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Original Research Article

In silico Evaluation of the Pharmacokinetics and Toxicity Profiles of Chitosan from Haruan Fish (*Channa Striata*) Scale as Anti-inflammatory Agent

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ABSTRACT

During periodontal inflammatory process, the enzyme cyclooxygenase-2 (COX-2) acts on arachidonic acid to produce prostaglandins, resulting in vasodilation, edema, and pain. The chitosan derived from haruan fish (*Channa striata)* scales has the potential to be used as anti-inflammatory agent due to its inhibitory effect on COX-2. The aim of this study was to evaluate the pharmacokinetics and toxicity profiles of chitosan derives (aminoethyl chitosan, carboxymethyl chitosan, and N-succinyl chitosan) from haruan fish scales as anti-inflammatory drug candidates. The 3D chemical structures of the chitosan derivatives and the reference ligand (diclofenac) were obtained from PubChem database. The 3D structure of COX-2 receptor (5IKR) was retrieved from Protein Data Bank. Molecular docking simulation of the ligands and protein was performed using PyRx software and the docking interaction was visualized using BIOVIA Discovery Studio. The pharmacokinetics and toxicity predictions were done using pkCSM software. The docking results showed that chitosan derivatives from haruan fish scales bind to the target protein (COX-2) via hydrogen bonds. From the data obtained from the pharmacokinetics prediction, chitosan derivatives were shown to be poorly absorbed through the mouth and intestines, but they had good metabolic ability, good excretion rate, and were not mutagenic. The toxicity prediction showed that N-succinyl chitosan and carboxymethyl chitosan had lower toxicity levels compared to aminoethyl chitosan and the reference ligand (diclofenac). In conclusion, the chitosan derivatives from haruan fish scales have been predicted to have anti-inflammatory effects in silico through molecular docking with Cyclooxygenase-2 enzyme.

Keywords: *In Silico*, Cyclooxygenase-2, Periodontal Inflammation, Chitosan, Haruan Fish Scale.

Introduction

The prevalence of periodontitis is about 74.1% in Indonesia according to the 2018 report of the Indonesian Ministry of Health.[1] According to a 2023 report, there are about 56.9% cases of periodontitis among people who do not receive dental and oral care.[2] Periodontitis begins with the interaction of microbial lipopolysaccharide (LPS) with host receptors to induce the production of proinflammatory cytokines such as IL-1, IL-6, and TNF-α.[3] *Porphyromonas gingivalis, Aggregatibacter actinomycete comitans, Tannerella forsythia, Prevotella intermedia, Campylobacter rectus, Fusobacterium nucleatum,* Peptostreptococcus *micros,* and *Treponema* are anaerobic gram-negative bacteria that are responsible for inflammation in the supportive tissue of the tooth.[4,5] The inflammatory response results in the synthesis of arachidonic acid by phospholipase A2 which is metabolized through two main enzyme pathways, namely; cyclooxygenase (COX-2) and lipoxygenase.[6] COX-2 is induced in inflammatory cells, activating prostaglandins that play an important role in the inflammatory response of periodontal tissues.[7]

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The clinical impact of acute inflammation due to a long-lasting inflammatory process is an extensive tissue damage, thus, one way of preventing prolonged inflammation is by inhibiting COX-2.[8] Inflammation involving periodontal tissues requires not only local action but also systemic interventions such as the administration of anti-inflammatory drugs. The use of selected anti-inflammatory drugs such as diclofenac is recommended because it is a standard first-line drug that effectively inhibits COX-2 and prevent the production of PGE2.[9] However, as with other non-steroidal anti-inflammatory drugs, the prolonged use of diclofenac is reported to increase the risk of ulceration and bleeding of the gastrointestinal mucosa and renal papillary necrosis. This is due to decreased production of COX-1 derived prostanoids.[10] Researchers have continued to device innovative approaches to overcome this challenge. One of such approaches is the development of drugs from biomaterials that are biocompatible, biodegradable, non-allergenic, non-toxic and help reduce the inflammatory process.[11,12] On of such biomaterials is chitosan. Some chitosan derivative compounds include aminoethyl chitosan, carboxymethyl chitosan, and N-succinyl chitosan.[13-15]

The haruan fish (*Channa striata*) is one of the animals believed to be a natural ingredient in traditional medicine that accelerates wound healing and has anti-inflammatory effects. Haruan fish is popularly consumed in South Kalimantan. Consumption of fish meat (edible portion) ranges from 40 to 50%, while the bones, heads, fins, and scales are discarded as waste.[16] Fish wastes including the scales are not optimally utilized, they are usually piled up in the environment causing environmental pollution such as unpleasant odours, littering of the surroundings, hindering residential activities, water contamination, thereby causing a threat to public health. Fish scale waste can be harnessed into useful products of economic value, for example, chitosan.[17] Research has shown that haruan fish scale chitosan has anti-inflammatory properties, which is exhibited by promoting angiogenesis through the binding of the N-acetylglucosamine monomers to mannose

receptor, resulting in macrophages activation and proliferation. Activated macrophages increases metabolic activity and increase the secretion of growth factors such as Vascular Endothelial Growth Factor (VEGF), which accelerates the formation of new blood vessels through increased proliferation and differentiation of endothelial cell. Haruan fish scale chitosan has also been shown to stimulate and increase the number of fibroblasts thereby accelerating wound healing. Increased fibroblasts proliferation is due to the stimulation of macrophages by the N-acetylglucosamine monomer of chitosan leading to increased cytokines and growth factors such as Interleukin-Iß (IL-Iß), Platelet Derived Growth Factor (PDGF), Fibroblast Growth Factor (FGF), and Transforming Growth Factor-beta (TGF-ß) which are important for fibroblast proliferation and differentiation.[18]
Studies have shown that haruan fish scale chitosan has little or no toxic effect *in vitro* and *in vivo*.[19,20] In addition to its anti-inflammatory effect, chitosan of haruan fish scale has also been shown to possess antibacterial property against *Porphyromonas gingivalis*, this could be attributed to its highly reactive and positively charged amino group (-NH_2), which binds to the negatively charged cell wall of *Porphyromonas gingivalis*.[21]

As the demand for new drug compounds continues to increase, researchers are motivated to develop novel drugs derived from natural ingredients, specifically chitosan from haruan fish scales. This study seeks to assess the pharmacokinetics and toxicity profiles of chitosan-derived drug candidates using *in silico* approach. This research represents a significant advancement in anti-inflammatory drug development using natural compounds.

Materials and Methods

Protein and ligand preparation
The 3D structure of COX-2 receptor (5IKR) was retrieved from Protein Data Bank (https://www.rcsb.org/) in .pdb format. Proteins were pretreated using BIOVIA Discovery Studio to remove built-in ligands and water molecules.[22] The 3D structures of chitosan-derived compounds were downloaded from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/) in SDF format. The ligands used were Aminoethyl chitosan, Carboxymethyl chitosan, N-succinyl chitosan and diclofenac. The ligands were prepared using PyRx application to minimize their energy and convert them into .pdb format.[23]

Assessment of physicochemical property and drug likeness
Prediction of toxicity and pharmacokinetics properties using Lipinski and ADMET rules was performed using the pkcsm website (https://biosig.lab.uq.edu.au/pkcsm/prediction).[24]

Validation of docking method
The docking method was validated by redocking method using natural ligand with COX-2 receptor. The validation results of native ligand with COX-2 receptor showed RMSD (root mean square deviation) value ≤ 2Å. The docking method is valid if the RMSD value ≤ 2 Å, so the docking method can be used to dock the test compound. This result shows that the docking method is valid and the parameter setting is in accordance with the validation criteria.[25]

Molecular docking and visualization of docking results
The docking between the ligand and the receptor was a blind docking, which involve the tethering of unknown active site between the receptor and ligand using PyRx application. After docking, docking visualization was performed using BIOVIA Discovery Studio application.[25]

Results and Discussion

Molecular docking results of haruan fish scale chitosan with COX-2
The binding affinity is used to describe the binding energy in the compound-receptor complex. Binding affinity represents the ability of a compound or ligand to bind to the receptor and greatly affects the stability of the interaction between the ligand and the receptor.[26] In this study, the strongest binding affinity of -9.0 kcal/mol was exhibited by the interaction of aminoethyl chitosan with COX-2 (Table 1). The negative value present in the docking results indicates that the ligand-receptor interaction occurred spontaneously. The more negative the binding affinity value, the more stable and stronger the interaction between the ligand and the receptor.[27,28] This study predicted that aminoethyl chitosan, a chitosan derivative has the potential to be a potent COX-2 inhibitor, as indicated by a more negative binding affinity compared to the binding affinity of the interaction of diclofenac with COX-2.

The binding of aminoethyl chitosan to COX-2 produced the highest number of hydrogen bonds with an average bond distance of < 3.5 Å compared to the receptor binding with the control ligand. The longest and shortest hydrogen bond distances were shown by the amino acid residues GLY225 (3.05Å) and GLY225 (1.75Å) of the aminoethyl chitosan bond with COX-2. The optimal requirement for hydrogen bond distance is between 2.5 Å to 3.5 Å.[29] The interaction distance between amino acid residues of the target protein and the ligand affects the bond strength and stability of the ligand and receptor interaction. If the interaction distance between ligands and proteins is too close or too far, the bond is considered weak, unstable and easily broken. [30,31]

The number and type of amino acid residues involved in the interaction affect the strength of the bond between the ligand and the receptor protein. Based on the docking results, it was predicted that chitosan derivatives from haruan fish scales and the reference ligand can interact with COX-2 via several amino acid residues. No similar amino acid residues were found between the ligands of chitosan derivatives and the reference ligand (diclofenac). This is because a compound will seek the most stable position or conformation on the active pocket of the target protein. This explains why the chitosan derivatives from haruan fish scales and the reference ligand, although interacting with COX-2 via different active pocket, but still produced a strong and stable interaction (Figure 1).

The docking results showed that all the test ligands interacted with the COX-2 receptor via several hydrogen bonds, followed by hydrophobic bonds. The interaction of aminoethyl chitosan with COX-2 had a total of 10 hydrogen bonds, the highest among all the ligands including the reference ligand (diclofenac). This indicates that the interaction between aminoethyl chitosan and COX-2 is stronger and more stable compared to the interaction between the reference ligand and COX-2. It is important to note that the number and type of bond determines the strength of the ligand-protein complex.[32] The formation of hydrogen bonds between ligands and proteins is necessary because it functions to maintain the molecular complex, and to trigger the biological response in the protein target.[27]

Aminoethyl Chitosan acts by inhibiting the production of PGE2, as seen from its anti-inflammatory activity in LPS-induced mouse macrophage cells through inhibition of the iNOS (inducible nitric oxide synthase) and COX-2 genes so that the production of pro-inflammatory cytokines including TNF-α, IL-1 and IL-6 through the NF-B pathway is also inhibited.[15,33] Carboxymethyl chitosan has been reported to have anti-inflammatory, antibacterial and antioxidant activities, making it useful as a wound healing agent.[34,35] A study by Liu *et al*. showed that Carboxymethyl chitosan could significantly reduce the expression of PGE2, NO, IL-6 and IL-8 in human gingival fibroblasts stimulated by lipopolysaccharide, and decrease the phosphorylation of NF-κB p65, IκB, and PI3K.[36] N-succinyl chitosan increases the solubility of poorly soluble drugs and can also be used to improve the oral and parenteral bioavailability of drugs. N-succinyl chitosan as a therapeutic agent is widely combined with proteins, as well as anti-inflammatory, anticancer, antihypertensive, antianginal, and antibacterial drugs in various NSC formulations to achieve controlled and site-specific drug delivery.[15] This derivative can potentially be used in the treatment of inflammation, and it can serve as a more tolerable and safer alternative to synthetic anti-inflammatory drugs.[17,37]
From the findings of this study, aminoethyl chitosan was found to have the best binding affinity with COX-2 receptor. This finding is in agreement with the work of Ngo *et al*. (2020), which has shown that aminoethyl chitosan exhibit anti-inflammatory by inhibiting COX-2 leading to reduced PGE2 production.[1]

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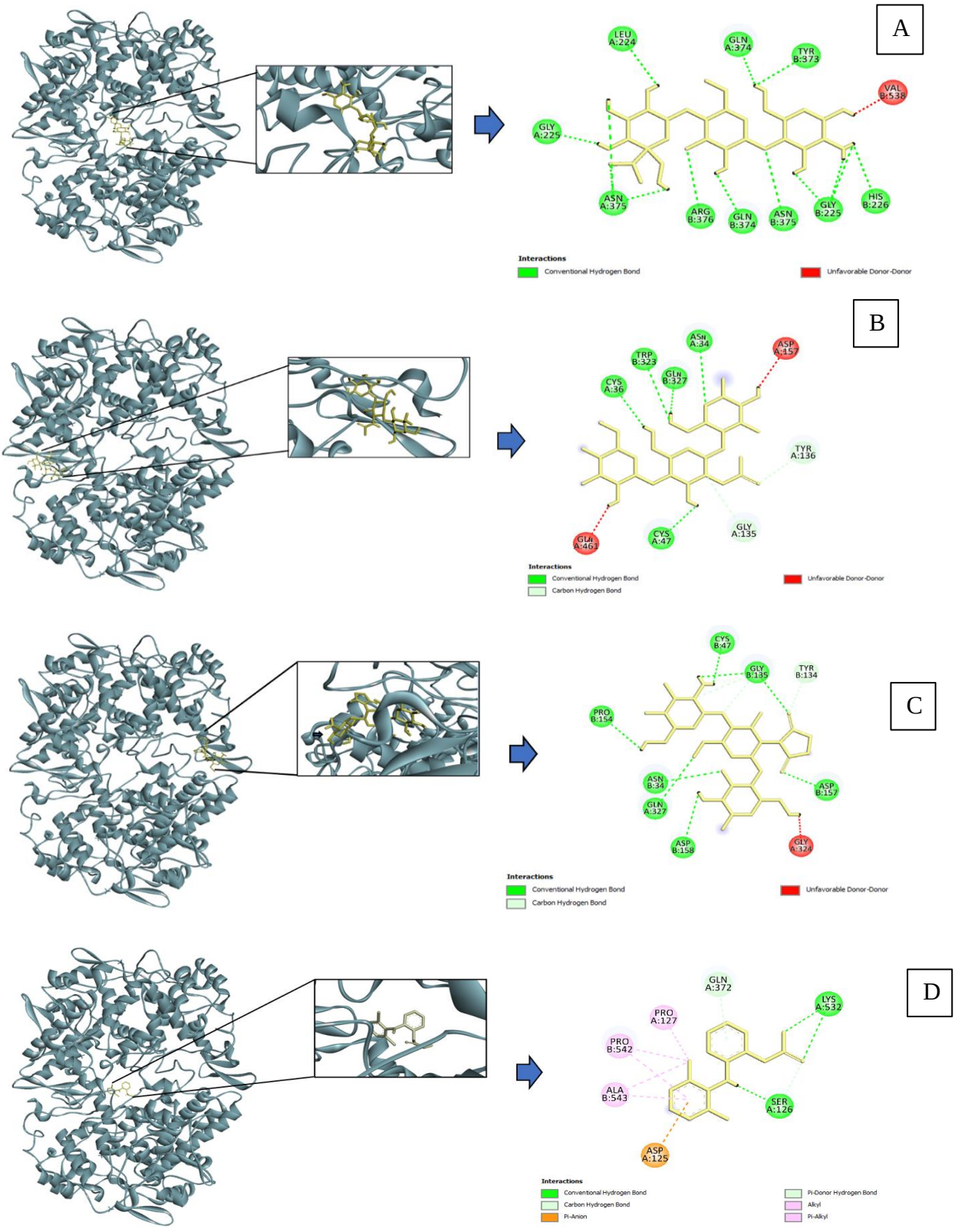

Figure 1: (A): Docking interaction between COX-2 and aminoethyl chitosan; **(B):** Docking interaction between COX-2 and carboxymethyl chitosan; **(C)** Docking interaction between COX-2 and N-succinyl chitosan; **(D)** Docking interaction between COX-2 and Diclofenac

Table 1: Docking results of chitosan derivatives of haruan fish scales (*Channa striata*) and diclofenac with cyclooxygenase-2

Compound Name	Binding Affinity (ΔG) kcal/mol	RMSD Upper and Lower Bound	Amino Acid Residues	Types of Bond Interactions	Bond Distance (Å)
Aminoethyl chitosan	-9.0	0	LEU224	Conventional Hydrogen Bond	2.22
			GLN374	Conventional Hydrogen Bond	2.13
			GLN374	Conventional Hydrogen Bond	2.44
			TYR373	Conventional Hydrogen Bond	2.13
			HIS226	Conventional Hydrogen Bond	2.58
			GLY225	Conventional Hydrogen Bond	1.75
			GLY225	Conventional Hydrogen Bond	3.05
			ASN375	Conventional Hydrogen Bond	2.47
			ARG376	Conventional Hydrogen Bond	2.16
			ASN375	Conventional Hydrogen Bond	2.64
			VAL538	Unfavorable Donor-Donor	1.52
Carboxymethyl chitosan	-8.6	0	CYS36	Conventional Hydrogen Bond	2.40
			TRP323	Conventional Hydrogen Bond	2.54
			GLN327	Conventional Hydrogen Bond	2.25
			ASN34	Conventional Hydrogen Bond	2.53
			CYS47	Conventional Hydrogen Bond	2.27
			TYR136	Carbon Hydrogen Bond	2.45
			GLY135	Carbon Hydrogen Bond	2.60
			ASP157	Unfavorable Donor-Donor	2.24
			GLN461	Unfavorable Donor-Donor	1.94
N-succinyl chitosan	-8.6	0	PRO154	Conventional Hydrogen Bond	2.53
			CYS47	Conventional Hydrogen Bond	1.78
			GLY135	Conventional Hydrogen Bond	2.03
			ASP157	Conventional Hydrogen Bond	2.02

			ASP158	Conventional Bond	Hydrogen	2.37
			GLN327	Conventional Bond	Hydrogen	2.22
			ASN34	Conventional Bond	Hydrogen	2.26
			TYR134	Carbon Hydrogen Bond		2.94
			GLY324	Unfavorable Donor-Donor		1.23
Diclofenac	-7.3	0	LYS532	Conventional Bond	Hydrogen	2.97
			SER126	Conventional Bond	Hydrogen	2.79
			ASP125	Pi-Anion		3.97
			GLN372	Pi-Donor Hydrogen Bond		3.02
			ALA543	Alkyl		3.89
			PRO127	Alkyl		5.35
			ALA543	Pi-Alkyl		4.70

Hence, the inhibition of COX-2 enzyme in periodontal disease can reduce the inflammatory response through decreased macrophage infiltration, and therefore can accelerate wound healing process.[15] COX-2 levels in patients with periodontitis is higher than in healthy individuals. Excessive and chronic inflammatory reactions cause periodontitis, and tissue damage due to the production of damaging reactive radicals. Suppression of COX-2 reduces the production of PGE2 from arachidonic acid, thereby reducing edema, pain and inflammation. Previous *in silico* studies have predicted the potential of chitosan derivatives as COX-2 inhibitors in periodontitis.[21,38] This assertion has also be been supported by previous *in vivo* study on the anti-inflammatory effect of haruan fish scale chitosan, where the anti-inflammatory has been attributed to its structural similarity with glucosamine.[39] One of the functions of glucosamine is to inhibit the production of COX-2 enzyme so that the synthesis of PGE2 is decreased.[32] It is hoped that chitosan derived from haruan fish scales will be useful in the development of new types of anti-inflammatory agents based on natural ingredients.[40]

Pharmacokinetics and drug-likeness profile of haruan fish scale chitosan

Factors that are considered in evaluating candidate molecules for their potentials to be used as drugs are their drug-likeness properties.[41] The results of the pharmacokinetics analysis and drug-likeness properties of chitosan derivatives and diclofenac based on Lipinski's Rule of Five are presented in Table 2. The result shows that the only compound that met the requirements and passed the drug-likeness test was diclofenac with the following properties: molecular weight of ≤ 500 g/mol, logP < 5, number of hydrogen bond donors < 5, and number of hydrogen bond acceptors < 10. Result of the prediction of the toxicity profile using ADMET (Adsorption, Distribution, Metabolism, Excretion, Toxicity) analysis is presented in Table 3.

The result showed that both the test ligands and the reference ligands are poorly absorbed or not well absorbed through the human intestine based on the HIA value.[41] The Caco-2 permeability value (< 0.9 x 10^{-6} cm/s) must be above 0.9 for good absorption of orally administered drug. In this study, the test ligands had Caco-2 values less than 0.9, hence they were predicted to be poorly absorbed when administered through the oral route. All the test ligands had VDss values of less than -0.15, which indicates that more of the compounds are distributed in plasma than in body tissues. For metabolism, the test ligands had good metabolic profile, and were predicted as CYP3A4 inhibitors.

Table 2: Drug-Likeness test result of test ligands (chitosan derivatives)

Compound Name	**Molecular weight**	**LogP**	**Hydrogen bond Donor**	**Hydrogen bond Acceptor**	**Molar Refractivity**	**Description**
Aminoethyl chitosan	544.555	-7.956	12	17	8	No
Carboxymethyl chitosan	543.523	-7.496	11	16	8	No
N-succinyl chitosan	583.544	-7.483	10	17	8	No
Diclofenac	**296.153**	**4.364**	**2**	**2**	**4**	**Yes**

Table 3: Pharmacokinetics (ADMET) profile of test ligands (chitosan derivatives)

No	Indicator	Aminoethyl chitosan	Carboxymethyl chitosan	N-succinyl chitosan	Diclofenac
1.	HIA	6.269	0	7.807	91.923
2.	CaCo-2	-0.523	-0.571	-0.59	1.379
3.	VDss	-0.97	-0.661	-0.595	-1.605
4.	Total Clearance	0.3673611	0.3506944	0.4041667	0.2020833
5.	CYP Inhibitor	No	No	No	No
6.	AMES toxicity	No	No	No	No
7.	Hepatotoxicity	No	No	No	No
8.	Oral Rat Acute Toxicity (LD50)	1.977	2.763	2.79	2.405
9.	Oral Rat Chronic Toxicity (LOAEL)	4.241	4.346	4.326	1.562

Among the test ligands and reference ligand, N-succinyl chitosan showed the highest total clearance value. The greater the estimated total clearance value, the better the excretion rate. Ames Toxicity prediction showed that both the test and reference ligands are not mutagenic. In addition, based on the hepatotoxicity prediction, the test and reference ligands do not have the ability to cause severe liver damage. The ligands with the highest LD_{50} and lowest-observed-adverse-effect level (LOAEL) doses are N-succinyl chitosan and carboxymethyl chitosan, respectively, so that these two compounds can be said to have a lower level of toxicity than the other test compound as well as the reference compound.[42]

Based on the drug-likeness test using Lipinski's rule, none of the chitosan derivatives met the requirements. However, a compound that meets Lipinski's rule does not guarantee that such compound would have good activity because this law (Lipinski's rule) is not related to the specific chemical structure of the compound.[43] Based on the ADMET test conducted, chitosan derivatives are poorly absorbed through the mouth and intestines, but they had good metabolic ability because they inhibited CYP3A4, had good excretion rate, and were not mutagenic. N-succinyl chitosan and carboxymethyl chitosan have a lower level of toxicity than aminoethyl chitosan and diclofenac. The findings from this study have shown the potential of chitosan derivatives to be used as candidate anti-inflammatory drug, however, it is necessary to modify their physical properties or combine them with other organic materials to improve their anti-inflammatory effect.[44]

Conclusion

The chitosan derivatives; aminoethyl chitosan, N-succinyl chitosan and carboxymethyl chitosan from haruan fish scales have been shown to be potent inhibitors of COX-2 *in silico*, and therefore have the potential to be used as anti-inflammatory agents.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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