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Evaluation of Angiotensin Converting Enzyme inhibitors by SPR biosensor and Theoretical Studies

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Highlights

- Angiotensin converting enzyme was immobilized on carboxymethyl dextran (CMD 500D) chip via its primary amine groups.
- Analysis of AFM images showed successful attachment of ACE onto the sensor surface.
- This SPR biosensor can be used for screening of natural products extract with greater sensitivity and no interferences with non-active compounds.
- The binding energies computed via the DFT binding energy analysis method was in agreement with the experimental results.

Abstract

Surface plasmon resonance (SPR) biosensor has been utilized for monitoring analyte-ligand interactions in modern drug discovery processes. SPR biosensors measure the change in refractive indexes over the course of analyte molecules' binding to a specific immobilized ligand on sensor chip. This effort highlights a comprehensive SPR study besides enzymatic assay for discovery of new Angiotensin Converting Enzyme (ACE) inhibitors via screening of medicinal plants. At first, five medicinal plants were selected as potential sources for developing new ACE inhibitors through hydrolyzing hippuryl-L-histidyl-L-leucine (HHL) assay. The interaction of selected extracts with immobilized ACE on the sensor chip (500D) confirmed that the *Onopordum acanthium* L. had the greatest ACE inhibition activity among the set of compounds and its active compound (onopordia) was isolated. SPR biosensor used to evaluate binding affinity of onopordia and ACE. Equilibrium constant (K_D), and changes in Gibb's free energy of the binding ($\Delta G_{binding}$) values for the interaction of onopordia with ACE were found to be 10.24 µM and -28.48 kJ/mol, respectively. Computational analysis supported the binding of onopordia to the ACE active site. Kinetic and thermodynamic parameters of binding revealed that onopordia is an acceptable ACE inhibitor and could treat hypertension. SPR biosensor can be used to improve the drug discovery process for many important classes of drug targets due to its great sensitivity.

Keywords: Angiotensin Converting Enzyme, Surface plasmon resonance, Inhibitor, Medicinal plants, Drug discovery

1. Introduction

Angiotensin converting enzyme (ACE, EC 3.4.15.1) is a zinc dependent dipeptidyl carboxypeptidase capable of cleaving His-Leu from angiotensin I to produce the potent vasopressor angiotensin II (AngII) [1]. In addition, ACE hydrolyzes the vasodilator bradykinin by cleaving Phe-Arg from its COOH-terminus to form inactive components [2]. Enhanced activation of ACE by narrowing of the vessels and increasing blood pressure leads to different pathological outcomes including cardiovascular and kidney diseases [3-5]. Therefore, development of ACE inhibitors could be considered as a therapeutic strategy. To date, numerous compounds have been investigated to inhibit ACE such as captopril, lisinopril and their families. These compounds are safe and commonly used to treat hypertension and to maintain the electrolyte balance [6,7]. But they have several adverse side effects associated, such as dry cough, hyperkalemia and angioedema due to their lack of selectivity [8]. Thus, seeking for new ACE inhibitors with novel structure and improved selectivity is compulsory to improve the quality of human life.

Medicinal plants are potential source drug leads, due to their chemical structural diversity [9,10]. However, false positive or negative results caused by Pan Assay Interference Compounds (PAINS) are common problems of plants extract screening by chemical methods. This interaction in assay conditions such as metal chelation, fluorescence effects, cysteine oxidation, and redox cycling are more common [11,12]. Therefore, development of efficient and effective methods for evaluating their pharmaceutical properties and avoid mentioned problems has been a challenge for many years. The use of an orthogonal screening approach, combining a biophysical and a biochemical assay, could be helpful to avoid PAINS problems [13].

To this end, surface plasmon resonance (SPR) has been developed [14] to analyze a direct, label free procedure, to investigate the binding of an analyte to a target in real time, without complex sample preparation steps [15,16].

Computational techniques such as molecular docking, molecular dynamics simulations, and quantum mechanical methods have been emerged to monitor and compare the microscopic characteristics of interaction of new lead compounds, during the last decades [17,18]. Application of informatics could help to better understand the interactions of ligand-protein and complement experimental data.

In the present study, a carboxymethyl dextran SPR sensor chip (CMD 500D) was used to immobilize ACE via amine coupling. Subsequently, the interaction of the immobilized ACE with extracts from five selected medicinal plants was studied. Among them, *Onopordon acanthium* L. extract and its isolated compound (Onopordia) were identified as ACE potent inhibitors. Onopordia inhibitory activity was then compared with captopril using *in vitro* assays followed by combination of computational methods to assess ACE inhibitors.

2. Material and Methods

2.1. Chemicals

The carboxymethyl dextran (CMD 500D) sensor chip and the amine-coupling kit containing N-hydroxysuccinimide (NHS), N-Ethyl-N′-(3-dimethylaminopropyl) carbodiimide and ethanolamine hydrochloride were obtained from Xantec Bioanalytics (Germany). Angiotensin converting enzyme (ACE) from rabbit lung (CAS Number: 9015-82-1), hippuryl-L-histidyl-L-

leucine (HHL) and 4-(2-hydroxyethyl)-1-piperazineethane- sulfonic acid (HEPES) buffer were purchased from Sigma-Aldrich Co. (England). Deionized water was used in all experiments and all other reagents were of analytical grade and purchased from Merck (Darmstadt, Germany).

2.2. Plant materials

Thirty Medicinal plants that were used to manage different diseases (see Table S1) obtained from herbal store located in Tehran, Iran (October 2014) and Herbaratum of Faculty of Pharmacy, Tehran University of Medical Sciences (June 2015). Prof. F. Mojab identified these plants by plant taxonomy method. The authenticated samples were entrusted in the Herbarium of Shahid Beheshti University of Medical Sciences.

2.3. Crude extracts preparation

1 g of air dried and powdered plant material was extracted in 10 mL methanol:water (80:20) at room temperature (25 ± 1 ºC) for 24 hours and then in an ultrasonic bath over 2 hours. The resulting liquid extract was then filtered by Whatman cellulose filters 15-18 µm and concentrated to dryness under reduced pressure and freeze dried. The dry extracts were stored at -20 ºC till used [19].

2.4. ACE inhibition assay

Hippuryl-L-histidyl-L-leucine (HHL) was used as an artificial substrate to determine the activity of ACE, and amount of produced hippuric acid (HA) has been measured by reversed-phase high-performance liquid chromatography (RP-HPLC) [20]. Inhibitor solutions were prepared by dissolving 1mg of plant extracts in 1 mL of solvent containing buffer/DMSO (90:10, v/v) and

diluted to the final concentration of 330 μg/mL. The substrate solution (9 mM) was prepared by dissolving HHL in 50 mM HEPES buffer solution, containing 300 mM NaCl (pH 8.3).

25 μL of the ACE (80 mU/mL) was added to 25 μL of inhibitor solution at 37 ºC for 10 min. Subsequently, 25 μL HHL (9 mM) solution was added and reaction mixture was incubated for 30 min at 37 ºC. The reaction was stopped by the addition of 0.1 mL trichloroacetic Acid (TCA) (20%). The resulting reaction mixture was analyzed using RP-HPLC. A mixture of 10 mM KH_2PO_4 (pH 3) and methanol (50:50, v/v) was used as an isocratic mobile phase system. The flow rate was 1mL/min. Detection carried out with a PDA detector at the wavelength of 228 nm.

2.5. ACE inhibition measurement and Data processing

Activity of uninhibited ACE was selected as a control. Percentage of inhibition computed using the below formula:

$$I (\%) = 100 - 100 * (T / C) \qquad (1)$$

Where I (%) is the inhibition of the ACE, T is area under curve (AUC) of the HA peak with inhibitor, and C is AUC of the HA peak without inhibitor (control). Each experiment was accomplished in triplicate and data were expressed as mean ± standard error (SD). The IC_{50} value obtained from dose-response curves generated by conducting linear regression, using Graphpad prism 5 software.

2.6. Immobilization of ACE onto CMD chip surface

In this study SPR SR7500DC instrument (XanTec bioanalytics GmbH, Germany) was used for immobilization and detection. Surface carboxyl groups were activated by injecting a 10 min pulse of EDC/NHS, and then 250 µL of ACE (0.1 mg. mL^{-1}) in 10 mM acetate buffer (pH 4.5) was injected over the activated chip to immobilize via its primary amine groups (lysine residues) [21]. The remaining active sites of the SPR sensor were blocked with 250 mL of 1 ethanolamine (pH 8.5). A change in the refractive index was proportional to the analyte interacting with the surface.

2.7. Atomic force microscopy (AFM) characterization of chip surfaces

The surface topology of the bared and immobilized CMD chip was analyzed using AFM apparatus (Veeco-Autoprobe-CP-research). The AFM imaging achieved in non-contact mode at room temperature. A resolution of 256 pixels was utilized for all AFM images.

2.8. SPR measurements

To study the interaction between analyte and immobilized ACE, the analyte flowed over the sensor surface. After optimizing the pH condition, each analyte in 10 mM phosphate buffer (pH 7.5) were individually injected over the ACE immobilized CMD surface and the reference protein, human serum albumin (HSA), for 5 min with a flow rate of 50 µL/min at 25 ˚C [22]. A blank control run was performed by injecting the above-mentioned buffers and bulk effects were modified using solvent correction. Because of transient interactions of analytes and ACE, a continuous flow of running buffer was used as regeneration solution. Reference sensorgrams were

subtracted from binding sensorgrams using the Scrubber analysis program (Biologic Software Pty. Ltd., Canberra, Australia). All buffers and solutions used in SPR experiments have been filtered (through 0.22 µM syringe) and degassed.

2.9. Docking studies

AutoDockTools (ADT; version 1.5.6) [23] was used to characterize the binding modes of captopril and onopordia with ACE active site in a grid box of 72×72×72 Å. The crystal Structure of Human Angiotensin Converting Enzyme in complex with lisinopril (PDB ID: 1O86) was obtained from the RCSB Protein Data Bank (www.rcsb.org). The water molecules were omitted, and polar hydrogens were added to the protein structure. The Kollman-united charges method [24] was employed to calculate the partial atomic charges for all atoms of the enzyme. The chemical structures of ligands were generated by Marvin Sketch version 5.7, ChemAxon, (http://www.chemaxon.com). To add Hydrogens and perform energy minimization we used HyperChem software (version 8.0). Each docking job consisted of 100 runs, all other parameters and docking validation was performed using previously published methods [25].

2.10. QM binding energy calculations

All QM calculations were carried out using the Gaussian 09 quantum chemistry Package [26]. The geometry of the ligands along with zinc ion and number of amino acid residues with strong interactions used as protein-ligand complex systems for calculation of binding energies. The density functional theory (DFT) method at the B3LYP /6-31G(d) level of theory was performed

for the whole complex systems. The binding energy (BE) for protein-ligand complexes calculated according to the equation (2):

BE = E (complex) - E (Zinc ion and selected amino acid residues) - E (Ligand). (2)

3. Results and Discussion

3.1. Immobilization of ACE on CMD chip surface

To design a SPR- based biosensor, CMD500D sensor chip surface was activated by EDC/NHS. Then ACE passed over the sensor chip and successfully immobilized on the surface via amine coupling. Steps of immobilization are schematically shown in Figure 1.

Figure 1

Atomic force microscopy (AFM) was used to analyze the topographic features of the bare and immobilized CMD surface. Two and three dimensional topographic images of the CMD chip clearly showed homogeneous distribution of the immobilized enzyme (Figure 2). The AFM images along with the height distribution diagrams indicated a height difference between the immobilized surface than the bare CMD surface. The average surface roughness (Ra) was raised from 0.433 nm to 0.910 nm that demonstrates appropriate covalent attachment of ACE to the sensor surface.

Figure 2.

3.2. Medicinal plants as ACE inhibitors

Medicinal plants, due to their active chemical constituents, can be considerable resources to develop new drugs [27]. In this study, the inhibitory activity of thirty medicinal plants, which were traditionally employed to treat different diseases, was investigated using HHL as a substrate. Information about selected plants are mentioned in table S-1 (Supporting Information). According to the obtained results, five extracts exhibited more than 80% inhibitory activity. These extracts were selected to determine their IC_{50} values and the result was re-evaluated via SPR assay. The IC_{50} values of potent plant extracts are shown in Table 1.

Table 1.

3.3. SPR binding analysis of the selected plant extracts with ACE

Investigating and eliminating the PAINS is very important in drug discovery process due to their impact on creating false positive results [14]. Therefore, the utilization of surface plasmon resonance biosensor (binding assay) accompanied by enzymatic assay could be useful to avoid problems created by PAINS. Therefore, in the current study, SPR biosensor was used to screen selected plant extracts. At first, ACE immobilized onto CMD500D by amine coupling method. To evaluate the activity and accessibility of the immobilized enzyme, HHL was injected over immobilized ACE. Analyzing the collected product by RP-HPLC, confirmed the catalytic activity of the enzyme after immobilization. Captopril as a known competitive inhibitor of ACE was also used as a positive control in this study. Then selected plant extracts with a concentration of 33 µg/mL were injected over the immobilized enzyme to study SPR based binding assay. Sensorgrams resulting from the interaction of selected plant extracts with ACE are shown in Figure 3. As shown in Figure 3, the corresponding SPR signal increased with the injection of plant extracts

over the sensor chip. When the binding process reached equilibrium, the running buffer was passed over the sensor, the analyte was dissociated from ACE and the SPR signal decreased. Eventually, the SPR signals returned back to the baseline, and showed that the binding of plant extracts is reversible.

Figure 3.

The results achieved from SPR assay were in good agreement with HHL hydrolysis assay (Table 1). Among the chosen extracts, *O. acanthium* exhibited the highest association constant (k_a) for binding and had a strong interaction with ACE without requiring regeneration solution (reversible binding); therefore, it has been selected for future studies.

3.4. Interaction study between immobilized ACE and onopordia

Onopordia (Figure 4A), as an ACE inhibitor, was extracted from *O. acanthium* seeds by a repeated column chromatography as described previously [25]. The inhibitory effect of onopordia was examined by HHL degradation assay and the IC_{50} value was determined to be 300 µM (Figure 4B). In order to study the binding kinetics, onopordia was injected concurrently over both the immobilized ACE and the reference channel (HSA). A fast and continuous response was observed. When the binding reached equilibrium, running buffer was passed over the sensor chip to dissociate and regenerate the chip, and eventually giving the baseline response. As shown in Figure 4C, the corresponding SPR signal increased upon injections with increasing concentration of onopordia from 1 nM to 1 mM. The results revealed that onopordia bound reversibly to ACE, with a clear association and dissociation phase.

Figure 4.

Detecting small molecular weight analytes (molecular weight <500 Da) in development of SPR biosensors is a big challenge [28]. Here, because of, proper signal intensity obtained from the interaction between ACE and onopordia (MW=326.7 Da), we ensure a measurable change in refractive index despite the low molecular weight of the drug.

The interactions between immobilized ACE and onopordia was then characterized by the equilibrium constant (K_D). To determine the association and dissociation constants (k_a and k_d), were calculated with the two state binding model, [A] + [B] ↔ [AB], where A is the injected analyte, B is the immobilized ligand, and AB is the analyte–ligand complex formed during the reaction. In the SPR system, the signal is proportional to the amount of [AB] and R_{max} is the maximum analyte [B] binding capacity of the surface [29]. The K_D value was used to compute the thermodynamic parameters, using Van't Hoff equations [30]:

$$\Delta G_{\text{binding}} = -RT \ln K_A \qquad (3)$$

$$K_A = 1/K_D \qquad (4)$$

Where R is the ideal gas constant, K_A is the affinity constant, T is temperature, and $\Delta G_{binding}$ is changes in Gibb's free energy of the binding of analyte with the immobilized ACE. In SPR assay captopril, a known ACE inhibitor, used as positive control. To study the binding affinity of captopril with immobilized ACE, various concentrations of this compound was injected over both the immobilized ACE and the reference channel (Figure 5A). The biosensor demonstrated a linear detection range from 0.1 to 5 nM (Figure 5B). The LOD (limit of detection) and LOQ (limit of quantification) based on 3 × (standard deviation of the blank/slope) and 10 × (standard deviation of the blank/slope) turned out to be 33 and 110 pM, respectively.

Figure 5.

Kinetic and thermodynamic parameters for interaction between captopril-ACE and onopordia-ACE have been illustrated in Table 2.

Table 2.

3.5. Docking studies

The molecular docking methodology was used to identify the position, orientation and interactions of captopril and onopordia with ACE binding pocket. As it is depicted in Figure 6, captopril strongly interacts with ACE, by forming hydrophobic bonds with Ala354, Ser355, Phe512, Tyr523, and hydrogen bonds with Ala356, His383, His387 and Glu411. Onopordia has a different mode of interactions in comparison with captopril by penetrating into hydrophobic pocket of ACE and establishing hydrophobic interactions with Ala356, Phe391, Tyr394. Furthermore, it was stabilized by forming hydrogen bonds with His353, Asp 358, Glu384 and His513.

Specific hydrogen bonding and hydrophobic interactions of captopril and onopordia with ACE active site confirmed the inhibitory activity of both inhibitors. Captopril showed more inhibitory activity against ACE when compared with onopordia. There is a good correlation between docking analysis and experimental values (K_D, IC_{50}).

Figure 6.

3.6. DFT Binding energy analysis

Further analysis of the complexes of captopril and onopordia with ACE revealed that the most important amino acids in the active site are His353, Ala354, Ser355, Ala356, Asp358, Tyr360, His383, Glu384, His387, Phe391, Tyr394, Glu403, His410, Glu411, Asp415, Phe512, His513,

Arg522 and Tyr523 that are involved in the ACE-ligand interactions. The DFT binding energy calculations were performed for the systems, including the captopril and onopordia as ligands, 19 selected amino acid residues, and the Zinc ions existing in the active pocket of ACE. The N- and C-termini of amino acids were terminated using hydrogen atoms in order to neutralize the charge of the residues. The binding energies of captopril and onopordia to ACE was calculated as -89.24 and -68.63 kJ/mol, respectively. The BEs computed via the abovementioned method was in agreement with the experimental results and demonstrated the stronger binding of captopril than onopordia to the ACE.

4. Conclusions

Hypertension, silent killer, causing several serious pathological conditions such as cardiovascular diseases, kidney diseases, and myocardial infarction. It is clear that ACE inhibitors are useful in the treatment of hypertension and heart failure, therefor it is necessary to create new robust approach to identify novel ACE inhibitors. To this end, several instrumental methods have been developed to analyze the ACE inhibitor activity of various synthetic or natural compounds [31-34]. However, these methods present a number of disadvantages, such as lack of sensitivity, high ACE consumption, and the involvement of organic solvents, which limit their applications. This report highlights the development of an enzyme-based SPR biosensor for discovering of ACE inhibitors via investigating its interaction with immobilized enzyme. At first, inhibitory activity of 30 medicinal plant extracts against ACE were examined by HHL hydrolyzing assy. Among them five of the extracts were chosen as potential sources for developing new ACE inhibitors. Immobilization of ACE on the sensor chip, followed by interaction with selected plant extracts confirmed that the *O. acanthium* extract had the greatest binding to ACE. The efficiency of the

biosensor was examined by captopril and onopordia. The K_D values of captopril and onopordia were 10 nM and 10.24 µM, respectively. $\Delta G_{binding}$ values revealed a spontaneous interaction between inhibitors and ACE. Comparison between K_D values showed that onopordia is a moderate ACE inhibitor and could be exposed for further optimization, to generate a more potent inhibitor. The results obtained from computational studies were in good agreement with SPR assay and confirmed that captopril and onopordia bound to the ACE active site. The most considerable finding of this study was that onopordia was responsible for the ACE inhibitory activity, and *O. acanthium* seeds extract could be used as a potent ACE inhibitor for treatment of hypertension.

In summary, we have used SPR technology combined with enzymatic assay to screen plant extracts and to evaluate the kinetic and thermodynamic binding parameters of ACE–inhibitors. SPR technique is a complementary device to enzymological examinations for elimination of false positive readout caused by PAINS. Therefore, these methods could be employed, to improve the drug discovery process and to select promising inhibitors due to its greater sensitivity, fast, label free and real time procedure.

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Legends

Figure 1. Different steps for covalent immobilization of ACE onto the activated SPR-CMD surface. Carrier solution: 10 mM phosphate buffer (pH 7.5), flow speed 25 µL/min. After washing the chip by NaCl/NaOH, the carboxyl group of surfaces activated by EDC/NHS, then ACE was injected over one channel (immobilized ligand channel) and finally, surfaces were blocked with ethanolamine. The solid lines representation; blue line: Sample side, red line: Reference and pink line: Difference.

Figure 2. Two and three-dimensional AFM micrographs of bare (A and B) and immobilized surface (D and E). Height distribution diagrams are shown at C and F.

Figure 3. SPR response between plant extracts and the immobilized ACE.

Figure 4. (A) Chemical structure of onopordia, (B) The IC_{50} value for onopordia, (C) SPR sensorgrams for the interaction of ACE and onopordia; carrier solution: phosphate buffer, flow speed: 50 µL/mim, flow duration: 5 mins.

Figure 5. (A) SPR sensorgrams for the interaction of ACE and captopril; carrier solution: phosphate buffer, flow speed: 50 µL/mim, flow duration: 5 mins, (B) Calibration curve in the linear detection range from 0.05 nM to 5 nM, (C) The IC_{50} value for captopril.

Figure 6. Interaction of the ligands (captopril and onopordia) with ACE active site. Two-dimensional representation; olive green arrow: H bond interaction, brick red representation: hydrophobic site, Nitrogen is blue, Oxygen is red, Carbon is black and Sulfur is yellow (Left). Three-dimensional representation; captopril and onopordia are presented in magenta and orange, respectively, and the residues in green colors (Right). (A) Captopril, (B) Onopordia.

Figures

Figure 1.

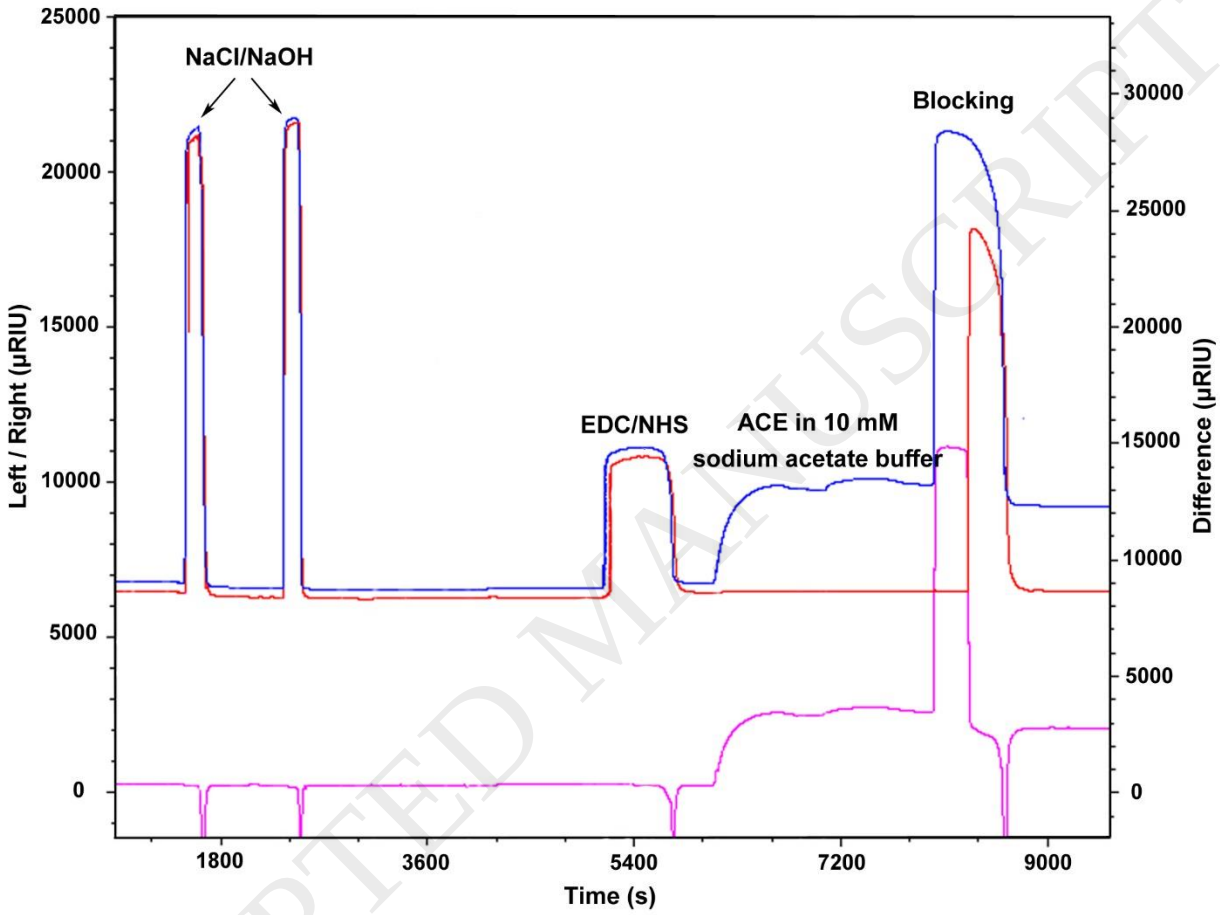


Figure 2.

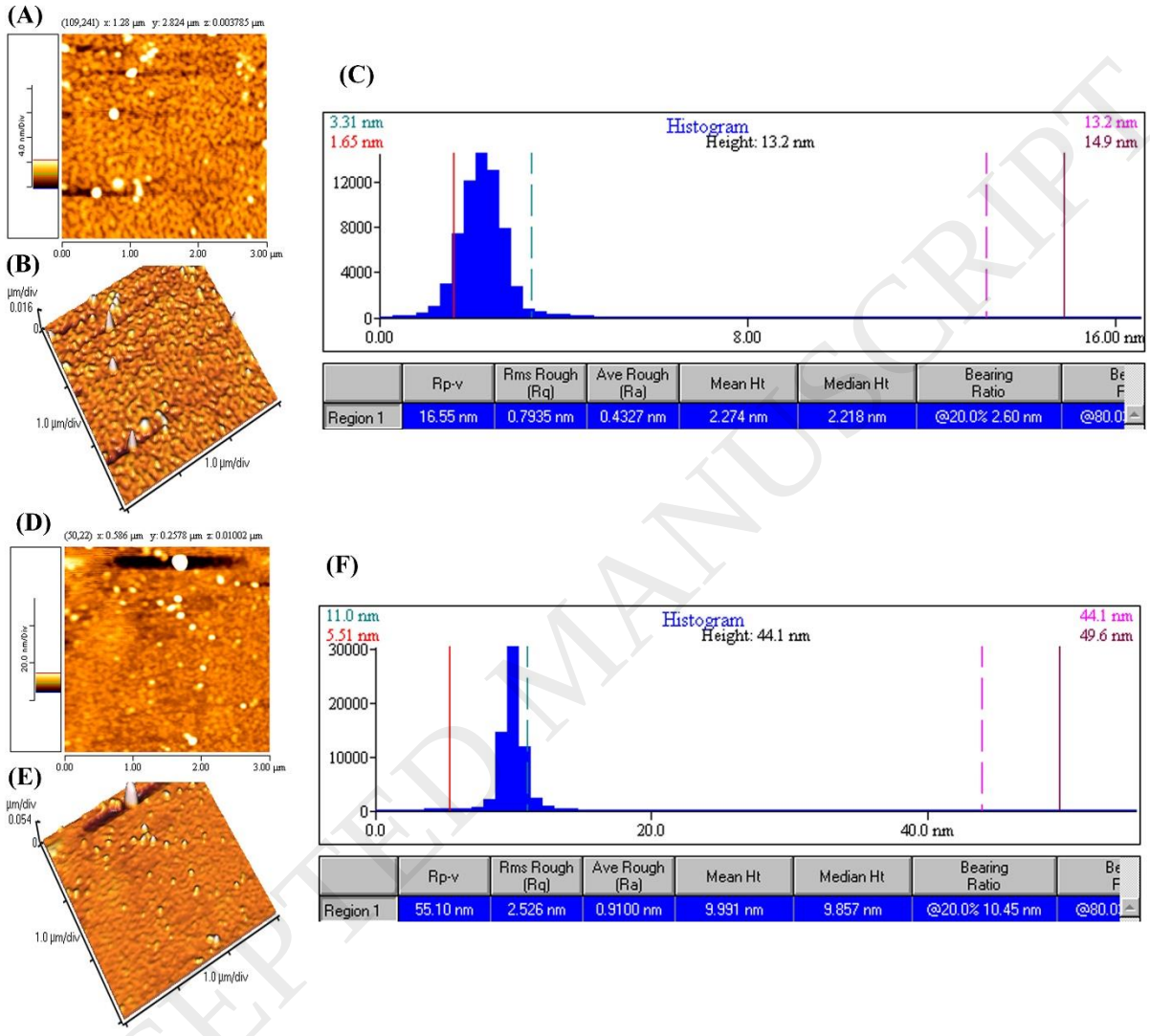


Figure 3.

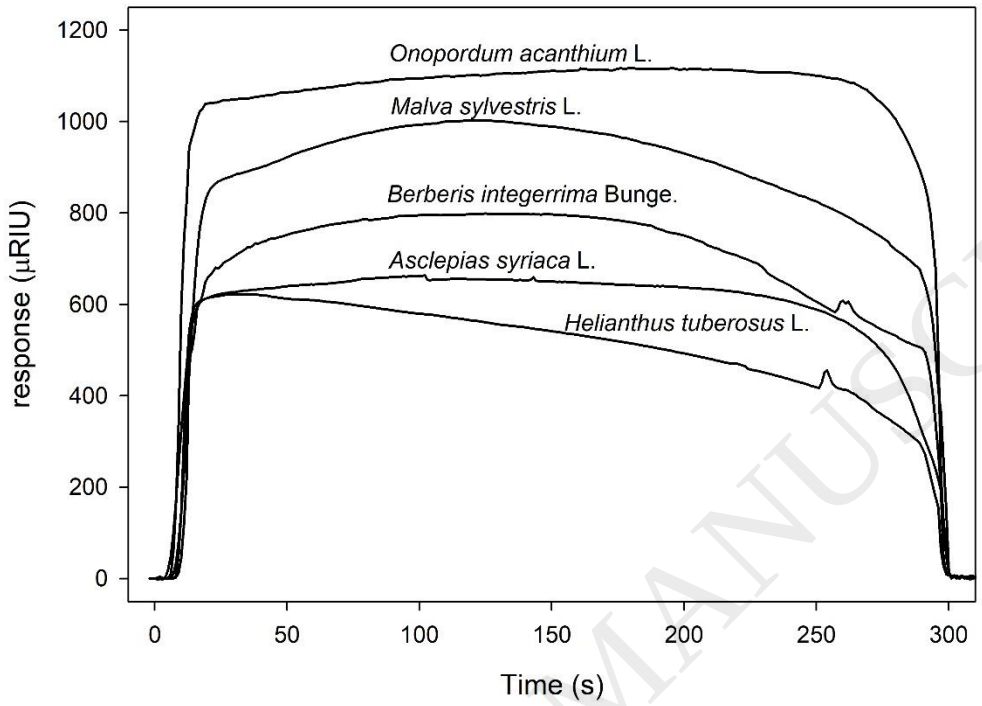


Figure 4.

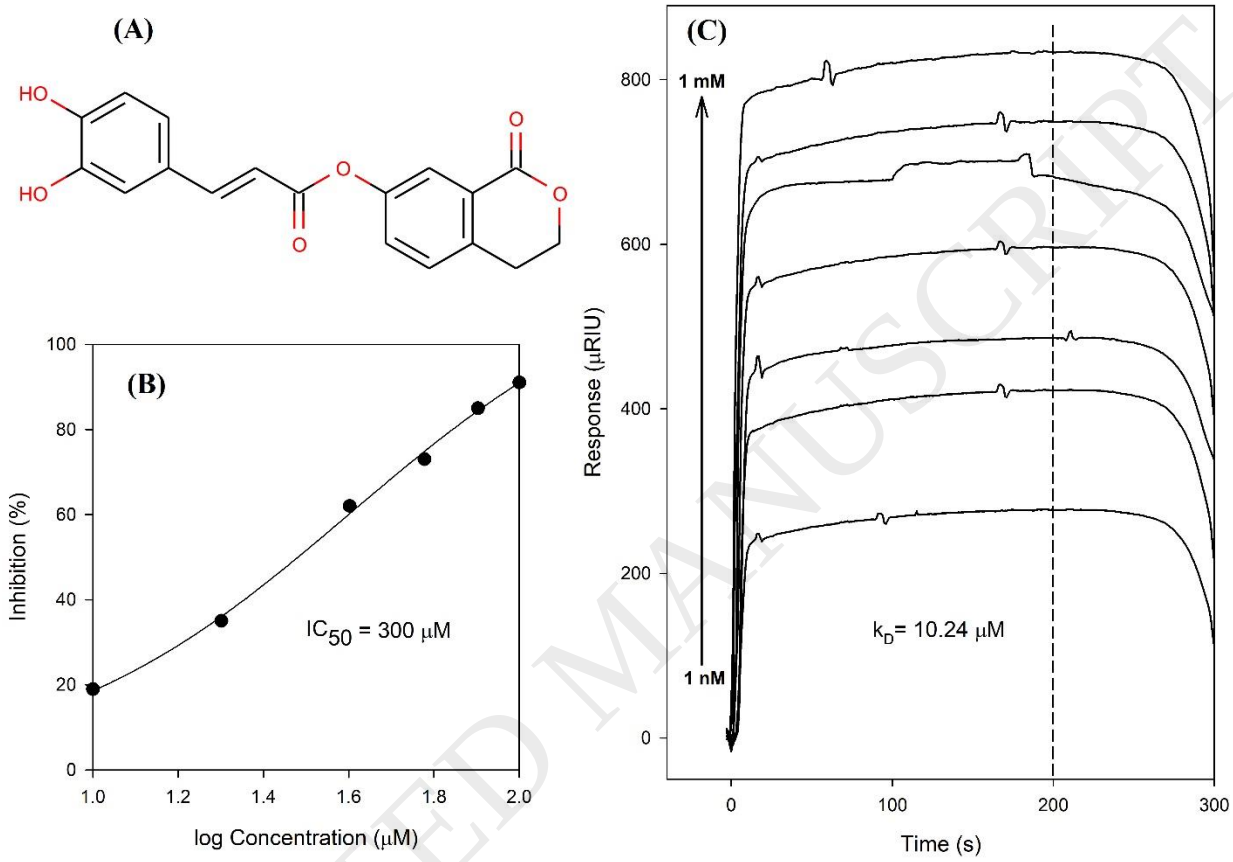


Figure 5.

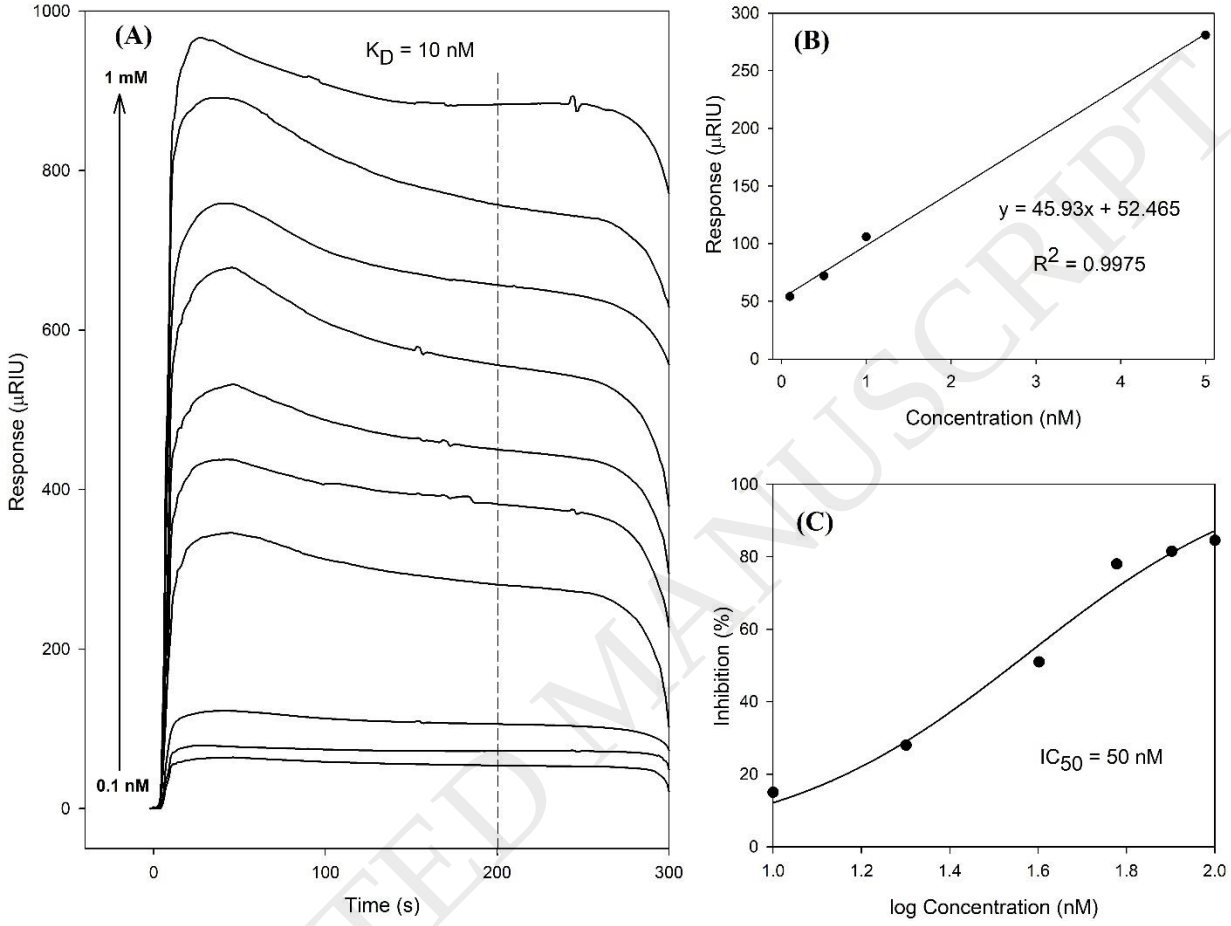
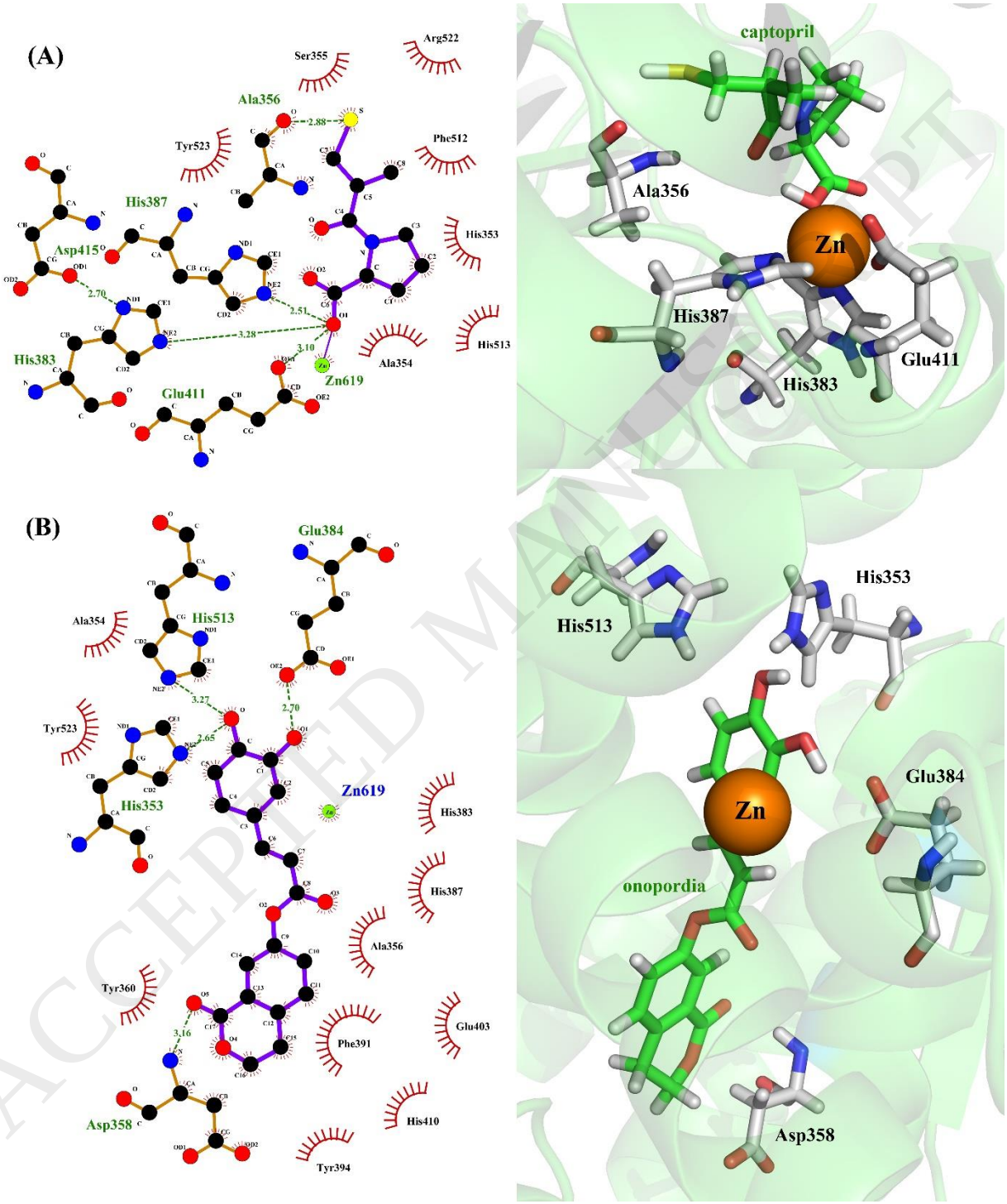


Figure 6.



Tables

Table 1. The percent of enzyme inhibition and the IC_{50} (µg/mL) of potent plant extracts.

Scientific name	**Percent of inhibition**	**IC_{50} (µg/mL)**
Asclepias syriaca L.	73.31 ± 0.28	76.05
Berberis integerrima Bunge.	78.15 ± 0.2	51.67
Helianthus tuberosus L.	69.13 ± 0.09	103.92
Malva sylvestris L.	85.07 ± 0.73	37.14
Onopordum acanthium L.	90.73 ± 1.21	23.16

Table 2. Kinetic analysis parameters of interaction between ACE and its inhibitors.

Parameter / Inhibitor	k_a (M^{-1} S^{-1})	k_d (S^{-1})	K_D (kinetic)	R_{max}	ΔG_b (kJ/mol)	Fitting model
Captopril	$7x10^6$	0.07	10 nM	2040	-45.66	1:1
Onopordia	$1.21x10^8$	1238	10.24 µM	1550	-28.48	1:1