.

2. MATERIALS AND METHODS

2.1. Chemistry

While carrying out this study, all chemicals used and planned to be used during the reaction and pathways were supplied from Sigma-Aldrich (Sigma-Aldrich Corp., USA) or Merck (Merck KGaA, Germany). ^{1}H-NMR spectra were recorded in DMSO-d_6 by a Bruker digital FT-NMR spectrometer (Bruker Bioscience, USA) at 300 MHz. MS experiments were planned and carried out on the LCMS-IT-TOF device (Shimadzu, Japan). Termination checks between reaction steps were checked with classical TLC applications on silica gel 60 F254 (Merck KGaA, Germany). Melting degree determination was determined with the Mettler Toledo-MP90 (Greifensee, Switzerland).

2.1.1. Synthesis of 2-chloro-N-(6-substitutedbenzo[d]thiazol-2-yl)acetamide derivaties (1a-d)

Chloroacetyl chloride was added slowly to a mixture of 6-substituted benzothiazole-2-amine (0.9 g, 0.006 mol) and triethylamine (TEA) (0.894 mL) in tetrahydrofuran (THF) (12 mL) in ice bath. After it was determined that the reaction was over, THF was removed and the compound was washed with water to remove the salt [15-16].

2.1.2. Synthesis of sodium 4-(4-methoxyphenyl) piperazine-1-carbodithioate (2a)

NaOH and carbon disulfide (0.05 mol) were added to the mixture of 1-(4-methoxyphenyl) piperazine (0.05 mol) dissolved in ethanol and stirred in a mixture of ice and water for 4 hours. When the reaction was monitored by TLC and determined to have ended, the precipitated substances were filtered, washed with diethyl ether and left to dry [15-16].

2.1.3. Synthesis of target compounds (3a-d)

2-chloro-*N*-(6-substitutedbenzothiazol-2-yl)acetamide (**1a-d**) (0.0011 mol), sodium 4-(4-substituted phenyl) piperazine-1 carbodithioate (**2a**) (0.0011 mol) were stirred for 6 hours in acetone. After detection of disruption of the reaction, acetone was removed with a rotary evaporator. It was cleaned with water to remove salts from the substances synthesized as a result of the reactions and allowed to dry. Then, recrystallization was performed with ethanol [15-16].

*2-(Benzo[d]thiazol-2-ylamino)-2-oxoethyl-4-(4-methoxyphenyl)piperazine-1-carbodithioate (**3a**)*

Yield: 79%, M.p: 225-227°C, ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 3.16 (4H, m, CH_2-CH_2), 3.69 (3H, s, -OCH_3), 4.12 (2H, brs, CH_2), 4.34 (2H, brs, CH_2), 4.45 (2H, s, -CH_2), 6.85 (2H, d, *J*=6.8 Hz), 6.94 (2H, d, *J*=7.1 Hz), 7.30 (1H, t, *J*=15.3 Hz, benzothiazole), 7.44 (1H, t, *J*=15.4 Hz, benzothiazole), 7.75 (1H, d, *J*=8.0 Hz, benzothiazole), 7.96 (1H, d, *J*=8.1 Hz, benzothiazole), 12.66 (1H, s, -NH). HRMS (ESI) (*m/z*): $[M+2H]^{2+}$ calculated for $C_{21}H_{22}N_4O_2S_3$: 230.0511; found 230.0525.

*2-((6-Methylbenzo[d]thiazol-2-yl)amino)-2-oxoethyl-4-(4-methoxybenzyl)piperazine-1-carbodithioate (**3b**)*

Yield: 84%, M.p: 237-239°C, ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 2.40 (3H, s, $-CH_3$), 3.16 (4H, m, CH_2-CH_2), 3.69 (3H, s, $-OCH_3$), 4.11 (2H, brs, CH_2), 4.34 (broad s, 2H, CH_2), 4.44 (2H, s, $-CH_2$), 6.84 (2H, d, *J*=9.0 Hz), 6.94 (2H, d, *J*=9.1 Hz), 7.25 (1H, d, *J*=8.5 Hz, benzothiazole), 7.63 (1H, d, *J*=8.2 Hz, benzothiazole), 7.76 (1H, s, benzothiazole), 12.56 (1H, s, -NH). HRMS (ESI) (*m/z*): $[M+H]^+$ calculated for $C_{22}H_{24}N_4O_2S_3$: 473.1127; found 473.1134.

*2-((6-Methoxybenzo[d]thiazol-2-yl)amino)-2-oxoethyl-4-(4-methoxybenzyl)piperazine-1-carbodithioate (**3c**)*

Yield: 81%, M.p: 242-244°C, ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 3.16 (4H, m, CH_2-CH_2), 3.69 (3H, s, $-OCH_3$), 3.80 (3H, s, $-OCH_3$, benzothiazole), 4.11 (2H, brs, CH_2), 4.34 (2H, brs, CH_2), 4.43 (2H, s, $-CH_2$), 6.84 (2H, d, *J*=8.8 Hz), 6.94 (2H, d, *J*=9.1 Hz), 7.03 (1H, d, *J*=8.8 Hz, benzothiazole), 7.57 (1H, s, benzothiazole), 7.64 (1H, d, *J*=8.8 Hz, benzothiazole), 12.53 (1H, s, -NH). HRMS (ESI) (*m/z*): $[M+H]^+$ calculated for $C_{22}H_{24}N_4O_3S_3$: 489.1072; found 489.1083.

*2-((6-Nitrobenzo[d]thiazol-2-yl)amino)-2-oxoethyl-4-(4-methoxybenzyl)piperazine-1-carbodithioate (**3d**)*

Yield: 73%, M.p: 259-261°C, ^{1}H-NMR (300 MHz, DMSO-d_6): δ =3.16 (4H, m, CH_2-CH_2), 3.69 (3H, s, $-OCH_3$), 4.11 (2H, brs, CH_2), 4.33 (broad s, 2H, CH_2), 4.49 (2H, s, $-CH_2$), 6.85 (2H, d, *J*=9.1 Hz), 6.94 (2H, d, *J*=9.1 Hz), 7.91 (1H, d, *J*=9.0 Hz, benzothiazole), 8.29 (1H, d, *J*=8.9 Hz, benzothiazole), 9.06 (1H, s, benzothiazole), 13.11 (1H, s, -NH). HRMS (ESI) (*m/z*): $[M+H]^+$ calculated for $C_{21}H_{21}N_5O_4S_3$: 504.0792; found 504.0828.

Table 1. IC_{50} (µM) values of the obtained compounds against MAO-A and MAO-B enzyme

Compounds	MAO-A IC_{50} (µM)	MAO-B IC_{50} (µM)
3a	0.198±0.008	>100
3b	0.104±0.004	0.120±0.005
3c	0.167±0.007	0.280±0.013
3d	>100	>100
Moclobemide	6.0613±0.2625	-
Selegiline	-	0.0374±0.0016

2.2. *In vitro* MAO inhibition assay

In vitro fluorometric enzymatic analysis, which allows accurate and accurate detection and observation of monoamine oxidase activities, was applied to investigate the inhibitory potential of **3a-d** coded compounds on hMAO-A and hMAO-B. Compounds were used at concentrations of 10^{-5} M-10^{-9} M to calculate their IC_{50} inhibition values of the obtained compounds were calculated as described in previous studies [17-20] (Table 1).

2.3. Prediction of ADME Parameters

The online SwissADME program was used to estimate ADME parameters. [21] (Table 2).

Table 2. Predicted ADME parameters of compounds **3a-d**

Comp	Physicochemical Properties							Lipo.	Druglikeness					Water Solubility		Pharmacokinetics	
	MW	Fsp3	RB	HBA	HBD	MR	TPSA	cLogP	Lipinski	Ghose	Veber	Egan	Muegge	LogS	Class	GI abs.	F
3a	458.62	0.29	8	3	1	136.39	143.33	3.43	+	-	-	-	+	-5.37	Moderately	Low	0.55
3b	472.65	0.32	8	3	1	141.36	143.33	3.76	+	-	-	-	+	-5.68	Moderately	Low	0.55
3c	488.65	0.32	9	4	1	142.89	152.56	3.57	+	-	-	-	-	-5.46	Moderately	Low	0.55
3d	503.62	0.29	9	5	1	145.22	189.15	2.71	+	-	-	-	-	-5.45	Moderately	Low	0.55

Comp: Compounds, MW: Molecular weight, Fsp3: Fraction Fsp3, RB: Number of rotatable bonds, HBA: Number of hydrogen bond acceptors, HBD: Number of hydrogen bond donors, MR: Molar refractivity, TPSA: Total polar surface area, Lipo: Lipophilicity, GI abs: Gastrointestinal absorption, F: Bioavailability score.

Table 3. Molecular docking scores, interaction types and estimated inhibition constants of synthesized compounds **(3a-d)** and MAO-A (PDB ID: 2Z5X)

Comp.	Autodock Results				Vina Results
	Interacting Residues	**Interaction Types**	**Estimated Inhibition Constant, K_i**	**Best Docking Score**	**Best Docking Score**
3a	-	-	16.31 mM	-6.53	-8.2
3b	PHE112	Pi-Pi Stacking	106.25 nM	-9.51	-8.8
	TYR124	Pi-Pi Stacking			
	TRP128	Pi-Pi Stacking			
3c	HIS488	Pi-Pi Stacking	144.15 nM	-9.33	-8.7
	ASP132	H-Bond			
3d	HİS488	Pi-Pi Stacking	7.13 nM	-11.11	-9.4
	ASP132	H-Bond			
	LYS136	Salt Bridge			

Table 4. Molecular docking scores, interaction types and estimated inhibition constants of synthesized compounds **(3a-d)** and MAO-B (PDB ID:2V5Z)

Comp.	Autodock Results				Vina Results
	Interacting Residues	**Interaction Types**	**Estimated Inhibition Constant, K_i**	**Best Docking Score**	**Best Docking Score**
3a	CYS172	H-Bond	36.35 nM	-10.15	-8.2
3b	PHE343	Pi-Pi Stacking	34.84 nM	-10.17	-8.8
3c	-	-	31.24 nM	-10.24	-8.6
3d	ILE199	H-Bond	12.81 nM	-10.77	-8.3

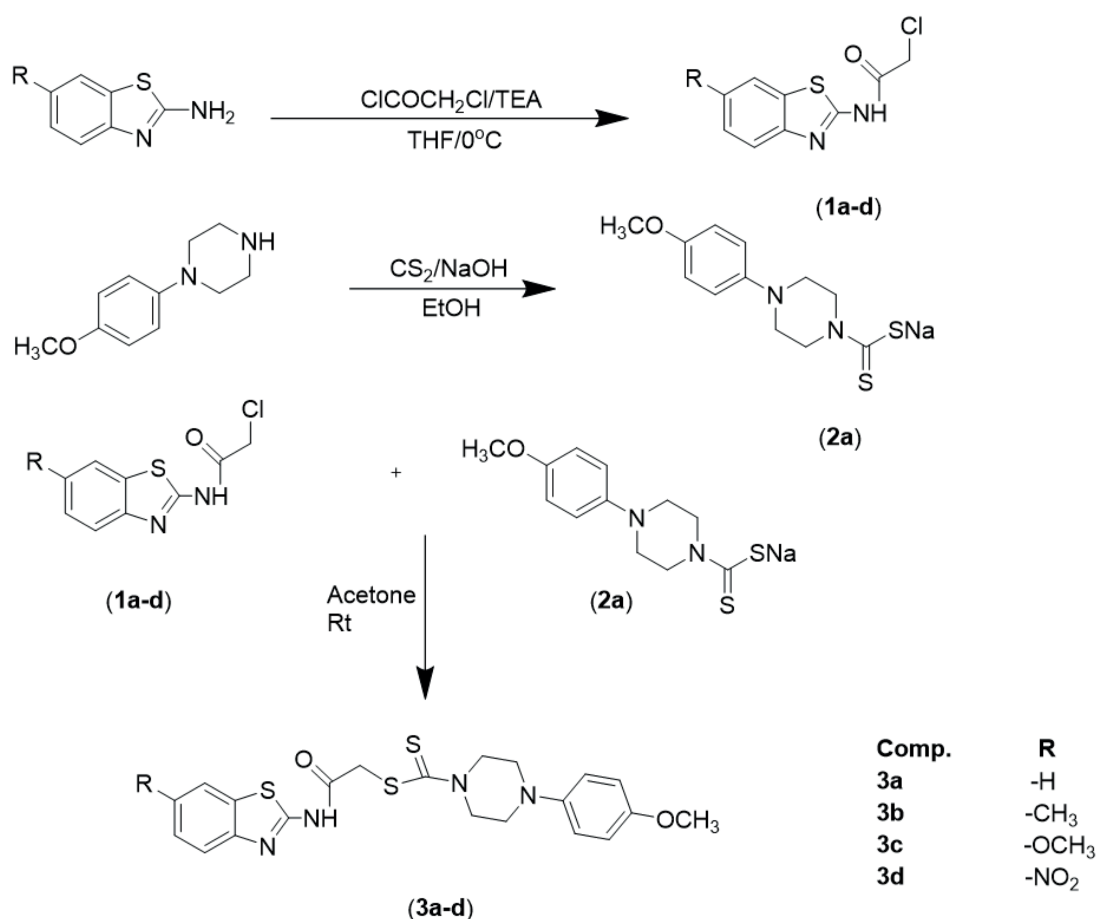
2.4. Molecular Docking Study

An *in silico* procedure was applied to investigate the residues and binding modes that interact with the docking of compounds **3a-d** to the active site of the hMAO-A (PDB ID: 2Z5X) [22], the hMAO-B (PDB ID: 2V5Z) [23] enzymes identified in the previous study. The macromolecular structure of hMAO-A crystallized with harmine was obtained from the Protein Data Bank and the molecular docking procedure was performed by researchers in our research group, as done in previous docking studies [24-26]. The Pdb file of the macromolecule was optimized using Maestro Version 6.4.135, Release 2023-4 [27]. In both receptors (MAO-A: 2Z5X and MAO-B: 2V5Z), the (6Å) waters around the previously determined active site (MAO-A: HRM700 and MAO-B: SAG601) were left and all other water molecules were removed. Preprocessing and H-Bond optimization for both receptors was done using Maestro. Then, the obtained pdb formatted macromolecules were edited with Autodock and saved in pdbqt format. The regular space of the Grid boxs are determined as 0.375 Å, centered on SAG601 (40*40*40 $Å^3$) and HRM700 (50*50*50 $Å^3$). Lamarckian Genetic Algorithm was preferred in all studies, detailed results such as docking scores were obtained using both AutoDock 4.2 [28] and AutoDock Vina programs [29] and results are presented in Table 3 and Table 4. To validate the molecular docking studies, redocking studies were performed with both HRM700 on MAO-A and SAG601 on MAO-B, and RMSD values were found to 0.92 and 1.08 respectively.

3. RESULTS AND DISCUSSION

3.1. Chemistry

Compounds **3a-d** were obtained as shown in Scheme 1. In this study, a synthesis involving dithiocarbamate salt and benzothiazole rings was carried out. The planned and realized synthesis consists of 3 steps.



Scheme 1. Synthesis pathway for obtained compounds **(3a-d)**

As the first step, 2-chloro-*N*-(6-substitutedbenzo[*d*] thiazol-2-yl)acetamide derivatives were obtained by acetylation (**1a-d**). As a second step, dithiocarbamate salt was obtained by the reaction of carbon disulfide with secondary amines (**2a**). In the third and last step, the two products obtained were dissolved in acetone, boiled under reflux, filtered and dried, and substances (**3a-d**) were obtained (Scheme 1). The structures of the compounds **3a-d** were confirmed by using spectroscopic methods (HRMS and ^{1}H-NMR).

When the NMR results of the synthesized compounds were examined, it was observed that the proton peaks of the piperazine ring appeared in 3 different forms (2H, 2H and 4H) between 3.16 ppm and 4.30 ppm. The proton peaks of the acetyl group attached to the piperazine ring were detected as singlets between 4.43 ppm and 4.49 ppm. It was observed that the protein belonging to the amine group was between 12.53-13.11 ppm and protons belonging to disubstituted benzene were observed between 6.84 ppm and 6.94 ppm. Proton peaks of benzothiazole are also observed between 7.03 ppm and 9.06 ppm. While the CH_3 group in compound **3b** was observed as a singlet at 2.40 ppm, the OCH_3 group of compound **3c** was observed to peak as a singlet at 3.80 ppm. Mass spectra were performed using high-resolution liquid chromatography. In the mass spectra taken using the electron sputtering method, all compounds were recorded as having excess molecular weights.

3.2. *In vitro* MAO inhibition assay

In vitro fluorometric enzymatic analysis, which allows accurate and accurate detection and observation of monoamine oxidase activities, was applied to investigate the inhibitory potential of **3a-d** coded compounds on hMAO-A and hMAO-B.

3a-d coded compounds were used at concentrations of 10^{-5} M-10^{-9} M to calculate their IC_{50} inhibition values of the obtained compounds were calculated [30]. The inhibition of MAO-A and MAO-B at the initial concentrations of the resulting compound and moclobemide and selegiline are shown in Table 1. When the results obtained were examined, compounds **3a**, **3b** and **3c** showed IC_{50} values of 0.198±0.008 µM, 0.104±0.004 µM and 0.167±0.007 µM on MAO-A, respectively. The reference drug moclobemide showed a value of 6.0613±0.2625 µM. On the other hand, compounds **3b** and **3c** showed values of 0.120±0.005 µM and 0.280±0.013 µM on MAO-B, respectively. The IC_{50} value of selegiline used as the reference drug was measured as 0.0374±0.0016 µM. Based on these results, it was observed that the synthesized compounds gave approximately 50 times better results on MAO-A than the reference drug, and were approximately 9 times less active on MAO-B than the reference drug. *In vitro* results mostly overlapped with the MAO-A enzyme site interactions examined *in silico* results.

3.3. Prediction of ADME Parameters

The online SwissADME was used and the estimated ADME parameters of the obtained compounds were calculated [21]. Looking at Table 2 showing the results, it is observed that none of the synthesized compounds violate the Lipinski rule [31]. Gastrointestinal absorption provides a preliminary result as to whether the obtained compounds can be used orally. When the table was examined, it was seen that the compounds had low absorption. Log S values of the compounds are between -5.37 and -5.68, and their solubility is estimated to be moderate. The F value, which shows the oral bioavailability of the compounds, is 0.55, which is the ideal value [32], in contrast to the result in gastrointestinal absorption.

3.4. Molecular Docking Studies

As stated in the *in vitro* MAO-A inhibition results, compounds **3b** and **3d** were found to be the 2 compounds with the highest inhibition activity on MAO-A enzyme among the 4 compounds synthesized. Among the synthesized and obtained compounds, compound **3b** with an IC_{50} value of 0.104±0.004 µM was found to be the best compound. By using X-ray crystal structure of MAO-A (PDB ID: 2z5x) docking studies were performed, and binding modes of compound **3b** were assigned (Figures 1 and 2). Molecular docking poses of all synthesized and obtained compounds are presented in 2D and 3D images in supp. mat. file.

The interaction domain of MAO-A and its cocrystal ligand Harmine (PDB ID: HRM700) has been previously revealed, TYR69, ILE180, ASN181, PHE208, GLN215, ILE335, LEU337, PHE352, TYR407 and TYR444 were emphasis to be important for the interaction (https://www.ebi.ac.uk/pdbe/entry/pdb/2z5x/bound/HRM#700A).

The interaction domain of MAO-B and its cocrystal ligand Safınamide (PDB ID: SAG601) has been previously revealed, PRO102, TRP119, LEU164, PHE168, ILE171, CYS172, ILE199, GLN206, ILE316, TYR326, PHE343, TYR398, TYR435, FAD600, HOH798, HOH808 and HOH839 were emphasis to be important for the interaction (https://www.ebi.ac.uk/pdbe/entry/pdb/2v5z/bound/SAG#601A).

Compound **3a** was sufficiently bound to the amino acid residues in the macromolecule and was also observed in a very close position to the FAD enzyme. Compounds **3b**, **3c**, and **3d** bind sufficiently to amino acid residues spanning the gap and are located close to DCX1 and DCX2 (Figure 2). When the docking poses of all compounds were examined, it was clearly seen that it had many interactions such as salt bride, pi-pi stacking and H-bond. The pi-pi stacking was detected in the benzothiazole ring of compounds **3b, 3c** and **3d**. Also, there was a H-bond interaction between the nitrogen atom of amide functional group and ASP132 (Figures 1 and 2). Moreover, there was a salt bridge interaction between the nitro group of benzothiazole and LYS136 (Table 3). In this study, it was determined that compound **3b** interacted with these residues of MAO-A in a similar way. When the docking poses of MAO-B was examined, compounds **3a** and CYS172 was observed to make hydrogen bonds. Compound **3b** and PHE343 were observed to exhibit pi-pi stacking. Additionally, compounds **3d** and ILE199 were observed to form hydrogen bonds.

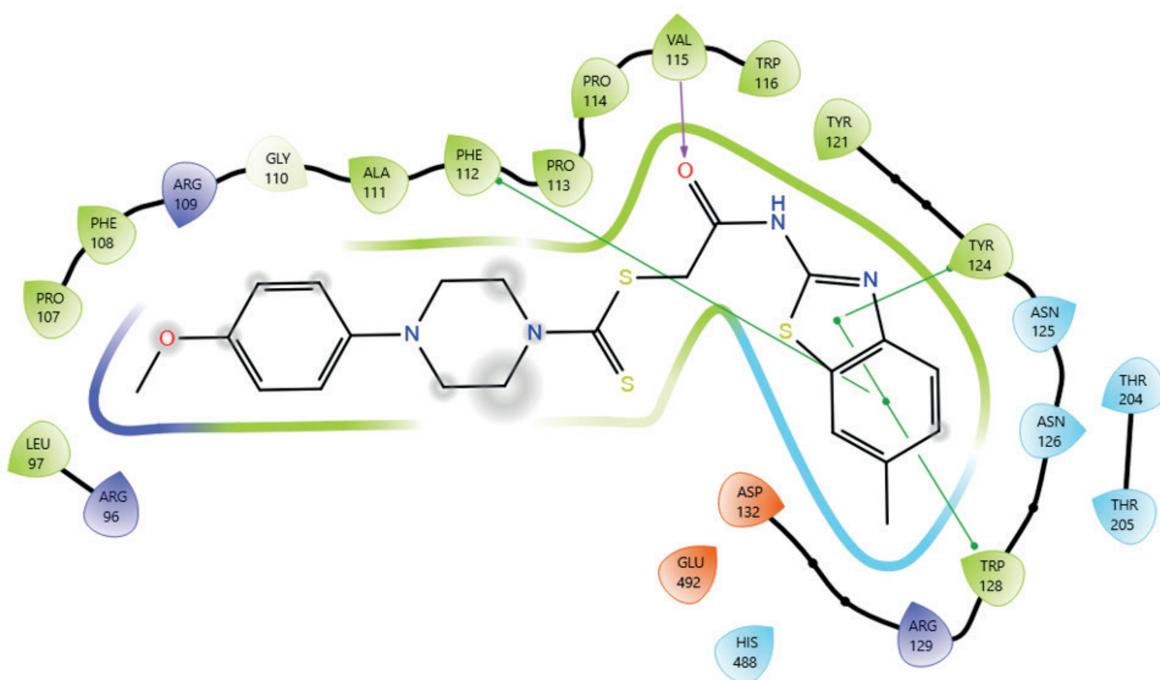


Figure 1. 2D interaction diagram with 2Z5X for compound **3b**

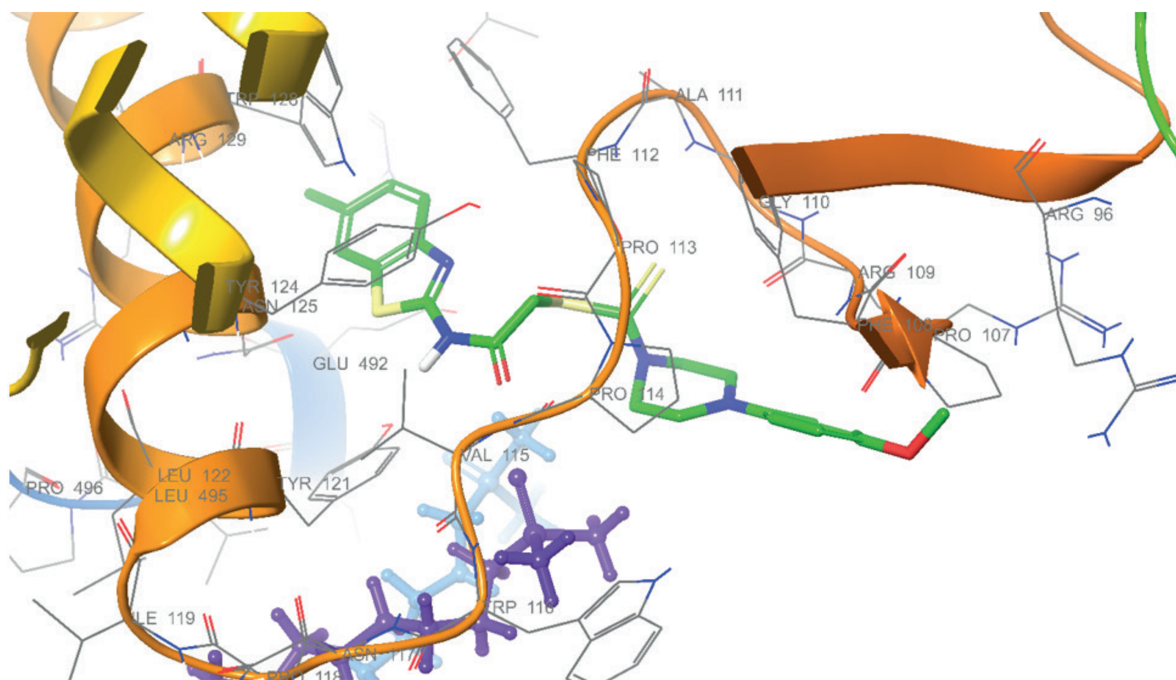


Figure 2. 3D interaction diagram with 2Z5X for compound **3b**

4. CONCLUSION

Compounds containing piperazine rings have previously been proven to be effective, have been synthesized and are still used today. In this study, compounds were synthesized with reference to a previous study. hMAO inhibition activities were investigated. ADME results showed that the synthesized compounds were moderate to good in terms of pharmacokinetics. When molecular docking studies were examined, it was observed that the compounds interacted with the residues in the active site. *In vitro* results show that the MAO-A inhibition of our compounds is better than the MAO-B inhibition. When the *in vitro* activity results were examined, compound **3b** showed the best inhibition value with an IC_{50} of 0.104±0.004 µM compared to the moclobemide reference drug. Moclobemide showed an IC_{50} value of 6.0613±0.2625 µM. Our compounds coded **3a**, **3b** and **3c** showed approximately 50 times better activity than the reference drug. The data obtained in this study can be used as a source for subsequent compound synthesis studies that can be used in the treatment of anxiety and depression.

Acknowledgements

As the authors of this study, we thank Anadolu University Faculty of Pharmacy Central Laboratory for their support and contributions.

Ethical approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Author contribution

Conceptualization, H.U., D.O. and Y.Ö.; Methodology, B.G., S.L. and B.N.S.Ö.; Software, H.U., D.O. and B.N.S.Ö.; Formal analysis, B.G., D.O. and S.L.; Investigation, B.G.; Resources, S.P.G.; Writing—original draft preparation, B.G., H.U., D.O. and S.P.G.; Writing—review and editing, Y.Ö.; Supervision, Y.Ö. All authors have read and agreed to the published version of the manuscript.

Source of funding

This research received no grant from any funding agency/sector.

Conflict of interest

The authors declare that there is no conflict of interest.

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ANADOLU UNIVERSITY
FACULTY OF PHARMACY

European Journal of Life Sciences 2024; 3: 82-92

Research Article

Synthesis of a new thiadiazole-benzodioxole derivative, investigation of acetylcholinesterase inhibition with *in vitro* and *in silico* studies

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https://doi.org/10.55971/EJLS.1497561

Received: 07.06.2024
Accepted: 16.08.2024
Available online: 30.08.2024

ABSTRACT

Alzheimer's disease is a progressive and degenerative brain disease that negatively affects people's lives and reduces cognitive and sensory human functions. Today, there are active ingredients that work on Alzheimer's disease, containing benzodioxole and thiadiazole rings. Acetylcholinesterase terminates neurotransmission in the nervous system and leads to the accumulation of acetylcholine, overstimulation of various receptors and consequent impairment of neurotransmission. Thiadiazole and benzodioxole rings are compounds that exhibit a wide range of biological activities, especially known to be effective on acetylcholinesterase. A new compound containing benzodioxole and thiadiazole rings was designed, synthesized and its chemical structure was revealed using spectroscopic methods such as HRMS, ^{13}C-NMR and ^{1}H-NMR. Acetylcholinesterase inhibition activities were investigated using *in vitro* methods. To elucidate the acetylcholinesterase inhibition of compound **4a**, it was subjected to *in silico* insertion procedure with 4EY7. Compound **4a** exhibited 0.114±0.005 µM against AChE. The above data is compared with data for donepezil (0.0201±0.0014 µM), the reference compound in our study.

Keywords: AChE, ADME, Benzodioxole, Molecular Docking, Thiadiazole

1. INTRODUCTION

Acetylcholinesterase is a cholinesterase group enzyme that can catalyze the decomposition reaction of acetylcholine, a neurotransmitter [1]. Cholinesterases, which cleave choline esters with varying efficiency, are a ubiquitous group of serine hydrolases. When vertebrates are analyzed, we come across forms encoded by two different genes, acetylcholinesterase and butyrylcholinesterase. While the biological role of butyrylcholinesterase is not fully defined, acetylcholinesterase hydrolyzes acetylcholine at cholinergic synapses [2]. Acetylcholinesterase is found in muscle, lungs, spleen, neurons, brain gray matter, bone marrow and placenta [3]. This enzyme is an elliptical α/β protein polymer with a 12-stranded central mixed β-sheet surrounded by 14 α-helices. When the catalytic domains of other serine proteases are analyzed, there

are similarities and the catalytic domain has a serine-histidine-glutamate triplet structure [4].

When we look at its role in the brain, acetylcholinesterase is the enzyme that hydrolyzes acetylcholine, which is responsible for the passage of the stimulus between neurons and prevents the post-synapse passage of the stimulus [5].

Looking at this role of acetylcholinesterase, it is an important enzyme despite its secondary position after and before the synapse [6,7]. This enzyme terminates neurotransmission in the nervous system and leads to the accumulation of acetylcholine, overstimulation of various receptors and consequent impairment of neurotransmission. Synthesized acetylcholinesterase inhibitor drugs have attempted to reverse these conditions by inhibiting this enzyme. These inhibitory drugs are either reversible or irreversible. Reversible inhibitors are generally used in Alzheimer's disease, which we call neurodegenerative disorders. In today's pharmaceutical market, the approved active ingredients donepezil, rivastigmine and galantamine stand out [8].

Alzheimer's disease is the most common form of dementia. It is a progressive neurodegenerative disease that occurs at the onset of dementia. In this case, there is an initial cognitive decline, followed by speech, visual changes, and motor systems are affected. It is an extremely bad condition [9]. This disease is associated with aging and causes severe deficiencies in choline acetyltransferase activity in the cerebral cortex. These patients have reduced cholinergic activity, so acetylcholinesterase inhibitors are used to increase cholinergic activity and improve cognitive function. The most promising treatment for this disease is to try to increase the acetylcholine neurotransmitter in the brain. But of course, side effects such as hepatotoxicity develop with the drugs [10].

Thiadiazole and benzodioxole rings are compounds that exhibit a wide range of biological activities, especially known to be effective on acetylcholinesterase. It is found in the structure of compounds can be used in the treatment of Alzheimer disease [11-15]. When previous studies were examined, acetylcholinesterase inhibition activity was observed in many compounds containing thiadiazole and benzodioxole rings [16, 17].

In this study, a new compound containing thiadiazole and benzodioxole rings was synthesized, its structure was determined, molecular docking studies and biological evaluation of their acetylcholinesterase inhibition were carried out.

2. MATERIALS AND METHODS

2.1. Chemistry

All reagents are purchased from chemical suppliers (Sigma-Aldrich Corp., USA or Merck KGaA, Germany). NMR spectroscopy was recorded ^{1}H-NMR 300 MHz FT-NMR spectrometer; ^{13}C-NMR, 75 MHz spectrometer (Bruker Bioscience, Billerica, MA, USA) in DMSO-d_6. In the NMR spectra splitting patterns were represented as follows: singlet (s); doublet (d); triplet (t). *J* values were expressed as Hertz. Mass spectra were recorded on a LCMS-IT-TOF (Shimadzu, Kyoto, Japan) using ESI. Melting degree determination was determined with the Mettler Toledo-MP90 (Greifensee, Switzerland).

2.1.1. Synthesis of N-(benzo[d][1,3]dioxol-5-ylmethyl)hydrazinecarbothioamide (1)

5-(Isothiocyanatomethyl)benzo[*d*][1,3]dioxole (0.012 mol, 2.32 g) and hydrazine hydrate (0.015 mol) were dissolved in separate beakers by adding ethanol. The hydrazine hydrated mixture was added dropwise to the other mixture in an ice bath environment. At the end of the reaction, the precipitated product was filtered, washed with ethanol and dried.

2.1.2. Synthesis of 5-((benzo[d][1,3]dioxol-5-ylmethyl)amino)-1,3,4-thiadiazole-2-thiol (2)

N-(Benzo[*d*][1,3]dioxol-5-ylmethyl) hydrazinecarbotioamide (**1**) (0.009 mol, 2.025 g) was dissolved in ethanol. Sodium hydroxide (0.012 mol) and carbon disulfide (0.012 mol) were added and this mixture was refluxed for 12h. Afterwards, 20% HCl was added dropwise in an ice bath and the pH was adjusted to 4. At the end of the reaction,

the precipitated product was filtered, washed with ethanol and dried.

2.1.3. Synthesis of 2-chloro-N-(4-trifluoromethylphenyl)acetamide (3)

Chloroacetyl chloride (0.009 mol) was added dropwise to a mixture of 4-(trifluoromethyl) aniline (0.009 mol) and triethanolamine (10 mL) in tetrahydrofuran (10 mL) in an ice bath. After the reaction occurred, the resulting substance was purified and washed with water to remove the salt.

2.1.4. Synthesis of the target compound (4a)

5-((Benzo[*d*][1,3]dioxol-5-ylmethyl)amino)-1,3,4-thiadiazole-2-thiol (**2**) (0.007 mol, 1.87 g) and 2-chloro-*N*-(4-trifluoromethylphenyl)acetamide (**3**) (0.007 mol) in acetone were dissolved. K_2CO_3 was added to the mixture and was refluxed for 12 h. At the end of the reaction, the precipitated product was filtered, washed with ethanol and dried.

*2-((5-((Benzo[d][1,3]dioxol-5-ylmethyl)amino)-1,3,4-thiadiazol-2-yl)thio)-N-(4-(trifluoromethyl)phenyl)acetamide (**4a**)*

Yield: 83%, M.p: 120-123°C ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 4.04 (2H, s, -CH_2), 4.34 (2H, s, -CH_2), 5.92 (1H, s, -NH), 5.98 (2H, s, -CH_2, 1,3-dioxole), 6.77 (1H, d, *J*= 4.6 Hz, benzodioxole), 6.81 (1H, s, benzodioxole), 6.85 (1H, d, *J*= 8.0 Hz, benzodioxole), 6.89 (1H, s, -CONH), 7.68 (2H, d, *J*= 8.7 Hz, trifluoromethylphenyl), 7.78 (2H, d, *J*= 8.5 Hz, trifluoromethylphenyl). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 37.28, 48.09, 48.92, 99.02, 101.35, 103.67, 107.42, 109.61, 118.40, 118.47, 120.30, 120.67, 122.42, 125.54, 127.68, 142.98, 146.90, 166.99, 170.07. HRMS (*m/z*): $[M+H]^+$ calcd for $C_{19}H_{15}F_3N_4O_3S_2$: 469.0622; found 469.0610.

Table 1. IC_{50} (µM) values of the obtained compound against AChE and BChE

Compound	AChE IC_{50} (µM)	BChE IC_{50} (µM)
4a	0.114±0.005	>100
Donepezil	0.0201±0.0014	-
Tacrine	-	0.0064±0.0002

2.2. Acetylcholinesterase Inhibition Assay

The inhibitory activities of compound **4a** against AChE and BChE were determined by modified Ellman method using 96-well plate. The reference drugs in the method were Donepezil and Tacrine. Pipetting in the method was performed by Biotek Precision XS robotic system (USA). Percent inhibition values were measured at 412 nm by BioTek-Synergy H1 microplate reader (USA) [18]. First, compound **4a** was prepared at two different concentrations (10^{-3} and 10^{-4} M) using 2% DMSO and the inhibition potentials were measured. Then the compound was tested at higher concentrations (10^{-5}-10^{-9} M). Inhibition potencies of synthesized compound and IC_{50} of selected derivatives were calculated as reported previously. The results obtained are shown as mean ± standard deviation (SD) [19] (Table 1).

2.3. Prediction of ADME Parameters

SwissADME (online) were used for the prediction of ADME parameters of our synthesized compound [20] (Table 2).

Table 2. Predicted ADME parameters of compound **4a**

Comp	Physicochemical Properties							Lipo.	Druglikeness					Water Solubility		Pharmacokinetics	
	MW	Fsp3	RB	HBA	HBD	MR	TPSA	cLogP	Lipinski	Ghose	Veber	Egan	Muegge	LogS	Class	GI abs.	F
4a	468.47	0.21	9	8	2	110.32	138.91	4.13	+	+	+	-	+	-5.47	Moderately	Low	0.55

Comp: Compounds, MW: Molecular weight, Fsp3: Fraction Fsp3, RB: Number of rotatable bonds, HBA: Number of hydrogen bond acceptors, HBD: Number of hydrogen bond donors, MR: Molar refractivity, TPSA: Total polar surface area, Lipo: Lipophilicity, GI abs: Gastrointestinal absorption, F: Bioavailability score.

Table 3. Molecular docking scores, interaction types and estimated inhibition constants of synthesized compound and AChE (PDB ID: 4EY7)

Comp.	Autodock Results				Vina Results
	Interacting Residues	**Interaction Types**	**Estimated Inhibition Constant, K_i**	**Best Docking Score**	**Best Docking Score**
4a	Trp86	Pi-Pi Stacking	358.32 nM	-8.79	-10.6
	Tyr337	Pi-Pi Stacking			

nM: nanomolar, Docking Score: Estimated Free Energy of Binding (kcal/mol)

2.4. Molecular Docking Study

Molecular docking studies were performed using *in silico* procedure to define the binding modes of compound **4a** in the active regions of enzyme X-ray crystal structures of acetylcholinesterase (PDB ID: 4EY7) [21] were retrieved from Protein Data Bank server (https://www.rcsb.org/structure/4ey7, accessed 20.05.2024). Molecular docking studies were performed as previously reported [22-24]. For docking validation, co-crystallized ligand were re-docked onto target site of 4EY7 and RMSD value have been determined to be less than 0.3 for existing ligand E20 in macromolecule. In the receptor, waters around the previously identified active site E20 (8Å) were left and all other water molecules were removed. Preprocessing and H-Bond optimization for the macromolecule was performed using the Maestro program. Then, the resulting pdb formatted macromolecule was edited with the AutoDock program and saved in pdbqt format. The regular spacing of the grid boxes was determined to be 0.375 Å centered on E20 (40*40*40 Å3). Lamarckian Genetic Algorithm was preferred in the study, detailed results such as docking scores were obtained using both AutoDock 4.2 and AutoDock Vina software [25, 26] and are presented in Table 3 and Figure 1, 2.

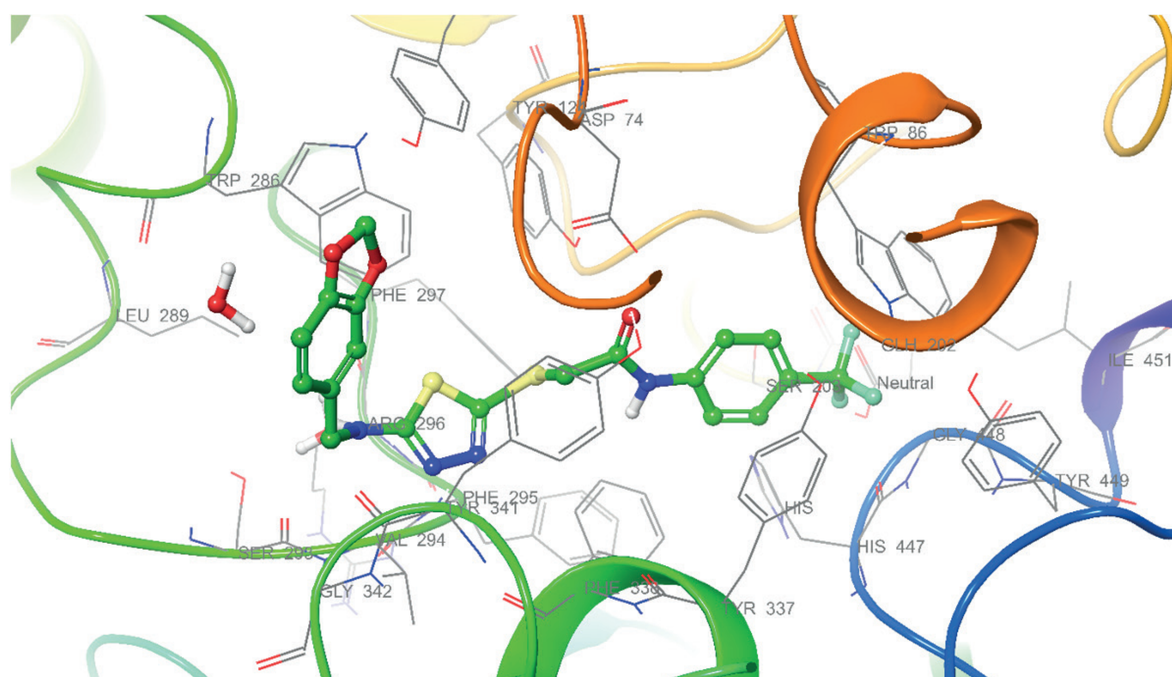


Figure 1. Localization of compound **4a** in the enzyme active site (PDB ID: 4EY7)

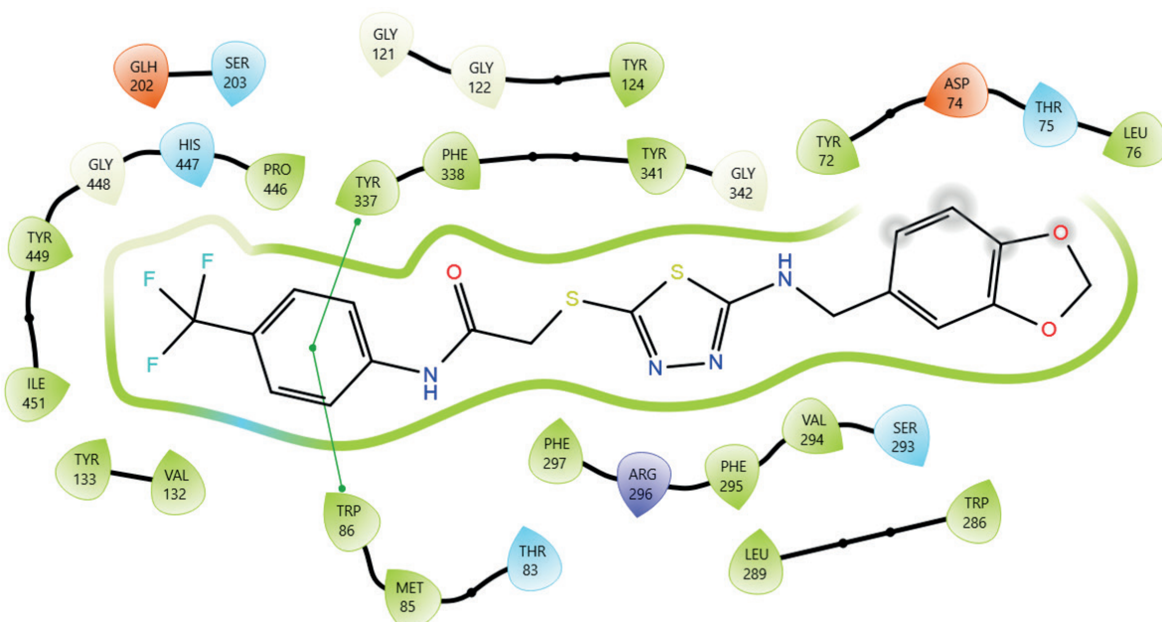
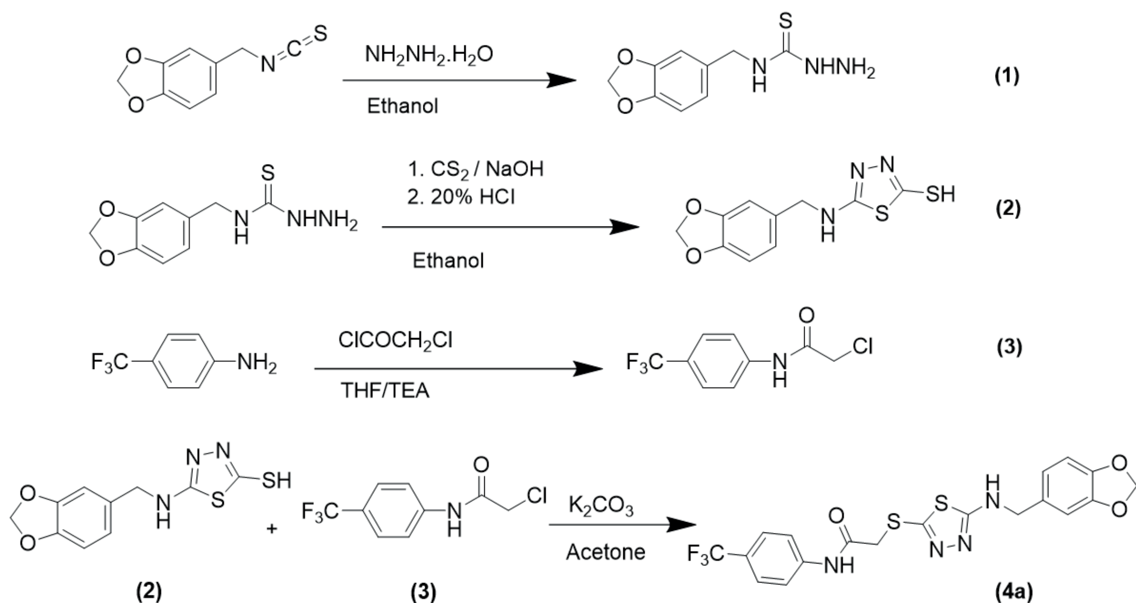


Figure 2. The three-dimensional interacting mode of compound **4a** in the active region of acetylcholinesterase enzyme (PDB ID: 4EY7)



Scheme 1. Synthesis pathway for compound **4a**

3. RESULTS AND DISCUSSION

3.1. Chemistry

Compound **4a** was obtained as presented in Scheme 1. Initially, hydrazinecarbothioamide derivative **(1)** was obtained as a result of the reaction of 5-(isothiocyanato-methyl)benzo[*d*][1,3]dioxol with hydrazine hydrate. Secondly, *N*-(Benzo[*d*][1,3]dioxol-5-ylmethyl)hydrazinecarbothioamide underwent ring closure reaction to obtain thiadiazole derivative (**2**). As the third, 4-trifluoromethylphenyl was reacted with chloroacetylchloride to obtain

2-Chloro-*N*-(4-trifluoromethyl phenyl)acetamide (**3**). Target compound **4a**, 5-((Benzo[*d*][1,3]dioxol-5-ylmethyl)amino)-1,3,4-thiadiazole-2-thiol (**2**) and 2-Chloro-*N*-(4-trifluoromethylphenyl)acetamide (**3**) was obtained by reaction. The structure of compound **4a** was evaluated using spectroscopic methods (HRMS, ^{1}H-NMR and ^{13}C-NMR) (Figures 3-5).

When the NMR data of the compound are examined, it is seen that the protons of methylene groups observed between 4.04 ppm and 4.34 ppm with 2H and 2H. Proton of the amine group were recorded singlet 5.92 ppm. Methyl group of inside benzodioxole come 5.98 ppm and 2H singlet. When benzodioxole ring was examined 3H protons were observed between 6.77 and 6.85 ppm. Trifluoromethylphenyl ring protons were detected as 2H-2H between 7.68-7.68 ppm. When carbon NMR peaks are examined its seen that all carbon peaks are detected as we expect. Aromatic carbon peaks were observed between 110-170 ppm. Moreover, the carbonyl peak we expected around 165 ppm was observed at 166.99 ppm. Most importantly our compound was examined with mass spectra, which is equipped with high resolution liquid chromatography system, and its HRMS data was found to be compatible with its molecular weight.

3.2. Acetylcholinesterase Inhibition Assay

Synthesized compounds were tested at 10^{-3} M and 10^{-4} M concentrations. The inhibition of acetylcholinesterase and butyrylcholinesterase at the initial concentrations of the resulting compound **4a**, donepezil and tacrine is shown in Table 1. According to the activity results, the obtained compound showed higher inhibition activity on AChE than BChE. The IC_{50} value was calculated as 0.0201±0.0014 µM for donepezil. The synthesized compound **4a** gave an IC_{50} value of 0.114±0.005 µM. The 4-substituted phenyl group activity also played an important role.

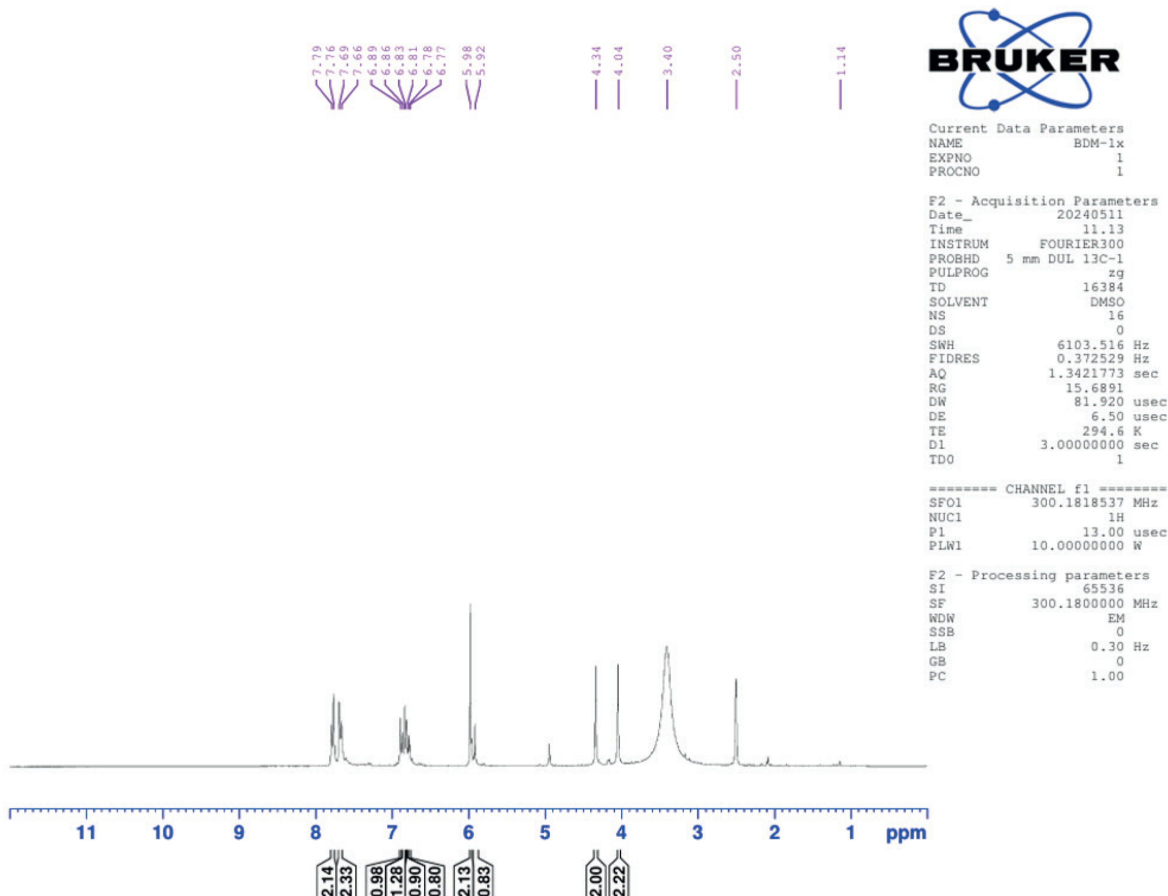


Figure 3. ^{1}H-NMR spectrum of compound **4a**

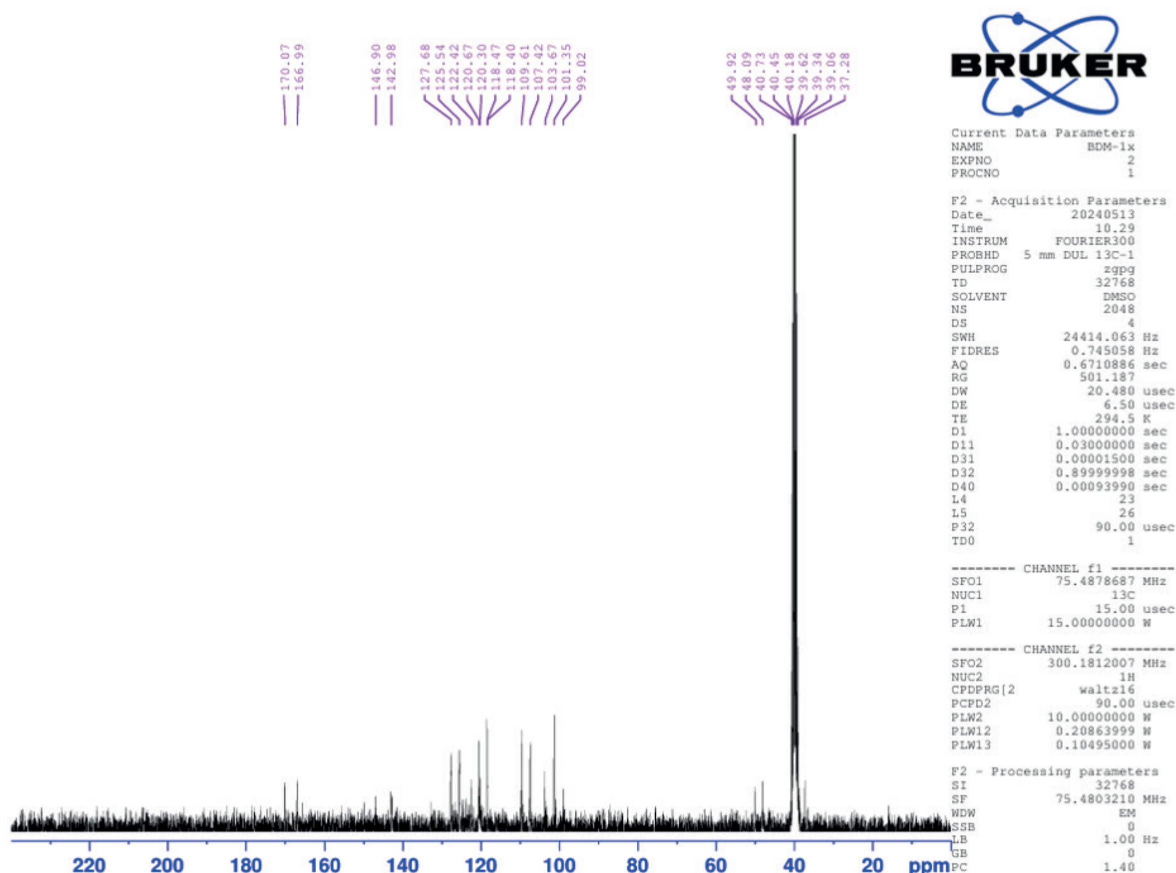


Figure 4. ^{13}C-NMR spectrum of compound **4a**

The results demonstrated that our compound IC_{50} values similar activities compared to the reference drug against AChE.

3.3. Prediction of ADME Parameters

The online SwissADME was used and the estimated ADME parameters of the obtained compound were calculated [20]. Looking at Table 2 showing the results, It was observed that none of the synthesized compounds violated any other drug rules except Egan's rule [27]. Absorption from the gastrointestinal tract provides a preliminary idea about the oral use of the synthesized compound. When the table was examined, it was seen that the absorption of the compound was low. Log S values of the compound is -5.47, and their solubility is estimated to be moderate. The F value, which shows the oral bioavailability of the compound, is 0.55, which is the ideal value [28].

3.4. Molecular Docking Studies

To elucidate the acetylcholinesterase inhibition of compound **4a**, it was subjected to *in silico* insertion procedure with 4EY7 [21]. Active site interactions of the synthesized compound are shown Figure 1-2.

The interaction domain of acetylcholinesterase and its cocrystal ligand Donepezil (PDB ID: E20) has been previously revealed, Tyr72, Trp86, Tyr124, Glu202, Trp286, Ser293, Phe295, Phe297, Tyr337, Phe338, Tyr341, His447 were emphasis to be important for the interaction (https://www.ebi.ac.uk/pdbe/entry/pdb/4ey7/bound/E20#604A).

When the docking poses of compound **4a** were examined, it was clearly seen that it had many interactions such as H-bond and pi-pi stacking with macromolecule. The pi-pi stacking was detected in the 4-trifluoromethylphenyl (Trp86 and Tyr337)

Data File: C:\LabSolutions\Data\Analiz\derya\BDM-1_611.lcd

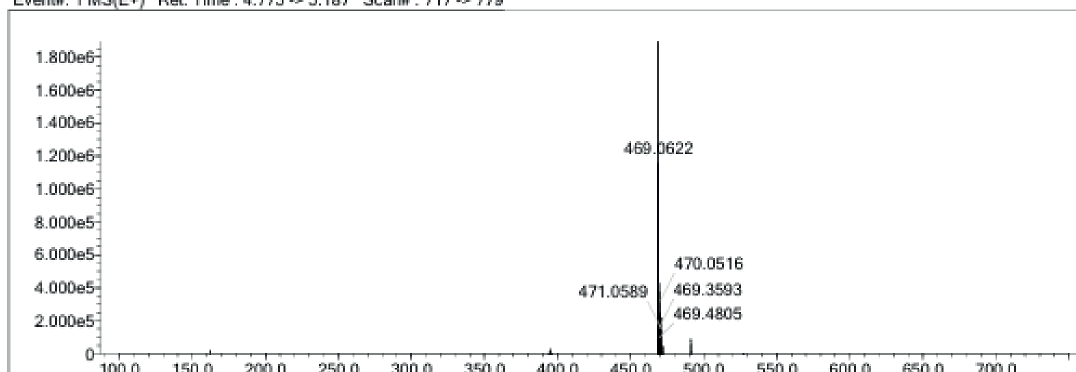
Elmt	Val.	Min	Max	Elmt	Val.	Min	Max	Elmt	Val.	Min	Max	Elmt	Val.	Min	Max	Use Adduct
H	1	8	33	O	2	0	5	S	2	0	2	Ru	2	0	0	H
C	4	4	19	F	1	0	3	Cl	1	0	0	Pd	2	0	0	
N	3	0	5	P	3	0	0	Br	1	0	0	I	3	0	0	

Error Margin (ppm): 5
HC Ratio: unlimited
Max Isotopes: 3
MSn Iso RI (%): 10.00

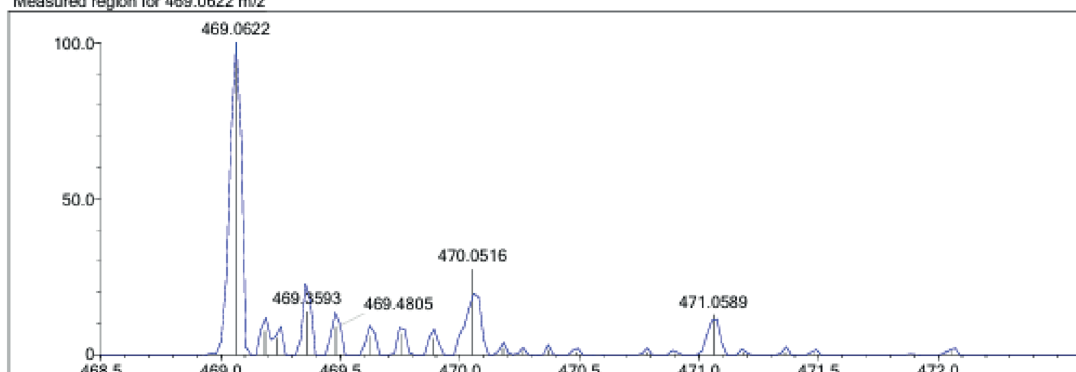
DBE Range: 0.0 - 20.0
Apply N Rule: no
Isotope RI (%): 1.00
MSn Logic Mode: AND

Electron Ions: both
Use MSn Info: yes
Isotope Res: 9000
Max Results: 50

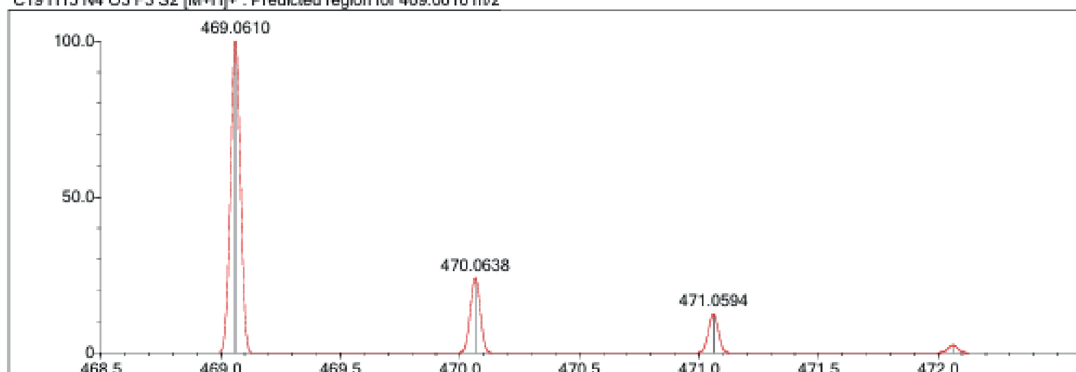
Event#: 1 MS(E+) Ret. Time : 4.773 -> 5.187 Scan# : 717 -> 779



Measured region for 469.0622 m/z



C19 H15 N4 O3 F3 S2 [M+H]+ : Predicted region for 469.0610 m/z



Rank	Score	Formula (M)	Ion	Meas. m/z	Pred. m/z	Df. (mDa)	Df. (ppm)	Iso	DBE
1	71.25	C19 H15 N4 O3 F3 S2	[M+H]+	469.0622	469.0610	1.2	2.56	74.14	13.0

Figure 5. HRMS report of compound **4a**

ring of compound **4a**. Also, there was an H-bond interaction between the benzo[*d*][1,3]dioxol ring and HOH953 (Figures 1, 2). In this study, it was determined that compound **4a** interacted with these residues of acetylcholinesterase in a similar way.

4. CONCLUSION

A decrease in this neurotransmitter in the brain also causes Alzheimer's disease. Previous studies have shown that compounds containing thiadiazole rings have an acetylcholinesterase inhibition effect. In this study, a compound containing benzodioxole and thiadiazole rings (**4a**) was designed, and synthesized, and its acetylcholinesterase and butylcholinesterase inhibition activities were compared with the active ingredients donepezil and tacrine. Compound **4a** showed IC_{50} value of 0.114±0.005 µM against AChE (IC_{50} value of donepezil: 0.0201±0.0014 µM). No significant values were noted for compound **4a** against butyrylcholinesterase. *In silico* studies, the interactions of compound **4a** with the active site of acetylcholinesterease were examined and it was observed that it interacted with Trp86 and Tyr337 residues, which are known to be important in inhibition. In conclusion, this study provides a result that may be important for the development of new agents for neurodegenerative diseases such as Alzheimer's disease.

Acknowledgements

As the authors of this study, we thank Anadolu University Faculty of Pharmacy Central Laboratory for their support and contributions.

Ethical approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Author contribution

Conceptualization, D.O., H.U. and Y.Ö.; Methodology, S.P.G., S.L. and B.N.S.Ö.; Software, D.O., B.N.S.Ö. and H.U.; Formal analysis, S.P.G., D.O. and S.L.; Investigation, S.P.G.; Resources, B.G.; Writing—original draft preparation, S.P.G., D.O., B.G. and H.U.; Writing—review and editing, Y.Ö.; Supervision, Y.Ö. All authors have read and agreed to the published version of the manuscript.

Source of funding

This research received no grant from any funding agency/sector.

Conflict of interest

The authors declare that there is no conflict of interest.

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