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RESEARCH ARTICLE

Investigation of the effect of catechol-o-methyltransferase gene rs4680 polymorphism on trigeminal neuralgia susceptibility

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Abstract

Research has been conducted to explore the genetic basis of trigeminal neuralgia, a persistent pain condition that impacts the trigeminal nerve. *COMT* is an enzyme responsible for inactivating substances and hormones containing catechol and catecholamines. Previous research has linked *COMT* gene polymorphism with various pain conditions, including migraine. Our research aimed to investigate the correlation between trigeminal neuralgia and the rs4680 polymorphism of the *COMT* gene. We conducted a research project which included 10 individuals diagnosed with trigeminal neuralgia and 30 healthy individuals as controls. Following collection of blood samples, we isolated DNA from the samples and then genotyping of *COMT* rs4680 polymorphism was performed with Real-Time PCR, using TaqMan SNP Genotyping Assay. Among the trigeminal neuralgia patients, 2 of them exhibited the AA genotype, 6 had the AG genotype, and 2 had the GG genotype for *COMT* rs4680. The AG genotype was notably prevalent. No statistically significant differences in the distributions of *COMT* genotypes and allele frequencies were found between the experimental (patients) and the control group. However, the AG genotype appeared to be more frequent in the patient group. Moving forward, we plan to expand our study by increasing the number of patients and control subjects. This will enable us to further elucidate the potential relationship between *COMT* gene polymorphism and trigeminal neuralgia.

Keywords: catechol-o-methyltransferase, polymorphism, trigeminal neuralgia

Introduction

Trigeminal neuralgia is a type of short-term pain that develops suddenly and very severely, upon stimulation of the 5th cranial nerve in the face. It is popularly classified as one of the most unbearable pains. It causes severe pain in the face and if untreated, it is a gravedisease that disrupts the patient's quality of life and social activity. For this reason, it is also known as suicidal illness (Merskey & Bogduk, 1994). Trigeminal neuralgia often manifests with dental pain, leading to treatments such as tooth extraction or root canal procedures. However, these treatments may not effectively alleviate the pain, as the source of the pain lies within the trigeminal nerve branches extending to the tooth's root. In some cases, the pain originates from the trigeminal nerve itself or even from the exit region of the brain stem (Zakrzewska, 2002). Therefore, conventional dental interventions may not address the underlying cause of trigeminal neuralgia.

The methylation of catechol fragments is an essential process carried out by the enzyme Catechol-O-methyltransferase (*COMT*), which serves to deactivate catechol hormones, neurotransmitters, and foreign catecholamines found within the body (Axelrod & Tomchick, 1958). In addition to its role in catecholamine regulation, *COMT* is also implicated in melanin biosynthesis (Pavel, 1993). Moreover, researchers have proposed associations between *COMT* and various mental disorders, including depression and schizophrenia (Murphy & Wyatt, 1975). The amino acid valine at codon 108/158 results in a heat-stable version of *COMT* with high activity, whereas methionine at the same position produces a heat-sensitive form of *COMT* with lower activity. This functional variation in the *COMT* gene leads to alterations in the activity of the *COMT* enzyme. Accordingly, the gene is thought to play a role in the pathogenesis of neuropsychiatric disorders, schizophrenia, migraine, Parkinson's disease and bipolar affective disorders (Daniels et al., 1996; Emin et al., 2001).

Recent research has suggested that various variants of the *COMT* gene are associated with cranial pain disorders (Meloto et al., 2015). Polymorphisms in the *COMT* gene have been associated with a variety of pain conditions, including fibromyalgia (Gürsoy et al., 2003), temporomandibular joint disorder (Brancher et al., 2021), migraine (Emin et al., 2001), irritable bowel syndrome (Karling et al., 2001) and ongoing surgical pain (Dharaniprasad et al., 2000). Anotherstudy has shown that the incidence of the disease in patients with trigeminal neuralgia is positively associated with genetic polymorphisms, such as familial inheritance (Panchagnula et al., 2019). Understanding the causes and providing treatment for trigeminal neuralgia has been one of theextensively studied fields With the increase in molecular genetic studies on trigeminal neuralgia, where early diagnosis and treatment are important, it is predicted that the causes of the disease will be better understood, and more permanent genetic solutions can be provided.

The aim of this study is to investigate the hypothesis that rs4680 (Val158Met) polymorphism of the *COMT* gene may cause trigeminal neuralgia disease, which has been supported by various molecular studies.

Materials and methods

Study group

The research encompassed 10 patients diagnosed with trigeminal neuralgia who sought treatment at the Department of Oral, Dental, and Maxillofacial Surgery at Marmara University, Faculty of Dentistry. 30 healthy individuals voluntarily participated in the study as control group. The research protocol was developed following the 2015 guidelines of the Helsinki Declaration and received approval from

the Clinical Research Ethics Committee of Marmara University's Faculty of Medicine (protocol code: 09.2021.324). Prior to the study, every participant signed consent forms that contained comprehensive details about the study's protocol, findings, and assessment of those findings.

Inclusion criteria for the study:

- Trigeminal neuralgia that has been diagnosed by a neurologist
- Episodic attacks of trigeminal neuralgia felt in the maxilla or mandible
- Unilateral neuralgia in the distribution of the second and/or third branches of the trigeminal nerve
- No presence of genetic disorders in their families or in themselves.
- Participants' age between 18- 65.

Exclusion criteria for the study:

- The existence of organic elements like tumors or various brain lesions, such asmultiple sclerosis.
- The presence of unusual facial pain, with symptoms that resemble trigeminal neuralgia.
- Presence of a family history of genetic disease.
- Individuals outside the age range of the study

DNA isolation and genotyping

Peripheral blood samples obtained from patient and control samples were used to isolate DNA (Kazancı et al., 2021). DNA isolation was conducted using the PureLink DNA isolation kit (Invitrogen, Carlsbad, CA, USA) Isolated DNA samples were stored at -20°C until gene region analysis.

Real time- PCR analyses

Real-Time PCR was used for the genotyping of the *COMT* rs4680 polymorphism, using StepOnePlus device, utilizing Taqman SNP Genotyping Assays kits in accordance with the protocols provided by the manufacturers. A and G alleles were determined using Taqman VIC and FAM primers, respectively (Figure 1).

Statistical analysis

Statistical analyses were conducted using the SPSS 25.0 software and the $\chi 2$ (chi-square) test. A p value of less than 0.05 was regarded as statistically significant.

Results and discussion

COMT rs4680 polymorphism analysis revealed that 2 out (20%) of 10 trigeminal neuralgia patients had AA genotype, 6 (60%) had AG genotype and 2 (20%) had GG genotype. When analyzing the distribution of alleles, it was found that 50% were allele A and 50% were allele G. In the control group (n=30), 5 patients had the AA genotype, 14 patients had the AG genotype, and 11 patients had the GG genotype. When analyzing the distribution of alleles, it was found that 40% were allele A and 60% were allele G. The distributions of genotypes and alleles in the patient and control groups were summarized in Table 1. At the same time, a statistical analysis was performed using the chi-square test to compare the distribution of genotypes and alleles of the *COMT* rs4680 polymorphism in the

patient and control groups. The obtained p-values indicate that there is no statistically significan difference between the frequency of the genotype (p=0.6202) and the allele (p=0.4334). These results indicate that there is no significant genetic difference between patients with trigeminal neuralgia and healthy controls regarding the *COMT* rs4680 polymorphism.

Table 1. Genotypic and allelic distribution of *COMT* rs4680 polymorphism in patients with trigeminal neuralgia and control group.

	Genotype				**Allelic Distribution**		
	AA	**AG**	**GG**	**p Value**	**A**	**G**	**p Value**
TN (n=10)	**2**	**6**	**2**		**10**	**10**	
Percentage	**20%**	**60%**	**20%**	**0,6202**	**50%**	**50%**	**0,4334**
Control (n=30)	**5**	**14**	**11**		**24**	**36**	
Percentage	**16%**	**47%**	**37%**		**40%**	**60%**	

- Significance was evaluated at a minimum level of p<0.05. The χ2 test was utilized to compare the results with the control group.

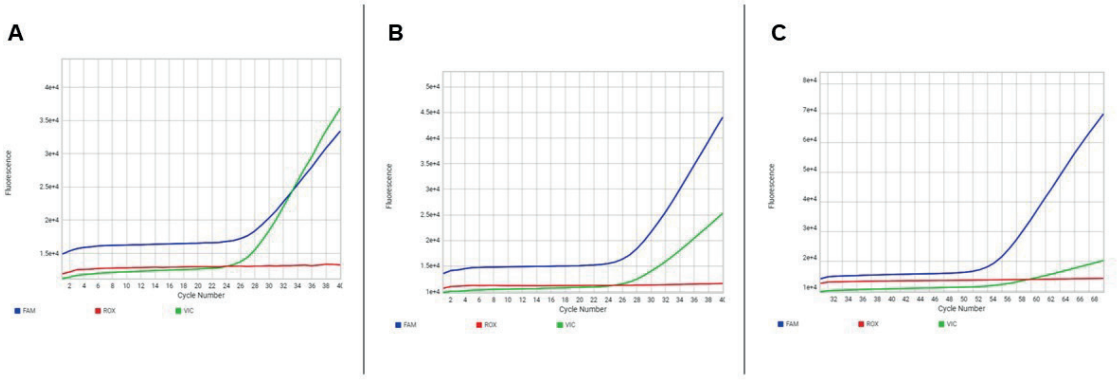

Figure 1. Real-Time PCR image of *COMT* rs4680 polymorphism. FAM represents the G allele (blue curve), whereas VIC signifies the A allele (green curve). The (A) single green curve represents the homozygous genotype AA, (B) the combination of green and blue curves signifies the heterozygous genotype AG, while (C) the single blue curve reflects the homozygous genotype GG.

COMT, encoded by a gene located on chromosome 22q11.2, catalyzes the degradation of catecholamines, particularly dopamine. It has been shown that a functional polymorphism in the 158th codon (Val158Met) of this gene, which involves substitution of methionine for valine, causes a 4-fold variation in enzyme activity (Taerk et al., 2004).

Several genetic studies have investigated the underlying causes of trigeminal neuralgia (TN) and identified potential genes associated with the condition. Costa et al. (2019) examined polymorphisms in the *SCN9A* gene, which encodes the NaV1.7 sodium channel, and the *NTRK1* gene, which encodes the TrkA receptor. Although they found no direct association between these polymorphisms and NTN, they suggested that other genotypes may still play a role in disease pathogenesis. Similarly, Tanaka et al. (2016) discovered the Met136Val mutation in the *SCN8A* gene encoding the sodium channel

NaV1.6, which may increase the excitability of trigeminal ganglion neurons and contribute to TN.

Siqueira et al. (2009) observed downregulation of the sodium channel NaV1.7 and upregulation of NaV1.3 in patients with TN, suggesting that TN may be a type of channelopathy, a condition caused by dysfunction of ion channels. Further studies by Gambeta et al. (2021) showed that mutations in *CACNA* genes encoding calcium channels can increase neuronal excitability and potentially increase susceptibility to TN. Cui et al. (2014) found that a polymorphism in the *SLC6A4* gene encoding the serotonin transporter (5-HTT) was associated with higher susceptibility to TN, increased pain severity, and better response to carbamazepine (CBZ) treatment. This suggests that serotonin transporter genotype may play a crucial role in modulating TN pain and treatment outcomes, as TN patients have a higher prevalence of this polymorphism compared to the general population.

A meta-analysis conducted by Barbosa et al. (2012) has revealed that individuals carrying the Met allele of the rs4680 polymorphism in the *COMT* gene exhibit a heightened susceptibility to fibromyalgia or widespread chronic pain. This association was evident across various Caucasian populations, as well as among Israelis and Turks. Furthermore, the study suggests a similar correlation between fibromyalgia and pain sensitivity with the rs4680 polymorphism in the Brazilian Caucasian population, which comprises a blend of Hispanic Caucasians, indigenous Brazilians, and individuals of African descent. Additionally, several studies propose that the rs4680 polymorphism may play a regulatory role in conditions linked to fibromyalgia pain (Finan et al., 2010,2011).

While the *COMT* rs4680 polymorphism may not be directly linked to migraine headache, it could still play a role in influencing the risk of other chronic headaches or even impact the migraine phenotype. Hagen et al. (2006) proposed that the Met allele might serve as a risk factor for headaches other than migraine among Norwegian women. Similarly, Park et al. (2007) demonstrated that Korean female migraine patients carrying the Met allele experienced more severe headaches, along with symptoms like nausea and vomiting. Considering *COMT*'s involvement in estrogen metabolism and the role of estrogen in migraine pathophysiology, *COMT* polymorphisms have been posited to predispose individuals to migraine. Although a meta-analysis examining the association between the *COMT* gene and musculoskeletal disorders found no significant link between the rs4680 polymorphism and musculoskeletal pain, it did reveal that the *COMT* rs4633 polymorphism may regulate the recovery of disability index scores after surgery (Dai et al., 2010). Moreover, *COMT* pain sensitivity haplotypes have been shown to impact pain ratings, including catastrophic pain ratings, suggesting a potential modulatory role for *COMT* in chronic pain (George et al., 2008).

Research exploring the link between *COMT* and fibromyalgia has primarily centered on a functional polymorphism that results in a single substitution of methionine for valine in exon 4 (Lachman et al., 1996). This amino acid sequence alteration affects the activity of the *COMT* enzyme, with homozygosity for the valine allele exhibiting 3-4 times higher activity compared to the methionine allele. Studies have demonstrated that lower activity associated with homozygosity for the methionine allele leads to significantly reduced dopamine levels in postsynaptic neurons (Egan et al., 2001). In temporomandibular disorder (TMD), chronic and persistent pain states have been found to induce notable changes in catecholamine physiology, which are closely linked to *COMT* enzyme activity (Nackley et al., 2007). The majority of genetic association studies related to *COMT* have concentrated on the commonly studied rs4680 SNP. The A allele of the rs4680 polymorphism has been linked to a higher likelihood of experiencing postoperative pain (Ahlers et al., 2013), fibromyalgia (Cohen et al., 2009), and arthritis (Van Meurs et al., 2009). Furthermore, the rs4680 polymorphism has been linked

to important intermediate phenotypes, including experimental pain (Zubieta et al., 2003), anxiety (Fernandez-de-Las-Penas et al., 2012), depression, and attention (Voelker et al., 2009).

Trigeminal neuralgia (TN) presents as a severe facial pain disorder characterized by an elusive etiology and uncertain genetic underpinnings. Unfortunately, TN often goes undiagnosed or misdiagnosed, contributing to its challenges in clinical management. Research indicates that the incidence of TN varies, with reported rates ranging from 4.3 to 27 new cases per 100,000 individuals annually. Moreover, TN is more commonly observed in women and tends to escalate with advancing age. Community-based studies estimate the lifetime prevalence of TN to be approximately 0.16-0.3%.

The typical onset age for classical TN is around 53 years, while secondary TN manifests earlier, around 43 years, although onset timing can vary considerably across age groups. Notably, secondary TN accounts for 14-20% of patients in tertiary care settings (Mannerak et al., 2021). A Brazilian study showed that there was no significant difference in the genotype distribution of *COMT* rs4680 polymorphism analyzed between TN patients and controls (Romero et al., 2021). This study compared the genotypic and allelic distribution of *COMT* rs4680 polymorphism between patients with trigeminal neuralgia and healthy controls. In our study, AG genotype was found to be more common (60%) in patients with trigeminal neuralgia and it was hypothesized that this genotype may affect susceptibility to the disease. However, the results of the chi-square test showed that this difference was not statistically significant (p=0.6202), suggesting that genotype distribution does not have a strong association with trigeminal neuralgia. There was also no statistically significant difference in allele distribution between A and G allele frequencies (p=0.4334).

Conclusions

These data suggest that *COMT* rs4680 polymorphism may not play a critical role in the pathogenesis of trigeminal neuralgia. However, due to the limited sample size of our study, these results need to be confirmed by studies with larger sample groups. Barbosa et al. (2012) found that *COMT* rs4680 polymorphism may have a significant association with other pain disorders, especially fibromyalgia and temporomandibular joint disorders. Therefore, larger and comprehensive genetic studies are needed to better understand the role of this polymorphism in neuropathic pain syndromes such as trigeminal neuralgia. Although the results of our study do not fully reveal the potential impact of *COMT* rs4680 polymorphism on the development of trigeminal nerve diseases. Patients with neuralgia emphasize the importance of genetic research in this field. In particular, a better understanding of the genetic factors involved in the pathogenesis of neuropathic pain syndromes may facilitate the development of more personalized treatment approaches in the future. In this context, examining larger patient groups and evaluating different genetic variants together in future studies may help to better understand the genetic basis of trigeminal neuralgia and other chronic pain disorders.

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Conflict of interest

The authors disclose no conflicts of interest related to the publication of this manuscript.

Data availability statement

Data can be obtained from the corresponding author upon a reasonable request.

Ethics committee approval

Marmara University Faculty of Medicine Clinical Research Ethics Committee, Istanbul, Türkiye (protocol code: 09.2021.324) approved all the experimental procedures.

Authors' contribution statement

The authors acknowledge their contributions to this paper as follows: **Study conception and design:** N.A., B.T.A., K.U.; **Data collection:** N.A., F.G., G.D.; **Analysis and interpretation of results:** Ö.Ö.Y., B.T.A.; **Manuscript draft preparation:** N.A., Ö.Ö.Y. All authors reviewed the results and approved the final version of the manuscript.

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RESEARCH ARTICLE

Electrochemical activation and characterization of carbon cloth

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Abstract

Here, carbon cloth (CC), which is a disposable, inexpensive, conductive substrate, was electrochemically activated for the formation of functional groups on the electrode surface. The electrochemical activation of commercial CC was achieved in various acidic solutions such as 0.1 M H_2SO_4, 0.1 M HCl and 0.1 M HNO_3 to create functional groups on the surface of the gas diffusion layer by applying a constant 100 mA current (galvanostatic) for 10 s, 20 s, and 30 s, respectively. The electrochemical measurements were conducted using a 3-electrode system, including disposable carbon cloth as a working electrode, saturated Ag/AgCl as a reference electrode and Pt wire as a counter electrode. The modified CCs were tested via cyclic voltammetry using 5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ redox probe. Electrochemical experiment results showed that acid treatment of CC resulted in a significant increase in peak current compared to bare CC, indicating formation of functional groups on the electrode surface and improved electrical conductivity.

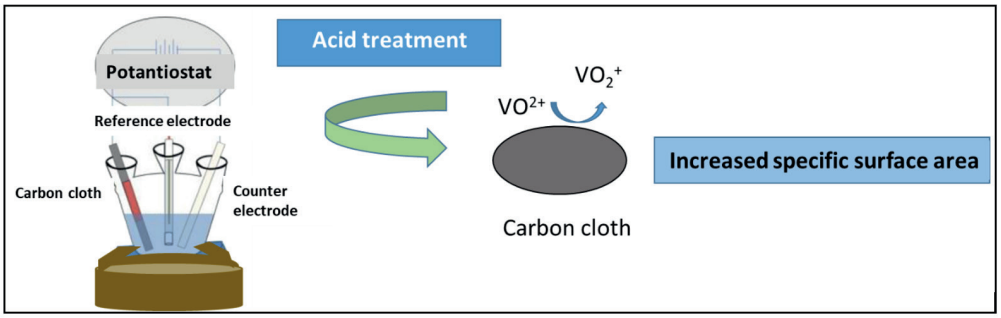
Keywords: *Carbon cloth, electrochemical activation, gas diffusion layer, voltammetry*

Introduction

Carbon cloth (CC), which is a conductive textile, has drawn attention due to its outstanding properties, such as good electrical conductivity, stability, high surface area, high capacitance (Bi et al., 2016; Shao et al., 2017). CC-based electrodes (CCE) are advantageous, because they are inexpensive, portable, disposable, and flexible. Since CC has a porous structure, it offers fast ion transport through increased number of channels, leading to improved diffusion of electrolyte into the electrode (Razali & Majid, 2019). In addition, CCE has a simple operation compared to the commercial glassy carbon electrode (GCE), since pretreatment steps such as polishing and ultrasonic cleaning are not necessary before using CCE. CC substrate is more advantageous over glass-based or metal-based electrodes due to its thin and soft nature, and ease of obtaining various shapes using scissors. Thus, CCEs have been widely used in various applications, such as double-layer capacitors (Galinski & Stepniak, 2009), super capacitors (Lewandowski, Olejniczak, Galinski, & Stepniak, 2010), microbial fuel cells (Tsai, Wu, Lee, & Shih, 2009) and wastewater treatment (Huang & Su, 2010).

Afkhami et al. modified the carbon cloth using distilled water and acid, and successfully applied it for removal of nitrate and nitrite from water samples at neutral pH (Afkhami, Madrakian, & Karimi, 2007). While CC sample was submerged in a washing container and eluted with conductivity water at 60°C for two days, nitrogen gas was applied in the washing container to prevent adsorption of CO_2 present in the water. Then, the CC was placed into 4.0 mol L^{-1} H_2SO_4 and HCl solutions to generate surface functional groups. Anions are able to electrostatically attach on the CC surface due to the protonation of -OH groups via acid treatment. Nitrite and nitrate are adsorbed from water by interaction between positive charges on the CC surface and negative charges of the anions. Similarly, Mo(VI) and W(VI) were removed from water using acid-treated high surface area CC (Afkhami, Madrakian, & Amini, 2009). In another study, acid treated carbon cloth was hydrothermally modified with porous -Fe_2O_3 nanoparticles by Tai group (Mahesh, Shown, Chen, Chen, & Tai, 2018). A mixture of HNO_3 and H_2SO_4 (3:1) was used to pretreat the CC surface at 80°C for 2 h. Then, synthesized α-Fe_2O_3 was attached to the CC surface due to the hydroxyl and carboxyl groups. Finally, they used highly conductive modified CC to detect dopamine. Xu et al. developed an electrochemical sensor based on Au nanoparticles/polyaniline/CC for detection of glucose (Xu et al., 2017). CC was incubated with the use of a 1.0 mM $HClO_4$ solution and PANI clusters were formed at 40 Acm^{-2} for 3 h. After that, PANI/CC was used as a supporting electrode to deposit AuNPs to the surface. The modified flexible sensor was successfully applied for detection of glucose in human serum. Thus, acid treatment of CC is useful to modify electrode surfaces with nanomaterials and developing sensors for detection of biomarkers and heavy metals.

Here, new, disposable, low-cost and simple pretreated CCEs with different functional groups using single step electrochemical activation are presented for the first time. The functionalized electrodes were characterized via cyclic voltammetry (CV) (Scheme 1).



Scheme 1. Schematic representation of the acid functionalized carbon cloth.

Materials and methods

Without further purification, all chemicals and reagents were used as obtained. CC was purchased from the Freudenberg Group (Germany). The electrochemical measurements were performed using a potentiostat (Gamry). A traditional 3-electrode system using disposable carbon cloth, a saturated Ag/AgCl electrode and Pt electrode were utilized for electrochemical studies. CV measurements were carried out using 5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ in 0.1 M KCl solution. Solutions were prepared with deionized (DI) water (18.2 MΩ).

Electrochemical activation of CC

The electrochemical activation process of commercial CC was conducted using three-electrode configuration in different acidic solutions, including 0.1M H_2SO_4, 0.1M HCl and 0.1M HNO_3, to obtain different functional groups on the surface of the gas diffusion layer (Fig.1). At first, the carbon cloths were placed into specially designed sample holders. 5 cm^2 surface area of the CCE had contact with the solution when it was immersed into the coating bath, and an electrochemical activation process occurred on this surface. The activation process was carried out by applying a constant 100 mA current (galvanostatic) for different durations. After the activation process, the gas diffusion layer was taken out from the sample holder, followed by washing with distilled water and drying under a halogen lamp. The geometric surface area of acid treated CC electrodes is around 0.196 cm^2.

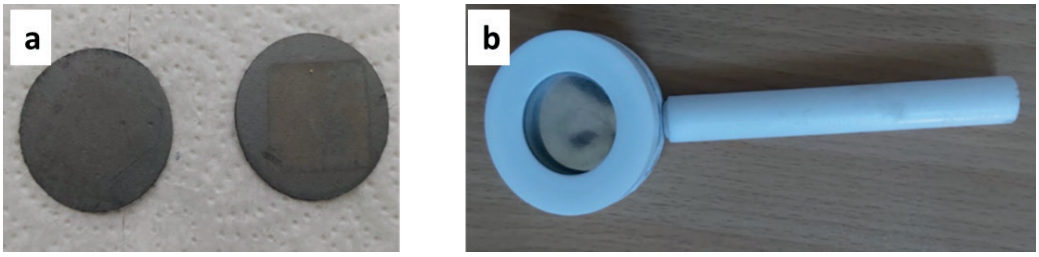


Figure 1. (a) Acid treated carbon cloth, **(b)** sample holder.

Results and discussion

Determination of electroactive surface area of the CCE

The commercial carbon cloth was electrochemically activated in acid solutions (H_2SO_4, HNO_3, HCl) at a constant current for 10 s, 20 s and 30 s, respectively. The acid treatment of the CCEs with H_2SO_4 and HNO_3 provides the hydroxyl and carboxylic groups on the surface of the electrodes, resulting in improvement of the CC hydrophilicity (Zhang, Yu, Shen, & Hu, 2020).

The electrochemical behavior of the bare and acid treated CCEs was investigated using cyclic voltammetry in 5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ in 0.1 M KCl solution. The voltammograms of the electrodes were recorded at various scan rates between 10 mV s^{-1} and 300 mV s^{-1} and are shown in Fig. 2-9a. The anodic peak current densities (I_{pa}) of bare CCE, H_2SO_4 treated CCE via electrodeposition for 10 s, 20 s and 30 s, HCl treated for 10 s, 20 s, HNO_3 treated for 10 s, 30 s at a potential window between -0.1 and 0.6 V with a scan rate of 50 mV/s are 0.32, 1.28, 1.0, 1.05, 0.66, 0.65, 1.28, and 1.22 mA cm^{-2}, respectively. The electroactive surface area of the bare and acid treated CCEs were calculated by using I_{pa} values in the Randles-Sevcik Equation (Equation 1) (Carvalho, Gouveia-Caridade, & Brett, 2010).

$$Ip = 2.69 \times 10^5 AD^{1/2}n^{3/2}V^{1/2}C \quad (1)$$

The electroactive surface area of the bare CCE was ~4 times lower than that of the acid treated CCE. Higher background current responses were obtained with the acid treated CC compared to the bare CC electrodes due to the nature of acid treated carbon materials (Luo, Shi, Li, Gu, & Zhuang, 2001). In addition, the optimum duration for acid treatment was found to be 20 s for H_2SO_4 whereas 10 s for HCl and HNO_3.

Previously, it was reported that different functional groups were generated, affecting adsorption or catalysis and the formation of electric double layers because of the interaction between carbons with different complexes (Nian & Teng, 2002; Noh & Schwarz, 1990). The capacitance increased due to the adsorption of ions (McCreery, Cline, McDermott, & McDermott, 1994). Oxygen functional groups are generated on carbon surfaces in the presence of oxidizing acid solutions, such as nitric acid or sulfuric acid via treatment. Therefore, oxygen functional groups offer redox activity to improve the pseudo-capacitance (Koresh & Soffer, 1977).

Electrochemical characterization of CCE

A 5 mM $[Fe(CN)_6]^{3-/4-}$ electrolyte solution including 0.1 M KCl was utilized in order to electrochemically characterize the CCE. The effect of the scan rate on the peak currents was evaluated in Fig. 2-6, which shows CV curves of bare CCE and acid treated CCE. As can be depicted in Fig. 2-6, current responses increased with increasing scan rate from 10 mV s^{-1} to 300 mV s^{-1} , demonstrating diffusion-controlled reaction process (Adhikari et al., 2017). While the reduction peaks were observed at negative potentials, the oxidation peaks were at positive potentials with the increase in the scan rates. The ion diffusion for electronic neutralization during a rapid faradic reaction might lead to an increase in internal diffusion resistance (Zhao et al., 2019).

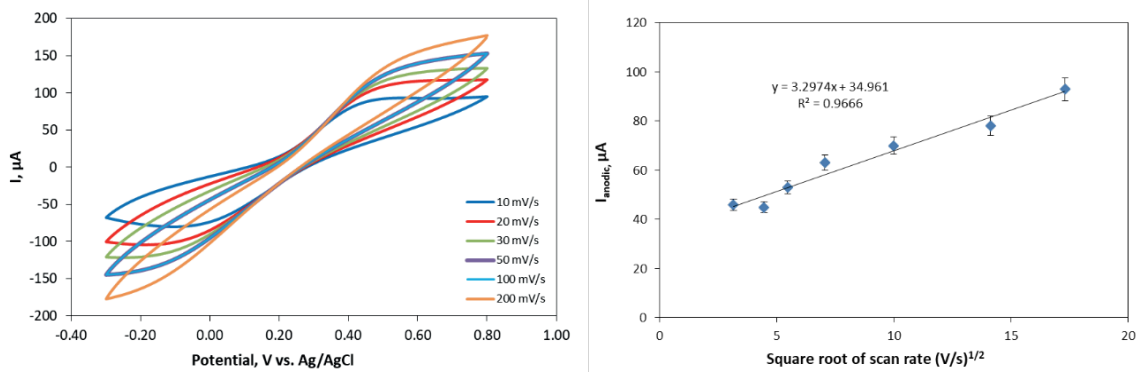


Figure 2. (a) CV of bare CCE versus scan rate from 10 to 300 mV s^{-1}, (b) The square root of the scan rate vs. Ipa at different scan rates for bare CCE.

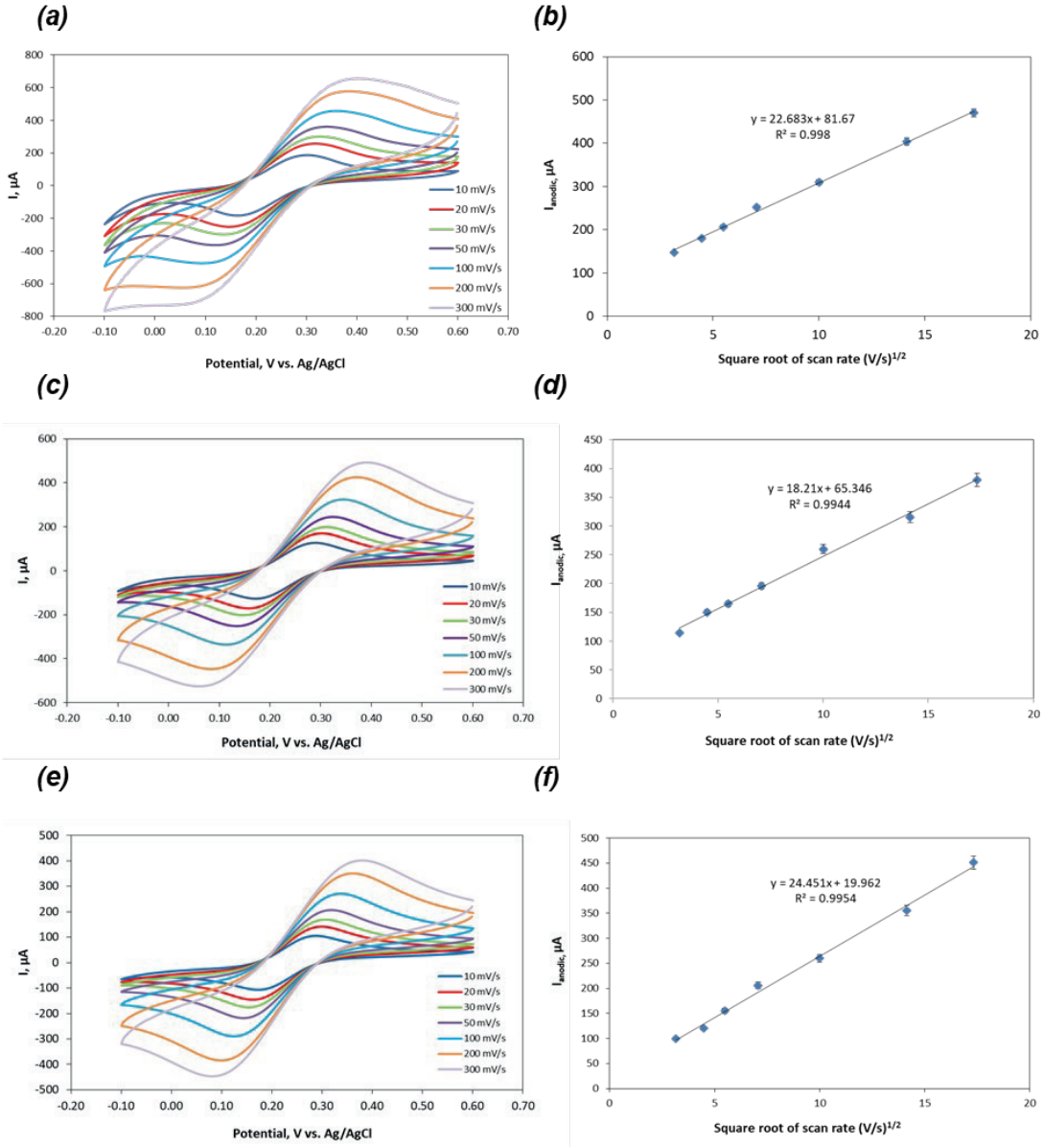


Figure 3. (a) CV of H_2SO_4 treated (10 s) CCE versus scan rate from 10 to 300 mV s^{-1}, (b) The square root of the scan rate vs. Ipa for 0.1 M H_2SO_4 treated (10 s) CCE. (c) CV of H_2SO_4 treated (20 s) CCE versus scan rate from 10 to 300 mV s^{-1}, (d) The square root of the scan rate vs. Ipa for 0.1 M H_2SO_4 treated (20 s) CCE. (e) CV of H_2SO_4 treated (30 s) CCE versus scan rate from 10 to 300 mV s^{-1}, (f) The square root of the scan rate vs. Ipa for 0.1 M H_2SO_4 treated (30 s) CCE.

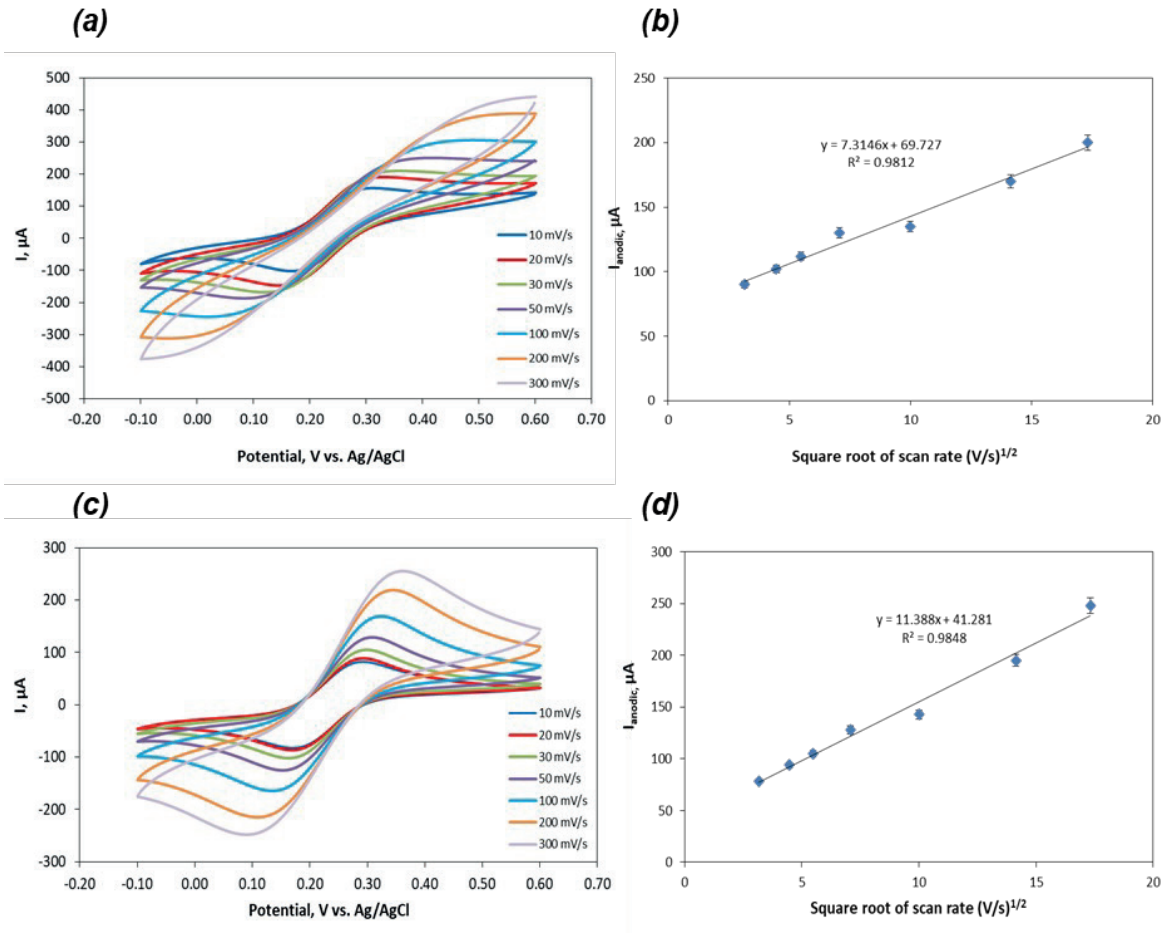


Figure 4. (a) CV of HCl treated (10 s) CCE versus scan rate from 10 to 300 mV s^{-1}, (b) The square root of the scan rate vs. Ipa for 0.1 M HCl treated (10 s) CCE. (c) CV of HCl treated (20 s) CCE versus scan rate from 10 to 300 mV s^{-1}, (d) The square root of the scan rate vs. Ipa for 0.1 M HCl treated (20 s) CCE.

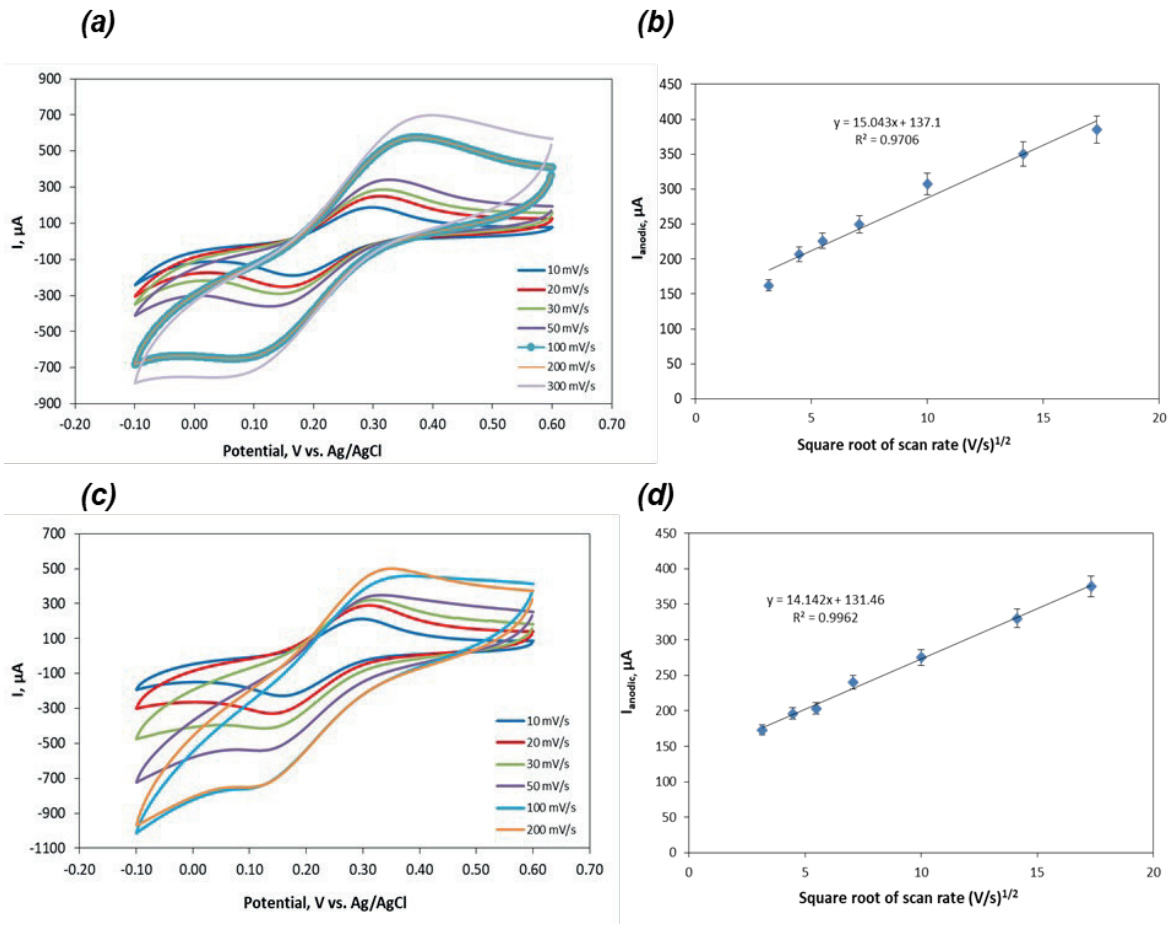


Figure 5. (a) CV of HNO_3 treated (10 s) CCE versus scan rate from 10 to 300 mV s^{-1}, (b) The square root of the scan rate vs. Ipa for 0.1 M HNO_3 treated (10 s) CCE. (c) CV of HNO_3 treated (30 s) CCE versus scan rate from 10 to 300 mV s^{-1}, (d) The square root of the scan rate vs. Ipa for 0.1 M HNO_3 treated (30 s) CCE.

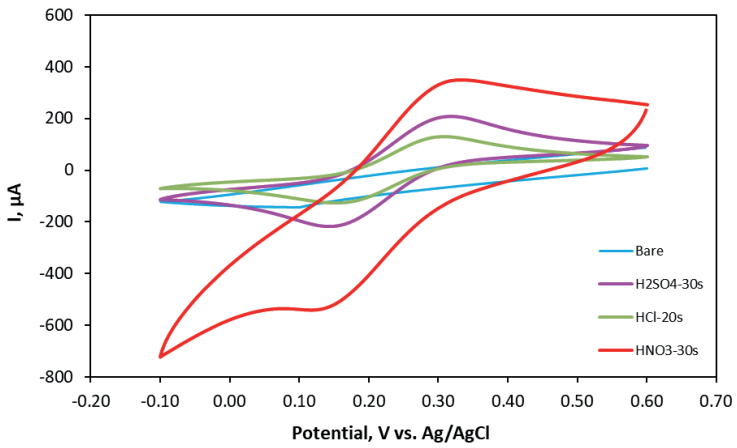


Figure 6. CV of bare CCE and acid treated CCEs at the scan rate of 50 mVs^{-1}.

Acid treated CCE exhibited a higher redox peak and improved electrochemical responses compared to bare CCE. Since CC has higher active surface area and better conductivity than bare CCE, the electron transfer process may be improved (Shi et al., 2019).

It was observed that the CV area of HNO_3 treated (30 s) CCE was much larger than those of the H_2SO_4 (30 s) and HCl (20 s) treated CCE and bare CCE (Fig.10). Moreover, HNO_3 (30 s), H_2SO_4 (30 s) and HCl (20 s) treated CCs demonstrated similar reaction reversibility and reaction kinetics behavior.

Conclusions

A simple and inexpensive electrodeposition method to functionalize carbon cloth substrate is presented in this study. Oxidation through nitric acid and sulfuric acid treatment increased the electrochemical capacitance of activated carbon cloth electrodes. Cyclic voltammetry demonstrated that the presence of the oxygen desorbing complexes enhanced the double-layer formation, as well as the capacitance. Thus, the capacitance improvement can be attributed to the increased presence of the CO-desorbing complexes. Consequently, the faradaic current increased with the total number of oxygen atoms on the electrode surface, enhancing the redox process. Also, the modified electrodes showed fast electron transfer and good reversibility (Wu et al., 2023). Acid modified disposable CC electrode holds the potential for the development of flexible biosensors to detect analytes in various application fields.

Funding

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

Data can be obtained from the corresponding author upon a reasonable request.

Ethics committee approval

Ethics committee approval is not required for this study.

Authors' contribution statement

Study conception and design: T.O.; Data collection: T.O.; Analysis and interpretation of results: T.O. Manuscript draft preparation: T.O. The author reviewed the results and approved the final version of the manuscript.

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RESEARCH ARTICLE

Entrapment of protease from *Bacillus* sp. in polyvinyl alcohol hydrogels

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Abstract

This study highlights the effective immobilization of protease from *Bacillus* sp. in polyvinyl alcohol hydrogels and its characterization. Both free and entrapped proteases exhibited optimal activity at pH 8.0 and 55°C, indicating that the immobilization did not significantly alter the enzyme's fundamental properties. The entrapment in polyvinyl alcohol hydrogels significantly enhanced thermal stability. After 24 hours at 55°C, the free protease retained only 19% of its initial activity, whereas the entrapped protease retained 72%. The entrapped protease showed a longer half-life of 53.3 hours compared to 10.6 hours for the free protease. The K_m and V_{max} values of free protease were determined to be 0.5 mg/mL and 23.3 U/mg protein, respectively, for casein. These values were found to be 0.2 mg/mL and 23.8 U/mg protein, respectively for the entrapped protease. The entrapped protease retained 58% of its initial activity after 5 reuses in a batch reactor. As a result, the entrapment of *Bacillus* sp. protease in polyvinyl alcohol is an effective immobilization method due to its simplicity, low cost, and ability to provide a 5-fold increase in thermal stability.

Keywords: *Bacillus sp.,* immobilization, polyvinyl alcohol, protease

Introduction

Proteases are used in various industrial fields, such as detergents, pharmaceuticals and medicine, occupying 60% of the worldwide enzyme market (Dyer & Weiss, 2022). Proteases catalyze the hydrolysis of peptide bonds, and the hydrolyzates obtained are used in the cosmetic, food and pharmaceutical industries owing to their antioxidant, antihypertensive, antidiabetic, and antimicrobial activities (Brandelli & Daroit, 2024; Elbira et al., 2024; Sun et al., 2024). Therefore, chemical, physical and enzymatic methods have been used to obtain protein hydrolyzates from different sources (Tang et al., 2023). Due to their low cost, safety and environmentally friendly nature, enzymatic methods are preferred over chemical and physical methods (Sun et al., 2024). However, the recovery problem of soluble enzymes from reaction medium, contamination of the final product with enzyme molecules as well as stability problems in organic solvents and/or at extreme pHs and temperatures make soluble enzymes economically unviable biocatalysts in many industrial processes. Enzyme immobilization is a practical and effective method to eliminate the disadvantages of using free enzymes in industrial applications. Moreover, many enzyme properties, such as activity, selectivity, specificity, and inhibition can be tuned by immobilization. Therefore, different natural and synthetic supports have been used for the immobilization of proteases (El-Shazly et al., 2024; Katić et al., 2024; Santos et al., 2024; Ungaro et al., 2024).

The use of polyvinyl alcohol (PVA) for the immobilization of enzymes and/or proteins by entrapment has been popular in recent years because of some advantages of this technique, such as ease of immobilization, low cost, protection the 3D structure of the enzyme from pH and temperature changes, and high protein loading (Toprak et al., 2021). Fernandes et al. (2009) reported that entrapped inulinase displayed high operational stability after 20 consecutive uses at 50°C. Toprak et al. (2021) entrapped nitrilase in PVA hydrogel and showed that 80% activity recovery and 100% immobilization yield were obtained. Alagöz et al. (2022) entrapped ene reductase in PVA hydrogel and demonstrated that entrapped ene reductase had 34.4-fold higher thermal stability than free ene reductase at 30°C.

In this study, a protease derived from *Bacillus* sp. was successfully entrapped in polyvinyl alcohol (PVA) hydrogel, and both free and entrapped forms of the enzyme were extensively characterized. The reuse stability of PVA@protease was evaluated for casein hydrolysis in a batch reactor.

Materials and methods

Materials

Protease from *Bacillus sp*., casein, polyvinyl alcohol, Folin Ciocalteu's phenol reagent, glutaraldehyde (50%, w/w), and trichloroacetic acid were purchased from Sigma-Aldrich Chemie GmbH (Germany). All other chemicals were of analytical grade.

Protease immobilization

The entrapment of protease in PVA hydrogel was performed as described by Toprak et al. (2021). Before protease immobilization, 10 g of solid PVA was added to 80 mL of distilled water, and the mixture was heated to 95°C to melt the solid PVA. The mixture was then cooled to room temperature. For the entrapment of protease, 2 mL of PVA solution and one 1 mL of protease solution (1 mg protein/mL at pH 8.0) were mixed. Subsequently, 100 µL of sample was carefully dripped onto the surface of a polystyrene plate. The plates were then placed in a ventilated evaporator for 24 hours

at 5°C. The entrapped protease samples (PVA@protease) were stored at 5°C until use. The amount of protein encapsulated in PVA hydrogel was determined as described by Lowry et al. (1951). The immobilization yield and recovered activity values were calculated according to Tülek et al. (2021).

Determination of protease activity

Protease activity was measured as described by Abdella et al. (2023). A 1% (w/v) casein solution was prepared in a 50 mM Tris-HCl buffer at pH 8.0. Subsequently, 0.5 mL of the casein solution was taken and incubated in a water bath at 50 °C for 1 min. The reaction was initiated by adding 50 µL of the protease solution with a concentration of 1 mg/mL. After a 5 min reaction time, 1.5 mL of TCA (10% m/v) solution was added to terminate the reaction. The mixture was then centrifuged at 10,000 x g for 15 minutes at room temperature to separate the precipitated casein from the supernatant. The absorbance of clear supernatant was measured spectrophotometrically at 280 nm.

Optimum pH and temperature

To determine the optimum pH for both free protease and PVA@protease, activity measurements were conducted using various buffer solutions across a pH range of 5.0 to 9.0. The buffers used were 100 mM acetate (pH 5.0 and 5.5), 100 mM citrate (pH 6.0), 100 mM phosphate (pH 6.5, 7.0, 7.5 and 8.0), 100 mM Tris-HCl (pH 8.5) and 100 mM borate (pH 9.0). For each pH condition, the enzyme activity was measured, with the highest recorded activity being set as 100%, and the activities at other pH levels calculated as relative values. In addition, the optimum temperature for each protease preparation was assessed by measuring their activity over a temperature range of 40°C to 70°C, maintaining pH 8.0. Similar to the pH optimization, the highest activity observed at any temperature was designated as 100%, with all other activities expressed as relative percentages.

Thermal stability

The free protease and PVA@protease were incubated in a buffer solution without substrate at 55°C to evaluate their thermal stability over time. The residual activity of each preparation was measured at specified time intervals, with the activity at zero time considered as 100%. The residual activities were then calculated relative to this initial activity. The deactivation constant (k_d), half-life time ($t_{1/2}$) and stabilization factor (SF) of each preparation were determined as described by Tülek et al. (2021).

Kinetic parameters

The activity of both free protease and PVA@protease was evaluated across a range of casein concentrations (2-40 mg/mL) to determine the apparent Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) for each enzyme preparation. The measurements were performed under the optimal conditions established for each protease form. To analyze the data and estimate K_m and V_{max} values, a Lineweaver and Burk plot was used.

Reuse stability

Reuse stability of PVA@protease was carried out in a batch reactor (1.1x5 cm) at the determined optimum pH and temperature. After each cycle, the PVA@protease was washed with distilled water and these processes were repeated 5 times.

Results and discussion

In this study, a protease from *Bacillus* sp. was encapsulated in PVA hydrogels and the immobilization yield and recovered activity values were determined to be 100% and 90%, respectively. Sinha

and Khare (2015) reported the immobilization yield was 15% for *Bacillus* sp. EMB9 protease was immobilized on silica nanoparticles by simple adsorption. When silica nanoparticles were activated by glutaraldehyde, the immobilization yield increased to 40%. Thakrar and Singh (2019) encapsulated protease from *Nocardiopsis alba* TATA-5 in calcium alginate and determined the immobilization yield and recovered activity values were 96.6% and 44.8%, respectively.

The changes in the activities of free protease and the PVA@protease with increasing pH were investigated in different buffer solutions in the pH range of 5.5-9.0 and the results are presented in Figure 1. Both proteases show their maximum activities at pH 8.0. As seen in Figure 1, the PVA@ protease exhibits higher relative activity at pH values below and above the maximum pH. These findings indicate that the stability of the protease against pH changes increases after immobilization.

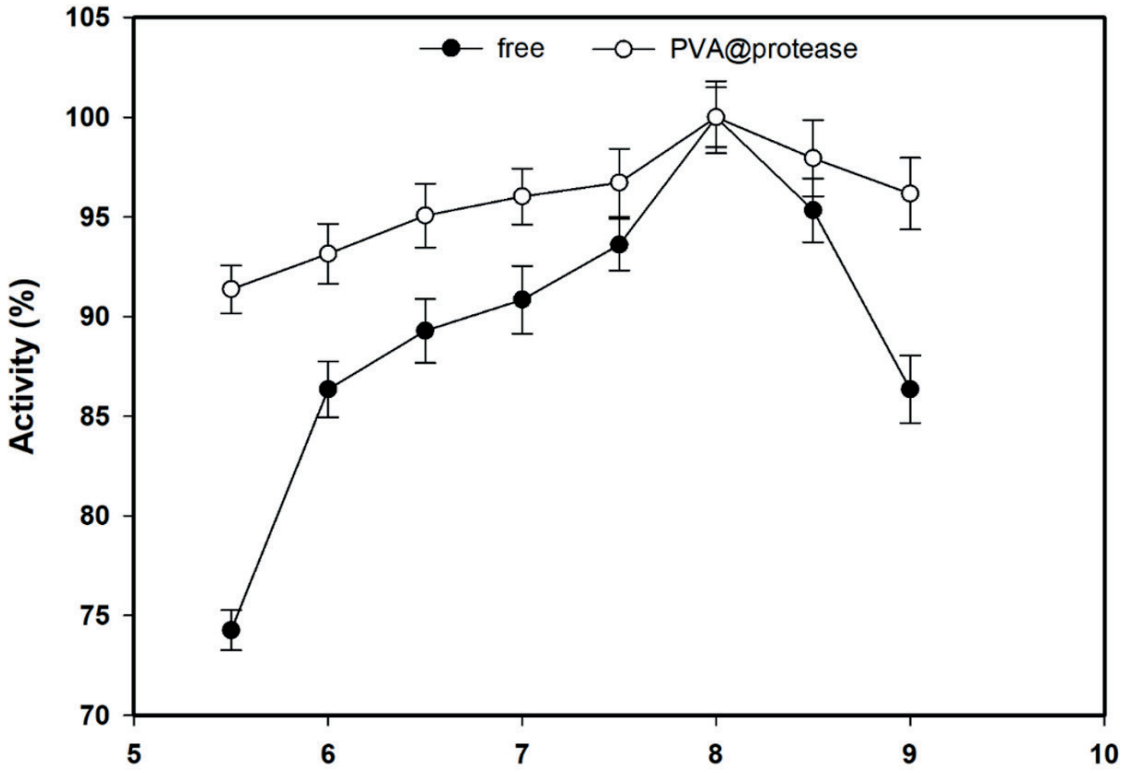


Figure 1. Effect of pH on the activity of free protease and PVA@protease at 55 °C.

The activity change of free and immobilized protease against temperature was measured at pH 8.0 in the range of 40-70°C and the results are given in Figure 2. Both free protease and the PVA@ protease showed their maximum activity at 55°C. Free protease and the PVA@protease displayed almost similar relative activity values in the range of 40-55°C, while the PVA@protease demonstrated higher relative activity values than free protease at 65°C and 70°C. This indicates that the resistance of the PVA@protease increased to denaturation at 65°C and 70°C.

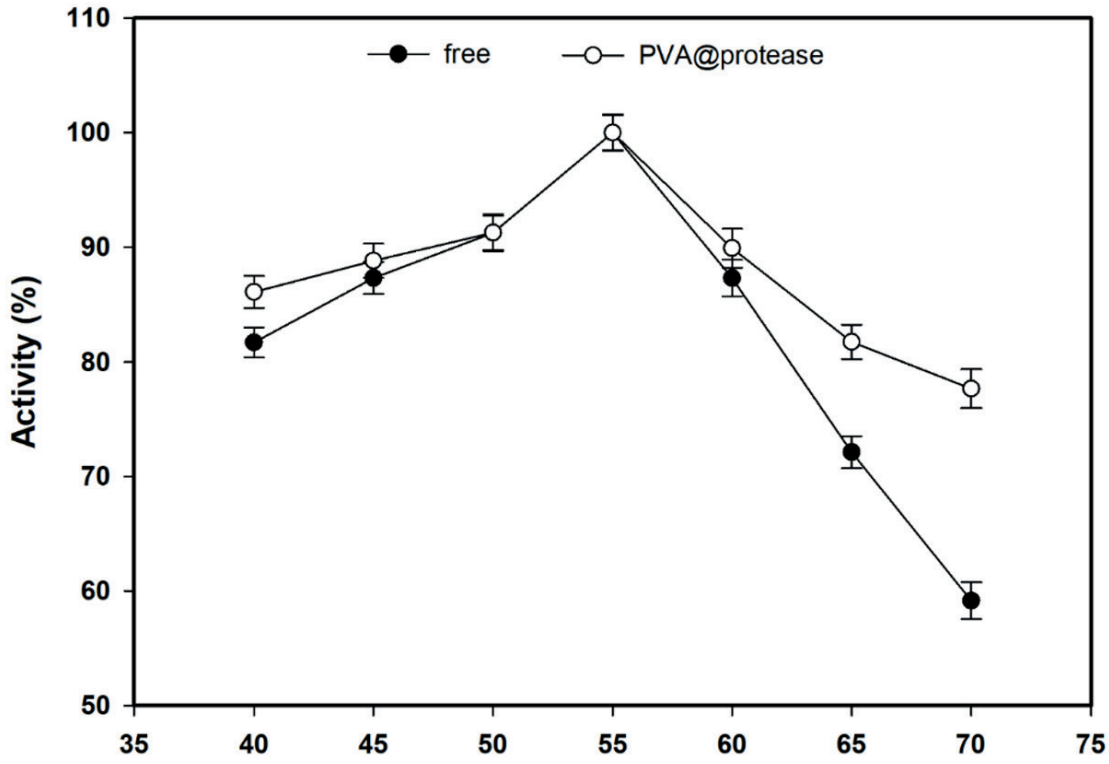


Figure 2. Effect of temperature on the activity of free protease and PVA@protease at pH 8.0.

Ferreira et al. (2003) immobilized *Bacillus licheniformis* protease onto 3-APTES-activated silica supports via glutaraldehyde spacer and found the optimum pH and temperature values as 8.0 and 60°C for both free and immobilized enzymes, respectively. Nakashima et al. (2006) reported the optimum pH value as 8.0 for free *B. licheniformis* protease. Anwar et al. (2009) immobilized *B. subtilis* protease in calcium alginate gel and found the optimum pH and temperature values 7.5 and 50°C for both the free and immobilized protease, respectively. Sinha and Khare (2015) covalently immobilized *Bacillus* sp. protease onto silica nanoparticles and determined the optimum pH and temperature as 9.0 and 55°C for both enzymes, respectively. Ramalho and de Castro (2023) determined the optimum pH and temperature values for both *B. licheniformis* protease immobilized on free and glutaraldehyde-modified chitosan as 9.0 and 60°C, respectively.

To determine the thermal stability of the free protease and thr PVA@protease, the remaining activities of the enzymes were measured for different incubation times at 55°C and the results are given in Figure 3. The activity of the free protease decreased linearly with increasing incubation time and its remaining activity was calculated as 19% at the end of the 24-hour incubation. The remaining activity was calculated as 72% for the PVA@protease under the same conditions. The results show that entrapping the protease in polyvinyl alcohol creates a protective effect against denaturation. The k_d and $t_{1/2}$ values of free protease were calculated to be $65x10^{-2}$ h^{-1} and 10.6 h, respectively (Table 1). These values correspond to $13x10^{-2}$ h^{-1} and 53.3 h for the PVA@protease. SF value was 5.0 at 55°C, showing that thermal stability of the free enzyme enhances 5.0-fold upon immobilization. Wahba (2022) indicated that *B. licheniformis* protease immobilized on functionalized gum tragacanth-agar (iBPR) protected 59.8% of its initial activity after 1 h incubation at 56°C, while

the free protease preserved only 13.80% of its initial activity under the same conditions. Ferreira et al. (2003) reported that *B. licheniformis* protease immobilized on free and 3-APTES-activated silica supports via glutaraldehyde spacer lost 50% and 10% of their initial activities after 2 h incubation at 50°C, respectively. Sinha and Khare (2015) reported that free *Bacillus* sp. EMB9 protease lost 70% of its initial activity after 12 h incubation at 70°C, and the $t_{1/2}$ value at this temperature was 3 h. However, the covalently immobilized *Bacillus* sp. EMB9 protease on silica nanoparticles lost 20% of its initial activity under the same conditions, and the $t_{1/2}$ value was 8.8 h.

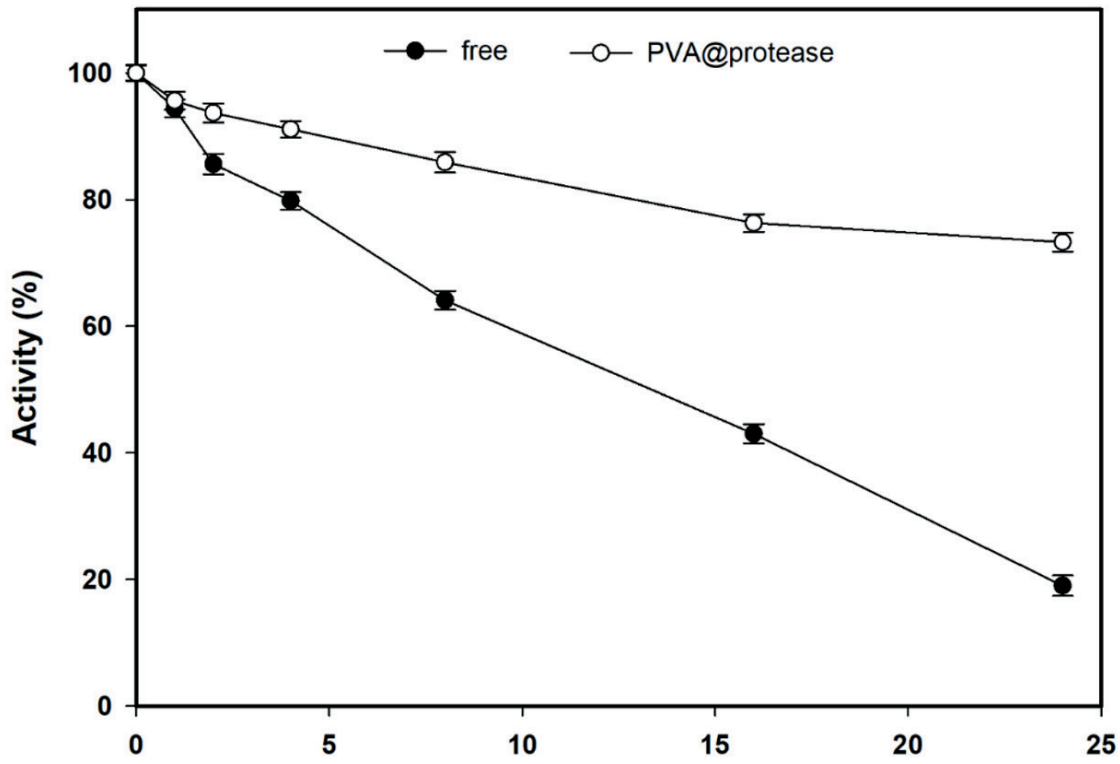


Figure 3. Thermal stability of free protease and PVA@protease at pH 8.0 and 55°C.

Table 1. Thermal stability parameters of free protease and PVA@protease at pH 8.0 and 55°C.

Enzyme form	k_d (h^{-1})	$t_{1/2}$ (h)	SF
Free protease	$65x10^{-2}$	10.6	-
PVA@protease	$13x10^{-2}$	53.3	5.0

Km values for free protease and PVA@protease were estimated as 0.5 and 0.2 mg/mL for casein, respectively. This result indicates that the affinity of the enzyme to substrate increases 2.5-fold upon immobilization. Encapsulating the protease in PVA hydrogel may have indeed promoted proper orientation and therefore, the affinity of the protease to its substrate may have increased. The corresponding V_{max} was determined as 23.3 and 23.8 U/mg (Figure 4).

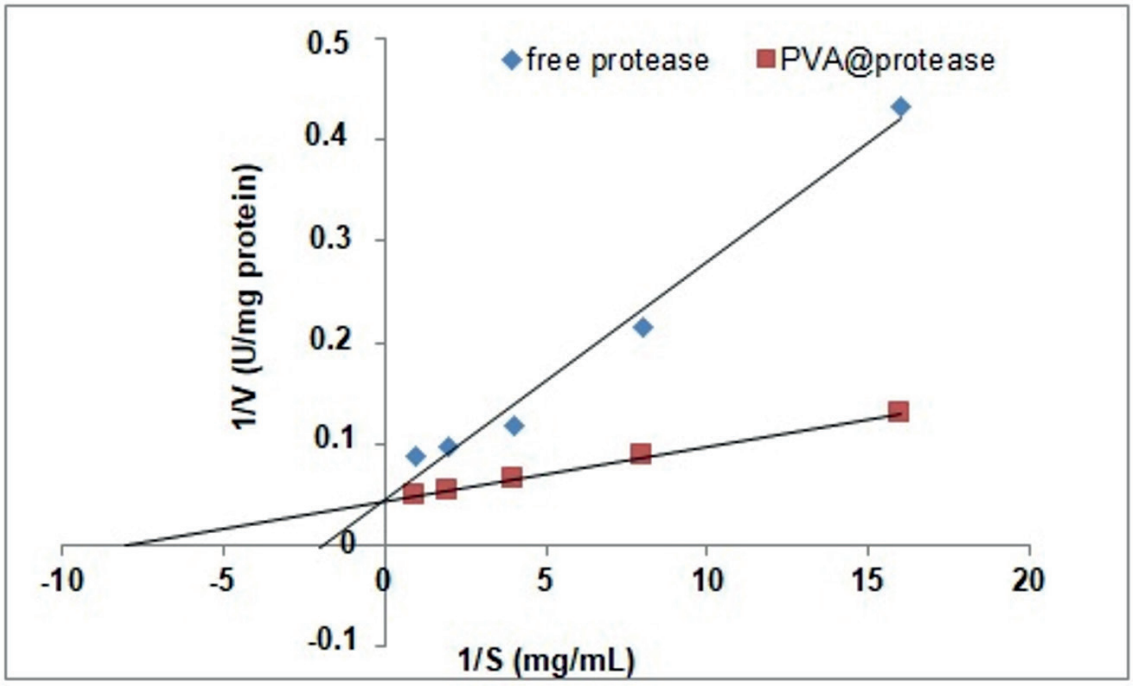
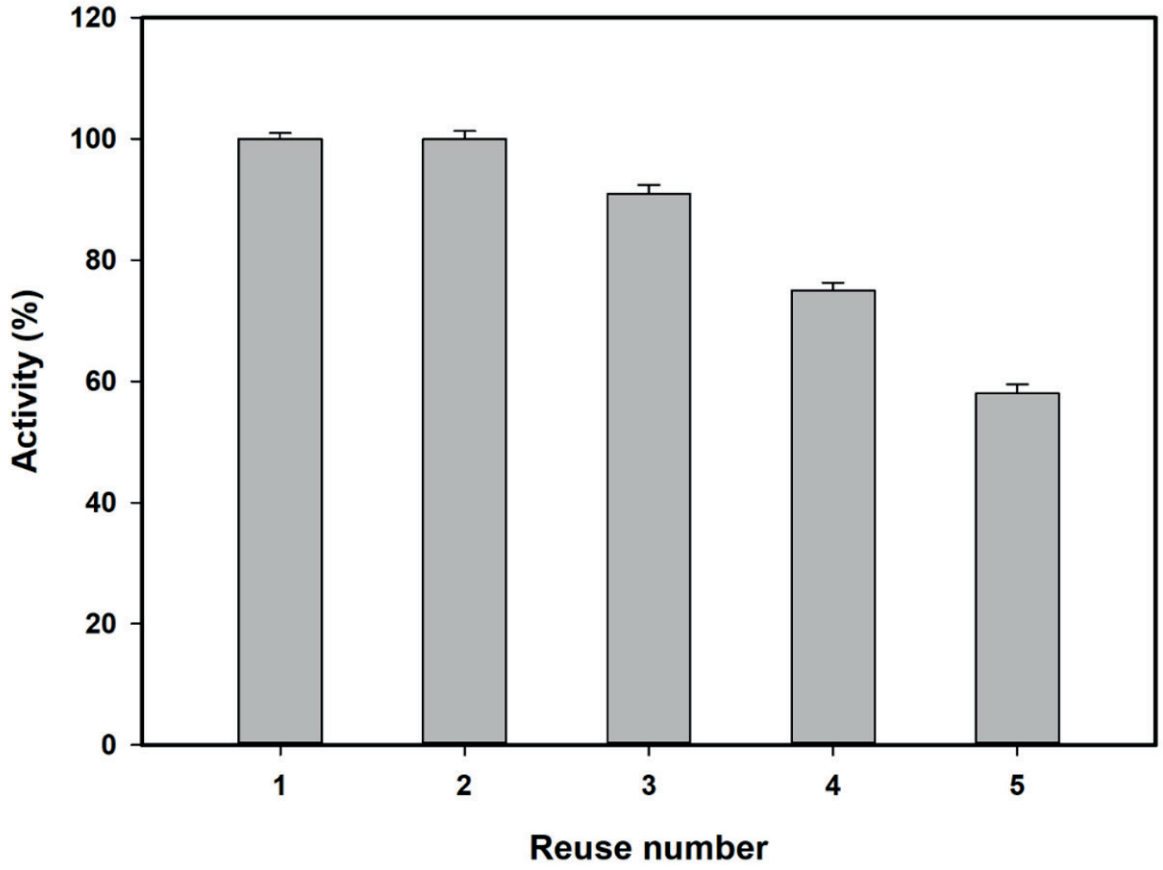


Figure 4. Lineweaver-Burk plot of free protease and PVA@protease at pH 8.0 and 55°C for different casein concentrations.

Wahba (2022) determined K_m and V_{max} values as 0.8 mg/mL and 7.8 U mg protein^{-1}, respectively for free *B. licheniformis* protease and 1.2 mg/mL and 3 U mg protein^{-1}, respectively for iBPR. Abdella et al. (2023) immobilized protease from *B. thuringiensis* on onto activated Alginate/dextrose (Alg/dex/protease) beads and reported that the K_m values of the free and Alg/dex/protease was 2.56 and 5.26 mg/mL, respectively towards casein. The corresponding V_{max} values were determined to be 121.95 and 149.52 U/mL/min. The K_m and V_{max} values of free protease from *B. pumilus* Y7 were 6.05 µM and 20.16 U min^{-1}, respectively towards casein. These values correspond to 27 µM and 34.13 U min^{-1} for its entrapped counterpart in poly(vinylimidazole)/clay hydrogel. Adetunji and Olaniran (2023) reported the K_m value of the free protease from *B. aryabhattai* was 2.023 mg/mL for casein, while the encapsulated protease in alginate showed a lower K_m value of 1.225 mg/mL. The corresponding V_{max} values were found to be 232.56 and 250 U/mL.

To evaluate the reuse stability of the immobilized protease, the enzyme was utilized in a batch reactor for casein hydrolysis across five successive cycles and the results are depicted in Figure 5. The PVA@protease retained 58% of its initial activity after 5 uses. Anwar et al. (2009) observed that a protease from *Bacillus subtilis* immobilized in calcium alginate gel retained only 35% of its activity after three uses. Sinha and Khare (2015) reported that a protease from *Bacillus* sp. EMB9, retained 75% of its initial activity after six uses when covalently immobilized on silica nanoparticles. Ramalho and de Castro (2023) reported that *B. licheniformis* protease immobilized on glutaraldehyde-modified chitosan retained 47% of its initial activity after 3 uses. The iBPR remained 37.6 % of its initial activity after 10 reuses (Wahba, 2022). Thakrar and Singh (2019) demonstrated that the protease encapsulated in alginate beads preserved 85% of its initial activity after 10 successive uses. Duman and Tekin (2020) immobilized protease from *B. pumilus* Y7 in poly(vinylimidazole)/clay hydrogel and showed the immobilized protease lost 35% of its initial activity after six cycles.



| **Figure 5.** Reuse stability of PVA@protease.

Conclusions

In this study, a protease from *Bacillus* sp. was successfully immobilized in PVA hydrogels. The free and PVA@protease were then characterized. Both the protease preparations showed their optimal activities at pH 8.0 and 55°C. The residual activities of free and PVA@protease were found to be 19% and 72%, respectively, after 24 h of incubation at 55°C. The SF value was calculated to be 5, indicating that stability of the protease increased 5-fold by encapsulation in PVA hydrogel at 55°C. PVA@protease protected 58% of its initial activity after 5 uses. In conclusion, entrapment of the protease in PVA hydrogels is indeed a straightforward and effective method due to mild immobilization conditions. Because PVA is biocompatible, the PVA@protease can be used in the preparation of protein hydrolyzates for the food industry.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this study.

Data availability statement

Additional information, data, or materials related to the study can be provided from the corresponding author upon reasonable request.

Ethics committee approval

This research does not involve human participants, animals, or sensitive data.

Authors' contribution statement

The authors acknowledge their contributions to this paper as follows: **Experiment design**: D.Y.; **Data collection**: F.M.H.; **Analysis of data**: F.M.H. and D.Y.; **Manuscript writing**: F.M.H. and D.Y. all authors agree with the findings and conclusions presented in the manuscript.

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RESEARCH ARTICLE

Dual role of natural molecules in bridging cancer and Alzheimer's disease: insights from *in silico* simulations

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Abstract

Cancer and Alzheimer's disease (AD) present significant socioeconomic challenges and remain without definitive cures. Existing chemotherapeutic and anti-Alzheimer drugs approved by the FDA offer limited efficacy and carry notable side effects, underscoring the need for safer, more effective therapies. Our research group has recently focused on identifying natural molecules to treat AD by targeting acetylcholinesterase. Building on this, the current study expands our approach through virtual screening of DrugBank and Zinc databases to discover natural compounds that inhibit Estrogen Receptor Alpha (ERα) for breast cancer treatment. Molecular docking and drugability analyses identified four promising compounds: Queuine, Thiamine, Galantamine, and Folic Acid. The docking scores for Galantamine, Thiamine, Queuine, and Folic Acid were -8.8, -8.3, -8.0, and -7.5 kcal/mol, respectively. These molecules demonstrate interactions with key residues in the ERα binding site such as Glu 353 and Phe 404 through hydrogen bonding and pi-pi stacking. Similar interactions are also maintained in the FDA-approved selective Estrogen Receptor Modulators, Raloxifene and Tamoxifen. ADMET analysis indicated that these natural molecules possess favorable drug-like properties and offer a safety advantage, as they are less likely to induce deep vein thrombosis or pulmonary embolism, which are the serious side effects commonly associated with Raloxifene and Tamoxifen. A thorough literature review further highlights these compounds' neuroprotective effects, suggesting they could serve as dual-purpose therapeutics to address both cancer and AD, paving the way for integrated treatment strategies.

Keywords: *Breast cancer, Queuine, Galantamine, molecular docking, virtual screening*

Introduction

Cancer and Alzheimer's disease (AD) impose a significant socio-economic burden on society, resulting in nearly 10 million deaths and 19.3 million new cancer cases diagnosed in 2020 (Sung et al*.,* 2021). Additionally, at least 50 million people worldwide are living with AD or other forms of dementia (Breijyeh & Karaman, 2020). While treatment options for both conditions have expanded in recent years, they all exhibit significant drawbacks, including toxicity and resistance. Therefore, there is an urgent need for the development of novel and more effective agents to combat these diseases.

Breast cancer is among the most prevalent cancers affecting women globally. It develops when cells within the breast tissue multiply uncontrollably, leading to the formation of a tumor, which can become malignant (cancerous). Breast cancer can be classified into various subtypes based on the presence or absence of specific receptors on the surface of cancer cells. These receptors are critical for guiding treatment options and determining prognosis. Three primary types of receptors play key roles in breast cancer: Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epidermal Growth Factor Receptor 2 (HER2). The estrogen receptor (ER) is a protein found in many breast cancer cells. Estrogen, a hormone primarily produced by the ovaries, binds to these receptors, promoting the growth and division of cancer cells. About 70-80% of breast cancers are estrogen receptor-positive (ER+), meaning they have ER proteins and respond to estrogen. ER+ breast cancers are often more responsive to hormone therapies because blocking estrogen from binding to these receptors can help slow or stop tumor growth (Weigel & Moore, 2010; Keenan et al., 2019).

The human estrogen receptor (ER) is a protein encoded by the ESR1 gene. It exists in ER-alpha (ERα) and ER-beta (ERβ) forms. ERα is more commonly implicated in breast cancer. When estrogen binds to the ERα receptor, it forms a complex that enters the cell nucleus and activates the transcription of genes that control cell proliferation, survival, and differentiation. The role of Estrogen in breast cancer development occurs via proliferation when Estrogen promotes cell division in breast tissue, and by overexposure to estrogen, which can lead to an increased risk of mutations, potentially resulting in cancer. If breast cancer is ER+, the cancer cells can use estrogen to grow and multiply, making it crucial to block estrogen's effects in treatment, which then leads to tumor growth. For ER+ breast cancer, therapies focus on blocking estrogen's ability to stimulate cancer growth. Common treatments targeting ER are based on selective Estrogen Receptor Modulators in which medications like Tamoxifen and Raloxifene block estrogen receptors in breast tissue, reducing the growth of ER+ tumors (Deal & Draper, 2006; Kumar & Kumar, 2018; Mason et al., 2020).

Recent research has investigated the complex relationship between cancer and Alzheimer's disease (AD), with some studies suggesting a positive correlation, while others indicate an inverse relationship. Although cancer and AD often show an inverse relationship in terms of risk, they share several molecular mechanisms, and this complex interplay continues to be an area of active research. Several studies suggest that individuals with AD may have a reduced risk of developing certain types of cancer, such as colorectal or breast cancer. Similarly a higher risk of AD is associated with a lower likelihood of developing breast cancer. (Shafi, 2016; Yuan et al., 2024). Numerous studies indicate that cancer patients exhibit a reduced risk of developing Alzheimer's disease (AD), and vice versa (Y. Li et al., 2024; R. Li et al., 2024; Wang et al., 2024). This inverse association is further reflected in the distinct pathological processes of both conditions: cancer is marked by uncontrolled cell proliferation, whereas AD involves progressive neuronal degeneration and cell death (Driver et al., 2012). An opposite finding reporting a positive correlation is based on selenium-containing compounds that

were synthesized and evaluated for their antioxidant and antiproliferative activity in breast, lung, prostate, and colorectal cancer cell lines. Several of these molecules were found to exhibit dual activity as both anticancer and anticholinesterase agents (Kisla et al., 2024). In conclusion, the exact mechanisms behind this relationship are still unclear, but they may involve shared genetic pathways and environmental factors. These findings suggest that while AD and cancer may share some underlying biological mechanisms, their interaction is complex and more research is required to fully understand this relationship.

Recent work of our study group has primarily been focused on the discovery of natural source molecules for the treatment of Alzheimer's Disease by targeting acetylcholinesterase and beta-site amyloid precursor protein-cleaving enzyme-1 (BACE-1) enzymes (Girgin et al., 2023; Girgin & Kantarci-Carsibasi, 2023). In the current study, a similar methodology will be employed to propose natural molecules as inhibitors of the ERα receptor protein through *in silico* virtual screening and molecular docking simulations. DrugBank (Wishart et al., 2006) and Zinc15 (Sterling & Irwin, 2015) databases are utilized to identify potential natural compounds with dual roles in treating both Alzheimer's Disease and cancer. These results may subsequently be validated through *in vitro* biological assays and *in vivo* cancer models for future research.

Materials and methods

Protein preparation

The crystal structure of the ERα receptor protein (PDB ID: 1ERR), bound to Raloxifene, was retrieved from the Protein Data Bank. The preparation was conducted using Schrödinger's Maestro Molecular Modeling Suite, utilizing the Protein Preparation Wizard module. The protein structure was refined during the process by correcting bond order and adding any missing hydrogen atoms. All heteroatoms, except the native ligand, were excluded, and water molecules within 5 Å of the binding site were retained. Missing loops or side chains were reconstructed using the Prime module. The protonation states were assigned using PROPKA at a pH of 7.0, and restrained minimization was performed with an RMSD threshold of 0.3 Å, applying the OPLS2005 force field for optimization (Jorgensen & Tirado-Rives, 1988).

Ligand preparation and virtual screening

Prior to docking simulations, the ligands were prepared with Schrödinger's LigPrep module (Schrödinger, 2018; Madhavi-Sastry et al., 2013). Plausible 3D conformations are generated and correctly optimized. Ionization states, tautomers, and stereoisomers are assigned. Additionally, missing hydrogen atoms and bond orders are corrected. Energy minimization is performed to prepare the ligands suitable for molecular docking. Epik is used to generate the ionization states and tautomers at pH 7.0 ± 2.0 (Shelley et al., 2007), and chiralities present in the ligands' 3D structures are used to obtain the stereoisomers. Natural source molecules, including metabolites (3093 molecules) and nutraceuticals (107 molecules), and also the FDA-approved drug subset (2619 molecules) were sourced from DrugBank separately. LigPrep was used to generate possible conformers from these molecules, making a total of 21.994 molecules which were subsequently docked into the ERα receptor protein binding site.

Molecular docking

Molecular docking was performed using the Glide SP (standard precision) algorithm in the Schrödinger Suite. A grid box was generated around the ERα receptor protein binding site, centered on the co-crystal ligand Raloxifene centroid, with a size chosen to accommodate ligands up to 20 Å in length. The same grid file was used throughout all docking simulations to ensure reliable comparisons. Ligands were kept flexible, and Epik state penalties were added to the docking scores. The docking protocol was validated by redocking the co-crystallized ligand Raloxifene, with an RMSD of 1.0 Å between the co-crystal and docked conformations. A total of 21.994 natural source molecules were directly docked, and molecules with high binding affinities were filtered and compared with the co-crystal drugs Raloxifene and also Tamoxifen, which are two commercially available drugs on the market.

Results and discussion

In this study, molecular docking simulations were performed to evaluate the binding affinity of various molecules to the ERα receptor protein (PDB ID: 1ERR). Docking simulations of nutraceutical molecules obtained from the DrugBank database revealed Galantamine, Thiamine, Queuine, and Folic Acid as the top four molecules with the highest docking scores. Docking simulations of metabolic molecules, on the other hand, identified metabolites of the control drugs Tamoxifen and Raloxifene, as well as several antidepressant drug metabolites that were deemed inappropriate. The FDA-approved drug subset of Drugbank yields Raloxifene, Astemizole (which is a withdrawn drug due to serious side effects), and Testosterone. Raloxifene is already the benchmark drug for treating breast cancer. By acting as an estrogen antagonist in breast tissue, Raloxifene lowers the risk of developing hormone receptor-positive breast cancer (Cummings et al., 1999). Testosterone is a hormone used to treat hypogonadism, breast carcinoma in women, hence already in use. Testosterone is reported to act as an anti-estrogen, particularly in hormone receptor-positive breast cancers. Competing with estrogen for binding to estrogen receptors, testosterone may inhibit estrogen's stimulatory effects on tumor growth (Glaser & Dimitrakakis, 2015). Finally, the docking simulations of the Zinc15 library subset composed of biogenic and the FDA-approved drugs resulted in Astenile (Prasterone), Alogliptin, Paroxetine, and Galantamine. Astenile, which is a major C19 steroid produced by the adrenal cortex. It is also produced in small quantities in the testis and the ovary, and this molecule is already known as an estrogen receptor binder. Alogliptin is prescribed to manage high blood sugar levels in individuals with type 2 diabetes. Paroxetine, a selective serotonin reuptake inhibitor, is used to treat conditions such as major depressive disorder and panic disorder. Hence, these molecules are not preferred to treat breast cancer ERα. As the literature evaluations, docking scores and safety considerations are taken into the picture, the molecules: Galantamine, Thiamine, Queuine, and Folic acid come forward. These molecules are mentioned to be involved in various cancer types before as well as AD. Hence, they may have a dual role in both diseases. Galantamine is a natural alkaloid primarily used to treat mild to moderate Alzheimer's disease. Originally, it was extracted from the bulbs and flowers of plants but can also be synthesized (Tariot et al., 2000). Queuine is a naturally occurring biochemical compound present endogenously in the human body. It plays a critical role in the synthesis of essential chemicals such as tyrosine, serotonin, dopamine, epinephrine, norepinephrine, nitric oxide, and various lipids, contributing to numerous physiological processes (Fergus et al., 2015). Thiamine, commonly known as vitamin B1, is essential for intracellular glucose metabolism and supports the normal function of vital systems, including the cardiovascular, nervous,

and digestive systems, in most organisms. It is regarded as one of the key vitamins necessary for maintaining overall health (Tylicki et al., 2018). Folic acid is the synthetic form of folate (vitamin B9) that is essential for numerous biological processes. It plays a crucial role in DNA synthesis, repair, and methylation, as well as in the production of red blood cells (Bailey, 2009). Figure 1 demonstrates the molecular structures of these molecules together with control-approved drugs.

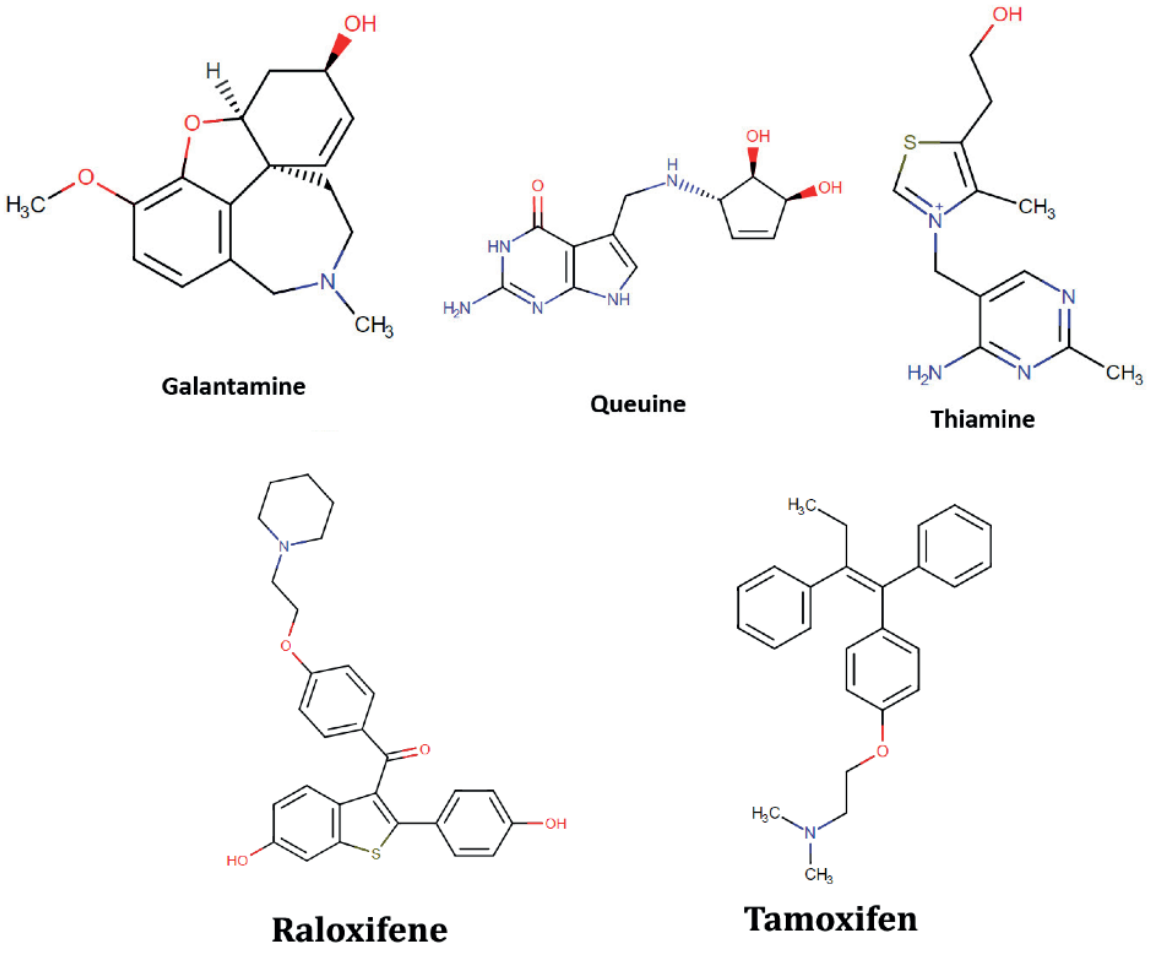

Figure 1. The chemical structures of three promising natural molecule candidates proposed for ERα receptor inhibition along with the FDA-approved drugs Raloxifene and Tamoxifen.

Figure 2 illustrates the virtual screening protocol conducted over the DrugBank and Zinc15 libraries to identify potential inhibitors of the ERα receptor protein. Subsets were downloaded from DrugBank, specifically the nutraceuticals, metabolites, and the FDA-approved molecule sets. Additionally, one subset from the Zinc15 library, consisting of the FDA-approved and biogenic molecules, was obtained. Consequently, four molecule sets underwent virtual screening, beginning with the LigPrep module to generate possible conformers. As shown in Figure 2, these subsets were docked directly into the ERα receptor binding site using Glide SP and ranked based on their docking scores, from the strongest binder to the weakest. Promising compounds identified from each set are also displayed. The green ticks indicate molecules not previously

recognized as ERα receptor protein inhibitors, suggesting the potential for further investigation due to their properties. In contrast, the red crosses mark molecules that are either already in use as ERα receptor inhibitors for breast cancer treatment, withdrawn for various reasons, deemed unsafe, or considered irrelevant and not essential for further studies as discussed above in detail.

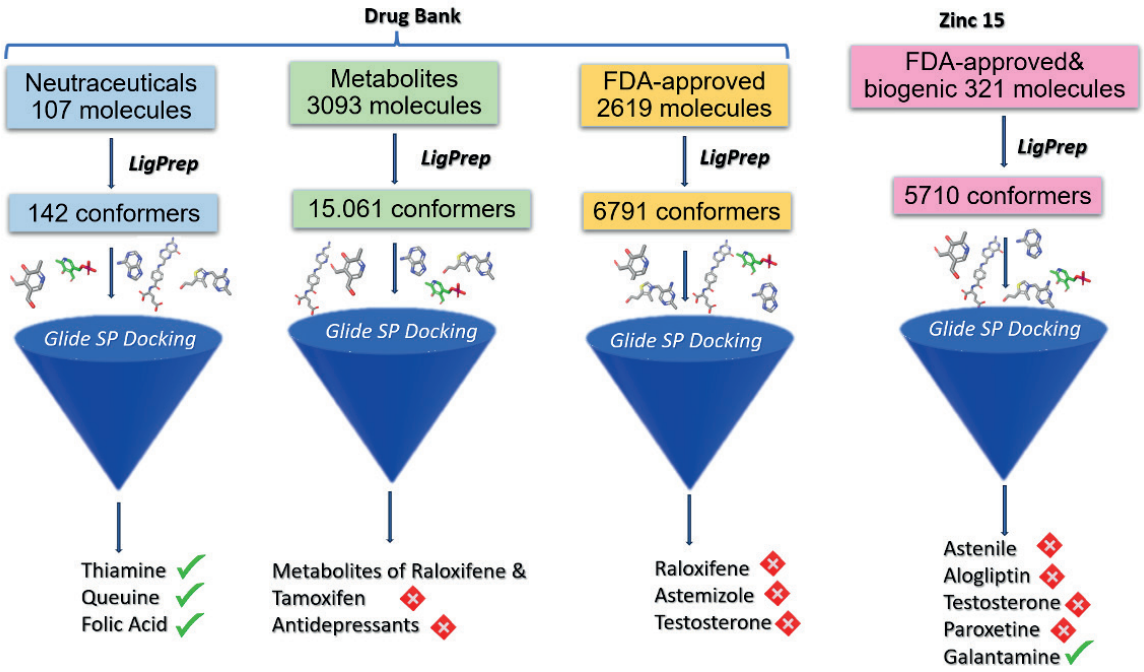

Figure 2. Schematic flow for the filtering procedure conducted on Drug Bank and Zinc Databases targeting ERα receptor protein.

Table 1 presents the properties of the natural hit molecules, alongside the reference control drugs Raloxifene and Tamoxifen. This table details the associated Drug Bank IDs, the origins of each molecule, their relevance to specific cancer types, and the corresponding references. From the table, it can be inferred that Thiamine, Folic acid and Galantamine have been previously linked to breast cancer. However, to the best of our knowledge, literature does not report a connection between Queuine and breast cancer. Nonetheless, all four leading natural molecules have been noted concerning various cancer treatments.

Table 1. Properties of lead natural molecules identified as potential ERα receptor protein inhibitors.

Molecule name	DrugBank ID	Origin	linked to cancer before?	Reference
Thiamine	DB00152	nutraceutical	breast, colon, pancreatic, and hematological cancers	Liu et al., 2018; Comín-Anduix et al., 2001; Bruce et al., 2003, Iimura et al., 2021

Queuine	DB14732	nutraceutical	colon, ovarian, brain, lung, leukemia, and lymphomas	Baranowski et al., 1994; Aytac & Gündüz, 1994; Huang et al., 1992; Fergus et al., 2015
Folic acid	DB00158	nutraceutical	colorectal, breast, pancreatic, and cervical cancers	Qin et al.,2013
Galantamine	DB00674	botanical FDA-approved	colorectal, and breast	İnce et al., 2023
Tamoxifen	DB00675	synthetic FDA-approved control drug	FDA-approved breast cancer drug (selective estrogen receptor modulators)	Jordan, 2003; Jordan, 2006
Raloxifene	DB00481	synthetic FDA-approved control drug	FDA-approved breast cancer drug (selective estrogen receptor modulators)	Deal & Draper, 2006

Our recent study on Alzheimer's disease and acetylcholinesterase enzyme inhibition highlighted the effects of Thiamine and Queuine. The efficacy of these compounds was compared to that of Galantamine, a benchmark natural drug for Alzheimer's treatment. Results demonstrated that Queuine exhibited comparable docking scores, binding affinity, and a more potent IC_{50} value than Galantamine. Conversely, Thiamine was found to be non-cytotoxic even at high concentrations (Girgin et al., 2023). Folic acid has also been recognized for its effects on both various cancer types and Alzheimer's disease. Therefore, we emphasize Queuine, as it has not been previously associated with breast cancer. Additionally, all four molecules listed in Table 1 have been reported to play a role in both cancer and Alzheimer's disease, suggesting a potential positive correlation for molecules that may have dual therapeutic roles in these conditions.

Figure 3 depicts the ERα receptor protein in complex with the FDA-approved drug Raloxifene, which is also the co-crystal ligand in Protein Data Bank structure pdb ID: 1ERR. The figure shows both 3D and 2D docked conformations of Raloxifene and interactions it accomplishes with the binding site. The hydrogen bonding with Glu 353 and Arg 394, and pi-pi stacking through Phe 404 are the most significant interactions.

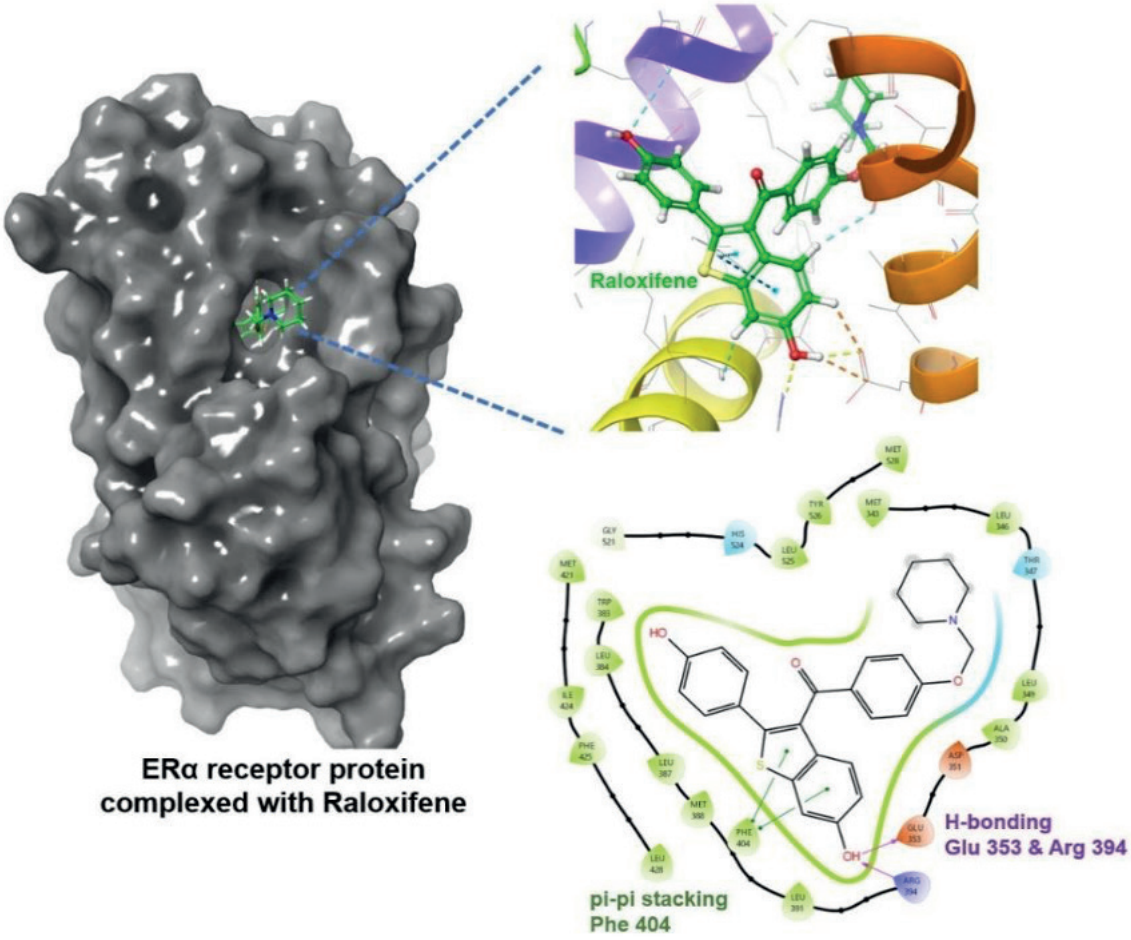

Figure 3. Raloxifene in complex with Erα receptor protein: 3D and 2D interactions in the active site.

Figure 4 depicts Queuine, Thiamine, Galantamine and Folic acid in complex with target receptor ERα. Interactions are displayed for Queuine (Fig 4a and 4b), Thiamine (Fig 4c and 4d), and Galantamine (Fig 4e and 4f) as 3D and 2D representations. Queuine forms hydrogen bonds with Glu 353 and Thr 347, achieving a docking score of -8 kcal/mol. Thiamine interacts via π-π stacking with Phe 404, resulting in a docking score of -8.3 kcal/mol. Galantamine, like Queuine, forms hydrogen bonds with Glu 353, with a docking score of -8.8 kcal/mol. Folic acid performs hydrogen bonds through Glu 353, Leu 387 and Tyr 526, in addition to pi-pi interaction conducted with Phe 404, all contributing to a docking score of -7.5 kcal/mol. The control drug Raloxifene shows interactions with Glu 353 and Arg 394 through hydrogen bonding, along with π-π stacking with Phe 404, and yields a superior docking score of -11.4 kcal/mol. The presence of hydrogen bonding with the Glu 353 residue appears to be a critical factor, as it is consistently observed among the top candidate molecules. The details of the residue interactions and interaction types are also tabulated in Table 2.

Table 2. Results of docking simulations: docking scores, interacting residues, and interaction types of proposed compounds compared with approved drugs.

Molecule	ERα receptor DScore(kcal/mol)	Interacting residues	Interaction type
Galantamine	-8.8	Glu 353	H-bond
Thiamine	-8.3	Phe 404	π-π stacking
Queuine	-8.0	Glu 353 Thr 347	H-bond H-bond
Folic acid	-7.5	Glu 353 Leu 387 Tyr 526 Phe 404	H-bond H-bond H-bond π-π stacking
Tamoxifen	-9.6	Asp 351 Asp 351	H-bond Salt bridge
Raloxifene	-11.4	Glu 353 Arg 394 Phe 404	H-bond H-bond π-π stacking

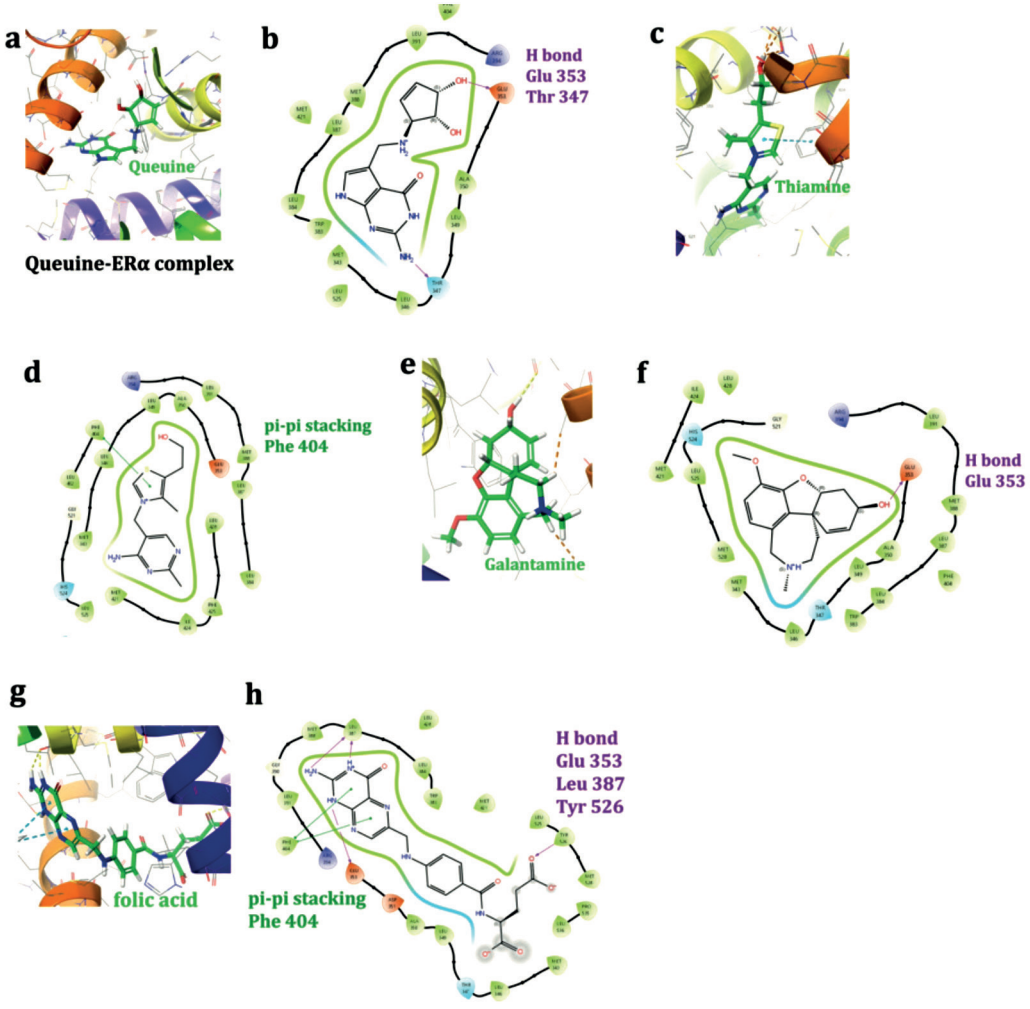

Figure 4. Queuine, Thiamine, Galantamine and Folic acid in complex with target receptor ERα: 3D and 2D interactions.

Table 3 presents the drug-like properties of the proposed natural compounds alongside those of the control drugs. Compared to the FDA-approved drugs Raloxifene and Tamoxifen, the natural molecules Queuine, Galantamine, Thiamine, and Folic acid exhibit significantly lower lipophilicity values. This suggests they are more water-soluble and hydrophilic, which may contribute to better bioavailability and distribution in aqueous environments. Additionally, all molecules passed the AMES test for mutagenic potential, indicating no toxicity or mutagenicity concerns.

Table 3. Predicted druglike and ADMET properties of proposed lead natural molecules compared to Raloxifene and Tamoxifen.

Molecule	MW[1] (g/mol)	log P[2]	HBD[3]	HBA[4]	TPSA[5] (Å)	HIA[6] (%)	AMES[7] toxicity
Queuine	277.3	-1.6	7	7	136.5	94.6	Non-toxic
Galantamine	287.4	1.2	1	4	41.9	99.9	Non-toxic
Thiamine	265.4	-2.1	2	4	75.9	79.7	Non-toxic
Folic Acid	441.4	-0.5	6	12	208.9	79.5	Non-toxic
Raloxifene	473.6	5.5	2	5	70	98.6	Non-toxic
Tamoxifen	371.5	5.9	0	2	12.5	99.7	Non-toxic

[1] Molecular Weight (from Pubchem, Kim et al., 2023)

[2] octanol/water partition coefficient (from ALOGPS, Tetko et al., 2005)

[3] Hydrogen bond donor (from Drug Bank, Daina, et. al., 2017; Wishart et al., 2006)

[4] Hydrogen bond acceptor (from Drug Bank, Wishart et al., 2006)

[5] Topological polar surface area (from Drug Bank, Wishart et al., 2006)

[6] Human intestinal absorption from (admetSAR, Cheng et al., 2012)

[7] Mutagenic potential (from admetSAR Cheng et al., 2012)

Conclusion

This study focuses on identifying new natural compounds to inhibit Estrogen Receptor Alpha (ERα) for the treatment of breast cancer. Although the FDA-approved drugs Raloxifene and Tamoxifen are effective in managing breast cancer, their use is often accompanied by side effects. The compounds proposed in this research aim to deliver comparable efficacy with reduced toxicity compared to these FDA-approved drugs. Through *in silico* virtual screening of extensive drug databases, Drug Bank and Zinc15, followed by molecular docking simulations, several promising molecules were identified. In particular, molecules such as Queuine, Thiamine, Galantamine, and Folic Acid exhibited strong binding affinity to Estrogen Receptor Alpha and demonstrated promising effects against Alzheimer's disease, opening doors for a dual-purpose therapeutic approach. It is important to note that molecular docking simulations have certain limitations. The docking simulations employed in this study considered the target protein as a rigid structure, whereas proteins undergo dynamic conformational changes in real biological systems. Therefore, the obtained results may not fully reflect the interactions occurring in a more realistic environment where the protein is flexible and surrounded by water molecules. The effect of water is often not explicitly modeled or is modeled using

simplified water models in virtual docking methods. This can lead to results that do not accurately represent the interactions in a real biological system. Water molecules play a significant role in protein-ligand interactions and can influence interactions such as hydrogen bonding. Therefore, in addition to molecular docking simulations, conducting molecular dynamics simulations would be crucial for gaining a deeper understanding of the dynamic interactions of these compounds with the protein and elucidating the binding mechanisms. For future work conducting molecular dynamics simulations can provide more in-depth information about the selectivity and side effects of these compounds, thus contributing to the identification of more reliable candidate molecules. The Log P values of the nutraceutical molecules are lower than those of the control drugs, indicating their hydrophilic nature. While this may offer an advantage in terms of oral bioavailability, it could pose a disadvantage to cell membrane penetration and interaction with the binding site, which favors more hydrophobic characteristics. These challenges can be addressed by employing appropriate drug delivery systems, such as formulating the compounds with more hydrophobic encapsulations to enhance binding affinity and cellular uptake. The findings of the present study constitute a significant step toward developing novel therapeutic strategies for both cancer and neurodegenerative diseases. However*, in vivo* efficacy and safety profiles of these compounds need to be further supported by more extensive studies.

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No funding has been provided for this project.

Conflict of interest

The authors declare there are no conflicts of interest.

Data availability statement

Data can be obtained from the corresponding author upon request.

Ethics committee approval

Ethics committee approval is not required for this study.

Authors' contribution statement

The authors acknowledge their contributions to this paper as follows: Concept and design: N.K.C and MG.; Data collection: N.K.C.; Analysis of data: N.K.C and M.G.; Manuscript writing: N.K.C and M.G. All authors agree with the findings and conclusions presented in the manuscript.

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RESEARCH ARTICLE

Antibacterial properties of substituted phenethylamine-based β-lactam derivatives in oral infections

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Abstract

Oral infections are a type of infection that occurs in and around the mouth, typically arising when proper oral hygiene is neglected. These infections manifest as symptoms such as mouth sores, dental caries, and periodontal diseases, with dental caries being the most common form. *Streptococcus and Lactobacillus* bactteria are the primary causative agents in dental caries. These bacteria act as opportunistic pathogens, potentially leading to serious diseases. Moreover, antibiotic resistance is developing in these pathogenic bacteria, limiting treatment options. β-lactam antibiotics are vital due to their broad spectrum and selective toxicity. This study synthesized novel phenethylamine-based β-lactam derivatives, and their antibacterial activities against oral pathogens were investigated. The antibacterial activities of the compounds were determined using agar well diffusion and microdilution assays. The study observed that β-lactam derivatives formed inhibitory zones against the growth of oral pathogens, while imine compounds did not create such zones. The diameter of the inhibition zones for the β-lactam compounds ranged from 0.9 to 2.1 cm. The MIC values were calculated to be between 12.5 and 100 µM. These data suggest that β-lactam derivatives could be potent therapeutic agents for oral infections.

Keywords: β-lactam, Imine, Phenethylamine, Oral pathogens, Antibacterial activity

Introduction

Oral infections are types of infections that occur in and around the mouth. These diseases are usually caused by inadequate oral hygiene and the uncontrolled proliferation of pathogens (bacteria and fungi) (Li et al., 2000). Oral infections manifest as symptoms such as mouth sores, dental caries, and periodontal diseases, with dental caries being the most common (Nyvad & Takahashi, 2020). Dental caries occur when acids erode the enamel layer of the teeth, a process usually triggered by bacteria converting sugary and starchy foods in the mouth into acids These acids lead to the demineralization of tooth enamel, resulting in cavities (Sato et al., 2021). *Streptococcus and Lactobacillus* species are the two most prevalent bacteria that cause dental cavities (Caulifeld et al., 2015; Spatafora et al., 2024). *Streptococci* play a significant role in the development of dental caries, with *Streptococcus mutans* (*S. mutans*) being the most common and well-known causative agent (Lemos et al., 2019). *S. mutans* is a gram-positive bacterium that colonizes the natural oral microbiota. It can adhere to tooth surfaces and plaques, metabolize sugars into acids, and dissolve tooth enamel, causing cavities (Zhu et al., 2023). Additionally, *S. mutans* is a pathogenic organism that exhibits antibiotic resistance, posing a significant challenge in treating dental caries and other oral infections (Nomura et al., 2020; Zhang et al., 2022). Other *Streptococcus* species in the oral flora, such as *Streptococcus mitis* (*S. mitis*), are also associated with dental caries, though not as prominently as *S. mutans (Bloch et al., 2024; Hohwy et al., 2001). S. mitis* is a gram-positive bacterium, part of the normal oral microbiota, that adheres to tooth surfaces and contributes to plaque formation. However, its ability to ferment sugars and produce acids is not as strong as that of *S. mutans*, resulting in slower acid production and less harmful effects (Seow et al., 2009). Nevertheless, *S. mitis* is a common causative agent of endocarditis and is highly resistant to antibiotics (Ayi, 2007). Other bacterial species that can lead to dental caries include *Lactobacillus s*pecies. *Lactobacillus* species are bacteria found in dairy products and the mouth. Due to their lactic acid fermentation, they cause tooth enamel erosion, leading to cavities (Wen et al., 2022). Specifically, *Lactobacillus casei (L. casei), Lactobacillus fermentum (L. fermentum),* and *Lactobacillus acidophilus (L. acidophilus)* are observed in dental caries. These bacteria can also cause nosocomial infections in immunocompromised individuals, and they exhibit antibiotic resistance, complicating treatment (Kullar et al., 2023).

β-lactam antibiotics are the most frequently prescribed broad-spectrum antibiotics, known for their minimal toxicity (Smith et al., 2018). The core structure of β-lactam antibiotics contains a four-membered saturated cyclic amide, known as the β-lactam ring, composed of one nitrogen and three carbon atoms (Fu et al., 2017). β-lactam group antibiotics were first discovered with the discovery of penicillin by Alexander Fleming in 1929. They were widely used to treat infectious diseases (Gaynes, 2017). These antibiotics are also recognized as biologically active molecules, exhibiting various activities such as antibacterial, antifungal, anti-inflammatory, and anticancer properties. The phenethylamine compound, a dopamine-like monoamine alkaloid, also possesses various biological activities (Yamase et al., 1996).

In this study, the antibacterial effects of previously synthesized phenethylamine-based β-lactam derivatives (7-12) (Figure 2) (Yildirim et al., 2022) on dental pathogens (*S. mutans, S. mitis, L. casei, L. fermentum, L. acidophilus*) were investigated. The antibacterial effect of the compounds used (1-12) on oral pathogens was reported for the first time in the literature in this study. In this respect, the study is quite original. This study contributes to the development of new approaches to the treatment of oral pathogens.

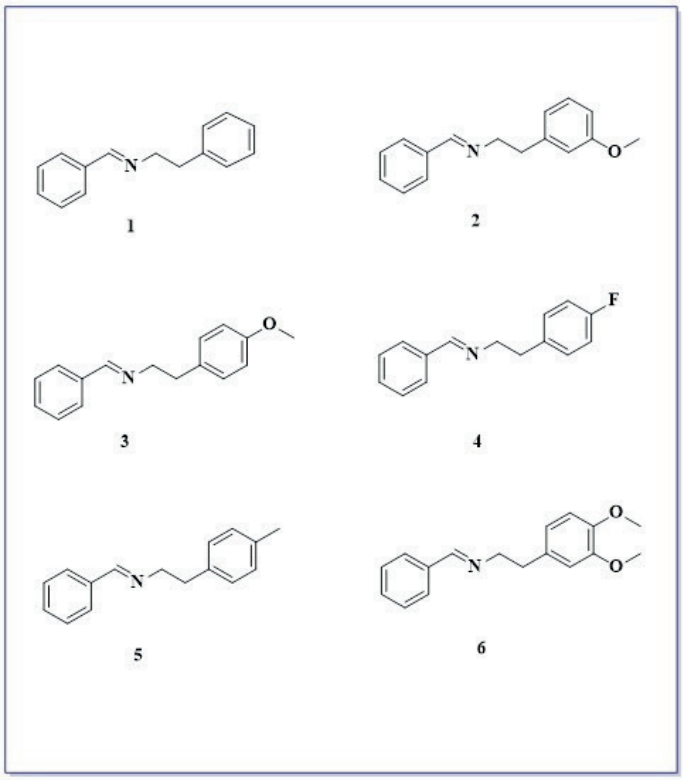

Figure 1. Imine derivatives (1-6), which are intermediate products used in the study.

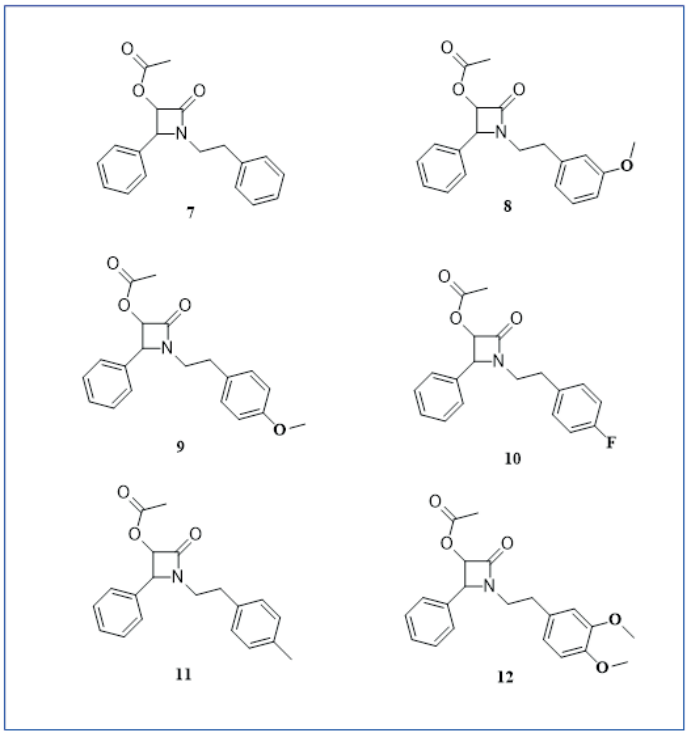

Figure 2. The target product used in the study was β-lactam derivatives (7-12).

Materials and methods

Materials

For the synthesis of β-lactam derivatives, the following chemicals were procured and used: Benzaldehyde (Sigma Aldrich), phenethylamine (Acros Organics), 3-methoxy phenethylamine (Alfa Aesar), 4-fluorine phenethylamine (J&K Scientific), 4-methyl phenethylamine (Acros Organics), 4-methoxy phenethylamine (J&K Scientific), acetoxy acetyl chloride (Acros Organics), ethyl acetate (TEKKİM), methylene chloride (Sigma Aldrich), molecular sieve 4Å (Sigma Aldrich), n-hexane (TEKKİM), sodium sulfate (MERCK), triethylamine (Sigma Aldrich), and chloroform D1 (Sigma Aldrich). For antibacterial studies, Luria Bertani Agar (LBA) (Miller MERCK), dimethyl sulfoxide (DMSO) (Merck), Mueller Hinton Agar (MHA) (Oxoid), Mueller Hinton Broth (MHB) (Biolife), and ampicillin were commercially purchased.

Synthesis design

In this study, β-lactam derivatives (7-12) and their imine intermediates (1-6) synthesized according to a previous report were used (Yildirim et al., 2022). Briefly, these compounds were synthesized as follows: Imines were obtained with a glass rod by adding substituted phenethylamines to benzaldehyde in the presence of a molecular sieve in a beaker. Then, it was dissolved in dichloromethane, and the molecular sieve was filtered on filter paper and removed. After the solution was evaporated, imine compounds (1-6) were obtained. The obtained imines were then reacted with acetoxy acetyl chloride in the presence of triethylamine and stirred overnight at room temperature. After the reaction, the mixture was extracted with water and dichloromethane. The desired compounds (7-12) were purified by column chromatography to obtain the pure product (Genc et al., 2016).

Antibacterial activity

In the biological assays conducted in the investigation, strains of *S. mutans* (ATCC 35668), *S. mitis* (NCIMB 13770), *L. acidophilus* (ATCC 4356), *L. casei* (ATCC 334), and *L. fermentum* (ATCC 9338) sourced from the repository of Erzurum Technical University Molecular Biology Laboratory were employed. The bacterial samples were cultivated in an LBA medium and utilized in investigations of antibacterial properties.

Agar well diffusion test

The agar well diffusion test is a widely used method in antibacterial tests. An agar well diffusion test was used to determine the antibacterial activity of imine and β-lactam derivatives. Bacterial inocula were prepared according to 0.5 McFarland standards and spread on the agar medium using a stick. Then, wells were created by piercing the agar with a cork borer. The wells were filled with compounds prepared at 200 µM. After 24 hours of incubation at 37°C, the diameters of the inhibition zones were measured to determine the antibacterial susceptibility of dental pathogens. To evaluate the activity of the synthesized compounds, Ampicillin at a concentration of 200 µM was used as a positive control and DMSO as a negative control. The diameters of the inhibition zones of the active compounds were measured as a result of the agar well diffusion test (Ozgeris, 2021).

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations of compounds (7-12) that were effective in the agar well diffusion test were determined to determine the minimum antimicrobial concentration required to inhibit bacterial growth. This method uses a liquid medium to prepare serial dilutions ranging from 200 µM

to 6.25 µM in a 96-well plate. Then, 100 µL of bacterial culture inoculum, adjusted to 0.5 McFarland standards, was added and incubated for 24 hours. After incubation, the lowest concentration at which no visible turbidity, indicating bacterial growth inhibition, was observed and recorded as the MIC value (Gormez et al., 2015).

Results and discussion

β-lactam antibiotics exhibit various biological activities and have garnered significant attention in bioorganic chemistry. Consequently, numerous methods for synthesizing β-lactam derivatives have been reported in the literature (Payili et al., 2018). Staudinger developed the first synthesis method for β-lactam derivatives, and many other methods have been introduced subsequently (Staudinger, 1907). The most common β-lactam synthesis approach involves the reaction of aromatic aldehydes with amines to form imines, which are then used to synthesize the corresponding β-lactam derivatives (Decuyper et al., 2018). This study successfully synthesized imine intermediates (1-6) from benzaldehyde and substituted phenethylamines using the given synthesis method. Subsequently, the target β-lactam derivatives (7-12) were obtained from the imines, with yields ranging from 48% to 91%. The synthesized compounds were evaluated for their antibacterial activities against pathogens responsible for oral infections.

The antibacterial activity of the synthesized compounds against oral pathogens was determined using the agar well diffusion method and the minimum inhibitory concentration (MIC) assay. The results of these studies are detailed in Table 1 and Table 2 below. According to the findings, while the target β-lactam derivatives exhibited antibacterial activity, the imine derivatives showed no antibacterial activity against oral pathogens.

Table 1. Agar well diffusion analysis results of novel compounds **(1-12).**

	Zone Diameter of Compounds (cm)												
Bacteria Strain	**Imine Derivatives (1-6)**						**β-lactam derivatives (7-12)**						**Control**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Ampicillin**
***S. mutans* (ATCC 35668)**	No Zone	No zone	No zone	No zone	No zone	No zone	2.1	1.5	1.9	1.7	2.0	0.9	2.0
***S. mitis* (NCIMB 13770)**	No zone	No zone	No zone	No zone	No zone	No zone	No zone	1.5	1.1	1.6	1.9	No zone	1.3
L. acidophilus (ATCC 4356)	No zone	No zone	No zone	No zone	No zone	No zone	2.3	1.4	1.5	1.6	1.6	No zone	1.3
L. casei (ATCC 334)	No zone	No zone	No zone	No zone	No zone	No z one	1.7	1.6	1.6	1.7	1.8	No zone	1.2
L. fermentum (ATCC 9338)	No zone	No zone	No zone	No zone	No zone	No zone	1.9	1.4	1.5	1.5	1.6	No zone	1.3

Table 2. MIC values of effect compounds **(7-12).**

Bacteria Strain	MIC value of β-lactam derivatives						
	7	**8**	**9**	**10**	**11**	**12**	**Ampicillin**
***S. mutans* (ATCC 35668)**	25 μM	25 μM	25 μM	25 μM	25 μM	12.5 μM	6.25 μM
***S. mitis* (NCIMB 13770)**	-	50 μM	50 μM	50 μM	100 μM	-	50 μM
***L. acidophilus* (ATCC 4356)**	25 μM	50 μM	50 μM	50 μM	25 μM	-	50 μM
***L. casei* (ATCC 334)**	25 μM	25 μM	50 μM	50 μM	50 μM	-	100 μM
***L. fermentum* (ATCC 9338)**	12.5 μM	25 μM	12.5 μM	25 μM	12.5 μM	12.5 μM	50 μM

In the literature, the antibacterial activity of imine derivatives against oral pathogens has been reported. For example, a study examined the antibacterial activities of imine derivatives against oral pathogens. As a result, it was reported that these compounds exhibited antibacterial activity (Sa'ad et al., 2022). In addition, another study included the synthesis of hemocompatible imine derivatives in which potent antimicrobial activity was observed (Altamimi et al., 2020). However, our study detected no antibacterial activity of imine derivatives against oral pathogens. This discrepancy may be attributed to the structural differences in the imine derivatives used in our study compared to those reported in the literature. It is well known that adding different side groups to the structure of organic compounds can significantly affect their biological activities. The imine compounds in our study have different side groups than those in the literature. This explains the lack of antibacterial activity of imine derivatives (1-6) against oral pathogens (Love & Ren, 1993).

The antibacterial activity of β-lactam derivatives against oral pathogens has garnered significant attention due to their effectiveness and the emergence of resistant strains. Recent studies highlight the potential of these compounds in combating oral infections, particularly those caused by resistant bacteria. Previous studies have investigated the effects of β-lactam derivatives on *S. mutans*. One study reported that β-lactam antibiotics are effective against antibiotic-resistant *S. mutans*, with a minimum inhibitory concentration (MIC) of 125 μM for aztreonam (Hirasawa & Takada, 2002). In our study, *S. mutans* was highly susceptible to compounds **(7-12)**, exhibiting low MIC values and higher antibacterial activity than ampicillin. Compound **7** showed a significantly larger inhibition zone and a lower MIC value, indicating potent activity.

The increasing resistance of *S. mitis* to β-lactam antibiotics poses significant challenges in clinical settings. Research indicates that mutations in penicillin-binding proteins (*pbp)* are a primary mechanism behind this resistance, leading to reduced antibiotic affinity and efficacy (Nakayama et al., 2006). In the literature, *S. mitis* has been identified as harboring β-lactam resistance genes. Studies show that *S. mitis* strains exhibit multiple mutations in *pbp* genes, significantly lowering their affinity for ampicillin and cefaclor, resulting in high resistance levels. Clinical *S. mitis* strains have demonstrated MICs as high as 64 μg/ml for benzylpenicillin and 128 μg/ml for cefotaxime, indicating severe resistance (Wajima et al., 2022). In our study, compounds **(8-11)** displayed antibacterial activity against *S. mitis*, with inhibition zones larger than those produced by ampicillin. The MIC values were calculated to be between 50-100 μM, suggesting that these compounds are considerably potent compared to commercial products. Additionally, structural analysis revealed that electron-withdrawing groups at the meta and para positions of the compounds enhanced their antibacterial activity.

Lactobacillus strains, commonly found in dairy products, are known for their probiotic properties and ability to secrete natural antibiotics against pathogens (Mann et al., 2021). However, recent studies have reported the development of antibiotic resistance mechanisms in *Lactobacillus* strains (Chen et al., 2019). Notably, strains like *L. casei* have resisted β-lactam antibiotics. The *blaTEM* gene in 80% of these strains suggests potential resistance mechanisms against β-lactam derivatives. Additionally, *L. casei* may exhibit intrinsic resistance to certain β-lactams, potentially due to the presence of β-lactamases that can hydrolyze these antibiotics (Anisimova & Yarullina, 2019). This resistance poses a significant challenge, as β-lactam antibiotics used for treating oral pathogens may be inactivated by the β-lactamases produced by *Lactobacillus* (Anisimova et al., 2022). In our study, *Lactobacillus* strains exhibited potent activity against ampicillin, demonstrating their significant resistance capabilities. This finding highlights the need for ongoing research to better understand and address antibiotic resistance mechanisms in these probiotic strains, particularly in the context of oral infections.

Conclusions

In conclusion, in this study, substituted phenethylamine-based β-lactam derivatives (7-12) imine intermediates (1-6) were successfully synthesized in two steps. The antibacterial activities of these synthesized compounds against oral pathogens were studied and showed strong antibacterial activity. Based on these results, it is anticipated that β-lactam derivatives may serve as effective therapeutic agents against oral infections. Furthermore, the study highlights the potential of these β-lactam derivatives in biotechnological applications, especially in developing innovative antimicrobial agents to address the increasing challenge of antibiotic resistance and improve oral health solutions.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Data availability statement

Data can be obtained from the corresponding author upon a reasonable request.

Ethics committee approval

Ethics committee approval is not required for this study.

Authors' contribution statement

MY, EA, TYB, HG, and EI conducted the analyses and prepared the initial draft of the manuscript. AG and BO provided supervision for the study and interpreted the results. All authors contributed to the revision of the manuscript and have read and approved the final version.

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