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Investigation of the Membrane-Stabilizing and Clot-Lysing Effects of Allyl Isothiocyanate Through *In Vitro* and *In Silico* Studies

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Abstract

This study sought to comprehensively explore the thrombolytic and membrane-stabilizing activities of allyl isothiocyanate (AITC) through both *in vitro* experimental assays and computational analysis. To investigate its thrombolytic activity, we conducted clot-lysis assays that mimic physiological clot formation and breakdown. In parallel, the membrane-stabilizing activity of AITC was assessed via human erythrocyte hemolysis assays, designed to cellular membrane damage that occurs during inflammation and oxidative stress. These assays allowed us to investigate the therapeutic relevance of AITC in conditions involving thrombosis and cell lysis. Additionally, molecular docking studies made us understand the potential interactions of AITC with two key molecular targets: plasminogen, which contributes significantly to the breakdown of fibrin clots, and cyclooxygenase-1 (COX-1), a major enzyme involved in the inflammatory responses. The *in vitro* results showed that AITC exerted concentration-dependent effects. At 100 µg/ml concentration, AITC achieved 82.18 ± 0.01% clot dissolution and 92.10 ± 0.01% membrane protection. The IC_{50} values were observed to be 41.70 ± 1.47 µg/mL for clot-lysis and 36.01 ± 1.71 µg/ml for membrane-stabilization, indicating potent bioactivity. Docking analysis demonstrated favorable interaction of AITC with plasminogen (−4.2 kcal/mol) and COX-1 (−4.5 kcal/mol), suggesting possible mechanisms underlying its observed effects. Taken together, these results suggest that AITC may function through dual fibrinolytic and anti-inflammatory pathways. Its ability to interact with key proteins involved in clot resolution and inflammation suggests it as a promising natural therapeutic candidate. However, additional clinical and pre-clinical investigations are required to establish its therapeutic safety and efficacy profile.

Key words: Inflammation; allyl isothiocyanate; membrane-stabilization; clot-lysis; egg albumin, cyclooxygenase-I.

Introduction

The stabilization of red blood cell (RBCs) membranes helps to prevent the escape of fluids and serum proteins into adjacent tissues and inhibits the release of inflammatory mediators, thereby reducing inflammation (Anosike *et al.*, 2012). This protective effect plays a vital role in preserving cellular integrity during inflammatory responses and contributes to preventing tissue damage (Bhol *et al.*, 2024; Viswanath *et al.*, 2019). Inflammation is a vital and complex biological process that defends against infection (Dobson *et al.*, 2020). Occasionally, the body triggers this defensive inflammatory response to eliminate harmful cells and initiate the normal healing process. However, chronic inflammation may contribute to various autoimmune disorders (Moore, 2015).

Membrane-stabilizing studies demonstrated anti-inflammatory mechanisms, as RBC lysis under hypotonicity and heat induces hemolysis and

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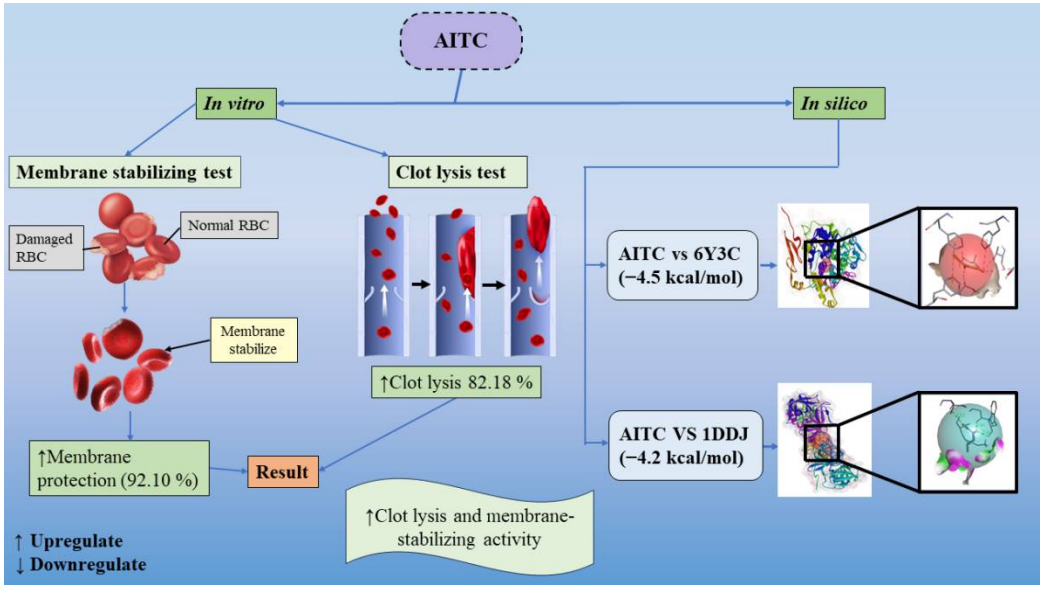
haemoglobin oxidation, triggered by infections, immune responses, drugs, or genetic disorders (Bhol *et al.*, 2024). Hypotonic solutions cause water influx, rupturing RBC membranes; damage heightens susceptibility to lipid peroxidation and free radical-induced injury (Sun *et al.*, 2018).

Thus, the inhibition of RBC lysis from heat and osmotic imbalance serves as an indicator of anti-inflammatory activity, as the structure of human RBC resembles that of lysosomal membranes (Yesmin *et al.*, 2020). Traditional NSAIDs cause gastrointestinal, kidney and heart complications; though, selective NSAIDs reduce such risks, but still cause stomach discomfort, dizziness, and the risk of cardiovascular complications (Suryavanshi & Rathore, 2019).



Blood defends against pathogens, clears toxins, transports oxygen and nutrients, regulates temperature, and forms clots via thrombosis to prevent blood loss (Litvinov & Weisel, 2023). Thrombosis, a common disease, can cause serious cardiovascular diseases like pulmonary embolism, heart attack, and stroke (Wendelboe & Raskob, 2016). Thrombolytics break down clots, restore circulation, and prevent organ damage, commonly used in emergencies for brain, heart, or lung blood flow (Xu *et al.*, 2023). Commercial thrombolytics like tissue plasminogen activators (tPA) are used clinically but are limited by poor absorption, off-target effects, and increased bleeding risk (Kumar & Sabu, 2019). Blood thinners like aspirin, clopidogrel, and warfarin raise haemorrhage risk, while statins cause digestive diseases, liver enzyme upregulation, and muscle pain (Mulchandani *et al.*, 2020).

Globally leading companies are driven to search for plant-based bioactive compounds that could be the basis for novel medications (Najmi *et al.*, 2022). WHO indicates that, about 80% of the globally people rely on plant-based remedies for managing serious diseases (Shakya, 2016). Plant-based compounds show numerous biological properties, like wound-healing, antioxidant, antidiabetic, antimicrobial, anti-inflammatory, anticancer, and anticoagulant effects (Shakya, 2016). Excessive use of costly synthetic drugs with side effects raises concerns (Abraham, 2023). Natural compounds offer safer alternatives, highlighting the need to identify anti-inflammatory agents from medicinal herbs (Cragg & Newman, 2013; Zhang *et al.*, 2019).

Allyl isothiocyanate (AITC), chemically known as 3-isothiocyanatoprop-1-ene, is a naturally occurring phytochemical ubiquitously distributed in plants, including mustard and horseradish belonging

to the Cruciferae family. AITC is also present in leafy greens of the Brassica genus, including cabbage (Eleimat, 2013). AITC possess numerous important biological properties, including antibacterial (Lin *et al.,* 2000), antioxidant (Bhushan *et al.,* 2021), neuroprotective (Hacet *et al.,* 2023), cardioprotective (Waz & Matouk, 2022), and anti-thrombotic properties (Kala & Khan, 2020). Previous research showed that AITC produces anti-inflammatory-like effects in laboratory animal models *(*Chang *et al.,* 2019*; Subedi et al.,* 2017; Wagner *et al.,* 2012). Currently, no published data exist on the membrane-stabilizing and clot-lysing effects of allyl isothiocyanate. Therefore, investigating these properties is essential to justify it's therapeutic potential as a traditional plant-based medicine. This study evaluates AITC's thrombolytic and membrane-stabilizing activities via clot-lysis and HRBC assays, alongside molecular docking analysis targeting plasminogen and COX-1 to elucidate its possible mechanisms.

Materials and Methods

In vitro studies: Selection of test concentration and preparation of test and controls: The maximum concentration of test sample was 100 µg/mL, substituted for the standard human dosage of AITC, followed by the other four sequential dilutions, which are 6.25, 12.5, 25 and 50 µg/mL. The reference and test samples were produced in a vehicle that included 0.05 percent tween 80 prepared in distilled water. In every investigation, the vehicle was used as a control.

Chemicals and reagents: AITC (CAS: 57-06-7, purity: 95 %, liquid) was bought from Sigma Aldrich (India), and tween 80 was obtained from Merck, India. Streptokinase (SK: DURAKINASE 15,00,000 IU) was acquired from South Kore (the Dongkook Pharmaceutical Co. Ltd.), whereas acetyl salicylic acid (ASA), known as aspirin was a generous gift from the ACME Laboratories Ltd. (Bangladesh).

Membrane-stabilization test (HRBC model): With a few minor modifications, the Shinde *et al.* model was employed in this study (Shinde *et al.,* 1999). This assay was conducted at the Pharmacology Laboratory, Department of Pharmacy, Gopalganj Science and Technology University, Gopalganj, Bangladesh. Five milliliters of recently drawn blood from a human donor in good health was mixed with 2.2 milligrams per milliliter of EDTA dipotassium salt. After the blood cells were collected and centrifuged for 10 minutes at 3000 × g, they were rinsed three times using a 10 mM sodium phosphate buffer with pH 7.4 containing an isotonic solution. Following another 10-minute centrifugation at 3000 × g, the prepared cell suspension was suspended again in an isotonic buffer solution's equal volume. As instructed, 0.5 mL of the cell suspension was combined with 5 mL of 50 mM NaCl solution and 100 µL of the test or reference solution (6.25, 12.5, 25, 50, and 100 µg/mL) in 10 mM sodium phosphate-buffered saline (pH 7.4). The control tube included only 100 µL of distilled water (DW) in the previously specified buffer, hypotonic solution 5 mL, and 0.5 mL of cell suspension. The mixture was allowed to incubate for ten minutes at room temperature before being centrifuged for ten minutes at 3000 × g. Finally, a colorimeter (LT-114, India) was used to measure the supernatant's optical density (OD) at 660 nm. The hemolysis inhibition % was estimated using the following mathematical formula:

$$\% \text{ Inhibition of hemolysis} = \{(OD_{control} - OD_{test\ samples})/OD_{control}\} \times 100$$

GraphPad Prism program was utilized to perform non-linear regression analysis and determine IC_{50}, or the half-maximal inhibitory concentration.

Clot-lysis test: The test model created by Prasad *et al.* served as the basis for this *in vitro* investigation (Prasad *et al.,* 2006). Here, we dispersed 0.5 mL of new blood from people who don'tuse contraception or anticoagulants into pre-weighed microcentrifuge tubes. The tubes were weighed after the blood sample was incubated at 37 °C for 45 minutes. Carefully, the serum was discarded without causing any disruption to the clot. 100 µL of the experimental sample was added to each tube at different concentrations (6.25, 12.5, 25, 50, and 100 µg/mL). The positive control and control marked tubes were filled with 100 µL of distilled water and 100 µL of Streptokinase (equal to

30,000 IU), respectively. Each tube's released fluid was carefully removed after incubation at 37 °C for 90 minutes, and the tubes were then weighed once again. The below mathematical equation was employed to compute % of clot-lysis:

% Clot-lysis = (clot weight after treatment ÷ clot weight before treatment) × 100

As previously stated, the IC_{50} value of AITC was also ascertained.

In silico studies (molecular docking)

Protein/selection and preparation: We have selected COX-1 (PDB ID: 6Y3C) and plasminogen (PDB ID: 1DDJ) enzymes as subjects for literature-based molecular docking as well as visualization research. The structure in three-dimensions (3D) of the COX-1 and plasminogen receptors were sourced from the RCSB Protein Data Bank (https://www.rcsb.org/) and downloaded as a PDB file. By deleting undesirable, lipids, water molecule, heteroatoms, and protein chains, receptors were tuned to avoid docking interference (Bappi *et al.*, 2024). Discovery Studio Visualizer v20.1.0.19295 was used for the optimization process. Next, the Swiss-PDB Viewer program (version 4.1.0) (Akbor *et al.*, 2023) was conducted to minimize the energy of protein structure.

Collection and preparation of ligands: The 3D structural conformers of the compound AITC (Compound CID: 5971), standard drug aspirin (ASA) (PubChem ID: 2244), and streptokinase (SK) (PubChem ID: 9815560) were acquired from the chemical database PubChem (https://pubchem.ncbi.nlm.nih.gov/) as SDF file. Subsequently, Chem3D 16.0 program were used in docking studies, the 3D structures of the compounds stored in SDF format (Al Hasan *et al.*, 2025). The 2D molecular depictions of AITC and ASA are shown in Figure 1.

Docking protocol and non-bond interactions: The docking was performed using PyRX to ascertain how much energy a ligand needs to interact with its receptor's active regions (Kondapuram *et al.*, 2021). The grid box size for the three axes (x-, y-, and z-) was found to be 22.35 × 28.42 × 25.00 Å in order to guarantee effective docking. In the docking calculations, two thousand steps were taken (Ibrahim *et al.*, 2022). The PDB format is used to assemble the protein-ligand complex. A comma-separated values (CSV) file was used to record the result of docking affinity. For additional investigation, the ligand is also gathered in PDBQT format (Sah *et al.*, 2024, 2025). The molecular interplay between the receptor's active site and ligand-proteins were depicted utilising PyMol (v2.5.8), and Discovery Studio Visualizer (v21.1.020298) (Islam *et al.*, 2024). Following that, the bond types, the entire quantity and duration of hydrogen bonds (HBs), as well as the residues of amino acids associated with each ligand-protein interaction.

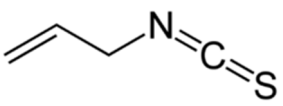
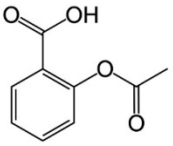
Allyl isothiocyanate	Aspirin
	

Figure 1. Chemical configurations of allyl isothiocyanate and aspirin.

Physicochemical, pharmacokinetic, and toxicity prediction: In order to justify the quality of medicine, computational methods were utilized to predict the ligands ADMET properties and their contaminants (Bhuia *et al.*, 2023). The ADMET and physicochemical traits of AITC, ASA, and SK were determined using SwissADME and pkCSM (Abdullah *et al.*, 2023). In the medication development process, toxicological prediction is essential because it assists in the determination and assessment of compounds with the maximum potential for ensuring safety and is helpful for usage by people (T Issa *et al.*, 2017). It is feasible to predict the possible toxicological properties of AITC, ASA, and SK ligands utilizing the online web servers Protox-3. Utilizing the online web servers Protox-3, the toxicological parameters of carcinogenicity, hepatotoxicity, mutagenicity, cytotoxicity and immunotoxicity, were primarily identified (Banerjee *et al.*, 2024). The pkCSM, SwissADME and web servers ProTox-3 were used to import the Canonical SMILES in order to determine the physicochemical,

toxicological and pharmacokinetic characteristics (https://tox.charite.de/protox3/), which was gathered from PubChem.

Statistical analysis: Data are shown as mean ± SEM (standard error of the mean). Statistical analysis was performed with One-way ANOVA (analysis of varianceANOVA followed by the Newman-Keuls post-host t-students test in GraphPad Prism (v9.5), with $p < 0.05$ considered statistically significant at the 95 % confidence level.

Results and Discussion

In vitro study

Membrane stabilization test: Inflammation is the protective response of immune system to dangerous stimuli, including infections, toxic substances, damaged cells, and radiation. Inflammation, therefore, plays a vital defensive mechanism for overall health (McKelvey et al., 2018). Chronic diseases, like rheumatoid arthritis, cancer, heart disease, neurological disorders and many other diseases, are closely linked with inflammation (Figus *et al.,* 2021). The five fundamental characteristics of acute inflammation are tumour or swelling, dolor or pain, calor or heat, rubor or redness, and loss of function (Lau, 2023). By engulfing and destroying harmful microbes, phagocytosis helps to suppress infection and gradually resolve inflammation (Gordon, 2016). Acute inflammation frequently heals and recovers tissue damage, as opposed to chronic inflammation can lead to scars, oxidative stress, tissue damage and impair organ function (El Ayadi *et al.,* 2020). Multiple factors and numerous mechanisms, including accidental trauma, oxidative stress, thermal attack, osmotic lysis, G6PD deficiency, parasitic infection, complement-induced hemolysis, and bacterial poisons, and G6PD deficiency, are responsible for hemolysis (Hay *et al.,* 2022; Kalfa, 2018). In the present study, the vehicle represents negligible inhibition of hemolysis (1.35 ± 0.03%), whereas AITC and ASA considerably ($p < 0.05$) suppressed hemolysis, increasing with dose. AITC affected most (92.10 ± 0.01% at 100 µg/mL) compared to ASA (73.09 ± 0.01%). Their IC_{50} values were 36.01 ± 1.71 µg/mL (AITC) and 42.59 ± 1.12 µg/mL (ASA) (Table 1). AITC showed slightly higher protection (79.66 ± 0.01%) than ASA (74.19 ± 0.01%) in the egg albumin assay, with comparable IC_{50} values of 36.01 and 42.59 µg/mL, correspondingly (Table 2).

Table 1. Membrane-stabilizing capacity of allyl isothiocyanate and control groups.

Sample/controls	Concentration	%Membrane protection	IC_{50} [CI, R^2]
Control (vehicle)	100 µl	1.35 ± 0.03	-
ASA	6.25 µg/ml	12.59 ± 0.02*	42.59 ± 1.12 µg/ml
	12.5 µg/ml	21.73 ± 0.02*	[35.10 – 52.51 µg/ml; 0.9893]
	25 µg/ml	31.36 ± 0.03*	
	50 µg/ml	55.80 ± 0.01*	
	100 µg/ml	73.09 ± 0.01*	
AITC	6.25 µg/ml	9.63 ± 0.02*	36.01 ± 1.71 µg/ml [26.57 – 48.44 µg/ml; 0.9773]
	12.5 µg/ml	17.28 ± 0.02*	
	25 µg/ml	32.35 ± 0.01*	
	50 µg/ml	59.75 ± 0.03*	
	100 µg/ml	92.10 ± 0.01*	

Values are mean ± SD (standard deviation) (n = 3), One-way ANOVA with student's t-test; *p <0.05 compared with control group (vehicle: 0.05% tween 80 solution in distilled water) group; AITC: Allyl isothiocyanate; ASA: Acetyl salicylic acid; IC50: Half-maximal concentration of inhibition; CI: Confidence interval; R2: Co-efficient of determination;

COX-1 enzyme drives prostaglandin production, promoting inflammation, platelet aggregation, vasoconstriction, and oxidative stress, which damage cell membranes (Ornelas *et al.,* 2017). Inhibiting COX-1 reduces these effects, enhancing membrane stability, while natural plant compounds show

promising potential in clot-lysis and membrane protection (Mukherjee & Chattopadhyay, 2022). AITC exhibits potent anti-inflammatory effects in macrophages and mouse models by downregulating COX-2 and inflammatory mediators (TNF-α, NO, IL-6, PGE2), inhibiting NF-κB via MAPK/JNK pathways, while enhancing NGF and promoting neurite growth in neuroblastoma cells (Subedi *et al.*, 2017; Wagner *et al.*, 2012). In *in vitro*, AITC showed substantial ($p < 0.05$) membrane-stabilizing action, though ASA was slightly higher. Nonetheless, AITC demonstrated superior efficacy likely by suppressing COX-1 and reducing different inflammatory modulators to protect cellular membrane integrity.

Table 2. Membrane-stabilizing egg-albumin protein denaturation by Allyl isothiocyanate and controls.

Sample/controls	Concentration	%Membrane protection	IC_{50} [CI, R^2]
Control (vehicle)	100 µl	1.43 ± 0.03	-
ASA	6.25 µg/ml	8.69 ± 0.03*	42.59 ± 1.12 µg/ml [35.10 – 52.51 µg/ml; 0.9893]
	12.5 µg/ml	14.03 ± 0.01*	
	25 µg/ml	23.70 ± 0.02*	
	50 µg/ml	42.64 ± 0.02*	
	100 µg/ml	74.19 ± 0.01*	
AITC	6.25 µg/ml	9.26 ± 0.03*	36.01 ± 1.71 µg/ml [26.57 – 48.44 µg/ml; 0.9773]
	12.5 µg/ml	15.00 ± 0.03*	
	25 µg/ml	24.82 ± 0.02*	
	50 µg/ml	43.2 ± 0.02*	
	100 µg/mL	79.66 ± 0.01*	

Values are expressed as mean ± SD (standard deviation) (n = 3), One-way ANOVA with student's *t*-test; *p <0.05 compared with the control group (vehicle: 0.05% tween 80 in distilled water) group; AITC: Allyl isothiocyanate; ASA: Acetylsalicylic acid; IC_{50}: Half-maximal inhibitory concentration; CI: Confidence interval; R^2: Co-efficient of determination;

Clot-lysis test: The *in vitro* clot-lysis assay assesses how well a compound dissolve blood clots, aiding early thrombolytic drug evaluation (Prasad *et al.*, 2006). Blood clot formation and breakdown are tightly regulated by pH, calcium, and platelets. Clot stability depends on fibrinolytic cofactors, while endothelial damage triggers thrombosis via collagen–factor VIII interactions and platelet activation (Yau *et al.*, 2015). The intrinsic and extrinsic pathways activate factor X, leading to convertion of prothrombin to thrombin, which then coverts fibrinogen into fibrin to stabilize clots (Krishnaswamy, 2013; Mackman, 2012). Fibrinolysis is mediated by plasmin, activated from plasminogen by tPA and uPA, with drugs like alteplase, streptokinase, reteplase, and tenecteplase enhancing this process (Zorio *et al.*, 2008). Blood clotting and lysis assays were evaluated by comparing fibrin formation and breakdown. The vehicle control showed negligible clot-lysis (1.12 ± 0.01%), compared to SK caused 81.37 ± 0.02% lysis at 100 µL ($p < 0.05$). AITC exhibited concentration-dependent clot-lysing activity with 82.18 ± 0.01% at 100 µg/mL followed by 52.71 ± 0.01%, 32.56 ± 0.02%, 15.02 ± 0.03%, and 8.33 ± 0.02% at the concentrations of 50, 25, 12.5, and 6.25 µg/mL, correspondingly. IC_{50} value of AITC was 41.70 ± 1.47 µg/ml (Table 3).

Plant phytochemicals, including alkaloids, glycosides, flavonoids, saponins, and tannins, showed clot-lysing activity, aiding thrombosis management. *In vitro* models streamline drug development, optimize clot-dissolving properties, and reduce early-stage animal testing (Kostewicz *et al.*, 2014). The clot-dissolving activity accelerates the identification of potential clinical trial candidates. In *in vitro*

experiments, AITC demonstrated significant ($p < 0.05$) dose-dependent thrombolytic activity, though SK showed higher potency. IC_{50} analysis indicates moderate clot-lysis potential. The mechanism likely involves converting inactive plasminogen into active plasmin, promoting fibrin breakdown (Figure 4). More research is needed to assess whether AITC initiates directly plasminogen or enhances tPA and uPA activity, clarifying its precise thrombolytic pathway and therapeutic potential for managing thrombotic disorders.

Table 3. Clot-lysis capacity of allyl isothiocyanate (AITC) and control groups.

Sample/controls	Concentration	%Clot-lysis	IC_{50} [CI, R^2]
Control (Vehicle)	100 µg/ml	1.12 ± 0.01	–
SK (15,00,000 U/Vial/10 ml)	100 µl	81.37 ± 0.02*	–
AITC	6.25 µg/ml	8.33 ± 0.02*	41.70 ± 1.47 µg/ml [34.39 – 50.85 µg/ml; 0.9897]
	12.5 µg/ml	15.02 ± 0.03*	
	25 µg/ml	32.56 ± 0.02*	
	50 µg/ml	52.71 ± 0.01*	
	100 µg/ml	82.18 ± 0.01*	

Values are the mean ± SD (standard deviation) (n = 3), One-way ANOVA with student's *t*-test; *p <0.05 when compared with the control group (vehicle: 0.05% tween 80 solution in distilled water) group; AITC: Allyl isothiocyanate; SK: Streptokinase; IC_{50}: Half-maximal concentration of inhibition; CI: Confidence interval; R^2: Co-efficient of determination;

In silico study

Molecular docking and visualization of ligand-protein interaction: In silico analysis revealed moderate binding affinity of AITC with receptor 6Y3C (−4.5 kcal/mol) forming one hydrogen bond with A:ALA302 (3.617 Å) and four hydrophobic interactions with A:LEU244 (3.926), A:LEU252 (4.114), A:PRO264 (4.178), and A:LEU306 (4.823). Whereas, ASA demonstrated higher binding affinity (−6.3 kcal/mol) to 6Y3C, establishing three hydrogen bonds with A:HIS43 (2.692), A:GLN44 (2.165), and A:GLU465 (2.476), and two hydrophobic bonds with A:LEU152 (4.664) and A:PRO153 (5.337). AITC showed modest binding energy (−4.2 kcal/mol) with 1DDJ receptor, and formed three hydrogen bonds with A:SER736 (3.062), A:GLU687 (3.778), and A:CYS737 (3.696 Å), and two hydrophobic interactions with A:ILE682 (4.580) and A:LYS698 (5.044). Conversely, SK showed stronger 1DDJ binding (−6.5 kcal/mol), forming two hydrogen bonds with A:GLY739 and A:GLY686, and one hydrophobic interaction with A:LYS698.

Overall, ASA and SK displayed better binding energies and more stable interactions compared to AITC. The parameters of binding are listed in Table 4, 2D and 3D views of non-bond interactions are shown in figure 2.

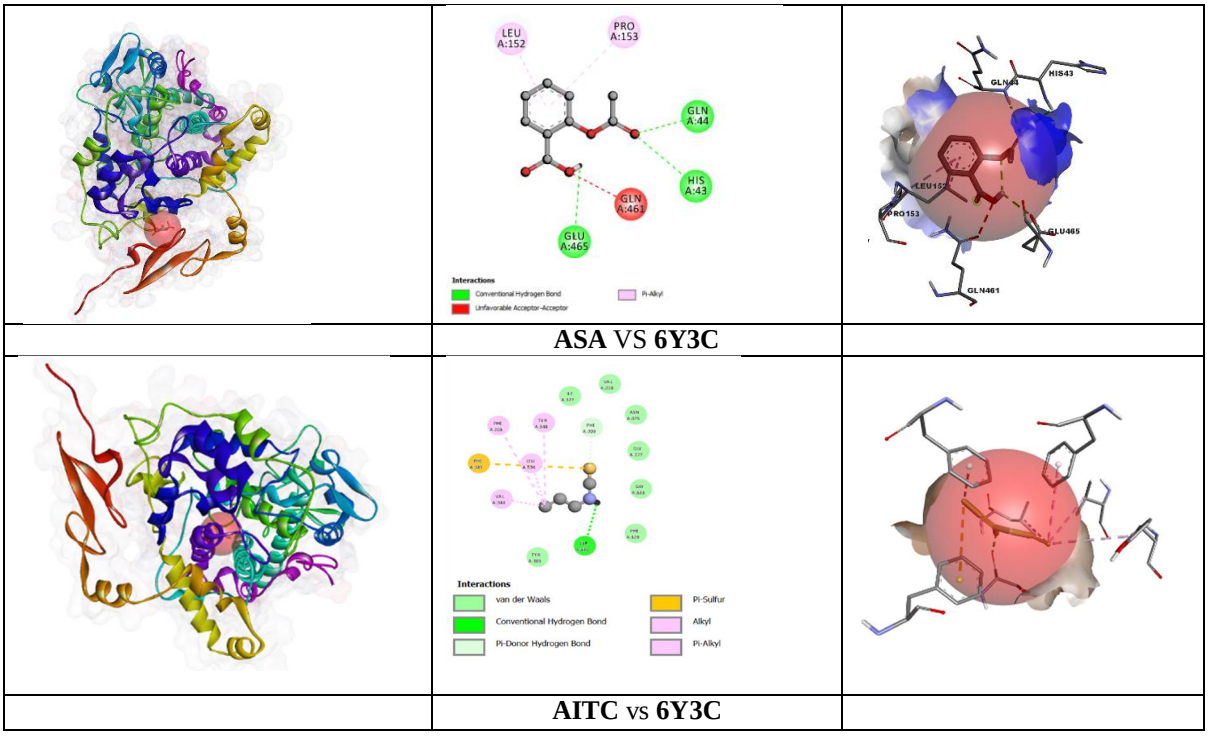
*Pharmacokinetic and drug-likeness and toxicity properties***:** Drug-likeness defines pharmacokinetics and therapeutic possibility of a ligand. All the ligands examined had MW < 500 g/mol, acceptable molar refractivity (≤140), HBD (≤5), and HBA (≤10). ASA showed the highest bioavailability score of 0.85 followed by 0.55 for AITC and SK. Both AITC and ASA were highly water-soluble. Log P values for AITC (2.41), ASA (1.19), and SK (0.27) indicated lipophilicity within the acceptable range (XLOGP3 < 4.15). The ligands showed good GI absorption; notably, AITC crossed the blood–brain barrier, suggesting CNS effects. Pharmacokinetic predictions showed compatibility with regard to Pgp substrate status, log Kp, CYP3A4/CYP2D6 inhibition, bioavailability, clearance, plasma protein binding, and half-life. *In silico* toxicity prediction estimated LD_{50} values of 112 mg/kg (AITC), 220 mg/kg (ASA), and 5000 mg/kg (SK). AITC and ASA

belonged to toxicity class III, while SK class V. AITC showed mutagenicity and neurotoxicity, while ASA indicated clinical toxicity. SK showed cardiotoxicity and neurotoxicity. AITC showed promising pharmacokinetic properties but needs to be properly evaluated for its mutagenicity and neurotoxicity (Table 5).

Table 4. Binding affinities and several types of bonds between ligands and proteins.

Receptor (PDB ID)	Ligands	Binding affinity (kcal/mol)	No of HB	AMA residues	
				HB (length) Å	Other bonds (types)
COX-1 (6Y3C)	ASA	-6.3	3	A: HIS43 (2.692), A: GLN44 (2.165), A: GLU465 (2.476)	A: LEU152 (4.664), A: PRO153 (5.337)
	AITC	−4.5	1	A: ALA302 (3.617)	A: LEU244(3.926), A: LEU252(4.114), A: PRO264(4.178), A: LEU306(4.823)
Human plasminogen catalytic domain (1DDJ)	AITC	-4.2	3	A: SER736 (3.062) A: GLU 687 (3.778) A: SYS737 (3.696)	A: ILE682 (4.580) A: LYS698 (5.044)
	SK	-6.5	2	A: GLY739 (2.926) A: GLY686 (3.644)	A: LYS698 (5.214)

ASA: Acetylsalicylic acid; AITC: Allyl isothiocyanate; SK: streptokinase; AA: Amino acid; HB: Hydrogen Bond; PDB ID (6Y3C): Cyclooxygenase-1; PDB ID (1DDJ): Human plasminogen



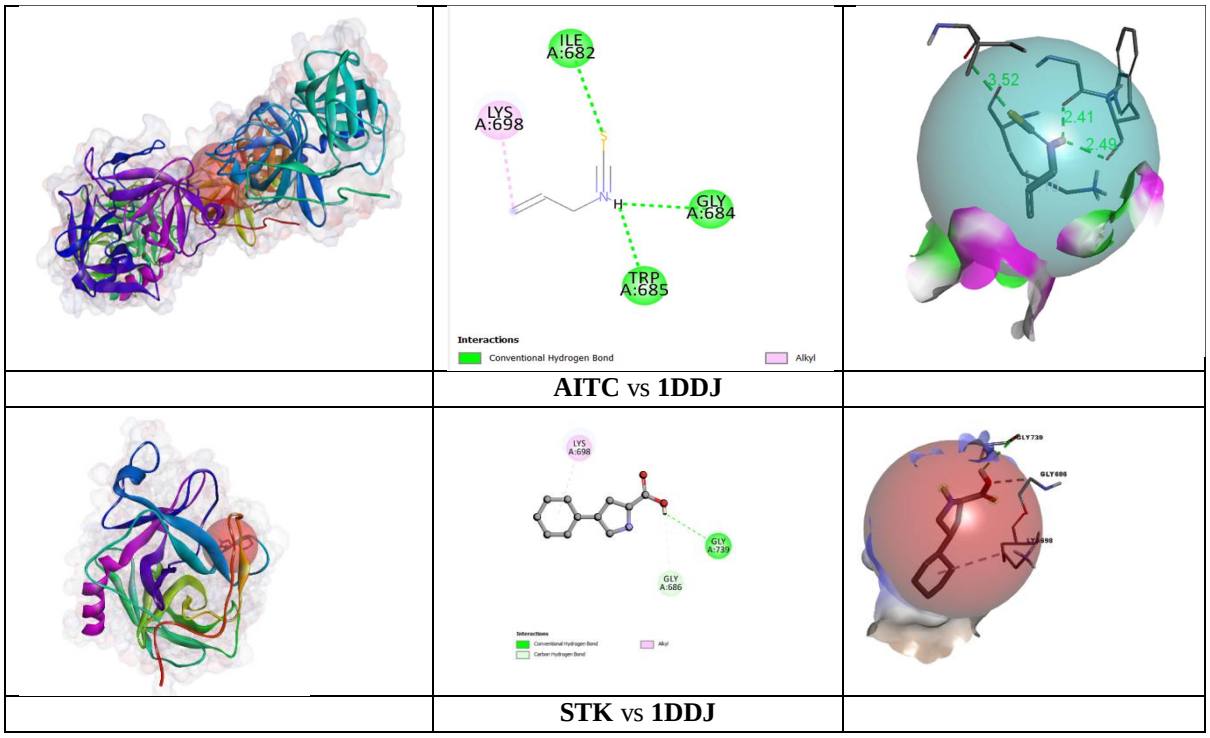
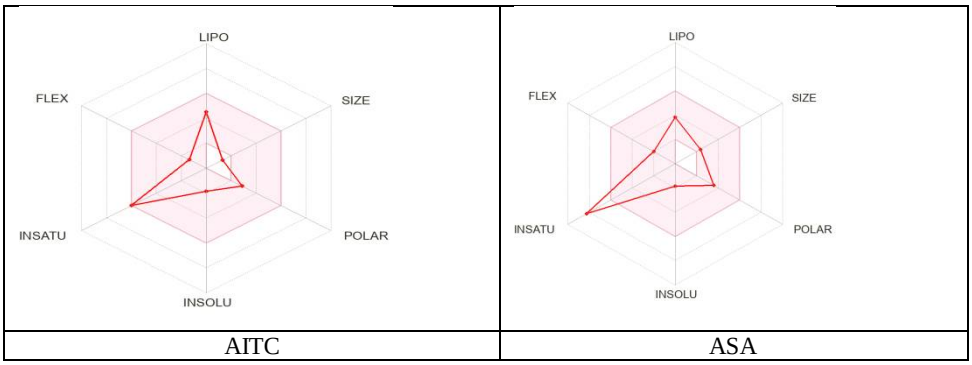


Figure 2. The 2D and 3D illustrations of the non-bond interactions between aspirin, Allyl isothiocyanate and streptokinase with 6Y3C, as well as aspirin, Allyl isothiocyanate and SK with 1DDJ. [ASA: Acetylsalicylic acid; AITC: Allyl isothiocyanate; SK: Streptokinase; PDB ID (6Y3C): Human plasminogen catalytic domain: Cyclooxygenase-1; PDB ID (1DDJ);].



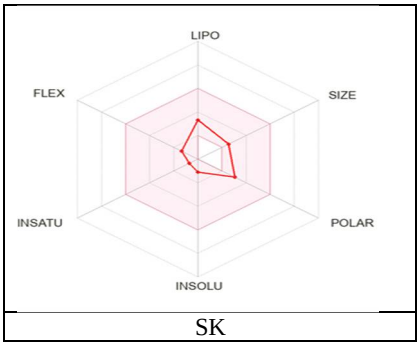


Figure 3. Bioavailability radar associated with the physicochemical characteristics of Allyl isothiocyanate [Lipophilicity (LIPO): –0.7 < XLOGP3 < +5.0, Insolubility (INSOLU): –6 < log S (ESOL) < 0, Insaturation (INSATU) 0.25 < Fraction Csp3 < 1, Polarity (POLAR): 20 $Å^2$ <TPSA <130 $Å^2$, Flexibility (FLEX): 0 < Num, rotatable bonds <9]

Table 5. Pharmacokinetic and drug-likeness properties of AITC, ASA and SK.

Properties	Factors	AITC	ASA	SK
Physicochemical properties	Formula	C4H5NS	C9H8O4	C11H19NO2
	Molecular weight (g/mol)	99.15 g/mol	180.16 g/mol	197.27
	Number of heavy atoms	6	13	14
	Number of aromatic heavy atoms	0	6	0
	Number of H-bond donors	0	1	2
	Number of H-bond acceptors	1	4	3
	Molar refractivity	30.03	44.90	59.25
Lipophilicity	Log Po/w (XLOGP3)	2.41	1.19	0.27
Drug-likeness	Lipinski	Yes; 0 violation	Yes; 0 violation	Yes; 0 violation
	Bioavailability score	0.55	0.85	0.55
Water solubility	Log S (ESOL)	-1.84	-1.85	-1.633
	Class	Very soluble	Very soluble	Soluble
Absorption	Caco2 permeability (log Papp in 10-6 cm/s)	1.378	0.09	1.235
	Intestinal absorption (human) numeric (%Absorbed)	100	76.938	91.77
	Skin permeability (log Kp cm/h)	-2.254	-2.715	-2.735
	P-glycoprotein I inhibitor	No	No	No
	P-glycoprotein II inhibitor	No	No	No
Distribution	BBB permeability (log BB)	0.105 yes	-0.332	0.254
	CNS permeability (log PS)	-2.476	-2.489	-2.455
	VDss (human) (log L/kg)	0.008	-1.716	-0.317
Metabolism	CYP2D6 substrate	No	No	Yes
	CYP3A4 substrate	No	No	No
	CYP2D6 inhibitor	No	No	No
	CYP3A4 inhibitor	No	No	No
Excretion	Total clearance (log ml/min/kg)	0.496	0.72	0.421
	Renal OCT2 substrate	No	No	No
Toxicity (ProTox 3.0)	Hepatotoxicity	Inactive	Inactive	Inactive
	Neurotoxicity	Active	Inactive	Active
	Immunotoxicity	Inactive	Inactive	Inactive
	Mutagenicity	Active	Inactive	Inactive
	Cardiotoxicity	Inactive	Inactive	Active
	Carcinogenicity	Inactive	Inactive	Inactive
	Clinical toxicity	Inactive	Active	Inactive
	Cytotoxicity	Inactive	Inactive	Inactive
	Toxicity class	3	3	5
	AMES toxicity	Yes	No	No
	Skin sensitization	No	No	No
	Predicted LD_{50} (mg/kg)	112 mg/kg	220	5000 mg/kg
	Maximum-tolerated dose (human) (log mg/kg/day)	0.932	1.016	0.689

Molecular docking analysis demonstrated moderate binding affinities with COX-1 (–4.5 kcal/mol) and human plasminogen (–4.2 kcal/mol) enzymes and favorable interaction profiles, supporting the mechanistic findings observed in *in vitro* experiments related to clot-lysis

and membrane-stabilization. These findings align with earlier studies suggesting that the conversion of plasminogen to its active form, plasmin, is central to thrombolytic processes, while inhibition of COX-1 plays a significant role in mediating anti-inflammatory effects. This dual mechanism implies that AITC may exert its therapeutic benefits by promoting plasminogen activation and suppressing COX-1 activity, thereby contributing to both clot-dissolving and membrane-stabilizing actions. To validate these proposed mechanisms, further investigations such as tPA activity assays and COX inhibition studies are essential. The ADMET analysis indicates that the AITC ligand is expected to have outstanding pharmacokinetic characteristics and meet Lipinski's criteria for prospective drug development. The *in silico* toxicity forecast showed that the recommended compound had no adverse impacts on hepatotoxicity, immunotoxicity, cardiotoxicity, carcinogenicity, cytotoxicity, or clinical toxicity. Furthermore, the AITC exhibits a similar toxicity class to ASA. Moreover, to validate its role in plasminogen activation and gain deeper insight into its molecular mechanism of action, advanced approaches such as molecular dynamics simulations, allosteric interaction analyses, and precise identification of AITC's binding site on target enzymes will be essential.

The *in vitro* and *in silico* studies collectively highlight the potential membrane-stabilizing and clot-lysing properties of AITC. The observed effects of AITC in both models demonstrate its potential as a valuable therapeutic agent for managing inflammation. The possible mechanism of AITC is represented in figure 4. These findings reinforce the growing body of evidence supporting the use of AITC during the progression of novel membrane-stabilizing and clot-lysing agents. Further studies, including investigations of dose-response relationships and underlying molecular mechanisms, are necessary to comprehensively evaluate the therapeutic potential and safety profile of AITC for clinical applications.

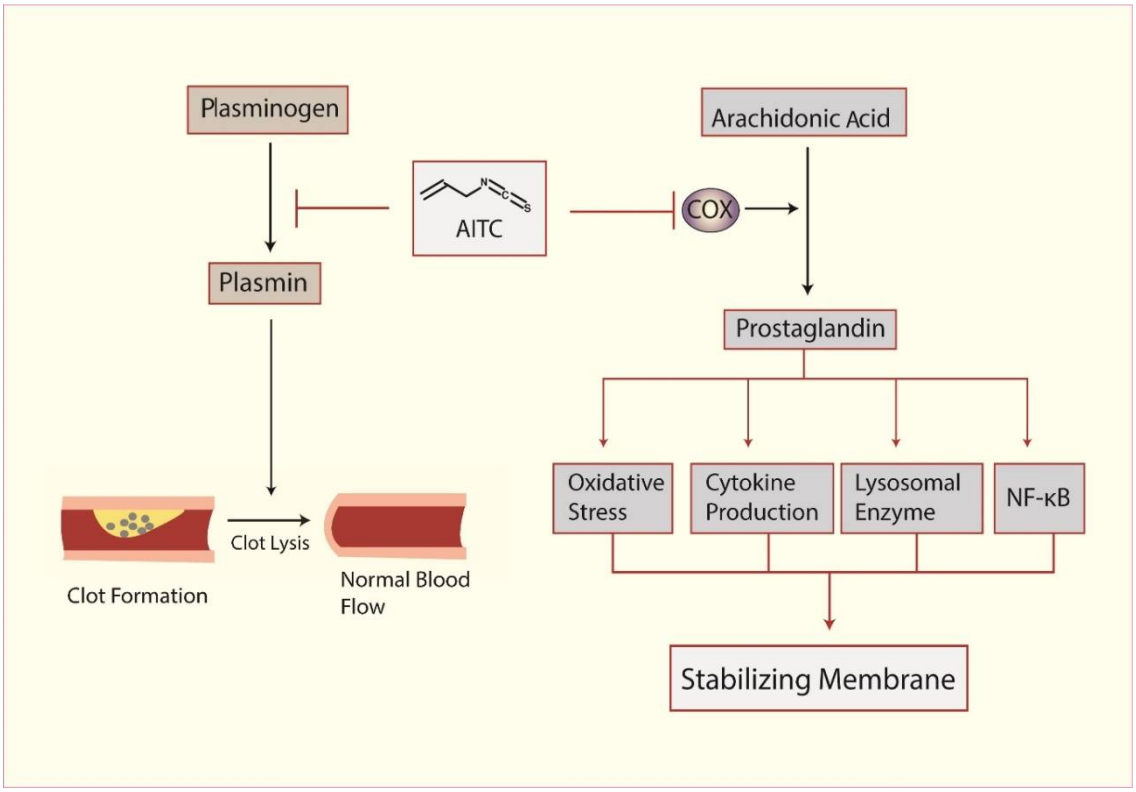


Figure 4. Proposed mechanism of action of AITC in clot-lysis and membrane-stabilization activity.

AITC exhibits dual functionality in both clot-lysis and membrane-stabilization. During the clot-lysis assay, AITC interacts with plasminogen, facilitating its conversion to plasmin, the key component of the degradation of clot, similar to the action of the standard thrombolytic agent streptokinase (SK). Plasmin, along with thrombin, fibrinogen cause fibrinolysis leading to the breakdown of clots into soluble products.

In the membrane-stabilization (Table 1.) and egg albumin protein denaturation assay (Table 2), AITC demonstrated superior membrane protection properties compared to the standard drug ASA. In line with these *in vitro* findings, the molecular docking study suggests that AITC, similar with the standard drugs ASA influences arachidonic acid activity by modulating the enzyme COX-I. This leads to reduced cytokine production, diminished oxidative stress, and suppression of lysosomal enzymes and the inflammation-associated protein complex NF-κB. Together, these effects helps stabilize the membrane through mitigating inflammation and oxidative responses.

Conclusion

Naturally occurring plant-based compounds are thought to be affordable, easily obtainable, generally safe, and are widely used for the treatment of numerous health disorders, particularly those involving inflammatory processes. In this study, AITC demonstrated notable clot-lysing and membrane-stabilizing effects in the HRBC model, suggesting its superior thrombolytic and membrane-protective efficacy over the standard drug. Supporting these *in vitro* findings, molecular docking analysis revealed moderate binding affinities between AITC and the key targets plasmin and COX-1, indicating an underlyin mechanism for its biological activity. Overall, the membrane-stabilizing, and clot-lysing properties of AITC reinforce its ability as a therapeutic candidate for managing thrombosis and maintaining RBC membrane integrity. However, additional research is necessary to fully elucidate its molecular mechanisms and evaluate its potential for clinical application in thrombolytic therapy and membrane-stabilization.

Data availability

Data will be made available on request.

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The list of contributor with their specific role during conducting research and preparing this manuscript is Fazley Rohan and Shoyaeb Ahammed: Investigation and Data Curation. Mst Sharmin Akter Juli: Methodology. Asratun Akter Tuli: Validation. MD. Tanvin Mridha: Resources. Muhammad Ali Khan: Writing – original draft, Supervision, Project administration and Funding Acquisition. This project was partially financed by the research center, GSTU, Bangladesh. The authors also extend their appreciation to the Department of Pharmacy, GSTU, Gopalganj, Bangladesh.

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Declaration of Generative AI and AI-assisted technologies in the writing process

The authors utilized ChatGPT (OpenAI) during the preparation of this manuscript to enhance the clarity, grammar, and overall readability of the text. All scientific interpretations, data analyses, and conclusions were solely conceived and developed by the authors. After using the AI tool, the manuscript was thoroughly reviewed, edited, and refined by the authors to maintain accuracy and uphold scientific standards. The authors assume full responsibility for the final content of the work.

Ethics approval and consent of participant

The blood samples used in the anti-inflammatory assay were voluntarily self-donated by the manuscript's first author, Fazley Rohan. According to the guidelines of the Human Ethics Committee of Gopalganj Science and Technology University, Gopalganj-8105, Bangladesh, ethical approval was

not required for self-donated blood, as studies involving minimal risk are exempt from ethical review. The authors affirm that all procedures were carried out in accordance with relevant institutional guidelines and regulations. Every effort was made to minimize the amount of sample used during testing. Furthermore, informed consent was obtained from all participants.

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