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Identification of Bioactive Peptide Inhibitors of Cyclooxygenase-2 from Peanut Worm (*Siphonosoma australe*): An *In Silico* Studies

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Abstract

The study was conducted to address the growing need for safer and more effective anti-inflammatory medications, given the side effects associated with conventional anti-inflammatory drugs. Natural sources, such as bioactive peptides, present a promising alternative. The research aimed to generate cyclooxygenase-2 (COX-2) inhibitory peptides from *Siphonosoma australe* and evaluate their potential as natural anti-inflammatory drug candidates using *in silico* methods. The PeptideCutter, SwissADME, SwissTargetPrediction, ToxinPred, and principal component analysis (PCA) were utilised to generate peptides, followed by molecular docking using Yasara-structure software. The study identified 113 peptides as suitable candidates based on their physicochemical properties, COX-2 inhibitory potential, and safety profiles. PCA narrowed these down to 5 peptides; Phenylalanine-Tryptophan (FW), Phenylalanine-Phenylalanine (FF), Phenylalanine-Proline-Phenylalanine (FPF), Leucine-Proline-Phenylalanine (LPF), and Valine-Proline-Phenylalanine (VPF) with similar characteristics to celecoxib. Among these, the FW peptide exhibited the best binding energy (–9.32 kcal/mol) and significant inhibitory interactions with COX-2 through hydrogen bonds with Tyr341 and Val509, hydrophobic interactions with Ile331, Val335, Leu345, Ala502, and Leu517, and plenty of amino acid residues in van der Waals forces. The findings highlight the therapeutic potential of peptides from *Siphonosoma australe* as natural COX-2 inhibitors, offering a safer alternative to synthetic drugs for managing inflammation. This research contributes to the development of natural anti-inflammatory therapeutics, addressing a critical need in pharmacological research.

Keywords: Anti-inflammatory, COX-2 inhibitor, *In silico*, Molecular docking, Peptide, Peanut worm, *Siphonosoma australe*

Introduction

Inflammation is a biological response to harmful stimuli, such as foreign bodies, irritants, and pathogens. COX-2 is a significant inflammatory mediator that causes pain, redness, swelling, heat, and loss of function. The enzyme COX-2 facilitates the conversion of arachidonic acid into prostaglandins. Several inflammatory disorders, such as cancer, stroke, cardiovascular disease, and arthritis, are associated with elevated levels of COX-2 [1,2]. Current anti-inflammatory drugs that target COX-2, such as nonsteroidal anti-inflammatory drugs (NSAIDs), are effective but are associated with significant adverse effects, including gastrointestinal toxicity, renal impairment, and cardiovascular risks [3]. These limitations necessitate the development of safer and more sustainable therapeutic alternatives. Natural sources, particularly bioactive compounds and peptides derived from plants and animals, have gained attention as promising alternatives for anti-inflammatory drug development [4].

Marine organisms with anti-inflammatory properties can be found in the Sipuncula phylum. The peanut worm (*Siphonosoma australe*) belongs to the Sipuncula phylum. It is used as a food source and a traditional medicinal ingredient [5]. Zhang *et al.* [6] found that extracts derived from the peanut worm

Sipunculus nudus possess properties that can decrease inflammation and relieve pain. Furthermore, Sangtanoo *et al.* [7] discovered that 2 novel peptides derived from *S. nudus* possess anti-inflammatory characteristics. The peptides derived from the peanut worm *S. nudus* have anti-inflammatory characteristics by influencing various mechanisms, including reducing pro-inflammatory agent expression and inhibiting COX-2 [8,9]. Despite these findings, no research has explored of *S. australe* as a source of bioactive peptides targeting COX-2. According to this, *S. australe* has the potential to be used to create and produce novel bioactive peptides as a substitute for current anti-inflammatory drugs.

Bioactive peptides are small segments of protein found in our food, composed of 2 - 20 amino acids with a molecular weight of less than 3 kDa, and they contribute to improving health [10]. Challenges in traditional bioactive peptide research, such as the complexity of isolation, high costs, and time constraints, make computational strategies an ideal solution. Using *in silico* proteins is a legitimate alternative strategy for conducting research studies to generate bioactive peptides [11]. Using molecular docking *in silico* allows for predicting interactions between peptides (ligands) and protein receptors using computational procedures [12]. Computer-based simulation will enable researchers to create, design, and simulate highly precise peptides. Furthermore, it can predict how peptides interact with their biological targets, allowing for faster screening of potential peptide candidates. Using *in silico* methods can reduce time and costs while facilitating the quick identification of numerous possible peptide structures for further development.

Until now, the peanut worm *S. australe* has not been used as a source of peptides that inhibit COX-2, especially *in silico* methods. The primary objective of this research is to comprehensively understand the potential of *S. australe* as a natural source of bioactive peptides with COX-2 inhibitory activity. The specific aims are to identify peptides generated from *S. australe* protein hydrolysates using computational tools; to evaluate the physicochemical properties, toxicity, and COX-2 inhibition potential of the identified peptides; and to perform molecular docking analysis to investigate the binding affinity and interaction mechanisms of the peptides with COX-2. This research will provide a solid foundation for further *in vitro* and *in vivo* studies to investigate the therapeutic potential of peanut worms as a natural anti-inflammatory drug candidate.

Materials and methods

Materials and tools

The materials utilized in this study included the fasta sequences of peanut worm *S. australe* (cytochrome b and c), obtained from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov), and the COX-2 enzyme structure identified with PDB ID: 3LN1, retrieved from the Protein Data Bank (https://www.rcsb.org/). The software tools employed in the analysis were Peptide Cutter (https://web.expasy.org), SwissADME (www.swissadme.ch), SwissTargetPrediction (http://www.swisstargetprediction.ch), ToxinPred (https://webs.iiitd.edu.in), PCA using Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA), NovoPro for generating 3D structures (https://www.novoprolabs.com/tools/smiles2pdb), Yasara-structure software version 23.8.19 (YASARA Biosciences), and Discovery Studio Visualizer (DSV) version 21.1.0.20298 (Dassault Systèmes). All software was operated with default configurations. The research was performed on a PC with an Intel Core i7-13700KF processor and 64 GB of RAM, utilizing the Linux operating system.

Methods

Target protein selection

The protein target used in this study is the COX-2 enzyme, retrieved from the protein data bank (https://www.rcsb.org/) [13] with PDB ID 3LN1 (**Figure 1**). Then, the enzyme structure was prepared by removing water, unnecessary ligands, and adding polar hydrogen using Yasara-structure software.

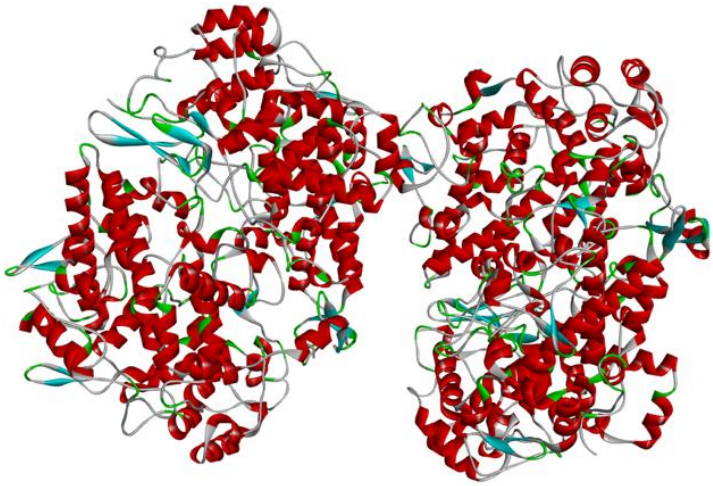

Figure 1 3D structure of COX-2 from NCBI.

Ligand preparation

Peanut worm (*S. australe*) fasta was obtained from website ncbi.nlm.nih.gov and consists of 2 parts: Cytochrome b, partial mitochondrion (accession number: UJZ05548.1), and cytochrome c oxidase subunit I, partial mitochondrion (accession number: UPO78953.1) (https://www.ncbi.nlm.nih.gov) [14]. Cytochrome b and c are subsequently enzymatically hydrolysed with digestive enzymes pepsin (pH 1.3 and pH >2), trypsin, and chymotrypsin (high and low specificity) using a peptide cutter (https://web.expasy.org) [15]. Enzyme cleavage produces small peptides, also known as ligands, which are then chosen for further stages.

Ligand selection

Ligands derived from enzymatic hydrolysis are then selected in several stages. First, using Lipinski's rule (500 Daltons molecular weight, 5 Log P for octanol-water partition coefficient, 10 H bond acceptors, 5 H bond donors) (http://www.swissadme.ch) [16]. Furthermore, by determining if the ligand has the potential target to inhibit COX-2 (http://www.swisstargetprediction.ch) [17]. The next stage is to determine if the ligand is toxic (https://webs.iiitd.edu.in) [18]. The final stage uses PCA to reduce the number of ligands into smaller and more correlated dimensions.

Principal Component Analysis (PCA)

The selected ligands or peptides were tabulated in MS Excel, and PCA was performed. The PCA analysis used Lipinski's rule of five (Ro5) to compare the peptides to celecoxib. Celecoxib is a selective COX-2 inhibitor and a positive control; hence, the peptide closest to celecoxib was chosen as a possible ligand for docking.

The selected peptide was converted into a 3D structure using the website novoprolab.com. Firstly, the peptide sequence is transformed into SMILES string format [19]. Next, the SMILES string of the peptides was converted into a 3-dimensional (3D) structure format [20]. Then, the 3D structure of peptides was prepared by minimising the energy using Yasara-structure software (YASARA Biosciences GmbH).

Molecular docking of the ligand and protein

Yasara-structure, employing a force field scoring functional approach, is the basis for the docking method utilised to calculate the binding energy value. The docking process uses a macro command (dock_run.mrc), which is available in Yasara-structure. The grid box was created with a radius of 5.0 Å around the native ligand. The vina docking method within the Yasara structure was utilised to compute binding energy and identify receptor residues involved in the interaction. There are 100 conformations in the docking experiment. Upon finishing the docking proses, the outcomes were stored in the PDB (.pdb) file format. Subsequently, the post-docking data underwent analysis and visualisation using Biovia Discovery Studio software (Dassault Systèmes).

Results and discussion

Ligand screening

The peanut worm (*S. australe*) was chosen for an *in silico* investigation based on Nurhikma *et al*. [21]. The extract of *S. australe* has been shown to have anti-inflammatory properties, with an IC_{50} of 3.80 µg/mL.

Using the keyword "*Siphonosoma australe*", the peanut worm protein fasta selection process was completed using the ncbi.nlm.nih.gov web service. Several protein fasta were produced; however, they were mostly composed of 2 types of sources: Cytochrome b and cytochrome c. Furthermore, samples of the 2 kinds of cytochromes were chosen based on the most extended amino acid fasta sequence. Cytochrome b has 330 amino acids of protein, whereas cytochrome c contains 300 amino acids. The chosen protein fasta was subsequently hydrolysed using digestive enzymes.

Enzymatic hydrolysis using digestive enzymes is used to acquire insight into gastrointestinal processes in the human body. They used pepsin, trypsin, and chymotrypsin, resulting in short-chain peptides that can act as COX-2 inhibitors. The results of enzyme cleavage revealed that cytochrome b produced more peptides than cytochrome c. This is because the length of the protein amino acid sequence influences the quantity of enzyme cleavage; the longer the sequence, the more peptides can be produced by the cleavage. Pepsin is the enzyme with the most peptide manufacturers in cytochrome b and cytochrome c, at 144 and 115, respectively. The quantity of peptides produced depends on the enzyme's selectivity in cleaving the protein polypeptide chain. Pepsin cleaves the telopeptide bond into smaller peptides with high specificity for hydrophobic amino acids [22].

After completing enzymatic hydrolysis, the peptides were filtered through several stages to determine their bioactivity. First, the SwissADME web tools were used to predict the peptides' physicochemical and drug-likeness. Only peptides that met the Ro5 criteria were included in the selection. The total number of peptides acquired the criteria of Ro5 from cleavage with pepsin, trypsin, and chymotrypsin enzymes of 49, 1, and 73, respectively (**Figure 2**). According to Ro5, a molecule is more likely to have poor absorption or penetration if it has more than 5 hydrogen bond donors, more than 10 hydrogen bond acceptors, a molecular weight larger than 500, and a predicted Log P (CLogP) more significant than 5 (or MlogP > 4.15) [23].

Furthermore, selected peptides are used to determine if the peptide has the potential to inhibit COX-2 using the SwissTargetPrediction web tool. SwissTargetPrediction uses reverse screening to predict the most probable protein targets for small molecules based on the similarity principle [24]. The next stage is to determine if the peptide is toxic using the ToxinPred web tool. ToxinPred is capable of predicting peptide or protein toxicity. It will help design the least harmful peptides and identify toxic protein areas [25]. According to the results, the total number of peptides from cleavage with pepsin, trypsin, and chymotrypsin enzymes is 47, 1, and 65, respectively (**Figure 2**). Considering the quantity of these outcomes are equal, all peanut worm peptides that have target COX-2 inhibition are safe and non-toxic to the human body.

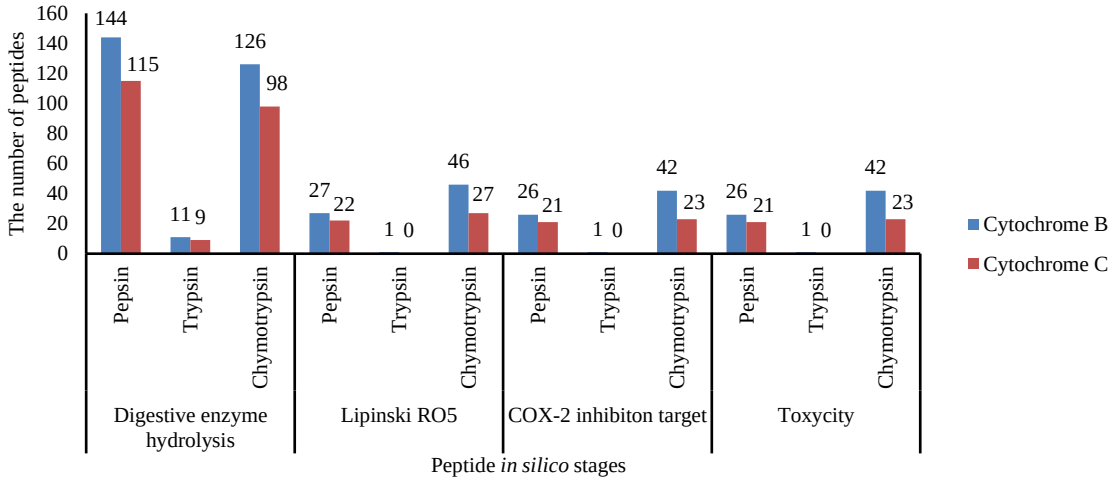

Figure 2 Number of peptides at each stage of *in silico* screening.

Selected ligand

The screening results obtained 113 peptides; thus, PCA was performed. The PCA was used to reduce and analyse the peptides based on their similarity to celecoxib as measured by the Ro5 value, which was then chosen for molecular docking. The PCA results revealed

that 5 peptides were selected that were similar to celecoxib (**Figure 3**), consisting of dipeptides (FW and FF) and tripeptides (FPF, LPF, and VPF) with molecular weights ranging from 312 to 409 Da. The selected peptides do not violate the Ro5 criteria, which means they are drug-likeness, have high gastrointestinal (GI) absorption ability, are non-toxic, and have a binding energy value similar to celecoxib [26] (**Table 1**). Celecoxib is a selective inhibitor of the COX-2 enzyme used as a nonsteroidal anti-inflammatory drug (NSAID) to relieve pain and fever. It is among the most effective nonsteroidal anti-inflammatory agents [27].

Before molecular docking, the selected peptides were transformed into SMILES and subsequently into 3D using the Novoprolab web tools (**Figure 4**). The 3D structure of peptides was created by removing water, adding hydrogen atoms, and minimising energy with Yasara-structure software.

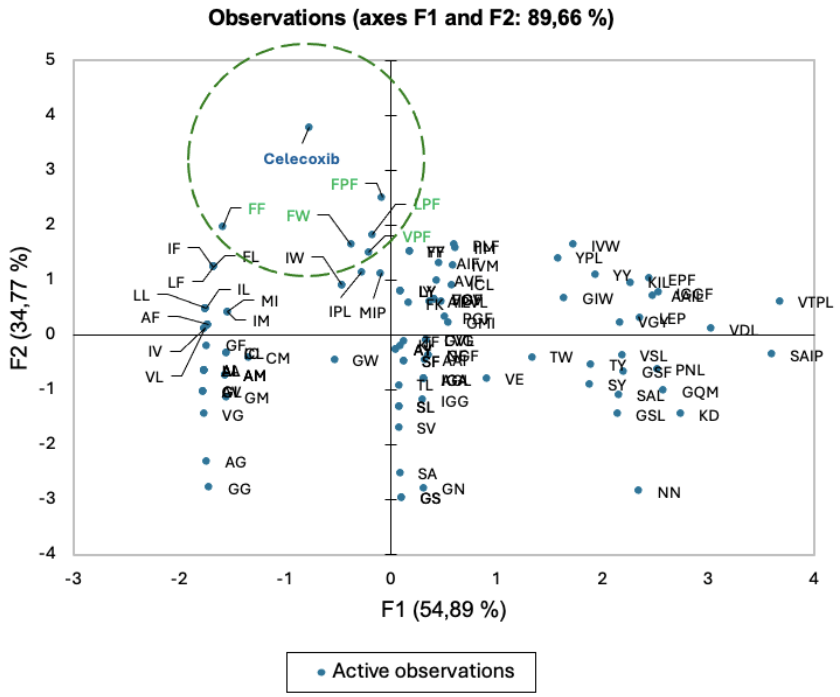

Figure 3 PCA of *S. australe* peptides.

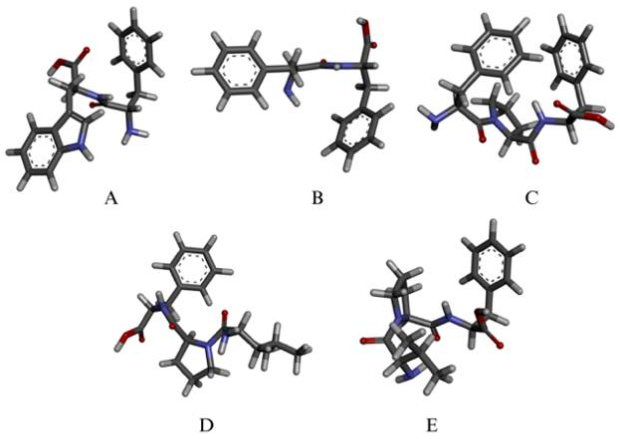

Figure 4 3D structure of selected peptides: (A) FW, (B) FF, (C) FPF, (D) LPF, and (E) VPF.

Molecular docking

The molecular docking revealed that the interaction between the protein (COX-2) and the selected ligands (peptides) is shown in **Figure 5**. The peptides' binding energy values ranged between –6.99 and –9.32 kcal/mol (**Table 1**). The FW peptide had the

highest binding energy of –9.32 kcal/mol, comparable to celecoxib's –10.9 kcal/mol [26]. Peptide FW interacts with COX-2 by forming hydrogen bonds with Tyr341 and Val509, hydrophobic interactions with Ile331, Val335, Leu345, Ala502, and Leu517, and Van der Waals interactions with His75, Met99, Val102, Gln178, Val330, Leu338, Ser339, Arg499, Ile503, Phe504, Met508, Gly512, Ala513, Ser 516, and Leu520 (**Figure 5(A)**). The hydrogen bond with Tyr341 and Val509 from the compound 149 had excellent inhibition against COX-2 (91 %) [28]. The interaction of hydrophobic amino acids with Val335 and Ala513, primarily established compound 9 at the active site of COX-2, with a binding energy value of –9.4 kcal/mol [29]. Peptide FW is a ligand candidate with the best ability among the other 4 candidates. The FW peptide comprises the hydrophobic amino acids phenylalanine and tryptophan, specifically Phe, at the N terminus. Hong *et al.* [30] found that tetrapeptides from walnut pulp with hydrophobic amino acid residues at the N terminus formed strong hydrogen bonds and hydrophobic interactions with residues within the COX-2 active site. Furthermore, due to the hydrophobic nature of the COX-2 active site, Gly, Ala, Val, Leu, and Phe were selected to synthesise COX-2 inhibitory peptides [31].

The interactions of the other selected peptides with the active side of COX-2 reveal similarities and differences in some of the enzyme's amino acid residues. Peptide FF formed hydrogen bonds with Tyr341 and Ala513, as well as hydrophobic interactions with Ile331, Val335, Leu338, Leu345, Val509, and Leu517 (**Figure 5(B)**). The FPF peptide formed hydrogen bonds with Ser339, Tyr341, and Val509, and hydrophobic interactions with Val102, Val335, Leu345, Arg499, Ala502, Ala513, and Leu517 (**Figure 5(C)**). The LPF peptide formed hydrogen bonds with His75 and Ser339. It also had hydrophobic interactions with Ile331, Val335, Leu345, Ala502, Phe504, Val509, Ala513, and Leu517 (**Figure 5(D)**). VPF peptides formed hydrogen bonds with Ser339, Met508, and Val509, and hydrophobic interactions with Ile331, Val335, Leu338, Tyr371, Trp373, Phe504, and Leu517 (**Figure 5(E)**). Their interactions with Leu338, Ser339, Arg499, and Phe504 residues in the active site are similar to celecoxib, which suggests that they can selectively inhibit COX-2 [32]. Furthermore, each peptide forms multiple van der Waals forces with amino acid residues on COX-2's active site. The van der Waals force primarily stabilises the COX-2 active site at the naphthoquinone 9-binding region [29]. These results imply that peanut worm peptides may be a natural source for substitute medications with strong COX-2 inhibitory actions, although more investigation is necessary.

Table 1 Characteristics of selected ligands.

No.	Selected ligand	Lipinski Ro5				GI Absorption	Targeting COX-2	Toxicity	Binding energy (kcal/mol)
		MW (Da)	MlogP	H-bond acceptors	H-bond donors				
1	FW	351.40	1.27	4	4	High	Yes	Non-Toxin	–9.32
2	FF	312.36	1.90	4	3	High	Yes	Non-Toxin	–7.64
3	FPF	409.48	1.34	5	3	High	Yes	Non-Toxin	–6.99
4	LPF	375.46	0.92	5	3	High	Yes	Non-Toxin	–8.43
5	VPF	361.44	0.69	5	3	High	Yes	Non-Toxin	–7.64
6	Celecoxib	381.37	2.65	7	1	High	Selective drugs	Non-Toxin	–10.9[26]

Legend: MW = Molecular weight, Ro5 = Rule of five, GI = Gastrointestinal, and COX-2 = Cyclooxygenase-2.

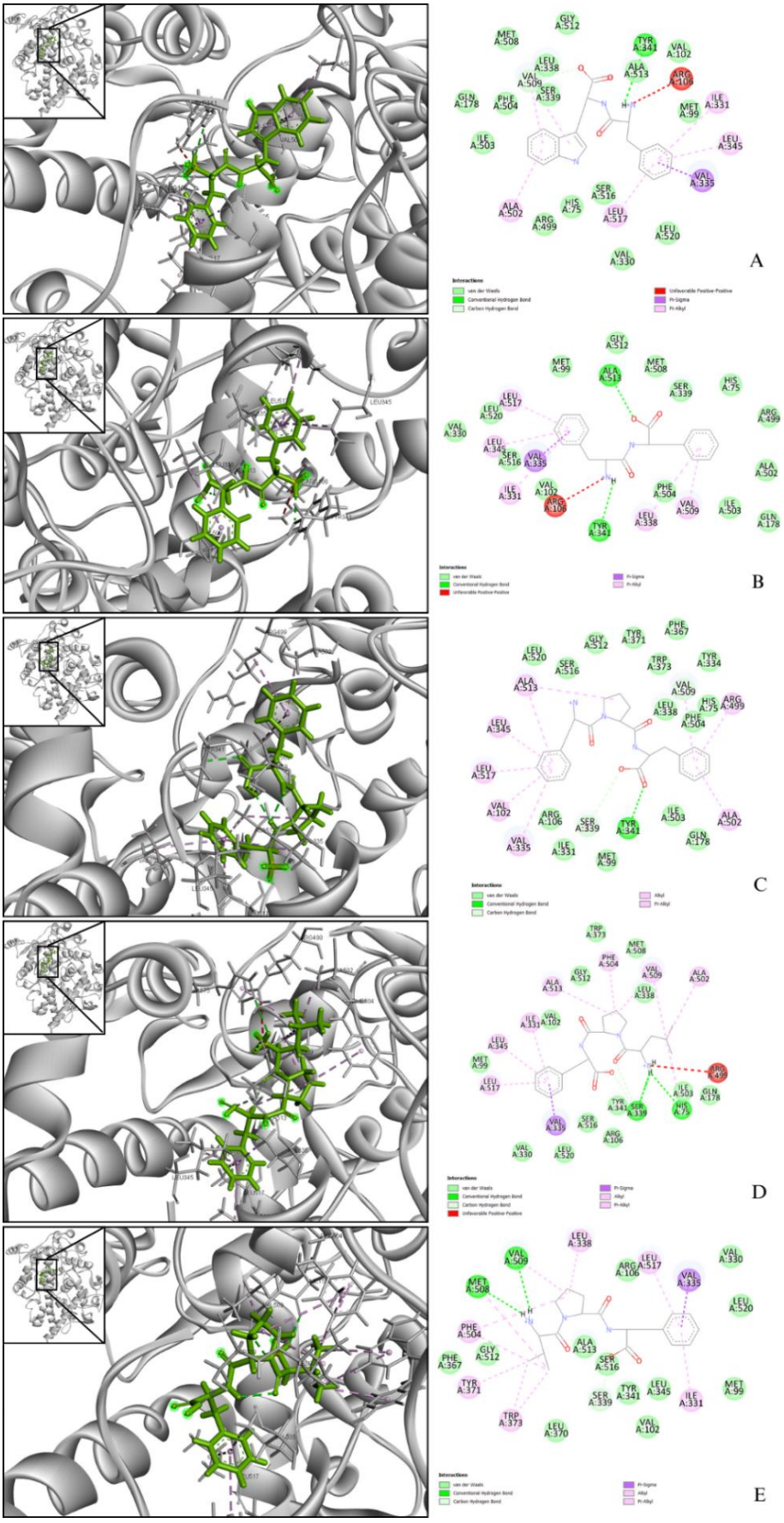

Figure 5 3D and 2D structure of peptide ligand interaction with COX-2: (A) FW, (B) FF, (C) FPF, (D) LPF, and (E) VPF. The grey ribbon was the visualization of COX-2, while green was the peptide.

Conclusions

The peptides produced from *S. australe* protein hydrolysate through cleavage by digestive enzymes using computational tools resulted in 503 peptides. Based on the peptide's physicochemical properties, toxicity, and COX-2 inhibition potential, 113 peptides were suitable for further development. Consequently, PCA was used to decrease and identify peptides similar to celecoxib. Five selected peptides composed of dipeptides and tripeptides were obtained. Molecular docking analysis showed that the formed inhibitory interactions included hydrogen bonds, hydrophobic interactions, and van der Waals forces. The most effective peptide is FW due to its binding energy being comparable to celecoxib. These results indicate that *S. australe* peptides have a lot of potential as a natural source of anti-inflammatory agents that can replace the side effects of anti-inflammatory drugs.

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