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Antioxidant activity and in vitro/in silico acetylcholinesterase and urease enzyme inhibition effects of amygdalin

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Abstract

This study investigated the acetylcholinesterase, urease enzyme inhibition and, these enzymes' molecular docking as well as the antioxidant activity of commercially available amygdalin. While Amygdalin displayed effective anti-urease activity compared to acetohydroxamic acid anti-acetylcholine esterase activity was a little ineffective compared to donepezil. The molecular docking was performed to check binding interactions between the amygdalin and the enzymes. DPPH· and FRAP assays were preferred to determine the antioxidant activity. The antioxidant activity (3.39 ± 0.33 µmol $Fe_2SO_47H_2O$ /g, SC_{50} 18.74 ± 0.72 mg/mL using the FRAP and DPPH assays, respectively. Amygdalin's *in vitro* and *in vivo* studies are needed to demonstrate that is a therapeutic agent for the treatment of various diseases.

Keywords: Acetylcholinesterase, urease, molecular docking, antioxidant activity, amygdalin

1. Introduction

Amygdalin is a naturally occurring chemical compound found in the kernels of various fruits, such as apricots, peaches, cherries, and plums, as well as in bitter almonds. It has a molecular weight of 457.42g/mol and, a chemical formula $C_{20}H_{27}O_{11}$. Amygdalin, also known as laetrile or Vitamin B17, has garnered attention in recent years due to claims of its potential anti-cancer, anti-inflammatory, antibacterial and antioxidant properties [1,2]. Amygdalin is classified as a cyanogenic glycoside, meaning it contains both a sugar molecule and a cyanide group. When metabolized, amygdalin breaks down into glucose, benzaldehyde, and hydrogen cyanide. While amygdalin itself is harmless, the release of hydrogen cyanide (HCN) during its enzymatic hydrolysis can be harmful. Recent studies have shown that HCN is released in normal cells, indicating that it may not be safe for the human body [3].

Amygdalin is a cyanogenic disaccharide [4]. There are numerous studies in the literature regarding its high therapeutic effects, such as anti-inflammatory and analgesic effects in neurodegenerative diseases [5]. Studies have shown positive effects of amygdalin in the treatment of various diseases such as leprosy, colorectal cancer, asthma, and bronchitis [6,7]. Numerous studies have also revealed its anticancer and anti-inflammatory effects [8,9]. Amygdalin has been used as a therapeutic agent in cancer treatment for many years [10,11,12]. For example, it has been reported to have a curative effect in the treatment of prostate cancer as well as a positive effect in the treatment of colon cancer.[13] In another study, it was found that amygdalin derived from Apricot (*Prunus armeniaca*) inhibited breast, lung, and bladder cancer cell [14].

Alzheimer's disease (AD) is the most common form of dementia, which is a progressive neurological condition characterized by the gradual degeneration and loss of brain cells Despite numerous studies on the treatment of this disease, a complete cure has not yet been found [15,16,17]. Acetylcholinesterase (AChE) is an enzyme responsible for breaking down the neurotransmitter acetylcholine in the nervous system. Inhibiting AChE activity is a key mechanism of action for drugs used in the treatment of Alzheimer's disease.

Helicobacter pylori (*H. pylori*) infection occurs when *H. pylori* bacteria infect your stomach. *H. pylori* is a widespread cause of stomach ulcers, also known as

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peptic ulcers, and it is estimated that it could be found in over half of the world's population [18]. Persistent infection with *H. pylori* has been linked to the onset of gastric cancer, primarily attributed to the formation of DNA damage caused by oxidative and nitrosative processes [19]. Urease is an enzyme that the breakdown down of urea into ammonia and carbon dioxide through hydrolysis. It holds significant importance as a target for the advancement of anti-ulcer medications.

In the treatment of Alzheimer's disease, drugs such as tacrine, donepezil, rivastigmine, and galantamine are used as they effectively inhibit the AChE enzyme [20]. Similarly, inhibitor drugs are also used for the urease enzyme, which plays a role in the development of diseases like stomach cancer and ulcers. However, due to the significant side effects of these drugs, natural products are preferred for the treatment of these diseases. In this study, we evaluated commercially purchased amygdalin for its *in vitro* inhibition of urease, and acetylcholinesterase enzymes, as well as its antioxidant activity. In our planned study, we investigated the inhibitory potential of amygdalin, which has limited studies in the literature, on acetylcholinesterase and urease enzymes, as well as its molecular docking. Additionally, its antioxidant activity was also determined.

2. Material and methods

2.1. Acetylcholinesterase ınhibition assay

The inhibition of acetylcholinesterase (AChE) was determined using Ellman's method [21] reported with slight modification as by Kantar et al. [22]. Initially, 50 µl of 2.5U/mL enzyme, 50 µL of the sample, 3 mL of pH:8 100 mM phosphate buffer was mixed and left for 5 minutes. Subsequently, the reaction was initiated by the addition of 100 µL of a 10 mM solution of 5,5-dithio-bis(2-nitrobenzoic) acid (DTNB) and, 20 µL of a 75 mM solution of acetyl thiocholine chloride (ATCl). After 30 minutes, absorbance values were recorded at 412 nm. Donepezil was used as the standard.

2.2. Urease enzyme inhibition assays

The inhibition effect of the urease enzyme was determined according to Weatherburn [23] with slight modifications. In brief, a reaction mixture comprising 500 µL of buffer solution (pH 8.2), 200 µL of urease enzyme solution, and 100 µL of the sample was incubated for 15 minutes in a tube. Then, 500 µL of a phenol reagent and 600 µL of an alkali reagent were added to each tube and incubated for 50 minutes in a dark room. The increase in absorbance at 625 nm was measured at the end of the incubation period using a UV-Vis spectrophotometer. Acetohydroxamic acid was used as the inhibitor. The IC_{50} value corresponds to the concentration of a compound that effectively inhibits 50% of the maximum activity observed.

2.3. Molecular Docking Studies

In order to investigate the potential binding modes of amygdalin against both human acetylcholinesterase (AChE) and urease enzymes, a molecular docking (MD) technique was employed using Auto Dock 4.2 software [24]. The three-dimensional structure of human acetylcholinesterase (AChE) complexed with donepezil was obtained from the Protein Data Bank (PDB) website (https://www.rcsb.org/) with the identifier 4EY7 (Chain A, Res: 2.35 Å). The other receptor, urease in complex with Acetohydroxamic Acid, was downloaded from Protein Data Bank, which was denoted 4H9M (Chain A, Res: 1.52Å). The 3D structures of the reference molecules, acetohydroxamic acid and donepezil, were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in sdf format and converted to pdb file format using OpenBabelGUI 2.4.1 software [25].

After energy minimization, the protein structures were prepared for docking by removing water molecules, ions, and other ligands, and adding polar atoms of hydrogen and Kollman charges. The prepared structures were then converted to PDBQT file format for docking. Possible docking modes between molecules and target proteins were studied using Auto Dock 4.2 software with the Lamarckian genetic algorithm employed for all docking simulations. For the standard docking procedure, the target protein was kept rigid while all ligands were kept flexible with torsion angles of 150 independent runs per ligand. Docking studies were performed with a population of 150 individuals, maximum energy evaluations of 2,500,000, and maximum generation of 54,000.

Additionally, the active site of AChE was covered with a grid box of dimension 60 Å x 60 Å x 60 Å and -14.1, -43.83, 27.66 points in the x, y, and z directions with a grid spacing of 0.375 Å. To cover the urease active site, a grid box of dimension 70 Å x 70 Å x 70 Å and -19.84, -58.46, -22.38 points in the x, y, and z directions with a grid spacing of 0.375 Å was applied. Default settings were applied for all other parameters. The results of the molecular calculations elucidated the binding affinity of each ligand to the targeted proteins, as determined by the docking score and hydrogen/hydrophobic binding interactions. The binding energies of docked conformations and post-docking analyses of each ligand against the target protein were assessed using BIOVIA Discovery Studio Visualizer 2018 [26].

2.4. Antioxidant activity assays

2.4.1. Ferric-reducing antioxidant power (FRAP)

The frap method is the most common method for determining antioxidant activity due to its simplicity and low cost. The ferric-reducing antioxidant capacity of amygdalin was determined according to the method described in the literature [27]. To create the FRAP reagent, a mixture was prepared using the following components: 2.5 mL of 10 mM (2,4,6-Tris(2-pyridyl)-*s*-triazine) TPTZ, 2.5 mL of 20 mM $FeCl_3$, and 25 mL of 0.3 M acetate buffer at pH 3.6. Then, 50 µL of the sample and 1.5 mL of the FRAP reagent were combined. After 4 minutes, the absorbance was measured at 595 nm. $Fe_2SO_4 7H_2O$ was used for calculating the antioxidant activity. FRAP result was expressed as µmol $Fe_2SO_4 7H_2O$ /g.

2.4.2. DPPH· assay

DPPH• (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was investigated using the method described in the literature [28]. The sample was prepared at six different concentrations. A 10 mM DPPH solution was prepared in methanol. Subsequently, 750 µL of each sample and 750 µL of the stock DPPH solution were combined and incubated for 50 minutes in a dark room. Following the completion of the incubation period, absorbance measurements were obtained at a wavelength of 517 nm. Lower absorbance values indicate greater free radical scavenging activity. Trolox® was employed as a positive reference in the experiment.

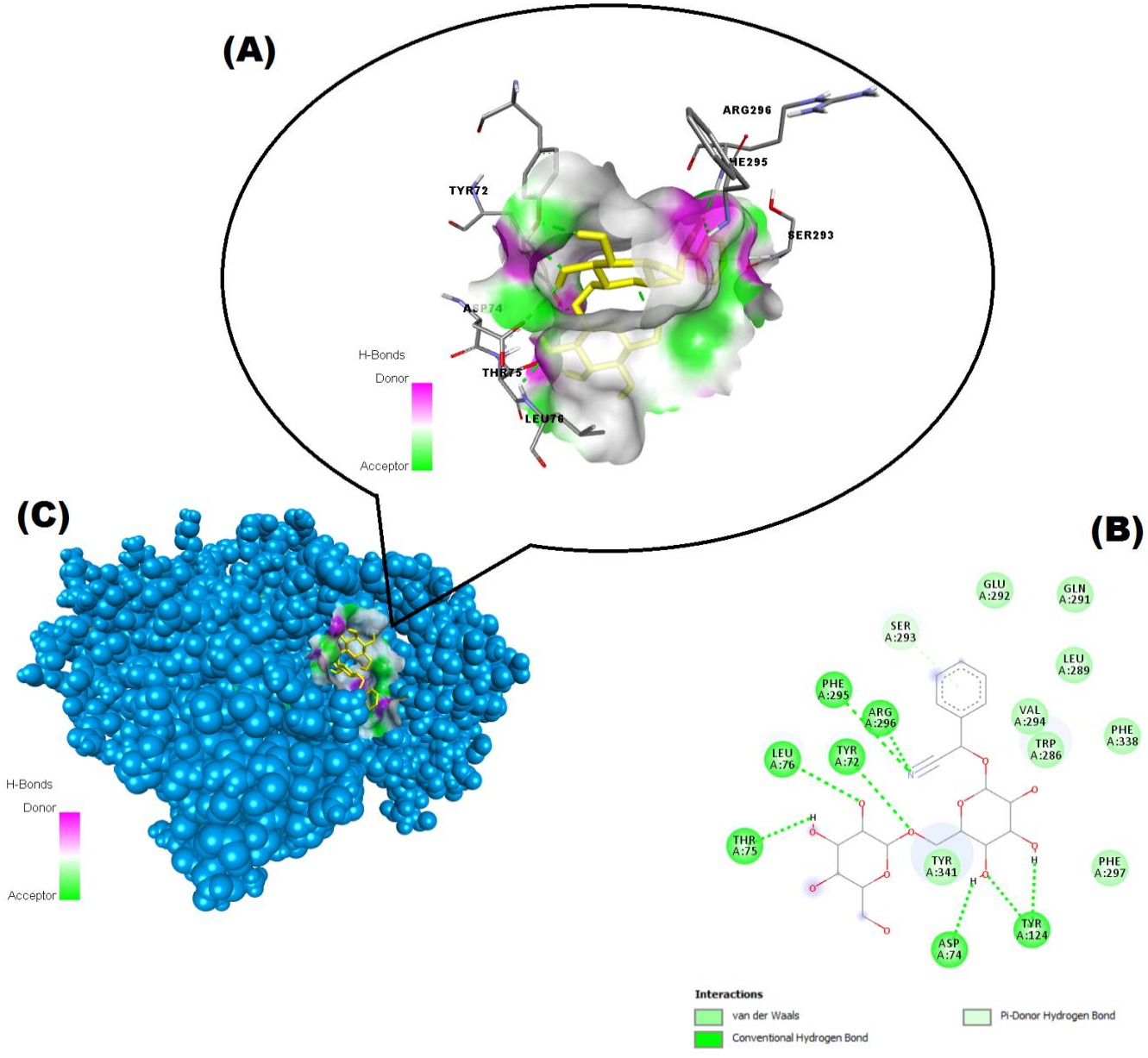

Figure 1. Binding pose profile of amygdalin in the target protein AChE (C), blue shaped molecule represents the receptor and yellow shaped molecule indicates the ligand. The two-dimension (2D) (B) and three-dimension (3D) (A) interactions analysis of human acetylcholinesterase (AChE) with amygdalin.

Table 1. Acetylcholinesterase and urease and enzyme inhibition of Amygdalin

Sample	Acetylcholinesterase Inhibition IC_{50} (M)	Urease Inhibition IC_{50} (M)
Amygdalin	>1	0.11 ± 0.01
Donepezil	39.84 ± 0.21	—
Acetohydroxamic acid	—	28.6 ± 0.78

2.5. Statistical analysis

All experiments were carried out in triplicates, and the results are presented as mean ± standard deviation (SD). SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) of the Windows software was executed for statistical analysis.

3. Results and discussion

3.1. Acetylcholinesterase and urease enzyme inhibition

Acetylcholinesterase is an enzyme that plays a vital role in the breakdown of the neurotransmitter acetylcholine. Acetylcholine is a critical molecule involved in the transmission of nerve signals within the nervous system, including the brain, spinal cord, and peripheral nerves. Inhibition of acetylcholinesterase can have therapeutic implications in the treatment of conditions such as Alzheimer's disease. Donepezil and rivastigmine are very widely used AD remedies targeting the AChE enzyme. However, both are known to have various side effects [29,30]. The use is widespread of synthetic AChE inhibitors (such as donepezil, rivastigmine, and galantamine) and urease inhibitors (like thio-ureas, hydroxamic acids, etc.) however, they are associated with several side effects. Therefore, there is a growing demand to explore natural sources for the development of AChE and urease inhibitors that exhibit strong therapeutic effects with minimal to no toxicity, aiming to treat diseases caused by these enzymes effectively. For this reason, amygdalin was investigated for its ability to inhibit AChE (acetylcholinesterase) and urease. According to our findings we may say that amygdalin has the potential to inhibit the activity of AChE, which may have therapeutic implications for the treatment of Alzheimer's disease, and also the urease enzyme, which is vital in addressing conditions like gastric cancer, gastritis, and peptic ulcers as a potential inhibitor. Table 1 shows that amygdalin exhibited an inhibition effect against acetylcholinesterase and urease.

Several studies have reported that Amygdalin has the potential to act as an acetylcholinesterase inhibitor. Vahedi-Mazdabadia et al. [31] reported that both sweet and bitter extracts of apricot kernels had inhibitory effects on cholinesterase (ChE) enzymes in vitro.

The aqueous extract of the bitter type had the best inhibition effect, with an IC_{50} value of 134.93 ± 2.88 μg/mL. Another study reported that kernels extracted from fruits of 20 different peach cultivars had IC_{50} values ranging from 0.67 to 5.85 (mg of dried seeds). A study was conducted to investigate the neuroprotective and neuritogenic effects of amygdalin. Initially, the study demonstrated that amygdalin enhanced the process of nerve growth factor (NGF)-induced neuritogenesis and also reduced the neurotoxicity induced by 6-hydroxydopamine (6-OHDA) in rat dopaminergic PC12 cells [32].

A camp formulation consisting of *P. armeniaca* L. has been investigated for its neuroprotective abilities and its potential to improve learning difficulties and synaptic loss in AD (Alzheimer's disease) patients. It was found that synergistic effects play a significant role in directly activating neurons [33].

The enzyme urease functions in the process of converting urea to ammonia and carbon dioxide through its catalytic activity. Inhibition of urease can have potential applications in the treatment of various medical conditions, including urinary tract infections and gastric ulcers. The inhibition of urease by amygdalin at a concentration of (IC_{50}) 0.11 M. As a result of, we suggest that amygdalin may have moderate potential as a urease inhibitor.

In a study, the methanol and aqueous extracts of five apricot cultivars were investigated for their effects against the urease enzyme involved in treating stomach conditions such as carcinomas and ulcers. In the study, it was found that both methanol and aqueous extracts exhibited inhibitory effects on the urease enzyme, with IC_{50} values ranging from 20.763 to 195.437 mg/mL [34].

Table 2. Description of amygdalin and reference molecules against different enzymes with binding energy, K_i and interacting residues in the binding site

Receptor Name	PDB ID	Ligand name	Binding Energy (kcal/mol)	K_i	No of H bonds	Interacted residues with ligand
Human acetylcholinesterase (AChE)	4EY7	Amygdalin	-9.35	140.66 nM	7	Asp74, Trp86, Asn87, Pro88, Tyr124, Gly126, Tyr341
		*Donepezil	-12.09	1.37 nM	2	Tyr72, Trp86, Trp286, Leu289, Ser293, Phe295, Tyr337, Phe338, Tyr341, His447
Urease	4H9M	Amygdalin	-6.13	32.19 μM	8	Arg439, His492, Met588 Leu589, Val591, His593, Arg609, Asp633, Ala636
		*Acetohydroxamic Acid	-5.04	200.49 μM	6	Glu547, Gly548, Ala549, Gly550, Glu618

*Reference molecules

3.2. In silico analysis

Molecular docking is widely used to investigate binding interactions between potential drugs and different sites or active sites on target molecules. Various types of interactions, such as H-bonds, π-π interactions, and amide-π interactions, are evaluated to determine the binding efficiency of a ligand molecule with a target. The hydrogen bonding pattern and the nature of residues present in the active site play a crucial role in explaining the binding affinity of a ligand with a target molecule. Binding free energy (kcal/mol) is used to examine and compare the binding affinity of different ligands with their respective target receptor molecules. When the binding energy is lower, it signifies a greater affinity of the ligand towards the receptor [35,36]. In this study, molecular docking was performed to evaluate amygdalin against AChE and urease enzymes. The simulation results provided predicted protein-ligand binding energies, Ki values, and identified potential ligand binding sites (Table 2). After successfully docking amygdalin and reference molecules (Table 2), significant interactions of the ligand with the receptor proteins were observed. When examining the interaction of amygdalin with AChE, it was found that the ligand effectively binds to the receptor with a low binding energy of -9.35 kcal/mol, while the reference molecule, donepezil, binds strongly with an even lower binding energy (-12.09 kcal/mol). Both the reference molecule and amygdalin appear to form strong interactions with Trp86 and His341 residues in the active site of AChE. Amygdalin forms five conventional hydrogen bonds, two carbon hydrogen bonds, two Pi-sigma bonds, and two donor-donor bonds with AChE, with one of these bonds having an atomic distance lower than 2 Å. On the other hand, when examining the docking poses with urease enzyme and amygdalin, it is evident that the ligand binds to the receptor more effectively than the reference molecule, acetohydroxamic acid. Amygdalin forms six conventional hydrogen bonds with urease, three of

Table 3. Antioxidant Activity of Amygdalin

Sample	FRAP (μmol $Fe_2SO_4.7H_2O$ /g)	DPPH SC_{50} (mg/mL)
Amygdalin	3.39±0.33	18.74 ± 0.72
Trolox	—	0.004 ± 0.00

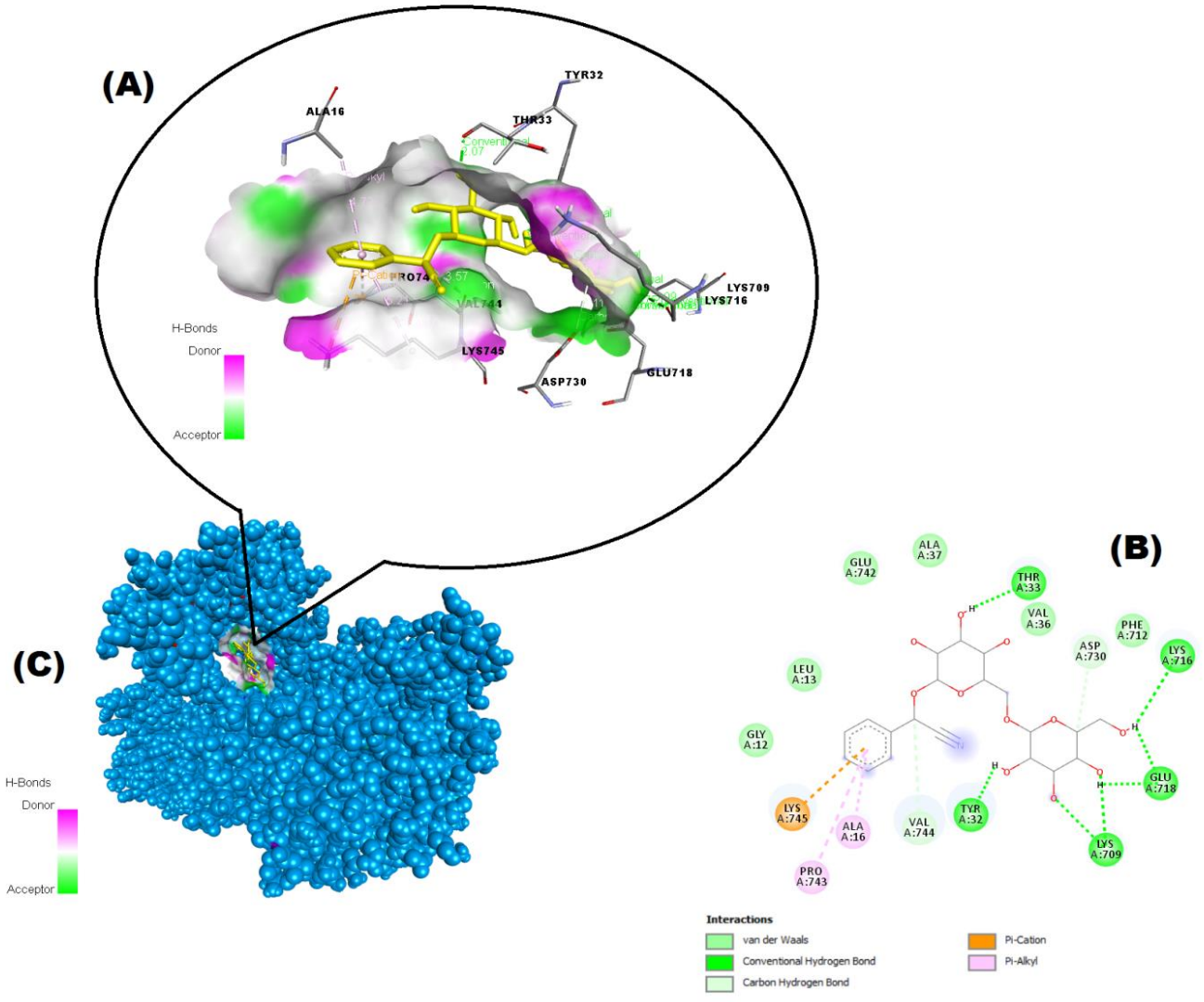

Figure 2. Binding pose profile of amygdalin in the target protein Urease (C), blue shaped molecule represents the receptor and yellow shaped molecule indicates the ligand. The two-dimension (2D) (B) and three-dimension (3D) (A) interactions analysis of Urease with amygdalin

which have atomic distances lower than 2 Å (Fig. 2). Additionally, the ligand forms two carbon hydrogen bonds, two pi-alkyl bonds, two donor-donor bonds, one pi-cation bond, one pi-anion bond, and one pi-pi t-shaped bond with the receptor. It appears that Met588 plays a crucial role by forming two conventional hydrogen bonds with lengths of 1.99 and 1.88 Å. Fig. 1 and Fig. 2 provide the docked poses of the best hits in each target receptor, the residues they interact with, and the corresponding interactions.

3.3. Antioxidant activity of amygdalin

Amygdalin has been observed to demonstrate antioxidant activity in numerous *in vitro* and *in vivo* studies. It is a natural compound found in the seeds of many fruits, such as apricots, peaches, and almonds, as well as in other plants. Apricot seeds contain antioxidant, Angiotensin I converting enzyme (ACE) inhibitor, and hypocholesterolemic peptides [37]. In our study, we performed DPPH and FRAP tests to assess the antioxidant activity of commercially purchased amygdalin. The FRAP value was determined as 3.39±0.33 µmol $Fe_2SO_47H_2O$/g, while the DPPH value was determined as SC_{50} 18.74±0.72 mg/mL (Table 3). When the numerical value of the DPPH value is low, it signifies a potent capability to scavenge free radicals. When comparing our study with the Trolox standard, we could see that the DPPH value is effective.

In an *in vivo* study, the effects of amygdalin at different doses on antioxidant gene expression and suppression of oxidative damage were evaluated in mice. The results of found that low and moderate doses of amygdalin did not cause toxicity in hepatic and testis tissues, and they did not have any adverse effects on the oxidative balance [38].

In a study conducted by Sushma et al. [39], amygdalin was extracted from *Prunus dulcis* and tested for its antioxidant and cytotoxic properties *in vitro.* Multiple antioxidant experiments revealed that the amygdalin extract from *P. dulcis* exhibited potent antioxidant characteristics. Zhang et al. [40] investigated the different varieties of apricot kernels. The researchers found that all the tested varieties had antioxidant activity, with some varieties showing higher levels than others. The study also suggested that the antioxidant activity of apricot kernels may be due to their high levels of phenolic compounds. A study was conducted to determine the amygdalin levels in different genotypes of bitter and sweet almonds. The results showed that bitter almonds had a higher amygdalin content compared to sweet almonds. Additionally, the study found that bitter almonds were rich in phenolic content. These findings suggest that it can be concluded that the amygdalin content affects the phenolic content [41].

Another study assessed the biological activities of amygdalin extracted from the organs of three cassava varieties that are commonly produced in Benin (BEN, RB, and MJ). HPLC analysis was employed to measure the amygdalin content in cassava organs and derivatives. The results indicated that the organs of all three cassava varieties contained glycosides, flavonoids, saponins, steroids, tannins, coumarins, and cyanogenic derivatives. Among these, young stems and fresh cassava leaves exhibited the highest concentrations of amygdalin, with 11,142.99 µg per 10 g and 9251.14 µg per 10 g, respectively. The antioxidant activity results showed that the amygdalin extracts were DPPH radical scavengers with IC_{50} values ranging from 0.18 mg/mL to 2.35 mg/mL [42]. Based on these results, it can be concluded that these extracts possess a considerably high concentration of amygdalin.

4. Conclusion

In summary, the purpose of this present study was to emphasize the enzyme inhibitory properties of amygdalin, as well as its antioxidant activity. In this study, the potential of amygdalin to act as an inhibitor for acetylcholinesterase and urease enzymes was investigated *in vitro,* as well as its molecular docking binding capacity. And the antioxidant capacity of amygdalin was determined. Based on the findings of our study, we can say that amygdalin holds significant potential as a valuable agent in pharmaceutical and food-related applications. Amygdalin, to be recommended for the treatment of human diseases, its safety and efficacy as a potential therapeutic agent must undergo thorough evaluation in clinical trials.

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Conflicts of Interest:

The authors declare no conflict of interest.

Authors Contributions:

ZC: Methodology, Investigation, Data curation, Writing – original draft.

YK: Investigation of in vitro enzyme inhibition and antioxidant activity.

HİG: Investigation of molecular docking, writing,

CB: Investigation of antioxidant activity and enzyme inhibition.

SK: Writing

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