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Assessment of clot-lysing and membrane-stabilizing capacity of ascorbic acid: *In vitro* approach with molecular docking

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ABSTRACT

This study aimed to evaluate the clot-lysing and membrane stabilizing capacities of ascorbic acid (AA) using *in vitro* and *in silico* methods. For this, we used *in vitro* clot lysis and hemolyzing tests to check the anti-atherothrombosis and membrane-stabilizing properties of AA, respectively. Additionally, molecular docking studies were performed to investigate AA's interactions with cyclooxygenase-1 (COX-1) and plasminogen enzymes. Findings suggest that AA exhibited a concentration-dependent effect, with 43.95 ± 1.27 % clot lysis and 64.46 ± 0.01 % membrane stabilization at 100 μg/mL. The IC_{50} values for clot lysis and membrane stabilization were 215.19 ± 1.09 and 57.21 ± 2.11 μg/mL, respectively. *In silico* analysis showed strong binding affinities of AA with COX-1 (−6.2 kcal/mol) and plasminogen (−5.8 kcal/mol), supporting its observed clot lysis and membrane protection activities. Taken together, AA showed moderate clot-lysing and robust membrane-stabilizing effects, which may be due to its strong antioxidant and anti-inflammatory properties. AA might be a good therapeutic agent for atherothrombosis and membrane damage, highlighting the need for further investigation into its underlying molecular mechanisms and potential clinical applications. AA shows promising clot-lysing and membrane-stabilizing effects, highlighting its therapeutic potential for atherothrombosis and membrane damage.

1. Introduction

Atherothrombosis is a significant and escalating global health concern that serves as a primary cause of cardiovascular diseases, such as coronary artery disease, stroke, and peripheral arterial disease [43]. This condition arises when thrombi develop at sites of ruptured or eroded atherosclerotic plaques [5]. Cardiovascular diseases account for approximately 17.9 million deaths annually, establishing them as the leading cause of death worldwide [26]. Key risk factors for atherothrombosis include hypertension, diabetes, dyslipidemia, and smoking [52]. Thrombolysis is a medical therapy designed to dissolve dangerous blood clots, improve circulation, and protect vital organs and tissues

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[44]. It is frequently used in emergencies to treat clots in the arteries of the heart and brain, which are the main causes of heart attacks and ischemic strokes, as well as in the arteries of the lungs in cases of acute pulmonary embolism [17]. This therapy works by promoting fibrinolysis through plasmin, which is activated by the infusion of tissue plasminogen activator (tPA) analogs, the protein that naturally initiates plasmin activation [11]. Thrombolytic agents, such as tPA, urokinase (Ukase), and streptokinase (SK), are widely used for treating atherothrombotic conditions like myocardial or cerebral infarction, though these conditions can sometimes result in death [40]. However, the immunogenic nature of SK can occasionally provoke severe allergic reactions, including urticarial, itching, flushing, and nausea, limiting the possibility of administering multiple doses during thrombolytic therapy [41]. Consequently, natural small molecules that activate the signal of inactive plasminogen to active plasmin are emerging as promising therapeutic targets for use as thrombolytic agents.

Hemolysis refers to the destruction of red blood cells (erythrocytes), resulting in the release of hemoglobin and other cellular byproducts into the bloodstream [79]. This process can occur either within blood vessels (intravascular) or outside them (extravascular). Various factors, including infections, immune reactions, medications, and genetic conditions, can trigger hemolysis [47]. The consequences of hemolysis can include anemia, jaundice, hemoglobinuria, hyperbilirubinemia, iron overload, splenomegaly, gallstones, and vascular complications [9]. However, acetylsalicylic acid (ASA) primarily functions by inhibiting the enzyme cyclooxygenase (COX), particularly COX-1 and COX-2, which are key enzymes in the synthesis of prostaglandins [8,71]. Prostaglandins are crucial mediators in inflammation, pain, and fever [55]. By inhibiting COX-1, ASA reduces prostaglandin production, thereby exerting its anti-inflammatory, analgesic, and antipyretic effects. To stabilize cell membranes, various medications are employed, though some come with unwanted side effects. Non-selective non-steroidal anti-inflammatory drugs (NSAIDs), for instance, can irritate and inflame the stomach lining, increasing the risk of ulcers, gastrointestinal ulcers, renal impairment, and cardiovascular risks [15,75]. Even selective NSAIDs may cause adverse effects, such as gastrointestinal discomfort, headaches, dizziness, and an elevated risk of cardiovascular events [16,48].

Natural products derived from plants, minerals, and animals have long served as a foundation of human medicine, providing a vast array of bioactive compounds [6]. Secondary metabolites from these natural sources have proven to be invaluable in the development of potential drugs for clot lysis and membrane stabilization [42]. Several plants are known to contain secondary metabolites with clot-lysing properties, including *Curcuma longa* [36], *Glycyrrhiza glabra* [30], *Zingiber officinale* [46], and *Camellia sinensis* [58]. Additionally, plants such as *Withania somnifera* [67], *Centella asiatica* [13], *Terminalia chebula* [7], and *Allium sativum* [64] are recognized for their membrane-stabilizing properties. Several marketed drugs are available for clot lysis (thrombolysis), including alteplase [33], tenecteplase [72], SK [62], and Ukase [24], which are used to treat conditions related to blood clot dissolution. Additionally, drugs such as lidocaine [76], procainamide [74], quinidine [70], hydroxychloroquine [65], and corticosteroids (e.g., Dexamethasone, Prednisone) [73] are employed for membrane stabilization. These medications are commonly utilized in clinical settings to manage conditions where thrombolysis or membrane stabilization is critical for treatment. Currently, most drugs are synthetic, which makes them costly and often associated with side effects. Therefore, natural products offer a promising alternative to existing medications.

Ascorbic acid ($C_6H_8O_6$), commonly known as vitamin C, is a water-soluble vitamin that plays a vital role in numerous physiological functions [53]. It naturally occurs in many fruits and vegetables, particularly in citrus fruits like lemons, grapefruits, oranges, and pomelos, as well as in strawberries, kiwifruit, and green leafy vegetables such as spinach and kale [21,50,69]. AA is renowned for its antioxidant properties, its role in collagen synthesis, immune system support, and its anti-inflammatory effects, all of which contribute to its wide-ranging pharmacological activities and therapeutic benefits [2,27].

AA prevents atherothrombosis by reducing oxidative stress and inflammation, which are major factors in the development of the condition [2]. It also improves endothelial function, thereby decreasing the risk of plaque formation, and inhibits platelet aggregation, which is crucial for preventing thrombus formation. Additionally, AA exerts an anti-hemolyzing effect by protecting red blood cells (RBCs) from oxidative damage, stabilizing cell membranes, and scavenging free radicals that could otherwise cause RBC lysis [77]. This protective effect is particularly beneficial in conditions characterized by elevated oxidative stress, such as certain hemolytic anemia or during inflammatory responses. Moreover, some medicinal plant-derived phytochemicals have demonstrated clot-lysing and membrane-stabilizing properties [4,14,29].

The study employs a combination of *in vitro* and *in silico* methods. We assess AA's thrombolytic potential *in vitro* using clot-lysis assays, and measure its membrane-stabilizing effects using human red blood cell (HRBC) hemolysis tests. These tests are performed across various concentrations of AA to establish dose-dependent responses and calculate IC_{50} values for clot lysis and membrane protection. Furthermore, molecular docking studies are conducted to examine AA's interactions with COX-1 and plasminogen. Binding affinities and interaction patterns are analyzed to understand the mechanistic basis of AA's effects.

Given these insights, our current study aims to evaluate the clot-lysing and membrane-stabilizing capacities of AA with possible molecular mechanisms behind these effects using *in vitro* and *in silico* study protocols.

2. Materials and methods

L (+)- AA (CAS: 50–81–7, purity: ≥98 %, crystalline) and tween 80 were bought from Merck (India). Streptokinase (SK: DURAKINASE Lyophilisate for solution for infusion 1 500 000 IU) was purchased from the Dongkook Pharmaceutical Co. Ltd. (South Korea), while acetyl salicylic acid (ASA) was kindly provided by the ACME Laboratories Ltd. (Bangladesh).

2.1. In vitro studies

2.1.1. Selection of test concentration and preparation of test and controls

Regular human dose for AA was converted to the highest test concentration of 100 μg/mL, then the other four successive dilutions, i.e., 50, 25, 12.5, and 6.25 μg/mL. We prepared the test sample and standards in the vehicle containing 0.05 % tween 80 in distilled water. The vehicle served as a control group for each study.

2.1.2. Membrane-stabilization test (HRBC model)

This study was conducted using the model developed by Shinde et al. [68] with some modifications. This study was approved by the Department of Pharmacy, Bangabandhu Sheikh Mujibur Rahman Science and Technology University, Gopalganj (#bsmrstu-18PHR069–01). Initially, 5 mL of fresh blood was collected from a healthy donor (human) and mixed with dipotassium salt of EDTA (2.2 mg/mL). The blood cells were then accumulated by centrifugation at 3000 × *g* for 10 minutes and washed thrice with an isotonic solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The resulting cell suspension was re-centrifuged at 3000 × *g* for 10 minutes and finally re-suspended in an equal volume of isotonic buffer solution. Next, 0.5 mL of the cell suspension was added to a mixture of 5 mL of hypotonic solution (50 mM NaCl) and 100 μL of the test or standard solution (6.25, 12.5, 25, 50, and 100 μg/mL) in 10 mM sodium phosphate-buffered saline (pH 7.4), as specified. The control tube contained only 0.5 mL of cell suspension, 5 mL of hypotonic solution, and 100 μL of distilled water (DW) in the above-mentioned buffer. The reaction mixture was incubated for 10 minutes at room temperature and centrifuged at 3000 × *g* for

10 minutes. Finally, the optical density (OD) of the supernatant was quantified at 540 nm using a colorimeter (AE-11M, Japan). The percentage inhibition of hemolysis was calculated using the following equation:

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

The half-minimal inhibitory concentration (IC_{50}) was measured utilizing non-linear regression analysis with the aid of Graph Pad Prism software.

2.1.3. Clot lysis test

This *in vitro* study was done according to the model developed by Prasad et al. [59]. In this case, we distributed 0.5 mL of fresh blood in pre-weighed microcentrifuge tubes from the non-contraceptive or anti-coagulant-receiving humans. After incubating the blood sample at 37 °C for 45 min, the serum was cautiously excluded without disquieting the clot, and tubes were weighed. 100 µL of the test sample at different concentrations (6.25, 12.5, 25, 50, and 100 µg/mL) was added into each tube. 100 µL of SK (Equiv. 30,000 IU) and 100 µL of DW were added to the positive control and control marked tubes, respectively. After incubation of the tubes at 37 °C for 90 min, the discharged fluid from each tube was carefully removed, and the tubes were reweighed. The percentage of clot lysis was calculated as follows:

%Clot lysis = (Weight of clot after treatment ÷ Weight of clot before treatment) × 100

The IC_{50} value for the AA was also determined as mentioned above.

2.2. Statistical analysis

Values are expressed as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was followed by Newman Keuls *post-host* t-students test using the Graph Pad Prism software (version 9.5), considering $p < 0.05$ at 95 % confidence intervals.

2.3. In silico studies

2.3.1. Macromolecule selection and preparation

We targeted the enzyme COX-1 for membrane stabilization and plasminogen for clot lysis, given their critical roles in these processes, to conduct molecular docking and receptor-ligand visualization. The 3D structures of COX-1 (PDB ID: 6Y3C, chain A) [54] and catalytic domain of plasminogen (PDB ID: 1DDJ, chain A–D) [35,78] were retrieved from the RCSB Protein Data Bank (https://www.rcsb.org/). To minimize potential docking issues, the enzymes were optimized by removing unnecessary protein chains, lipids, water molecules, and heteroatoms from the protein sequence using the PyMol software tool (version 2.4.1) ([57] a). Energy minimization and receptor geometry optimization were then performed with the SwissPDB Viewer software using the GROMOS96 force field, after which the PDB file was saved for molecular docking [60].

2.3.2. Ligand preparation

In the study, we selected AA as the ligand due to its clot lysis and membrane stabilization properties. ASA was used as a standard drug to validate docking results with the COX-1 enzyme. The 3D structures of AA and ASA (PubChem CID: 54670067 and 2244, respectively) were obtained from the PubChem online chemical database (https://pubchem.ncbi.nlm.nih.gov/, accessed on August 21, 2024) in SDF format. The ligands were optimized using Chem3D Pro 20.1.1 software, employing Allinger's force field (MM2) technique [61]. Fig. 1 depicts the two-dimensional structure of AA.

2.3.3. Molecular docking and visualization

We performed molecular docking studies of AA with the enzymes COX-1 and plasminogen. A standard drug was used to validate the docking and bond interactions. The docking simulations were carried out using the PyRx software package (Version 0.8) to predict the binding affinities of the drugs at the receptor's active sites [37]. The process involved setting up a grid box around both the receptor and the ligand, with the grid dimensions optimized along the x, y, and z axes, followed by 200 calculation steps [18]. The docking results were saved in '.csv' format, while the best ligand-protein complex pose was extracted in PDB format, with the ligand saved separately in PDBQT format. The interactions between the ligand and the receptor's active site were analyzed using Discovery Studio Visualizer (v21.1.020298) and PyMol (v2.4.1) [28,57]. This analysis detailed the involved amino acid (AMA) residues, types of bonds, hydrogen bond lengths, and other interactions for each ligand-receptor pair.

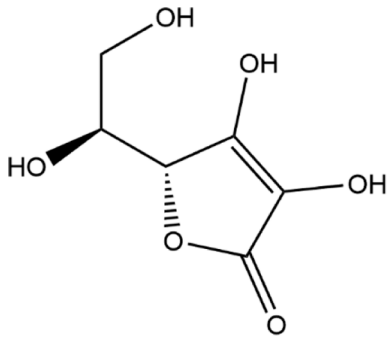

Fig. 1. The two-dimensional structure of ascorbic acid.

3. Results

3.1. Clot-lysing capacity

The plasma-based clot formation and lysis assay enables a detailed assessment of fibrin production and breakdown capabilities. The clot lysis activity of the AA and controls was evaluated at varying concentrations. The vehicle group exhibited minimal clot lysis of 1.15 ± 0.01 %. The standard drug SK at a concentration of 100 µL demonstrated a significant ($p < 0.05$) clot lysis of 84.57 ± 0.02 %. On the other hand, AA showed concentration-dependent clot-lysing capacity, with the highest activity ($p < 0.05$) observed at 100 µg/mL by 43.95 ± 1.27 %, then followed by 28.19 ± 4.63, 13.50 ± 3.11, 10.86 ± 3.81, and 6.64 ± 2.19 % at 50, 25, 12.5, and 6.25 µg/mL, respectively. The IC_{50} value for AA was calculated to be 215.19 ± 1.09 µg/mL, with a confidence interval (198.11–227.18 µg/mL) and an R^2 value of 0.93 (Table 1).

3.2. Membrane-stabilizing capacity

Membrane stabilizing activities prevent action potentials from propagating across the membrane. This action also inhibits human erythrocyte hemolysis and protein denaturation. The anti-hemolyzing capacity of AA and ASA was assessed at different concentrations. The vehicle control demonstrated minimal membrane protection of 2.03 ± 0.01 %. ASA showed significant ($p < 0.05$) and concentration-dependent membrane protection, with the highest concentration (100 µg/mL). AA showed membrane-stabilizing power of 72.61 ± 0.02 %, then followed by 55.25 ± 0.02, 31.31 ± 0.01, 20.52 ± 0.01, and 10.79 ± 0.01 % at 50, 25, 12.5, and 6.25 µg/mL, respectively. The IC_{50} value for ASA was 45.41 ± 1.03 µg/mL with a confidence interval (36.21 – 59.34 µg/mL) and an R^2 value of 0.91. AA also exhibited significant ($p < 0.05$) anti-hemolysis activity, with the highest membrane protection observed at 100 µg/mL (64.46 ± 0.01 %), followed by 50 µg/mL (41.57 ± 0.02 %), 25 µg/mL (32.62 ± 0.01 %), 12.5 µg/mL (26.31 ± 0.02 %), and 6.25 µg/mL (14.73 ± 0.01 %), respectively. The IC_{50} value for AA was calculated as 57.21 ± 2.11 µg/mL, with a confidence interval (47.13 – 71.61 µg/mL) and an R^2 value of 0.92 (Table 2).

Table 1
Clot lysis capacity of ascorbic acid and control groups.

Sample/controls	Concentration	%Clot lysis	IC_{50} [CI, R^2]
Control (Vehicle)	100 µg/mL	1.15 ± 0.01	–
SK (15,00,000 U/Vial/10 mL)	100 µL	84.57 ± 0.02*	–
AA	6.25 µg/mL	6.64 ± 2.19*	215.19 ± 1.09 µg/mL [198.11 – 227.18 µg/mL; 0.93]
	12.5 µg/mL	10.86 ± 3.81*	
	25 µg/mL	13.50 ± 3.11*	
	50 µg/mL	28.19 ± 4.63*	
	100 µg/mL	43.95 ± 1.27*	

Values are the mean ± standard error of the mean (SEM) (n = 3), One-way ANOVA followed by *t*-student post hoc test;
*p <0.05 when compared to the control (vehicle: 0.05 % tween 80 dissolved in distilled water) group; AA: Ascorbic acid; SK: Streptokinase; IC_{50}: Half-minimum inhibitory concentration; CI: Confidence of interval; R^2: Co-efficient of determination;

Table 2
Anti-hemolyzing capacity of ascorbic acid and control groups.

Sample/controls	Concentration	%Membrane protection	IC_{50} [CI, R^2]
Control (Vehicle)	100 µL	2.03 ± 0.01	-
ASA	6.25 µg/mL	10.79 ± 0.01*	45.41 ± 1.03 µg/mL [36.21 – 59.34 µg/mL; 0.91]
	12.5 µg/mL	20.52 ± 0.01*	
	25 µg/mL	31.31 ± 0.01*	
	50 µg/mL	55.25 ± 0.02*	
	100 µg/mL	72.61 ± 0.02*	
AA	6.25 µg/mL	14.73 ± 0.01*	57.21 ± 2.11 µg/mL [47.13 – 71.61 µg/mL; 0.92]
	12.5 µg/mL	26.31 ± 0.02*	
	25 µg/mL	32.62 ± 0.01*	
	50 µg/mL	41.57 ± 0.02*	
	100 µg/mL	64.46 ± 0.01*	

Values are mean the ± standard error of the mean (SEM) (n = 3), One-way ANOVA followed by *t*-student post hoc test;
*p <0.05 when compared to the control (vehicle: 0.05 % tween 80 dissolved in distilled water) group; AA: Ascorbic acid; ASA: Acetyl salicylic acid; IC_{50}: Half-minimum inhibitory concentration; CI: Confidence of interval; R^2: Co-efficient of determination;

3.3. Molecular docking and visualization of ligand-protein interaction

The *in silico* docking processes are being utilized to examine the molecular complementarity between a ligand and a protein target. In the *in silico* analysis, AA displayed remarkable binding affinities with the 1DDJ and 6Y3C receptors (−5.8, and –6.2 kcal/mol, respectively). In addition, AA formed five hydrogen bonds (HBs) interactions with GLY739 A: (Bond distance: 1.971 Å), GLU687 A: (2.649 Å), GLY565 A: (3.290 Å), GLY686 A: (3.387 Å), and CYS737 A: (3.484 Å) AMA residues at the 1DDJ receptor binding site, each varying in bond length. Moreover, AA also established two HBs with HIS388 A: (3.058 Å) and THR206 A: (2.945 Å) AA residues with the COX-1 receptor. ASA, as standard with the 6Y3C receptor, formed three HB with AMA residues HIS43 A: (3.073 Å), GLN44 A: (3.103 Å), and GLN461 A: (2.064 Å) along with pi-alkyl bonds. The binding energy, number of hydrogen bonds, interacting AMA residues, and other bond types between AA and the enzymes 6Y3C and 1DDJ are detailed in Table 3. Furthermore, the 2D and 3D visualizations of the non-bond interactions of AA and ASA with 6Y3C, as well as AA with 1DDJ, are displayed in Fig. 2.

Table 3
Binding affinities and several types of bonds between ascorbic acid and the specific receptors 1DDJ and 6Y3C.

Receptor (PDB ID)	Ligands	Binding affinity (kcal/mol)	No of HB	AMA residues	
				HB (Length) (Å)	Other bonds (Types)
Human plasminogen catalytic domain (1DDJ)	AA	−5.8	5	GLY A: 739 (1.971), GLU A: 687 (2.649), GLY A: 565 (3.290), GLY A: 686, (3.387), CYS A: 737 (3.484)	-
COX−1 (6Y3C)	AA	−6.2	2	HIS A: 388 (3.058), THR A: 206 (2.945)	-
	ASA	−6.3	3	HIS A: 43 (3.073), GLN A: 44, (3.103), GLN A: 461 (2.064)	LEU A: 152 (Pi-Alkyl), PRO A: 153 (Pi-Alkyl)

AA: Ascorbic acid; ASA: Acetylsalicylic acid; AMA: Amino acid; HB: Hydrogen Bond; PDB ID (6Y3C): Cyclooxygenase-1; PDB ID (1DDJ): Human plasminogen catalytic domain;

4. Discussion

Clot lysis and blood clotting are crucial physiological hemostatic processes within the body, influenced by factors such as pH, calcium levels, and platelet counts. The stability of a clot is maintained by a complex interplay of cofactors, inhibitors, and receptors that regulate fibrinolysis. Endothelial injury or dysfunction is a primary trigger for thrombosis, exposing sub-endothelial collagen and von Willebrand factor (vWF) upon damage [80]. This exposure leads to platelet adhesion and activation. Both intrinsic and extrinsic pathways converge to activate factor X, which then converts prothrombin to thrombin [39,45]. Thrombin plays a pivotal role in transforming fibrinogen into fibrin, creating a stabilizing mesh over the platelet plug [34]. Fibrin strands interlace with the platelet plug, reinforcing the clot. Plasmin, the main enzyme responsible for fibrinolysis, is activated from plasminogen by tPA or UK plasminogen activator (uPA) [82]. While tPA is produced and released by endothelial cells, uPA is generated by monocytes, macrophages, and the urinary epithelium. Both activators have short half-lives in circulation (4–8 minutes) due to high levels of inhibitors like plasminogen activator inhibitor-1 (PAI-1). Consequently, plasmin serves as the primary regulator of clot lysis, and FDA-approved drugs such as alteplase, SK, reteplase, and tenecteplase are used to activate plasmin.

Hemolysis can occur through various mechanisms, including mechanical trauma, thermal injury, osmotic lysis, oxidative stress, complement-mediated hemolysis, parasitic infections, bacterial toxins, and G6PD deficiency [25,32]. However, COX-1 is an enzyme that catalyzes the conversion of arachidonic acid into prostaglandins, such as thromboxane A2, which mediate inflammation, platelet aggregation, and vasoconstriction. Inflammatory processes often result in increased oxidative stress, leading to cell membrane damage through lipid peroxidation [56]. Inhibiting COX-1 can reduce the production of these prostaglandins and thromboxane, potentially decreasing inflammation and associated membrane damage while also indirectly contributing to membrane stabilization by reducing oxidative stress.

Phytochemicals derived from natural sources have garnered significant interest in recent years for their potential in a broad spectrum of therapeutic applications, including clot lysis and membrane stabilization [51]. Various studies, such as those by Sardar et al. [66], emphasize the relevance of natural molecules in modulating health outcomes. *Vigna unguiculata*, for instance, is highlighted for its phytochemical content

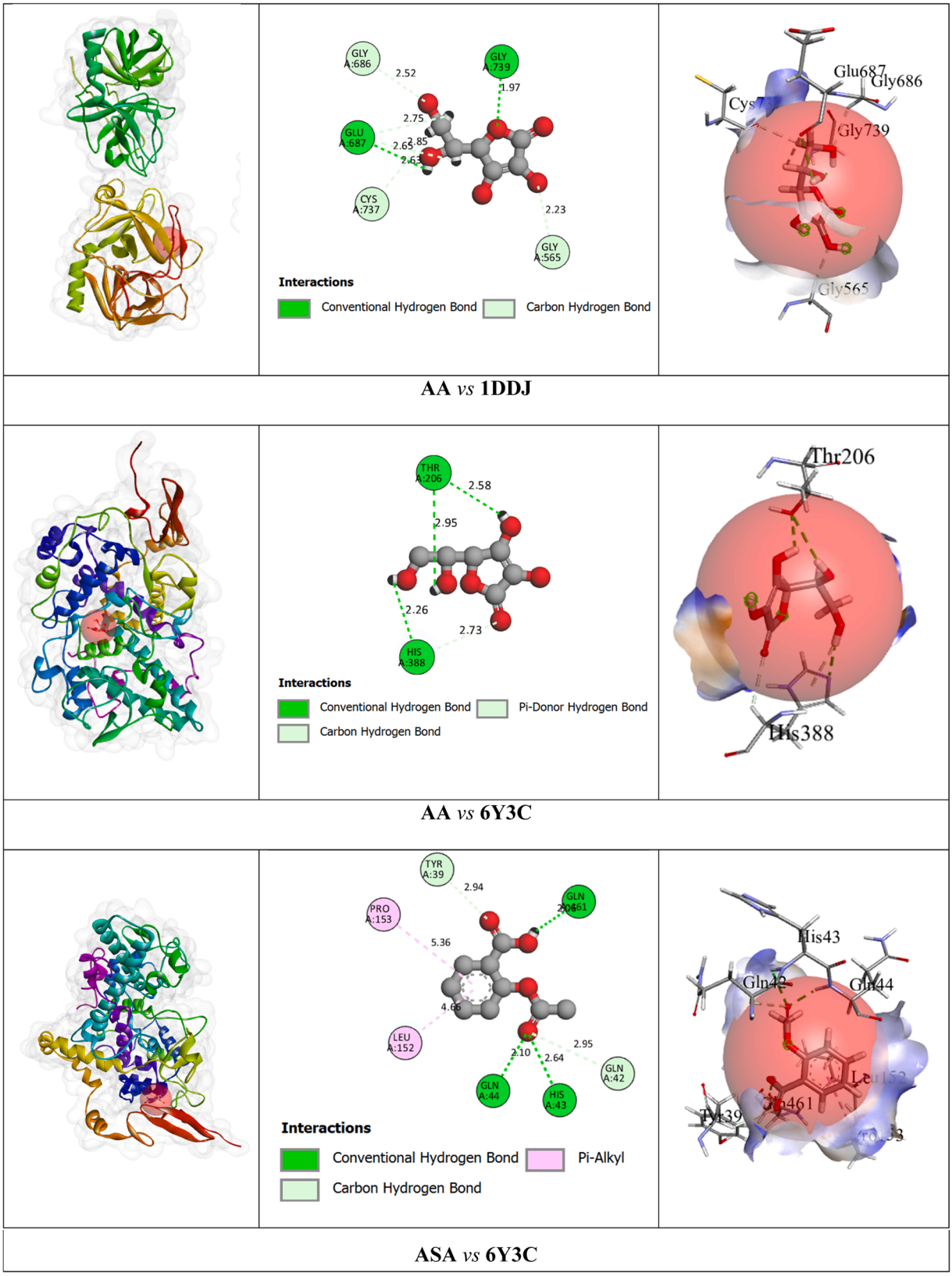

Fig. 2. The 2D and 3D representations of the non-bond interactions between ascorbic acid and acetyl salicylic acid with 6Y3C, as well as ascorbic acid with 1DDJ. [AA: Ascorbic acid; ASA: Acetylsalicylic acid; PDB ID (6Y3C): Cyclooxygenase-1; PDB ID (1DDJ): Human plasminogen catalytic domain;].

and its capacity to influence health through its antioxidative properties. Similarly, other studies, like that by Ferrarini et al. [19], demonstrate how cannabis oil can modulate fibromyalgia symptoms through its bioactive compounds. Natural vitamins and fatty acids act against chemotherapy-induced intestinal mucositis and asthma features, and also recent research suggests the nutritional immunomodulation of cancer-related microRNAs [3,10,63]. Natural flavonoids and other classes of compounds modulate neurodegenerative disease [1]. Apoptosis-inducing anti-proliferative bioactive phytochemicals used as an anti-cancer, and anti-oxidant efficacy [49]. These findings support the growing body of evidence on natural compounds being beneficial for inflammatory and oxidative stress-related conditions, reinforcing the role of AA in our study.

The clot lysis test is a laboratory technique used to assess the ability of a substance to dissolve blood clots (fibrinolysis) [59]. It is crucial in new drug development for thrombolysis as it provides an early assessment of a compound's ability to dissolve clots effectively [59]. This *in vitro* model allows for the evaluation of the thrombolytic potential of new drugs, guiding optimization and reducing the need for initial *in vitro* testing [38]. By demonstrating efficacy in clot dissolution, this test accelerates the identification of promising candidates for further clinical development. In our *in vitro* study, AA demonstrated significant ($p < 0.05$) membrane-stabilizing activity at a concentration of 100 µg/mL, resulting in maximum clot lysis. Conversely, SK exhibited greater clot lysis activity at a dose of 100 µL. However, at lower concentrations (6.25, 12.5, 25, and 50 µg/mL), AA showed a dose-dependent decrease in thrombolytic activity. The IC_{50} value for AA was determined to be 215.19 ± 1.09 µg/mL.

In the hypotonic solution, the hemolytic effect occurs due to the rupture of the cell membrane caused by excessive fluid accumulation inside the cell [23]. Membrane stability is crucial in preventing the leakage of fluids and serum proteins into the tissues, particularly during periods of increased permeability triggered by inflammatory mediators. In our *in vitro* study, AA demonstrated significant ($p < 0.05$) membrane-stabilizing activity at a concentration of 100 µg/mL, providing the highest level of membrane protection. Conversely, ASA exhibited even greater membrane-stabilizing activity at the same concentration. However, at lower concentrations (6.25, 12.5, and 25 µg/mL), AA outperformed ASA in terms of membrane-stabilizing activity, which is a positive outcome for our study. The IC_{50} value for AA was determined to be 57.21 ± 2.11 µg/mL, while for ASA, it was 45.41 µg/mL.

Molecular docking is essential in structure-based drug design, enabling the prediction of how small molecule ligands bind to specific target sites on receptors or enzymes [20]. These ligand-protein interactions can either activate or inhibit the enzyme's activity. Among the non-covalent forces, HBs stand out due to their strength and directionality, offering higher specificity in drug-target interactions compared to van der Waals interactions [81]. In our *in silico* analysis, we found that AA exhibited a significant binding affinity of −6.2 kcal/mol with the COX-1 enzyme, comparable to the standard binding affinity of −6.3 kcal/mol. Additionally, AA demonstrated a good binding affinity of −5.8 kcal/mol with the catalytic domain of plasmin, which is crucial for plasmin activation and subsequent clot lysis. Notably, AA binds to its receptor sites exclusively through HBs, indicating strong interactions. Similarly, the binding of AA to the COX-1 receptor also involves HBs, comparable to the standard drug. Overall, our study suggests that the test compound exhibits remarkable binding interactions and properties, potentially contributing to clot lysis and membrane stabilization. However, a possible mechanism of AA in clot lysis and membrane stabilization activity is shown in Fig. 3.

However, AA has long been explored for its various medicinal characteristics, which include antioxidant and anti-inflammatory actions. Additionally, the evidence for its role in blood clotting varies, and some studies did not show a significant beneficial impact. For example, certain studies showed that AA could not have significant fibrinolytic or anticoagulant activity under certain conditions, notably in human trials or clinical settings where confounding factors such as diet and underlying health conditions may impact results [12,22,31]. To address this, we used a unique combination of *in vitro* and *in silico* approaches to

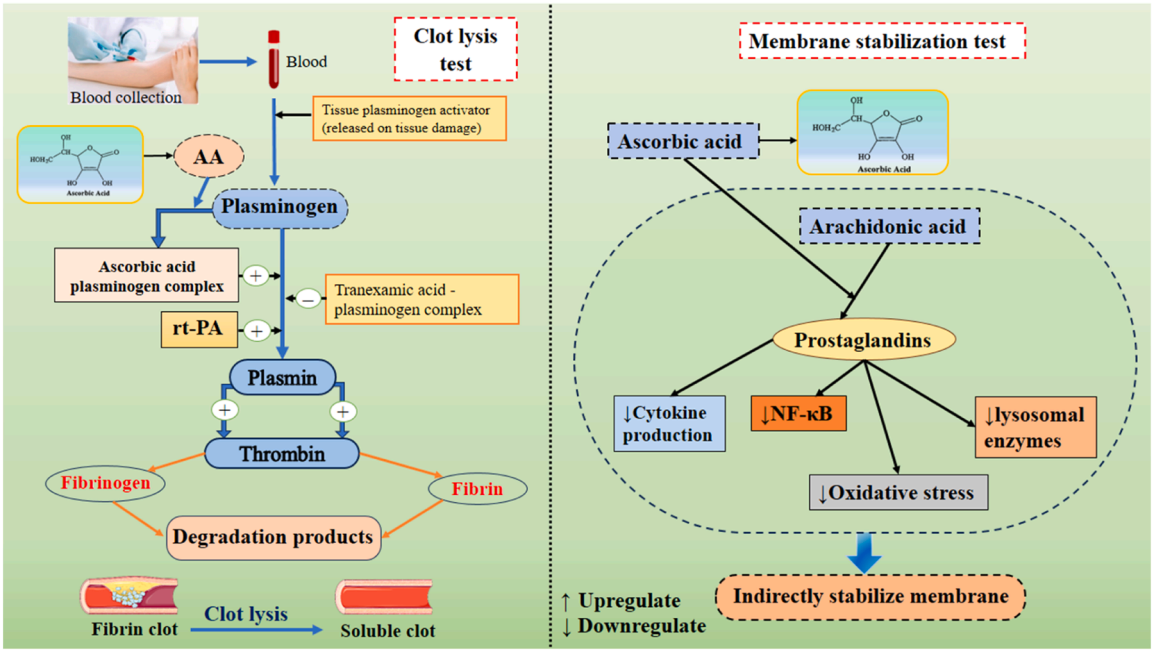

Fig. 3. Possible mechanism of ascorbic acid in clot lysis and membrane stabilization activity [AA plays a dual role in clot lysis and membrane stabilization. In the clot lysis test, AA forms a complex with plasminogen, promoting its activation into plasmin alongside rtPA, which, with thrombin, targets fibrinogen and fibrin, breaking down clots into degradation products. In the membrane stabilization test, AA modulates the activity of arachidonic acid, impacting the prostaglandin pathway, leading to decreased cytokine production, reduced oxidative stress, and inhibition of lysosomal enzymes and NF-κB, an inflammation-related protein complex. These actions collectively contribute to membrane stabilization by lowering inflammatory and oxidative factors; AA: Ascorbic acid; NF-κB: Nuclear factor kappa B; rtPA: Recombinant tissue plasminogen activators].

assess AA's thrombolytic capability. In our study, we observed AA at higher concentrations showed dose-dependent clot-lysis capabilities, while AA's effect may not always be pronounced in clinical settings and under controlled conditions. The novelty of our study lies in the integration of molecular docking with COX-1 and plasminogen enzymes to propose mechanisms for AA's moderate thrombolytic activity. Further clinical trials focusing on specific patient groups may clarify the exact therapeutic potential of AA in cardiovascular disease prevention.

The clinical significance of AA's clot-lysing and membrane-stabilizing properties is especially evident in diseases such as atherothrombosis and hemolytic anemia. Cardiovascular illnesses remain a primary cause of death worldwide, therefore discovering cost-effective, safe, and accessible medicines is crucial. AA, being a naturally occurring and commonly available molecule, holds promise in this area due to its multifaceted bioactivity. The observed clot lysis and membrane protection at clinically relevant doses indicate that AA could be used as an adjunct therapy in thrombolytic therapies, minimizing the requirement for synthetic medicines, which are frequently associated with adverse effects. Furthermore, AA's membrane-stabilizing effect may aid in the treatment of disorders defined by oxidative stress-induced hemolysis, such as sickle cell anemia and autoimmune hemolytic anemia.

In our *in vitro* study, we observed that AA demonstrated significant clot lysis activity compared to the control (vehicle) group, although the standard group exhibited even higher activity than the test group. Additionally, AA showed greater membrane-stabilizing activity compared to the standard group, with effects increasing in a dose-dependent manner. Our molecular docking study also revealed strong bond interactions and favorable binding properties. While the specific mechanisms of clot lysis and membrane stabilization were not directly observed in our *in vitro* study, previous research suggests that converting plasminogen into plasmin plays a key role in thrombolytic activity and that inhibiting COX-1 activity contributes to anti-inflammatory effects. This indicates that AA may exert its clot-lysing and membrane-stabilizing effects through the activation of plasminogen into plasmin and the inhibition of COX-1 activity. Further research, including tPA activity assays and COX inhibition assays, is needed to precisely elucidate the mechanisms underlying AA's membrane stabilization and clot lysis. Additionally, detailed molecular dynamics (MD) simulations, analysis of allosteric effects, and identification of the binding pocket site are crucial to confirm AA activation of the plasminogen enzyme.

5. Conclusion

This study revealed that AA possesses significant clot-lysing and membrane-stabilizing properties. At a concentration of 100 μg/mL, AA-induced 43.95 ± 1.27 % clot lysis and provided 64.46 ± 0.01 % membrane protection in the HRBC model. The observed dose-dependent effects across various concentrations further highlight AA's role as a moderate thrombolytic and a potent membrane-stabilizing agent. The IC_{50} values for AA's clot-lysing and membrane-stabilizing activities were determined to be 215.19 ± 1.09 and 57.21 ± 2.11 μg/mL, respectively. Molecular docking studies corroborated these findings, demonstrating strong binding interactions between COX-1 and plasmin with AA, suggesting a mechanistic basis for its biological effects. Collectively, AA's antioxidant and anti-inflammatory properties underline its potential as a therapeutic agent for atherothrombosis and red blood cell membrane protection. Further research is needed to explore the precise molecular mechanisms and potential clinical applications of AA in thrombolysis and membrane stabilization.

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CRediT authorship contribution statement

Carolina Domiciano: Writing – original draft, Validation. **Imam Rakib:** Investigation, Formal analysis. **Fazley Rohan:** Resources, Investigation. **Siddique Akber Ansari:** Software, Investigation. **Irfan Aamer Ansari:** Validation, Investigation. **Henrique Coutinho:** Supervision, Project administration. **Shuv Yadav:** Conceptualization. **Muhammad Torequl Islam:** Writing – original draft, Supervision. **Md. Sakib al Hasan:** Investigation, Data curation. **Balaram Das:** Methodology. **Md. Shadin:** Investigation, Data curation. **Md. Shimul bhuia:** Writing – original draft, Supervision. **Micheline Lima:** Writing – original draft, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interestsor personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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