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In Silico Study of Neoagaro-Oligosaccharides (NAOS) Anti-Inflammatory Activity: Molecular Docking with iNOS and COX-2 Proteins

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ABSTRACT

Neoagaro-Oligosaccharides (NAOS) arise from the enzymatic hydrolysis of agarose employing β-agarases enzymes. Comprising diverse monomers such as neoagarobiose (NA2), neoagarotetraose (NA4), neoagarohexaose (NA6), and neoagaroоctaose (NA8), NAOS are characterised by their Degree of Polymerization (DP). Extensive investigations have delineated the potential of various NAOS monomers, particularly anti-inflammatory agents, owing to their capability to impede iNOS and COX-2, pivotal mediators of inflammation. Nevertheless, the molecular interplay between NAOS and inflammatory mediators remains unexplored. Thus, this study aimed to elucidate the interaction dynamics between NAOS with iNOS and COX-2. Employing ligands neoagarobiose (ID: 275080182), neoagarotetraose (ID: 130476782), neoagarohexaose (ID: 131485243), and neoagaroоctaose (ID: 54758640) in conjunction with target proteins iNOS (3E7G) and COX-2 (5F19), analyses were conducted utilising ProTox-II and SwissADME. Protein preparation was carried out using Discovery Studio, while ligand preparation entailed PyRx, with docking facilitated by CBDock2.0. Absorption, distribution, metabolism, and excretion (ADME) evaluations revealed that neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagaroоctaose did not adhere to Lipinski's Rule of Five. Docking simulations exhibited the capacity of all ligands to engage with the binding site of iNOS, forming diverse bond types. Notably, neoagarobiose, neoagarotetraose, and neoagarohexaose demonstrated enhanced affinity towards COX-2, whereas neoagaroоctaose exhibited heightened binding affinity towards iNOS.

Keywords: COX-2, iNOS, inflammation, molecular docking, NAOS

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INTRODUCTION

Inflammatory responses represent orchestrated actions by the immune system to combat pathogenic infection and restore homeostasis (Bennett et al., 2018; Chen et al., 2018). The manifestations of inflammation arise from the activities of diverse mediators, including but not limited to Nitric Oxide (NO), pro-inflammatory cytokines, prostaglandins (PGs), histamines, reactive oxygen species (ROS), and reactive nitrogen species (RNS), engendered during the inflammatory cascade (Patel & Patel, 2015).

Nitric Oxide (NO) assumes a pivotal role as a free radical species in modulating inflammatory responses, with its synthesis catalysed by members of the nitric oxide synthase (NOS) family, namely endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), and inducible nitric oxide synthase (iNOS) (Vishwakarma et al., 2019). The biosynthesis of NO commences with the enzymatic hydrolysis of L-Arginine by NOS, yielding N-hydroxy-L-arginine, subsequently oxidised to L-Citrulline and NO. Upon interaction with superoxide (O_2•−), NO undergoes conversion to peroxynitrite (ONOO-), eliciting deleterious effects on lipids, proteins, and deoxyribonucleic acid (DNA) integrity (Batra et al., 2007; Forstermann & Sessa, 2012).

Prostaglandins (PGs), derivatives of arachidonic acid (AA) metabolism catalysed by the cyclooxygenase (COX) enzyme, notably manifest the interplay between COX-1 and COX-2 isoenzymes. While both isoforms orchestrate PG production, COX-1 primarily regulates physiological homeostasis, whereas COX-2 assumes prominence in pathophysiological states such as inflammation (Rawat et al., 2019).

The shift from acute inflammation to a chronic condition signifies serious health consequences (Bennett et al., 2018; Chen et al., 2018). While traditional anti-inflammatory medications like aspirin and glucocorticoids relieve chronic inflammation, their extended usage is associated with various negative effects (Coutinho & Chapman, 2011; Harirforoosh et al., 2014; Sherwood et al., 2010). Thus, exploring natural alternatives to mitigate inflammation is imperative, among which Neoagaro-oligosaccharides (NAOS) have emerged as promising candidates.

Agarose, derived from red algae (Rhodophyta), is highly valued for its diverse applications due to its unique chemical composition, which includes (1–4)-linked 3,6-anhydro-α-L-galactose and (1–3)-linked β-D-galactopyranose components (Fu & Kim, 2010). Widely employed as a gelling agent across food, cosmetic, and research domains, agarose undergoes enzymatic or chemical hydrolysis to yield NAOS and Agaro-oligosaccharides (AOS) (Pandey et al., 2019; Xu et al., 2018).

Neoagaro-oligosaccharides (NAOS), enzymatic breakdown products of agarose facilitated by β-agarases, harbour β-D-galactose residues at their reducing ends (Cheong et al., 2018; Fu & Kim, 2010; Higashimura et al., 2013; Xu et al., 2018; Yun et al., 2017). Distinguished by their Degree of Polymerization (DP), NAOS encompass a spectrum of

oligomeric forms such as neoagarobiose (NA2), neoagarotetraose (NA4), neoagarohexaose (NA6), neoagaroоctaose (NA8), neoagarodecaose (NA10), neoagarododecaose (NA12), and others (Qu et al., 2020; Wang et al., 2017).

The industrial, cosmetic, and pharmaceutical sectors recognise the utility of Agaro-oligosaccharides (AOS) and NAOS, leveraging their diverse bioactivities (Qu et al., 2020). Noteworthy among these are the antioxidant and prebiotic properties attributed to NAOS, along with their purported anti-diabetic and skin-whitening efficacies mediated through modulation of α-glucosidase expression and melanin/tyrosine production, respectively (Hong et al., 2017; Zhang et al., 2019).

While existing literature has explored the anti-inflammatory potential of various NAOS monomeric forms based on their DP, investigations into their interactions with key inflammatory mediators, particularly iNOS and COX-2 proteins, remain nascent. Hence, this study aimed to elucidate the drug likeness profiles of diverse NAOS monomers based on their DP and their putative interactions with iNOS and COX-2 proteins via molecular docking analyses.

MATERIAL AND METHODS

Protein Data Acquisition and Preparation

Data pertaining to iNOS (PDB code: 3E7G) and COX-2 (PDB code: 5F19) were acquired from the Protein Data Bank (PDB) website (http://www.rcsb.org/pdb). Subsequently, the Ramachandran plots of both proteins were scrutinised utilising PDBSum (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/). These proteins' binding active sites were analysed using the PrankWeb platform (https://prankweb.cz/). The removal of water molecules and ligands from the protein structures was executed using the Discovery Studio 2016 Client application, followed by preserving the modified structures in (.pdb) format.

Ligand Data Retrieval and Preparation

This investigation employed four ligands—neoagarobiose (ID: 275080182), neoagarotetraose (ID: 130476782), neoagarohexaose (ID: 131485243), neoagaroоctaose (ID: 54758640), and aspirin (ID: 2244)—serving as positive controls. The three-dimensional (3D) conformers and two-dimensional (2D) structures of these ligands were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/). A subsequent ligand toxicity assessment was performed using the ProTox-II website (https://tox-new.charite.de/protox_II/). The ligands' drug-likeness parameters and pharmacokinetic characteristics were evaluated via the SwissADME website (http://www.swissadme.ch/). Following, energy minimisation of the ligands was carried out utilising the PyRx application, and the resultant structures were saved in (.pdb) format.

Molecular Docking

Blind docking simulations were executed utilising the CB-Dock2.0 website (https://cadd.labshare.cn/cb-dock2/), which facilitates blind docking of protein-ligand complexes based on AutoDock Vina. The docking procedure entails uploading the pre-processed protein and ligand structures, followed by cavity detection and blind docking exploration.

RESULTS AND DISCUSSION

Protein Characteristic

The study herein focused on the characterisation of proteins iNOS (3E7G) and COX-2 (5F19) as designated targets, being predicated upon Ramachandran Plot analyses (Jordan et al., 2023; Md Idris et al., 2022). Protein iNOS exhibited 90.6% of its residues within the Ramachandran plot's favoured regions (Ali et al., 2023), accompanied by a G-Factor normality value of 0.31 and a resolution of 2.20 Å. Conversely, protein COX-2 portrayed 90.7% of its residues nestled within the favoured regions of the Ramachandran plot, with a G-Factor normality value of 0.28 and a resolution of 2.04 Å. Notably, the normality values for both proteins marginally exceed the prescribed range. Per PROCHECK standards, the G-factor is ideally between 0 and 0.5, with optimal quality models reflecting values proximal to zero (Elengoe et al., 2014). P2Rank analysis elucidated 23 binding site pockets for iNOS (Figure 1A) and 16 for COX-2 (Figure 1B).

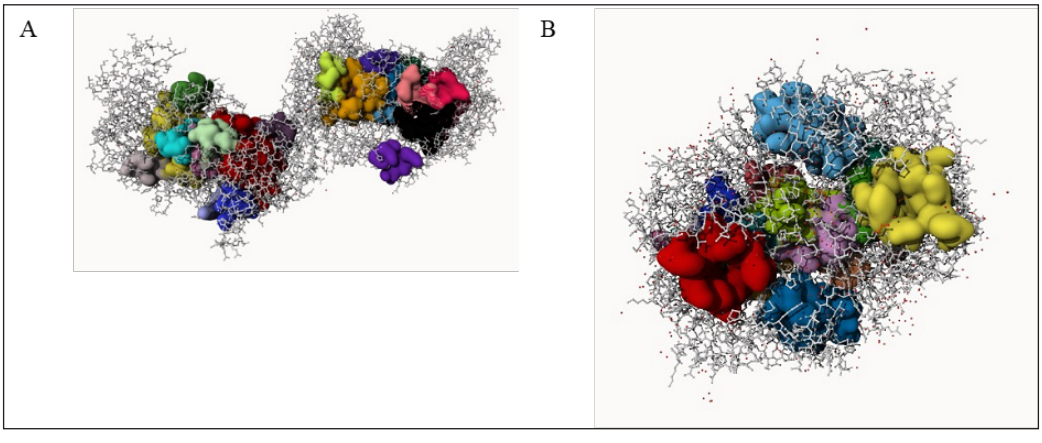

Figure 1. Protein binding sites pocket of (A) iNOS (3E7G) and (B) COX-2 (5F19)

ADME Analysis

All ligands' toxicity and absorption, distribution, metabolism, and excretion (ADME) properties were assessed using ProTox-II and SwissADME (Table 1). Neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagarooctaose exhibited identical toxicity

profiles, classified as level three toxicity with an LD50 value of 648 mg/kg. Furthermore, hepatotoxicity and carcinogenicity assays across all ligands demonstrated negligible activity, with a high probability of 0.91. Similarly, immunotoxicity assessment for these ligands indicated minimal activity, with a probability of 0.87. Moreover, all four ligands exhibited no mutagenic potential, with a probability of 0.75. Cytotoxicity analysis also revealed minimal activity for these ligands, with a probability of 0.63. Based on the toxicity evaluation, neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagarooctaose share similarities with aspirin in their toxicity profiles.

Based on the toxicity assessment outcomes, neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagarooctaose are included within the "danger if swallowed" category as per the classification on the ProTox-II website (Banerjee et al., 2018). Furthermore, the toxicity evaluation indicates that these four compounds are ostensibly safe according to their predicted lack of hepatotoxicity, carcinogenicity, mutagenicity, cytotoxicity, and immunotoxicity (Banerjee et al., 2018).

The outcomes of ADME physiochemical analysis using SwissADME for each ligand are shown in Table 2. The purpose of ADME analysis is to evaluate the pharmacokinetic properties of potential drug molecules and to determine the efficacy and safety of a drug candidate. Using tools like SwissADME makes it possible to assess various parameters such as molecular weight, lipophilicity, solubility, bioavailability, and likelihood of gastrointestinal absorption. These parameters provide insights into how efficiently a drug candidate can be absorbed into the bloodstream, distributed to the target tissues, metabolised by the body, and ultimately excreted. These are all critical considerations in drug development (Morak-Młodawska et al., 2023).

As shown in Table 2, neoagarobiose exhibited an molecular weight (MW) < 500 g/mol, whereas neoagarotetraose, neoagarohexaose, and neoagarooctaose surpassed this threshold. Consequently, neoagarobiose is postulated to undergo oral absorption more readily, while the latter compounds may be absorbed through alternative routes. Molar refractivity (MR) and Rotatable Bond Number (RBN) serve as parameters in silico for probing the pharmacokinetic properties of targeted compounds (Ibrahim et al., 2021). According to Lipinski's five rules, MR values falling between 40–130 are acceptable, whereas compounds with an RBN < 10 suggest favourable oral bioavailability. The convergence of acceptable MR and RBN values signifies robust intestinal absorption and oral bioavailability of the substance (Ibrahim et al., 2021). Notably, neoagarobiose and neoagarotetraose demonstrated MR values ranging from 64.77 to 126.18 and RBN values of 3–8, indicative of favourable intestinal absorption and oral bioavailability.

Hydrogen Bond Acceptor (HBA) and Hydrogen Bond Donor (HBD) indices are pivotal for analysing intermolecular interactions between macromolecules and chemicals, thereby influencing oral absorption (Ibrahim et al., 2021). As stipulated by Lipinski's 5 rules, the acceptable count of HBA should not exceed 10, with HBD not surpassing 5. However, all targeted compounds in Table 2 exhibited HBA counts exceeding 10, and HBD counts

Table 1
Toxicity prediction using ProTox-II

Ligand	Toxicity Tests					
	Toxicity class	**Hepato-toxicity**	**Carcinogenicity**	**Immuno-toxicity**	**Mutagenicity**	**Cyto-toxicity**
Neoagarobiose	3 (LD_{50}: 648mg/kg)	Inactive (prob: 0,91)	Inactive (prob: 0,91)	Inactive (prob: 0,87)	Inactive (prob: 0,75)	Inactive (prob: 0,63)
Neoagarotetrose	3 (LD_{50}: 648mg/kg)	Inactive (prob: 0,91)	Inactive (prob: 0,91)	Inactive (prob: 0,87)	Inactive (prob: 0,75)	Inactive (prob: 0,63)
Neoagarohexaose	3 (LD_{50}: 648mg/kg)	Inactive (prob: 0,91)	Inactive (prob: 0,91)	Inactive (prob: 0,87)	Inactive (prob: 0,75)	Inactive (prob: 0,63)
Neoagarooctaose	3 (LD_{50}: 648mg/kg)	Inactive (prob: 0,91)	Inactive (prob: 0,91)	Inactive (prob: 0,87)	Inactive (prob: 0,75)	Inactive (prob: 0,63)
Aspirin	3 (LD_{50}: 250mg/kg)	Inactive (prob: 0,51)	Inactive (prob: 0,86)	Inactive (prob: 0,99)	Inactive (prob: 0,97)	Inactive (prob: 0,94)

Table 2
Ligand physiochemical analysis using SwissADME

	Physiochemical analysis				
	Neoagarobiose	**Neoagarotetraose**	**Neoagarohexaose**	**Neoagarooctaose**	**Aspirin**
Molecular weight (g/mol)	324,28	630,55	936,81	1243,08	180,16
Number of heavy atoms	22	43	64	85	13
Number of rotatable bonds	3	8	13	18	3
Number of hydrogen bond acceptors	10	19	28	37	4
Number of hydrogen bond donors	6	10	14	18	1
Molar refractivity	64,77	126,18	187,60	249,01	44,90
TPSA (A^2)	158,30	285,37	412,44	539,51	63,60
Lipophilicity (Log P)	-2,93	-5,28	-7,99	-10,17	1,28
Water solubility (Log S / ESOL)	0,70 (highly soluble)	0,86 (highly soluble)	1,02 (highly soluble)	1,18 (highly soluble)	-1,85 (very soluble)

surpassing 5, suggesting potential challenges in oral absorption due to increased interactions with biological targets. Further investigation and optimisation to determine HBA and HBD compounds such as O, N, and H atoms using nuclear magnetic resonance (NMR) may be required to address these issues and improve the compounds' pharmacokinetic profiles (Wang et al., 2021).

As gauged by the Log P value, lipophilicity delineates a compound's solubility and permeability characteristics. Lipinski posits that compounds with a Log P < 5 hold promise as potential drugs. As per the results in Table 2, all four compounds exhibited a Log P value < 5, indicating their capacity to dissolve in both water and lipids. The negative Log P values denoted their hydrophilic nature, rendering them soluble in water, tolerable in the gastric milieu, and conducive to proper renal excretion (Al Mogren et al., 2020; Kadela-Tomanek et al., 2021). These findings align with the log S (water solubility) values, portraying the compounds as highly soluble in water.

According to Lipinski's criteria, a compound warrants consideration as a drug if it adheres to specific thresholds concerning molecular weight, lipophilicity, HBA, HBD, and molar refractivity (Riyadi et al., 2021). Furthermore, Lipinski stipulates that an orally active drug should not exceed one violation of these criteria (Ibrahim et al., 2017; Riyadi et al., 2021; Sen et al., 2021). Drug likeness analysis based on Lipinski's 5 rules suggested that neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagarooctaose lacked the requisite chemical and physical properties to qualify as orally active drugs for human use.

Table 3 presents the drug-likeness analysis based on Lipinski's 5 rules. Lipinski's test aims to ascertain whether a target compound with known biological activity possesses chemical and physical characteristics conducive to oral consumption as medicine in humans. As shown in Table 3, it is evident that neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagarooctaose failed to satisfy several Lipinski indicators. Specifically, neoagarobiose fulfilled 3 out of 5 Lipinski indicators, while neoagarotetraose satisfied only 2 out of 5. Neoagarohexaose and neoagarooctaose, however, fulfilled only 1 of the 5 indicators established by Lipinski.

Molecular Docking

Using CBDock2.0, molecular docking simulations employed AutoDock Vina for ligand-protein interactions. The resultant binding affinities, quantified as Vina scores, reflect the strength of interaction between the ligands and the protein receptors (Hasan et al., 2023). Docking analyses of neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagarooctaose with iNOS protein are depicted in Figure 2. These analyses revealed that neoagarooctaose exhibited the highest binding affinity, with a value of -10.6 kcal/mol, followed by neoagarohexaose (-9.2 kcal/mol) and neoagarotetraose (-8.8 kcal/mol). In contrast, neoagarobiose demonstrated the lowest binding affinity at -6.6 kcal/mol (Table 3).

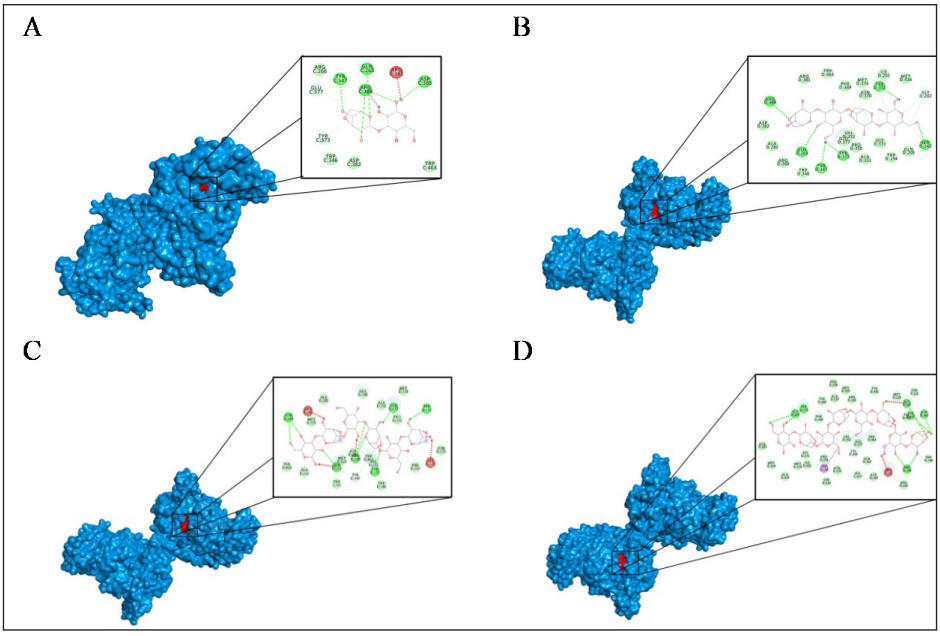

Figure 2. Molecular docking of interaction between (A) neoagarobiose and iNOS, (B) neoagarotetrose and iNOS, (C) neoagarohexaose and iNOS, (D) neoagarooctaose and iNOS

The outcomes of molecular docking analyses revealed the binding of neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagarooctaose within the binding site pocket of the inducible nitric oxide synthase (iNOS) protein. Various bonds were formed between each ligand and iNOS, encompassing van der Waals interactions, conventional hydrogen bonds, carbon-hydrogen bonds, Pi-Sigma bonds, and bonds deemed unfavourable (Figure 3). Specifically, the interaction between neoagarobiose and iNOS was characterised by one unfavourable bond at residue ARG381. Likewise, in the case of neoagarooctaose, an unfavourable bond at residue ARG381 was observed, along with a Pi-Sigma bond at residue TRP194. Neoagarohexaose's interaction with iNOS was marked by two unfavourable bonds at residues ARG266 and GLY371. Conversely, the interaction involving neoagarotetraose did not exhibit any unfavourable bonds.

Figure 4 provides a visual representation elucidating the interaction dynamics between neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagarooctaose with the COX-2 protein. Concurrently, Table 3 outlines the binding affinity values associated with the interactions of the ligands mentioned above with the COX-2 protein. Notably, neoagarohexaose exhibited the most favourable binding affinity value upon binding to the COX-2 protein, registering at -10.7 kcal/mol. Following this, neoagarotetraose demonstrates a binding affinity value of -10.3 kcal/mol, neoagarooctaose at -9.8 kcal/mol, and neoagarobiose exhibited the least favourable binding affinity value at -6.6 kcal/mol (Table 3).

Table 3
Binding affinity and hydrogen form

Protein	Ligand	Binding affinity (Kcal/mol)	Hydrogen bond
iNOS (3E7G)	Neoagarobiose	-6,6	GLN263, TYR347, TYR373, GLU377, ASP385, ARG388
	Neoagarotetrose	-8,8	GLY202, SER242, GLN263, TYR347, TRP372, TYR373, ARG388
	Neoagarohexaose	-9,2	GLN263, TYR347, ASN354, TRP372, TYR373, GLU377, ARG388
	Neoagarooctaose	-10,6	CYS200, GLY202, GLN263, TRP372, TYR373, ASP382, ARG388
	Aspirin	-7,3	TRP194
COX-2 (5F19)	Neoagarobiose	-7,1	GLY225, ASN375, GLY533
	Neoagarotetrose	-10,3	GLY225, GLY227, ASN375, GLY533, VAL228, GLN374, ASN375, GLY536
	Neoagarohexaose	-10,7	GLY225, GLY235, GLN241, TYR373, ASN375, ARG376, VAL538
	Neoagarooctaose	-9,8	SER143, GLY225, GLU236, ASN375, GLU140, ASP229, ARG376
	Aspirin	-6,6	ALA202, THR206, TRP387

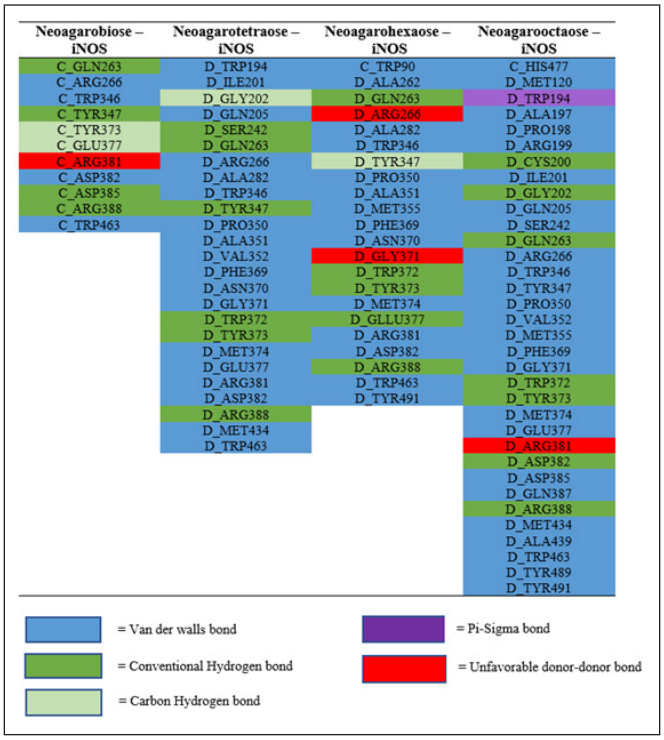

Figure 3. Residue bonds formed through interaction between neoagarobiose, neoagarotetraose, neoagarohexaose, neoagarooctaose interaction and iNOS protein (3E7G)

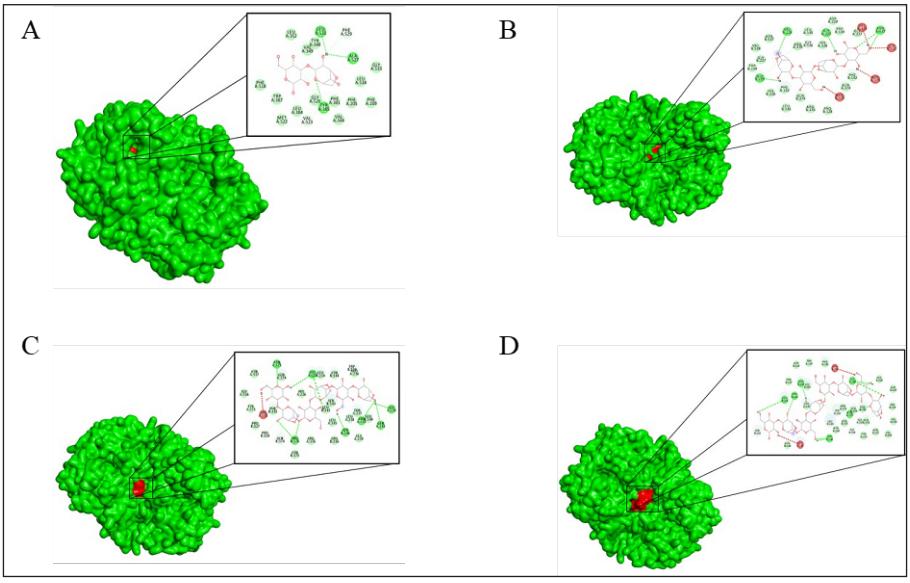

Figure 4. Molecular docking between (A) neoagarobiose and COX-2, (B) neoagarotetrose and COX-2, (C) neoagarohexaose and COX-2, (D) neoagarooctaose and COX-2

The binding interactions of neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagarooctaose with the COX-2 binding site pocket resemble those observed with all ligands and iNOS. These interactions manifest through the formation of diverse bonds, including van der Waals bonds, conventional hydrogen bonds, carbon hydrogen bonds, and unfavourable donor-donor bonds, as depicted in Figure 5. Specifically, as illustrated in Figure 5, the interactions of neoagarobiose and neoagarooctaose with COX-2 lacked residues exhibiting unfavourable donor-donor bonds. Conversely, the interaction between neoagarotetraose and COX-2 entails one unfavourable donor-donor bond located at residue GLY225. Notably, the interaction of neoagarohexaose with COX-2 involved two unfavourable donor-donor bonds situated at residues LEU238 and SER143.

The outcomes of molecular docking analyses reveal the presence of hydrogen bond interactions between neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagarooctaose with iNOS and COX-2, characterised by specific residues within the protein structures. Neoagarobiose exhibited hydrogen bonding with iNOS residues GLN263, TYR347, TYR373, GLU377, ASP385, and ARG388. Similarly, neoagarotetraose engaged in hydrogen bonding interactions with iNOS residues GLY202, SER242, GLN263, TYR347, TRP372, TYR373, and ARG388, while with COX-2, it formed hydrogen bonds at GLY225, GLY227, ASN375, GLY533, VAL228, GLN374, ASN375, and GLY536 residues. Likewise, neoagarohexaose established hydrogen bonds with iNOS at GLN263, TYR347, ASN354, TRP372, TYR373, GLU377, and ARG388 residues, and with COX-2 at GLY225, GLY235, GLN241, TYR373, ASN375, ARG376, and VAL538 residues.

Additionally, neoagarooctaose interacted via hydrogen bonding with iNOS residues CYS200, GLY202, GLN263, TRP372, TYR373, ASP382, and ARG388, and with COX-2 residues SER143, GLY225, GLU236, ASN375, GLU140, ASP229, and ARG376. These hydrogen bond formations signify robust binding of the ligands to the respective proteins, enhancing their stability (Wibowo et al., 2019; Wibowo et al., 2020; Wibowo et al., 2021; Wibowo et al., 2022).

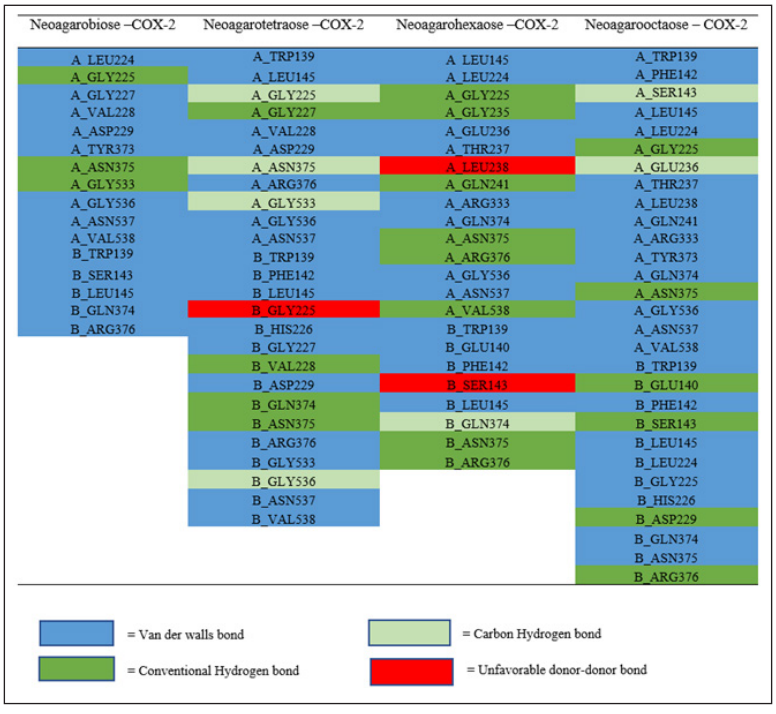

Figure 5. Residues bond formed through interaction between neoagarobiose, neoagarotetraose, neoagarohexaose, neoagarooctaose and COX-2 (5F19)

Docking simulations revealed that all four target compounds fit within the binding pockets of iNOS and COX-2 proteins. Molecular docking analyses further unveiled the presence of unfavourable bonds at specific residues, potentially compromising protein stability (Wibowo et al., 2019). Additionally, hydrogen bonds formed between the compounds and iNOS/COX-2 proteins played pivotal roles in fortifying their interactions. Neoagarobiose exhibited a propensity to inhibit COX-2 production to a greater extent than iNOS, as evidenced by its stronger binding affinity with COX-2 (-7.1 Kcal/mol) compared to iNOS (-6.6 Kcal/mol). Analogously, neoagarotetraose, neoagarohexaose, and neoagarooctaose demonstrated stronger binding affinities with COX-2 relative to iNOS. These findings suggest a potential therapeutic advantage in targeting COX-2 over iNOS inhibition.

In summary, the comprehensive evaluation of neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagarooctaose underscores their promising pharmacological attributes, particularly in gastrointestinal drug delivery and anti-inflammatory therapy.

CONCLUSION

Based on the findings presented, the proteins iNOS and COX-2 have been thoroughly characterised, revealing their structural attributes and potential binding sites. The ligands neoagarobiose, neoagarotetraose, neoagarohexaose, and neoagarooctaose exhibited favourable toxicity profiles with negligible hepatotoxic, carcinogenic, mutagenic, cytotoxic, and immunotoxic properties. However, they failed to fulfil Lipinski's criteria for drug-likeness due to certain physicochemical characteristics. Molecular docking simulations highlighted the strong binding affinities of these ligands with both iNOS and COX-2 proteins, indicating their potential as inhibitors. Notably, neoagarobiose preferred to inhibit COX-2, suggesting a therapeutic advantage in targeting this pathway for anti-inflammatory purposes.

Future perspectives may involve further in vitro and in vivo studies to validate the therapeutic potential of these ligands, exploring their efficacy and safety profiles in relevant disease models. Additionally, structural optimisation efforts could be undertaken to enhance their drug-likeness properties and improve their pharmacological utility. Further investigations into the specific mechanisms underlying their interactions with iNOS and COX-2 would also contribute to a deeper understanding of their mode of action and potential clinical applications.

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REFERENCES

Al Mogren, M. M., Zerroug, E., Belaidi, S., BenAmor, A., & Al Harbi, S. D. A. (2020). Molecular structure, drug likeness and QSAR modeling of 1,2-diazole derivatives as inhibitors of enoyl-acyl carrier protein reductase. *Journal of King Saud University - Science*, *32*(4), 2301–2310. https://doi.org/10.1016/j.jksus.2020.03.007

Ali, A., Mir, G. J., Ayaz, A., Maqbool, I., Ahmad, S. B., Mushtaq, S., Khan, A., Mir, T. M., & Rehman, M. U. (2023). In silico analysis and molecular docking studies of natural compounds of *Withania somnifera* against bovine NLRP9. *Journal of Molecular Modeling*, *29*(6), 171. https://doi.org/10.1007/s00894-023-05570-z

Banerjee, P., Eckert, A. O., Schrey, A. K., & Preissner, R. (2018). ProTox-II: A webserver for the prediction of toxicity of chemicals. *Nucleic Acids Research*, *46*(W1), W257–W263. https://doi.org/10.1093/nar/gky318

Batra, J., Chatterjee, R., & Ghosh, B. (2007). Inducible nitric oxide synthase (iNOS): Role in asthma pathogenesis. *Indian Journal of Biochemistry & Biophysics*, *44*(5), 303–309.

Bennett, J. M., Reeves, G., Billman, G. E., & Sturmberg, J. P. (2018). Inflammation–Nature's way to efficiently respond to all types of challenges: Implications for understanding and managing "the epidemic" of chronic diseases. *Frontiers in Medicine*, *5*, 316. https://doi.org/10.3389/fmed.2018.00316

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., & Zhao, L. (2018). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, *9*(6), 7204–7218. https://doi.org/10.18632/oncotarget.23208

Cheong, K.-L., Qiu, H.-M., Du, H., Liu, Y., & Khan, B. (2018). Oligosaccharides derived from red seaweed: Production, properties, and potential health and cosmetic applications. *Molecules*, *23*(10), 2451. https://doi.org/10.3390/molecules23102451

Coutinho, A. E., & Chapman, K. E. (2011). The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments, and mechanistic insights. *Molecular and Cellular Endocrinology*, *335*(1), 2–13. https://doi.org/10.1016/j.mce.2010.04.005

Elengoe, A., Naser, M., & Hamdan, S. (2014). Modeling and docking studies on novel mutants (K71L and T204V) of the ATPase domain of human Heat Shock 70 kDa Protein 1. *International Journal of Molecular Sciences*, *15*(4), 6797–6814. https://doi.org/10.3390/ijms15046797

Forstermann, U., & Sessa, W. C. (2012). Nitric oxide synthases: Regulation and function. *European Heart Journal*, *33*(7), 829–837. https://doi.org/10.1093/eurheartj/ehr304

Fu, X. T., & Kim, S. M. (2010). Agarase: Review of major sources, categories, purification method, enzyme characteristics and applications. *Marine Drugs*, *8*(1), 200–218. https://doi.org/10.3390/md8010200

Harirforoosh, S., Asghar, W., & Jamali, F. (2014). Adverse effects of nonsteroidal antiinflammatory drugs: An update of gastrointestinal, cardiovascular and renal complications. *Journal of Pharmacy & Pharmaceutical Sciences*, *16*(5), 821. https://doi.org/10.18433/J3VW2F

Hasan, T. N., Naqvi, S. S., Rehman, M. U., Ullah, R., Ammad, M., Arshad, N., Ain, Q. U., Perween, S., & Hussain, A. (2023). Ginger ring compounds as an inhibitor of spike binding protein of alpha, beta, gamma and delta variants of SARS-CoV-2: An in-silico study. *Narra J*, *3*(1). https://doi.org/10.52225/narra.v3i1.98

Higashimura, Y., Naito, Y., Takagi, T., Mizushima, K., Hirai, Y., Harusato, A., Ohnogi, H., Yamaji, R., Inui, H., Nakano, Y., & Yoshikawa, T. (2013). Oligosaccharides from agar inhibit murine intestinal inflammation through the induction of heme oxygenase-1 expression. *Journal of Gastroenterology*, *48*(8), 897–909. https://doi.org/10.1007/s00535-012-0719-4

Hong, S. J., Lee, J.-H., Kim, E. J., Yang, H. J., Chang, Y.-K., Park, J.-S., & Hong, S.-K. (2017). In vitro and in vivo investigation for biological activities of neoagarooligosaccharides prepared by hydrolyzing agar with β-agarase. *Biotechnology and Bioprocess Engineering*, *22*(4), 489–496. https://doi.org/10.1007/s12257-017-0049-8

Ibrahim, E. I., Omer, R. A., Elsadig, A. H., Elkhatim, M., & Mohamed, S. B. (2017). In silico analysis of the structural and biochemical features of the Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), Interleukin-3 (IL-3) and Interleukin-5 (IL-5) receptors subunit α. *American Journal of Bioinformatics Research*, *7*(1), 25–47. https://doi.org/10.5923/j.bioinformatics.20170701.03

Ibrahim, Z. Y., Uzairu, A., Shallangwa, G. A., & Abechi, S. E. (2021). Pharmacokinetic predictions and docking studies of substituted aryl amine-based triazolopyrimidine designed inhibitors of *Plasmodium falciparum* dihydroorotate dehydrogenase (PfDHODH). *Future Journal of Pharmaceutical Sciences*, *7*(1), 133. https://doi.org/10.1186/s43094-021-00288-2

Jordan, P., Costa, A., Specker, E., Popp, O., Volkamer, A., Piske, R., Obrusnik, T., Kleissle, S., Stuke, K., Rex, A., Neuenschwander, M., Von Kries, J. P., Nazare, M., Mertins, P., Kettenmann, H., & Wolf, S. A. (2023). Small molecule inhibiting microglial nitric oxide release could become a potential treatment for neuroinflammation. *PLOS ONE*, *18*(2), e0278325. https://doi.org/10.1371/journal.pone.0278325

Kadela-Tomanek, M., Jastrzębska, M., Marciniec, K., Chrobak, E., Bębenek, E., & Boryczka, S. (2021). Lipophilicity, pharmacokinetic properties, and molecular docking study on SARS-CoV-2 target for betulin triazole derivatives with attached 1,4-Quinone. *Pharmaceutics*, *13*(6), 781. https://doi.org/10.3390/pharmaceutics13060781

Md Idris, M. H., Mohd Amin, S. N., Mohd Amin, S. N., Nyokat, N., Khong, H. Y., Selvaraj, M., Zakaria, Z. A., Shaameri, Z., Hamzah, A. S., Teh, L. K., & Salleh, M. Z. (2022). Flavonoids as dual inhibitors of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX): Molecular docking and in vitro studies. *Beni-Suef University Journal of Basic and Applied Sciences*, *11*(1), 117. https://doi.org/10.1186/s43088-022-00296-y

Morak-Młodawska, B., Jeleń, M., Martula, E., & Korlacki, R. (2023). Study of lipophilicity and ADME properties of 1,9-Diazaphenothiazines with anticancer action. *International Journal of Molecular Sciences*, *24*(8), 6970. https://doi.org/10.3390/ijms24086970

Pandey, S. P., Shukla, T., Dhote, V. K., K. Mishra, D., Maheshwari, R., & Tekade, R. K. (2019). Use of polymers in controlled release of active agents. In Tekade, R. K. (Eds.), *Basic fundamentals of drug delivery* (pp. 113–172). Elsevier. https://doi.org/10.1016/B978-0-12-817909-3.00004-2

Patel, H., & Patel, V. H. (2015). Inflammation and metabolic syndrome: An overview. *Current Research in Nutrition and Food Science Journal*, *3*(3), 263–268. https://doi.org/10.12944/CRNFSJ.3.3.10

Qu, W., Wang, D., Wu, J., Chan, Z., Di, W., Wang, J., & Zeng, R. (2020). Production of neoagaro-oligosaccharides with various degrees of polymerization by using a truncated marine agarase. *Frontiers in Microbiology*, *11*, 574771. https://doi.org/10.3389/fmicb.2020.574771

Rawat, C., Kukal, S., Dahiya, U. R., & Kukreti, R. (2019). Cyclooxygenase-2 (COX-2) inhibitors: Future therapeutic strategies for epilepsy management. *Journal of Neuroinflammation*, *16*(1), 197. https://doi.org/10.1186/s12974-019-1592-3

Riyadi, P. H., Romadhon, Sari, I. D., Kurniasih, R. A., Agustini, T. W., Swastawati, F., Herawati, V. E., & Tanod, W. A. (2021). SwissADME predictions of pharmacokinetics and drug-likeness properties of small molecules present in *Spirulina platensis*. *IOP Conference Series: Earth and Environmental Science*, *890*(1), 012021. https://doi.org/10.1088/1755-1315/890/1/012021

Sen, D. J., Nandi, K., & Saha, D. (2021). Rule of five: The five men army to cross the blood brain barrier for therapeutically potent. *World Journal of Advance Healthcare Research*, *5*(3), 206–211.

Sherwood, A. R., Kurihara, A., Conklin, K. Y., Sauvage, T., & Presting, G. G. (2010). The Hawaiian Rhodophyta biodiversity survey (2006-2010): A summary of principal findings. *BMC Plant Biology*, *10*(1), 258. https://doi.org/10.1186/1471-2229-10-258

Vishwakarma, A., Wany, A., Pandey, S., Bulle, M., Kumari, A., Kishorekumar, R., Igamberdiev, A. U., Mur, L. A. J., & Gupta, K. J. (2019). Current approaches to measure nitric oxide in plants. *Journal of Experimental Botany*, *70*(17), 4333–4343. https://doi.org/10.1093/jxb/erz242

Wang, H., Zhao, Y., Zhang, F., Ke, Z., Han, B., Xiang, J., Wang, Z., & Liu, Z. (2021). Hydrogen-bond donor and acceptor cooperative catalysis strategy for cyclic dehydration of diols to access O-heterocycles. *Science Advances*, *7*(22), eabg0396. https://doi.org/10.1126/sciadv.abg0396

Wang, W., Liu, P., Hao, C., Wu, L., Wan, W., & Mao, X. (2017). Neoagaro-oligosaccharide monomers inhibit inflammation in LPS-stimulated macrophages through suppression of MAPK and NF-κB pathways. *Scientific Reports*, *7*(1), 44252. https://doi.org/10.1038/srep44252

Wibowo, S., Costa, J., Baratto, M. C., Pogni, R., Widyarti, S., Sabarudin, A., Matsuo, K., & Sumitro, S. B. (2022). Quantification and improvement of the dynamics of human serum albumin and glycated human serum albumin with astaxanthin/astaxanthin-metal ion complexes: Physico-Chemical and computational approaches. *International Journal of Molecular Sciences*, *23*(9), 4771. https://doi.org/10.3390/ijms23094771

Wibowo, S., Sumitro, S. B., & Widyarti, S. (2020). Computational study of Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, and Mn^{3+} binding sites identification on HSA 4K2C. *IOP Conference Series: Materials Science and Engineering*, *833*(1), 012052. https://doi.org/10.1088/1757-899X/833/1/012052

Wibowo, S., Widyarti, S., Sabarudin, A., Soeatmadji, D. W., & Sumitro, S. B. (2019). The role of astaxanthin compared with metformin in preventing glycated human serum albumin from possible unfolding: A molecular dynamic study. *Asian Journal of Pharmaceutical and Clinical Research*, *12*(9), 276–282. https://doi.org/10.22159/ajpcr.2019.v12i9.34617

Wibowo, S., Widyarti, S., Sabarudin, A., Soeatmadji, D. W., & Sumitro, S. B. (2021). DFT and molecular dynamics studies of astaxanthin-metal ions (Cu^{2+} and Zn^{2+}) complex to prevent glycated human serum albumin from possible unfolding. *Heliyon*, *7*(3), e06548. https://doi.org/10.1016/j.heliyon.2021.e06548

Xu, S.-Y., Kan, J., Hu, Z., Liu, Y., Du, H., Pang, G.-C., & Cheong, K.-L. (2018). Quantification of neoagaro-oligosaccharide production through enzymatic hydrolysis and its anti-oxidant activities. *Molecules*, *23*(6), 1354. https://doi.org/10.3390/molecules23061354

Yun, E. J., Yu, S., & Kim, K. H. (2017). Current knowledge on agarolytic enzymes and the industrial potential of agar-derived sugars. *Applied Microbiology and Biotechnology*, *101*(14), 5581–5589. https://doi.org/10.1007/s00253-017-8383-5

Zhang, Y.-H., Song, X.-N., Lin, Y., Xiao, Q., Du, X.-P., Chen, Y.-H., & Xiao, A.-F. (2019). Antioxidant capacity and prebiotic effects of *Gracilaria* neoagaro oligosaccharides prepared by agarase hydrolysis. *International Journal of Biological Macromolecules*, *137*, 177–186. https://doi.org/10.1016/j.ijbiomac.2019.06.207